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In Vitro Study of Endophytic Bacteria Isolated from Tomato Plant against *Fusarium oxysporum*

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In this study, 43 isolates of endophytic bacteria isolated from healthy tomato plants against *Fusarium oxysporum*, which causes Fusarium wilt disease of tomato, was studied. Initially effects of endophytic bacteria on the growth of tomato seedlings were tested. The results showed that most endophytic bacteria did not affect the growth of tomato seedlings. Characterization by gram staining revealed that most of them were gram-positive bacteria. Subsequently they were tested on the antagonistic activity against *Fusarium oxysporum* by dual culture technique. It was found that only seven isolates showed the ability to inhibit the pathogen more than 30 percent. The best isolates including SuRW02 SuRW01 and LbRW03 were highest inhibition percentage of 71.94, 68.33 and 68.19%, respectively. The potential isolates found in this study will be further study and develop for coating tomato seed which an alternative method to control Fusarium wilt disease in the future.

Keywords: endophytic bacteria, *Fusarium oxysporum*, Tomato

Introduction

Fusarium oxysporum causes Fusarium wilt in tomato is a major pathogen affecting tomato production. The symptoms of this disease include wilting, chlorosis, and stunted seedling. As a result, the plants die or got lower yields (Hussain *et al.*, 2016). Agriculturists had many controlled measures by using several methods, including cultural technique and chemical application. Especially the use of chemicals has been widely used. Although the use of chemicals is effective in controlling the disease, this medthod is harmful to organisms and the environment. Therefore, safe strategies would be used in the management of this disease.

Biological control has been reported as a potential for the management of several disease. It consists of a variety of antagonistic microorganisms which have activity for controlling of various plant pathogens, including Fusarium

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wilt pathogen (Larkin and Fravel., 1998). Among of that endophytic bacteria is one of the benefit microbial, which is a group of microorganisms that live in healthy plant tissue and did not negative effect on plant (Bacon and White, 2000; Hundley, 2005). Several studies of biological control by endophytic bacteria have shown that they were able to suppress the pathogen of bacterial wilt disease in tomato (Purnawati *et al.*, 2014). Also, inoculation with bacterial endophytes has been demonstrated to reduce disease symptoms caused by vascular wilt pathogens such as *Verticillium dahlia* and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) (Nejad and Johnson., 2000). Nandhini *et al*. (2012) also reported that endophytic bacteria isolated from root, stem, leaves and fruits of healthy tomato plants can control *Fusarium oxysporum* f. sp. *lycopersici*. Therefore, this research presents the results of in vitro antagonistic activity of endophytic bacteria against *Fusarium oxysporum* and useful information for alternative biological control strategy in the future.

Objectives: Isolation and screening of endophytic bacteria that has potential to control *Fusarium oxysporum*. Selection potential isolate for further study as seed bio-coating of Fusarium wilt management in the future.

Materials and methods

Isolation of Fusarium oxysporum and pathogenicity tests

Fusarium oxysporum was isolated from tomato plant exhibiting symptoms of Fusarium wilt by tissue transplanting technique. Identification was checked based on morphological characteristics.

Pathogenicity tests: Fungal pathogens were grown for 7 days on Potato dextose agar (PDA). Then the spore suspension at concentration of 10^6 spore / ml was prepared for this test. Tomato seedlings at 3 weeks of age were test by root dip technique (cut roots and dip into the spore suspension for 20 minutes), before transplanted into planting bag, and compared with dipping in sterile distilled water (control). Disease severity was evaluated at 2 week after inoculation by 0-3 scoring which modified from Marlatt *et al.* (1996); where 0 = healthy, 1 = temporary wilt, 2 = Permanent wilt and 3= plant die. Most violent isolate was selected to be used in next experiments. The experimental design was completely randomized design (CRD) with 5 replications.

Isolation and screening of beneficial endophytic bacteria for tomato plant

Isolation of endophytic bacteria was done from root, stem and leaves of healthy tomato plants. All parts of plants were surface disinfected by soaking in

70 % ethyl alcohol for 30 sec., washed with sterile distilled water, follow by soaking in 5% sodium hypochlorite for 2 min then washed again with sterile distilled water and dried on sterile filter paper. After surface disinfected, each sample was ground by sterile mortar and prepared the suspension to 10^{-1}-10^{-4}. Then each dilution of suspensions were cultured by pour plate technique on nutrient agar (NA) and incubated for 48 h at room temperature. Single colony occurred on the culture was move onto NA by streak plate technique to obtain pure colony. Morphological examination consists of colony shape, colony color, cell shape, and gram test by using 3%KOH and gram stain were also examined.

Screening for beneficial endophytic bacteria: All isolates obtained from healthy tomato plant was cultured in nutrient broth (NB) and incubated on rotary shaker for 48 hr. The culture was collected and centrifuged at 5000 rpm for 10 minutes to obtain the bacterial pellet and prepared to bacterial suspension which adjusted the concentration equal to 0.5 Mcfarland standard solution turbidity. A 10 ml of bacterial suspensions were added to the pots of tomato seedlings at 3 day of age grown in sterilized peat moss. The experimental design was completely randomized design (CRD) with 4 replications. After 7 days of inoculation with endophytic bacteria, the seedlings survived and the growth data was collected. The growth data including stem height, stem weight, root weight, total weight and number of leaves. Data were calculated for growth index of seedling vigor index (svi) as following formula:

$$\text{svi} = \text{Average germination percentage x Average weight per plants}$$

$$\%\ \text{svi} = \frac{\text{svi of treatment}}{\text{svi of control}} \text{ X } 100$$

In vitro* antagonistic activity of endophytic bacteria against *Fusarium oxysporum

Fusarium oxysporum was cultured on PDA for 7 days. Endophytic bacteria were cultured on NA for 2 days**.** Antagonistic activity was evaluated by using dual culture technique on petridis containing PDA medium. The agar plug of pathogen was placed at the center of culture medium and endophytic bacteria were parallel streaked on the left and right sides of the pathogen at 2 cm. length from the edge of the plate then incubated at room temperature. Control plates were streaked with sterile distilled water. Evaluation of mycelial growth inhibition when pathogen grown full in control plate. The mycelial growth inhibition rate (IR) was calculated using the formula as follow: [(C2-C1)/ C2 x 100 where C2: diameter of the pathogen colony on control plate. and C1: diameter of the pathogen colony on the antagonist plate. The experimental design was completely randomized design (CRD) with 4 replications.

Statistical analysis

The results were subjected to the analysis of variance and means were separated according to the Duncan's multiple range test at $P \leq 0.05$.

Results

Pathogen and pathogenicity

Fusarium oxysporum isolated from tomato wilt disease plants were found the mycelium is delicate white to pink and produce charmadospore microconidia and macroconidia. Macroconidia have three-septate. (Figure1). In pathogenicity tests, the tomato showed symptom of permanent wilting to dead and browning of the vascular tissues after 7 days of inoculation (Figure2). The evaluation showed that the disease severity was 2.4 and the disease incidence was 80%.

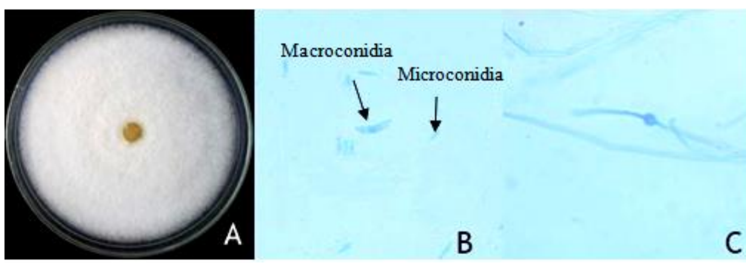


Figure 1. Morphology of *Fusarium oxysporum* (A =colony B = microconidia and macroconidia C = charmadospore)

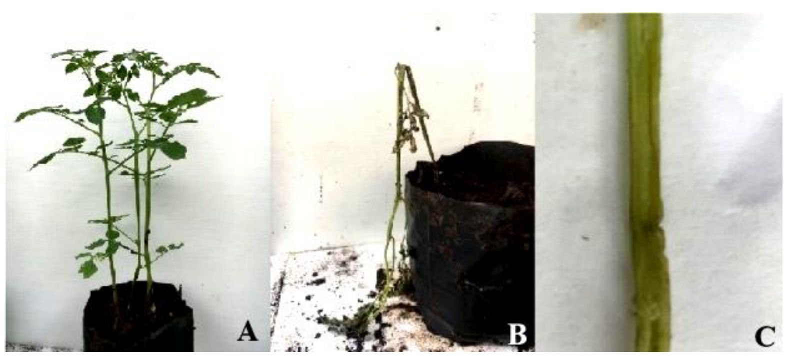


Figure 2. Symptom of wilt disease on tomato seedling at 7 days after inoculation (A =control B = inoculation with *Fusarium oxysporum* C = browning of the vascular tissues)

Morphological characteristics and effects on growth and seedling vigor of endophytic bacteria

Fourty three isolates of endophytic bacteria isolated from each part of healthy tomato plants (root leaves and stem) was found to have a variety of morphological characteristics. Most of them were gram-positive bacteria, which was divers in colony characteristic as shown detail in Table 1 and Figure3.

In addition, 43 isolates of endophytic bacteria were tested on tomato seedlings. The results showed that most endophytic bacteria did not affect the growth of tomato seedlings. Several endophytic bacteria were found that can be growth promoting and had seedling vigor more than 20 percentages when compare with control. Those isolates including LaRW01, LaKY03, LaLW02, LaLY02, LaTY01, LaTY02, LaTO01, SuLW01, SuLY03, SuRW02, SuRW04, SuRY01, SuRY02, SuRB01, SuKY01, LbTW01, LbLW02, and LbRW02. The details of data were shown in Table 2.

Table 1. Characteristic of endophytic bacteria isolated from healhy tomato plants.

Isolate	**colony**				**3%KOH test**	**Gram staining**	**shape**
	color	shape	margin	surface			
LaRW01	white	circular	entire	mucoid	+	+	coccus
LaRW02	cloudy white	circular	erose	smooth	-	-	coccus
LaRW03	white	irregular	undulate	smooth	+	+	bacillus
LaRY01	white	circular	erose	rough	+	+	bacillus
LaKY01	yellow	irregular	entire	mucoid	+	+	bacillus
LaKY02	yellow	circular	entire	mucoid	+	+	coccobacilli
LaKY03	light yellow	circular	entire	mucoid	+	+	coccobacilli
LaKW01	white	circular	entire	smooth	+	+	coccobacilli
LaKW02	white	circular	entire	mucoid	+	+	coccus
LaLW01	white	circular	entire	rough	-	-	coccus
LaLW02	white	circular	entire	mucoid	-	-	coccus
LaLW03	white	circular	entire	mucoid	-	-	bacillus
LaLY01	light yellow	circular	entire	mucoid	+	+	bacillus
LaLY02	light yellow	circular	entire	mucoid	+	+	coccus
LaLY03	light yellow	circular	entire	mucoid	+	+	bacillus
LaTY01	yellow	circular	entire	mucoid	+	+	coccus
LaTY02	yellow	circular	entire	mucoid	-	-	coccus
LaTW01	white	circular	entire	mucoid	-	-	coccus
LaTO01	orange	circular	entire	mucoid	+	+	bacillus
SuLW01	cloudy white	circular	erose	smooth	+	+	bacillus
SuLW02	light yellow	circular	entire	mucoid	+	+	bacillus
SuLW03	cloudy white	circular	entire	mucoid	+	+	bacillus
SuLW04	cloudy white	circular	entire	mucoid	+	+	bacillus
SuLY01	light yellow	circular	entire	mucoid	+	+	coccus
SuLY02	yellow	circular	entire	mucoid	+	+	bacillus

Table 1. Characteristic of endophytic bacteria isolated from healhy tomato plants (continue).

Isolate	colony				3%KOH test	Gram staining	shape
	color	shape	margin	surface			
SuLY03	light yellow	circular	entire	mucoid	+	+	bacillus
SuLO01	orange	circular	entire	smooth	+	+	bacillus
SuRW01	cloudy white	circular	undulate	rough	+	+	bacillus
SuRW02	cloudy white	irregular	undulate	rough	+	+	bacillus
SuRW03	cloudy white	circular	entire	mucoid	+	+	coccobacilli
SuRW04	white	irregular	undulate	rough	+	+	bacillus
SuRY01	light yellow	circular	entire	mucoid	+	+	bacillus
SuRY02	light yellow	circular	entire	smooth	+	+	bacillus
SuRY03	light yellow	circular	entire	mucoid	-	-	coccus
SuRB01	egg	circular	undulate	smooth	+	+	bacillus
SuTW01	white	irregular	entire	mucoid	-	-	coccobacilli
SuKY01	light yellow	irregular	entire	mucoid	+	+	bacillus
LbTW01	cloudy white	irregular	erose	rough	+	+	bacillus
LbLW01	cloudy white	irregular	erose	rough	+	+	bacillus
LbLW02	cloudy white	irregular	erose	smooth	-	-	coccus
LbRW01	cloudy white	irregular	erose	smooth	+	+	bacillus
LbRW02	cloudy white	irregular	erose	rough	+	+	bacillus
LbRW03	cloudy white	circular	entire	smooth	+	+	bacillus

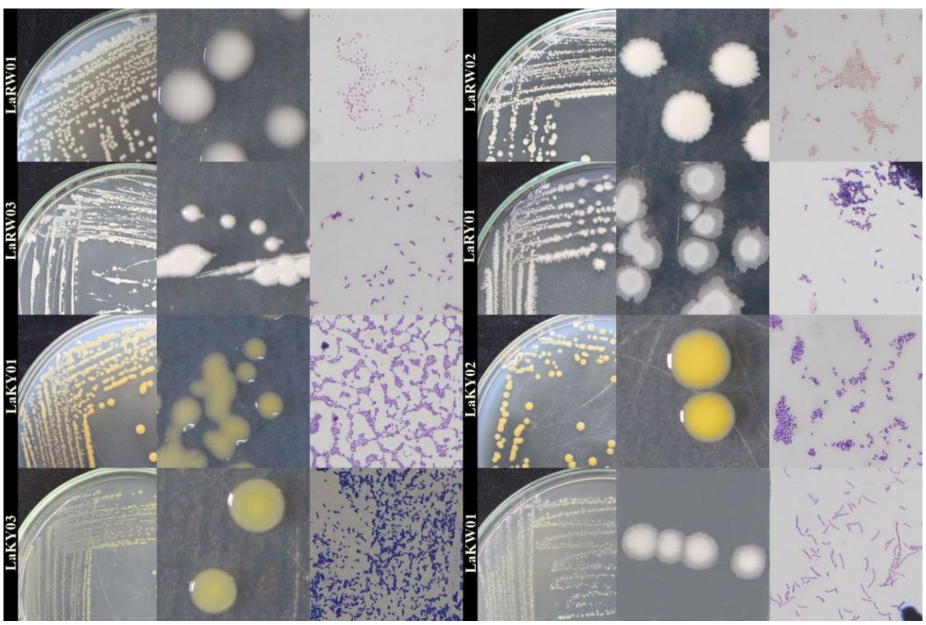
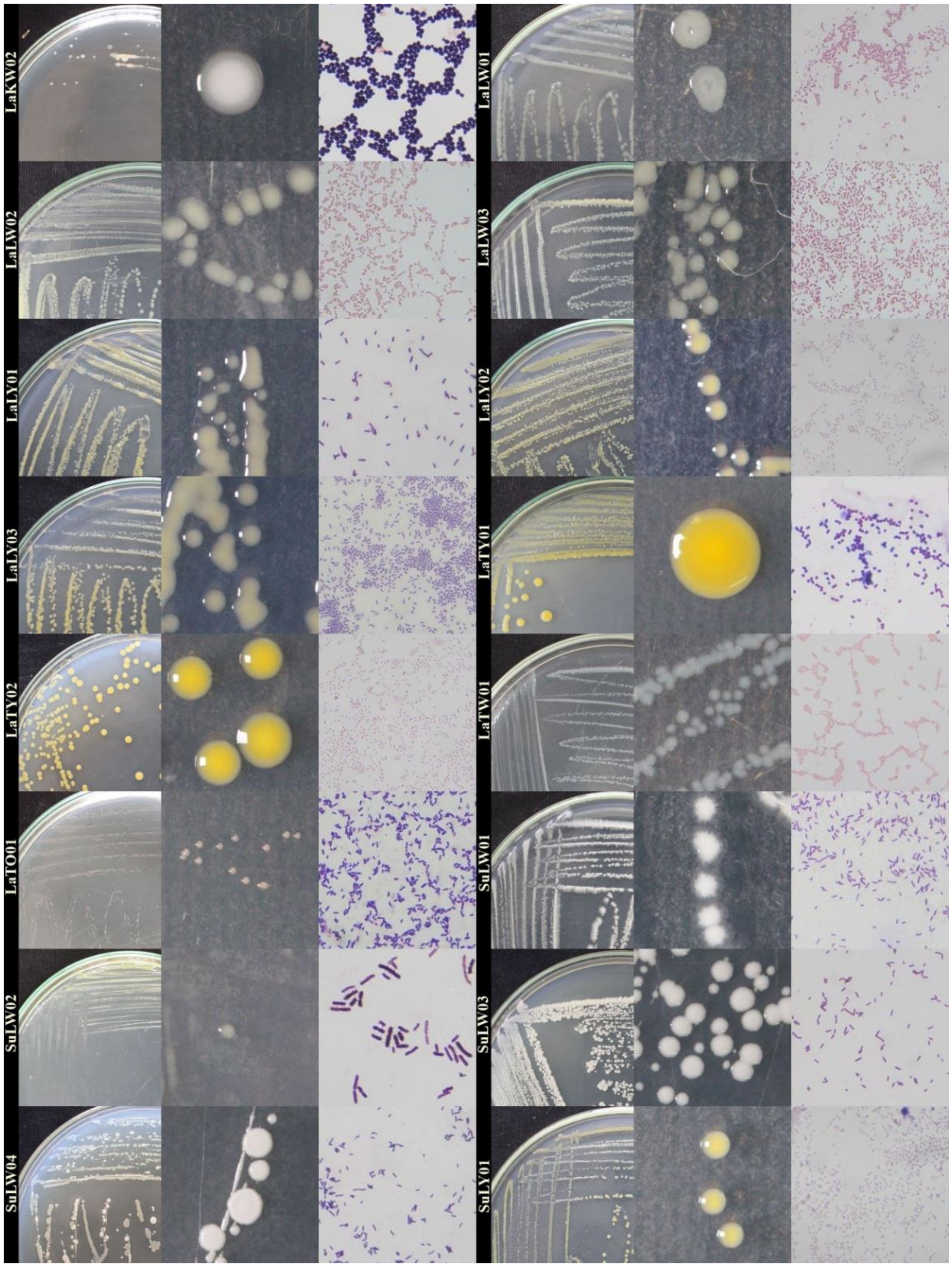


Figure 3. Morphology of endophytic bacteria isolated from healthy tomato plants (colony on NA (left), colony at 6.7X and gram staining (right)).



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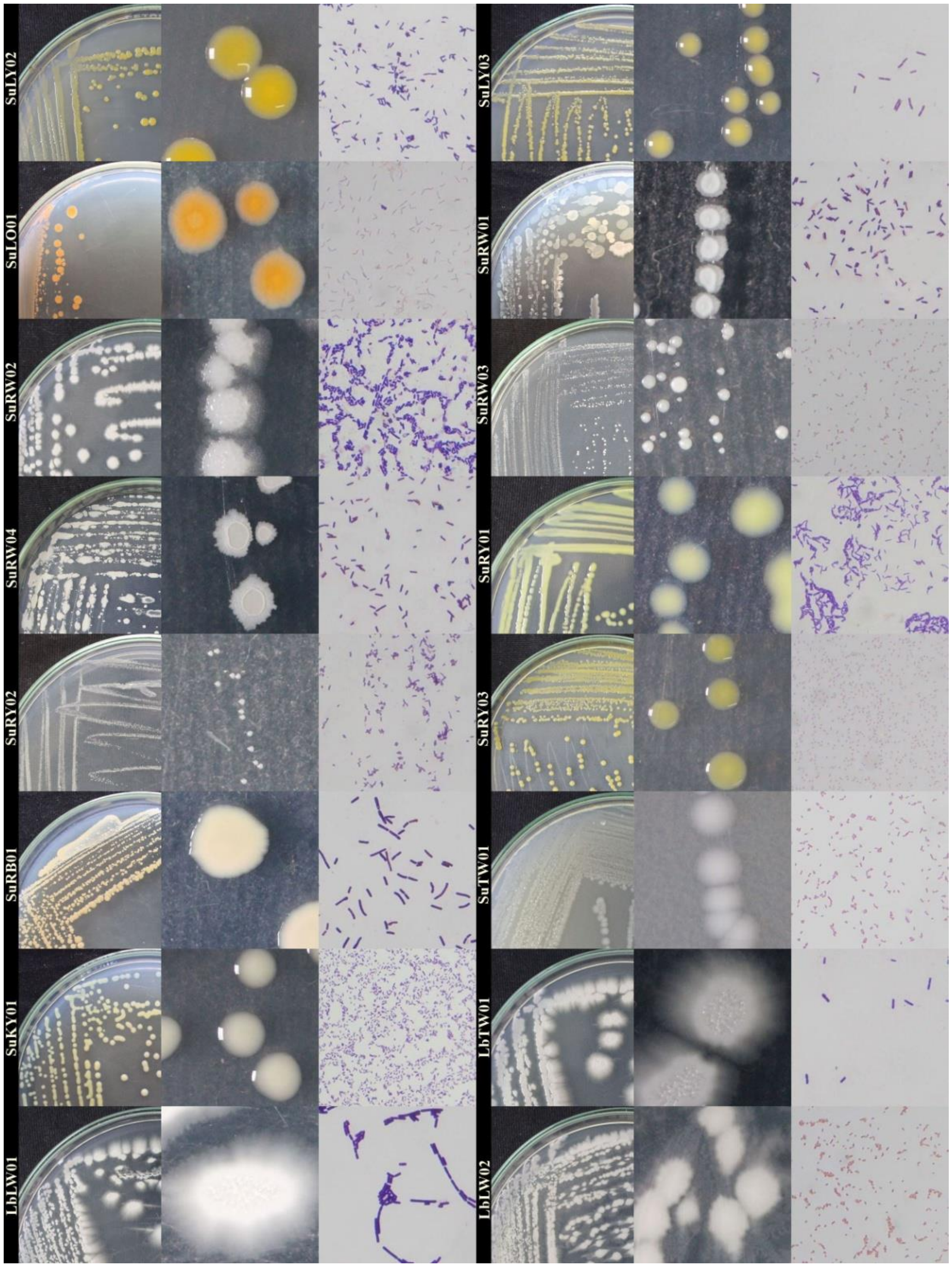

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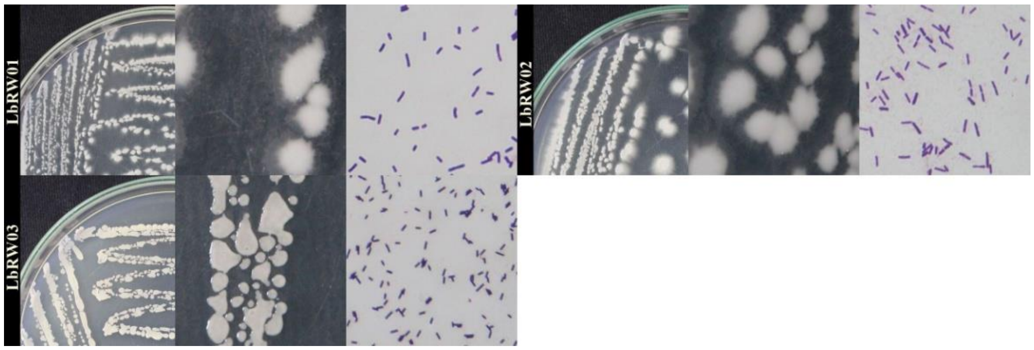

Figure 3. Morphology of endophytic bacteria isolated from healthy tomato plants (colony on NA (left), colony at 6.7X and gram staining (right)). (Continue)

Table 2. Effect of endophytic bacteria isolated from healhy tomato plants on the growth of tomato seedlings.

Isolates	Suvival of seedling	Number of leaves	Heigh of shoot (cm.)	Fresh weight (g)			% svi
				root	shoot	total	
control	100a[1/]	2.00a	6.30ab	0.089cd	0.452f	0.541ghij	100.00
LaRW01	100a	1.95a	6.00 ab	0.136a	0.562bcd	0.698cbd	129.09
LaRW02	95ab	1.90a	5.37ab	0.095c	0.470ef	0.565ghij	99.16
LaRW03	95ab	2.00a	6.50ab	0.119ab	0.436 f	0.555ghij	97.41
LaRY01	100a	1.90a	5.50ab	0.073def	0.466ef	0.537ghij	99.30
LaKY01	100a	1.95a	5.50ab	0.073def	0.518de	0.591fgh	109.23
LaKY02	100a	1.90a	5.37ab	0.071def	0.437f	0.508j	93.99
LaKY03	100a	2.00a	5.62ab	0.086cdef	0.561bcd	0.647def	119.63
LaKW01	100a	1.90a	6.00ab	0.095c	0.465ef	0.560ghij	103.46
LaKW02	85c	1.90a	5.87ab	0.067f	0.437f	0.505j	79.30
LaLW01	100a	1.95a	5.87ab	0.121ab	0.443f	0.565ghij	104.38
LaLW02	100a	1.95a	5.50ab	0.120ab	0.562bcd	0.682cd	126.09
LaLW03	100a	1.90a	6.12ab	0.124ab	0.4675ef	0.591fgh	109.23
LaLY01	100a	1.90a	5.75ab	0.086cdef	0.436f	0.522ij	96.53
LaLY02	100a	2.00a	5.50ab	0.126ab	0.561bcd	0.687cd	127.02
LaLY03	100a	1.90a	5.25ab	0.095c	0.4385f	0.533hji	98.61
LaTY01	100a	2.00a	5.62ab	0.116b	0.562bcd	0.678cd	125.40
LaTY02	100a	1.95a	5.87ab	0.118ab	0.578bc	0.696cbd	128.63
LaTW01	100a	1.95a	6.12ab	0.069ef	0.428f	0.497j	91.91
LaTO01	100a	2.00a	6.00ab	0.115b	0.632a	0.747ab	138.10
SuLW01	100a	1.95a	6.37 ab	0.116b	0.536cd	0.652de	120.55
SuLW02	100a	1.95a	5.87 ab	0.071def	0.441f	0.512j	94.68
SuLW03	100a	2.00a	5.75 ab	0.121ab	0.476ef	0.597efg	110.39
SuLW04	100a	2.00a	5.75 ab	0.0675f	0.477ef	0.545ghij	100.69

[1/]: Means in the same column with different letter are significant difference at P=0.05, according to Duncan's Multiple Range test.

Table 2. Effect of endophytic bacteria isolated from healhy tomato plants on the growth of tomato seedlings (Continue).

Isolates	Suvival of seedling	Number of leaves	Heigh of shoot	Fresh weight			% svi
				root	shoot	total	
SuLY01	100a	1.85a	6.00ab	0.079 cdef	0.4725ef	0.551 ghij	101.84
SuLY02	100a	1.95a	6.00ab	0.086cdef	0.442f	0.528ij	97.69
SuLY03	100a	1.90a	6.00ab	0.123ab	0.606ab	0.728abc	134.64
SuLO01	100a	2.00a	6.37ab	0.0905c	0.466ef	0.561 ghij	103.69
SuRW01	95ab	2.00a	5.75ab	0.068ef	0.442f	0.511j	89.73
SuRW02	100a	1.90a	5.37ab	0.128ab	0.518de	0.646def	119.39
SuRW03	95ab	2.00a	6.37ab	0.0685ef	0.437cd	0.506j	88.85
SuRW04	100a	2.00a	6.00ab	0.115b	0.538cd	0.653de	120.78
SuRY01	100a	1.95a	6.25ab	0.126ab	0.630a	0.756a	139.72
SuRY02	100a	2.00a	6.87a	0.086cdef	0.605ab	0.691cbd	127.71
SuRY03	100a	2.00a	6.62ab	0.118ab	0.466ef	0.583ghi	107.85
SuRB01	100a	1.95a	6.12ab	0.116b	0.537cd	0.653de	120.78
SuTW01	100a	1.90a	6.12ab	0.128ab	0.577bc	0.705abcd	130.24
SuKY01	100a	2.00a	6.62ab	0.118ab	0.537cd	0.655de	121.01
LbTW01	100a	1.90a	5.75ab	0.069ef	0.441f	0.510j	94.22
LbLW01	90bc	1.95a	5.75ab	0.086cde	0.475ef	0.562 ghij	93.53
LbLW02	100a	1.90a	5.75ab	0.126ab	0.537cd	0.663d	122.63
LbRW01	100a	1.85a	5.7ab	0.071def	0.436f	0.507j	93.76
LbRW02	100a	1.95a	5.87ab	0.086cdef	0.576bc	0.662d	122.40
LbRW03	100a	1.95a	5.87ab	0.065ef	0.443f	0.512j	94.68

[1/]: Means in the same column with different letter are significant difference at P=0.05, according to Duncan's Multiple Range test.

Effect of endophytic bacteria against Fusarium oxysporum

The result of antagonistic activity of 43 isolates endophytic bacteria against *Fusarium oxysporum* were found that 25 isolates were significantly different compared with control. Among of these, only seven isolates showed the ability to inhibit the pathogen growth more than 30 percent, including LaLW03, LaLY01, SuLW03, LaRY01, SuRW01, SuRW02, and LbRW03 (Figuer 4). The best isolates were SuRW02 SuRW01 and LbRW03, which had the highest inhibition percentage of 71.94, 68.33 and 68.19%, respectively (Table 3).

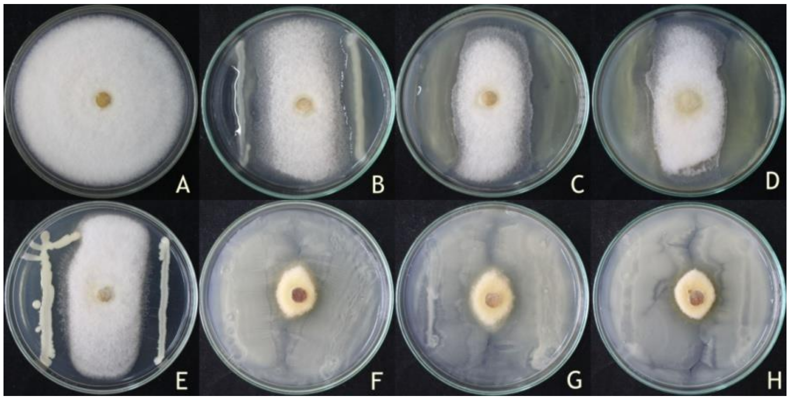

Figure 4. Dual-culture of seven isolates of endophytic bacteria that showed ability to inhibit the pathogen growth more than 30 percent (A= control, B= LaRY01, C=LaLW03, D=LaLY01, E= SuLW03, F= SuRW01, G= SuRW02, H= LbRW03)

Table 3. Antagonistic trait of 43 isolates of endophytic bacteria isolated from healthy tomato plants to suppress the growth of *Fusarium oxysporum.*

Isolates	Inhibitory agiast Fusarium oxysporum	
	Diameter of colony (cm.)	% Growth inhibition
control	9.00	0.00k[1/]
LaRW01	7.00	22.22 defg
LaRW02	6.95	22.78defg
LaRW03	6.42	28.61cd
LaRY01	5.71	36.53b
LaKY01	8.77	2.50jk
LaKY02	8.95	0.00k
LaKY03	8.93	0.00k
LaKW01	8.75	2.72jk
LaKW02	8.23	8.47ij
LaLW01	7.62	15.28ghi
LaLW02	8.03	10.69hi
LaLW03	6.26	30.42bc
LaLY01	6.25	30.47bc
LaLY02	7.53	16.25fgh
LaLY03	7.50	16.67fgh
LaTY01	8.92	0.00k
LaTY02	8.87	0.00k
LaTW01	6.56	27.08cde
LaTO01	7.35	18.33fg
SuLW01	8.97	0.00k
SuLW02	7.15	20.56 efg

[1/]: Means in the same column with different letter are significant difference at P=0.05, according to Duncan's Multiple Range test.

Table 3. Antagonistic trait of 43 isolates of endophytic bacteria isolated from healthy tomato plants to suppress the growth of *Fusarium oxysporum.* (Continue)

Isolates	Inhibitory agiast Fusarium oxysporum	
	Diameter of colony (cm.)	% Growth inhibition
SuLW03	6.16	31.53bc
SuLW04	8.92	0.00k
SuLY01	8.87	0.00k
SuLY02	7.10	21.06efg
SuLY03	6.90	23.33def
SuLO01	9.00	0.00k
SuRW01	2.85	68.33a
SuRW02	2.52	71.94a
SuRW03	8.08	10.14hi
SuRW04	9.00	0.00k
SuRY01	7.00	22.22 defg
SuRY02	9.00	0.00k
SuRY03	8.76	0.00k
SuRB01	7.57	15.83fgh
SuTW01	6.98	22.36 defg
SuKY01	9.00	0.00k
LbTW01	7.12	20.83 efg
LbLW01	7.28	19.028fg
LbLW02	6.97	22.50 defg
LbRW01	7.12	20.83 efg
LbRW02	7.15	20.56 efg
LbRW03	2.86	68.19a

[1/]: Means in the same column with different letter are significant difference at P=0.05, according to Duncan's Multiple Range test.

Disscustion

The morphological characteristic of *Fusarium* sp. isolated in this research was similar to *Fusarium oxysporum* according to Nirmaladevi *et al.* (2016) reported that the mycelia of *Fusarium oxysporum* isolates appeared delicate, white to pink, often with purple tinge. The fungus produced macroconidia, microconidia and chlamydospores. Macroconidia have 3-5 septate. Microconidia usually has non-septate or single septate. Chlamydospores, both smooth and rough walled, were abundant and formed terminally or on an intercalary basis. They are generally solitary, but occasionally form in pairs or chains.

Endophytic bacteria are bacteria that live in plant tissues. This bacterium is not harmful to plants and some species can help promote plant growth. (Bacon and White, 2000; Hundley , 2005). The results in this research showed that most endophytic bacteria did not harm tomato seedlings, also promote the growth and seedlings vigor. According to Khan *et al.* (2014), Tomato plants inoculated with endophytic bacteria showed significantly increased growth attributes (shoot length, chlorophyll contents, shoot, and root dry weights). In addition, this study has shown that endophytic bacteria can be inhibiting the growth of *Fusarium oxysporum.* Several researches have reported the use of endophytic bacteria for controlling many pathogens such as *Sclerotium rolfsii*, *Colletotrichum capsici*, *Pythium* sp., *Verticillium dahlia*, include *Fusarium oxysporum* causes Fusarium wilt in tomatoes. (Nejad and Johnson. 2000; Amaresan *et al*., 2012 and Nandhini *et al.*, 2012)**.**

This research showed that endophytic bacteria isolated from healthy tomato plants tissue are capable of promoting the growth of tomato seedlings and inhibit the growth of Fusarium oxysporum. The results of this study are the guideline for further study on the control of Fusarium sp. by biological method.

Acknowledgement

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