The effectiveness of biopesticide formulation *Bacillus subtilis* BNt8 as biocontrol agent of banded leaf and sheath blight (*Rhizoctonia solani*) disease on corn (*Zea mays* L.)

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Abstract. The role of synthetic pesticides to control banded leaf and sheath blight (BLSB) disease on corn are still very dominant, therefore still many efforst are needed in order to minimize the negative impacts. One way of controlling this disease more prudent at this time is the use of antagonistic bacteria as the biological control agent like *Bacillus subtilis*. The present study aimed to determine the effectiveness of a biopesticide formulation *B. subtilis* BNt8 on a different frequency and dosage to control BLSB disease (*Rhizoctonia solani*) on corn plants in the field. Another effect of the application of a biopesticide formulation of *B. subtilis* can be observed by monitoring the growth of plants. The research was conducted in the plant pathology laboratory and greenhouse of Indonesian Cereals Research Institute at Maros and in Bajeng experimental farm. In the field study, the treatments were arranged in randomized factorial design. Result of experiment showed that in vitro test, treatment with concentration 2 g/L provided relatively better levels of inhibition in suppressing the development of fungal mycelia of *R. solani*. In green house trial, seed treatment with concentrations of 3% was able to suppress BLSB disease, so it can be recommended for field tests. In the field trial, application intervals of 3 weeks tend to give a better effect in suppressing disease and improve yields. Applications of biopesticide formulation *B. subtilis* could suppress BLSB disease up to 18.5% and the yield potential to reach 5.3 tons/ha.

Key Words: seed-borne pathogen, biological control, seed treatment, corn pathology, *Z. mays*.

Introduction. All part of the corn plant (*Zea mays* L.) are susceptible to a wide variety of diseases that can reduce the yield and quality of its products. One of the important diseases that became serious in recent years is banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani* Kuhn. BLSB has become increasingly severe and economically important disease of *Z. mays* in several countries of Asia (Sharma et al 2002). The occurrence of the disease has also been reported from other parts of the world. The use of fungicides to control BLSB in *Z. mays* is limited and has adverse ecological implication, while the use of resistant varieties has not progressed much because of the limited host genetic variability for resistance to *R. solani* (Sharma et al 2002).

Effective, compatible, and sustainable technique in controlling BLSB disease that is environmentally friendly is required. One method of controlling the disease that more prudent at this time is the use of antagonistic bacteria as a biological agent (Rustam et al 2011). Biological control of diseases caused by the fungus *R. solani* using antagonistic fungi has been widely studied. Biological control is an important method in the management of plant pathogens. Advantages include reduction of dependence of high-risk chemicals for diseases, control or other ecological and economic benefits (Bale et al 2008). BLSB could also be controlled with antagonistic fungi. Mulyati (2009) used several species of fungi as biological control agent in controlling BLSB. Soenartiningsih (2013) suggested that arbuscular mycorrhizal fungi *Glomus* sp. and *Acaulospora mellea* could inhibit the development of *R. solani*, the causal agent of BLSB disease. Similarly, the isolates of the fungus *Trichoderma* spp. and *Gliocladium* spp. can suppress the development of BLSB disease (Soenartiningsih et al 2014).

Antagonistic bacterium that has been used in controlling BLSB is *Bacillus subtilis* (Ehrenberg) Cohn. Suriani & Muis (2016) reported that *B. subtilis* is a biological agent that is effective in controlling various types of plant pathogens, including soil-borne pathogens in *Z. mays*. The mechanism of biocontrol bacteria to provide protection for the plant is through the production of secondary metabolites with antifungal activity, antibiotic activity and competition for nutrients (Jalgaonwala et al 2010). Factors that affect the antimicrobial activity include bacterial metabolic activity, the amount of inoculum, and differences in environmental pH (Dutta et al 2013).

The use of *B. subtilis* alone or in combination with other biological agent shown to be effective to control the growth and development of *R. solani* in vitro (Ali & Nadarajah 2013). Khaeruni et al (2014) proved that the application of rhizobacteria *B. subtilis* ST21b, *B. cereus* ST21e, and *Serratia* strain SS29a promote plant growth, yield, and resistance to *R. solani* of soybean. Suryadi et al (2015) reported that the secondary metabolites produced from extracted *B. cereus* 11UJ at a concentration of 1000 ppm could inhibit the growth of fungi *R. solani* and *Pyricularia oryzae*. Goudjal et al (2014) reported that a strain of *Streptomyces* sp. CA-2 and AA-2 isolated from the roots of tomato plants effectively control damping-off disease (*R. solani*) and spur the growth of tomato seeds. Ashwini & Srividya (2014) reported that the treatment of the chilli seed with co-inoculation of the pathogen with *Bacillus* sp. culture showed 65% reduction in anthracnose disease incidence by the treatment as compared to the seed treated with pathogen alone (77.5%).

This study is a continuation of the research that has been done before, such as: virulence test of some putative *B. subtilis* (Ehrenberg) Cohn as biological control agents of plant diseases *Z. mays* (Muis et al 2015), the evaluation of several inert carrier and formulation of *B. subtilis* (Muis et al 2015).

This study aimed to obtain the frequency and dose of formulated *B.* subtilis that is effective in controlling BLSB disease on *Z. mays* in the field.

Material and Method. The research was conducted from January to September 2016 in Plant Pathology Laboratory, green house of Indonesia Cereals Research Institute (ICERI), and Bajeng experimental farm of Gowa.

Test for volatile toxic compounds of B. subtilis BNt8 formulation in vitro. The materials used in this test were *B. subtilis* BNt8 formulation, fungal pathogen *R. solani*, and *Z. mays* seeds of Anoman variety. *B. subtilis* BNt8 formulation used in this study was in powder formed enriched with additives (Muis et al 2014). *R. solani* isolate was taken from the collection of Plant Pathology Laboratory of ICERI.

The treatments were arranged in completely randomized design with three replications. The treatments consist of 0.01, 0.02, 0.03, 0.04, 0.05 g formulation, each dissolved in 10 mL of sterile distilled water in a test tube and prepared a serial dilution up to 10^{-7}. Each treatment was taken as much as 0.1 mL and streaked on PDPA plates and incubated for 24 h at room temperature. A bottom plate with PDA inoculated at the center with a mycelial plug of *R. solani* was placed on top of the bottom plate with the 24 h old *Bacillus* isolate. The two bottom plates were sealed together with parafilm and incubated at room condition. For the control, the plate with *R. solani* mycelial plug was placed on top of the bottom plate containing PDPA only (no bacteria). Two days after incubation, the radial growth of the fungus was measured.

Preparation of R. solani inoculum. *Z. mays* plants showing banded leaf and sheath blight (BLSB) disease symptoms were collected from Bajeng Experimental Farm in Gowa. These were brought to the laboratory and cleaned in running water. Three to four sq mm tissue (½ diseased, ½ healthy) sections from the BLSB lesion were disinfected in 1% sodium hypochlorite for 2-3 minutes, washed 3 x in SDW for about 30 seconds, blotted dry in filter paper, and transferred to Petri plates containing half-strength potato dextrose agar (PDA). The Petri plates were sealed with cellophane tape and incubated in inverted position.

If sclerotial bodies were found from plant tissues, these were surface disinfected with 10% chlorox, rinsed three times with SDW, and placed on acidified PDA. The Petri plates were sealed with cellophane tape and incubated in inverted position.

The culture plates were incubated for 48 hours at room temperature. Mycelial strands growing from sclerotial bodies or tissue sections were transferred to PDA slant, labeled properly and allowed to grow for 5 days at room temperature. Cultures were kept in the refrigerator for further use.

For field experiment use, *R. solani* inoculum was prepared by culturing it in rice hull-rice grain (RHRG) substrate. RHRG substrate consisted of 80 g rice hull, 130 g rice grain and 150 mL tap water, packed in autoclavable plastic bags of 30 cm long and 15.5 cm wide or in 1-L dextrose bottles. The substrate was sterilized for two hours at 15 psi. After sterilization, a 3-mm agar disk from the margins of actively growing cultures of *R. solani* was placed into the substrate and incubated for two weeks at room temperature before use.

In vivo test of B. subtilis BNt8 formulation. Tests using a randomized completely block design with four replications. The treatment includes a formulation concentration level of 1%, 2%, 3%, 4% and 5%. The first phase of testing is a seed treatment. Each 100 g of *Z. mays* seeds mixed with 1 g, 2 g, 3 g, 4 g, 5 g formulation, fungicides heksakonazol 1 mg/g seed used as control. The treatments were given 2 hours before planting, then air dried at room temperature. For the treatment of positive and negative control, seeds were not mixed with formulation. Corn seeds were then planted in plastic trays (24 x 32 x 8 cm) containing sterile soil that has been mixed with a culture of *R. solani* on RHRG substrate. Each tray consists of 20 *Z. mays* seeds. Seed germination was observed in 7 days after planting (DAP). Plant height was measured at 14 DAP, root length was measured at 21 DAP. The percentage of seed germination calculated using the formula:

$$P = \frac{a}{b} \times 100\%$$

Where:

P = percentage of seed germination;
a = number of germinated seed;
b = number of planted seed.

Field test of B. subtilis BNt8 formulation. This experiment was conducted at Bajeng experimental farm in Gowa. The treatments were arranged in factorial design, where the first factor was frequency of application:

F2: application of formulation in every 2 weeks;
F3: application of formulation in every 3 weeks;
F4: application of formulation in every 4 weeks.

While the second factor was dosage formulation in each application:

D3: 1 kg/ha
D4: 2 kg/ha
D5: 3 kg/ha

Two hours before planting, the seeds were treated by mixing 3 g *B. subtilis* BNt8 formulation with 100 g of *Z. mays* seeds, hexaconazol fungicide was used as control with the dosage of 1 mg/g seed. There were three control treatments in this experiment: seed treatment with heksakonazol fungicide 1 mg/g; inoculated plot with *R. solani*; uninoculated plot. The seeds from each treatment were planted in a 1 x 5 m plot, planting space was 75 x 20 cm with one seed per hole. Fertilizer application was done at 14 and 21 DAP using Urea and Phonska (300 kg/ha).

At 4 weeks after planting (WAP), plants were inoculated with 10 g RHRG with *R. solani* by placing it at the bottom of plant stems, exept uninoculated control plot. Then the solution of *B. Subtilis* BNt8 formulation was sprayed at the same day base on the treatment frequency and the dosage of application.

BLSB scores were recorded at 2, 4, and 6 days after inoculation using the following 1-9 scale of Ahuja & Payak (1983) (Table 1).

Table 1

BLSB scores according to Ahuja & Payak (1983) 1-9 scale

Scale 1	Disease on one leaf sheath only; few small, non-coalescent lesions present
Scale 2	Disease on two sheaths; lesions large and coalescent
Scale 3	Disease up to four sheaths, lesions many and always coalescent
Scale 4	As in scale 3 + rind discolored with small lesions
Scale 5	Disease on all sheaths except two internodes below the ear
Scale 6	Disease up to one internode below the ear shoot; rind discoloration on many internodes with large depressed lesions
Scale 7	Disease up to internode bearing the ear shoot but shank not affected
Scale 8	Disease on the ear; husk leaves show bleaching, bands and caking among themselves as also of silk fibers; abundant fungal growth between and on kernel rows; kernel formation normal except their being lusterless; ear size less than normal; some plant prematurely dead
Scale 9	In addition to scale 8, shrinkage of stalk; reduced ear dimensions; wet rot and disorganization of ear; kernel formation absent or rudimentary; premature dead plants common; abundant sclerotial production on husk leaves, kernels, ear tips or silk

The scores reading were transformed to percent disease severity by using formula Townsend & Heüberger (Agrios 2005):

$$P = \frac{\Sigma (n \times v)}{Z \times N} \times 100\%$$

Where:

P = Disease severity;
n = number of sample in each category;
v = numerical value of each category;
Z = the highest numerical value of scale;
N = total number of sample.

The percentage of disease suppression was calculated by formula proposed by Meera et al (1995):

$$Ps = \frac{K - P}{K} \times 100\%$$

Where:

Ps = percentage of disease suppression;
K = mean value of percentage of disease incidence on control;
P = mean value of percentage of disease incidence on treatment.

Statistical analysis. The data were subjected to two-way ANOVA followed by a Least Significant Difference Test comparison of means test was used to determine if means were significantly different (α=0.05) from each other. Count data was transformed using percentage data arcsine-square root transformed. Statistical analysis was conducted with the software package STAR Ver 13 for Windows (IRRI 2013). In graphs and figure, the original data and their standard errors are presented.

Results and Discussion

Test for volatile toxic compounds of B. subtilis BNt8 formulation in vitro. Results of in vitro test are shown in Table 2. The best inhibitory potency on PDA was 84.2% shown by the application concentration of 3 g/L in the incubation period of 24 hours and at 48 hours after incubation, treatment with application concentration 2 g/L could inhibit the growth of *R. solani* to 72.5%. Muis et al (2015) reported that the antagonistic bacteria secrete an enzyme that is capable of inhibiting the mycelia growth of fungal pathogens. Inhibitory potency shown by the bacteria *B. subtilis* is also supported by media propagation and growth of bacteria used the yeast extract and the PDPA. According to Muis (2006) yeast extract is the best stimulator for bacterial growth, while the culture medium containing vitamins, nitrogen, amino acids and carbon.

Table 2

Relative inhibitory level (THR) of formulation *B. subtilis* BNt8 to growth of *Rhizoctonia solani* in vitro (Plant Pathology Laboratory of ICERI, Maros, 2016)

Concentration of application	*Relative inhibitory level (%) at ….. hai*	
	24	*48*
1 g/L formulation of *B. subtilis*	27.4 bc	47.1 ab
2 g/L formulation of *B. subtilis*	75.8 ab	72.5 a
3 g/L formulation of *B. subtilis*	84.2 a	61.3 a
4 g/L formulation of *B. subtilis*	32.6 abc	49.2 ab
5 g/L formulation of *B. subtilis*	66.3 ab	58.8 a
Control	0.0 c	0.0 b

hai - hour after incubation.

In vivo test of B. subtilis BNt8 formulation. Result of test of *B. subtilis* BNt8 formulation in the greenhouse showed that seed treatment with *B. subtilis* BNt8 formulation gave positive effect on growth and development of plants (Table 3). The result also showed that seed germination was 100% compared to the untreated seeds were only 97%, likewise its effect on plant height and root length. Plant height of treated seeds reached 41.2 cm, whereas the plant height on untreated seeds was only 32.3 cm. The root length of treated plants was 41.3 cm, while the root length of untreated plants was only 26.4 cm.

Table 3

The effect of seed treatment with *Bacillus subtilis* BNt8 formulation on germination, plant height, and root length of corn plant in the greenhouse of ICERI, Maros, 2016

Treatments	*Germination (%) 7 DAP*	*Plant height (cm) 14 DAP*	*Root length (cm) 21 DAP*
K+ (inoculated control)	97	32.3	26.4 b
K-(uninoculated control)	97	36.8	32.3 ab
KP (fungisida)	98	36.9	33.1 ab
1 % formulation	100	40.2	34.9 ab
2 % formulation	100	38.5	33.3 ab
3 % formulation	100	41.2	41.3 a
4 % formulation	100	38,5	36.1 ab
5 % formulation	100	37,1	34.2 ab
CV (%)	2.7	14.8	17.8

From the results of this study we found that plant growth in seed treatment formulation at a concentration of 3% was better than those in the other concentration treatments. These results concur with those of Wartono et al (2012) that the application of *Burkholderia cepacia* isolates E76 formulation at a concentration of 3% through seed

treatment effectively and more efficiently enhance the growth, shoot length and root length of rice plants in vitro.

Field test of B. subtilis BNt8 formulation. The result of field test showed that application of *B. subtilis* BNT8 formulation in once in a three weeks with the dosage of 1 kg/ha gave a better result in controlling BLSB disease. In this treatment, the BLSB incidence was 51.5% (Table 4). At a certain growth phase, transmission of the disease was relatively high, but on the next phase might be higher. Development of the disease in the field is influenced by biotic and abiotic factors (Wartono et al 2014). According to Budi et al (2011), plant defense mechanisms can be induced by agents that are endophytic antagonists. Muis & Quimio (2006) reported that *B. subtilis* BR23 formulation used as seed treatment potentially control BLSB disease and increased yield of *Z. mays*.

Table 4

Disease incidence of BLSB at 2, 4, and 6 weeks after inoculation (WAI)

Treatments	*Incidence of BLSB disease (%) at …… WAI*		
	2	*4*	*6*
F2D3	6.9 (14.4)	29.3 (31.5)	58.7 (50.1)
F2D4	8.5 (16.4)	31.1 (33.8)	67.9 (55.7)
F2D5	11.8 (19.1)	32.6 (34.4)	72.2 (58.3)
F3D3	8.4 (16.8)	26.8 (31.1)	51.5 (45.9)
F3D4	8.1 (16.2)	31.8 (34.2)	61.7 (51.8)
F3D5	6.3 (14.3)	33.5 (34.6)	61.8 (52.0)
F4D3	10.6 (19.0)	31.5 (35.8)	73.1 (58.8)
F4D4	9.6 (17.6)	28.7 (35.6)	67.7 (55.6)
F4D5	6.6 (11.5)	32.8 (33.6)	65.2 (54.3)
K+	7.5 (15.5)	34.4 (32.5)	63.1 (52.8)
K-	6.7 (14.6)	8.7 (19.2)	58.9 (50.4)
KP (Fungicide)	1.2 (6.1)	34.4 (32.3)	56.5 (48.8)
CV (%)	33.7	24.9	12.5

Data in brackets was the result of arcsine-square root transformation.

Effect of *B. subtilis* BNt8 formulation in suppressing BLSB in the field is presented in Figure 1. Figure 1 shows that, the higher the concentration, the better the suppression of disease.

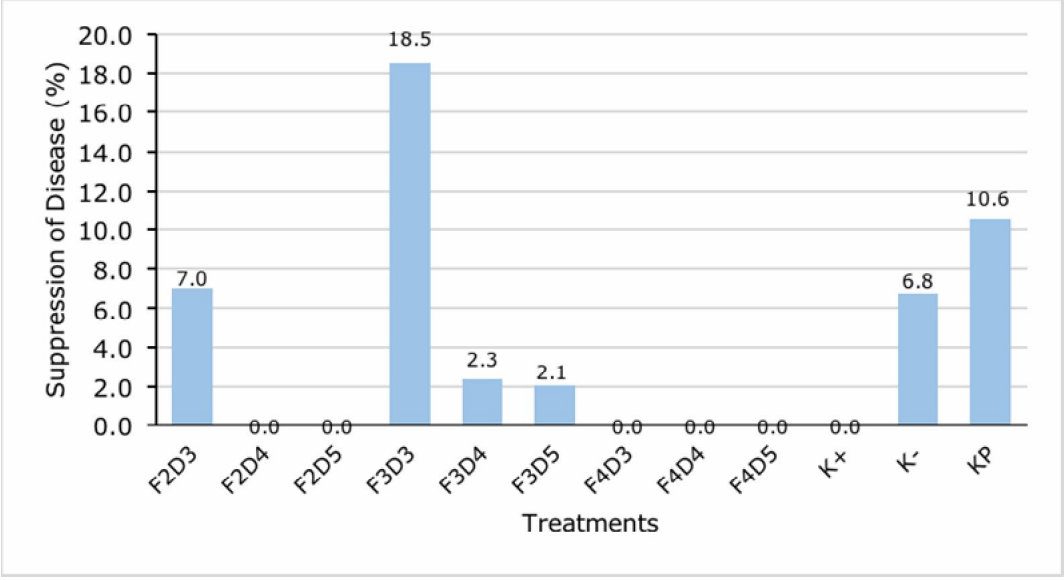

Figure 1. Effect of combination between frequency and dosage of *Bacillus subtilis* BNt8 formulation to BLSB disease suppression in the field.

The best suppression of disease was demonstrated by the treatment F3D3 i.e. 18.5%, followed by treatment F2D3 i.e. 7.0%. The suppression of this disease occurs presumably because *B. subtilis* BNt8 used produced antibiotics. Several *Bacillus* species produce a variety of antibiotics, which can enhance the antifungal activity and control of plant pathogens (Kumar et al 2009). Awais et al (2010) reported that *B. subtilis* produces main antibiotics such as polymyxin, difisidin, subtiline, micobasilin, and bacitracin.

The results of observations also show that the effectiveness of the formulation of *B. subtilis* BNt8 did not rely on the high frequency and dosage of formulation applications. Based on the increase in plant height (Figure 2) and yields (Figure 3), F3D3 treatment tends to be better. So it can be recommended that the application once in every three weeks is more ideal for the application interval of *B. subtilis* BNt8 formulation. As stated by Istifadah et al (2014), for the development of microbial antagonist treatment for further use it is need to consider a variety of things including efficiency in propagation and the use of antagonist isolates.

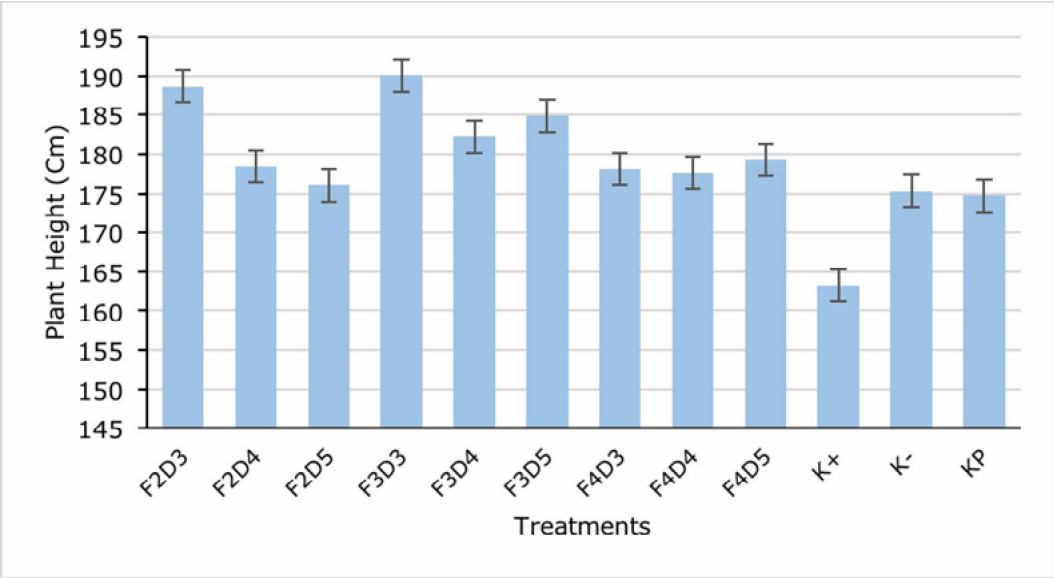

Figure 2. Effect of combination between frequency and dosage of *Bacillus subtilis* BNt8 formulation to plant height at 8 WAP.

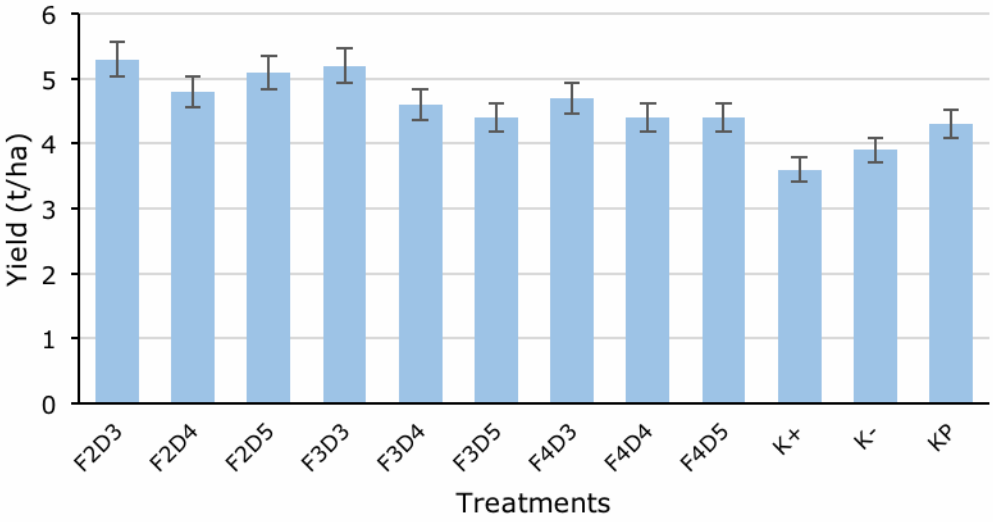

Figure 3. Effect of combination between frequency and dosage of *Bacillus subtilis* BNt8 formulation upon yield of *Z. mays*.

Applications of *B. subtilis* BNt8 formulation through seed treatment and spraying generally improve the productivity of *Z. mays* with a relatively small difference (Figure 3). However, treatment frequency of applications in every two weeks at a dose of 1 kg/ha (F2D3) tend to exhibit relatively better effect. Besides having antifungal effect, *B. subtilis* also produce compounds that can stimulate plant growth. Zongzheng et al (2009) reported that *B. subtilis* SY1 had a good effect in shortening the time of germination and growth of the embryo and radically increase crop yield (Zongzheng et al 2009).

Frequency and excessive concentration formulation applications, in addition to inefficient feared to give the opposite effect on plant growth and development of bacteria (Wartono et al 2014). Based on our observation, we found that the higher the concentration of formulation, the more is the bacterial populations, so that there is a competition between individuals of bacteria and increase growth regulators.

Conclusions. In the greenhouse test, the best treatment was a seed treatment formulation at a concentration of 3%. Spraying *Z. mays* plants with *B. subtilis* BNt8 formulations in the field can suppress the development of BLSB disease up to 18.5%. Applications of *B. subtilis* BNt8 formulation with a frequency of every two and three weeks at a dosage of application 1 kg/ha gave the best results in controlling BLSB disease on *Z. mays*.

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