Clavibacter michiganensis subsp. *michiganensis* MANAGEMENT STRATEGIES IN TOMATO (*Solanum lycopersicum* L.)

Valeria **Roldán-Guzmán**[1], Sergio **Aranda-Ocampo**[1*],
Lauro **Soto-Rojas**[1], Joel **Pineda-Pineda**[2], Cristian **Nava-Díaz**[1]

[1]Colegio de Postgraduados Campus Montecillo. Carretera México-Texcoco km 36.5, Montecillo, Texcoco, State of Mexico, Mexico. C. P. 56230.
[2]Universidad Autónoma Chapingo. Carretera México-Texcoco km 38.5, Texcoco, State of Mexico, Mexico. C. P. 56230.
* Author for correspondence: arandasergio63@gmail.com

Citation: Roldán-Guzmán V, Aranda-Ocampo S, Soto-Rojas L, Pineda-Pineda J, Nava-Díaz C. 2025. *Clavibacter michiganensis* subsp. *michiganensis* management strategies in tomato (*Solanum lycopersicum* L.). **Agrociencia.** https://doi.org/ 10.47163/agrociencia.v59i6.3089

Editor in Chief:
Dr. Fernando C. Gómez Merino

Received: February 10, 2025.
Approved: September 03, 2025.
Published in Agrociencia: September 18, 2025.

ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is the causal agent of bacterial canker, which is the most destructive disease of tomato (*Solanum lycopersicum* L.). This study compared the effectiveness of two nitrogen fertilization regimes, Steiner 100 % NO_3^- and Steiner 85/15 NO_3^-/NH_4^+, in supplying total nitrogen for tomato nutrition under greenhouse conditions. These treatments were tested in combination with the plant activators Romel (*Brevibacillus* sp.), Fungifree (*Bacillus* sp.), and Messenger Gold (Harpin protein αβ 3 %) to evaluate their effects on Cmm severity and yield in a completely randomized factorial experiment. Each fertilization regime was applied daily in drip irrigation, while plant activators were sprayed weekly. Inoculation of Cmm in plants was performed by cutting the petiole with scissors soaked in a suspension with 10^8 CFU mL^{-1}. Yield and severity were assessed by evaluating the nutrition and plant activator factors, as well as their interaction. A two-way analysis of variance (treatment and nutrition) and Tukey's comparison of means ($p \leq 0.05$) were performed. The Steiner 100 % NO_3^- nutrition regime showed significant differences ($p \leq 0.05$) in yield, fresh and dry weight of leaves, number of bunches, and number and diameter of fruits. Significant differences ($p \leq 0.05$) were found in the weighted percentage of leaf damage and whole-plant wilting severity; the highest severity was recorded with the Steiner 85/15 NO_3^-/NH_4^+ + Romel treatment and the lowest with Steiner 100 % NO_3^- + Messenger Gold. The application of Harpin protein and exclusive nitrogen fertilization with nitrates (NO_3^-) is an efficient management strategy that promotes higher yield and fruit quality while reducing the severity of Cmm under greenhouse conditions.

Keywords: Bacterial canker, Harpin protein, nitrate, ammonium, management.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is an international quarantine pathogen by the European and Mediterranean Plant Protection Organization (EPPO). This microorganism causes bacterial canker in tomato (*Solanum lycopersicum* L.), which is considered one of the most destructive diseases worldwide (Chalupowicz *et al*., 2016). It affects plants by causing wilting, necrosis, and canker on stems and petioles. This results in a nutritional imbalance and a decrease in physiological activity, which impacts fruit quality and yield (Kolomiiets *et al*., 2020). To date, there are no tomato varieties or hybrids with complete resistance to Cmm, making it difficult to control (Nandi *et al*., 2018).

The nutritional status of plants influences the plant-pathogen interaction (Ding *et al*., 2021). It has been demonstrated that the supply of nitrogen in the form of nitrate (NO_3^-) fertilization increases resistance through a hypersensitive response as a defense mechanism against pathogens and abiotic stress (Planchet, 2022). In tomato cultivation, fertilization with NO_3^- activates the salicylic acid pathway, inducing plant defense against the bacterial pathogens *Pseudomonas syringae* and *Ralstonia solanacearum* (Ding *et al*., 2021), while the supply of ammonium (NH_4^+) in fertilization acts as a cofactor for antioxidant enzymes and an activator of systemic acquired resistance as a defense response against *P. syringae* (González-Hernández *et al*., 2019).

Plant activators with commercial formulations based on species from the genera *Bacillus* and *Brevibacillus* as biocontrol agents, inducers of systemic resistance, and plant growth promoters have been extensively studied, inducing different defense responses in the plant against phytopathogens (Tiwari *et al*., 2019). Previous research has shown that the application of *Bacillus* species reduced the severity of diseases caused by bacterial pathogens such as *R. solanacearum* and *Xanthomonas perforans*; specifically, the application of *B. amyloliquefaciens* in tomato crops provides protection against infection by Cmm (Dame *et al*., 2021).

The Harpin protein is a plant activator used as an inducer of natural plant defenses, known as systemic acquired resistance against bacterial phytopathogens. It is also related to the promotion of plant growth and increased fruit yield and quality (Liu *et al*., 2020). Previous studies have concluded that the application of the PeBL1 protein induces a hypersensitive response and systemic resistance in *Nicotiana benthamiana* Domin against *P. syringae* pv. *tabaci* (Wang *et al*., 2015). The BAR11 protein obtained from *Saccharothrix yanglingensis* stimulated systemic resistance in *Arabidopsis thaliana* L. against *P. syringae* pv. *tomato* (Zhang *et al*., 2018).

In this study, it is assumed that nitrogen fertilization with NO_3^- or NH_4^+, combined with the spraying of plant activators, improves yield and reduces the severity of Cmm in greenhouse tomato crops. Therefore, the objective of this study was to evaluate the efficacy of two fertilization regimes in supplying total nitrogen, in combination with plant activators, on tomato yield and Cmm severity under greenhouse conditions.

MATERIALS AND METHODS

Location and plant material

The experiment was conducted in an overhead greenhouse located at the Postgraduate College, Montecillo Campus (19° 27′ 51′′ N, 98° 54′ 15′′ W) in Texcoco, State of Mexico,

Mexico. A total of 120 seedlings of the Cmm-susceptible hybrid tomato OptiMax (Sakata, Mexico) at the five-true-leaf phenological stage were used. Seedlings were transplanted into black plastic bags (25 × 25 cm) with 125 cm^3 of coarse tezontle at the base of the bags and a mixture of peat and perlite (2:1) as a substrate. Seedlings were maintained in the greenhouse with a relative humidity > 70 % and a temperature between 22 and 33 °C.

Nitrogen fertilization and plant activators

Two fertilization regimes were used to supply total nitrogen (N): 100 % Steiner NO_3^- and 85/15 modified Steiner NO_3^-/NH_4^+ (Table 1). Both regimes were applied daily via drip irrigation 5 d after transplant. Four 3-min irrigations were carried out daily until two months of vegetative development; then, irrigations were increased to 10-min irrigations until crop completion.

Table 1. Chemical composition (meq L^{-1}) of the Steiner 85/15 NO_3^-/NH_4^+ and Steiner 100 % NO_3^- fertilization regimes.

Nutrient solution	NO_3^-	$H_2PO_4^-$	SO_4^-	K^+	Ca^{2+}	Mg^{2+}	NH_4^+
Steiner 85/15 NO_3^-/NH_4^+	10.2	1	8.8	6.37	8.19	3.64	1.8
Steiner 100 % NO_3^-	12	1	7	7	9	4	0

Values expressed in milliequivalents per liter (meq L^{-1}). NO_3^-: nitrate; H_2PO_4-: phosphate; SO_4-: sulfate; K^+: potassium; Ca^{2+}: calcium; Mg^{2+}: magnesium; NH_4: ammonium.

For each fertilization regime, plant activators Romel (*Brevibacillus* sp., 2.5 mL L^{-1}) (Teza, Agricultura Sustentable S.A. de C.V., Mexico), Fungifree (*Bacillus* sp., 2.5 mL L^{-1}) (FMC Agroquímica, Mexico), and Messenger Gold (Harpin αβ protein 3 %, 1 g L^{-1}) (PHC, Mexico) were sprayed weekly at the doses recommended on the label.

Every two months, the variables evaluated were the number of bunches, fresh and dry leaf weight, number of small fruits, total number of fruits, fruit diameter, and kilograms of fruit per plant until harvest. Fresh and dry leaf weight (g) was determined using a D-Weight DGIT-01 analytical scale (Stay Elit), and fruit diameter (mm) using a None electronic vernier caliper (REXQualis). Fruit size was classified according to Mexican standard NMX-F-009-1982 (DOF, 1982).

Severity assessment

Cmm inoculation was performed by cutting petioles with scissors soaked in a bacterial suspension containing 10^8 colony-forming units per milliliter (CFU mL^{-1}) on the third apical leaf when more than 50 % of the plants had 15 true leaves. The pathogenic CP_Cmm-1 strain, previously identified as highly aggressive (Rivera-Sosa *et al*., 2022), was used. For the control, the cut was made with scissors soaked in sterile distilled water.

Since the first week of inoculation, severity was assessed using the weighted percentage of leaf damage (WPLD) and whole-plant wilting severity (WPWS) ratings. For WPLD, a severity scale was used, where 0 = healthy leaf; 1 = leaves with epinasty or loss of turgor; 2 = leaves with loss of turgor in half of the leaflet and the other half apparently healthy; 3 = wilting and browning in ¾ of the leaf; 4 = wilting and browning of the entire leaf and interveinal chlorosis; and 5 = presence of stem canker (Rivera-Sosa *et al*., 2022).

For WPWS, an arbitrary whole-plant wilting severity scale was used, where 0 = healthy plant, 1 = up to 20 % of the area shows wilting symptoms, 2 = 21 to 40 %, 3 = 41 to 60 %, 4 = 61 to 80 %, and 5 = more than 81 % of the area shows symptoms. The experiment ended 112 d after inoculation, when all fruits reached maturity Grade 6 according to the Mexican standard NMX-F-009-1982 (DOF, 1982).

Experimental design

The experiment was conducted using a completely randomized design with a factorial arrangement and 15 replicates. A first factor was represented by two nutrient solution regimes: the 100 % NO_3^- Steiner solution and the modified Steiner solution with an 85/15 NO_3^-/NH_4^+ ratio. Another factor was represented by four treatments applied by foliar spraying of plant activators: the application of Romel (*Brevibacillus* sp.), Fungifree (*Bacillus* sp.), Messenger Gold (3 % Harpin αβ protein), and the spraying of sterile distilled water as a control. The experimental unit consisted of one tomato plant corresponding to each combination of fertilization regime and plant activator treatment.

Plant performance and canker disease severity were assessed by comparing the effects of nutrition and plant activator factors, as well as their interaction. Data were assessed using two-way analysis of variance (ANOVA) for treatment and nutrition; normality and homogeneity data were analyzed using the Shapiro-Wilks and Levene tests, and means were compared with the Tukey test ($p \leq 0.05$) using the R v4.2.1 programming language (https://www.R-project.org/).

Reisolation and identification of the pathogen

From plants with characteristic symptoms due to Cmm infection, vascular tissue samples (3 cm in length) were taken and macerated in sterile distilled water. Around 100 µL of the macerate was seeded in Petri dishes with nutrient agar culture medium (Difco, USA) and incubated at 28 °C for 48 h. Colonies with Cmm-like morphology were purified and characterized by Gram staining and infiltration of a suspension containing 10^8 CFU mL^{-1} on the underside of four-o'clock (*Mirabilis jalapa* L.) and tobacco (*Nicotiana tabacum* L.) leaves. The identity of the isolated strains was confirmed with the CMM5F-5′ and CMM6R-5′ primers specific for Cmm and PCR conditions described by Rivera-Sosa *et al*. (2022).

RESULTS AND DISCUSSION

Reisolation and identification of the pathogen

Yellow-orange, mucoid, Gram-positive bacterial colonies were isolated from plant tissue samples from each treatment with Cmm symptoms and used to induce hypersensitivity reactions on tobacco and four-o'clock leaves. The identity of the cultured Cp_Cmm-1 strain was confirmed by comparing the amplified product to the GenBank sequence. This demonstrated a 97 % identity with the *C. michiganensis* strain NCPBB 382 plasmid pCM2 (accession number AM711866.1).

Vegetative development and yield

At 78 d after transplant, results showed significant differences ($p \leq 0.05$) between treatments. Higher values were obtained with the Steiner 100 % NO_3^- nutrient solution regime for the variables fresh leaf weight ($F_{1, 14} = 5.7$, $p = 0.031$), dry leaf weight ($F_{1, 14} = 7.18$, $p = 0.017$), number of bunches ($F_{1, 14} = 7$, $p = 0.019$), number of fruits ($F_{1, 14} = 5.2$, $p = 0.038$), and fruit diameter ($F_{1, 16} = 9.77$, $p = 0.0065$), and had fewer small fruits ($F_{1, 16} = 16.98$, $p < 0.01$) (Table 2).

Table 2. Vegetative growth, fruit quality, and yield of the OptiMax tomato hybrid (*Solanum lycopersicum* L.) under two nitrogen fertilization regimes.

Nitrogen regime	FD *	NSF *	NB *	TNF *	FLW *	DLW *	Y *
Steiner 100 % NO_3^-	53.90 a	34.16 a	4.58 a	27.75 a	1286.17 a	77.51 a	00.76 a
Steiner 85/15 NO_3^-/NH_4^+	51.47 b	20.00 b	4.08 b	23.16 b	912.85 b	51.79 b	00.70 b
Pr(>F)	0.006	0.001	0.02	0.04	0.03	0.01	0.81

FD: fruit diameter; NSF: number of small fruits; NB: number of bunches; TNF: total number of fruits; FLW: fresh leaf weight (g); DLW: dry leaf weight (g); Y: yield (kg plant^{-1}). *Means followed by the same letter are not significantly different (Tukey, $p \leq 0.05$).

The results of this study are consistent with those reported by Nawarathna *et al*. (2021), who concluded that nitrate as a nitrogen source in tomato plants under greenhouse conditions increased plant biomass compared to ammonium supplementation. Biomass content is an indicator of nitrogen uptake in crops. The results suggest that nitrogen supplementation via nitrates in the OptiMax tomato hybrid is a better fertilization strategy than ammonium supply, promoting higher plant biomass content in terms of fresh/dry weight of leaves, number of bunches, and larger fruit diameter with export quality (62–71 mm in diameter) in accordance with the Mexican standard NMX-F-009-1982 (DOF, 1982).

Interaction between plant activator and nutrition

Plant activators showed differences between treatments for the variable small fruit size ($F_{7, 16}$ = 4.26, p = 0.007). A greater number of small fruits were recorded with the Steiner 85/15 NO_3^-/NH_4^+ + Fungifree treatment and a lower number with Steiner 100 % NO_3^- + Fungifree (Table 3).

Table 3. Fruit quality and yield of the OptiMax tomato hybrid (*Solanum lycopersicum* L.) under different nitrogen fertilization regimes and plant activator applications.

Nitrogen regime	Plant activator	Number of small-sized fruits	*	Yield (kg plant^{-1})	*
Steiner 100 % NO_3^-	Romel	22.7	ab	2.3	a
	Fungifree	19.0	b	2.3	a
	Messenger Gold	22.7	ab	2.3	a
	Control	15.7	b	2.3	a
Steiner 85/15 NO_3^-/NH_4^+	Romel	38.7	ab	2.0	a
	Fungifree	43.3	a	2.1	a
	Messenger Gold	33.3	ab	2.2	a
	Control	21.3	ab	2.2	a
Pr(>F)		0.008		0.999	

*Means followed by the same letter are not significantly different (Tukey, $p \leq 0.05$).

These results highlight the influence of the nutrition regimes, since the only difference between treatments was the form of nitrogen supplied. It has been shown that most plants absorb and assimilate nitrogen better in the form of nitrate than ammonium (Boschiero *et al*., 2019). The effect of nitrogen nutrition on the yield of small-sized fruits (38–52 mm in diameter) in tomato crops is consistent with that reported by Carreras-Sempere *et al*. (2021), who concluded that nitrogen fertilization with ammonium nitrate promotes the production of a higher proportion of small fruits and, consequently, a lower yield of fruits with commercial quality.

Likewise, Howard *et al*. (2021) showed that fertilization with a nitrate/ammonium (NO_3/NH_4^+) combination also reduced the quality and size of tomato fruits. This was also observed in this research, as results suggest that fertilization with the nitrate/ammonium combination is not a cost-effective strategy for the OptiMax tomato hybrid, as it promotes the production of small-sized fruits over commercial-quality fruits. Other studies have shown that ammonium (NH_4^+) can affect plant development due to some toxicity, depending on the plant genotype and environmental conditions (Bittsánszky *et al*., 2015).

Ammonium has been shown to acidify the root zone and decrease the absorption of Ca^{2+} and Mg^{2+}. This could explain the effect on fruit size in treatments with the Steiner 85/15 NO_3^-/NH_4^+ fertilization regime, since these nutrients (Ca^{2+} and Mg^{2+}) promote the development of larger fruits (Alcántar-González *et al*., 2016). However, the optimal nitrate/ammonium ratio for tomato cultivation in relation to the absorption of Ca^{2+} and Mg^{2+} differs, since it can be influenced by factors such as the plant genotype and the type of soil (Rivera-Espejel *et al*., 2014).

Severity of *Clavibacter michiganensis* subsp. *michiganensis*

The severity results at 78 d after inoculation showed significant differences in weighted percentage of leaf damage (WPLD) ($F_{5, 60}$ = 3.41, p = 0.0087) and whole-plant wilting severity (WPWS) scores ($F_{2, 60}$ = 5.43, p = 0.0067); the highest WPWS was recorded with the Steiner 85/15 NO_3^-/NH_4^+ + Romel treatment and the lowest with Steiner 100 % NO_3^- + Messenger Gold (Table 4). The source of nitrogen fertilization has implications for plant-pathogen interactions and varies depending on the pathogen and plant species (Ding *et al*., 2021). The results of this study are similar to those obtained by Gupta *et al*. (2013), who concluded that nitrate fertilization induced greater resistance against *Pseudomonas syringae* in tobacco.

Table 4. Whole-plant wilting severity (WPWS) caused by *Clavibacter michiganensis* subsp. *michiganensis* in the OptiMax tomato hybrid (*Solanum lycopersicum* L.) under different nitrogen fertilization regimes and plant activator applications.

Nitrogen regime	Plant activator	WPWS*	
Steiner 100 % NO_3^-	Romel	27.2	ab
	Fungifree	30.2	ab
	Messenger Gold	13.5	b
Steiner 85/15 NO_3^-/NH_4^+	Romel	42.8	a
	Fungifree	31.8	ab
	Messenger Gold	17	ab
	Pr(>F)	0.007	

*Means followed by the same letter are not significantly different (Tukey, $p \leq 0.05$).

Likewise, Zimerman-Lax *et al*. (2016) reported a significant reduction in the severity of *Acidovorax citrulli* in melon crops grown under greenhouse conditions. The contribution of nitrate as a source of nitrogen is reported to increase plant resistance by increasing the levels of salicylic acid and producing polyamines as defense signals, unlike ammonium, which increases the level of γ-aminobutyric acid that can be a food source for some pathogens (Mur *et al*., 2017).

The results of this study showed that the application of the Harpin protein (Messenger Gold), with the addition of nitrates in nutrition, significantly reduced WPLD ($F_{5, 60}$= 3.41, p = 0.0087) and the severity of the plant disease caused by Cmm (Table 5).

The application of the Harpin protein (Messenger Gold) with nitrate supplementation resulted in higher yields and lower severity of bacterial canker disease. It also promoted

Table 5. Frequency distribution and weighted percentage of leaf damage (WPLD) caused by *Clavibacter michiganensis* subsp. *michiganensis* in the OptiMax tomato hybrid (*Solanum lycopersicum* L.) under different nitrogen fertilization regimes and plant activator applications.

		Severity scale							
Nitrogen regime	Plant activator	Grade 0	Grade1	Grade 2	Grade 3	Grade 4	Grade 5	WPLD	*
									
Steiner 100 % NO_3^-	Romel	13.33	6.67	20	53.33	6.67	0	13.4	ab
	Fungifree	6.67	6.67	20	20	26.67	20	18.7	ab
	Messenger Gold	13.33	60	26.67	0	0	0	5.2	b
Steiner 85/15 NO_3^-/NH_4^+	Romel	6.67	6.67	6.67	13.33	20	46.67	22.7	a
	Fungifree	13.33	6.67	6.67	20	13.33	40	17	ab
	Messenger Gold	13.33	46.67	6.67	13.33	13.33	6.67	6	b

*Means followed by the same letter are not significantly different (Tukey, $p \leq 0.05$).

better fruit set and filling and a higher proportion of fruits classified as medium and large. The Harpin protein induces plant defenses against different pathogens, in addition to promoting plant growth (Rajwade *et al*., 2020; Yuan *et al*., 2020). Its effect has been linked to the production and accumulation of salicylic acid and systemic acquired resistance (Nazarov *et al*., 2020; Sheikh *et al*., 2022). Fertilization in general influences the susceptibility response of tomato to Cmm and is reported to depend on factors such as genotype, growth stage, and amounts of fertilizer used in production systems (Brochu *et al*., 2023).

Likewise, differences in disease severity have been observed in tomato genotypes due to the inoculation of different Cmm strains, showing variability in virulence (Rivera-Sosa *et al*., 2022). To date, no research has addressed the direct relationship between the nitrogen source in fertilization and the degree of disease severity caused by Cmm in tomato crops. However, in this study, the exclusive nitrogen source with NO_3 and the application of the Harpin protein reduced severity after inoculating a strain identified as highly aggressive (Rivera-Sosa *et al*., 2022) in a susceptible tomato genotype (OptiMax). The above could suggest that the relationship between the nitrogen source in fertilization together with the application of the Harpin protein should be evaluated in genotypes with greater tolerance to Cmm infection to promote a better resistance response and reduce disease severity.

Plant activators formulated with *Bacillus* and *Brevibacillus* spp. showed higher degrees of severity (Grade 5) than the treatment with the application of the Harpin protein. Although species of the genera *Brevibacillus* and *Bacillus* are generally recognized for their efficient and broad-spectrum antibacterial activity (Song *et al*., 2012; Dame *et al*., 2021), in this study, the formulation based on *Brevibacillus* sp. (Romel) resulted in the highest WPWS (Table 4, Figure 1) and WPLD (Table 5) scores. At severity Grade 5, plants in this treatment exhibited completely collapsed leaflets.

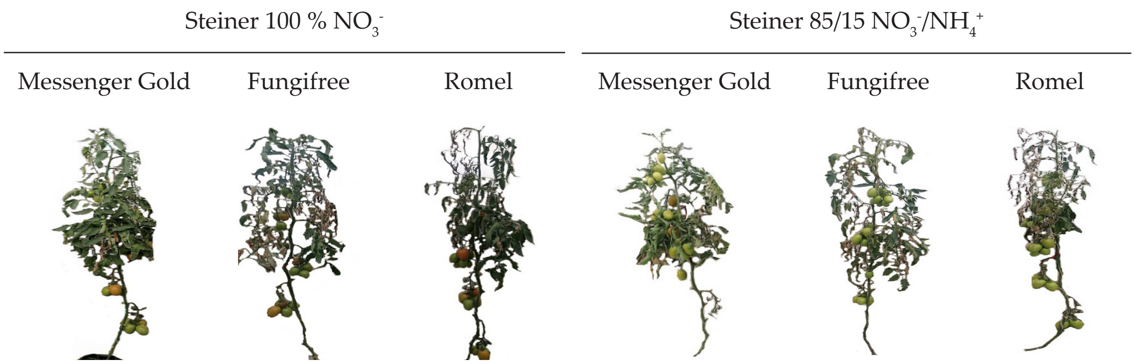

Figure 1. Whole-plant Grade 5 severity of *Clavibacter michiganensis* subsp. *michiganensis* in the OptiMax tomato hybrid (*Solanum lycopersicum* L.) under two nitrogen fertilization regimes and different plant activator treatments.

Biocontrol agent-plant-pathogen interactions are inherently complex. Several studies have demonstrated that *Brevibacillus* and *Bacillus* species can effectively reduce the severity of pathogen-induced diseases. However, commercial strains and formulations based on these biocontrol agents have also been reported as ineffective in certain pathosystems. This has been attributed to multiple factors, including the persistence of the agent in the phyllosphere over time and space (for foliar applications), inoculum concentration, frequency of application, and the genetic characteristics of the agent related to the production of metabolites involved in pathogen suppression. Additional factors include the plant species, its genotype, and susceptibility to the pathogen, the inoculation method, and the genetic variability and virulence of the pathogen itself (Lahlali *et al*., 2022; Mahapatra *et al*., 2022; Sedighian *et al*., 2025).

Treatments with the application of the Harpin protein resulted in the lowest severity, with photosynthetically active areas visible on the leaves and leaflets up to Grade 3. Plants receiving this treatment did not reach severity Grade 5 (Table 5). Across the whole plant, this treatment also exhibited lower overall severity (Figure 1) and better yields, producing fruits with larger diameters and better commercial quality. The Harpin protein induces a range of metabolic responses, including the natural expression of systemic acquired resistance (SAR) genes in response to pathogen infection (Nazarov *et al*., 2020; Sheikh *et al.,* 2022). It also activates jasmonic acid/ethylene-dependent defense mechanisms as well as plant growth-related systems. These mechanisms have been extensively studied against various fungal and bacterial pathogens. To date, there are no reports of resistance induction in phytopathogens, making the Harpin protein a promising strategy to enhance crop protection without causing adverse damage to the environment (Li *et al*., 2023).

CONCLUSIONS

The nitrogen fertilization regime influenced tomato fruit yield and quality. Under greenhouse conditions, the exclusive application of nitrate (NO_3^-) increased yield and fruit production within export quality standards, in contrast to the combined application of nitrate/ammonium (NO_3^-/NH_4^+). The application of plant activators formulated with *Bacillus* and *Brevibacillus* did not protect against infection by *Clavibacter michiganensis* subsp. *michiganensis* in the OptiMax tomato hybrid. The Harpin protein application and exclusive nitrogen fertilization with nitrates proved to be an efficient management strategy that promotes higher yield and fruit quality and reduces the severity of this pathogen under greenhouse conditions.

ACKNOWLEDGEMENTS

We would like to thank the Secretariat of Science, Humanities, Technology and Innovation (SECIHTI) and Postgraduate College's Program in Phytosanitary-Phytopathology, Montecillo Campus, for funding this research.

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