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In *vitro* study of plant growth promoting rhizobacteria (PGPR) and endophytic bacteria antagonistic to *Ralstonia solanacearum* formulated with graphite and silica nano particles as a biocontrol delivery system (BDS)

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ABSTRACT

Antagonistic *Lysinibacillus* sp., *Bacillus subtilis*, and *Pseudomonas fluorescens* were proven to control *Ralstonia solanacearum* that causes bacterial wilt on potatoes. Present study reports in vitro evaluation of a formulation containing PGPR and endophytic bacteria with the novel biocontrol delivery system (BDS) consisted of graphite and silica NPs. Crystal structure, morphology, elemental analysis and size distribution of particles in the suspension were respectively observed by XRD, SEM, EDS, and PSA. While the morphology and bacterial existence were observed by SEM, UV–vis, and photoluminescence spectroscopy. The ability of above BDS to form synergistic effect and inducing antagonistic interactions against *R. solanacearum* was evaluated by measuring the diameter of inhibition zone (DIZ). It is highlighted that the widest inhibition zones were resulted from biocontrol with graphite + silica NPs, indicating that BDS significantly affects the biocontrol mechanism. Compared to control, the inhibition zones caused by *Lysinibacillus* sp. were widest in the formulation with 3% graphite +5% silica NPs; by *B. subtilis* were in the formulation with 5% graphite +5% silica NPs, and 5%; by *P. fluorescens* were in the formulation with 5% graphite +5% silica NPs.

1. Introduction

Potato (*Solanum tuberosum* L.) is an important food security crop with great potential for poverty alleviation and combating malnutrition in the developing world (FAO, 2008). However, significant harvest failure can be caused by several constraints such as poor seed quality, soil fertility, and plant pathogens (FAO, 2009). One of the most destructive diseases limiting potato production in many potato growing area, including the developing countries in Asia and Africa, is bacterial wilt caused by *Ralstonia solanacearum* (Allen et al., 2004; Gildemacher et al., 2011). This bacterium also affects many plant species including tomato, tobacco, banana, peanut, ginger, and other solanaceous crops (CABI, 2018). Diverse mechanism of the pathogen affecting the plant such as causes wilt on potato plants and rot on potato tubers. The bacterium is difficult to control because it survives in residues of infected plants in the soil (Hayward, 1991; Pradhanang and Momol, 2013). The spread of asymptomatic, latently infected seed tubers promotes the establishment of this pathogen in new areas (Allen et al., 2004). Crop rotation is useful to decrease the inoculum of bacterial wilt pathogen and control the disease. One of the other environmentally friendly control methods is biocontrol by using antagonistic agents, such as plant growth promoting rhizobacteria (PGPR) and endophytic bacteria.

Root surface and root system are colonized by a wide range of soil bacteria which are able to stimulate plant growth and health (Saharan et al., 2011). These plant growth-promoting rhizobacteria (PGPR) enhance the growth of plants and crops. Besides this direct effect, many PGPR increases crop production indirectly by their antagonistic effect against phytopathogens. PGPR affect plant growth through several mechanisms, such as nitrogen fixation, phosphate and potassium solubilization, siderophore production, antibiosis, and protective enzymes synthesis (Shaikh et al., 2016). PGPR that is capable of enhancing the growth of vegetables such as potato, carrot, onion, etc. belong to genera Azotobacter, Azospirillum, Pseudomonas, Bacillus, and many more (Rai and Nabti, 2017; Gouda et al., 2018). Boukerma et al. (2017) reported the activity of PGPR *Pseudomonas fluorescens* and *P. putida* in the

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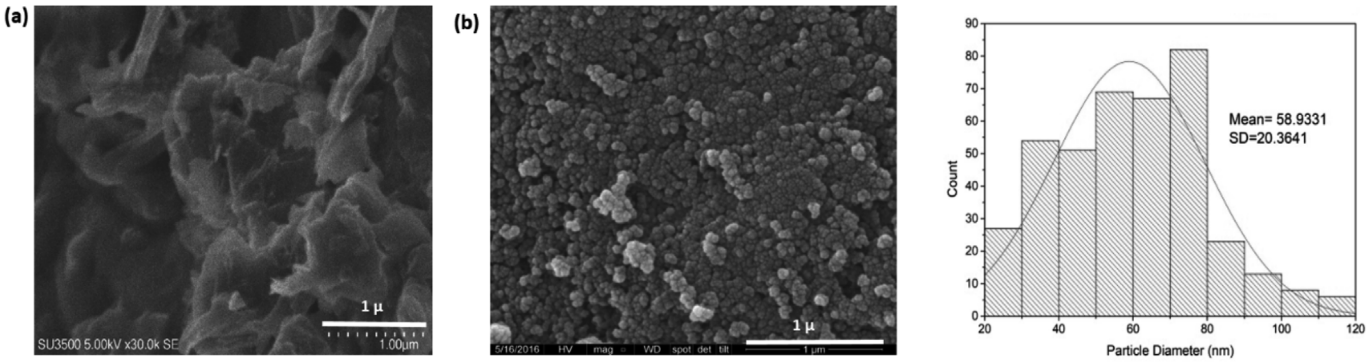

Fig. 1. Scanning Electron Microscope (SEM) images of (a) graphite and (b) SiO_2 nanoparticles and corresponding size distribution with average size of 58.9 nm.

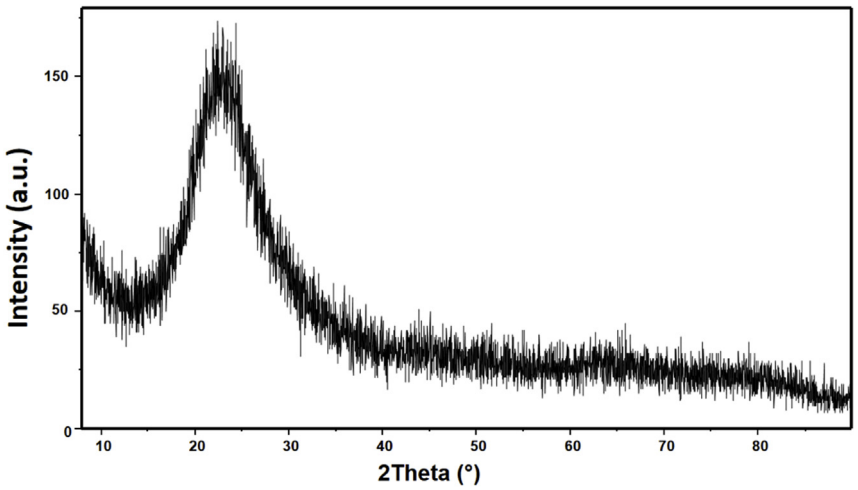

Fig. 2. The obtained X-ray Diffraction (XRD) spectroscopy of amorphous SiO_2 nanoparticles.

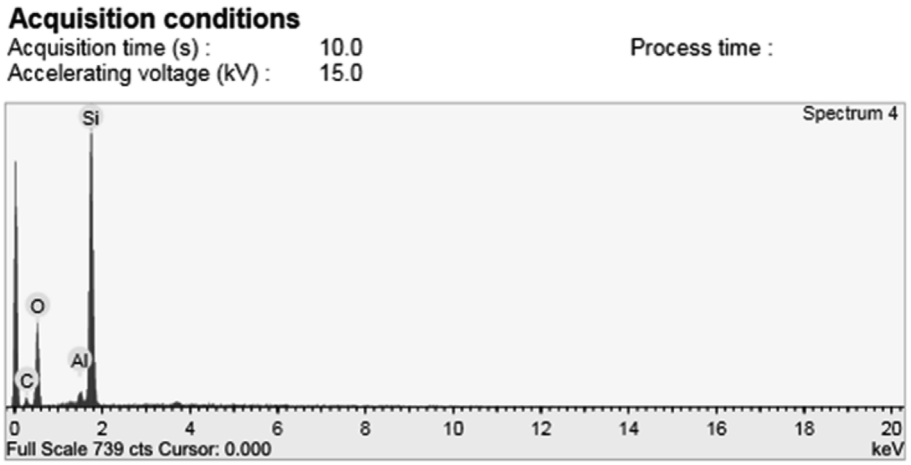

Fig. 3. The obtained elemental analysis by energy dispersive spectroscopy (EDS) of SiO_2 nanoparticles.

biocontrol of tomato Fusarium wilt.

Endophytic bacteria colonize plant tissues intracellularly as well as intercellularly without causing disease symptoms on the plant. These microbes have important roles in plant health. They colonize an ecological niche similar to that colonized by plant pathogens but do not cause disease or damage to their hosts. Several bacterial endophytes have been reported to support growth and improve the health of plants (Nair and Padmavathy, 2014) and therefore may be important as sources of biocontrol agents. The mechanisms by which endophytic bacteria control phytopathogens are a competition of space, siderophore production, inhibitory substances production such as antibiotics or HCN which may end up to disease suppression (Ramesh and Phadke, 2012). The mechanism of indirect disease control is by modulating the plant immune response, including the induction of systemic acquired resistance.

In some earlier screening studies, an endophytic isolate of *Lysinibacillus*, isolated from potato root, and PGPR isolates of *Bacillus subtilis* and *Pseudomonas fluorescens*, isolated from the rhizosphere of chrysanthemum were collected since they were able to control bacterial wilt disease on potato plants (Hersanti et al., 2009). This endophytic

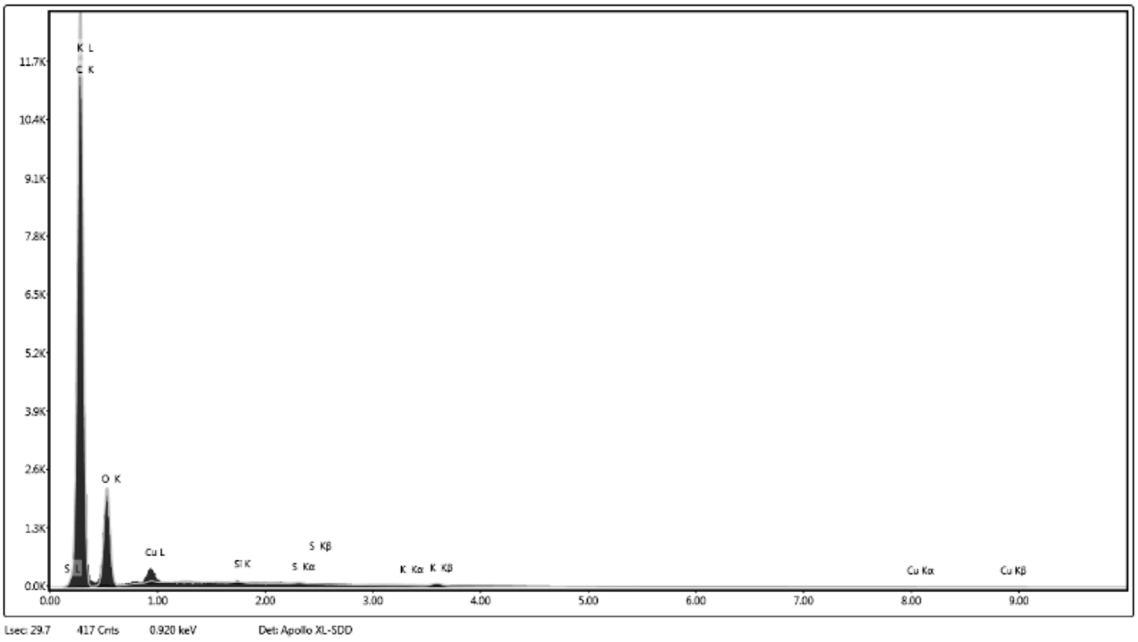

Fig. 4. The obtained elemental analysis by energy dispersive spectroscopy (EDS) of graphite.

Table 1
EDS elemental analysis of silica NPs.

Element	Weight %	Weight % σ	Atomic %
C K	15.780	2.405	23.749
O K	45.275	1.729	51.152
Al K	1.334	0.222	0.893
Si K	37.611	1.391	24.206

Table 2
EDS elemental analysis of graphite.

Element	Weight %	Error %	Atomic %
C K	72.98	3.78	78.34
O K	26.77	10.18	21.57
Si K	0.06	61.77	0.03
S K	0.1	57.65	0.04
K K	0.02	68.29	0.01
Cu K	0.08	76.54	0.02

Lysinibacillus was also able to reduce potato tuber soft rot (Istifadah et al., 2016). These three antagonistic isolates were intentionally formulated to be prepared as a biopesticide. Biocontrol agents must be formulated in a suitable delivery system to maintain their condition and to provide easier handling in field application (Naakeeraan et al., 2005). These antagonists were formulated with graphite suspension enriched with silica NPs. In the earlier study, the bacterial isolates were also proven to be viable in the formulation. Graphite is a composite black material, rigid, strong and light, synthetically produced, and environmentally friendly as the toxicity is very low. Graphite has a larger surface for the bacteria and silica NPs to cling onto in a formulation.

The incorporation of nanotechnology by means of nanoparticles as a delivery system is in the early stage of development. The main idea behind the application of nanoparticles is to lower the indiscriminate use of conventional pesticides to be in line with safe environmental applications. In the early stage of the development of the delivery system, polymer-based formulations have received the greatest attention such as application of biopolymer chitosan (Prem et al., 2015; Perez et al., 2018) followed by formulations containing inorganic

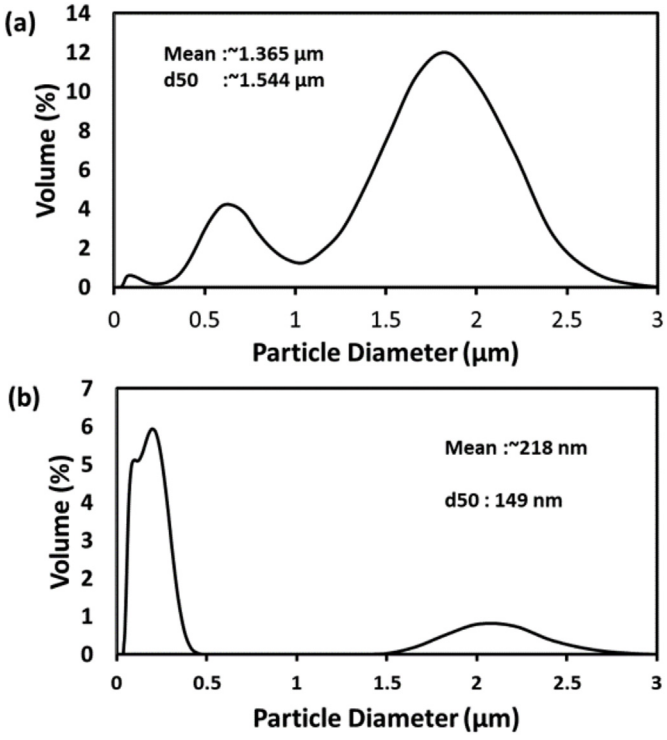

Fig. 5. Particle size distribution of SiO_2 nanoparticles: (a) without dispersing agent (b) with dispersing agent PEG 400.

nanoparticles (e.g., silica, titanium dioxide) and nanoemulsions (Xu et al., 2016). The detailed review related to a smart formulation of the biocontrol delivery system for agriculture can be found in the recent review elsewhere (Sandeep et al., 2019).

The role of carbon in plant growth is extremely valuable. The source of carbon in plants is used to create healthier specimens and some are converted into carbon dioxide, but some of the carbon is locked into the soil which plays important roles in adsorption of various minerals resources. Thus, application of graphitic which is one of the most stable allotropes of carbon as a biocontrol delivery system has promising

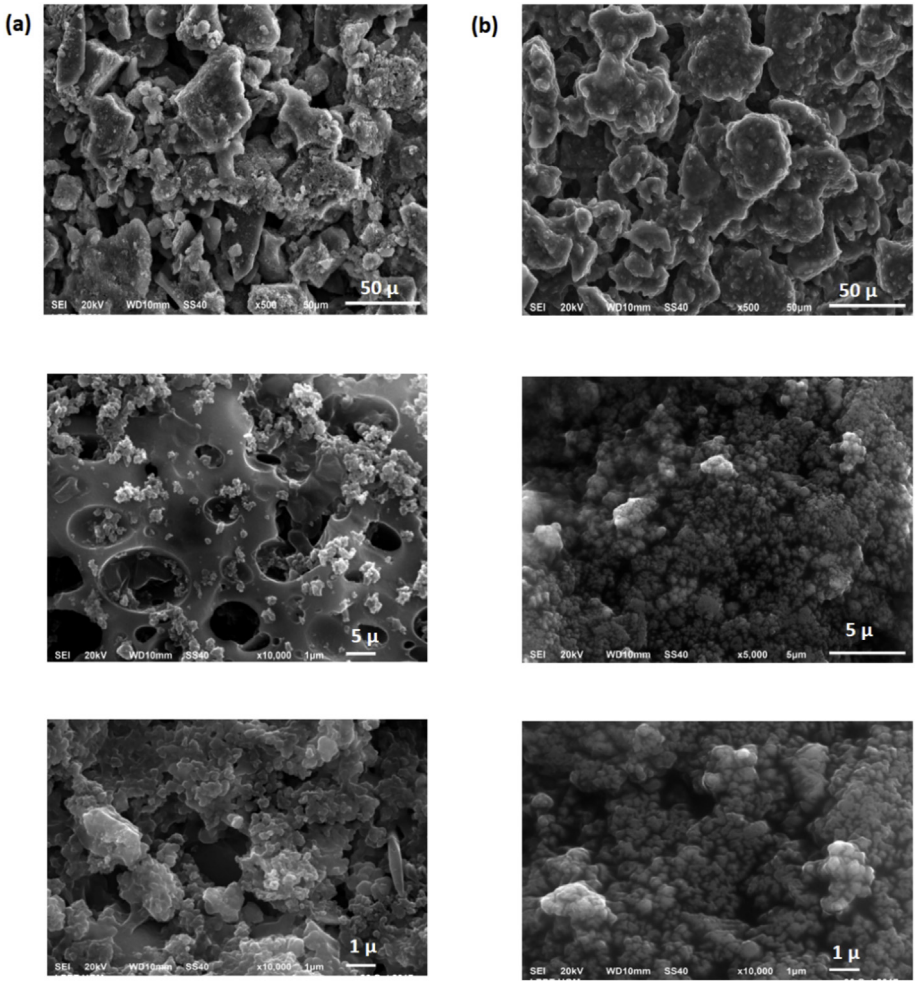

Fig. 6. Scanning Electron Microscope (SEM) images of bacterial isolate *P. Fluorescens* at different delivery formulation and image magnification of10,000: (a) only SiO_2 nanoparticles 3 wt% and (b) SiO_2 nanoparticles 3 wt % and graphite 5 wt %.

advantages such as a host for various bacteria and also adsorbed soil minerals.

Silica plays an important role in soil and shows beneficial effects on plants include disease and insect resistance, plant structural fortification, and regulation of the uptake of other plant nutrients. Silica stimulates photosynthesis and graphite dioxide translocation, reduces abiotic stress such as temperature, radiation, nutrient deficiency, and toxicity, and increases plant resistance to biotic stress. Silica has also a role in strengthening plant tissues, so the plant becomes more resistant to pests and pathogens (Etesami and Jeong, 2018). Silica NPs is useful in overcoming some limitation in the use of biopesticide, such as variability in the performance of PGPR and endophyte due to various environmental factors that affect their growth and multiplication in the plants (Gouda et al., 2018).

Therefore in the present study, the delivery system of PGPR and endophytic bacteria were formulated using graphite and silica NPs. The objective of the present investigation is the in vitro study on the formulation of delivery biocontrol system with graphite and silica NPs on the PGPR and endophytic bacteria. The evaluation was carried out on various treatments such as the in vitro antagonism between isolates of *Lysinibacillus, Bacillus subtilis*, and *Pseudomonas fluorescens*, in graphite + silica NPs formulation, against *R. solanacearum*. In addition, the investigation was also carried out on the highest inhibition due to varying in the concentration of silica NPs in the formulation.

2. Material and methods

2.1. Bacterial isolates

Lysinibacillus, the CKU3 isolate, is an endophytic bacterium isolated from potato root grown in a potato field in Garut, West Java, Indonesia, and became a collection in the Laboratory of Phytopathology, Universitas Padjadjaran. This isolate was screened by challenging the in vitro growth of *Erwinia carotovora* and *R. solanacearum*, and caused the highest inhibition (Istifadah et al., 2016). PGPR *B. subtilis* and *P. fluorescens* were isolated from the root of chrysanthemum at the Ornamental Plants Research Center, Segunung, West Java, Indonesia. These isolates were also collected by the Laboratory of Phytopathology, Universitas Padjadjaran. *R. solanacearum* was isolated from the basal stem of potato that showed wilt symptoms in a potato field in Lembang, West Java, Indonesia.

2.2. Biocontrol delivery system (BDS)

2.2.1. Preparation of silica NPs and graphite suspension

Amorphous silica particle was prepared using 350 ml of Na_2SiO_3 (Technical grade, PT Bratachem, Indonesia) and mixed with 700 ml of water and 100 ml of HCl 12 M as a catalyst. The stirring was done at room temperature until the clear homogeneous mixture was obtained. The final product was recovered by filtration, followed by cleaning with distilled water in order to neutralize the acid until the pH became neutral. The product was dried at 60 °C for 12 h to obtain a white

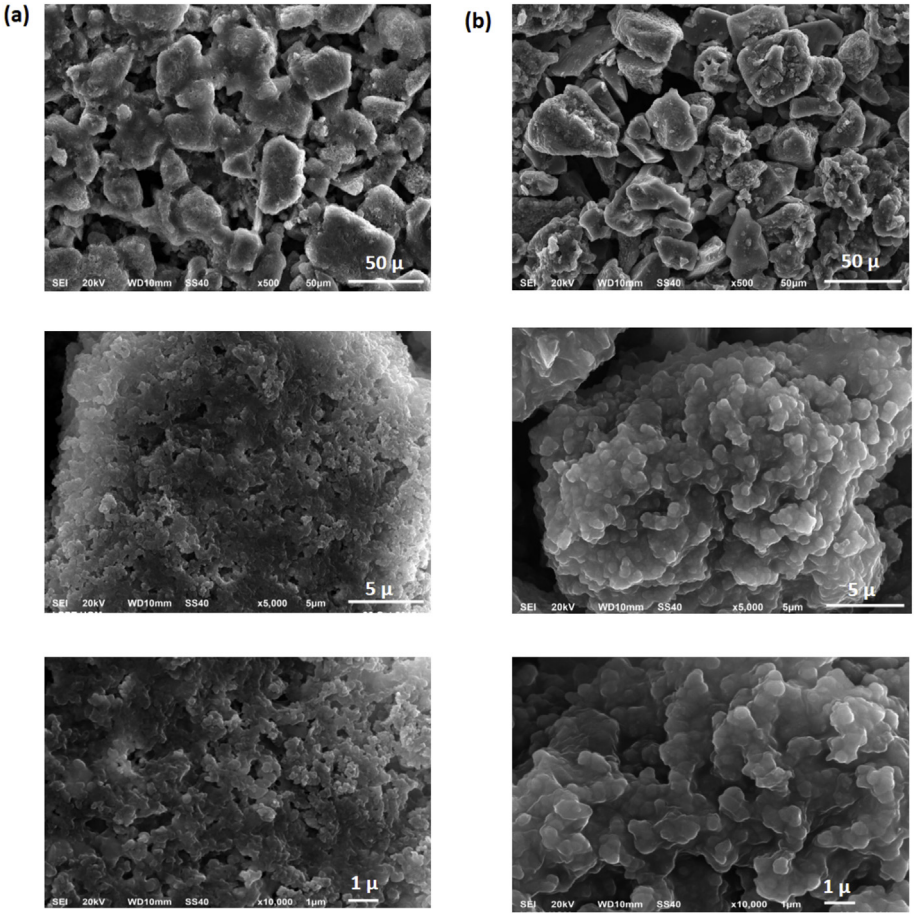

Fig. 7. Scanning Electron Microscope (SEM) images of bacterial isolate *Lysinibacillus* at different delivery formulation and image magnification of 5000 and 10,000: (a) only SiO_2 nanoparticles 3 wt% and (b) SiO_2 nanoparticles 3 wt % and graphite 5 wt %.

powder of silica particle. The PEG 1.5 wt % was dissolved in distilled water and mixed by a magnetic stirrer for 60 min, then 3 wt% of silica NPs was added to the mixture and stirred for 60 min. The suspension was ultrasonically agitated to obtain well-dispersed silica NPs suspension. The graphite powder was obtained from low-grade graphite with 60–80% carbon content (Merch KGaA, Germany). In order to observe another mineral in the graphite powder, EDS observation was carried out. The suspension of graphite and silica NPs were used to formulate the BDS as required for the various treatments.

2.2.2. Characterization of delivery system

The morphology and size of obtained silica NPs and graphite powder were evaluated by means of scanning electron microscopy (SEM) using Hitachi SU 3500, FEI, Inspect-S50. The crystallinity of the silica NPs was measured by XRD Rigaku (Cu Kα with λ = 1.54060 Å) PANanalytical X'Pert. The impurities of both silica NPs and graphite were investigated using EDS elemental analysis (Energy Dispersive X-rays Fluorescence Spec, Rigaku). The particles size of silica NPs suspension in distilled water was observed using PSA Beckman Coulter LS 13 320. While the BDS were observed by means of SEM with a similar instrument, UV–vis (Spectrostar Omega) and photoluminescence spectroscopy (PerkinElmer LS55) to observe the morphology and the existence of the bacteria, respectively. The contact angle of BDS suspension was observed in glass plates and potato surface to determine the hydrophobicity of the suspension by using a high-resolution camera adopted from (Lamour et al., 2010).

2.3. Viability test of the bacterial isolates in the formulation

The viability test was carried out to evaluate the viability of the antagonists in the formulation with graphite + silica NPs. Suspension of the antagonist only, suspension of the antagonist + silica NPs (0.5%, 1%, 3%, and 5%), and suspension of antagonist in 5% graphite + silica NPs (0.5%, 1%, 3%, and 5%) were serially diluted and plated on nutrient agar on day 1, and day 3 after mixing the antagonist with the graphite + silica NPs. The three antagonist populations were prepared to 10^7 cfu/ml when mixing. The total plate counts were assessed by counting the colony number on the medium after 3 days of incubation.

2.4. In vitro antagonism between the bacterial isolates in the formulation against R. solanacearum

To evaluate the antagonism between the bacterial isolates in the formulation against *R. solanacearum*, the experiment was arranged in a completely randomized design with 9 treatments and 3 replicates. The data were statistically analyzed by analysis of variance, continued with Duncan's multiple range test (5%), using SPSS21.0 application. To prepare the antagonism test, 100 µL diluted suspension of *R. solanacearum* was spread inoculated onto nutrient agar in a Petri dish by using L shape glass, and air-dried for several minutes. Filter paper discs (8 mm diameter) were dipped in antagonist suspensions (formulated with graphite + different concentration of silica NPs). Then they were placed on the inoculated agar plates, 4 discs per plate. The antagonism was shown by inhibition zones around the filter paper discs, and measured by the width of the clear zones. The inhibition zones on treatments (IHT) were also compared to the control (IHC) by

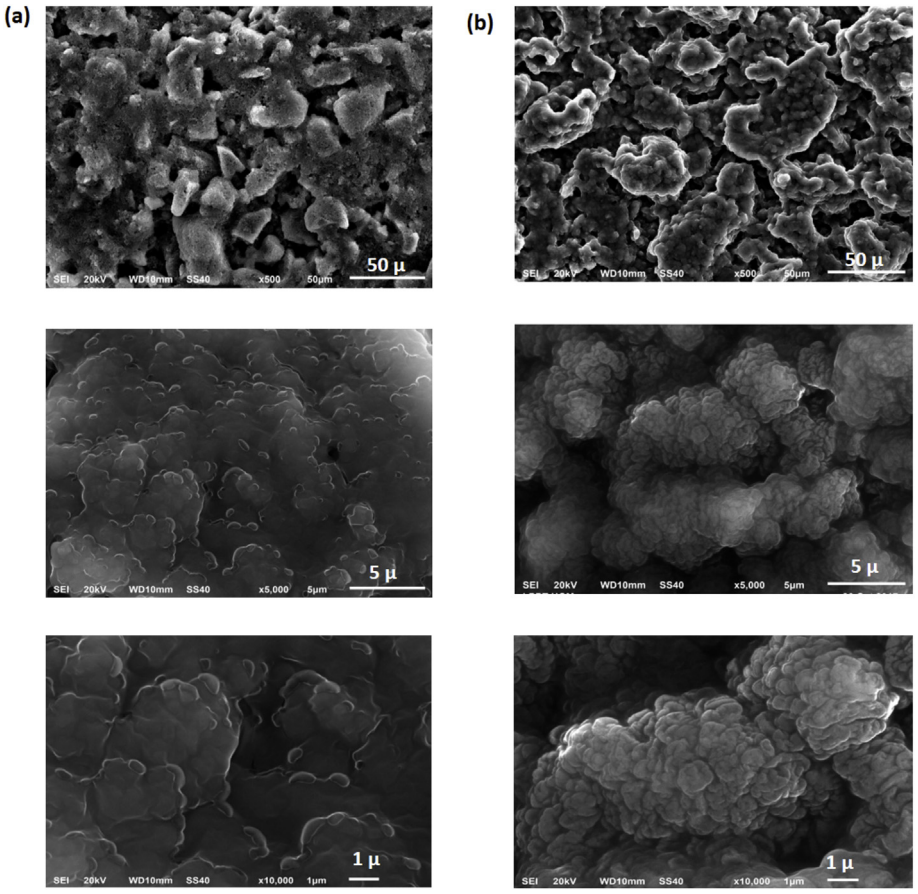

Fig. 8. Scanning Electron Microscope (SEM) images of bacterial isolate *B. subtilis* at different delivery formulation and image magnification 5000 and 10,000: (a) SiO_2 nanoparticles 3 wt% and (b) SiO_2 nanoparticles 3 wt % and graphite 5 wt%.

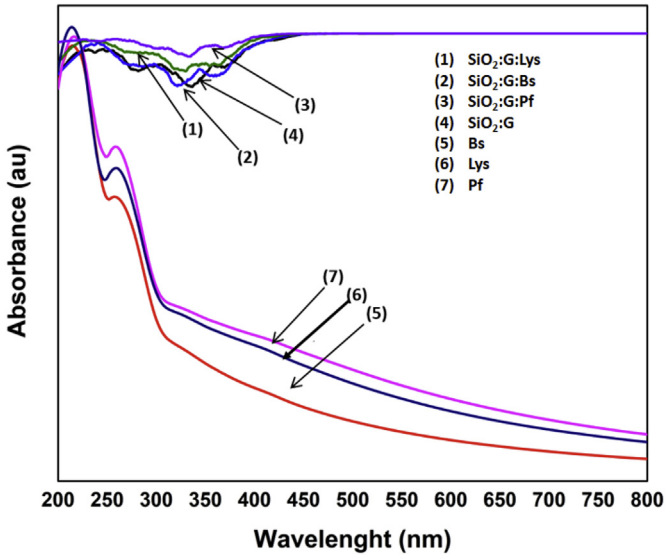

Fig. 9. UV–Visible spectroscopy of various delivery formulation: (1) SiO_2:G (graphite): Bs (*Bacillus subtilis*): Lys (*Lysinibacillus*), (2) SiO_2:G:Bs, (3) SiO_2:G:Lys, (4) SiO_2:G, (5) only Bs, (6) only Lys, and (7) Bs + Lys.

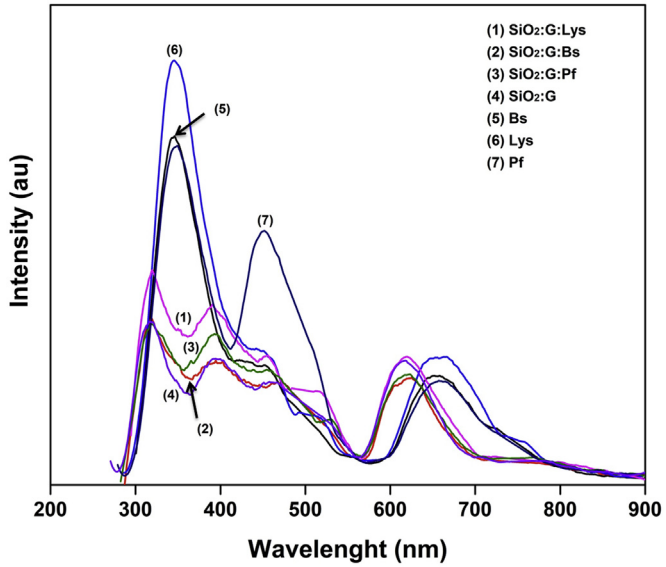

Fig. 10. Photoluminescence (PL) spectroscopy of various delivery formulation: (1) SiO_2:G (graphite): Bs (*Bacillus subtilis*): Lys (*Lysinibacillus*), (2) SiO_2:G:Bs, (3) SiO_2:G:Lys, (4) SiO_2:G, (5) only Bs, (6) only Lys, and (7) Bs + Lys.

subtracting IHT with IHC and dividing the result by IHC, as follow:

$$\text{Comparison of inhibition} = \frac{\text{IHT} - \text{IHC}}{\text{IHC}} \quad (1)$$

3. Results

3.1. Graphite and silica NPs

Fig. 1a shows the SEM image of graphite powder after ball milled

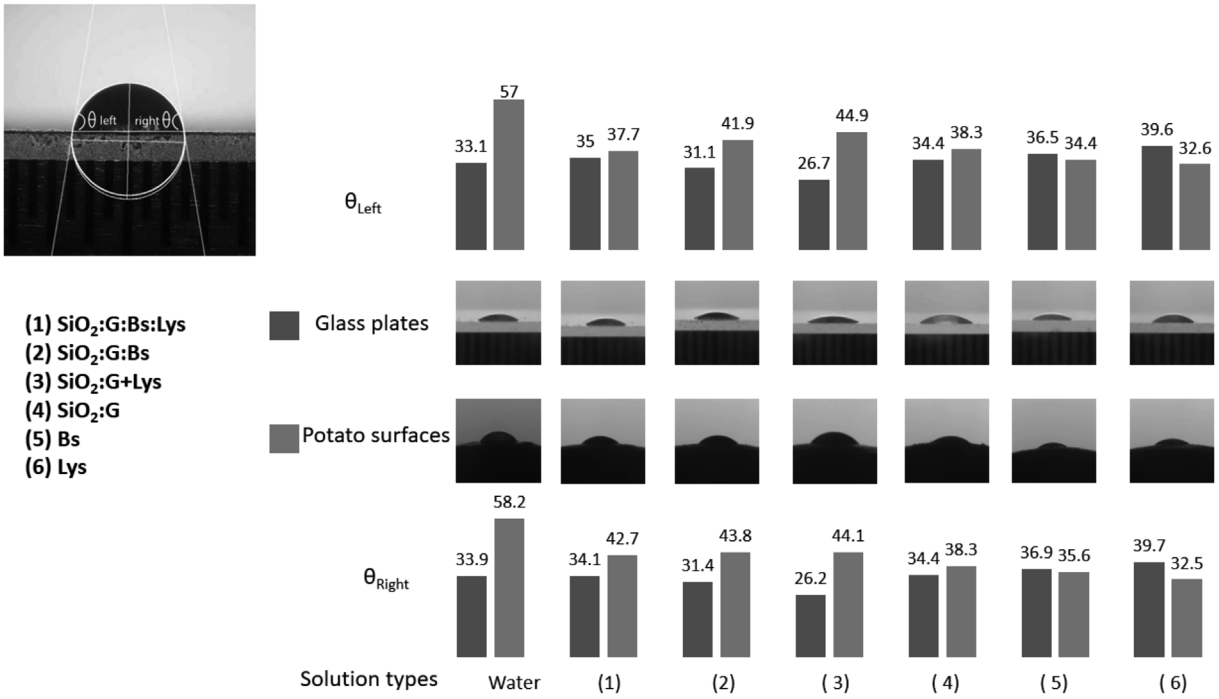

Fig. 11. Contact angle of suspension of various delivery formulation compare with water at glass plates and potato surfaces: (1) SiO_2:G (graphite): Bs (*Bacillus subtilis*): Lys (*Lysinibacillus*), (2) SiO_2:G:Bs, (3) SiO_2:G:Lys, (4) SiO_2:G, (5) only Bs, and (6) only Lys.

and filtered by mesh 400. The SEM images of silica as shown in Fig. 1b shows nano-sized particles with average size of 58.9 nm and relatively homogenous in size. X-ray diffraction disclosed the presence of crystalline domains due to silica nanoparticles content in an amorphous structure as shown in Fig. 2.

Figs. 3 and 4 show the EDS elemental analysis of silica NPs and graphite powder, respectively. The elemental analysis for amorphous silica NPs shows 75 atomic % contain Si and O with impurity dominated by carbon 23 atomic % and a small amount of aluminum 0.89 atomic % as shown in Table 1. While for the graphite powder consist of 78.3 atomic % carbon and another small amount of impurities includes Si, S, K, and Cu (Table 2**)** which is common existing minerals in the soil and considered safe for the plant.

Fig. 5 shows the particle size distribution of 5 wt% silica NPs suspension in distilled water without dispersing agent and with dispersing agent PEG. Silica NPs were agglomerated in suspension without dispersing agent as shown in Fig. 5. a with average size of 1.5 µm. The average size of suspension close to their primary size after dispersing agent of PEG was added 50 wt% of silica NPs weight. Similar treatment for the graphite was applied in preparing the suspension for the BDS with properly diluted according to the various experimental design of the treatments.

3.2. Characteristics of the delivery system

Based on the observation on the morphology of as-prepared BDS, the PEG acting as a dispersing agent in suspension, and also as biopolymer binder to both silica NPs and graphite. It is clearly observed from Fig. 6a equipt with higher magnification of SEM images, silica NPs were bound by PEG to form agglomerated microsize-sized particles which are consisted of primary nanosized silica particles. This type of morphology allows the bacterial isolate *P. fluorescens* to be either encapsulated or absorpted on the surface of agglomerated particles. In addition, when graphite was added to the system, more dense agglomeration was observed as shown in Fig. 6. b. It is highlighted that the presence of graphite prevents the PEG to form biopolymer film as shown in Fig. 6 a at an image magnification of 10.000.

In contrast, the SEM image of BDs with *Lysinibacillus* showed different morphology which was higher agglomerated sized both with or without graphite as shown in Fig. 7a and b. This figures indicated that the bacterium also plays an important role in the formation and morphology of the BDS. Similarly, the SEM images of *B. subtilis* (Fig. 8), also indicated the same role. However, denser agglomerated silica NPs embedded with graphite and PEG was observed. This results indicated that the isolate of bacteria clearly affects the morphology of the BDS which is in agreement with SEM images of BDS for the *P. fluorescens* and *Lysinibacillus.*

In order to prove that the bacteria exist in the agglomerated particles of BDS, the samples were subjected to UV–Vis and PL analysis (Figs. 9 and 10). The observation was carried out for the suspension of (1) SiO_2:G (graphite): Lys (*Lysinibacillus*), (2) SiO_2:G:Bs (*Bacillus subtilis*), (3) SiO_2:G:Pf (*P. fluorescens*); (4) SiO_2:G, (5) only Bs; (6) only Lys, and (7) Pf.

UV–vis spectroscopy shows a weak absorption maximum at 270–280 nm for bacteria *Bacillus subtilis* (5), *Lysinibacillus* (6) and *P. fluorescens* (7) (Fig. 9). The highest intensity resulted from the absorption of *P. fluorescens* (Fig. 9) (6). In contrast, the SiO_2:G shows strong two weak absorptions at 240–250 and 280–290 nm. It was highlighted that when BDS was incorporated into the BDS (sample 1, 2 and 3), the peaks at 270–280 nm was significantly increased, this indicated that the BDS hosted the bacterias. Similarly also was observed in photoluminescence spectroscopy (Fig. 10). All samples are excited with wavelength 254 nm aims to excite the bacteria.

Photoluminescence spectroscopy for the bacteria shows two peaks at 400 and 450 nm for the *Bacillus subtilis* (5), *Lysinibacillus* (6) and *P. fluorescens* (7) (Fig. 10). The highest intensity resulted from the absorption of *Lysinibacillus* (6). When samples of suspension contained SiO_2:G (4) without bacteria, the intensity was very low. In contrast, when the bacteria embedded with the BDS (SiO_2:G), the intensity of luminescence spectroscopy was significantly decreased compared to only bacteria, however, the two peaks assign for bacteria still appeared. This indicated that the silica and graphite have properly hosted bacteria.

The contact angle of BDS suspension (Fig. 11) were observed in

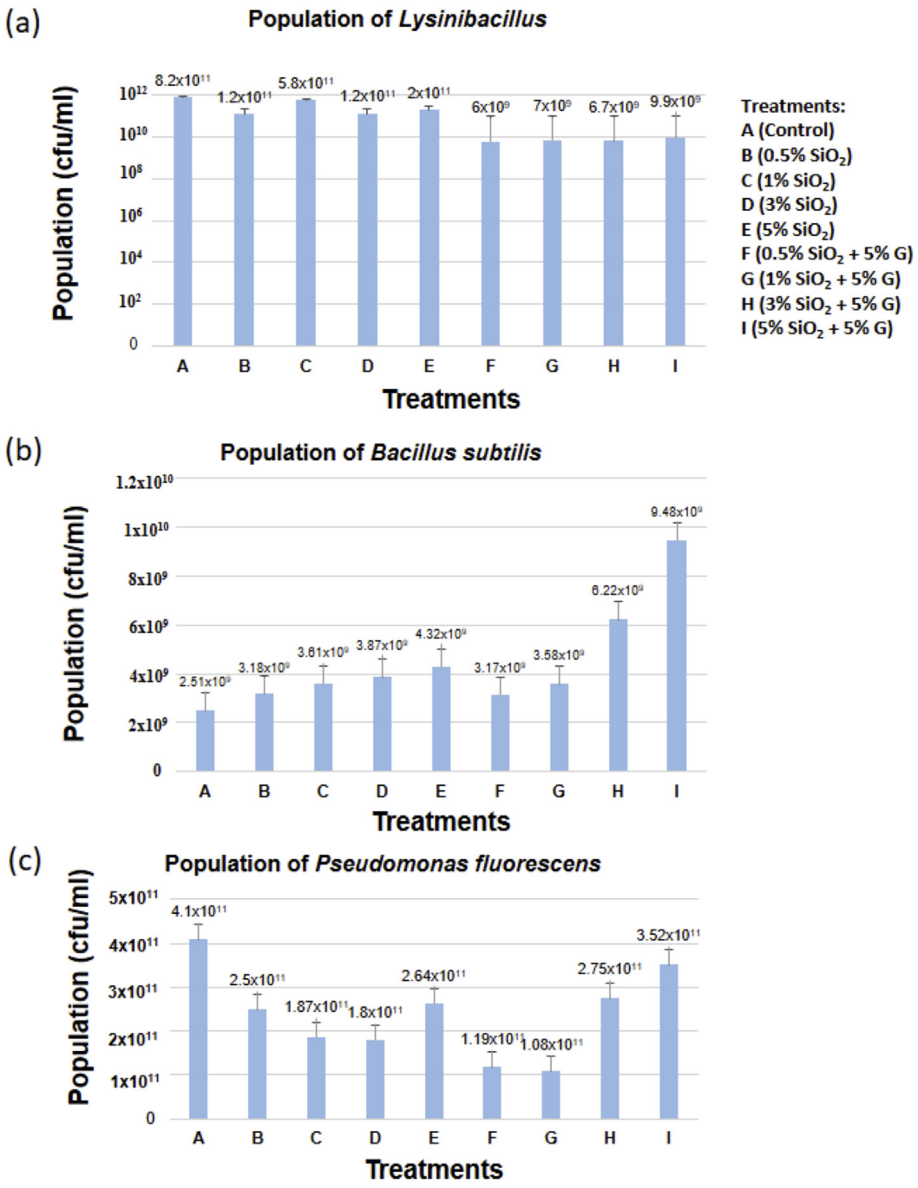

Fig. 12. The population of the bacteria that represent the viability of (a) *Lysinibacillus*, (b) *B. subtilis,* (c) *P. fluorescens*.

glass plates and potato tuber surface to determine the hydrophobicity of the suspension in comparison to water. This result shows that all samples were hydrophilic (contact angle less than 90°) in both types of surfaces indicated that the BDS easily adsorped on the surface of potato tuber. This means BDS formulation is suitable for the potato tuber surface application.

3.3. Viability test of the bacterial isolates in the formulation

Fig. 12 shows the population of the bacteria on day 3 that represent the viability of *Lysinibacillus* (a), *B. subtilis* (b), and *P. fluorescens* (c). This results showed that three bacterial isolates were viable in the BDS of graphite and silica NPs. In contrast, the viability of the *Lysinibacillus* and *P. fluorescens* compared to the control were not significantly different, but the viability of the *B. subtilis* was significantly different compared to control. The highest viability of *B. subtilis* was obtained from the formulation of 5% graphite +5% silica NPs with a population of 9.48×10^9 cfu/ml on day 3. This result indicated that the existent of SiO_2 and graphite (G) did not affect the viability of all bacteria. Furthermore, the survival of the bacteria in the formulation needs to be investigated on more than day 3 and this will be a subject for further study.

3.4. In vitro antagonism between the bacterial isolates in the BDS against R. .Solanacearum

The antagonism between the antagonist isolates against *R. solanacearum* was observed as the means of inhibition zone width around the filter paper discs on the *R. solanacearum* culture (Table 3). The largest inhibition zones caused by endophytic *Lysinibacillus*, 5.51 mm, was at 3% concentration of silica NPs in graphite formulation; *B. subtilis*, 6.69 mm, at 5% silica NPs in the graphite formulation; *P. fluorescens*, 4.56 mm, was caused also by 5% silica NPs in graphite formulation. The inhibition zones caused by the three antagonists indicated the antibiosis mechanism of antagonism. Compared to control, inhibition zones of the treatments of *Lysinibacillus* sp. were 0.69–10.24 times wider, and treatments of *Bacillus* sp. were 0.37–12.6 times respectively (Table 3). However, two treatments of *Pseudomonas fluorescens* (without graphite, with 0.5 and 1% of silica NPs) caused smaller inhibition zones compared to control, although the others were 0.05–0.85 times wider (Table 3). Generally, the formulation of BDS by graphite and silica NPs showed wider inhibition zones compare to control, indicating that the

Table 3
Inhibition zone width on the *R. solanacearum* culture.

Treatments	Inhibition zone width (mm)			Comparison of Inhibition (compare to control)		
	Lys	Bs	Pf	Lys	Bs	Pf
A (control)	0.49 a	0.49 a	2.46 bc	–	–	–
B (0.5% SiO_2)	1.94 a	0.67 a	1.53 a	2.96	0.37	−0.38
C (1% SiO_2)	1.03 a	0.74 a	2.37 ab	1.10	0.51	−0.04
D (3% SiO_2)	0.87 a	1.02 b	2.6 bcd	0.78	1.08	0.06
E (5% SiO_2)	1.80 a	0.80 ab	3.45 de	2.67	0.63	0.40
F (0.5% SiO_2 + 5% G)	5.11 b	1.50 c	2.59 bcd	9.43	2.06	0.05
G (1% SiO_2 + 5% G)	3.84 b	1.67 c	3.39 cde	6.84	2.41	0.38
H (3% SiO_2 + 5% G)	5.51 b	5.46 d	3.79 e	10.24	10.1	0.54
I (5% SiO_2 + 5% G)	0.83 a	6.69 e	4.56 f	0.69	12.6	0.85

- Lys (*Lysinibacillus*), Bs (*Bacillus subtilis*), Pf (*Pseudomonas fluorescens*).
- Numbers followed by a different letter in a column were significantly different according to Duncan's multiple range test (5%).

delivery system significantly affects the mechanism of biocontrol. In addition, Fig. 13 shows the diameter of inhibition zone (DIZ) from the best treatment of *Lysinibacillus* (a), *B. subtilis* (b), and *P. fluorescens* (c).

4. Discussion

It was shown that the width of the inhibition zones caused by the antagonist in the formulation of graphite and silica NPs was higher than the treatment of silica NPs only. This result indicated that the graphite has a role as the site for the bacteria as well as the silica NPs. Application of BDS was able to deliver a higher bacterial population. Therefore, it was produced a higher antibiotic compound to inhibit the pathogen's growth. In addition, silica NPs is also antibacterial which reduces the growth of *R. solanacearum*. It was also reported that the addition of up to 5000 ppm silica NPs inhibited the bacterial growth (Singh, 2016). Moreover, Karunakaran (2013) reported that silica NPs is not toxic to soil bacterial community. Remarkably silica NPs triggers the growth of PGPR and increases the total soil bacterial population. Thus, nanosilica can be included for fertilizer formulations to improve beneficial soil bacterial community.

The antibiosis mechanism indicated by inhibition zones around the filter paper disc + *Lysinibacillus*, filter paper disc + *B. subtilis,* and filter paper disc + *P. fluorescens* was not the only antagonism mechanism. In the present study, however, only the antibiosis mechanism was observed. The indirect mechanism of PGPR in increasing crop yield varies, including the production of antibiotics, other secondary metabolites, bacteriocin, hydrogen cyanide, and siderophore, competitive interaction in root colonization, obtaining nutrients and suitable niche, and induced systemic resistance (Gupta et al., 2000).

Lysinibacillus produces adenosine deaminase (Kathiresan et al., 2014). Adenosine deaminase is a toxic metabolite, may cause disruption on the development and growth, and the viability of other bacterial cells. *B. subtilis* produces antibiotics which inhibit the pathogen growth. Chen et al. (2008) isolated three antibiotics produced by *B. subtilis* JA. They were identified to the lipopeptide families, surfactin, iturin, and fengycin. These compounds could function as biocontrol agents against a large spectrum of pathogens. The mechanism of *P. fluorescens* in inhibiting the growth of *R. solanacearum* was caused also by the antibiotic compound produced by *P. fluorescens*. Some antibiotics that inhibit *Rhizoctonia solani* and *Pythium ultimum* produced by *P. fluorescens* were identified by Howell and Stipanovic (1979, 1980).

The primary mechanism of biocontrol by PGPR involves the production of antibiotics such as phenazine-1-carboxyclic acid, 2,4-diacetyl phloroglucinol, oomycin, pyoluteorin, pyrrolnitrin, kanosamine, zwittermycin-A, and pantocin (Fernando et al., 2005). However, Dwimartina et al. (2017) reported that endophyte and rhizobacteria, identified as *B. subtillis* and *B. cereus* could produce IAA, dissolve phosphate and inhibition zones against *R. syzygii* subsp. *syzygii*, the cause of Sumatra disease on the clove.

In our study, a novel material of graphite was used as carrier formulated in the biocontrol delivery system. It was found that this material did not cause significant reduction of the population of the antagonistic bacteria. This study suggested the in vitro abilities of the three antagonistic isolates, formulated in graphite as the BDS, in inhibiting the growth of *R. solanacearum*. The antagonistic bacteria proved effective under in vitro conditions have to be screened for their performance in natural conditions before they can be used as biocontrol agents. It is because in the natural conditions (in vivo) they have to effectively colonize in the rhizosphere and tissue of the host plant, and they need to compete with other soil microflora for nutrients (Kloepper et al., 1988). In this study, the presence of silica NPs in the formulation was able to maintain the population of the three antagonistic isolates, indeed it increased the initial population in the case of *B. subtilis.* Karunakaran (2013) concluded that silica NPs had a favourable effect on beneficial bacterial population and nutrient value of soil, the colony forming unit (cfu) was doubled in the presence of silica NPs.

As a comparison to our study on the formulation of the BDS, talc is used widely as a BDS in controlling plant pathogens. Ramesh and Phadke (2012) used talc in the formulation as the BDS for both in vitro as well as a field study of the control of *R. solanacearum* using PGPR and endophyte. They collected 17 antagonistic bacterial isolates against *R. solanacearum* in vitro and in vivo on eggplant. Most of the isolates were identified as *Pseudomonas* and *Bacillus*. Suryadi et al., 2013 used a talc-based delivery system of bacterial consortium formulation consisted of *Pseudomonas aeruginosa* + *Bacillus cereus* and some other composition to control several rice pathogens. Lumsden et al. (1995) used *Gliocladium virens* formulated in alginate prill as the delivery system and incorporated into soilless potting media for the control of the damping-off pathogens *Pythium ultimum* and *Rhizoctonia solani* on vegetable and ornamental seedlings. However, some BDS did not use any carrier in the formulation. Nawangsih et al. (2011) selected some endophytic

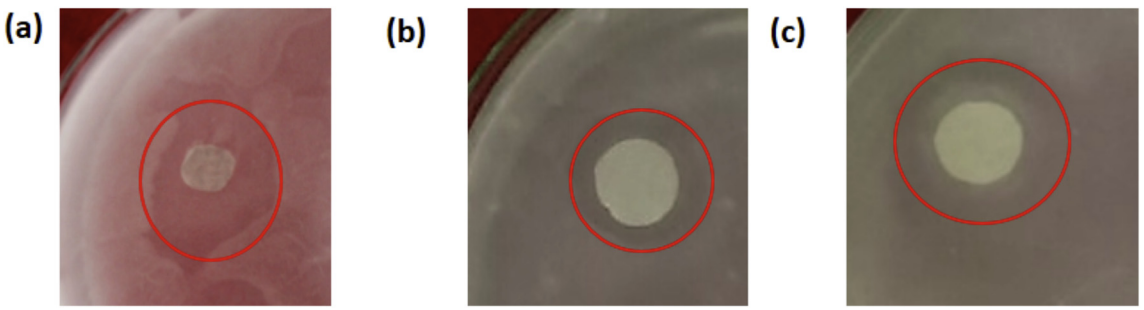

Fig. 13. The selected picture of diameter of inhibition zone for the best treatments against *R. solanacearum* by (a) *Lysinibacillus, (b) B. subtilis (c) P. fluorescens.*

bacteria to control tomato bacterial wilt disease and obtained 2 isolates that reduced the wilt incidence identified as *Staphylococcus epidermidis* and *Bacillus amyloliquefaciens*. They applied the endophytes directly from the culture on agar medium, without carrier delivery system. Rado et al. (2015) suggested 3 actinomycete strains efficient as biocontrol agents against potato bacterial wilt. They did not involve a carrier in the delivery system. Okigbo and Osuinde (2003) reported that *B. subtillis* reduced fungal leaf spot on mango, they apply the antagonist also with no carrier formulation or BDS.

5. Conclusions

Isolates of endophytic *Lysinibacillus* and PGPR *B. Subtilis,* and *P. fluorescens* that reduce the wilt disease incidence caused by *R. solanacearum* on potato plant were viable in the biocontrol delivery system formulated with graphitic and silica NPs. Those individual bacterial isolates in the formulation were also able to inhibit the in vitro growth of *R. solanacearum*. The widest inhibition zones were shown by the formulation with graphite and silica NPs, indicating that the delivery system significantly affects the mechanism of biocontrol.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101153.

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