

SCREENING AND CHARACTERIZATION OF RHIZOBACTERIA FOR ENHANCING GROWTH AND CHLOROPHYLL CONTENT OF SWEET SORGHUM (*Sorghum bicolor* L. Moench)

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ABSTRACT

Sweet sorghum (*Sorghum bicolor* L. Moench) is a potential source of new and renewable energy as it has high adaptability in marginal land. In addition, development of sweet sorghum as a source of bioethanol in marginal lands is not competing with food production. Utilization of potential microbes such as arbuscular mycorrhizal fungi (AMF) and rhizobacteria is a solution to maintain productivity level of sweet sorghum. Screening of rhizobacteria for enhancing growth and chlorophyll content of sweet sorghum was conducted at the Biotech Center, Agency for the Assessment and Application of Technology, Puspiptek Serpong Tangerang, Indonesia from March to November 2012. One hundred and forty four rhizobacteria were isolated from 40 sample soils of rhizosphere of sorghum, rice and maize plants. Based on selection results, twenty five rhizobacteria isolates showing highest performance for increasing growth and chlorophyll content of sweet sorghum were characterized on their ability to fix N₂, solubilize phosphate and produce phytohormone such as indole-3-acetic acid (IAA), gibberellic acid (GA) and 6-benzylaminopurine (cytokinin). The highest concentration of fixed N₂ was about 337.51 nmol h⁻¹ mL⁻¹ produced by SL66 isolate. The highest concentration of solubilized phosphate was about 67.32 μmol mL⁻¹ produced by SL64 isolate. Twenty four isolates could produce IAA and twenty one isolates could produce GA, but only eight isolates were able to produce cytokinin. The highest concentration of IAA was produced by LR73 isolate, while ML14 and LR73 isolates produced the highest concentration of GA. The highest concentration of cytokinin was produced by LR73 isolate. The best results in increasing growth and chlorophyll content of sweet sorghum were plants inoculated with LR73 and JR80 isolates. Molecular identification based on 16S rRNA gene sequence showed that LR73 isolate was similar to *Mycobacterium senegalense*, while JR80 was similar to *Bacillus firmus*.

Key words: chlorophyll, phosphate solubilizing, N₂ fixing, phytohormone production

INTRODUCTION

Development of sorghum cropping systems, especially in marginal lands showed a low level of productivity (El-Lattief, 2011). One of the solutions to increase sorghum productivity is the use of soil microbes that live in the rhizosphere of sorghum and interact with plant roots system. It has been reported that rhizobacteria could be associated with *Sorghum bicolor* L. (Basaglia et al. 2003). Increasing crop productivity by rhizobacteria take place through direct or indirect mechanisms. The

direct mechanisms involve N₂ fixing, phosphate solubilizing, siderophore production and phytohormone production, while the indirect mechanism involve production of chemical compounds that can control pathogens. A bacterium could have different abilities at different times in the plant life cycle (Timmusk et al, 2003). It is widely reported that many species of rhizobacteria are able to colonize sorghum, maize, cassava and sugarcane and increase the growth and productivity of plants (Dobereiner et al. 1997; Boddey et al. 2003; Cocking, 2003; Herschkovitz et al. 2005).

Some rhizobacteria, such as *Azospirillum brasilense*, *Acetobacter*, *Herbaspirillum* and *Azotobacter* sp. could increase productivity of plants by fixing N₂ or producing phytohormones (Basaglia et al. 2003; Kennedy et al. 1997). Several other rhizobacteria such as *Arthrobacter ureafaciens*, *Phyllobacterium myrcinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. have been reported as phosphate solubilizing bacteria after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by secreting organic acids (Chen et al. 2006). *Pseudomonas fluorescens* has the ability to solubilize three type of inorganic phosphate such as tricalcium phosphate, aluminum phosphate and ferric phosphate (Henri et al. 2008). Synthesis of phytohormones by rhizobacteria was probably responsible for increasing root proliferation and consequently increase nutrients uptake from the soil (Fallik et al. 1994). A number of rhizobacteria have ability to produce IAA (indole-3-acetic acid) (Ravi et al. 2002) while *Bacillus pumilus* and *Bacillus licheniformis* produce GA (gibberellic acid) (Gutierrez-Manero et al. 2001).

IAA is a phytohormone which is known to be involved in root initiation, cell division and cell enlargement (Salisbury, 1994). Most commonly, IAA produced by rhizobacteria are believed to increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from the soil (Vessey, 2003; Zakharova et al. 1999). GA is a phytohormones most commonly associated with modifying plant morphology by extension of plant tissue, particularly stem tissue (Salisbury, 1994). Some rhizobacteria could also produce cytokinin, such as *Halomonas desiderata*, *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *B. megaterium*, *B. cereus*, *B. subtilis* and *Escherichia coli* (Hussain and Hasnain, 2009). Ryu et al. (2003) reported that cytokinin production by bacteria could increase the growth of *Arabidopsis* by stimulating cell division in plants. However, the recent discoveries of cytokinins and GA produced by rhizobacteria opened the possibility that even more plant growth-regulating substances may be involved in the promotion of plant growth by some rhizobacteria.

This study sought to determine the ability of several rhizobacterial strains isolated from sorghum, rice and maize plants to fix N₂, solubilize insoluble phosphate and produce phytohormones (particularly IAA, GA and cytokinin) and their effects on the growth and chlorophyll content of sweet sorghum (*Sorghum bicolor* L. Moench).

MATERIALS AND METHODS

The experiments were conducted in a greenhouse at the Biotech Center, Agency for the Assessment and Application of Technology, Puspiptek Serpong Tangerang, Indonesia from March to November 2012.

Isolation of Rhizobacteria

Soil samples were collected from the rhizosphere of sorghum, rice and maize plants located in six agro-ecological zones of Indonesia, such as Jasinga Bogor, Sulusuban Lampung, Jember Jawa Timur, Malang Jawa Timur, Sukabumi Jawa Barat and Medan Sumatera Utara.

Rhizobacteria were isolated by using Trypticase Soy Agar (TSA) medium. The serially diluted soil samples were plated on TSA medium. After three days of incubation at 28°C, colonies that

showed good growth were subcultured in fresh TSA medium. One hundred and forty four bacterial isolates were screened for the ability to increase plant height and chlorophyll content index (CCI) of sorghum plants compared to uninoculated plant as control.

Preparation of Inoculants

Bacterial inoculants were prepared by transferring a loopful of each culture from 48 h old culture to 50 mL nutrient broth. The flasks were incubated at 28°C for three days. The standard population of each bacterium was 1.0×10^8 CFU per mL. Ten mL of each broth was centrifuged at 7000 xg for five minutes. Each of bacterial cell was dissolved with distilled water and then used as inoculant.

Effect of Bacterial Inoculation on Plant Growth

The experiments were done in pot culture in a green house. The soil media used was sand and soil (1:1). All materials were sterilized by fumigation using tetrahydro-3,5,-dimethyl-2H-1,3,5-thiadiazine-2-thione. Each pot was filled with 2000 g of soil media. Each treatment was replicated 3 times.

Seeds of sweet sorghum were surface sterilized with 95% ethanol and 2.5% sodium hypochlorite. At two days after germination, 1 mL of bacterial culture (1.0×10^9 CFU per mL) was inoculated to the rhizosphere of sorghum. Plant height was examined every two weeks, whereas CCI (Chlorophyll Content Index) was examined 60 days after treatment.

Twenty five isolates that showed the best impact to increase plant height and chlorophyll content were characterized on their ability to fix N_2 , solubilize insoluble phosphate and produce phytohormones (IAA, GA and cytokinin).

Determination of Chlorophyll Content Index (CCI)

Chlorophyll content index (CCI) was measured using a chlorophyll content meter CCM-200 plus (Ghasemi *et al.*, 2011). The CCM-200 plus used absorbance to estimate chlorophyll content in the leaf tissue. Two wavelengths used for absorbance determinations were 660 and 940 nm. One wavelength was the chlorophyll absorbance range while the other served to compensate for mechanical differences such as tissue thickness. The meter measured the absorbance of both wavelengths and calculated a chlorophyll concentration index (CCI) value that was proportional to the amount of chlorophyll in the sample.

Determination of N_2 Fixing

The ability to fix N_2 for each isolate was measured by acetylene reduction assay (ARA) technique as described by Bergersen (1980). A number of researches related to fixed biological nitrogen showed that nitrogenase enzyme could reduce acetylene gas to ethylene gas. Both gases could be measured at low concentration using gas chromatography. The ARA method was reliable to estimate the nitrogenase activity.

Bacterial inoculants were prepared by transferring a loopful of culture from 48 h old culture to 50 mL N-free bromothymol broth. The content in flasks were incubated at 28°C for five days. Incubation vessel used to assay was determined based on size and composition of sample. During sample incubation there should be no exposure of sample to oxygen. A standard curve of standard acetylene were prepared prior to acetylene measurement. A Shimadzu 17A gas chromatograph was

used with a capillary column (30 m x 0.32 mm), SH-Rxi-17 with crossbond diphenyl dimethyl polysiloxane. Temperatures used were 35°C (oven), 40°C (injector) and 200°C (FID detector). Carrier gas used was helium.

Determination of Phosphate Solubilizing

Modification of methods described by Alam et al. (2002), He and Honeycutt CW (2005) and Dudhagara et al. (2008) were used. One milliliter suspension of inoculant in sterile aquadest was inoculated to 100 mL Pikovskaya medium in 250 mL flask. It was incubated at 150 rpm in a shaker incubator for seven days at 35°C. Medium without inoculant was made as negative control. The pH was recorded daily using Digital pH meter. After 7 days of incubation, 10 mL sample was centrifuged for 15 minutes at 1500 xg. The supernatant was extracted using filter paper. The supernatant was used to measure the pH and soluble phosphate content. Supernatant was reacted by reagent A (ascorbic acid 0.1 M and trichloroacetic acid 0.5 M) and reagent B (ammonium molybdate 0.01 M). Absorbance was measured using UV Spectrophotometer (UV 160A Shimadzu) at 850 and 700 nm in a plastic cuvette (He and Honeycutt, 2005).

Determination of IAA

The method for determining of IAA production was described by Naher et al. (2009). Isolates were inoculated to Jensen's broth (Nagananda et al. 2010) with addition of tryptophan (2 mg mL⁻¹) and incubated at 28 ± 2°C for 48 h. The culture was centrifuged at 7000 xg for 7 min and 1 mL of supernatant was mixed with 2 mL of Salkowsky's reagent. The IAA concentration was determined using UV spectrophotometer (UV 160 A Shimadzu) at 535 nm.

Determination of GA

Jensen medium (Nagananda et al. 2010) was used to cultivate microbial isolates. The extraction and measurement of GA were based on method described by Berrios et al. (2004). Broth samples were centrifuged to remove cell biomass. An aliquot expected to contain between 2 and 6 mg of GA was transferred to a 100 mL separating funnel. Distilled water was added to make up to 10 mL. The pH of the solution was adjusted between 1 and 2 using 0.1 M HCl. Twenty mL of ethyl acetate was added and vigorously shaken for 60s. The aqueous phase was then transferred to a second separating funnel and the extraction procedure was repeated by adding another 20 mL ethyl acetate. The aqueous phase was then discharged and organic phase was transferred to the first separating funnel. The GA was re-extracted from ethyl acetate with successive portions of 20, 15 and 10 mL phosphate buffer (pH 7.4), shaken each time for 60s, and combined each extract in a 50 mL volumetric flask. The volume of this flask was made up with phosphate buffer.

For determination of GA, five mL standard solution and sample were placed in a 25 mL volumetric flask. Five mL 3.75 M HCl was added to the flask, vigorously mixed for 10s and then incubated at room temperature for 75 min. The absorbance of the resulting solution was measured by spectrophotometer (160 A Shimadzu) at 254 nm. The concentration of GA in the sample was determined using linear regression equation of standard graph.

Determination of Cytokinin

Cytokinin was determined using a modified method described by Hussain and Hasnain (2009) and Patel et al. (2012). Each bacterial isolate was maintained in a liquid medium M9 with addition of 0.2% tryptophan, 0.01% thiamin, 2 µg L⁻¹ biotin and 9g L⁻¹ bacto agar. That medium was incubated on a shaker 100 rpm at 28°C for 96h.

Bacterial culture was centrifuged at 16000 xg at 4°C for 10 min. The pH of supernatant was adjusted to 7.0 using 7 M NaOH and 7 M HCl. This solution was transferred to a funnel and the extraction proceeded by using 15 mL ethyl acetate. The ethyl acetate solution was dried in air and the final residue was re-suspended in 1 mL methanol.

The ethyl acetate fraction was plated on TLC plates (silica gel 60F - f 254, thickness 0.25 mm) and developed in n-butanol: acetic acid : distilled water (12:3:5) v/v. Spot with R_f values identical to standard (6-benzyl aminopurin) was identified under UV light (254 nm). Bands detected were scraped from the TLC plated and dissolved in methanol and analyzed by HPLC. 6-benzyl aminopurin was separated on a reversed phase C-18 column (25 cm x 3.0 mm) HPLC (Shimadzu). Sample was analyzed under isocratic condition with 70% methanol. The UV detector was set at 254 nm. The total run time for separation was approximately 30 min at a flow of 1 mL min⁻¹.

Identification of Bacterial Strain

Identification of bacterial isolates were based on analysis of 16S rRNA. DNA was isolated using the FastPrep, special kits for DNA isolation. DNA amplification was done using PCR with specific primers (Kary Mullis, 1987). 16S rRNA genes obtained were sequenced using Dye terminator sequence by V.3.1 cycle sequencing kit. Sequences were compared with a database that was available in NCBI using Blast search engines (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was created using the program ClustalW (Mega 3.1) by comparing the DNA sequences of multiple bacterial species obtained from the genes in the NCBI database.

Statistical Analysis

Relationships between two or more parameters were determined using correlation analysis by SPSS 17.

RESULTS AND DISCUSSION

One hundred and forty four rhizobacterial strains were isolated from the rhizosphere of sorghum, rice and maize plants. Inoculation to sorghum showed that all isolates were able to increase plant height and chlorophyll content index (CCI) compared to the control (without inoculant). Twenty five isolates showing the best enhancing of plant height and chlorophyll content index (Table 1) were tested for their ability to fix atmospheric nitrogen, solubilize insoluble phosphate and produce phytohormones (IAA, GA and cytokinin).

All isolates were able to increase the height of plants and chlorophyll content index compared to the control (without inoculant). Plants inoculated by LR73 and JR80 isolates showed the highest increase in plant height and chlorophyll content index. Based on correlation analysis of two or more parameters above, results found that correlation between plant height and chlorophyll content was significant at the 0.01 level (0.973) and also between plant height and shoot weight (0.530), but correlation between shoot weight and chlorophyll content was significant at the level 0.05 (0.437). These results showed that plant height and chlorophyll content parameters had the significant relationship with the high level compared to other correlations.

Shoot weight could be a parameter determined by chlorophyll content, but in this case correlation between shoot weight and chlorophyll content was lower than the correlation between plant height and chlorophyll content. According to Biljana Bojovic (2005), the determination of chlorophyll content as an indirect method of estimating the productivity of vegetation represent a good way to gain an understanding of the photosynthetic regime of plants. So that, the growth of plant

was related to rate of photosynthesis took place on the leaf of plants, especially sorghum. However, the biggest part of the yield of cultivated plants is known to result from work of the photosynthetic apparatus, in which the chlorophyll molecule occupies a key place.

Table 1. The effects of 25 isolates of rhizobacteria on plant height, chlorophyll content and shoot weight of sweet sorghum at 60 days after treatment.

Code of Isolates	Host Plants	Plant Height (cm)	Chlorophyll Content Index	Shoot Weight (g)
Control	-	72.40 ± 2.26	17.00 ± 1.41	3.98 ± 0.09
LR74	Sorghum	86.00 ± 0.35	16.00 ± 0.71	5.48 ± 0.78
MR114	Sorghum	86.00 ± 0.64	16.90 ± 2.12	5.20 ± 0.98
LL118	Maize	86.00 ± 2.83	22.60 ± 1.41	2.50 ± 0.12
BR98	Sorghum	88.00 ± 2.67	21.80 ± 1.87	5.85 ± 0.66
ML59	Maize	89.00 ± 2.12	23.00 ± 1.41	5.08 ± 0.76
SL62	Maize	89.00 ± 0.71	24.20 ± 2.12	4.59 ± 0.24
ML29	Maize	90.00 ± 0.71	23.00 ± 2.12	10.31 ± 0.45
MDL68	Maize	90.00 ± 1.06	24.00 ± 0.00	7.53 ± 0.62
SL67	Maize	91.00 ± 0.71	23.90 ± 0.00	4.51 ± 0.04
MDR115	Sorghum	91.00 ± 1.06	23.90 ± 0.14	9.24 ± 0.91
JL57	Maize	91.00 ± 1.41	24.70 ± 0.13	6.27 ± 0.23
SL31	Maize	92.00 ± 0.71	25.80 ± 0.49	3.96 ± 0.35
JL53	Maize	92.00 ± 1.41	25.80 ± 0.47	6.22 ± 0.35
JR77	Sorghum	93.00 ± 1.41	26.00 ± 0.27	11.16 ± 0.78
BL116	Maize	93.00 ± 1.06	26.90 ± 0.71	7.67 ± 0.88
ML14	Maize	93.00 ± 4.95	26.60 ± 0.00	6.20 ± 0.56
SL19	Maize	94.00 ± 3.54	31.40 ± 0.00	3.91 ± 0.06
SL15	Maize	95.00 ± 0.35	31.30 ± 0.71	11.60 ± 0.56
MDR113	Sorghum	95.00 ± 0.35	31.70 ± 0.04	7.41 ± 0.65
JL41	Maize	96.00 ± 0.35	35.30 ± 1.41	6.43 ± 0.66
SL66	Maize	97.00 ± 0.35	35.20 ± 1.41	4.58 ± 0.32
LL37	Maize	98.00 ± 0.71	35.40 ± 1.14	8.17 ± 0.65
SL64	Maize	100.00 ± 0.00	40.50 ± 0.71	14.20 ± 0.89
JR80	Sorghum	102.00 ± 2.12	40.50 ± 2.12	11.74 ± 0.68
LR73	Sorghum	103.00 ± 2.83	42.40 ± 1.41	11.98 ± 0.99

Each of rhizobacterium was varied in its ability to fix N₂, solubilize insoluble phosphate, and produce phytohormones (IAA, GA and cytokinin). By using acetylene reduction assay (ARA) technique, strains of nitrogen fixing bacteria could fix N₂ at about 9.33 nmol h⁻¹ mL⁻¹ to 337.51 nmol h⁻¹ mL⁻¹ measured at 5 days after incubation (Fig.1). The highest N₂ fixing was demonstrated by SL66 isolate (337.51 nmol h⁻¹ mL⁻¹), whereas the highest value on solubilizing insoluble phosphate was showed by SL64 isolate (67.32 μmol) (Fig.2).

The plant growth promoting substances (phytohormones) were produced as a result of bacterial activity with tryptophan as precursor. Twenty four isolates could produce IAA and twenty one isolates could produce GA, but only eight isolates could produce cytokinin (Fig.3). The IAA, GA and cytokinin concentrations from each isolate varied widely. The highest IAA concentration was produced by JR80 isolate, whereas ML14 and LR73 isolates produced the highest GA, respectively. The highest cytokinin concentration was produced by LR73.

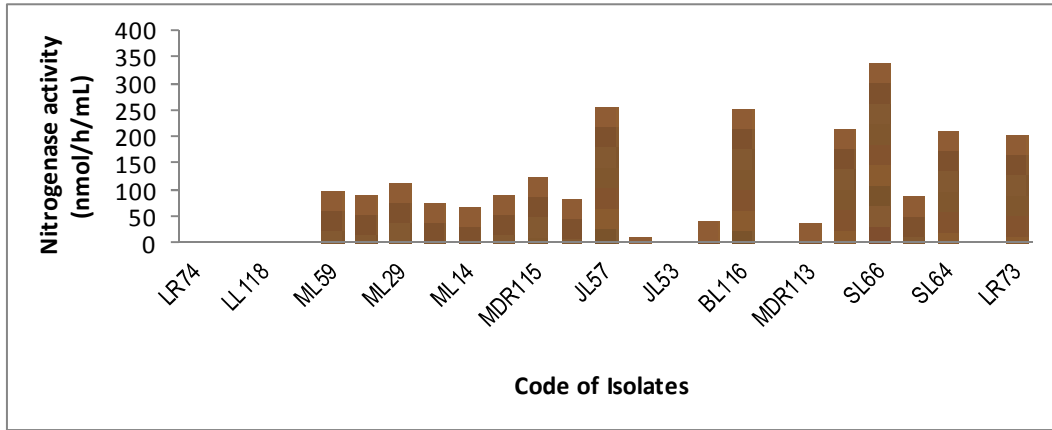


Fig. 1. Nitrogenase activity of each isolate

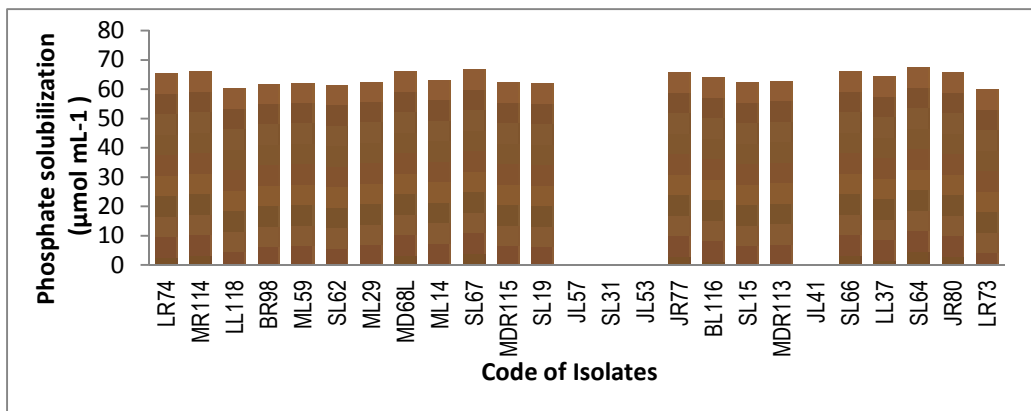


Fig. 2. Phosphate solubilization of each isolate

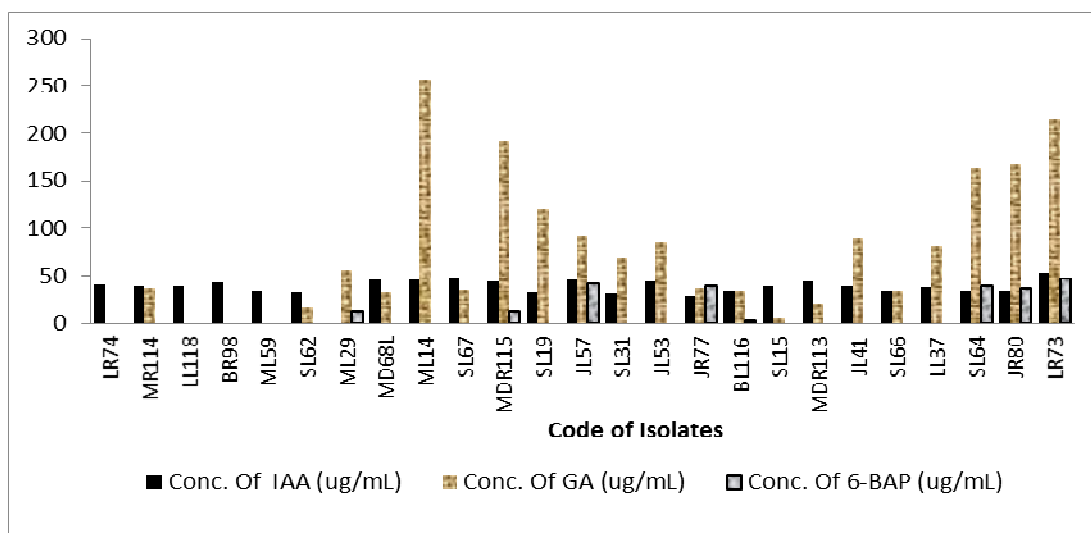


Fig. 3. Phytohormone production of each isolate

Based on correlation analysis between two parameters, only correlation between GA and cytokinin (6-BAP) concentrations was significant. The values of plant height and CCI of sorghum were not directly related to nitrogenase activity (ARA), insoluble phosphate solubilization and the concentration of phytohormones (IAA, GA and 6-BAP) produced by these isolates. These results suggested that there was considerable influence from the ability of these isolates to fix N₂, solubilize insoluble phosphate and produce phytohormones.

The difference on the concentration of phytohormone produced by each isolate and the diversity of bacterial strains producing these substances caused different effect on the growth of sorghum plant. According to Takahashi (1986), these mechanisms were quite complex. The concentration of phytohormone was one of the variables that can cause differences in response. IAA could induce and stimulate root and stem cell elongation, cell dividing, xylem and phloem element differentiating, and root formation. Biochemically IAA could activate specific enzymes and stimulate biosynthesis of other compound which could act on the processes of plant growth. Whereas, GA (gibberelic acid) could act on cell elongation and the processes of cell dividing of plant tissue cell. Fried and Hademenos (2006) explained that cytokinin was compounds with a structure resembling adenine (adenine derivatives) which can stimulate cell division in plants. Cytokinin can interact with auxins (IAA) to determine the differentiation of meristematic tissues. Cytokinin was also necessary for the formation of such a chloroplast organelle and may play a role in flowering, fruit development and seed dormancy termination. In this study, the highest cytokinin (6-benzyl aminopurine) concentration was produced by LR73 isolate. This isolate had the highest CCI and plant height suggesting that the ability to produce cytokinin by LR73 isolate was related to increased chlorophyll content and plant height. However, the ability of LR73 and JR80 isolates to produce IAA, GA, cytokinin and the combination of these phytohormones might be related to increased chlorophyll content and plant height.

Molecular identification based on 16S rRNA gene sequence showed that LR73 isolate was similar to *Mycobacterium senegalense* with the similarity level at 99%, while JR80 was similar to *Bacillus firmus* with the similarity level at 100% (Fig.4).

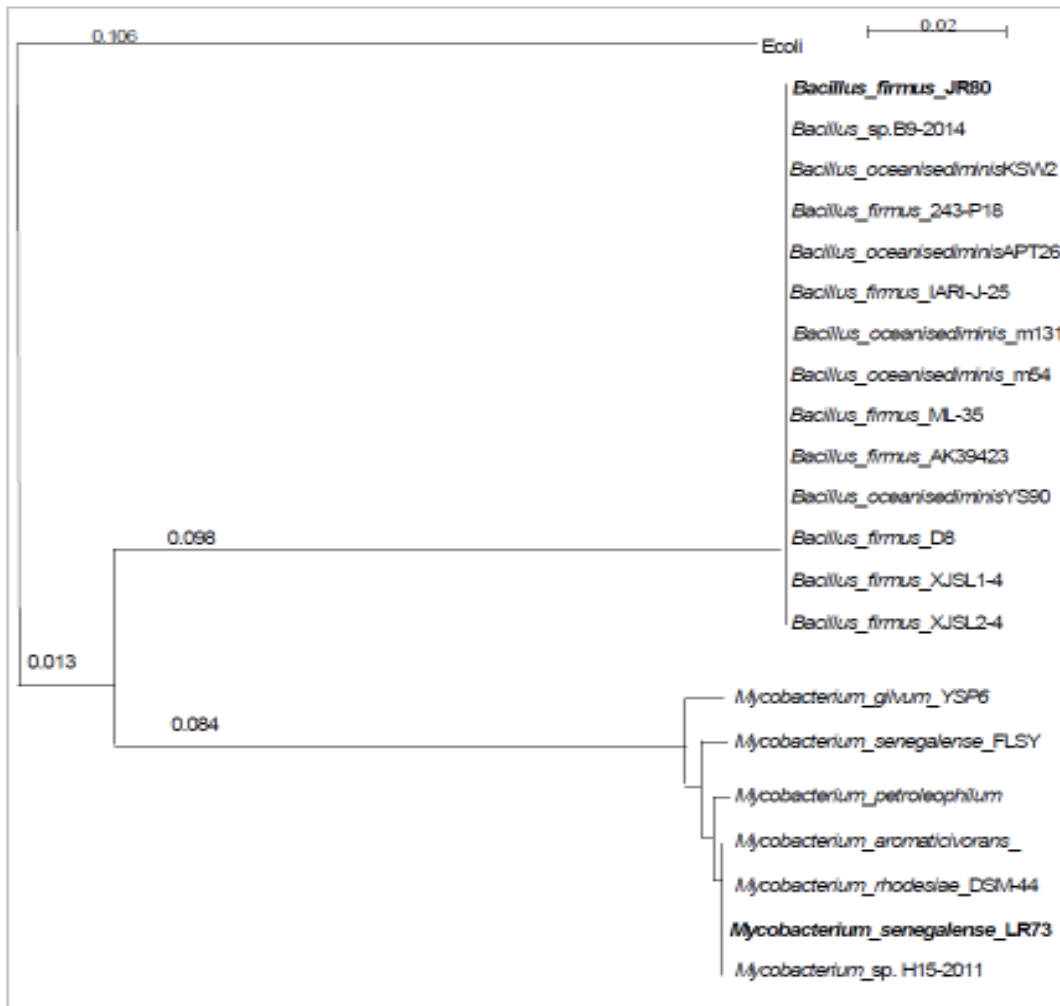


Fig. 4. Phylogenetic tree of *Mycobacterium senegalense* LR73 and *Bacillus firmus* JR80

CONCLUSION

The ability of each rhizobacterial isolate to fix N₂, solubilize insoluble phosphate and produce phytohormones (IAA, GA and cytokinin) varied among isolates. These results might improve the growth and chlorophyll content of sorghum plants.

The ability of isolates *Mycobacterium senegalense* LR73 and *Bacillus firmus* JR80 to produce IAA, GA and cytokinin or the combination of these phytohormones could be responsible for increasing chlorophyll content and plant height indirectly.

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