

Occurrence of *Xanthomonas campestris* pv. *campestris* (Pammel, 1895) Dowson 1939, on Brassicas in Montenegro

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SUMMARY

Brassicas form the most important group of vegetable crops in Montenegro. The cabbage (*Brassica oleracea* var. *capitata*) is most commonly grown, although other brassicas, particularly kale, Brussels sprout, cauliflower and broccoli, have been increasingly produced since recently. One of the specialties of vegetable production in Montenegro is growing of collard (*Brassica oleracea* var. *acephala*), which is the simplest variety of the *Brassica oleracea* species and in the nearest relation with their wild ancestor – the *sylvestris* variety.

Diseases are the main restrictive factors for successful production of these vegetables. Susceptibility of the cultivars and inadequate control often result in more or less damaged crops in some plots.

Causal agents of brassica diseases, especially bacterial, have not been investigated in Montenegro until 2009. Since the symptoms observed in 2009 were „V” shaped leaf edge necrosis and black rot of vascular tissue, it was assumed that they were caused by plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*.

Samples of the infected plants were collected from different localities in Montenegro. Isolation and identification of the bacterium were performed using laboratory methods according to Schaad (1980), Lelliott and Stead (1987) and Arsenijević (1997). Examination of chosen bacterial isolates was conducted using both, classical bacteriological methods (examination of their pathogenic, morphological, cultivation and biochemical and physiological characteristics), and ELISA test.

The obtained results confirmed the presence of *X.campestris* pv. *campestris* (Pammel, 1895) Dowson 1939, on cabbage, kale, broccoli and collard in Montenegro. This is the first experimental evidence that collard is the host of *X. campestris* pv. *campestris* in Montenegro.

Keywords: Montenegro; Collard; *Xanthomonas campestris* pv. *campestris*; *Brassica oleracea*

INTRODUCTION

Production of vegetables has a long tradition in Montenegro. The largest and most important production regions are situated in central part of the country, in Zeta Plain. Besides potatoes, brassicas are the most important vegetable crops in Montenegro, which have been traditionally grown in Zeta Plain for many years (Figures 1 and 2). According to the data regarding the year 2009 available in Statistical Annual of Montenegro, vegetable crops occupied a total of 18403 ha, of which cabbage and kale were grown on 1968 ha, with the average yield of 25.44 t/ha.

Cabbage (*Brassica oleracea* var. *capitata*) is most often grown in this area, while larger production of other brassicas, especially kale, Brussels sprout, cauliflower and broccoli, started only recently. Examining the influence of different planting conditions on cabbage yield, Mirecki (1999) emphasized the weather and agro-ecological conditions in Zeta Plain as very favourable for growing of this vegetable in open fields, all the year round.



Figure 1. Plot of cabbage in Zeta Plain



Figure 2. Plot of kale in Zeta Plain

One of the specialties of this region is growing of collard (*Brassica oleracea* var. *acephala*), the simplest variety of the species *Brassica oleracea*, which is in the nearest relation with their wild ancestor – the *sylvestris* variety, and is an endemic species and a specificity of agricultural production in Montenegro (Pajović, 2005).

There are two types of collard in this region (Figures 3 and 4). Continental type has a stem of medium height and smaller leaves. Its life cycle lasts two years. In contrast, maritime type has a tall stem and dark- to purple-green leaves of rich, sweet taste. Its life cycle is three to four years long.



Figure 3. Continental type of collard in Zeta Plain



Figure 4. Maritime type of collard in Zeta Plain

Because of mild climate in this region, brassicas are grown in shifts during the whole year. Their production is most often monocultural and usually limited to relatively narrow areas, using intensive agro-technical measures, all of which favor the occurrence of fungal, bacterial and viral diseases.

Bacterial diseases are particularly significant as one of the limiting factors in the production of brassicas. According to Balaž (2005), many factors contributed the spread of bacterial diseases on brassicas, especially the production of new, quality and high-yielding hybrids, susceptible to phytopathogenic bacteria on large areas, using intensive agro-technical measures.

Xanthomonas campestris pv. *campestris* (Pammel, 1895) Dowson 1939 is the most important and widely distributed plant pathogenic bacterium on brassicas. It is the causal agent of black rot, vascular bacterial disease, one of commercially the most important diseases on brassicas (Janse, 2005). It causes the greatest damage in tropical and subtropical regions with humid climate, but is also very harmful in colder, coastal areas in northern Europe and North America. Plants can be infected during the whole vegetation period from the stage of springing until the end of vegetation, and even later, during transportation and storage. Yield can be reduced by 40-50%, and a total destruction of crops is also possible.

X. c. pv. *campestris* is commercially the most harmful bacterium on brassicas in Serbia (Arsenijević, 1988; Balaž, 2005; Obradović, 2010). It was first described in 1964, when it caused losses of about 80% in fodder kale crops in central Serbia (Perišić and Panić, 1964, cit. Balaž, 2001). At the beginning of 1990s, Balaž (1989) observed the occurrence of *X. c.* pv. *campestris* on several brassicas in Vojvodina: cauliflower, kale, cabbage, Brussels sprout and broccoli. Obradović and Arsenijević (1999) described this bacterium as a very common parasite on cabbage, cauliflower and kale, which could cause significant losses in the production of heads, as well as in the seed crops of these plants. Obradović et al. (2000b) carried out detailed researches of pathogenic, cultivation, biochemical and physiological characteristics of this bacterium in Serbia, by examining its isolates obtained from the samples of diseased cabbage, cauliflower and kale plants from various regions in Serbia, during the period 1995-1999.

In Montenegro, a high incidence of the bacterium was first observed on cabbage in 1949, when the disease caused rotting of heads on 50% cabbage plants in one plot of the former State Farm in Podgorica (Mijušković, 2002). Since then, no data on the occurrence and spreading of *X. c.* pv. *campestris* in Montenegro have been reported.

During the summer 2009, some symptoms typical of bacterial diseases, such as marginal, chlorotic yellow

to brown lesions and blackening of the veins were observed on most brassicas (cabbage, kale, broccoli and collard) in Zeta Plain. Pathogen caused leaf drying and plant under development and, in some plots, complete wilting and decay of the infected plants. In order to identify the causal agent of these pathological changes, plant samples with clearly expressed symptoms were collected, and pathogen isolation and identification of the obtained isolates were carried out in the laboratory.

MATERIAL AND METHODS

Plant material and isolation

In 2009, samples with clearly expressed symptoms of „V” shaped leaf edge necrosis were collected at the localities of Tuzi and Golubovci, from four *B. oleracea* varieties: cabbage (*B. o.* var. *capitata* L.), kale (*B. o.* var. *sabauda* L.), collard (*B. o.* var. *acephala* D.C.) and broccoli (*B. o.* convar. *botrytis* L. Alef var. *italica* Plenk). Isolation and identification of the bacteria were carried out at the Laboratory for Plant Pathology of the Biotechnical Faculty in Podgorica, Montenegro, and the Laboratory for Plant Bacterial Diseases of the Faculty of Agriculture in Novi Sad, Serbia.

The bacterium was isolated from the leaf, petiole and stem tissue. Plant material was first rinsed under running water and dried, and then cut by a sterile scalpel into small fragments which were macerated in sterilized distilled water and left for 15-20 minutes to enable diffusion of the bacteria. According to the standard method of isolation, the obtained suspension was struck on yeast extract, glucose and CaCO₃ medium (YDC) in Petri dishes (Schaad, 1980).

Petri dishes were incubated at 28°C in a thermostat and the appearance and growth of the bacterial colonies were monitored after 3-5 days. Pure cultures were obtained by picking out the individual colonies and transferring them onto the slanting YDC media in test tubes. In this way the strains chosen for investigation were obtained. Pure bacterial cultures old 24h, grown on the YDC medium at 28°C were used in all tests. Vitality of the obtained strains was maintained by transferring them periodically and storing at 4°C, in a fridge.

Among a large number of the obtained isolates, 12 isolates of this plant pathogenic bacterium were collected for further investigation. The isolation success was determined proportionally (in percentage terms), on the basis of the number of Petri dishes in which the expected bacterial colonies developed in relation to the number of unsuccessful isolations.

Characteristics of the isolated strains were compared with the control strain of the bacterium *X. c. pv. campestris* (code name NCPPB 1144), obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB) in Great Britain.

Pathogenicity test and inoculation

Pathogenicity of all the obtained isolates was tested by inoculation of cabbage leaves. The suspension of 48h old bacterial strains (10^7 cells/ml) was infiltrated into cabbage leaf mesophyll and leaf nerves (Obradović and Arsenijević, 1999).

The leaves infiltrated with water were used as a negative control, while the leaves infiltrated with the suspension of the control *X. c. pv. campestris* strain (NCPPB 1144) were used as a positive control.

Both, the inoculated and control plants were incubated in a moist chamber for 48 hours, and then kept at 25°C and relative air humidity of 35-40%.

Morphological characteristics

Gram reaction

Gram reaction was carried out with 3% KOH. Using a wooden toothpick, 24h old bacterial culture was removed onto a microscopic plate where it was homogenized in a drop of 3% KOH and the appearance of a thin "thread", produced by cell wall degradation, was monitored.

Cultivation characteristics

Time of the occurrence of bacterial colonies, as well as their appearance, colour, size and shape were monitored during their cultivation on nutrient media. The isolation of *X. c. pv. campestris* is usually carried out on the medium of yeast extract, glucose and CaCO_3 (YDC), on which this bacterium produces mucoid, convex and shiny colonies of intensive yellow colour (Arsenijević, 1997).

On glucose media, *X. c. pv. campestris* produces a yellow extra-cellular polysaccharide, called xanthan gum. Yellow colour of the colonies comes from the pigment xanthomonadine.

Moffett and Croft (1983) emphasize that it is a slow-growing bacterium which needs even 7-14 days to develop colonies.

Biochemical and physiological characteristics

The following biochemical and physiological characteristics of the obtained isolates were examined: oxidative-fermentative metabolism of glucose (O/F test),

catalase and oxidase activity, hydrolysis of starch, gelatin, esculin and Tween-80, reduction of nitrates, tolerance of 0.1% and 0.02% TTC, production of indole and hydrogen sulfide and growth at 35°C (Lelliott and Stead, 1987; Schaad, 1980, 1988).

Serological characteristics

Serological characteristics of the obtained isolates were examined by conducting the indirect enzyme-linked immunosorbent assay (PTA ELISA) on a microtiter plate, using a polyclonal antiserum, specific for the detection of *X. c. pv. campestris* (Identikit PTA General Y, ADGEN Phytodiagnostics, Neogen Europe, Ltd., Scotland, UK). Testing was carried out according to the manufacturer's instructions.

The results were read on the ELISA automatic reader for serological plates (Universal Microplate Reader EL x 800 BioTek Instruments, USA). Absorption values which were twice or more the values of the negative control were considered as positive reactions.

RESULTS AND DISCUSSION

Disease symptoms and presence of the bacterium in Montenegro

During the summer 2009, the disease symptoms were observed on brassica plants in open fields in Zeta Plain. The first symptoms occurred on leaf edges as characteristic chlorotic spots which got narrower towards the main leaf nerve, forming the shape of „V” letter. Black necrosis occurred within chlorotic spots and the diseased parts of the leaves were drying out (Figures 5-8). The blackness of nerves, characteristic of this bacterial disease, was particularly intense on kale leaves. Dried parts of the leaves resembled parchment. Finally, the diseased leaves completely turned yellow, dried up and fell off.





Different types of symptoms were observed on the infected plant leaves (Figures 9 and 10). During August and September, at temperature of about 30°C , which is characteristic of the region, symptoms were expressed in the form of large spots in the middle parts of the leaves. At the beginning of October, at lower temperatures of about 15-18°C , only marginal leaf necrosis could be observed. Koike et al. (2007) also reported the dependence of symptom types upon air temperature, emphasizing that large spots develop in the central leaf parts at temperatures between 20°C and 28°C , marginal necrosis at about 16°C , and the absence of symptoms in cold weather despite the presence of the bacterium in the infected plant.



Figures 5-8. Chlorosis and blackness of nerves on cabbage, collard, kale and broccoli leaves

Figures 9 and 10. Different types of symptoms on a cabbage leaf in Zeta Plain

There were no visible external changes on infected petioles and stem. However, on their cross and longitudinal sections dark-coloured vascular vessels which exuded yellow, mucoid matter, full of bacteria could be observed (Figures 11 and 12).



Figures 11 and 12. Dark-coloured vascular vessels of cabbage petiole and stem – longitudinal section –

Systemic plant infection appeared as the result of presence of the bacteria in vascular tissues. After the infection through leaf hydathodes, the bacteria moved from intercellular space of parenchyma to vascular vessels (xylem), and then spread into petioles, stems and finally, root.

Because of the destructive activity of the bacteria, infected plants grew slowly, became stunted and dried out. Large number of diseased plants did not form heads which resulted in reduced yield. After the first rains, systemically infected plants were flattened over

the plots. Further development of secondary parasites caused rotting and decay of heads. In some plots, over 80% of the plants were destroyed (Figures 13 and 14). The greatest damage was recorded in the plots on lower ground, in depressions.



Figures 13 and 14. Drying and decay of infected cabbage plants in Zeta Plain caused by *X. c. pv. campestris*

During 2009, there were favorable environmental conditions for occurrence and growth of the bacterium *X. c. pv. campestris* in Zeta Plain. First of all, optimal temperature and humidity values contributed to its intensive reproduction. The symptoms on brassica plants were particularly expressed during August and September, at characteristic temperatures of 25-30°C. Adequate humidity for the disease development was provided by surface irrigation with sprayers, which is the most common way of irrigating brassica crops in this region.

The same environmental preferences of *X. c. pv. campestris* were reported by many authors, who emphasized that it is a thermophilic bacterium with optimal growth temperature of 30-32°C and that warm and wet weather favours the epiphytotic disease occurrence (Moffett and Croft, 1983; Janse, 2005). According to Agrios (2005), under warm and wet weather conditions which favours reproduction of parasites, infection of plants is rapid and visible symptoms appear within only several hours.

Identification of bacterial isolates

Isolation of the bacterium from diseased plant parts (leaves, petioles and stems) was successfully carried out on nutrient media. Five days after inoculating the media, shiny, convex, round colonies of yellow colour developed, which is characteristic of the genus *Xanthomonas*. A large number of isolates was obtained, and 12 isolates were chosen for further investigation: from collard – RŠ 1, RŠ 2 and RŠ 3; from broccoli – BR 1, BR 2 and BR 3; from cabbage – KP 1, KP 2 and KP X-7; and from kale – KLJ 1, KLJ 2 and KLJ V-8.

The most successful isolation was from kale (91.6%), and then from collard (74.9%), cabbage (66.6%) and broccoli (58.3%).

Investigated bacterial strains are presented in Table 1.

Table 1. Investigated bacterial strains originating from brassica leaves, petioles and stems

Number	Strain	Host plant	Locality
1	RŠ 1	collard	Tuzi
2	RŠ 2	collard	Tuzi
3	RŠ 3	collard	Tuzi
4	BR 1	broccoli	Golubovci
5	BR 2	broccoli	Golubovci
6	BR 3	broccoli	Golubovci
7	KP 1	cabbage	Tuzi
8	KP 2	cabbage	Tuzi
9	KP X-7	cabbage	Tuzi
10	KLJ 1	kale	Tuzi
11	KLJ 2	kale	Tuzi
12	KLJ V-8	kale	Tuzi

Pathogenicity of all investigated isolates was tested on cabbage leaves. All the isolates caused the occurrence of watery, light brown spots around the pricks, 48 hours

after the infiltration of bacterial suspension (Obradović and Arsenijević, 1999). These spots expanded, and the tissue within them softened and began to rot six days after inoculation (Figures 15 and 16).



Figures 15 and 16. Symptoms on cabbage slices inoculated in laboratory (RŠ-2 and Xcc NCPPB 1144)

All the investigated isolates were gram-negative, which was determined using 3% KOH in which a thin “thread” was formed as the result of complete degradation of the bacterial cell wall and releasing of mucoic DNA.

After five days of development on the medium of yeast extract, glucose and CaCO₃ (YDC), all the investigated isolates formed large (5-6 mm in diameter),

muroid colonies of rapid growth. They were round, slightly convex and shiny, of yellow-brown colour which turned into intense yellow after 5-6 days of development (Figures 17 and 18).



Figures 17 and 18. *X.c. pv. campestris* – appearance of colonies on YDC medium after 5 days of development (RŠ-2 and Xcc NCPPB 1144)

All the isolates investigated in this study had the same biochemical and physiological characteristics as those described by Obradović et al. (2000b), which are also in agreement with the characteristics of *X.c.pv.campestris* reported in literature by other authors (Lelliott and Stead, 1987; Schaad, 1980, 1988; Arsenijević, 1992).

For all the investigated isolates, including the control isolate Xcc NCPPB 1144, metabolism of glucose was

oxidative, the activity of catalase was positive, and the activity of oxidase was negative. All isolates hydrolyzed starch, gelatin, esculin and Tween 80, while none of the isolates reduced nitrates. Medium with 0.1% and 0.02% TTC inhibited the growth of the bacteria. All the isolates produced indole and hydrogen sulfide and grew at 35°C.

The results of biochemical tests are presented in Table 2.

Table 2. Biochemical and physiological characteristics of investigated strains

Test	Investigated strains	Reference strain Xcc NCPPB 1144
Origin Host	Montenegro cabbage, collard, kale, broccoli	Great Britain cabbage
Gram reaction	–	–
Mucoid growth on YDC	+	+
Glucose (O/F) metabolism	O	O
Catalase production	+	+
Oxidase activity	–	–
Starch hydrolysis	+	+
Gelatin liquefaction	+	+
Aesculin hydrolysis	+	+
Hydrolysis of Tween 80	+	+
Nitrate reduction	–	–
Growth on: 0.02% TTC	–	–
0.1% TTC	–	–
Indol production	+	+
H ₂ S production	+	+
Growth at 35°C	+	+

Legend: + = positive reaction
 – = negative reaction
 O/F = oxidative-fermentative metabolism of glucose
 TTC = triphenyl tetrazolium chloride

Indirect ELISA test, conducted on a microtiter plate, confirmed that all the investigated isolates reacted with the specific antibodies and had the same serological characteristics as the bacterium *X. c. pv. campestris*. All isolates, together with the control isolate Xcc NCPPB 1144, showed positive reaction, i.e. their absorption values were twice or more the value of negative control.

Serological method proved to be rapid and precise in identification of *X. c. pv. campestris* strains. Besides reducing the time needed for identification of bacteria, this method also enables simultaneous examination of a large number of samples (Schaad et al., 2001). On the basis of the obtained results it was concluded that the symptoms observed on brassicas were caused by plant pathogenic bacterium *X. c. pv. campestris* whose presence was confirmed for the first time on cabbage, kale, broccoli and collard in Montenegro.

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Pojava *Xanthomonas campestris* pv. *campestris* (Pammel, 1895) Dowson 1939, na kupusnjačama u Crnoj Gori

REZIME

Kupusnjače predstavljaju grupu najznačajnijih povrtarskih kultura u Crnoj Gori. Najviše se gaji kupus glavičar (*Brassica oleracea* var. *capitata*), a u novije vreme uvode se u proizvodnju i ostale kupusnjače, u prvom redu kelj, kelj pupčar, karfiol i brokola. Jedna od specifičnosti povrtarske proizvodnje u Crnoj Gori je gajenje raštana (*Brassica oleracea* var. *acephala*), najprostijeg varijeteta vrste *B. oleracea*, najrodnijeg divljem pretku – varijetetu *sylvestris*.

Bolesti predstavljaju glavni ograničavajući faktor uspješne proizvodnje ovih biljaka. Zbog osjetljivosti sortimenta i neadekvatne zaštite, često se dešava da usev na pojedinim parcelama bude u manjoj ili većoj meri oštećen.

U Crnoj Gori do sada nisu sprovedena istraživanja o prouzročivačima bolesti na kupusnjačama, a posebno ne istraživanja bakterijskih oboljenja. S obzirom da su tokom 2009. godine na biljkama konstatovani simptomi nekroze ivice lista u obliku latiničnog slova V i crna trulež sudovnog sistema, pretpostavka je bila da je prouzročivač ovih patoloških promena fitopatogena bakterija *Xanthomonas campestris* pv. *campestris*.

Uzorci sa obolelih biljaka prikupljeni su sa različitih lokaliteta u Crnoj Gori. Izolacija i identifikacija bakterije urađena je laboratorijskim metodama prema Schaad-u (1980), Lelliott i Stead-u (1987) i Arsenijeviću (1997). Ispitivanja odabranih bakterijskih izolata obuhvatala su primenu klasičnih bakterioloških metoda (ispitivanje patogenih, morfoloških, odgajivačkih i biohemijsko-fizioloških osobina dobijenih izolata) i ELISA test.

Dobijeni rezultati potvrdili su prisustvo i pojavu *X.campestris* pv. *campestris* (Pammel 1895) Dowson 1939 u Crnoj Gori, na kupusu, kelju, brokoliju i raštanu. Ovo je prvi eksperimentalni podatak da je raštan domaćin *X. campestris* pv. *campestris* u Crnoj Gori.

Ključne reči: Crna Gora; raštan; *Xanthomonas campestris* pv. *compestris*; *Brassica oleracea*