Potential of bacteria consortium as growth controller of pathogenic fungi Fusarium oxysporum F. sp. cubense (Foc)

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Submission date: 23-Apr-2021 02:21PM (UTC+0700)
Submission ID: 1567451035
File name: Hadi_2021_IOP_Conf._Ser.__Earth_Environ._Sci..pdf (667.15K)
Word count: 4182
Character count: 22036

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Potential of bacteria consortium as growth controller of pathogenic fungi *Fusarium oxysporum* F. sp. *cubense* (Foc)

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Abstract.. Utilization of rhizosphere bacteria as agents for controlling of soilborne pathogenic soil microbiomes has been reported in many studies since decades. The aim of the research is identification and characterization the potential of isolated bacteria consortium for controlling of *Fusarium oxysporum* f.sp. *cubense* (Foc). The bacteria were selected through the antagonistic test, pathogenic test, and compatibility test. The effectiveness of inhibition of selected bacteria were tested in-vitro through the greenhouse-scale of suppressive soil experiments. The result showed that the *Bacillus cereus* strain CCM 2010 and BS 3–4B have the highest percentage of inhibition to the Foc (25.68–29.02%). Non-pathogenic bacteria with a percentage of inhibition above 20%, consists of 3 consortia by compatibility testing. Three bacterial consortiums were obtained, the first consortium (BS 3–4B, *Bacillus cereus* strain CCM 2010, *Staphylococcus arlettae* strain ATCC 43957, *Bacillus cytotoxicus* strain NVH 391–98 and *Bacillus pseudomycoides* strain NBRC 101232), 2nd consortium (*Bacillus cereus* strain CCM 2010 and *Lysinibacillus xylanilyticus strain* XDB9), and 3rd consortium (*Lysinibacillus xylanilyticus* strain XDB9 dan *Bacillus pseudomycoides* strain NBRC 101232). The data showed application of selected single bacteria able to delay fusarium disease in banana plants more than 45 days post infection in comparison to control plants.

1. Introduction

Indonesia is one of the bananas producing country in Southeast Asia, which has more than 200 varieties scattered around Indonesia regions [1]. One example for the banana plants producers with the huge amount of harvested bananas in Indonesia is PT Perkebunan Nusantara (PTPN) VIII Parakansalak, Sukabumi. However, due to the attack of a disease, the bananas in the Parakansalak PTPN VIII is decreasing [2]. The disease of banana plants that can lower the quality of banana plants production besides wilt disease (Fusarium wilt and bacteria wilt), is leaf spot disease (Black Sigatoka and yellow Sigatoka), a disease which caused by viruses especially banana dwarf virus (Banana Bunchy Top Virus/BBTV). The wilt disease is a main limiting factor in the production and the quality of banana plants. Furthermore, Foc fungi is one of the most dangerous confounded organisms that can disrupt the industry and banana plantations in Indonesia [3].

The Fusarium Wilt can be controlled through the use of fungicides and technical culture. However, it uses has not been able to suppress the growth of Foc in plantations. One of alternative treatment to control the Foc is the use of biological agents in the form of bacteria, which can inhibit the growth of pathogens. The bacterias such as *Bacillus*, *Enterobacter*, *Pseudomonas*, *Micrococcus Serratia,* and *Vibrio* [4], will help the inhibitions of the Foc. Bacteria can degrade chitin on the fungus cell wall,

protect the plants against the pathogens, increasing the plant resistance and produce the antifungal compounds that cause swelling of Foc hyphae in vitro [5]. Therefore, this research was done to obtain to find a biological agent from a single isolate that has the highest inhibitory against Foc and to control The Panama Wilt on banana plants through the use of a bacterial consortium that controls the growth of the Foc.

2. Methods

2.1. Foc culture

Foc was being cultured by taking colonies on Petri dish. The Foc colony was inoculated using the streak method on a petri dish containing PDA media. The culture was incubated for 24 hours at 30°C and stored for being used in antagonistic tests.

2.2. Culture of soil bacterial isolates

Soil bacterial isolate was cultured in 6 ml sterile Luria-Bertani medium into a test tube. Bacterial isolates were inoculated using the streak method and incubated for 24 hours at 32°C. Isolate culture was stored for use in antagonistic and compatibility tests [6].

2.3. Antagonistic test

The antagonistic test was being done by a dual culture technique on PDA media. A 5-mm agar disc of an actively growing culture of Foc was placed in the center of each plate with agar blocks method. Each isolate was streaked 2 cm away from the agar disc towards the edge of the petri dish. In the control plate, the petri dish was not given bacterial isolates. Petri dish were stored and incubated at 32°C for 5 days to determine inhibition of colony growth (Figure 1) [7]. Colony growth was measured using ImageJ software [8]. Colony growth inhibition (%) was calculated by using the formula [9] :

$$\%\, Percentage = \frac{C - T}{C} \times 100\%$$

Note :
C : The colony growth of pathogen in control
T : The colony growth of pathogen in dual culture

2.4. Patogenic test

The patogenic test was being done by the streak plate method on blood agar base medium to detect the ability of bacterial hemolysis. Blood agar base medium as much 40 g was adding with 1,000 ml of distilled water into Erlenmeyer. The medium was homogenized and sterilized. Sterile blood agar base cooled to 45 to 50°C and then adding 10% sterile defibrinated blood and homogenized. Each isolate was streaked into a petri dish and incubated at 32°C. Petri dish observed at 24 hours, 48 hours, and 72 hours [10].

2.5. Compatibility test

The selected soil bacteria were tested for compatibility by cross streak method. Two different bacterial isolates were streaked vertically and horizontally on Luria-Bertani medium into petri dish. Petri dish were incubated for 48 hours and observed for lysis at the juncture of the streaks (Figure 2) [11].

2.6. Preparation of pathogen inoculation and soil bacteria isolates

Foc was inoculated on PDB media for ±7 days at 28°C with 90 rpm in the shaker incubator [12]. Then, fungal cultures were filtered using Whatman filter paper No.1 and washed using sterile aquades 3 times [13]. Conidia are counted using a hemocytometer to get the conidia amount of 10^6 conidia ml^{-1}. Fungal pathogen suspension of 25×10^6 conidia is dissolved in 5,000 ml of distilled water to infected root of banana plants [14].

The selected singular soil bacteria or consortium bacteria were cultured on Luria-Bertani Broth medium and incubated for 24 hours at 32°C with 120 rpm in the shaker incubator [15]. Isolate culture was centrifuged at 8,000 rpm for 5 minutes. Then, separate pellets from supernatant. Pellets were resuspended using 0.9% sterile NaCl. Each treatment using 9 ml of isolate culture to made into 1×10^8 CFU/isolates based on 0.5 McFarland standard [16].

2.7. Greenhouse-scale of suppressive soil experiments

The banana plants are from Seedling Culture Laboratory, Department of Maritime and Agriculture of DKI Jakarta province. Banana plants are adapted in the greenhouse University of Al Azhar Indonesia for ±45 days. Banana plants are able to adjust to different environmental temperatures. Banana plants that have been adapted, the roots of the plants will be cleaned using water. Then, the treatment of banana plants grouped into 4 treatments. The first, second, third and fourth treatments used bacterial isolate BS 3–4 B, *Bacillus vallismortis* strain NBRC 101236, *Bacillus cereus* strain CCM 2010 and *Lysinibacillus xylanilyticus* strain XDB9. Each treatment uses 5 banana plants and the remains are used as a control [17, 18].

Table 1. Consortium of compatibility test results

Consortium	Bacteria
Consortium 1	BS 3–4 B, *Bacillus cereus* strain CCM 2010, *Staphylococcus arlettae* strain ATCC 43957, *Bacillus cytotoxicus* strain NVH 391–98 and *Bacillus pseudomycoides* strain NBRC 101232
Consortium 2	*Bacillus cereus* strain CCM 2010 and *Lysinibacillus xylanilyticus* strain XDB9
Consortium 3	*Lysinibacillus xylanilyticus* strain XDB9 and *Bacillus pseudomycoides* strain NBRC 101232
Consortium 4	*Bacillus thuringiensis* strain NBRC 101235 and *Bacillus cytotoxicus* strain NVH 391–98

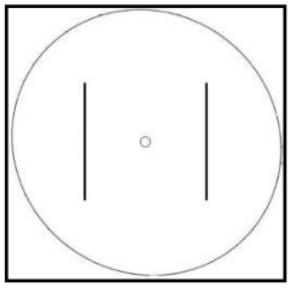

Figure 1. Scheme of antagonistic test.
Note: —— : Bacterial isolates
O : Fungal pathogen

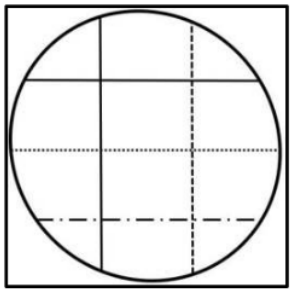

Figure 2. Scheme of compatibility test.
Note: type of line indicate the use of different isolates during testing.

The roots of banana plants that have been cleaned, then the roots are soaked for 30 minutes in 10^6 conidia of Foc suspension for 25 plants. Furthermore, each treated plant-soil uses bacterial isolate culture. isolate culture 5×10^8 CFU was homogenized into 5,500 g of soil in 250 ml of distilled water for 5 plants, each plant containing 1,100 g of soil. Meanwhile, control banana plants were only given treatment in the form of soaking the roots of plants in the Foc suspension. Banana plants were incubated at 21–28°C for 0, 1, 3, 5, 7, and 14 days after planting [18, 19].

Meanwhile, the consortium test plants used 30 plants which were grouped into 3 consortia, namely consortium 1, consortium 2, and consortium 3 (Table 1). Each treatment used 6 banana plants and the

rest were used as controls. Banana plants consortium test using soil with a mixture of consortium 1 (30×10^8 CFU), consortium 2 (12×10^8 CFU) and consortium 3 (12×10^8 CFU). Soil that was mixed with consortium isolates was incubated for 1 week, then the banana plant was planted in a 25×25 cm polybag whose plant roots had been soaked with Foc suspension [19].

3. Results and discussion

3.1. Bacterial isolate antagonist test against Fusarium oxysporum f. sp. cubense in vitro

In vitro antagonist test results found 24 bacterial isolates that have the antagonistic ability of Foc from 34 bacterial isolates with inhibitory power varying between 6% to 29%. The percentage is obtained from the average percentage of each test isolate used and derived from growth measurements in the Foc test with Foc control. The highest antagonistic ability is shown by BS 3–4 B which is shown in table 2 of 29.02%, while the lowest antagonist ability is shown by BS 2–4 A with a percentage of 6.03%. Antagonistic ability marked by inhibition zone was shown by *Bacillus vallismortis* strain NBRC 101236 with a percentage of 26.1735%, while isolates without antagonistic ability were shown by *Bacillus cereus* ATCC 14579 (Figure 3).

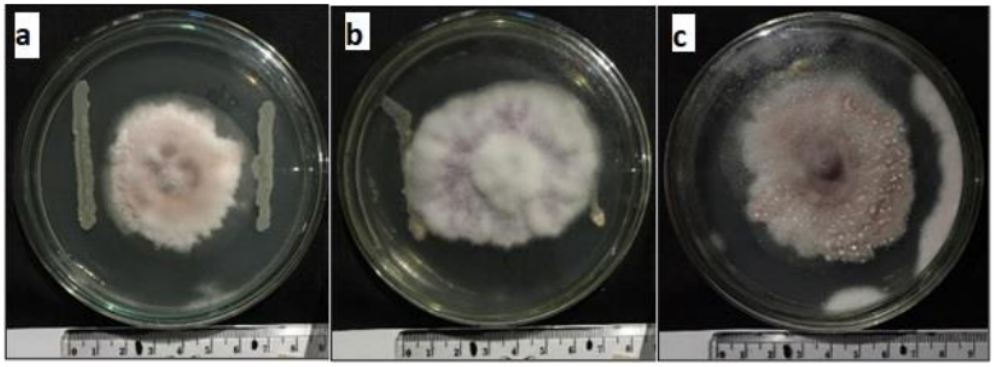

Figure 3. Observation of the 16th day. *Bacillus vallismortis* strain NBRC 101236 which has antagonistic ability (a), *Bacillus cereus* ATCC 14579 which does not have antagonistic ability (b), Foc control (c).

Table 2. Percentage of bacterial inhibition against Foc.

No	Name of isolates	Percentage inhibition
1.	BS 3–4 B	29.0243
2.	*Bacillus vallismortis* strain NBRC 101236	26.1735
3.	*Bacillus cereus* strain CCM 2010	25.6820
4.	*Lysinibacillus xylanilyticus* strain XDB9	24.6744
5.	*Bacillus thuringiensis* strain NBRC 101235	22.6837
6.	*Staphylococcus arlettae* strain ATCC 43957	22.3028
7.	*Bacillus cytotoxicus* strain NVH 391-98	21.5164
8.	*Bacillus pacificus* strain MCCC 1A06182	21.2583
9.	*Bacillus pseudomycoides* strain NBRC 101232	21.2583
10.	*Bacillus cereus* strain IAM 12605	20.4048

Isolates that do not have the antagonistic ability are characterized by the growth of pathogenic fungi that meet the isolates [20]. Isolates that have the potential as antagonistic agents against Foc have inhibition levels above 20%. These isolates can be used as good biocontrol candidates because they can carry out an antibiotic mechanism to control the growth of Foc pathogenic fungi [21]. Table

2. shows that 10 isolates of rhizosphere bacteria were able to inhibit Foc growth with the inhibition percentage of more than 20%. This percentage indicates that the rhizosphere bacteria have a strong antagonistic ability so that they can produce extracellular enzymes such as chitinase, protease, and cellulase. Besides, *Bacillus* sp. rhizosphere bacterium has an antagonistic mechanism in the form of antibiosis by producing antifungal compounds that can cause hyphal growth to become abnormal (malformations) and the presence of chitinase enzyme activity that causes fungal cell walls to undergo lysis [22].

3.2. Bacterial patogenic test

Patogenic test using blood base agar with the addition of sheep blood defibration as much as 5–10%. The blood contains anticoagulants to prevent clotting and detect the ability of bacterial hemolysis using the streak plate technique [23]. Perfect hemolysis is marked by a clear zone, partial hemolysis is characterized by changes in the color of the media to greenish or brownish. A clear zone formed in β hemolysis showed that the isolate was pathogenic [24]. While isolates that do not undergo media hemolysis will not change. Figure 4. shows partial hemolysis (α), complete hemolysis (β), and no hemolysis (γ) [25].

(a) (b) (c)

Figure 4. Hemolysis on blood base agar + 10% blood sheep for 72 hours. Partial hemolysis, *Bacillus velezensis* strain FZB42 (a); complete hemolysis, BS 2-5 A (b); no hemolysis, *Staphylococcus arlettae* strain ATCC 43957 (c).

3.3. Bacterial isolate compatibility of biological agents

Compatibility test using bacterial isolates from antagonistic test selection with a percentage above 20% and partially hemolysis as many as 7 bacterial isolates. compatible bacterial isolates (synergism) are characterized by the absence of lysis at the point of intersection of the line. The lysis of the intersection of the line is seen based on the growth between isolates. Isolates that synergize will experience the same growth so that the resulting size is the same. Meanwhile, isolates that do not synergize will appear at an intersection point lysis which is marked by differences in the size of the isolates, overlapping between isolates and there are isolates that cone at the intersection. Figure 5 shows the isolate of rhizosphere bacteria that synergizes and does not synergize [11].

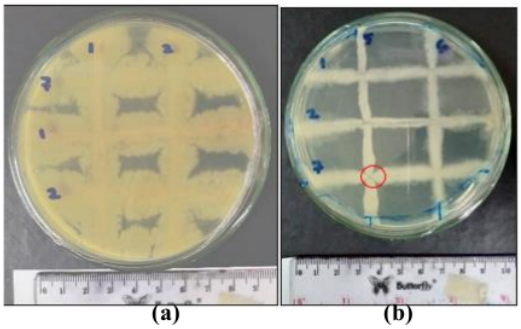

Figure 5. Isolates that synergize with each other (a), Isolates (5.7) do not synergize (b).
Note: 1. BS 3-4 B; 2. *Bacillus cereus* strain CCM 2010; 5. *Staphylococcus arlettae* strain ATCC 43957; 7. *Bacillus pseudomycoides* strain NBRC 101232

Table 3. shows the synergism between isolates and form a group of bacterial isolates that synergize with each other (consortium). The bacterial consortium interacts synergistically in providing nutrients, eliminating inhibitory products, and stimulate and inhibit each other physically and biochemically to increase the effectiveness between isolates [26]. The design of consortium obtained will be tested using a greenhouse-scale of suppressive soil experiments.

Table 3. Compatibility test between rhizosphere bacteria

	BS 3-4 B	*Bacillus cereus* strain CCM 2010	*Lysinibacillus xylanilyticus* strain XDB9	*Bacillus thuringiensis* strain NBRC 101235	*Staphylococcus arlettae* strain ATCC 43957	*Bacillus cytotoxicus* strain NVH 391-98	*Bacillus pseudomycoides* strain NBRC 101232
BS 3-4 B	✓	✓	O	O	✓	✓	✓
Bacillus cereus strain CCM 2010	✓	✓	✓	O	O	✓	O
Lysinibacillus xylanilyticus strain XDB9	O	✓	✓	O	✓	O	✓
Bacillus thuringiensis strain NBRC 101235	O	O	O	✓	O	✓	O
Staphylococcus arlettae strain ATCC 43957	✓	O	✓	O	✓	✓	O
Bacillus cytotoxicus strain NVH 391-98	✓	✓	O	✓	✓	✓	✓
Bacillus pseudomycoides strain NBRC 101232	✓	O	✓	O	O	✓	✓

Note: ✓: isolates synergism with each other, O: isolates are not synergistic.

3.4. Greenhouse-scale of suppressive soil experiments

Twenty-four bacterial isolates that have antagonistic ability against *Fusarium oxysporum* f. sp. *cubense* can be used as a biocontrol agent. Biocontrol agents related to the use of microbes in plants to control plant diseases. The use of microbes in plants is done in two ways, namely a single isolate and a consortium. Based on the antagonist test, obtained 10 single isolates with an antagonistic capability above 20% and 4 consortium isolates. However, consortium 4 was not used for a limited test of greenhouse scale suppressive soil because *Bacillus thuringiensis* strain NBRC 101235 was only

compatible with *Bacillus cytotoxicus* strain NVH 391-98 so this consortium was not used. BS 3-4 B (29.02%), *Bacillus vallismortis* strain NBRC 101236 (26.17%), *Bacillus cereus* strain CCM 2010 (25.68%), *Lysinibacillus xylanilyticus* strain XDB9 (24.67%), consortium 1, consortium 2 and consortium 3 were single isolates and consortium of biocontrol agents that can inhibit the growth of Foc in plants. The isolate was evaluated using a greenhouse scale limited suppressive soil test method to determine the mechanism of Foc inhibition in plants.

Figure 6f. shows that the banana plants are not given antagonistic bacteria on the soil show symptoms of Foc wilt that characterized by the occurrence of leaf chlorosis, namely discoloration of the oldest leaves to yellow [27]. Meanwhile, the addition of antagonistic bacteria to the soil of Foc infected plants can stunt contact and penetration of pathogens to its host, elongate the incubation period and suppress the growth of pathogenic fungi. Pathogens in plants must compete first with antagonistic microorganisms to obtain nutrients and food (Figure 6a-d) [19]. Besides, antagonistic bacteria also secrete an antibiotic compound that is capable of destroying the membranes of pathogenic fungi and disrupting its metabolic system so that pathogenic fungi lose the ability to infect host plants [28].

The addition of a single isolate antagonist bacteria and a consortium in Foc infected plant soils has a high Foc growth-inhibitory ability. Meanwhile, the lowest inhibition is showed by controls using sterile distilled water (Figure 7). The bacterial consortium has a better inhibition mechanism than a single isolate because there is a mechanism of interaction between microbial antagonists to inhibit the development of Foc [29]. Besides, the bacterial consortium has a superior effect on plants, because synergistic interactions between bacteria can provide nutrients, eliminate inhibitory products and stimulate one another through physical or biochemical activities that can affect physiological effects [26].

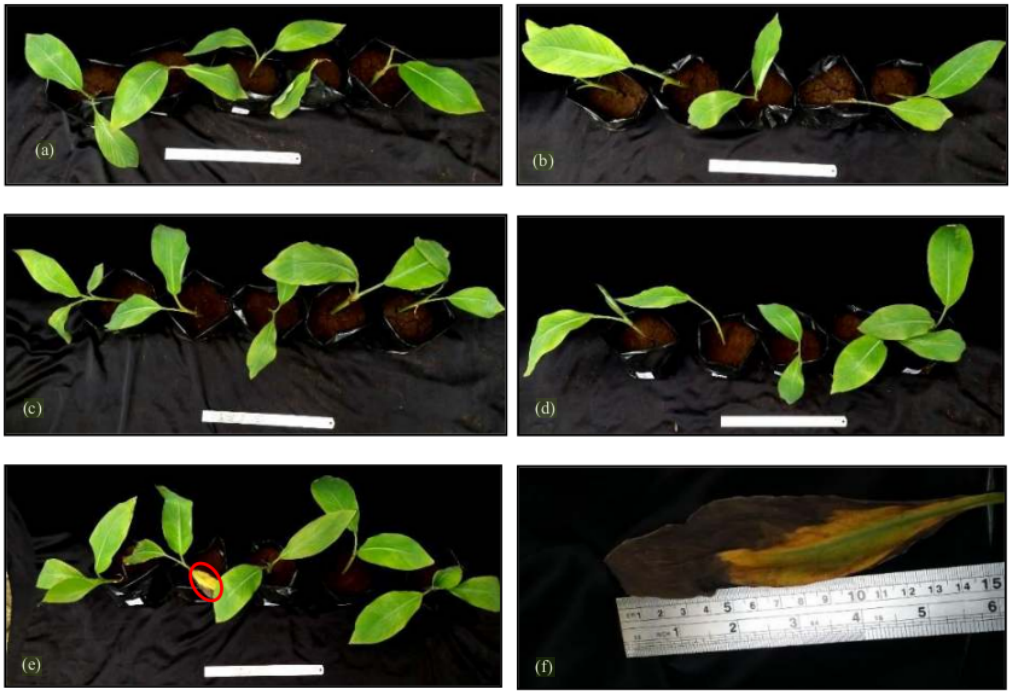

Figure 6. Observation of the banana plant on the 5th day. Banana plant treatment (a-d); control banana plants (e); Foc infected leaves in control banana (f).

Note: (a) BS 3-4 B; (b) *Bacillus vallismortis* strain NBRC 101236; (c) *Bacillus cereus* strain CCM 2010; (d) *Lysinibacillus xylanilyticus* strain XDB9.

Figure 7. Observation of the banana plant on the 5th day. Control banana plants (a); Foc control banana plants (b); banana plant consortium (c, e, g), single isolate banana plant (d, f, h).
Note : (c) Consortium 1; (d) BS 3-4 B; (e) Consortium 2; (f) *Bacillus vallismortis* strain NBRC 101236; (g) Consortium 3; (h) *Bacillus cereus strain* CCM 2010.

On the 45th day after Foc infection, banana plants showed symptoms of Foc wilt, which is marked by a change in a rhizome, pseudostem changes to blackish brown, and leaves will be through dryness (Figure 8a, c) [30]. The genus antagonist of *Bacillus* sp. produces phytohormone that has the potential to develop sustainable farming systems. Indirect phytohormone from bacteria inhibits the activity of pathogens in plants, while the effect of phytohormone directly is to increase plant growth and act as a facilitator in the absorption of nutrients from the environment [31].

(a) (b)

Figure 8. *Fusarium oxysporum* f.sp. wilt symptoms cubense day 45 on stems and tubers (a); leaf (b); (a,b) Control Foc.

4. Conclusion

Isolate BS 3-4 B and *Bacillus cereus* strain CCM 2010 are non-pathogenic isolates that are antagonistic and able to inhibit the growth of fungal pathogenic *Fusarium oxysporum* f.sp. *cubense* (Foc) with a percentage of inhibition of 29.02% and 25.68%. The use of bacterial rhizosphere bacteria *Bacillus* sp. and *Staphylococcus* s[illegible]he limited test of greenhouse scale suppressive soil shows that the genus can suppress the growth of *Fusarium* wilt in banana plants. The use of consortium in the greenhouse-scale of suppressive soil experiments provides the best effectiveness in inhibiting Foc growth.

Acknowledgements
The research was partially funded by PTUPT research grant from The Ministry of Research Technology and Higher Education-Republic of Indonesia with Dr. rer. nat. Yunus Effendi as the PIC of the research project and research grant from LPPM Universitas Al Azhar Indonesia.

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