Beneficial Microbes and Basal Fertilization in Antagonism of Banana Fusarium Wilt

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Abstract: Fusarium Wilt Tropical Race 4 (TR4) is spreading rapidly all over the world and threatens banana production, especially the Cavendish variety, which is the main commercial cultivar in Asia. This work aims to use beneficial microbes and fertilizers to control TR4. Both Bacillus mycoides NP02 (BM) and Bacillus amyloliquefaciens BaPD1 (BA) antagonized TR4 growth in plate tests. In addition, basal nutrients (BN) in combination with BM and BA were used to treat 1-month-old banana seedlings infected by Fusarium Wilt; plant growth and nutrients of leaves were investigated after 6-month cultivation in the field. The seedling survival rates for uninfected and infected controls without the microbes were 89.4 ± 14.8% and 50 ± 10.7%, respectively. In contrast, the corresponding rates for 2BM, 5BM, 1BA, F1 (5BM + 4BN + 1BA) and G1 (1BM + 4BN + 1BA) in the infected plants were 80 ± 9.4%, 83 ± 4.3%, 85 ± 14.2%, 96.7 ± 1.9% and 96.7 ± 3.3%, respectively. The 2BM treatment promoted the growth of both uninfected and infected banana plants and the 5BA treatment significantly reduced the severity index by 1.45-fold. Plants infected with TR4 showed significantly reduced contents of nitrogen and potassium, but the contents of iron, copper and zinc were higher than those of healthy plants. Additionally, 1BM treatment stimulated the accumulation of nitrogen and zinc ions in the leaves of uninfected plants. Both the 1BA and 2BN treatments increased the iron (Fe) and nickel (Ni) metal ion levels of TR4 infected plants. The F2 (5BM + 2BN + 1BA) treatment significantly maintained the growth of banana plants under TR4 stress with increased contents of nickel and zinc in banana leaves, suggesting that these ions may play a key role in stimulating the growth of banana plants under the threat of TR4. This work shows the potential of applying BM, BN and BA in the control of Fusarium wilt in field conditions.

Keywords: banana; Fusarium wilt; biological control agent; Bacillus mycoides; Bacillus amyloliquefaciens; field trial; Fusarium oxysporum f. sp. cubense; basal nutrients

1. Introduction

Bananas (Musa spp.) are one of the main crops grown in the world. In Southern and Central Asia, Cavendish bananas are mainly grown to be resistant to Fusarium oxysporum f. sp. cubense tropical race 1 (TR1) in place of the dominant cultivar Gros Michel, which is severely affected by TR1. However, the Cavendish banana cultivar is currently being threatened by a virulent fungus, F. oxysporum f. sp. cubense tropical race 4 (TR4) [1]. The diseases caused by Fusarium oxysporum are known as Fusarium wilt, which strictly limits the production of bananas [2]. Fusarium wilt is one of the most destructive soil-borne diseases of banana orchards in the world [3]. So far, several key strategies to control banana Fusarium wilt disease have been proposed, including breeding disease-resistant varieties through tissue culturing [4], genetic modification [5], biological [6-8] and chemical controls [9,10]. In addition, integrative plant nutrition is an essential part, and because of the development of agriculture within a sustainable system, the control of plant diseases is more cost-effective and environmentally friendly, creating crops that are more nutritious and do not contain pesticides. Proper nutrition can reduce diseases
to acceptable levels, while cultural practices or conventional organic biocides are more successful, environmentally friendly, and less expensive [11]. Some reports indicate that controlling the levels of soil nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), manganese (Mn) and zinc (Zn) levels may reduce the incidence of Fusarium disease [11–15].

Microbial biological control methods are also an important component of sustainable agricultural development. Some reports indicate that Bacillus species can effectively promote plant growth and may be a candidate for a biological control agent (BCA) [16]. Jia et al. [17] reported that the colonization of B. subtilis and suppression of F. oxysporum in the rhizosphere are important for Fusarium wilt disease control. Chen et al. [18] investigated two sets of bacterial samples: Bacillus licheniformis (X-1) and Bacillus methylotrophicus (Z-1) both with a strong inhibitory effect on Fusarium wilt caused by F. oxysporum f. sp. fragariae in strawberry crops. Agarwal et al. [19] reports that chitinolytic enzymes and antibiotic surfactin demonstrated differential biocontrol of F. oxysporum and R. solani by B. pumilus MSUA3. Bacillus amyloliquefaciens as a component of bio-organic fertilizer significantly decreased the incidence of Fusarium wilt and promoted the growth of banana plants compared with that of organic fertilizer [20]. Bacillus amyloliquefaciens BaPD1 strain has the effect of antagonism on pathogenic microorganisms [21,22]. After applying this strain in the field as biocontrol reagents, it can reduce rice blast disease by Pyricularia oryzae Cavara [21]. B. mycoides BM02 produces a series of biologically active compounds, including PAA and MPA, to inhibit plant diseases caused by F. oxysporum f. sp. lycopersici and other pathogenic microorganisms [23]. Bacillus mycoides NP02 produced a zoosporicidal biosurfacant, which inhibits cucumber Pythium damping-off pathogen (Pythium aphanidermatum). The biosurfacant is a kind of surfactin A compound that inhibits the germination of migrating spores [24]. These studies indicate that Bacillus species could be used as good biological control agents against Fusarium infection in field trials.

This work showed the application of biocontrol reagents containing B. amyloliquefaciens strain BaPD1 (BA), B. mycoides strain NP02 (BM) and basal nutrients (BN) on banana plants infected by Fusarium, with foci on (1) plant growth morphology after BM, BN, BA and combined treatments under TR4 stress and (2) the content of essential and nonessential nutrients in banana leaves, including nitrogen (N), phosphorus (P), potassium (K), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn), chromium (Cr), lead (Pb) and cadmium (Cd).

2. Materials and Methods

2.1. Materials

Tai-Chiao No.6 cv. Cavendish bananas were purchased from Taiwan Banana Research Institute (TBRI, Pingtung, Taiwan) [25]. Bacillus amyloliquefaciens BPD1 strain was obtained from Taiwan Agricultural Chemicals and Toxic Substances Institute (Taichung, Taiwan). Bacillus mycoides NP02 (BM) and F. oxysporum f. sp. cubense race 4 were obtained from Chung Hsing University (Taichung, Taiwan). Ammonium nitrate, potassium dihydrogen phosphate and magnesium sulfate were purchased from Chenfeng Chemical Co., Ltd. (Laizhou City, Shandong, China). Ferric ethylenediaminetetraacetic acid and ethylenediaminotetraacetic acid copper(II) disodium salt were purchased from BASF (Ludwigshafen, Germany). Sodium tetraborate was purchased from INKABOR S.A.C (Cerro Colorado, Peru). Luria–Bertani broth (LB) and Potato dextrose agar (PDA) were purchased from Difco Laboratories (Detroit, MI, USA). Fertilizer (N:P:K, 11:5.5:22) and organic fertilizer (N:P:K, 20-5-10-60) with peat, humic acid and 60% organic matter was purchased from Taiwan Fertilizer Co., Ltd. (Taipei, Taiwan).

2.2. Antagonistic Assay in Culture Plate

The antagonism assay in the culture plate is modified from the method previously published by Getha and Vikineswary [26]. The aim is to evaluate the growth rate of mycelium of foc4 and understand the inhibition ability of the isolated bacteria in order to evaluate the antifungal activity. For antifungal bioassays, we cultured the bacterial isolates in an incubator at 30 °C and shook them at 150 rpm for 24 h to increase the bacterial
amount. Next, 20 µL of test bacteria is spread on the center of the culture plate in a line, then the antagonistic foc4 fungus is placed on the center side of the potato dextrose agar (PDA) plate 2 cm apart. Next, 20 µL of microbial culture medium LB is added as a control group. On the PDA medium, two 8 mm precultured Foc TR4 mycelium plugs are placed with a distance of 2 cm between them. After 7 days of cultivation at 30 °C, the growth length of the fungus is calculated to evaluate the efficiency. The inhibition efficiency (IE) is calculated by IE = (Dck-Dexp)/Dck * 100%; here, Dck is the distance from the control and Dexp is the distance from the experimental group to the center.

2.3. Banana Cultivation and Irrigation Management

Banana plant growth requires proper irrigation. If the soil moisture content is too high, it will damage the root system. In tropical and subtropical regions, the annual yield response to irrigation is variable, but water efficiency should be 40 kg ha⁻¹ mm⁻¹ (fresh fruit/water application) and the summer irrigation interval should not exceed 2–3 days [27]. The amount of irrigation can be reduced during the seedling stage. During the rainy season, irrigation can be stopped. For example, before it rains in early summer or before the dry season in winter, water supply should be increased to twice a week, moisture in the rhizosphere soil should be maintained and the osmotic stress in rhizosphere soils that are too dry and too wet must be avoided. In the banana seedlings stage, flower bud differentiation stage and fruit development stage, more water is needed, which requires timely and appropriate water supply.

2.4. Banana Cultivation Management and Fertilizer Management

Fertilizer was modified from methods described by Al-Harthi and Al-Yahyai [28]. Bananas were applied with compound fertilizer No. 4 (N:P:K, 11:5.5:22), which was applied six times as a side dressing between planting and flowering with a total amount of around 1.5 kg/plant. A total of 15% was applied for the first month after planting, then once a month at the banana flowering period (usually five to seven months after planting). The number of applications and the amount of each application can be adjusted according to the rainfall. In addition, the application of green manure and organic manure in the soil of banana gardens can improve soil conditions. Organic fertilizers (N:P:K, 20-5-10-60) with peat and humic acid are premixed with the soil. Applying them in furrows during plant growth should be avoided.

2.5. Fusarium Wilt Inoculation

The inoculation methods were modified from Lichtenzveig’s method [29]. F. oxysporum (TR4) was cultured in a 500 mL flask with 200 mL PDB broth, incubated at 28 °C, and shaken at 150 rpm for 4 days to acquire macroconidia. The macroconidia were collected after filtering with 200 mesh (75 µm) filter and centrifuged for 6000 rpm for 20 min. The conidia were washed twice by resuspending the spores in sterile distilled water and centrifuging, and conidia were resuspended in 2–3 mL water. PDB was inoculated with a conidial suspension to a final concentration of 10⁵ spores/mL. The macroconidia were used as the inoculation of Tai-Chiao No.6 (Yu Fong) (Cavendish, AAA). The amount of inoculated macroconidia per plant was 500 mL. First symptoms (leaf yellowing and wilting) started appearing at about 14 days post inoculation.

2.6. Experimental Design of Field Test

Bacillus mycoides strain concentrations reached a maximum of 1 × 10⁸ CFU/mL after 3 days of fermentation. Bacillus mycoides (BM) 1 × 10⁸ CFU/mL, diluted 200-fold to 5 × 10⁷ CFU/mL (5BM); 500-fold to 2 × 10⁷ CFU/mL (2BM) and 1000-fold to 1 × 10⁶ CFU/mL (1BM); basal nutrient (BN) diluted 500-fold (4BN), 1000-fold (2BN) and 2000-fold (1BN); Bacillus amyloliquefaciens (BA) 1 × 10¹¹ CFU/mL diluted 200-fold to 5 × 10⁸ CFU/mL (5BA); 500-fold to 2 × 10⁷ CFU/mL (2BA) and 1000-fold to 1 × 10⁸ CFU/mL (1BA) dilution. Tap water was used for the infected site and the un-
infected site control (CK). Beneficial microorganisms and basal nutrients can be used as antagonists and nutritional modifiers. *Bacillus mycoides* (BM), *Bacillus amyloliquefaciens* (BA) and basal nutrient (BN) were used in the base of the banana and used for irrigation. Three different concentrations of beneficial microorganisms were used, each time using 500 mL per plant. The basal nutrient is applied to the banana plant by foliar spraying. The interval of application is 1–2 times per month.

### 2.7. Severity Level of the External Symptoms of Fusarium Wilt

The severity rating of each plant was assigned according to Inibap’s Technical Guidelines [30]. According to the different growth stages of bananas, different standards are set for observation. In the early stage of the middle banana plant, the survey was conducted as follows: five lower leaves of each banana plant without leaf yellowing gives a score of 0, one or two leaves with drooping or curling leaves gives a score of 1, one or two leaves being yellowed results in a score of 2, three to four leaves being yellowed is a score of 3, three to four leaves being yellowed and the leaf stalk being bent and drooped is a score of 4, and all five leaves drooping or the plant being scorched and dead is a score of 5. In the middle and late stages of bananas, the ratio of the area of yellow leaves to the total area is calculated as (1) 0–20%, (2) 20–40%, (3) 40–60%, (4) 60–80%, and (5) 80–100% or completely withered.

### 2.8. Basic Nutrient Determination of Banana Leaf

The determination of nitrogen, phosphorus, potassium and heavy-metal levels in leaves is carried out in accordance with the inspection methods of fertilizer inspection items published by the Agriculture and Food Agency of Council Agriculture Executive Yuan (Taiwan). The sampling position of the banana leaf is the middle part of the fifth leaf from bottom to top. Different elements are analyzed according to the following notification methods of the Agriculture Fertilizer Standards (ASF) [31]: total phosphorus anhydride (No. AFS1120-1), total nitrogen (No. AFS1110-1), potassium oxide (No. AFS1130-1), total ferric (No. AFS1105-1), total cadmium, chromium, copper, nickel, lead, zinc and titanium (No. AFS1293~9-1). These methods are described in detail in Supplementary method S1.

### 2.9. Field Test of Benefical Microorganisms and Basal Elements on Fusarium Wilt

We used a total of 32 treatment groups of Cavendish cv. Tai-Chiao No.6 (Yu Fong) banana plantlets inoculated with strains BA, BM and BN. The field trials were performed in a randomized complete block design (RCBD) with fifteen replicates per treatment. A total of 480 plants were used for field trials. There were two areas, one was an uninfected area, and the other was a Foc TR4 severely infested farm at Taichung, the two areas were separated by a distance of about 100 m (GPS location at 24.2297.895 N, 120.6999.166 E from April to December 2017). All banana plants were managed in the same way. Seven months after the field test, the plant height, pseudostem circumference, number of healthy leaves and number of infected plants in each plot were recorded.

### 2.10. Statistical Analysis

Comparison between groups was analyzed via a Student’s t-test and ANOVA using statistical software (R-language 4.04). All data are presented as mean ± standard deviation with $p$ values < 0.05 for significant differences. PCA analysis was conducted using the built-in R functions `prcomp()` and PCA results were displayed graphically through the library R functions `ggbiplot()`.

### 3. Results

#### 3.1. TR4 Mycelium Inhibition Test

In order to evaluate the inhibition of *F. oxysporium* race 4 (TR4) pathogen growth by BM or BA, we used an in vitro plate assay to examine the inhibitory ability. After 4 days of incubation, the inhibition distance of TR4 mycelium was measured by a dual-culture
method, and compared with the TR4-only group, the inhibition rate of BA and BM was
evaluated. Figure 1 shows the diameter of the zones of mycelium growth inhibition as
determined and recorded in vitro. Figure 1a–c is the growth inhibition of the TR4 growth
inhibition test. Figure 1a is a negative control, TR4 only, where the growth value after
quantification is 100%. Figure 1b shows that the BA strain antagonizes the growth of TR4,
after quantification, the growth rate of TR4 is reduced to 43.9 ± 2.7%. Figure 1c shows
that the BM strain antagonizes the growth of TR4, after quantification, the growth rate of
TR4 is reduced to 66.1 ± 3.3%. In Figure 1d, BA was shown to inhibit the growth of TR4
mycelium significantly more than BM (p < 0.01).

![Figure 1](image1.png)

**Figure 1.** TR4 mycelium antagonism test. In vitro antagonism of strains BA and BM against *F. oxysporium* race 4. After 4 days of incubation, the antagonism between strains BA, BM and *Fusarium oxysporum* TR4 was tested by a dual culture assay on PDA medium. (a) Control, (b) BA, (c) BM and (d) Quantification of TR4 mycelium growth rate.

### 3.2. Field Trial on Banana Growth

The field test is divided into two areas. One is the uninfected area and the other is the
infected area, where banana plants are infected with *Foc TR4*, and the plants that initially showed symptoms of yellowing and wilting of the leaves began to appear about 14 days after inoculation with *Fusarium* spores. In the uninfected area, we used 10 treatments, namely A1-C3 and CK (tap water). In the infected area we used 22 treatments, namely A1-G3 and CK (tap water). A total of 32 treatments were used in the field trial. The treatment list is in our supplementary data (Table S1). Fifteen plants per treatment for a total of 480 plants were used for field trials. The BM, BN and BA treatments were applied to field trials in banana nursery trials for six months.

#### 3.2.1. Survival Rate

Figure 2 Shows the survival rate of bananas with different treatments. The seedling survival rates for uninfected and infected controls was 89.4 ± 14.8%, among which the survival rate of 1BN and 2BN treatments were 100%; the BM treatment exceeded 90%, and the BM treatment varied from 66% to 95%. The seedling survival rates for infected controls was 50 ± 10.7%, and in the 2BM, 5BM and 1BA treatments, the seedling survival
rate was significantly increased by 80 ± 9.4%, 83 ± 4.3%, and 85 ± 14.2%, respectively. The combined treatment is shown in Figure S1. The higher survival rate of the combined treatment is such that F1 (5BM + 4BN + 1BA) and G1 (1BM + 4BN + 1BA) have a 96.7 ± 1.9% and 96.7 ± 3.3% survival rate in the infected area, respectively, as shown in Figure 2c.

Figure 2. Comparison of banana survival rates under different treatment conditions. (a) Comparison of plant survival rates under different treatments in the uninfected area. (b) Comparison of plant survival rates under different treatments in the infected area. (c) Combination treatments.

3.2.2. Banana Growth Height

The two field growth results of the six-month nursery experiment from July to December are as follows. The average growth height of the uninfected area from July to December, as seen in Figure S2a, was 69.0 ± 2.4 cm, 125.1 ± 3.3 cm, 151.0 ± 3.8 cm, and 158.7 ± 3.5 cm, respectively. The average height increase of bananas of the infected area from July to December in Figure S2b was 30.0 ± 0.9 cm, 64.7 ± 1.6 cm, 89.6 ± 2.3 cm, and 93.9 ± 2.2 cm, respectively. There are significant differences in the growth of these two areas. Figure 3a shows that in the uninfected area, there are different effects on banana nurseries with different treatments and doses. Growth in the case of 2BM was significantly higher than the other groups. The Foc TR4 infected field trial is designed by random complete module design (RCBD), and the major factors in Figure 3b were analyzed. Figure 3b shows the infected area with enhanced effects on banana nurseries with different treatments and doses. Growth with the use of 2BM was significantly higher than the other groups. The combined treatment showed that F2 (5BM + 2BN + 1BA) and G2 (1BM + 2BN + 1BA) were significantly different from the infection site in Figure 3c, p < 0.05, and were 116.5 ± 8.3 cm and 124.4 ± 12.2 cm, respectively.
3.2.3. Total Number of Banana Leaves

The number of leaves of a banana plant can reveal the influence of pathogen infection characteristics. In the different treatment methods in the uninfected site, number of leaves was 11.8 ± 0.2, as shown in in Figure 3a. The 1BM, 5BM and 2BA treatments were significantly different (p < 0.05), where the number of leaves was 5.4 ± 0.4, 10.0 ± 0.4 and 6.5 ± 0.8, respectively. In the TR4 infected area, the number of leaves in the infected site was 7.0 ± 0.5, and the 1BA, 4BN, 2BM, and 5BM treatments were significantly higher (p < 0.05) than the TR4 infected site, which were 9.2 ± 0.4, 8.2 ± 0.8, 8.2 ± 0.5, and 8.0 ± 0.5, respectively. The average number of banana leaves in the uninfected area was 10.6 ± 0.4, and the average number of banana leaves in the infected area was 7.5 ± 0.6. In the combined treatment of the infected area, the F2 (5BM + 2BN + 1BA) and G2 (1BM + 2BN + 1BA) treatments have more leaves, as shown in Figure 4c, which are 10.5 ± 0.6 and 10.4 ± 0.8, respectively.

3.2.4. The Length and Width of the Banana Leaf

Figure 5a shows the leaf width of the uninfected area. The leaf width of the uninfected site is 41.5 ± 1.3 cm. The 1BM and 2BN treatments are significantly wider than the
uninfected site at 48.0 ± 1.9 cm and 48.5 ± 2.5 cm, respectively. Figure 5b shows the leaf width when infected by TR4, which at the infected site is 19.8 ± 0.9 cm, and under the 5BM treatment is 24.3 ± 2.2 cm, which is significantly wider than the infected site (p < 0.05). Figure 5c shows the leaf length in the uninfected area. The leaf length of the uninfected site was 101 ± 3.9 cm, and under the 2BM treatment is significantly longer than the uninfected site (p < 0.001), at was 128 ± 6 cm. Figure 5d shows the TR4 infected area. The leaf length of the infected site is 48 ± 2 cm, and under 5 BM treatment is significantly longer than the infected site, at 61 ± 27 cm (p < 0.05). In the combined treatment of the infected area, the F2 (5BM + 2BN + 1BA) and G2 (1BM + 2BN + 1BA) treatments have much wider and longer leaves, as shown in Figure 5c,f, at are 31.8 ± 2.2 and 79.9 ± 6.5, respectively.

![Leaf width and length comparison](image)

**Figure 5.** After 6 months of field trials, the leaves’ widths and lengths under different treatments. (a) Leaf width under different treatments compared with the uninfected area. (b) Leaf width under different combinations compared with the infected area. (c) Leaf width under combination treatments. (d) Leaf length under different treatments compared with the uninfected area. (e) Leaf length under different combinations compared with the infected area. (f) Leaf length under combination treatments.

3.2.5. Yellow Ratio of Banana Leaves

The leaves are the main photosynthetic organs of the banana plant. When the leaves become the orange–yellow of old leaves, it is a symptom of nitrogen deficiency, excessive watering, and poor soil drainage. Yellow leaves also show physiological obstacles to banana growth. We checked the proportion of yellow leaves to know which treatments were effective. In the uninfected area, the data show significant differences between the 1BM, 1BA and 2BA treatments in the uninfected area, which are 63.9 ± 4.7%, 66.2 ± 2.3%,
and 65.1 ± 3.2% in Figure S6a, respectively. In the infected area, these results show no significant difference from the infected site in Figure S6b,c.

3.2.6. Banana Leaf Symptom Severity Index

A basic principle of severity index is that they define banana leaf symptoms with the expectation of similar outcomes given some standard of treatments. The survey was conducted with five lower leaves of each banana plant. The total score ranges from 0 to 5, such as without leaf yellowing, which is 1 point, one or two leaves with drooping or curling is 2 points, one or two leaves are yellowed is 3 points, three to four leaves are yellowed is 4 points and the leaf stalk is bent and drooped and all five leaves are drooping or the plant is scorched and dead being 5 points. The higher the score, the more severe the plant disease symptoms, and vice versa. Figure 6a showed the severity index of bananas in the uninfected area treated with different doses of BM, BN and BA. The results showed that there was no significant difference between these three treatments in the uninfected site, which was 2.4 ± 0.2. The value of the severity index of banana and the severity index of the infected site is 3.6 ± 0.1. The severity values of 5BA and 1BM were 2.5 ± 0.2 and 2.6 ± 0.3, respectively, which were significantly different from the infection site in Figure 6b. The 5BA treatment significantly reduced the severity index by 1.45-fold. In addition, when treated with 1BM or 5BA, there was no difference from uninfected areas. Comparing the severity index of banana plants under different treatment combinations in the infected area, the values of D1 treatment (5BM + 4BN + 5BA) and F2 treatment (5BM + 2BN + 1BA) were lower, which were 2.7 ± 0.2 and 3.0 ± 0.2, respectively, as seen in Figure 6c.

![Figure 6](image.png)

Figure 6. Comparison of the severity index of bananas (a) in uninfected area (b) in TR4 infected area. (c) Combination treatments.

3.3. Nutrient Analysis of Banana Leaves

We randomly selected 5 banana plant leaves from every 32 groups and obtained 160 samples for analysis. We analyzed macronutrients such as nitrogen, phosphorus and potassium in each sample, along with four essential micronutrients—iron, zinc, copper and nickel, and the following heavy metals: cadmium, chromium and lead. Analysis of the effects of various treatments on plant growth will be affected by ion conditions.

3.3.1. NPK of Banana Leaves

Nitrogen, phosphorus and potassium are macronutrients that promote plant growth. They are essential to the growth and development of plants. These elements participate in the formation and movement of sugar during cell division and the development of new tissues and are necessary for cell division and expansion during all growth processes. Table 1 shows the average value of NPK for uninfected areas and infected areas. Compared with the infected area, the nitrogen content of the uninfected area was significantly
higher, \( p < 0.001 \); phosphate is slightly lower than the infected area, \( p = 0.23 \); potassium is significantly higher than the infected area, \( p < 0.05 \).

**Table 1.** Comparison of the average content of nitrogen, phosphate and potassium in uninfected area and infected area.

| Variables of significance (* \( p \leq 0.05 \), ** \( p < 0.01 \), *** \( p \leq 0.001 \)).

| Nitrogen (%) | Phosphate (%) | Potassium (%) |
|--------------|--------------|--------------|
| Uninfected area | 2.9 ± 0.1 *** | 0.45 ± 0.01 | 3.07 ± 0.16 * |
| Infected area  | 2.4 ± 0.1    | 0.50 ± 0.02 | 2.6 ± 0.11   |

Nitrogen

Figure 7a displays the banana leaf nitrogen content in the control area. The 1BM, 1BN and 5BM treatments are significantly higher than the uninfected site, at 3.28 ± 0.16%, 3.3 ± 0.06% and 3.3 ± 0.15%, respectively, \( p < 0.05 \). In the infected area, Figure 7d shows that only 2BN is significantly higher, \( p < 0.05 \), and the nitrogen content is 3.0 ± 0.15%. The combined treatment is shown in Figure S8a, where E3 treatment (5BM + 4BN + 1BA) was 3.0 ± 0.10%, which is significantly higher than the infected site, \( p < 0.05 \).

![Figure 7](image)

**Figure 7.** The NPK content of banana leaves. (a-c) uninfected area and (d-f) infected area.

Phosphate

We analyzed the phosphate accumulated in the banana leaves. In the uninfected area, Figure 7b shows that only the 1BN treatment, with a phosphate level of 0.61 ± 0.03%, was significantly higher than that of the uninfected area, \( p < 0.05 \). In the infected area, Figure 7e shows that there was no significant difference from the infected site. The combined treatment is shown in Figure S8b, where E3 treatment (5BM + 4BN + 1BA) was 3.0 ± 0.10%, which is significantly higher than the infected site.
Potassium

We analyzed the potassium level of banana leaves. In the uninfected area, Figure 7c shows that there is no significant difference in potassium content between different treatments. In the infected area, Figure 7f shows that the 1BM treatment value is $5.3 \pm 0.4\%$, which is significantly higher than the infected area, $p < 0.05$. Combination treatments are shown in Figure 8c, and all combination treatments are significantly lower than untreated infection sites, $p < 0.05$.

3.3.2. Analysis of Essential Metal Elements in Banana Leaves

Essential metals can provide trace elements for plant growth, especially protein binding elements such as cofactors. These functions are related to basic physiological functions such as DNA replication, respiration and photosynthesis. Here, we analyzed iron, nickel, copper and zinc. Figure 8a–d shows the analysis of uninfected area results and Figure 8e–h shows the infected area results. Table 2 shows the average content of Fe, Ni, Cu and Zn in the uninfected and infected areas. Compared to the uninfected area, the content of Fe, Cu and Zn in the infected area increased significantly, $p < 0.001$.

![Figure 8](image)

**Figure 8.** Fe, Ni, Cu and Zn content of banana leaves. (a–d) use treatments in the uninfected area and (e–h) use treatments in the infected area.

|               | Fe (ppm) | Ni (ppm) | Cu (ppm) | Zn (ppm) |
|---------------|----------|----------|----------|----------|
| Uninfected area | $118.2 \pm 2.7$ | $4.0 \pm 0.2$ | $9.4 \pm 0.4$ | $13.8 \pm 0.3$ |
| Infected area  | $149.6 \pm 3.7$ *** | $4.2 \pm 0.1$ | $14.1 \pm 0.7$ *** | $16.1 \pm 0.4$ *** |

*Variables of significance (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).*

Iron

The iron content of banana leaves under different treatments was analyzed. In the uninfected area, only the BN treatment did not have a significant difference, and the $p$-value = 0.0126 was tested by ANOVA. In Figure 8a, compared with the uninfected site, when using 2BN, the Fe content of banana leaves is significantly higher, at $136 \pm 11$ ppm, $p < 0.05$. In the field infected area, the Fe content of banana leaves in Figure 9e was not significantly different from the infected site. In the combined treatment of the infected area shown in Figure S9a, E3 (1BM + 1BN + 5BA), F1(5BM + 4BN + 1BA), and G2(1BM + 2BN + 1BA)
were 120.5 ± 3.5 ppm, 121.4 ± 4.0 ppm, and 95.3 ± 22.9 ppm, respectively, which were significantly lower than the infected area.

![Cr Content](image)

![Cd Content](image)

![Pb Content](image)

Figure 9. Cr, Cd and Pb content of banana leaves. (a–d) in the uninfected area, and (e–f) in the infected area.

Nickel

The nickel content of banana leaves was analyzed. Compared with the uninfected area, the uninfected area in Figure 8b is not significantly different. In Figure 8f, compared with the infected site, the Ni content of the 2BN and 1BA treatments is significantly different from the infected site, which are 4.2 ± 0.5 ppm and 5.7 ± 0.6 ppm, respectively. In Figure S9b, the Ni content of banana leaves treated with various combinations in the infected area is displayed. When treated with E3 (1BM + 1BN + 5BA), F2 (1BM + 1BN + 5BA) and G1 (1BM + 1BN + 5BA), the Ni content is 6.0 ± 0.5 ppm, 6.2 ± 0.5 ppm and 4.8 ± 1.0 ppm, respectively. Compared with the infected site, the Ni content of E3, F2 and G1 treatments was significantly higher.

Copper

Figure 8c shows the copper content of the uninfected area. When using 1BN, 2BN and 4BN, the copper content of banana leaves in the uninfected area was significantly higher than that in the uninfected area, and the copper content was 10.5 ± 0.4 ppm, 11.5 ± 1.5 ppm and 16.5 ± 1.2 ppm, respectively. Figure 8g shows the copper content in the infected area. When 1BN, 2BN and 4BN is used, the banana leaf copper content in the infected area is also significantly higher than that in the infected area, where the copper content was 12.8 ± 1.1, 12.9 ± 3.9 and 21.1 ± 3.9 ppm, respectively. In Figure S9c, the copper content of banana leaves treated with various combinations in the infected area is displayed. When treated with D1 (5BM + 4BN + 5BA), E1 (1BM + 4BN + 5BA), E2 (1BM + 2BN + 5BA), F1 (5BM + 4BN + 1BA), F2 (5BM + 2BN + 1BA), G1 (1BM + 4BN + 1BA) and G2 (1BM + 2BN + 1BA), the copper content is 15.0 ± 0.4 ppm, 19.7 ± 3.6 ppm, 27.1 ± 2.4 ppm, 14.4 ± 0.6 ppm,
22.6 ± 0.9 ppm, 14.7 ± 0.8 ppm and 14.2 ± 3.3 ppm, respectively. Compared with the infected site, the copper content of E3, F2 and G1 treatments was significantly higher.

Zinc

Figure 8a shows that for zinc levels the treatments of 1BM, 2BM, 5BM, 1BN, 4BN, 1BA and 2BA are significantly higher than the uninfected site, where the zinc content was 14.6 ± 0.9 ppm, 14.8 ± 0.6 ppm, 14.4 ± 0.6 ppm, 14.4 ± 0.3 ppm, 13.9 ± 1.1 ppm, 14.8 ± 0.6 ppm and 15.0 ± 0.8 ppm, respectively. In the infected area, Figure 8b shows that only the 2BA treatment was significantly lower than the infected area, where the zinc content was 13.7 ± 0.8 ppm. The combined treatment of the infected area in Figure S9d shows that E2 (1BM + 2BN + 5BA) and F2 (5BM + 2BN + 1BA) produce significantly higher zinc levels than the infected area, and the zinc content is 27.1 ± 2.4 ppm and 22.6 ± 0.9 ppm, respectively.

3.3.3. Analysis of Nonessential Metal Elements in Banana Leaves

Nonessential elements can affect the growth of banana plants when they accumulate in plant cells. The accumulation of nonessential metal elements can induce the formation of reactive oxygen species (ROS) in plant cells, affect plant growth and even make banana leaves yellow. Table 3 shows the comparison of the nonessential metal elements Cr, Cd and Pb in the two areas. In the table, only Cd is significantly higher in the uninfected than in the infected area. Figure 9 shows the analysis of nonessential metal ions in banana leaves, where Figure 9a–c is the uninfected area and Figure 9d–f is the infected area.

|              | Cr (ppm) | Cd (ppm) | Pb (ppm) |
|--------------|----------|----------|----------|
| Uninfected area | 3.21 ± 0.16 | 0.09 ± 0.01 *** | 0.67 ± 0.14 |
| Infected area  | 3.58 ± 0.14 | 0.04 ± 0.01 | 0.63 ± 0.03 |

Variables of significance (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

Chromium

Figure 9a shows the chromium content of banana leaves in the uninfected area. When treated with 2BN, 1BA and 2BA, the chromium content was significantly higher than that of the uninfected area. Figure 9d shows the chromium content of the infected site. When treated with 5BM and 1BN, the chromium content was significantly lower than the infected site. As shown in Figure 9a, the chromium content following E2 (1BM + 2BN + 5BA) treatment was significantly higher than that of the infected site.

Cadmium

Figure 9b shows the cadmium content of banana leaves in the uninfected area. The 1BN treatment results in significantly higher cadmium levels than in the uninfected site. There was no significant difference in the cadmium content of different treatments in the uninfected area. Figure 9b shows that in the infected areas, E1 (1BM + 4BN + 5BA), E2 (1BM + 2BN + 5BA), E3 (1BM + 1BN + 5BA), F2 (5BM + 2BN + 1BA) and G2 (1BM + 2BN + 1BA) treatments were significantly higher than the infected site, where the cadmium content was 0.089 ± 0.034 ppm, 0.179 ± 0.008 ppm, 0.101 ± 0.020 ppm, 0.115±0.018 ppm and 0.128 ± 0.038 ppm, respectively.

Lead

Figure 9c shows the lead content in the uninfected area. The 1BN treatment was significantly higher than the uninfected site, with a value of 2.8 ± 0.7 ppm. In the infected area, the lead content of various treatments was not significantly different from other treatments. In the combined treatment shown in Figure 9d, E2 (1BM + 2BN + 5BA) and F2 (5BM + 2BN + 1BA) were significantly higher than the infected site, which were 1.19 ± 0.05 ppm and 0.92 ± 0.10 ppm, respectively.
4. Discussion

The antifungal properties of beneficial microbes against *Fusarium* sp. have been investigated by many workers [19,32,33]. A possible mechanism is the release of biochemical substances that can diffuse outside of the cell as an effective antagonist to inhibit the growth of pathogenic mycelium, for example, surfactin [34], chitinase [19] and β-1,3-glucanase [35] may be involved in the degradation of fungal cell membranes or cell walls. Microbial populations living in a common environment will compete for nutrients and other resources in the environment. As competition for nutrition and space intensifies, highly competitive microorganisms generally prefer this mode of action of biological control agents [36]. It was also observed that the isolated BA and BM showed strong antifungal activity in the dual culture experiment with *F. oxysporum*. The antifungal activity of BA against *F. oxysporum* has also been reported before [37]. In this study, *F. oxysporum* TR4 have been introduced for their pathogenicity with Cavendish banana plantlets. Their macromorphological structures were also consistent with the previous review [2]. The observed small yellowing surface formed by *F. oxysporum* can be explained, as *F. oxysporum* mainly penetrates the root and moves upward from the rhizome to the pseudostem xylem [38]. Therefore, current work attempts to apply antagonistic bacterial strains that can be used as biological control agents in field trials. In the previous screening, two local isolates of *B. amyloliquefaciens* strain BaPD1 (BA) and *B. mycoides* strain NP02 (BM) were believed to have mycelial growth inhibitory effects on *Fusarium oxysporum* TR4. It was observed that after 3–7 days of culture at 28 °C, the pathogen-inhibition rate was 40–70%. Some papers have reported that the addition of specific biological control agents to biological fertilizers will produce substrates with a broader inhibitory effect [39–41], and adding compost to the soil always stimulates microbial activity, while *F. oxysporum* is susceptible to competition for nutrients [42]. In the current study, we assessed the impact of BM, BN and BA application on the suppression of Fusarium wilt and explored the response of the macronutrients, essential elements, and nonessential elements in banana leaves. Our field trial results show that BM, BN and BA application, especially 2BM, 5BM, 1BA, F1 (5BM + 4BN + 1BA) and G1 (1BM + 4BN + 1BA) effectively controlled the survival rate of banana plantlets under TR4 infection. These results were roughly in agreement with many previously published works showing that the application of organic fertilizer or microorganisms can well inhibit soil-borne diseases [43,44]. The possible mechanism of microbial control of Fusarium wilt is related to certain compounds secreted by *Bacillus mycoides*, which also contribute to biological activity against pathogens. For example, phenylacetic acid and methylphenyl acetate from the biological control *Bacillus mycoides* can inhibit the spore germination of *F. oxysporum* [8], and NP02 used in this research could produce surfactin A suppressed by the zoospores germination of *Pythium aphanidermatum* [24]. *B. mycoides* produced an array of bioactive compounds including chitinase, β-1,3-glucanase, peroxidase, PAA and MPA to suppress plant diseases [23,45]. In addition, *B. amyloliquefaciens* is a strong antagonistic strain, and when used alone, it can control the survival rate, maintain the plant height, the number of leaves, and reduce the severity index.

The growth and reproduction speed of bananas is believed to be related to mineral availability and mineral absorption rate [46]. *Bacillus* is used as a PGPR with plant growth promoting properties, it also helps to dissolve inorganic phosphate, potassium, zinc and other nutrients [47]. Rhizosphere bacteria retain soil organic nitrogen and other nutrients in the soil–plant system, thereby reducing the need for fertilizers and promoting the release of nutrients from native or mineral sources [47]. The enhanced nutrient absorption of minerals in inoculated plants is considered to be a possible mechanism for PGPR to promote plant growth. The major element involved in nitrogen fixation is a form of nitrate in banana plants [48,49]. Other elements such as P and K have also been suggested to play a key role in this plant–bacterial interaction [50]. The production and management of biological fertilizers containing potassium solubilizing bacteria can be an effective alternative to chemical fertilizers [50]. In the present study, the results showed that nutritional structure observed under treatment with BM, BN and BA differed from those found in infected
areas. In addition, macro and trace element analysis showed that the application level of biological fertilizers is also related to changes in nutrient structure. This finding was also similar to the results of previous work showing that different level of organic amendment application result in different element structures [46]. Hence, different levels of biofertilizer application can alter the nutritional levels of banana plants in different degrees. The banana plants grown in noninfected areas show significantly higher soil nitrogen and potassium concentration, as well as significantly higher leaf phosphorus concentration, than banana plants grown in infected areas, and the banana plants grows significantly bundle weight and bundle cluster numbers [10]. The combined application of organic fertilizer and inorganic fertilizer can effectively improve soil fertility and reduce the incidence of banana diseases [9]. Changes in the NPK ratio will significantly affect the yield of commercially grown bananas [28]. Our study also shows a variety of results, when 2BN is applied to bananas, the survival rate can be improved, while 1BN applied to bananas can increase the NPK level of banana leaves. In addition, the use of 2BM contributes to the plant height, leaf width and leaf length, as seen in Figure 3a, Figure 5a,c, indicating that BM may be helpful to plant growth. BM is a beneficial microorganism that can stimulate the growth of lateral roots of plants by secreting plant growth hormones or beneficial compounds, and help plants grow in various environments [51,52]. B. mycoides can secrete plant growth-promoting hormones to help plants grow. B. mycoides is a rhizosphere-related bacterium that has good endophytic properties and the potential to promote plant growth [53]. These results indicate that sidero-bacterin participates in the growth-promoting activity of plants and can affect the root colonization of Bacillus mycoides, indicating the important role of B. mycoides in plant growth promotion and root colonization [54].

Mineral uptake is also an important issue for the contribution of soil microorganisms. The nutrient increase observed in the PGPR system appears to be due to a general increase in root area rather than a specific absorption rate [55]. Bacillus may improve the efficiency of mineral nutrient application by helping plants to remove restrictive nutrients, and its effect is similar to that of mycorrhiza in phosphate recovery. PGPR inoculation changed many root and shoot parameters. These changes are directly attributable to the positive effects of bacteria on plant mineral absorption. Some reports show that proper nutrients can help banana plants resist Fusarium wilt [13]. Minerals can provide the nutrients needed for plant growth. In our experiments, the survival rate with 2BN is much higher than other treatments. The iron content in banana leaves is higher when 2BN is applied. Iron is a key element in plants, playing an important role as a cofactor in multiple metabolic processes, and is essential in heme and iron–sulfur protein [56], which are essential for maintaining the structure of chloroplasts, which are important and photosynthetic properties [57]. Some reports [58–60] showed that when iron is supplied as a nutrient, it reduces the accumulation of cadmium. In this study, Cadmium accumulation in banana leaves was noted. It inhibit absorption of iron in banana leaf. As we can see in Figure S12, the PCA diagram shows that the Fe and Cd are in opposite and the Pearson correlation coefficient is $-0.40$ in Table S2. The Fe and zinc are used to adjust the Cd toxicity of micropropagated banana branches [60]. This basal nutrient is important for maintaining the physiological characteristics of bananas for normal growth [61]. Zinc and nickel can improve the growth of banana plants in infected fields. In our PCA results in Figure S12, the zinc and nickel contents of the F2 treatments are higher than other treatments. In the morphological study, F2 treatment was superior to other treatments in terms of height, leaf number, leaf width and leaf length of the infected area. In infested fields, after applying 1BA to bananas, the Ni level in Figure 8b is seen to be significantly increased, thereby increasing the survival rate and plant height, and reducing the severity index. Some works have shown that nickel ions play a key role in plant defense responses. Zinc plays an important role for plant defense to against pathogens [62]. Nickel (Ni) plays a key role in the metabolism of some profitable crops, such as soybeans, because it is a component of a variety of biomolecules and is necessary for the catalytic process of a variety of enzymes [63]. Nickel can enhance the resistance of soybeans to Phakopsora pachyrhizi [63]. Nickel (Ni) level and growth, photosynthesis, and physiological
and biochemical properties, have potential applications in the phytoremediation of Ni through the application of salicylic acid [64]. When combined with BM + BN + BA, it has been shown that the combination of F2 (5BM + 2BN + 1BA) or G2 (1BM + 2BN + 1BA) can maintain plant height, leaf number, leaf length, leaf width and reduce the severity index. After F2 (5BM + 2BN + 1BA) treatment, the shape is closer to the uninfected control, indicating that the F2 treatment has a significant effect, indicating that the combination treatment improves the growth conditions of banana plants under infection stress. The content of Ni and Zn in F2 (5BM + 2BN + 1BA) treatment is higher than other treatment groups. This shows that when infected with Fusarium, the accumulation of Ni and Zn in plants can promote plant growth. The possible mechanism of Ni, Zn and plant growth under the stress of Fusarium wilt needs to be further studied. Zn deficiency was observed to change the ultrastructure of both chloroplasts and mitochondria in the leaves, and Fusarium wilt appeared to be more severe under Zn-deficient conditions [58]. Nickel is a component of urease and hydrogenase, and it is the latest nutrient to be recognized as an essential element for plants. Nickel is applied to the soil to improve nitrogen metabolism [65]. In addition, nickel nanoparticles can be applied for control of Fusarium wilt [65]. In the infected area, F2 (5BM + 2BN + 1BA) treatment showed significantly higher of Ni content, also indicating the Ni element does not compete with nonessential elements. The Pearson correlation coefficients of nickel in Table S2 are related to nitrogen, zinc, iron, and chromium in the infected area. The use of BM and BA in the soil as a fertilizer possibly increased the exchangeable form of microelements in the soil [14].

Our research shows that the application of BM, BN and BA has a comparative advantage in banana plant growth. In particular, the combination of BM + BN + BA can improve the growth conditions of banana plants and the nutrient content of Ni and Zn and prevent Fusarium wilt. However, the mechanism of control Fusarium wilt is unclear. How do Ni and Zn fight pathogens and Fusarium wilt in banana plants? This is a complicated mechanism that requires research in different fields to find a truly effective method. In future work, whether a specific ion concentration of Ni or Zn turns on a gene cluster or specific genes to enhance banana growth or resistance to Fusarium wilt should be investigated. According to our findings, we can use combined treatment to control Fusarium wilt. We can also monitor the level of nickel or zinc to achieve the nutrients needed for banana plant defense or banana growth. Although biological control may be found to be effective under laboratory or greenhouse conditions, the use of biological control agents usually fails to control diseases in the field. This work provides a real field test result, which comes from an assay, and finally presents the best combination of two different Bacillus species. Therefore, in order to obtain successful biocontrol products. The products must be applied in accordance with good ecological requirements. A successful biocontrol product requires a lot of work to make the biocontrol agent more effective and practical under field trials.

5. Conclusions

Our results showed BM and BA strongly antagonize TR4 growth in vitro. Consistently, field trials showed that BM treatment promoted plant growth and BA treatment can increase the survival rate of infected banana seedlings. In addition, when basal nutrients are used at the same time, the increases in survival rate are significant, indicating that proper nutrient supply is important. These data indicate that BM can increase the nitrogen and zinc content in banana leaves and help plant growth. In particular, the F2 (5BM + 2BN + 1BA) treatment can increase the infected seedling survival rate, leaf number, leaf height, leaf length and leaf width. When treated with F2, the content of Ni and Zn increase, indicating the importance of these elements for the control of Fusarium wilt and promoting banana plant growth. Thus, this work provides the concept of basic nutrients and biological fertilizers for banana management in both uninfected and infected fields.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11102043/s1. Table S1: The treatments each group in the uninfected area and the infected area; Table S2: Pearson correlation coefficient between elements; Figure S1: Compare
the survival rate of plants under different treatment combinations in the infected area; Figure S2: In six months of nursery trials, the growth results of the two fields in the uninfected area and the infected area from July to December; Figure S3: Compare the banana height of plants under different treatment combinations in the infected area; Figure S4: Compare the number of banana leaves under different treatment combinations in the infected area; Figure S5: Compare the leaf of banana plant leaves under different treatment combinations in the infected area; Figure S6: Compare leaf yellow ratio of banana plant under different treatment combinations in the uninfected area; Figure S7: Compare Severity index of banana plant under different treatment combinations in the infected area; Figure S8: Compare the NPK of banana plants under the combined treatment of the infected area; Figure S9: Compare the trace elements of banana plants under the combined treatment of the infected area; Figure S11: PCA of banana plant morphology; Figure S12: Principal component analysis (PCA) of nutrients and elements: method S1: Basic nutrient determination of banana leaf.

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