Pak. J. Biotechnol. Vol. 14 (2) 238 – 244 (2017)
www.pjbt.org

ISSN Print: 1812- 1837
ISSN Online: 2312- 7791

EXPLORING ENDOPHYTIC BACTERIA ORIGIN FROM *Jatropha curcas* L. AND THEIR POTENTIAL TO ENHANCE PLANT GROWTH IN EGGPLANT

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Article received 31.3.2017, Revised 18.6.3017, Accepted 22.6.2017

ABSTRACT

Endophytes are often microorganisms that live within plants without causing apparent diseases. They are ubiquitous and have been found in all species of plants studied to date. However, most of the endophyte to plant relationships is not well understood. In this research, we isolated and studied the properties of the endophytic bacteria from *Jatropha curcas*. L. as plant growth promoter's agents. In this study, were isolated 195 isolates of endophytic bacteria, of which 44 passed the hemolytic and hypersensitive test using blood agar media and tobacco leaves host as an indicator. Twenty-three of the 44 endophyte bacteria isolates exhibited different biochemical properties. Out of these 44 isolates, 12 were able to dissolve phosphate and 5 isolates were able to fix nitrogen through in *vitro* test making them 23 candidates for plant growth promoting bacteria (PGPB). These 23 isolates were developed as PGPB to enhance the eggplant seedlings under *in vivo* test. Based on SAS multiple range test data analysis, two of the isolates FJS23 and FJS24 showed higher result among the rest of the isolates. Our results, therefore, indicated that these two isolates assayed are perfect candidates to be used as bio-control and growth enhancer for eggplant.

Keywords— endophyte, *Jatropha curcas*, biochemical assay, candidates, phenotypic characteristics.

INTRODUCTION

Jatropha curcas L. (Euphorbiaceae), is a shrub or small plant, semi-evergreen tree found and cultivated in the tropics and subtropics region of the World mostly found in part of Asia, Central America and Africa. Some research has reported that Jatropha curcas is a toxic plant and its height can reach to 6 m (Janick *et al*., 2008). In Indonesia *jatropha* is called "jarak pagar" and it was brought to Indonesia by Portuguese traders. *J.curcas* can be easily planted in low and high rainfall areas of saline and marshy lands. It has been reported that Jatropha grows well in the marginal land which indicates it potential to solve the problem of soil erosion of degraded land approximately 20 million hectares in Indonesia (Afiff and Suraya, 2014).

People in Indonesia are often confused to differentiate between castor and *jatropha* because castor is also called jarak (Afiff and Suraya, 2014). *Jatropha* is one of the promising biodiesel crops and can be cultivated on marginal lands and in soil with low nutrition content, thus it has demonstrated to be strong and high tolerance to drought and unfertilized soil (Madhaiyan *et al*., 2015). It also plays an important role in phytoremediation in polluted soil (Openshaw and Keith, 2000) and help to control soil erosion.

Endophyte microbes live inside the plant without causing any negative impacts on the plant; rather they provide a protection for the host plant from pathogens, insects, and other pests (Saikkonen *et al*., 2004). In addition, the plant gets more benefits from this interaction with endophyte bacteria such as phytohormones production to stimulate the plant growth, atmosphere nitrogen fixation, delayed sene-scence through suppression of ethylene biosynthesis by secreting 1-aminocyclop-ropane-1-carboxylate (ACC) deaminize; alteration of sugar sensing mechanisms. Moreover, endophyte bacteria have the ability to produce hydrolytic enzymes to inhibit pathogens that attack the plant, competition for nutrients and place, and bacteria that induce the plant systemic resistance mechanisms for plant (Madhaiyan *et al*., 2015 and Sana Hanif *et al*., 2014)

Our investigation in this research was to explore the endophyte bacteria and determine the biochemical properties of endophyte bacteria from *Jatropha curcas*.L to be used as plant growth promoters.

MATERIALS AND METHODS

A. Isolation of Endophyte Bacteria: *Jatropha* roots, stem, fruits, and leaves were collected from a non-symptomatic plant from the research institute for industrial crops– sukabumi (BALITTRI). The plant roots were then washed in running tap water to remove soil particles. Surface sterilization procedure was conducted using 70% ethanol for 2 min, and sodium hypochlorite solution (1%, w/v, of chlorine) containing 20% for 3 min. We rinsed each sample five times in two dishes containing sterile distilled water to remove surface sterilization agents and finally, the samples were dried on sterile paper towels. The samples were split into 1cm diameter each and then pressed onto *tryptic soy agar* (TSA) media as a disinfection control to confirm that the disinfection process was successful. The plates were incubated at 28≠°C for 3 days using Hallmann *et al*., (2001) method. Five gram

of each sample was homogenized using sterile pestle and mortar in 12.5 mm potassium phosphate buffer (7.0 pH). All diluted homogenates samples were placed separately on 20% of TSA with three repli-cations. The plates were incubated at 28°C for 7 days to allow growth of endophyte bacteria. Single colonies were further sub-cultured in respective media (100% TSA) and homogenized sample of endophyte bacteria was selected randomly, purified, and grouped on the basis of phenotypic characteristics such as colony morphology, colony colour, cell shape, motility, growth rate, and gram reaction test (El-deeb *et al*., 2013).

B. Haemolysis Test: To confirm if the bacteria were pathogenic for human or mammals, a hemolysis test was used. Bacteria were plated into the petri dish containing 5% sheep blood media agar and each of the plates were then incubated for 24-48 hours at 37^{o}C using Sorokulova *et al*., (2008) method.

C. Hypersensitive reaction (HR) test: Tobacco cultivar (*Nicotiana tabacum* L) leaves were used to test the HR-inducing ability. Inoculation by injecting the intercostal area of young and fully expanded leaves was carried out with an 18-gauge sterile syringe using sterile water as a control. Routinely, the tobacco plants were kept in a growth chamber under laboratory conditions. Under these conditions, were investigated the pathogenic strain that showed developed necrotic symptoms or a typical HR within 12 hours (Vanneste *et al*., 1990).

D. Phosphate Solubilizing Activity: Phosphate solubilizing microorganisms were routinely screened by a plate measure technique utilizing *Pikovskaya* (PVK) agar medium supplemented with 1.5% Bacto-agar. Four bacterial strains for each plate were placed in triplicate using sterile toothpicks. Colony diameters and halo clear zone were measured 4 days after the bacteria were plated on PVK media at 28^{o}C. Halo clear zone size was calculated by subtracting colony diameter from the total diameter (Srivastava *et al*., 2010).

E. Nitrogen Fixation: Nitrogen fixation ability was tested by growing isolates as much as 1mL in 10mL of semi-solid Nitrogen Fixing Bacteria (NF B) medium. Incubation was performed between 4-7 days until the colour changed from green to greenish blue media, as well as emerging of pellicle/ring bacteria in the media (Grobelak *et al*., 2015).

F. In *Vivo* Assay: Effects on seedlings development: to study the ability of our endophyte strains as plant growth promoters Bacteria (PGPB) we used the modified method described by Nejad *et al*., (2000). Twenty-three bacterial strains were examined to induce the eggplant seeds growth in in *vivo* conditions. Eggplant seeds steeped into bacterial suspension were cultured into 100% *Neutron Broth* (NB) medium and shaken for 48 hours, and then the seed were treated by bacteria suspension and plated onto sterile soil mixed with plant compost with ratio 1:2 in the experimental growth trial (2 seeds/trial units, with 5 replications /strains). Distilled sterilized water was used as control treatment. Seeds were handled with bacterial suspensions (20 ml/seed) and incubated under greenhouse condition. 3 weeks after incubation the effect of bacteria germination was evaluated by measuring the stem height and root length and comparing the outputs with the control for each isolate.

G. Data Analysis: Data analysis in this research was done using the SAS software. All data was analysed by Duncan's multiple range tests at 5% probability level (Sana Hanif *et al*., 2014).

RESULTS AND DISCUSSION

Isolation of Endophyte Bacteria: After the procedure of exploration in this research, we found about 195 isolates of endophyte bacteria which were isolated from several parts of the *Jatropha curcas* L. (Jarak Pagar). These included 95 isolates from the root, 45 isolates from the stem, 8 isolates from the leaves, and 47 isolates from the fruits as showed in Table 1. The abundant of endo-phytic bacteria isolated from the root was more than any of the other parts because most endo-phytes belong to the rhizospheric bacteria group. The average population of endophyte bacteria colonies and colony forming units (CFU) per gram of plant material was 10^{-4}-10^{-5} from plant part for each sample. The purification of isolated bacteria was performed using 100% TSA media. Previous researchers have demonstrated that the population density of endophytic bacteria is better developed in TSA medium than in other media (Hung and Annapurna 2004). Lopez *et al*., (2011) reported a lower population of endophyte bacteria in wild types plant growing in the deserts than the ones cultivated in plantations which support Munif *et al*., (2012) assertion that the dynamics of microbial populations is affected by various abiotic and biotic factors. Many endophytic bacteria invade plant tissues using mechanisms similar to pathogens, the use of hydrolytic enzymes, or herbal (e.g. Stomata) or synthetic (wound) openings; but their population density is usually lower than pathogens. Most of these endophyte bacteria are yet to be diagnosed through the aid of vegetation but rather they are seen to be potential pathogens. Endophyte bacteria depend heavily on nutrients furnished by the host plant. For this reason, variables affecting plant nutrition also influence the endophyte communities (Bashan *et al*., 2014).

Table 1 Total number of collected isolates of Endophytic Bacteria from *Jatropha curcas* L.

No	Plant parts	∑ Endophyte	Hemolysis test			∑ HR		∑Selected isolates
			α	β	λ	+	-	
1	Roots	95 isolates	9	0	86	65	21	12
2	Stem	45 isolates	2	28	15	5	10	5
3	Leaves	8 isolates	0	0	8	4	4	3
4	Fruits	47 isolates	0	0	47	38	9	3
	Total	195 isolates	11	28	156	112	44	23 isolates

∑= Total Number

Biosafety Assays:

The obtained bacterial isolates from the different plant parts were examined and the pathogenicity of the bacterial strains hypersensitive reaction was tested as described in the methodology. These bacteria were divided into two groups according to their pathogenicity ability reaction in tobacco leaves as shown in Figure 1. Tobacco (*Nicotiana tabacum*.L) leaves were used as host indicator for necrotic reaction. After the bacterial suspension was incubated for 48 hours, they were then injected into the tobacco leaves. After 48 hours of inoculation, For the hypersensitive test, we obtained 112 positive isolates due to the fact that injected leaves showed necrotic symptoms (positive hypersensitive) and the rest of the 44 isolates were negative hypersensitive. One of the requirements of a bacterium to be used as a biological agent is that it should not be pathogenic. By this, the microbes are not pathogenic or are not potentially pathogenic to the plant. Baker and Cook (1974) mentioned that antibiotics (secondary metabolites) that produced an antagonist agent should not cause damage to their host. Other hand, according to the hemolysis test there were about 156 isolates were assumed to be non-pathogenic bacteria for mammalians based on the hemolytic test result. Another requirement of a microbe/bacterium is it should be an antagonist agent to other nematodes or other bacteria but also not pathogenic to humans or mammalians, therefore require a hemolysis test to ascertain the status of erythrocytes in blood cells (Segel 2011). Bauman (2007) reported that some hemolysin enzymes which are from both gram-negative bacteria such as *Escherichia coli, Pseudomonas aeroginosa, Serratia* sp and gram-positive including *Streptococcus* spp., *Staphylococcus aureus*, *Listeria* sp. can lead to lysis of erythrocytes.

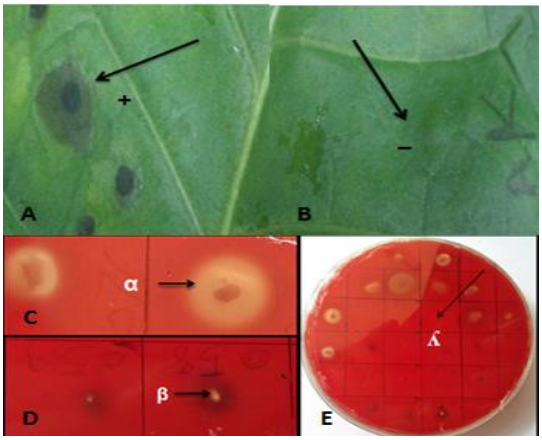

Fig.-1: Hypersensitive reaction result: A) Positive hypersensitive assay causes necrotic area, B) negative hypersensitive non-pathogenic bacteria; C and D) Haemolysis assay in endophyte bacteria isolates from *jatropha curcas*; arrows indicating that α-hemolysis and β-hemolysis are pathogenic bacteria for mammalians and E) λ-hemolysis indicate non-pathogenic bacteria for mammalians.

In *Vivo* Assay and Bacteria biochemical properties: The PGP test showed that some of the bacteria had a good result to enhance plant height and root length as compared with the control. From our experiment, some of the strains produced a higher result that promoted eggplant growth, and the best isolate were FJS23 and FJS24. After a biochemical examination on selective medium under laboratory condition, some of the isolates exhibited different attitudes. For instance, FJS23 was positive for phosphate solubilizing and negative for nitrogen fixing test while FJS24 showed positive for phosphate solubilizing test and negative for nitrogen fixing. We can, therefore, conclude that the microorganism interaction with their surrounding environment can affect the plant. On the other hands, some of the isolates also showed a moderate response to enhance the eggplant seedlings height and root length. These responses were good after been examined biochemically. Twelve isolates out of the 23 selected endophytic bacteria had the ability to solubilizing phosphate when they were examined using the Pikovskaya's selective media with 5 isolates detected as nitrogen fixing bacteria.

Table 2: PGPB result of examined eggplant seedlings height, root length, Phosphate Solubilizing and nitrogen fixation test.

Strains code	Plant height	Root length	Phosphate Solubilizing	Nitrogen fixing
RJS175	5.70^{defg}	3.98^{abc}	+++	-
RJS176	5.10^{efgh}	3.98^{abc}	+	-
RJS177	5.56^{defg}	4.10^{ab}	+++	-
RJS178	7.24^{b}	4.44^{ab}	+	-
RJS179	5.98^{bcdef}	3.80^{abcd}	+	-
RJS181	5.08^{efgh}	3.62^{abcd}	-	-
RJS184	4.82^{fgh}	3.58^{abcd}	++	-
RJS185	6.22^{bcde}	3.86^{abcd}	+	-
RJS186	6.52^{bcd}	4.48^{ab}	+++	++
RJS188	6.64^{bcd}	3.96^{abc}	+	-
RJS189	5.86^{cdefg}	3.30^{bcde}	+++	-
RJS161	5.74^{cdefg}	2.86^{bcde}	-	+
FJS16	4.58^{gh}	3.04^{bcde}	-	-
FJS17	7.07^{bc}	4.10^{ab}	-	-
FJS37	5.32^{defgh}	3.60^{abcd}	-	+
SJS54	5.06^{efgh}	2.36^{cdef}	-	+++
SJS57	4.12^{h}	2.28^{def}	-	-
SJS60	4.72^{fgh}	3.00^{bcde}	-	+
LJS67	4.54^{gh}	1.94^{ef}	-	-
LJS69	5.06^{efgh}	1.88^{ef}	-	-
LJS70	4.18^{h}	1.30^{f}	-	-
FJS23	8.64^{a}	4.22^{ab}	+	-
FJS24	8.54^{a}	5.06^{a}	++	-
CONTROL	6.24^{bcde}	2.86^{bcde}		

Digits followed by the same letters are not significantly different. Data have been analyzed by SAS software using Duncan Multiple Ranges Test (DMRT). α=5%. (+) means positive result; (++) means moderate positive result; (+++) means strongly positive result; (-) means negative result for test.

Some known PGPB endophyte bacteria such as rhizosphere, rhizoplane, and phyllosphere found in free soil are beneficiary bacteria for plant growth. Some of these beneficiary bacteria have been studied and their role to enhance plant growth using different mechanisms has increased the ability of plants to produce some nutrient compounds such as fixing nitrogen and help the plant to absorb phosphate from the soil and produce IAA hormones and also activate the plants resistance system against pathogenic infection by inducing the systemic resistance ISR and SAR. Such function beneficiary bacteria are defined as plant growth-promoting bacteria (Bashan and de-Bashan 2005). SAS – Duncan Grouping test was used to analyze our data to show the differences among bacterial strains that were examined to enhance the plant growth.

Due to agrochemical compounds residues used in agriculture, there is an urgent need for our essential crops to have increase resistance to viruses, insect, and pests, tolerance to drought and warmth, salt, for better fine grain and other merchandise and for the maximum and to improved systems of nitrogen fixation (Cocking and Edward 2003). Although there are many p-solubilizing microorganism and fungi, the suppression of pathogens by using these organisms is yet to be reported (Rudresh *et al*., 2005). *Jatropha curcas* can be developed on marginal land where the soil is poor in nutrients; it also has high demand for nitrogen more than others vegetables and crops, therefore, there are some suspected bacteria strains such as R4368 (*Enterobacter* sp. strain) that are related to rhizosphere known as nitrogen-fixing bacteria was used to fixed atmospheric nitrogen in *vivo* and improves the growth and seed productivity of *J. curcas* as reported by Madhaiyan *et al*., (2014). Some of the free-soil bacteria are capable of solving the soil phosphate to available to uptake by plant roots. This microorganism's Microbial biomass assimilates soluble P and prevents it from adsorption or nitrogen fixation (Niaz Ali Sial *et al*., 2015).

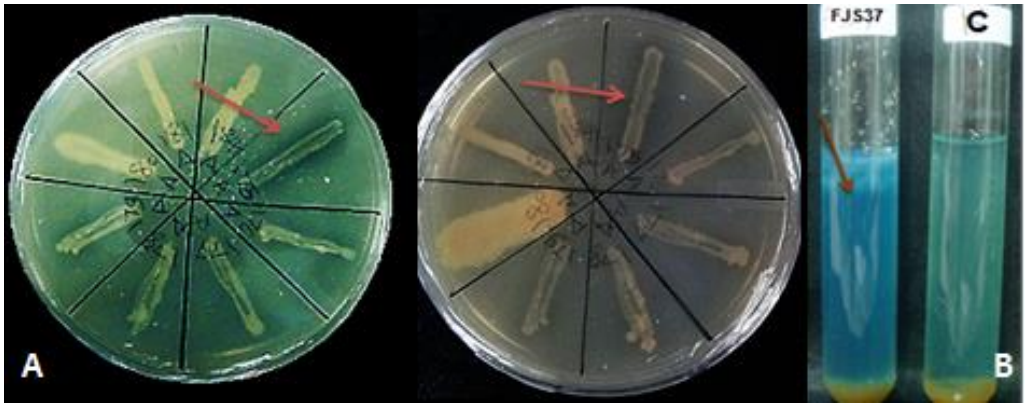

Fig. 2: A. the phosphate solubilizing bacteria colony has been surrounded by clear zone in the selective medium; B. The fixing nitrogen bacteria formed pellicle/ring into the NFB media into the test tube.

Based on the genotypic biotic system experiments result, we found out that some of our bacteria did not give perfect response to increasing the seedlings height when they were applied directly into the plant seed treatment; yet, other isolates had stronger potential for promoting seedling growth (stem and root) due to the assertion that growth - regulating substances might have been involved. Madhaiyan *et al*., (2015) in his research, isolated 1017 endophytic bacteria from different parts of jatropha and examined them for nitrogen fixing; 69.1% of these bacteria were isolated from leaves. The genera *methylobacterium* were identified to have colonized the leaf tissue and can serve as an important trait for jatropha, and it may be signifycant in contributing to increasing the plant drought and low soil nutrition tolerant.

CONCLUSIONS

In this study, were finding out that *Jatropha curcas*. L. is a unique plant that can be utilized by a human in different fields of life. We also screen this plant for best bio-control and growth promoter endophytic bacteria agents. From different parts of the Jatropha plant, Twenty-three out of 44 selected isolates were examined for their biochemical property and their ability to enhance plant growth of eggplant seeds. From the selective mediums result of biochemical assays, 12 isolates had good response/result as it relates to phosphate solubilizing while 5 isolates had a good response for nitrogen fixing. These isolates are therefore assumed as best candidates when they will use to stimulate the plant's growth.

ACKNOWLEDGMENT

I would like to thank Dr. Abdul Munif and Dr. Kikin Hamzah Mutaqin for the support and pieces of advice during this research. Special thanks to my parents, my colleagues, and friends who always motivated me during this study.

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