Journal of Agricultural, Food and Environmental Sciences Vol 78 No 1 (2024) 87-92
DOI:

Original scientific paper

THE PRESENCE OF BACTERIAL LEAF SPOT CAUSED BY *XANTHOMONAS ARBORICOLA* PV. *PRUNI* IN THE REPUBLIC OF SRPSKA

Biljana Lolić[1*], Stefani Tepić[1], Mirela Kajkut Zeljković[1,2]

[1]University of Banja Luka, Institute of genetic resources, University City, Bulevar vojvode Petra Bojovića 1A, Republic of Srpska
[2]University of Banja Luka, Agricultural faculty, Bulevar vojvode Petra Bojovića 1A, Republic of Srpska
[*]corresponding author:biljana.lolic@igr.unibl.org

ABSTRACT

Xanthomonas arboricola pv. *pruni* (*Xap*), the causal agent of bacterial leaf spot disease of stone fruit, cause disease of stone fruits and almond worldwide. *Xanthomonas arboricola* pv. *pruni* (*Xap*) is regulated as quarantine pathogen in the European Union and the European and Mediterranean Plant Protection Organization (EPPO, A2 list). The bacterium can be latent and can be transmitted by plant material, and visual inspections are used for monitoring plants in orchards and nurseries. Monitoring was continuously conducted in period 2021-2023 in commercial orchards and registered nurseries on the territory of the Republic of Srpska. Leaves, twigs and branches of hosts (peach, nectarine, plum, apricot, cherry and sour cherry) were inspected and samples were taken for laboratory analysis. Detection and identification were done according to EPPO diagnostic protocols PM 7/64(1) and PM 7/100 (1), with slight modifications. As a reference material it was used freeze dried bacteria CFBP 2535 (producer CIRM, France). Out of 321 analyzed samples, 21 samples (plum, cherry, peach and apricot) confirmed as positive. Further studies on *Xanthomonas arboricola* pvs. are planned to be conducted in the following period.

Key words: quarantine bacterium, stone fruits, orchards, nurseries.

INTRODUCTION

Xanthomonas arboricola pv. *pruni* (Vauterin, *et al.,* 1995), was previously named *Xanthomonas campestris* pv. *pruni* (Smith, 1903). The disease caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*) was described for the first time in the USA (Michigan) by Smith in 1903 on Japanese plum. *X. arboricola* pv. *pruni* attacks only *Prunus* species. Bacterial leaf spot is reported from all continents where stone fruits are grown, most known as a pathogen of plum, nectarine and peach (Stefani *et al*., 1989), apricot (Scortichini & Simeone, 1997), almond (Young, 1977) and cherry in particular, and their hybrids (Rosello *et al.*, 2012). *X. arboricola* pv. *pruni* is differentially virulent on stone fruit species, being common and severe on peach and plum but causing less damage on apricot (Du Plesis, 1988). Ornamental *Prunus* species are also attacked. The disease is widespread on cultivated stone fruit, probably have developed due to the spread of endemic populations from wild stone fruit (Vauterin *et al.*, 1993).

The disease was first described in North America in 1903 (Smith, 1903). Since its initial description in North America, the disease has been observed on four continents and several European countries. In Europe, the disease first was detected in Italy in 1920 (Petri,

1934; Zaccardelli *et al*., 1998; Stefani, 2010) and considered endemic there since the 1970s (Battilani et al., 1999). It also was reported in Romania in 1941 and the first documented observation of the disease in France was in 1995 (Boudon et al., 2005).

The disease was very severe in 2000 and has expanded to new stone fruit orchards every year. *X. a.* pv. *pruni* (*Xap*), the causal agent of bacterial spot disease, is a quarantine organism in the EU phytosanitary legislation (Anonymous, 2000) and in the European and Mediterranean Plant Protection Organization list in EPPO A2 List of pest recommended for regulation (Anonymous, 2003a).

Symptoms of bacterial spot of stone fruits can be observed on leaves, young and fully developed fruits, twigs, branches and trunks (EPPO, 2003, 2006). It can enter the host before full healing of the leaf scar, or through stomata, during the following growing season (Zaccardelli *et al.,* 1995). The leaf spots are water-soaked circular or angular with darkening to deep-purple, brown or black color and surrounding pale-green to yellow irregular areas. This disease is also called shot-hole and black spot (Jami *et al.,* 2005). In orchards, although affected trees can develop symptoms on different parts, they may appear healthy if not inspected closely. However, in nursery plantlets, heavy infections can be detected easily, mainly on *P. persica* (peach and nectarine), because of heavy defoliation and chlorosis of the leaves (OEPP/EPPO, 2021). The symptoms of bacterial spot become more obvious during warm seasons with temperatures of 19–28°C, frequent rain, wind and heavy dew, which are all favourable conditions for severe infection (Fahy & Persley, 1983; Bradbury, 1986; Du Plessis, 1988; Zehr and Shepard, 1996).

The inconsistent pattern of bacterial epidemics observed in different countries or areas may be related to differential pathogenicity features of bacterial strains, variations in susceptibility of stone fruit species and cultivars, and cropping conditions, such as irrigation, fertilization and pruning time and frequency (Stefani, 2010). Control methods are limited to copper sprays supplemented with cultural practice modifications such as optimization of irrigation to reduce extended wetting of the foliage. Although less-susceptible cultivars are available, none have complete resistance (Boudon *et al.,* 2005).

In most countries, including EU and EPPO region, *Xap* is regulated as a quarantine pathogen (Anonymous, 2000, 2006; EPPO/CABI, 1997). Also, the Ministry of Agriculture, waters and forestry supported Special surveillance program for the presence of quarantine pest organisms in stone fruits on the territory of the Republic of Srpska in 2019. Therefore in 2019, monitoring, sample collection, examination of the health condition of stone fruits and laboratory analysis were carried out. The aim of this work was to obtain a view of health status of stone fruit production both in commercial and in nursery production.

MATERIALS AND METHODS

During the period 2021-2023 observation of the symptoms of bacterial leaf spot were done on leaves, fruits, twigs and branches on Prunus hosts in orchards and also inspected in nurseries. Symptoms are first apparent on the lower surface on leaves but soon become more evident on the upper surface. During the field monitoring, sampling of symptomatic stone fruit leaves were monitored from June to September, collected in plastic bag, marked and store in fridge condition before laboratory analyses.

Disease symptoms were monitored in commercial orchards and registered nurseries of 7 regions (Prijedor, Banja Luka, Doboj, Bijeljina, Istočno Sarajevo, Gradiška and Trebinje) in the Republic of Srpska.

Detection and identification were done according to EPPO standard diagnostic protocols PM 7/64(1), with slight modifications. The symptomatic and asymptomatic leaf samples were taken for laboratory analysis for isolation and cultivation in non-selective media YDC (yeast extract-dextrose-calcium carbonate agar) and molecular methods PM

7/100 (1). As a reference material it was used freeze dried bacteria CFBP 2535 (producer CIRM, France).

The leaves are briefly washed under running tap water to remove dirt and disinfected for 40-60 seconds with 70% ethanol. They are rinsed repeatedly in sterile water and immediately used for further analysis. A few small pieces of a couple of leaves tissue (1–2 cm) were taken from the margin of the healthy tissue and necrotic lesion and crushed in a mortar. After crushing, adding a few drops of sterile water (2-3 ml) and the suspension is left to macerate no longer than 1-2 min. The dilution-plate method was used to streak 100 µL of the suspension onto YDC agar plates (yeast extract-dextrose-calcium carbonate agar). YDC agar plates were prepared by adding yeast extract 10 g, dextrose (glucose) 20 g, calcium carbonate (light powder) 20 g, agar 15 g and distilled water to 1 liter. Colonies of each sample are cultivated in five dilutions. The plates are incubated at approximately 28°C for 2–3 days. *Xanthomonas*-like colonies were observed on agar plates after 2 days of incubation. Suspend 1⁄3 loopful of bacteria from 48–72 h culture on nutrient agar (NA) or yeast peptone glucose agar (YPGA) in 100 lL R⁄DNAse free water. Vortex to acquire a homogeneous suspension, lyse bacteria and extract DNA.

DNA extraction was done from the plant material and from suspected colonies when the morphology of the bacterial culture was typical for the pathovar. Extraction was carried out using commercial kit for bacterial DNA extraction (Blirt, Gdansk) according to the manufacturer's protocol and analysed by Rep-PCR. DNA extracted from suspected colonies and leaves have been stored at -20°C. Quality and quantity of DNA was evaluated on a 0.7% agarose electrophoresis gel. DNA was amplified by Rep-PCR (EPPO PM 7/100 (1). Rep-PCR was carried out in a total volume of 25 µl containing 14.05 µl nuclease-free water, 2.5 µl 10X Buffer, 0.75 µl 50mM $MgCl_2$, 0,5 µl 10mM dNTP mix, 2.5 µl of each primers, 0,2 µl 5 U^{-1}Platanium Taq Polymerase. ERIC-PCR conditions: initial denaturation at 95˚C 7 min followed by 30 cycles (94˚C 1 min, 52˚C 1 min, 65˚C 8 min) and one final step at 65˚C 16 min before cooling at 4˚C. The PCR products were separated by electrophoresis in 2% agarose gel (15×20 cm) for 16 h at a constant voltage of 90V in 1×TBE at 4°C. The Rep-PCR profiles were visualized after staining with ethidium bromide under ultraviolet light.

Young fully expanded leaves (3–6th leaf from the top) were detached from apricot and peach cultivar known to be susceptible to *X. a. pruni* for the pathogenicity test. The leaves were briefly washed under running tap water to remove upper dirt and disinfected for 40–60s with 70% ethanol. They were rinsed repeatedly in sterile water and immediately used for inoculation of bacteria suspension at concentration of 10^5. Leaves side upward, were placed on several Petri dishes. The inoculum was infiltrated by using a syringe without a needle and by applying gentle and steady pressure until a 2-4 mm diameter area of mesophyll tissue is water-soaked. Each leaf were inoculated on 6 sites approximately 1 cm apart. The leaves were lightly blotted to remove any excess of inoculum. Negative controls were prepared using sterile water. All inoculated leaves were placed on wet paper and incubated for 5-7 days at 25°C.

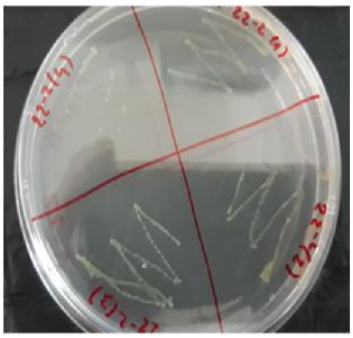
RESULTS AND DISCUSSION

X. arboricola pv. *pruni* can be isolated from immature fruits showing water-soaked angular spots, or from twigs and branches with cankers, but that isolation of the pathogen can be more difficult than to take the leaves for patogen isolation. So, leaf samples were collected in orchards and nurseries of stone fruit and taken for laboratory analysis. Isolation and cultivation was done in non-selective media YDC (yeast extract-dextrose-calcium carbonate agar).

During the last tree years, out of 321 analyzed samples. All samples were isolated and cultivated in non-selective media. After incubation period, bacterial colonies that appeared

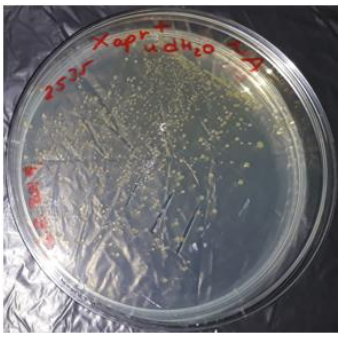
was yellow (bright and creamy), translucent, circular, raised, convex and mucoid, with a tendency to darken a little (turning yellow-orange) with age or depending on the media. Typical colonies were re-streaked onto suitable agar plates to obtain pure cultures for further identification (Fig. 1and 2). At the same time, a reference culture was grown for comparison and further analyses (Figure 3.). Pure cultures of presumptive *X. arboricola* pv. *pruni* should be identified with at least one more tests based on different biological principles and known *X. arboricola* pv. *pruni* reference strains should be included in each test.

1)



2)

Figure 1. and Figure 2. Pure cultures on agar medium: shining, convex, yellow and mucoid



3)

Figure 3. Positive control, CFBP 2535

For routine detection, confirmation and identification of *X. arboricola* pv. *pruni* (Xap), it is recommended to use Rep- PCR. In this study, the moleculat analyses confirm positive results of isolated bacteria on media. Summarised, 20 samples (plum, cherry and peach) confirmed as positive and originated from orchards and apricot confirmed as positive and originated from nursery production. The plum assumes as the most sensitive with 16 positive samples, 3 peach positive and 1 cherry positive samles in orchard comercial production. In the mention period, 80 samples were analysed from nursery production and only 1 apricot confirmed as positive.

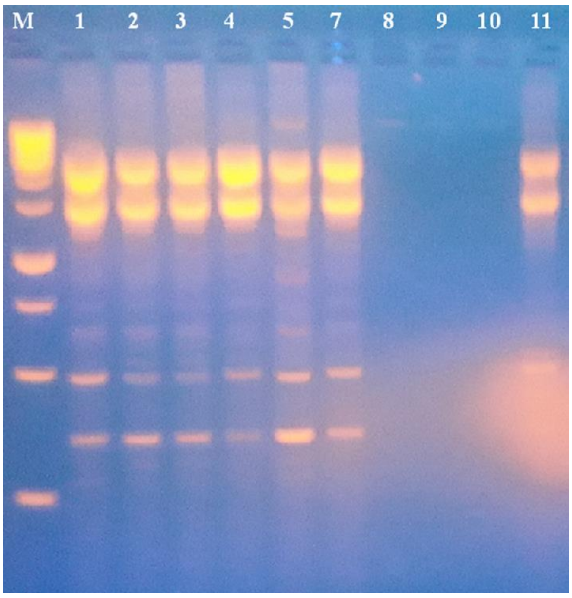


Figure 4. Agarose gel (2%) with ERIC primers: M-marker (1kb), lanes 1-10 refer to Xap isolated from leaves of stone fruits, lane 11 corresponds to the Xap reference CFBP 2535 (producer CIRM, France)

Pathogenicity tests carried out at young leaves by observed symptoms in the injured infected region, whereas no symptoms were observed in the control (Fig 5,6,7). Approximately 5-7 days after inoculation, injured leaves induced water-soaked angular leaf spots. These spots turned into brown lesion during next 5 days.


5)

6)

7)

Figures 5-7. Pathogenicity test on apricot and peach cultivars

Symptoms caused by all *X. arboricola* pv. *pruni* strains were similar on detached leaves, as well as on the leaves infected wirh referent strain CFBP 2535, thus confirming pathogenicity of all isolates of the pathogen previously identified as *X. arboricola* pv. *pruni*. The negative control with sterile water did not cause any symptom on tested leaves.

CONCLUSION

As the causal agent of a major disease of stone fruits, Xap is a quarantine organism for the most of Europe counties as well as for the Republic of Srpska. Therefore it is necessary to continuously implement eradication measures to prevent further spread to new areas and/or new hosts. YDC as non-selective media can be usful for Xap detection but for routine detection and confirmation, it is recommended to use Rep- PCR as a rapid method.

In period 2021-2023, out of 321 analyzed samples, 21 samples (plum, cherry, peach and apricot) confirmed as positive and originated from orhards and nurseries. Further studies on *Xanthomonas arboricola* pvs. are planned to be conducted in the following period.

REFERENCES

Battilani, P., Rossi, V. and Saccardi, A. (1999). Development of *Xanthomonasarboricola* pv. *pruni* epidemics on peaches, Journal of Plant Pathology, 81:161-171.

Boudon S., Manceau C., Notteghem, J-L., 2005. Structure and origin of *Xanthomonas arboricola* pv. *pruni* populations causing Bacterial spot of stone fruit trees in Western Europe, The American Phytopathological Society, Vol.95, No.9: 1061-1088.

Bradbury JF (1986) Guide to Plant Pathogenic Bacteria. CAB International, Farnham Royal (UK): 332.

Du Plessis, H.J. (1988) Bacterial spot disease of stone fruits: overview of findings. Deciduous-Fruit Grower 38, 128–132.

Jami, F., Kazempour, M.N., Elahinia, S.A., Khodakaramin, G. (2005): First report of *Xanthomonas arboricola* pv. *pruni* on stone fruit trees from Iran, Phytopathology 153: 371-372.

EPPO/CABI (1997) Xanthomonas arboricola *pv.* pruni. In: *Quarantine Pests for Europe*, 2nd edn, pp. 1096–1100. CAB International, Wallingford (GB).

EPPO (European and Mediterranean Plant Protection Organization) (2006): *Xanthomonas arboricola* pv. *pruni* . EPPO Bull. 36: 129–133.

EPPO (European and Mediterranean Plant Protection Organization) (2010): Rep-PCR tests for identification of bacteria. EPPO Bull. 40: 365–368.

Fahy, P.C. and Persley, G.J. (1983): Plant Bacterial Diseases: A Diagnostic Guide. Academic Press, New South Wales, Australia: 393.
OEPP/EPPO (2021): PM 7/064 (2) Xanthomonas arboricola pv. pruni, EPPO Bulletin, 51:240–266.
Roselló, M., Santiago, R., Palacio-Bielsa, A., García-Figueres, F., Montón, C., Cambra, M.A., López, M.M. (2012): Current status of bacterial spot of stone fruits and almond caused by *Xanthomonas arboricola* pv. *pruni* in Spain. *Journal of Plant Pathology*, 94: 15-21.
Smith, E. F. 1903. Observations on a hither to unreported bacterial disease, the cause of which enters the plant through ordinary stomata. Science 17: 456-457.
Stefani E, 2010. Economic significance and control of bacterial spot/canker of stone fruits caused by Xanthomonas arboricola pv. pruni. *Journal of Plant Pathology*, 92 (Suppl. 1), 99–103.
Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45: 472-489.
Vauterin, L., Hoste, B., Yang, P., Alvarez, A., Kersters, K., and Swings, J. 1993. Taxonomy of the genus *Xanthomonas*. Pages 157-192 in: *Xanthomonas.* J. G. Swings and E. L. Civerolo, eds. Chapman & Hall, London
Zaccardelli M., Consiglio M.F., Mazzucchi U. (1995): Detection of *Xanthomonas campestris* pv. *pruni* in symptomless peach trees in winter. *Phytopathologia Mediterranea* 34: 199–203.
Zehr, E.I. and Shepard, D.P. (1996): Bacterial spot of peach as influenced by water congestion, leaf wetness duration, and temperature, Plant Disease 80: 339–341.