Development of the PGPR and Cyanobacteria Consortium for Growth Promotion and Control Ralstonia syzigii subsp. indonesiensis of Tomato

Yulmira Yanti*, Hasmiandy Hamid, Reflin
Lecture at Plant Protection Department, Agriculture Faculty, Andalas University, Indonesia; Padang, West Sumatera, 25163
*Coresponding author: yy.anthie79@gmail.com; mira23@agr.unand.ac.id

Abstract. Plant growth-promoting rhizobacteria (PGPR) are known in various cropping systems to increase plant growth, vigour and plant nutrient contents. cyanobacteria are known to possess the ability to form associations with vascular/non-vascular plants and produce growth-promoting substances. The present work was undertaken to formulate the best effective consortium consist of plant-growth-promoting rhizobacterium and cyanobacteria to promote growth rate of tomato and control R. syzigii subsp. indonesiensis. Our previous study had screened 7 best rhizobacteria (Bacillus thuringiensis strain RBI 2AB1.1, Bacillus cereus strain HYM 88 RBI 2AB 2.1, Bacillus cereus strain APSB-03 RBI 2AB 2.2, Bacterium JP60 RBI 2 AB 2.3, Bacillus sp. M6 RBI KDA 1.2, Bacillus cereus strain JN23 RBI KDA 2.2 and Bacillus subtilis BSn5 RBI IPBL 2.3) and 4 cyanobacteria strain (RZ2AB2.1, RZ1AB2.3, RZ1BPL2.3, RZSD1.1) to promote growth of tomato. To increase those strains’ ability both for suppress pathogen attack and promote growth, development of suitable consortium is necessary. Our research consists of two phase which were in vitro dual culture studies for interaction assay, and in planta consortium assay. Results shown that almost all strains were had a good compatibility to growth together. We develop 12 consortiums based on its previous ability and the compatibility. All consortiums shown capability to reduce bacterial wilt disease development and also promote growth of tomato. Consortium consist of Bacillus thuringiensis strain RBI 2AB1.1, Bacillus cereus strain APSB-03 RBI 2AB 2.2, Bacillus subtilis BSn5 RBI IPBL 2.3 and cyanobacteria RZ2AB2.1) shown best ability to reduce disease development and promote growth and yields of tomato.

1. Introduction

Bacterial wilt cause by a soil-borne vascular pathogen, Ralstonia syzygii subsp. indonesiensis (Rs) Safni et al. [1] (formerly Ralstonia solanacearum [2]) and were very common in the field. Rs is a soil-borne gram-negative bacterium that causes bacterial wilt disease in over 200 families of plants, including tomatoes [3,4]. This pathogen causes wilt by infecting plants through roots and colonizing stem vascular tissue and the vascular tissues in the lower stem of the wilted plants usually show a brown discoloration [5]. Approximately 450 crop species were reported as hosts of Ralstonia [6]. Attention has been paid to minimize the disease infestation through cultural practices, development of resistant varieties and use of chemicals, but most of them have a limited success [7]. Bacterial wilt management in tomato and in other crops has been difficult. Even though integrated management, including cultural practices, crop rotation, and use of resistant cultivars, provides some limited success, the disease still threatens commercial tomatoes [8]. Once this pathogen is existed in a field, it will be difficult to control [9]. Control of R. syzigii subsp. indonesiensis is difficult due to high variability of the pathogen, limited
possibility for chemical control, high capability of pathogen to survive in diverse environments and its extremely wide host range.

Biological control has emerged as one of the important methods in the management of soilborne plant pathogens. Biological control reduces the dependence on high-risk chemicals for disease management and is ecologically sound and environmentally friendly technique [10]. Biological control is one of a desirable approach because control with other methods gives variable results [11] and since biological control is a key component of integrated disease management, it is important to search for biological control as promising alternatives to replace chemical pesticide and fertilizer in sustainable and organic agricultural systems [12].

Plant growth promoting rhizobacteria (PGPR) are potential agents for biological control of plant pathogens [13]. PGPR bring about disease suppression by various modes of action such as antagonism, competition for space and nutrients, and induction of systemic resistance [14]. Plant growth promoting activity by rhizobacteria may be associated with secretion of auxins, gibberellins, and cytokinins [15] and suppression of deleterious microorganisms in the rhizosphere [16]. The use of rhizosphere bacteria for increasing yield and for crop protection is an attractive approach in the modern system in developing a sustainable agriculture. PGPR are also known to rapidly colonize the rhizosphere and suppress deleterious microorganisms as well as soilborne pathogens at the root surface [17]. These organisms can also be beneficial to the plants by stimulating growth [18].

PGPR are antagonistic to the pathogens and incorporate them into successful disease management as biocontrol agents. A key feature of such organisms is their ability to adjust to the rhizosphere and to aggressively colonize the host roots [19]. Therefore, it was recommended that to achieve greater efficiency of biocontrol agents they should be isolated from the environment where they would be required to function [20]. Application of biocontrol agents has been tested in a limited manner. Bacillus spp. provided complete control of black rot on crucifer [21]. In greenhouse studies, E. herbicola and Bacillus subtilis suppressed X. axonopodis pv. vignicaeow and X. axonopodis pv. Vignearandiatae on mungbean [22]. Some of the naturally antagonistic microorganisms isolated successfully against R. syzigii subsp. indonesiensis are Bacillus species and Pseudomonas fluorescens [23], Stenotrophomonas maltophilia [24], Streptomycetes setonii [25].

Other potential biocontrol agents candidate were Cyanobacteria. Cyanobacterial (blue-green algae) inoculation is known to enhance the growth, nitrogen fixation and yields in the rice-wheat cropping sequence [26]. Cyanobacteria are prominent inhabitants of many agricultural soils and are the most natural colonizers of rice roots [27] where they potentially contribute towards biological nitrogen fixation [28], phosphate solubilization [29] and mineral release to improve soil fertility and crop productivity [30]. Besides naturally fertilizing and balancing mineral nutrition in the soils, many organisms are known to produce growth promoting substances that enhance plant health by a plethora of mechanisms [31]. Cyanobacterial inoculation is known to enhance the growth, nitrogen fixation and yields in the rice-wheat cropping sequence [32,33]; however, very few reports are available on their role in disease reduction and protection against fungal diseases [34,35].

Most of biocontrol agents used single biocontrol agent against a pathogen in controlling plant disease. This approach had partially reported for its inconsistent performance due to the use of single biocontrol agent is not likely to be active in all ecosystems it was applied or against all the host pathogens. Nowadays, more attention are being concerned on the use of mixed strains of PGPR [36]. The microbial consortium is a group of species of microorganisms that act together as a community. In a consortium, the organisms work together in a complex and synergistic way [37]. According to [38], a consortium of bacteria that interacts synergistically can give better results than a single bacterial application. Each bacterial species has different mechanisms so that the bacterial consortium can provide various control mechanisms simultaneously, thereby it is more effective in controlling pathogens [39]. Evaluation of the compatibility and synergism of microbial components is essential in the ability of a microorganism consortium of as biocontrol agents and biofertilizers [40]. Ouhaibi-Ben Abdejalil et al. [41] used a consortium of 3 types of selected rhizobacteria to control wilting of tomato plants by Sclerotinia sclerotiorum and to promote the growth of tomato plants. Shanmugam et al. [42] study showed that the PGPR strain consortium treatment could promote the plant growth and enhanced production of ginger rhizome by 45.8%. In field experiments, the PGPR strain consortium could also
reduce yellow and rhizome rot incidences on ginger by about 50.5%, which was comparable to that of a carbendazim and mancozeb fungicide mixture.

The present work was undertaken to formulate the best effective consortium consist of plant-growth-promoting rhizobacterium and cyanobacteria to promote growth rate of tomato and control R. syzigii subsp. indonesiensis.

2. Materials and Methods

2.1. Study area

The study was conducted in February-July 2019 at the Laboratory of Microbiology and Experimental Fields, Faculty of Agriculture, Andalas University, Padang.

2.2. Methodology

The research designed in experimental methods and was arranged in a completely randomized design. Our research consists of two phase which were in vitro dual culture studies for interaction assay (I), and in planta consortium assay (II). The strains used in this study were 7 best rhizobacteria (Bacillus thuringiensis strain RBI 2AB1.1, Bacillus cereus strain HYM 88 RBI 2AB 2.1, Bacillus cereus strain APSB-03 RBI 2AB 2.2, Bacterium JP60 RBI 2 AB 2.3, Bacillus sp. M6 RBI KDA 1.2, Bacillus cereus strain JN23 RBI KDA 2.2 and Bacillus subtilis BSn5 RBI IPBL 2.3) and 4 cyanobacteria strain (RZ2AB2.1, RZ1AB2.3, RZ1BPL2.3, RZSD1.1) from previous study. The consortium was designed based on the first stage compatibility assay.

2.3. Procedures

2.3.1. Rejuvenation and confirmation of PGPR and cyanobacteria isolates

The strains were obtained from Yanti’s collection (Unpublished). The bacteria were rejuvenated by the scratch method on Nutrient Agar (NA) medium and incubated for 2x24 hours. For the cyanobacteria isolates, the selected isolates growth in BG-11 medium agar according to modified methods of [43] and incubated for 48 h at incubated at 27 oC in an incubator with light/dark cycles (16:8 h) with white light (50–55 mmol photons m\(^{-2}\)). Furthermore, the bacteria were characterized by Gram test with 3% KOH solution and hypersensitive reaction on Mirabilis jalapa leaves.

| Strain                                      | Code |
|---------------------------------------------|------|
| Bacillus thuringiensis strain RB2AB1.1      | R1   |
| Bacillus cereus strain HYM 88 RBI 2 AB2.1   | R2   |
| Bacillus cereus strain APSB-03 RBI 2 AB2.2  | R3   |
| Bacterium JP60 RBI 2 AB 2.3                 | R4   |
| Bacillus sp. M6 RBI KDA 1.2                 | R5   |
| Bacillus cereus strain JN23 RBI KDA 2.2     | R6   |
| Bacillus subtilis BSn5 RBI IPBL 2.3         | R7   |
| RZ2AB2.1                                    | C1   |
| RZ1AB2.3                                    | C2   |
| RZ1BPL2.3                                   | C3   |
| RZSD1.1                                     | C4   |

2.3.2. Compatibility test of PGPR and Cyanobacteria isolates

Compatibility between each PGPR and cyanobacteria isolates was tested by crossing the endophytic bacteria on mixed NA medium & BG-11 medium (1:1) in a petri dish, which were incubated for 48 h at incubated at 27 oC in an incubator with light/dark cycles (16:8 h) with white light (50–55 mmol photons). Furthermore, the inhibition zones that appeared between isolates were observed.
2.3.3. Test of the ability of the consortium to control *R. syzigii* subsp. *indonesiensis* on tomato

2.3.3.1 Preparation of the consortium

The consortium was prepared by combining 2-3 compatible species (from the results of stage I). For pre-culture, the bacteria were cultured in 5 mL of Nutrient Broth (NB) (for PGPR) and BG-11 broth (for cyanobacteria) incubated in a rotary shaker at 150 rpm for 24 hours at 27 °C in an incubator with light/dark cycles (16:8 h) with white light (50–55 mmol photons). Furthermore, for the main culture, 1 mL pre-culture suspension was put into 24 mL of sterile coconut water and incubated in the same way for 2x24 hours. The consortium designed were shown on table 2.

### Table 2. PGPR and cyanobacteria consortium for the treatment

| Code | Strain |
|------|--------|
| C1   | *Bacillus thuringiensis* strain RBI 2AB1.1, *Bacterium JP60* RBI 2 AB 2.3, cyanobacteria RZ2AB2.1 |
| C2   | *Bacillus thuringiensis* strain RBI 2AB1.1, *Bacillus cereus* strain APSB-03 RBI 2AB 2.2, *Bacillus subtilis* BSn5 RBI IPBL 2.3 and cyanobacteria RZ2AB2.1 |
| C3   | *Bacillus cereus* strain APSB-03 RBI 2AB 2.2, *Bacterium JP60* RBI 2 AB 2.3, cyanobacteria RZ2AB2.1 |
| C4   | *Bacillus thuringiensis* strain RBI 2AB1.1, *Bacillus cereus* strain JN23 RBI KDA 2.2, cyanobacteria RZ1AB2.3 |
| C5   | *Bacillus sp.* M6 RBI KDA 1.2, *Bacillus subtilis* BSn5 RBI IPBL 2.3, cyanobacteria RZ1AB2.3 |
| C6   | *Bacillus cereus* strain HYM 88 RBI 2AB 2.1, cyanobacteria RZ1AB2.3 |
| C7   | *Bacillus cereus* strain APSB-03 RBI 2AB 2.2, *Bacterium JP60* RBI 2 AB 2.3, *Bacillus subtilis* BSn5 RBI IPBL 2.3, cyanobacteria RZ1BPL2.3 |
| C8   | *Bacillus sp.* M6 RBI KDA 1.2, R2 *Bacterium JP60* RBI 2 AB 2.3, cyanobacteria RZ1BPL2.3 |
| C9   | *Bacillus cereus* strain JN23 RBI KDA 2.2, *Bacterium JP60* RBI 2 AB 2.3, cyanobacteria RZ1BPL2.3 |
| C10  | *Bacillus subtilis* BSn5 RBI IPBL 2.3, *Bacillus cereus* strain HYM 88 RBI 2AB 2.1, *Bacterium JP60* RBI 2 AB 2.3, cyanobacteria RZSD1.1 |
| C11  | *Bacillus thuringiensis* strain RBI 2AB1.1, *Bacillus cereus* strain APSB-03 RBI 2AB 2.2, *Bacillus sp.* M6 RBI KDA 1.2, cyanobacteria RZSD1.1 |
| C12  | *Bacillus cereus* strain HYM 88 RBI 2AB 2.1, *Bacterium JP60* RBI 2 AB 2.3, *Bacillus sp.* M6 RBI KDA 1.2, cyanobacteria RZSD1.1 |

2.3.3.2 The introduction of PGPR and cyanobacteria consortium

The PGPR and cyanobacteria consortium was introduced into the seeds and tomato seeds two times. The Tomato seeds were sterilized with 1% NaOCl-distilled water - each distilled for 1 minute. The seeds were immersed in the consortium of *Bacillus* spp. for 15 minutes then sowed in the seed tray. Tomato seeds were maintained for 21 days.

Tomato seedlings then were planted in 10 kg-polybags containing soil mixed with manure (2: 1, v / v). Before the tomato seedlings were planted, they were first soaked in the consortium treatments for 15 minutes, and the control was soaked in sterile distilled water. The maintenance of tomato was carried out by following the recommended cultivation techniques.

2.3.3.3 Pathogens Innoculation

The *Ralstonia syzygii* subsp. *indonesiensis* was isolated from infected plants by dipping the stem in sterilized water and the suspension then streaked in Tryphenyl tetrazolium (TZC) agar medium. The isolates were then assayed on 2 weeks old tomato plants to select the most virulence pathogens by injecting 1 ml of *Ralstonia* suspension (108 CFU/ml) to the tomato plant base stem. The most virulence bacteria (the fastest wilt disease development) was then re-cultured in TZC agar and used for plant disease infection. The pathogen was innoculated on the 2 weeks old plants by root wounding methods.
described by Yanti et al., (2018a). The roots were cutted in 2 sides of the plants using scissors and poured with 10 mL (108 CFU/mL) of the R. syzigii subsp. indonesiensis suspensions.

2.3.4. Data Collection
Parameter observed in this research were disease development such as incubation time (observed when the first symptoms of bacterial wilt disease appear) and disease incidence (total of plant diseased until last observation days), and plant growth such as plant height, number of leaves, first flowering time and yields at the first harvest.

Data were analysed by analysis of variance (ANOVA) at 0.05 probability level. The difference between two means were analysed using Least Significance Difference (LSD) at 0.05 probability level. Effectivity of all treatments towards control also calculated using formula of [45].

3. Result and Discussions
Microbial inoculants are promising components for integrated solutions to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants [46]. Our present study had shown that compatible strains developed as consortiums could increase plant growth and have biocontrol activity. The rhizosphere is a rich habitat for complex communities of soil microorganisms [47], some of which are plant growth-promoting rhizobacteria (PGPR) which stimulate plant growth by efficient root colonization, synthesizing hormones such as IAA and mobilizing nutrients [48]. PGPR are known to increase the availability of nutrients in the rhizosphere, which may be through solubilisation of unavailable forms of nutrients and/or siderophore production and facilitating transport of nutrients [49].

Table 3. Compatibility of PGPR strains

| Strain | R1 | R2 | R3 | R4 | R5 | R6 | R7 | C1 | C2 | C3 | C4 |
|--------|----|----|----|----|----|----|----|----|----|----|----|
| R1     |    |    | √  |    |    |    |    |    |    |    |    |
| R2     |    |    |    |    |    |    |    |    |    |    |    |
| R3     | √  |    |    |    |    |    |    |    |    |    |    |
| R4     |    | √  |    |    |    |    |    |    |    |    |    |
| R5     |    | √  |    |    |    |    |    |    |    |    |    |
| R6     |    |    |    |    |    |    |    |    |    |    |    |
| R7     |    |    |    |    |    |    |    |    |    |    |    |
| C1     |    |    |    |    |    |    |    |    |    |    |    |
| C2     |    |    |    |    |    |    |    |    |    |    |    |
| C3     |    |    |    |    |    |    |    |    |    |    |    |
| C4     |    |    |    |    |    |    |    |    |    |    |    |

*Note: √= compatible (no inhibition zone appear); - = incompatible (inhibition zone appear)

The consortiums developed in this study had showed to increase the plant height and number of leaves of tomato. The growth increase had better results compared to previous study that use single strains. This may due to the synergistic ability of the strains to increase the growth of tomato. Earlier reports had shown that combined inoculation of Azospirillum, Azotobacter chrococcum, Pseudomonas fluorescens and B. megaterium for sorghum was significantly increased grain yield. The stimulatory effects of this PGPR strains on the yield and growth of the crops were attributed to the N2 fixation ability, plant growth regulator production and phosphate solubilizing capacity [50-53].
Table 4. Growth Promoting activity of the PGPR and Cyanobacteria consortium on tomato

| Consortium | Plant height (cm) | Effectivity (%) | Number of leaves | Effectivity (%) | First Flowering time | Effectivity (%) | Yields (g) | Effectivity (%) |
|------------|------------------|-----------------|-----------------|-----------------|---------------------|-----------------|-----------|-----------------|
| C2         | 129.50 a         | 62.61           | 62.60 a         | 75.84           | 28.60 g             | 34.40           | 850.00 a  | 60.88           |
| C1         | 120.60 a         | 51.43           | 60.00 a         | 68.54           | 30.50 f             | 30.05           | 825.50 a  | 56.25           |
| C3         | 115.72 b         | 45.30           | 58.40 ab        | 64.04           | 33.00 e             | 24.31           | 837.20 ab | 58.46           |
| C5         | 110.26 b         | 38.45           | 54.60 b         | 53.37           | 35.00 d             | 19.72           | 800.62 b  | 52.54           |
| C4         | 109.60 b         | 37.62           | 54.00 b         | 51.60           | 37.50 c             | 13.99           | 786.24 b  | 48.82           |
| C6         | 105.72 bc        | 32.75           | 52.00 b         | 46.07           | 38.00 b             | 12.84           | 732.65 c  | 38.67           |
| C10        | 100.40 c         | 26.07           | 50.60 bc        | 42.13           | 39.00 b             | 10.55           | 698.32 cd | 32.17           |
| C11        | 98.52 c          | 23.71           | 48.80 c         | 37.08           | 39.00 b             | 10.55           | 655.29 d  | 24.03           |
| C12        | 97.00 cd         | 21.80           | 47.60 c         | 33.71           | 41.00 ab            | 5.96            | 623.45 de | 18.00           |
| C7         | 96.50 d          | 21.17           | 42.60 d         | 19.66           | 41.50 ab            | 4.82            | 601.80 e  | 13.91           |
| C8         | 94.20 d          | 18.28           | 40.00 d         | 12.36           | 42.00 a             | 3.67            | 598.20 c  | 13.22           |
| C9         | 82.70 e          | 3.84            | 39.80 de        | 11.80           | 42.00 a             | 3.67            | 587.60 ef | 11.22           |
| Control    | 79.64 e          | 35.60 e         | 35.60 e         | 35.60 e         | 43.60 e             | 35.60 e         | 528.33 f  | 35.60 e         |

Our study also showed that the consortiums could accelerate flowering and increase yields of tomato. This effect may due to the increased growth of tomato by consortiums could also lead to the accelerate the flowering time and increase yields. The yields increase response also showed better when treated in consortia compared to single strain inoculants. The beneficial effect of seed inoculation with bacterial consortia on shoot, dry weight and yield of maize was also reported by [54]. Son et al. [55] also found that combination treatment of rhizobacteria strains of Bradyrhizobium japonicum and Pseudomonas spp. could increase yields of soybean.

The combinations of PGPR strains consistently reduced the bacterial wilt disease of tomato under field conditions. The disease incidence varied from 0 to 100% (Table 5). No disease symptom was observed in the six consortia (C2, C1, C3, C5, C4 and C6) until the last day of observations. The results provide evidence that the compatibility of PGPR strains in the consortia effectively suppress disease development of R. syzgii subsp. indonesiensis. Combination of biocontrol agents is a strategic approach to control the plant disease and pest [56]. Furthermore, interactions among the bacterial strains may have synergistic effects that could induce systemic. This results could happen due to consortium may also improve their efficacy, reliability and consistency under diverse soil and environmental conditions [57].

Table 5. Consortium ability as biocontrol agents of R. syzgii subsp. indonesiensis on tomato

| Consortium | Incubation time (dpi) ± sd | Effectivity (%) | Disease incidence (%) | Effectivity (%) |
|------------|---------------------------|-----------------|-----------------------|-----------------|
| C2         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C1         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C3         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C5         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C4         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C6         | 42.00*                    | 28.04           | 0.00                  | 100.00          |
| C10        | 41.00                     | 28.04           | 40.00                 | 50.00           |
| C11        | 41.20                     | 25.60           | 40.00                 | 50.00           |
| C12        | 39.60                     | 20.73           | 40.00                 | 50.00           |
| C7         | 37.60                     | 14.63           | 60.00                 | 25.00           |
| C8         | 37.60                     | 14.63           | 60.00                 | 25.00           |
| C9         | 37.00                     | 12.80           | 60.00                 | 25.00           |
| Control    | 32.80                     | 0.00            | 80.00                 | 0.00            |

There are many reports on the interactions of plant and bacteria, including cyanobacteria [58,59]. Gotz et al. [60] also revealed that the efficient colonization of bacteria was related to the availability of...
nutrients on the root surface or to the root exudation. The interaction of cyanobacteria was studied with pea plants [61]. This is supported by earlier studies using cyanobacteria which revealed their significant role in nutrient cycling and biofortification of wheat crop [62, 63].

3. Conclusion
In conclusions, the application of PGPR and cyanobacteria consortiums found to be effective in controlling the bacterial wilt disease caused by R. syzygii subsp. indonesiensis. The present study clearly indicated that combination of biocontrol agents showed the maximum effects on reduction of R. syzygii subsp. indonesiensis disease development, compared to individual agents used as the previous study suggesting the synergistic effect of consortiums against the pathogen. The PGPR combinations of C2 (Bacillus thuringiensis strain RBI 2AB1.1, Bacillus cereus strain APSB-03 RBI 2AB 2.2, Bacillus subtilis BSn5 RBI IPBL 2.3 and cyanobacteria RZ2AB2.1), C1 (Bacillus thuringiensis strain RBI 2AB1.1, Bacterium JP60 RBI 2 AB 2.3, cyanobacteria RZ2AB2.1) and C3 (Bacillus cereus strain APSB-03 RBI 2AB 2.2, Bacterium JP60 RBI 2 AB 2.3, cyanobacteria RZ2AB2.1) were the promising consortia for the management of wilt disease and enhance the growth of tomato plants.

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