

## An Ability of Endophytes from Blackboard Tree (*Alstonia scholaris*) in Increasing Chili Performance

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**Endophytes are a group of bacteria live in plant tissues, and have been numerously reported to promote plant growth. The aim of this study was to observe bacterial endophytes from blackboard tree (*Alstonia scholaris*) in inhibiting pathogenic fungal growth on chili plants, promoting plant growth and producing indole acetic acid, as well as in solubilizing phosphate. Sixteen endophytic bacteria were isolated from blackboard tree root and leaf samples, in which two, A1 and A3, out of sixteen showed to highly inhibit pathogenic fungi *Fusarium* sp. and *Colletrotichum* sp., to produce indole acetic acid, and to solubilize phosphate. 16S rRNA gene sequences showed that A1 was relatively closed to *Carnobacterium gallinarum* strain DSM4847 and A3 was relatively closed to *Carnobacterium maltaromaticum* strain JCM 1154 with similarity of 84 and 78%, respectively.**

**Keywords:** *Alstonia scholaris*, blackboard tree, chili, endophytes, indole acetic acid, phosphate.

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Modern agriculture are heavily dependent on chemical compounds such as fertilizers and pesticides that may cause environmental stress, reducing soil fertility, and even causing environmental instability due to the change in energy flow in the ecosystem (Amilia *et al.*, 2016). Pesticides residue on agricultural products can also decrease product quality that causes the selling price of these products to decrease (Supardi, 1994). Thus, the urge for precise, cheap, safe, and innovative control is now in demand. To address this issue, utilization of endophytic microorganisms producing phytohormones such as indole acetic acid (IAA) that can stimulate plant growth, phosphate solubilizing microorganisms, as well as the use of microorganisms to control plant pests and diseases. In general, the role of microbes

is to increase the availability of nutrients in the soil, decomposition and mineralization of organic matter in the soil, rhizosphere-endophytic bacteria promoting plant growth by forming rhizosphere – endophytes symbiosis and protect plant roots from pathogenic microbes, and plays a role as a natural pest and disease control agents. Various chemical reactions in the soil also occur with the help of soil microbes.

Microbes enter plant tissue in various ways, such as through injuries to plant tissue, leaf stomata, or through the pores of the roots. The ability of bacteria to penetrate the plant's internal tissue can be due to the extracellular enzyme produced by endophytic bacteria (Saraswati *et al.*, 2004). Endophytic bacteria can produce bioactive compounds whose character is similar to the compounds produced by their host plants (Eliza *et al.*, 2007). A number of endophytic microbes are known to significantly produce phytohormones, especially IAA (Tan and Zou, 2001). Host plants

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infected with endophytes have many advantages, such as accelerated growth and increased resistance to drought and pest attacks. The role of host plants is to provide the nutritional needs for endophytic microbes. The condition of host plants, soil conditions i.e. temperature and humidity can greatly affect the number and type of endophytic microbes. The presence of endophytic microbes in plant tissue can also help plants to compete in the nature.

Blackboard tree (*Alstonia* sp) is categorized as a fast growing plant native to Indonesia (Pamungkas *et al.*, 2006). The genera *Alstonia* itself belongs to the family Apocynaceae, similar to *Rauvolfia canescens*, *Rauvolfia serpentina*, *Alstonia boonei*, and *Vinca rosea*, which had been commercially produced as herbal medicine in China and India (Pratyush *et al.*, 2011). A study on endophytic bacteria isolated from blackboard tree leaf (*Alstonia scolaris*) has previously been conducted. This study showed that the endophytic bacteria had a potential to produce antibacterial compounds against *Escherichia coli* and *Staphylococcus aureus* (Fatiqin, 2009). However, no study was carried out in their ability to produce indol acetic acid (IAA), to solubilize phosphate, and to inhibit of pathogenic fungi in chili.

## MATERIALS AND METHODS

### Isolation of Endophytic Bacteria from Blackboard Tree

Endophytic bacterial isolation from roots and leaves of blackboard tree was performed as described by Radu *et al.* (2002). Samples were immediately washed with water to remove dirt, then dried and wrapped in newspaper and put into a plastic bag containing ice blocks (Zinniel *et al.*, 2002). Samples were then brought to the laboratory for isolation. Samples were initially washed with running water. Sterilization of the root surface area was done by immersing the following solution successively: 75% ethanol for 5 minutes, 5.3% sodium hypochlorite solution for 5 minutes, and 75% ethanol for 30 seconds. Subsequently, the roots of the plant were rinsed with sterile distilled water then dried with sterile filter paper. Afterwards, the root part is cut to 1-1.5 cm, and each piece of root was placed on NA medium mixed

with antifungal ketoconazole (0.3 gram/100 ml) incubated for  $\pm$  5 days. Similar procedure was done for leaf samples. The colonies were subcultured into a new NA medium to be purified.

### Characterization of Morphology and Biochemistry of Bacterial Endophytes

Bacterial isolates were morphologically characterized by observing their colony shape, color, elevation and edge. Gram staining was performed to observe their cell shape, arrangement and bacterial gram reaction. Biochemical tests including starch hydrolysis, gelatin hydrolysis, citrate utilization test, hydrogen sulfide test, and motility test were conducted to observe their biochemical activity.

### Antagonistic Assay of Endophytic Bacteria against Pathogenic Fungi

Inhibition of endophytic bacterial against pathogenic fungi was carried out in vitro. Pathogenic fungi used in this study were *Colletotrichum* sp. and *Fusarium* sp. Active growing mycellia of the pathogenic fungus was taken using cork borer, and subsequently inoculated in the middle of Mueller's Hinton Agar medium. Endophytic bacteria were carefully inoculated using a sterile toothpick 3 cm away from the pathogenic fungal cultures, Cultures were incubated for 3 days.

### Growth of Endophytic Bacterial Isolates

Endophytic bacteria cultures previously grown on nutrient agar (NA) were inoculated into 5 ml of sterile physiological saline solution. Bacterial suspension of 5% was introduced into 50 ml of sterile Luria Bertani (LB) containing tryptophan, and spectrophotometry measurement was done at 600 nm wavelength. The initial absorbance of LB + tryptophan was used as blank, and initial measurement of LB containing bacteria was conducted aseptically. Culture was incubated on orbital shaker at 27°C of 100 rpm. The absorbance of the culture was measured every 4 hours to know the bacterial growth until it reached its stationary phase.

### Solubilizing Phosphate Assay of Endophytic Bacteria

Endophytic bacterial isolates were assayed for their ability in solubilizing phosphate. This was done by inoculating the bacterial isolate on Pikovskaya agar aseptically using sterile toothpick. Culture was incubated for 7 days. Phosphate Solubilization Index (PSI) was determined by

comparing the diameter of clear zone formed in the agar against the diameter of the growing bacterial colony.

Phosphate Solubilization Index (PSI) = diameter of clear zone/diameter of colony

#### **Assay of Endophytic Bacterial Isolates in Producing IAA**

An assay on endophytic bacterial isolate ability to produce IAA was carried out as described by Dewi *et al.* (2016). Bacterial isolates were subcultured on NA for 24 hours. A 5 ml of culture suspension was adjusted to 0.5 McFarland standard ( $H^{10^8}$  cell/ml). Suspension of 5% was inoculated into LB broth + tryptophan and then incubated in orbital shaker at 27°C of 100 rpm. Measurement of IAA was done every 4 hours using centrifugation method at 5000 rpm for 10 minutes. Analysis of IAA production was determined using spectrophotometric method. A 0.5 ml of Salkowsky reagent was dropped into 2 ml of supernatant at a ratio of 4:1. Solution was incubated at ambient temperature for 15 minutes. Its absorbance was measured using spectrophotometer at 530 nm. Measurement of IAA curve was terminated after the bacterial culture reached stationary phase.

#### **Application of Potential Endophytic Bacteria on Red Chili Seeds**

Suspension of potential endophytic bacteria with cell density of  $10^8$  CFU/ml was inoculated to LB broth to make 5% culture suspension. Culture was incubated at orbital shaker for 4 hours at ambient temperature. Chili root were soaked into the suspension for 15 minutes and planted in a 3 kg-polybag previously filled with soil compost. Observation was done 30 days after plantation by measuring plant height, number of leaves, fresh weight, dry weight, and number of chlorophylls (Dey *et al.*, 2004).

#### **Identification of Endophytic Bacteria Based on 16S rRNA**

Extraction of DNA of potential endophytic bacteria was done using freeze and thaw method. A 1.5 ml of pure bacterial culture was centrifuged for 10 minutes. Supernatant was removed. Cell pellet was added aseptically with 100  $\mu$ L of sterilized water and suspended. Cell suspension was subsequently frozen at -10°C and then heated at 90°C for 10 minutes for 5-10 times (Nusyirwani, 2007).

To make a 25  $\mu$ L of PCR reaction

solution, the following materials were added into 0.2 mL eppendorf tube: 12.5  $\mu$ L of Master Mix 2X GoTaqGreen (Promega), 1  $\mu$ L of forward primer (10 pmol), 1  $\mu$ L of reverse primer (10 pmol), 2  $\mu$ L of template DNA, and 8.5  $\mu$ L of nuclease free water. PCR machine was programmed and run using the following temperature: pre-denaturation at 94°C for 2 minutes, denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, primer elongation at 72°C for 1 minute, and post-PCR at 72°C for 5 minutes. This process was run for 30 cycles. Amplification of 16S rRNA gene of broken cell was conducted using Polymerase Chain Reaction (PCR) machine (Labcycler Gradient) with a 16S rRNA-specific universal primer for prokaryotes, 63f (5'- CAG GCC TAA CAC ATG CAA GTC - 3') and 1387r (5' - GGG CGG WGT GTA CAA GGC 3') (Marchesi *et al.*, 1998). PCR product was analyzed with electrophoresis. Analysis was performed at 80 volts and 400 mA for 90 minutes, and then visualized using UV-transilluminator.

The amplified DNA was commercially purified and sequenced. DNA sequence was compared to that of GenBank database of The National Center for Biotechnology Information (NCBI), using Basic Local Alignment Search Tool (BLAST).

## **RESULT AND DISCUSSION**

### **Characterization of Endophytic Bacteria from Roots and Leaves of Blackboard Plant**

Sixteen endophytic bacteria were isolated in this study, of which 8 isolates were obtained from the root and 8 isolates were from the leaves of blackboard tree. Other isolated 9 isolates of endophytic bacteria from blackboard tree leaves (Fatiqin, 2009). Characterization on the bacterial isolates was carried out, including cell morphology, bacterial colonies and biochemical tests (Table 1). Of this characterization, it showed that all isolates might be different.

### **Assay on Endophytic Bacteria of Blackboard Tree to Inhibit *Colletotrichum* sp. and *Fusarium* sp. Growth**

The ability of endophytic bacterial isolates to inhibit fungal growth is determined by the presence of inhibition zone at the intersection area between bacterial isolates and pathogenic

Table 1. Morphological and Biochemical Characteristics of Endophytic Bacterial Isolates of Blackboard Tree

Source	Isolate code	Shape	Colony morphology			Color	Gram staining	Starch metabolism	Gelatin hydrolysis	Biochemical Traits		
			Edge	Elevation	Metabolism					Citrate	H <sub>2</sub> S production	Motility
Root	A1	Circular	Entire	Flat	Cream	Positive	-	-	-	+	+	
	A2	Circular	Entire	Flat	Cream	Negative	+	-	+	+	+	
	A3	Circular	Entire	Raised	Cream	Positive	-	-	+	+	+	
	A4	Irregular	Undulate	Flat	Pink	Negative	-	-	+	+	+	
	A5	Irregular	Entire	Convex	Cream	Negative	-	-	+	+	+	
	A6	Circular	Entire	Flat	Cream	Negative	-	-	+	+	+	
	A7	Irregular	Undulate	Flat	Pink	Negative	+	-	+	+	+	
	A8	Circular	Entire	Flat	Cream	Positive	-	-	-	-	+	
Leaves	D1	Irregular	Undulate	Flat	Cream	Negative	-	-	+	+	+	
	D2	Circular	Entire	Convex	Cream	Positive	-	-	+	+	+	
	D3	Circular	Entire	Convex	Cream	Negative	+	-	+	+	+	
	D4	Circular	Entire	Convex	Cream	Negative	-	-	+	+	+	
	D5	Circular	Entire	Flat	Cream	Negative	-	+	+	+	+	
	D6	Circular	Entire	Convex	Cream	Negative	-	+	+	+	+	
	D7	Circular	Entire	Convex	Cream	Negative	-	+	+	+	+	
	D8	Irregular	Undulate	Flat	Cream	Negative	-	-	+	+	+	

fungi. The ability of endophytic bacteria to inhibit pathogenic fungi is based on their ability to produce metabolites such as cell wall degradation enzymes or other antifungal compounds (Ramamoorthy *et al.*, 2002).

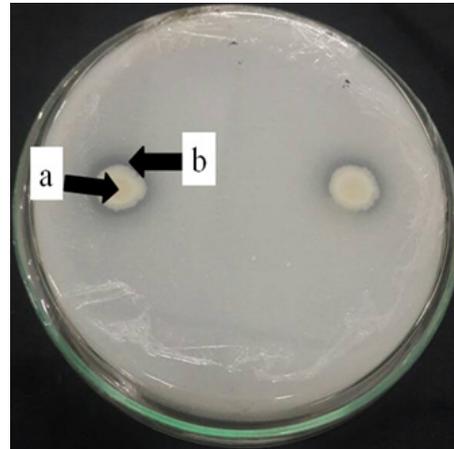
Two out of 16 isolates were observed to have the ability to inhibit both pathogenic fungi, ten were able to inhibit only either one of the pathogenic fungi, while the other four was not able to inhibit pathogenic fungi (Table 1). Previous

study on endophytic bacteria from blackboard tree leaf showed that all 9 species of the endophytic bacterial isolates had the potential to produce antibacterial compounds against *Escherichia coli* and *Staphylococcus aureus* (Fatiqin, 2009). One characteristic of antagonistic microbes is that their growth rate is faster than pathogens and/or produce antibiotic compounds that can inhibit the growth of pathogens (Shehata, 2008).

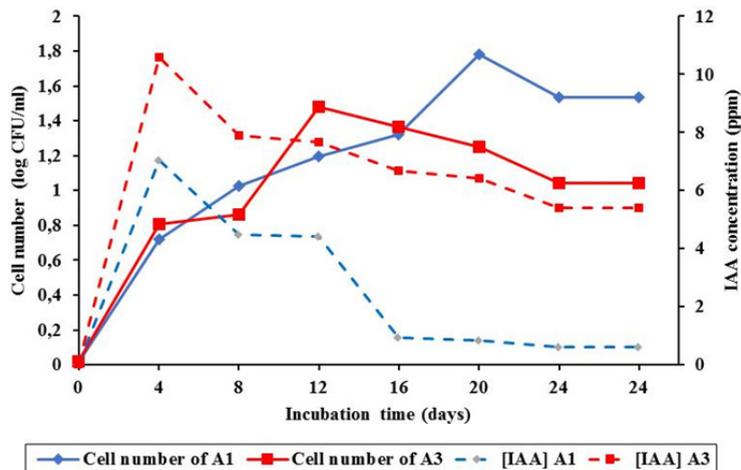
The size of the inhibition zone produced is highly dependent on the type and stability of the antifungal metabolites produced by different bacterial species. This *in vitro* inhibition ability may be affected by the type, solubility, and stability

**Table 2.** Endophytic bacterial isolate ability to inhibit growth of *Fusarium sp.* and *Colletotrichum sp.*

Isolates	Inhibition zone in cm	
	<i>Fusarium sp.</i>	<i>Colletotrichum sp.</i>
A1	0,5	0,4
A2	0	0,9
A3	0,2	0,8
A4	0	0,5
A5	0	0
A6	0	0
A7	0	0,6
A8	0	0,2
D1	0	0,6
D2	0	0
D3	0,6	0
D4	0	1,4
D5	0	1,4
D6	0	1,0
D7	0	0
D8	0	1,2



**Fig. 1.** Endophytic bacterial ability to solubilise phosphate shown by (a) clear zone around (b) bacterial colonies on Pikovskaya agar after 7 days incubation at ambient temperature



**Fig. 2.** Bacterial growth and IAA production of A1 and A3 for 24 hours

of the antimicrobial compounds produced by endophytic bacteria. The stronger inhibition ability against pathogenic fungi is expected to be more effective in promoting plant growth by inhibiting pathogen attack plants.

#### Ability of Endophytic Bacteria of Blackboard Tree in Solubilizing Phosphate

The ability of endophytic bacteria in solubilizing phosphate may be due to their ability to produce phosphatase or extracellular organic acids. The organic acid may be citric acid, glutamate, succinate, lactate, oxalate, glyco-oxalate, malate, fumarate, tartaric or alpha-ketobutyric acid (Maryanti, 2006), formic, acetic, propionic, lactic, glycolic, and succinate acids capable of

forming chelates with cations such as Al and Fe (Rao, 1994).

Six isolates A1, A3, A4, D1, D3, and D6 demonstrated to have ability in solubilizing phosphate showed by their PSI. A3 was observed to have higher PSI. The formation of clear zones around bacterial colony in Pikovskaya agar added with  $\text{Ca}_3(\text{PO}_4)_2$  (Figure 1) indicated that the isolates are capable of producing phosphatase or extracellular organic acids binding to Ca ions in the medium, liberating  $\text{H}_2\text{PO}_4^-$  ions to form a clearer colored area compared to areas with bound P (George *et al.*, 2002).

Many studies were conducted to isolate endophytic bacteria from plants but blackboard.

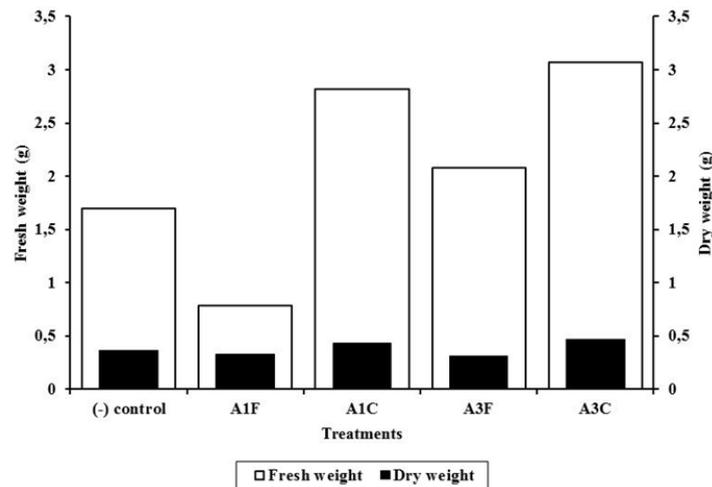


Fig. 3. Fresh and dry weight of infected chili plant treated with endophytic bacteria of blackboard tree

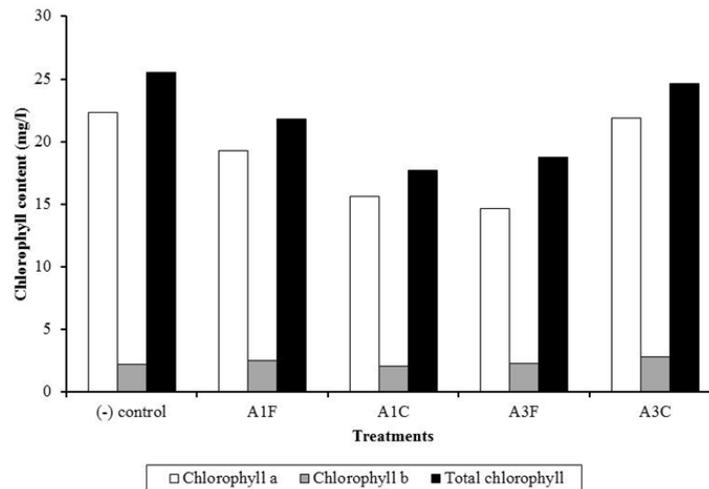


Fig. 4. Chlorophyll content of chili plants treated with endophytic bacteria of blackboard tree

For examples 373 endophytic isolates was isolated from soybean plants, in which 49% were able to solubilize phosphate (Long *et al.*, 2008). Similar study reported that of 77 endophytic bacteria isolates from *Solanum nigrum* (leunca), only six isolates were able to solubilize the inorganic phosphate (Rao, 1994).

**Bacterial Growth and Production of IAA**

A1 was observed to reach its maximum growth 20 hours after incubation. On the other hand, A3 was shown to reach its maximum growth earlier with 12 hours after incubation before entering stationary phase (Figure 2).

The process of elongation, cleavage and cell differentiation contribute by hormon such as IAA is better to support plant growth. In this study, IAA from endophytic isolates was measured using spectrophotometric method. IAA produced by A1 and A3 was varied. It was shown that both A1 and A3 produced high IAA in the first 4 hours and started to decrease there after. A1 and A3 might produce IAA with concentration of 7.041 ppm and 10.579 ppm respectively 4 hours after incubation. Decrement of IAA production together with increment of bacterial colonies indicated that some nutrients in the medium decreased during

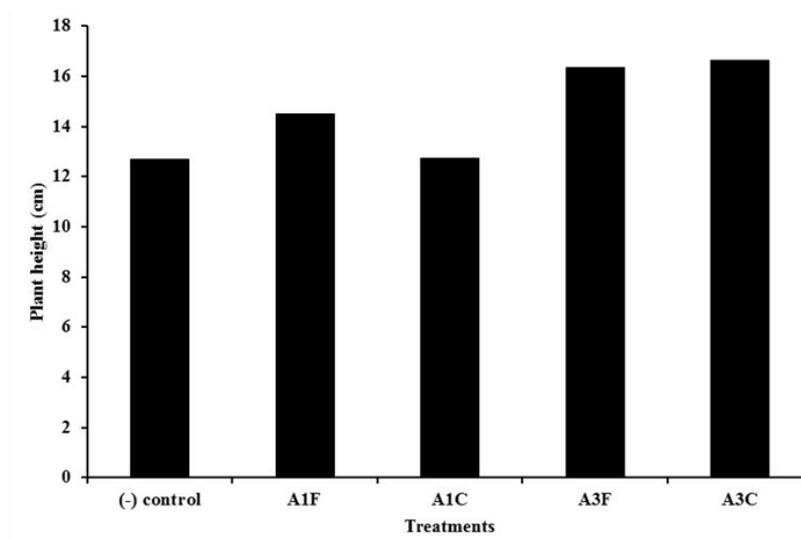


Fig. 5. Plant height of chili plants treated with endophytic bacteria of blackboard tree

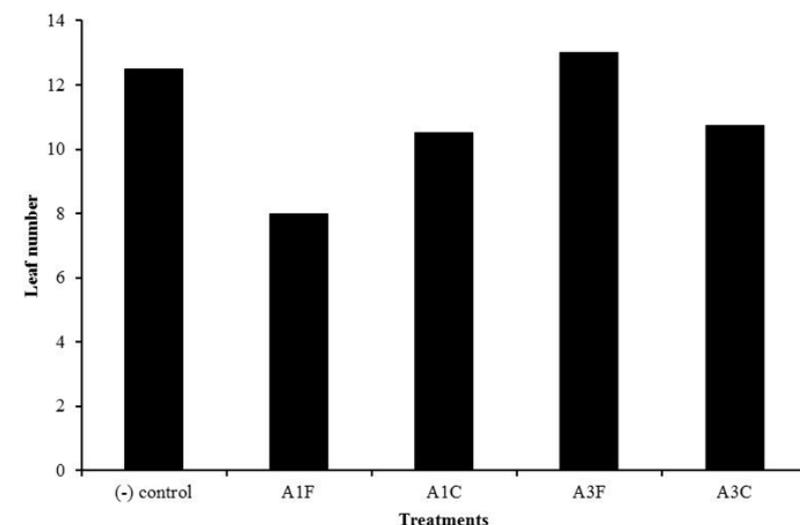


Fig. 6. Leaf number of chili plants treated with endophytic bacteria of blackboard tree

**Table 3.** Bacterial count before and after application

Code Isolates	PCA Medium		Pikovskaya Agar Medium	
	Number of cells before application (CFU/g)	Number of cells after application (CFU/g)	Number of cells before application (CFU/g)	Number of cells after application (CFU/g)
A1F	1,5 x 10 <sup>9</sup>	2,3x 10 <sup>13</sup>	2,1 x 10 <sup>5</sup>	0,4 x 10 <sup>13</sup>
A1C	1,3x 10 <sup>9</sup>	1,9 x 10 <sup>13</sup>	1,8 x 10 <sup>5</sup>	0,8 x 10 <sup>13</sup>
A3F	1,6 x 10 <sup>9</sup>	2,5x 10 <sup>13</sup>	1,3 x 10 <sup>5</sup>	0,3 x 10 <sup>13</sup>
A3C	1,9x 10 <sup>9</sup>	2,8 x 10 <sup>13</sup>	2,8x 10 <sup>5</sup>	1,1 x 10 <sup>13</sup>

the period of IAA production (Lestari *et al.*, 2007). Simultaneously, the bacteria might reuse IAA due to nutrition depletion (Lay, 1994). Many studies were reported to isolate hormone producing endophyte bacteria. For example endophytic bacteria from corn roots produce plant growth regulator (Retnowati *et al.*, 2011). Other study also reported that pepper plant fresh weight can be improved by soaking their roots in several endophytic bacterial isolates from pepper plant (Harni *et al.* 2011). Similarly, endophytic bacteria from potato (Suriaman, 2010) and sweet potato (Hidayatullah, 2012) plants can produce IAA hormone at varied concentration to promote plant growth.

#### Assay on Application of Potential Endophytic Bacterial Isolates in Chili

Crop production is more accurately expressed by measuring the dry weight than fresh weight. This is due to that fresh weight can strongly be affected by moisture conditions (Sitompul *et al.*, 1995). Furthermore, the increment of fresh weight is influenced by the availability of sufficient and balanced nutrients due to increased cell division activity (Goldsworth *et al.*, 1986). Dry weight results however are a balanced measurement between photosynthesis and respiration (Gardner *et al.*, 1991).

Except that of A1F, other treatments of endophytic bacteria to chili increased fresh and dry weight of the plant. However, it varied among treatments with higher shown by A3C (Figure 3). This might be due to differences in nutrient availability and microorganism in the soil. Disease severity caused by pathogenic fungi might also contributed to fresh and dry weight. Compared to that of *Colletotrichum* sp. infestation, *Fusarium* sp. infestation might affect more to fresh weight

that in turn affected dry weight. This fungus might decrease water intake by clogging xylem.

It was reported that many endophytic bacteria from different plants were able to solubilise phosphate to make it available for plants. Application of phosphate solubilizing bacteria increased paddy rice production by 5.4% (Thakuria *et al.*, 2004). Moreover phosphate solubilizing bacteria applied in soil increased the fresh weight of red chili (Yanti *et al.*, 2009). Another study showed similar result in which endophytic bacteria increased fresh weight and length of tomato roots (Munif *et al.*, 2013). Pepper root and plant weight was increased by soaking the roots of pepper seedlings in the endophytic bacterial solution isolated from pepper plants (Harni, 2011). Fresh and dry weight of plant is the total weight of the plant showing the results of its metabolic activity. The availability of nutrients during plant growth leads to actively running photosynthesis (Sarief, 1986).

A1F and A3C were observed to have high content of chlorophyll a and b, however they were lower to that of (-) control. *Colletotrichum* might cause leaf necrotics (antrachnose). In A3C, endophytic bacteria A3 probably decreased disease severity. Previous data showed that A3 was more capable to inhibit *Colletotrichum* rather than A1. Early symptoms of *Fusarium* wilt are pale leaf midrib particularly on apical regions, followed by epinasty on older leaves due to the of leaf petiole that causes the plants to completely wilt. *Fusarium* infection on young plants may cause immediate death due to the damages at stem base, while older plants could survive and but with very low production and small fruits (Semagun, 2000).

All treatments were demonstrated to increase plant height compared with (-) control

(Figure 5). This suggested that application of endophytic bacteria were to promote plant growth. Plant height increment on treated plants might be due to IAA effect and phosphate solubilization by applied endophytic bacteria. Endophytic bacteria may increase plant growth by increasing the availability of plant nutrients such as nitrogen, phosphate, and other minerals, and stimulate plant growth by producing growth hormones such as ethylene, auxin, and cytokines (Bacon, 2007). Other results also reported that some endophytic bacteria can associate and promote chili plant growth (Sundaramoorthy *et al.*, 2012).

Number of leaves was varied between each treatment during observation. The highest average number of leaves increment was obtained from A3F treatment (Figure 6). Other treatments had lower average number of leaves increment compared to negative control. This indicates that A3F treatment is the best compared to other treatments.

Application of endophytic bacteria was able to increase nutrient uptake of N, P and K. This increment was in line with the production of dry matter in plants. Nutrient absorption in plants is affected by nutrient content in the plant and the production of dry matter. Application of endophytic bacteria can promote the dry materials in plants (Gusmaini *et al.*, 2013).

Bacterial cell number was counted before and 30 days after application using total plate count method on PCA and Pikovskaya medium. increased in all treatments, showed both on PCA and Pikovskaya agar (Table 3). Bacterial growth of phosphate solubilizing bacteria in this study was considerably low compared to that of Marista *et al.* (2013).

#### **Identification of Endophytic Bacterial Isolates Based on 16S rRNA gene**

Both selected isolates, A1 and A3, were identified using 16S rRNA gene. Identification showed that A1 was relatively closed to *Carnobacterium gallinarum* strain DSM4847 with 84% gene similarity, while A3 was relatively closed *Carnobacterium maltaromaticum* strain JCM 1154 with 78% gene similarity. So far, these two bacteria have never been reported as IAA-producing bacteria nor phosphate solubilizing bacteria. Both species are gram-positive, lactic acid, non-spore forming bacteria. Previous study reported that

*Carnobacterium* isolated from the atropic salmon fish intestinal was capable of controlling the growth of *Vibrio anguillarum* (Verschuere *et al.*, 2000).

### **CONCLUSION**

Sixteen isolates of endophytic bacteria were found from leaves and roots of blackboard tree (*Alstonia scholaris*). Two potential isolates, A1 and A3, showed to inhibit both *Fusarium* sp. and *Colletotrichum* sp. A3 was observed to have relatively high Phosphate Solubilizing Index and in producing IAA. It also showed that in general A3 promoted more chili performance, even with infestation of fungal pathogen. Identification using 16S rRNA gene sequence demonstrated that A1 was relatively closed to *Carnobacterium gallinarum* strain DSM4847 with 84% gene similarity, while A3 was relatively closed *Carnobacterium maltaromaticum* strain JCM 1154 with 78% gene similarity.

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