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Epidemiology studies of *Pseudomonas syringae* pathovars associated with bacterial canker on the sweet cherry in Serbia

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Abstract: This study was conducted to examine the epidemiological characteristics of two *Pseudomonas syringae* pvs. – *syringae* (*Pss*) and *morsprunorum* race 1 (*Psm*) on the sweet cherry in both field and laboratory conditions. Cycled inoculations of one-year-old branches indicated that the sweet cherry became sensitive to *Psm* race 1 earlier in the season (October) compared to *Pss* (November). The most severe infections occurred in the dormancy period (November), while the necroses formed in January and March were less in their length. Inoculations of the two-/three-year-old branches performed in November resulted in necrosis, with those induced by *Pss* being more aggressive. The January inoculations, however, resulted in less formed necroses in length. Laboratory tests performed on excised sweet cherry branches confirmed the results yielded by the field experiments, indicating that the dormancy period poses the greatest risk for the *P. syringae* pvs. infection. The isolation of *Pss* and *Psm* bacteria from naturally infected sweet cherry samples (cankers) during the summer indicated that the bacteria that persists in cankers in low numbers became active during autumn. Significant *Pss* and *Psm* race 1 epiphytic bacterial populations were noted during the spring, but decreased in the summer and increased again in the autumn, indicating the presence of inoculum reservoirs. The greater understanding of the control strategies aimed at the epidemiological factors should, thus, facilitate better disease outbreak management.

Keywords: bacterial dieback; field and laboratory inoculation; natural infection; sweet cherry

The sweet cherry (*Prunus avium* Linnaeus) was among the first fruit species to be cultivated in Serbia. In recent years, its production has become more intensive, due to which the dieback of young trees caused by bacterial pathogens *Pseudomonas syringae* pv. *syringae* (*Pss*) and *Pseudomonas syringae* pv. *morsprunorum* (*Psm*) race 1 has become more common (Balaž et al. 2016; Iličić et al. 2019). The symptoms caused by these pathogens are known as bacterial cankers, dieback and gummosis of all plant organs, including the trunks, branches, shoots, buds, flowers, leaves and fruits (Konavko et al. 2014; Hulin et al. 2018).

Controlling the bacterial canker is very difficult and is limited to the use of copper-based fungicides during the leaf fallout and bud burst stages. Empirical evidence suggests that the timing of the copper application is essential for bacterial canker control especially when treating infections of the wooden parts of the tree (Hinrichs-Berger 2003). Thus, Konavko et al. (2014) posited that, due to the complex pathogen epidemiology, disease prevention and use of less susceptible cultivars is the only practical approach to bacterial canker control.

Epidemiology of *P. syringae* on stone fruit is rendered more complex by the fact that the pathovars *syringae*, *morsprunorum* (race 1 and 2), *persicae*, *avii* and the species *P. cerasi* can cause bacterial canker, even though it is usually attributed to pvs. *syringae*

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and *morsprunorum* (Crosse 1966; Crosse & Garrett 1970; Lattore & Jones 1979; Hattingh et al. 1989; Kennelly et al. 2007; Scortichini 2010; Kaluzna et al. 2016). Crosse (1966) was the first to describe the epidemiology of *P. syringae* pathovars on stone fruit, recognising a bacterial winter and summer phase. Infections of wood parts (winter phase) occur during autumn and winter through wounds, or are induced by pruning or injuries caused by freezing temperatures and leaf scars. The active canker formation and spread occurs during spring, and stops in the early summer when the bacteria present in the affected tissues die due to being surrounded by calluses formed by the host (Crosse 1966; Crosse & Garrett 1970; Spotts et al. 2010). During the vegetative (summer) phase, the epiphytic *P. syringae* population infects the green, fresh plant organs (flowers, leaves and fruits), as confirmed by studies conducted in the UK (Crosse 1966; Crosse & Garrett 1966, 1970), Canada (Cao et al. 2013), the US (Cameron 1962; Sundin et al. 1988), Europe (Klement et al. 1974, 1984; Hinrichs-Berger 2003), South Africa (Roos & Hattingh 1986; Hattingh et al. 1989) and Australia (Wimalajeewa 1987). Crosse & Garrett (1966) emphasised the complex nature of plant pathogenic Pseudomonads on stone fruits, suggesting that different factors determine the growth and rate of tissue colonisation after the infection and induction of the defensive host reaction.

Earlier epidemiological work on bacterial cankers in the sweet cherry have been carried in countries with climatic conditions different from those prevalent in Serbia, i.e., colder continental (UK, Canada) or warmer tropical (Africa, Australia) climatic conditions. In Serbia and its surroundings (Hungary), only the *P. syringae* epidemiology on the apricot has been studied (Klement et al. 1974, 1984; Arsenijević 1980), while no prior investigations involving the sweet cherry have been conducted. Consequently, owing to the significance of the sweet cherry production in Serbia, the epidemiological features of *P. syringae* pvs. (*Pss* and *Psm* race 1) have been examined as a part of the present investigation, aimed at determining (*i*) the phenophases when the sweet cherry is most susceptible to the pathogen and its spreading through cyclic branch inoculations, (*ii*) the persistence of the bacteria in cankers on naturally infected sweet cherry trees, and (*iii*) the epiphytic pathogen populations on healthy sweet cherry buds and leaves. By gaining a better understanding of the pathogen epidemiology, the goal of this study is to contribute to a more adequate disease control.

MATERIAL AND METHODS

Sweet cherry susceptibility towards to *P. syringae* during its lifecycle. In order to determine the period when the sweet cherry is most susceptible to *P. syringae* pvs., as a part of the two-year-long experiment, inoculations of the branches, shoots, buds, scars, flowers and fruits were performed under field conditions, as well as on excised sweet cherry branches under laboratory conditions.

Two strains of *Pss* (KBNS87 and KBNS93) and *Psm* race 1 (KBNS74 and KBNS79) isolated in 2012 from the sweet cherry in Serbia (Balaž et al. 2016) were used for the inoculations. Bacterial suspensions adjusted to a final concentration of 10^9 CFU/mL were prepared from strains grown on nutrient agar (NA) at 26 °C for 48 hours.

Shoots, as well as one- and two-/three-year-old branches were inoculated by wounding the bark with a scalpel on three sites (Sobiczewski & Jones 1992; Spotts et al. 2010). The inoculations performed in the field experiments involved the cultivars Bigarreau Burlat, Drogan's Yellow, Germerzdorfer and Hedelfinger growing in a 20-year-old sweet cherry orchard. The shoots were inoculated in June and September, and the one-year-old branches were inoculated in October, November, January, March and April. The inoculations of the two-/three-year-old branches were carried out in June, September, October, November, January, March and April and involved trees growing in a seven-year-old orchard (cv. Summit).

The laboratory experiments were conducted on excised sweet cherry branches using the method described by Lattore and Jones (1979). The assays were carried out by cycled inoculations of the two-year-old excised branches of four sweet cherry cvs. (B. Burlat, Summit, Germerzdorfer and Hedelfinger) during the November – January period for two consecutive years. The excised branches (40–50 cm in length) were collected from an orchard free from bacterial canker. The branches were disinfected with 70% alcohol before wounding three sites to induce the inoculation. The inoculated branches were placed in a box with moist sterile quartz sand maintained at room temperature (20–22 °C) for the first seven days, after which the boxes were transferred to a botanical garden under field conditions, high humidity was ensured by wrapping the wounds with wet wool and aluminium foil.

Experiments involving the inoculations of sweet cherry leaf scars were performed in October,

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November and December, while fresh scars (buds forcibly removed with a scalpel) were inoculated in February and March, the flowers were inoculated in April and fruit in May. All the inoculations were performed by spraying the trees growing in a seven-year-old sweet cherry orchard (cv. Summit).

In all the experiments (performed in triplicate), sterile distilled water (SDW) served as a negative control. No mineral fertilisers or pesticides were applied.

The time of the pathogen inoculation was considered as 0 days post-inoculation (dpi). The results were evaluated at 30-day intervals and symptom development was rated on the basis on the lesion length (measured and expressed in cm). The gathered data were analysed by two-way ANOVA (Duncan's Multiple Range Test, $P < 0.05$). The re-isolations of the bacteria were performed from the developed lesion margins on nutrient agar supplemented with 5% w/v sucrose (NSA). The re-isolates were identified for their congruence with the original ones by LOPAT (Lelliott et al. 1966), GATTa and pathogenicity tests conducted on immature sour and sweet cherry fruitlets (Latorre & Jones 1979).

Persistence of *P. syringae* in cankers on naturally infected sweet cherry trees. In order to determine the persistence of the bacteria in cankers, branches with active/inactive cankers were collected on a monthly basis over a one-year period from four locations (Ljutovo, Mikićevo, Selenča and Gornji Tavankut) where the presence of *Pss* and *Psm* race 1 was previously confirmed (Balaž et al. 2016; Iličić et al. 2016). The diseased branches were first disinfected with 70% alcohol, after which the bark was aseptically removed and the tissue was excised from the canker margins. The macerated tissues were incubated at room temperature in SDW for 2 h, and were subsequently plated on NSA at 26 °C for 48 hours.

Epiphytic population of *P. syringae* pvs. Since *P. syringae* is known to be epiphytic in one part of its lifecycle, when performing the isolations from the healthy sweet cherry buds and leaves, the same locations were used as those chosen for the study of the bacterial persistence in the cankers. None of the collected samples had visible symptoms of the disease.

The isolations from sweet cherry buds were performed during February, March and October, as recommended by Roos and Hattingh (1983). The collected branches were disinfected with 70% alcohol before aseptically removing the buds. Approximately 100 buds were cut into smaller fragments and were immersed in 100 mL of a 0.01M Phosphate Buffer Saline (PBS, pH 7) before being placed on an orbital rotary shaker for 2 hours.

Healthy sweet cherry leaves were collected during August using the method described by Crosse (1959). Each sample was immersed in 4 L of the PBS and was kept at room temperature for 6 hours.

The obtained extracts were serially diluted in PBS and then plated on NSA. After incubation at 26 °C for 48 h, whitish, round, Levan-positive bacterial colonies formed and were counted.

The selected bacterial colonies were identified using LOPAT and GATTa tests and polymerase chain reaction (PCR) using specific primer pairs PRIMER1/2 (Bereswill et al. 1994) for the rapid identification of *Psm* race 1 and primer pairs B1/B2 and SyD1/SyD2 for *Pss* (Sorensen et al. 1998; Bultreys & Gheysen 1999). As positive controls, the reference strains *Pss* CFBP1582 and CFBP2119 *Psm* race 1 obtained from the Plant Associated Bacteria collection (France) were used.

RESULTS

Sweet cherry susceptibility towards to *P. syringae* during its lifecycle. On the one-year-old branches, the first positive result was yielded by the October inoculations, but only with *Psm* race 1, 90 dpi. Moreover, in the third rating conducted 120 dpi, the necrosis caused by *Psm* race 1 continued to enlarge and ranged from 1.36 to 2.70 cm in length, as shown in Table 1. The *Pss* inoculations performed in October were negative during the entire rating period. The November inoculations of both bacteria were positive on all the tested cvs. 120 dpi. In the third rating conducted 180 dpi, the necrosis reached 2.17–3.35 cm in length (Table 1, Figure 1). The first symptoms after the January inoculations with both tested *P. syringae* were observed 60 dpi in all the sweet cherry cvs. At the time of final rating (120 dpi), the necrosis length ranged from 1.44 to 2.31 cm (Table 1). The March inoculations produced a similar trend as those yielded by the January inoculations. The first symptoms were recorded 30 dpi, and the necrosis subsequently spread, reaching 1.22–2.13 cm in length 90 dpi (Table 1). During the vegetative phase (in April, June, and September), negative inoculations were noted for all the tested bacterial strains and cvs.

The inoculations performed with both bacteria on the two-/tree-year-old sweet cherry branches (cv. Summit) in November gave positive results

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Table 1. Field experiment: necrosis (cm) formed on one-year old branches of sweet cherry inoculated by *Pseudomonas* syringae pvs. (2013/2014)

Strain	Cultivar	October inoculations			November inoculations			January inoculations			March inoculations		
		30 dpi	90 dpi	120 dpi	120 dpi	150 dpi	180 dpi	60 dpi	90 dpi	120 dpi	30 dpi	60 dpi	90 dpi
KBNS74	*B. Burlat*	0^{a}	1.71^{d}	2.13^{d}	1.63^{cd}	2.98^{g}	3.03^{hij}	1.23^{bcde}	1.56^{cde}	1.85^{def}	1.13^{cde}	1.27^{b}	1.37^{bc}
	D.Yellow	0^{a}	1.26^{b}	1.75^{c}	1.27^{b}	2.81^{fg}	2.95^{fghij}	1.10^{bc}	1.34^{bc}	1.44^{b}	1.12^{cde}	1.23^{b}	1.32^{bc}
	Germerzdorfer	0^{a}	1.17^{b}	1.39^{b}	1.28^{b}	1.83^{b}	2.52^{cde}	1.33^{cde}	1.94^{fg}	2.31^{h}	0.97^{bc}	1.30^{b}	1.40^{bc}
	Hedelfinger	0^{a}	1.26^{b}	2.16^{d}	1.24^{b}	$2.12^{b, c, d}$	2.72^{efgh}	1.17^{bcd}	1.80^{efg}	1.87^{ef}	1.10^{bcde}	1.22^{b}	1.22^{b}
KBNS79	*B. Burlat*	0^{a}	1.87^{e}	2.70^{e}	1.77^{de}	$2.88^{f, g}$	3.20^{k}	1.16^{bcd}	1.44^{bcd}	1.94^{efg}	1.22^{e}	1.73^{d}	1.76^{d}
	D.Yellow	0^{a}	1.48^{c}	2.12^{c}	1.38^{bc}	$2.24^{c, d e}$	2.77^{efghi}	1.03^{b}	1.28^{b}	1.56^{bcd}	1.10^{bcde}	1.27^{b}	1.40^{bc}
	Germerzdorfer	0^{a}	1.13^{b}	1.36^{b}	1.40^{bc}	1.90^{b}	2.17^{b}	1.41^{de}	1.91^{fg}	2.08^{fgh}	0.92^{b}	1.27^{b}	1.35^{bc}
	Hedelfinger	0^{a}	1.26^{b}	2.22^{d}	1.17^{b}	$2.31^{d e}$	2.65^{ef}	1.10^{bc}	1.36^{bc}	1.51^{bc}	1.10^{bcde}	1.28^{b}	1.33^{bc}
KBNS87	*B. Burlat*	0^{a}	0^{a}	0^{a}	1.62^{cd}	$2.26^{c, d, e}$	3.06^{ijk}	1.44^{e}	1.70^{def}	1.79^{cdef}	1.14^{cde}	1.35^{bc}	1.57^{d}
	D.Yellow	0^{a}	0^{a}	0^{a}	1.20^{b}	$2.13^{b, c, d}$	2.87^{fghij}	1.05^{b}	1.37^{bc}	1.47^{b}	1.06^{bcde}	1.38^{bc}	1.44^{bc}
	Germerzdorfer	0^{a}	0^{a}	0^{a}	1.33^{b}	$2.02^{b, c, d}$	2.40^{bcd}	1.11^{bc}	1.61^{cde}	2.06^{fgh}	0.94^{bc}	1.38^{bc}	1.45^{bc}
	Hedelfinger	0^{a}	0^{a}	0^{a}	1.16^{b}	$2.30^{d, e}$	2.98^{ghij}	1.06^{bc}	1.95^{fg}	2.01^{fg}	1.01^{bcd}	1.41^{bc}	1.46^{bc}
KBNS93	*B. Burlat*	0^{a}	0^{a}	0^{a}	1.98^{e}	$2.58^{e, f}$	3.35^{k}	1.26^{bcde}	1.48^{bcd}	1.80^{cdef}	1.20^{de}	1.55^{cd}	2.13^{e}
	D.Yellow	0^{a}	0^{a}	0^{a}	1.17^{b}	$2.26^{c, d, e}$	2.72^{efgh}	1.06^{bc}	1.56^{cde}	1.65^{bcde}	1.07^{bcde}	1.42^{bc}	1.49^{c}
	Germerzdorfer	0^{a}	0^{a}	0^{a}	1.36^{bc}	$1.92^{b, c}$	2.28^{bc}	1.25^{bcde}	1.50^{bcd}	1.87^{ef}	0.96^{bc}	1.40^{bc}	1.47^{c}
	Hedelfinger	0^{a}	0^{a}	0^{a}	1.81^{de}	$2.72^{f, g}$	3.08^{ijk}	1.17^{bcd}	2.00^{g}	2.23^{h}	1.13^{cde}	1.72^{d}	1.74^{d}
Control	*B. Burlat*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	D.Yellow	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Germerzdorfer	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Hedelfinger	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values followed by the same letter are not significantly different ($P < 0.05$)

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Figure 1. Necrosis formed on one-year-old branches on a sweet cherry inoculated by *Pseudomonas syringae* pvs. 2013/2014

120 dpi (Table 2). Moreover, six months after inoculation, the necrosis reached 2.48–2.58 cm and 2.30–2.33 cm in length for *Pss* and *Psm* (Table 2), respectively. When the inoculations were performed in January, the lesion length reached 1.60–1.82 cm 120 dpi (Table 2), whereas the inoculations conducted during the vegetative phase (March, April, June and September) and the leaf fallout (October) period were negative.

The bacteria were re-isolated in June from all the sites that had developed lesions in both experiments. The bacterial strains recovered were subjected to LOPAT, GATTa and pathogenicity tests and all were shown to correspond to the original strains used in the previous October inoculations

Table 2. Field experiment: necrosis (cm) formed on two-year old branches of sweet cherry cv. Summit inoculated by *Pseudomonas* syringae pvs. (2013/2014)

Strain	November inoculations (dpi)			January inoculations (dpi)		
	120	150	180	60	90	120
KBNS74	1.60^{b}	2.01^{bc}	2.33^{b}	0.98^{b}	1.30^{b}	1.60^{b}
KBNS79	1.44^{b}	1.90^{b}	2.30^{b}	1.02^{b}	1.25^{b}	1.65^{b}
KBNS87	1.58^{b}	2.09^{c}	2.58^{c}	1.04^{b}	1.34^{b}	1.76^{b}
KBNS93	1.44^{b}	2.03^{bc}	2.48^{c}	0.99^{b}	1.28^{b}	1.82^{b}
Control	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values followed by the same letter are not significantly different ($P < 0.05$)

Table 3. Laboratory experiment: necrosis (cm) formed on two-years old excised branches of sweet cherry inoculated by *Pseudomonas* syringae pvs. (2013/2014)

Strain	Cultivar	November inoculations (dpi)			December inoculations (dpi)			January inoculations (dpi)		
		60	90	120	30	60	90	30	60	90
KBNS74	*B. Burlat*	0.34^{bcde}	1.03^{bc}	2.14^{efg}	0.41^{bc}	1.43^{efg}	2.66^{de}	0.47^{g}	1.39^{de}	2.77^{fg}
	Summit	0.30^{bc}	1.10^{cdef}	2.15^{efg}	0.36^{b}	1.28^{bcd}	2.31^{bc}	0.41^{cdef}	1.33^{bcde}	2.51^{cde}
	Germerzdorfer	0.29^{b}	0.97^{b}	1.35^{b}	0.39^{bc}	1.25^{bc}	2.36^{bc}	0.38^{bcd}	1.25^{bc}	2.34^{c}
	Hedelfinger	0.37^{de}	1.07^{bcde}	1.76^{cd}	0.41^{bc}	1.20^{b}	2.20^{b}	0.38^{bcd}	1.25^{bc}	2.13^{b}
KBNS79	*B. Burlat*	0.34^{bcde}	1.12^{cdefg}	2.21^{fg}	0.41^{bc}	1.52^{g}	2.80^{e}	0.45^{efg}	1.34^{bcde}	2.66^{efg}
	Summit	0.31^{bcd}	1.08^{cdef}	1.98^{cde}	0.36^{b}	1.27^{bcd}	2.18^{b}	0.41^{cdef}	1.28^{bcd}	2.58^{def}
	Germerzdorfer	0.30^{bc}	1.04^{bcd}	1.46^{b}	0.41^{bc}	1.42^{efg}	2.45^{cd}	0.39^{bcdef}	1.37^{cde}	2.32^{bc}
	Hedelfinger	0.32^{bcd}	1.09^{cdef}	1.83^{cd}	0.38^{bc}	1.37^{cde}	2.45^{cd}	0.39^{bcdef}	1.35^{bcde}	2.30^{bc}
KBNS87	*B. Burlat*	0.38^{e}	1.14^{defgh}	2.28^{g}	0.43^{c}	1.49^{fg}	2.66^{de}	0.45^{efg}	1.42^{de}	2.68^{efg}
	Summit	0.37^{de}	1.11^{cdef}	2.18^{efg}	0.41^{bc}	1.26^{bcd}	2.66^{de}	0.42^{cdef}	1.40^{de}	2.68^{efg}
	Germerzdorfer	0.36^{cde}	1.16^{efgh}	2.33^{g}	0.43^{c}	1.38^{cdef}	2.73^{e}	0.41^{cdef}	1.32^{bcd}	2.39^{cd}
	Hedelfinger	0.33^{bcde}	1.20^{fgh}	2.02^{def}	0.39^{bc}	1.36^{cde}	2.50^{cd}	0.40^{bcdef}	1.29^{bcd}	2.35$^{c, d}$
KBNS93	*B. Burlat*	0.38^{e}	1.25^{h}	2.35^{g}	0.39^{bc}	1.39^{def}	2.63^{de}	0.45^{efg}	1.47^{e}	2.81^{g}
	Summit	0.31^{bcd}	1.23^{gh}	2.17^{efg}	0.37^{bc}	1.33^{cde}	2.72^{e}	0.42^{cdef}	1.38^{cde}	2.66^{efg}
	Germerzdorfer	0.31^{bcd}	1.08^{cdef}	2.26^{fg}	0.39^{bc}	1.18^{b}	2.70^{e}	0.35^{b}	1.30^{bcd}	2.38^{cd}
	Hedelfinger	0.33^{bcde}	1.13^{cdefg}	1.83^{cd}	0.40^{bc}	1.35^{cde}	2.64^{de}	0.35^{b}	1.22^{b}	2.35^{cd}
Control	*B. Burlat*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Summit	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Germerzdorfer	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Hedelfinger	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values followed by the same letter are not significantly different ($P < 0.05$)

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Table 4. Laboratory experiment: necrosis (cm) formed on two-year old excised branches of sweet cherry inoculated by *Pseudomonas syringae* pvs. (2014/2015)

Strain	Cultivar	November inoculations (dpi)			December inoculations (dpi)			January inoculations (dpi)		
		60	90	120	30	60	90	30	60	90
KBNS74	*B. Burlat*	0.39^{efgh}	1.08^{cd}	2.24^{i}	$0.44^{bcd\ e}$	1.53^{e}	2.43^{cdef}	0.49^{ij}	1.46^{defg}	2.65^{g}
	Summit	0.40^{efgh}	1.26^{f}	2.18^{hi}	0.39^{b}	1.33^{b}	2.29^{bcde}	0.47^{ghij}	1.42^{cdef}	2.34^{de}
	Germerzdorfer	0.29^{b}	0.96^{b}	1.35^{b}	0.43^{bcde}	1.33^{b}	2.38^{bcdef}	0.37^{bc}	1.27^{b}	2.15^{bcd}
	Hedelfinger	0.36^{cdef}	1.06^{bcd}	1.75^{cd}	0.41^{bcd}	1.33^{b}	2.15^{b}	0.42^{cdef}	1.31^{bc}	2.30^{cde}
KBNS79	*B. Burlat*	0.40^{efgh}	1.14^{cdef}	2.30^{j}	0.47^{e}	1.54^{e}	2.52^{efg}	0.46^{fghij}	1.53^{fg}	2.60^{g}
	Summit	0.34^{bcde}	1.10^{cd}	2.02^{efgh}	0.39^{b}	1.37^{b}	2.40^{cdef}	0.48^{hij}	1.48^{efg}	2.40^{ef}
	Germerzdorfer	0.32^{bc}	1.03^{bc}	1.47^{b}	0.45^{cde}	1.50^{cde}	2.47^{def}	0.40^{bcde}	1.33^{bcd}	2.11^{bc}
	Hedelfinger	0.36^{cdef}	1.08^{cd}	1.88^{cdef}	0.40^{bc}	1.42^{bcde}	2.24^{bcd}	0.42^{cdef}	1.30^{bc}	2.20^{bcd}
KBNS87	*B. Burlat*	0.42^{gh}	1.16^{cdef}	2.19^{hi}	0.45^{cde}	1.52^{de}	2.57^{fg}	0.44^{efghi}	1.42^{cdef}	2.68^{g}
	Summit	0.41^{fgh}	1.13^{cdef}	2.08^{ghi}	0.43^{bcde}	1.49^{cde}	2.72^{g}	0.47^{ghij}	1.55^{g}	2.67^{g}
	Germerzdorfer	0.35^{cdef}	1.16^{cdef}	1.71^{c}	0.44^{bcde}	1.42^{bcde}	2.58^{fg}	0.39^{bcd}	1.34^{bcd}	2.20^{bcd}
	Hedelfinger	0.37^{cdefg}	1.24^{ef}	1.91^{defg}	0.42^{bcde}	1.38^{bc}	2.41^{cdef}	0.43^{defgh}	1.30^{bc}	2.25^{cde}
KBNS93	*B. Burlat*	0.44^{h}	1.24^{ef}	2.30^{j}	0.46^{de}	1.50^{cde}	2.56^{fg}	0.51^{j}	1.50^{fg}	2.61^{g}
	Summit	0.38^{efg}	1.17^{def}	2.05^{fghi}	0.47^{e}	1.50^{cde}	2.74^{g}	0.49^{ij}	1.56^{g}	2.70^{g}
	Germerzdorfer	0.33^{bcd}	1.12^{cde}	2.01^{efgh}	0.43^{bcde}	1.40^{bcde}	2.53^{efg}	0.36^{b}	1.37^{bcde}	2.11^{bc}
	Hedelfinger	0.35^{cdef}	1.14^{cdef}	1.85^{cde}	0.43^{bcde}	1.40^{bcd}	2.19^{bc}	0.35^{b}	1.27^{b}	2.01^{b}
Control	*B. Burlat*	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Summit	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Germerzdorfer	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	Hedelfinger	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values followed by the same letter are not significantly different ($P < 0.05$)

and in the other months in which positive results were obtained.

The inoculations conducted in the laboratory conditions during the two-year-long experiment involving the excised sweet cherry branches (i.e., during the dormancy period) yielded the same results as those obtained under the field conditions, confirming that the sweet cherry is most susceptible to *Pss* and *Psm* race 1 during the dormancy period. On the branches inoculated in November, the first disease symptoms appeared 60 dpi, while they registered 30 dpi on branches inoculated in December and January (Tables 3 and 4, Figure 2).

Considering that bacteria infect the buds through leaf scars, these served as inoculation sites in the experiments conducted in October, November and December. For both *Pss* and *Psm* race 1, the results were negative. Similarly, the inoculation of fresh leaf scars (forcibly removed by a scalpel) in February and March yielded negative results. Although we inoculated the flowers (April) and fruits (May), all the results were negative as well.

Persistence of *P. syringae* in cankers on naturally infected sweet cherry trees. The bacteria *Pss* and *Psm* race 1 were found to be persistent in the cankers in the April–July period, and were detected in September, November, January, February or March in some cases. Considering that both *Pss* and *Psm* race 1 were obtained from cankers (Table 5) throughout most of the year, our results indicate that small bacteria populations remain in the

Figure 2. Necrosis formed on two-year-old excised sweet cherry branches inoculated by *Pseudomonas syringae* pvs.

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Table 5. *Pseudomonas syringae* pvs.: isolation from cankers and determination of their epiphytic population

Locality	Isolation from canker												No. of determined isolates/pathovar*		Epiphytic population (buds, leaves)			No. of determined isolates/pathovar*	
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	*Pss*	*Psm* race 1	Spring	Summer	Autumn	*Pss*	*Psm* race 1
GornjiTavankut	–	–	–	+	+	+	+	–	+	–	+	–	15	0	+	+	+	10	4
Mikićevo	–	+	+	+	+	+	+	–	–	–	–	–	12	12	+	+	+	9	11
Ljutovo	+	+	+	+	+	+	+	–	–	–	+	–	18	0	+	+	+	12	6

+positive isolation; – negative isolation; *determined based on LOPAT, GATTa and conventional PCR (presence of cfl, or syrB and syrD genes)

cankers during the summer and become active in the autumn. In general, all on the examined sites, *Pss* was found to be present in the active or inactive sweet cherry cankers, while *Psm* race 1 was determined only in two locations (Table 5).

Epiphytic population of *P. syringae* pvs. In all the completely healthy sweet cherry buds and leaves collected during 2016, both *Pss* and *Psm* race 1 were confirmed as epiphytes. A significantly higher abundance of bacteria was found in the isolations from the buds during the spring (10^3–10^4 CFU/mL) and autumn (10^6 CFU/mL) compared to those obtained from the leaves in the summer months (10^1–10^2 CFU/mL). Table 5 presents *P. syringae* isolates collected from the epiphytic bacterial populations along with the identification test results. As can be seen, the number of isolates obtained from the epiphytic population was higher for pv. *syringae*.

DISCUSSION

The findings yielded in the present study indicate that sweet cherries growing under the agroecological conditions prevalent in Serbia are most susceptible to bacterial infection during the dormancy period and in the early stages of the vegetative phase, i.e., from October (*Psm* race 1)/November (*Pss*) to March. The fact that positive October inoculations of the one-year-old branches were only obtained with *Psm* could be attributed to the findings reported by Crosse (1966), who found that, in the UK, in this period, *P. morsprunorum* predominantly infects branches through the leaf scars or sites of mechanical damage of various origins. The first positive results obtained by *Pss* in the November inoculations in our study could be ascribed to the well-studied ice nucleation activity (INA) of this bacterium under freezing temperatures, and the increasing bacterial canker severity (Sobiczewski & Jones 1992; Cao et al. 2013). In our study, after the November inoculations, the temperatures initially declined to 8–10 °C, reducing further to –2 to –5 °C, thus inducing *Pss* to cause disease symptoms [Figure S1 in the electronic supplementary material (ESM)]. Similar findings were reported by Arsenijević (1980), who obtained the greatest success when November and December apricot *Pss* inoculations were performed at 15–18 °C followed by a period of low temperatures (–10 to –15 °C). Hinrichs-Berger (2003) suggested that frost without wounding was sufficient

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to introduce *Pss* into the plum bark, as bacteria can multiply, spread and cause symptoms. Klement et al. (1984) demonstrated that *Pss* cannot induce the disease in apricots in the absence of low winter temperatures. Therefore, it seems that sweet cherry infections with *Pss* are also promoted by frost and low temperatures. The shorter necrosis length stemming from the March inoculations compared to the November ones could be explained by the physiological state of the trees as they transition from dormancy to the vegetative phase, which affects the host resistance. Indeed, all the inoculations of the branches during the vegetative phase (April, June and September) were negative, owing to the plant defence system and warmer weather conditions (Figures S1 and S2 in the ESM). These results are supported by the findings reported by other authors (Crosse & Garret 1970; Hinrichs-Berger 2003).

The November inoculations of the two-/three-year-old branches showed the highest *Pss* aggressiveness, which is in line with the argument put forth by Bultreys & Kaluzna (2010) that *Pss* mainly infects thicker branches. Therefore, as the frost facilitated the aggressiveness of *Pss* on the sweet cherry inoculated during the winter months (Figures S1 and S2), the tissue damage promoted furthered the bacterial development, as reported by other authors (Sobiczewski & Jones 1992; Cao et al. 2013). Garrett (1979) stated that inoculations made from December until the middle of the dormant season ultimately caused the longest cankers, which is in line with the results obtained in our study.

In the tests conducted on the excised sweet cherry branches, the *Pss* strains were more aggressive than *Psm* race 1. Sobiczewski & Jones (1992) similarly demonstrated that, although both *P. syringae* pathovars exhibited pathogenicity when introduced to dormant branches exposed to freezing, more extensive necroses was produced by *Pss*.

In this study, inoculations of leaf scars with both *P. syringae* bacteria were negative, whereas positive inoculations through cherry leaf scars were confirmed by Hulin et al. (2018). However, the authors noted significantly less prominent symptoms than those yielded by the wound inoculations. This finding could also be ascribed to the leaf scars acting as a physical barrier to infection (Crosse 1966). Hinrichs-Berger (2003) suggested that plum buds are rarely infected by *P. syringae* through leaf scars, and this observation can be correlated with the results obtained in our study. The negative inoculation could also be attributed to the tree age, since our trials were performed in a seven-year-old orchard, but *Pss* and *Psm* introduced through leaf scars mainly caused symptoms in the young (1–3 years old) sweet cherry trees (Crosse 1956; Iličić et al. 2019). All the inoculations of the sweet cherry flowers and fruits performed in our study were negative, indicating that the summer phase is not an obligatory stage of the *P. syringae* lifecycle. This assertion is supported by the resistance of fruit trees during the vegetative phase, and partly by the specificity of the Serbian agroecological conditions (low precipitation and high temperature; Figure S2), which leads to the weakening of infectious pressure and virulence. Our results further confirm that weather plays a significant role in the disease occurrence and transition of bacteria from the epiphytic to the parasitic phase of its lifecycle. Considering that *Psm* race 1 isolations were positive during the summer and autumn, we believe that the bacterial persistence can be conditioned by various agroecological factors, inoculum pressure, as well as certain characteristics and the physiology of the sweet cherry cvs. Our results also align with the findings reported by Erickson (1945) and Dye (1954) who isolated *P. morsprunorum* from cankers during a rainy summer period, providing evidence that the pathogen persists in cankers in this part of the year. According to Prunier (1977), *P. morsprunorum* can survive in cankers up to eighteen months post-inoculation. Our findings are also supported by the observations made by other authors, according to whom epiphytic bacteria populations reach their maximum during spring and autumn, but decrease in size in summer due to the prevalence of high temperatures (Latorre & Jones 1979; Roos & Hattingh 1986; Scortichini 2010). The population level on leaves is mainly influenced by the climatic factors (Roos & Hattingh 1986), which is in line with the results obtained in this study (Figure S3). Significant *Pss* and *Psm* race 1 populations collected from buds during the spring and especially in autumn will promote infection in the wooden parts under the Serbian agroecological and climatic conditions. Thus, to reduce the epiphytic *P. syringae* population, a copper spray should be applied before and after the leaves fall out (in late winter and early spring). It is also important to protect the sweet cherry trees from freezing or any kind of damage or stress. This was the first study to describe the seasonal lifecycle of *P. syringae* on the sweet cherry in Serbia and its surroundings. By identifying the periods during

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which the sweet cherry is most susceptible to the most serious bacterial infections, in addition to estimating the pathogen prevalence in the cankers and its surface populations in field conditions during the growing season, our work has contributed to the better understanding of the pathogen epidemiology and can ultimately lead to a better disease control.

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