

Periodic isolation of *Xanthomonas campestris* pv. *campestris* from soil

Periodična izolacija *Xanthomonas campestris* pv. *campestris* iz zemljišta

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ABSTRACT

Xanthomonas campestris pv. *campestris* (Xcc) is a major pathogen of brassicas in all production areas worldwide. The bacterium is primarily seed-borne but is also capable of persisting in soil. The duration of survival and seasonal fluctuations of soil populations play a key role in disease epidemiology. This study aimed to determine the seasonal dynamics of Xcc presence in soil under continuous cabbage monoculture. Soil sampling was conducted monthly over a two-year period (2015–2017) at the Futog locality. Samples were collected from a depth of up to 15 cm, and Xcc isolation was performed using SX selective medium. The identity of isolates was confirmed by biochemical tests and multiplex PCR analysis. The presence of the bacterium was recorded during spring (April–June), late summer (August), and autumn (September–November), whereas it was not detected during winter (December–March). This pronounced seasonal pattern indicates a clear relationship between Xcc survival in soil and temperature and moisture conditions. The results emphasize the key role of soil as an inoculum reservoir and a source of primary infections in the epidemiology of black rot in *Brassicaceae*.

Keywords: *Xanthomonas campestris* pv. *campestris*, soil, seasonal dynamics, inoculum, epidemiology

SAŽETAK

Xanthomonas campestris pv. *campestris* (Xcc) predstavlja značajnog patogena kupusnjača u svim područjima gajenja. Bakterija se prvenstveno prenosi semenom, ali je sposobna da perzistira i u zemljištu. Dužina opstanka i sezonske fluktuacije populacija u zemljištu imaju ključnu ulogu u epidemiologiji bolesti. Cilj istraživanja bio je da se utvrdi sezonska dinamika prisustva Xcc u zemljištu pod monokulturom kupusa. Uzorkovanja su sprovedena mesečno tokom dvogodišnjeg perioda (2015–2017) na lokalitetu Futog. Uzorci zemljišta prikupljeni su sa dubine do 15 cm, a izolacija je vršena na selektivnoj SX podlozi. Identitet izolata potvrđen je biohemijskim testovima i multiplex PCR analizom. Prisustvo bakterije utvrđeno je tokom proleća (april–jun), krajem leta (avgust) i tokom jeseni (septembar–novembar), dok tokom zimskog perioda (decembar–mart) nije detektovana. Izražen sezonski obrazac ukazuje na jasnu povezanost opstanka Xcc u zemljištu sa temperaturnim i vlažnim uslovima. Dobijeni rezultati potvrđuju značaj zemljišta kao rezervoara inokuluma i potencijalnog izvora primarnih infekcija, što je od epidemiološkog značaja za crnu trulež kupusnjača.

Ključne reči: *Xanthomonas campestris* pv. *campestris*, zemljište, sezonska dinamika, inokulum, epidemiologija

INTRODUCTION

Phytopathogenic bacteria are capable of surviving in the absence of a host, within various ecological niches, including epiphytic and endophytic habitats, but frequently also as saprophytes in soil (Sobiczewski and Iakimova, 2022). Among these, *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot of *Brassicaceae*, represents an economically important pathogen with a worldwide distribution. Infected cabbage heads lose their market value, while rot commonly develops both in the field and during post-harvest storage (Massomo et al., 2003). According to Kong et al. (2021), annual cabbage yield losses can reach 50–60%, and in cases of early field infection shortly after transplanting, yield reduction may be as high as 90% (Dey et al., 2015).

The bacterium is primarily seed-borne but also survives in soil and on certain *Brassicaceae* weed species, which considerably contributes to its persistence within agroecosystems (Cook et al., 1952; Dane and Shaw, 1996; Vicente and Holub, 2013; Vlajić et al., 2022). Schaad and White (1974a) and Dane and Shaw (1996) demonstrated that Xcc can persist in soil in the absence of a host for up to 40 days during winter and up to 20 days during summer. In infected plant residues, the pathogen can remain viable until its complete decomposition, from several months in tropical climates to as long as two years in dry and cold regions (Strandberg, 1973; Schultz and Gabrielson, 1986). From the soil, the bacterium can infect healthy plants through root injuries (Cook et al., 1952). Weed species within the *Brassicaceae* family may also be latently infected, without exhibiting visible disease symptoms (Schaad and Dianese, 1981).

Infection typically manifests as chlorotic lesions along leaf margins that taper toward the central nerve, forming a characteristic V-shaped pattern (Williams, 1980; Vicente and Holub, 2013). Understanding the mechanisms and environmental factors that enable Xcc survival outside the host is essential for developing effective management strategies and eliminating potential inoculum sources (Silva et al., 2017).

Integrating climatic data with disease epidemiology is crucial for understanding pathogen survival and seasonal dynamics, as well as for developing predictive models and effective control strategies. Highlighting these interactions provides a broader framework for interpreting the occurrence and persistence of Xcc under local agro-ecological conditions.

Although the survival of Xcc in soil has been investigated in different climatic regions, data on its seasonal dynamics in Serbia remain limited. This knowledge gap highlights the need for more detailed studies on the periodic occurrence of Xcc in soil across different months and climatic conditions, which represents the main objective of the present study.

MATERIALS AND METHODS

Experimental site

The experiments were conducted at the Futog locality (45°14'17" N; 19°42'25" E), in the Republic of Serbia. This area is well known for the traditional cultivation of the local cabbage variety "Futoški kupus".

Sample collection

To assess the seasonal fluctuation of Xcc presence in soil, 55 sampling events were performed on the same field plot, at monthly intervals over a 24-month period between 2015 and 2017. Based on successful isolations, i.e. presence or absence of the bacterium in soil samples, seasonal fluctuations of Xcc were determined for each calendar month. The trial was conducted on a 0.57 ha field plot located within the Futog cadastral municipality, under continuous cabbage monoculture for three consecutive years.

Soil sampling and isolation

Soil samples were collected using a soil probe to a depth of 15 cm at 12 diagonal points across the plot. Composite samples of 0.5 kg were prepared, placed in PVC bags, and processed within 24 h. Subsamples of 5 g of soil were suspended in 50 ml of sterile physiological solution, followed by preparation of serial dilutions (1:10,

1:100, and 1:1000). Aliquots of 100 µl from each dilution were plated onto the surface of SX selective medium (Schaad and White, 1974b), according to the method of Đukić et al. (2010).

For positive control and validation, sterilized and artificially inoculated soil was used. A bacterial suspension with a concentration of 3×10^8 CFU/ml (determined by McFarland standard) was added to 10 g of soil. After 5 days of incubation at 27 °C, isolation was performed using the same procedure as for field samples. Incubation on SX medium lasted 3–4 days, depending on the sampling period. Colonies were subsequently subcultured, and a collection of isolates was established.

Pathogenicity test of isolates

The pathogenicity of isolates was evaluated by artificial inoculation of host leaves (Obradović and Arsenijević, 1999; Radunović and Balaž, 2012). A bacterial suspension (10^6 – 10^7 CFU/ml) prepared from 48-hour cultures grown on YDC medium was injected into the vein of cabbage leaves (cv. Futoški) using a medical syringe and needle. The reference strain *Xanthomonas campestris* pv. *campestris* NCPPB 1144 served as a positive control, while sterile distilled water was used as a negative control. Inoculated leaves were placed in PVC boxes lined with moistened filter paper and incubated at 25 °C in a growth chamber. Symptom development was evaluated on the third, fifth, and seventh day after inoculation. Upon completion of the experiment, reisolation and reinoculation were performed to confirm isolate identity and pathogenicity.

Biochemical and physiological characteristics of isolates

All isolates were examined for the following physiological and biochemical traits: catalase and oxidase activity; hydrolysis of starch, gelatine, esculin, and Tween-80; citrate utilization; milk proteolysis; nitrate reduction; tolerance to 0.02% and 0.1% TTC; oxidative–fermentative metabolism of glucose (O/F test); indole and hydrogen sulfide production; and growth at 35 °C (Schaad, 1988; Arsenijević, 1997).

Molecular identification methods

DNA extraction for multiplex-PCR

DNA was extracted following the ISTA protocol (Roberts and Koenraad, 2006 - Annexe to Chapter 7 – 019a: Seed Health Testing Methods). Forty-eight-hour-old single colonies were suspended in 1 ml of sterile physiological solution in 1.5 ml microtubes. After centrifugation for 5 min at 8000 rpm at 25 °C, the supernatant was discarded, and the precipitate was resuspended in 500 µl of 0.5 N NaOH. Following incubation at 65 °C for 10 min and vortexing, 5 µl of the suspension was transferred into 495 µl of DNA-free water. Samples were stored at –20 °C until use.

Multiplex PCR

For Xcc identification, multiplex PCR was performed using two sets of specific primers: DLH120/DLH125 (Berg et al., 2005) and Zup2309/Zup2310 (Rijlaarsdam et al., 2004). The reaction mixture (20 µl) consisted of 10 µl Master Mix, 0.25 µl of each primer, 2 µl CL, 3 µl DNA template, and 4 µl sterile water.

PCR program:

1. Initial denaturation: 95 °C, 3 min (1 cycle)
2. DNA denaturation: 95 °C, 40 s (6 cycles)
3. Primer annealing: 63 °C (+1 °C/cycle), 40 s; extension: 72 °C, 40 s; denaturation: 95 °C, 40 s (29 cycles)
4. Extension: 58 °C, 40 s; 72 °C, 40 s
5. Final extension: 72 °C, 5 min

PCR products (7 µl) were separated by electrophoresis on 2% agarose gels in TBE buffer (BlueMarine™ 200) at 90 V for 90 min. Gels were stained with ethidium bromide (0.5 µl/10 ml) for 30 min, discoloration for 20 min, and visualized under UV light. Molecular fragment sizes were determined using SERVA FastLoad 100 bp DNA Ladder. Positive samples were identified by amplification of 370 bp and 619 bp fragments. The reference strain Xcc NCPPB 1144 was used as a positive control. No specific amplification was observed in negative controls (*Escherichia coli* ATCC 25922 and *Xanthomonas vesicatoria* KFB 29).

Meteorological data

Meteorological data for the period from October 2015 to November 2017 were obtained from the Republic Hydrometeorological Service of Serbia (RHMZ, 2015-2017).

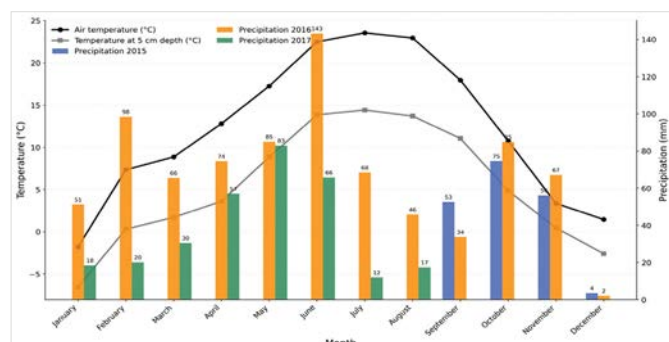


Figure 1. Monthly dynamics of air temperature, temperature in 5 cm depth, and precipitation (2015-2017)

RESULTS

Isolation of the bacterium from soil

Following the isolation procedure from soil samples on SX selective medium and incubation for three days at 27 °C, small colonies surrounded by clear zones were observed, indicating starch hydrolysis. After five days of incubation, colony diameters reached 2-3 mm, while hydrolysis zones extended to 7-9 mm. Consistent colony development was recorded from artificially inoculated soil, confirming the reliability of the applied isolation method. The colonies were mucoid, convex, and bluish-purple in colour, which is characteristic of the genus *Xanthomonas*. A total of 37 isolates were obtained, all showing phenotypic traits consistent with this genus (Table 1).

Table 1. Number of soil samples, successful isolations of *Xcc*, and isolation dates per month and year (2015-2017)

Month	2015 – Date / Result	2016 – Date / Result	2017 – Date / Result	Total samples
January	– *	22 Jan / –	24 Jan / –	0
February	–	23 Feb / –	24 Feb / –	0
March	–	25 Mar / –	20 Mar / –	0
April	–	20 Apr / + (Z-2016/4)	26 Apr / –	5
May	–	23 May / + (Z-2016/5)	17 May / + (Z-2017/5)	6
June	–	14 Jun / + (Z-2016/6)	26 Jun / + (Z-2017/6)	3
July	–	21 Jul / –	24 Jul / –	0
August	–	24 Aug / + (Z-2016/8)	28 Aug / + (Z-2017/8)	4
September	17 Sep / + (Z-2015/9)	20 Sep / + (Z-2016/9)	–	7
October	20 Oct / + (Z-2015/10)	17 Oct / –	–	2
November	18 Nov / + (Z-2015/11)	22 Nov / + (Z-2016/11)	–	2
December	22 Dec / –	18 Dec / –	–	0
Total	/	/	/	29

* “+” indicates successful isolation of *Xcc*; “–” indicates unsuccessful isolation. Isolation codes (e.g. Z-2016/5) correspond to isolate labels used in m-PCR identification (Figure 2)

Pathogenicity test of isolates

Pathogenicity testing was conducted by infiltrating bacterial suspensions into the veins of cabbage leaves. Three days after inoculation, 29 isolates induced discoloration of leaf tissue and the formation of water-soaked lesions that spread from the injection site along the petiole. By the fifth day, the affected tissue had expanded considerably and turned brown. After seven days, a distinct black line was observed at the boundary between healthy and diseased tissue, accompanied by the appearance of yellowish exudate and the onset of tissue maceration. No symptoms were recorded on control plants inoculated with sterile distilled water (SDW) during the entire incubation period.

Biochemical and physiological characteristics of isolates

The examination of 29 isolates revealed a high level of homogeneity in their biochemical and physiological traits. All isolates metabolized glucose strictly aerobically (oxidative pathway), produced indole, and generated hydrogen sulphide from peptone. The oxidase test was negative, whereas catalase activity was positive. All isolates hydrolysed starch, esculin, Tween-80, and gelatine, utilized citrate, and showed proteolytic activity on milk. None reduced nitrate to nitrite, nor did they exhibit tolerance to 0.02% and 0.1% TTC. Growth was observed at 35 °C. All test results were identical to those obtained for the reference strain *Xcc* NCPPB 1144, providing preliminary confirmation of their identity as *Xanthomonas campestris* pv. *campestris*.

Molecular identification of isolates

Multiplex PCR analysis using two sets of specific primers, DLH120/DLH125 (619 bp) and Zup2309/Zup2310 (370 bp), confirmed the identity of all isolates and reisolates. All tested samples yielded amplification products of the expected sizes (619 and 370 bp), identical to those of the reference strain *Xcc* NCPPB 1144 (Figure 2).

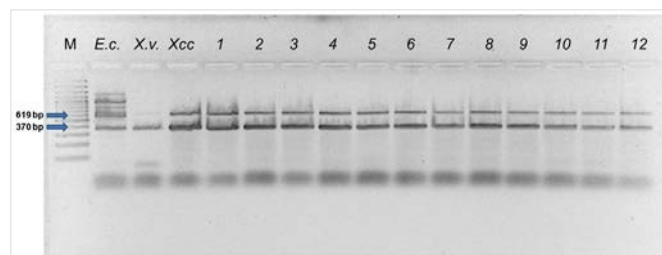


Figure 2. Identification of *Xcc* isolates from soil by multiplex PCR (m-PCR). M – molecular size marker; E.c. – *E. coli* strain ATCC 25922; X.v. – *X. vesicatoria* strain KFB 29; Xcc – reference strain Xcc NCPPB 1144; 1–12 – tested isolates (1: Z-2015/9; 2: Z-2015/10; 3: Z-2015/11; 4: Z-2016/4; 5: Z-2016/5; 6: Z-2016/6; 7: Z-2016/8; 8: Z-2016/9; 9: Z-2016/11; 10: Z-2017/5; 11: Z-2017/6; 12: Z-2017/8.)

Seasonal distribution of isolations

Periodic soil sampling over a two-year period confirmed the presence of *Xcc* in September, October, and November of 2015 and 2016, as well as in April, May, June, and August of 2016, and in May, June, and August of 2017. The bacterium was not detected in December of 2015 and 2016, nor during the winter months (January–March) of 2016 and 2017, and was also absent in April 2017. These findings indicate a pronounced seasonal pattern of *Xcc* occurrence in soil, strongly associated with temperature and moisture conditions (Figure 3).



Figure 3. Seasonal pattern of *Xcc* isolation from soil

DISCUSSION

The climatic conditions recorded during the study period (2015–2017) largely corresponded to the biological requirements of *Xanthomonas campestris* pv. *campestris* (*Xcc*) for survival and infection. The optimal temperature range for *Xcc* infection is approximately 20–30 °C, with high relative humidity and the presence of free water playing a critical role (Vicente and Holub, 2012). Recent

studies have confirmed these findings and further emphasized the importance of the combined effects of temperature and precipitation under field conditions, which directly influence inoculum dynamics and disease development (Liu et al., 2022).

Moderate air temperatures and soil temperatures at a depth of 5 cm (15–25 °C), in combination with increased precipitation during spring and autumn, created favourable conditions for bacterial multiplication and spread. The spring period, characterized by frequent rainfall and a gradual increase in temperature, coincides with the early growth stages of brassica crops (Červenski et al., 2022; 2025), and represents the most critical period for primary infections. Under warm and humid conditions, *Xcc* enters the plant vascular system through hydathodes or wounds along the leaf margins, producing characteristic disease symptoms (Köhl and van der Wolf, 2005). In most cases, infections are systemic, resulting in wilting or rotting of plants (Greer et al., 2023).

Periodic soil isolations conducted over the two-year period confirmed a pronounced seasonal dynamic of *Xcc* occurrence. Successful isolations were obtained during September, October, and November (2015 and 2016), as well as during spring and early summer (April–June 2016, May–June 2017, and August 2016–2017). These results indicate a strong association between *Xcc* fluctuations in soil and climatic factors, consistent with findings for *X. campestris* pv. *citri* in tropical and subtropical regions (Graham et al., 1989). These authors reported that the bacterium survives in soil for only a few days, whereas it can persist for several months in plant debris, confirming the role of crop residues as an important inoculum reservoir.

In contrast, isolations conducted during the winter months (January–March and December) and during the summer peak (July) were largely unsuccessful, highlighting the influence of unfavourable climatic conditions on the survival of *Xcc* populations in soil. Extremely low temperatures during winter had a particularly negative effect on bacterial presence. Soil temperatures at 5 cm depth in January and December 2016 were -3.4 °C and

-5.1 °C, respectively, while in December 2017, temperatures reached -9.6 °C (RHMZ). Such conditions are unsuitable for active bacterial growth and likely lead to a substantial decline in soil populations. Conversely, very high summer temperatures and intense heating of the soil surface during July also negatively affected inoculum persistence.

In addition to climatic conditions, microbiological interactions play an important role in the survival and ecology of *Xcc*. The activity of indigenous soil microorganisms can significantly influence bacterial population dynamics. López et al. (1999) showed that *Xcc* cell numbers decline much more rapidly in fresh soil than in sterilized soil due to competitive interactions with native microbiota. Habte and Alexander (1975) also demonstrated the role of protozoa in reducing *X. campestris* populations in soil, confirming the impact of biotic factors on inoculum suppression.

The phyllosphere and rhizosphere of weeds represent key ecological niches influencing bacterial survival, with the phyllosphere acting as a transient inoculum reservoir and site for population persistence (Silva et al., 2017). In contrast, the rhizosphere provides a more stable microenvironment, offering greater protection from climatic fluctuations while simultaneously supplying carbon and mineral nutrients necessary for bacterial survival (Brencic and Winans, 2005).

Pronounced interannual differences, particularly in the amount and distribution of spring precipitation, may explain variations in pathogen detection and disease occurrence between seasons. Warm and humid springs favour earlier and more intense disease development, whereas dry or cooler springs may delay or reduce pathogen activity. Understanding the interactions between climatic factors, microbial communities, and soil inoculum dynamics is crucial for the epidemiological interpretation of disease occurrence and for the development of effective disease management strategies. The ecology of certain plant pathogens, including *Xcc*, remains insufficiently understood, partly due to the lack of sensitive methods for monitoring pathogens in soil.

CONCLUSIONS

Periodic soil sampling conducted over a two-year period confirmed the presence of *Xcc* during spring (April–June), late summer (August), and autumn (September–November), whereas the bacterium was not detected during the winter period (December–March). This pronounced seasonal pattern is clearly associated with temperature and moisture conditions, indicating the ecologically dependent nature of *Xcc* survival in soil and the seasonal fluctuation of the inoculum.

Future research should focus on the quantitative monitoring of *Xcc* populations in different soil types and under varying climatic conditions, as well as on elucidating the interactions between the pathogen, plant residues, and the soil microbiota, in order to more precisely define the role of soil in the epidemiology of black rot of crucifers.

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