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Biocontrol Delivery System of *Bacillus subtilis* and *Trichoderma harzianum* Formulated with Graphite and Silica Nano Particles to Control *Phytophthora infestans* in vitro

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ABSTRACT
A novel biocontrol delivery system by using graphite was proven effective in controlling bacterial plant pathogens. In this study, a consortium of biocontrol agents, consisting of *Bacillus subtilis* and *Trichoderma harzianum*, was formulated in graphite added with silica nano particles (NPs). *B. subtilis* is an endophytic bacterium, while *T. harzianum* is a rhizosphere fungus. The formulation was in vitro tested on *Phytophthora infestans*, the cause of leaf blight on tomatoes. The objective of the experiment was to study the ability of single and consortium of *B. subtilis* and *T. harzianum*, formulated in graphite and silica NPs, in inhibiting the in vitro growth of *P. infestans* compared to the non-formulated ones. The experimental design was the randomized complete block design comprised of eight treatments and replicated four timens. The eight treatments were dual cultures of the consortium and the pathogen on V-8 medium as follow: *B. subtilis* only, *B. subtilis* + graphite + silica NPs, *T. harzianum* only, *T. harzianum* + graphite + silica NPs, *B. subtilis* + *T. harzianum*, *B. subtilis* + *T. harzianum* + graphite + silica NPs, graphite + silica NPs, and a control. It was concluded that the abilities of both single and consortium of *B. subtilis* and *T. harzianum*, formulated with graphite and silica NPs, in inhibiting the in vitro growth of *P. infestans* were the same as the non-formulated ones. This proved that the formulation in graphite added with silica NPs in the biocontrol delivery system did not reduce the ability of the biocontrol agents to control *P. infestans* in vitro. The pathogen growth inhibitions caused by the single and consortium of *B. subtilis* and *T. harzianum* formulated with graphite and silica NPs were ranged from 35 - 54%, compared to the non-formulated ones 24.19 – 49.48%.

Keywords: *Biological control agents, bio pesticide, late blight disease*

1. INTRODUCTION

Biological control of plant pathogens by using microbes is considered safer and less harmful to the environment. Antagonistic bacterium, *Bacillus subtilis*, and fungus, *Trichoderma harizianum*, were proven to control several plant pathogens. *B. subtilis* was able to control soil borne as well as air borne pathogens. Prihatiningsih [1] found that the mechanisms of *B. subtilis* B315 as biocontrol agents were antibiosis and systemic induced resistance, and the strain could be used to control bacterial wilt and promote potato plant growth. *B. subtilis* produces secondary metabolite compounds such as antibiotics, siderophore and enzymes. Moreover, the endospore formed by *B. subtilis* enhances its ability to resist the unfavorable environmental condition, and make it easier to preserve in a formulation [2]. El Naggar [3] reported that *B. subtilis* suppressed the in vitro growth of *Phytophthora infestans* by 84.44%. Similarly, Suriani [4] reported that *B. subtilis* reduced the in vitro growth of *Fusarium vertillicioides* up to 24,6%. Istiqomah and Kusumawati [5] also reported that *B. subtilis* was able to inhibit the in vitro growth of *Ralstonia solanacearum* up to 50%. In the other hand, *T. harzianum*, a Plant Growth Promoting Fungus (PGPF) that colonizes plant rhizosphere, is antagonistic to many plant pathogens, such as *Phytium, Rhizoctonia, Sclerotium, Sclerotinia, Fusarium*, and *Phytophthora*, because of the production of chitinolytic enzymes [2, 6, 7]. *Trichoderma harzianum* formulated in carbon fiber and silica NPs was able to control

Fussarium oxysporum in vitro [8]. The antagonism mechanism performed by *T. harzianum* against plant pathogens are competition in space and nutrition, and also production of antibiotics and enzymes that breakdown the cell wall of pathogens [6]. Fatima [9] reported also that the mechanism of antagonism between *T. harzianum* against *P. infestans* was competition and colonization, and the *in vitro* inhibition was 86%.

The antagonism between *B. subtilis* and *T. harzianum* against plant pathogens can be improved by combining them in a consortium. They can make a good combination as they have different characteristics. *B. subtilis* is an endophytic bacterium, whilst *T. harzianum* is a rhizosphere inhabitant fungus or PGPF. Synergistic antagonism produced by the two biocontrol agents, with different mechanism, may give better results than application of single agent [10].

To obtain longer shelf life and more practical application methods, biocontrol microbes are prepared in formulations. Wijayanti [11] developed a biopesticide formulation of *B. subtilis* AB89 and *Staphylococcus epidermis* BC4 to control bacterial wilt on tomato. Many biocontrol formulations are already available commercially [12]. A biocontrol formulation contains microbes as the active ingredients, a carrier, and other additives. In this study, graphite was used as the carrier, and the formulation was added with silica NPs, a plant micro nutrient [13, 14]. Silica NPs has favourable effect on beneficial bacterial population and nutrient value of soil. [15].

In earlier studies, it was found that 5% of graphite 80 mesh and 3% of silica NPs in a formulation maintained the viability of *B. subtilis* [16]. *T. harzianum* in 5 % of graphite 80 mesh and 1% of silica NPs was able to inhibit the in vitro growth of *Phytophthora nicotianae* up to 74,63% [17]. Djaya [14] also reported that endophytic *Lysinibacillus* and Plant Growth Promoting Rhizobacteria (PGPR) isolates of *B. subtilis* and *Pseudomonas fluorescens*, formulated with graphite and silica NPs as a biocontrol delivery system, were able to reduce the in vitro growth of *Ralstonia solanacearum*, the cause of plant bacterial wilt. Based on those results, the formulation of *B. subtilis* and *T. harzianum* in 5 % of graphite 80 mesh and 1% of silica NPs was tested against *Phytophthora infestans* to find out its ability in inhibiting the in vitro growth of the pathogen, compared to the non-formulated ones.

2. MATERIALS AND METHODS

To find out the effect of the formulation of *B. subtilis* and *T. harzianum*, in 5 % graphite 80 mesh and 1% silica NPs, on the growth of *P. infestans* (in vitro), an experiment was carried out in the laboratory. The isolate of endophytic *B. subtilis* was the collection of Noor Istifadah (Laboratory of Phytopathology, Universitas Padjadjaran), and the isolate of *T. harzianum* (PGPF) was the collection of Hersanti from the same laboratory. *P. infestans* was isolated from diseased tomato plant. Graphite and silica NPs were prepared at Functional Nano Powder Centre of Excellence (FiNder CoE), Universitas Padjadjaran.

B. subtilis was cultured on nutrient agar (20 g agar + 8 g Oxoid nutrient broth added with distilled water to 1 liter). *T. harzianum* was cultured on potato dextrose agar (200 g diced potato + 20 g dextrose + 20 g agar per liter of medium). *P. infestans* was isolated and cultured on V8 juice agar (200 ml V8 juice + 3 g $CaCO_3$ + 15 g agar per liter medium). The culture media were sterilized in autoclave for 15 minutes at 121^{o}C. The cultures were incubated at room temperature.

The in vitro antagonism was tested by dual culture method (randomized complete block design) with 8 formulation treatments i.e.: A) *B. subtilis* only, B) *B. subtilis* + graphite + silica NPs, C) *T. harzianum* only, D) *T. harzianum* + graphite + silica NPs, E) *B. subtilis* + *T. harzianum*, F) *B. subtilis* + *T. harzianum* + graphite + silica NPs, G) graphite + silica NPs, and a control (sterile distilled water). They were replicated 4 times. The radial of *P. infestans* was measured daily up to the maximum growth in the Petri dish (up to 9 days incubation) to compare the pathogen's growth in treatments and control. The data were analyzed by analysis of variance, and continued with Duncan multiple range test since there was differences in the effect of treatments.

Suspension of *B. subtilis* was prepared by collecting the 48 hours culture at room temperature on NA, and suspended in sterile distilled water. The density was measured by total plate count method. *T. harzianum* was cultured on PDA, 3-5 days incubation at room temperature, and suspended in sterile distilled water. The spore density was measured by using hemocytometer. These suspensions were mixed with graphite and silica NPs suspensions. Concentrations in the mixture were 10^8 cfu/ml *B. subtilis*, 10^8 spores/ml *T. harzianum*, 5% graphite 80 mesh, and 1% silica NPs.

Filter paper discs, 0.5 cm diameter, were soaked in the formulation treatments for 30 minutes, and placed on the V8 agar medium to confront with the pathogen. Colony of *P. infestans* on V8 agar was picked by using cork borer, 5 mm diameter, and placed in the opposite of the filter paper on the V8 agar (Figure 1).

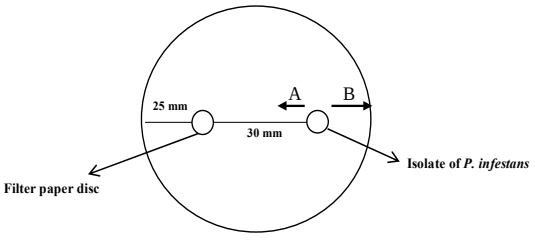


Figure 1. Dual culture on V8 agar in Petri dish

The radial growth of the pathogen toward (A) and fromward (B) the treatment was measured everyday up to 9

days incubation. The growth inhibition was calculated by formula as follow:

$$\text{Inhibition} = \frac{B - A}{B} \text{ X } 100\ \%$$

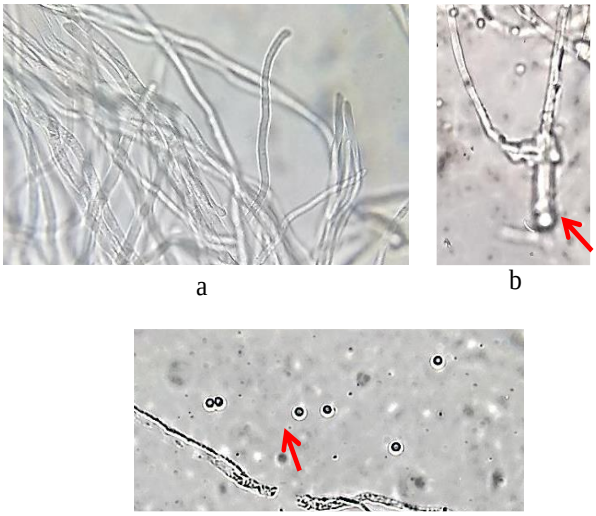
where A is radial of *P. infestans* toward the treatment (mm) and B is radial of *P. infestans* fromward the treatment (mm).

3. RESULTS AND DISCUSSION

The growth of *P. infestans* on the dual culture medium was observed and measured daily. At day 9, the radial of *P. infestans* toward the treatment (Figure 1: A) in all formulation treatments were less than control (Table 1). This result indicated that all formulation treatments were able to inhibit the growth of *P. infestans*. The inhibition was ranged between 24.19 to 54%. *B. subtilis* has antibiosis mechanism against pathogens [2]. Antibiosis in antagonism involved metabolites that caused cell lysis, enzymes, volatile and nonvolatile compounds, and toxin produced by the antagonistic agents [18]. The antibiotics produced by *B. subtilis* are streptavidin, bacitracin, surfactin, polymixin, difisidin, subtilin and subtilosin [19]. Besides, *B. subtilis* also produces enzymes such as amylase, protease, pullulanase, chitinase, xylanase and lipase that degrade the cell wall of pathogens (Kunts and Rapoport, 1995 in [19]).

The antagonism mechanism by *T. harzianum* was competition in space and nutrition, antibiosis, and hyper parasitic [18]. Macroscopic and microscopic observations revealed those mechanisms in this experiment. Space and nutrition competition were detected from the faster and larger growth of *T. harzianum* compared with the growth of *P. infestans*. Similar results were reported by [20] that the space and nutrition competition produced by *T. harzianum* dominated the culture medium, so that the pathogen ran out of space to grow. *T. harzianum* also produces antibiotics such as alkyl pyrones, isonitrin A-D, and harzianolida that disrupt pathogen germination [18]. Harman [21] overviewed that *T. harzianum* produced exoglycanase (β-1.4 glycanohydrolase) and cellubiase (β-glucosidase) which caused disruption of the cell wall of *P. infestans* that contained cellulose. Hyper parasitic mechanism was revealed by the microscopic image as shown on Figure 2.

The lytic hyphae of *P. infestans* looked clear and empty (Figure 2b). It seemed that the cell content of *P. infestans* was digested by *T. harzianum* as reported by Sunarwati and R. Yoza [22]. Berlian et al. [18] explained that the initial hyphal growth of *T. harzianum* was elongated, then twisted around the pathogen's hyphae, penetrated into the pathogen's hyphae and caused vacuolation, lysis and break down.



a b c

Figure 2. Microscopic of *P. infestans* (400x)
(a) Whole hyphae of *P. infestans* in control.
Malformation of *P. infestans*: (b) swollen hyphae of *P. infestans*, (c) lysis hyphae of *P. infestans*

B. subtilis and *T. harzianum* were compatible in a mixture or consortium as shown by their normal growth when cultured together on a petri dish. There was no indication of antagonism between *B. subtilis* and *T. harzianum* on the culture medium. The ability of consortium of *B. subtilis* + *T. harzianum* to inhibit the in vitro growth of *P. infestans* was the same as the single microbe (Table 1). It can be predicted that the in vivo result will be different, since the two microbes has different niche. The isolate of *B. subtilis* used is endophytic, and *T. harzianum* is rhizosphere inhabitant. Endophytic *B. subtilis* protects tomato plant from the infection of air borne *P. infestans*, and retard the infection. *T. harzianum* protects the plant by competing the pathogen before infection through roots. It was suggested that this study should be continued with the in vivo experiment. The formulation with graphite and silica NPs was also resulted in the same inhibition as the non-formulated one (Table 1). This condition indicated that graphite and silica NPs did not reduce the antagonistic ability of *B. subtilis* and *T. harzianum* against *P. infestans*. Djaya et al. [14] reported the use of graphite in biocontrol delivery system of some bacteria antagonistic to *Ralstonia solanacearum*. Graphite is potential to utilize as carrier in a bio-pesticide formulation.

Table 1 Effect of treatments on the radial growth of *P. infestans*

Code	Treatments	Radial of *P. infestans* (mm)	Inhibition (%)
A	*B. subtilis*	20.13 c	24.19
B	*B. subtilis* + graphite + silica NPs	18.75 cd	35
C	*T. harzianum*	12.63 d	49.48
D	*T. harzianum* + graphite + silica NPs	11.50 d	54
E	*B. subtilis* + *T. harzianum*	18.50 cd	26
F	*B. subtilis* + *T. harzianum* + graphite + silica NPs	15.75 cd	39.4
G	Graphite + silica NPs	46.62 b	-
H	Control (sterile distilled water)	58.50 a	-

4. CONCLUSION

From the results of the experiment, it was concluded that the formulation of *B. subtilis* and *T. harzianum* in 5 % of graphite 80 mesh and 1% of silica NPs was able to equally inhibit the in vitro growth of *P. infestans* as the non-formulated one. The biocontrol delivery system by using graphite added with silica NPs can be applied for the consortium of *B. subtilis* and *T. harzianum* as the antagonistic agents. *T. harzianum* caused malformation on the hyphae of *P. infestans* such as cell swollen and lysis

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