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Bio-management of anthracnose disease in chilli with microencapsulates containing *Bacillus subtilis* B298

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Abstract. The objectives of this research were to evaluate the *Bacillus subtilis* B298 strain as antagonist of *Colletotrichum* sp. pathogens in vitro, and to evaluate the ability of microencapsulated *B. subtilis* B298 strain formula to suppress anthracnose disease of chilli in the fields. Methods for antagonism test of *B. subtilis* B298 against *Colletotrichum* sp. was conducted by dual culture on potato dextrose agar medium. Microencapsulated formula 2g. $L^{-1}$of *B. subtilis* B298 spray was used as control of anthracnose disease in the fields. The four treatments were arranged by Randomized Completely Block Design consist of control, *B. subtilis* B298, fungicide, and combination of *B. subtilis* B298 and fungicide with six replications. The measured variable in vitro was inhibition percentage, and that of in field were; disease intensity, infection rates and plant total phenol. Results showed that *B. subtilis* B298 strain inhibited 56% growth of *Colletotrichum* sp. in vitro, micro capsulated *B. subtilis* B298 reduced disease intensity by 48% with infection rates of 0,02 unit.day^{-1}. Microencapsulated *B. subtilis* B298 induced plant systemic disease resistance on chili as total phenol of the treated plant increased.

1. Introduction

The production of chili still needs to be increased, considering that the community needs for chili has not been fulfilled nationally. In 2017 the national chili productivity reached 8.35 tons/ha, which decreased compared to 8.65 tons/ha in 2016. This is caused by several constraints such as plant diseases. Anthracnose is a major disease in chili, because the intensity of the disease can reach more than 75% which can reduce both quantity and quality bymore than 50% [1]. *Colletotrichum* sp. as a fungus that causes anthracnose chili disease, easily dispersed by the wind and capable to survive in the plants remain and in the soil for a relatively long time, so that anthracnose always accompanies each of chili plant; primarily *C. capsici* and *C. gloeosporioides* [2].

The symptoms of anthracnose are small brown spots then getting bigger with orange to pink fungus spore in the surface and sometimes forms black aservulus body on the spot surface. Symptoms of anthracnose caused by *C. capsici* and *C. gloeosporioides* produce small, circular black spots or spots with concentric rings consisting of black aservulus body on the spot surface. Chili anthracnose disease is also called “patek”, dry rot, dieback [3;2]. The control of anthracnose which is commonly done by farmers is using fungicide. However, if carried out continuously can cause resistance to the pathogen, therefore the fungicide does not work anymore, not to mention the negative impacts on the environment and humans as consumers. Therefore an environmentally friendly control alternative is needed, such as biological agent *Bacillus subtilis*. The *B. subtilis* isolate rhizosphere potatoes as antagonistic bacteria known as Gram positive bacteria, have character as a plant growth promoter because is capable of producing a growth hormone IAA, phosphate solubilization, siderophore producer, nitrogen producer, and resistant to rifampicyn antibiotics [4;5,6,7]. This shows that *B. subtilis* has the ability as a biofertilizer which can stimulate plant growth or as PGPR (*Plant Growth Promoting Rhizobaceria*). *B. subtilis* as biological control agent of plant diseases because its ability to produce amylase and chitinase enzymes which degrade pathogenic fungal cell walls whose cell wall consists of chitin [6, 8, 9].

The application of *B. subtilis* in a liquid formula is by soaking the seeds, as a basic fertilizer and as a supplementary fertilizer splashed around the planting hole in 10 days after tranplanting (*dat*) intervals carried out on the chili to reduce the incidence of anthracnose and bacterial wilt. The formulation and application of *B. subtilis* B298 in the microencapsulation formula intended to prolong the shelf life and maintain its effectiveness. Microencapsulation is a process of coating on an active ingredient both liquid and relatively thin solids on small particles of solid or liquid into very small particle sizes in the range 1-5000 µm. This small particle causes the material to be spread evenly and extends to the location of the target application. The material inside it is called the core or internal phase or content, while the wall is called a protector or coating or membrane. The diameter of microcapsules is several micrometers (µm) to millimeters (mm). The reasons for microcapsules formulation are; 1) to protect active ingredient from the environment, 2) to convert liquid active component into a dry solid form, 3) to separate the incompatible components, 4) maintain the active ingredient components, and 5) to protect and control the release of components active so that it is released slowly (slow release) and long and ongoing [10].

Aims of the study was to evaluate the antagonism of *B. subtilis* B298 against *Colletotrichum* sp. in vitro, and evaluate the ability of *B. subtilis* B298 strains in microencapsulation formulas to suppress anthracnose in the field.

2. Materials and methods

B. subtilis B298 was prepared on Yeast Pepton Glucose Agar (YPGA) media (5g yeast extract, 10g peptone, 10g glucosa and 20g agar), and on YPG broth medium. The encapsulant materials are maltodextrin and gum arabic in ratio of 3: 2. *Colletotrichum* sp. isolated from anthracnose symptomatic chili on Potato Dextrose Agar (PDA) media (200g potato, 15g dextrose, 20g agar). The study was conducted in two stages; *in vitro* to evaluate the antagonism of *B. subtilis* B298 against *Colletotrichum* sp. on PDA media, and then to suppress the anthracnose disease in the fields. The study was carried out for 6 months, initiated by formulation of microencapsulates, followed by examinations.

2.1. Microencapsulates preparation

B. subtilis B298 strain was isolated from the potato rhizosphere, grown on YPGA media; after 2 days of age then transferred to YPG broth media and shaked for 2 days at 150 rpm 28+2^{o}C temperature. The microencapsulant formula of maltodextrin and gum arabic with a ratio of 3: 2 is mixed and stirred while sterile water added, until paste is formed then sterilized in autoclave at 120^{o}C, 15 psi for 25 minutes, after cooling 0.2 v / w suspension *B. subtilis* B298 was added, stirred evenly and put in a refrigerator at 15^{o}C for 10 minutes and dried with freeze drying benchtop type, VLC Mode 100 mT at -73.6^{o}C for 10-14 hours. The dried formula obtained then smoothed and sifted to uniform µm-sized particles.

2.2. In vitro antagonism assay

The method used is dual culture [11]; *Colletotrichum* sp. was cut with cork borer, placed on a solidified PDA media on a Petri dish then at a distance of 3 cm *B. subtilis* B298 streaked, incubated for 2-5 days, then the percentage inhibition observed with measured of clear zona.

The percentage of inhibition is calculated based on [12] with formula , I = (CT) / C x 100%, with I: percentage inhibition, C: radius of pathogenic fungal colonies that grow opposite the antagonist direction, T: radius of pathogenic fungal colonies that grow towards the direction of antagonistic bacteria.

2.3. On field antagonism assay
Application of microencapsulated *B. subtilis* B298 was carried out by watering the planting hole with 10-days intervals and spraying with 7-days intervals. Concentration of microencapsulated *B. subtilis* B298 used is 2 g formula /L, with population density 10^8 cfu/g. The treatments were arranged using Complete Randomized Block Design with 4 treatments; control, *B. subtilis* B298 2gformula. L^{-1}, fungicide with active ingredients karbendazim 2g. L^{-1}, and a combination of *B. subtilis* B298 and fungicides with a concentration of 1 g. L^{-1}; respectively. The treatment was repeated 6 times. The variables observed were disease intensity with the formula $IP = \sum (nv) / ZN \times 100\%$, the rate of infection [13] $Xt = Xo.e^{rt}$, and the percentage of suppression and total phenol to detect affected systemic resistance using the DPPH = diphenyl-1-picrylhydrazyl method [14].

3. Results and discussion
The microencapsulated *B. subtilis* B298 resulted is white powder with particle size of 403.9 x 343.9 µm, has a solubility and durability of up to 7 weeks. Microcapsules as a result of microencapsulation processes have sizes between 1-5,000 µm, have high solubility and stability [10, 15].

3.1. In vitro inhibition of Colletotrichum sp. by B. subtilis B298
Results of inhibition of *B. subtilis* B298 against *Colletotrichum* sp. *in vitro* can be seen in Table 1. *B. subtilis* B298 in both the microencapsulated form and its colonies can inhibit the growth of *Colletotrichum* sp. with inhibitory effectiveness ranging from 22-56%. *In vitro* inhibition was higher in *B. subtilis* B298 colonies compared to microencapsulated form. This is caused by the slow release of the active ingredient *B. subtilis* B298 in microencapsulated form. The purpose of microencapsulation is to prolong its shelf life, retain effectiveness were maintained its resistant to environmental changes, therefore is more flexible and can extend shelf life [16].

Table 1. *B. subtilis* B298 inhibition against *Colletotrichum* sp. *in vitro*

Treatment	Colony diameter (mm)	Inhibition (%)
Control	88	-
B. subtilis B298 Mic	62	22
B. subtilis B298	58	56

Note: *B. subtilis* B298 Mic: (microencapsulated form)

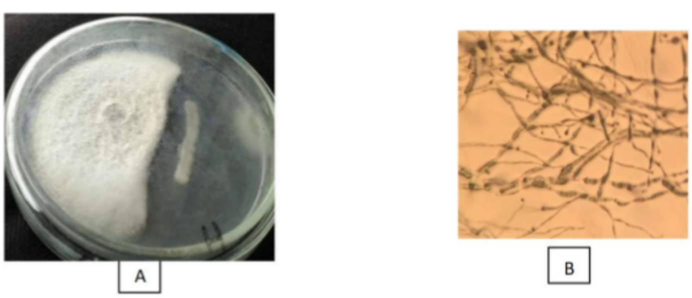

Figure 1. Mechanism of *B. subtilis* B298 inhibition against *Colletotrichum* sp. *in vitro*.
A. Inhibition of *B. subtilis* B298 against *Colletotrichum* sp., B. Swelling and lysis of hypha, its mechanism if inhibition

Inhibition *Colletotrichum sp*. growth by *B. subtilis* B298 which caused by the production of bioactive compounds, chitinase enzymes and antibiotics. *B. subtilis* B298 produced chitinase enzyme with activity of 6,937 U / mL at 15 hours incubation and 5,764 U / mL at incubation temperature of 40°C and 6,813 U / mL at pH 5 [7]. *B. subtilis* B298 inhibition mechanism against *Colletotrichum* sp. is antibiosis, lysis which is characterized by swelling of the hyphae. Five *B. subtilis* isolates from the rhizosphere of potatoes were capable to inhibit the growth of *C. gloeosporioides* and *C. capsici* with the mechanism of hyphae swelling, twisting, distortion and lysis [17]. In direct treatment of colonies *B. subtilis* B298 indeed, the inhibition is greater *in vitro*, but if this colony is applied on field it can be sensitive to the environment, and less practical in storage and transportation, therefore the microencapsulated form is considered more flexible. Microencapsulated form of *B. cereus* in the can be resistant to UV light and rain, and this formula can improve viability during application by spraying [18].

3.2. Suppression of chili anthracnose with B. subtilis B298 microencapsulate

The application of combination *B. subtilis* B298 microencapsulated form and fungicide resulted the lowest disease intensity reduction, while the infection rate as same as microencapsulated form *B. subtilis* B298 treatment. This is in accordance with the results of the research that the treatment of *B. cereus* strain CIL microencapsulated form was capable to prevent leaf blight in Lily flowers, and the most effective was the application of maneb fungicides followed by the application of *B. cereus* strain CIL microencapsula\ted form [16]. Probenazole pesticides and *B. cereus* strain CIL microencapsulated form shows the same effectiveness in controlling leaf blight.

Table 2. Effect of *B. subtilis* 298 microencapsulates form to disease intensity, infection rate and inhibition effectivity

Treatment	Disease Intensity (%)	Infection rate (r) unit.day^{-1}	Inhibition effectivity (%)
Control	70.60	0.30	-
B. subtilis B298 Me	36.71	0.02	48
Fungicide	44.23	0.12	37.35
B. subtilis B298 Me - Fungicide	26.88	0.02	61.93

B. subtilis B298 strain inhibited 56%growth of *Colletotrichum* sp. *in vitro*, microcapsulated *B. subtilis* B298 reduced disease intensity by 48% with infection rates of 0,02 unit.day^{-1}. This result shows that *B. subtilis* B298 is capable to suppress disease in the field because of its potential to produce the enzyme chitinase [7] which inhibits the growth of *Colletotrichum* fungi therefore that the disease intensity is reduced compared to control (sprayed with water only). The treatment of the combination of *B. subtilis* B298 with fungicides showed a greater effectiveness of disease suppression, namely 61.93% with the rate of infection with similar to the treatment of *B. subtilis* B298 alone, which was 0.02 unit.day-1. This shows that *B. subtilis* B298 microencapsulated form is flexible, therefore it is compatible with fungicides.

3.3. B. subtilis B298 microencapsulate as an inducing systemic resistance

Microencapsulated form of *B. subtilis* B298 induced plant systemic disease resistance on chili as total phenol of the treated plant was increased (Table 3), which showed that BF treatment (combination of *B. subtilis* B298 microencapsulated form with fungicide) was the most effective treatment for systemic resistance to chili with total phenolic content of the plant is the highest, which is 54.24% with the effectiveness increase by 10.21%. This result is in accordance with another research that of spectrophotometric antioxidant examination of plant extracts showed the plant phenol content protect plants from disease [12]. The higher the phenol content the plant, the more resistant to attack by pathogens, which is indicated by the decrease disease intensity.

Table 3. Total phenol compound in root and plant canopy of chilli

Treatment	Root Phenol Total (%)	Effectivity (%)	Plant Canopy Phenol Total (%)	Effectivity (%)
K: control	18.22	-	48.70	-
B: B.s B298 Me	20.14	9.53	51.96	6.27
F: Fungicide	22.64	19.52	52.68	7.55
BF: B.s B298 Me + Fungicide	20.66	11.81	54.24	10.21

4. Conclusion

B. subtilis B298 strain inhibited 56% growth of *Colletotrichum* sp. *in vitro*, microcapsulated *B. subtilis* B298 reduced disease intensity by 48% with infection rates of 0,02 unit.day^{-1}. Combination of microencapsulated *B. subtilis* B298 and fungicide is the best treatment in suppressing anthracnose disease and inducing systemic resistance to the disease with the disease suppression effectiveness of 61.93% and the total phenolic compounds increased by 10.21%.

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