



Early detection of Tomato bacterial canker (Cmm) using the qPCR molecular method

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ABSTRACT

Background: Tomato (*Solanum lycopersicum* L.) is not only one of the key vegetable crops worldwide but also an important source of bioactive compounds, including lycopene, vitamin C, and polyphenols, which are associated with antioxidant and health-promoting properties. In the Republic of Armenia, the intensive development of greenhouse vegetable production contributes to increased crop productivity; however, it simultaneously increases the risk of bacterial diseases. One of the most problematic diseases is Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), which can lead to massive plant losses and significant yield reduction. Early diagnosis using quantitative polymerase chain reaction (qPCR) is crucial for effective disease control and the prevention of epiphytotics in greenhouses.

Objective: To study the manifestation of bacterial canker of tomato under commercial greenhouse conditions and to evaluate the effectiveness of a qPCR method for the early and latent detection of the Cmm pathogen.

Methods: The studies were conducted in commercial hydroponic greenhouses of the Kotayk and Ararat regions of Armenia. DNA was extracted from infected tomato stems and leaves using the CTAB method with Genetic Analysis Strategies S.L. kits and was spectrophotometrically analysed ($A_{260/280} = 1.78-1.92$). Identification of Cmm was performed by qPCR using species-specific primers, and quantitative assessment of the pathogen was carried out using a standard curve based on serial tenfold dilutions. The reliability of the method was confirmed by analysis of sensitivity, linearity, repeatability, and reproducibility (Efficiency 98.1%, $R^2 = 1.0$, LOD $3.1 \pm 0.4 \times 10^1$ copies/ μ l).

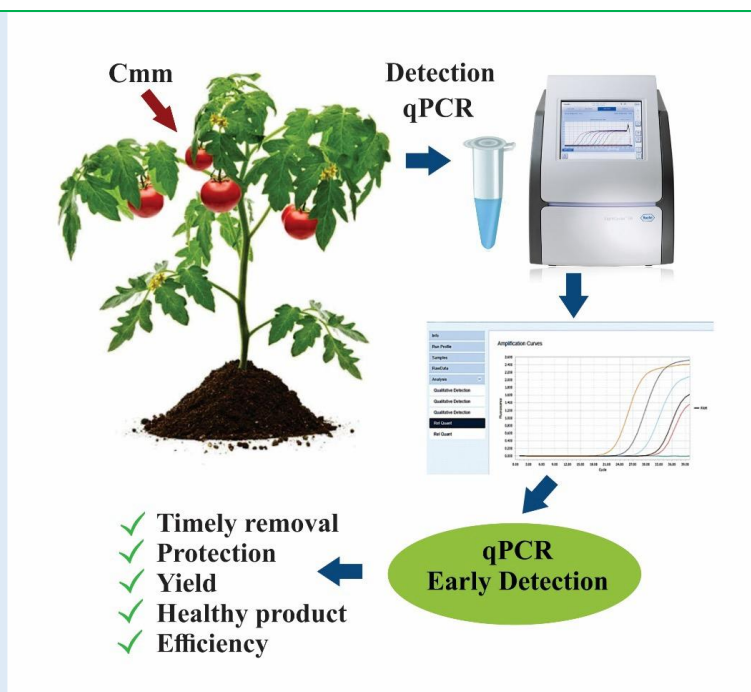
Results: In commercial greenhouses, the first manifestations of bacterial canker in tomato include unilateral leaf wilting, darkening and necrosis of the stem vascular system, as well as alterations in fruit structure. The qPCR method enabled the detection of Cmm in all 12 examined samples with high amplification efficiency (97.5%). Samples with low Ct values (12.5-17.5) corresponded to a high number of pathogen DNA copies and exhibited pronounced visual disease symptoms. In contrast, samples with higher Ct values (26.7-36.8) contained low pathogen concentrations and, in most cases, showed no symptoms, indicating a latent stage of infection.

Application of qPCR for early diagnosis reduced the proportion of infected plants from 13.2-14.9% to 4.8-5.1%, and yield losses from 9.4-10.1% to 3.5-3.9%. In addition to yield reduction, bacterial infection may affect the physiological processes of fruit development. In particular, the present study revealed a decrease in average fruit weight from 165.4 to 134.2 g, corresponding to a reduction of 18.9%. At the same time, accelerated fruit ripening was observed. The obtained results show that qPCR ensures effective early detection of infection and limits its spread under protected cultivation conditions, leading to reduced production losses and contributing to the production of healthier and high-quality plant products.

Novelty: This study presents, for the first time, a comprehensive diagnosis of Cmm in commercial greenhouses in Armenia using qPCR to detect both symptomatic and latent tomato infections. The data demonstrates a direct correlation between bacterial load and symptom severity and confirms that early molecular detection of the pathogen can significantly reduce yield losses. The results highlight the practical value of implementing qPCR in greenhouse monitoring systems and provide a basis for developing a rapid phytosanitary control strategy, which has not previously been documented for the study region.

Conclusion: The qPCR method demonstrated high efficiency for the early diagnosis of bacterial canker in tomato, enabling the detection of the pathogen at latent stages and preventing widespread infection. Early detection of Cmm allows timely implementation of protective measures, minimizes yield losses, and enhances the resilience of greenhouse tomato production. Thus, early molecular detection of Cmm is important not only for plant disease management but also for preserving the bioactive compounds and functional food value of tomato.

Keywords: tomato, *Clavibacter michiganensis* subsp. *michiganensis*, bacterial canker, qPCR, greenhouse, early detection



Graphical Abstract: Early detection of Tomato bacterial canker (Cmm) using the qPCR molecular method.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the leading vegetable crops in global agriculture and ranks first among vegetables in terms of cultivated area and production volume. Due to its high nutritional value, rich biochemical composition, and versatility, tomatoes are widely used both fresh and in processed products, highlighting their key role in food security and functional nutrition [1-2]. Tomato fruits are characterized by a high content of essential micronutrients, including copper, manganese, zinc, and selenium. They represent a valuable source of natural antioxidants, such as carotenoids (notably β -carotene and lycopene), ascorbic acid (vitamin C), tocopherols (vitamin E), and phenolic compounds [3-5]. The presence of this complex of bioactive constituents underlies their strong antioxidant capacity and contributes to the mitigation of oxidative stress in the human body. Regular consumption of tomatoes is associated with beneficial health effects, including enhancement of immune function, prevention

of cardiovascular diseases, and a reduced risk of cancer and diabetes [6-8].

In the Republic of Armenia, tomato production in recent years has shown a stable positive trend, driven by a combination of favorable soil and climatic conditions, the active development of greenhouse vegetable cultivation, the implementation of modern agrotechnologies, and the expansion of high-yielding varieties and hybrids [9]. The main cultivation areas are concentrated in the Armavir, Ararat, and Kotayk regions, which serve as the country's key vegetable-growing zones. A significant contribution to this process was made by the implementation of the State Strategy for Agricultural Development of the Republic of Armenia for 2020-2030, aimed at increasing agricultural efficiency, developing infrastructure, and introducing innovative and resource-saving technologies in crop production [10]. At the same time, with the increase in production volumes, the importance of vegetable product quality is growing, as it determines its nutritional and functional

value. In this regard, ensuring the quality and safety of tomato production is of particular importance as a factor contributing to a balanced diet and the maintenance of public health [11-13].

The physiological health status of plants is a critical factor determining the accumulation and stability of bioactive compounds in agricultural crops. Biotic stress may induce metabolic disturbances, the development of oxidative stress in plant tissues, as well as alterations or reductions in the synthesis of key nutritional components. Intensive tomato cultivation technologies, particularly under protected conditions, contribute to increased yields but simultaneously elevate the risk of infectious diseases, among which bacterial diseases occupy a prominent position. One of the most dangerous bacterial diseases of tomato is bacterial canker, caused by *Clavibacter michiganensis subsp. michiganensis* (Cmm) -a non-motile rod-shaped bacteria (occasionally coccoid) measuring $0.6-0.7 \times 0.7-1.0 \mu\text{m}$, Gram-positive aerobes [14].

The first description of tomato bacterial canker is from the United States, specifically the state of Michigan, where Erwin Smith first identified this previously undescribed disease in 1910 and isolated the causative agent, naming it after the region. Later, this bacterial disease reached alarming proportions, spreading across other U.S. states and, in some areas, affecting up to 80% or more of tomato crops [15]. In Armenia, tomato bacterial canker was first recorded in 1942 and 1943 in Leninakan and Kirovakan regions; however, since the disease occurred only sporadically, it did not initially receive significant attention.

Currently, Cmm is widely distributed across all major tomato-growing regions. This pathogen poses a serious threat to agricultural production and is subject to quarantine regulations in Europe. According to the European and Mediterranean Plant Protection Organization (EPPO), it is listed as an A2 quarantine pest [14,16].

Bacterial canker of tomato is particularly dangerous under protected cultivation. The primary source of Cmm transmission is contaminated seeds, with disease

development occurring even when the proportion of infected seeds does not exceed 1% [17]. In greenhouse conditions, the pathogen can persist for many years, causing outbreaks in nearly every cropping cycle. Infection can also spread through contact with infected plants, contaminated tools, and personnel [18]. In greenhouse settings, tomato bacterial canker can reach epiphytotic levels, leading to the death of 30–70%, and in some cases up to 96%, of plants [19-20].

Bacterial canker is one of the most complex tomato diseases, and its occurrence is difficult to predict and monitor, making control challenging due to the high variability in symptom expression. It should also be noted that cases of mixed infections have become more frequent, where overlapping symptoms obscure the primary source of infection [21]. This complicates pathogen identification and, consequently, the selection of appropriate protective measures. Therefore, in all suspected cases, laboratory testing is necessary for accurate pathogen identification. Early pathogen detection, in turn, allows for the timely implementation of necessary control measures and ensures the production of healthy and high-quality crops.

MATERIAL AND METHODS

Pathogen identification: Samples were collected from infection sites under natural infection in commercial greenhouses in the Ararat and Kotayk regions during 2025-2026. A total of 12 samples were analyzed in this study.

Sample preparation and analysis were conducted at the Laboratory of Plant Biotechnology, Phytopathology and Biochemistry of the Scientific Center. Infected tomato plant parts (stems and leaves) were used as diagnostic material.

Pathogen DNA was extracted using Genetic Analysis Strategies S.L. (Spain) reagent kits according to the manufacturer's protocol, employing the CTAB method. The DNA extraction kit included the following buffers: Lysis Buffer CTAB, Lysis/Binding Buffer, Proteinase K, Disinhibition Buffer, Wash Buffer, and Elution Buffer. Extraction was performed using spin columns (MiniSpin

column). The concentration and purity of the extracted DNA were determined spectrophotometrically using a Carry 60 UV-Vis spectrophotometer (Agilent Technologies, USA) by measuring the optical density ratio at 260 and 280 nm. A260/280 values ranged from 1.78 to 1.92, indicating high-quality DNA suitable for qPCR analysis.

Pathogen DNA was identified by qPCR using Genetic Analysis Strategies S.L. kits. The final reaction mixture volume was 20 µl, consisting of: DNase/RNase-free water (10 µl), MixStable qPCR 5× (4 µl), TargetSpecies dtec-qPCR mix (1 µl), and sample DNA (5 µl).

Amplification was performed on a LightCycler 96 instrument (Roche, Germany). The principle of the thermocycler is based on the detection of a fluorescent signal using the 6-FAM (6-carboxyfluorescein) dye, which binds to oligonucleotides and emits intense fluorescence at a wavelength of 488 nm. The thermal cycling program was as follows: 94 °C-5 min (1 cycle); 94 °C-30 sec, 72 °C-10 sec (40 cycles); 62 °C -10 sec (1 cycle). The cycles were optimized according to the manufacturer's protocols for the specific primer set.

For quantitative pathogen assessment, standard solutions with known nucleic acid concentrations were

prepared through six serial tenfold dilutions, which were used to generate calibration curves. A linear regression model, constructed using the instrument software, was employed to calculate the standard curve, its slope, the correlation coefficient (R^2), and amplification efficiency. A test was considered positive if an exponential amplification curve was observed with a Ct value ≤ 37 . Samples with Ct ≥ 40 were considered negative.

To ensure assay reliability, controls were included: a positive control (PC) containing a known amount of target pathogen DNA, and a negative control (No Template Control, NTC) containing all reaction components except DNA.

qPCR method validation: To confirm the analytical reliability of the qPCR method for Cmm detection, laboratory validation was performed using serial dilutions of the positive control (10^6 - 10^1 copies/µl, with threefold replication). The main method parameters were assessed: sensitivity, linearity, repeatability, and reproducibility. All tests were conducted on standard positive control samples. The validation results are presented in Table 1.

Table 1. Validation of the qPCR method for Cmm detection.

Parameter	Value
Efficiency	98.1%
R^2	1.0
LOD (limit of detection)	$3.1 \pm 0.4 \times 10^1$ copies/µl
LOQ (limit of quantification)	$1.0 \pm 0.2 \times 10^2$ copies/µl
Intra-assay CV	0.8-1.9%
Inter-assay CV	1.5-3.2%

RESULTS AND DISCUSSION

Disease Symptoms of Cmm: The first symptoms of bacterial canker in tomato under greenhouse conditions appeared on the apical leaves as unilateral wilting, leading to leaf distortion (Fig. 1, A1-A2). The observed symptoms are consistent with previous studies, in which Cmm causes similar localized damage. Nandi et al. (2018) and Sen et al. (2013) reported that the pathogen enters

plants through stomata or wounds, inducing necrosis at leaf margins and partial wilting of leaflets [18, 22].

In our study, necrosis was observed in the stems of infected plants, accompanied by discoloration of the vascular tissues (Fig. 1, B). Characteristic canker lesions appeared as light yellow or brown streaks, which became deeper and darker as the infection progressed. In severe cases, longitudinal stem cracking was observed with the formation of long brown cankers, while the upper parts

of the shoots began to wilt and die back. Upon transverse sectioning of stems and petioles, brown streaking was clearly visualized in the vascular system, along with reddish-brown discoloration of the vascular tissues and pith, indicating severe damage to the conducting system. Ultimately, this leads to vascular wilt and premature plant death (Fig. 1, C1-C3).

The obtained results are consistent with literature data indicating that, during the development of bacterial canker of tomato, progressive destruction of vascular bundles occurs, accompanied by browning of the pith, its granular or spongy transformation, and the formation of internal cavities within the stem [21,23].

The disease can manifest in various forms, with fruits exhibiting flat or slightly raised spots, snow-white at the periphery and dark in the center. On green fruits, the spots are white and arranged in clusters. On ripening fruits, the spots turn yellow, with small brown dots appearing in the center, resembling a “bird’s eye”- a

symptom widely described in the literature [24]. In our study, infected fruits were characterized by a netted or marbled pattern, accompanied by internal discoloration of the vascular and surrounding tissues (Fig. 1, A1), which was also previously reported by Mekina-Mora et al. (2001) [25]. According to Peritore-Galve et al. (2021), the severity of symptoms on fruits does not always correlate with the severity of vascular infection, suggesting differences in the mechanisms of pathogen colonization in various plant tissues [26].

In our research, disease outbreaks occurred during the autumn–winter period, likely correlating with greenhouse conditions that favor infection development: shortened photoperiod, high humidity (80-85%), and temperatures optimal for pathogen growth (+25-27 °C). These factors create a conducive environment for rapid pathogen spread. This is further supported by Nandi et al. (2018), who emphasized the importance of climatic conditions in activating infection [18].

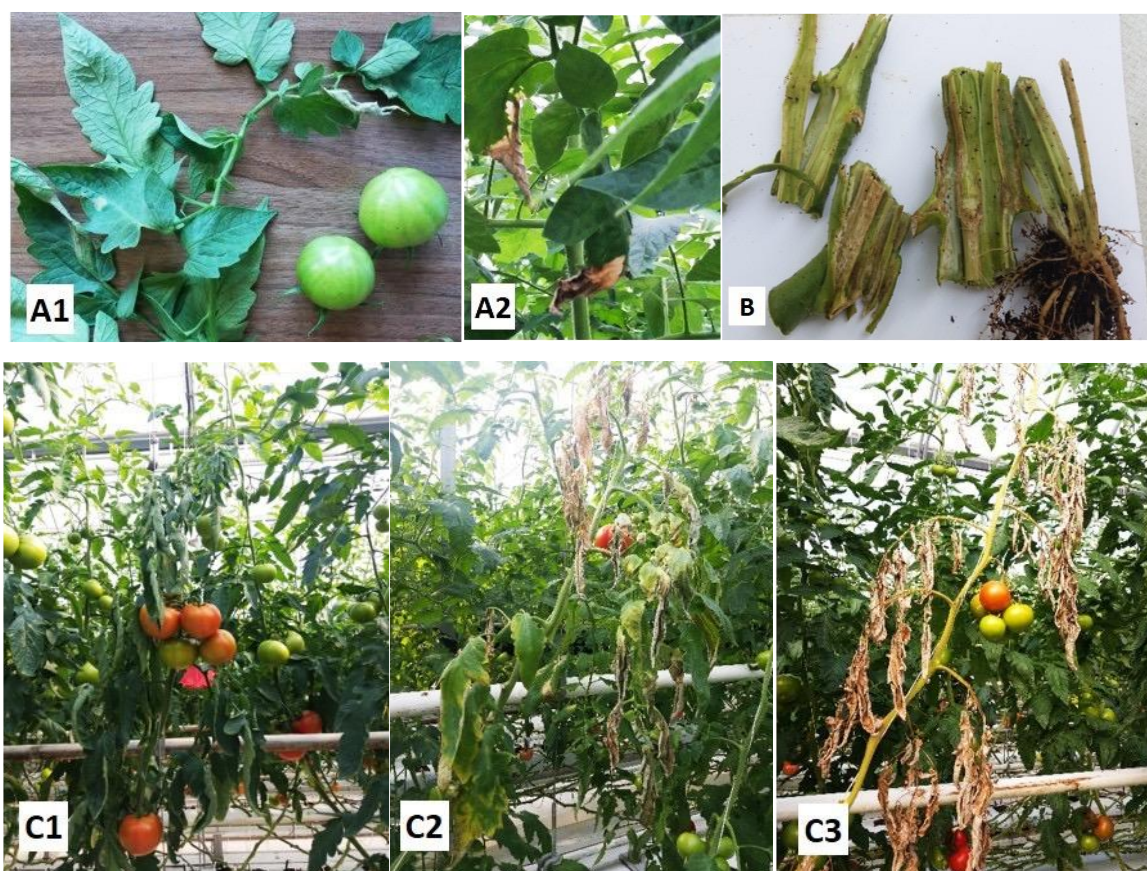


Figure 1. Infected tomato plants: A1-A2- leaves and fruits of infected plants, B- necrosis of the stem vascular system, C1-C3- wilted tomato plants.

Identification and Diagnosis of Cmm: The qPCR method is characterized by high sensitivity and specificity, which significantly reduces the risk of false-positive and false-negative results typical of conventional diagnostic methods [27]. Detection of the fluorescent signal at each amplification cycle allows quantitative assessment of the initial target content based on the threshold cycle (Ct) value, while performing the reaction in a closed system minimizes the risk of contamination and shortens analysis time. Accordingly, qPCR is a more informative and reliable method, particularly when working with plant tissues that have low pathogen concentrations and high levels of PCR inhibitors [28].

The qPCR method represents an effective tool for the identification and monitoring of tomato bacterial canker, including under protected cultivation conditions, where the pathogen can persist for extended periods. Timely diagnosis of *Cmm* enables rapid localization of

infection foci and prevents widespread plant infection. The results obtained are consistent with other studies in which qPCR demonstrated high sensitivity and specificity for the detection of various phytopathogens [29].

In our study, a qPCR standard curve for Cmm detection was constructed, including analysis of serial dilutions as well as the tested tomato samples - Samples № 1-12 (Fig. 2). A linear relationship was obtained between Ct values and the $\log_{10}$ DNA concentration. Amplification efficiency was 97.5%, the slope of the standard curve was -3.382, and the coefficient of determination (R^2) was 1.0, indicating high efficiency and reproducibility of the method. All tested samples amplified within the range of the standard curve. Amplification was observed in the positive control (PC), whereas no signal was detected in the negative control (NTC).

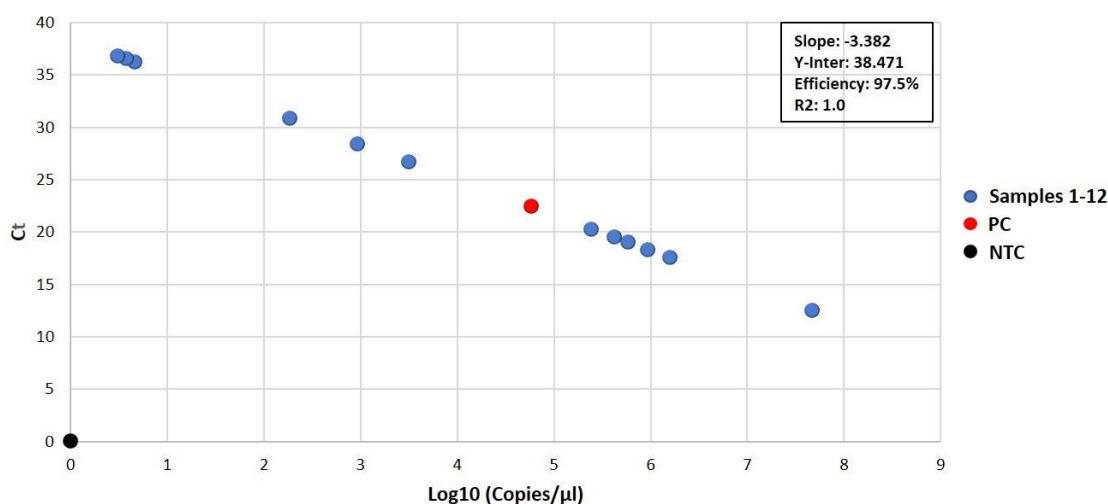


Figure 2. Linear relationship between Ct values and $\log_{10}$ DNA concentration of Cmm pathogen.

The qPCR results for the tomato samples, including Ct values and the calculated number of Cmm DNA copies per sample, are presented in Table 2. As shown, Ct values range widely from 12.5 to 36.8, indicating substantial variability in pathogen DNA content among the tested samples.

Samples N7 and N1 were characterized by low Ct values (12.5 and 17.5, respectively) and high bacterial

loads (4.78×10^7 and 1.59×10^6 copies/μl), indicating a high level of infection. The plants from which these samples were taken exhibited pronounced symptoms of bacterial canker, including wilting and overall plant decline, confirming the correlation between high pathogen DNA concentration and the severity of clinical disease manifestations. The obtained results are consistent with the literature data, according to which under high

infection pressure ($>10^8$ CFU/g of tissues), plant survival and yield are sharply reduced, while the productivity of infected plants accounts for only 51–63% compared to the control [30].

Samples N6 and N8-12 exhibited high Ct values (26.7-36.8) and low Cmm DNA content (from 3.12×10^1 to 3.02×10^3 copies/ μ l), indicating a latent stage of infection or a low level of pathogen presence. These plants did not

show visible disease symptoms. These results highlight the diagnostic value of qPCR, as the absence of visual infection signs can make pathogen detection difficult with conventional methods. Latent infections can remain undetected for extended periods and pose a significant epidemiological risk, particularly under protected cultivation conditions, which is consistent with literature data.

Table 2. qPCR results for tomato samples (data from 2024-2025).

Sample №	Region	Ct value	DNA copies, copies/ μ l	Disease development stage
1	Ararat	17.5	1.59×10^6	Severe symptoms
2	Ararat	18.3	9.21×10^5	Moderate symptoms
3	Ararat	19.0	5.72×10^5	Moderate symptoms
4	Kotayk	19.5	4.07×10^5	Moderate symptoms
5	Kotayk	22.4	5.65×10^4	Moderate symptoms
6	Kotayk	26.7	3.02×10^3	Latent stage (no symptoms)
7	Kotayk	12.5	4.78×10^7	Severe symptoms
8	Kotayk	30.8	1.85×10^2	Latent stage (no symptoms)
9	Kotayk	36.2	4.69×10^1	Latent stage (no symptoms)
10	Ararat	36.5	3.12×10^1	Latent stage (no symptoms)
11	Ararat	36.8	3.12×10^1	Latent stage (no symptoms)
12	Ararat	28.4	9.50×10^2	Latent stage (no symptoms)

Samples N2-N5, with intermediate Ct values (18.3-22.4), had moderate pathogen DNA concentrations (from 5.65×10^4 to 9.21×10^5 copies/ μ l), corresponding to early stages of disease development. These plants exhibited initial infection symptoms, including leaf damage and necrotic changes in the stem. For comparison, according to Kawaguchi (2022), the concentration of Cmm in asymptomatically infected plants may reach 3×10^6 CFU/g of plant tissue, confirming the possibility of latent (hidden) infection development at early stages [31].

Detecting infection at this stage is crucial for timely phytosanitary intervention, significantly reducing the risk of further pathogen spread and yield losses. The results obtained corroborate previous studies emphasizing the importance of regular monitoring and early diagnosis of phytopathogens using qPCR [32-34].

Based on the qPCR results and their correlation with symptom severity, Ct value ranges were classified into practical diagnostic categories for disease management in greenhouses (Table 3).

Table 3. Interpretation of Ct values and recommended phytosanitary actions.

Ct Range	Interpretation	Biological status	Recommended actions
<18	High infection load	Active infection, severe symptoms	Immediate removal of plants, sanitary treatment
18–25	Early stage infection	Initial symptoms	Enhanced monitoring, local isolation of infection foci
25–35	Latent infection	Asymptomatic carrier	Preventive measures, regular qPCR monitoring
>35	Borderline value	Low-level detection	Retesting and dynamic monitoring

The results indicate that asymptomatic tomato plants with high Ct values (25-35) pose a significant epidemiological threat under greenhouse production conditions. Despite the absence of visible disease symptoms, these plants contain detectable levels of Cmm DNA and can serve as sources of secondary infection. Under high planting density and intensive agronomic practices, latent infections play a key role in pathogen spread. This highlights the limited informativeness of visual monitoring and underscores the necessity of

regular qPCR surveillance for the early detection of infection foci.

Evaluation of diagnostic efficiency: Analysis of the data presented in Table 4 showed a similar trend of reduced infection levels and yield losses in commercial greenhouses in Kotayk and Ararat regions when transitioning from visual diagnosis to molecular diagnosis using qPCR. Visual diagnosis was carried out by the agronomists of the farms.

Table 4. Detection of infections and yield losses in commercial greenhouses of Kotayk and Ararat regions, Armenia (extended vegetation, 2023–2026).

Year	Infection detection stage	Diagnostic method	% of infected plants		Yield loss, %		Description
			Kotayk	Ararat	Kotayk	Ararat	
2023-2024	Late	Visual	13.2	14.9	9.4	10.1	Mass plant mortality
2025-2026	Latent/ asymptomatic	qPCR	4.8	5.1	3.5	3.9	Timely removal of foci, immediate implementation of protective measures

In 2023-2024, with late detection of infection, the proportion of infected plants in Kotayk was 13.2%, and in Ararat, 14.9%, with corresponding yield losses of 9.4 and 10.1%, respectively. In 2025-2026, using qPCR for detection at the latent stage, a significant reduction in both indicators was observed in both regions. The proportion of infected plants decreased to 4.8% in Kotayk and 5.1% in Ararat, while yield losses dropped to 3.5 and 3.9%, respectively.

Overall, the efficiency of early molecular diagnosis was comparable in both regions and resulted in a reduction of production losses by approximately 2.5-3 times. Early pathogen identification allowed rapid removal of primary infection foci and immediate implementation of protective measures, effectively limiting further disease spread.

In addition to yield reduction, bacterial infection may affect the physiological processes of fruit development, which is reflected in quality parameters such as fruit size, ripening dynamics, and the content of bioactive compounds that determine the nutritional and functional value of tomato. A decrease in average fruit

weight from 165.4 to 134.2 g was observed in the present study, corresponding to a reduction of 18.9%. At the same time, accelerated fruit ripening was recorded. The obtained results are consistent with the literature, where reductions in fruit weight of up to 50% have been reported in cases of bacterial canker development in tomato plants [14].

Thus, the results clearly demonstrate that the use of real-time PCR for early diagnosis of bacterial infections in tomatoes significantly reduce phytosanitary risks and allows for the minimization of production losses. The integration of molecular methods into greenhouse monitoring systems not only improves disease control efficiency but also contributes to preserving product quality as a functional food, ensuring the production of healthier and biologically more valuable plant-derived food products.

CONCLUSION

The results of this study confirm the high efficiency of quantitative PCR as a tool for early and reliable diagnosis of tomato bacterial canker under protected cultivation

conditions. The qPCR method enables the detection of *Clavibacter michiganensis subsp. michiganensis* at latent stages of the disease, when visual symptoms are absent, allowing timely implementation of phytosanitary measures and preventing widespread infection. The integration of qPCR monitoring into greenhouse management systems contributes to a significant reduction in the proportion of infected plants and yield losses, thereby enhancing biosafety and the resilience of intensive tomato production. In addition, early detection and control of the pathogen are important not only for improving agronomic efficiency but also for preserving product quality as a functional food. Maintaining the phytosanitary status of plants contributes to the preservation of the level and stability of bioactive compounds in tomato fruits, which directly determines their nutritional value and potential beneficial effects on human health.

List of abbreviations: SCVIC - Scientific Centre of Vegetable and Industrial Crops, MEofRA – Ministry Economy of the Republic of Armenia, Cmm- *Clavibacter michiganensis subsp. Michiganensis*, qPCR- quantitative Polymerase Chain Reaction, CTAB- Cetyltrimethylammonium Bromide, PC - Positive Control, NTC - No Template Control, LOD- Limit of detection, LOQ- Limit of quantification.

Competing interests: The authors declare that they have no financial, professional, or personal competing interests that could have appeared to influence the work reported in this manuscript.

Authors' contributions: IV and GS conceived and designed the study. MAA provided consultation on qPCR methodology. IV and HM performed the qPCR analyses. GS and AP edited the manuscript. DS performed the statistical analyses. All authors read and approved the final version of the manuscript.

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