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RAPID COMMUNICATION

First Detection of *Xanthomonas campestris* in Georgian hazelnuts: A threat to economic production

Iveta Megrelishvili[1]*, Tinatin Elbakidze[2], Stefaan Werbrouck[3], Nino Maziashvili[1], Zurab Khidesheli[1], Amaal Rabaaoui[3], Levan Ujmajuridze[1]

[1] Integrated Plant Protection Research, LEPL Scientific Research Center of Agriculture, Tbilisi, Georgia

[2] School of Science and Technology, University of Georgia, Tbilisi, Georgia

[3] Department of Plants and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

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*Corresponding address:
ivetameg@yahoo.com

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Abstract

Bacterial blight, caused by *Xanthomonas campestris* is a major disease of hazelnut (*Corylus avellana*) worldwide. Georgia is a significant hazelnut producer, but the presence of *X. campestris* has not been previously confirmed. In 2022–2023, hazelnut plants in nurseries across western Georgia were observed with symptoms of bacterial blight. Samples, including 538 symptomatic samples of both 'Anakliuri' (Georgian) and 'Tonda di Giffoni' (Italian) hazelnut cultivars, were collected from the Samegrelo and Guria regions in Georgia. DAS-ELISA with polyclonal antibodies identified 34 potentially positive for *X. campestris*. Additionally, samples were cultured on YPGA and King's medium B for presumptive *X. campestris* identification based on yellow colonies with subsequent confirmation of 28 samples by a TaqMan triplex real-time PCR assay using species-specific primers. Sanger sequencing of the 16S rRNA gene, performed on 24 of these, with further BLAST analysis, revealed four isolates as *X. campestris* (GenBank Accession Numbers: PP437082.1, PP434581.1, PP434578.1, PP434556.1) showing up to 100% nucleotide identity to *X. campestris* pv. *campestris* strains isolated from different countries (India, Serbia, and Mexico) and sources. Pathogenicity was confirmed by inoculating young shoots (20–30 cm long) of 2-year-old potted hazelnut plants ('Hall's Giant') with a bacterial suspension (10^8 CFU · ml^{-1}) of each isolate. Bacterial cultures were re-isolated, fulfilling Koch's postulates. This represents the first report of *X. campestris* in Georgia, highlighting the need for disease management strategies and the use of disease-free planting material to protect this economically important crop.

Keywords: bacterial blight, detection, economic impact, hazelnut, Georgia, *Xanthomonas*

Xanthomonas arboricola pv. *corylina* (formerly *Xanthomonas campestris* pv. *corylina*) (Miller *et al.* 1940; Vauterin *et al.* 1995), the causal agent of bacterial blight, poses a significant threat to hazelnut (*Corylus avellana*) production worldwide. It prevails in Europe (Pulawska *et al.* 2010; Mikiciński *et al.* 2012; Lamichhane and Varvaro 2014) and Turkey's Black Sea region (EPPO 2021). First described by Barss (1913) in the early 20th century, bacterial blight symptoms include bud and shoot death, branch and trunk cankers, leaf spots, dark brown nut spots, and bacterial exudates on lesions. The disease can inflict severe economic losses (Webber *et al.* 2020).

Young orchards (1–4 years old) are particularly vulnerable, with potential losses of up to 10% (EPPO 2004). In older trees (15 years), mortality can reach 100% (Miller *et al.* 1949). The losses are even more severe in nurseries where multiplication by layering is widely practiced on densely planted mother trees. This condition favors high moisture and low aeration, ideal for the multiplication of the pathogen (Barss 1913; Miller *et al.* 1949).

International trade has led to the dissemination of *X. campestris* through contaminated materials used for propagation. The European and Mediterranean Plant Protection Organization (EPPO) and European

Union phytosanitary regulations (Council directive 2000/29/EC) have classified *X. arboricola* pv. *pruni* as a quarantine organism due to its detrimental economic impact. However, it was recently reclassified as a Regulated Non-Quarantine Pest (RNQP) (European Union 2019). Currently, bacterial blight caused by *X. campestris* has been reported in nearly every hazelnut-producing country (EPPO 2004).

According to the EPPO database in just the last decade, 10 disease outbreaks caused by *X. campestris* have been reported in nine different countries (EPPO 2004; Lamichhane *et al.* 2012). Due to the high rate of outbreaks, additional future outbreaks can be expected. It is crucial to understand the biology, epidemiology, and underlying causes of the disease of which not much is currently known to address the effects of its emergence.

Georgia is a major global hazelnut producer, averaging 32.8-45.4 tons annually between 2020–2022. Production centers in Samegrelo (42.2%), Guria (20.7%), Kakheti (12.9%), Adjara (12.0%), and Imereti (10.5%), with 66% of the crop exported primarily to Italy and Germany (https://pmcg-i.com/publication/hazelnut-production-sector-in-georgia/). Due to its economic importance, the European and Mediterranean Plant Protection Organization (EPPO) designated *X. arboricola* a quarantine pathogen for Georgia in 2018 along with other quarantine organisms (Megrelishvili *et al.* 2022). The disease's potential for yield reduction makes its spread a critical concern for Georgian hazelnut production. This study investigated the presence of *X. arboricola* in Georgia's western regions.

Symptomatic hazelnut plants indicative of bacterial blight were observed in nurseries across Georgia's western regions. A comprehensive survey was conducted in 2022-2023 (spring and summer), encompassing 2000 hazelnut samples from the Samegrelo (Zugdidi, Khobi, Tsalenjikha, Senaki) and Guria (Ozurgeti) regions. Samples exhibiting potential *X. arboricola* symptoms (538 total) were collected for laboratory analysis (Fig. 1, 2)

Both 'Anakliuri' (Georgian) and 'Tonda di Giffoni' (Italian) hazelnut cultivars were tested. Initial screening employed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams 1977) with polyclonal antibodies (Loewe® biochemica GmbH, Germany). According to the manufacturer's instructions leaves were crushed [(w/v) 1:5] in extraction buffer (pH 8.2) containing 2% polyvinylpyrrolidone (PVP MW 24,000), 0.02% NaN_3 and 0.05% Tween 20. Absorbance was determined at 405/450 nm on ELX800 Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) and the sample was considered positive if its optical density was three times higher than the negative control.

Tissue samples were also cultured on nutrient agar and King's medium B, with presumptive *X. arboricola*

Fig 1. Xanthomonas arboricola Anakliuri

Fig. 2. Xanthomonas arboricola Tonda di Giffoni

identification based on the development of small yellow colonies after two to three days (25°C). DNA extraction (GeneJET, USA) enabled a triplex real-time PCR assay with specific primers (Palacio-Bielsa *et al.* 2011).

Total DNA was extracted using GeneJET Genomic DNA Purification Kit (GeneJET, USA) according to the manufacturer's instructions. DNA was eluted in 100 µl Elution Buffer. The concentration of DNA was measured using Qubit™ 4 Fluorimeter (Invitrogen, USA).

PCR assays TaqMan® triplex real-time PCR using TaqMan®MGB™ probes (Applied Biosystems) were used to detect Xantomonas strains. Probes were 5'-labelled with FAM™ and TAMRA™ reporter dye, respectively. Reactions were performed in a final volume of 20 µl containing 11 µl of TaqMan® Fast Advanced Master Mix (ThermoFisher Scientific), primers and probes, at a final concentration of 0.4 µM each, and 2 µl of extracted DNA as template. Amplification and detection were done using the 7500 Fast Real-Time PCR System (Applied Biosystems) with optical 96-well

Table 1. Distribution of DAS-ELISA and Triplex RT-PCR positive samples in different regions

Regions						
Cultivar	Samegrelo			Guria		
	number of samples tested	DAS-ELISA positive	triplex RT- PCR positive	total number of samples	DAS-ELISA positive	triplex RT- PCR positive
Tonda di Giffoni	195	8	5	45	2	2
Anakliuri	218	16	15	100	8	6
Total number of samples	413	24	20	125	10	8
Totally	538					

plates and adhesive covers. The thermal cycle consisted of a pre-step of 5 min at 95°C for Hot Start Taq DNA polymerase activation, followed by 45 cycles of 60 s denaturation at 95°C and 60 s hybridization and elongation at 59°C. The software v2.0 or 2.3 of 7500 fast Real Time PCR system (Applied Biosystems) was used for fluorescence acquisition and evaluation of threshold cycles (Ct) (Mori *et al*. 2015).

Of the 538 samples, 24 positives in both ELISA and PCR underwent further sequencing. Sanger sequencing targeted the 16S RNA ribosomal gene, and PCR--positive amplicons were deposited in GenBank (Accession Numbers: PP437082.1, PP434581.1, PP434578.1, PP434556.1).

Out of 538 symptomatic hazelnut samples, initial DAS-ELISA screening identified 34 potential *X. arboricola* positives. Subsequent TaqMan triplex real-time PCR confirmed the pathogen's presence in 28 samples. Sanger sequencing was conducted on 24 samples, ultimately identifying four isolates as *X. arboricola*. All sequenced samples exhibited characteristic bacterial blight symptoms (Fig. 1, 2). BLAST analysis of the 16S RNA ribosomal gene sequences revealed the presence of *X. campestris* in Georgia, demonstrating high nucleotide identity with relevant worldwide isolates. Specifically: PP437082.1: 100% nucleotide identity to *X. campestris* pv. *campestris* strains from India and Serbia (KF270091, JQ818431), PP434581.1: 99.8% nucleotide identity to *X. campestris* pv. *campestris* strains from Mexico (MT645261.1, MT645246.1), PP434578.1 and PP434556.1: 100% nucleotide identity to the same Mexican strains.

Out of 538 symptomatic hazelnut samples, initial DAS-ELISA screening identified 34 potential *X. arboricola* positives. Subsequent TaqMan triplex RT- PCR confirmed the pathogen's presence in 28 samples (Table 1). Sanger sequencing was conducted on 24 samples, ultimately identifying four isolates as *X. arboricola*. All sequenced samples exhibited characteristic bacterial blight symptoms. BLAST analysis of the 16S RNA ribosomal gene sequences revealed the presence of *X. campestris* in Georgia, demonstrating high nucleotide identity with relevant worldwide isolates. Specifically: PP437082.1: 100% nucleotide identity to *X. campestris* pv. *campestris* strains from India and Serbia (KF270091, JQ818431), PP434581.1: 99.8% nucleotide identity to *X. campestris* pv. *campestris* strains from Mexico (MT645261.1, MT645246.1), PP434578.1 and PP434556.1: 100% nucleotide identity to the same Mexican strains. The pathogenicity test was done for all isolates deposited in GenBank. The young shoots (20–30 cm long, with up to 7 leaves) of 2-year-old potted hazelnut plants (cv. Hall's Giant) were sprayed with the 10^8 CFU/ml bacterial suspension resuspended in sterile tap water using a hand-held sprayer. For each bacterial culture three hazelnut plants were used. For the negative control sterile distilled water (SDW) was applied to all of the test plants each in triplicate while PP437082.1, PP434581.1, PP434578.1, and PP434556.1 Xanthomonas strains cultured in SDW were used as positive controls, respectively. To create an appropriate environment the inoculated shoots were placed in a greenhouse at 22 to 26°C for 72 h and covered with plastic bags in order to provide high humidity conditions. Symptoms in all of the inoculated shoots were seen five to six weeks after inoculation as lesions surrounded by a halo on leaves. In addition, leaves for the negative control sprayed with SDW remained symptomless. Further isolation of the bacterial cultures from the necrotic plant tissues was performed in order to confirm Koch's postulates.

This study represents the first confirmed report of *X. campestris* pv. *campestris* in Georgia. Implementing reliable laboratory assays is crucial in managing the spread of hazelnut diseases within the country. These findings highlight the importance of using disease-free planting materials when establishing new hazelnut nurseries.

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