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RESEARCH ARTICLE

First Report of Bacterial Wilt Caused by *Clavibacter michiganensis* subsp. *michiganensis* Affecting Tomato in Iğdır

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ABSTRACT

Tomato wilt disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is one of the most destructive tomato diseases and causes significant crop loss in both greenhouse and field tomato production areas worldwide. In this study, the presence of the causal agent of bacterial wilt disease in tomato plants was investigated in Aras Valley. Isolation was made from diseased plant samples and it was determined whether the strains were pathogenic by cellulase activity and HR test. The virulence, morphological and biochemical characteristics of the strains were determined. Strains that fatty acid methyl ester extraction, isolation and purification were performed were identified at species and subspecies level with % similarity index using gas chromatography system. The diagnosis was confirmed with the Biolog Gen III System and all strains were identified at the subspecies level with a % similarity index. As a result of this study, 57 strains were obtained in the isolation, and 39 of the strains were determined not to be pathogenic. Strain 18 was determined as the pathogen causing the most damage to tomato plants with 100 % disease severity. Strains were identified as Cmm at subspecies level with a similarity index of 71-87 % using gas chromatography system and 54-75 % similarity index with Biolog Gen III System. According to the heat map created, it was determined that the strains consisted of two main clusters. The presence of pathogen in Aras Valley was proven for the first time by this study.

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable species grown worldwide, with high production and consumption and commercial value. Bacterial diseases are of great importance among the factors limiting the cultivation of tomato plants, and the production of quality tomatoes is made possible by the diagnosis and control of these disease-causing organisms. Among these diseases, tomato wilt disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is one of the most destructive tomato diseases and causes significant crop loss in both greenhouse and field tomato production areas worldwide (Gautam et al., 2020). The disease still remains a serious source of concern for the tomato industry worldwide with 50-80 % yield and quality losses (Takishita et al., 2018).

The aerobic, gram-positive pathogen penetrates the plant through natural openings and wounds, then passes into the xylem and creates characteristic symptoms such as light brown colouring of the vascular bundles, wilting of one-sided leaves, necrotic lesions on the stem and petioles (Eichenlaub & Gartemann, 2011). When the pathogen infects the plant during the seed and seedling period, systemic infection occurs and leads to the death of the plant. When the infection occurs in the later stages of plant development, bird's eye symptom occurs

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on the fruit. The pathogenicity is manifested by transcriptional regulators and virulence factors encoded by chromosomes and two plasmids. Virulence factors consist of serine proteases and cell wall degrading enzymes (cellulases, xylanases, pectinases). In studies, it was detected that the virulence genes viz. Cel A encoding cellulase enzyme and pat1, chp C and ppa A encoding serine protease were stimulated after the entry of the pathogen into the host (Nandi et al., 2018).

The successful use of fatty acid methyl ester (FAME) analysis and Biolog Gene III System in the diagnosis of bacteria was reported by different researchers (Anak et al., 2021; Belgüzar et al., 2016; Çakmakçı et al., 2010; Sunyar et al., 2021). Vauterin et al. (1995) used the Biolog System as one of the classification criteria for pathovars belonging to the genus *Xanthomonas*. Mirik et al. (2011) successfully identified *Pseudomonas cichorii* causing disease in tomato by FAME analysis with a similarity index of 84-97%. Aysan and Uygur (2005) detected mainly 12 fatty acids as a result of FAME analysis and identified the pathogen as *Pseudomonas viridiflava* with 81-96% similarity index. This study was carried out with the aim of investigating the presence of Cmm, which causes the disease in tomato plant that has economic value in Aras Valley, and determining the morphological, biochemical and pathological characteristics of the strains obtained and detecting their diagnosis by fatty acid methyl ester analysis and Biolog Gen III system.

2. Materials and Methods

2.1. Collection and Isolation of Diseased Plant Samples

In 2020, tomato fields were visited in Iğdır, Aralık, Tuzluca and Karakoyunlu districts and Kasımcan, Oba and Melekli villages of Iğdır city center, and diseased plant samples were taken according to the simple random sampling method by selecting 3 fields from the district and each village. The surveys were carried out 2 times during seedling and fruiting periods of the plants. Fruits showing bird's eye symptoms and stems with browning of vascular bundles were taken as isolation material. Bacteria were isolated from the samples and yellow coloured colonies were purified from the bacteria growing on YDC (Yeast Dextrose Carbonate Agar) medium. Stock cultures of the strains were prepared in 500 ml Luria Bertani Broth and 500 ml 30% glycerol and stored at -80°C.

2.2. Identification of Non-Pathogenic Strains

Cellulase activity of the strains was tested to identify non-pathogenic bacteria. For this test, a medium consisting of four different solutions recommended by Yin et al. (2010) was used. The prepared medium was inoculated with bacterial cultures cultivated on Nutrient Agar (NA) medium for 24-48 hours and incubated at 26°C for 4 days. After incubation, the petri surface was covered with 10 ml of 0.1% congo red solution and waited for 20 minutes. After this duration, the solution in the petri dish was removed from the medium. The medium was then covered with 10 ml of 1M NaCl and waited for 5 minutes. The yellow coloured open area formed around the colonies in the red coloured medium was evaluated as a positive result. The pathogenicity of strains with positive cellulase activity was confirmed by the hypersensitive reaction test in tobacco (*Nicotiana tabacum* L. Samsun) specified by Lelliot and Stead (1987).

2.3. Evaluation of Virulence of Bacterial Strains

The pathogenicity test was carried out using super 5656 tomato variety. Plants were grown for five weeks in pots containing sterile sand + soil mixture prepared at a ratio of 1:4. Cells of the strains grown on NA medium were transferred to Nutrient Broth (NB) medium with a sterile core and incubated overnight at 150 rpm/min on a shaker set at 26°C. At the end of the incubation period, the inoculum density was set as 10^8 CFU ml^{-1} by turbidimeter. Tomato seedlings were inoculated with 100 µl of bacterial suspension using stem inoculation assay. The plants in the negative control group were treated with sdH_2O. After inoculation, polythene bags were placed over the plants and incubated at 26°C for 48 hours. At the end of the period, the bags were removed and the occurrence of disease symptom was monitored daily for 14-21 days. The study was carried out according to random plots experimental design with 3 repetitions. Disease appearance was evaluated according to a 0-5 scale (0: no disease symptoms; 1: wilting on 1-10% of the leaves; 2: wilting on 11-25% of the leaves; 3: wilting on 26-49% of the leaves; 4: wilting on 50-74% of the leaves; 5: all leaves of the plant wilted) (Soylu et al., 2003). The % disease severity was determined using the formula of Townsend and Heuberger (1943) (Formula 1).

$$\% \text{ Disease Severity} = \frac{\Sigma \text{ (scale value} \times \text{number of plants evaluated on the scale)}}{\text{highest scale value} \times \text{total number of plants}} \text{ x } 100 \quad (1)$$

2.3.1. Determination of colony and mobility characteristics of bacterial strains

Colony characteristics of bacterial strains were determined in YDC medium. For the mobility test of bacterial strains, 10 g tryptone, 5 g NaCl and 5 g agar were added to one litre of sdH_2O. The pH of the mixture was adjusted to 7.2 and 5 ml of this mixture was placed in tubes and sterilised in autoclave at 121°C for 15 minutes. After inoculation of bacterial strains into the prepared media, bacterial growth was checked at 8^{th}, 24^{th} and 48^{th} hours. Colony growth from the inoculation point to the surrounding area was recorded as a positive result (Schaad et al., 2001).

2.4. Biochemical Characterization of Bacteria Strains

The gram reaction test was performed with 3 % potassium hydroxide (KOH) solution (Hyder et al., 2020), the catalase test with 7% H_2O_2, the oxidase test with discs containing 1% tetra

methyl-p-phenylendiamine dihydrochloride (Narayanasamy, 2001). The arginine dehydrolase test was determined by the pinkish red colour of Thornley 2A medium, the levan test by the presence of convex, mucoid colonies on Nutrient Sucrose Agar medium, starch hydrolysis by the detection of a transparent zone around the colonies on Nutrient Starch Agar medium, and the pectolytic activity of bacterial strains by the formation of wells or watery appearance on Crystal Violet Pectate Agar (CVP) medium (Hélias et al., 2012).

2.5. Identification of Microorganisms by Fatty Acid Methyl Ester Analysis

Bacterial strains maintained as pure cultures at -80°C were cultivated on Tryptic Soy Agar medium for 24-48 hours for fatty acid methyl ester extraction, isolation and purification. The colonies of the bacterial strains were collected with a sterile core and placed in glass test tubes with Teflon caps and each tube was treated with cell lysis solution [150 ml methyl alcohol (HPLC Grade, 45 g sodium hydroxide (ACS Grade), 150 ml sdH_2O]. 1 ml was added to free fatty acids. Then 2 ml of methylation solution [325 ml hydrochloric acid (6N), 275 ml methyl alcohol (HPLC Grade)] was added to the test tubes and methyl was added to the free fatty acids with ester bonds and fatty acid methyl esters were obtained and fatty acids were given high temperature volatility. After this process, the tubes were cooled rapidly and 2.5 ml of the purification solution [200ml methyl-tert-butyl-ether (HPLC Grade) 200 ml hexane (HPLC Grade)] was added. Meanwhile, the organic phase formed in the tube was retained and the acidic phase remaining at the bottom was discarded with a pasteur pipette. In the last step, 3 ml of basic washing solution [10.8 g solid sodium hydroxide (ACS Grade) 900 ml sdH_2O] was added to the test tubes and free fatty acid methyl esters were obtained in the pure form. At this stage, the phase containing fatty acid methyl esters collected at the top of the tube was collected with a pasteur pipette and transferred to 2 ml gas chromatography tubes, then the caps were firmly sealed and placed in the sample storage tray on the device. The cultured strains were identified at the species and subspecies level using the Microbial Identification System (Agilent 7890A GC System, MIDI, Inc., Newark, DE, Sherlock Software Version 6.1), which is a computer-controlled gas chromatography system. Fame profiles were compared with the RTSBA 6 library (Sasser, 1990). Analysis conditions of gas chromatography were presented in Table 1.

Table 1. Gas chromatography analysis conditions.

System	Agilent 7890A GC System
Column	HP-Ultra 2 (25 m x 199 µm x 0.33 µm)
H_2 Flow	30 ml/min
Air Flow	350 ml/min
N_2 Flow	28.771 ml/min
Total Flow	53.37ml/min
Septum Purge Flow	3 ml/min
Pressure	20 psi
Oven Temperature	120-210°C
Equilibration Time	0.25 min
Injection Temperature	250°C
Column Temperature	60°C for 2 min, 10°C/min to 200°C, 5°C/min to 240°C, hold 240°C for 7 min
Split Ratio	1/40
Split Flow	100 ml/min
Flame Ionization Detector	250°C

2.6. Diagnosis of Bacterial Strains with Biolog Gene III System

The obtained bacterial strains were grown on BUG (Biolog Universal Growth Agar) medium to determine their metabolic profiles. The bacterial cultures were suspended in IF-A buffer solution and the bacterial concentration in the tubes was adjusted by turbidimeter with a transmittance value of 92-95%. 100 µl of the adjusted bacterial suspensions were added to each well on the microplates and the plates were and the plates were incubated.

After incubation, the microplates were read on a Biolog reader. The metabolic profiles obtained for the test microorganisms were compared with the metabolic profiles of the microorganisms in the package programme of the system (MicroLogTM3 MicroStationTM Software, Version 5.2.2) (Saygılı et al., 2006).

2.7. Statistical Analysis

The data obtained as a result of the pathogenicity test were subjected to analysis of variance in SPSS statistical programme (16.0). Significant values were grouped by using the Duncan multiple comparison test at $p \leq 0.01$ significance level.

Depending on the differences in the fatty acid profiles and metabolic profiles of the strains, a heat map was created by using the "heat map.2" command in the "glots" library in the R package programme.

3. Results and Discussion

As a result of isolation from the stems and fruits of diseased tomato plants, 57 bacterial strains were obtained. By using the cellulase activity test, 39 non-pathogenic strains were identified from the bacteria. The pathogenicity test was carried out with the remaining 18 strains and the results are given in Table 2 and Figure 1. The differences between the strains in terms of scale values were determined to be statistically significant ($p \leq 0.01$). When the disease severity values were analysed, it was determined that 12 Cmm strains had high virulence.

Table 2. The pathogenicity test results and virulence of bacterial strains.

Strain	Scala Value	Virulence Level	Strain	Scala Value	Virulence Level
Cmm 1	1.7^{BC}	Low	**Cmm 10**	4.7^{A}	High
Cmm 2	3.0^{AB}	Moderate	**Cmm 11**	4.3^{A}	High
Cmm 3	1.7^{BC}	Low	**Cmm 12**	3.0^{AB}	Moderate
Cmm 4	3.0^{AB}	Moderate	**Cmm 13**	3.0^{AB}	Moderate
Cmm 5	4.0^{A}	High	**Cmm 14**	4.3^{A}	High
Cmm 6	4.0^{A}	High	**Cmm 15**	4.3^{A}	High
Cmm 7	4.0^{A}	High	**Cmm 16**	4.3^{A}	High
Cmm 8	4.3^{A}	High	**Cmm 17**	3.0^{AB}	Moderate
Cmm 9	4.3^{A}	High	**Cmm 18**	5.0^{A}	High
			Control	0^{C}	

^{A,B,C}The values indicate the average of 3 replications, according to the Duncan multiple comparison test, the difference between the averages shown with different letters in the same column is significant with respect to $p \leq 0.01$.

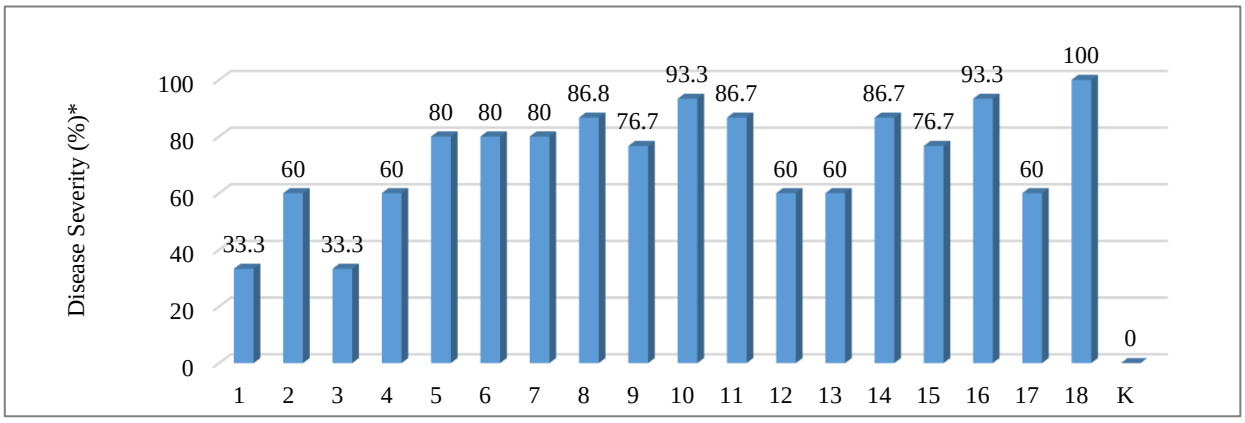

Figure 1. The disease severity index of Cmm strains.

The morphological and biochemical characteristics of the bacterial strains are given in Table 3. It was observed that all of the strains formed mucoid, yellow coloured colonies on YDC medium. The bacterial strains were identified as rod-shaped, gram positive bacteria by using the Biolog System. However, the strains were detected to be gram-negative in the KOH test. The results of the catalase and starch hydrolysis tests were positive, while the levan colony formation and pectolytic activities were negative.

Table 3. Morphological and biochemical characteristics of bacterial strains.

SN	Colony Description	M	GR	BG	CT	OX	AD	LC	SH	PA
1	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
2	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
3	Yellow, mucoid, rod	-	-	+	W^+	-	-	-	+	-
4	Yellow, mucoid, rod	-	-	+	+	-	-	-	W^+	-
5	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
6	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
7	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
8	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
9	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
10	Yellow, mucoid, rod	-	-	+	+	W^+	-	-	+	-
11	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
12	Yellow, mucoid, rod	-	-	+	+	-	-	-	W^+	-
13	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
14	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
15	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
16	Yellow, mucoid, rod	-	-	+	+	-	-	-	+	-
17	Yellow, mucoid, rod	-	-	+	W^+	-	-	-	+	-
18	Yellow, mucoid, rod	-	-	+	+	W^+	-	-	+	-

SN: Strain No; M: Motility; GR: Gram reaction; BG: Biolog System gram reaction result; CT: Catalase; OX: Oxidase; AD: Arginine Dihydrolase; LC: Levan Colony; SH: Starch Hydrolysis; PA: Pectolytic Activity; +: Positive result; -: Negative result; W^+: Weak positive.

The fatty acid types and their % amounts in 18 Cmm strains were determined by the gas chromatography system. The identification results, the similarity index and the number of fatty acids contained in the strains are given in Table 4. The strains obtained were identified as Cmm at subspecies level with 71-87% similarity index. It was determined that the number of fatty acids detected varied in the strains. The highest number of fatty acids (18) was detected in Cmm 2 and Cmm 12.

Table 4. Diagnostic results of bacterial strains according to FAME profiles.

SN	FAME Identification Result	SI (%)	NFA
1	*Clavibacter michiganensis* subsp. *michiganensis*	72	11
2	*Clavibacter michiganensis* subsp. *michiganensis*	72	18
3	*Clavibacter michiganensis* subsp. *michiganensis*	76	11
4	*Clavibacter michiganensis* subsp. *michiganensis*	78	15
5	*Clavibacter michiganensis* subsp. *michiganensis*	71	12
6	*Clavibacter michiganensis* subsp. *michiganensis*	82	15
7	*Clavibacter michiganensis* subsp. *michiganensis*	87	14
8	*Clavibacter michiganensis* subsp. *michiganensis*	72	13
9	*Clavibacter michiganensis* subsp. *michiganensis*	83	13
10	*Clavibacter michiganensis* subsp. *michiganensis*	74	11
11	*Clavibacter michiganensis* subsp. *michiganensis*	85	14
12	*Clavibacter michiganensis* subsp. *michiganensis*	72	18
13	*Clavibacter michiganensis* subsp. *michiganensis*	72	13
14	*Clavibacter michiganensis* subsp. *michiganensis*	83	13
15	*Clavibacter michiganensis* subsp. *michiganensis*	71	12
16	*Clavibacter michiganensis* subsp. *michiganensis*	82	15
17	*Clavibacter michiganensis* subsp. *michiganensis*	78	15
18	*Clavibacter michiganensis* subsp. *michiganensis*	74	11

SN: Strain Number; FAME: Fatty Acid Methyl Ester; SI: Similarity Index; NFA: Number of Faty Acid.

When the FAME profiles of the strains were analysed, it was observed that 10 fatty acids (14:0, 14:0 iso, 15:0 iso, 15:0 anteiso, 16:0, 16:0 iso, 17:0, 17:0 iso, 17:0 anteiso, 18:1 w9c) were common in all strains. Fatty acids 13:0, 14:0 anteiso and 17:1w8c were present in Cmm strain 2 and 12, while 18:0 3OH and 20:0 iso were present only in Cmm strain 7. It was determined that 15:0 anteiso (42.4-52.4%) and 17:0 antesiso (26.45-34.60%) fatty acids were present in all strains. The presence of 15:1 anteiso A fatty acid, having a diagnostic value for Cmm, was determined at a rate of 2.3-4.9%. A heat map was created based on the fatty acid types and % amounts determined in the strains with the R package programme (Figure 2). In this map, the relationship between the data of fatty acids that enable the grouping of bacterial strains is represented by colours. In the heat map, X axis represents fatty acids and Y axis represents bacterial strains. When the heat map is analysed, it is seen that 18 Cmm strains consist of 2 main clusters, A and B. The first cluster consists of two subgroups, A1 (Cmm strain 2-12) and A2 (Cmm strain 4-17). The second cluster consists of B1, which is represented only by Cmm 7, and B2 (Cmm strain 8-13, 14-9, 11, 5-15, 16-6, 18-10, 3-1) subgroups that contain a wide variety.

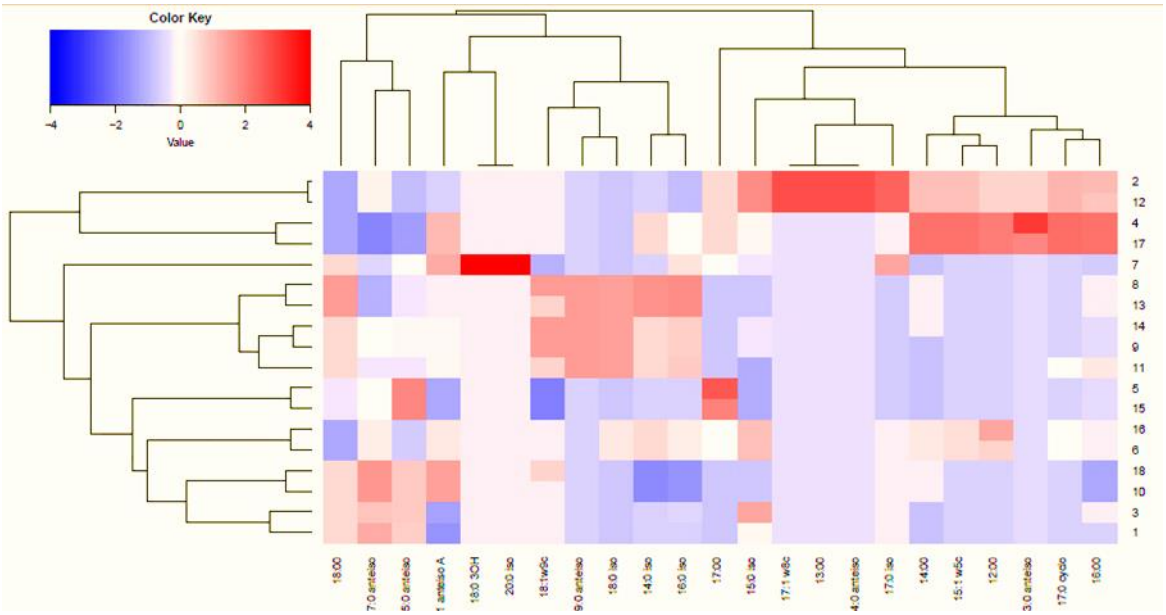

Figure 2. Heat map indicating the grouping of bacterial strains based on fatty acids.

The properties of the bacterial strains obtained in the study to utilise 71 different carbon sources and sensitivity tests for 23 chemicals were evaluated in the Biolog Gen III MicroPlate system and profiles were obtained accordingly. MicroPlates were evaluated visually, optically and spectrophotometrically. Visual reading revealed that redox reaction occurred in some of the wells, indicating that Cmm was metabolically active and as a result the wells were coloured purple. Optical reading was performed on the Biolog reader provided with a video camera that captured images at specific times. Spectrophotometric reading was carried out with a MicroStationTM reader at absorbance values of 590 nm and 750 nm at two intensities. Columns 1-9 were used for the carbon source utilisation tests. All reactions in these columns were compared to the reaction in the well A1, the negative control well. Therefore, reactions in wells in the columns 1-9 that gave a similar colour to the well A1 (absence of any colour) indicated that the respective carbon sources were not used and were considered negative. The purple coloured wells indicated that the strains metabolised the respective substrates using the carbon sources and were considered positive. The columns 10-12 were used for the chemical sensitivity tests. The reactions in these wells were compared with the reaction in the well A10, which was the positive control reaction. Since the colour of the positive control was purple, the reactions in the purple coloured wells in the columns 10-12 were considered positive and indicated that the strains were resistant to the respective chemical. The colourless well were considered negative as they demonstrated no growth and indicated that the strains exhibited a significant sensitivity to the chemical inhibitor. All of the strains were identified as *Clavibacter michiganensis* subsp. *michiganensis* at subspecies level. When the diagnosis report of the strains was analysed, the lowest similarity index was 54% and the highest was 75%. The diagnostic results of the strains are presented in Table 5.

Table 5. Identification of Cmm by Biolog Gen III System.

Identification	Strain No	Prob*(%)	Sim (%)	Dist
Cmm	1	100	97	0.3
	2	100	80	2.4
	3	99	80	2,7
	4	99	82	2.3
	5	99	88	1.6
	6	98	78	1.5
	7	98	76	1.4
	8	98	74	3.0
	9	98	54	1.2
	10	96	80	1.1
	11	94	71	1.6
	12	91	68	1.9
	13	87	75	0.2
	14	85	76	1.5
	15	75	72	1.2
	16	75	64	1.4
	17	75	75	1.7
	18	70	56	3.0

*Prob: Probability of correct identification; Sim: Similarity index value indicating the quality of each match; Dist: Distance rating indicating the number of mismatches.

When the profiles of Cmm strains were evaluated, it was determined that all of them used 3 carbon sources, namely Sucrose, D-Fructose and D-Mannitol. It was detected that only strains 9 and 14 grew in Tween 40 and the other strains did not grow in the presence of Tween 40. While all strains were observed to grow in 1% NaCl in the well B10, none of them were observed to grow in 8% NaCl in the well B12. In 4% NaCl in well B11, only 6 and 16 of the strains were observed to grow. When the tolerance of the strains to pH was analysed, it was detected that all of the strains grew at pH 6 and none of them grew at pH 8. When the reactions of Cmm strains against antibiotics were evaluated, it was observed that all of the strains did not grow in the wells containing troleandomycin, rifamycin, minocycline, lyncomycin and vanomycin, so they were sensitive to these antibiotics. In the wells containing Aztreonam antibiotic, all strains grew and therefore were resistant to this antibiotic. In the well containing nalidixic acid antibiotic, it was determined that all of the strains except 9 and 14 grew. A heat map was created based on the metabolic profiles determined with the R package programme (Figure 3). In the heat map, the relationship between the data belonging to the metabolic profiles that enable the grouping of Cmm strains is expressed in colours. In the heat map, the horizontal axis represents the bacterial strains and the vertical axis represents the wells in the Biolog Gen III MicroPlate. When the heat map is evaluated, it is seen that 18 Cmm strains consist of 2 main clusters, namely A and B. The first cluster consists of two subgroups, which are A1 (Cmm strain 9 and 14) and A2 (Cmm strain 6 and 16). The second main cluster (B) consists of two subgroups, namely B1 and B2. It is observed that the diversity in B1 is considerably high.

It is observed that the FAME and the Biolog Gen III System grouped Cmm strains differently based on their own characteristics. The results obtained demonstrate the value of both systems in the diagnosis of bacterial strains.

In this study, 57 bacterial strains were isolated from the stems and fruits of the diseased tomato plants. Jahr et al. (2000) reported that the endoglucanase gene celA of Cmm was an important virulence factor required for wilt induction on tomato. Therefore, cellulase activity test was used to determine the non-pathogenic *Clavibacter* strains. The results showed that 39 of the strains lacked cellulase activity, which was the proof of the non-pathogenicity. In the pathogenicity test, the disease-causing properties of 18 strains were investigated and it was determined that bacterial strains with cellulase activity caused wilt symptom on tomato plants. The results of the cellulase activity test were confirmed by determining that 12 of the bacterial strains had high, 4 of them had moderate and 2 of them had low levels of virulence. Załuga (2013) also investigated the presence of cellulase activity in the non-pathogenic Clavibacter strains and found that 15 non-pathogenic *Clavibacter* strains showed no cellulase activity, while the LMG 5616 and PD 5707 strains had high cellulase activity. It was determined that the strains formed yellow, mucoid colonies on the YDC medium. This result was found to be homogeneous with the results obtained in the study conducted by Li et al. (2018). In parallel with the findings of Cristina et al. (2018), it was determined in

this study that the bacterial cells were rod-shaped and immobile. As a result of biochemical tests, catalase, amylase, oxidase and levan colony formation of the strains were detected positive and negative. These test results were consistent with the findings of Kolomiets et al. (2017). In this study, while the strains were determined as gram positive bacteria by the Biolog System, they were determined as gram negative as a result of the 3% KOH test. In the study conducted by Tripathi et al. (2022), Cmm strains were found to be positive as a result of gram staining, but negative in the gram reaction test conducted with KOH. As stated in EPPO (2016), it was determined that Cmm strains developed in 6% NaCl.

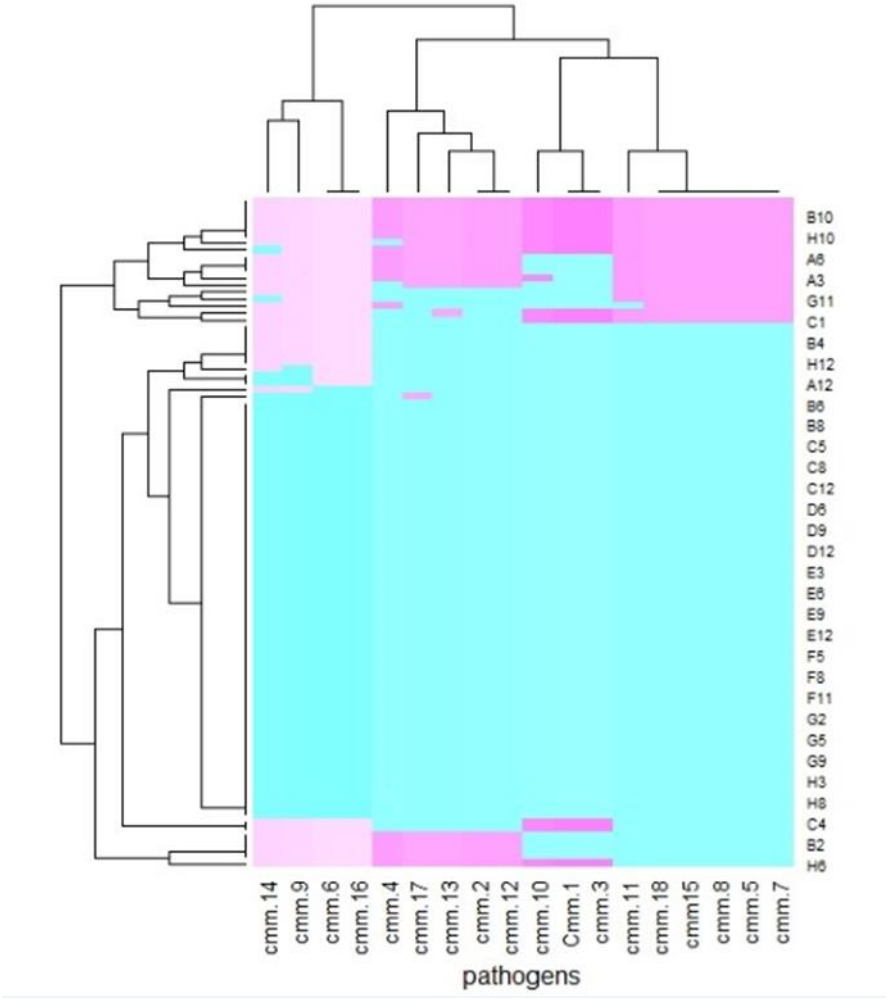

Figure 3. The heat map indicating the grouping of bacterial strains based on their metabolic profiles.

In numerous studies, the FAME analysis was used to classify microorganisms. A large library of fatty acid profiles including the Cmm fatty acid profile was established and the taxonomic significance of the FAME content in Coryneform bacteria, including *Clavibacter*, was reported. The anteisopenladecenoic acid, an unsaturated branched chain fatty acid with a carbon number of 15 and a double bond, was identified as an important criterion for the diagnosis of Cmm (Gitaitis & Beaver, 1990). In this study, the presence of 15:1 anteiso A fatty acid, which had a diagnostic value for Cmm, was identified in all strains at a rate of 2.33-4.95 %. In all strains, 15:0 anteiso (42.4-52.4%) and 17:0 antesiso (26.4-34.6%) fatty acids were detected at high rates and were identified as Cmm with a similarity index of 72-87%. These values were similar to other studies. For example, in the study conducted by Şahin et al. (2002) in the Eastern Anatolia Region of Türkiye, 16 strains were identified as Cmm with a similarity index of 47-89% as a result of the FAME analysis in the study in which the pathogen causing heavy yield losses up to 100% in tomato production areas was investigated. In the study conducted by Çetinkaya Yıldız (2007), 6 strains obtained from Mersin and Adana provinces were identified as Cmm with a similarity index of 41-81% and the 13 strains obtained from tomato production areas in Tokat province by Belgüzar et al. (2016) were identified as Cmm with a similarity index of 58-82% by using the Microbial Identification System. When the rates of fatty acids were analysed, it was observed that the strains were largely similar and 14:0 iso, 14:0, 15:1 anteiso A, 15:0 iso, 15:0 anteiso, 16:0 iso, 16:0, 17:0 iso, 17:0 anteiso and 17:0 fatty acids were detected in all strains. Especially 15:0 anteiso and 17:0 anteiso fatty acids were detected at high rates in the strains. It was determined that 15:1 anteiso A, which is

characteristic for Cmm, was found in strains at an average rate of 2.2%. Basım and Basım (2018) isolated a total of 118 strains from diseased tomato samples in the Western Mediterranean region of Türkiye. As a result of the FAME analysis, it was determined that the anteisoheptadeconic acid (a15:0, 41.8-55.8%), palmitic acid (i16:0, 7.1-15.4%) and anteisoheptadeconic acid (a17:0, 24.8-30.9%) were the major components in all strains and were detected in high amounts. These results were consistent with the characteristics of the *Clavibacter* genus members. It was stated that the number, diversity and % amount of bacterial fatty acids remained unchanged as long as the environmental conditions were the same, therefore differences in fatty acid profiles were an indirect indicator of genetic kinships between strains (Yang et al., 1993). As a result, cellular fatty acid profiles were suggested to determine the differences between microorganisms. It was stated that quantitative and qualitative changes in cellular fatty acids could be used as an indicator of differentiation between species. However, in the comparison of fatty acid profiles, it was stated that standardisation of cultivation conditions was important since the composition of the medium, age of the culture, temperature and oxygen availability had a strong effect on fatty acids (Schumann et al., 2009).

The Biolog System is described as the gold standard for bacterial diagnosis (Morgan et al., 2009). However, most of the studies using this system did not focus on gram-positive corineform phytopathogens, i.e., bacteria belonging to the genus *Clavibacter*. In this study, *Clavibacter michiganensis michiganensis*, which causes wilt in tomato, was identified by using the Biolog Gen III system. When the profiles of bacterial strains obtained with the Biolog Gen III System were evaluated, 18 of them were identified as Cmm. It was determined that all of the strains used 3 carbon sources (Sucrose, D-fructose and D-mannitol). It was determined that they did not use 47 carbon sources in the MicroPlate and yielded negative results in the sensitivity test against 6 chemicals. Stancu and Mitrea (2020) conducted a study on the identification of Cmm strains obtained from different regions of Romania by using the Biolog Microbial Identification System. The bacterial strains were identified as *C. michiganensis* at the species level, but subspecies could not be identified. It was determined that all of the identified strains used α-D-glucose, dextrin, D-mannose, D-mannitol, D-fructose, D-galactose and sucrose carbon sources and 27 carbon sources were not used. It was determined that D-mannitol, D-fructose and sucrose were also used by the strains in this study, but the strains exhibited differences in the use of α-D-glucose, dextrin, D-mannose and D-galactose carbon sources. Regarding the chemical sensitivity tests, the growth of the strains in the presence of 1% NaCl, nalidixic acid, aztreonam, potassium tellurite and pH 6 was detected as positive, while their tolerance to 8% NaCl was detected as negative. In this study, the same results were obtained for all tests except growth in potassium tellurite in well G12. The strains were found to be sensitive to troleandomycin, lincomycin, vancomycin, fusidic acid, rimfamycin SV, guanidine HCl, D-serine, minocycline and niaproof 4. As reported in the study by Yasuhara-Bell and Alvarez (2015), it was determined in this study that Cmm strains did not hydrolyse gelatin. In the study conducted by Korus (2011) on the identification of subspecies of 32 *C. michiganensis* strains, only one strain was identified as Cmm by using the GEN III OmniLog ID System. The similarity index of the strain was relatively low (31%). In the study conducted by Yasuhara-Bell and Alvarez (2015) on the differentiation of *Clavibacter* subspecies from Cmm, Cmm strains were identified with the Biolog System with an average similarity index of 0.748 and 0.898 and a probability index of 0.564 and 0.686. Ialacci et al. (2016) used the Biolog System for the diagnosis of 63 strains obtained from samples taken from 17 farms during tomato bacterial cancer outbreaks in Sicily and identified 21 of the strains as Cmm with a similarity index of 0.50-0.70 and a probability index of 83-100%. In this study, 18 strains were identified as Cmm with a similarity index of 54-97% and a probability index of 70-100% and the result was confirmed by the pathogenicity test. Ialacci et al. (2016) and Stancu and Mitrea (2020) emphasised that the identification would be correct if the similarity index was greater than 0.5. In this study, it was determined that the similarity index of the strains was greater than 0.5. Harris-Baldwin and Gudmestad (1996) reported that the accuracy of the strain identification increased when the profiles of the known strains of a particular species were added to the Biolog System database. In this study, the strains were successfully identified with the Biolog Gen III System. However, in most of the studies, it was found that the differences between the strains studied with the Biolog System database did not alter their classification at the genus and species level, but were inadequate for their classification at the subspecies level (Morgan et al., 2009). The results indicated that *C. michiganensis* subspecies shared very similar dietary characteristics and therefore produced very similar profiles, which could pose a problem when trying to use profiles for subspecies differentiation. Yasuhara-Bell (2014) suggested that differences in metabolic profiles of strains could reflect loss or gain of genetic material and/or adaptation to a more specific niche.

4. Conclusion

In this study, all bacterial strains isolated from tomato plants were identified based on the FAME analyses and the metabolic profiles obtained using the Biolog Gene III System. The obtained morphological and biochemical test results provided valuable information for the diagnosis of Cmm. The results showed that morphological and biochemical properties could be used together to make a distinction between species, but it was necessary to use MIS and Biolog Gen III System to determine subspecies categories. It is thought that the profile

data obtained in this study will be useful in the development of databases of systems for the diagnosis of Cmm.

Conflict of Interest

The author has no conflict of interest to declare.

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