Effectiveness of *Bacillus subtilis* TM4 biopesticide formulation as biocontrol agent against maydis leaf blight disease on corn

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**Abstract.** Maydis Leaf Blight, caused by Bipolaris maydis, the disease on maize results not only in yield reduction but also deteriorate the value and quality of the grain. During this time the control is carried out by farmers using synthetic pesticides. But dependence on the use of synthetic pesticides has not been able to solve the problem of leaf blight disease, instead it often leads to new problems, such as the occurrence of environmental damaged. Needed an environmentally friendly alternative that is a biological control to control the pathogen. *Bacillus subtilis* is one of the many bacteria developed as biological agents to control plant pathogens. This study was aimed to examine the effectiveness of *B. subtilis* biopesticide formulation by mean of seed treatments and foliar sprays, using different concentrations and frequencies to control maydis leaf blight (MLB) disease on maize, caused by *Bipolaris maydis*, and to evaluate its function as a plant growth promoter. The experiments were conducted at the greenhouse and in the field using factorial design. At the greenhouse trial, seed treatment and foliar spray application at 3 weeks interval produced better result in controlling MLB, and better result on promoting maize plant growth. In the field experiment, using a concentration of 3 g/l showed a better effect on suppressing the disease and on increasing yield. Application of the formulation of *B. subtilis* TM4 biopesticide reduced MLB disease by 21%.

1. **Introduction**

Maydis blight disease in corn plants caused by *Bipolaris maydis* is one of the main diseases in corn. *B. maydis* is a fungus of ascomycetes, produces toxins that attack mitochondria and weaken the ability of plants to capture energy from metabolic processes. The severity of the disease varies from season to season, the symptoms spread from the lower leaf to the middle of the growing middle leaf and then head to the flag leaves to all parts of the plant. The infected tissue is covered with spotting and chlorosis so it is not productive [1], [2]. In addition to susceptible host resistance, pathogenic virulence and environmental conditions are beneficial; the amount of inoculum also plays an important role in determining the severity of the disease and the reduction of plant yield parameters [3].

Maydis leaf blight disease has now become one of the most common and severe corn diseases in Indonesia, Pakistan, India, Nepal, Cambodia, Philippines, Vietnam and China [1]. Maydis leaf blight disease is most serious in temperate and tropical hot and humid regions of the world, where temperatures ranging from 20-30°C during planting period and yield losses of almost 70% have been reported due to this disease. The symptoms and severity of the disease depend on the race of the
pathogen and the germplasm host. Some of the \(B. \text{maydis}\) races are pathogenic to maize. Three races (C, O, and T) have been identified in corn [4], [1], [5].

The use of synthetic pesticides has not been able to solve the problem of plant diseases, instead it can induce pathogens to be resistant to pesticides used [6]. Therefore, an alternative control method such as the application of biocontrol agents and environmental engineering to eco-friendly approaches in controlling diseases caused by various plant pathogens is needed [7].

Control of corn diseases focused on biological control by utilizing antagonistic microorganisms. One of the microorganisms that is antagonistic to pathogenic fungi is \(B. \text{subtilis}\). This bacteria have been successfully used for the protection of plants and their commercialized products in the world for the control of important plant diseases such as Avogreen®, Bio safe®, Biosubtilin, Serenade®, Rhizo Plus®, Rhapsody®, Companion®, Ecoshot, Kodiak®, Cease® [8]. \(B. \text{subtilis}\) shows antifungal ability in some plant pathogens by inhibiting the reproduction of the fungus. In addition to the effect of increasing plant growth, \(B. \text{subtilis}\) also plays an important role in improving stress tolerance in host plants [9].

\(B. \text{subtilis}\) TM4 is a bacterial isolate derived from corn planting rhizosphere. The bacteria have been tested in vitro capable of suppressing the development of \(B. \text{maydis}\), \(Rhizoctonia \text{solani}\), and \(Fusarium \text{moniliforme}\) [10]. The potential of \(B. \text{subtilis}\) TM4 in suppressing the pathogens then developed in further tests in the greenhouse and field. This study aimed to develop biopesticide formulation of \(B. \text{subtilis}\) TM4 by determining the proper concentration and frequency of application to control maydis leaf blight disease and its effect on the corn crop.

2. Materials and Methods

The study was conducted from April to August 2017 at the Greenhouse of Indonesian Cereals Research Institute (ICERI) in Maros, and Bajeng Experimental Farm in Gowa.

2.1. Green House Trial of \(B. \text{subtilis}\) TM4 Formulation

The formulation used was \(B. \text{subtilis}\) TM4. The experiment was arranged in a complete randomized factorial design with three replications. Factors tested consisted of two factors, namely seed treatment and application frequency (spraying). The first factor is B0 (without seed treatment) and B1 (seed treatment). The second factor is A0 (without spraying), A1 (application every one week), A2 (application every two weeks), and A3 (application every three weeks).

Testing was done in four steps. The first step was seed treatment. Each 100 g of Anoman varieties seed was mixed with 3 g of formulation. The formulation is given 2 hours before planting. Meanwhile for those without seed treatment was not given formulation. In the second phase, seeds were planted in a polybag of 30 cm in diameter containing 10 kg soil/polybag, three plants per polybag. The third step was plant inoculated with \(B. \text{maydis}\) pathogen suspension that was previously propagated in the PDA media. The inoculation was carried out by spraying the suspension (10⁶ spores/ml) on the surface of the plant at 2 weeks after planting (WAP). At 3 WAP, the plant observed its root length by taking two plants per polybag. Furthermore, one remaining plant is preserved for subsequent testing. Fertilization was performed on 15 days after inoculation with urea + NPK fertilizer (2:2 g/polybag). The fourth step was application (spraying) of the formulation by dissolving \(B. \text{subtilis}\) formulation in water (10⁸ cfu/ml), then sprayed on the surface of the plant evenly (15 ml/plant) in the afternoon and the morning at intervals once a week, once in two weeks, and once in three weeks (according to each treatment).

Further observations including plant height at 8 WAP, the severity of maydis leaf blight disease a week after the first formulation application, repeated at once a week interval, and dry weight of the plant at harvest. Observation of the disease was done by scoring the severity of the disease. The disease scores were then transformed to the severity formula (DS) calculated based on the Townsend and Heüberger equations [11]:

\[
\text{DS} = \frac{1/(2W + 1)}{\sum (W_i/2^n)}
\]

\(W_i\) is the frequency of spraying, and \(n\) is the number of spraying at each interval.
\[ DS = \frac{\sum (n_i \times v_i)}{Z \times N} \times 100\% \]

\( DS \) = disease severity
\( n_i \) = number of plant infected to i.
\( v_i \) = score with category infection to i.
\( Z \) = the highest score.
\( N \) = number of plant observed.

Score severity of maydis leaf blight disease on corn are based on [12] that is: 0 = no symptoms, 1 = very mild infection, one to two lesions spread on lower leaves, 2 = mild infection, number of lesions is on lower leaves, 3 = medium infections, large number of lesions in lower leaves, some in middle leaves, 4 = severe infections, large number of lesions in lower leaves, middle and extends to upper leaves, 5 = very severe infections, lesion is abundant in almost all leaves, the plants dry up to death.

Cumulative disease infection is calculated based on the area under the disease developmental curve (AUDD) with the equation \( AUDD = \left[ \frac{Y_i}{100 + Y_i + 1/100} \right] \frac{1}{2} [t_i + 1-t_i] \) [13]; \( Y_i \) = disease severity on the 1st observation; and \( t_i \) = time of i-th observation.

The data then analyzed its diversity with GLM (General Linear Model) procedure with 5% confidence interval. Data processed using STAR 2.0.1 for windows [13].

2.2. Field Trial of B. subtilis TM4 Formulation

The treatments were arranged in randomized completely block design in factorial with three replications. Factors tested consisted of two factors, namely: 1) application concentration (spraying) formulation to the plant and 2) corn varieties. The first factor was the concentration of application of biopesticide \( B. \) subtilis TM4 formulation to a plant consisting of four application concentrations: K0 (application concentration 0 g/l), K1 (application concentration 1 g/l), K2 (application concentration 2 g/l), and K3 (application concentration 3 g/l). The second factor was two corn varieties (Anoman and Bima 4).

Two hours before planting, seeds were treated by mixing 3 g of \( B. \) subtilis TM4 biopesticide formulation with 100 g of seeds. Furthermore, seeds were planted in the treatment plot with the size of 3.75 m x 5 m, one plant per hole with a planting distance of 75 cm x 25 cm. Fertilization was done at age 3 and 4 WAP using urea and NPK Phonska (300 kg/ha). Inoculation of plants with \( B. \) maydis spore suspension was done by spraying the suspension on plant surface at 4 WAP. Application of \( B. \) subtilis biopesticide at 15 days after inoculation of \( B. \) subtilis TM4 formulation was done by dissolving it 1 g/l, 2 g/l, and 3 g/l aquadest (in accordance with treatment), then sprayed onto the plant surface evenly. Control of weeds and other disease pests is done by spraying using pesticides in accordance with the recommended dosage.

Observation on plant height was done at 15 days after application of \( B. \) subtilis formulation and disease severity at 8, 10, and 12 WAP. Observation of the disease is done by scoring. The severity and cumulative severity rates were calculated based on the same equation as the greenhouse test. The data obtained then analyzed its diversity with GLM (General Linear Model) procedure and the average treatment was differentiated by Tukey test (\( P = 0.05 \)). Data processing uses the STAR 2.0.1 for windows program [14].

3. Results and Discussion

3.1. Green House Trial of Formula B. subtilis TM4 Formulation

Test results showed that seed treatment with \( B. \) subtilis TM4 formulation had a positive effect on plant growth (Table 1). In Table 1 it appears that the root length reaches 20 cm or longer than the plant without seed treatment which only reaches 15.5 cm. Similarly, the effect on plant height, it showed that plant height treated with bacterial formulation reached 178.8 cm, while those not treated only
163.7 cm. This condition is thought to be due to \textit{B. subtilis} TM4 capable to produce a growth regulator which inducing root growth. The results of this study are in line with those reported by [9], that \textit{B. subtilis} bacteria are capable of producing growth regulators.

\textbf{Table 1.} The effect of \textit{B. subtilis} TM4 formulation to root length and plant height of corn seedling Anoman variety in the greenhouse of ICERI.

| Treatments                                           | Root length at 21 DAP (cm) | Plant height at 56 DAP (cm) |
|------------------------------------------------------|----------------------------|-----------------------------|
| Seed treatment with \textit{B. subtilis} TM4 formulation. | 20.0                       | 178.8                       |
| Without seed treatment                               | 15.5                       | 163.7                       |

Seed treatment and spraying of \textit{B. subtilis} TM4 formulations on application frequency can increase plant height (Figure 1). Similar results have been reported by [15], that the treatment of seed and spraying of biological agents at several frequencies can increase plant height. [16] stated that \textit{B. subtilis} isolates as antagonistic bacteria can produce growth hormone that is siderophore, Indole Acetic Acid (IAA) and ammonia. In this study, combination of seed treatment with application frequency every 3 weeks (p 2x) is better than combination with spraying in every 1 week (p 5x) and 2 weeks (p 3x).

\textbf{Figure 1.} Interaction between seed treatment with the frequency of application of \textit{B. subtilis} TM4 formulation to plant height at 56 DAP. Greenhouse, Maros. tp = without seed treatment; p = with 0-5x seed treatment. Initial formulation = 108 cfu/ml, spray volume = 15 ml/plant.

In the application frequency treatment, spraying of formulations at every 1, 2, and 3 weeks was not significantly different in pressing maydis leaf blight, but the disease incidence was lower than that in control treatment (without application). The effect of the application of biopesticide formulation of \textit{B. subtilis} TM4 in suppressing maydis leaf blight disease is seen from the value of area under the disease development curve (AUDDC) (Table 2 and Figure 2). The disease pressure gets better when the value of AUDDC is smaller.
Table 2. Effect of application frequency of *B. subtilis* TM4 formulation on the development of maydis leaf blight disease on Anoman variety. Greenhouse, Maros.

| Treatments                  | the area under the disease development curve (AUDDC) |
|-----------------------------|------------------------------------------------------|
| Without application         | 7.5                                                  |
| Application in every 1 week  | 6.7                                                  |
| Application in every 2 week  | 6.8                                                  |
| Application in every 3 week  | 5.4                                                  |

The suppression of maydis leaf blight disease is influenced by the interaction between seed application and application frequency. The combination of seed treatment and frequency of application of *B. subtilis* TM4 formulation in every 1, 2, and 3 weeks interact positively in suppressing the development of maydis leaf blight disease. According to [17], *B. subtilis* bacteria isolated from rhizosphere is antagonistic to various phytopathogenic fungi.

![Figure 2](image_url) **Figure 2.** The interaction between seed treatment and the frequency of application of *B. subtilis* TM4 to the development of disease (AUDDC). Greenhouse, Maros. tp = no seed treatment; p = with seed treatment; application frequency in every 1-3 weeks.

Seed treatment is an early strategy that needs to be developed in the use of biopesticide formulations, as it proves able to suppress maydis leaf blight disease and trigger plant growth. [18] reported that *B. subtilis* applied as seed treatment has an antibiotic inhibitory as antibiosis mechanism, it also has an inhibitory mechanism through competition of nutrition or space competition so as to have the ability to inhibit the growth of pathogens. Application of *B. subtilis* TM4 formulation through seed treatment was supposed to induce corn plant resistance against maydis leaf blight disease. Application of *Bacillus* spp. can reduce disease severity by various pathogens by inducing plant resistance [19]. The effectiveness of *B. subtilis* RB14 is reported to decrease the incidence of damping off of tomato plants by up to 80% due to the antifungal content of lipopeptide A contained there in [20].

### 3.2. Field Trial of *B. subtilis* TM4 Formulation

The effect of the application of *B. subtilis* TM4 formulation in suppressing maydis leaf blight disease is seen from the value of AUDDC. The smaller the value of AUDDC, the higher the suppression of the disease. Separately, seed treatment had a better effect in suppressing maydis leaf blight with a lower AUDDC value than without seed treatment (Figure 3). In the spraying treatment of *B. subtilis* TM4
formulation at the dosage of 1 g/l, 2 g/l, and 3 g/l were able to suppress the development of maydis leaf blight disease. This is indicated by lower AUDDC values. The value of AUDDC in corn plants applied with bacterial formulation with the dosage of 1 g/l, 2 g/l, and 3 g/l was 17.7; 17.9; and 16.1 respectively or disease suppression of 7.9 - 21.5% compared with controls (without application) (Table 3). This indicate that spraying application of *B. subtilis* TM4 formulation has positive effect in controlling maydis leaf blight disease in both Anoman and Bima 4. The test results also showed that spraying formulations with doses of 3 g/l gave better results in controlling leaf blight disease.

**Table 3.** The effect of *B. subtilis* TM4 formulation to development of maydis leaf blight in the field.

| Treatments         | AUDDC | Percentage of disease suppression |
|--------------------|-------|-----------------------------------|
| Anoman (0 g/l)     | 21.3  | 0.0c                              |
| Bima-4 (0 g/l)     | 18.6  | 0.0c                              |
| Anoman (1 g/l)     | 19.8  | 8.0b                              |
| Bima-4 (1 g/l)     | 15.6  | 16.7a                             |
| Anoman (2 g/l)     | 20.1  | 7.9b                              |
| Bima-4 (2 g/l)     | 15.7  | 15.9a                             |
| Anoman (3 g/l)     | 16.9  | 15.1a                             |
| Bima-4 (3 g/l)     | 15.2  | 21.5a                             |

The values in the same column followed by the same letter are not significantly different based on the DMRT at 5% level.

0-3 g/l = concentration of formulation application

Application of *B. subtilis* TM4 formulation causes AUDDC value to be smaller than control (without application). [21] reported that *Bacillus subtilis* E1R-j strain not only inhibits germination of conidia and appressorial formation but also inhibits the development of haustoria and extension of mycelium. The results of a consortium of two antagonistic agents namely *B. subtilis* and *Pseudomonas aeruginosa* KUC1d showed a significantly lower incidence of disease up to 45 days after pathogen inoculation [22].

![Figure 3](image-url)  
**Figure 3.** Effect of application concentration of *B. subtilis* TM4 formulation on development (AUDDC) of maydis leaf blight in the field.  
TA = no application.
Application of biopesticide formulation *B. subtilis* TM4 gives a positive response to plant height. Spraying of *B. subtilis* TM4 formulation at concentrations of 3 g/l resulted in higher plant height than other treatments (Table 4). [23] reported that *B. subtilis* SL-13 strains can stimulate plant growth and control disease in greenhouses and fields. Further [24] found that Bacillus and Pseudomonas bacteria have the ability to hydrolyze P compounds by removing enzymes so that P releases as organic P.

**Table 4.** Effect of application concentration of *B. subtilis* TM4 formulation on plant height.

| Treatments                  | Plant height at 56 DAP (cm) |
|-----------------------------|-----------------------------|
| Without application         | 171.7                       |
| 1 g/l formulated *B. subtilis* TM4 | 172.7                       |
| 2 g/l formulated *B. subtilis* TM4 | 184.2                       |
| 3 g/l formulated *B. subtilis* TM4 | 188.5                       |

In the spraying treatment formulations with concentrations of 1, 2, and 3 g/l were not significantly different from the dry weight of ear, but higher than the control treatment (without application) (Table 5). The results of this study are similar to those obtained by [25] which states that the dry weight of tomato plants increased after inoculation with *B. subtilis* bacteria compared with those not inoculated with antagonistic microbes.

**Table 5.** Effect of spraying concentration of *B. subtilis* TM4 formulation to dry weight of ear in the field.

| Treatments                  | The dry weight of ear (kg/10 ears) |
|-----------------------------|-----------------------------------|
| Without application         | 1.9                               |
| 1 g/l formulated *B. subtilis* TM4 | 2.0                               |
| 2 g/l formulated *B. subtilis* TM4 | 2.0                               |
| 3 g/l formulated *B. subtilis* TM4 | 2.0                               |

According to [26], high concentrations of formulations and tight application spacing resulted in a growing population of bacteria, resulting in competition among individual bacteria and increased growth regulators. In addition, spraying formulations with excessive powder carriers is feared will cover the surface of the plant, so the process of photosynthesis is disrupted.

**Table 6.** Effect of application of *B. subtilis* TM4 formulation to the yield.

| Treatments                  | Number of ears | Yield (kg/10 plant) | Percentage increase |
|-----------------------------|----------------|---------------------|---------------------|
| Anoman (0 g/l)              | 32             | 2.3d                | 0.0c                |
| Bima-4 (0 g/l)              | 35             | 5.3b                | 0.0c                |
| Anoman (1 g/l)              | 25             | 3.1cd               | 22.2bc              |
| Bima-4 (1 g/l)              | 35             | 6.9a                | 40.7ab              |
| Anoman (2 g/l)              | 23             | 4.1c                | 43.9ab              |
| Bima-4 (2 g/l)              | 30             | 6.7a                | 47.6a               |
| Anoman (3 g/l)              | 26             | 3.5cd               | 34.5ab              |
| Bima-4 (3 g/l)              | 32             | 6.7a                | 39.2ab              |

The values in the same column followed by the same letter are not significantly different based on the DMRT at 5% level.
Increased plant growth can not be separated from mutually beneficial interaction between \textit{B. subtilis} TM4 with plants. \textit{B. subtilis} colonizes root system because it requires metabolite compounds produced by plants as nutrients. Once accumulated in the root system, the bacteria will produce growth regulators, which can induce roots to grow well. With good rooting, the penetrating power and root absorption of nutrients will be better [26]. According to Singh and Srivastava (2012) [1], Mmydis leaf blight disease results in significant yield losses in cultivars developed from subtropical or moderate germplasm ranging from 9.7\% to 11.7\% depending on weather conditions.

4. Conclusion

Application of \textit{B. subtilis} TM4 biopesticide formulation through seed treatment and spraying on plants gives a positive response to suppression of leaf blight and increased production of corn plants. In greenhouse trial, the best treatment is by seed treatment where the frequency of application is every two and three weeks. Spraying of corn plants with biopesticide formulation \textit{B. subtilis} TM4 can suppress the development of maydis leaf blight disease up to 21.5\%. In field trial, application of formulation with the concentration of 3 g/l tends to give the best result in controlling maydis leaf blight disease and also gave high yield on Bima 4 variety.

Further testing is still needed to determine the effectiveness of this \textit{B. subtilis} biopesticide formulation against downy mildew on maize plants because the resistance of varieties to downy mildew disease is an absolute requirement for the release of new improved varieties of corn.

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