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Selection of Potential Rhizobacteria as Biofertilizer and *In Vivo* Application to Promote Bird's Eye Pepper (*Capsicum frutescens*) Seed Germination in North Central Timor Regency, Indonesia

Gergonius Fallo[1,*], Anna Tefa[2] and Lukas Pardosi[1]

[1]Study Program of Biology, Faculty of Agriculture, University of Timor, East Nusa Tenggara, Indonesia
[2]Study Program of Agrotechnology, Faculty of Agriculture, University of Timor, East Nusa Tenggara, Indonesia

([*]Corresponding author's e-mail: gergofallo@yahoo.com)

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Abstract

North Central Timor (NCT) Regency, located on Timor Island, East Nusa Tenggara Province (NTT) Indonesia, has dry climate conditions with rainfall of 1,500 milli meters (mm) every year. This holds the potential for microbial diversity including rhizobacteria. This research aimed to select and identify potential isolates as biological fertilizers and *in vivo* application for bird's eye pepper (*Capsicum frutescens*) germination. Selection of rhizobacteria from 20 isolates collected by researchers was obtained 5 isolates solubilizer phosphate, 4 isolates fixed nitrogen, and 5 isolates produced Indole Acetic Acid (IAA). Two isolates of CN03 and RTCR01 have potential as biofertilizers. The results of biochemical identification demonstrate that CN03 and RTCR01 isolates ferment glucose, lactose, and sucrose, are positive for citrate and catalase tests, while the molecular identification of 16S rRNA isolates CN03 99 % is similar to *Pseudomonas aeruginosa* and isolates RTCR01 99 % is similar to *Lysinibacillus boritolerans*. Meanwhile, the application with the immersion treatment of bacterial isolates on the bird's eye pepper seed germination results in immersion treatment (K1) of *Pseudomonas aeruginosa;* (K3) the combination of *P. aeruginosa* and *L. boronitolerans* significantly ($p > 0.05$) affected germination, vigor index, length of root, and growth of sprouts, but treatment (K2) *L. boronitolerans* is not significantly different ($p < 0.05$) from the control treatment (KO) using distilled water.

Keywords: *Capsicum frutescens*, *Lysinibacillus boronitolerans*, Biofertilizer, *Pseudomonas aeruginosa*; Rhizobacteria

Introduction

Indonesia is a mega-biodiversity country due to its large diversity of biological resources in the world. Biodiversity includes plants, animals, and microbes. The high biodiversity is inseparable from the climatic background. North Central Timor (NCT) is a regency in East Nusa Tenggara (ENT) which has dry climate conditions with rainfall of 1,500 milli meters (mm) every year [1]. These conditions hold tremendous potential for biodiversity, including microbial biodiversity.

In Indonesia, especially in the dry climate of the NCT Regency, microbial diversity has not been widely explored. In fact, microbes are known to have a lot of potentials and are useable in the fields of industry, health, and agriculture. One of the microbes that need to be explored and utilized properly in the NCT Regency is the Rhizobacteria group.

Rhizobacteria are a group of bacteria living and colonizing in the rhizosphere. The diversity of microbes in the rhizosphere is tremendously high. The study by Fulthorpe *et al.* [2] analyzed 139,819 bacterial strains from 4 regions having different geographical locations using the 16S rRNA marker method. The results of 16S rRNA analysis showed that the most abundant bacterial species in the rhizosphere are *Chitinobacteria spp., Acidobacterium spp*. with an abundance between 13 - 20 %. Research results of Kielak *et al.* [3] revealed that 8 genera are always found in various rhizosphere, namely *Bacillus, Flavobacterium, Pseudomonas, Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes,* and *Gemmatimonades*.

Rhizobacteria have been widely applied to various food crops because many bacteria of the Bacillus and Pseudomonas genera are known to be able to produce IAA, dissolve phosphate, and inhibit *in vitro* fungal growth [4]. Several strains of the Pseudomonas genus are also known to produce chitinase enzyme

[5]. Moreover, Pseudomonas is known to be able to degrade and use several organic and inorganic compounds, interact with plants, and associate in the rhizosphere, making Pseudomonas beneficial in agriculture.

According to Aloo *et al.* [6], when microbes are applied to plants or soil, they can stimulate plant growth, increase soil fertility, grow crop production, and do not cause side effects on farmers and the environment. The use of biofertilizers according to Djajadi *et al.* [7] can increase the levels of nutrients in plants, such as nitrogen (N), phosphorus (P), and potassium (K). Another advantage is that the microbes applied to plants can encourage increased growth of root hairs so that the absorption of water and mineral nutrients becomes more efficient [8]. Biofertilizer also becomes biological control agent against plant diseases up to an efficiency level of 78.2 % [9].

Given the substantial role of rhizobacteria to overcome agriculture in the dry land, NCT Regency, ENT Province, the presence of these bacteria needs to be explored. Therefore, this study aimed to select rhizobacteria as nitrogen fixers, phosphate solvents, and plant growth promoters, as well as identify potential isolates biochemically and 16S rRNA molecular as well as in vivo application of bacterial isolates against bird's eye chili pepper (*Capsicum frutescens*) seed germination.

Materials and methods

Isolates rejuvenation

This study used 20 isolates rhizobacteria from the results of study collection at Biology laboratory of Timor University. Isolates were rejuvenated on Nutrient agar (NA) media using the streak plate method and they were incubated for 24 h.

Selection of phosphate solubilizing bacteria isolates

Phosphate solubilizing bacteria were selected using pikovskaya media. The selection was conducted by spotting on a petri dish containing sterile solid pikovskaya media. After incubation at room temperature for 3 days, the clear zone around the colony was observed and the phosphate solubilization index (IP) was measured [10] based on the formulation:

$$\text{Phosphate Solubilizing Index} = \frac{\text{diameter of the clear zone - diameter of the colony}}{\text{diameter of colony}}$$

Selection of IAA-producing bacteria isolates

The selection of IAA-producing isolates was measured by the colorimetric method using the Salkowski's reagent. The isolates were cultured in 50 mL of NB liquid medium added with 1.0 mM L-trp. One mL of the culture was taken and reacted with 4 mL of Salkowski's reagent (7 mL of 0.5 M $FeCl3.6H_2O$, 250 mL of distilled water, and 150 mL of concentrated H_2SO_4). The mixture was incubated for 20 min and its color changes were observed. If the color of the isolate turns pink, this indicates that the isolate produces IAA [11].

Selection of nitrogen-fixing bacteria isolates

The selection of nitrogen-fixing bacteria isolates was carried out qualitatively using *Jensens agar* selective media [12]. The purified bacterial isolates were selected by scratching the isolates on *Jensens agar* selective media and incubated at 37 °C for 96 h. If the isolate grows and a clear zone is seen, this indicates that the isolate has the potential to fix nitrogen.

Biochemical identification of potential isolates based on 16S rRNA

The selected isolates were identified biochemically in the form of citrate, TSIA, and catalase tests. Each isolate test was scratched on a specific medium and the changes were observed in the medium after a 24-h incubation period at 37 °C. Molecular identification of potential isolates was carried out through the freezing and thawing processes. Pure bacterial culture aged 24 h on NB media, taken 1.5 mL and then centrifuged for 10 min. The supernatant was discarded and added with 100 µL of distilled water under aseptic conditions. The cell suspension was then frozen at –10 °C until the solution crystallized and then it was thawed at 90 °C for 10 min. The cycle was repeated 5 - 10 times for efficient cell breakdown [13]. The isolated DNA was used for amplification of the 16S rRNA gene using a Polymerase Chain Reaction (PCR) machine (Veriti® 96-Well Therm Cycler 4375786), Applied Biosystems Singapore with a universal primer specific for 16S rRNA for prokaryotes, namely 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC 3') [14].

The effect of bacterial isolate on red chili seed germination

The effect of bacterial isolates on the growth of red chili seed germination was tested. The isolates were rejuvenated using NA media for 24 h, then 1 loop of the bacterial colonies was taken, and put into 30 mL of NB, then incubated for 48 h. Chili seeds that would be used were first disinfected using 2 % sodium hypochlorite for 5 min and then rinsed 7 times using sterile distilled water.

This study used a non-factorial completely randomized design (CRD) i.e. immersion with 4 treatments and 3 replications. Treatments include: K0: Control using distilled water, K1: Bacteria isolates, K2: Bacteria isolates, and K3: Isolate combinations. The seeds were soaked for 24 h with the prepared bacterial culture. Seed germination test was conducted by using the Top of Paper Test (TPT) method and by planting 30 seeds per repetition. Each variable was repeated 3 times so as to generate 90 seeds per variable of observation. Parameters observed in this germination test were:

Germination (G): The calculation of germination ability was based on the percentage of normal seedlings on day 14. Germination is calculated using the formula:

$$\text{Germination (G): } \frac{\sum \text{Normal seedling evaluation}}{\sum \text{Seed planted}} \times 100\ \%$$

Vigor index (VI) calculation was based on persentase the percentage of normal seedlings that grow on the 7 days. Abnormal seed (%). Measurement based on observing the number of seeds that germinate abnormally. Observation of abnormal seeds is carried out on the day 14 after the nursery. Abnormal seeds are calculated using the formula:

$$\text{Abnormal seeds (\%): } \frac{\sum \text{Abnormal seeds}}{\text{Number of seedlings planted}} \times 100\ \%$$

Maximum Growth Potential (MGP). Maximum growth population was calculated based on the percentage of all seedlings that grow both normal and abnormal until the end of the observation. Maximum growth potential was calculated using:

$$\text{MGP: } \frac{\sum \text{Seed grow until the end}}{\sum \text{Seed planted}} \times 100\ \%$$

Observation of the growth of bird's eye pepper (*Capsicum frutescens*) sprouts and the length of root of bird's eye pepper (*Capsicum frutescens*) seeds after 14 days

Statistical analysis

All the data were analyzed using the SAS statistical software and the treatment means were compared using the least significant difference at $p \leq 0.05$ level of significance

Results and discussion

This study selection of rhizobacteria were obtained 6 potential isolates capable of dissolving phosphate in pikovskaya medium were selected. Furthermore, 4 nitrogen-fixing isolates grew on Jansen's agar after 96 h of incubation, and 5 isolates produced IAA (**Table 1** and **Figure 1**).

Table 1 Results of the selection of bacterial isolates as a phosphate solubilizer, a nitrogen fixer, and IAA producer.

Isolate code	Characterization of potential rhizobacteria		
	Phosphate solubilizer	**Nitrogen fixer**	**Indole acetic acid (IAA)**
CMB01	-	-	-
CMB02	+	-	-
CMB03	+	-	-
CMB04	+	-	+
CMB 05	-	+	-

Isolate code	Characterization of potential rhizobacteria		
	Phosphate solubilizer	**Nitrogen fixer**	**Indole acetic acid (IAA)**
CMB 06	-	-	-
CN01	-	-	-
CN02	-	-	-
CN03	+	+	+
CN04	+	-	+
CN05	-	-	-
CN06	-	-	+
RTCR01	+	+	+
RTCR02	-	-	-
RTCR03	-	-	-
RTCR04	-	-	-
RTCR05	-	-	-
RTCR06	-	+	-
RTCR07	-	-	-
RTCR08	-	-	-

Note: (+) Isolate phosphate solubilizer, fixes nitrogen, and produces IAA, (-) Isolate does not dissolve phosphate, fix nitrogen, and produce IAA.

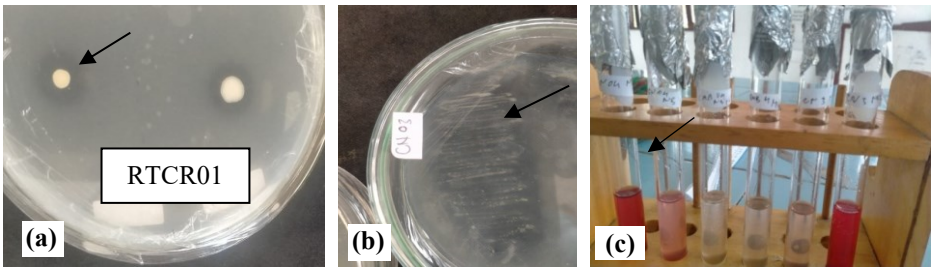

Figure 1 (a) Test results of RTCR01 isolates as phosphate solubilizer on pikovskaya medium after 48 h incubation, (b) CN03 isolates grow on Jensen's agar media after 96-h incubation, (c). CN03 produce IAA.

The phosphate solubilizing bacteria were selected using pikovskaya media. The clear zone formed around the isolated colonies on the media indicated the presence of bacterial activity in dissolving bound P. The ability of isolates to release bound P, such as Ca-phosphate, caused the isolates to function to provide available phosphate for plant needs.

The fixing bacteria were chosen through Jensen's agar media. This medium is selective because it does not contain nitrogen, so only bacteria having the ability to fix nitrogen can grow. This Jansen media composition consists of sucrose functioning as a source of energy for bacteria, while sodium molybdate is useful for increasing the activity of nitrogen-fixing. Furthermore, sodium chloride also serves to maintain the balance of the osmotic pressure of the medium. Calcium is useful for stimulating nodule growth when it appears in the form of chloride or sulfate, so isolates growing in this medium can be confirmed as nitrogen-fixing bacteria [15].

The selected isolates produced IAA using Nutrient Broth (NB) medium with the addition of L-tryptophan reacted with Salkowski's reagent. The Salkowski reagent used in this IAA production test became an indicator of IAA biosynthesis. This reagent will react with indole pyruvic acid causing a red color. Six isolates namely CMB04, CN04, CN03, CN06, and RTCR01 produced IAA without the addition of l-tryptophan or the presence of tryptophan in the medium. Sutariati *et al.* [16] said that IAA production

was strongly influenced by the level of availability of l-tryptophan substrate. The presence of IAA synthesis with tryptophan indicates that isolates are inducible by tryptophan compounds through the IpyA pathway. Meanwhile, those that synthesized IAA without using l-tryptophan as a precursor used the indole-3-glycerol phosphate (IGP) pathway.

The isolate selection resulted in 2 isolates, namely CN03 and RTCR01, showing their potential as phosphate solvents, nitrogen fixers, and IAA producers so that both isolates were classified into the plant growth promoting bacteria (PGPB) group. The biochemical test of CN03 and RTCR01 isolates was to determine the ability of isolates to produce H_2S gas and carbohydrates (catalase test and TSIA). The CN03 and RTCR01 isolates are known to be able to ferment glucose, lactose, and sucrose, positive for citrate and catalase tests. Moreover, the cell morphology of CN03 isolates is known to be gram-negative with the rod shape, while the cell morphology of RTCR01 is gram-positive with the rod shape (**Table 2**).

Table 2 Biochemical and morphology tests of CN03 and RTCR 01 bacterial cell isolates.

Isolate code	TSIA	Biochemical test		Morphology of cell	
		Citrate	Catalase	Gram	Shape of cell
CN03	Fermenting glucose, lactose, and sucrose	+	+	-	Rod
RTCR01	Fermenting glucose, lactose, and sucrose	+	+	+	Rod

Notes: (+) positive, (-) negative

The molecular identification of 16S rRNA using the BLAST program revealed that the CN03 isolate had 99 % similarity to the bacterium *Pseudomonas aeruginos* strain LC069033, while the RTCR01 isolate had 99 % similarity to the bacterium *Lysinibacillus boronitolerans* (**Table 3**; **Figure 2**).

Table 3 Identification results of CN03 and RTCR01bacterial isolates based on the 16S rRNA gene.

Isolate code	Accession	Description	Max score	Total score	Cove rage	e value	Identity %
CN03	cp 012001.1	*Pseudomonas aeruginosa*	1,475	1,476	98	0	99
RTCR01	NR_114207.1	*Lysinibacillus boronitolerans*	1,466	1,478	100	0	99

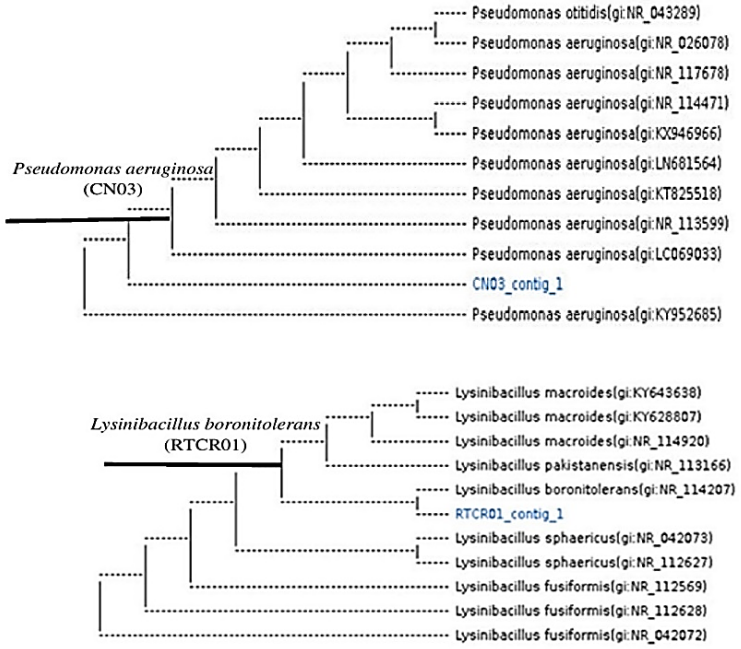

Figure 2 CN03 and RTCR01 bacterial isolate phylogenetic tree.

According to Agustian *et al.* [17] bacteria, such as *Pseudomonas* sp., *Azotobakter* sp., and *Bacillus* sp. act as phosphate solubilizers and producers of phytohormones such as IAA. This organisme Bacteria *Lysinibacillus boronitolerance*, was previously considered a member of the genus *Bacillus* and in 2007 was changed to a new genus *Lysinibacillus* based on its peptidoglycan composition. The cell wall constituents of the peptidoglycan *L. boronitolerance* are lysine, aspartate, and glutamic acid. Based on the distinctive peptidoglycan, the strain becomes a new species in a new genus. *Pseudomonas aeruginosa* belongs to a group of phosphate solubilizing and IAA-producing bacteria that have been widely studied. However, *L. boronitolerance* has not been reported. *Lysinibacillus* species reported by Ahsan *et al.* [18] have potential as bioremediation, biostimulant, and biocontrol agent. Specifically, for *Lysinibacillus* species, it is reported as a phosphate solubilizer, nitrogen fixer, and producer of IAA hormones, namely *Lysinibacillus sphaericus, Lysinibacillus fusiformi*, and *Lysinibacillus mangiferihumi* [19]. The research of Ahmed *et al.* Ahmed *et al.* [20] has characterized the genus *Lysinobacillus* which has a cell length of 3.0 - 5.0 mm and a diameter of 0.8 - 1.5 mm. Colonies are circular with flat elevations. The growth temperature is 16 - 45 °C. Furthermore, the results of the biochemical test using API 20E were found to be positive for the Voges-Proskauer test, urease, L-arginine dihydrolase, tryptophan deaminase, using citrate, and negative for hydrolysis of gelatin and L-lysine, and L-ornithine decarboxylase.

The effect of the treatment of bacterial isolates of *P. aeruginosa* CN03 and *L. boronitolerans* RTCR01 on red chili seed germination. The immersion by using (K1) *P. aeruginosa,* (K2) *L. boronitolerans* RTCR01, (K3) the combination of *P. aeruginos* CN03 and *L. boronitolerans* RTCR01 ($p < 0.05$) significantly affected germination power, vigor index (VI), abnormal germination, and ungerminated seeds. Meanwhile, the results of observations of sprout height and root length of red chili seeds after 14 days showed that the effect of immersion using (K1) *P. aeruginosa* CN03, (K3) the combination of *P aeruginos* CN03 and *L. boronitolerans* RTCR01 ($p < 0.05$) significantly affected root length and sprout height. However, the immersion treatment of (K2) *L. boronitolerans* RTCR01 ($p > 0.05$) was not significantly different from the control treatment of KO using distilled water (**Table 3**, **Figure 3**).

Table 3 Effect of bacterial isolates on germination power, vigor index, abnormal sprouts, and non-germinating seeds.

Treatment	Germination (G) % Day 14	Vigor index (VI) % Day 7	Abnormal seeds (%) Day 14	Maksimum growth potential (MGP) % Day 7
K0	83.2b	50c	27.7a	4a
K1	96.7a	76.5b	6.7b	3.2a
K2	94a	84ab	5b	4a
K3	97.5a	87.5a	1.5b	2.5a

Notes: K0: Distilled Water; K1: *P. aeruginos* CN03, K2: *L. boronitolerans* RTCR01, K3: *P. aeruginos* CN03 and *L. boronitolerans* RTCR01. Numbers that are followed by the same letter in the same column show results that are not significantly different at the 5 % level based on Duncan's multiple range test.

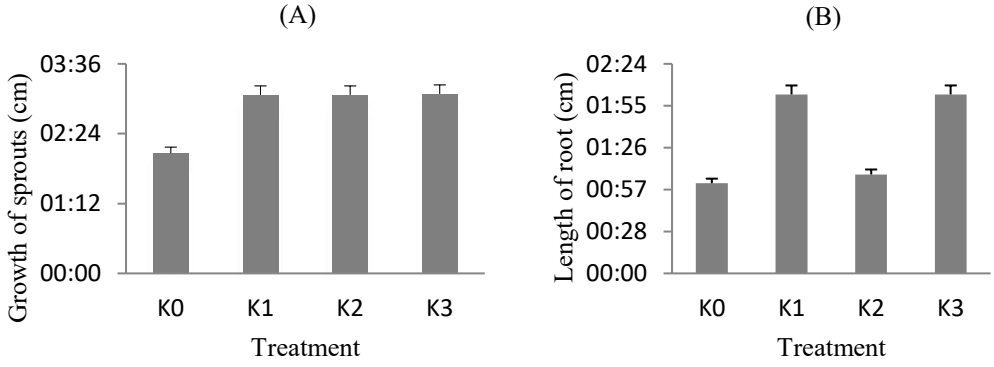

Figure 3 Observation of the growth of promote bird's eye chili pepper (*Capsicum frutescens*) sprouts (A) and the length of root (B) after 14 days. K0: distilled water, K1: *P. aeruginosa* CN03, K2: *L. boronitolerans* RTCR01, K3: *P. aeruginos* CN03 and *L. boronitolerans* RTCR01.

The experiment results showed that *P. aeruginosa* CN03 and the combination of *P. aeruginos* CN03 and *L. boronitolerans* RTCR01 significantly affected germination power, vigor index (VI), and could stimulate root elongation. The research results of Sembiring and Sumanto [21] regarding the effect of isolates on the viability of red chili seeds also revealed that isolates of IAA-producing bacteria in the range of 7.96 - 47.23 ppm could increase germination power by 33 % and vigor index (VI) by 10 %. This proves that the production of IAA produced by bacteria does not only function as a hormone in the bacterial cell, but rather leads to the development of the interaction relationship between bacteria and plants. Patten and Glick [22] stated that a low concentration of IAA, which was around 10^{-9} - 10^{-12} M, stimulated lateral root elongation, whereas a high concentration of IAA produced by high-density inoculum stimulated lateral and adventitious root formation. The IAA produced does not only affect plant growth through cell differentiation but also regulates various physiological responses affecting seed germination.

Conclusions

The selection of rhizobacteria resulted in 2 isolates, namely CN03 and RTCR01, having potential as biological fertilizers. The results of the molecular identification of 16S rRNA showed that CN03 isolates had 99 % similarity to *P. aeruginosa*, while RTCR01 had 99 % similarity to *L. boronitolerans*. The effect of isolates on red chili seed germination was identified by immersion treatment of (K1) *P. aeruginosa*, (K2) *L. boronitolerans*, (K3) *P. aeruginos* and *L. boronitolerans* that significantly affected germination power and vigor index (VI). Meanwhile, observations of sprout height and root length after 14 days were identified through immersing *P. aeruginosa*, (K3) *P. aeruginosa* and *L. boronitolerans* that significantly affected root length and growth height. However, the treatment of (K2) *L. boronitolerans* was not significantly different from the control treatment (KO) using distilled water.

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