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Determination of Resistance Levels to Clavibacter michiganensis subsp. michiganensis in Some Solanum Species

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ABSTRACT: ***Clavibacter michiganensis*** *subsp.* ***michiganensis*** *(Cmm), is a devastating bacterial disease agent causing bacterial wilt and canker in tomatoes. There is no definitive solution to prevent yield losses by Cmm in tomatoes. Moreover, there is currently no commercially successful Cmm resistant tomato cultivar on the market. Therefore, we aimed to determine the tolerance level of some tomato accessions to Cmm in the present study. For this purpose, we screened seven tomato accessions representing four species (****Solanum arcanum, S. habrochaites, S. pennellii,*** *and* ***S. peruvianum****) from Peru, Ecuador, and Mexico against the highly virulent isolates Cmm-244 and Cmm-9. A root immersion method was used to identify new sources of resistance to this important disease. Two accessions,* ***S. habrochaites LA1777****, and* ***S. arcanum LA2157*** *were found to be moderate and highly tolerant, respectively, and could serve as tolerance resources for tomato breeding in Türkiye. These materials can also be investigated more extensively to determine their intrinsic Cmm tolerance mechanism.*

Keywords: ***Solanum*** *spp., wild species, bacterial canker, cultivar resistance.*

Bazı Solanum Türlerinin Clavibacter michiganensis subsp. michiganensis'e Dirençlilik Seviyelerinin Belirlenmesi

ÖZ: ***Clavibacter michiganensis*** *subsp.* ***michiganensis*** *(Cmm), domateste bakteriyel solgunluk ve kansere neden olan yıkıcı bir hastalık ajanıdır. Domateste Cmm'e karşı verim kayıplarını önlemek için kesin bir çözüm yoktur. Ayrıca, günümüz piyasasında Cmm'e karşı dayanıklı, başarılı bir ticari domates çeşidi bulunmamaktadır. Bu nedenle, bu çalışmada bazı domates çeşitlerinin Cmm'ye karşı toleranslık düzeylerini belirlenmesi hedeflenmiştir. Bu önemli hastalığa karşı yeni direnç kaynaklarını belirlemek amacıyla Peru, Ekvador ve Meksika'dan, dört domates türünü (****Solanum arcanum, S. habrochaites, S. pennellii*** *ve* ***S. peruvianum****) temsil eden yedi domates çeşidi son derece virülent Cmm-244 ve Cmm-9'a karşı kök daldırma yöntemi kullanılarak test edilmiştir. Çalışmamızın sonucunda iki çeşit,* ***S. habrochaites*** *LA1777 ve* ***S. arcanum*** *LA2157, sırasıyla orta ve yüksek toleranslı bulunmuştur ve Türkiye'de domates yetiştiriciliği için önemli tolerans kaynağı olarak kullanılabileceklerdir. Bu malzemeler ayrıca sahip oldukları Cmm tolerans mekanizmalarının ortaya çıkarılması amacı için daha kapsamlı bir şekilde ileriki çalışmalarda araştırılabilir.*

Anahtar Kelimeler: ***Solanum*** *spp., yabani türler, bakteriyel kanser ve solgunluk, çeşit dayanıklılığı.*

INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important fruit due to its high nutritional content including vitamins and antioxidants. In recent years, tomato production has steadily increased worldwide with a greater than 65% increase since the 2000s. Türkiye ranks first in Europe and fourth worldwide with a 10% share of tomato production (Anonymous, 2021). Tomato yield and quality are limited by various biotic and abiotic stress factors such as pathogenic viruses, fungi, and bacteria.

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is a serious disease agent causing bacterial wilt and canker in tomatoes. Bacterial canker has been considered a major problem since it was first reported in 1909 in the USA (Razdan and Mattoo, 2007). It causes death in 46 to 93% of plants, resulting in global yield losses ranging from 10 to 80% in tomatoes (Peritore-Galve *et al.*, 2021). Cmm is a soil-borne disease agent and can maintain its vitality in plant residues for more than two years after infection (Fatmi and Schaad, 2002). This pathogen is generally transmitted by infected seeds (Belgüzar *et al.*, 2016) and can infect seedlings even at a density as low as five bacterial cells per seed (Lelis *et al.*, 2014). Moreover, it can cause an epidemic even when only 0.01% of plants are infected (Chang *et al.*, 1991). Cmm is responsible for the wilting and death of plants due to the systemic movement of the bacterium through plant tissues and the clogging of vascular bundles. Due to the late appearance of symptoms (Pine *et al.*, 1954), plants cannot be rescued by intervention and completely die. As a result, bacterial wilt is considered the most devastating bacterial disease of tomatoes and can cause yield losses of up to 99% during the production season (Francis *et al.*, 2001). In Turkiye, the disease is especially prevalent in the Aegean (Karaca and Saygılı, 1982; Özyılmaz and Benlioğlu, 2001), Mediterranean (Kahveci and Gürcan, 1993; Yıldız and Aysan, 2008), and Central Anatolia regions (Öktem and Benlioğlu, 1993) where tomato production is most intense. In the Western Mediterranean region in 2003, it was reported that the disease incidence of Cmm was between 26% and 65% (Basim *et al*., 2004). In Türkiye, the tolerance of this disease agent in tomato seeds is zero (Uyar, 2011) which makes it a high risk for tomato production.

Although biological control (*Bacillus subtilis*) and chemicals (copper compounds, antibiotics) are used to prevent the spread of Cmm, these treatments are neither fully effective nor environmentally friendly. The most common way to prevent yield losses by Cmm is to remove infected plant material from the field because the bacterium can survive within the plant. However, this traditional method is not completely effective because laboratory analysis may be needed for detection of the bacterium due to possible confusion with other disease factors, and this can be time consuming and costly. The most effective way to control Cmm is to develop tomato varieties that are resistant or tolerant to the pathogen. However, such work has not yet yielded stable field tolerance due to the variability of Cmm isolates and their different virulences (Razdan and Mattoo, 2007; Gartemann *et al.*, 2003; Yuqing *et al.*, 2018). Therefore, it is essential to explore new sources of resistance to different Cmm isolates.

Although several *Solanum* species are reported to have tolerance to Cmm such as *S. habrochaites* (Francis *et al.*, 2001; Hassan *et al.*, 1968), *S. arcanum*, *S. chilense, S. peruvianum* (Sotirova *et al.*, 1994), *S. pimpinellifolium* (Thyr, 1976) and some *S. lycopersicum*-derived lines (Crino *et al.*, 1995; Poysa, 1993) transferring resistance to new cultivars has not been accomplished due to the complexity of Cmm resistance. Sen *et al.* (2012) tested 25 wild tomato accessions representing ten different species against isolate Cmm-542 and reported three new tolerant accessions: *S. pimpinellifolium* GI.1554, *S. parviflorum* LA735, and *S. parviflorum* LA2072. Moreover, there are a few genetic transformation studies aimed at generating Cmm resistant cultivars. Wittmann *et al.* (2015) used *Agrobacterium*-mediated transformation to transfer the endolysin *lys* gene, a peptidase specific for the murein peptidoglycan of Cmm isolates, into a tomato line and showed that transgenic plants were resistant to Cmm. In another study, two Cmm resistance quantitative trait loci (QTL) from *S. habrochaites* LA407 were introgressed into a tomato cultivar which then demonstrated resistance to Cmm (Kabelka *et al.*,

2002). Despite this work, there is currently no commercially successful Cmm resistant tomato cultivar on the market (Peritore-Galve *et al.*, 2019).

In the present study, we screened seven tomato accessions representing four species (*S. arcanum, S. habrochaites*, S. *pennellii,* and *S. peruvianum*) from Peru, Ecuador, and Mexico against *C. michiganensis* subsp. *michiganensis* isolates Cmm-244 and Cmm-9 to identify new sources of resistance to this important disease.

MATERIALS and METHODS

Plant material

A total of seven tomato accessions including three *S. arcanum,* two *S. habrochaites*, one *S. pennellii,* and one *S. peruvianum* accession were used in this study and supplied by the Tomato Genetics Resource Center (TGRC, Davis CA, USA) (Table 1). *S. lycopersicum* SC2121, a local variety highly susceptible to Cmm, was used as a control.

Seeds of the seven tomato accessions and SC2121 were sterilized with 1% NaOCl. Seeds were sown at a depth of 0.5 cm in seedling trays containing peat. Seedlings were grown in a climate cabinet at 25 °C with a 16 h daylight period.

Pathogenicity assay

Clavibacter michiganensis subsp. *michiganensis* isolates Cmm-244 (Ege University Faculty of Agriculture, Department of Plant Protection, Bacteriology Laboratory) and Cmm-9 (Adnan Menderes University Faculty of Agriculture, Department of Bacteriology Laboratory) are the most virulent isolates (Cmm-9, Özyılmaz and Benlioğlu, 2015; Cmm-244, based on unpublished doctoral thesis) in their respective culture collections and were used for inoculation.

The isolates were grown in King B medium (20 g L^{-1} peptone, 1.5 g L^{-1} K_2HPO_4, 1.5 g L^{-1} $MgSO_4.7H_2O$, 10 g L^{-1} glycerol, 16 g L^{-1} agar) (King *et al.*, 1954) at 25 °C for 48 h. The two isolates were adjusted to an OD600 of 0.1, corresponding to 10^8 CFU ml^{-1} and mixed in a 1:1 ratio. The mixture of isolates was used to survey for broader spectrum coverage.

Cmm inoculation was done at the three true leaf stages by root immersion. For this, the plants were removed from the seedling tray and the roots were carefully cleaned with running water. The ends of lateral roots were then injured with scissors and incubated in the bacterial suspension for 30 min. After inoculation, the plants were replanted into pots (10 x 10 x 10 cm) containing peat and grown at 25 °C under 16 h daylight period with 70% relative humidity in a climate chamber.

Disease evaluation

The experiment was conducted as two trials with a randomized plot design. Each accession was represented by four replicate plants in each trial. SC2121 plants inoculated with the disease agent were the positive control and uninoculated plants were the negative control group. When the SC2121 plants showed the most disease symptoms, six weeks after inoculation, the disease severity of all accessions was evaluated based on a 0-4 scale. Plants with no symptoms received a score of 0, those with ¼, ½, or ¾ of the plant withered (from the bottom up) received scores of 1, 2, or 3, respectively. A score of 4 indicated withered and dead plants (Klement *et al*., 1990). The results were converted to % disease incidence with the Townsend-Heuberger (1943) formula.

Table 1. Tomato accessions used in this study.
Çizelge 1. Bu çalışmada kullanılan domates aksesyonları.

Accession Aksesyon	Species Tür	Origin Orijin
LA1708 (O10759)	*Solanum arcanum*	Chamaya to Jean, Cajamarca, Peru
LA2172	*Solanum arcanum*	Cuyca, Cajamarca, Peru
LA2157	*Solanum arcanum*	Tunel Chotano, Cajamarca, Peru
LA1223 (PI365903)	*Solanum habrochaites*	Aiausi, Chimborazo, Ecuador
LA1777	*Solanum habrochaites*	Rio Casma, Ancash, Peru
LA716 (PI246502)	*Solanum pennellii*	Atico, Arequipa, Peru
LA3640 (PI270435)	*Solanum peruvianum*	Mexico City, Mexico

$$\text{Disease incidence (\%)} = \frac{\sum(\text{\# of plants in scale category x scale value})}{(\text{Total \# of plants x max value of evaluation scale})} x\ 100$$

To determine the pathogen load in the plant, the upper part of the plant (stem and leaves) showing disease symptoms were homogenized in 10 ml 0.8% FTS (physiological water). The resulting suspension was diluted with a 9-step dilution process. Samples from each dilution series were inoculated into semi-selective SCM medium (Selective *Clavibacter* Medium) (0.1 g L^{-1} yeast extract, 10 g L^{-1} sucrose, 1.5 g L^{-1} H_3BO_3, 0.122 g L^{-1} $MgSO_4$, 2 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 18 g L^{-1} agar, 30 mg L^{-1} nalidixic acid sodium salt, 100 mg L^{-1} nicotinic acid, 100 mg L^{-1} nystatin, 0.01 g L^{-1} Chapman's potassium tellurite, pH 7.3), using the point inoculation method to calculate the colony-forming bacteria per gram (CFU ml^{-1} gr^{-1}). This procedure was performed for all replicates of each accession.

The disease severity (%) results were evaluated using R statistical software and RStudio (RStudio Team, 2020) using the "agricolae" and "ggplot2" libraries included in R. One-way analysis of variance (ANOVA) in the Agricolae library with 95% confidence and Tukey's Test were used for multiple comparisons.

RESULTS and DISCUSSION

According to one-way ANOVA performed with 95% confidence intervals in both trials, the difference among accessions was statistically significant ($p<0.05$) (Table 2). All uninoculated controls had no symptoms and no pathogen colonization as expected (data not shown). Based on the intensity of pathogen symptoms, *S. arcanum* LA2157 showed the highest tolerance to Cmm. Moreover, the difference in average disease severity between accession LA2157 ($\overline{x}$ = 12.5%) and the negative control group ($\overline{x}$ = 0.0%) was statistically insignificant. *S. habrochaites* LA1777 and *S. peruvianum* LA3640 were moderately tolerant ($\overline{x}$ = 53.1% and $\overline{x}$ = 59.4%, respectively) compared to SC2121 ($\overline{x}$ = 90.6%). On the other hand, *S. habrochaites* LA1223 ($\overline{x}$ = 68.7%), *S. arcanum* LA1708 ($\overline{x}$ = 81.2%), *S. arcanum* LA2172 ($\overline{x}$ = 93.7%) and *S. pennellii* LA716 ($\overline{x}$ = 100.0%) were highly susceptible. There were no statistically significant differences in the average disease severity between these four accessions and the positive *S. lycopersicum* SC2121 control ($\overline{x}$ = 90.6%).

In the pathogen density assay conducted using the leaves and stems of each replicate plant, statistically significant accession differences were determined ($p<0.05$). The lowest pathogen densities were measured in *S. arcanum* LA2157 and *S. habrochaites* LA1777 (Table 3). The highest inoculum density was observed in *S. pennellii* LA716 with values even higher than in tissues from the positive control (SC2121) (Table 3).

Table 2. Average disease severity of accessions for first and second trials. Accessions are ordered from most to least susceptible.
Çizelge 2. Birinci ve ikinci deneme için aksesyonların ortalama hastalık şiddetleri. Aksesyonlar en duyarlıdan en az duyarlıya doğru sıralanmıştır.

Accession* Aksesyon	Species Tür	1st trial disease severity (%) 1. deneme hastalık şiddeti (%)	2nd trial disease severity (%) 2. deneme hastalık şiddeti (%)	Average disease severity (%) Ortalama hastalık şiddeti (%)
SC2121	*Solanum lycopersicum*	100.0 ± 0.0 a	81.2 ± 12.5 abc	90.6 ± 12.9 ab
LA716	*Solanum pennellii*	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
LA2172	*Solanum arcanum*	100.0 ± 0.0 a	87.5 ± 14.4 ab	93.8 ± 11.6 a
LA1708	*Solanum arcanum*	93.7 ± 12.5 ab	68.7 ± 23.9 abc	81.2 ± 22.2 abc
LA1223	*Solanum habrochaites*	75.0 ± 0.0 abc	62.5 ± 14.4 abc	68.7 ± 11.6 bcd
LA3640	*Solanum peruvianum*	68.7 ± 23.9 bc	50.0 ± 35.3 bcd	59.4 ± 29.7 cd
LA1777	*Solanum habrochaites*	62.5 ± 32.8 c	43.7 ± 37.5 cd	53.1 ± 33.9 d
LA2157	*Solanum arcanum*	12.5 ± 14.4 d	12.50 ± 14.4 de	12.5 ± 14.4 e

* Values followed by the same letter are not significantly different at P< 0.05 (Tukey test). Accessions were shown with the same letters. *Aynı harf taşıyan değerler P<0,05 (Tukey testi) güvenilirlik seviyesinde önemli ölçüde farklı değildir. Aksesyonlar aynı harflerle gösterilmiştir.

Table 3. Pathogen density in leaf and stem tissue of Cmm-inoculated accessions.
Çizelge 3. Cmm ile inokule edilmiş aksesyonların yaprak ve gövde dokusundaki patojen yoğunluğu.

Accession* Aksesyon	Species Tür	1st trial colonization (CFU g^{-1}) 1. deneme kolanizasyon (CFU g^{-1})	2nd trial colonization (CFU g^{-1}) 2. deneme kolonizasyon (CFU g^{-1})
SC2121	*Solanum lycopersicum*	6.3×10^{10} b	1.2×10^{7} bc
LA716	*Solanum pennellii*	1.2×10^{13}a	3.5×10^{8} a
LA2172	*Solanum arcanum*	6.3×10^{10} b	9.3×10^{7} ab
LA1708	*Solanum arcanum*	8.1×10^{12} a	3.5×10^{6} cd
LA1223	*Solanum habrochaites*	7.2×10^{12} a	1.5×10^{5} d
LA3640	*Solanum peruvianum*	5.1×10^{12} a	3.3×10^{5} b
LA1777	*Solanum habrochaites*	2.7×10^{8} c	3.3×10^{5} d
LA2157	*Solanum arcanum*	2.7×10^{8} c	3.2×10^{3} e

* Values followed by the same letter are not significantly different at P<0.05 (Tukey test). Accessions were shown with the same letters. *Aynı harf taşıyan değerler P<0,05 (Tukey testi) güvenilirlik seviyesinde önemli ölçüde farklı değildir. Aksesyonlar aynı harflerle gösterilmiştir.

Wild species of tomato are known to be useful sources of resistance to biotic and abiotic stresses and can be used in breeding programs to develop desired stress-tolerant tomato cultivars. In the present study, we screened seven different tomato accessions representing four wild species for the determination of their tolerance to Cmm. Although none of the accessions were completely resistant to Cmm, accessions with different levels of tolerance were identified. High susceptibility to Cmm was observed in both *S. arcanum* accessions (LA1708 and LA2172). As expected based on their disease symptoms, plants of these two accessions had some of the highest levels of colonization indicating that the pathogen replicated freely in their tissues. On the other hand, *S. arcanum* LA2157 had the lowest density of Cmm in plant tissues, consistent with its appearance as the most tolerant accession. These results suggest that this accession may have a pathogen-inhibiting mechanism against Cmm that is not present in other accessions of the same species. Previous studies of *S. arcanum* accessions reported that LA1708, LA2172, and LA2157 carry resistance to various diseases. LA1708, LA2172, and LA2157 are resistant to root-knot nematode disease caused by *Meloidogyne arenaria* (Veremis and Roberts 2000; Seifi *et al.*, 2011; El-Sappah, *et al.*, 2019); LA1708 has tolerance to *Alternaria solani*, the fungus that causes tomato early blight (Chaerani *et al.*, 2007); and LA2172 is resistant to the tomato powdery mildew pathogen *Leveillula taurica* (Seifi *et al.*, 2011). Further study of LA2157 indicated that it carries alleles for the genes Mi-1 and Cf2 that provide resistance to root-knot nematode (*Meloidogyne* spp.) and tomato leaf mold disease (*Cladosporium fulvum*), respectively (Jablonska *et al.*, 2007). Similar to our results, previous studies reported that LA2172 was highly sensitive (Peritore-Galve *et al.*, 2019) while LA2157 was resistant to Cmm (Francis *et al.*, 2001; Sandbrick *et al.*, 1995). However, in our experiments, LA2157 exhibited only a high level of tolerance to Cmm, not resistance, a result that is similar to the findings of Kabas *et al.* (2018).

S. habrochaites LA1223 exhibited moderate tolerance to Cmm. The colonization assay revealed the presence of significantly less pathogen than the control indicating that there may be a degree of tolerance in this accession. In the literature, LA1223 is reported to have resistance against potato Y potyvirus, the causative agent of potato Y disease (Toprak *et al.*, 2009), tomato spotted wilt virus (Maluf *et al.*, 1991), and heterogeneous resistance to cucumber mosaic virus (Balci, 2005). However, no information on this accession's tolerance to Cmm was found in the literature. Therefore, our work is the first report that LA1223 has moderate tolerance to Cmm. LA1777, another *S. habrochaites* accession used in the study, was the second-best performing accession showing moderate tolerance consistent with reduced pathogen density in plant tissues. Decreased colonization in this accession suggests that an interaction to reduce pathogen

replication might occur in this accession. In the literature, LA1777 is reported to have resistance to many disease pathogens including, tomato downy mildew pathogen, *Phytophthora infestans* (Li *et al.*, 2011), bacterial spot disease agent *Pseudomonas syringae* pv. tomato race-1 (Thapa *et al.*, 2015), tomato yellow leaf curl begomovirus, tomato mottle mosaic disease pathogen, tomato mottle tobamovirus (Momotaz *et al.*, 2007), and potato Y potyvirus (Toprak *et al.*, 2009). The same accession was indicated to be effective against the insect pests tobacco whitefly (*Bemisia tabaci*) (Momotaz *et al.*, 2010) and *Tuta absoluta* (Bitew, 2018) and was reported to have moderate tolerance to *Orobanche aegyptiaca,* a parasitic plant. Moreover, this accession was reported as moderately tolerant to Cmm by Kabas *et al.* (2018). Thus, our work confirmed this previous result by examining both disease severity and colonization. Therefore, LA1777 is a good source of multiple disease tolerances for tomato improvement.

S. pennellii LA716 is another valuable genetic source conferring tolerance to various biotic and abiotic stresses. For example, it is tolerant to abiotic stresses such as salt (Frary *et al.*, 2010; Li *et al.*, 2010) and drought (Borba *et al.*, 2017), Moreover, LA716 showed high resistance to the obligate parasitic plants (*Orobanche aegyptiaca*) (El-Halmouch *et al.*, 2006) and *Phelipanche aegyptiaca* (Bai *et al.*, 2020). While previous work indicated that LA716 has resistance to different biotic stresses such as whitefly (*Bemisia tabaci*) (Baldin *et al.*, 2005; Oriani and Vendramim, 2010); tomato leaf miner, *Tuta absoluta* (Bitew, 2018); a bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* (Astua-Monge *et al.*, 2000) and a wilt fungal disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Scott and Jones,1989), LA716 was shown to be susceptible to Cmm for the first time in our study.

Moderate tolerance was observed against Cmm in *S. peruvianum* LA3640 in our work. Likewise, the colonization of the pathogen in plant tissue confirmed this result. LA3640 is mentioned in the literature as having tolerance to tomato spotted wilt tospovirus (Gordillo *et al.*, 2009) which is the causative agent of tomato spotted wilt disease, and resistance to root-knot nematode disease (Yang *et al.*, 2011). However, there is no information about its tolerance to Cmm. Thus, this work is the first to report moderate tolerance of LA3640 to Cmm.

Given the present study results, *S. arcanum* LA2157 and *S. habrochaites* LA1777 should be more extensively studied for their tolerance to different isolates of Cmm. The identification of such tolerance sources is important for the development of new tolerant materials by backcrossing. Moreover, these materials may be used to identify genes for Cmm tolerance and to shed light on the molecular mechanism of this tolerance.

Conflict of interest/competing interests

The authors declare that they have no conflict of interest or competing interests.

Ethics approval and consent to participate

The authors declare that they have followed all necessary ethical guidelines. Human and animal subjects were not used in this work

Consent for publication

All authors and associated institutes have consented to publication of this work.

Authors' Contributions

US*: pathogen tests, data interpretation and analysis, AAB*: manuscript drafting and revision; HÖ: experimental design; pathogenicity test and evaluation of the test results, AF: manuscript revision and interpretation of the data; SD: experimental design, manuscript revision; All: final approval of the version to be published. *These authors contributed equally to this work.

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