The effectiveness of eight bacteria formulations of *Bacillus subtilis* (Ehrenberg) Cohn. on maydis leaf blight (*Bipolaris maydis* (Nisik. & Miyake) Shoemaker) in corn (*Zea mays* L.)

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Abstract. Utilization of biological control agents (BCA) against plant pathogens is an important component of integrated pest control management. *Bacillus subtilis* (Ehrenberg) Cohn as plant growth promoting rhizobacteria (PGPR) bacteria with one of its functions namely bioprotector has been shown to suppress the attack of several kinds of pathogens. This study aimed to test the effectiveness of some isolates of *B. subtilis* formulated in the form of flour to control maydis leaf blight (*Bipolaris maydis* (Nisik. & Miyake) Shoemaker) in corn (*Zea mays* L.). The experiment was conducted in the plant pathology laboratory with co-cultivation test and in the greenhouse of Indonesia Cereals Research Institute (ICERI) from February 2015 to December 2016. In the laboratory test, test for diffusible toxic metabolite(s) was done by agar co-cultivation test method. In the green house experiments, the treatments were arranged in a completely randomized design consists of 10 treatments; i.e. Seed treatments and spraying suspension of *B. subtilis* formulation BS-TLB1, BS-BJ6, BS-M3, BS-TM4, BS-BNt4, BS-BNt5, BS-BNt6, BS-BNt8 and two controls (K1 with the use of synthetic fungicides and K2 with steril destiled water). Data collected were percentage of inhibition of BCA(s), percentage of seed germination, plant height and disease incidence of maydis leaf blight. The results showed that formulation of *B. Subtilis* TM4 and BJ6 performed the best in co-cultivation test, application of *B. subtilis* formulations by means of seed treatment can stimulate plant growth and suppress maydis leaf blight incidence. Spraying plants with suspension of *B. subtilis* a formulation was more effective in suppressing maydis leaf blight incidence than the seed treatment method. Formulation of *B. subtilis* TM4 showed the best efficacy in promoting plant growth and its ability to suppress the maydis leaf blight incidence either through seed treatment and spraying the suspension.

Key Words: diseases management, bio control agent, fruit color, seed treatment, spray suspension.

Introduction. Corn (*Zea mays*) is one of the important agricultural commodities as a staple food after rice, so research and development continues to be done. In Indonesia, corn became a staple food every day, especially in some areas, including Nusa Tenggara, Madura, some of Maluku and Papua (Najiyati & Danarti 1999). Besides being a source of food ingredients, corn is used as raw material for food, feed and fuel industries. Therefore it is natural that corn demand over time is increasing along with population growth and development of the food industry and livestock.

The decline in crop productivity is constrained by biotic and abiotic factors. Biotic constraints usually with the emergence of several important diseases that attack either in vegetative and generative stages of the crops. One of the main disease in corn is maydis leaf blight (*Bipolaris maydis*). The disease attacks the plant from vegetative to the generative stages and can lead to yield losses up to 70%. In some corn production centers of Indonesia, maydis leaf blight is reported to have a high virulence that is capable of causing plants death (Pakki 2005).

To overcome the problem, appropriate control measures is needed, for example by using a cheap and environmentally friendly control methods such as antagonistic microbes. *Bacillus subtilis* is one of antagonistic microorganism used as a biocontrol agent against soil-born disease and air borne disease (Prihartiningsih et al 2014; Muis et al 2015a; Suriani & Muis 2016). The bacteria can produce antibiotic compounds such chitinase enzyme that can hydrolyze the cell walls of fungi, siderophores and other

antibiotics that can inhibit the development of pathogens (Wang & Chang 1997). *B. subtilis* volatile toxic compounds have been reported to suppress the mycelial growth of *Fusarium verticillioides* and *B. maydis* in vitro (Muis et al 2015a).

B. subtilis serves as plant growth promoting rhizobacteria (PGPR) that actively colonize plant roots by having three main roles for the plant: as biofertilizer, biostimulant and bioprotectant (Karlidag et al 2013). On that basis, some researchers are interested in exploring the *B. subtilis* to increase crop productivity. Results of research conducted by Wartono et al (2015) showed that apllication of spores formulation of *B. subtilis* could suppress leaf blight of rice by 21% and has the potential to increase yield up to 50%. Seeing the effectiveness of *B. subtilis* in the control of pathogens, the researchers formulated the biological agents either in liquid or solid formula for easy application in the field and extend the shelf life. Muis et al (2015b) found that the talc is the best inert carrier for formulation of *B. subtilis* because it has a very small particle size, easy to spread and in the water it form a condensed suspension and easily bind to the bacterial suspension.

Based on the experiences and results of some studies, the research was conducted in order to test the effectiveness of several *B. subtilis* isolates which have been formulated in the form of flour to control maydis leaf blight and their effect on the growth of corn plants.

Material and Method. The study was conducted at the plant pathology laboratory and greenhouse of the Indonesian Cereals Research Institute from February 2015 to December 2016.

Preparation of pure culture of B. maydis. Infected corn leaves were taken and brought to the lab to be identified. The sample was cut in 1-2 mm and then sterilized by soaking in sterile destilled water (SDW) for 3-5 minutes then transfer to a 70% alcohol solution for 1-3 minutes and finally washed again with SDW for 1-3 minutes. The samples were grown on potato dextrose agar (PDA) medium and incubated for 2-3 days. After three days, the mycelia were taken and transferred to other PDA medium for purification and incubated for 7-10 days as stock for further use.

Test for diffusible toxic metabolite(s). This agar co-cultivation test was conducted as follows: five millimeter agar mycelial disc of *B. maydis* was seeded at the center of a 9-cm PDPA plate. Each *B. subtilis* formulation which has been dissolved at a concentration of 0.001 g/mL streaked lengthwise on PDA media that already contains agar mycelial disc of *B. maydis.* The plates were sealed with parafilm and incubated at room temperature for five days. Radial growth of the fungus away from or towards to bacterial antagonists was measured. Treatments arranged in a completely randomized design (CRD) with three replications.

The percentage inhibition of BCA was calculated using the following formula (Nielsen et al 1998):

$$P = \frac{\mathrm{r1} - \mathrm{r2}}{\mathrm{r1}} X\ 100\%$$

Where: P - percentage inhibition of BCA (%);
r1 - radial growth of the fungus towards to the edge of the plates (cm);
r2 - radial growth of the fungus towards to bacterial antagonists (cm).

The effectiveness of B. subtilis formulation against B. maydis through seed treatment. The experiment was conducted on a 2 x 4m plots planted with corn seeds at a distance of 20 x 20 cm with one seed per hole (hill). Usual caring of the plants were done by applying furadan for insect pests control at planting time and N-P-K fertilizer at a rate of 150 N/ha, 150 P_2O_5/ha, and 100 K_2O/ha.

The treatments were arranged in RCBD, each treatment with 3 replicates, each replicate with 20 hills. The treatments were as follows:

P1 = seed treatment with *B. subtilis* TLB1 formulation

P2 = seed treatment with *B. subtilis* BJ6 formulation
P3 = seed treatment with *B. subtilis* TM3 formulation
P4 = seed treatment with *B. subtilis* TM4 formulation
P5 = seed treatment with *B. subtilis* BNt4 formulation
P6 = seed treatment with *B. subtilis* BNt5 formulation
P7 = seed treatment with *B. subtilis* BNt6 formulation
P8 = seed treatment with *B. subtilis* BNt8
K1 = seed treatment with synthetic fungicide Dithane (control)
K2 = untreated control.

Inoculation with *B. maydis* spore suspension (10^6) was done at 2 WAP.

The effectiveness of B. subtilis formulation against B. maydis through spray suspension. This experiment was carried out using a completely randomized design (CRD). Corn seeds (Anoman) were planted in plastic pails. Each treatment was replicated in 3 plastic pails with 10 seeds per pail. The treatment consists of 8 *B. subtilis* formulations and two controls (fungicide Dithane and SDW). Bacterial suspension was prepared by taking three grams of each formulation diluted into 1 L SDW. The treatments tested were:

P1 = spray suspension of *B. subtilis* TLB1 formulation
P2 = spray suspension of *B. subtilis* BJ6 formulation
P3 = spray suspension of *B. subtilis* TM3 formulation
P4 = spray suspension of *B. subtilis* TM4 formulation
P5 = spray suspension of *B. subtilis* BNt4 formulation
P6 = spray suspension of *B. subtilis* BNt5 formulation
P7 = spray suspension of *B. subtilis* BNt6 formulation
P8 = spray suspension of *B. subtilis* BNt8
K1 = spray suspension of synthetic fungicide Dithane (control)
K2 = untreated control.

Thinning was done one week after planting (WAP), leaving 3 plants per pale. Inoculation with *B. maydis* spore suspension (10^6) was done at 2 WAP.

Data collection. Data collected were: seed germination at 1 WAP, plant height at 2 WAP, and disease incidence at 4, 5, and 6 WAP using following 1-5 scale of Sharma (1983):

Table 1

Scoring system of disease caused by *Bipolaris maydis* (Sharma 1983)

Scale	*Description*
1	No disease symptom
2	1-2 lesion spread on lower leaves
3	Number of lesion moderate on lower leaves
4	Number of lesion many on lower leaves; some lesion on middle leaves
5	Number of lesion many on lower and middle leaves, spread up to upper leave

The scores reading were transformed to percent disease severity by using formula:

$$I = \frac{\sum (ni \times vi)}{Z \times N} \times 100\%$$

Where: I - disease severity (%);
ni - number of sample in each category;
vi - 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. Statistical analysis was conducted with the software package SAS Ver. 6.12 (SAS Institute, Cary, NC). In graphs and figure, the original data and their standard errors are presented.

Results and Discussion

Test for diffusible toxic metabolite(s). This test was done to the ability of each *B. subtilis* formulation in inhibiting the mycelial growth of *B. maydis.* The test results showed that all the formulations showed inhibition of *B. maydis* (Table 2).

Table 2

Percentage of inhibition of *Bacillus subtilis* formulations against mycelial growth of *Bipolaris maydis* after incubation for five days

Treatments	*Percentage of inhibition*
Formulation of *B. subtilis* TLB1 vs *B. maydis*	19.89^{bc}
Formulation of *B. subtilis* BJ6 vs *B. maydis*	31.79^{ab}
Formulation of *B. subtilis* TM3 vs *B. maydis*	24.67^{abc}
Formulation of *B. subtilis* TM4 vs *B. maydis*	34.33^{a}
Formulation of *B. subtilis* BNt4 vs *B. maydis*	31.83^{ab}
Formulation of *B. subtilis* BNt5 vs *B. maydis*	23.31^{abc}
Formulation of *B. subtilis* BNt6 vs *B. maydis*	26.74^{abc}
Formulation of *B. subtilis* BNt8 vs *B. maydis*	13.45^{c}
B. maydis alone (control)	0.00^{d}

Results of statistical analysis showed that all formulations of *B. subtilis* tested had different inhibitory effect on *B. maydis*. Formulation of *B. subtilis* TM4 showed the highest percentage of inhibition with 34.33%, the percentage of inhibition by this formulation showed significant differences with the formulation of *B. subtilis* TLB1 and BNt8 which have lower inhibitory effect at 19.89% and 13.45% respectively. Differences in percentage inhibition of each *B. subtilis* formulation were due to differences in the types and proportions of secondary metabolic content. Killani et al (2011) reported that *B. subtilis* produces at least five different antimicrobial compounds, which are subtilin, bacitracin, bacilin, subtenolin, and bacilonycin. Further stated by Chen et al (2013) that the microbial isolates originating from different regions show different properties, especially the colony morphology, formation of biofilms, biocontrol activity, competence, and the production of pigment secreted. Moreover, the process of production of antibiotics a species of bacteria is influenced by several factors, including the nutrient content of nitrogen and carbon as well as environmental factors including temperature and pH (Islam et al 2012).

Visually, there is a difference between *B. maydis* mycelium growing close to *B. subtilis* with mycelium opposite to the antagonist (Figure 1). Mycelium grows toward antagonist more thickened than other side. This is presumably because their secondary metabolic produced by *B. subtilis* which suppress the growth of pathogens. Metabolic compounds produced by biological agents are capable of damaging the cell wall causing

radial growth of fungal pathogens to be slow and not developed (Radji 2005). Khaeruni et al (2013) reported that *Rhizoctonia solani* mycelial growth adjacent to *B. subtilis* is thicker and shorter due to their antifungal activity chitinolytic enzymes that degrade the cell walls of fungi so that growth is not optimal. In addition to producing the enzyme, these antagonistic microbes also produce other enzymes that play a role in breaking down the cell wall like *B. subtilis* ST21e that capable of producing the protease and chitinase enzymes (Khaeruni et al 2010).

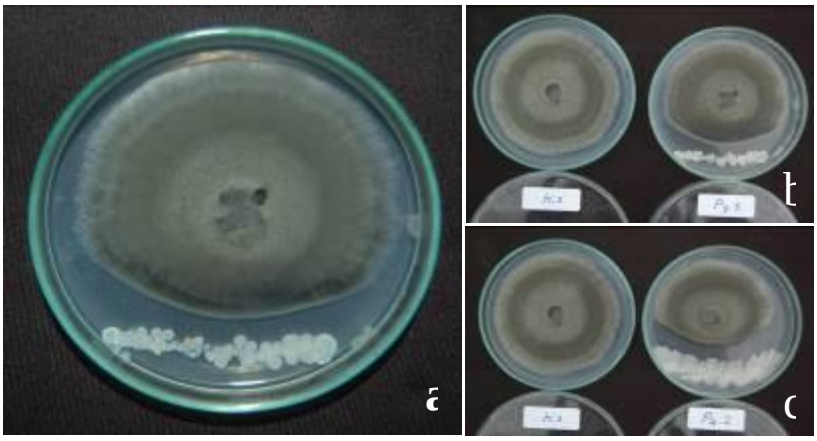

Figure 1. Appearance of inhibition of bacterial antagonist *Bacillus subtilis* against radial growth of *Bipolaris maydis* in vitro (a); inhibition of *Bacillus subtilis* TM4 against radial growth *B. maydis* (b) and inhibition of *B. subtilis* BJ6 against radial growth *Bipolaris maydis* (c) (original).

The effectiveness of B. subtilis formulation against B. maydis through seed treatment. Result of observations on seed germination showed that the average seed germination vary between treatments with a range of 89-100% (Figure 2). Figure 2 shows that the lowest seed germination is shown by formulation of *B. subtilis TLB1*, while the highest (100%) is shown by the treatment formulation of *B. subtilis* TM3, Bnt4, BNt6, BNt8, and Dithane fungicide.

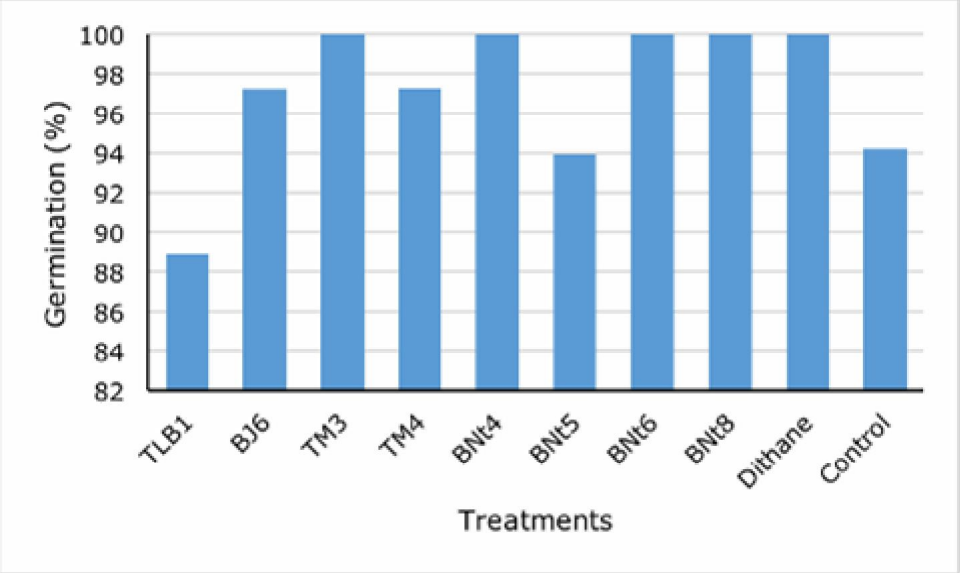

Figure 2. Average of percentage of seed germination on 8 formulations of *Bacillus subtilis*, Dithane fungicide, and untreated control.

Seed treatment with formulation of *B. subtilis* gave a positive and significant influence on the growth of corn (Figure 3). There were 5 formulations having plant height significantly different from untreated control. But among them, BS-TM4 formulation showed the highest ratio of plant height compare to control treatment. The average of plant height on treatment *B. subtilis* TM4 formulation at 2 WAP was 43.19 cm. Another *B. subtilis* formulation showed significant difference with control treatment was *B. subtilis* BNt8. This condition is suspected as *B. subtilis* contained in the formulation produce a plant growth regulator that is able to induce the growth of plants.

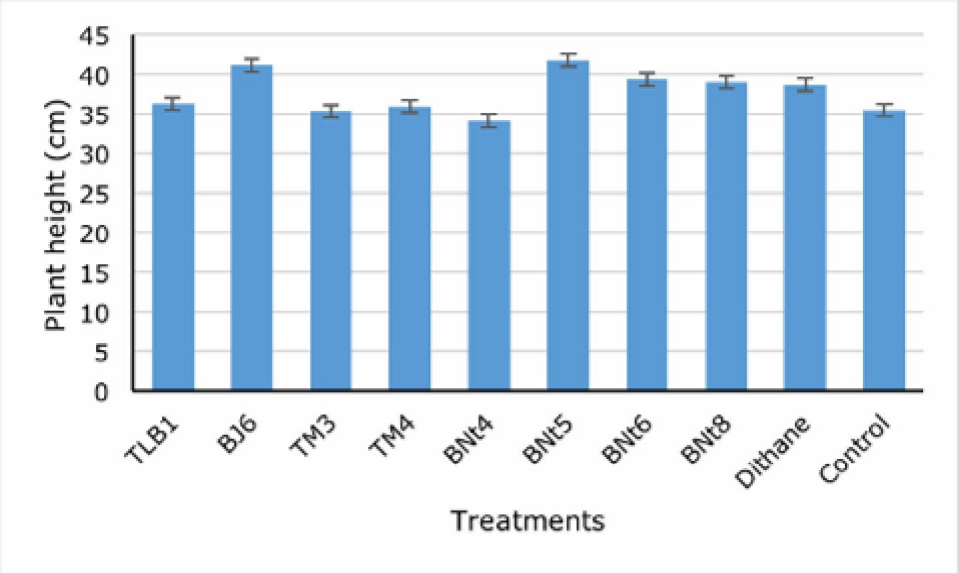

Figure 3. The effect of seed treatment with *Bacillus subtilis* formulations on plant height at 2 WAP in the greenhouse.

Plant growth is influenced by growth regulator substances that regulate physiological crops such as enlargement of cells, tissue differentiation, in response to light and gravity. The growth regulator substances are produced by the plant itself and there are also produced by soil bacteria such as *B. subtilis* (Swain et al 2006; Teale et al 2006). These bacteria colonize plant roots and help the absorption of plant nutrients. Another effect of the application of *B. subtilis* is the development of crops through increased concentrations of chlorophyll and carotene which is an important element in photosynthesis. Results of research conducted by Zongzheng et al (2009) showed that the application of *B. subtilis* SY1 able to increase the concentration of chlorophyll and plant carotene as much as 34% and 22% respectively.

Application of several *B. subtilis* formulations either through seed treatment or by spraying into the *B. maydis*-infected plants showed that the formulations were able to inhibit the development of maydis leaf blight disease significantly (Table 3 & 4). Disease incidence of maydis leaf blight at 4 WAP on treatment *B. subtilis* TM4 formulation showed that the disease incidence was not significantly different with the synthetic fungicide treatment. Likewise, at the next observation at 5 and 6 DAP, but the disease incidence on treatment synthetic fungicide was still lower than that on formulation *B. subtilis* treatments (Table 3).

Table 3

The average of disease incidence of maydis leaf blight in seed treatment with several *Bacillus subtilis* formulations at 4, 5, and 6 WAP in greenhouse of ICERI

Treatments	*Disease incidence of maydis leaf blight at*			*Inhibition (%)*
	4 WAP	*5 WAP*	*6 WAP*	*6 WAP*
Seed treatment with BS TLB1 formulation	31.85	46.67^{bcd}	65.92^{ab}	10.11
Seed treatment with BS BJ6 formulation	28.89	52.59^{abc}	54.07^{bcd}	26.27
Seed treatment with BS TM3 formulation	25.93	46.67^{bcd}	52.59^{bcd}	28.28
Seed treatment with BS TM4 formulation	17.04	43.70^{cd}	49.63^{cd}	32.32
Seed treatment with BS BNt4 formulation	30.37	57.04^{abc}	62.96^{abc}	14.14
Seed treatment with BS BNt5 formulation	33.33	62.96^{ab}	67.41^{ab}	8.07
Seed treatment with BS BNt6 formulation	36.30	68.89^{a}	57.04^{bc}	22.21
Seed treatment with BS BNt8 formulation	31.85	55.56^{abc}	64.44^{abc}	12.12
Seed treatment with synthetic fungicide	15.56	30.37^{d}	39.26^{d}	46.46
Untreated control	39.26	51.11^{abc}	73.33^{a}	0.00

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

The effectiveness of B. subtilis formulation against B. maydis through spray suspension. The incidence of maydis leaf blight on the treatment of applications by spraying on infected plants at 6 WAP showed that the use of formulation of *B. subtilis* TM4 and BNt4 have lower disease incidence than the use of synthetic fungicides which was 38.27% (Table 4). This shows that in the control of maydis leaf blight, spraying formulations of *B. subtilis* is more effective than the seed treatment applications. With the application of *B. subtilis* formulation by spraying, there is direct contact between the BCA and *B. maydis* growing on the leaves. Thus, the BCA compete directly with pathogens both in terms of space and nutrients.

Table 4

The average of disease incidence of maydis leaf blight in spraying application of with several *Bacillus subtilis* formulations at 4, 5, and 6 WAP in greenhouse of ICERI

Treatments	*Disease incidence of maydis leaf blight at*			*Inhibition (%)*
	4 WAP	*5 WAP*	*6 WAP*	*6 WAP*
Spraying with BS TLB1 formulation	35.80^{abc}	43.21	65.43a	0.00
Spraying with BS BJ6 formulation	40.74^{a}	43.21	55.56^{ab}	15.09
Spraying with BS TM3 formulation	25.93^{cd}	30.86	50.62^{abc}	22.64
Spraying with BS TM4 formulation	25.92^{cd}	35.80	38.27^{c}	41.51
Spraying with BS BNt4 formulation	28.39^{bcd}	33.33	38.27^{c}	41.51
Spraying with BS BNt5 formulation	40.74^{a}	43.21	53.09^{abc}	18.86
Spraying with BS BNt6 formulation	18.51^{de}	38.27	48.15^{bc}	26.41
Seed treatment with BS BNt8 formulation	35.80a^{bc}	38.27	45.68^{bc}	30.19
Spraying with synthetic fungicide	11.11^{e}	33.33	45.68^{bc}	30.19
Unsprayed control	35.80^{abc}	43.21	65.43^{a}	0.00

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Previous research conducted by Nurhayati & Agustin (2012) to test the effectiveness of *Trichoderma virens* to control downy mildew disease in caisin through several methods of application showed that spraying *T. virens* directly onto the leaves gives the lowest percentage of disease severity of 13.88% compared with the application by watering around the roots and root immersion with the percentage of disease severity was 18.90% and 55.72% respectively.

Overall, the observation of the disease incidence at 4 WAP showed that the application of the *B. subtilis* formulation by spraying showed higher disease incidence compared with seed treatment, however, with increasing time, the condition is reversed. Factors affecting this condition are that because the formulation of *B. subtilis* was sprayed 3 WAP where the symptoms of maydis leaf blight has appeared. Zamzani et al (2014) suggested that BCA takes time to adapt and evolve achieve optimum population to colonize the plant. Unlike the case with the application by seed treatment, BCA can induce plant resistance to spur the formation of compounds in plants that are anti pathogens.

Effectiveness of eight *B. subtilis* formulations in suppressing maydis leaf blight disease through the application as seed treatment or spraying the infected plants showed that *B. subtilis* TM4 formulation provide the largest percentage of inhibition, 32.32% and 41.51% respectively. Inhibition of the fungal pathogen by *B. subtilis* TM4 formulation in the greenhouse in line with ability of *B. subtilis* TM4 formulation inhibit the development of *B. maydis* resulted in in vitro testing (Table 1). This is presumably because the formulation of *B. subtilis* TM4 produces greater antibiotic compounds that suppress the growth of *B. maydis*. Zhao et al (2014) reported that *B. subtilis* SG6 application significantly reduced the incidence of diseases caused by *Fusarium graminearum* by producing compounds such as chitinase, fengycin and surfactins that can destroy the cell structure of the pathogen. Chitinase compound is known to break down the cell walls of pathogens. When the pathogen cell wall is damaged, then the organelles and cytoplasm in the cells will disperse and cause cell death.

The rate of disease progression can be reduced by reducing the level of the initial inoculum of pathogens through enhancement of chemical compounds that able to prevent the growth and development of the pathogen. One of them is through the mechanism of induced resistance. Induced resistance mechanisms pre inoculating the plants with various physical agents, chemical and biological reactions which can cause changes inoculation of diseases caused by pathogens (Misaghi 1982). Efforts in induced resistance mechanism against maydis leaf blight on corn can be done through seed treatment with formulation of *B. subtilis* TM4. This treatment is an initial strategy as evident in this way we can increase the percentage of seed germination and plant height and can suppress the incidence of maydis leaf blight in the early planting. The ability of *B. subtilis* in suppressing the development of pathogens through seed treatment because *B. subtilis* can survive, associated, and continues to grow on plant roots and is able to compete and suppress pathogens (EPA 2003; Wartono et al 2015).

Conclusions. Formulation of *B. subtilis* TM4 and BJ6 performed the best in co-cultivation test. Application of *B. subtilis* formulations by means of seed treatment can stimulate plant growth and suppress maydis leaf blight incidence. Spraying plants with suspension of *B. subtilis* a formulation was more effective in suppressing maydis leaf blight incidence than the seed treatment method. Formulation of *B. subtilis* TM4 showed the best efficacy in promoting plant growth and its ability to suppress the maydis leaf blight incidence either through seed treatment and spraying the suspension.

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