Diversity of Indonesian Bacterial Biocontrol Agents Against Bacterial Wilt and Damping-off of tomato

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ABSTRACT

As a mega biodiversity country, Indonesia possesses a great potential resource for bacterial biocontrol agents against various plant pathogens. Increasing concern regarding demands for food safety and quantity, environmental pollution and detrimental effects of agrochemicals on a variety of non target organisms has generated an interest in biocontrol agents (BCAs) to protect plants from crop pathogens.

The objectives of this study were to isolate and characterize possible bacterial BCAs from Indonesian peat soils, various local composts and plants. In the beginning of this research work, BCAs were isolated from peat soils in a tropical peat swamp forest in Kelampangan zone, central Kalimantan and various local composts around Bogor city. Forty seven bacterial isolates from peat soils and composts were screened for *Rhizoctonia solani*. Seven out of thirteen peat soil isolates, and six out of thirty three composts isolates showed an antagonistic activity against *R. solani* in potato dextrose agar. The culture filtrates of the antagonistic bacterial isolates in a medium of trypticase soy broth were analyzed with a high performance liquid chromatography (HPLC) column. The HPLC analysis indicated that the antagonistic isolates produced an antifungal iturin A. Macroscopic observation of isolates colonies showed that their colony forms were amuboid, myceloid, curled, circular, rhizoid, irregular and filamentous.

Bacteria having ability to form antibacterial and antifungal substances can be isolated easily from soil samples and compost. Lievens *et al*. (1989) and Leyns *et al*. (1990) found about 30% of all bacteria isolated from soils were able to produce antifungal inhibition zones *in vitro*. Soil of peat swam forest and composts are good samples for searching of BCAs. As organic material degrades, compost may contain various genus or species bacteria that have antagonistic acticity towards various plant pathogens.While soil of peat swamp forest that is acidic, may contain acidopihilic bacteria which has its own advantages to found BCAs the acidic tolerant. There are many researchers have searched BCAS from soils and composts, while BCAs isolated from plants are still rare until now.

Since Indonesian plants exhibit a high diversity, and at this moment, no report has been published on BCAs isolated from healthy plants in Indonesia that suppress bacterial wilt and damping-off of tomato so that for further study BCAs were isolated and characterized from

various healthy plants in organic farming in Bedugul in Bali island, Sukabumi, and Kepulauan Seribu in Java island, Indonesia. One hundred bacterial strains isolated from the various plants grown organically were assessed for their potential biocontrol ability. Phylogenic analysis based on the 16S rRNA analysis showed that Gram positive and negative bacteria were distributed in the host plants. About 43% of them belonged to *Bacillus* spp. and the other genera were *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Brevibacterium, Enterobacter, Leucobacter, Microbacterium, Paenibacillus, Pseudomonas, Serratia*, and *Stenotrophomonas*. The screening results showed that strains EB13, EB45, and EB53 isolated from *Brassica chinensis*, *Fragaria vesca*, and *Ipomea aquatic*, which were identified as *B. amyloliquefaciens*, *B. cereus*, and *Alcaligenes* sp., increased the survivability of tomato in bacterial wilt (BWT) significantly (P<0.05) by 67%, 83%, and 72%, respectively. Two strains, EB13 and EB45, also increased the survivability of tomato in damping-off significantly (P<0.05) by 45%, while EB53 and EB87 identified as *Enterobacter gergoviae* showed 23% and 34% disease suppression, respectively, although the differences were not significant. EB13rifkan, EB53rif and EB87rif, spontaneous antibiotics mutants of the parent strains, were confirmed to colonize tomato roots and suppress the population of *R. solani* in soil and root. A seven-day culture broth of strains *B. amyloliquefaciens* EB13 and *E. gergoviae* EB87 and its butanol extract showed antibiosis to *R. solani* and *R. solanacearum*. HPLC analysis revealed the productions of iturin and surfactin by EB13 and an iturin like compound by EB87. These results indicate that peat swamp forest soils and plant-derived bacteria not only offer potential biocontrol agents for the tomato diseases but also provide a new source for antibiotics.

Dissertation Summary

論文要旨

Diversity of Indonesian Bacterial Control Agents Against Bacterial Wilt and Damping-off of tomato

トマト青枯病ならびに苗立枯病に対するインドネシア産拮抗細菌の多様性

インドネシアは非常に生物多様性に富む国なので、様々な植物病原菌に対する拮抗菌の分離現源としての可能性がある。食の安全性に対する需要拡大、環境汚染や農薬の様々な非標的生物に対する悪影響などへの関心が高まりつつある中、病原菌から作物を守る手段として生物防除への興味が増している。

本研究の目的は、インドネシアの泥炭土壌、地元産堆肥、植物から拮抗細菌を単離し、特徴付けを行う事である。カリマンタン島の熱帯湿地林の泥炭土壌などから、48 菌株を分離し、*Rhizoctoniasolani* に対する抑止能を評価した。泥炭土壌から分離した 13 菌株の内の 7 株ならびに堆肥分離株 33 株中の 6 株が PDA 培地上で *Rhizoctonia solani* に対して阻止円を形成した。それらの培養濾液を HPLC で分析したところ、阻止円形成株は抗カビ性のイチュリンを生産していた。それらのコロニーは amuboid、myceloid といった特徴的な性状を示した。

トマト青枯病と苗立枯病を抑制する細菌株に関して、インドネシアからこれまでのところ報告例がないので、バリ島およびジャワ島の有機農園で栽培されている健全な作物から細菌株を分離した。100 株の分離株について 16S rRNA 配列情報を基に系統関係を見たところ、43%が *Bacillus* に、残りは *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Brevibacterium, Enterobacter, Leucobacter, Microbacterium, Paenibacillus, Pseudomonas, Serratia, Stenotrophomonas* といった多様な属に分類された。これらの菌株の内、EB13（*B. amyloliquefaciens*、分離源：*Brassica chinensis*）、EB45（*B. cereus*、分離源：*Fragaria vesca*）、EB53 （*Alcaligenes* sp.、分離源：*Ipomea aquatic*）株がトマト青枯病を有意にそれぞれ 67%、83%、72%抑制した。EB13 と EB45 株はトマトの苗立枯病も有意に 45%抑制した。EB53 株と *Enterobacter gergoviae* と推定された EB87 株は、有意ではなかったが、苗立枯病をそれぞれ 23%、34%抑制した。EB13、EB53、EB87 株の抗生物質耐性変異株を用いて環境中での動態を追跡したところ、これら菌株はトマトの根に定着し、土壌ならびに根における病原菌の増殖を抑制したことが推察された。EB13 株はイチュリンとサーファクチンを、EB87 株はイチュリン用の化合物を生産していることが HPLC 分析よりわかった。

これらの結果は、熱帯泥炭湿地林と植物由来細菌はトマトの各種病気に対する拮抗菌の、さらには新規抗生物質の単離源ともなる可能性を示唆した。

List of Publications:

Publications of the papers constituting PhD dissertation;

(1) **Yuliar**, Koki Toyota, and Kenji Yokota. 2015. Characterization of possible bacterial biocontrol agents, isolated from various plants in Indonesia, against bacterial wilt and damping-off of tomato. Soil Microorganisms 69:39-47.

(2) **Yuliar**, Yanetri Asi Nion, and Koki Toyota. 2015. Recent trends in control methods for bacterial wilt diseases caused by *Rasltonia solanacerum*. Microbes Environ.30:1-15

(3) Yuliar, Zaenal Abidin, and Wibowo Mangunwardoyo.2011. Potency of biocontrol agents

isolated from compost and peat soil of tropical peat swamp forest in Kelampangan zone, Central Kalimantan. Journal of Forestry Research Vol.8 (2): 144-157.

Publications of the papers related, but not constituting PhD dissertation;

(1) Yuliar. 2014.The effect of endophytic mangrove bacteria on leaf blight of rice caused by *Xanthomona oryzae* pv. Oryzae. Global Journal of Biology, Agriculture and Health Sciences.Vol.3(1):1-7.

(2) Yuliar, Suciatmih, D.Supriyati, and M. Rahmansyah. 2013. Biodiversity of endophytic bacteria and their antagonistic activity to *Rhizoctonia solani* and *Fusarium oxysporum*.Global Journal of Biology, Agriculture and Health Sciences.Vol.2(4):111-118.

(3) Yuliar. 2009. Damping- off of chilli (*Capsicum annum* L) management by using biocontrol agents of *Bacillus brevis* and *Bacillus pantothenticus*. Proceeding of international conference on biological science. Yogyakarta 16-17 October 2009: 926-928.

(4) Yuliar. 2009. Influence of *Bacillus pantotheinticus* inoculant on total microbial activity in soil and growth of *Glycine max* (L.Merr) var Baluran. Journal of Nature (special edition):39-42.

(5) Yuliar. 2008. Screening of bacteria isolates for biocontrol agent of *Rhizoctonia solani* and surfactin producer. Journal of Biological Diversity, 9(2): 70-74.

(6) Yuliar. 2007. Biocontrol of *Rhizoctonia solani* damping-off of tomato with antagonistic bacteria. Environmental Journal, 9(1): 61-65.

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Chapter 1. GENERAL INTRODUCTION

1-1. CURRENT SITUATION IN CROP PRODUCTION

Global crop production needs to double by 2050 to meet the demands from rising world population that continuosly increase every year. However, current estimates are far below that is needed (Ray *et al*. 2013). Expansion of crop land, improvement of cultivation methods, breeding for beneficial crops, increasing irrigation, and improvement of crop productivity are all of possible methods of increasing the world food supply that are being pursued (Agrios 2005).

Soilborne pathogens cause a significant reduction in yield and quality of crops and they are difficult to manage. They are particularly challenging since these pathogens often survive in soil for many years. The term soilborne pathogens can be defined as pathogens that cause plant diseases via inoculum that infects to the host plant by the way of the soil. The most familiar diseases caused by soil borne pathogens are probably rots that affect belowground tissues, including damping-off of seedlings, roots and crown rots, seed decay, and vascular wilts. Soil borne diseases are estimated to reduce the production of major crops such as corn, potato, rice, sugar cane, tomato, weed, and wheat (Fiers *et al*. 2012; Grosch *et al*. 2005; Gross *et al*. 1998; Hebbar *et al*.1998; Larkin 2008; Mao *et al*. 1997; Nandakumar *et al*. 2001; Viswanathan, 200; Yin *et al*. 2013).

Tomato soil borne diseases as important as soil borne diseases in alliums, asparagus, carrot, celery, lettuce, cole,spinach, cucumber, melons, squash, bean, pea, pepper, and potato (Koike, 2003 in http://anrcatalog.ucdavis.edu).

Tomato (*Solanum lycopersicum)* is one of the most important vegetables in the world, with global production reaching almost 160 million tons in 2011 (FAOSTAT, 2013). Tomato production is hampered by various plant pathogens and pests, with fungal and oomycete pathogens in particular posing serious yield restraints (Foolad *et al*. 2008).

Major diseases of tomato are caused by 7 bacteria, 24 fungi and 10 viruses and several nematodes (Jonez *et al*. 1991). Phytopathogens that attack tomato include *Phytophthora infestans, Alternaria solani, Sclerotium sclerotiorum, Rhizoctonia solani, Fusarium oxysporum, Pseudomonas syringae, Ralstonia solanacearum* and *Pectobacterium carotovorum* (de Oliviera *et al*. 2010).

1-2. Importance of tomato

Tomato (*Solanum lycopersicum* L.) is one of the most popular vegetables and is the most widely grown worldwide with up to 5 million hectares. China is the world' top tomato grower and has more than 25% of the world's tomato acreage. Asia and Africa account for about 79% of the global tomato areas, with about 65% of the world yield (FAOSTAT, 2008). Tomato unquestionably occupies a significant position in world vegetable production owing to its world wide consumption (Sahu *et al*. 2012). As fruit which is often considered a vegetable, it is ranked second behind potato in production (FAOSTAT, 2010). Tomato unquestionably occupies a significant position in world vegetable production owing to its world wide consumption (Sahu *et al*. 2012). Top 25 tomato producers in 2012 have been listed in Table 1. Tomato belongs to Solanaceae family, which includes more than 3,000 species, occupying a wide variety of habitat (Knapp, 2002). Recent taxonomic revision of the Solanaceae has reintegrated *Lycopersicon* into the genus *Solanum* with a revised new nomenclatur (Peralta and Spooner, 2001; Spooner *et al*. 2005; Peralta *et al*. 2008). The majority of taxonomists as well as most plant breeders and other users have accepted the reintegration of tomatoes to *Solanum* (Caicedo and Schaal 2004; Fridman *et al*. 2004; Schauer *et al*. 2005; Mueller *et al*. 2009). The wild relatives of the cultivated tomato are native to Western South America, from Northern Ecuador through Peru to Northem Chile, including the Galapagos islands (Darwin *et al*. 2003; Peralta and Spooner, 2005). They are spread throughout diverse habitats that include the desert of the Pacific coast at sea level, the gree inter-Andean valleys and mountainous Andean regions at an altitude of 3,300 meters (Rick and Holle. 1990; Warnock.1991). This peculiar ecological diversity in the Andean region has contributed to the variability of the tomato related to wild species (Warnock, 1991). The tomato has been continually subjected to human selection for a wide array of application in both science and commerce (Bai and Lindhout, 2007). During selection for a variety with high yield and quality of tomato, various diseases may appear besides vitamins and antioxidans. Tomato is an important source of vitamins and antioxidants. It is rich in the carotenoids lycopene and ß-carotene (provitamin A), which are reported to have anticancer properties. Tomatoes are also an important source of vitamin C: ca.10% of total dietary intake of vitamin C in the USA (Gerrior and Bente, 2002). Among more than 600 carotenoids in plant, only about 14 are found in human tissues (Khachik *et al*. 1995). Tomato and tomato products contribute to nine of these 14 carotenoids and are the prodominant source of lycopene, neuorosporene, gamma-carotene, phytoene, and phytofluene (Sies *et al*. 1995). Frequent consumption of tomato products is

associated with a lower risk of prostate cancer (Giovannucci *et al*. 2002; Ambrosini *et al*. 2008). In plant pathology research, tomato is also very important and useful plants. Arie *et al*. (2007) mentioned that tomato has been a good model plant to analyze plant-pathogen interactions and its prospects for the future are promising. An international consortium named International Solanaceae Genomic Project (SOL) is proceeding with whole genome sequencing of tomato.

Table 1.1. Top 25 tomato producers by countries in 2012

Rank	Country	Productions (tons)
1	China	41,864,750
2	USA	12,902,000
3	India	11,979,700
4	Turkey	10,052,000
5	Egypt	8,544,990
6	Italy	6,024,800
7	Iran	5,256,110
8	Spain	4,312,700
9	Brazil	3,691,320
10	Mexico	2,997,640
11	Uzbekistan	2,347,000
12	Russian Federation	2,000,000
13	Nigeria	1,860,600
14	Ukraina	1,824,700
15	Greece	1,406,200
16	Portugal	1,406,100
17	Morocco	1,277,750
18	Tunisia	1,100,000
19	Syrian Arab Republic	1,052,200
20	Irag	1,013,180
21	Chile	900,000
22	Indonesia	891,616
23	Netherlands	815,000
24	Romania	768,532
25.	Jordan	737,261

Source: FAOSTAT, 2012

According to the references (Asaka and Shoda, 1996; Kelman, 1994; Kondoh *et al*. 2001; Sabaratnam andTraquair, 2002; Lievens *et al*. 2003; Schwarz and Grosch, 2003; Elphinstone, 2005; Borrero *et al*. 2006; Taiwo *et al*. 2007; Moretti *et al*. 2008 Thanh *et al*. 2009; Xu *et al*. 2009; Srivastava *et al*. 2010; Wei *et al*. 2011,2013; Nunez, 2012 ; Radwan *et al*. 2012; Adam *et*

al. 2014; and Goudjala *et al*. 2014), the following are listed as one of major tomato diseases caused by soilborne pathogens such as bacteria, fungal, and nematodes.

1-3. Major soil borne diseases and their control measures

1-3-1. Bacterial wilt

Based on scientific and economic importance in plant diseases, top ten bacterial species have been listed recently: 1) *Pseudomonas syringae* pathovars, 2) *Ralstonia solanacearum*, 3) *Agrobacterium tumifaciens*, 4) *Xanthomonas oryzae* pv. *oryzae*, 5) *X. campestris* pathovars, 6) *X. axonopodis* pathovars, 7) *Erwinia amylovora*, 8) *Xylella fastidiosa*, 9) *Dickeya* (former *Erwinia*) (*dadantanii* and *solani*), 10) *Pectobacterium* (former *Erwinia*) *carotovorum* (and *Pectobacterium atrosepticum*) (Mansfield *et al*. 2012).

Ralstonia solanacearum Yabuuchi *et al.* (syn. *Pseudomonas solanacearum* (Smith) Smith, *Burkholderia solanacearum* (Smith), Yabuuchi *et al.* 1995), ranked as the second important bacterial pathogen, cause a vascular wilt disease and are one of the most destructive pathogens with rapid and fatal wilting symptoms. This pathogen infects an extensively wide range of host plants over 200 species, distributes worldwide and induces destructive economic impact (Kelman, 1998). Direct yield losses by the pathogen vary widely according to the host, cultivar, climate, soil type, cropping pattern and strain. For example, yield losses vary from 0 to 91% in tomato, 33 to 90% in potato, 10 to 30% in tobacco, 80 to 100% in banana and up to 20% in groundnut (Elphinstone, 2005). It is very difficult to control this pathogen due to its abilities to grow endophytically, survive in soil, especially in a deeper layer, travel along water and associate with weeds (Wang and Lin, 2005).

Many reports have described development in the control methods against bacterial wilt diseases caused by *R. solancearum*, such as biological, physical, chemical, cultural or integral ones.

1-3-2. Biological control method

1-3-2-1. Biological control agent (BCA)

Interest in biological control has increased since public has been concerned over the use of chemicals in general (Whipps, 2001). The benefits of BCAs are 1) potentially self-sustaining, 2) spread on their own after initial establishment, 3) reduced inputs of non-renewable resources and

4) long-term disease suppression in an environmentally manner (Quimby *et al*. 2002; Whips and Gehardson, 2007).

Mechanisms of BCAs are sustained by various interactions such as competition for nutrient and space, antibiosis, parasitism and induced systemic resistance (Agrios, 2005; Cook and Barker, 1983). According to our reference survey, BCAs have been dominated by bacteria (90%) and fungi (10%). According to Montesinos (2003), most of BCAs patented are made of bacteria. Topics on the biocontrol agents to bacterial wilt are separated into the following categories: isolation, screening and identification of BCA, application methods of BCA, improvement of BCA, suppression mechanisms of BCA, and effect of BCA on environment.

Recent studies have shown the potential value of some promising BCAs for controlling bacterial wilt, which are dominantly avirulent strains of *R. solanacearum* and *Pseudomonas* spp., followed by *Bacillus* spp., *Streptomyces* spp. and other species. Recently, 109 strains of endophytic or rhizobacteria were screened for their antibacterial activity against *R. solanacearum*. Effective isolates (total 22) consisted of *Pseudomonas* spp. (18) and then *Bacillus* sp. (2) (Ramesh and Phadke 2012).

Several new or uncommon BCAs have been reported to control bacterial wilt such as *Acinetobacter* spp. (Xue *et al*. 2009), *Burkholderia nodosa, B. sacchari, B. tericola, B. pyrrocinia* (Nion and Toyota 2008*),* bacteriophage (Alvarez, *et al*. 2007; Yamada *et al*. 2007:), *B. thuringiensis (*Zhou *et al*. 2008*), Chryseobacterium indologenes* (Hoa *et al*. 2004), *Chryseomonas luteola* (Hoa *et al*. 2004), *Clostridium* sp. (Momma *et al*. 2007), *Enterobacter* sp. (Xue *et al*. 2009), *Myroides odoratimimus* (Yang *et al*. 2012), *Paenibacillus marcerans* (Li *et al*. 2011), *P. polymyxa* (Ling *et al*. 2006; Li *et al*. 2011), *Pseudomonas brassicacearum* (Zhuo *et al*. 2012*), Ralstonia pickettii* (Wei *et al.* 2013), *Serratia* sp. (Guo *et al*. 2004; Xue *et al*. 2013), *Sphingomonas paucimobilis* (Hoa *et al*. 2004), *Staphylococcus auricularis* (Hoa *et al.* 2004), *Stenotrophomonas maltophilia* (Messiha *et al*. 2007*), Streptomyces rochei* (Liu *et al*. 2013), *Streptomyces virginiae* (Tan *et al*. 2011) and *Xenorhabdus nematophila* (Ji *et al*. 2004).

The possible suppression mechanisms of these species are competition, induced systemic resistance, antibiosis, and production of enzymes degrading cell wall and siderophores. Successful trials using BCA in field are introduced in Table 1-2. Recently, Hyakumachi *et al*. (2013) revealed that *B. thuringiensis*, a famous bioinsectice producing bacterium, induces defense-related genes, such as PR-1, acidic chitinase and beta-1,3-glucanase and shows

resistance against direct inoculation with *R. solanacearum*. It was confirmed that the expression of several salicylic acid-responsive defense-related genes was specifically induced (Takahashi *et al*. 2014), and that the suppression by *B. thuringiensis* may be different from ISR elicited by many PGPRs, in which jasmonic acid and ethylene-dependent signaling pathways mediate plant resistance to pathogen (Hyakumachi *et al*. 2013).

Table 1-2. Various biocontrol agents that have been tested in field for controlling bacterial wilt diseases caused by *Ralstonia solanacearum* (2005 -2014)

Microorganisms	Inoculation method and application rate	Mechanisms	BE (%)	Yield*	Ref
1. *Bacillus amyloliquefaciens* SQR-7 and SQR-101 and *B. methylotrophicus* SQR-29	Pouring, 6.8 x 10^{10} cfu/plant (SQR-7), 7.5 x 10^{10} cfu/plant (SQR-101), 8.2 x 10^{10} cfu/plant (SQR-7)	Production of indole acetic acid and siderophores	18-60% in tobacco	25-38%	Yuan *et al*. (2014)
2. *Ralstonia pickettii* QL-A6	Stem injection, 10 µL of 10^7 CFU mL^{-1}	Competition	73% in tomato	NA	Wei *et al*. (2013)
3. *Pseudomonas monteilii* (A) + *Glomus fasciculatum* (B)	Stem cuttings were dipped in A (9.1 x 10^8 mL^{-1}), then B (53 infective propagules) was added to each cutting and again A was poured	Increased plant nutrient uptake (N, P, K) and reducing the pathogen population	56-75% in herb (*Coleus forskohli*)	54%	Singh *et al*. (2013)
4. *Brevibacillus brevis* L-25 +*Streptomyces roche* L-9 +*organic fertilizer*	Mixed with soil at a density of 7.3 x 10^7 (L-25) and 5.0 x 10^5 (L-9) cfu g^{-1} of soil	Decreased root colonization by the pathogen	30-95% in tobacco	87-100%	Liu *et al*. (2013)
5. *Bacillus amyloliquefaciens*+bio-organic fertilizer (BIO23) *B. subtilis*+bio-organic fertilizer (BIO36)	Mixed with soil at a density of 5.5 x 10^6 (BIO23) and 7.0 x 10^6 (BIO36) cfu g^{-1} of soil	Plant growth promotion	58-66% in potato	64-65%	Ding *et al*. (2013)
6. *Bacillus* sp. (RCh6) *Pseudomonas mallei* (RBG4)	3x10^8 cfu g^{-1} (talc formulation). Seedlings were dipped in antagonist suspension (25 g talc formulation L^{-1}). Leftover suspension was poured around the root zone of the seedling (50 mL $plant^{-1}$)	Production of inhibitory compounds and siderophores	81% in eggplant	60-90%	Ramesh *et al*. (2012)
7. *Trichoderma viride* (A), *B. subtilis* (B), *Azotobacter chroococcum* (C), *Glomus fasciculatum* (D), *P. fluorescens* (E)	D (53 infective propagules) was added to each stem cutting that was dipped in A (1.2x10^6 CFU mL^{-1}), B (1.8x10^8 CFU mL^{-1}), C (2.3x10^7 CFU mL^{-1}), E (2.5x10^8 CFU mL^{-1}). Then, 5 ml of A,B,C,E was poured into 200 g	Competition for nutrient uptake (NPK) and reducing *R. solanacearum* population	7-43% in herb (*Coleus forskohlii)*	159 - 227%	Singh *et al*. (2012)

	soil.				
8. *B. amyloliquefaciens* QL-5, QL-18 + organic fertilizer	Mixed with soil at a density of 1 x 10^7 (QL-5) or 1 x 10^7 (QL-18) cfu g^{-1} of soil	Decreased root colonization by the pathogen	17-87% in tomato	NA	Wei *et al.* (2011)
9. *B. amyloliquefaciens* Bg-C31	Poured 10 mL of bacterial suspension $plant^{-1}$ (potato dextrose broth culture).	Production of antimicrobial protein	60-80% in Capsicum	NA	Hu *et al.* (2010)
10. *Acinetobacter* sp. Xa6, *Enterobacter* sp. Xy3	Poured 20 mL of the bactetial suspension (1×10^9 cells mL^{-1}) $plant^{-1}$. Or seedling roots were soaked in the bacterial suspension.	Rhizocompetence and root colonization	57-67% in tomato	32-41%	Xue *et al.* (2009)
11. *B. vallismortis* ExTN-1	Bacterial suspension was mixed into an organic fertilizer (10^6 cfu mL^{-1}) and poured to soil.	Induction of systemic resistance	48-49% in tomato	17%	Thanh *et al.* (2009)
12. *Glomus mossease*	30 g of inoculum (650-700 spores of *G. mossease* 100 $g^{-1}$of soil) was added to a planting hole.	Competition for nutrients and decreased pathogen population	25% in tomato	16%	Taiwo *et al.* (2007)

BE: biological control efficacy, NA: not applicable, Yield*: increase in yield

1-3-2-2. Organic matter

Organic amendments to soil stimulate the activities of microorganisms that are antagonistic to pathogens (Akhtar and Malik, 2000). In addition, organic amendments often contain biologically-active molecules such as vitamins, growth regulators, toxins and low molecular mass humic substances which can affect the soil microorganisms.

Organic matter has come from recently living organisms and is capable of decay or the product of decay. It is categorized into plant or animal origins, and simple organic carbons. In the previous references related to *R. solanacearum* study, different organic matters, such as plant residue (80%), animal waste (10%), and simple organic matters (10%), controlled bacterial wilt disease. Larkin (2008) stated that in general, biological amendments can effectively deliver microorganisms to natural soil, resulting in a wide variety of effects on soil microbial communities depending on the particular types, numbers, and formulations of organisms added. A new approach is the suppression of bacterial wilt in an organic hydroponic system through a rhizosphere biofilm formed on roots only in the organic system (Fujiwara *et al*. 2012).

1-3-2-2-1. Plant residue controlling bacterial wilt

Several researchers reported that bacterial wilt was suppressed by plant residue deriving from, e.g. chili (*Capsicum annum*) (Teixeira *et al*. 2006), Chinese gall (*Rhus chinensis*) (Yuan *et al*. 2012), citronela (*Cymbopogon nardus*), clove (*Szygyum aromaticum*) (Amorim *et al*. 2011), eggplant (*Solanum melongena*) (Almeida *et al*. 2007), eucalyptus (*Eucalyptus globules*) (Paret *et al*. 2010, 2012), geranium（*Geranium carolinianum*）(Ooshiro *et al*. 2004), guava (*Psidium guajava* and *P. quineense*) (Acharya and Srivastana, 2009), hinoki (*Chamaecyparis obtuse*）（Yu and Komada, 1999), Japanese cedar（*Cryptomeria japonica*) (Hwang *et al*. 2005; Matsushita *et al*. 2006), lemongrass (*Cimbopogon citratus*) (Pare *et al*. 2010, 2012), marigold（*Tagetes patula*）(Terblance and de Villiers, 1998), neem (*Azadirachta indica*) (Pontes *et al*. 2011), palmarosa (*Cimbopogon martini*), (Pare *et al*. 2010, 2012), pigeon pea（*Cajanus cajan*), sunn hemp (*Crotalaria juncea*）(Cardoso *et al*. 2006), tamarillo (*Cyphomandra betacea*）（Ordóňez *et al*. 2006), thyme (*Thymus* spp.), (Ji *et al*. 2005; Pradhanag *et al*. 2003), wood wax tree (*Toxicodendron xylvestre*) (Yuan *et al*. 2012), and worm killer（*Aristolochia bracteata*）(Shimpi *et al*. 2005). The possible mechanisms in the plant residues are considered mainly antimicrobial activity, then indirect suppression of the pathogen through the improvement of physical, chemical and biological soil properties (Cardoso *et al*. 2006).

1-3-2-2-2. Animal waste controlling bacterial wilt

Many researchers already reported that animal waste can control plant disease, but there are few papers reporting that animal waste suppresses bacterial wilt disease. For example, application of pig slurry decreased the population of *R. solanacearum* in the soil (Gorissen *et al*. 2004). The mechanism behind the enhanced decline of population and disease suppressiveness was unclear but shifts in bacterial community profiles might be related to them. Another experiment suggested that suppression of bacterial wilt by poultry and farmyard manure are relating to higher microbial activity and higher numbers of cultural bacteria and fungi (Islam and Toyota, 2004). In the study, a lower disease index was related to a poor survival of the pathogen. There are, however, limitations to a wide use of organic wastes. Janvier *et al*. (2007) explained that the major key-points for efficiency of organic matter in suppressing plant pathogens depend

on: 1) the combination of plant and pathogen , 2) the rate of application, 3) the nature/type of amendment, and 4) the degree of maturity of decomposition stage of crop residues.

1-3-2-3. Simple organic compounds controlling bacterial wilt

Efficacy of simple organic compounds, including amino acids, sugars, organic acids, on bacterial wilt of tomato was evaluated in pot experiments. Among them, the application of lysine into pumice culture medium (0.25 mg g^{-1}) and soil (2.5 mg g^{-1}l) reduced bacterial wilt of tomato by 85-100% (Igawa *et al*. 2008; Nion, 2008) and by 58-100% (Posas *et al*. 2007), respectively. The possible suppression mechanism was not due to induced systemic resistance, but to shifts in the soil microbial community structure leading to more rapid death of the pathogen (Posas *et al*. 2010). In contrast, riboflavin induced a series of defense responses and secondary metabolism in cell suspensions and thus protected tobacco against *R. solanacarum* (Liu *et al*. 2010). D,L-3-aminobutyric acid (BABA) also increased polyphenol oxidase activity and decreased catalase in tomato plants, suggesting induction of resistance to bacterial wilt of tomato (Hassan *et al*. 2013). Another type of report is that methyl gallate strongly exhibited bactericidal effect on *R. solanacearum* (Fan *et al*. 2014).

1-3-3. Physical method, including biofumigation

A number of physical control methods, e.g. solarization and hot water treatment, have proved effective against *R. solanacearum*. Recently, there is growing attention to biofumigation, which refers to the agronomic practice of using volatiles chemicals released from plant residue to suppress soil-borne plant pathogens (Kirkegaard *et al*.1996).

Lower moisture conditions (20-30% maximum water holding capacity) and pre-incubation at lower temperatures (4°C) reduced bacterial wilt and had a negative impact on the survival of *R. solanacearum* (Islam and Toyota, 2004). Scherf *et al*. (2010) found that *R. solanacearum* survived for 6 months in infected geranium at a constant temperature, while declined rapidly in repeated winter temperature cycles of 2 days at 5°C followed by 2 days at -10°C.

Both of heat treatment at 45°C for 2 days or a minimum temperature of 60°C for 2 h to the infected soil prior to tomato planting reduced the total bacterial population by 60-97%, that of *Ralstonia* spp. from 2 to 7 x 10^8 cfu g^{-1} to 0 to 115 cfu g^{-1}, and the bacterial wilt incidence by 50-75% (Kongkiattikajorn and Thepa, 2007).

Vinh *et al*. (2005) reported that soil solarization using transparent plastic mulches for 60 days prior to planting of tomato reduced bacterial wilt incidence. Other researcher reported rhizome solarization on ginger seed for 2 to 4 h reduced by 90-100% of bacterial wilt at 120 days after planting, and when ginger seed sterilized with discontinuous microwaving (10 s pulse) at 45^{o}C reduced 100% wilt incidence (Kumar *et al*. 2005). Baptista *et al*. (2006, 2007) studied the effect of soil solarization that reduced bacterial wilt of tomato. In the experiment soil solarization reduced soil pH, K, Na, B and Zn contents, microbial biomass and microbial respiration in soil, while did not affect significantly the other soil chemical properties.

Mechanism in the suppression of bacterial wilt by physical methods is generally in killing pathogen with high temperatures or low. In biological soil disinfection (BSD), the production of organic acid or heavy metal ions was involved (Momma, 2008). In the control with high voltage electrostatic field and radio frequency electromagnetic field, inducing systemic resistance was involved (Wu *et al*. 2007). Silver coated non woven cloth filter and visible light source (Bando *et al*. 2008) or electrostatic spore precipitator ozone saturated water (Zhou *et al*. 2007) was developed as a sterilization device and inactivated the pathogen.

To a wide use of soil solarization, several parameters should be carefully considered: how to control temperature or release of volatile compounds and economical and/or practical feasibility in field.

1-3-4. Chemical methods (pesticides and non-pesticides)

World pesticide use exceeds 5.0 billion pounds in 2000 and 2001 (Kiely *et al.* 2004). Herbicides account for the largest portion of the total use, followed by insecticides and fungicides. Plant disease control has been largely dependent on the use of pesticide (Whipps and Gerhardson, 2007). Schreinemachers *et al*. (2012) reports that pesticide use per hectare, especially herbicide and fungicide/bactericide, has generally increased more than porportionally with crop output per hectare, and reveal that a 1% increase in crop output per hectare is associated with 1.8% increase in pesticide use per hectare.

Pesticides such as algicide (3-(3-indolyl) butanoic acid), fumigants (metham sodium, 1,3-dichloropropene, chloropicrin), and plant activators generating systemic resistance on tomato (valydamycin A and validoxylamine) have been used to control bacterial wilt. The combination of methyl bromide, 1,3-dichloropropene or metham sodium with chloropicrin significantly

reduced bacterial wilt in the field ranging from 72% to 100% and increased the yield in tobacco and tomato. The pesticide treatment increased the yield of tomato by 1.7 to 2.5 times compared to untreated control (Fortnum and Martin, 1998; Santos *et al*. 2006).

Edwards-Jones (2008) reported that pesticides will offer greater net benefits than other control methods, but this will not always be the case. For example, if farmers use pesticide carelessly or without proper knowledge, a part of pesticides would remain in the environment for many years (Gadeva and Dimitrov, 2008) and become a contaminant in soil and/or groundwater (Acero *et al*. 2008), and can be poisonous to farmers (Dasgupta *et al*. 2007).

Bacteriocides (triazolothiadiazine (0.5 to 12 mM) (Khanum *et al*. 2005), streptomycine sulphate (600 ppm) and streptocycline (500 ppm), Khan *et al*. 1997), other chemicals such as bleaching powder 86.8% (25 kg ha^{-1}) and formaldehyde as sterilizers or weak acidic electrolyzed water (Yamasaki *et al*. 2006) are also effective to kill microorganisms.

Acibenzolar-S-methyl (ASM) is proposed to induce systemic resistance (Hacisalihoglu *et al*. 2007). Combination of ASM and thymol significantly reduced disease and increased the yield of tomato, whereas ASM or thymol alone did not significantly reduce disease or increase yield (Hong *et al*. 2011). Silicon (Dannon and Wydra, 2004; Wydra and Dannon, 2006) or Si and chitosan (Kiirika *et al*. 2013) reduced bacterial wilt through induced resistance. Wang *et al*. (2013) reported Si-mediated resistance associated with the changes of soil microorganism amount and soil enzyme activity. Soaking of seeds in a low sodium chloride solution increased the seedling vigor and tolerance to *R. solanacearum* in tomato (Nakaune *et al*. 2012).

Mechanism of the non-pesticide chemicals that suppress bacterial wilt is considered either induced systemic resistance or antibacterial activity. Some new mechanisms to suppress bacterial wilt are to capture microbial cells alive with 10 g kg^{-1} of coated sawdust with 1% of an equimolar polymer of N-benzyl-4-vinylpyridinium chloride with styrene (PBVP-co-ST) (Kawabata *et al.* 2005a), to coagulate pathogenic bacterial cells in the soil with 10 mg kg^{-1} a copolymer of methyl methacrylate with N-benzyl-4-vinylpryridium chloride in a molar ratio 3:1 (PMMA-co-BVP) (Kawabata *et al*. 2005b), to protect plants from infection through a bacteriostatic action with a phosphoric acid solution (Norman *et al*. 2006).

A variety of non-pesticide chemicals have a good future for application into field to control bacterial wilt disease because their effects on environment are considered less, but the economic consideration often influences the choice of the chemicals. Expensive chemicals and repeated

applications are possible only for valuable crops that might incur substantial economic losses in the absence of treatment. Considering that crop yield and quality is not damaged when disease severity is rather low or in the absence of pathogens, diagnosis on an economic threshold should be essential to determine whether or not chemical treatment is needed.

1-3-5. Cultural practices

1-3-5-1. Cultivar resistant

Growing of cultivars resistant to bacterial wilt will be the most economic, environmentally friendly and effective method of disease control. Worldwide, breeding for resistance to bacterial wilt has been concentrated on crops of wide economic importance such as tomato, potato, tobacco, eggplant, peppers and peanut. Breeding for resistance to bacterial wilt has usually been influenced by factors like availability of resistance sources, their diversity, genetic linkage between resistance and other agronomic traits, differentiation and variability in pathogenic strains, the mechanism of plant-pathogen interactions, and breeding or selection methodology (Boshou, 2005; Elphinstone, 2005; Hanson *et al*. 1996). For example, the *Arabidopsis* NPR1 (nonexpresser of *PR* genes) gene was introduced into a tomato cultivar, by which resistance to bacterial wilt (BW) was enhanced and wilt incidence reduced by approximately 70% at 28 days after inoculation (Lin *et al*. 2004). Potato genotype BP9, that is a somatic hybrid between *Solanum tuberosum* and *S. phureja,* successfully reduced bacterial wilt by 90-100% (Fock *et al*. 2000). Somatic hybrids between *S. melongena* cv. Dourga and two groups of *S. aethiopicum* were produced by the electrical fusion of mesophyll protoplasts and found tolerant to *R. Solanacearum* (Collonnier, 2001).

Prior *et al.* (1996) showed that the resistant plants were heavily invaded by *R. solanacearum* without exhibiting wilt symptoms. Bacterial multiplication in the stems of resistant tomato plants was suppressed owing to the limitation of pathogen movement from the protoxylem or the primary xylem to other xylem tissues (Nakaho *et al*. 2004). Preotemics approach was done to elucidate the molecular interactions in the cell wall of resistant and sensitive plants inoculated with *Ralstonia solanacearum* (Dahal *et al.* 2010). Generally, resistance to bacterial wilt in many crops is negatively correlated with yield and quality. Thus, release of resistant cultivars may be poor because of the other agronomic traits and are not widely accepted by farmers or consumers. In the future we hope to breed for good resistant cultivar by making more efforts in genetic

enhancement of bacterial wilt resistance through biotechnology approaches in order to improve yield crop.

1-3-5-2. Crop rotation, multi-cropping

Benefits of crop rotation are the maintenance of soil structure and organic matter, and a reduction in soil erosion that is often associated with continuous row crops (Janvier *et al*. 2007). While continuous cropping with the same susceptible host plant will lead to the establishment of specific plant pathogenic populations, crop rotation avoids this detrimental effect and is often associated with a reduction in plant diseases caused by soil-borne pathogens (Janvier *et al*. 2007; Kurle *et al*. 2001). For example, the onset of bacterial wilt was delayed by 1 or 3 weeks and wilt severity was reduced by 20-26% when a susceptible tomato variety was grown after corn, lady's fingers, cowpea or resistant tomato (Adhikari and Bansyat, 1998). Potato cultivation rotated with wheat, sweet potato, maize, millet, carrots, sorghum or phaseolus beans showed that wilt incidence was reduced by 64 to 94% and the yield of potato increased by 1 to 3 times compared to that in monoculture potato (Katafiire *et al*. 2005). In an example of multi-cropping, Yu *et al.* (1999) explained suppression mechanisms of Chinese chive (*Allium tuberosum*), which suppressed the occurrence of bacterial wilt of tomato (approximately 60%) because root exudates of Chinese chive may prevent *R. solanacearum* from infecting tomato plants.

1-3-5-3. Soil amendment

Previous studies have revealed that fertilizer application reduced bacterial wilt. Calcium is most famous fertilizer inducing the disease suppression. A famous story is Ca. Increased Ca concentrations in plant reduced the severity of bacterial wilt and the populations of *R. solanacearum* in the stems of tomato (Yamazaki and Hoshina, 1995; Yamazaki, 2001), and that the increase of Ca uptake in tomato shoots was correlated with lower levels of disease severity (Yamazaki *et al.* 1996, 2000). Lemaga *et al.* (2005) reported that application of nitrogen + phosphorus + potassium and nitrogen + phosphorus (application rates of each fertilizer = 100 kg/ha) reduced bacterial wilt by 29% and 50%, respectively, and increased the yield of potato up 18.8 t ha^{-1} and 16.6 t ha^{-1}, respectively, compared to that in untreated control (11.2 t ha^{-1}). Hacisalihoglu *et al.* (2007) reported that bacterial wilt induced the changes in nutrient distribution, especially Ca, B and P in tomato leaves. Li and Dong (2013) revealed that a

combined amendment of a rock dust and commercial organic fertilizer reduced the bacterial wilt of tomato. A single amendment of rock dust was also effective and the raised soil pH and Ca content were the key factors for the rock dust amendment controlling bacterial.

Many soil elements found in cell walls influence the susceptibility or resistance of plants to pathogen infections, among them silicon is considered to be a beneficial element for plants and higher animals (Epstein, 1999). Kiirika *et al*. (2013) reported that combined application of silicon with chitosan reduced bacterial wilt of tomato through induced resistance. Si and chitosan gave the synergistic effects against the disease.

1-3-6. Integrated Pest Management (IPM)

Agrios (2005) explained that the main goals of an integrated plan for disease control are to (1) eliminate or reduce the initial inoculums, (2) reduce the effectiveness of initial inoculums, (3) increase the resistance of the host, (4) delay the onset of disease, and (5) slow the secondary cycles.

IPM reduced bacterial wilt disease ranging 20-100% in the field or laboratory condition, and usually combines two or three methods among cultural practices, chemical and biological method. For example, bacterial wilt incidence in tomato was monitored in a soil infested with *R. solanacearum* and added with S-H mixture (contains agricultural and industrial wastes such as bagasse, rice husk, oyster shell powder, urea, potassium nitrate, calcium superphosphate, and mineral ash) or Actigard (a.i.: acibenzolar-*S*-methyl). The addition of S-H mixture decreased BWT by 32%, while that of Actigard decreased BWT by 5%. In contrast, the addition of S-H mixture and Actigard decreased BWT by 53% (Anith *et al.* 2004).

To develop IPM, it is necessary to assess the relative importance of factors accounting for production losses. Combinations in cultural practice methods, such as a combination of crop rotation with a resistant cultivar or a soil amendment, or a combination of organic matter with a non-pesticide chemical such as formaldehyde or bleaching powder look effectively to reduce bacterial wilt and increase crop yield (Sharma and Kumar, 2000; Adhikari and Bansyat, 1998; Vinh *et al*. 2005; Lemaga *et al.* 2005). Combined application of ASM and *P. fluorescens* Pf2 resulted in the highest reduction of bacterial wilt of tomato, while either application of ASM or Pf2 was effective (Abo-Elyousr *et al*. 2012).

While a combination of endophytic bacteria (*Bacillus* sp. and *Serratia marcescens*, both of which had no antibiosis) with resistant cultivars of tomato reduced bacterial wilt (*Barretti et al*. (2012)

Grafting is an important integrated pest management strategy to manage soil borne pathogens. Important diseases management by grafting are caused by fungal pathogens (such as *Verticillium*, *Fusarium*, *Pyrenochaeta* and *Monosporascus*), oomycete pathogens (*Phytophthora*), bacterial pathogens (particularly *Ralstonia*), root knot nematodes and several soil-borne virus pathogens (Louws *et al*. 2010).

We should select methods which are easy, practical, profitable and also environmentally healthy to control disease and improve yield.

1-4.Major soil borne diseases and their control measures -Damping-off-

1-4-1 Damping-off caused by *Rhizoctonia solani*

R. solani Kühn (teleomorph, *Thanatephorus cucumeris* (A. B. Frank) Donk) is an important cosmopolitan necrotophic soil-borne fungus. Damping-off caused by *R. solani* results in yield losses in more than 200 crops globally (Lee *et al*. 2008). The widespread soilborne pathogen *R. solani* is also responsible for serious damage to trees worldwide (Grosch *et al*. 2006). High yield losses were reported, e.g., up to 50% for sugar beet (Kiewnick *et al*. 2001), up to 70% for field-grown lettuce (Davis *et al*. 1997), and about 20% for potato (Grosch *et al*. 2005). The host range is extremely broad and its dormant organ sclerotia survive for longer periods under various environmental conditions (Grosch *et al*. 2006). Besides, cultivars with complete resistance are not available at present (Li *et al*. 1995). Therefore, efficient strategies to control the pathogen are urgently required. On the other hand, increasing use of chemical inputs causes several negative effects, such as the development of pathogen resistance to the applied agents and their non-target environmental impacts (Gerhardson, 2002). A growing awareness of agricultural practices in using chemicals has a great impact on human health and on the environment and has spawned research into the development of effective biocontrol agents to protect crop against diseases. Wang *et al*. (2009) reported that the use of an antagonistic microorganism, a *Bacillus* sp. strain CHM1, against *R. solani* on horsebean (*Vicia faba*), and Kumar *et al*. (2013) also reported a *Bacillus* sp. strain N antagonized *R. solani*, *F. oxysporum*, and *Penicillium expansum*.

Among others of host range of *R. solani* are; bottom rot on lettuce, black scurf on potato, damping-off of cucumber, pine, and tomato (Tunlid *et al*. 1989; Huang *et al*. 2013; Golinska and

Dahm, 2013; Asaka and Shoda., 1996), late sugar beet rot (Mahr *et al*. 1986; Wolf and Verreet, 2002), root and hypocotyl diseases of snap bean (Sumner *et al*. 1992), root rot of wheat (Gill *et al*. 2001), sheath blight of rice (Belmar *et al*. 1987; Mutuku and Nose, 2012), sore shin and spot of tobacco (Csinos and Stephenson, 1999; LaMondia, 2012), stem and crown rot of tomato (De Curtis, 2010).

1-4-2 Biological control methods

Methods which have been used to control *R. solani* disease are as follow;

1-4-2-1 Biological control agents

As agents to control *R.solani* diseases the following were reported: *Rhizophagus intraradices, Rhizophagus clarus* and *Claroideoglomus etunicatum* which belong to arbuscular mycorrhizal fungi (AMF) group; in fungi, *Penicillium brevicompactium, P. expansium, P. pinophilum* (Nicoletti *et al*. 2004), *Trichoderma hamatum, T. veride, T. virens, Laetisaria arvalis,* (Lewis *et al*. 1990; Grosch *et al*. 2006; Almeida *et al*. 2007) and; in bacteria, *Bacillus amyloliquefaciens*(Yu *et al*. 2002), *B. Subtilis* (Kondoh *et al*. 2001), *Burkholderia cepacia, Flavobacterium balustinum, P. fluorescens* (Howell and Stipanovic, 1979), *Pseudomonas putida* , *Rhizobium, Rhizophagus intraradices, Rhizophagus clarus and Claroideoglomus etunicatum, and Serratia marcescens* (Song *et al*. 2013), *Streptomyces cyaneofuscatus, S.mutabilis,* and *Streptomyces* sp. Di-944 (Sabaratnam and Traquair, 2001; Goudjal *et al*. 2014).

1.4.2.2. Animal waste

Lima *et al*. (2009) stated that the application of farmyard manure results in a higher content of organic matter derived from angiosperms. An application of farmyard manure shows an increase in lignin and lignin-like products in the soil organic matter. Lignin, a highly aromatic polymer, when it is released in soils by decomposition of plant tissues is highly resistant to microbial decomposition. Spectroscopic studies showed an increase of lignin and lignin-like products in the organic matter fraction of the soil, which may derive from the cereal straw supplied with farmyard manure. Comparing the three organic amendments (sewage sludge, farmyard manure, and compost), the most significant differences were observed after long-term application of farmyard manure, with an increase in lignin and lignin-like products in the soil,

and compost, which appears to contribute to an increase of protein and protein-like, as well as carbohydrates content in the soil.

In mature compost, where concentration of free nutrients are low (Chen *et al*. 1988), sclerotia of *R. solani* are killed by hyperparasite, and biocontrol prevail (Nelson *et al*. 1983).

Abbasi *et al*. (2004) reported that the incorporation of 0.5% (*v*/*v* soil) fish emulsion into soil 5 days before planting radish provided effective control of cucumber damping-off diseases caused by *R. solani* and *Pythium aphanidermatum*.

Suppression of damping-off caused by *R. solani* in compost-amended container media has been most frequently related to the presence of specific microbial antagonists (Kuter *et al*. 1983; Nelson *et al*. 1983; Kwok *et al*. 1987; Krause *et al*. 2001).

Damping-off of cress (*Lepidium sativum*) caused by *R. solani* was significantly reduced by compost residues obtained from a viticulture and enological factory and composted cow manure (Pane *et al*. 2011). The effectiveness of manure amendments against the disease depends on the type of manure, soil, and other factors.

1-4-2-3. Plant residue

Alfano *et al*. (2011) revealed that the disease suppressive effect of olive waste compost seems to be due to the combined effects of suppression phenomena caused by the presence of microorganisms competing for both nutrient and space as well as by the activity of specific antagonistic microorganisms. Kasuya *et al*. (2006) observed that the incidence of damping-off of sugar beet caused by *R. solani* was significantly and consistently suppressed in the soils amended with residues of clover, peanut, and *Brassica rapa* subsp. *rapifera* ‘Saori. Tomato and escarole green manure were reported as the most suppressive ones in suppressing *R. solani* damping-off on *Lepidium sativum* (Pane *et al*. 2013). Compost is often reported as a substrate that is able to suppress soilborne plant pathogens, but suppression varies according to the type of compost and pathosystem (Termorshuizen *et al*. 2006). Composts prepared from agricultural waste and used in container media or as soil amendments may have highly suppressive effects against diseases caused by a variety of soilborne plant pathogens such as *Rhizoctonia* spp. (Tuitert *et al*. 1998**;** Rivera *et al*. 2004), *Pythium* spp. (Mandelbaum and Hadar, 1990; Pascual *et al*. 2000), *Phytophthora* spp. (Hoitink and Boehm, 1999; Widmer *et al*. 1999), and *Fusarium* spp. (Cotxarrera *et al*. 2002).

Plant extracts that can control the growth of *R. solani* are the extract of garlic bulb with saponins (Lanzotti *et al*. 2012), extract of *Picea neoveitchii* with four flavonoids (Song *et al*. 2011), cauliflower with caulilexins (Soledade *et al*. 2006), extract of *Anemarrhena asphodeloides* rhizomes with nyasol (Z)-1,3-bis (4-hydroxyphenyl)-1.4-pentadiene (Park *et al*. 2003). *Brassica juncea, B. napus,* and *Sinapis alba* added to the soil protected wheat from rot root of *R. solani* (Handiseni *et al*. 2013).

1-4-3. Chemical control methods

Efficiency of chemical pesticides are often low and the legal regulations restrict their uses. Schreinemachers *et al*. (2012) studied about levels and trends in agricultural pesticides used for large cross-section of countries using FAO data for the period 1990-2009. Their analysis shows that a 1% increase in crop output per hectare is associated with 1.8% increase in pesticide used per hectare but that the growth in intensity of pesticide used levels off as countries reach a higher level of economic development. Sumner *et al*. (1992) observed in their research that pentachloronitrobenzene (PCNB) was as effective as the newer fungicides flutolanil, tolclofos-methyl and mepronil in improving yield of snap bean, but flutolanil had a consistently more efficacy in reducing symptoms of disease and population densities of *R. solani* AG-4 in soil. Motoba *et al*. (1988) mentioned that flutolanil is a systemic fungicide that is highly specific for basidiomycetes, such as *Rhizoctonia*, *Corticium*, *Thyphula* and *Gymnosporangium*. In addition, Csinos (1987) reported that flutolanil has been effective in reducing limb and pod rot diseases caused by *R. solani* AG-4 and *S. rolfsii* in peanut and its effectiveness may be equal to, or better than PCNB in controlling diseases caused by *R. solani* in vegetables. Win and Sumner (1988) revealed that the lack of efficacy of seed treatment with metalaxyl plus carboxin against *R. solani* and the increased incidence of diseases when metalaxyl was used alone compared with combinations with tolclofosmethyl and flutolanil. Furthermore Grosch *et al*. (2004) reported a new fungicide with the code name BAS 516 00 F, developed by BASF, containing two active ingredients, boscalid and pyraclostrobin. The biochemical consequences of boscalid and pyraclostrobin are the breakdown of essential membrane potentials and concentration gradients and the inhibition of nucleic acid and protein biosynthesis. Fungal spore germination, mycelial growth and the development of infection structures are prevented. Therefore both active ingredients inhibit the fungal growth and colonization of plant tissue.The fungicide BAS 516 00

F was effective against *R. solani* in the field as well as in the leaf-disc bioassay and under various conditions in the climate chamber. BAS 516 00F reduced disease incidence (DI) significantly from 94 to 0 on bottom rot of lettuce.

Under greenhouse conditions, sedaxane showed high levels and consistent protection against *Ustilago nuda*, *Pyrenophora graminea* and *Rhizoctonia* spp. Under field conditions, efficacy against *Rhizoctonia* spp. resulted in increased yield compared with the untreated check (Zeun *et al*. 2013).

1-4-4. Physical control method

Temperature, moisture level and biofumigation are used in physical methods to control *R. solani* diseases. Temperature and moisture strongly affect *R. solani* diseases through its effect on the biotic and abiotic components of the disease (Katan, 1981). Moisture is important in relation to leaf diseases and damping-off seedlings, when crops are particularly susceptible to disease at certain stages of their growth e.g. web blight caused by *R. solani* (Palti and Katan, 1997).

Moisture levels have been observed to affect *Rhizoctonia* diseases. *R. solani* AG-3 caused greater stem canker to potatoes under relatively dry soil conditions than under wet soil conditions (Lootsma and Scholte, 1997; Hide and Firmager, 1989). Kumar *et al.* (1999) studied the effect of temperature and moisture on the pathogenicity of *R. solani* AG-11 on lupins. Gross *et al.* (1998) reported that lesion incidence on perennial ryegrass caused by *R. solani* (AG-1 IA) was increased as hours of leaf wetness at all temperatures tested.

There was a significant difference in the number of hypocotyls lesions that developed in the soybean plants across the temperatures (at 20, 24, 28, and 32^0C), and was not in root rot severity of soybean. The hypocotyls lesions were the lowest at 20^0C and the highest at 32^0C. Moisture contents affected the root rot disease caused by *R. solani*: 50 and 75% soil moisture holding capacity (MHC) gave significantly($P<0.001$) higher survivability of soybean by 86% than 25% and 100% MHC by survivability 74-75% of soybean (Dorrance *et al*. 2003).

1-4-5. Cultural control method

1-4-5-1. Cultivar resistant

Another environmentally safe method for controlling plant diseases is the use of resistant cultivars. Sugarbeet varities for *Rhizoctonia* resistance have been reported by Gaskill (1968),

then Hecker and Ruppel (1977), who have continued this germplasm enhancement effort. Resistance to *R. solani* varied widely among the *Capsicum* accessions. The most resistant chili accessions to *C. baccatum* were PI 439410 and PI 555611 (Muhyi and Bosland, 1995).

1-4-5-2. Crop rotation

Cereal crops (e.g., wheat, barley, corn) are considered non-hosts for *R. solani* AG 2-2 and thus, are recommended for rotation with broadleaf crops (sugar beet, soybean, sunflower) in the upper Midwest. Rotation with cereal crops decreases the populations of *R. solani*. Reports from Europe, however, indicate that *R. solani* AG 2-2 IIIB causes root and stalk rot of corn and also is the primary cause of *Rhizoctonia* root and crown rot of sugarbeet (Ithurrart, 2004). In addition Belmar *et al*. (1987) reported that cropping systems for 34 rice fields had a significant effect (P=0.05) on the preplant inoculum density of *R. solani* and the percentage of sheat blight incidence. Mean numbers of sclerotia recovered (per kg of soil) were 4.02, 1.43, and 0.07 with an average disease incidence of 5.4, 2.7, and 0.4% for rice-soybean-rice, soybean-soybean-rice, and pasture-pasture-rice cropping systems, respectively. Significantly (P = 0.05) higher inoculum densities and disease incidence were found in alternate year rotations out of rice (rice-soyben-rice) than in 2-yr rotations out of rice (pasture-pasture-rice).

1-4-5-3. Soil amendment

Epstein (1994, 2009) mentioned that mineral elements found in crop plants are nitrogen, phosporus, sulfur, potasium, calcium, magnesium, iron, manganese, zinc, copper, nickel, boron, chlorine, molybdenum, cobalt, sodium, silicon, and aluminium. Nakata *et al*. (2008) revealed that silicon (Si) is the second most abundant element in soil and considered as an absolutely useful element for a large variety of plants. In additon Fauteux *et al*. (2005) reported that Si is a bioactive element associated with beneficial effects on mechanical and physical properties of plants. Silicon alleviates biotic and biotic stresses, and increases the resistance of plants to pathogenic fungi. Chen *et al*. (2000) revealed that in nature, silicon is found in the form of silica (SiO_2), aluminium silicates, or iron or calcium silicates and is absorbed by plant as mono-silicic acid ($Si(OH)_4$. Total number of sheath blight lesions, total area under the relative lesion extension progress curve, severity of sheath blight, and the highest relative lesion height on the main tiller decreased by 24% to 52%, as the rate of Si increased from 0 to 0.096%.

Silicon may offer a viable method to control sheath blight in areas where soil is deficient in Si and cultivars with sheath blight resistance are not commercially available (Rodrigues *et al*. 2003). It is probably because Si increased the resistance of rice to a pathogenic fungus, *R. solani*.

In field peas, soil amendment with nitrogen plus phosphorus fertilizer, the application of nitrogen plus phosphorus, phosphorus plus potassium or nitrogen plus phosphorus plus potassium were effective in reducing severity of root rot caused by *R. solani* (Srihuttagum & Sivasithamparam, 1991). Plant productivity and disease incidence are influenced by soil nutrients (Ali *et al*. 2008).

1-4-6. Nanotechnology methods

The study of nanoparticles dates back to 1831 when Michael Faraday investigated gold colloids. It was more than 125 years later in 1959, that the potential benefits of fabricating matter at nano-level were visualized by Noble Laurete Richard Feynman. One Japanese researcher Norio Taniguchi finally engineered materials at nanometer scale in 1974 and coined the term nanotechnology. The term nanotechnology, buzzword of present day science owes its origin from the Greek word “nano” literally meaning dwarf. When it is expressed in terms of dimension one nanometer equals to one billionth of a matter (1 nm=10^{-9} m) (Banik and Sharma, 2011).

Some of nanoparticles that have entered into the crop protection are nano forms of copper, iron, silver, silica silver, and carbon. Nano silver was tested for antimicrobial effects against plant diseases by Jo *et al*. (2009) who used two fungal pathogens of cereals viz. *Bipolaris sorokiana* (spot blotch of wheat) and *Magnaporthe grisea* (rice blast).

Nanosized silica-silver (Si-Ag) particles were produced and tested against a number of fungal and bacterial pathogens (Park *et al*. 2006). Si-Ag inhibited the growth and development of both gram-positve and negative bacteria.

Besides, nano-copper was reported to be highly effective in controlling bacterial diseases viz. bacterial blight of rice (*Xanthomonas oryzae* pv. oryzae) and leaf spot of mung bean (*X. campestris* pv. *phaseoli*) (Gogoi *et al*. 2009).

The antifungal activity of six carbon nanomaterials has been evaluated, the result showed that single-walled carbon nanotubes (SWCNTs) possessed the strongest antifungal activity against *Fusarium graminearum* and *F. poae*, followed by multiwalled carbon nanotubes (MWCNTs), graphene oxide (GO), and reduced praphene oxide (rGO), while fullerene (C_{60}) and activated

carbon (AC) showed no significant antifungal activities. The antifungal mechanisms of carbon nano materials (CNMs) were deduced to target the spores in three steps;(i) depositing on the surface of the spores, (ii) inhibiting water uptake and (iii) inducing plasmolysis (Wang *et al*. 2014). The development of plant protection by nanotechnology method such as application of Si-Ag at 10 ppm showed 100% growth inhibition of *R. solani* (Park *et al*. 2006).

However synthesis of nanoparticle physically and chemically have a limitation in maintaining the shape, size, and monodispersivity compared with biologically synthesized nanoparticles while also lower cost and friendlier environment. Thereby the biosynthesis of nanoparticle using microbes and plants have been developed, such as in plants; leaf extract of *Peltophorum pterocarphum* containing quercetin-3-O-β-D-galactopyranoside, which reacted with silver nitrate enhanced the antifungal property by forming silver nano cubes (Sivakumar *et al*. 2013). In case of bacteria, the extracellular biosynthesis of silver nanoparticles by the culture supernatant of *Bacillus licheniformis* (Kalishwaralal *et al*. 2008) and the biosynthesis of silver and gold nanoparticle using *Brevibacterium casei* have been reported (Kalishwaralal, *et al*. 2008). However they did not report about the antimicrobial activy of these two nanoparticles against plant pathogens.

1-4-7. Integrated method

A combinatiom of T.harzianum and sheep manure at all concentration tested reduced *R.solani* damping-off after manure incubation in the soil for 0-24 months compared to the control at zero time.

A combination of *Trichoderma* and manure amendment to the soil for 24 months incubation time, at 6 and 10% significantly ($P \leq 0.005$) reduced damping-off by 33 and 50%, respectively (Barakat, 2008). Integration of a fungicide, banodanil and *T. harzianum* were found to be effective for the control of *Rhizoctonia* pre-emergence damping-off of radish (Lishitz *et al*. 1985).

A granulate biofungicide named PBGG was developed by combining *Pseudomonas boreopolis* with *Brassica* seed pomace, glycerin and sodium alginate. Results of greenhouse tests showed that the most effective treatment was the amendment of pathogen-infested soil with *Streptomyces padanus* + 1% (w/w) PBGG which resulted in a disease incidence of 6.5–8.6%, compared to 27.8–31.7% for the treatment of *S. xantholiticus* + 1% (w/w) PBGG, 36.9–38.6%

for the treatment of 1% (w/w) PBGG alone, and 61.8–64.8% for the treatment of control (unamended soil) (Chung *et al*. 2006).

Noble and Coventry (2005) reviewed the various combinations of biological control agents (including *T. harzianum*) and organic amendment that were reported to control soilborne plant pathogens. They stated that such combinations could significantly reduce the disease caused by *R. solani*. Six out of 36 composts tested showed significant suppression in damping-off by *R. solani*, and those were the hemlock bark (*Tsuga heterophylla* bark), dairy fir-bark compost (*Pseudotsugamenziesii* bark+gravity belt separated dairy solids (3:1 vol/vol), mushroom compost (straw+chicken manure+seed meal + others), and nursery regrind compost, and consistent suppression was observed over three repeated bioassays (Scheuerell, 2005). In greenhouse experiments, disease control obtained with a combination of *B. subtilis* NJ-18 and fungicide (jinggangmycin) that was better than the control obtained with the bacterium or fungicide alone, and some combinations of bacterium plus fungicide demonstrated a small synergistic effect in reducing disease (Peng *et al*. 2014).

1-5 . Major soil borne diseases and their control measures -Fusarium wilt-

Fusarium wilt is an economically important disease, devastating tomato worldwide, is considered as one of the main soil-borne systemic diseases and the major limiting factor in the production of tomato (Borrero *et al*. 2006; Lievens *et al*. 2003; Moretti *et al*. 2008: Schwarz and Grosch, 2003; Srivastava *et al*. 2010). It causes significant losses in tomato production both in greenhouse and field-grown tomatoes (Ozbay and Steven 2004). On tomato, two symptomologically distinct forms of pathogen cause either a vascular wilt (*F. oxysporum* f. sp. *lycopersici* W.C. Snyder & H.N. Hans) or a crown and root rot (*F. oxysporum* f. sp. *radicis-lycopersici*(FORL) W.R. Jarvis & Shoemarker). There are three races of *F. oxysporum* f. sp. *lycopersici*. Race 1 is widespread and common in many, if not most, tomato growing regions of the world. Race 2 and Race 3 are each represented by more than one unique genotype and occur in scattered areas around the world (Nunez, 2012). Initial symptoms are wilt of individual branches and mild to severe chlorosis. Yellowing of individual branches creates a flagging effect. Occasionally, wilt is limited to the leaflets on one side of a leaf. Internally, a reddish-brown discoloration of the vascular system extends the length of the plant. The diseases caused by *F. oxysporum* f. sp. *lycopersic*i are favored by warm temperature. The optimal temperature for

diseases development is approximately 28^0C. The fungus survives in tomato residue as thick-walled chlamydospores and on the surface of roots of many crops and weeds (Nunez, 2012).

Numerous strategies on the disease are available e.g. cultural technique, biocontrol, resistant cultivar, crop rotation and chemical control (Abo-Elyousr and Mohamed, 2009). Chemical control of Fusarium wilt of tomato in greenhouse was examined repeatedly. Fungicides including benomyl, captafol, imazalil, thiram, and prochloraz MN, provided inconsistent control of *Fusarium* crown and root rot on tomatoes, leaving problematic residues in the fruit tissues (Hartman and Fletcher, 1991). In contrast the application of methyl bromide and chloropicrin reduced *Fusarium* crown and root rot of tomato (Mc Govern and Vavrina, 1998). In addition Mandal and Sinha (1992) found out that copper chloride, ferric chloride and manganese sulfate controlled *Fusarium oxysporum* f. sp. *lycopersici* by inducing resistance in susceptible tomato plants. El-Shami *et al*. (1993) reported that vitavax (carboxin)-thiuram or vitavax-captan, applied as fungicidal seed treatment, was effective in controlling *Fusarium* wilt disease and that the latter gave better diseases control than the former. Further Dwivedai *et al*. (1995) demonstrated that thiram and topsin-M were the most effective at reducing the populations of *F. oxysporum* f. sp. *lycopersici* by 83.4% after 45 days at a rate of 800 mg/g soil. Cohen *et al.* (1992) observed that pretreatment of tomato seedlings with acetochlor reduced the disease incidence of Fusarium wilt by 58-62%. Benhamou (1992) reported that the application of chitosan to tomato plants prior to inoculation with *F. oxysporum* f. sp. *radicis-lycopersici* enhanced the resistance of tomato plants to the crown and root rot.

As with other vascular plant diseases, chemical control is less effective, besides sanitation measures are not easy to apply, due to the occurrance of the disease inside the plants (Brayford, 1992; Borrero *et al*. 2006). Furthermore chemical control is being restricted as the result of increasing public concern related to the danger for human health, environment, and appearrance of resistant strains.

The use of resistant cultivars is the most effective method of controlling *Fusarium* wilt (Beckman 1987), however, new races of the pathogen appear to overcome resistance genes in currently grown cultivars. Simons *et al.* (1998) mentioned that resistant cultivars are not always available, or the resistance is rapidly overcome by new races of the pathogen.

As an alternative approach, biocontrol agents are being used for the management of various diseases (Kavino *et al*. 2008; Harish *et al*. 2009).

Numerous microorganisms have been reported to be very effective against many soilborne plant pathogens, including *Fusarium oxysporum* f. sp. *lycopersici*. They can be *Achromobacter xylosoxydans* MM1 (Morreti *et al*., 2008), *Alcaligenes* sp. (Yuen and Schroth,1986), *Bacillus subtilis* strain S2BC-1 and *B. subtilis* strain GIBC-Jamong, (Shanmugam and Kanoujia, 2011), *Burkholderia cepacia* (Larkin and Fravel, 1998), *Chaetomium globosum*, *C. lucknowense* (Charoenporn *et al*. 2010), *Enterobacter* sp. (Akkopru and Demir, 2005), *Gliocladium virens* (Larkin and Fravel, 1998), *Pseudomonas* sp., *Trichoderma harzianum* and *Glomus intraradices* (Srivastava *et al*. 2010), nonpathogenic *F. oxysporum* (Alabouvette and Couteaudier, 1992; Larkin and Fravel, 1998; Monda, 2002; da Silva and Bettiol, 2005; Shishido *et al*. 2005), *Penicillium oxalicum* (De Cal *et al*. 1997), *Streptomyces corchorusii*, *S. mutabilis* (El-Shanshoury *et al*. 1996), and *Trichoderma asperellum* (Cotxarrera *et al*. 2002).

Efficacy of soils and composst to suppress *Fusarium* wilt has been also evaluated. For example, Borrero *et al*. (2004) reported that grape marc compost was the most effective medium to suppress *Fusarium* wilt of tomato, and cork compost was intermediate. In addition Cotxarrera *et al*. (2002) mentioned that the use of some composted sewage sludge in the plant growth medium is effective for suppression of *Fusarium* wilt.

The use of antagonist mixtures may also provide improved disease control over the use of single organisms (Larkin and Fravel, 1998). Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action, a more stable rhizosphere community and effectiveness over a wider range of environmental condition. Several researchers have observed improved disease control using various combinations of multiple compatible biocontrol organisms (de Boer *et al*. 2003; Akkopru and Demir, 2005; Leeman *et al*. 2005). Given that the establishment of a threshold density of an antagonist is a key factor in biocontrol, the rationale of using a mixture of antagonist isolates is a logical approach, because the use of mixtures more closely mimics microbial communities and has multiple mechanism of disease suppression (Duffy *et al*. 1996; Raaijmaker, 1995; Schisler *et al*. 1997; Sneh *et al*. 1984). The mixture of two antibiotic-producing bacteria or of a nonpathogenic *F.oxysporum* and fluorescent pseudomonads enhanced the efficacy of biocontrol of *Fusarium* wilts as compared with individual strains (Alabouvette *et al*. 1996; De Boer *et al*. 1999; Duijff *et al*. 1999).

Several mechanisms have been proposed to be involved in the suppression of *F. oxysporum* by these antagonistic microorganisms. Alabouvette *et al*. (1996) concluded that pathogen

suppression was due to nutrient competition between pathogenic and saprophytic *F. oxysporum*, whereas Schneider (1984) and Mandeel and Baker (1991) proposed that suppression was due to competition for the infection sites at the root surface. Competition for iron between pathogenic *F. oxysporum* and fluorescent pseudomonads was proposed to contribute to the suppression of *Fusarium* wilt, although these bacteria also produced several antifungal metabolites (Weller, 1988; Alabouvette *et al*. 1996, Dwivedi and Johri, 2003). Later, induced systemic resistance was reported to be a mechanism in the biological control of *Fusarium* wilt of watermelon and radish by bacteria (Leeman *et al*. 1995; Larkin *et al*. 1996). Chitinolytic enzymes have been considered important in the biological control of soilborne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin (Garcia, 1968; Chet, 1987).

1-6. Major soil borne diseases and their control measures-root knot nematodes

Many species of root-knot nematodes, *Meloidogyne incognita, M. arenaria, M. javanica* and *M. hapla,* are very important plant parasites affecting tomato production in all vegetable-growing regions (Sasser and Freckman, 1987; Netcher and Sikora, 1990). Chemical method has been used to control the diseases using soil fumigants or systemic nematicides (Heald*,* 1987; Johnson, 1985). New biological control methods including rhizobacteria and arbuscular mycorrhizal fungi (AMF) have been tested. AMF was shown to reduce root-knot nematode population densities on tomato and other vegetables (Sikora and Schonbeck, 1975; Sikora, 1992). However, many of the numerous reports of such experiments are contradictory (Kellam and Schenk, 1980; MacGuidwin *et al*. 1985; Smith *et al.* 1986; Lingaraju and Goswami, 1993). Similarly, *Trichoderma* spp. are known nematicidal substances producers (Sharon *et al*. 2001). Tomato genotype was evaluated for resistance to root-knot nematodes among 33 genotype screened, and tomato Mongal T-11 and tomato beef master were found to be highly resistant to *Meloidogyne* spp. (Jaiteh *et al*. 2012). Ethyl acetate extract (1 mg/mL) of *Pseudomonas aeruginosa* and *Paecilomyces lilacinus*, respectvely, caused 100% and 64% mortality of *Meloidogyne javanica* larvae after 24 hours (Siddiqui *et al*. 2000).

1-7. Purpose of this study

Various control measures have been practiced to manage soilborne plant diseases, including sanitary measures, the use of cultivar resistant, physical methods including biofumigation, and

other integrated management methods. Chemicals are also widely utilized for the management of diseases. However, indiscriminate use of chemicals are known to cause health hazards to human beings besides the cause of imbalances in the soil microbial community, which may be unfavorable to the activity of beneficial organisms and may also lead to the development of resistant strains of pathogen. As an alternative approach, biocontrol agents (BCAs) are being used, and they seem to become a promising way to manage the soilborne diseases. BCAs isolated from soils and plants are considered to perform better in the soil-crop environment, since they may adapt easier than microorganisms from other origins. Plants are constantly involved in the interaction with a wide range of microorganisms, especially bacteria. At present, there are no report that has been published on BCAs isolated from healthy plants in Indonesia that suppress bacterial wilt and damping-off of tomato, though Indonesia has diverse plants. This study was conducted with the following objectives; (1) to isolates BCAs candidates from peat soils of tropical peat swamp forests, composts, and plants, and screen them that would inhibit *R. solani* growth *in vitro* test, (2) to test whether the BCAs candidates produce an antifungal iturin A, (3) to screen the potential BCAs to suppress bacterial wilt of tomato using growth chamber, (4) to verify the BCAs efficacy to suppress bacterial wilt and damping-off of tomato under greenhouse experiment, (5) to analyse of suppression mechanisms of BCAs and to identify them by based on the 16S rRNA sequences.

1-8. Outline of the thesis

The thesis composed of 3 chapters. Chapter 1 presents a general introduction, chapters 2 and 3, present isolation, screening, identification, and characterization of BCAs isolated from peat soil of tropical peat swamp forest, compost, and plants. The outline of each chapter is as follows;

Chapter 1 is the general introduction. It deals with eight sections; (1) Current situation in crop production, (2) Importance of tomato, (3) Major soil borne diseases and their control measures-bacterial wilt, (4) Major soil borne diseases and their control measures-damping-off, (5) Major soil borne diseases and their control measures- *Fusarium* wilt, (6) Major soil borne diseases and their control measures-root knot nematodes, (7) Purpose of this study, (8) Outline of this thesis.

Chapter 2 has a title “Biocontol agents, isolated from composts and peat soil of peat swamp forest in Kalampangan zone, central Kalimantan, Indonesia”. It deals with isolation and purification of candidates of bacterial BCAs from composts and peat soil of peat swamp forest. It

also presents screening test of by antagonistic activity of them towards a fungal pathogen of *R. solani,* and an extraction and measurement of iturin A from the selected of the BCAs by high performance liquid chromatography (HPLC).

Chapter 3 has a title" Characterization of possible bacterial biocontrol agents, isolated from from various plants in Indonesia, against bacterial wilt and damping-off of tomato". It deals with four section; (1) the isolation and identification of 100 bacterial isolates based on 16S rRNA analysis, (2) the effectivenes of 20 BCAS against bacterial wilt of tomato under climatron experiment, (3) the efficacy of the selected single strain against bacterial wilt of *R. solanacearum* and damping-off of *R. solani* in greenhouse experiment, (4) the efficacy of the selected of four single bacteria against bacterial wilt and damping- off, and suppression of *R. solani* population in the root and the rhizosphere soil by colonization of EB13rifkan, EB53rif and EB87rif.

Chapter 4 is conclusion. It consists of two sections; (1) conclusion to the thesis, and (2) prospect for future research.

Chapter 2. BIOCONTROL AGENTS ISOLATED FROM COMPOSTS AND PEAT SOIL OF TROPICAL PEAT SWAMP FORESTS IN KELAMPANGAN ZONE, CENTRAL KALIMANTAN

2-1. ABSTRACT

Rhizoctonia solani is a soilborne pathogen that causes diseases in a wide range of hosts of agricultural, horticultural and flower crops. Biological control measure is the most promising way for the diseases management since it is environmental friendly. The objective of this chapter was to isolate biocontrol agents (BCAs) from peat soils tropical peat swamp forests in Kelampangan zone, Central Kalimantan and various local composts around Bogor city. Forty seven isolates from peat soils and composts were screened for in vitro antibiosis against *R. solani*. Seven out of thirteen peat soil isolates, and six out of thirty three compost isolates showed antagonistic activity against *R. solani* in potato dextrose agar. The culture filtrate of the antagonistic isolates in trypticase soy broth (TSB) was obtained and analyzed with a high performance liquid chromatography (HPLC) column. The HPLC analysis indicated that the antagonistic isolates produced an antifungal iturin A. Macroscopic observation of isolates colonies showed that forms of their colonies were amoeboid, myceloid, curled, circulair, rhizoid, irregular, and filamentous. These achievements indicate that peat swamp forests not only offer potential biocontrol agents of damping-off but also provide a new source for antibiotics producers.

2-2. INTRODUCTION

Composts may act as a growth medium or a source of benefecial organisms. A compost rich in microorganisms has proven to have a suppressive effect on plant pathogens. Several microorganisms isolated from different composts have turned out to be strongly antagonistic against certain plant pathogenic fungi. Numerous antagonistic microorganisms towards soilborne plant pathogens have been isolated from disease suppressive composts (Kuter *et al*., 1983; Kwok *et al.* 1987).

Bacteria in peat forest soils play an important role in global carbon cycling, since the peat forest soils show high bacterial diversity and species richness (Sun *et al*. 2014). Peat contains substantial reservoirs of carbon and nitrogen, accumulating as much as one-third of global

terrestrial carbon pool (Gorham, 1991; Limpens *et al*. 2008). Microorganisms in peatlands can control the turnover of organic carbon and contribute to global carbon cycling (Winsborough and Basiliko, 2010). They are instrumental in nutrient mineralization and uptake, which can feedback on plant productivity and overall ecosystem. (Andersen *et al*. 2013).

Damping-off disease not only causes a problem in horticultural crops, but also often makes predicament in forest plant nurseries. As reported by Hood *et al*. (2004), *Milicia regia* seedlings had a higher probability of dying due to damping-off disease in low-light conditions, which are characteristic of tropical forests under storey, as opposed to higher light conditions that may be found in light gaps.

Different mechanisms are involved in the interactions between bacteria, used as a biocontrol agent, and fungal plant pathogens, such as a parasitism, cross protection, antibiosis and competition. The antibiotic mechanism are said to operate when the metabolic products (antibiotics) produced by one species inhibit or suppress the growth of another species (Shoda, 2000).

The main sources of microbial antibiotics are *Streptomyces* (Actinomycetes), *Bacillus* (bacteria) and *Penicillium* (fungi) (Madigan *et al*. 1997). These bacteria are used commercially and intensively studied. Bacteria having the ability to form antifungal metabolites can be isolated easily from soil samples. Lievens *et al*. (1989) and Leyns *et al*. (1990) found about 30% of all bacteria isolated from soils were able to produce antifungal inhibition zones *in vitro*. Soil of peat swamp forest and compost are good samples for searching bacteria for biocontrol agents. As organic material decomposes, composts are able to stimulate bacteria activities (Aryantha *et al*. 2000). Therefore, compost may contain various genus or species of bacteria. Since soil of peat swamp forest is acidic, acidophilic bacteria may be contained. This chapter will explore the importance of peat swamp forests as a source of bacterial control agents against damping-off and a new source of peptide antibiotic producer.

More specifically, members of the genus *Bacillus* produce a variety of antifungal peptide antibiotics (Katz and Demain, 1977). Strains of *B. subtilis* have been also studied as biological control agents of plant pathogens. But, only a few of them were isolated and identified in Indonesia. Indonesia is a mega biodiversity country, and there is a great potential

to utilize many antifungal agents especially from genus *Bacillus*. Increasing concern regarding food safety, environmental pollution and detrimental effects of agrochemical on a variety of non-target organisms has generated an interest in biological control agents to prevent and control plant diseases. The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides (Phae *et al*. 1990; Phae and Shoda, 1990). It has been tested for control of a variety of fungi in pure cultures, and during composting (Phae *et al*. 1990). The objective of this chapter was to isolate soil bacteria, especially *Bacillus* spp., as biocontrol agents from composts and peat soils of tropical peat swamp forests. These bacteria produce antifungals (iturin A). This antibiotic has a strong antifungal activity on a large variety of yeast and fungi, but its activity is limited to a few bacteria, especially *Micrococcus luteus* (Besson *et al*. 1978).

2-3. MATERIALS AND METHODS

A. Samples for bacteria isolation

Samples used for the isolation of bacteria are as follows:

1. Peat soils from Kelampangan, Palangkaraya (2^0 04'51.21"S, 114^0 02' 04.18"E) with vegetation *Callophyllum canum*, *Capnosperma squamatum*, *Ctenotophon parvifotius*, *Cratoxylum glaucum*, and *Elaeocarpus petiolatus* were collected in different plots (plots number; A1 (pH=6.5), E6 (pH=6.0), G5 (pH=5.4), J1 (pH=4.4), and J5 (pH=3.2) (Fig. 1). Leaves on the soil surface were removed. The soil for about 200 g were collected at depths of 0-10 cm by using shovel, and put it into a black plastic bag (polybag).

2.Various brands of composts (Ratna Cibedug compost (KA), microbiolgy division, Reserach center for Biology-LIPI Biogi, (KB), Enka saritani Bogor (KC), Depok compost (KD), Sinar Katel Perkasa Bogor (KE), and anonim compost (KF). The pH of the compost were about 6.8. All of the composts (KA,KC,KD, KE, and KF) were purchased from local dealers around Bogor city, West Java, Indonesia, except for microbiology division compost (KB).

Fig.2-1. Sampling was performed in the forest area around the station JSPS-LIPI Research cooperation, Kelampangan, Central Kalimantan. This area located between Kahayan and Sebangau river, approximately 40 km Southeast Palangkaraya (HAG: unburnt natural forest, plots in which sampling has been done, where A1, E6, G5, J1 and J5).

B. Bacteria isolation procedure

Bacteria isolation was carried out by the method described by Steubing (1993), and personal communication with Dr. Shinji Miyadoh. Two grams of each sample (peat soil or compost) were heated at 100^0C for 15 minutes to kill non-spore-forming bacteria. After the heating, the sample was diluted with 2 ml of 0.85% NaCl and mixed thoroughly. One hundred µl of 1/4000 dilution sample was spread over the sterilized Nutrient Agar (NA) medium on petridish, and incubated at room temperature for five days.

C. Purification of bacteria isolates

A single colony of bacteria was streaked onto a sterilized NA medium plate. Then, it was incubated for two days (Steubing, 1993).

D. Preincubation

Five mL of sterilized LB medium in a test tube were inoculated with one loop of bacterial isolate. Then, it was incubated in an incubator shaker at 37^0C (temperature optimum for *Bacillus subtilis*, Ohno *et al*. 1995), 124 rpm for about 16 hours (Yuliar, 2002).

E. Incubation

Fifty mL of sterilized TSB medium in an Erlenmeyer flask were inoculated with 500 µl of pre-incubated isolate, then it was incubated in the incubator shaker at room temperature for seven days (Yuliar, 2002).

F. Antagonistic test (*in vitro* test)

Rhizoctonia solani (sized 5 mm x5 mm) was inoculated onto the center of sterile PDA medium in petridish. After that, two holes in the PDA medium were made using a cork borer (the position of two holes was at the same distance from the center of the medium, where the *R. solani* plug was placed). One hundred µl of a 7-day incubated isolates was put into one hole. For the negative control distilled water was added to another hole. Finally, the plates were incubated for five days and growth inhibition area was observed (Yuliar, 2002).

G. Extraction and measurement of iturin A

One mL of the incubated isolates for each of these three samples (KB6, KC3, and A13) was transferred to a sterile eppendorf tube (1.5 mL) and acidified with 2 N HCl to adjust to pH around two. After that, the samples were kept overnight at 4^0C and centrifuged at 4000 rpm for 15 minutes. Subsequently, the samples were re-suspended and extracted with one mL methanol for about one hour at room temperature. Then, the samples were centrifuged at 4,000 rpm for 15 minutes, and the supernatant was filtered through a 0.20 µm PTFE membrane filter (Albet-JPT 020, Hahnemuhle company, Barcelona, Spain). Twenty µl of the filtrate was injected to HPLC (WATER, WATER corporation, Milford, USA) and was monitored by a UV detector at λ205 nm. Conditions of HPLC were as follows: Mobile phase; acetonitrile: ammonium acetate = 35:65, column C18, flow rate = 2 mL/minute. Iturin A detection was performed in the Chemistry Laboratory, Bogor Agricultural University.

H. Measurement of cell concentration and pH

Cell concentration was measured with optical density using a spectrophotometer (Perkin Elmer, Perkin Elmer, Cambridge, USA) at OD_{660nm}. pH was measured with a pH meter (Horiba Ltd, Kyoto, Japan).

2-4. RESULTS

A. Number of bacterial isolates

Fourteen bacterial strains were isolated from peat soils, and 33 from composts (Table 2-1). The results showed that soils of peat swamp forest contained less number of bacterial isolates than compost. Macroscopic observation showed that 11 isolates were amoeboid, 15 isolates circular, 5 isolates curled, 1 isolate filamentous, 5 isolates irregular, 5 isolates myceloid, and 5 isolates rhizoid (Fig.2-2 and Table 2-2). The colour of colonies varied from white to yellow.

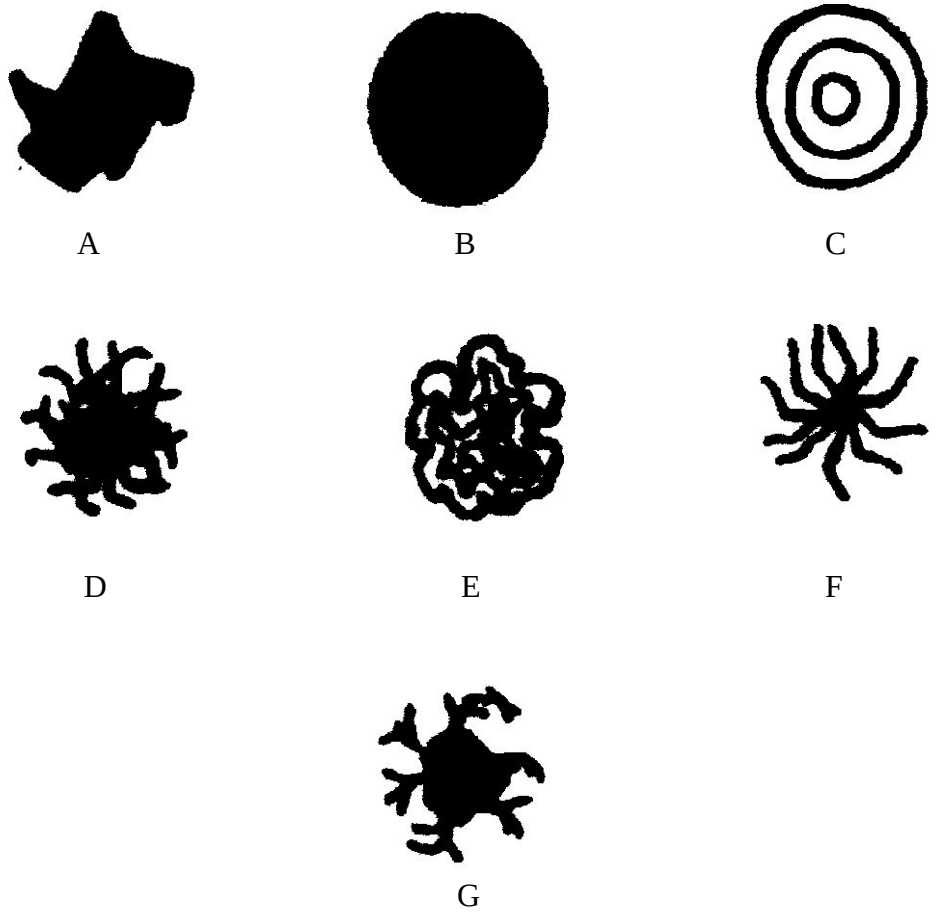


Figure 2-2. Macroscopic shape of bacterial isolates; (A) amoeboid; (B) circular; (C) curled; (D) filamentous; (E) irregular; (F) myceloid; (G) rhizoid. Source Thomson (1983)

B. Cell concentration and pH of isolates cultivation in Tryptic Soy Broth medium

Isolates A11, KB6, and KC3 entered to the end of exponential growth phase on days two and days three of incubation, and the end of stationary phase occurred on days five of incubation. pH of cultivation medium increased from seven on days three to nine on days five of incubation time (data not shown).

C. *In vitro* test

In vitro test indicated that 13 isolates (A12, A13, A14, J11, J13, J51, J52, KB2, KB4, KB6, KC2, KC3, and KC) inhibited *R. solani* growth (Fig. 3A). Their diameter of inhibition zones in mm were 16, 20, 11, 14, 17, 12, 14, 19, 12, 32, 11, 30, and 14, respectively.

D. Detection of Iturin A

Iturin A fraction is characterized by the peaks with the retention times of 4.317, 5.642, 5.892, and 9.092 minutes (Fig. 2-4). In this experiment, only four peaks of the iturin A were found, with area numbers are 699684, 267647, 365363, and 109323, respectively. HPLC analysis was performed on three samples (KB6, KC3, and A13), and their concentrations were about 3.7 ppm for KB 6 and KC3, and 3.1 ppm for A13 (Fig. 2-5, 2-6, and 2-7).

HPLC analysis showed that the extraction of KB6 culture broth had two peaks (peak 1 and 3), identified as iturin A fraction with retention time 4.350 minutes and the area of 43477 (peak 1), and iturin A fraction with the retention time 5.867 minutes and its area of 6310 (peak 3), (Fig.2-5). Based on the area of these two peaks , the production of iturin A was calculated 1.5 ppm (peak 1), and 2.2 ppm (peak3), the total was 3.7 ppm. HPLC analysis of KC3 culture broth (Fig.2-6) indicated that its had only one peak, that was peak 1. Its retention time was 4.333 minutes and the area was 107210, and iturin A production was 3.7 ppm. HPLC analysis of A13 culture broth (Fig. 2-7) had only one peak (peak 1), the retention time was 4.342 minutes and the area was 87776, and its iturin A production was 3.1 ppm.

Table 2-1. Bacterial isolates (*Bacillus* spp.) from peat soils and composts

No.	Soil samples	Samples codes	pH	Isolates codes
1	Soil of peat swamp forest	A1	6.5	A11
2				A12
3				A13
4				A14
5		E6	6.0	E61
6				E62
7		G5	5.4	G51
8				G52
9		J1	4.4	J11
10				J12
11				J13
12		J5	3.2	J51
13				J52
14				J53
15	Compost Pondok Ratna Cibedug	KA	6.8	KA1
16				KA2
17				KA3
18	Compost Microbiology,Research Center For Biology,LIPI	KB	6.8	KB1
19				KB2
20				KB3
21				KB4
22				KB5
23				KB6
24				KB7
25				KB8
26	Compost Enkasaritani Bogor	KC	6.8	KC1
27				KC2
28				KC3
29				KC4
30				KC5
31				KC6
32	Compost Depok	KD	6.8	KD1
33				KD2

34	Compost Sinar Katel Perkasa Bogor	KE	6.8	KE1
35				KE2
36				KE3
37				KE4
38				KE5
39				KE6
40	Compost Bogor (unlabelled)	KF	6.8	KF1
41				KF2
42				KF3
43				KF4
44				KF5
45				KF6
46				KF7
47				KF8

Table 2-2. Macroscopic observation of *Bacillus* spp. colonies

Bacillus spp. codes	Elevation	edge	Surface	Form	Colours
A11	low convex	undulate	irregular	amoeboid	cream
A12	low convex	lobate	irregular	amoeboid	cream
A13	raised	lobate	irregular	amoeboid	cream
A14	effuse	lobate	irregular	amoeboid	cream
E61	low convex	erose	smooth	myceloid	cream
E62	low convex	undulate	irregular	myceloid	cream
G51	convex	erose	smooth	circular	light yellow
G52	effuse	lobate	verrucoser	myceloid	white
J11	convex	undulate	undulate	curled	light cream
J12	raised	erose	smooth	myceloid	light cream
J13	effused	entire	smooth	circular	light yellow
J51	low convex	erose	smooth	circular	light cream
J52	raised	lobate	smooth	circular	cream
J53	effuse	crenate	irregular	rhizoid	light yellow
KA1	raised	undulate	smooth	circular	lightcream
KA2	effuse	lobate	smooth	amoeboid	cream
KA3	raised with concave beaded edge	erose	smooth	circular	light yellow

KB1	raised	crenate	verrucose	amoeboid	cream
KB2	convex	lacerate	undulate	rhizoid	cream
KB3	low convex	undulate	smooth	curled	light brown
KB4	low convex	entire	verrucose	irregular	white
KB5	low convex	undulate	smooth	irregular	light cream
KB6	low convex	entire	smooth	circular	light brown KB7
	low convex	undulate	verrucose	circular	cream
KB8	low convex	entire	verrucose	circular	cream
KC1	effuse	ramose	smooth	rhizoid	cream
KC2	raised	entire	smooth	circular	light cream
KC3	low convex	erose	smooth	circular	cream
KC4	low convex	crenate	smooth	irregular	cream
KC5	low convex	entire	smooth	circular	cream
KC6	effuse	lacerate	smooth	myceloid	cream
KD1	effuse	crenate	irregular	amoeboid	light crem
KD2	low convex	lobate	verrucose	rhizoid	light cream
KE1	low convex	crenate	smooth	circular	light cream
KE2	low convex	lobate	verrucose	irregular	cream
KE3	low convex	undulate	smooth	irregular	white
KE4	effuse	erose	smooth	filamentous	white
KE5	raised	entire	smooth	curled	light cream
KE6	low convex	lobate	smooth	circular	white
KF1	effuse	lobate	smooth	rhizoid	dark cream
KF2	effuse	entire	irregular	amoeboid	cream
KF3	low convex	entire	smooth	circular	white
KF4	effuse	erose	smooth	amoeboid	dark yellow
KF5	effuse	lobate	smooth	amoeboid	light cream
KF6	low convex	undulate	undulate	curled	white
KF7	low convex	smooth	smooth	amoeboid	light cream
KF8	effuse	smooth	smooth	curled	light cream

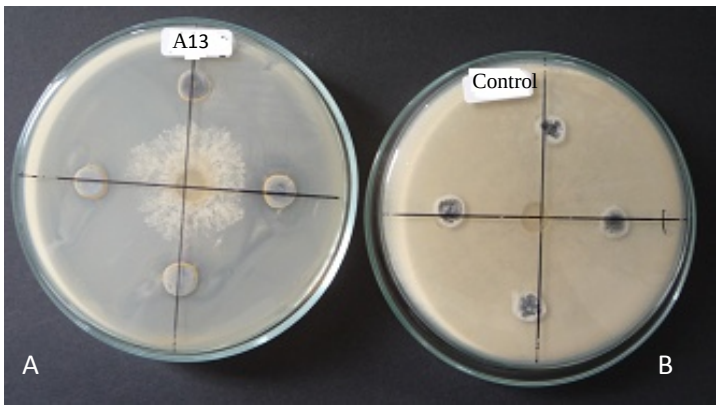


Fig.2-3.(A) Culture broth of the isolates A13 inhibited *Rhizoctonia solani* growth, its mycelia keep away from the holes. (B) Sterile distilled water as control did not inhibit *R. solani* growth, its mycelia by pass the holes.

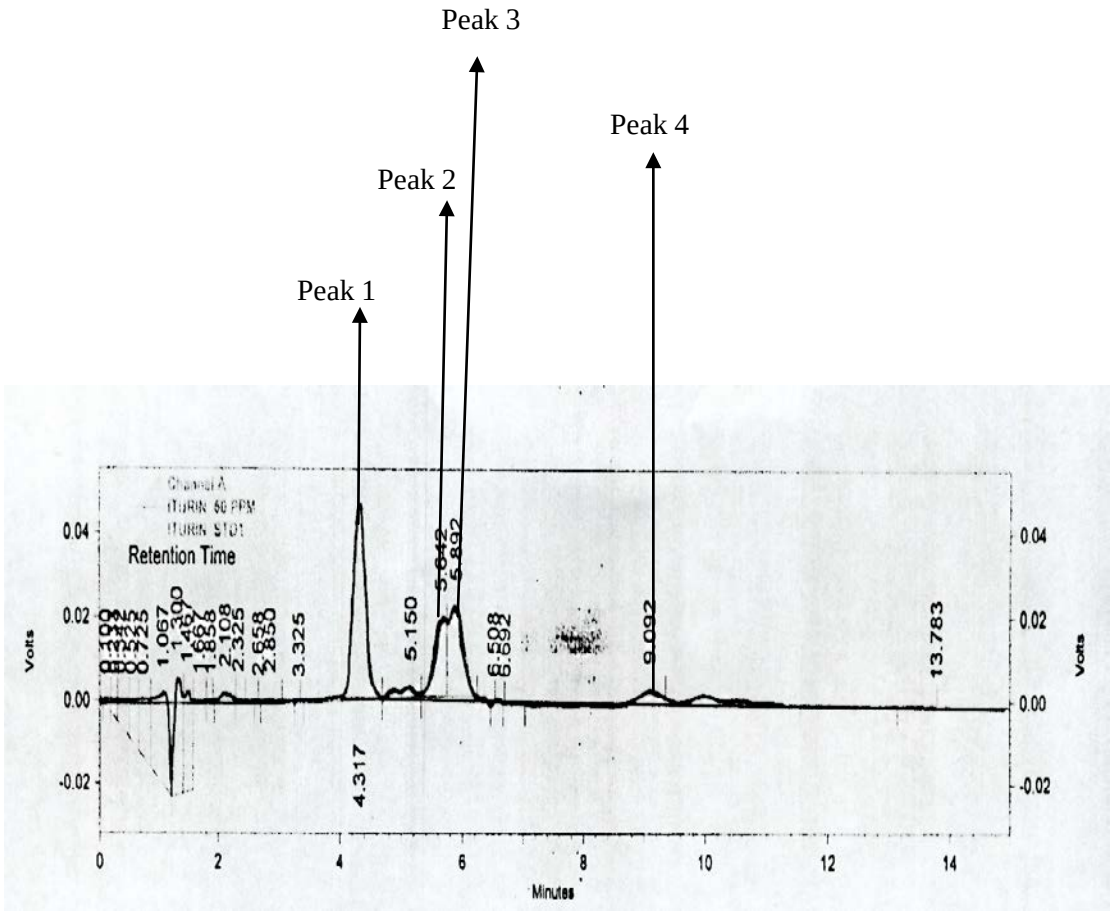


Fig. 2-4. HPLC pattern of iturin A standar (50 ppm), detector A-1 (205nm)

Peak 3

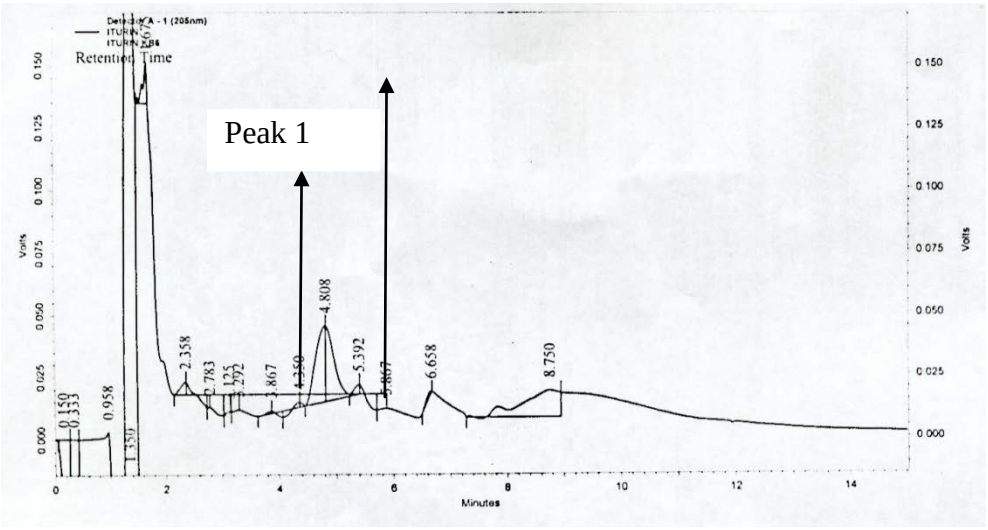


Fig. 2-5. HPLC pattern of iturin A of KB6 isolate, detector A-1(205nm)

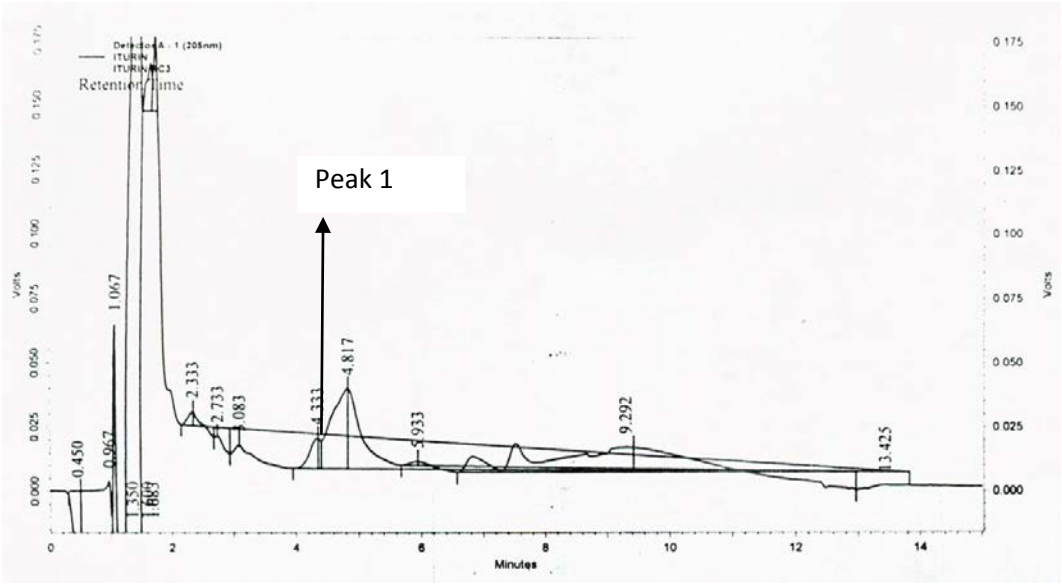


Fig.2-6.HPLC pattern of iturin A of KC3 isolate, detector A-1(205 nm)

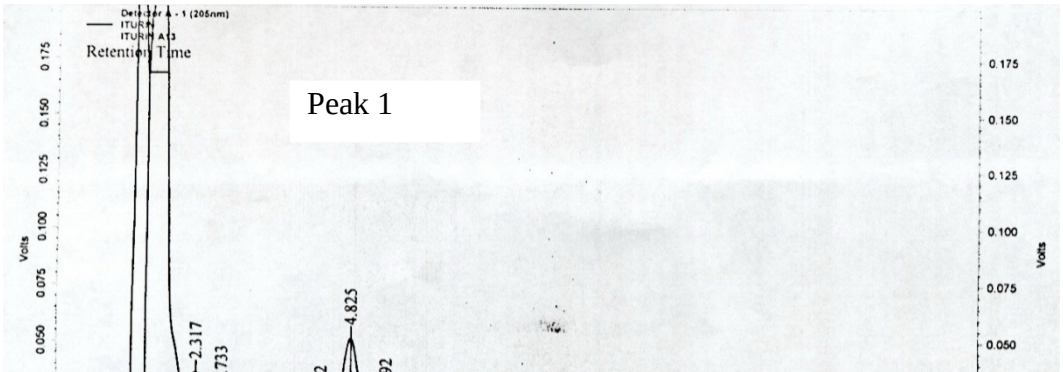
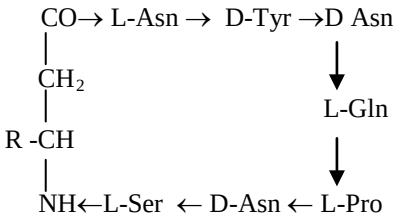


Fig. 2-7. HPLC pattern of iturin A of A13 isolate, dtector A-1(205nm)

2-5. DISCUSSION

Peat swamp forest contains less numbers of bacterial isolates than compost. It was probably because the pH of soil of peat swamp forest was lower than the compost pH. The pH range of peat soil samples were 3.2 to 6.5 and compost samples pH were about 6.8. The result was in agreement with Rosenburgh *et al*. (2013) who reported that pH of peat soils ranged 3.9-4.1 and Benito *et al*. (2006) who also mentioned that the peat soil pH was about 5.9.

The pH in the cultures of isolates A11, KB6 and KC3 increased from seven on days three to nine on days five of incubation time. The pH increased in the medium occurred because the isolates produced the secondary metabolite compounds namely iturin A (see HPLC analysis). Huang *et al.* (1993) described that the chemical structure of iturin A is a cyclic peptidolipid, that contains L-amino acids (asparagine and glutamine) (Fig.2-8). These amino acids are bases, so that the pH of medium increased.



$R=CH_3(CH2)_{10}$-,$CH_3CH_2(CH_3)CH(CH_2)_8$,$CH_3)_2(CH)(CH_2)_9CH_3(CH_2)_{12}$, $(CH_3)_2CH(CH_2)_{10}$

Fig. 2-8. Chemical structure of iturin A.

The ability of the isolates KB6, KC3, and A13 to inhibit *R. solani* was because the isolates produce an antifungal of iturin A. Iturin A fraction was detected by peaks of retention times of 4.317, 5.642, 5.892, and 9.092 minutes (Fig. 4). Yuliar (2002) reported that the iturin A fraction of *Bacillus subtilis* strain RB14-CS has the chromatogram pattern with five peaks. This is in agreement with Phae *et al*. (1990) and Yu *et al*. (2002) that detected iturin A with the same peaks pattern. In this experiment, only four peaks of the iturin A were found, with area numbers are 699684, 267647, 365363, and 109323, respectively.

Iturin A belongs to polypeptide antibiotic that is secreted by *Bacillus* spp. to the cultivation medium (Shoda, 2000; Yuliar, 2002). Phae *et al*. (1990) also reported that four isolates (*Bacillus* spp.) out of 204 isolates inhibited *R. solani* growth. Some other possible inhibition mechanisms of biocontrol agent of the plant diseases in vitro test are chitinase (Huang *et al*. 2005) and protease production (Olajuyigbe and Ajele,2005) and siderophore production (de Boer *et al*., 2003). The other possibilities to produce antibiotics are fengycin and surfactin, as reported by Ongena and Jacques (2005) and Ongena *et al*. (2008). Mechanisms of biological control agents to antagonize plant pathogens use multiple actions. For instance, *Pseudomonas* known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses (Iavicoli *et al*. 2003). Additionally, DAPG producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients (Raaijmakers and Weller, 2001). Many researchers reported a potential use of biocontrol agents as an agent stimulating plant growth (Bottini *et al*. 2004), fixing nitrogen (Khan and Doty, 2009) and solubilizing phosphate (Malboobi *et al*. 2009). These reports implied that biocontrol agents are not only important for agronomic crops but also applicative and necessary for plant forest nurseries. Furthermore the biocontrol agents A12, A13, A14, J11, J13, J51, J52 will adapt easier to the environment, i.e. plants of peat swamp forests, because of their original sources. Additionally their application results may be better than those with another biocontrol agent isolated not

from peat soil. To achieve an optimal function of them, they should be in optimal growth conditions as well as. An optimal growth can be reached when they can adapt well to their environment.

HPLC analysis was performed on three samples (KB6, KC3, and A13), and their concentrations were about 3.7 ppm for KB6 and KC3, and 3.1 ppm for A13 (Figures 2-5, 2-6, and 2-7). The iturin A production of the three isolates was lower than that by *B. subtilis* S499 (140 mg/l) (Jacques *et al*. 1999). The highest iturin A production was 3300 mg/L that was produced by *B. subtilis* RB14-CS in soybean meal medium (Yuliar, 2002). The lowest of iturin A production was observed by the isolates KB6, KC3, and A13, and it was probably because TSB was not a good medium for iturin A production. As Theobald *et al*. (2000) declared that antibiotic production is dependent on the medium composition, especially on the carbon and nitrogen sources, and the fermentation condition. Antibiotic production is also dependent on culture conditions like temperature, pH, aeration, agitation and cultivation methods. Lynch and Bushell (1995) reported that erythromycin production was significantly enhanced in a cyclic feed batch culture compared to batch culture. Batch culture is a method for cultivation of cells, usually on a large-scale, in a closed system for the purpose of producing cells or cellular products to harvest. Furthermore, microelements also influence the antibiotics production. Wei and Cu (1998) were successful in the enhancement of surfactin productivity by adding 2-4 mM iron to the medium. The supplementation of iron to the culture highly improved the production of surfactin as high as 3500 mg/L which was almost ten times of the control.

2-6. CONCLUSION

This chapter shows that seven out of 13 peat soil isolates, and six out of 33 compost isolates were antagonistic against *R. Solani* in PDA plate.Thirteen isolates (A12, A13, A14, J11, J13, J51, J52, KB2, KB4, KB6, KC2, KC3, and KC) are potential isolates to inhibit *R. solani* growth. The highest inhibition zone was observed for the isolate number KB6, with 32 mm of the clear zone diameter. The antagonistic isolates produced an antifungal iturin A in TSB medium. It also needs further research especially in greenhouse test whether the potential isolates suppress damping-off of agricultural crops and in a plantation forest.

Chapter 3. CHARACTERIZATION OF POSSIBLE BACTERIAL BIOCONTROL AGENTS, ISOLATED FROM VARIOUS PLANTS IN INDONESIA, AGAINST BACTERIAL WILT AND DAMPING-OFF OF TOMATO

3-1. ABSTRACT

One hundred bacterial strains isolated from various plants grown organically in Indonesia were assessed for their potential biocontrol ability. Phylogenic analysis based on the 16S rRNA analysis showed that Gram positive and negative bacteria were distributed in the host plants. About 43% of them belonged to *Bacillus* spp. and the other genera were *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Brevibacterium, Enterobacter, Leucobacter, Microbacterium, Paenibacillus, Pseudomonas, Serratia, and Stenotrophomonas.* The screening results showed that strains EB13, EB45, and EB53 isolated from *Brassica chinensis, Fragaria vesca*, and *Ipomea aquatica*, which were identified as *B. amyloliquefaciens, B. cereus*, and *Alcaligenes* sp., increased the survivability of tomato in bacterial wilt (BWT) significantly (P<0.05) by 67%, 83%, and 72%, respectively. Two strains, EB13 and EB45, also increased the survivability of tomato in damping-off significantly (P<0.05) by 45%, while EB53 and EB87 identified as *Enterobacter gergoviae* showed 23% and 34% disease suppression, respectively, although the differences were not significant. EB13rifkan, EB53rif and EB87rif, spontaneous antibiotics mutants of the parent strains, were confirmed to colonize tomato roots and suppress the population of *Rhizoctonia solani* in soil and root.

A seven-day culture broth of strains EB13 and EB87 and its butanol extract showed antibiosis to *R. solani* and *R. solanacearum*. HPLC analysis revealed the productions of iturin and surfactin by EB13 and an iturin-like compound by EB87. These results indicate that plant-derived bacteria not only offer potential biocontrol agents for the two tomato diseases but also provide a new source for antibiotics iturin and surfactin.

3-2. INTRODUCTION

Indonesian government launched a policy for introducing organic farming in 1984 (Pracaya, 2007). However, the practical application of this policy requires intensive and integrated studies to support organic farming. One of the important agricultural crops is tomato. Tomato (*Solanum*

lycopersicum) is highly susceptible to pathogens, and tomato crops are among those most intensively treated with agrochemicals (de Oliviera *et al*. 2010). Phytopathogens of tomatoes are include *Phytophthora infestans, Alternaria solani, Sclerotium sclerotiorum, Rhizoctonia solani, Fusarium oxysporum, Pseudomonas syringae, Ralstonia solanacearum* and *Pectobacterium carotovorum* (de Oliviera *et al*. 2010). Among these, bacterial wilt caused by *R. solanacearum* is one of the important diseases and also a major constraint in the production of eggplant and many other solanaceous crops in tropical, subtropical, and warm temperate regions of the world. This pathogen is present world-wide and has a wide host range, including several hundreds of susceptible species in at least 50 different plant families, which makes the pathogen most destructive and most difficult to control (Kelman *et al*. 1994). *R. solani* is also an important cosmopolitan necrotophic soilborne fungus. Damping-off caused by *R. solani* results in yield losses in more than 200 crops globally (Lee *et al*. 2008). Although many studies have been published on biological control agents (BCAs) isolated from various soils and composts, there is limited information on BCAs isolated from plants. Wang *et al*. (2009) reported that an endophytic bacterium of a *Bacillus* sp. strain CHM1 isolated from rice possessed antifungal activity against *R. solani* on horsebean (*Vicia faba*). Moreover, Naik *et al*. (2009) isolated from rice BCAs that inhibited the growth of *R. solani, Nigospora oryzae, Macrophomina phaseolina, Phoma sorghin*a, and *A. alternate* in *in vitro* test. An endophytic bacterium from mangrove along the coast line of Guangdong province, China, which was identified as *B. amyloliquefaciens,* possessed biocontrol ability for capsicum bacterial wilt (Hu *et al*. 2010). At this moment, no report has been published on BCAs isolated from healthy plants in Indonesia that suppress bacterial wilt and damping-off of tomato. Since Indonesian plants exhibit a high diversity, there will be chances to get novel and best performing strains from these sources.

Plants are constantly involved in the interaction with a wide range of bacteria. These plant associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissue (endophytes). Plant-associate microbes have been isolated from leaf, flower, stem, fruit, and seed of various plant species (Ferreira *et al*. 2008; Mano and Morisaki, 2008). Many authors have well documented the important roles of endophytes, such as in reducing disease severity (Sturt and Nowak, 2000; Kloepper *et al*. 2004), inducing plant defense mechanisms (Bakker *et al*. 2007), increasing plant mineral uptake (Malinowski *et al*. 2000), promoting plant growth (Kang *et al*. 2007), and biologically fixing nitrogen (Martinez *et al*.

2003).

The strategy for searching microbes for biocontrol agents is as follows:

1. Select plants growing in great biodiversities. For this end, Indonesian plants were selected as exhibiting a high diversity of the microorganisms associated with a plant (Shibuya *et al*. 2005; Agusta *et al*. 2006; Kumala and Siswanto, 2007; Susilowati *et al*. 2010).
2. Select plants surrounded by pathogen-infected plants and showing no symptoms, since they might lodge endophytic bacteria possessing natural biocontrol ability.

The objectives of this study was to isolate and characterize potential antagonistic bacteria from Indonesia plants against *R. solanacearum* and *R. solani* and to evaluate their biocontrol efficiency in tomato plants using a non sterilized soil. In addition, the mechanism of BCAs on suppression of the diseases was studied.

3-3. MATERIALS AND METHODS

Isolation of bacteria

Various healthy plants in organic farming in Bedugul in Bali island, Sukabumi, and Kepulauan Seribu in Java island, Indonesia, were collected, and kept in an ice box and brought to laboratory.

Various parts of the plants such as stems, leaves, roots, and tubers were washed in running tap water and surface-sterilized with step wise washings in 70% ethanol for 1-2 minutes and in 5% sodium hypochlorite for 2 minutes, followed by three rinses with sterile distilled water. After removing extra water on a sterilized tissue paper, the plant parts were dried for 3-4 hours in a clean bench. After drying, they were cut into pieces about 5-10 mm, and about six of plant pieces were put into ½ strength nutrient agar (NA: Eiken Chemical Co, Tokyo, Japan) supplemented with 100 μg mL^{-1} of cycloheximide. Plates were incubated at room temperature for about 10-14 days. Two to three dominant colonies that grew on the NA plates was purified by repeated transfers to new NA.

Identification of bacterial strains

Bacterial strains were cultured in 1/10 strength nutrient broth (NB: Eiken Chemical Co, Tokyo, Japan) overnight and their DNA were extracted using a conventional method (Miyashita, 1992). The primers 27f (5'AGAGTTTGATCCTGGCTCAG-3') and 1378r (5'-CGG TGT GTA CAA GGC CCG GGA-ACG-3') were used to amplify the segment of bacterial 16S rDNA from nucleotides 27 to 1378 (*Escherichia coli* numbering). Each 25 µl PCR reaction contained 1 µl DNA template plus 24 µl of amplification mixture (7.5 µl sterile distilled water, 2 µl BSA (5 mg ml^{-1}), 1 µl 27f (10 pmol μl^{-1}), 1 µl 1378r (10 pmol μl^{-1}), and 12.5 µl GoTaq (Promega KK, Tokyo, Japan)).

The thermocycling consisted of an initial denaturing step at 94^0C for 3 min, 30 amplification cycles of 94^0C for 1 min, 55^0C for 1 min, 72^0C for 3 min, and final step at 72^0C for 7 min with a GeneAmp PCR System (PCR Thermal Cycles, PERSONAL, Takara Bio Inc., Otsu, Japan). PCR products were confirmed in electrophoresis using agarose gel with 0.5 µg mL^{-1} of ethidium bromide. PCR samples were purified with Suprec™-PCR (Takara Bio Inc.) and sent for sequencing at Takara Bio Inc. The resulting 16S rDNA sequences were deposited in the database (AB714640 to AB714704) and examined using the DDBJ homology search system BLAST (http://blast.ddbj.nig.ac.jp).

The first screening of bacteria for BCAs of *Ralstonia solanacearum* YU1Rif43 (climatron experiment)

The bacterial pathogen used was *R. solanacearum* YU1Rif43, an UV- induced resistant mutant to rifampicin (Toyota and Kimura, 1996).

Five strains of bacteria were combined (2 mL overnight culture using 1/10 NB × 5 = 10 mL) and poured into a vinyl pot (9 cm in diameter and 7 cm in height) containing 70 g pumice supplemented with 1.3 g of CaCO3 for neutralization and with pathogen 1×10^4 CFU g^{-1} pumice. Three tomato seeds (*Solanum lycopersicum* Mill cv. Momotaro) pre-germinated for two days were transplanted in each pot (3 seeds/pot) by using sterile forceps. The seeds were grown for four weeks in a climatron (LPH200, Nippon Medical & Chemical instruments Co., Ltd., Osaka, Japan; day:night=12h:12h), at 28^0C. Watering was done daily using a mixture of Otsuka House No.1 (1.5 g L^{-1}) and No.2 (1.0 g L^{-1}) (Otsuka AgriTechno Co., Ltd., Tokyo, Japan). In this screening, control- (no inoculations of BCAs and pathogen) and control + (inoculation with

pathogen only) were also prepared. This experiment was performed in triplicate. After four weeks of planting, the following parameters were recorded; Percentage of wilted plants and disease index, fresh weight, tomato height, total number of bacteria in pumice using 1/10 NA, number of *R. solanacearum* YU1Rif43 in a selective medium (1 L medium consisting of nutrient broth 1.8 g, agar 15 g, rifampicin 50 μg mL^{-1}, polymyxin B 50 μg mL^{-1}, benomyl 100 μg mL^{-1}, and cycloheximide 100 μg mL^{-1}. Disease percentage and index was expressed as means of triplicate pots, each consisting of three plants.

The second screening of bacteria for BCAs of *Ralstonia solanacearum* YU1Rif43 (greenhouse experiment)

Soil was collected from the top layer (0 to 10 cm) in the experimental area of Research Center for Biology, Indonesian Institute Science, Cibinong, Regency of Bogor, West Java Province, Indonesia. Some of the characteristics of the Cibinong soil were as follows: pH (H_2O) 5.7; total C and N 22.6 and 2.1 g kg^{-1}; texture, silty clay (sand 7%, silt 50%, and clay 43%); soil taxonomy Latosol. The soil was dried and sieved through a 2-mm mesh sieve and then used for the following experiments.

Fifteen bacterial strains were preincubated in 5 mL sterilized LB medium (10 g polypepton, 5 g yeast extract, 5 g NaCl, 1 L distilled water) at 30^0C with shaking (124 rpm) overnight. Forty mL of a sterile No.3 medium (10 g polypepton, 10 g glucose, 1 g KH2PO4, 0.5 g $MgSO_4 \cdot 7H_2O$, pH 6.8, 1 L distilled water) (Asaka and Shoda, 1996) in a 200 mL Erlenmeyer flask was inoculated with each strain preincubated in LB and incubated at 30^0C with shaking (124 rpm) for 5 days. No.3 medium was used because it is known as an antibiotic producing medium (Asaka and Shoda, 1996).

YU1Rif43 was cultured overnight at room temperature (28^0C). Afterwards the bacterial cells were washed thrice with sterile distilled water and collected by centrifugation at 13,000 × *g* for 5 min. The cell precipitate was resuspended in sterile water to a final density of 10^8 CFU mL^{-1}. Two hundred grams of soil was mixed with 40 mL sterile distilled water to adjust the moisture content to 60% of maximum water holding capacity (MWHC) and with 30 mL of a 5-day culture of bacteria in No.3 medium. Then, the mixture was inoculated with

pathogen at a rate of 10^6 cfu g^{-1} soil and transferred to a plastic pot (Hammy production, Jakarta, Indonesia) 9.5 cm in diameter and 6.5 cm in height. Six tomato seeds (*Solanum lycopersicum)* variety San Marino, PT. Sang Hyang Seri, Jakarta, Indonesia) pre-germinated for 2 days were transplanted to the pot by using forceps. The seeds were grown for three weeks in a greenhouse. In this screening, control- (no inoculations of BCAs and pathogen) and control + (inoculation with pathogen only) were also prepared. This experiment was performed in triplicate. After three weeks of planting, the percentages of tomato plants survivability were recorded.

The third screening of bacteria for BCAs of *Rhizoctonia solani* (greenhouse experiment)

Two hundreds grams of dried soil was mixed with 70 mL sterile distilled water and inoculated with a 1/2 part of 3-4 days of *R. solani* mycelia grown in a Petri dish with 9 cm in diameter containing 10^{-1} PDA at room temperature, and then transferred to a plastic pot (Hammy production, Indonesia: 9.5 cm in diameter, 6.5 cm in height), and incubated for 2 days at room temperature (about 28^0C). After incubation, 30 mL of a 5 day-culture of fifteen bacterial strains in No.3 medium was added to the pot and mixed. Six tomato seeds (*Solanum lycopersicum* variety San Marino) pre-germinated for two days were transplanted to the pot using forceps. The seeds were grown for 3 weeks in a greenhouse. In this screening, control- (no inoculations of BCAs and pathogen) and control + (inoculation with pathogen only) were also prepared. This experiment was performed in triplicate. After three weeks of planting, the survivability of tomato in damping-off was recorded.

The fourth screening of bacteria for BCAs of bacterial wilt caused by *Ralstonia solanacearum* YU1Rif43 (greenhouse experiment).

The fourth screening was performed as same as the second screening, by selecting the best performing strains among those screened in the previous trial.

The fifth screening of bacteria for BCAs of damping-off caused by *Rhizoctonia solani* (greenhouse experiment, trial 1)

The fifth screening was done as same as the third screening, by selecting the best performing strains among those screened in the previous trial.

The fifth screening, trial 2

Colonization of biocontrol agents in the root and rhizosphere soil of tomato plants was monitored. For this experiment ultra violet induced antibiotic resistant mutants were obtained, by following procedures; EB13, EB45, EB53, and EB87 were incubated in 5 mL sterilized NB medium with shaking overnight. After the incubation each of the BCAs were diluted to 10^{-1} to 10^{-5} . 0.1 mL of the BCAs dilution were spreaded into 1/100 NA plate. Each of plates was opened and exposed uv for 20, 40, and 60 seconds. The plates were incubated at room temperature for about 2 days, and the growing colonies were counted. The uv exposure time was estimated for 0.5% of the survival BCAs. After the exposure time (20 seconds) was known, the next was to get antibiotics resistant mutants by subsequent method. Each BCA was incubated in 5 mL sterilized NB medium, with shaking overnight. The serial dilution of BCAs were spreaded onto 1/100 NA plates containing antibiotic streptomycin, rifampicin, and kanamycin at various levels of concentration 100, 75, 50, and 25 µg ml^{-1}. The plates were incubated at room temperature for about 2-3 days. The growing BCAs colonies were purified into a new sterilized 1/100 NA medium with the antibiotics. UV induced antibiotics resistant mutants of EB13 were found when 1/100 $NB10^{-2}$ medium containing 25 µg $mL^{-1}$of rifampicin and kanamycin. While EB53 and EB87 were resistant to 25 µg mL^{-1} of rifampicin. Uv-induced antibiotic resistant mutant of EB45 was not found by using kanamycin, rifampicin, and streptomycin. Therefore, colonization experiments were done except for this strain. The other kinds of antibiotics were suggested for obtaining this mutant strain in the future. The population of EB13rifkan, EB53rif, and EB87rif were counted in selective media containing rifampicin (rif, 25µg mL^{-1} for EB53rif and EB87rif) or rif and kanamycin (25 µg mL^{-1}) for EB13 rifkan. Number of EB13rifkan colonies were calculated by subtracting those in positive control treatment from those in the inoculated treatment. Numbers of EB53rif as well as EB87rif colonies were also calculated in the same way by subtracting those in positive control treatment from those in the inoculated treatment. Experiments were conducted in the same way as trial 1, except for the use of mutants. Population of *R. solani* was assessed using a selective medium (1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 mg $FeSO_4.7H_2O$, 90 mg Dexon, 0.2 g $NaNO_3$, 0.4 g gallic acid, 50

mg chloramphenicol, and 50 mg streptomycin sulfate, 20 g agar, and 1 liter distilled water), (Ko and Hora, 1971). Tomato plants with no symptoms were carefully harvested from the pots at 10 days and 30 days after each treatment and roots were gently shaken in air to remove all but the tightly adhering soil. Adhering rhizosphere soil from 3 pots of the same treatment (2 to 3 g pot^{-1}) was collected by gentle brushing with fine paintbrush, and then mixed together, subsequently one gram of it was put into a test tube with 9 mL of sterile distilled water. Roots after the brushing were used as rhizoplane samples after cutting into 1-2 mm pieces. In this experiment, a total amount of root in a pot was very small and therefore all the roots from three replicate pots were combined. The root macerates were made by adding 0.9 mL of 0.85% NaCl to 0.1 g of the root pieces and vigorously grinding the mixture in a sterilized mortar and pestle and 100 µl of the samples were pipetted onto a selective medium for BCAs or *R. solani,* as described above. A glass rod was used to spread the dilution sample evenly over the surface of the medium plate. Plates were incubated at room temperature for about 3-5 days, the population density of the fungi was counted (colony forming units/g soil or g root). For BCAs the plates were incubated for about 2-3 days.

Antibiosis test

One loop of *R. solanacearum* colonies was suspended into sterile distilled water and100 µl of the bacterial suspension was spread onto 10^{-1} NA plate with a sterile glass rod. Then, bacterial strains were spotted on the center of the plate and incubated at room temperature for 2-3 days. Antibacterial activity was judged by the clear zone around the colonies of endophytic strains.

R. solani was inoculated onto the center of a sterile PDA plate, three holes (diameter 9 mm) were made using a cork borer (the position of the holes were at the same distance from the center of the medium, where the *R. solani* plug was placed). Seventy µl of a 7-day culture of bacterial strains in No.3S medium (the same composition as No.3, except for polypeptonS instead of polypepton) was put into each hole. Medium No.3S was used since Tsuge *et al*., (2001) reported that this medium containing Polypeptone S enhanced iturin A production. For the negative control distilled water was used. The plates were incubated for 5 days and growth inhibition area was observed.

Measurement of iturin and surfactin production

Polypeptide antibiotics from the culture broth of BCAs were extracted by the butanol extraction method. Two mL of bacterial culture in No.3S medium was transferred to a microtube, and centrifuge at 4,500 × *g* for 5 min. No.3S medium is a medium which is able to produce more iturin and surfactin as compared with No.3 medium (Tsuge *et al*. 2001). Then 1,167 µl of supernatant was collected and transfer to a new microtube. After that, 267 µl of 1- butanol (*n*-butyl alcohol) was added to it and the mixture was mixed on a vortex for 20 sec, centrifuged at 6,000 × *g* for 5 min. After centrifugation, the upper layer was transferred to a new microtube, and 100 µl of 1-butanol was added and the mixture was mixed on a vortex for 20 sec, centrifuged at 6000 × *g* for 5 min., and the upper layer was transferred to a new microtube. The combined butanol fractions were dried up using a centrifugal concentrator (VC-15SP, TAITEC Co., Ltd., Koshigaya, Japan). After drying, 100 µl of methanol was added and filtered through a 0.2 µm PTFE membrane filter. Finally, the filtrate was subjected to HPLC (LC-10A system, Shimadzu) and was monitored by an UV detector at 205 nm (Shimadzu Corporation, Kyoto, Japan). Conditions for HPLC were as follows: mobile phase: 0.05% trifluoroacetic acid (eluent A) and acetonitrile-isopropyl alcohol (3:7[vol/vol]) plus 0.02% trifluoroacetic acid (eluent B), ODS column (Mightysil RP-18 GP 250-4.6 (5 µm), Kanto Chemical, Tokyo, Japan), column temperature was 40^{0}C.

Data analysis

Data were subjected to analysis of variance (ANOVA) with SPSS software, version 13. Significance of mean differences was determined using the Duncan's test. And responses were judged significant at 5% level.

3-4. RESULTS

Identification of bacterial strains

One hundred bacteria were isolated from various healthy plants. The closest species of the each strain were composed of the following genera, *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Bacillus, Brevibacterium, Enterobacter, Leucobacter, Microbacterium, Ochrobactrum, Paenibacillus, Providencia, Pseudomonas, Serratia*, and

Stenotrophomonas, based on the 16S tRNA genes sequences (Table 3-1). Among them, about 43% of the strains were *Bacillus* sp., followed by *Microbacterium*, *Ochrobacterium*, and *Pseudomonas* (6%) and then *Alcaligenes* (5%).

Bacillus were isolated from *Ageratum conyzoides, Ananas comosus*, *Beta vulgaris, Brassica chinensis, Calliandra calothyrsus*, *Camellia sinensis*, *Capsicum frutescens*, *Cayratia geniculata, Colocasia* sp., *Costus megalobractea, Ficus benyamina*, *Fragaria vesca*, *Gravillea robusta*, *Hibiscus rosasinensis, H. sabdariffa*, *Ipomea aquatic, I. batatas*, *Lummitzera racemosa, Solanum lycopersicon*, *Melia ozedarach*, *Morindra citrifolia, Oryza sativa, Phempis acidula, Solanum lycopersicum, S. melongena,* and *Syzygium aquem.* While *Microbacterium* which were succeded to be obtained from *Capsicum frutescens, Cyphomandra betaceae, and Melastoma* sp., and *Solanum lycopersicum* and *Ochrobactrum* were from *Agave sisalana, Allium fistulosum, Cayratia geniculata, and Melastoma* sp. *Pseudomonas* sp. that have been succesfully isolated from *Coffea* sp., *Cyphomandra betaceae,* and *Solanum lycopersicum*. The source isolates of *Alcaligenes* sp., were *Exocaria agallocha ,Glycine max, Ipomea aquatica,* and *Oryza sativa*.

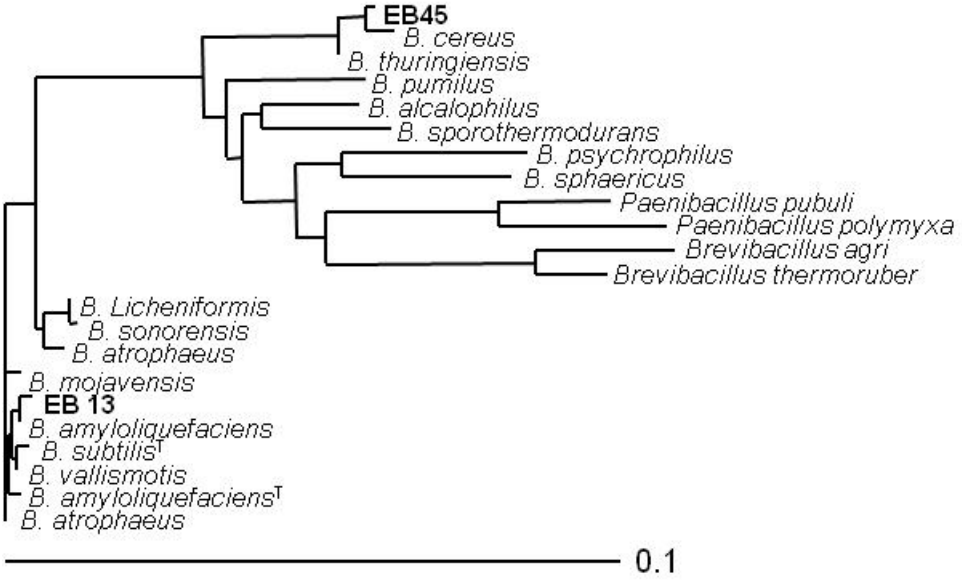
Strains EB13, 45, 53, and 87 were nearest to *Bacillus amyloliquefaciens*, *B. cereus*, *Alcaligenes faecalis*, and *Enterobacter cancerogenus*, respectively (Fig.3-1).

Table 3-1 Phylogenetic position of endophytic bacteria isolated from different plants in Indonesia

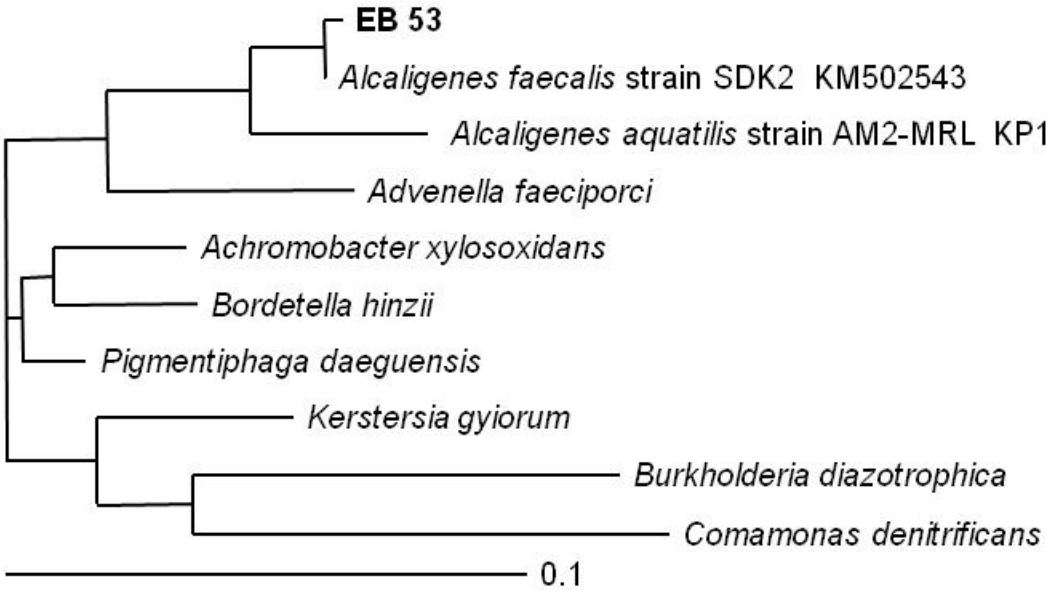
Strain (accession number)	Closest species (similarity %)	Host plants (common name)
EB01 (AB714681)	*Ochrobacterium oryzae* (100%)	*Agave sisalana* L Perr (agave, hemp-plant)
EB02 (AB714658)	*Bacillus cereus* (100%)	*Ageratum conyzoides* L (billygoat-weed)
EB03 (AB714650)	*O. intermedium* (100%)	*Allium fistulosum* L (green onion)
EB05 (AB714672)	*B. cereus* (100%)	*Ananas comosus* L (pineapple)
EB09 (AB714695)	*Paenibacillus favisporus* (99%)	*Asparagus officinalis* L (asparagus)
EB11 (AB714693)	*B. cereus* (100%)	*Beta vulgaris* L (beet)
EB12 (AB714659)	*B. thuringiensis* (100%)	*B. vulgaris* L
EB13 (AB714642)	*B. amyloliquefaciens* (100%)	*Brassica chinensis* L (Chinese cabbage, petsai)
EB15 (AB714673)	*B. pumilus* (100%)	*Calliandra calothyrsus* Meissner (red caliandra)
EB16 (AB714641)	*B. thuringiensis* (100%)	*Camellia sinensis* L Kuntze (tea plant)
EB18 (AB714645)	*B. pumilus* (100%)	*C. sinenesis* L Kuntze
EB20 (AB714694)	*B. cereus* (100%)	*Capsicum frutescens* L (chili)
EB21 (AB714696)	*Microbacterium foliorum* (99%)	*C. frutescens* L
EB22 (AB714674)	*B. cereus* (100%)	*Cayratia geniculata*

EB23 (AB714685)	*O. intermedium* (100%)	*C. geniculata*
EB24 (AB714 675)	*Brevibacterium* sp. (100%)	*Citrus grandis* L Osbeck (pomelo)
EB25 (AB714697)	*Serratia marcescens* (99%)	*Coffea* sp. (coffee plant)
EB26 (AB714686)	*Pseudomonas fluorescens* (100%)	*Coffea* sp.
EB27 (AB714699)	*B. thuringiensis* (100%)	*Colocasia* sp. (black radish)
EB28 (AB714660)	*B. thuringiensis* (100%)	*Costus megalobractea* K Schum
EB31 (AB714661)	*Agrobacterium larrymoorei* (98%)	*Cucurbita maxima* (pumpkin)
EB33 (AB714646)	*Achromobacter xylosoxidans* (100%)	*Cyphomandra betaceae* (tamarillo)
EB34 (AB714700)	*P. fulva* (99%)	*C. betaceae*
EB35 (AB714662)	*Stenotrophomonas maltophilia* (100%)	*C. betaceae*
EB36 (AB714647)	*M. paraoxydans* (100%)	*C. betaceae*
EB41 (AB714663)	*Alcaligenes* sp. (98%)	*Exocaria agallocha* (milky mangrove)
EB42 (AB714698)	*Rhodobacter sphaeroides* (98%)	*E. agallocha*
EB43 (AB714648)	*B. cereus* (100%)	*Ficus benyamina* L (weeping fig)
EB45 (AB714643)	*B. cereus* (100%)	*Fragaria vesca* L (strawberry)
EB46 (AB714676)	*B. megaterium* (100%)	*F. vesca* L
EB47 (AB714678)	*Alcaligenes* sp. (100%	*Glycine max* L Merr (soybean)
EB48 (AB714682)	*Enterobacter* sp. (100%)	*G. max* L Merr
EB49 (AB714651)	*B. cereus* (100%)	*Gravillea robusta* A Cunn (silk oak)
EB50 (AB714664)	*B. cereus* (100%)	*Hibiscus rosasinensis* (shoe flower)
EB51 (AB714652)	*B. thuringiensis* (100%)	*H. sabdariffa* L
EB52 (AB714683)	*B. thuringiensis* (100%)	*Ipomea aquatica* (water spinach)
EB53 (AB714653)	*Alcaligenes* sp. (98%)	*I. aquatica*
EB55 (AB714684)	*B. thuringiensis* (100%)	*I. batatas* L Lamk (sweet potato)
EB59 (AB714677)	*B. cereus* (100%)	*Lummitzera racemosa* (black mangrove)
EB60 (AB714640)	*P. putida* (100%)	*Solanum lycopersicum* L (tomato)
EB61 (AB714680)	*B. amyloliquefaciens* (100%)	*S. lycopersicum* L
EB62 (AB714689)	*Erwinia* sp. (100%)	*S. lycopersicum* L
EB63 (AB714665)	*Leucobacter tardus* (100%)	*S. lycopersicum* L
EB65 (AB714666)	*Brevibacterium* sp. (99%)	*Manihot esculenta* Crantz (cassava)
EB66 (AB714667)	*Microbacterium* sp. (99%)	*Melastoma* sp. (Singapore rhododendron)
EB67 (AB714688)	*O. intermedium* (100%)	*Melastoma* sp.
EB68 (AB714690)	*B. cereus* (100%)	*Melia ozedarach* L (Chinaberry, white cedar)
EB70 (AB714649)	*B. cereus* (100%)	*Morindra citrifolia* (noni, Indian mulberry)
EB74 (AB714668)	*B. pumilus* (100%)	*Oryza sativa* L (rice)
EB75 (AB714677)	*Rhodobacter sphaeroides* (100%)	*O. sativa* L
EB76 (AB714654)	*A. xylosoxidans* (100%)	*O. sativa* L
EB78 (AB714655)	*S. maltophilia* (100%)	*O. sativa* L
EB82 (AB714687)	*B. thuringiensis* (100%)	*Phempis acidula*
EB85 (AB714669)	*Acinetobacter schindler* (100%)	*Pyrrosia nummularifolia* (Swatz) Ching (creeping button fern)
EB84 (AB714701)	*Brachybacterium* sp. (100%)	*Pisum sativum* (pea)

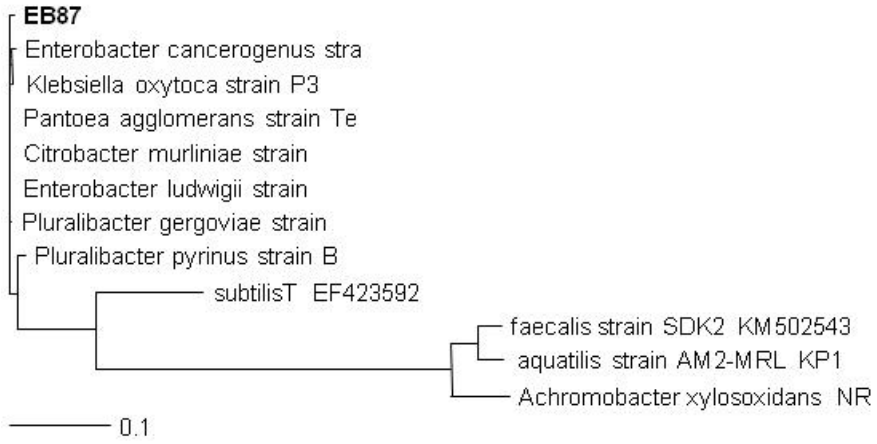
EB86 (AB714670)	*Agrobacterium* sp. (100%)	*Raphanus sativus* L (radish)
EB87 (AB714644)	*E. gergoviae* (100%)	*R. sativus*
EB91 (AB714671)	*B. cereus* (100%)	*Solanum lycopersicum* (cherry tomato)
EB93 (AB714703)	*M. testaceum* (100%)	*S. lycopersicum*
EB94 (AB714692)	Gamma proteobacterium (100%)	*S. lycopersicum*
EB95 (AB714656)	*B. cereus* (100%)	*S. melongena* (eggplant)
EB97 (AB714702)	*B. cereus* (100%)	*Syzygium aquem* (water cherry)
EB98 (AB714691)	*Providencia vermicola* (100%)	*Theobroma cacao* L
EB99 (AB714657)	*P. plecoglossicida* (100%)	*Toona sinensis* M Roem (chinese mahogany)
EB77 (AB714704)	*Providencia* sp. (100%)	*O. sativa* L



(A)



(B)



(B)

(C)

Fig.3-1. Phylogenic tree based on the 16S rDNA gene sequence analysis; (A) EB13 and EB45; (B) EB53; and (C) EB 87. Scale bare indices 10% sequences divergence

The first screening of BCAs for *Ralstonia solanacerum*

In the first screening, five each of bacterial strains were mixed together and tested for the efficacy of them against BWT. The highest BWT suppression was observed in three combinations (BCAs05, BCAs13, and BCAs18) and their suppressiveness was significantly higher than that of control positive (Table 3-2). The three combinations decreased the density of *R. solanacearum* in the culture medium significantly (P<0.05).

Table 3-2 Effect of consortia of biocontrol agents (BCAs) on bacterial wilt of tomato (BWT), densities of *Ralstonia solanacearum* and total bacteria in pumice (first screening)

Treatments	BWT (%)	*R. solanacearum* ($\times 10^3$ g^{-1})	Total bacteria ($\times 10^6$ g^{-1})
BCAs 01 (EB09,14,37,27,58)	78 a	3 def	9 defg
BCAs 02 (EB02,63,65,99,100)	67 ab	6 cde	5 hij
BCAs 03 (EB04,54,68,71,89)	67 ab	24 b	12 bcd
BCAs 04 (EB06,08,15,17,39)	44 abc	6 cde	5 hij
BCAs 05 (EB25,30,34,45,51)	22 bc	2 ef	12 bcd
BCAs 06 (EB11,31,36,46,86)	56 abc	22 b	8 efgh
BCAs 07 (EB16,32,49,64,80)	45 abc	5 cdef	7 fgh

BCAs 08 (EB03,44,48,76,83)	56 abc	2 def	6 gh
BCAs 09 (EB01,05,43,60,75)	67 ab	3 def	2 j
BCAs 10 (EB47,50,55,78,95)	100 a	4 def	2 j
BCAs 11 (EB21,38,57,67,84)	56 abc	3 def	13 bc
BCAs 12 (EB22,40,52,72,82)	89 a	7 cde	8 efgh
BCAs 13 (EB26,33,35,87,94)	22 bc	5 cdef	17 a
BCAs 14 (EB10,66,70,77,97)	44 abc	8 cd	7 fgh
BCAs 15 (EB12,19,61,88,98)	67ab	5 cdef	3 ij
BCAs 16 (EB23,42,59,73,90)	44abc	5 cdef	6 gh
BCAs 17 (EB28,62,93,92,91)	44 abc	10 c	6 gh
BCAs 18 (EB13,41,53,69,24)	22 bc	4 def	13 b
BCAs 19 (EB07,18,29,56,79)	45 abc	3 def	2 j
BCAs 20 (EB20,96,85,74,81)	78 a	7 cde	11 bcde
Control +	78 a	54 a	10 cdef
Control –	0c		18 a

Means in any column with different letters are significantly different (*P<0.05*). Control + : inoculated only with *R. solanacearum,* Control - : not inoculated with *R. solanacearum* and BCAs

The second and the third screening of bacteria for BCAs of *Ralstonia solanacearum* YU1Rif43 and *Rhizoctonia solani* (greenhouse experiment)

In the second screening, the tomato plants treated with EB33, EB53, and EB87 showed significant disease suppression effect against BWT ($P<0.05$) (Fig.3-2).

In the third screening, statistical analysis of the tomato plants treated with EB13, EB33, EB35, EB45, EB53, EB69, and EB87 showed increased disease suppression effect against damping-off of tomato for about 22 to 44% as compared to positive control. (Fig.3).

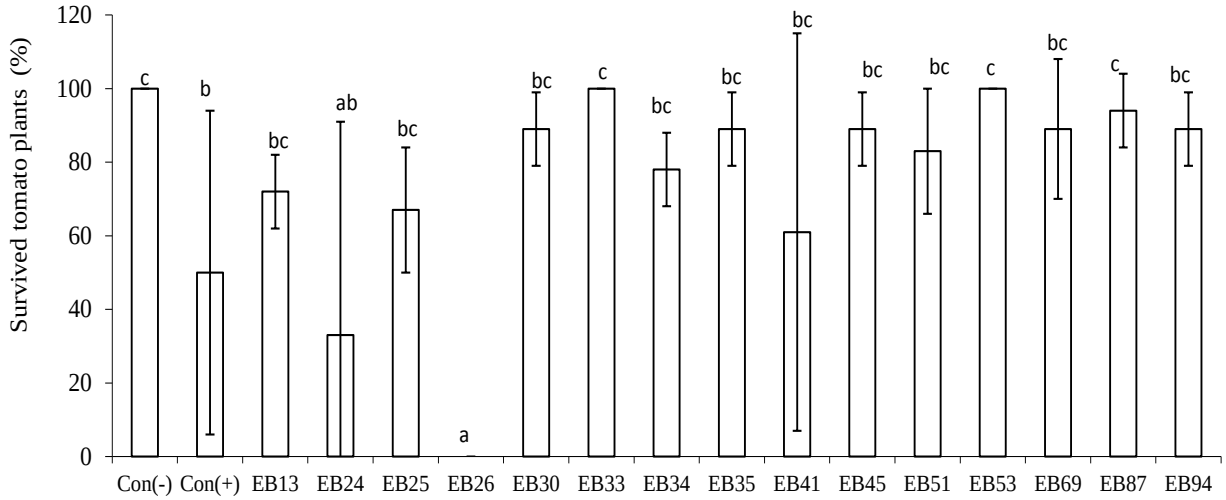


Fig. 3-2. Suppressive ability of the selected single strains to inhibit bacterial wilt of tomato in Cibinong (Indonesia) soil, caused by *Ralstonia solanacearum* YU1Rif43 (second screening). Means with different letters are significantly different (*P<0.05*).
Con(-) : not inoculated with *R. solanacearum* and BCAs, Con(+) : inoculated only with *R. solanacearum*.

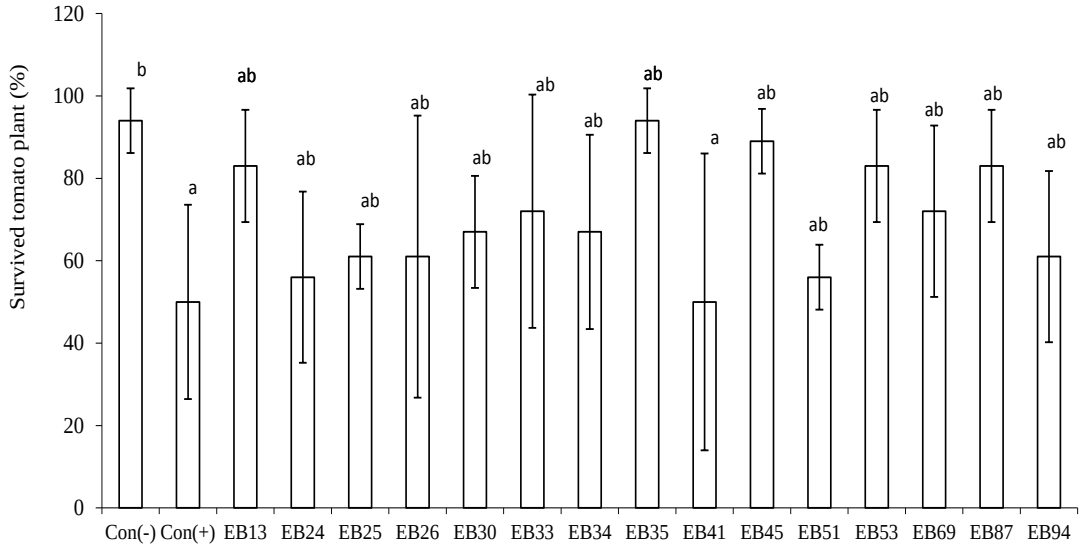


Fig. 3-3. Ability of the selected single strains to inhibit damping-off of tomato in Cibinong (Indonesia) soil, caused by *Rhizoctonia solani* (third screening)

The fourth screening of bacteria for BCAs of bacterial wilt caused by *Ralstonia solanacearum* YU1Rif43 (greenhouse experiment).

In the fourth screening, tomato plants treated with EB13, EB45, and EB53 showed the high disease suppression effect at levels of 67-83% against BWT significantly (*P*<0.05) (Fig.3-4).

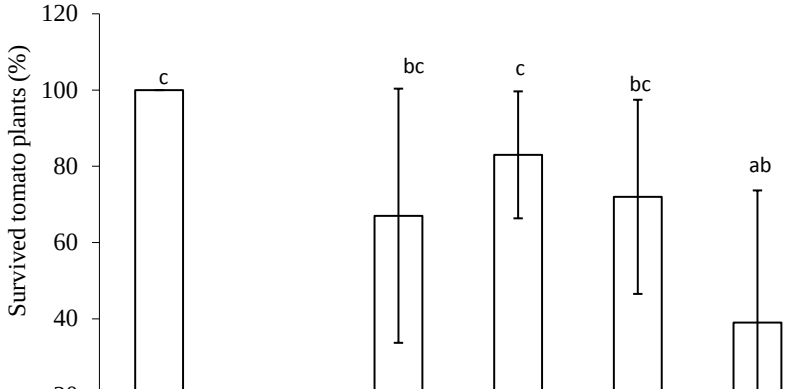


Fig.3- 4. Ability of the selected single strains to inhibit bacterial wilt of tomato in Cibinong (Indonesia) soil, caused by *Ralstonia solanacearum* YU1Rif43 (Fourth screening)

The fifth screening of bacteria for BCAs of damping-off caused by *Rhizoctonia solani* (greenhouse experiment, trial 1 and 2)

In the fifth screening, trial 1 and 2, strains EB13, EB45, EB53, and EB87 showed the increased disease suppression effect of tomato in damping-off significantly as compared to positive control (P<0.05) (Fig.3-4)

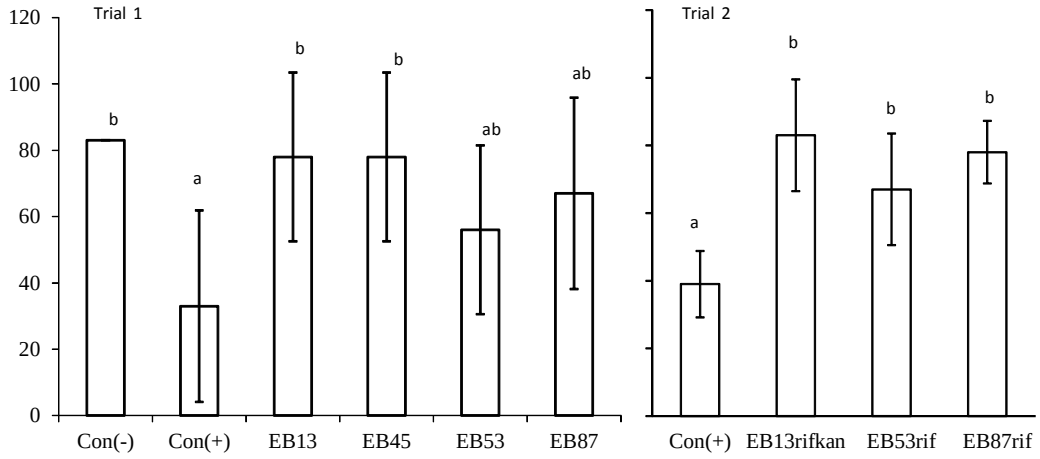


Fig.3-5. Ability of the selected bacterial strains to inhibit damping-off to tomato in Cibinong (Indonesia) soil, caused by *Rhizoctonia solani* (Fifth screening, trials 1 and 2)

Table 3-3 Colonization of biocontrol agents (BCAs) on tomato root and rhizosphere soil and their effect on *Rhizoctonia solani* population (fifth screening, trial 2)

Treatments	Population of BCA	Population of *R. solani*

	Day 10		Day 30		Day 10		Day 30	
	Root	Soil	Root	Soil	Root	Soil	Root	Soil
R. solani					15	120	140	80
R. solani + EB13	31	1	233	12	0	90	0.5	0.1
R. solani + EB53	2	0	103	36	0	0.5	0.5	0.1
R. solani + EB87	4	31	132	7	0	0.5	0	0

($\times 10^4$ CFU g^{-1} fresh root or soil)

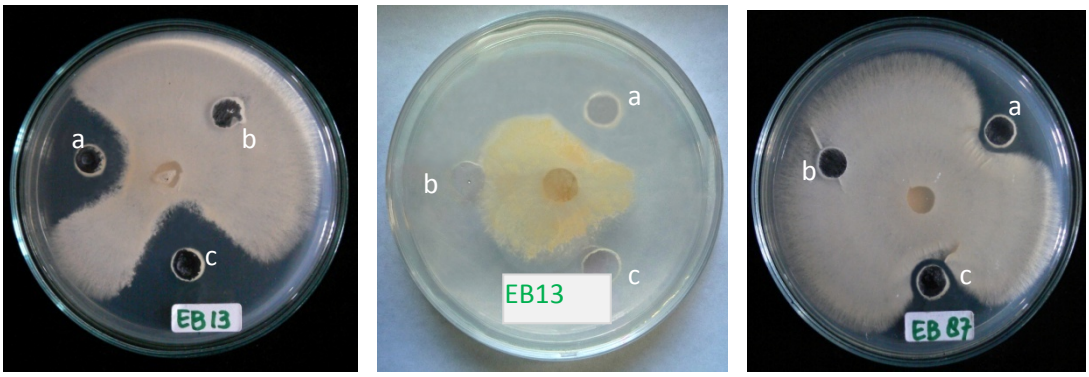
Antibiosis test

Eight strains (EB13, EB24, EB25, EB26, EB41, EB51, EB87, and EB94) produced antibacterial compounds (Table 3-4). HPLC analysis showed that two strains (EB13 and EB87) produced the bioactive compounds of lipopeptide antibiotics. EB13 produced iturin (0.56 mg mL^{-1}) and surfactin (0.40 mg mL^{-1}) and EB87 produced an iturin like compound (0.01 mg mL^{-1}) (Table 3-4). The other strains did not produce antibiotics of iturin and surfuctin.

Table 3-4 Antibiosis test of the selected bacteria, the pH of the medium 6 days after incubation, and productivity of antibiotics by HPLC analysis

Strains	Antibacterial	Antifungal	pH	Iturin (mg mL^{-1})	Surfactin
EB13	+++	+++	8.7	0.56	0.40
EB24	++	-	8.8	ND	ND
EB25	++	-	9.1	ND	ND
EB26	+	-	8.6	ND	ND
EB30	-	-	6.0	ND	ND
EB33	-	-	8.3	ND	ND
EB34	-	-	8.6	ND	ND
EB41	++	-	8.8	ND	ND
EB45	-	-	8.8	ND	ND
EB51	+	-	8.0	ND	ND
EB53	-	-	8.8	ND	ND
EB69	-	-	6.0	ND	ND
EB87	++	+	8.6	0.01	ND
EB94	+	-	5.0	ND	ND

Note: +++=high activity, ++=fair, +=low, -=no activity
Initial pH of the medium = 6.8, ND= not detected



(A) (B) (C)

Fig. 3-6. Growth inhibition o*f R.solani* by *B. amyloliquefaciens* EB13 culture broth (A), its butanol extract (B), and *E. gergoviae* EB87 culture broth (C) on PDA medium.
a and c (the holes were filled with the culture broth of the strain), b (the hole was filled with sterile distilled water).

3-5. DISCUSSION

This study is the first paper that different bacteria were isolated from healthy plants of orga nic farming in Indonesia and they showed their potential for suppressing both bacterial wilt caused by *R. solanacearum* and damping-off by *R. solani* in tomato plants. In this study we isolated one hundred possible bacterial strains from different plants, which belonged to different genera; *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*. The identification results revealed that both Gram-positive and Gram-negative bacteria were distributed in the host plants, and about 43% of them belonged to *Bacillus* spp. *Bacillus* spp. as plant-colonizing bacteria, colonized tomato roots, stems, leaves (Yan *et al*. 2003), and leaves, twigs of coffee (Nair *et al*. 2002). The reasons that *Bacillus* spp. were found in many host plants are probably because this type of bacteria forms endospore, and enable to survive under conditions of a wide range of temperature and pH (Olsson *et al*. 2003; Mendo *et al*. 2004). *Bacillus* spp. share several features that make them attractive candidates for BCAs, including their abundance in soil (Kilian *et al*. 2000), the production of various biologically active metabolites (Shoda, 2000), and the ability to form endospore (Setlow, 2005).

The highest BWT suppression was observed in the combinations BCAs 05, 13, and 18 and their suppressivenes was significantly higher than that of control positive (Table 3-2). Application of three highest suppressiveness BCAs 05, 13, and 18 increased coloni number of total bacteria in pumice. It is probably because the consortia growth in pumice, so that the

population increased, and they reduced diseases to 22%. One of the strain that belongs to the combination of BCAs 05 was identified as *Bacillus cereus* EB45. *Bacillus* spp. have several advantages over other plant growth promoting bacteria in that they posses several growth promoting traits such as production of phytohormones, secretion of antibiotics, induction of systemic resistance and their use as biopesticides (Reva *et al*. 2004; Swiecika *et al*. 2008). Two of the strains that belong to the combination of BCAs 13 were identified as *Enterobacter gergoviae* EB87 and *Stenotrophomonas maltophilia* EB35.

S. maltophilia produces various antibiotics, such as maltophilin (Berg *et al*. 1996; Jakobi *et al*. 1996), xanthobaccin A,B,C (Nakayama *et al*. 1999). Its also produces lytic enzyme such as chitinase and protease(Dunne *et al*. 2000). These the antibiotics and lytic enzyme production may also be active against *R. solanacearum.*

Two of the strains that belong to combination of BCAs 18 were identified as *B. amyloliquefaciens* EB13, and *Alcaligenes sp*. EB53.
Bacillus amyloliquefaciens SQR-7 and SQR-101 reduced bacterial wilt in tobacco (Yuan *et al*. 2014); and *B. amyloliquefaciens* reduced bacterial wilt in potato(Ding *et al*. 2013). There was no report on *Alcaligenes sp.* suppressing bacterial wilt.

Three (EB13, EB45, and EB53) of 100 strains significantly increased the survivability of tomato for BWT. EB13 (*B. amyloliquefaciens*) and EB45 (*B. cereus*) further increased the survivability of tomato for damping-off significantly at level 78 - 83%. EB 13 and EB53 strains markedly reduced *R. solani* population in the root and the rhizosphere soil by more than 200 times (Table 3-3). Although there was no significant difference, EB87 increased the survivability of tomato for damping-off. Therefore, the increase of tomato survivability might be because the BCAs colonized the tomato root and thereby suppressed the *R.solani* population in the tomato root, resulted in lower disease incidence (Table 3-3). This lower disease incidence was supported by the result that *R. solani* population in the roots was suppressed by EB13 and EB53, by more than 150 times. Previous studies also reported that the suppressive effects on the density of *R. solani* may increase the survivability of tomato plants. For example, Sumner *et al*. (1992) found that the reduction in the population of *R. solani* AG-4 might be the reason of the usefulness of a biocontrol agent *Gliocladium virens.* According to Naiki and Ui (1977), the populations of *R. solani* were markedly higher (1.43-2.5 sclerotia g^{-1}) in rhizosphere from the most severely infected roots of sugar beet plants

than those (0.04-0.12) from healthy ones. In EB13 and EB87, *in vitro* antibiosis was observed to *R. solanacearum* and *R. solani,* and iturin and surfactin were produced by EB13 and an iturin like compound by EB87 *(Enterobacter gergoviae).* Therefore, iturin and surfactin production might be responsible for suppressing damping-off of tomato and BWT through the suppression of the pathogens. Suppression of pathogen by the production of iturin and surfactin would improve plant growth, since such phenomenon have been reported by Ongena *et al*. (2005a,b), who found that *B. subtilis* S499 produced an antibiotic lipopeptide and induced an elevated state of resistance in various plants. Kloepper *et al*. (2004) also revealed that specific strains of the species of *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides,* and *B. sphaericus* elicit significant reduction in the incidence or severity of various diseases. *Enterobacter* species which have been reported as biosurfactant producers were *E. cloacae* (Sarafzadeh *et al.* 2013) and *E. asburiae* (Hoskova *et al*. 2013). By contrast, no production of iturin and surfactin was observed in EB45 and EB53, although both strains increased the survivability of tomato for BWT and EB45 did that for damping-off in a similar degree to an iturin producer EB13. This result suggests that the other mechanisms, such as competition for nutrient and space and induced systemic resistance, might work in EB45 and EB53, and remains to be solved.

Several researchers have reported the use of *B. amiloliquefaciens* for controlling multiple plant diseases caused by fungi, bacteria, and nematodes. Souto *et al*. (2004) stated that *B. amiloliquefaciens* BNM122 is a potential microbial biocontrol agent that produces surfactin and iturin-like compounds, and able to control damping-off caused by *R. solani*. Sutyak *et al*. (2007) revealed that the cell-free supernatant (CFS) of a *B. amyloliquefaciens* strain had antimicrobial activity against a wide range of bacterial species, including the pathogens *Listeria monocytogenes*,

Gardnerella vaginalis and *Streptococcus agalactiae*. Tan *et al*. (2013) reported that *B. amyloquefaciens* strain CM-2 and T-5 reduced bacterial wilt of tomato by 70.1 and 79.4%, respectively. Bacillomycin, iturin, fengycin, surfactin, unknown pepetide, bacillaene, chlorotetaine, difficidin, and macrolactin have been reported as bioactive compounds produced by *B. amyloliquefaciens* (Arquelles-Aries *et al*. 2009; Chen *et al*. 2009; Alvarez *et al*. 2011). Ability of *B. amyloliquefaciens* to produce multiple antibiotics confers this bacterium potential as a microbial biocontrol agent. This study showed that *B. amyloliquefaciens* EB13 produced

iturin and surfactin. The biocontrol activity of EB13 might be enhanced by the combination of iturin and surfactin, since Hiraoka *et al*. (1992) reported that the antifungal activity of iturin A is synergistically enhanced by the presence of surfactin.

In plant disease control by *B. cereus* UW 85, two antibiotics were identified that might be responsible for the suppression of damping-off of alfalfa caused by *Phytophthora medicaginis*: zwittermicin A, an aminopolyol group and an antibiotic B, an aminoglycoside containing a disaccharide (Silo-suh *et al.*1994). El-Hamshary *et al*. (2008) stated that *B. cereus* inhibited *F. oxysporum* growth in King's B medium. Romeiro *et al*. (2010) reported that the supernatant from a culture of a *B. cereus* strain suppressed the pathogens *P. syringae* pv. tomato, *Xanthomonas vesicatoria*, *Alternalia solani*, and *Corynespora cassiicola*. These results strongly suggest that *B. cereus* strains produce antimicrobial compounds. However, *B. cereus* EB45 showed no antibiosis to *R. solanacearum* and *R. solani* in this study, although disease suppression was observed. This result suggests that the other mechanisms, such as competition for nutrient and space and induced systemic resistance, might work in EB45 and EB53, and remains to be solved.

3-6. CONCLUSIONS

Conclusions to the chapter 3 are as follow;

1.Three strains (*B. amyloliquefaciens* EB13, *B. cereus* EB45, and *Alcaligenes* sp. EB53) out of one hundred bacterial strains isolated from various plants in Indonesia were potential biological control agents that increased survivability of tomato for bacterial wilt caused by *R. solanacearum*.

2. Four strains (*B. amyloliquefaciens* EB13, *B. cereus* EB45, *Alcaligenes* sp. EB53, and *E. Gergoviae* EB87) isolated from various plants in Indonesia were potential biological control agents that increased survivability of tomato for damping- off caused by *R. solani.*

3. *B. amyloliquefaciens* EB13 and *E. gergoviae* EB87 showed *in vitro* antibiosis, while two other bacterial strains, *B. cereus* EB45 and *Alcaligenes* sp. EB53 did not. This result suggests that the other mechanisms, such as competition for nutrient and space and induced systemic resistance, might work in EB45 and EB53, and remains to be solved.

Chapter 4. CONCLUSIONS

4-1. CONCLUSIONS TO THE THESIS

1. Seven out of 13 peat soil isolates, and six out of 33 compost isolates were antagonistic against *R. solani* in PDA plate.Thirteen isolates (A12, A13, A14, J11, J13, J51, J52, KB2, KB4, KB6, KC2, KC3, and KC) are potential isolates to inhibit *R. solani* growth. The highest inhibition zone was observed for the isolate number KB6, with 32 mm of the clear zone diameter. The antagonistic isolates produced an antifungal iturin A in TSB medium. It also needs further research especially in greenhouse test whether the potential isolates suppress damping-off of agricultural crops and in a plantation forest.
2. Three strains (*B. amyloliquefaciens* EB13, *B. cereus* EB45, and *Alcaligenes* sp. EB53) out of one hundred bacterial strains isolated from various plants in Indonesia were potential biological control agents that increased survivability of tomato for bacterial wilt caused by *R. solanacearum*.
3. Four strains (*B. amyloliquefaciens* EB13, *B. cereus* EB45, *Alcaligenes* sp. EB53, and *E. gergoviae* EB87) isolated from various plants in Indonesia were potential biological control agents that increased survivability of tomato for damping- off caused by *R. solani.*
4. *B. amyloliquefaciens* EB13 and *E. gergoviae* EB87 showed *in vitro* antibiosis, while two other bacterial strains, *B. cereus* EB45 and *Alcaligenes* sp. EB53 did not. This result suggests that the other mechanisms, such as competition for nutrient and space and induced systemic resistance, might work in EB45 and EB53, and remains to be solved.

4-2. PROSPECT FOR FUTURE RESEARCH

Biological control of plant pathogens continues to inspire research and development in crop protection.This study found four species bacterial, *B. amyloliquefaciens* EB13, *B. cereus* EB45, *Alcaligenes* sp. EB53, and *Enterobacter gergoviae* EB87 isolated from *Brassica chinensis*, *Fragaria vesca*, *Ipomea aquatica*, and *Rhapanus sativus* , respectively, grown organically in Indonesia. The bacteria showed they suppressed bacterial wilt and damping-off of tomato. *B. amyloliquefaciens* EB13 , and *Enterobacter gergoviae* EB87 produce iturin and surfactin, and an iturin-like compound, respectively.

To my knowledge, there are reports, *E. gergoviae* is neither as BCAs nor produces an iturin nor other polypeptides antibiotics. To study further about the kinds of antibiotics that are produced by *E. gergoviae* and the possibility of the antibiotic to control others plant diseases not only BWT and DOT, and to enhance its antibiotic production both in traditional and by genetic engineering approach, has good future prospect.

Bacillus amyloliquefaciens EB13, *Alcaligenes* sp. EB53, and *E. gergoviae* EB87 showed they colonized tomato root, and rhizospere in greenhouse test, to study its BCAs colonizatio in rhizoplant and rhizospere in field experiment is also exciting and challenging research. To enhance the BCAs suppression to the bacterial wilt and the damping-off diseases, it need further study, such as use of mixtures of BCAs (bacteria, fungi, and actinomycetes) and integration method BCAs combined with fungicide or organic matter (animal manure or green manure). This research works seem interesting in sustainable agricultural sector, based on previous studies (studied by other researchers), combination of BCAs with fungicide, can reduce the concentration of fungicide used, at the same level of disease index result.

Besides research on wider application as well as interesting, such as the BCAs formulation using a local agricultural byproduct (straw, sawdust, powdered rice bran). Simple and cheap formulation of BCAs is important for their wider application. Anothers promising way for wider application of BCAs are by introduction of foreign genes (such as chitinase gene, and Bt gene) to BCAs.

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Appendix

1. Iturin A concentration in chapter 2

Peaks No.	Iturin A standard (50 ppm)		KB6		KC3		A13	
	Retention time (min.)	Area	Retention time (min.)	Area	Retention time (min.)	Area	Retention time (min.)	Area
1	4.317	699684	4.35	43477	4.333	107210	4.342	87776
2	5.642	267674	-	-	-	-	-	-
3	5.892	365363	5.867	63210	-	-	-	-
4	9.092	109323	-	-	-	-	-	-
Total		1442044						

Concentration of iturin A for each peak;

1. Standard of iturin A;

   Peak1: $\frac{699684}{1442044}$ x 50 ppm= 24.26 ppm

   Peak2: $\frac{267674}{1442044}$ x 50 ppm= 9.28 ppm

   Peak3: $\frac{365363}{1442044}$ x 50 ppm= 12.67 ppm

   Peak4: $\frac{109323}{1442044}$ x 50 ppm= 3.79 ppm

   Total of iturin A concentration (peak1+peak2+peak3+peak4)=24.26 ppm+9.28 ppm+ 12.67 ppm+ 3.79 ppm= 50 ppm

2. Isolate KB6;

   Peak1: $\frac{43477}{699684}$ x 24.26 ppm= 1.5 ppm

Peak3: 63210 x 12.67 ppm= 2.2 ppm
365363
Total of iturin A concentration (peak1+peak3)= 1.5 ppm + 1.2 ppm= 3.7 ppm

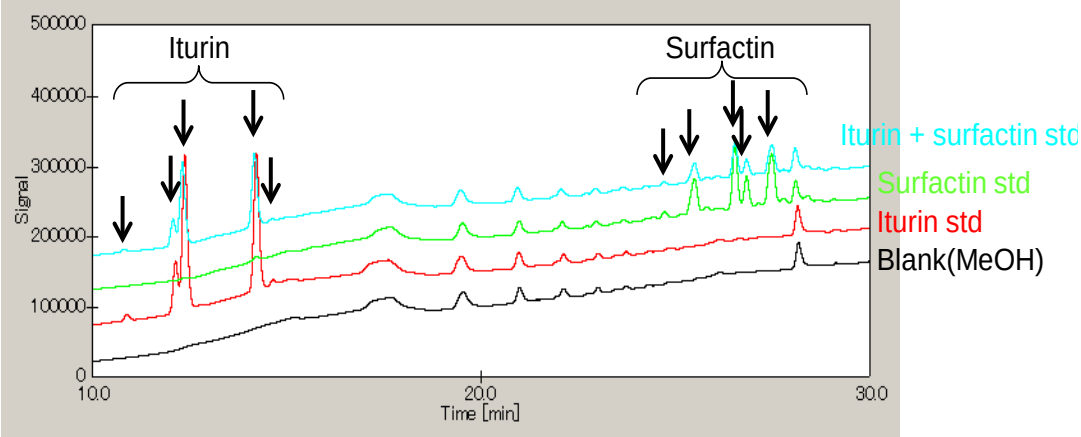
3. Isolate KC3;

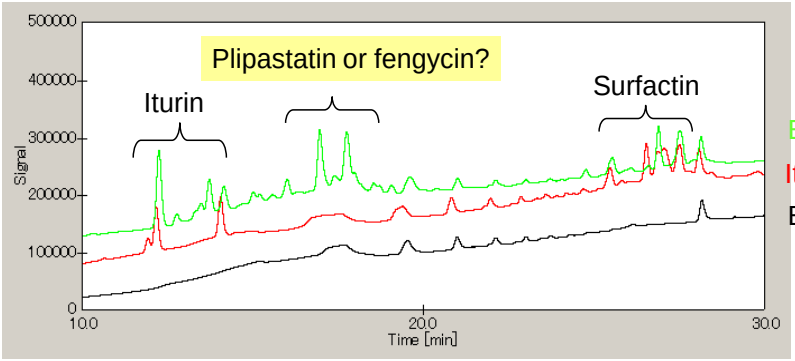
Peak1: 107210 x 24.26 ppm= 3.7 ppm
699684
Total of iturin A concentration= 3.7 ppm

4. Isolate A13;
Peak1: 87776 x 24.26 ppm= 3.1 ppm
699684

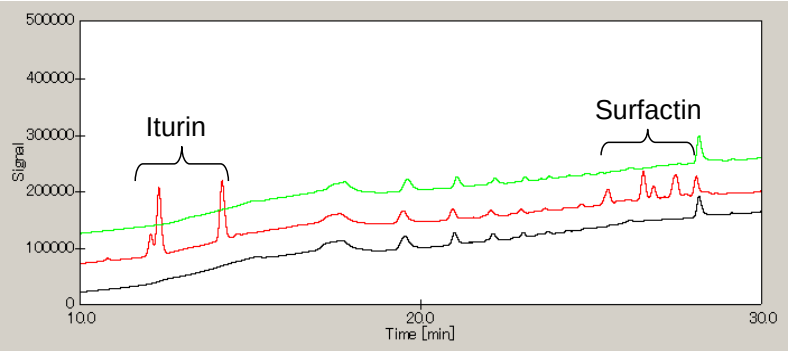
2. HPLC analysis for the culture broth extraction of strain EB13, 24, 25, 26, 33, 34, 41, 45, 51, 53, and 87 in chapter 3

Arrows showed the candidate of lipopeptides production by the strains culture broth. The peaks were found by subtraction of the peaks of blanks (MeOH) from samples.


Iturin
Surfactin
Iturin + surfactin std
Surfactin std
Iturin std
Blank(MeOH)
Signal
500000
400000
300000
200000
100000
0
10.0
20.0
30.0
Time [min]

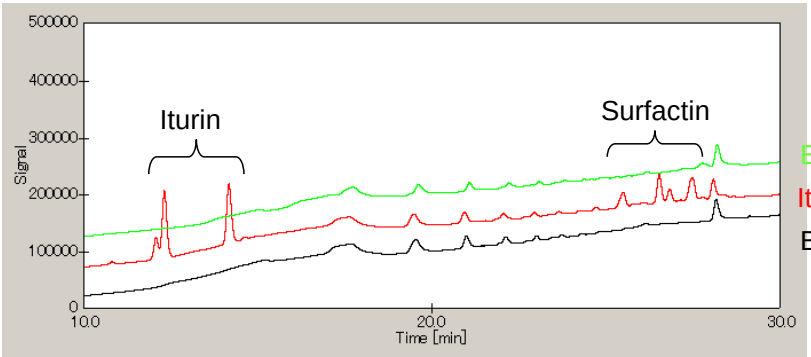


EB13	
Iturin	+
Surfactin	+
Other(s)	+



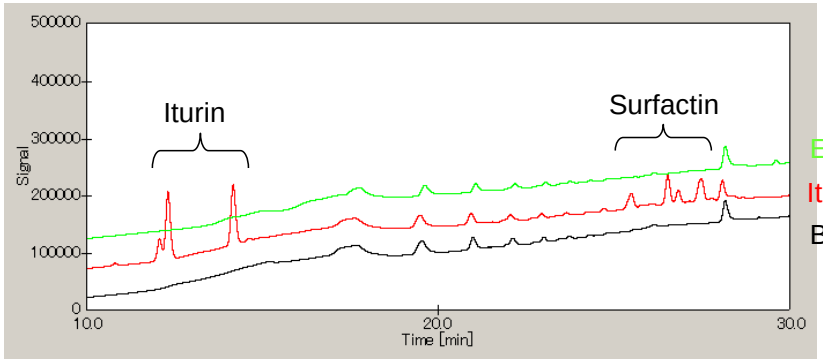
EB24
Iturin + surfactin std
Blank (MeOH)

EB24
Iturin -
Surfactin -
Other(s) -

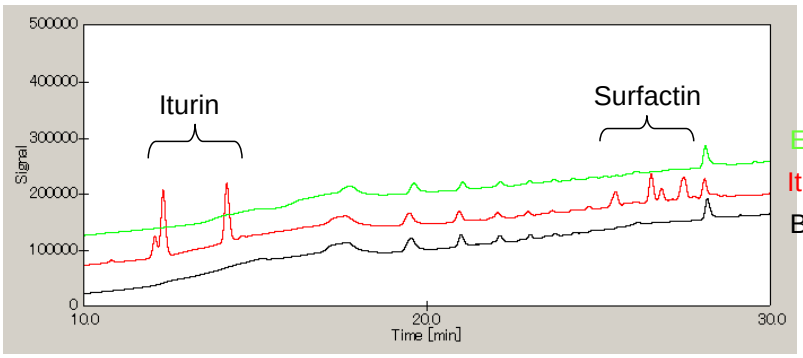


EB25
Iturin + surfactin std
Blank (MeOH)

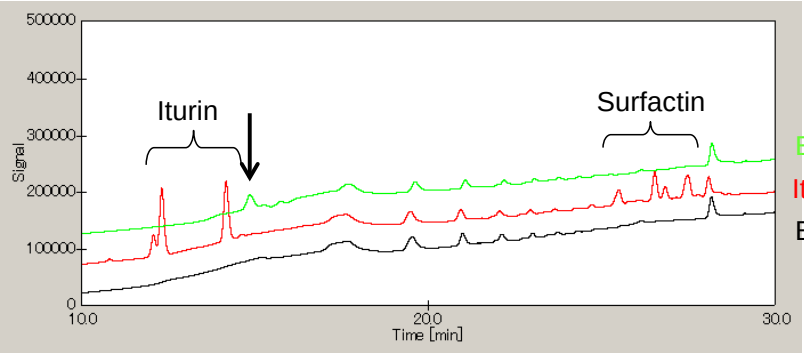
EB25
Iturin -
Surfactin -
Other(s) -



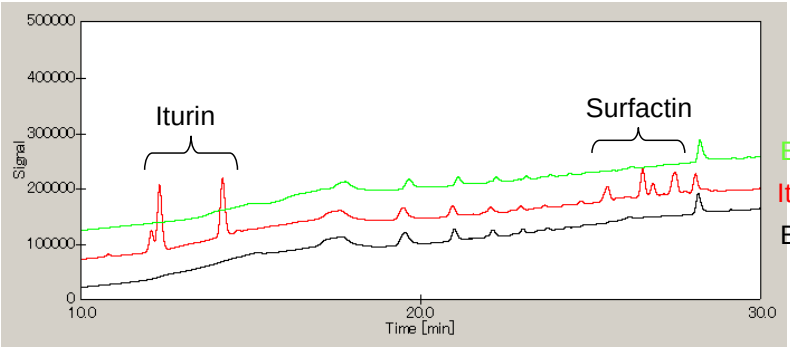
EB26
Iturin -
Surfactin -
Other(s) -



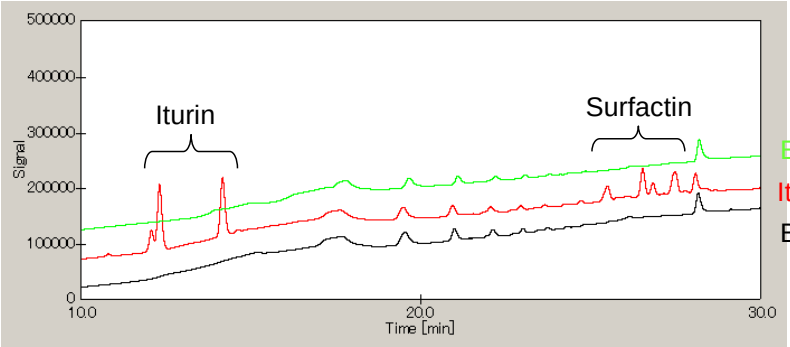
EB33
Iturin -
Surfactin -
Other(s) -



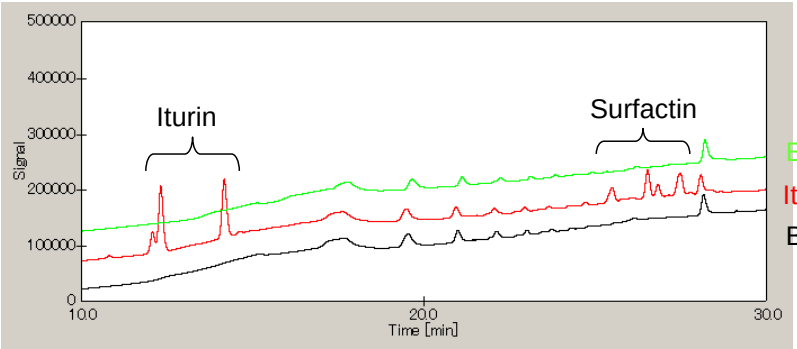
EB34	
Iturin	-
Surfactin	-
Other(s)	+?



EB41
Iturin -
Surfactin -
Other(s) -

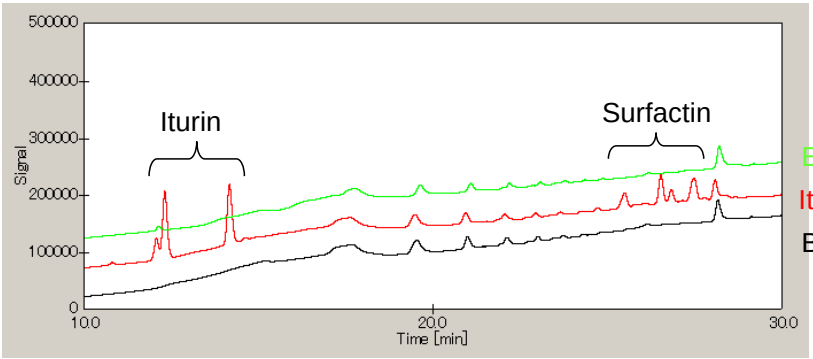


EB45
Iturin -
Surfactin -
Other(s) -



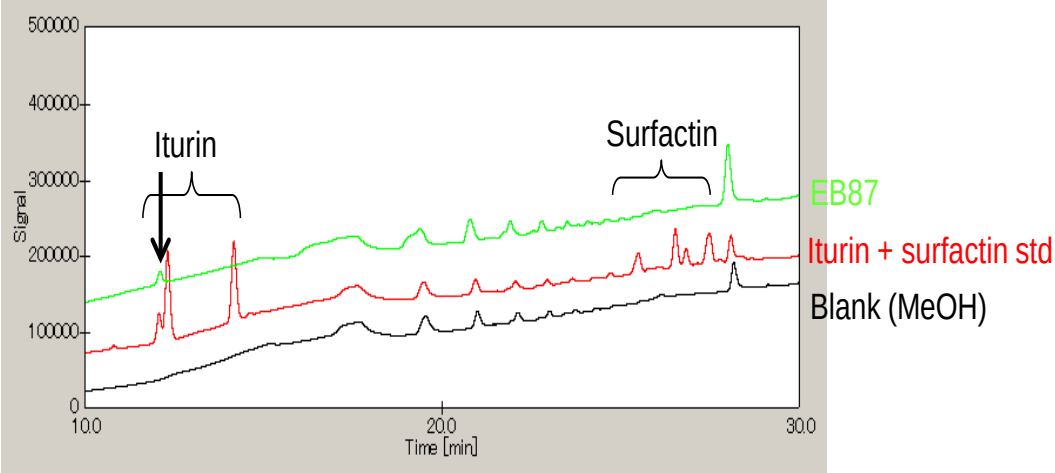
EB51

Iturin	-
Surfactin	-
Other(s)	-



EB53

Iturin	-
Surfactin	-
Other(s)	-



EB87	
Iturin	+
Surfactin	-
Other(s)	-

3. Photos at the first screening of consortia BCAS for bacterial wilt of tomato

(A)

(B)

(C)

(D)

Photo1:The first screening of consortia BCAs for *Ralstonia solanacearum*(climatron experiment), (A);BCAs 1-10,(B); BCAs11-15, and (C) BCAs16-20. Far left and right in each photo A-C is positive and negative control; (D); Thre pots on the left are positive control and on three pots on the right are negative control.

4. Photos at the second screening of the selected single strains to suppress bacterial wilt of tomato

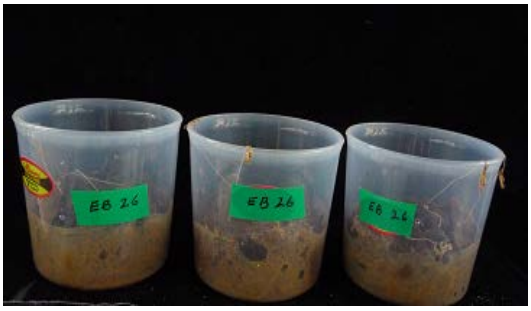
Control (+)

Control (-)

EB13

EB24

EB25



EB26

EB30

EB33

EB34

EB35

EB41

EB45

EB51

EB53

EB69

EB87

EB94

Photo 2. The second screening of the selected single strains (EB13, 24, 25, 26, 30, 33, 34, 35, 41, 45, 51, 53, 69, 87, and 94) to inhibit bacterial wilt of tomato in Cibinong (Indonesia) soil caused by *R.solanacearum* YU1Rif43

5. Photo at the perform of tomato by application of EB13rifkan, EB53rif, and EB87rif for suppression of tomato damping-off.

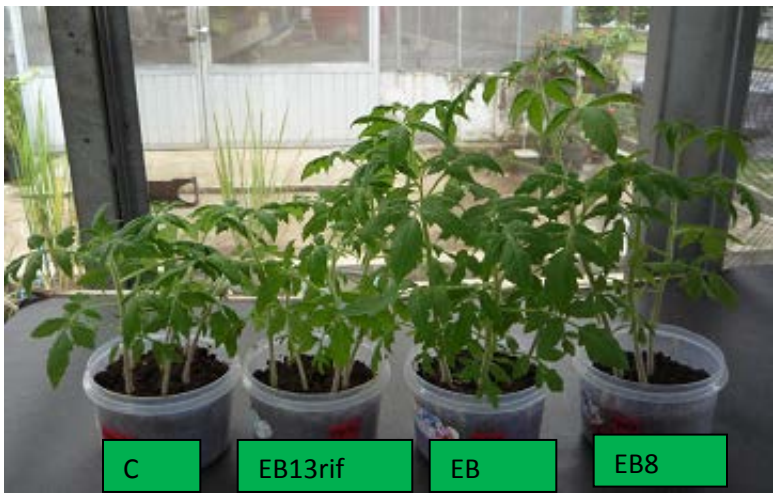


Photo 3. Monitoring colonization of EB13rifkan, EB53rif, and EB87rif in the root and rhizosphere soil of tomato against *Rhizoctonia solani* population.