Synergistic Effect of Combined Application of a New Fungicide Fluopimomide with a Biocontrol Agent *Bacillus methylotrophicus* TA-1 for Management of Gray Mold in Tomato

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Abstract

Gray mold caused by *Botrytis cinerea* can be a severe disease of tomato infecting leaves and fruits of tomato plants. Chemical control is currently the most effective and reliable method; however, application of fungicides has many drawbacks. The combination of biological control agents with newly developed fungicides may be a practicable method to control *B. cinerea*. Fluopimomide is a newly developed fungicide with a novel mode of action. *Bacillus methylotrophicus* TA-1, isolated from rhizosphere soil of tomato, is a bacterial strain with a broad spectrum of antimicrobial activities. Little information is currently available about the effect of fluopimomide and its integrated effect on *B. cinerea*. Therefore, laboratory, pot, and field experiments were carried out to determine the effects of fluopimomide alone and in combination with *B. methylotrophicus* TA-1 against gray mold on tomato. The in vitro growth of *B. methylotrophicus* TA-1 was unaffected by 100 mg liter⁻¹ fluopimomide. Inhibition of *B. cinerea* mycelial growth was significantly increased

under combined treatment of fluopimomide and *B. methylotrophicus* TA-1. In greenhouse experiments, efficacy against gray mold was significantly greater by an integration of fluopimomide and *B. methylotrophicus* TA-1 than by either alone; control efficacy of fluopimomide at 50 and 100 g ha⁻¹ in combination with *B. methylotrophicus* TA-1 at 10⁸ colony-forming units (cfu) ml⁻¹ reached 70.16 and 69.32%, respectively, compared with the untreated control. In both field trials during 2017 and 2018, control efficacy was significantly higher for the combination of fluopimomide at 50 and 100 g ha⁻¹ in combination with *B. methylotrophicus* TA-1 than for either treatment alone. The results from this study indicated that integration of the new fungicide fluopimomide with the biocontrol agent *B. methylotrophicus* TA-1 synergistically increased control efficacy of the fungicide against gray mold of tomato.

Keywords: synergistic effect, tomato gray mold

Tomato (*Lycopersicon esculentum* L.) is an economically important vegetable crop globally. Gray mold, one of the most economically important diseases on tomato, causes serious losses every year in most tomato production regions (Williamson et al. 2007). In China, 56,860,000 t of tomatoes were harvested on 1,100,000 ha in 2016, which ranked first worldwide (MOA 2017). Losses of 20 to 30% are typical, and economic losses can exceed 50% in years of serious gray mold.

Gray mold, caused by *Botrytis cinerea*, can be a devastating disease in tomato. It is difficult to control because of its broad host range and multiple modes of attack (Dean et al. 2012). Traditionally, breeding of resistant cultivars is a promising solution. However, currently available resistant tomato cultivars against gray mold possess undesirable horticultural traits (Bestfleisch et al. 2015). The use of synthetic fungicides is still the primary strategy to control this disease (Romanazzi et al. 2016). In China, the active ingredients (a.i.) of chemical control for *B. cinerea* mainly include iprodione, procymidone, and pyrimethanil (Sun et al. 2010). However, control efficacy

has been reported to be decreasing owing to the developed resistance in *B. cinerea* resulting from the overuse of the fungicides (Liu et al. 2016). Therefore, there is an urgent need to develop novel and environmentally friendly *B. cinerea* control methods.

Fluopimomide [formerly LH-2010A, *N*-(3-chloro-5-trifluoromethylpyridin-2-yl)methyl-2,3,5,6-tetrafluoro-4-methoxy-benzamide]; chemical abstracts service (CAS) number: 1309859-39-9] is a new fungicide that was developed by Shandong Sino-Agri Union Biotechnology Co. Ltd. in 2010 (Zhang et al. 2014a). Fluopimomide was derived from fluopicolide, a pyridinylmethyl-benzamide fungicide, which can control diseases caused by oomycete pathogens (Toquin et al. 2007). Compared with fluopicolide, fluopimomide has a broader spectrum of antimicrobial activities, and it is more efficient in managing plant diseases, including *Rhizoctonia solani* and *B. cinerea* (Zhang et al. 2013a).

Increasing concern about the negative side effects of synthetic fungicides on human health and the environment has prompted the development of alternative application methods. Application of biological control agents (BCAs) to manage tomato gray mold is considered a promising alternative to synthetic fungicides (Bi and Yu 2016; Jassbi et al. 2017). Many *Bacillus* strains can form endospores and survive in hostile conditions (Kloepper et al. 2004), which have been reported to be used in management of gray mold (Ye et al. 2018). *Bacillus methylotrophicus* is well known for its methanol-utilizing and plant growth-promoting properties, and it has been considered to be a promising BCA against different plant diseases (Frikha-Gargouri et al. 2017). *B. methylotrophicus* TA-1, isolated from the rhizosphere of tomatoes in China, is a potential biocontrol agent against maize stalk rot; however, no information is currently available about its effects on *B. cinerea* (Cheng et al. 2019).

Although BCAs may decrease the frequency and total amount of fungicide spray, thus reducing residues and resistance risk, their effectiveness is usually inferior to that of chemical fungicides because of the complexity of the field environment (Jacobsen et al. 2004). The

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combination of BCAs with newly developed fungicides may develop a practicable method to control *B. cinerea* in tomato fields. This study aimed to (1) evaluate the compatibility of fluopimomide and the antagonistic strain *B. methylotrophicus* TA-1 and (2) determine the efficacy of fluopimomide and *B. methylotrophicus* TA-1 alone and in combination for control of *B. cinerea* in laboratory, greenhouse, and field experiments. Results from this study can provide clues to develop new approaches to controlling *B. cinerea* in tomato by combined application of a new fungicide fluopimomide with a BCA *B. methylotrophicus* TA-1.

Materials and Methods

Fungicides. Fluopimomide (98% a.i.) and its commercial formulation (Kanuoz; 50% a.i., water-dispersible granule) were purchased from Shandong Sino-Agri Union Biotechnology. The structure of fluopimomide is shown in Figure 1. Stock solutions of fluopimomide (20, 50, and 100 mg liter⁻¹) were prepared with ethyl acetate and stored at 4°C before use. Procymidone (98.5% a.i.), used as a positive control in laboratory tests, was purchased from Zhejiang Heben Pesticide & Chemicals Co., Ltd.

Antifungal bacteria TA-1. The bacterial strain *B. methylotrophicus* TA-1 used in this work was previously isolated from the rhizosphere of field-grown tomato in Tai'an, Shandong, China. The bacterial strain TA-1 was identified as *B. methylotrophicus* based on morphological, biochemical, and molecular characterization (Cheng et al. 2019). *B. methylotrophicus* TA-1 was stored in 20% glycerol at -80°C. Nutrient agar (NA; 5 g peptone, 5 g sodium chloride, 1.5 g beef extract, 1.5 g yeast extract, and 15 g per liter agar) plates were used as the standard growth medium for *B. methylotrophicus* TA-1, and it was cultured at 30°C until colonies formed.

A bacterial suspension of *B. methylotrophicus* TA-1 was prepared as previously described (Cheng et al. 2019). Bacterial cells were added to nutrient broth (NB; NA without agar) medium and incubated for 24 h at 30°C with shaking (180 rpm) until the logarithmic growth phase was reached (Lin et al. 2014). Then, bacterial cells were harvested by centrifugation at 5,000 rpm for 5 min in a centrifuge (CS150GXL; Hitachi), the supernatant was discarded, and the pellets were washed and resuspended in sterile distilled water to obtain a final bacterial population density of 10⁹ colony-forming units (cfu) ml⁻¹. Finally, isotonic saline water (0.85% NaCl) was used to dilute the suspension to the concentration required (Zhou et al. 2011).

Causal agent *B. cinerea*. *B. cinerea*, originally isolated from naturally infected tomato plants, was maintained at 25°C on potato dextrose agar (PDA; 1 liter potato leachate, 20 g glucose, and 15 g agar). Conidia of *B. cinerea* were obtained by washing the surface of the culture with sterile distilled water containing Tween-80 (0.1% vol/vol). Four layers of sterile cheesecloth (100-μm pore size) were used to screen the suspension, and a hemocytometer (Hausser Scientific) was used to count the spores and obtain a final concentration of 10⁵ spores ml⁻¹.

Compatibility of fluopimomide with *B. methylotrophicus* TA-1. After *B. methylotrophicus* TA-1 was prepared in NB medium in cultures of shake flasks at 28°C for 48 h, 150 μl of the culture was added to each of 40 flasks (100 ml) containing 20 ml of NB medium. Ten flasks without fluopimomide were used as control. The other 30 flasks were equally supplemented with 20, 50, and 100 mg liter⁻¹

fluopimomide. After 6, 12, 24, and 36 h of shake incubation (28°C at 180 rpm), a 1-ml solution from each flask was serially diluted 10-fold and plated on NA for colony enumeration.

In vitro tests of fluopimomide in combination with *B. methylotrophicus* TA-1 for inhibiting mycelial growth of *B. cinerea*. Plugs (5 mm in diameter) were cut using a sterile cork borer from the edge of *B. cinerea* colonies growing on PDA plates; then, the plugs were transferred onto fresh PDA plates containing fluopimomide alone, *B. methylotrophicus* TA-1 alone, or both fluopimomide and *B. methylotrophicus* TA-1. Doses of fluopimomide were 0.625, 1.25, 2.5, 5, and 10 mg liter⁻¹, and the concentration of *B. methylotrophicus* TA-1 was 10⁸ cfu ml⁻¹. PDA plates without fluopimomide or *B. methylotrophicus* TA-1 were included as controls. Three replicates were performed per treatment, and the plates were incubated in the dark at 25°C. When the mycelia of *B. cinerea* colonies in the controls had covered one-half of the agar surface of the control plates (about 3 days), the diameters (minus the plug diameter of 0.5 cm) of the *B. cinerea* colonies were determined. Inhibition was expressed as a percentage of the control: 1 - [(diameter of treated colonies - 0.5)/(diameter of control colonies - 0.5) × 100]. The experiments were conducted three times.

Greenhouse pot experiments. Pot experiments were conducted under greenhouse conditions in square 12-cm-diameter pots containing a silt loam soil collected from a greenhouse at Shandong Agricultural University in Tai'an, China. The soil had an organic matter content of ~21.2 g kg⁻¹, a bulk density of 1.2 g cm⁻³, and a pH of 7.2. *B. methylotrophicus* TA-1 inoculum was produced in NB in flasks on a shaker (Guohua Electric Appliance Co., Ltd.) at 180 rpm and 28°C for 48 h. The suspension of *B. methylotrophicus* TA-1 in NB (10⁹ cfu ml⁻¹) was diluted with sterile distilled water to obtain a final concentration of 10⁸ cfu ml⁻¹ for application. Fluopimomide was applied with or without *B. methylotrophicus* TA-1 at 50 and 100 g ha⁻¹.

Forty-day-old tomato (cultivar Jinpengwuxian) seedlings of similar growth were purchased from Xi'an Jinpeng Seedling Co., Ltd. and transplanted to the pots. Then, the seedlings were treated with *B. methylotrophicus* TA-1 alone (10⁸ cfu ml⁻¹), fluopimomide alone (50 or 100 g ha⁻¹), or *B. methylotrophicus* TA-1 (10⁸ cfu ml⁻¹) plus fluopimomide (50 or 100 g ha⁻¹). Each treatment was sprayed uniformly with 100 ml solution until runoff, and 0.1% Tween-80 was included as a surfactant. Sterile water with 0.1% Tween-80 was used as an untreated control. All tomato plants were inoculated with *B. cinerea* 1 day after treatment by spraying 30-ml *B. cinerea* spore suspensions (10⁵ spores ml⁻¹). The experiment was repeated three times, with 10 pots per treatment in each replication. All plants were maintained in a greenhouse at 25°C and 90% relative humidity with a 16-h:8-h photoperiod of light to dark, respectively.

Seven days after *B. cinerea* inoculation (when gray mold in the untreated control plots reached 100% incidence), the disease severity index and effectiveness were calculated. The disease severity index of *B. cinerea* on tomato leaves was rated on a scale of 0 to 4, where 0 = no disease symptom, 1 = 0.1 to 5%, 2 = 5.1 to 20%, 3 = 20.1 to 40%, and 4 = 40.1 to 100% (the percentage of diseased leaf area) (Lee et al. 2006). The disease severity value was calculated using the following formula: Disease severity (%) = (Σ(the number of diseased leaves × disease severity index)/(4 × the number of leaves rated)) × 100. The disease control efficacy was calculated using the following formula: Disease control efficacy (%) = ((A - B)/A) × 100, where A is the disease severity of control and B is the disease severity of treatment. Plant height and fresh root weight were measured 30 days after *B. cinerea* inoculation, and fruit yields were measured when tomatoes matured. The experiments were conducted three times.

Field trials. Field trials were conducted on a commercial tomato farm during the growth seasons of 2017 and 2018 in Tai'an (Shandong, China), which had a warm temperate climate with a yearly average temperature of 12.8°C, total illumination of 2,627.1 h, and precipitation of 697 mm. The farm had been managed for conventional tomato production for 12 years before this experiment, and it had severe natural *B. cinerea* infestation. The farm soil was silt loam with an organic matter content of ~20.1 g kg⁻¹ soil, a pH of 7.1, and a

Fig. 1. The diagram of fluopimomide chemical structure.

bulk density of $\sim 1.5 \text{ g cm}^{-3}$. The field was disked twice; the beds were spaced at 0.5 m apart, and center to center was 0.8 m wide $\times$ 0.2 m high.

Individual 30-m² consisting of four neighboring rows were laid out in a randomized complete block design with three replicates. Twenty 40-day-old Jinpengwuxian tomato seedlings of similar growth were selected and transplanted on the top of the beds at in-row spacing of 0.5 m; ~ 95 tomato plants were grown in each plot. Each treatment was sprayed with treatments or water at 450 liters ha⁻¹ amended with 0.1% Tween-80. Treatments were (a) fluopimomide at 50 g ha⁻¹, (b) fluopimomide at 100 g ha⁻¹, (c) *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹, (d) fluopimomide at 50 g ha⁻¹ plus *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹, (e) fluopimomide at 100 g ha⁻¹ plus *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹, and (f) an untreated control (water with 0.1% Tween-80). Inoculum of *B. methylotrophicus* TA-1 was prepared in NB as described above.

All sprays were with a Jacto HD 400 sprayer (Agrolex) for the treatments. The first sprays for 2017 and 2018 were applied on April 15, 2017 and April 10, 2018, respectively, when the tomato plants started showing gray mold symptoms. The treatments were repeated three times at 7-day intervals, and the disease severity was assessed on the seventh day after the final spray. No other fungicides were applied during the experiment. Herbicides and fertilizers were applied according to the standard farm practices. Plants were tied and irrigated as needed. The disease severity index of gray mold and control efficacy were calculated as described previously. Marketable tomato fruits were harvested twice (12 and 14 weeks after transplanting) when tomatoes were mature.

Data analysis. Data were transformed to percentage of reduction from the untreated control and the synergistic or antagonistic responses of the two treatments calculated according to the Colby formula: $E = I_L + I_T - I_L I_T / 100$, where I_L is the percentage inhibition of growth by fluopimomide at dose C_L and I_T is the percentage inhibition of growth by *B. methylotrophicus* TA-1 at dose C_T (Colby 1967). E is the expected percentage inhibition of growth by *B. methylotrophicus* TA-1 plus fluopimomide at the dose of $C_L + C_T$. When the expected response is less than the observed, the combination is synergistic; when it is greater than observed, it is antagonistic.

The SPSS statistic program (V17; IBM SPSS Statistics) was used for the analysis of variance by the Student–Newman–Keuls test at the significant level of $P \leq 0.05$.

Results

Compatibility of fluopimomide with *B. methylotrophicus* TA-1.

The growth of *B. methylotrophicus* TA-1 was not affected by fluopimomide in NB medium. *B. methylotrophicus* TA-1 was able to grow in NB medium containing 20, 50, and 100 mg liter⁻¹ fluopimomide after 6, 12, 24, and 36 h of incubation, and the viable count of *B. methylotrophicus* TA-1 was not different compared with that of the untreated control (Table 1). Growth of *B. methylotrophicus* TA-1 in the NB medium with 100 mg liter⁻¹ fluopimomide was similar to that in the control, which suggested that the *B. methylotrophicus* TA-1 and fluopimomide were compatible as an integrated treatment.

In vitro tests of fluopimomide in combination with *B. methylotrophicus* TA-1 for inhibiting mycelial growth of *B. cinerea*. On PDA plates, both fluopimomide and *B. methylotrophicus* TA-1 alone significantly ($P < 0.05$) inhibited the mycelial growth of *B. cinerea* (Table 2). Inhibition was significantly increased when

fluopimomide and *B. methylotrophicus* TA-1 were combined. For example, inhibition was 67.09% with *B. methylotrophicus* TA-1 alone at 10^8 cfu ml⁻¹ and 21.96% with fluopimomide alone at 1.25 mg liter⁻¹, but it was 90.17% when both treatments were applied together. The combination of fluopimomide and *B. methylotrophicus* TA-1 showed a synergistic effect according to the Colby formula (Colby 1967).

Greenhouse experiments. In the greenhouse experiments, fluopimomide at 50 and 100 g ha⁻¹ each in combination with *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹ significantly reduced tomato gray mold severity and exhibited remarkable control effect against the disease with control efficacies of 70.16 and 69.32%, respectively (Table 3). Treatment of fluopimomide at 50 and 100 g ha⁻¹ and *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹ each inhibited the disease, with control efficacies of 56.10, 64.32, and 58.80%, respectively. Control efficacy was significantly higher for the combination of fluopimomide and *B. methylotrophicus* TA-1 than either alone. In addition, the combination of both treatments significantly increased plant height and fresh root weight compared with the treatment of fluopimomide at 50 g ha⁻¹. All treatments were able to improve tomato fruit yield; however, the combination of fluopimomide and *B. methylotrophicus* TA-1 displayed the greatest yield-increasing effect, which reached 47.25%. There was no significant difference between fluopimomide at 50 and 100 g ha⁻¹ each in combination with *B. methylotrophicus* TA-1.

Field trials. Field trials conducted in 2017 and 2018 demonstrated that all five treatments could reduce the disease severity and provide substantial disease control (Fig. 2). Among these treatments, *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹ combined with fluopimomide at 50 or 100 g ha⁻¹ resulted in lower disease severities, which were 6.39 and 4.17% in 2017, respectively, and 8.33 and 5.56% in 2018, respectively, whereas in the untreated control, disease severities were 27.22% in 2017 and 27.98% in 2018. In 2017, according to the Colby formula, the expected inhibition of *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹ combined with fluopimomide at 50 g ha⁻¹ was 73.98%, which was lower than the observed control efficacy (78.57%), indicating that a synergistic effect existed between these two treatments (Table 4). Similarly, the control efficacy of fluopimomide at 100 g ha⁻¹ in combination with *B. methylotrophicus* TA-1 at 10^8 cfu ml⁻¹ reached 85.71%, higher than the expected inhibition (83.16%), with no significant differences to the combination of fluopimomide at 50 g ha⁻¹ and *B. methylotrophicus* TA-1; the combinations also showed a synergistic effect. All treatments were able to improve tomato fruit yield, and the combination of fluopimomide at 100 g ha⁻¹ and *B. methylotrophicus* TA-1 displayed the greatest effect of yield increasing, which reached >40%, compared with the untreated control. The results of 2018 field trials exhibited a similar trend as those of 2017 trials. The observed control efficacy of the combined treatments was higher than control as expected, indicating a synergetic effect of the combination and that fluopimomide combined with *B. methylotrophicus* TA-1 could be used to control tomato gray mold effectively.

Discussion

Constant development of pathogen adaptability and resistance to routine fungicides has prompted urgent needs for new fungicides in vegetable production. Fluopimomide is a highly efficient and broad-spectrum fungicide with a new mode of action targeting many

Table 1. Effect of fluopimomide on growth of *Bacillus methylotrophicus* TA-1 in nutrient agar medium

Concentration of fluopimomide (mg liter ⁻¹)	Total viable count of TA-1 ($\times 10^8$ cfu ml ⁻¹) ^y			
	6 h	12 h	24 h	36 h
0	65.67 $\pm$ 0.88 a ^z	91.33 $\pm$ 2.73 a	113.67 $\pm$ 1.20 a	88.3 $\pm$ 2.03 a
20	65.33 $\pm$ 1.45 a	84.33 $\pm$ 2.33 a	116.67 $\pm$ 3.38 a	86.67 $\pm$ 1.45 a
50	68.67 $\pm$ 1.45 a	87.67 $\pm$ 2.33 a	119.67 $\pm$ 0.88 a	93.33 $\pm$ 2.96 a
100	64.33 $\pm$ 1.86 a	82.67 $\pm$ 1.45 a	118.67 $\pm$ 0.88 a	86.33 $\pm$ 1.45 a

^y cfu, colony-forming unit.

^z Values in columns followed by the same letters are not significantly different according to Student–Newman–Keuls ($P = 0.05$).

plant diseases. Zhang et al. (2013a) reported that fluopimomide had a broader spectrum of fungicidal activities than fluopicolide and possessed high fungicidal activity against *R. solani* and *B. cinerea*, with an EC₅₀ value of <1.0 mg liter⁻¹. Fluopimomide also demonstrated great efficacy in field trials to control cucumber downy mildew and rice sheath blight (Zhang et al. 2013b, 2014b). The primary mechanism of fluopimomide was investigated, and the results suggested that fluopimomide exhibited multiple modes of action, including the impairment of the energy generation system and the influence on the cell membrane permeability (He et al. 2016; Zhang et al. 2013a). To provide guidance for the application of this novel fungicide, multiple studies have been conducted to investigate the dissipation and residue of fluopimomide in different matrixes (Feng et al. 2017; Zhang et al. 2014a). The Quick, Easy, Cheap, Effective, Rugged, and Safe method using gas chromatography and electron capture detection was also

used to determine the dissipation kinetics and residue levels of fluopimomide in cucumber (Li et al. 2015). Results indicated that the dissipation of fluopimomide followed pseudofirst-order kinetics, with the half-life of 2.8 to 4.2 days and a maximum residue limit of 0.5 mg kg⁻¹.

Development of resistance to chemical fungicides in the pathogen populations and associated environmental issues may arise after long-term use of chemical fungicides. Biological control is widely recognized as a safe and reliable solution to these concerns. *Bacillus* species-based BCAs are pesticides of natural origin, and their complex mechanisms minimize the risk of potential resistance under selection pressure (Kloepper et al. 2004). Wang et al. (2013) reported that the bioorganic fertilizers BIO6 produced with *Bacillus amyloliquefaciens* W19 strain could effectively suppress Fusarium wilt disease of banana and promote plant growth. *B. methylotrophicus* has been considered to be a promising BCA with varied capabilities.

Table 2. Growth of *Botrytis cinerea* on potato dextrose agar plates as affected by fluopimomide and *Bacillus methylotrophicus* TA-1 alone and in combination^w

Treatment and concentration (mg liter ⁻¹ or cfu ml ⁻¹)	Colony diameter (cm) ^x	Inhibition observed (%) ^y	Inhibition expected (%)	Difference ^z
Fluopimomide				
0.625	7.31 b	6.29 n	—	—
1.25	6.09 d	21.96 l	—	—
2.5	4.23 f	45.73 j	—	—
5	3.04 h	61.03 h	—	—
10	0.73 m	97.08 b	—	—
TA-1				
10 ⁷	5.03 e	35.47 k	—	—
10 ⁸	2.57 j	67.09 f	—	—
2.5 × 10 ⁸	1.17 l	85.04 d	—	—
Fluopimomide + TA-1				
0.625	1.90 k	75.64 e	69.16	+6.48
1.25	0.77 m	90.17 c	74.32	+15.85
2.5	0.5 n	100 a	82.14	+17.86
5	0.5 n	100 a	87.17	+12.83
10	0.5 n	100 a	99.04	+0.96
Procymidone				
2.5	7.36 b	5.63 n	—	—
5	6.20 c	20.47 m	—	—
10	4.10 g	47.42 i	—	—
20	2.84 i	63.58 g	—	—
40	0.76 m	96.70 b	—	—
Control				
0	7.80 a	—	—	—

^w Values in columns followed by the same letters are not significantly different according to Student–Newman–Keuls ($P = 0.05$). cfu, colony-forming unit.

^x Minus the diameter of the initial plug. Each value is the mean of three independent experiments.

^y Relative to the control. Each value is the mean of three independent experiments.

^z Differences (the percentage reduction observed minus the percentage reduction expected) with a plus sign indicate synergistic effect.

Table 3. Effect of fluopimomide and *Bacillus methylotrophicus* TA-1 (10⁸ colony-forming units ml⁻¹) against tomato gray mold in the greenhouse alone and in combination

Treatment	Plant height (cm)	Root fresh weight (g)	Disease severity (%) ^y	Control efficacy (%)	Fruit yield (g plant ⁻¹)	Yield growth rate (%)
TA-1	40.1 ± 0.81 c ^z	1.27 ± 0.03 b	21.40 ± 0.55 bc	58.80 ± 1.06 bc	537.1 ± 10.65 c	31.29
Fluopimomide (50 g ha ⁻¹)	41.1 ± 0.84 bc	1.21 ± 0.05 b	22.80 ± 1.03 b	56.10 ± 1.97 c	530.6 ± 10.08 c	29.70
Fluopimomide (100 g ha ⁻¹)	42.9 ± 0.18 ab	1.31 ± 0.02 ab	18.53 ± 1.07 cd	64.32 ± 2.06 b	568.5 ± 7.67 b	38.50
Fluopimomide (50 g ha ⁻¹) + TA-1	43.3 ± 0.67 a	1.40 ± 0.04 a	15.50 ± 0.70 d	70.16 ± 1.35 a	602.5 ± 10.78 a	47.25
Fluopimomide (100 g ha ⁻¹) + TA-1	44.4 ± 0.45 a	1.42 ± 0.03 a	15.93 ± 0.45 d	69.32 ± 0.86 a	589.7 ± 2.32 ab	44.14
Control	36.4 ± 0.49 d	0.94 ± 0.05 c	51.94 ± 1.74 a	—	409.2 ± 10.99 d	—

^y Relative to the control.

^z Values are the means ± standard error of 10 replicates. Values in columns followed by same letters are not statistically different by Student–Newman–Keuls ($P = 0.05$).

B. methylotrophicus L7 was reported to be a gram-positive bacterial strain that exhibited efficient heterotrophic nitrification-aerobic denitrification ability (Zhang et al. 2012). *B. methylotrophicus* BC79 produced phenaminomethylacetic acid, and this bacterium was used to control rice blast (Shan et al. 2013). *B. methylotrophicus* KE2

produced gibberellins, promoting plant growth and enhancing nutritional metabolites and pigment contents of lettuce (Radhakrishnan and Lee 2016). Our previous study demonstrated that *B. methylotrophicus* TA-1 was a potential biocontrol agent against maize stalk rot (Cheng et al. 2019).

Fig. 2. Effect of fluopimomide and *Bacillus methylotrophicus* TA-1 alone and in combination against tomato gray mold in the field in **A**, 2017 and **B**, 2018. CK, control; 50, fluopimomide at 50 g ha⁻¹; 100, fluopimomide at 100 g ha⁻¹; TA-1, TA-1 at 10⁸ colony-forming units (cfu) ml⁻¹; TA-1 + 50, TA-1 at 10⁸ cfu ml⁻¹ in combination with fluopimomide at 50 g ha⁻¹; TA-1 + 100, TA-1 at 10⁸ cfu ml⁻¹ in combination with fluopimomide at 100 g ha⁻¹.

Table 4. Effect of the combination of fluopimomide and *Bacillus methylotrophicus* TA-1 (10⁸ colony-forming units ml⁻¹) on control tomato gray mold in the field in 2017 and 2018^a

Treatment	2017				2018			
	Control effect ^y (%)	Control expected (%)	Difference ^z	Yield (t ha ⁻¹)	Control effect (%)	Control expected (%)	Difference	Yield (t ha ⁻¹)
TA-1	57.14 ± 6.18 cd	—	—	64.98 ± 0.92 d	58.01 ± 6.45 bc	—	—	61.88 ± 1.21 b
Fluopimomide (50 g ha ⁻¹)	39.28 ± 3.57 d	—	—	63.80 ± 0.83 d	32.17 ± 5.59 d	—	—	58.50 ± 4.29 b
Fluopimomide (100 g ha ⁻¹)	60.71 ± 9.45 bc	—	—	69.63 ± 1.72 c	54.83 ± 4.38 c	—	—	62.18 ± 1.86 b
Fluopimomide (50 g ha ⁻¹) + TA-1	78.57 ± 6.19 ab	73.98	+4.59	71.88 ± 0.91 b	77.39 ± 8.54 ab	71.52	+5.87	69.09 ± 1.54 a
Fluopimomide (100 g ha ⁻¹) + TA-1	85.71 ± 3.57 a	83.16	+2.55	75.60 ± 0.98 a	83.85 ± 6.46 a	81.03	+2.82	70.76 ± 1.71 a
Control	—	—	—	52.90 ± 1.32 e	—	—	—	49.18 ± 4.83 c

^a Values in columns followed by the same letters are not significantly different according to Student–Newman–Keuls ($P = 0.05$).

^y Relative to the control.

^z Differences (the percentage reduction observed minus the percentage reduction expected) with a plus sign indicate synergistic effect.

Antimicrobial peptides (AMPs) are the major antimicrobial compounds secreted by *Bacillus* species, and they play key roles in the control of plant pathogens (Ongena and Jacques 2008; Sun et al. 2018). Many studies have been reported that *B. methylotrophicus* strains can produce one or more antifungal lipopeptides, which have been proven to effectively suppress pathogenic plant fungi (Zhang et al. 2015). Tanaka et al. (2015, 2017) demonstrated that lipopeptides and prumycin were major compounds responsible for suppression of cucumber gray mold and powdery mildew diseases by *B. amyloliquefaciens* SD-32. In our previous study, the presence of AMP biosynthetic genes *Sfp*, *ItuD*, *SrfC*, *FenD*, and *BamC* in *B. methylotrophicus* TA-1 was determined by polymerase chain reaction. Furthermore, surfactin and iturin were detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry techniques (Cheng et al. 2019). The broad range of antifungal activities and other advantages of BCAs as mentioned previously make them ideal substitutes to synthetic fungicides (Kim et al. 2015; Liu et al. 2015).

Although BCAs alone are effective against gray mold and reduce the risk to the environment, their efficacy is usually unstable under field conditions and can be affected by many factors (Peng et al. 2014; Yu et al. 2017). Knowledge of the persistence and behavior of *B. methylotrophicus* TA-1 under field conditions is important to determine the timing and method of application (Vilanova et al. 2018). In addition, using BCAs in integrated pest management systems is often made difficult by their incompatibility with synthetic fungicides. In this study, the growth of *B. methylotrophicus* TA-1 in the NB liquid medium was not affected by fluopimomide at up to 100 mg liter⁻¹ (Table 1), indicating the full compatibility of *B. methylotrophicus* TA-1 with fluopimomide. Furthermore, a synergistic effect was detected in this study by integration of fluopimomide with *B. methylotrophicus* TA-1 (Tables 2 and 4). Two hypotheses have been proposed that could be used to explain the synergistic effect (Liu et al. 2018). First, fluopimomide may facilitate the biofilms formation of *B. methylotrophicus* TA-1 on plant surfaces, increasing the bacterial resistance to stress under varying conditions. Second, another hypothesis is that tomato plants may produce substances that promote *B. methylotrophicus* TA-1 survival in the presence of fluopimomide. Although the risks of environment pollution and resistance development to synthetic fungicides in the pathogens exist, plant disease control is largely based on chemical fungicides (Walters et al. 2013). Integration of chemicals and biocontrol agents could be a more promising strategy than the use of fungicides or BCAs alone (Rotolo et al. 2018). Our results also demonstrated that control of gray mold of tomato can be significantly improved by combining application of fluopimomide with *B. methylotrophicus* TA-1.

In conclusion, results from this study revealed that the biocontrol agent *B. methylotrophicus* TA-1 was fully compatible with the novel fungicide fluopimomide and that the integrated use of fluopimomide and *B. methylotrophicus* TA-1 showed a synergistic effect in laboratory and field trials. Integration of fluopimomide with *B. methylotrophicus* TA-1 provides the opportunity to reduce the amounts of fluopimomide application. Control efficacy of fluopimomide at 50 g ha⁻¹ in combination with *B. methylotrophicus* TA-1 at 10⁸ cfu ml⁻¹ was higher than the expected inhibition, with no significant differences in the combination of fluopimomide at 100 g ha⁻¹ combining *B. methylotrophicus* TA-1 at 10⁸ cfu ml⁻¹. Results presented here indicated that the use of fluopimomide in combination with *B. methylotrophicus* TA-1 could be a useful tool against *B. cinerea*. Additional investigation to monitor the environmental fate and behavior of *B. methylotrophicus* TA-1 in the field can help to determine the optimal timing and the method of this BCA application to control gray mold in tomato production.

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