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Etiology of hazelnut (*Corylus avellana*) bacterial blight in Montenegro

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Abstract

In Montenegro, production of hazelnuts is continually increasing due to market demands and favorable climate. However, the production is occasionally affected by occurrence of hazelnut diseases. Bacterial blight is one of the most damaging worldwide. In June 2021, leaf spot, bud and twig necrosis were observed on young hazelnut plants (*Corylus avellana*) cultivar Hall's Giant, near Cetinje, Montenegro. From the symptomatic samples, fourteen bacterial strains, forming yellow, convex and mucoid bacterial colonies, were isolated. Pathogenicity of the strains was preliminary tested by their hypersensitivity in pelargonium, and by spraying young leaves of potted hazelnut plants with the bacterial suspension (10^8 CFU/mL SDW). The reference strain *Xanthomonas arboricola* pv. *corylina* (Xac) NCPPB 3037 and sterile distilled water (SDW) were used as positive and negative control, respectively. Small, irregular lesions appeared on the leaves of all inoculated plants within 5 to 6 weeks after inoculation, while the leaves sprayed with SDW remained symptomless. All the strains were Gram-negative, catalase positive, oxidase negative, obligate aerobic, hydrolyzed starch, gelatin and esculin, did not reduce nitrate and did not grow at 37°C and in the presence of 5% NaCl, showing the same characteristics as the reference Xac strain. PCR with XarbQ-F/XarbQ-R primers (Pothier et al. 2011) produced a fragment of 402 bp in 14 strains and the reference Xac, confirming their affiliation to *X. arboricola* species. Additionally, PCR analysis with primers XapY17-F/ XapY17-R (Pagani 2004; Pothier et al. 2011), produced a single band of 943 bp. Amplification and sequencing of the partial *rpoD* gene (Hajri et al., 2012) of two selected strains (GenBank Nos. OQ271224 and OQ271225), showed that they share 99.47% to 99.92% *rpoD* sequence identity with Xac strains CP076619.1 and HG992342.1 isolated from hazelnut in France and HG992341.1 in USA. According to the results, *Xanthomonas arboricola* pv. *corylina* was identified as the causal agent of hazelnut bacterial blight in Montenegro.

***Key words*:** *Xanthomonas arboricola* pv. *corylina*, leaf spot, bud necrosis, identification, *RpoD*

INTRODUCTION

The global demand for hazelnut (*Corylus avellana*) is increasing, which has caused a growing interest in production worldwide. Turkey is the world's leading hazelnut producer and exporter, accounting for about 70% of the total world supply (Castro Ramos & Swart, 2017). Due to market demands and favorable climate, commercially hazelnut production is rapidly increasing in Montenegro.

Hazelnut production is compromised by a numerous hazelnut pathogens. Among them, *Xanthomonas arboricola* pv. *corylina* (Xac) the causal agent of bacterial blight of hazelnut, under favorable environmental conditions, can cause significant crop losses.

Xac has rapidly spread into new geographic areas in recent years, due to climate change and intensive trade of seedling material.

Symptoms of hazelnut bacterial blight are visible on leaves and fruits as necrotic lesions, and on branches and trunk as cankers. The nuts are rarely affected and do not drop up. (Kałużna et al., 2021). Symptoms of affected plants can be different in orchards and nurseries as a result of different growing systems (Lamichhane et al., 2012). The main way for introduction and spread of the pathogen into new areas is by trade of latently infected plant material while in short distances can be spread by rain and wind.

During the survey of hazelnut orchard, leaf spot, bud and twig necrosis were observed on young hazelnut plants. The aim of this study was to identify of the bacterial strains isolated from diseased hazelnut plants in Montenegro.

MATERIALS AND METHODS

Isolation of bacteria

In June 2021, leaf spot, bud and twig necrosis were observed on 6-year-old hazelnut plants (*Corylus avellana*) cultivar Hall's Giant, in a 0.3 ha plantation near Cetinje, central Montenegro. The intensity of infection on hazelnut plants was more than 80%. Numerous, small, irregular, brown, necrotic spots, sometimes surrounded by a chlorotic halo, were observed on leaves (Figure 1a), while longitudinal brown lesions developed on twigs and branches (Figure 1b). In the orchard dieback of twigs and branches is observed (Figure 1c). Dark brown, necrotic buds did not often open in the spring (Figure 1d). Symptomatic plant material was collected during the late spring.

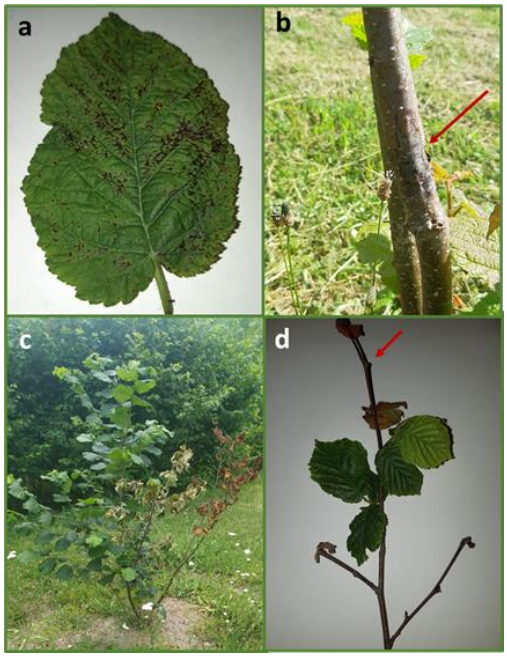

Figure 1. *Xanthomonas arboricola* pv. *corylina*: leaf spot (a); branch necrosis (b); twig and branch dieback (c); bud necrosis (d) of hazelnut cultivar Hall's Giant. Natural infection. (Photo: T. Popović)

For the pathogen isolation small fragments tissue were taken and macerated in 1 mL of sterile distilled water (SDW). The macerate was streaked onto nutrient agar (NA) plates and incubated at 27°C for 48 h. Single yellow colonies were picked and re-streaked on yeast extract-dextrose-CaCO3 (YDC) medium to ensure purity (Schaad et al., 2001). Fourteen bacterial isolates were further characterized. Xac strain NCPPB 3037 isolated from *Corylus avellana* in United Kingdom, was used as a reference.

Pathogenicity tests

In order to differentiate pathogenic isolates, pelargonium (*Pelargonium zonale*) hypersensitivity was used as discriminatory test. Appearance of the tissue necrosis within 24 to 48 hours after inoculation

was considered positive reaction. Pathogenicity of the isolates was also tested by artificial inoculation of the 2-year-old potted hazelnut plants (cv. Hall's Giant). It was tested by spraying young shoots (20 to 30 cm long, with 5 to 7 leaves) using a handheld sprayer with the bacterial suspension (10^8 CFU/mL of sterile tap water), in three replicates. Xac strain NCPPB 3037 was used as positive control, and SDW as a negative control. The inoculated shoots were incubated under plastic bags, providing high humidity conditions, in an acclimatized greenhouse at 22-26°C, for 72 h. Appearance of necrosis was monitored up to 7 weeks after inoculation. Koch's postulates were fulfilled by re-isolation of the pathogen from the necrotic test plant tissue and identification of bacterial isolates by the PCR.

Physiological and biochemical characteristics

The following physiological and biochemical characteristics of the strains were studied: Gram reaction, oxidase and catalase activity, oxidative-fermentative metabolism of glucose, gelatin, esculin and starch hydrolysis, nitrate reduction, grow in the presence of 5% NaCl and at 37°C (Lelliott & Stead, 1987; Schaad et al., 2001). In all tests Xac strain NCPPB 3037 was used as a control. All tests were carried out in two replicates.

Molecular characterization

Total genomic DNA was extracted from all studied isolates and Xac NCPPB 3037 reference strain using Dneasy Mericon Food Kit (Qiagen, Germany). Quality of extracted DNA was checked by gel electrophoresis on 0.8% agarose gel. The DNA samples were stored at -20°C until use.

The presence of the qumA gene characteristic for *Xanthomonas arboricola* species, was detected by PCR analysis, using primer pair XarbQ-F/XarbQ-R, by amplifying a product of 402 bp (Pothier et al., 2011). Additionally, the strains were further identified by PCR analysis, using primer pair XapY17-F/XapY17-R by amplifying a product of 943 bp (Pagani, 2004; Pothier et al., 2011). Based on pathogenic and biochemical characteristics, two strains RKFB 1375 and RKFB 1370 were selected for amplification and sequencing of the partial *rpoD* gene using a set of primers described by Hajri et al. (2012). Amplified DNA fragments of the selected strains were sequenced in both directions (Macrogen Europe, Amsterdam, The Netherlands). Sequences have been deposited in The National Center for Biotechnology (NCBI) database.

RESULTS AND DISCUSSION

Isolation of bacteria from symptomatic plant material

From the diseased leaf, bud and twig bark tissue, 14 strains were isolated on yeast extract dextrose $CaCO_3$ medium. The strains formed yellow, convex, and mucoid colonies, characteristic for *Xanthomonas* genus (Figure 2).

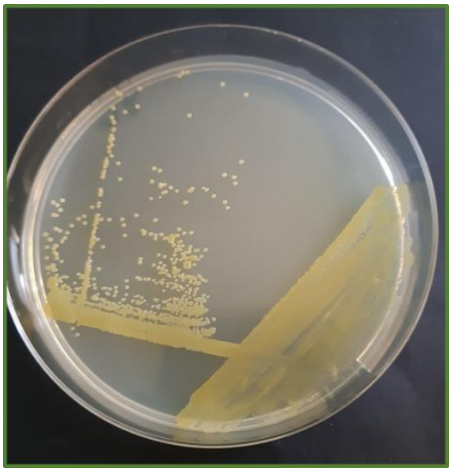

Figure 2. *Xanthomonas arboricola* pv. *corylina*. Colony morphology on nutrient agar medium. (Photo: T. Popović)

Pathogenicity of the isolates

All studied isolates induced hypersensitive reaction in pelargonium leaves (*Pelargonium zonale*) after 24 - 48h after inoculation (Figure 3a, b). Pathogenicity was confirmed by reproducing the symptoms on leaves of the potted hazelnut plants (cv. Hall's Giant) (Figure 3c, d). Lesions appeared on leaves of all inoculated shoots within 5 to 6 weeks after inoculation. Reference Xac strain, used as a positive control, also caused the same symptoms while tissue inoculated with SDW remained symptomless. Koch's postulates were confirmed by the re-isolation of the pathogen from the necrotic test plant tissue and identity checked by PCR using the primer set of Pothier et al. (2011).

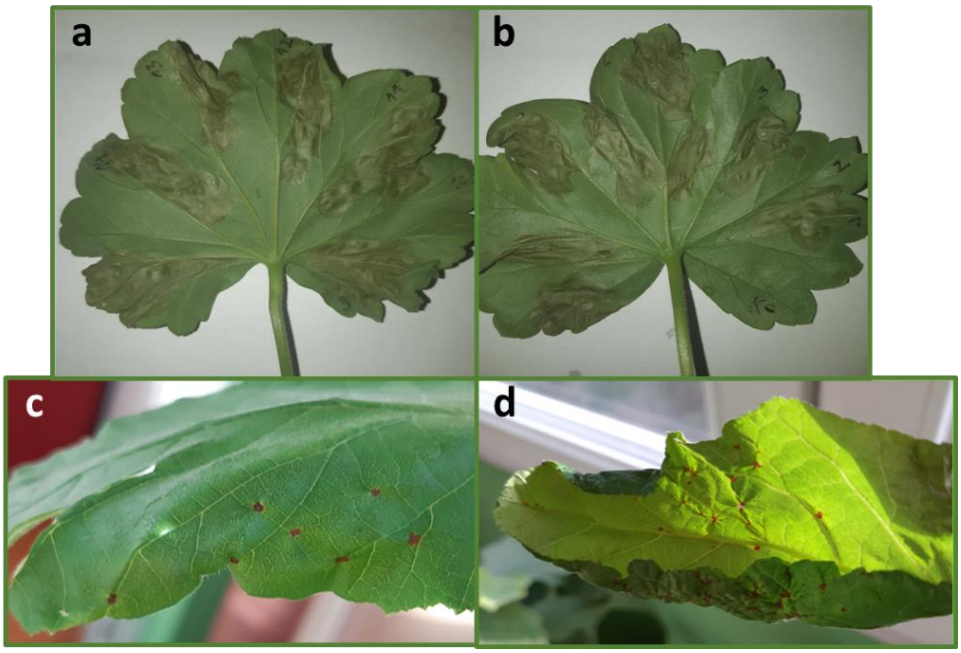

Figure 3. *Xanthomonas arboricola* pv. *corylina.* Hypersensitive reaction on leaves of pelargonium (a, b); necrotic spots on leaves of hazelnut observed in pathogenicity tests (c, d). (Photo: T. Popović)

Physiological and biochemical characteristics

All strains were Gram-negative, catalase positive, oxidase negative, obligate aerobic, hydrolyzed starch, gelatin and esculin, did not reduce nitrate and did not grow at 37°C and in the presence of 5% NaCl, showing so the same biochemical profile of the reference strain Xac NCPPB 3037. These results showed that the studied isolates are homogeneous in terms of biochemical and physiological characteristics.

Molecular characterization

The presence of the *qumA* gene was detected by PCR analysis, using primer pair XarbQ-F/XarbQ-R, by amplifying a product of 402 bp (Pothier et al., 2011) in all 14 isolates and Xac reference strain, confirming their affiliation to *X. arboricola* species. (Figure 4). The isolates were further identified by PCR analysis, using primer pair XapY17-F/XapY17-R (Pagani, 2004; Pothier et al., 2011), resulting in a single band of 943 bp characteristic for Xac (Figure 5). The exception were the isolates 13 and 14 (Figure 5) where two additional fragments were amplified, indicating heterogeneity of the isolates' population, The amplification and sequencing of the partial *rpoD* gene sequence of two selected isolates RKFB 1370 and RKFB 1375, were performed using a set of primers described by Hajri et al. (2012). The obtained partial DNA sequences showed that the isolates (GenBank Nos. OQ271224 and OQ271225) share 99.47% to 99.92% *rpoD* sequence identity with Xac strains CP076619.1 and HG992342.1 isolated from hazelnut in France and HG992341.1 in USA.

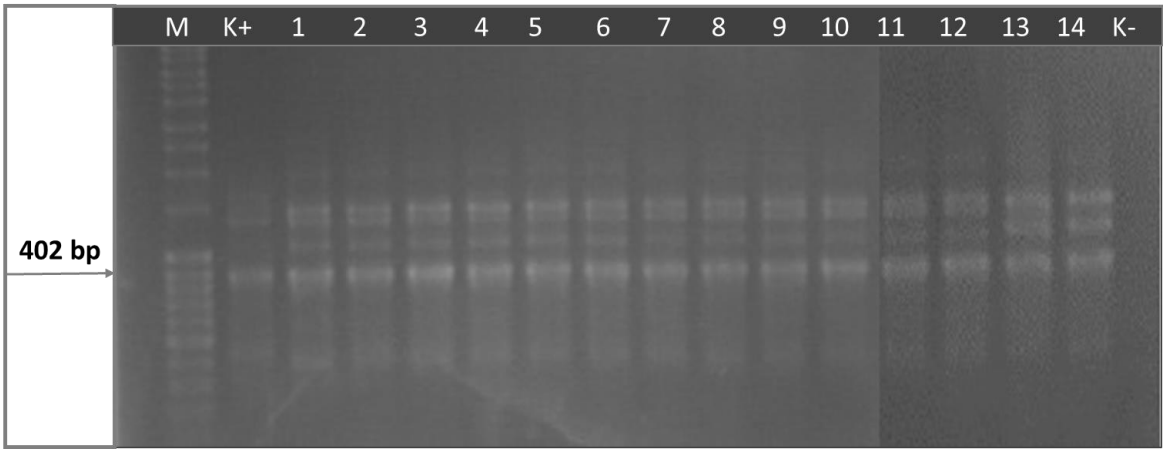

Figure 4. PCR detection of *qumA* gene in tested isolates. 1 – 14 - isolates from hazelnut (RKFB 1369, 1370, 1371, 1372, 1373, 1374, 1375, 1376, 1377, 1378, 1379, 1380, 1381, 1382); K-negative control, K+ positive control Xac NCPPB 3037; M - MassRuler Low Range DNA Ladder, Fermentas, Lithuania.

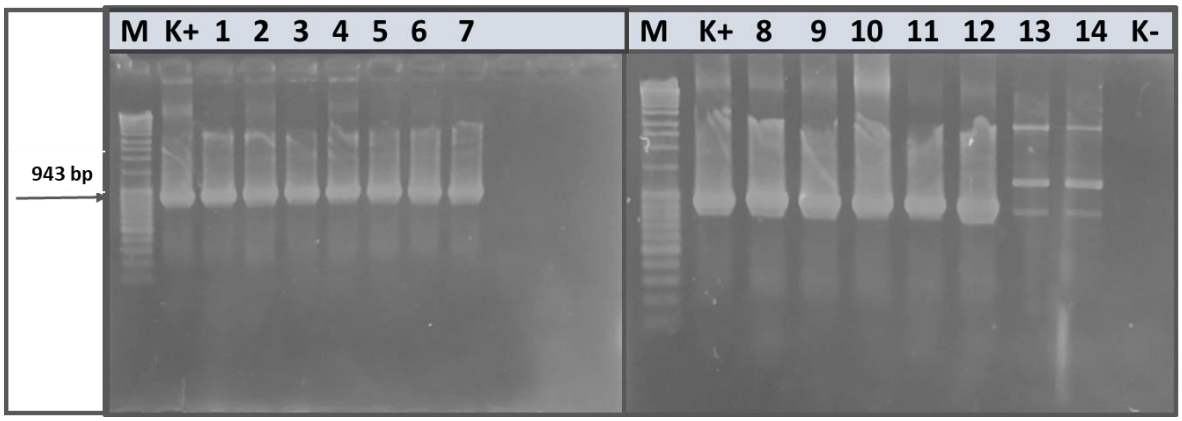

Figure 5. PCR detection of *ftsX* gene in tested isolates. 1 – 14 - isolates from hazelnut (RKFB 1369, 1370, 1371, 1372, 1373, 1374, 1375, 1376, 1377, 1378, 1379, 1380, 1381, 1382); K-negative control, K+ positive control Xac NCPPB 3037; M - MassRuler Low Range DNA Ladder, Fermentas, Lithuania.

CONCLUSIONS

Based on pathogenic, biochemical, and molecular characteristics, the isolates from hazelnut plants in Montenegro were identified as *X. arboricola* pv. *corylina*. The results of this study indicated that Xac is present in hazelnut commercial orchard in central part of Montenegro and in favorable climate conditions, pathogen causes significant economic losses. In order to prevent spreading pathogen in other areas phytosanitary measures should be established for the eradication of the Xac within this area. On the other side, hazelnut planting material is intensively imported and increases the risk of introduction of latently infected material. Therefore, strict import control have to be implemented to prevent introduction and spread of the pathogen to other areas.

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