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Biological Control of Downy Mildew and Yield Enhancement of Cucumber Plants by *Trichoderma harzianum* and *Bacillus subtilis* (Ehrenberg) under Greenhouse Conditions

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Abstract: The downy mildew disease of cucurbits is considered the most economically damaging disease of Cucurbitaceae worldwide. The causal agent, *Pseudoperonospora cubensis* (Berkeley & Curtis), may cause complete crop losses of cucurbits. Few commercial cucurbit cultivars are resistant to this disease. Commercially, *P. cubensis* is controlled primarily with synthetic fungicides that inhibit or eliminate the pathogen. Several biological agents have also been identified that provide some level of control. In our study, foliar applications of three strains of *Trichoderma harzianum* and two native strains of *Bacillus subtilis* were evaluated for the control of the disease on cucumber plants grown under commercial greenhouse conditions. The study was conducted using a completely randomized design with six individual treatments during two production cycles: fall 2015 and spring 2016. The response variables included disease incidence and severity, plant height, total yield, fruit quality, and weight. *B. subtilis* provided the best control over the incidence and severity of the disease in both production cycles. Interestingly, while *T. harzianum* was less effective at controlling the disease, it enhanced plant growth and productivity, and produced a higher number of better-quality fruits per plot. This increased yield with higher quality fruits may result in higher profit for the growers.

Keywords: beneficial microorganisms; *Cucumis sativus* L.; integrated pest management; *Pseudoperonospora cubensis* (Berkeley & Curtis); resistance; virulence

1. Introduction

Cucumber (*Cucumis sativus* L.) is the third most important vegetable crop produced under protected agriculture conditions in Mexico. Currently, 10 percent of the total greenhouse area is used for cucumber production, after tomatoes (70%) and bell peppers (16%). Under greenhouse conditions, the yield of cucumber plants is affected by several biotic and abiotic factors [1,2].

The cucumber crops are affected by the downy mildew disease of cucurbits. This disease is the most economically damaging disease of Cucurbitaceae worldwide [3]. The causal agent is *Pseudoperonospora cubensis* (Berkeley & Curtis), an obligate oomycete [4]. This pathogen may cause complete crop losses in cucumber, melon, watermelon, and pumpkin [5,6]. Over the past three decades, *P. cubensis* has resurged around the world. New genotypes, races, pathologists, and mating types have been identified [4,7,8]. During the last decade, the pathogen has become more detrimental, and currently, it causes greater disease severity. Weather factors affect the infection and disease development of the downy mildew. Foliar necrosis appears more quickly under hot and dry weather. However, low temperature and high humidity conditions do not stop the infection process [8,9]. The exact influence of these factors on the daily infection of the pathogen has not been fully determined [10].

P. cubensis has plant specialization that affects a wide range of Cucurbitaceae hosts. Pathogen virulence can be classified into pathogenic types based on their compatibility with the differential set of cucurbit hosts. The genetic basis of the specialization of the hosts of *P. cubensis* is not yet known. Nonetheless, the diversity and high virulence complexity of *P. cubensis* within the pathogen population indicate that host resistance is not effective in controlling downy mildew of the cucurbits for the available commercial Cucurbitaceae cultivars [3,4,11].

Control of the downy mildew disease of cucurbits requires an integrated approach that involves a combination of synthetic and biological fungicides, along with the introduction of resistant cultivars [3]. Currently, few commercial cultivars are resistant to the downy mildew disease. Thus, synthetic fungicides that inhibit or eliminate the pathogen are the primary method of control [7,12]. The widespread use of fungicides has created problems that include water and soil pollution, toxicity to animals and humans, and the generation of resistance by *P. cubensis* [13]. Recently, several antagonistic beneficial microorganisms of the pathogenic fungus have been identified. Among them, several species and strains of the genus *Trichoderma* spp. and *Bacillus subtilis* have been shown to control downy mildew under experimental laboratory conditions [14]. *Trichoderma* spp. has been reported to increase plant immunity against invasive pathogens [15]. The microorganisms used for the biological control of downy mildew present different modes of action for pathogen contention. These mechanisms include mycoparasitism, competition for space and nutrients, induced systemic resistance (ISR), and antibiosis mediated by the secretion of cell wall degrading enzymes. Reports on *Trichoderma* and *Bacillus subtilis* indicate that both microorganisms use all these mechanisms to control fungal diseases in plants under in vitro and greenhouse conditions [16,17].

The objective of this research was to evaluate the effectiveness of three strains of *Trichoderma* spp. and two of *Bacillus subtilis* for the control of *Pseudoperonospora cubensis* (downy mildew of cucurbits) and their effects on the yield and quality of cucumber crops grown under commercial greenhouse production conditions.

2. Materials and Methods

2.1. Study Area

The experiments were carried out under protected agriculture conditions in a plastic greenhouse located in Jaral del Progreso, Guanajuato (20.37° N, 101.067° W, and altitude 1735 m). This area has a humid subtropical climate according to the Köppen–Geiger weather classification system. Average temperatures are 18.5 °C, with minimum and maximum temperatures of 5 °C and 35.2 °C, respectively. The annual average rainfall is 687 mm, with February as the driest month (7 mm on average) and August with the highest precipitation (148 mm on average).

2.2. Crop Management and Application of Microorganisms

Two different cucumber crops were established during the fall–winter 2015–2016 (FW) and the spring–summer 2016 (SS) production cycles. The cucumber cultivars used for this study were the American type ‘Paraiso’ for the FW cycle and the Persian type ‘Kathrina’ for the SS cycle (Enza Zaden, http://www.enzazaden.com.mx, accessed on 15 September 2015). Seeds were planted in 50 cavity trays in August and March for the FW and SS cycles, respectively. Plants were transplanted 15 d later directly into the soil of the greenhouse. The greenhouse soil was a clay loam texture with a pH of 7.36, electrical conductivity of 2.32 $dS{\cdot}m^{-1}$, and 2.04% of organic matter. The soil contained 92.3 ppm of P, 25.7 ppm of NO_3^-, 597 ppm of K^+, 3244 ppm of Ca, 896 ppm of Mg, and 237 ppm of Na. Both cultivars were transplanted at a 2 m distance between rows and 0.4 m between plants in a double row, at a planting density of 2.5 plants·m^{-2}. Plant nutrition was administered using the Steiner nutrient solution using: $Ca(NO_3)_2{\cdot}4H_2O$, KNO_3, $MgSO_4{\cdot}7H_2O$, K_2SO_4, KH_2PO_4, and H_2SO_4. The concentrations of anions and cations of each nutrient expressed in mol$_c$·m^{-3} present in the Steiner solution are shown in Table 1 [18]. Fertilizer solutions were applied daily at rates of 0.5 and 1.2 L·plant^{-1} from 10 DAT to first anthesis, and from the first flower onward, respectively.

Table 1. Steiner nutrient solution.

Ions [1]	Cations molc m^{-3}	Anions molc m^{-3}	Total Ions
K^+	7		
Ca^{2+}	9		20
Mg^{2+}	4		
NO_3^-		12	
SO_4^{2-}		7	20
$H_2PO_4^-$		1	

[1] Ions needed to make the Steiner solution after the ions naturally occurring in the irrigation water were considered. The osmotic potential of the solution was 0.072 MPa, and the EC value was 2.0 dS m^{-1}. The commercial fertilizers used in the nutrient solution were $Ca(NO_3)_2{\cdot}4H_2O$, KNO_3, $MgSO_4{\cdot}7H_2O$, K_2SO_4, KH_2PO_4, and H_2SO_4.

All experimental plants were treated using the commercial practices for the control of the downy mildew disease used by the growers (biweekly applications of Serenade max®, Apolo®, and hydrogen peroxide (Q Basic®). The biological control treatments consisted of two native strains of *Bacillus subtilis* (VOB1 and VOB2) and three strains of *Trichoderma* spp. (VOT1, QLT, and BKNT). Both VOB1 and VOB2 *B. subtilis* strains and the VOT1 *Trichoderma* spp. strain were donated by Dr. Víctor Olalde (CINVESTAV, Unidad Irapuato). QLT was obtained from QLT by Química Lucava S.A. de C.V. (Grupo Lucava, http://grupolucava.com, accessed on 9 May 2015), and BKNT from Biokrone S.A. de C.V., (Biokrone, http://www.biokrone.com, accessed on 24 June 2015). Biological control treatments were also applied on a biweekly basis.

The biological treatments were applied by determining the dose of each strain for each treatment, and for each application, the strain or product was diluted in 5 L of water [19]. Also, Cosmocel®, a penetrating surfactant INEX-A®, was included (1 mL·L^{-1}) (Table 2). The solution was sprayed manually using a number three conical nozzle. All treatments were applied weekly during the phenological cycle of the crop.

Table 2. Treatments applied in cucumber cultivation in the Fall-Winter (2015–2016) and Spring-Summer (2016) cycles.

Treatment		Inoculum (Active Ingredient)	Dosage	Concentration
Control †	(a) Serenade max®	*Bacillus subtilis*	0.8 g·L^{-1}	1×10^9 UFC·g^{-1}
	(b) Apolo®	*Bacillus subtilis*	0.8 g·L^{-1}	1×10^8 UFC·g^{-1}
		Trichoderma harzianum		1×10^7 esp·g^{-1}
		Trichoderma viridae		1×10^7 esp·g^{-1}
		Streptomyces lydicus		1×10^8 UFC·g^{-1}
	(c) Hydrogen peroxide	H_2O_2	0.4 mL·L^{-1}	50%
VOT1		*Trichoderma harzianum*	0.8 g·L^{-1}	1×10^7 UFC·g^{-1}
QLT		*Trichoderma harzianum*	0.32 g·L^{-1}	1×10^7 UFC·g^{-1}
BKNT		*Trichoderma harzianum*	0.32 g·L^{-1}	1.1×10^7 UFC·g^{-1}
VOB1		*Bacillus subtilis*	0.8 mL·L^{-1}	1×10^9 UFC·mL^{-1}
VOB2		*Bacillus subtilis*	0.8 mL·L^{-1}	1×10^7 UFC·mL^{-1}

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® (Bayer: strain QST 713 4.6%. Wettable powder (PH) 1×10^9 UFC·g^{-1}), Apolo® (Arvensis, https://arvensis.com.mx/, accessed on 19 August 2015, *Bacillus subtilis* 1×10^8 UFC·g^{-1}; *Trichoderma harzianum* 1×10^7 esp·g^{-1}; *Trichoderma viridae* 1×10^7 esp·g^{-1}; *Streptomyces lydicus* 1×10^6 UFC·g^{-1}; plant extracts 60.0% p·p^{-1}; Si 2.0% p·p^{-1}), and hydrogen peroxide (Q Basic). UFC: unit-forming colonies.

2.3. Experimental Design

The study was conducted in a commercial greenhouse in which *Pseudoperonospora cubensis* was prevalent. The experimental design consisted of a completely randomized design with six treatments (T) (Table 2) and 100 randomly distributed repetitions per treatment. The biological control strains for each treatment were assigned randomly in both production cycles (FW and SS). The harvest dates of the FW crop cycle for the 'Paraiso' cultivar were at 30, 60, 90, and 120 days after transplant (dat), while the 'Kathrina' cultivar during the SS cycle were at 30, 60, and 90 dat. The experimental units consisted of individual cucumber plants in each of the production systems. Due to the severity of the disease, a control treatment with no chemical applications was not viable for a study under commercial conditions. This situation caused the need for control treatments to reduce the expansion of the disease, senescence of the plant, and yield loss. These treatments allowed us to identify the effectiveness of the proposed microorganisms under commercial conditions in the greenhouse.

The downy mildew disease was evaluated by classifying cucumber plants from each treatment by the level of disease symptoms according to the method described by Ruiz Sánchez et al. (2008) [20]. Disease data were taken at 30, 60, 90, and 120 dat for the FW cycle, and at 30, 60, and 90 dat for the SS cycle, due to the duration of the production cycles. Disease incidence was determined by counting the number of plants with symptoms relative to the total number of plants in each experimental plot. A severity scale was developed using the Horsfall–Barratt method. This method is based on assigning a numerical value based on the percentage of foliar area with disease symptoms. In our study, these percentages were t: 1 = 0%, 2 = 0–3%, 3 = 3–6%, 4 = 6–12%, 5 = 12–25%, 6 = 25–50%, 7 = 50–75%, 8 = 75–88%, 9 = 88–94%, 10 = 94–97%, 11 = 97–100%, 12 = 100% [21] (Figure 1).

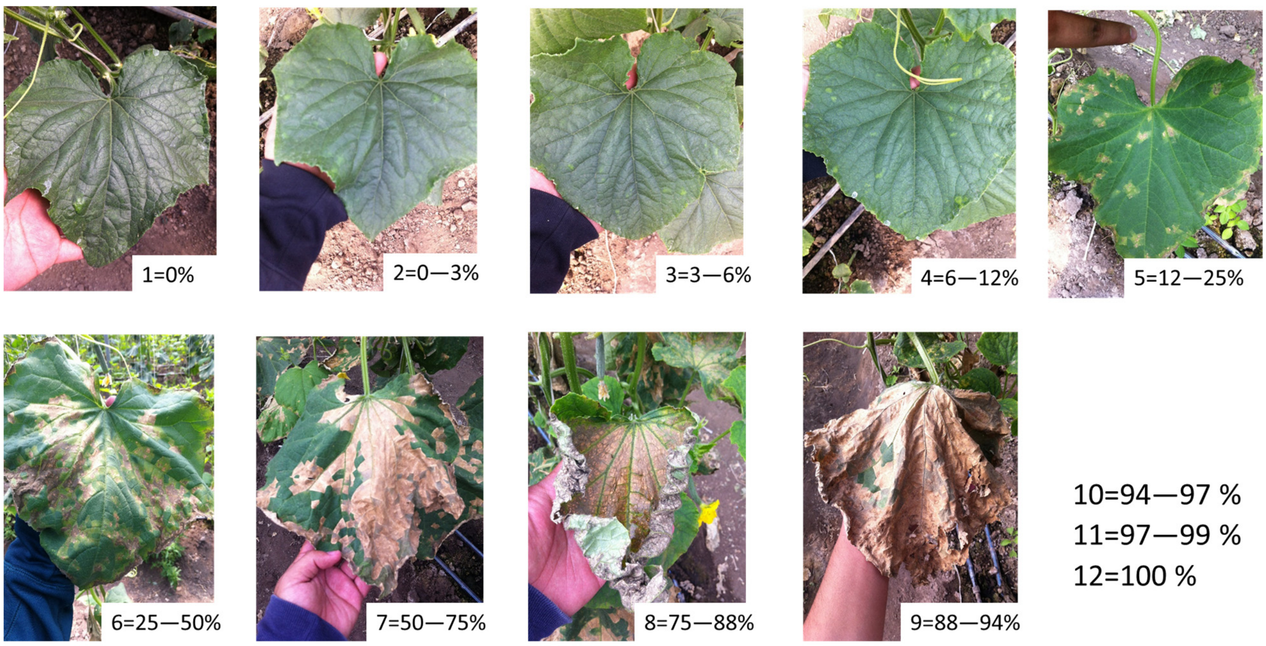

Figure 1. Horsfall–Barratt method for the establishment of severity index of downy mildew in cucumber leaves. Percentage represents the fraction of damage in the leaves.

The yield of cucumber plants for each treatment was determined by harvesting and weighing the cucumbers during the phenological cycle. Harvesting for the 'Kathrina' Persian-type finished at 90 dat and for the 'Paraiso' American-type cucumber at 120 dat. Fruit quality was determined using the standards of a commercial packinghouse (INTEBAJ, http://www.intebaj.com/, accessed on 17 May 2015) (Table 3). Plant height was measured from the base to the apex of the plant.

Table 3. Quality standards for cucumber fruits of the INTEBAJ commercial packinghouse.

Size [†]	'Paraiso'			'Kathrina'		
	First	Second	Third	First	Second	Third
Length (cm)	13.5–15	12–13.5, 15–17	<12, >17	23–25	18–23, 25–30	<18, >30
Width (cm)	3.3–3.5	3–3.3, 3.5–4	<3, >4	5.6–6	5–5.6, 6–6.6	<5, >6.6
Curvature (degree)	0°	10–20°	>20°	0°	20–30°	>30°
Damages (%)	0	<30	>30	0	<30	>30

[†] Based on cucumber fruit quality standards of Terra Bella (California, USA).

2.4. Statistical Analysis

The statistical analysis performed for the variables of yield per cucumber plant, plant height, and individual weight of the cucumbers in a factorial design with a completely randomized design was an analysis of variance, followed by the comparison of means by the Tukey method ($\alpha = 0.05$). Disease severity was evaluated using the Kruskal–Wallis test, followed by the Dunn's method for the comparison of means ($p \leq 0.05$). All analyses were carried out using the statistical analysis system program (SAS Institute, Cary, NC, USA).

3. Results and Discussion

The degree of disease severity was significantly different for both the American and the Persian type cucumbers at 60 dat. The treatments with the best controlling effect were *B. subtilis* VOB1, *B. subtilis* VOB2, and *T. harzianum* QLT, followed by the *T. harzianum* VOT1 strain at 60, 90, and 120 dat. These same strains also showed adequate disease control at 60 and 90 dat for the SS cycle (Table 4). By contrast, the plants that had the highest incidence of downy mildew in cucumber plants were the control and the BKNT strains treatments.

Table 4. Effect of treatments on the degree of severity of downy mildew on cucumber crops during the FW and SS cycles.

Treatment [†]	Cycle FW ('Paraiso')				Cycle SS ('Kathrina')		
	30 dat	60 dat	90 dat	120 dat	30 dat	60 dat	90 dat
Control	1.52 a	2.08 ab	2.2 b	2.49 b	1.58 a	4.57 a	7.91 a
VOT1	1.51 a	1.83 bc	2.0 bc	2.21 bc	1.54 a	3.69 bc	6.15 b
QLT	1.50 a	1.76 c	1.9 c	2.25 bc	1.55 a	3.52 c	6.34 b
BKNT	1.54 a	2.36 a	2.9 a	3.10 a	1.58 a	4.06 ab	
VOB1	1.50 a	1.71 c	1.8 c	2.10 c	1.51 a	3.42 c	6.05 b
VOB2	1.50 a	1.64 c	1.9 c	2.17 c	1.51 a	3.31 c	5.77 b

[†] Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; H_2O_2; VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2. Severity scale: 1 = 0%, 2 = 0–3%, 3 = 3–6%, 4 = 6–12%, 5 = 12–25%, 6 = 25–50%, 7 = 50–75%, 8 = 75–88%, 9 = 88–94%, 10 = 94–97%, 11 = 97–100%, 12 = 100% [21]. The data within the columns with different letters show significant differences in the Dunn's test ($p \leq 0.05$). Dat = days after transplant.

The highest disease severity of downy mildew was observable in the cucumber plants of the control treatments of both American- and Persian-type cucumbers (FW and SS cycles, respectively). In the control treatments, no microorganism types were applied (neither strains of *Trichoderma* and *Bacillus* nor BKNT of *T. harzianum)*. Increased severity of the disease could be observed in the 'Kathrina' cucumber plants, which indicates their low resistance to the presence of *P. cubensis* (Figure 2). These findings are consistent with previous reports in which plant pathogens can be controlled using microbial antagonists [16].

'Kathrina' cultivar plants show the greatest disease severity compared to the plants of the cultivar 'Paraiso' (Table 4). These different susceptibilities could be related to genomic differences between the cultivars. Environmental conditions may also have had an important effect on disease severity as summer was warmer and more humid than the fall, which was drier.

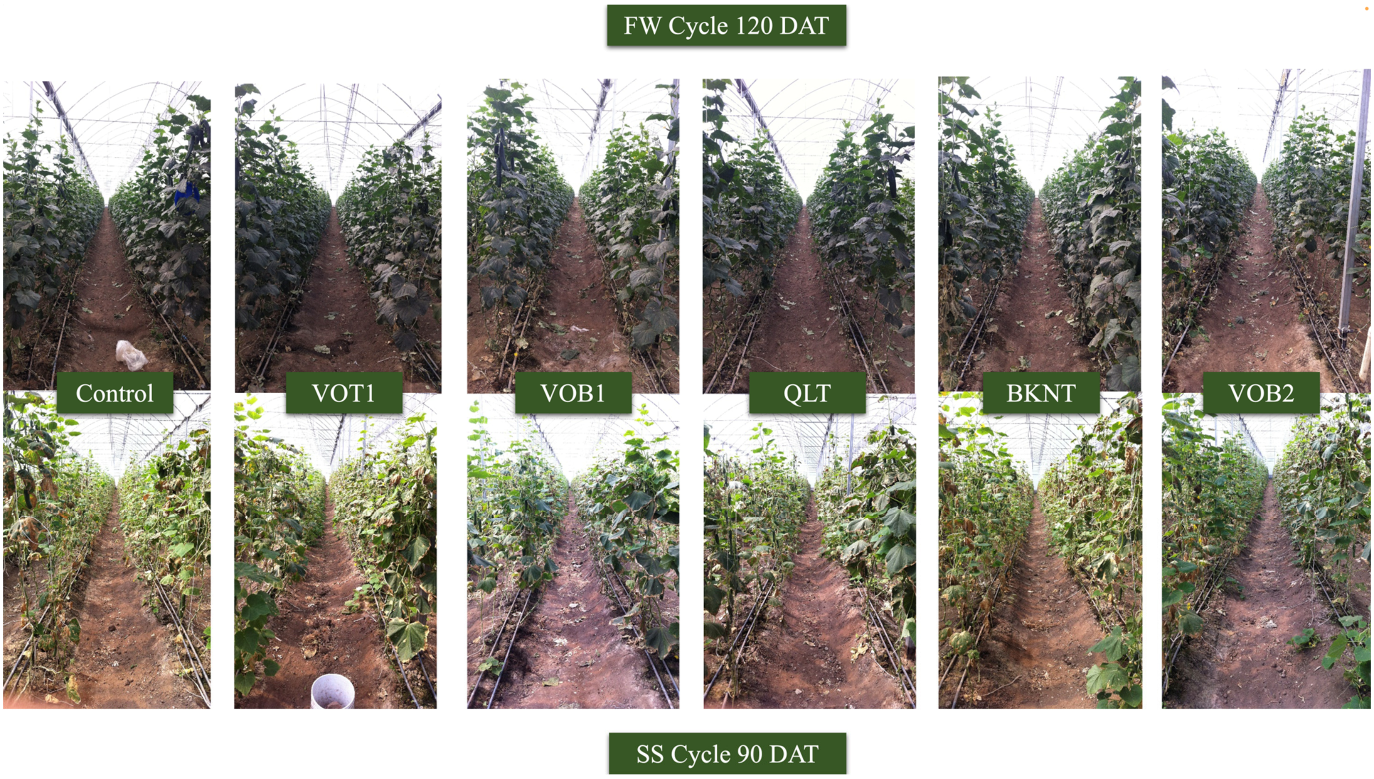

Figure 2. Greenhouse view of each treatment at the end of the fall–winter 2015–16 and spring–summer 2016 production cycles. The treatments were control: Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2.

According to the severity scale developed for downy mildew of cucurbits [21], the VOB1 strain of *B. subtilis* presents the best control of the disease. Similar data were obtained for the VOB2 of *B. subtilis* for the FW and SS cycles.

Bacillus subtilis is considered a broad-spectrum disease-resistant microorganism capable of controlling different strains of pathogens of cucurbits [22]. The suppressive effects on plant pathogens by *B. subtilis* could be related to several mechanisms, including antibiosis, secretion of degrading enzymes, and competition for space and nutrients. *B. subtilis* might also induce the plants to generate systemic resistance and have other positive effects such as enhanced nutrient absorption (mainly N uptake), phosphate solubilization, production of phytohormones and siderophores, and increased plant growth. Enhanced plant nutrient absorption caused by *B. subtilis* may increase the capacity to tolerate the infection. Resistance may be improved by enzymes, or other metabolites independent of the direct action of *B. subtilis* on the pathogen. These factors might influence the improvement in the resistance of the cultivars to colonization by the pathogen [22,23]. To fully understand the mechanism by which *B. subtilis* enhances disease resistance, future studies should consider determining the expression of plant defense resistance genes.

By contrast, the treatment that presents the least amount of control over the downy mildew in the FW cycle is the BKNT strain *of T. harzianum*; and for the SS cycle, the BKNT strain is comparable to the control (Table 5). This indicates the susceptibility of the pathogen to strains of *B. subtilis*, but not to *T. harzianum.* Therefore, the genetic resistance of the host is not effective for the control of the mildew [4].

Fruit yield of cucumber plants is significantly different for the American and Persian types. Interestingly, while the *T. harzianum* VOT1 strain is not the strain that provides the best disease control, it causes a yield increase during both production cycles (FW and SS). (Table 6).

Table 5. Severity percentage of the downy mildew of cucurbits in cucumber crop, during the FW and SS cycles, according to the Horsfall–Barratt method [21] for the development of a severity scale.

Treatment †	Cycle FW ('Paraiso')				Cycle SS ('Kathrina')		
	30 dat	60 dat	90 dat	120 dat	30 dat	60 dat	90 dat
Control	1	29	45	62	5 *	88 *	100 *
VOT1	1	29	43	61	3	78	95
QLT	0	23	37	60	3	80	97
BKNT	2 *	43 *	85 *	100 *	5 *	85	100 *
VOB1	0	18	31	52	1	77	90
VOB2	0	13	31	54	1	76	90

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2. * = greater incidence percentage. Dat = days after transplant.

Table 6. Fruit yield of cucumber plants during FW and SS cycles.

Treatment †	Yield ($kg \cdot m^{-2}$)	
	Cycle FW ('Paraiso')	Cycle SS ('Kathrina')
Control	10.81 bc	8.11 c
VOT1	12.02 a	12.35 a
QLT	10.72 c	8.84 b
BKNT	10.89 bc	8.25 c
VOB1	11.05 b	8.84 b
VOB2	10.16 d	9.04 b

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2. Data in the columns with different letters indicate significant differences in the Tukey test ($p \leq 0.05$).

The VOT1 strain of *T. harzianum* generates the largest cucumber plants in both production cycles. In addition, VOT1-treated plants produce the largest individual fruit weights and total yield of cucumber plants, and the greatest number of fruits per harvest. In the SS cycle, the increase in yield of the VOB2 treatment is 36% higher than the control (Table 7).

Table 7. Effect of treatments on height (cm) of cucumber plants during the FW and SS cycles.

Treatment †	FW Cycle ('Paraiso')				SS Cycle ('Kathrina')		
	30 dat	60 dat	90 dat	120 dat	30 dat	60 dat	90 dat
Control	35.4 bc	88.6 b	134.8 b	187.9 b	129.1 bc	209.0 b	288.1 bc
VOT1	41.2 a	93.2 a	144.2 a	196.5 a	136.3 a	223.6 a	314.8 a
QLT	38.2 ab	88.8 b	132.6 b	186.0 b	140.0 abc	206.8 b	290.0 bc
BKNT	38.7 a	88.5 b	135.7 b	189.5 b	134.2 ab	211.5 b	291.6 b
VOB1	33.2 c	81.8 c	130.1 b	183.1 b	129.0 bc	206.1 b	290.3 b
VOB2	32.4 c	83.7 c	129.3 c	182.9 b	127.4 c	207.2 b	284.3 c

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2. Data in the columns with different letters indicate significant differences in the Tukey test ($p \leq 0.05$). Dat = days after transplant.

The VOB1 and VOB2 strains of *B. subtilis* cause the lowest growth of cucumber plants. The treatments do not significantly affect the number of fruits per harvest. Nevertheless, the VOT1 treatment produces the largest number of fruits per harvest (102.9 fruits at 120 dat in the FW cycle and 152.4 fruits at 90 dat in the SS cycle), followed by the VOB1 strain. The increase in number of fruits per cut induced by the VOT1 strain is 7.5% in the FW cycle and 33% in the SS cycle when compared to the control treatments.

The VOT1 treatment causes plants to produce fruits with the largest individual weights, even at the first harvests, which has a direct impact on cucumber plant yield (Table 8).

Table 8. Effect of treatments on weight (g) of individual cucumber fruits during the FW and SS cycles.

Treatment †	Cycle FW ('Paraiso')		Cycle SS ('Kathrina')		
	90 dat	**120 dat**	**30 dat**	**60 dat**	**90 dat**
Control	346.1 c	347.1 c	95 e	95 e	94.5 d
VOT1	359.6 a	359.6 a	108 a	108 a	108 a
QLT	350 b	349.4 b	98 d	98 d	97.9 c
BKNT	343 d	343.6 d	95 f	95 f	95 d
VOB1	346.5 c	347.8 bc	98 c	98 c	97.9 c
VOB2	340 d	340 e	99 b	99 b	99 b

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* strain VOB2. Data in the columns with different letters indicate significant differences in the Tukey test ($p \leq 0.05$). Dat = days after transplant.

However, the higher yield of cucumber plants of the VOT1 strain is more related to the larger number of fruits than to their individual fruit weights. In the case of 'Kathrina', the number of fruits increases by 33%, compared to an increase of 8% in their weights (Table 9). Previous studies reported similar differences in yield and quality due to changes in the use of varieties during different cycles, even within the same production system [24]. As for the fruit quality variable, we did not find a consistent response in both production cycles (SS and FW) because the quality classification for the type of cucumber (Persian or American) had a considerable influence on our results as quality standards are more rigorous for American than for Persian cucumber.

Table 9. Effect of the treatments on the first ('Premium') and second quality cucumber fruits during the FW and SS cycles.

Treatment †	Yield (kg m^{-2})									
	Cycle FW ('Paraiso')				Cycle SS ('Katrina')					
	90 dat		120 dat		30 dat		60 dat		90 dat	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Control	95.2 e	4.9 a	91.6 a	8.4 a	70.8 a	26 a	73.9 d	23.7 a	74.4 d	22.9 a
VOT1	99.1 a	0.1 e	95.9 a	4.1 c	78 a	20 a	83.9 a	14.3 b	80.3 a	13.9 c
QLT	97.6 bc	2.4 cd	94.8 a	5.2 ab	75.3 a	22.3 a	77.7 bcd	20.4 a	78.1 bc	20.0 a
BKNT	95.9 de	4.1 ab	92.8 a	7.2 ab	70.8 a	25.8 a	75.4 cd	22.9 a	75.9 cd	22.2 a
VOB1	96.6 cd	3.4 bc	93.4 a	6.8 b	75.5 a	21.8 a	78.5 bc	19.2 a	78.9 bc	18.8 b
VOB2	98.4 ab	1.5 de	95.2 a	4.8 bc	76.3 a	21.5 a	80 ab	18.5	80.3 b	18.3 b

† Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* cepa VOB2. Data in the columns with different letters indicate significant differences in the Tukey test ($p \leq 0.05$). Dat = days after transplant.

In addition, adverse weather conditions during the FW cycle caused greenhouse damage and affected the final stage of the crop. This condition caused a reduction in fruits of 'premium' quality and no statistically significant differences were found in the treatments at 120 dat. Nevertheless, the most notable strains were the VOT1 of *T. harzianum* and the VOB2 de *B. subtilis*, which caused a similar response, with the exception that the latter case was at 90 dat during the SS cycle (Table 9).

The commercial value (price) of first quality cucumber (or 'premium') can be up to 50–100% higher than those fruits of second quality. Therefore, the economic profit of cucumber cultivation is directly related to the quantity and quality of the obtained fruits.

In both cycles, the VOT1 strain of *T. harzianum* produced a higher quantity of 'premium' fruits in the SS cycle and a reduced number of second quality fruits. This higher quality crop represents a greater economic gain for the producer since, for the FW cycle, 95% of the 120 tons were of first quality, while for the SS cycle, 80% of 123 tons were also of prime quality. In the FW cycle, plants treated with synthetic fungicides had a yield of 108 t, of which 91% were of first quality, while during the SS cycle 81 t was obtained, 74% of which were of first quality. The treatments that presented the greatest amount of second quality fruits were the control, QLT, and BKNT. The latter (QLT and BKNT) were treated with strains of *T. harzianum* (Table 9). The effects of the treatments on third quality fruits were not significant (Table 10).

Table 10. Effect of treatments on third quality cucumber fruits during the FW and SS cycles.

	Yield (kg m^{-2})				
Treatment *	**Cycle FW (Paraiso)**		**Cycle SS ('Kathrina')**		
	90 dat	**120 dat**	**30 dat**	**60 dat**	**90 dat**
Control	1.69 a	2.787 a	1.813 a	1.514 a	1.338 a
VOT1	2.14 a	3.087 a	1.437 a	1.341 a	1.136 a
QLT	2.78 a	4.589 a	1.553 a	1.783 a	1.643 a
BKNT	2.59 a	3.9 a	1.609 a	1.507 a	1.444 a
VOB1	2.54 a	4.424 a	3.21 a	1.957 a	1.767 a
VOB2	2.67 a	4.91 a	1.525 a	1.454 a	1.371 a

* Control, biweekly applications of commercial products as applied by the growers. Control treatments consisted of Serenade Max® 14.6; Apolo®; hydrogen peroxide); VOT1: *Trichoderma harzianum* strain VOT1; QLT: *T. harzianum* strain QLT; BKNT: *T. harzianum* strain BKNT; VOB1: *Bacillus subtilis* strain VOB1; VOB2; *B. subtilis* cepa VOB2. Data inside the columns with different letters indicate significant differences in the Tukey test ($p \leq 0.05$). Dat = days after transplant.

Our results indicate that the application of strains of microorganisms as biological control products (in particular, *T. harzianum*) for the control of the downy mildew of cucurbits can increase the amount of 'premium' quality fruits by approximately 15 additional t per hectare. The results obtained in this study seem to coincide with previous studies' findings, in which some strains of *Trichoderma* improved the performance of several horticultural crops [19]. A similar study using cucumber plants treated with *T. harzianum* also produced cucumbers with higher contents of soluble carbohydrates, soluble protein, and vitamin C compared to the untreated plants, which correlates directly with a higher quality fruit [25].

The increased yield of cucumber plants and improvements in fruit quality could be related to the beneficial microorganism-plant relationship that occurs when *Trichoderma* invades the plant rhizosphere. This beneficial interaction is associated with the enhancement of plant growth by the microorganism and an increase in systemic resistance [26–28]. The fungus produces auxins to facilitate fungal colonization and increases plant nutrient uptake. These changes in the metabolism of the crops enhance productivity and fruit quality [29–31].

Applications of *Trichoderma* increased fruit yield of cucumber plants in treated crops even though disease control may not be as efficient. In our study, an increase in fruit production was observable in the plants treated with the *T. harzianum* VOT1 even though this treatment was not the best for disease control. These effects could be related to the secretion of harzianic acid (HA) and 6-pentyl-a-pyrone (6PP) as significant secondary metabolites by *T. harzianum*. These compounds directly enhanced fruit production in different crops, resulting in higher quality fruit with an increase in fruit size [32]. Yield improvements could also be related to an increment in the synthesis of volatile organic compounds (VOC), which are lipophilic compounds of low molecular weight and may act as promotors of plant growth [33–35].

T. harzianum strains may also improve the uptake of plant nutrients, with an enhancing effect on the efficiency of nitrogen use of the crop. This effect improves photosynthetic efficiency, which might also contribute to the increment in fruit yield and quality in cucumber plants treated with VOT1 when compared to the crops treated with *B. subtilis* [36–38].

4. Conclusions

Foliar applications of native strains of *T. harzianum* (VOT1) and *B. subtilis* (VOB1 and VOB2) can be considered viable alternatives for the control of downy mildew of cucurbits, as they provided better control than other commercial products, including Serenade Max®, Apolo®, and hydrogen peroxide. The best strains of microorganisms for the control of downy mildew of the cucurbits are the *Bacillus subtilis* strains VOB1 and VOB2. The VOT1 strain of *T. harzianum* provides adequate control over the disease and induces the highest yield of cucumber plants in comparison to the other strains. Further research is recommended to identify the mechanism by which *T. harzianum* enhances fruit yield and quality.

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References

1. Jaime, M.; Lucero, J.M.; Sánchez, M. *Innovación Tecnológica de Sistemas de Producción y Comercialización de Especies Aromáticas y Cultivos Élite en Agricultura Orgánica Protegida con Energías Alternativas de bajo Costo: Inteligencia de Mercado de Pepino*; Centro de Investigaciones Biológicas del Noreste S. C.: La Paz, Mexico, 2012.
2. SIAP. Sistema de Información Agropecuaria y Pesquera. Servicio de información Agropecuaria y Pesquera. 2016. Available online: http://www.siap.gob.mx/agricultura-produccion-anual/ (accessed on 1 May 2020).
3. Thomas, A.; Carbone, I.; Choe, K.; Quesada-Ocampo, L.M.; Ojiambo, P.S. Resurgence of cucurbit downy mildew in the United States: Insights from comparative genomic analysis of Pseudoperonospora cubensis. *Ecol. Evol.* **2017**, *7*, 6231–6246. [CrossRef]
4. Thomas, A.; Carbone, I.; Cohen, Y.; Ojiambo, P.S. Occurrence and distribution of mating types of Pseudoperonospora cubensis in the United States. *Phytopathology* **2017**, *107*, 313–321. [CrossRef]
5. Holmes, G.; Ojiambo, P.S.; Hausbeck, M.; Quesada-Ocampo, L.M.; Keinath, A.P. Resurgence of cucurbit downy mildew in the United States: A watershed event for research and extension. *Plant Dis.* **2015**, *99*, 428–441. [CrossRef]
6. Runge, F.; Choi, Y.; Thines, M. Phylogenetic investigations in the genus Pseudoperonospora reveal overlooked species and cryptic diversity in the P. *cubensis species cluster. Eur. J. Plant Pathol.* **2011**, *129*, 135–146. [CrossRef]
7. Cohen, Y.; Whener, T.C.; Ojiambo, P.; Hausbeck, M.; Quesada-Ocampo, L.M.; Lebeda, A.; Sierotzki, H.; Gisi, U. Resurgence of Pseudoperonospora cubensis the agent of cucurbit downy mildew. *Phytopathology* **2015**, *105*, 998–1012. [CrossRef]
8. Savory, E.A.; Granke, L.L.; Quesada-Ocampo, L.M.; Varbanova, M.; Hausbeck, M.K.; Day, B. The cucurbit downy mildew pathogen Pseudoperonospora cubensis. *Mol. Plant Pathol.* **2011**, *12*, 217–226. [CrossRef]
9. Lebeda, A.; Cohen, Y. Cucurbit downy mildew (Pseudoperonospora cubensis)—Biology, ecology, epidemiology, host-pathogen interaction and control. *Eur. J. Plant Pathol.* **2011**, *129*, 157–192. [CrossRef]
10. Neufeld, K.N.; Keinath, A.P.; Ojiambo, P.S. A model to predict the risk of infection of cucumber by Pseudoperonospora cubensis. *Microb. Risk Anal.* **2017**, *6*, 21–30. [CrossRef]

11. Thomas, A.; Carbone, I.; Lebeda, A.; Ojiambo, P.S. Virulence structure within populations of Pseudoperonospora cubensis in the United States. *Phytopathology* **2017**, *107*, 777–785. [CrossRef]
12. Call, A.D.; Criswell, A.D.; Wehner, T.C.; Klosinska, U.; Kozik, E.U. Screening cucumber for resistance to downy mildew caused by Pseudoperonospora cubensis (Berk. *and Curt.) Rostov. Crop Sci.* **2012**, *52*, 577–592. [CrossRef]
13. Hewitt, H.G. *Fungicides in Crop Protection*, 1st ed.; CABI Publishing, CAB International: Oxon, UK, 1998.
14. Olalde, V. *Centro de Investigaciones Avanzadas*; Personal Communication: Irapuato, Mexico, 2013.
15. Shoresh, M.; Harman, G.E. The molecular basis of shoot responses of maize seedlings to Trichoderma harzianum T22 inoculation of the root: A proteomic approach. *Plant Physiol.* **2008**, *147*, 2147–2163. [CrossRef]
16. Heydari, A.; Pessarakli, M. A review on biological control of fungal plant pathogens using microbial antagonists. *J. Biol. Sci.* **2010**, *10*, 273–290. [CrossRef]
17. Perazzolli, M.; Moretto, M.; Fontana, P.; Ferrarini, A.; Velasco, R.; Moser, C.; Delledonne, M.; Pertot, I. Downy mildew resistance induced by Trichoderma harzianum T39 in susceptible grapevines partially mimics transcriptional changes of resistant genotypes. *BMC Genomics.* **2012**, *13*, 660. [CrossRef]
18. Steiner, A. The universal nutrient solution. In Proceedings of the Sixth International Congress on Soilless Culture. International Society for Soilless Culture, Lunteren, The Netherlands, 29 April–5 May 1984; pp. 633–649.
19. Martínez, B.; González, E.; Infante, D. Nuevas evidencias de la acción antagonista de Trichoderma asperellum Samuels. *Rev. Prot. Veg.* **2011**, *26*, 131–132.
20. Ruiz-Sánchez, E.; Tún-Suárez, J.M.; Pinzón-López, L.L.; Valerio-Hernández, G.; Zavala-León, M.J. Evaluación de fungicidas sistémicos para el control del mildiú velloso (*Pseudoperonospora cubensis* Berk. & Curt.) Rost. en el cultivo de melón (*Cucumis melo* L.). *Rev Chapingo Ser Hortic RCSH* **2008**, *14*, 79–84.
21. Horsfall, J.G.; Barrat, R.W. An improved grading system for measuring plant disease. *Phytopathology* **1945**, *35*, 655.
22. Zeriouh, H.; Romero, D.; García-Gutiérrez, L.; Cazorla, F.M.; Vicente, A.; Pérez-García, A. The iturin-like lipopeptides are essential components in the biological control arsenal of Bacillus subtilis against bacterial diseases of cucurbits. *Mol. Plant Microbe Interact.* **2011**, *24*, 1540–1552. [CrossRef]
23. Elad, Y. Biological control of foliar pathogens by means of Trichoderma harzianum and potential modes of action. *Crop Prot.* **2000**, *39*, 709–714. [CrossRef]
24. Soria, F.M.J.; Suárez, J.M.T.; Trejo, R.J.A. *Tecnología para Producción de Hortalizas a Cielo Abierto en la Península de Yucatán*; SEP-DGETA, Centro de Investigación y Graduados Agropecuarios, Instituto Tecnológico Agropecuario no.2: Conkal, México, 2000.
25. Mei, L.I.; Guang-shu, M.A.; Hua, L.; Xiao-lin, S.U.; Ying, T.; Wen-kun, H.; Jie, M.E.I.; Xi, J. The effects of Trichoderma on preventing cucumber fusarium wilt and regulating cucumber physiology. *J. Integr. Agric.* **2019**, *18*, 607–617. [CrossRef]
26. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. Trichoderma species-opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* **2004**, *2*, 43–56. [CrossRef]
27. Keswani, C.; Mishra, S.; Sarma, B.; Singh, S.; Singh, H. Unraveling the efficient applications of secondary metabolites of various *Trichoderma* spp. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 533–544. [CrossRef]
28. Shoresh, M.; Harman, G.E.; Mastouri, F. Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* **2010**, *48*, 21–43. [CrossRef]
29. Contreras-Cornejo, H.A.; Macías-Rodríguez, L.; Cortés-Penagos, C.; López-Bucio, J. Trichoderma virens, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiol.* **2009**, *149*, 1579–1592. [CrossRef]
30. Grossmann, K. Auxin herbicides: Current status of mechanism and mode of action. *Pest Manag. Sci.* **2010**, *66*, 113–120. [CrossRef]
31. Lombardi, N.; Caira, S.; Troise, A.; Scaloni, A.; Vitaglione, P.; Vinale, F.; Marra, R.; Salzano, A.; Lorito, M.; Woo, S.L. Trichoderma applications on strawberry plants modulate the physiological processes positively affecting fruit production and quality. *Front. Microbiol.* **2020**, *11*, 1364. [CrossRef]
32. Pascale, A.; Vinale, F.; Manganiello, G.; Nigro, M.; Lanzuise, S.; Ruocco, M.; Marra, R.; Lombardi, N.; Woo, S.L.; Lorito, M. Trichoderma and its secondary metabolites improve yield and quality of grapes. *J. Crop Prot.* **2017**, *92*, S11–S12. [CrossRef]
33. Garnica-Vergara, A.; Barrera-Ortiz, S.; Muñoz-Parra, E.; Raya-González, J.; Méndez-Bravo, A.; Macías-Rodríguez, L.; Ruiz-Herrera, L.F.; López-Bucio, J. The volatile 6-pentyl-2H-pyran-2-one from Trichoderma atroviride regulates Arabidopsis thaliana root morphogenesis via auxin signaling and Ethylene Insensitive 2 functioning. *New Phytol.* **2015**, *209*, 1496–1512. [CrossRef]
34. Lee, S.; Yap, M.; Behringer, G.; Hung, R.; Bennett, J.W. Volatile organic compounds emitted by Trichoderma species mediate plant growth. *Fungal Biol. Biotechnol.* **2016**, *3*, 7. [CrossRef]
35. Paul, D.; Park, K.S. Identification of volatiles produced by Cladosporium cladosporioides CL1, a fungal biocontrol agent that promotes plant growth. *Sensors* **2013**, *13*, 13969–13977. [CrossRef]
36. Benítez, T.; Rincón, A.M.; Limón, M.C.; Codón, A.C. Biocontrol mechanisms of Trichoderma strains. *Int. Microbiol.* **2004**, *7*, 249–260.
37. Kumar, S. Trichoderma: A biological weapon for managing plant diseases and promoting sustainability. *Int. J. Agric. Vet. Sci.* **2013**, *1*, 106–121.
38. Sood, M.; Kapoor, D.; Kumar, V.; Sheteiwy, M.S.; Ramakrishnan, M.; Landi, M.; Araniti, F.; Sharma, A. Trichoderma: The "secrets" of a multitalented biocontrol agent. *Plants* **2020**, *9*, 762. [CrossRef]