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MOLECULAR IDENTIFICATION OF LOCAL CYANOBACTERIUM AND SCREENING FOR BIOHERBICIDE ACTIVITY IN MODEL PLANT

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Abstract

Cyanobacteria produce a wide range of secondary metabolites with promising possible applications in agriculture, human health, or industry. This study aims to identify cyanobacterium using the *rbcL* gene as a molecular barcode and screen for the herbicidal activity of water and ethanol crude extract of the strain. DNA of cyanobacterium strain was extracted with modified CTAB method and then amplified using 1AB_rbcL primers and sent for sequencing. BLAST analysis showed that the strain belongs to *Synechocystis* sp. The strain was then cultured in 2 L of f/2 culture medium for harvesting. Harvested *Synechocystis* sp. was extracted in water and 70% ethanol with the ratio of 1:3 (w/v) in an 80°C water bath for 2 hours and ethanol extract was evaporated at room temperature. The herbicidal activity was assayed by germinating sorghum in 0.5% extract with glyphosate (Roundup) as positive control and water as a negative control in 5 replications. Germination rate, shoot, and root length were analyzed statistically with one-way ANOVA in GENSTAT software, Duncan post hoc analysis was performed. The local axenic isolate of cyanobacterium from Indonesia was identified as *Synechocystis* sp. based on the rbcL gene sequence. The ethanol extract of *Synechocystis* sp. promotes germination in sorghum seeds compared to water extract, but both were still lower compared to the sorghum seeds germination under glyphosate-based herbicide treatment. The ethanol extract also showed higher root growth inhibition compared to the water extract, but both extracts had no activity on shoot inhibition of sorghum seedling compared to the negative control.

INTRODUCTION

Cyanobacteria are one of the most morphologically and genetically diverse groups of prokaryotes with various forms, from unicellular to colonial and filamentous forms. The traditional approach to classifying cyanobacteria includes morphological characteristics such as cell size, sheath feature, and colony form. Therefore, the identification of cyanobacteria based upon morphology sometimes becomes difficult as morphological traits may vary due to changes in environmental conditions. At present, the use of PCR is considered the most reliable means used to identify cyanobacteria [1]. Sequencing of 16S rRNA has confirmed the existence of several morphologically uniform and well-defined traditional genera including *Microcystis, Cylindrospermum*, *Planktothrix, Tychonema, Arthrospira, Microcoleus,* and others [2].

Cyanobacteria are prokaryotic bacteria. However, the cells developed the plant-type photosynthetic apparatus that included chlorophyll a and both photosystems. The *rbcL* gene is a part of the DNA sequence located in chloroplast DNA encoding the large subunit of ribulose 1, 5 bisphosphate carboxylase/oxygenase, an enzyme which involved in the first major step of carbon fixation during photosynthesis. The rbcL gene has been developed to be used as a DNA barcode in plants and other photosynthetic

organisms. The sequence data of the rbcL gene are widely used in the phylogeny study for plants. Singh et al. reported that phylogenomic and evogenomic analyses of the heterocystous cyanobacteria using the rbcL gene as a molecular marker provides better understanding in the evolution study of cyanobacteria and proposed the need for classificatory reamendments in the cyanobacterial taxonomy, the intermixing amongst both the orders and the polyphyletic origin of the Stigonematales [3].

Cyanobacteria dominate photoautotrophic, oxygen-producing microbes on earth and have long been gained attention on bioactive compound research in recent years. Cyanobacteria produce a wide range of secondary metabolites, some of the known metabolites exhibit algaecides, herbicides, and insecticides [4], or antifungal, antibacterial, and antiviral activities, with promising possible applications in agriculture, human health, or industry [5].

Allelochemicals, bioactive compounds for the inhibition of rival organisms, play a major role in a cyanobacterial niche competition, having an all-inclusive distribution and occupying a vast array of terrestrial and aquatic environments. Their origin since the Early Precambrian was one of the most important steps in evolution [2]. In scientific literature, there is an appreciable amount of studies on the potential use of cyanobacterial compounds in agriculture. Recent research by Brilisauer et al. on antimetabolite 7-deoxy-sedoheptulose produced by *Synechococcus elongatus* blocks the shikimate pathway to inhibit the growth of phototrophic organisms, making it potential for organic herbicide [5]. One of the most prominent antimetabolites that target the shikimate pathway is the synthetic herbicide glyphosate [6].

Since firstly synthesized in 1970, glyphosate usage has grown steadily and become the main component of various herbicides applied in agriculture in amounts of >800,000 tons per year [7]. The International Agency for Research on Cancer (IARC) in 2015 reported that glyphosate belongs in a 2A category as probably carcinogenic to humans. Therefore, several governments are still considering establishing restrictions or limits on the use of glyphosate in agriculture, including Indonesia. Glyphosate used by Indonesia reached 9.67 million kilograms of active ingredient per year. Glyphosate is mainly used for oil palm plantation by 63% in Indonesia [8] and has been reported to cause a major weed *Eleusine indica* classified as glyphosate-resistant in 5 out of 6 population in Tapanuli Selatan Regency, Sumatera Utara-Indonesia [9].

The present work was undertaken to identify local cyanobacteria isolate based on rbcL marker and to study the possibility of utilizing water, acidic, and alkali extract of the local cyanobacterium, as bioherbicide by inhibiting the growth of the phototrophic organism, in this study *Sorghum bicolor* was used as a model plant. The effects of the cyanobacterial extract were investigated on the germination and early growth of the model plant.

MATERIALS AND METHODS

Cyanobacterium Culture

Cyanobacterium strain was obtained from the Laboratory of Bioprocess, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor-Indonesia. Cells of cyanobacterium were sub-cultured with 30% (v/v) inoculum amount of in f/2 liquid media [10] in sterile conditions at temperatures between 21°C under the illumination of 800–1000 lux for approximately 1 week for DNA extraction. The same condition was applied for cyanobacterium extract production. Cyanobacterium cultivation was carried out in 250 ml batch in Erlenmeyer with continuous bubbling and in-line sterilized through a 0.45 µm membrane filter.

Molecular Identification

DNA Isolation and PCR Amplification of the rbcL Gene

Cells were harvested from 50 mL cyanobacterial culture by centrifugation 10,000 rpm 15 mins. DNA was extracted with CTAB method described previously by Orozco-Castillo et al. with some modification, then supplemented with 1% β-mercaptoethanol into lysis buffer [11]. CTAB lysis buffer was added (500 µl) into the sample and then vortex and incubated for 30 mins at 65°C. DNA was extracted with 500 µl CI and then precipitated in isopropanol. The amount and purity were determined in Nanodrop 2000 spectrophotometer (Thermo Scientific). DNA concentration was adjusted to 100 ng/µl and stored at -20 °C.

The rbcL gene was amplified using the primer pairs of 1AB_rbcL forward 5'-TCIGCIAARAACTAYGGTCG-3' and reverse 5'-GGCATRTGCCAIACRTGRAT-3 as described by Ghosh and Love [12]. This set of primers amplify DNA from Chlorophyta, Euglenozoa, and certain bacteria including cyanobacteria with product size ~615 bp. The PCR protocol included an initial denaturation of 95 °C for 3 mins followed by 40 cycles of 95 °C for 1 mins, 55 °C for 1 mins and 72 °C for 1 mins 30 s, and 72 °C for 15 mins final extension step. The PCR products were visualized on 1% agarose gel electrophoresis stained by GreenSafe DNA Gel Stain (Canvax Biotech). The purified DNA was commercially sequenced (First Base Lab, PT. Genetika Science).

Cyanobacterium Extract Production

The cyanobacterium extracts were made in two methods modified from Paulet et al., which were water extract and ethanolic extract [13]. The water extraction was performed with aquadest. A total of 1 L Cyanobacterium culture was harvested by centrifugation at 10,000 rpm for 10 mins. The

biomass was placed in a conical tube and diluted with aquadest in the ratio of 1:3. The mixture was incubated in a water bath at 80°C for 2 hours, chilled in room temperature, and followed by centrifugation at 10,000 rpm for 10 mins. The supernatant was withdrawn and stored at 4 °C until further use in the bioassay. The ethanolic extract was made in the same method but the cyanobacterium biomass was diluted in 70% ethanol and the solvent was evaporated in desiccator at the end of the process.

Bioassay on Model Plant and Data Analysis

The bioassay of water and ethanol extract was performed by germination test and early growth of sorghum in 5 replications modified from Mijani et al. [14]. Treatments including water extract 0.5% (v/v), ethanol extract 0.5% (v/v), aquadest (negative control), and Roundup® 0.5% (v/v) (positive control). Roundup® (Bayer-USA) was chosen to be positive control because it is a widely used broad-spectrum herbicide with glyphosate as active ingredient. The sorghum seeds (10 seeds per petri dish 9.0 cm) were placed on double-layered paper towel that had been moistened by dropping with each treatment solution using a syringe. A seed was considered as germinated when the radicle stood out through the seed coat. The seeds were germinated for 5 days and the germination rate was calculated by the ratio of germinated seeds per total seeds per petri dish. The seeds were left in the petri dish for 2 more days and small amounts of treatment solution were added as needed to prevent from drying. A picture of sorghum sprouts per petri dish was taken for image analysis.

Data Analysis

DNA sequence analysis was performed in Geneious Prime software 2021.2.2 (https://www.geneious.com). The consensus sequence was created from forward and reverse direction sequencing and then was BLAST (Basic Local Alignment Search Tool) against GenBank database integrated to the software. The percentage of identical sites and E-value were observed, and phylogenetic analysis was carried out by constructing the neighbour-joining tree with Tamura-Nei distances with the bootstrap method of 1,000 replicates, and *Microcystis* sp. was selected as the outgroup.

Image analysis was performed for the shoot and root length in ImageJ software. The bioassay results including germination percentage, shoot, and root length were analyzed using One-way ANOVA in GenStat software version 12.1.0.3338 with α 0.05 and Duncan post-hoc analysis was chosen.

RESULTS AND DISCUSSIONS

Molecular Identification of Cyanobacterium Strain

In this research, a local cyanobacterium strain from Indonesia was cultured and identified using molecular approach. The genomic DNA was extracted and quantified with spectrophotometry analysis. The DNA concentration was quite low which is 56.2 ng/µl with high contamination. The low ratio of 260/280 indicates the presence of contaminant by protein, phenol, or other contaminants that absorb at around 280 nm. The contamination of salt in the extracted DNA was also present by a low ratio of 260/230 at 0.73, the appropriate ratio should in the range of 2.0-2.2. The low absorbance ratio of 260/230 indicates the presence of contaminants that absorb light spectrum at 230 nm, such as salt or EDTA from the culture media or carbohydrates from the cell extract [15].

Quality check was performed for extracted DNA by running DNA in agarose gel electrophoresis and compare the band to λ DNA marker. The result of DNA quality check was shown in Figure 1(A). the extracted DNA from cyanobacterium strain Nsp showed the faint band at the lower size than λ DNA marker. This result indicates that the extracted DNA may be degraded during the extraction process and may also indicate a low amount of extracted DNA, which also correlates to nanodrop reading for low DNA concentration. Johnson and Kemp explained that extracted DNA with the presence of inhibition, especially ancient, degraded, and low copy number DNA extracts can be challenging for PCR. Some other inhibitors from DNA extraction, such as salts (e.g., sodium or potassium chloride) and phenol may also reduce the PCR efficiency, for the reason that PCR is dependent on enzymes, it is subject to inhibition which causes a failure to copy available DNA molecules [16].

A portion of the rbcL coding regions of a cyanobacterium strain Nsp was amplified and sequenced (Figure 1B). The visualization of the PCR amplification was a single bright band, which indicates that the primers could successfully bind to the template DNA and copy the partial rbcL gene region of the cyanobacterium strain. Sequence data analysis was performed in Geneious Prime software including trimming with error probability limit of 5%, assembly forward and reverse sequence, building consensus sequence, BLAST analysis, and generate a phylogenetic tree. A 730 bp consensus sequence was created and BLAST was performed and the result showed that Nsp strain has the closest similarity to *Synechocystis sp.* CACIAM 05 (GenBank accession number CP019225) at the level of

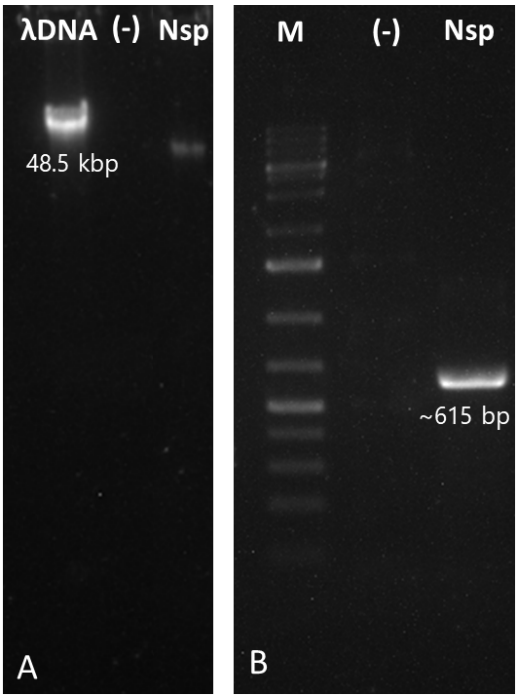

Figure 1: Visualization of (A) Quality check of genomic DNA against lambda DNA and (B) PCR amplification of cyanobacterium strain Nsp using the rbcL gene. λ DNA = 48.5 kbp. Marker (M) used is 1 kb + ladder and the target PCR product amplified sequence of ~615 bp (PCR product size is higher than bright band indicates the size is over 500 bp). Based on the agarose gel electrophoresis of genomic DNA extracted from *Synechocystis* sp., the DNA quality was fair with faint band below λ DNA marker (Figure 1A). The band over 500 bp showed in Figure 1B indicated that Nsp culture may belong to Chlorophyta, Euglenozoa, or certain bacteria including cyanobacteria. The amplification product was sent for DNA sequencing for species identification

99.6% and e-value 0.0. The percentage of similarity at >97.5% identity in DNA nucleotide sequence from a single gene locus defines as one species [17]. This, indicating that the set of primers flanking between 730 bp region of rbcL gene used in this study successfully identify local cyanobacterium from Indonesia as *Synechocystis sp.*

Synechocystis sp. CACIAM 05 has been defined as a unicellular coccoid, non-nitrogen (N2)-fixing cyanobacterium and a ubiquitous isolated from freshwater lake in Brazil. The full genome has published in NCBI in 2017 with 3,580,992 bp in length and contains 3,376 genes (https://www.ncbi.nlm.nih.gov/nuccore/1805894008).

Generally, the first *Synechocystis* to be firstly sequenced is *Synechocystis* sp. PCC 6803 in 1996 and became the first phototrophic organism to be fully sequenced which revealed the structure of the ~3.6-Mbp genome encoding 3,172 proteins [18, 19]. It has been one of the most popular organisms for physiological and genetic studies of photosynthesis for naturally transformable by exogenous DNA and grows heterotrophically at the expense of glucose [18].

Taxonomy and classification of cyanobacteria species have been extremely difficult work for long. The stable morphological markers, which were traditionally used for distinguishing taxa at the generic level, face great challenges for the relative morphological simplicity, rapid adaptations to various environmental conditions and combined with asexual reproduction [20]. Thus, made the physical or morphological characteristics not reliable for a sole-based-sources in species identification. The rbcL marker in this study was able to identify local cyanobacteria to a genus or strain level but not to a species level. There have been not many research using rbcL gene for identification marker in cyanobacteria. The most commonly used marker for assessing cyanobacterial diversity and phylogenetic designs is 16S rRNA [2, 3]. Therefore, 16S rRNA represents only a part of the whole genome and in some cases, the gene is too conservative and sequence identity masks species diversity [21]. Thus, rbcL gene is a potential alternative DNA marker for cyanobacteria identification.

Photographs of *Synechocystis* sp. under microscope showed round shape and small diameter. *Synechocystis* sp. cells in exponential growth showed to live either unicells or in colony of two cells (Figure 2A). There is no other clear morphological character identified under 400x magnification. Physiological characters of Cyanobacteria need longer time to observe, which became another challenge for species identification. In this study, growth profile of *Synechocystis* sp. was provided in Figure 3. It was

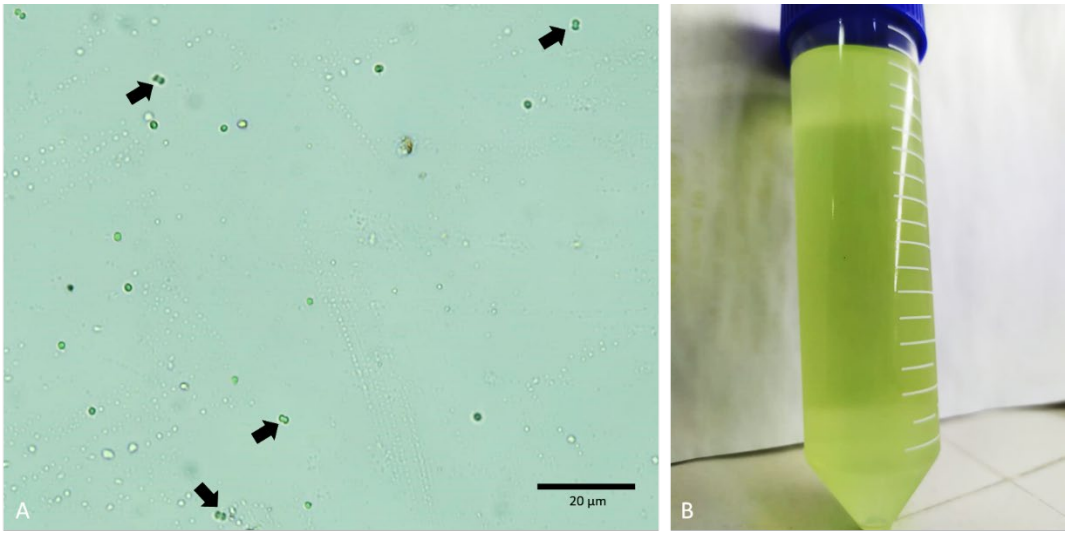

Figure 2. (A) Microscopic photographs of *Synechocystis* sp., black arrows show colony of two cells formation and (B) a 50 ml conical flask of 5 days *Synechocystis* sp. culture. The 5 days culture of *Synechocystis* sp. showed green in color. The microscopic photograph showed small size of unicellular *Synechocystis* sp. with some of them formed colonies of two cells

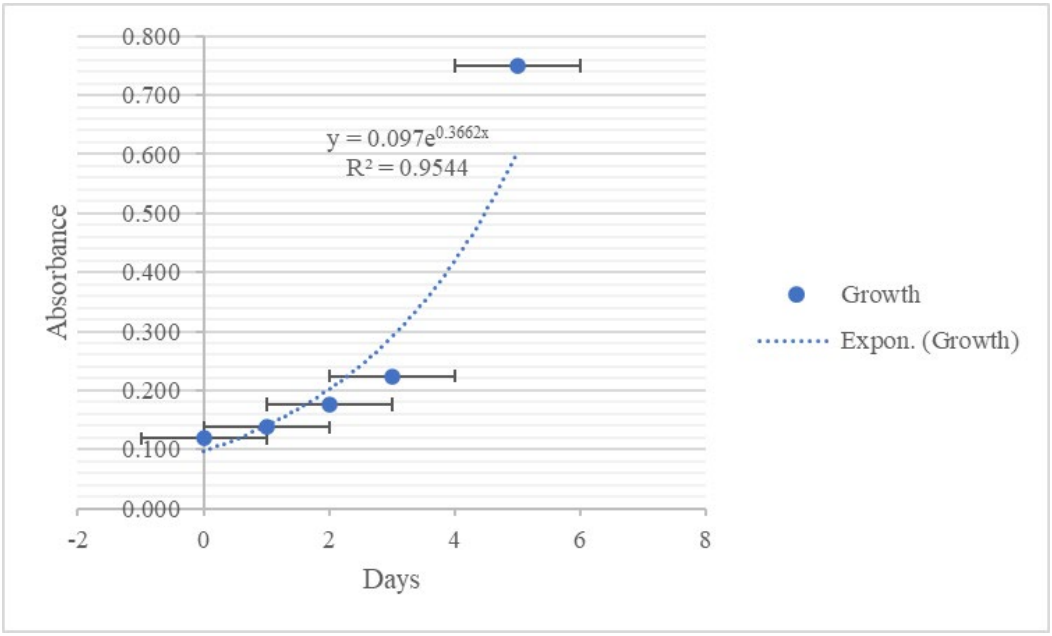

Figure 3. *Synechocystis* sp. growth curve under F/2 culture medium. Growth profile of *Synechocystis* sp. entering lag phase at day-3 and was in high exponential growth in dau-5 and was ready for harvesting for DNA extraction and production of crude extracts

shown that by day-3 of culture, *Synechocystis* sp. has enter lag phase and at day-5, it reaches of cyanobacteria crude extract. Therefore, to overcome these challenges, molecular approach is promising and becoming widely applied in the identification of cyanobacteria, either as the sole method of identification or in combination with morphological or physiological characterization.

The neighbor-Joining tree obtained from the analysis of the partial rbcL gene sequences showed the distinction of *Synechocystis* at high accuracy over another genus (Figure 4). This tree also placed *Synechocystis* into the expected correct clade (Clade B), clearly distinguished from *Synechococcus* clade (A) by bootstrap support value of 100%, which means that *Synechocystis* and *Synechococcus*

shared distinct taxon. Singh et al. also used rbcL gene sequence for phylogeny and evogenomics of heterocystous cyanobacteria [3]. Analysis of rbcL sequence from cyanobacteria in their study proposed the need for change in the cyanobacterial taxonomy of the Stigonematales and proved that *Nostocales* has a greater evolutionary pace as compared to Stigonematales, thus proving that rbcL gene was conserved enough in cyanobacteria for evolutionary analysis and supporting rbcL gene as a marker for cyanobacteria analysis. Moreover, rbcL plays a very important functional gene role in cyanobacteria having a single copy gene, approximately 1,430 base pairs in length, which free from length mutations except at the far 3' end and is known to have a fairly conservative rate of evolution [3].

Molecular parameters based on PCR based methods, such as the 16S rRNA gene sequences, phycocyanin encoding locus, ITS, rpoB, rpoC1, recA, rpoD1, nifH gene(s), and RNA polymerase gene have been used as genetic markers for assessing cyanobacterial diversity and phylogenetic designs [3, 22, 23]. Molecular approaches using DNA markers and gene sequencing should be used as much as possible to distinguish the separated taxonomic units, which also can be used as an obligatory basic method for further cyanobacterial classification. Therefore, the research on the development of high resolutions DNA markers was important for cyanobacterial identification, revealed previously hidden diversity, and re-arrangement of overestimated morphologically-diverse genera.

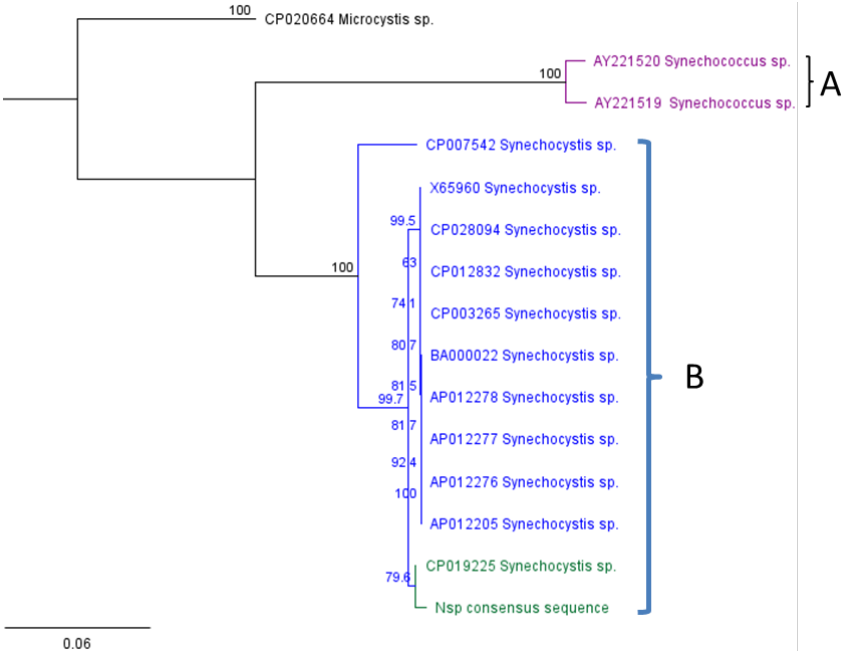

Figure 4. Neighbour-Joining (NJ) phylogenetic tree of rbcL gene of local *Synechocystis* strain from Indonesia (Nsp consensus sequence). The tree was constructed by the neighbor-joining (NJ) method in Geneious Prime software based on the multiple sequence alignment by Geneious alignment program. Bootstrap support values of 1000 replicates (%) are shown at the nodes. Phylogenetic tree of rbcL gene of *Synechocystis* sp. indicated that the strain belongs to the same clade with strains from the same genus and clearly separated with the sister species phylogenetic tree of rbcL gene of local *Synechococcus* sp.

Bio-herbicide Assay of *Synechocystis* Extracts

The biomass concentration of *Synechocystis* culture at lag phase (day 5 growing in f/2 medium) was varied between 1.48 g L-1 and 1.52 g L-1 (absorbance varied at 0.753 and 0.746 observed at λ 680 nm as seen in Figure 3) for production of ethanol and water extract, respectively. The bioherbicidal activity of the ethanol and water extract was tested against the germination and early growth of sorghum seeds with Roundup as positive control and water as a negative control (Table 1). The result showed that both extracts showed root inhibition growth but did not show inhibition on germination and shoot growth.

The germination rate of sorghum seeds differs between positive control, negative control, and *Synechocystis* extracts (Figure 5). Positive control showed the highest germination rate, followed by *Synechocystis* extracts which were not significantly different, and the lowest germination rate is a negative control. Based on Table 1, it was indicated that both *Synechocystis* extracts and glyphosate were promoting germination of sorghum seeds. Therefore, the germination was calculated based on the emergence of radicle from the seed at >1 mm, while there were seeds that are germinated

but stop growing further (Figure 5). Such a case happened in all glyphosate treatment seeds and several seeds in the *Synechocystis* extracts treatments.

Helander et al. reported different responses of glyphosate residues in soil towards crop plant germination and growth, including oats, turnip rapes, and faba beans [24]. The result indicated that glyphosate promotes germination but the plant

Table 1. Germination and early growth of *Sorghum bicolor* under *Synechocystis* sp. extracts. The germination of sorghum under *Synechocystis* sp. extracts was not significant to each other but significantly higher than negative control and lower than positive control. Ethanol extract gave higher inhibition of root growth compared to water extract. There was no significant different between treatment of *Synechocystis* sp. extracts with negative control in term of shoot growth

Treatments	Parameter		
	Germination rate (%)	Root length (mm)	Shoot length (mm)
Water	66±5.48 [a]	9.72±2.23 [d]	11.75±2.66 [b]
Water extract	72±19.24 [ab]	7.29±0.95 [c]	13.76±2.26 [b]
Ethanol extract	78±10.95 [ab]	5.16±0.83 [b]	13.45±3.48 [b]
Glyphosate	84±5.48 [b]	2.55±0.34 [a]	0.00±0.00 [a]

*Means in the same column followed by the same letters are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$

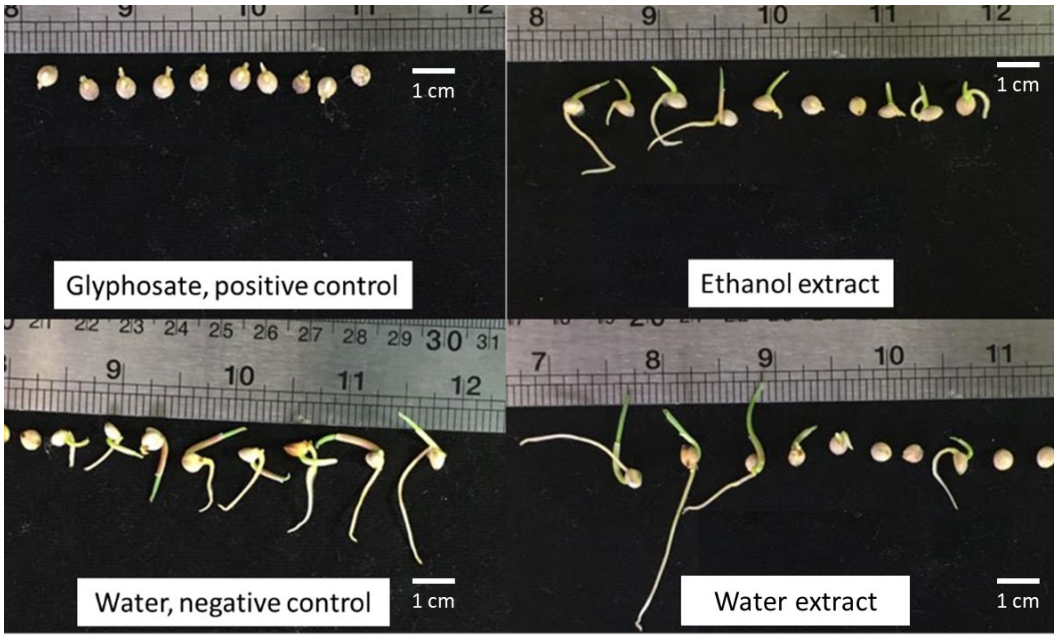

Figure 5. *Synechocystis* sp. extracts activity on germination and early growth of sorghum seeds. The germinated sorghum seeds under positive control failed to grow further, which is in contrast to negative control. Germination and early growth of sorghum under water and ethanol extracts tend to be lower than negative control but higher than positive control

height and biomass were lower in all plants. It was estimated that the glyphosate-based herbicide used in this study contains a surfactant that helps soften the seed coat and gave more moisture to swell for the seeds under positive control [25]. Rodríguez et al. reported a similar effect of *Scytonema hofmanni* extracellular extract with Gibberellic acid-3 (GA3) in the term of salinity tolerance in *Oryza sativa* L., which indicates the extract may counteract altered hormone homeostasis of rice seedlings under salt stress by producing gibberellin-like plant growth regulators [26]. Toribio et al. also reported that cyanobacteria release varied amounts of phytohormones, such as gibberellins which play a big role in seed germination. Therefore, this finding supports the potential of *Synechocystis* extracts in promoting seed germination in sorghum, but differently as a glyphosate-based herbicide [27].

The inhibition of root and shoot growth from sorghum seeds under glyphosate treatment by inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate acid pathway, interfering with the production of tryptophan, tyrosine, or phenylalanine, which are precursors of proteins and other molecules, including growth promoters

like indoleacetic acid (IAA) or secondary compounds which important for plant defense against herbivores, including tannins, anthocyanins, flavonoids and lignin [24, 28]. Lower root inhibition compared to glyphosate treatment was seen on ethanol *Synechocystis* extract, followed by water extract. The result is supported by Rodríguez et al. which showed that ethanolic extract of *Parkia biglobosa* pods has a higher toxicity than water extract for higher secondary metabolites extracted from the samples with ethanol maceration than water maceration [26]. Recent research by Brilisauer et al. found that a small antimetabolite from *Synechococcus elongatus* called 7-deoxy-sedoheptulose (7dSh) blocked shikimate pathway, with the different mechanism as glyphosate, by inhibiting the accumulation of 3-Deoxy-d-arabinoheptulosonate-7-phosphate (DAHP) is in accordance with 3-dehydroquinate (DHQ) synthase, one of the first enzymes in the shikimate pathway [5].

Both cyanobacterium extracts from *Synechocystis* had no activity on shoot inhibition compared to the negative control, though the shoot length was higher, the values were not significantly different (table 1). Shoot growth is highly regulated by phytohormones activity, especially auxin [29]. Sergeeva et al. reported the occurrence of naturally produced auxin, indole-3-acetic acid (IAA), in 21 of the 34 cyanobacterial strains screened including *Nostoc*, *Calothrix*, and *Gloeothece* [30]. Another report by Hussain and Krischke also reported production of exogenous and endogenous IAA from isolated cyanobacteria strains, including *Synechocystis* sp., *Chroococcidiopsis* sp, *Anabaena* sp., *Phormidium* sp. and *Oscillatoria* sp. Therefore, the endogenous and exogenous IAA production in ethanol and water extract of *Synechocystis* may contribute to the higher level of shoot length in the early growth of sorghum seedlings [31].

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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