Bioactive Compounds of Rice Phyllosphere Bacteria that are Antagonistic toward *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

There are several acts of antagonisms by bacteria toward phytopathogen and one of them is by producing antimicrobial compounds such as antibiotics. In dual plate tests, Pseudomonadaceae SH2a and *Pantoea* sp. MO22g showed ability to inhibit the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) which causes bacterial leaf blight disease of rice. These antagonistic bacteria have been isolated from rice phyllospheres from Wonogiri and Sukoharjo Regency, Central Java, Indonesia. This activity may suggest that those bacteria produce antibiotics as antimicrobial agents. Therefore, extraction and identification of their bioactive compounds were conducted to confirm the validity of antibiotics production. Stratified extraction based on polarity of solvents was performed using n-hexane, ethyl acetate, and methanol. Furthermore, column chromatography and UV-VIS spectrophotometer were used to classify groups of bioactive compounds. Disc diffusion method was also used to determine anti-*Xoo* activity of bioactive compounds in each fraction. The results showed that bioactive compounds from the methanol fraction of Pseudomonadaceae SH2a showed greater inhibitory activity against *Xoo* than its crude extract and the n-hexane fraction of *Pantoea* sp. MO22g. The Methanol fraction of Pseudomonadaceae SH2a contains bioactive compounds such as polyketide (pyoluteorin and 2,4-diacetylphloroglucinol), phenazine, pyrrolnitrin, peptides, terpenoids, alkaloids, and lipopeptide groups, while the n-hexane fraction of *Pantoea* sp. MO22g contains bioactive compounds such as polyketide, terpenoids, and alkaloids groups.
Key words: antagonist, bioactive compounds, phyllosphere, rice, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a bacteria that causes bacterial leaf blight (BLB) disease in rice. This phytopathogen can infect rice plants from the beginning nursery stage until the harvest (Sudir 2011). Attacks on young plants cause kresek (Suparyono *et al*. 2004), which is the most severe stage of the BLB disease and can cause harvest failure. Infection by *Xoo* that occurs in mature plants can lead to symptoms of blight that caused losses of 50-70% (Sudir 2011).

Biocontrol by using an antagonist agent is an alternative control for BLB disease that is environmentally friendly (Nurhayati 2011). Pseudomonadaceae SH2a and *Pantoea* sp. MO22g show antagonistic activity against *Xoo*. These antagonistic bacteria have been isolated from rice phyllosphere in Wonogiri and Sukoharjo Regency, Central Java Province, Indonesia, respectively.

According to Mishra *et al*. (2013) the production of antibiotics (antibiosis) is an antagonist mechanism that plays a crucial role on the inhibition of microbial phytophatogen growth by antagonistic bacteria. Research conducted by Ji *et al*. (2008) showed that *Lysobacter antibioticus*

13-1 strain significantly inhibited growth of *Xoo*. *Xoo* growth inhibition is suspected to be due to bioactive compounds that were produced by *L. antibioticus* 13-1. A related study was also conducted by Velusamy *et al*. (2013) which showed that *P. fluorescens* has antibacterial activity against *Xoo* through production of 2,4-diacetylphloroglucinol (DAPG).

In this research, extraction and identification of bioactive compound classes, as well as anti-*Xoo* activity tests of the extracted bioactive compounds produced by Pseudomonadaceae SH2a and *Pantoea* sp. MO22g were conducted to determine if the antagonistic mechanism that is applied by those bacteria is by producing antibiotics.

MATERIALS AND METHODS

Extraction of bioactive compounds antagonistic bacteria

Antagonistic bacteria were cultured in 2 mL of Nutrient Broth (NB) medium for 3 days at 27^{o}C (Shirzad *et al*. 2012). The liquid culture was poured in a 1.5 mL micro centrifuge tube and then was centrifuged at a speed of 8,000 rpm for 10 minutes (Beric *et al*. 2012). Resulting supernatant was used as a crude extract of bioactive compounds of the antagonistic bacteria.

Antagonistic bacteria were cultured in 150 mL of NB medium for 3 days at 27^{o}C (Shirzad *et al*. 2012). The liquid culture was poured into the tube and then centrifuged at 3,000 rpm for 10 minutes. Resulted supernatant was used for the stratified extraction. Extraction was done following Apriliany *et al*. (2013) with some modifications. Stratified extraction began by extraction using non-polar solvent (n-hexane p.a.), followed by semi polar solvent (ethyl acetate p.a.) and the final using polar solvent (methanol p.a.). Culture supernatant was combined with 150 mL n-hexane p.a., shaken vigorously for 10 minutes and then allowed to stand to form two layers. The upper layer (n-hexane fraction) was taken, while the lower layer was mixed with another 150 mL of n-hexane p.a., shaken vigorously for 10 minutes and then allowed to stand to form two layers. The upper layer is taken and combined with the first n-hexane fraction. The lower layer was mixed with 150 mL ethyl acetate p.a., shaken vigorously for 10 minutes and then allowed to stand to form two layers. The upper layer (ethyl acetate fraction) was taken, while the lower layer was mixed with another 150 mL of ethyl acetate p.a., shaken vigorously for 10 minutes and then allowed to stand to form two layers. The upper layer was taken and combined with the first ethyl acetate fraction. The lower layer was mixed with 300 mL of methanol p.a. (methanol fraction) then shaken vigorously for 10 minutes. The fractions of n-hexane, ethyl acetate, and methanol were heated at 45^{o}C to evaporate the solvents. The stratified extraction produced n-hexane, ethyl acetate and methanol fractions. Furthermore, fractions were identified into classes of compounds using column chromatography and UV-VIS spectrophotometer.

Identification of the classes of antagonistic bacteria bioactive compounds

Each fraction and standard compound was mixed with a suitable solvent and then put into different columns. Compounds that had detached and located in the same position as the standard compounds were taken. These compounds were combined with solvent to measure the absorbance. The absorbance values were compared with the absorbance value of the standard compounds (i.e. standard curve of correlation between absorbance toward concentration), from here the concentrations of these compounds can be known.

Test of the anti-*Xoo* activity of extracted antagonistic bacteria bioactive compounds

Tests of the anti-*Xoo* activity of the crude extract, n-hexane, ethyl acetate, and of methanol fractions of the antagonistic bacteria were conducted using the disc diffusion method. *Xoo* liquid culture was swabbed on Nutrient Agar (NA) medium in a petri dish by using cotton buds. For each extract as much as 15 µL was dripped on the 6 mm diameter paper disc (Murniasih and Rasyid 2010). The dried paper disc was placed on a petri dish containing NA medium already swabbed with *Xoo*. As a positive control, 30 µg of chloramphenicol antibiotic was used, while the negative control used NB medium and distilled water (for crude extract), n-hexane (for of n-hexane fraction), ethyl acetate (for ethyl acetate fraction), or methanol (for of methanol fraction). The cultures were incubated at room temperature for 48-72 hours. Anti-*Xoo* activity was shown by the inhibition zone or clear zone around the paper disc.

RESULTS

Identification of the classes of bioactive compounds antagonistic bacteria

Pseudomonadaceae SH2a produces bioactive compounds of polyketide, peptides, terpenoids, alkaloids, and lipopeptide groups with various concentrations. Polyketide and terpenoids were more soluble in n-hexane although some were also dissolved in methanol and ethyl acetate. This occurs because methanol is a common solvent and ethyl acetate is a solvent that can dissolve the compounds having ethyl and methyl groups. Compound classes of peptide, alkaloid, and lipopeptide dissolved more in methanol. The compound class with the greatest concentration was lipopeptide (Table 1).

Table 1. The bioactive compounds produced by Pseudomonadaceae SH2a

Bioactive compound group	Concentration (mg/L)		
	N-hexane fraction	Ethyl acetate fraction	Methanol fraction
Polyketide	7	4	3
Pyoluteorin	5	2	3
2,4- diacetylphloroglucinol	1	6	3
Phenazine	6	0	3
Pyrrolnitrin	0	4	2
Peptide	4	2	8
Terpenoid	5	2	1
Alkaloid	3	4	7
Lipopeptide	2	6	10

Pantoea sp. MO22g produces bioactive compounds from the classes of polyketide, peptides, terpenoids, alkaloids and lipopeptides with various concentrations. Polyketide and terpenoids are more soluble in n-hexane, whereas peptides, alkaloids, and lipopeptides are more soluble in methanol. The class of compounds with the greatest concentration produced by *Pantoea* sp. MO22g is peptide, whereas the smallest concentrations are polyketide and lipopeptide (Table 2).

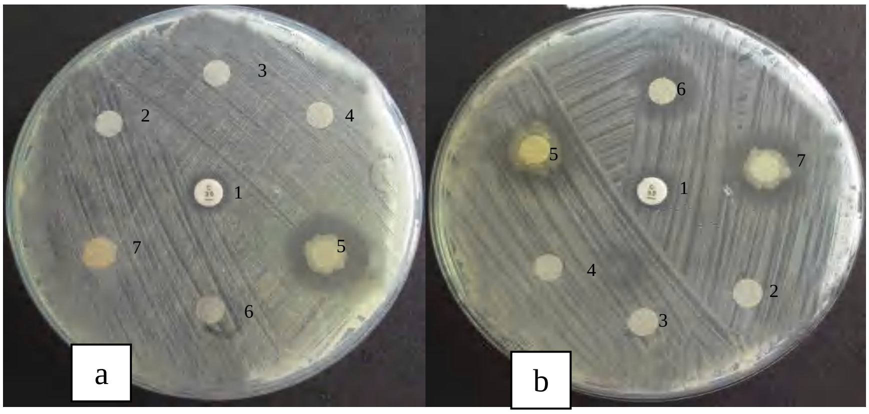
Table 2. The bioactive compounds produced by *Pantoea* sp. MO22g

Bioactive compound group	Concentration (mg/L)		
	N-hexane fraction	Ethyl acetate fraction	Methanol fraction
Polyketide	2	0	1
Peptide	0	2	3
Terpenoid	2	1	1

Bioactive compound group	Concentration (mg/L)		
	N-hexane fraction	Ethyl acetate fraction	Methanol fraction
Alkaloid	1	1	2
Lipopeptide	0	1	2

Test of the anti-*Xoo* activity of the bioactive compounds extracts

Disc diffusion test results showed that extracts of antagonistic bacteria bioactive compounds that produced clear zones indicating antibiotic activity were the crude extract and methanol fraction of Pseudomonadaceae SH2a, as well as the crude extract and n-hexane fraction of *Pantoea* sp. MO22g.The diameter of the clear zone formed by the crude extract of *Pantoea* sp. MO22g was larger than the diameter of the clear zone formed by the crude extract of Pseudomonadaceae SH2a (Table 3). The diameter of the clear zone formed by the crude extract of Pseudomonadaceae SH2a was smaller than that formed by its methanol fraction (Figure 1.a). This methanol fraction contained polyketide (pyoluteorin and 2,4-DAPG), phenazine, pyrrolnitrin, peptides, terpenoids, alkaloids, and the lipopeptide groups. The phenazine concentration in this methanol fraction was 3 µg/mL which could sufficient to form a clear zone of 11.17 mm (Shanmugaiah et al., 2010). It has yet to be ascertained if the inhibition zone is only the result of phenazine activity or from the activities of the entire class of compounds present in the methanol fraction. The diameter of the clear zone diameter formed by the crude extract of *Pantoea* sp. MO22g was greater than that formed by its n-hexane fraction (Figure 1.b). According to Sugijanto *et al*. (2014) this is possible because the anti-*Xoo* activity of the crude extract was produced from a combination of various compounds, while in the fractions of the extract, the compounds had been divided.



Note: 1 = chloramphenicol (positive control), 2 = n-hexane (negative control), 3 = ethyl acetate (negative control), 4 = methanol (negative control), 5 = methanol fraction, 6 = ethyl acetate fraction, 7 = n-hexane fraction

Figure 1. The anti-*Xoo* activity of Pseudomonadaceae SH2a (a) and *Pantoea* sp. MO22g extracts (b)

Table 3. The anti-*Xoo* activity of antagonistic bacteria bioactive compounds extracts

Antagonistic bacteria	Clear zone (mm)			
	Crude extract	Methanol fraction	Ethyl acetate fraction	n-hexane fraction
Pseudomonadaceae SH2a	5,33	11,17	-	-
Pantoea sp. MO22g	13,5	-	-	8,33

Note: - = not form a clear zone

DISCUSSION

Pyoluteorin and 2,4-DAPG were detected in equal concentrations from the compounds class of polyketides produced by the Pseudomonadaceae SH2a. According to Souza and Raaijmakers (2003) pyoluteorin is a phenolic polyketide with bactericidal, herbicidal and fungicidal activity, while 2,4-DAPG is a phenolic compound with broad-spectrum antifungal, antibacterial, anthelmintic, and phytotoxic activity (Schnider-Keel *et al*. 2000). Other compounds detected were phenazine and pyrrolnitrin. Phenazine was present in greater concentration than pyrrolnitrin. Phenazine and pyoluteorin are more soluble in n-hexane, while 2,4-DAPG and pyrrolnitrin are more soluble in ethyl acetate. Phenazine results from the shikimic acid pathway (Weller *et al*. 2007), and is a derivative of chorismic acid and is a broad-spectrum antibiotic that can fight bacteria, fungi, and eukaryotes (Fitzpatrick 2009). Pyrrolnitrin [3-chloro-4- (2`-nitro-3`-chloro-phenyl) Pyrrole] is also a broad-spectrum antibiotic that is effective against a variety of fungal pathogens (Kievit *et al*. 2011).

Research conducted by Zhou *et al*. (2012) revealed that *Pseudomonas brassicacearum* J12 generates 2,4-DAPG, hydrogen cyanide (HCN), siderophores, and proteases. *P. aeruginosa* MML2212 produces phenazine-1-carboxamide (PCN) (Shanmugaiah *et al*. 2010), and Williams (2003) showed that *P. aeruginosa* produces pyoluteorin. *P. fluorescens* synthesizes phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine (2-OH-PHZ) (Sujatha and Ammani, 2013), siderophores, HCN, chitinase, β-1,3-glucanase, cellulase (Shivalingaiah and Umesha 2013), as well as pyrrolnitrin (Howell and Stipanovic 1979). In line with the research that has been done, this study shows that Pseudomonadaceae SH2a also produces the antibiotics 2,4-DAPG, pyoluteorin, phenazine, and pyrrolnitrin.

Pantoea sp. MO22g produces bioactive compounds from the class of polyketides, peptides, terpenoids, alkaloids and lipopeptide with various concentrations. Terpenoid antibiotics such as phenalinolactones (terpene glycosides) are produced by *Streptomyces* sp. Tü 6071 (Durr *et al*. 2006) and terpentecin is produced by *Streptomyces griseolosporus* MF730-N6 (Hamano *et al*. 2001). Alkaloids with antimicrobial activity made by bacteria include norharman (an alkaloid β-Karbolin) produced by *Pseudoalteromonas piscicida*, a marine bacteria associated with *Hymeniacidon perleve* sponge (Zheng *et al*. 2005) and hapalindoles that is produced by cyanobacteria (Abad *et al*. 2011).

Peptide antibiotics such as polymyxin, colistin, and circulin are produced by the genus *Bacillus* that can inhibit the activity of Gram-negative bacteria (Katz and Demain 1977). A lipopeptide that has antibacterial activity is tauramamide formed by *Brevibacillus laterosporus* PNG276 which can inhibit *Enterococcus* sp. (Abad *et al*. 2011). Research conducted by Wright *et al*. (2001) showed that *P. agglomerans* Eh318 synthesizes pantocin A and B.

Research conducted by Shanmugaiah et al. (2010) showed that phenazine-1-carboxamide (PCN) at a concentration of 4-10 μg/mL effectively inhibits the growth of *Xoo* with inhibition zones on plates ranging from 30-54 mm. Phenazine has a mechanism of action by way of diffusion into the cell membrane and acts as a reducing agent, generating superoxide and hydrogen peroxide toxic radicals intracellularly that are harmful to the organism (Chin-A-Woeng *et al*. 2003). In vitro, 2,4-DAPG can damage the cytoplasmic membrane and inhibit the growth of *Azospirillum brasilense* (Couillerot *et al*. 2011). Pyrrolnitrin potentially inhibits respiration pathways and causes osmotic adjustment as a broad-spectrum antibiotic against fungi and protista and is lethal against *Amoeba* at very low concentrations (Jousset *et al*. 2010). According to Kievit *et al*. (2011), the pyrrolnitrin mechanism of action is through inhibition of endogenous and exogenous respiration and glucose metabolism.

The diameter of the clear zone diameter formed by the crude extract and fractions of the bioactive compounds Pseudomonadaceae SH2a and *Pantoea* sp. MO22g were larger than the diameter of the clear zone formed by chloramphenicol (positive control). Chloramphenicol produced a clear zone with a diameter of 1.91 mm. Chloramphenicol is a broad-spectrum antibiotic that can be used against Gram positive and Gram negative bacteria. Chloramphenicol inhibits peptide bond formation by binding to the 50S subunit of the prokaryotic ribosome in the peptidyl transferase region and interferes by competing in 3`aminoasil end-tRNA binding to the A site of the ribosome (Kostopoulou *et al*. 2011).

Research to exploration for biocontrol agents has been carried out. Species of the genus *Pseudomonas* have great potential to be developed into biocontrol agents for various phytophatogenic microbes. This is evidenced by the activity of P*. fluorescens* 1100-6 against *Rhizobium vitis* that causes tumors in wine (Eastwell *et al*. 2006); P*. fluorescens* and *P. putida* fight against *P. solanacearum* that causes wilt disease on mulberry (Nuraeni and Fattah 2007); *P. rhizosphaerae* JAN which acts against *Erwinia amylovora* that causes fire blight disease (Paternoster *et al*. 2010); and *P. brassicacearum* J12 which acts against *Ralstonia solanacearum* that causes bacterial wilt in tomatoes (Zhou *et al*. 2012). Research related to the use of *Pantoea* as an antagonistic bacteria to fight against phytophatogenic microbes includes the use of *Pantoea agglomerans* against *Erwinia amylovora* (Sammer *et al*. 2009) and *P. agglomerans* PTA-AF1 in combating *B. cinerea* that causes gray mold disease on grape (Throttle-Aziz *et al*. 2008).

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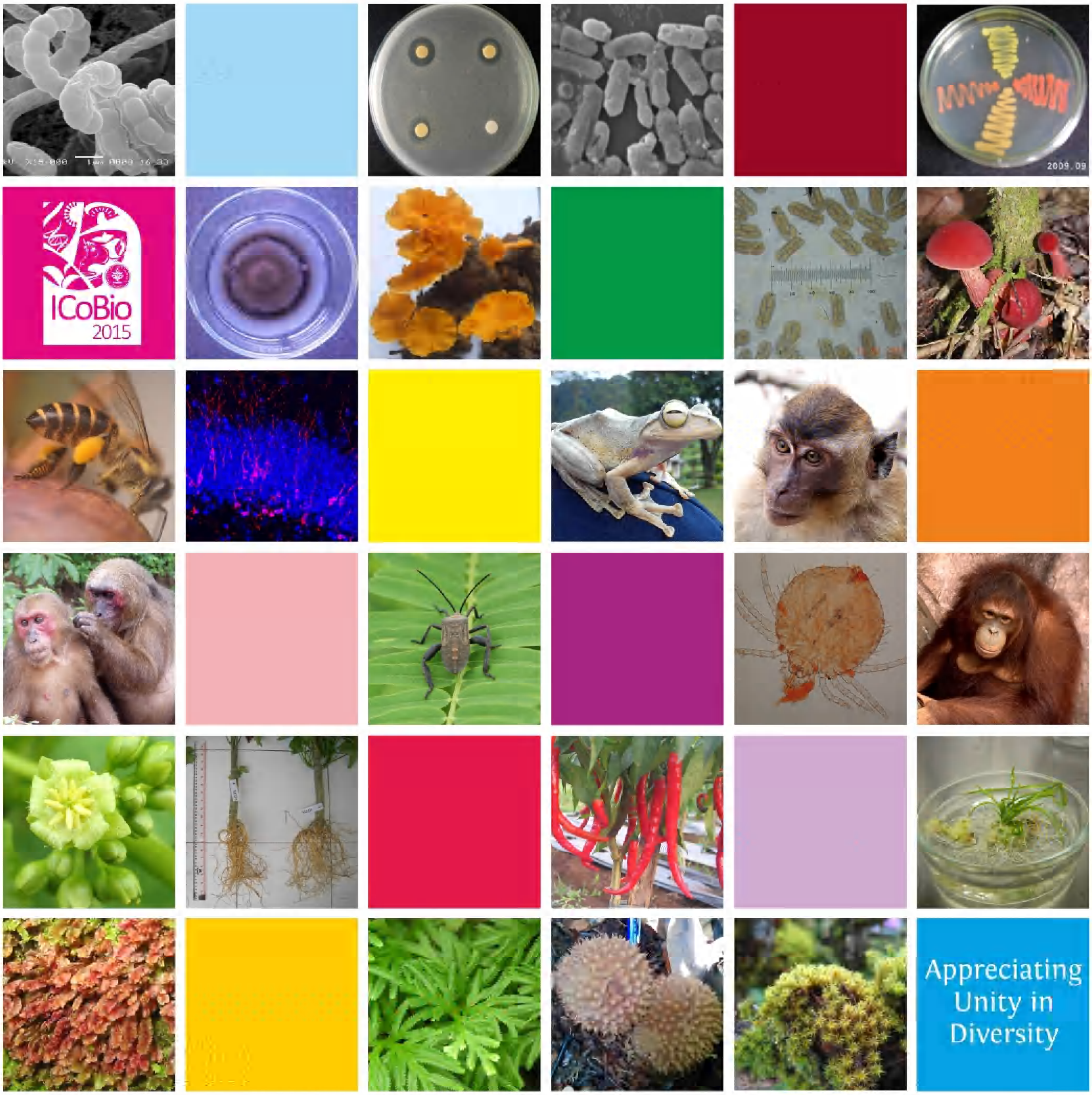

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Foreword

International Conference on Biosciences, ICoBio 2015, took place in Bogor, Indonesia, on August 5-7, 2015. The ICoBio 2015 have the theme of "Appreciating Unity in Diversity". This conference is intended to gain insight into current trends in research and teaching related to biology, such as interdisciplinary approaches that are important for understanding the biology and its applications. Moreover, to encourage the formation of networks between biologists and relevant stakeholders to accelerate our efforts to understand the biological phenomena and their applications.

The ICoBio 2015 is attended by more than 200 participants from several countries including Japan, Malaysia, India, Pakistan, Germany, Thailand, and Indonesia. The conference is the first international conference organized by the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Indonesia and is expected to serve as an initial step to be held continuously every two years (bianually). This activity is also the first step in the framework of collaboration between the Faculty of Mathematics and Natural Sciences (especially Department of Biology) Bogor Agricultural University, Indonesia with the Faculty of Science, Kasetsart University, Thailand.

One of the activities in this conference is the preparation of the proceeding. We received 9 keynote papers and more than hundred papers from oral presentations, workshops, and poster presentations. To collect paper we communicate with the authors and reviewers. One paper was reviewed by a competent reviewer. Reviewers provide comments and further authors revise his/her paper and return it to the editor of this proceeding. Therefore we highly indebted and appreciated to the reviewers who have taken the time, energy, and experience to review the papers.

Finally, there are the 16 accepted papers from oral presentations published in this book. Their topics cover a wide range of biosciences. In the conference, they presented the papers in the main four groups focusing on Biodiversity, ecology, and evolution (group 1); physiological, developmental, and behavioral sciences (group 2); Molecular biology, biotechnology, and omic technology (group 3); and Applied and interdisciplinary biology (group 4).

We do hope that this proceeding will provide you, the reader, the opportunity to get acquainted in greater detail with the ideas and results of the conference participants and also, perhaps, to recall some of the friendly and inspiring atmosphere of ICoBio 2015.

Bogor-Indonesia, August 24, 2015

Prof. Aris Tri Wahyudi
Conference Chairperson

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The International Conference on Biosciences (ICoBio) 2015

Organized by Department of Biology, Faculty of Mathematics and Natural Sciences Bogor Agricultural University

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