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Fluorescent pseudomonads from plant rhizosphere as biological agent to control white root disease and growth-promoting on rubber plants

HUSDA MARWAN♥, SRI MULYATI, S. SARMAN, ISLAH HAYATI
Program of Agroecotechnology, Faculty of Agriculture, Universitas Jambi. Jl. Jambi-Muara Bulian Km 15, Muaro Jambi 36361, Jambi, Indonesia.
Tel./fax.: +62-741-583051, ♥email: husda_marwan@unja.ac.id

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Abstract. *Marwan H, Mulyati S, Sarman S, Hayati I. 2020. Fluorescent pseudomonads from plant rhizosphere as biological agents to control white root disease and growth-promoting on rubber plants. Biodiversitas 21: 5338-5343.* White root disease (*Rigidoporus lignosus)* is a serious problem of rubber plantation in Indonesia, including in Sumatera. The fungal infection is often difficult to detect, infection develops rapidly and can cause plant death if left untreated since the early stage, and thus using biological agents might be an efficient way to overcome the disease. This research was aimed to explore fluorescent pseudomonads from rubber plant rhizosphere that can inhibit the growth of the fungal pathogen, characterize fluorescent pseudomonads isolated as biological control agent and plant growth promoter, and apply the isolate to rubber plants to control the disease and growth of plants. Twelve out of the 76 fluorescent pseudomonads isolated from several rhizospheres of rubber plants were found to be antagonistic to white root fungi. Fluorescent pseudomonads have various characteristics such as chitinolytic activity, phosphate solvent, and nitrogen fixation. The application of the antagonistic isolates on rubber seedlings was able to increase growth and suppress the colonization of *R. lignosus* on rubber plant roots.

Keywords: Biological control, pseudomonads, rhizobacteria, *Rigidoporus lignosus*

INTRODUCTION

White root disease is one of the obstacles in rubber plantations in Indonesia. The disease is caused by a fungus *Rigidoporus lignosus* (Nandris et al. 1987). This disease can result in plant death, thus reducing in production. The area of white root disease attacks in Indonesia was around 26.000 ha with an estimated yield loss of IDR 75.67 billion (Directorate General of Plantations 2016).

The initial stage of white root fungal infection is often difficult to detect because the initial location of the infection is below the soil surface, and if the plant is left untreated the infection develops rapidly and causes death (Mohd et al. 2009). The disease sign (rhizomorphs) are developed and only visible in late season. The disease cycle involves infection and colonization of fungi mycelia as primary inoculums on the root systems of rubber plant seedlings growing from stumps of infected forest trees. The fungus attack roots and collar regions of taproot causing white root rot. The growth of pathogens may progress from infected trees (second inoculum) to roots as well as healthy rubber surrounding them. The pathogen mycelia can grow about 2.5 m per year (Nandris et al. 1987; Kaewchai and Soytong 2010). These cause management of white root fungus disease often unable to suppress disease progression as there is difficulty in assessing the pathogens that have colonized plant roots. Early inoculation of beneficial rhizobacteria in rubber plants can protect plant roots from white root fungal infection.

The rhizosphere was defined as the zone around the root where microorganisms and processes important for plant growth and health are located (Bakker et al. 2013). Many plant rhizospheres are inhabited by microbial groups that colonize the surface of plant roots. This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria. The plant-associated microbiomes consist of beneficial organisms like plant growth-promoting (Beneduzi et al. 2012; Herrmann et al. 2016; de Vrieze et al. 2018) and biocontrol agents (Mohanram and Kumar 2019).

Rhizobacteria colonize the rhizosphere, the rhizoplane (root surface), or the root itself (within radicular tissues) (Gray and Smith 2005). Bacteria of diverse genera have been identified as Plant Growth Promoting Rhizobacteria (PGPR), of which *Bacillus* and *Pseudomonas* spp. are predominant. The genera of *Bacillus* and *Pseudomonas* are well known for their antagonistic effects and their ability to trigger Induce Systemic Resistance (ISR) (Beneduzi et al. *20*12; Pieterse et al. 2014). Fluorescent pseudomonads are suppressed the soil-borne pathogens through rhizosphere colonization, antibiosis, iron chelation by siderophore production and ISR (Vanitha and Ramjegathesh 2014).

The use of biological control agents on rubber seedlings prior to planting can be carried out to protect rubber plant roots from early white root fungus infection. Fluorescent pseudomonads are one of rhizobacteria that have the potential to grow as biological control agents against plant pathogenic fungi. Fluorescent pseudomonads are suitable for application as biological control agents due to their abundant population in natural soils and plant root systems and their capability to utilize many plant exudates as nutrient (Panpatte et al. 2019). This group of bacteria has

been reported to be able to inhibit the growth of several plant pathogenic fungi, such as *Fusarium oxysporum* f. sp. *lycopersici* in tomato (Manikandan et al. 2010), *Colletotrichum gloeosporioides* strains in pepper (Anees et al. 2019), *Rigidoporus* sp. in rubber plant (Hasanuddin 2011; Nasrun and Nurmansyah 2015; Damiri et al. 2019).

This study aims to: (i) explore fluorescent pseudomonads from rubber plant rhizosphere, which can inhibit the growth of the fungus *R. lignosus*: (ii) isolation of fluorescent pseudomonads which has characters that promoter plant growth; (iii) utilize the antagonistic fluorescent pseudomonads isolate on rubber plants as biological control agent.

MATERIALS AND METHODS

Isolation of fluorescent pseudomonads bacteria from rubber plant rhizosphere

Soil samples were taken from the rubber plantation of PT. Lestari Asri Jaya, Tebo District (Jambi Province, Indonesia) and smallholder rubber plantations in Muaro Jambi District. The soil samples were obtained from the rhizosphere of well-grown rubber plants among several symptomatic plants. A total of 50 g of soil samples were suspended in 450 ml of sterile distilled water in an Erlenmeyer and shake at a speed of 150 rpm for 30 minutes (Khaeruni et al. 2010). After deposited, 1 ml of the supernatant was taken and diluted. A total of each 0.1 ml of 10^{-7} and 10^{-9} dilutions were spread (platting) using glass beads on King's B medium which was specific for the fluorescent Pseudomonas bacteria group. The inoculated plate was then incubated for 48 hours at room temperature. The bacteria that grew on the media were observed under UV light, fluorescent colonies were a group of fluorescent Pseudomonas bacteria, and then transferred to the new King's B media (Vanitha and Ramjegathesh 2014).

Inhibition test of fluorescent pseudomonads isolates against *Rigidoporus lignosus*

Rigidoporus lignosus obtained from the Laboratory of Plant Diseases, Faculty of Agriculture, Jambi University, was cultured on Potato Dextrose Agar (PDA) and incubated for 7 days until the fungal mycelium had covered the PDA surface on the Petri dish.

Fluorescent pseudomonads isolates were tested for their ability to inhibit the growth of the fungus *R. lignosus* on PDA media with a dual culture method. Bacterial isolates were streaked with a distance of 3 cm from the edge of the plate on the PDA medium and 3 cm from the pieces of the fungal colony (0.5 cm in diameter). The cultures were incubated at room temperature for 4 days. Observations were made on the diameter of the fungal colony *R. lignosus.* The percentage of bacterial inhibition against *R. lignosus* was calculated using the following formula.

$$I = \frac{R1 - R2}{R1} \text{ X } 100\%$$

Where:

I: Percentage of inhibition (%)

R1: Colony radius of *R. lignosus* which grows in the opposite direction to the bacteria

R2: Colony radius of *R. lignosus* which grow towards the bacteria

Characterization of fluorescent pseudomonads isolates

Characterization was carried out to determine some of the physiological properties of fluorescent pseudomonads isolates which relate to their potential as biological control agents and plant growth promoters, namely hypersensitivity reactions, chitinolytic activity, phosphate solvents, and nitrogen fixation. Characterization was also carried out on isolates that showed inhibitory power against the fungus *R. lignosus.*

Hypersensitivity test was performed by infiltrating the suspension of each bacterial isolate into tobacco leaves (*Nicotiana tabacum* L.) at 2-3 months of age as indicator. Pseudomonads fluorescent bacteria isolate, *Xanthomonas oryzae* pv. *oryzae* (as a pathogen control) was cultured on Nutrient Broth (NB) medium for 48 hours. As much as 0.1 ml (population 10^8 cells/mL) of each bacterial suspension was taken using a 1 ml syringe (without a needle) then infiltrated into the lower surface of tobacco leaf slowly until the bacterial culture does not enter in leaf tissue. Hypersensitivity symptoms were observed 48 hours after inoculation. Leaves experiencing hypersensitivity symptoms showed a change in leaf color from green to yellowish (necrotic) in the inoculation area. The necrotic symptom indicates that the inoculated bacteria had the potential to become plant pathogens or have a positive reaction. In contrast, bacteria that react negatively do not show necrotic symptoms in tobacco leaves or not pathogenic to plants. The chitinolytic activity of isolates was tested using Lingappa and Lockwood method (1962). Chitin agar containing 0.2% colloidal chitin (pH 6.2) was sterilized, and then poured into a Petri dish (9 cm diameter). After the agar was frozen, Whattman filter paper with a diameter of 6 mm was placed on the surface of the agar medium. A total of 5 µl of liquid culture of each bacterial isolate (population 108 cells/mL) was poured onto filter paper, and then incubated at room temperature. This procedure was repeated twice. Observations were made on the clear zone formed around the filter paper after incubation for 7 days. A chitinolytic property of bacteria was expressed by measuring isolate's chitinolytic index (CI), ratio between diameter of clear zone (purple color), and diameter of bacterial colony (Zhou et al. 2002).

The ability of the fluorescent pseudomonads isolates as a phosphate solvent was tested by growing bacterial culture on Pikovskaya media (Paul and Sinha 2016). The Pikovskaya media was poured into 15 mL Petri dishes and then placed Whatman paper with a diameter of 6 mm on the media surface. A total of 5 µL of bacterial suspension (population 10^8cfu /ml) was inoculated on filter paper using a micropipette, and then incubated at room temperature. Clear zone measurements were carried out up to 7 days after incubation. The halo zone formations around the growing colony show a sign of phosphate solubilization. The soluble index was evaluated according to the ratio of

the total diameter (colony + halo zone) and the colony diameter (Edi-Premono et al. 1996).

The ability of pseudomonads fluorescent isolates to fix nitrogen was tested by growing bacterial cultures on Biological Nitrogen Fixation (BNF) media in test tubes. BNF media that already contains the bacteria culture was shaken at a speed of 120 rpm at room temperature for 48 hours (Harca et al. 2014). Observations were made by observing the turbidity of the BNF media. If the BNF medium turns cloudy, the tested isolates are able to fix nitrogen.

Application of fluorescent pseudomonads test on rubber plants

Fluorescent pseudomonads isolates that were able to inhibit the growth of the fungus *R. lignosus* with different characteristics were applied to rubber seedlings, using a randomized block design (RBD). Healthy plants were used as negative control (plants without bacterial isolate and *R. lignosus*), and diseased plant as positive control (Plants inoculated with *R. lignosus*). A total of 20 rubber seeds were used for each treatment.

One-month-old rubber seedlings roots were soaked in a bacterial suspension for 6 hours (Marwan 2011). The seedlings were planted on soil media (a total of 5 kg of humic soil and 500 gr of sterile manure) in a polybag. Two pieces of block rubber woods measuring 2 x 2 x 5 cm were placed next to each seedling. The woods were pretreated with *R. lignosus*. The plants were acclimated for 1 month before being placed in the field of experiment.

Observations were made on plant growth variables (number of leaves, stem height, stem diameter), root weight, and the percentage of colonization of the fungus *R. lignosus*. Observations of plant growth were carried out every 3 months after planting until the plants were 9 months old. Colonization of *R. lignosus* was observed 9 months after planting by dismantling all the plants, and then the roots were cleaned with running water and weighed to determine the root weight. The data for observing the growth of rubber plants was carried out by analysis of variance (ANOVA) and the Duncan Multiple Range Test at the 5% level.

RESULTS AND DISCUSSION

Pseudomonas fluorescent bacteria from rubber plant

A total of 76 isolates of fluorescent pseudomonads were observed, based on single colony testing. A single colony of fluorescent pseudomonads showed a yellowish-green color of fluorescent pigment under ultraviolet light (Figure 1.A). The 12 antagonist isolates showed a percentage of inhibition 15.8-61.9% (Table 1). The fluorescent pseudomonads antagonist was able to inhibit the growth of the fungal mycelium *R. lignosus* and a clear zone was formed between the fungal colony and the fluorescent pseudomonads colony (Figure 1.B).

Table 1. Antagonistic ability of fluorescent pseudomonads isolates against *Rigidoporus lignosus* and hypersensitivity reactions on tobacco leaves

Fluorescent pseudomonads isolates code	Inhibition against *R. lignosus* (%)	Hypersensitivity reactions
A1P13	45.8	Negative
A1P26	61.9	Negative
A2P13	46.7	Negative
A2P16	41.5	Negative
A3P4	43,7	Negative
A4P15	17.1	Negative
A4P16	15.8	Negative
A5P3	31.6	Negative
A5P3	40.8	Negative
K2P5	16.8	Negative
K3P6	17.1	Negative
K4P9	15.8	Negative

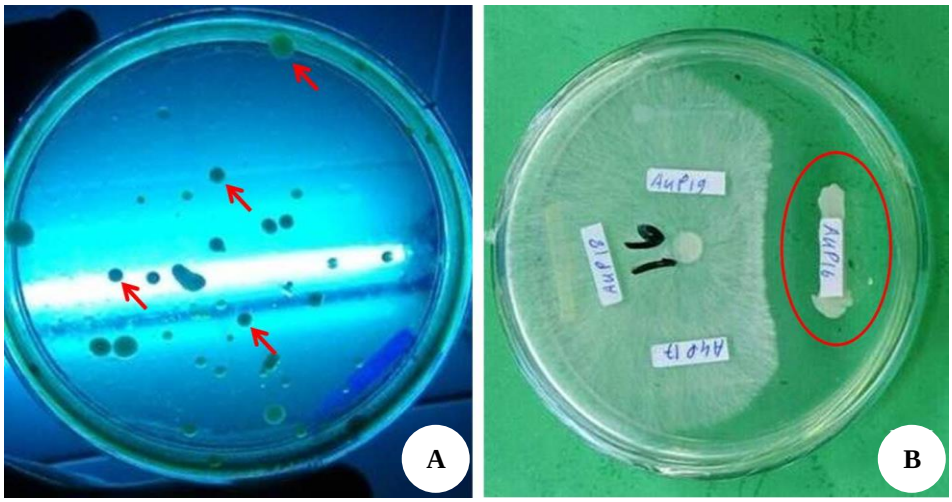

Figure 1. Isolation and selection of pseudomonads fluorescent against the fungus *Rigidoporus lignosus*: A. Single colony of fluorescent pseudomonads (red arrow) on Kings' medium observed under UV lamp; B. Pseudomonads fluorescent isolate which is able to inhibit the growth of *R. lignosus* (inside the red circle)

Table 2. Characteristics of fluorescent pseudomonads antagonists against *Rigidoporus lignosus*

Fluorescent pseudomonads isolates code	Chitinolytic activity (CI)[1)]	Phosphate solubility (SI)[2)]	Nitrogen fixation
A1P13	2.14	3.54	No
A1P26	2.67	3.79	No
A2P13	0	3.33	No
A2P16	0	3.25	No
A3P4	2.04	3.58	No
A4P15	3.49	2.71	No
A4P16	3.71	2.54	No
A5P3	3.38	3.46	Yes
A5P3	0	2.92	Yes
K2P5	3.25	2.04	Yes
K3P6	4.08	2.13	Yes
K4P9	4.75	0	Yes

Note: [1)] CI: Chitinolytic index, [2)] SI: Solubilization index

The ability of the fluorescent pseudomonads to inhibit the growth of the fungus *R. lignosus* was related to anti-fungal compounds, such as chitinolytic enzymes, produced by these bacteria. The results showed that all isolates inhibiting the growth of *R. lignosus* showed chitinolytic activity, except A2P13, A2P16, and A5P3 isolates (Table 2). The existence of chitinolytic activity indicates that bacteria produce enzymes that play a role in degrading chitin compounds as constituents of fungal cell walls. Chitin degradation is an important biological control mechanism for the fungal phytopathogens. Chitin is a major structural polysaccharide and is abundant in the cell walls of the majority of fungi. The β-1-4 glycosidic bonds in chitin are responsible for the cell wall integrity and are targeted by chitinases, the chitin degrading enzymes (Beier and Bertilsson 2013). The chitinolytic bacterial strains *P. fluorescens* Pf1 inhibited the mycelia growth of *Alternaria solani* by 43-48%, *Fusarium oxysporum* by 30-49% (Manikandan et al. 2010) and *P. fluorescens* HN1205 inhibited the colony growth of *Colletotrichum gloeosporioides* ranging from 28-41% (Anees et al. 2019).

Results indicated that the chitinolytic index value among isolates was not related to the percentage of bacterial inhibition against the growth of *R. lignosus*. K4P9 isolate with the highest chitinolytic index value (4.75) was only able to inhibit the growth of *R. lignosus* by 15.8%, in contrast, to isolate A1P26 with the highest inhibitory power (61.9%), only has a chitinolytic index value of 2.67. This shows that the ability of fluorescent pseudomonads to inhibit fungal growth was not only determined by chitinolytic activity. According to Haas and Defago (2005), fluorescent Pseudomonad strains that effectively suppress root diseases caused by soil-borne fungi were able to synthesize one or several antibiotic compounds. Several antibiotics that function in fungal suppression, were the phenazines (PHZ), phloroglucinols (PHL), for eg, 2, 4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), pyrrolnitrin, hydrogen cyanide (HCN), and non-ribosomal peptides (NRPs) including cyclic lipopeptides (Michelsen and Stougaard 2011). Hernandez-Leon et al. (2015) reported that the *P. fluorescens* UM16, UM240, UM256, and UM270 strains exhibited several antifungal traits (potential presence of phenazines, DAPG, HCN, ACC deaminase, production of biofilm, IAA, siderophores, and proteases), which could be acting during the antagonism toward *Botrytis cinerea*.

Fluorescent pseudomonads isolates which showed antagonistic ability against *R. lignosus* have several characters in common (Table 2). Based on the hypersensitive reaction test, 12 antagonist isolates did not show necrosis in tobacco leaves so they were included in the negative hypersensitive group. A total of 3 bacterial isolates (A3P5, K2P5, K3P6) showed chitinolytic activity, phosphate solvent, and nitrogen fixation. Whereas, 5 isolates (A1P13, A1P26, A3P4, A4P15, A4P16) showed chitinolytic activity and phosphate solvent, 3 isolates (A2P13, A2P16, A5P3) as a phosphate solvent, while K4P9 showed chitinolytic activity and nitrogen fixation.

The diversity of characters of fluorescent pseudomonads antagonists isolated from several rubber plant rhizospheres can be influenced by differences in land vegetation, land management, types of rubber plants cultivated, and the interaction between microbes in the rhizosphere. According to Ross et al. (2000), the rhizosphere has indicated that individual plants harbor mixed populations of pseudomonads, many of which can suppress plant pathogens *in vitro*. Diverse plant host and their rhizosphere microbes can secrete multiple metabolites that can alter gene expression and the physiology of biocontrol pseudomonads (Pierson and Pierson 2007). Phase variation in some pseudomonad populations can regulate secretion of secondary metabolites that can play role in plant-pathogen suppression (van den Broek et al. 2003).

Application of fluorescent pseudomonads antagonists to rubber seedlings before planting was able to maintain plant growth on planting media infested by *R. lignosus*. Results showed that fluorescent pseudomonads treatment before planting had an effect on plant height and root weight at 9 months after planting, but had no effect on plant stem diameter (Table 3). Treatment of isolates A1P13, A4P16, and K4P9 consistently showed that plant height, stem diameter and root weight were not significantly different from the positive control treatment.

Fluorescent pseudomonads can affect plant growth directly and indirectly. Fluorescent pseudomonads have a direct effect on growth occurs because bacteria are able to provide the compounds needed for plant growth. This is related to the ability of bacteria to dissolve phosphate, fix nitrogen, and produce various plant growth hormones. Species of fluorescent pseudomonads are known to produce phytohormones like indole-acetic acid (IAA), cytokinins, gibberellins, and inhibitors of ethylene production, which may indirectly help in increasing the absorptive surface of plant roots for uptake of water and nutrients. Fluorescent pseudomonads may act directly on the growth and physiological and nutritional status of the plant they colonize (Nihorimbere et al.. 2011).

Table 3. Effect of fluorescent pseudomonads application on rubber plant growth and mushroom colonization of *Rigidoporus lignosus* at 9 months after planting

Fluorescent pseudomonads isolates code	Plant height (cm)*)	Plant stem diameter (mm)ns)	Root weight (gram)*)	Root colonization by *R. lignosus* (%)*)
Positive control	89.0 a	7.4	17.1 b	0
Negative control	75.1 d	6.8	13.8 c	65
A1P13	90.2 a	7.5	23.2 a	0
A1P26	79.5 c	6.6	17.4 b	5
A2P13	86.0 ab	6.9	19.6 b	5
A2P16	79.4 c	7.5	17.4 b	10
A3P4	80.0 c	7.2	19.8 b	10
A4P15	86.0 ab	6.9	19.9 b	10
A4P16	89.6 a	7.6	17.2 b	5
A5P3	84.6 b	6.8	20.4 ab	0
A5P3	79.0 c	6.5	18.4 b	10
K2P5	79.2 c	6.4	18.3 b	10
K3P6	86.6 ab	6.9	18.6 b	5
K4P9	88.3 a	7.5	17.8 b	0

Note: *)The numbers followed by the same letter are not significantly different at $p \leq 0.05$ DMRT. ns) The ANOVA test results were not significantly different

This work suggests that several fluorescent pseudomonads isolates have the character of a phosphate solvent and nitrogen fixation. Phosphorus is one of the most important nutrients for plant growth. The majority of soil P is found in insoluble forms and only two soluble forms. the monobasic ($H_2PO_4^{-1}$) and the dibasic (HPO_4^{-2}) ions.thatcan be absorbed by plants (Bhattacharyya and Jha. 2012). Phosphate-solubilizing bacteria are considered promising biofertilizers since they can increase the availability of P to plants. Rhizobacteria released organic acids into the soil which solubilize the phosphate complexes converting them into ortho-phosphate which is available for plant uptake and utilization (Oteino et al. 2015). The ability of fluorescent pseudomonads isolates to fix nitrogen can directly increase plant growth. Pseudomonas strains can also benefit plant growth by providing plants with macro-and micro-nutrients, including nitrogen, iron, and phosphorus (Pieterse et al. 2014).

The indirect effect of fluorescent pseudomonads on rubber plant growth occurred because the bacteria were able to protect plant roots from the colonization of the fungus *R. lignosus* which can damage the roots, thereby disrupting the function of the roots in absorbing nutrients. *R. lignosus* penetrates the root system and colonizes the tissues. The mycelium of the pathogen degrades the host's cell structures. The pathogen of *R. lignosus* must repeatedly carry out penetration and colonization of their host cell wall by enzymatic digestion of the tissues characterized by differentiation of specialized structures ((Kaewchai and Soytong 2010).

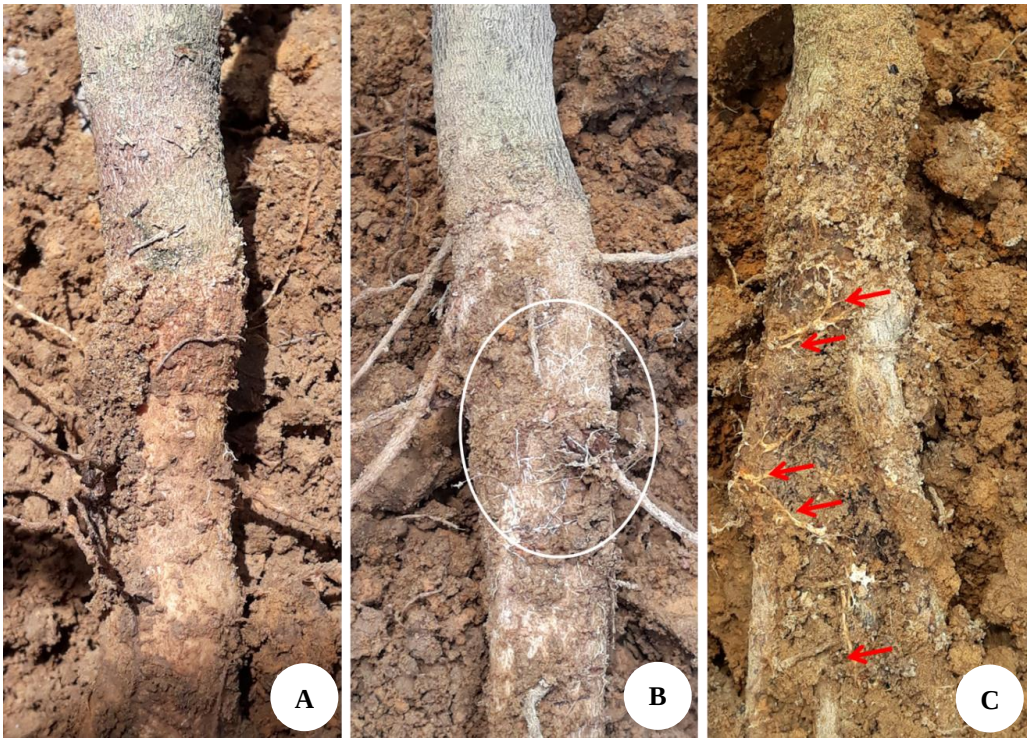

Figure 2. Effect of pseudomonads fluorescent isolate application on the colonization of *R. lignosus* on the roots of rubber plants: A. The root parts of plants were not found in the fungal colony of *R. lignosus* (healthy plants); B. White rhizomorph of the *R. lignosus* on the root neck (white circle). the part of the plant above the soil surface has not shown any disease symptoms; C. Brownish rhizomorphs found in all plant roots (red arrows)

Result showed that the application of fluorescent pseudomonads to rubber seedlings before planting was able to maintain plant growth. Application before planting had an effect on plant height and root weight at 9 months after planting, but had no effect on plant stem diameter (Table 3). This was also able to protect the roots from the colonization of the pathogen. The *R. lignosus* colonization in rubber plant without fluorescent pseudomonads treatment (negative control)was 65% compared to 0-10% in fluorescent pseudomonads treatment. Moreover, 35% rubber plants were dead in negative control compare to 0-5% of rubber plants in fluorescent pseudomonads treatment. Almost all root parts of the negative control plants showed *R. lignosus* rhizomorphs and some were brownish, causing the above-ground plant parts to show symptoms of wilting, yellowing, and falling leaves. There is some rubber plant with fluorescent pseudomonads treatment and without showed *R. lignosus* rhizomorph on the roots neck (Figure 2).

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