Utilization of antagonistic bacteria *Bacillus subtilis* to control *Fusarium verticilloides* on corn

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**Abstract.** Among a number of important diseases in maize plants, the disease caused by fungi *Fusarium verticilloides* needs attention because this fungus can cause decay on corn stalks and cobs causing economic losses. The aim of this study was to test several levels of the dosage formulations of *B. subtilis* BNt4 and TM3 to suppress the incidence of *F. verticilloides* in corn plants. *F. verticilloides* isolates were propagated by toothpick contamination method. The experiment was conducted in Bajeng Experimental Farm in 2018. The treatment was arranged in Factorial Randomized Block Design (RBD) with 3 replications. The first factor was the formulation of *B. subtilis* consisting of 2 strains namely *B. subtilis* BNt4 (F1) formulation and *B. subtilis* TM3 (F2) formulation. The second factor was the dosage application for antagonistic bacterial formulations. The results of the study showed that the application of the *B. subtilis* formulation, in general, is able to suppress the incidence of *F. verticilloides* on corn plants both on the stem and on the seeds. The application of *B. subtilis* TM3 formulation at a dose of 2.5 kg/ha gave the highest inhibition to the severity of Fusarium stem rot at 53.49%. The effect is implicated to the quality of seeds by showing the lowest percentage of ear rot which is 3.81%. This disease control technique is able to reduce the yield loss and the decreased quality of the maize grain due to *F. verticilloides* infection.

1. **Introduction**

*Fusarium verticilloides* (teleomorph *Gibberella moniliformis*) is one of the important disease pathogens in corn plants that can be transmitted through seeds and soil [1]; [2]. This pathogen causes decay on corn stalks and cobs causing economic losses at the farmer level [3]. [4] reported that in 14 corn-producing districts in eastern Indonesia, *F. verticilloides* was the cause of low seed quality and corn selling value. This fungus contamination in seeds affects the quality and determines the selling value of corn at the farmer level. The higher the contamination of *F. verticilloides*, the lower the selling value of corn farmers.[5] further reported that *F. verticilloides* infection in corn plant caused yield loss of up to 1.8 tons/ha.

Another thing to be aware for infection with *F. verticilloides* is the production of fumonisin mycotoxins which accumulate in seeds [6]; [7]. Fumonisin is one of the five mycotoxins that have received worldwide attention because of its impact on human and animal health [8]. Based on this, *F. verticilloides* needs to be controlled earlier because the infection of the pathogen in the stem can extend to the cob and infect the seeds. Systemic infections of seeds are pathogenic to corn, so that the infection cycle occurs from seed to stem and then cob and return to the seeds [9].
The control technique for *F. verticilloides* that has been carried out is crop rotation and the use of synthetic fungicides. Crop rotation is sometimes ineffective because these pathogens can survive in the soil even though there is no host [10]. While control with synthetic fungicides can pose a hazardous to human and livestock. Therefore biological control is needed by utilizing biological agents that have high virulence for this pathogen. [11] reported that soil microbes from a group of bacteria that have the potential to suppress soil-borne pathogens, including the Fusarium genus, which both attack corn and other plants, iare *Pseudomonas flourescens* and *Bacillus subtilis*. The effectiveness of *B. subtilis* in suppressing the intensity of plant pathogen incidence has been tested by several researchers including their ability to suppress the incidence of *Rhizoctonia solani* causing banded leaf and leaf blight in corn plants, *Colletotrichum panacicola*, and *Pseudomonas syringae* [12]; [13]; [14]. This bacterium can be applied to seeds to prevent infection with soil-borne pathogens. Test results that have been carried out in greenhouses show the formulation of *B. subtilis* BNt4 and TM3 has the highest inhibition of the attack of Fusarium stem rot in corn plants.

Based on the abovementioned, this study was conducted to test several levels of the dosage formulations of *B. subtilis* BNt4 and TM3 to suppress the incidence of *F. verticilloides* in corn plants.

2. Materials and Methods
The study was conducted at the Bajeng Experimental Farm in Gowa Regency from April to August 2018 and the Plant Pathology Laboratory of Indonesian Cereals Research Institute in August to September 2018. The research material used was *F. verticilloides* isolates which had been propagated by toothpick contamination method, 2 antagonistic bacterial formulations namely *B. subtilis* BNt4 and TM3 formulation, Anoman variety. Propagation of *F. verticilloides* begins with inserting a toothpick into the 800 ml E-flask which has been filled with 300 ml of sterile distilled water (SDW) and then covered. Then autoclaved at 1160C for 15 minutes. After cooling, the toothpick was rinsed with SDW and autoclaved again. This stage was repeated 5 times. After being autoclaved 5 times, the toothpick was rinsed with SDW and then put into the 400 ml E-flask which was filled with 100 ml of Potato Dextrose Broth (PDB). Then autoclaved at 1160C for 15 minutes. Toothpicks stored in PDB media for 3 days. Then twenty toothpicks were placed on DifcoTM Potato Dextrose Agar (PDA) media in petri dishes then inoculated with 4 ml of *F. verticilliodies* suspension. Incubated for 2 weeks under a 12 hour light cycle and 12 dark hours at room temperature [15]. While the formulation of *B. subtilis* used was taken from Plant Pathology Laboratory of ICERI in the form of flour with talc as a carrier and enriched with other additives such as CMC (Carboxymethyl cellulose) and yeast extract [16].

2.1. Effectiveness test of *B. subtilis* Formulation on the pathogenicity of *F. verticilliodies* in corn plants
The treatment was arranged in Factorial Randomized Block Design (RBD) with 3 replications. The first factor was the formulation of *B. subtilis* consisting of 2 strains namely *B. subtilis* BNt4 (F1) formulation and *B. subtilis* TM3 (F2) formulation. The second factor was the dosage application for antagonistic bacterial formulations with 4 levels, namely a dose of 1 kg/ha (D1), a dose of 1.5 kg/ha (D2), a dose of 2 kg/ha (D3), a dose of 2.5 kg/ha (D4). From these two factors 8 combinations of treatments and 3 controls were obtained, namely F1D1 (Application of formulation *B. subtilis* BNt4 at a dose of 1 kg/ha); F1D2 (Application of formulation *B. subtilis* BNt4 at a dose of 1.5 kg/ha); F1D3 (Application of formulation *B. subtilis* BNt4 at a dose of 2 kg/ha); F1D4 (Application of formulation *B. subtilis* BNt4 at a dose of 2.5 kg/ha); F2D1 (Application of *B. subtilis* TM3 formulation at a dose of 1 kg/ha); F2D2 (Application of *B. subtilis* TM3 formulation at a dose of 1.5 kg/ha); F2D3 (Application of *B. subtilis* TM3 formulation at a dose of 2 kg/ha); F2D4 (Application of *B. subtilis* TM3 formulation at a dose of 2.5 kg/ha); Control Fungicides with active ingredients difenconazole; Positive control (Inoculation of *F. verticilliodies* without pesticides); Negative control (without inoculation of *F. verticilliodies*).

The seeds were planted in each treatment with 4 rows of 5 m long, planting space of 75 x 20 cm. Each hole was planted two seeds and given Carbofuran 3G to prevent ants or leaf eaters. Thinning
plants was carried out at 7 weeks after leaving one plant perforated. The first fertilization was applied at 10 days after planting (DAP) with Urea and Phonska fertilizer 150 kg/ha and 200 kg/ha respectively. Furthermore, the second fertilization was carried out at the age of 30 HST using Urea fertilizer with a dose of 150 kg/ha.

*F. verticilloides* inoculation was carried out at 49 DAP by making a hole of 0.25 cm on the second stem segment and then inserting a toothpick overgrown with *F. verticilloides*. Meanwhile, the formulation treatment of *B. subtilis* was applied in two stages, namely coating the seeds with formulation for 24 hours before planting and spraying a day after inoculation of *F. verticilloides* and at 70 DAP. The parameters observed were the severity of Fusarium stem rot, Fusarium cob rot, the percentage of seeds infected with *F. verticilloides* and production.

### 2.2. The severity of Fusarium stalk rot disease

Observation on the severity of Fusarium stem rot was carried out when plants aged 70, 85 and 100 DAP by giving a score of 5 randomly selected plant samples at each observation. Samples of the plant were cleaved and scoring the level of decay symptoms that appear. The score of the score is then transformed into a severity level as follows:

\[
KP = \frac{\sum (n_i \times v_i)}{Z \times N} \times 100\%
\]

- KP = Severity of disease
- \( n_i \) = Scoring value of each stem sample
- \( v_i \) = Number of infected stems in the scoring
- \( N \) = Number of bars observed
- \( Z \) = The highest score

The scoring symptom of Fusarium stem rot attacks on corn plants is based on the [17], which is a score of 1 = a healthy color change or slightly inoculated segments; 2 = up to 50% of the inoculated segments change color; 3 = 51-75% of the inoculated segments change color; 4 = 76-100% of the inoculated segments change color; 5 = Less than 50% change in color from adjacent segments; 6 = more than 50% change in color of adjacent segments; 7 = discoloration of three segments; 8 = discoloration of four segments; 9 = discoloration of five or more segments and plants die early.

### 2.3. Percentage of Cob Infected by *F. verticilliodies*

This observation was carried out at harvest time by taking 10 cob samples per treatment unit. Percentage of *F. verticilliodies* incidence was calculated based on the scoring of cob damage then accumulated into the following formula:

\[
I = \frac{\sum (n_i \times v_i)}{Z \times N} \times 100\%
\]

- \( v \) = Number of infected cobs in the scoring
- \( N \) = Number of cobs observed
- \( Z \) = The highest score

The score for cob damage was assessed based on the modification of [18] namely scoring 1 = 1-3% of corn kernels infected with disease; 2 = 4-10% of corn kernels infected with disease; 3 = 11-25% of corn kernels infected with disease; 4 = 26 - 50% of corn kernels infected with disease; 5 = 51 - 75% of corn kernels infected with disease; 6 => 75% of corn kernels infected with disease.
2.4. Percentage of Seeds Infected with *F. verticilloides*

This stage was carried out at the Plant Pathology Laboratory of ICERI. A total of 100 seeds from each treatment unit were grown using a Plastic Rolled Paper Test method (PRPT) and stored for 9 days. The percentage of infected seeds is calculated based on the following formula:

\[
I = \frac{A}{B} \times 100\%
\]

- \( I \) = Percentage of Seeds infected with *F. verticilloides*
- \( A \) = Amount of Seeds infected with *F. verticilloides*
- \( B \) = Number of seeds observed

2.5. Yield

At harvest time, observations were made on harvest weight, the number of cobs and water content, then converted to yield/ha. Data were analyzed using variance. To see the comparison between treatment and control, it was continued with the LSD test.

3. Results and Discussion

3.1. Severity of Fusarium Stalk Rot

*F. verticilloides* cause rot on corn stalks and cobs. The initial symptoms of stem rot found in the field was the change in the color of the stem to brown around the area inoculated with *F. verticilloides*. If the stem is cleaved, it will show a decay of pink to brownish tissue (Figure 1).

![Figure 1](image)

*Figure 1.* Non-spreading inoculated scars of *F. verticilloides* on stems (a); Symptoms of stem root around the inoculation area of *F. verticilloides* (b); Discoloration of stem tissue from pink to brownish (c).

The result of the experiment showed that the plants that were applied with *B. subtilis* formulation had lower severity of Fusarium stem rot than the control plants inoculated with *F. verticilloides* without pesticides. Control plants showed symptoms of disease incidence quite high even some plants die. The type of *B. subtilis* formulation was statistically significant in suppressing stem rot disease at 100 DAP (Table 1).
Table 1. Effect of application of the 2 B. subtilis formulation on the severity of Fusarium stem rot in corn plants at 100 DAP in Bajeng Experimental Farm, 2018.

| Bacterial Formulation | Percentage of Stem Rot (%) |
|------------------------|-----------------------------|
| *Bacillus subtilis* BNt4 | 48.27 a                     |
| *Bacillus subtilis* TM3 | 36.67 b                     |
| LSD 5%                 | 9.02                        |

The dose level factor of each formulation used for the severity of Fusarium stem rot did not show statistically significant differences. However, the application of *B. subtilis* TM3 formulation at doses of 2 kg/ha and 2.5 kg/ha showed the percentage of stem rot was significantly different from control plants (without pesticides) at 70, 85, and 100 DAP except for the application of a dose of 2 kg/ha at 70 DAP (Table 2).

Table 2. Effect of application of *B. subtilis* BNT4 and TM3 formulation at 4 dose levels on the severity of Fusarium stem rot in corn plants at Bajeng Experimental Farm, 2018.

| Treatments                              | 70 DAP | 85 DAP | 100 DAP |
|-----------------------------------------|--------|--------|---------|
| *B. subtilis* BNt4 with the dosage of 1 kg/ha | 22.96 a | 31.85 ab | 49.38 b  |
| *B. subtilis* BNt4 with the dosage of 1.5 kg/ha | 21.85 a | 30.37 ab | 48.89 b  |
| *B. subtilis* BNt4 with the dosage of 2 kg/ha | 22.22 a | 30.37 ab | 48.89 b  |
| *B. subtilis* BNt4 with the dosage of 2.5 kg/ha | 21.48 ab | 27.41 ab | 45.93 bc |
| *B. subtilis* TM3 with the dosage of 1 kg/ha | 21.48 ab | 34.81 ab | 45.93 bc |
| *B. subtilis* TM3 with the dosage of 1.5 kg/ha | 21.11 a | 31.11 ab | 38.52 cd |
| *B. subtilis* TM3 with the dosage of 2 kg/ha | 21.48 ab | 25.19 b  | 32.59 d  |
| *B. subtilis* TM3 with the dosage of 2.5 kg/ha | 19.63 b  | 22.96 b  | 29.63 d  |
| Fungicide with active ingredient difenokoanazol | 21.85 a | 24.07 b  | 29.63 d  |
| Inoculation of *F. verticilloides* without pesticide | 22.96 a | 40.00 a  | 63.70 a  |
| Without inoculation of *F. verticilloides* | 0.00 b  | 0.00 c   | 0.00 e   |
| Nilai LSD 5%                           | 1.94   | 14.17   | 9.50     |

Note: The lane number followed by the same letter is not significantly different based on the LSD test at the 5% level.

The results of this study indicated that the formulation of *B. subtilis* TM3 has the ability to suppress the incidence of Fusarium stem rot higher than the formulation *B. subtilis* BNt4. The formulation of *B. subtilis* TM3 at a dose of 2.5 kg/ha was able to reduce the incidence of stem rot disease by 53.49%. This is in line with the previous study that has been carried out in vivo, the TM3 formulation has the ability to suppress stem rot disease attacks by 33.33% [19].

Unlike the effectiveness of the formulation of *B. subtilis* BNt4 which in the in vivo test it was able to suppress the incidence of Fusarium stem rot up to 38.27% [19]. The decrease in the effectiveness of the formulation can be caused by the low adaptability of microbes contained therein. This is in accordance with the results of the study by [20] which tested the effectiveness of *Trichoderma* spp. and *Glioglaedium* spp. in suppressing the incidence of BLSB, in greenhouse test showed the ability of *Trichoderma* spp. suppresses the disease by 70%, but decreases when testing in the field which ranges from 54-67%.
3.2. Percentage of Cob Infected by F. verticilliodies

*F. verticilloides* is known that in addition to causing stem rot in corn plants can also attack the cob and cause seed decay (Figure 2). In general, the percentage of attacks on ear rot disease found during the study was quite low, ranging from 1.91 to 9.52%. Control plants that were not inoculated with *F. verticilloides* were found in 1.91% of the symptoms of ear rot. This shows that *F. verticilloides* can be spread through the wind so that healthy plants close to the source of the inoculum can be infected. In addition to the wind, the conidia of the fungus are also spread by corn stalk pests, the higher the damage by pests on the cob the chance of this disease incidence is also high [21]; [22]. Conidia are attached to the body of stem borers so that infected parts of the plant will spread to other plants.

The percentage of cobs infected with *F. verticilloides* showed no significant difference in all application formulations, but the application of TM3 formulation at a dose of 2.5 kg/ha had the lowest percentage of ear rot disease incidence of 3.81% (Table 3).

**Table 3.** Effect of the application of *B. subtilis* BNT4 and TM3 formulations at 4 dose levels against the incidence of ear rot on corn plants at Bajeng Experimental Farm, 2018.

| Treatments                                      | Percentage of Ear Rot (%) |
|------------------------------------------------|----------------------------|
| *B. subtilis* BNT4 with the dosage of 1 kg/ha   | 5.24 bc                    |
| *B. subtilis* BNT4 with the dosage of 1.5 kg/ha | 6.67 ab                    |
| *B. subtilis* BNT4 with the dosage of 2 kg/ha   | 4.29 bc                    |
| *B. subtilis* BNT4 with the dosage of 2.5 kg/ha | 5.24 bc                    |
| *B. subtilis* TM3 with the dosage of 1 kg/ha    | 4.76 bc                    |
| *B. subtilis* TM3 with the dosage of 1.5 kg/ha  | 5.24 bc                    |
| *B. subtilis* TM3 with the dosage of 2 kg/ha    | 5.24 bc                    |
| *B. subtilis* TM3 with the dosage of 2.5 kg/ha  | 3.81 bc                    |
| Fungicide with active ingredient difenoconazole| 5.71 b                     |
| Inoculation of *F. verticilloides* without pesticide | 9.52 a                    |
| Without inoculation of *F. verticilloides*     | 1.91 c                     |

LSD 5% 3.39

Note: The lane number followed by the same letter is not significantly different based on the LSD test at the 5% level.

3.3. Percentage of Seeds Infected with F. verticilliodies

The percentage of infected seeds from 100 seeds tested varied between treatments for the application of *B. subtilis* formulations in the field (Table 4). Statistically, the percentage of infected seeds between the treatment of application of bacterial formulations did not show a significant difference, but the application of *B. subtilis* TM3 formulation at a dose of 1.5 kg/ha showed the lowest percentage of infected seeds which was 3.33% then followed by *B. subtilis* TM3 application with a dose 2.5 kg/ha which was 4.00%.
Table 4. The average percentage of seeds infected with *F. verticilloides* grown with the Paper Test Method Rolled using plastic from the seeds of each treatment application of *B. subtilis* formulation in the corn plant.

| Treatments                             | Percentage of infected seeds (%) | moisture content of harvested seeds(%) |
|----------------------------------------|----------------------------------|---------------------------------------|
| *B. subtilis* BNt4 with the dosage of 1 kg/ha | 6,33b                            | 29,73                                 |
| *B. subtilis* BNt4 with the dosage of 1.5 kg/ha | 8,00ab                           | 28,4                                  |
| *B. subtilis* BNt4 with the dosage of 2 kg/ha | 6,00b                            | 29,5                                  |
| *B. subtilis* BNt4 with the dosage of 2.5 kg/ha | 4,33b                            | 29,87                                 |
| *B. subtilis* TM3 with the dosage of 1 kg/ha | 7,00b                            | 32,33                                 |
| *B. subtilis* TM3 with the dosage of 1.5 kg/ha | 3,33b                            | 28                                    |
| *B. subtilis* TM3 with the dosage of 2 kg/ha | 5,00b                            | 30,9                                  |
| *B. subtilis* TM3 with the dosage of 2.5 kg/ha | 4,00b                            | 28,27                                 |
| Fungicide with active ingredient difenoconazole | 2,67b                            | 28,7                                  |
| Inoculation of *F. verticilloides* without pesticide | 13,00a                           | 30,93                                 |
| Without inoculation of *F. verticilloides*               | 4,33b                            | 28,8                                  |

Note: The lane number followed by the same letter is not significantly different based on the LSD test at the 5% level.

The application of *B. subtilis* TM3 formulation has an influence on *F. verticilloides*. This can be seen from the three observation aspects that have been done, namely the percentage of Fusarium stem rot attack, the percentage of cob and seeds infected with *F. verticilloides* showed that the *B. subtilis* TM3 formulation, especially the use of 2.5 kg/ha level has a high ability to inhibit the development of these pathogens.

![Figure 2](image.png) The difference between *f. verticilloides* infected seeds (a) with healthy seeds (b) after 9 days of growth on plastic rolled paper.

The percentage of *F. verticilloides* incidence on seeds in post-harvest was not only influenced by the control mechanism in the field but also the growth and development of the fungus in storage. This can be seen from the results of the research conducted, on the water content of 28% the percentage of infected seeds was about 3.33%, while in the moisture content of 30.93%, the percentage of infected seeds was quite high at 13%. According to [22], the higher the water content of stored corn seed, the greater the chance of the spread of fungi so that the corn becomes rotten.
3.4. Yield

The incidence of Fusarium stem rot on corn plants can reduce yield to 38.15% (Table 5). The incidence of stem rot which begins when the plant enters the generative phase results in the maximum flow of nutrients to the cob. In general, the application of *B. subtilis* formulation can reduce the decline in yield due to the incidence of Fusarium stem rot. This can be seen in the application of *B. subtilis* BNt4 formulation with the dose of 2 kg/ha and *B. subtilis* TM3 with the dose of 2.5 kg/ha showed almost the same yield as uninoculated control treatment. The use of *B. subtilis* antagonistic bacteria in addition to control diseases in the field can also prevent the spread of pathogens for subsequent crops. *F. verticillioides* is known to be transmitted through soil, so the application of *B. subtilis* in the area of plant rhizosphere is expected to be able to multiply and colonize the roots of new plants so that they can cause systemic resistance [7].

**Table 5.** The average of yield applied by *B. subtilis* BNT4 and TM3 formulations at 4 dose levels at Bajeng Experimental Farm, 2018.

| Treatments                                      | Yield (t/ha) |
|------------------------------------------------|--------------|
| *B. subtilis* BNt4 with the dosage of 1 kg/ha  | 7.11         |
| *B. subtilis* BNt4 with the dosage of 1.5 kg/ha| 6.72         |
| *B. subtilis* BNt4 with the dosage of 2 kg/ha  | 8.03         |
| *B. subtilis* BNt4 with the dosage of 2.5 kg/ha| 6.82         |
| *B. subtilis* TM3 with the dosage of 1 kg/ha  | 7.69         |
| *B. subtilis* TM3 with the dosage of 1.5 kg/ha| 6.85         |
| *B. subtilis* TM3 with the dosage of 2 kg/ha  | 7.73         |
| *B. subtilis* TM3 with the dosage of 2.5 kg/ha| 7.92         |
| Fungicide with active ingredient difenoconazole| 6.90         |
| Inoculation of *F. verticillioides* without pesticide | 4.96     |
| Without inoculation of *F. verticillioides*    | 8.02         |

4. Conclusion

Based on the results of the research, it can be concluded that the application of the *B. subtilis* formulation, in general, is able to suppress the incidence of *F. verticillioides* on corn plants both on the stem and on the seeds. The application of *B. subtilis* TM3 formulation at a dose of 2.5 kg/ha gave the highest inhibition to the severity of Fusarium stem rot at 53.49%.

The effect is implicated to the quality of seeds by showing the lowest percentage of ear rot which is 3.81%. The application of *B. subtilis* formulation in plants infected with Fusarium stem rot did not significantly affect yield, but the application of *B. subtilis* TM3 formulation showed the highest yield of 7.92 tons/ha. The next plan is to integrate the other control technique with *B. subtilis* formulation to achieve higher inhibition of disease attacks.

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