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ORIGINAL ARTICLE

An outbreak of *Verticillium dahliae* on sycamore maple in a forest stand in Slovenia

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Abstract

Verticillium dahliae Kleb., the causal agent of Verticillium wilts, is a devastating plant disease affecting many plant species. Fungus *V. dahliae* was detected in a partially artificially established *Acer pseudoplatanus* L. forest stand in central Slovenia. This finding incited further study about the risk of different sources of *V. dahliae* isolates for maples in forests and the pathogenicity of three *V. dahliae* isolates of different origins was tested on saplings of *A. pseudoplatanus*, *A. platanoides* L., and *A. campestre* L. The inoculated saplings exhibited disease symptoms, i.e., leaf necrosis and wilting. At the end of the pathogenicity test, typical xylem browning was visible on the cross-sections, and the pathogen was successfully re-isolated. The isolates showed significant differences in their pathogenicity to specific maple hosts, with the agricultural isolate (originated from bell pepper) being the most aggressive on all three maple species. The disease severity index (DSI) and relative area under the disease progress curve (rAUDPC), as well as the success of re-isolation, indicate that *A. platanoides* is the most susceptible to inoculation with *V. dahliae*. In addition, significant differences in sapling biomass were observed between treated and control plants. These results suggest that maples in forest stands are threatened by *V. dahliae*, and biosecurity measures should be considered and implemented in forest management to reduce the transmission and potential spread of the pathogen.

Keywords Verticillium wilt · *Acer* spp. · Pathogenicity test · Disease severity index (DSI) · Area under the disease progress curve (AUDPC) · Biosecurity

Introduction

Verticillium wilts are important vascular diseases of plants caused by soil-borne fungi from the genus *Verticillium*, which currently includes 10 plant pathogenic species (Inderbitzin et al. 2011). Among them, *Verticillium dahliae* Kleb. is a major pathogen causing severe economic losses by infecting numerous plant species worldwide (Neubauer et al. 2009). It has been detected on over 50 crop plants, as well as over 60 genera of woody and herbaceous ornamentals (Harris 1998a; Pegg and Brady 2002). Although disease symptoms vary among hosts, they typically include wilting, chlorosis, necrosis, vascular discoloration, and decline (Hiemstra 1998; Klosterman et al. 2009). Two distinct symptom complexes have been observed in woody plants infected with *V. dahliae*, with one being an acute/lethal form (apoplexy) and the other being a chronic form caused by mild pathotypes (slow decline) (Jiménez-Díaz et al. 2017; Keykhasaber et al. 2018b).

Maples (*Acer* spp.) are known for being susceptible to Verticillium wilt (Keykhasaber et al. 2018a), with Norway maple (*Acer platanoides* L.) being particularly vulnerable (Harris 1998a). While Verticillium wilt in maples has been widely reported in the USA, it is less frequent in northwestern Europe (Harris 1998b). Young trees, especially those in nurseries, are most affected by the disease (Bedwell and Childs 1938; Chandelier et al. 2003; Neubauer et al. 2009; Goud et al. 2011). While maple trees often experience severe dieback, ash trees are able to completely recover from Verticillium wilt (Hiemstra 1998). Some species, such as ash, olive, and stone fruit, may limit the spread of wilt

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from one year's xylem growth to the next because of their highly compartmentalised structure and production of secondary xylem (Shigo and Marx 1977; Keykhasaber et al. 2018b). The severity of the disease in a given year is primarily determined by the incidence and development of new root infections during that year (Hiemstra 1998). If new root infections do not occur, infected trees may simply outgrow the disease due to the limited radial spread ability of *V. dahliae*, as noted above (Sinclair et al. 1981).

Verticillium wilt is controlled by a combination of measures, such as using resistant cultivars, reducing soil-borne inoculum through crop rotation, and selecting planting sites free of *V. dahliae* (Neubauer et al. 2009). More recent methods include biocontrol with antagonistic bacterial strains, which have been demonstrated to be quite effective in a study on potatoes in Canada (El Hadrami et al. 2011). These measures primarily relate to agriculture, while they are usually more difficult to implement in forests. However, the challenge with *V. dahliae* is that even after infected plants are removed, the fungus can persist in the soil as microsclerotia formed in the tissue of dying infected plants (Schnathorst 1981). These microsclerotia can survive in the soil for up to 14 years, waiting for favourable conditions (Wilhelm 1955). They germinate in response to root exudates, the hyphae invade the roots, and the fungus grows into the xylem (Harris 1998a). Once the fungus has entered the vessels, it begins producing conidia that are distributed throughout the tree with the flow of xylem fluid, resulting in systemic colonisation of the upper parts of infected plants, which impedes water flow through the xylem and leads to the development of water stress symptoms (Baidez et al. 2007; Keykhasaber et al. 2018a). In perennial species such as maple, large amounts of inoculum can be released when leaves containing microsclerotia and hyphae are shed from diseased trees. Detached leaves can be carried by the wind, spreading the disease over long distances (Hiemstra 1998).

In 2017, an extensive outbreak of wilt disease was reported in a sycamore maple (*Acer pseudoplatanus* L.) forest stand in central Slovenia. The initial mycological analyses revealed the presence of *V. dahliae*, among other endophytes and saprophytic fungi. As maples are an important tree species in Slovenia, and this was the first reported large-scale outbreak in forest stands in the country, the objectives of this study were threefold: (i) to carry out a comprehensive analysis of the outbreak, (ii) to determine the pathogenicity and virulence of *V. dahliae* isolates, and (iii) to assess the risk of *V. dahliae* spreading to other forest stands.

Materials and methods

Outbreak analysis

In 2017 an extensive wilting of sycamore maple was observed in a partially artificially established sycamore maple stand (approximately 0.17 ha in size) located adjacent to an agricultural landscape (45.8763° N, 14.8476° E, 329 m a.s.l.) with non-intensive, private fields of various crops. The report was received from the local forester, who explained that the stand was partially artificially established once between 1998 and 2009 with maple saplings sourced from a nearby forest stand (about 10 km apart). The symptoms of wilting had already been observed previously but were initially misidentified as being the consequence of mechanical damage. A visual inspection of the stand was carried out at the time of the first and the second sampling, while an assessment of the percentage of damaged trees was carried out only at the time of the second sampling in 2019.

Plant material, fungal isolation, and molecular identification

The forest stand described above was visited on two occasions, in October 2018 and May 2019, with one and three symptomatic trunk samples collected from four sycamore maple trees for further analyses, respectively. After surface sterilisation, the samples were halved and cut into smaller subsamples using sterilised equipment. Fungal isolations were made from wood that was evenly plated on 3.9% (w/v) potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, USA) and incubated at 24 °C. In the second sampling, a total of 80 wood pieces from four subsamples, obtained from three symptomatic trunk samples (three sycamore maple trees), were cultured. Based on visual and growth characteristics, cultures were grouped into morphotypes. In addition, macroscopic observation of the samples under the Olympus SZX16 stereomicroscope (Olympus, Tokyo, Japan) revealed the presence of *Fusarium*-like fruiting bodies (one subsample), which were also plated on PDA. Genomic DNA was extracted from all obtained morphotypes. Mycelium was scraped from the PDA plates and DNA was extracted using a NucleoSpin® Plant II (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions, after homogenising the fungal material with a Lysing Matrix A tube (MP Biomedicals, Solon, OH, USA) using a Precellys Evolution device (Bertin Technologies, Montigny-le-Bretonneux, France). The preliminary characterisation of isolates was done based on the ITS rDNA region, which was amplified and sequenced using primer pairs ITS1 and ITS4 (White et al. 1990). For isolates from *Fusarium* genus, additional marker was used for

species characterisation (translation elongation factor 1, *tef*1), amplified and sequenced using primer pair EF1 and EF2 as described by Crous et al. (2021). For isolates from *Verticillium* genus, part of the calmodulin gene (*cal*) was amplified and sequenced using primer pair CAL-228 F and CAL-737R (EPPO 2021). All obtained PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA) kit according to the manufacturer's protocol and sequenced at the DNA sequencing facility of Eurofins Genomics (Köln, Germany) in both directions using Sanger sequencing. Sequences were visualised and manually edited using Geneious Prime® version 2022.2.2 (Biomatters Ltd., Auckland, New Zealand) following the principles of consensus sequence preparation described in the diagnostic protocol of EPPO (2021). Consensus sequences were preliminary compared to the GenBank nr/nt database using BLASTn and for the final identification compared to reference sequences (see Supplemental 1). Identity of *V. dahliae* isolates was additionally confirmed by conventional PCR, using species specific primers Df and Dr as described by Inderbitzin et al. (2013) and the diagnostic protocol of EPPO (2021).

Pathogenicity assay

In Slovenia, Verticillium wilt has not been reported to cause damage to forest stands. Since there is known variability in *V. dahliae* pathogenicity and virulence among different strains, our study addressed the question of pathogenicity and aggressiveness of *V. dahliae* isolates, obtained from the maple stand (see 2.1). The pathogenicity of two representative *V. dahliae* isolates, ZLVG 774 and ZLVG 909, isolated from the studied forest stand, was evaluated on two-year-old potted saplings of sycamore, Norway, and field maple (*Acer campestre* L.). In addition, a *V. dahliae* isolate highly aggressive to bell pepper (*Capsicum annuum*) (isolate designation: PAP-19, culture collection of the Slovenian Institute of Hop Research and Brewing– IHPS; GenBank accession Nos. OP536145 and OR353421) was included to assess its potential risk to maples. The maple plants were inoculated using the root-dip method (Flajšman et al. 2017), with the inoculum prepared by growing the isolate cultures on PDA for 14 days in the dark at room temperature. The cultures were rinsed with sterile distilled water and the conidial suspension was adjusted to 5×10^6 conidia/ml using a Thoma counting chamber. Eight saplings of each maple species were inoculated with each of the three *V. dahliae* isolates, and six saplings of each maple species were used as controls, except Norway maple with 5 control saplings. One block corresponds to one maple species with four treatments (i.e., inoculation with three isolates and control) that were spatially separated (1 m). The blocks (i.e., the different maple species) were placed at a distance of 2 m from each other and kept on trays to collect the drainage water. After inoculation, the plants were repotted into fresh commercial substrate (Gramoflor S04-2004 Topf/Pikier + TonXL + Fe) in 2 L pots and grown for 14 days in a Kambič RK-13,300 growth chamber (Kambič, Semič, Slovenia). Growth conditions were a 12-h photoperiod of fluorescent light (L 58 W/77; Fluora, Osram) at a temperature of 22 °C and relative humidity of 65% during the light period and 18 °C and 70% during the dark period. After 14 days, the plants were transferred from the growth chamber to the experimental plot at the IHPS research station, where they were maintained in pots under field conditions with the same positioning as in growth chamber.

The first observed leaf yellowing was the starting point for symptom scoring for all plants, which was conducted visually at 6- week intervals on a scale of 0 to 5, where 0 = no leaf symptoms, 1 = 1 to 20%, 2 = 21 to 40%, 3 = 41 to 60%, 4 = 61 to 80%, and 5 = 81 to 100% wilted leaf area.

Disease severity was assessed for each plant by a disease severity index (DSI), calculated according to the formula of Jakše et al. (2013). The DSI was calculated as the mean wilt score of the infected plants at each assessment time point. The area under the disease progress curve (AUDPC) was calculated for each tested isolate and maple species from average disease indices (Simko and Piepho 2012):

$$AUDPC = \sum_{i}^{n-1} \frac{(y_i + y_{i+1})}{2 \times (t_{i+1} - t_i)}$$

where n represents the total number of observations, y represents the DSI index for each plant, and t represents the number of days post inoculation. The AUDPC was also expressed as a relative value (Simko and Piepho 2012):

rAUDPC = actual AUDPC value/maximum potential AUDPC value.

To identify differences in average rAUDPC values between different isolates of *V. dahliae*, a non-parametric Kruskal-Wallis test was used. This was followed by a post-hoc multiple comparison Dunn test with Bonferroni correction. The homogeneity of variances was tested using the Levene test.

At the last visual assessment (i.e., 4 months post inoculation), plant biomass was determined by measuring above-ground height (from the base) and total fresh weight. Prior to weighing, plants were removed from the pots and the soil was removed from the roots by gentle washing to remove all soil particles. The plants were air dried on paper towels and then weighed using a Mettler-Toledo Mono Block PB3002S balance (Mettler Toledo, Columbus, OH, USA).

To re-isolate the pathogen, four xylem parts per plant were taken from the base of the stem and placed on PDA

with antibiotics (streptomycin sulphate, neomycin, chloramphenicol; 100 mg/ml each). During the re-isolation process, the presence or absence of vascular discoloration on inoculated plants was recorded. After seven days of incubation at room temperature in the dark, the growing cultures were examined by light microscopy. A plant was confirmed positive for *V. dahliae* if the pathogen grew out of at least one xylem sample on the plate.

To compare height and mass values between control and inoculated saplings, a non-parametric Kruskal-Wallis test was conducted, followed by a post-hoc multiple comparison Dunn test with Bonferroni correction. The homogeneity of variances was tested using the Levene test. Additionally, the Pearson correlation coefficient was calculated to investigate the relationship between successful re-isolation and the presence of browning in cross-sections. All calculations, graphs, and statistical analyses were performed using Microsoft Excel version 1908 and the R software environment for statistical computing (R Core Team 2019) with "car" (Fox and Weisberg 2019), "DescTools" (Signorell et al. 2019), "dplyr" (Wickham et al. 2019), and "ggplot2" (Wickham 2009) packages.

Results

Outbreak analysis

We estimate that 60% of the trees in the observed forest stand, which was about 0.17 ha in size, were affected at the time of the second sampling in 2019. Affected trees exhibited cracks in the bark, white (fresh) to black (dried) exudate, wilting, extensive necrosis, and white mycelium on the bark. A distinct colour change of the wood (brown-yellow with green margins) was noted on a cross-section (see Supplemental 2a), and the samples had a characteristic fish-like odour. Based on callus formation, we estimate that the first cracks appeared three years prior to sampling. However, there was also some fresh damage from the same year.

Fungal identification

Isolations from the wood and fruiting bodies, and subsequent molecular identification of obtained fungal isolates revealed the presence of six different fungal species: *Biatriospora* sp. K.D. Hyde & Borse, *Cladosporium* sp. Link, *Eutypa maura* (Fr.) Fuckel, *Fusarium sambucinum* Fuckel, *Peroneutypa scoparia* (Schwein.) Carmarán & A.I. Romero, and *Verticillium dahliae* (see Supplemental 1). Because *V. dahliae* was the most frequently isolated species and due to its known pathogenicity of numerous plant species, including maples, *V. dahliae* isolates were selected for further analysis. Representative cultures of *V. dahliae* were deposited in the culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute (accession Nos. ZLVG 774 and ZLVG 909) and associated ITS rDNA and calmodulin sequences were deposited in the GenBank database (accession Nos. OP536143, OP536144, OR353420, and OR353422).

Pathogenicity assay

All *V. dahliae* isolates, included in our pathogenicity assay, caused disease symptoms in all tested maple species, but to varying degrees and at different time points after inoculation. Symptoms included leaf yellowing and necrosis, slowed growth, and leaf defoliation. The first symptoms clearly caused by *V. dahliae* were observed two months after inoculation (i.e., during the first visual assessment) with the isolate from bell pepper (PAP-19) on sycamore and Norway maple. The other two isolates (ZLVG 774 and ZLVG 909) showed almost no symptoms at this time on either maple species (only one Norway maple sapling inoculated with ZLVG 774 was assessed with score 1, i.e., 1 to 20% of the leaf area was wilted). By the second assessment three months after inoculation, a slightly higher percentage of inoculated saplings showed mild symptoms of wilt disease (Table 1). By the final assessment four months after inoculation, symptoms were generally more severe, especially in plants inoculated with isolate PAP-19, which showed the highest levels of disease symptoms (Table 1, see Supplemental 2b). At the end of the pathogenicity test, only two field maple plants inoculated with isolate ZLVG 909 remained symptom-free. Only one Norway maple plant inoculated with isolate PAP-19 was assessed as having the worst score of 5. All control samples remained symptomless, without any wilting symptoms, at 2- and 3-months post inoculation. At the final assessment, up to 20% of the leaf area was wilted in all three control maple species (Table 1).

At the end of the pathogenicity test, 75% the saplings of each maple species inoculated with isolate ZLVG 774 exhibited vascular discoloration. All field maple saplings inoculated with isolates ZLVG 909 and PAP-19 showed brown discoloration when viewed in cross-section. Sycamore maple plants inoculated with isolates ZLVG 909 and PAP-19 also exhibited discoloration in 87.5% of cases (Fig. 1). In Norway maple, 87.5% of plants inoculated with ZLVG 909 and 100% of those inoculated with PAP-19 showed browning. The pathogen was re-isolated from inoculated plants at varying levels, but never from plants without vascular discoloration (Table 2). Control plants showed no vascular discoloration and no cultures of *V. dahliae* were obtained from re-isolations.

Table 1 Percentage of symptomatic maples at different time points after inoculation with different *V. dahliae* isolates

Isolate		ZLVG 774			ZLVG 909			PAP-19			Control		
Maple species	DS[a]	2 mpi[b]	3 mpi	4 mpi	2 mpi	3 mpi	4 mpi	2 mpi	3 mpi	4 mpi	2 mpi	3 mpi	4 mpi
Sycamore maple	0	100	25	37.5	100	37.5	37.5	37.5	25		100	100	66.7
	1		75	50		50	25	37.5	62.5	37.5			33.3
	2			12.5		12.5	37.5	12.5		25			
	3									25			
	4												
	5												
Norway maple	0	87.5	25		100	12.5	37.5	25	12.5		100	100	
	1	12.5	50	37.5		75	25	25	12.5	12.5			80
	2		25	62.5		12.5	37.5	25	75				
	3							25		25			
	4									50			
	5									12.5			
Field maple	0	100	50	75	100	37.5	25	37.5	37.5	12.5	100	100	50
	1		50	25		62.5	50	50	37.5	75			50
	2						25	12.5	25	12.5			
	3												
	4												
	5												

[a] DS, disease symptoms rated on a scale of 0 to 5 according to the proportion of foliage affected by wilt symptoms (0 = no leaf symptoms, 1 = 1 to 20% leaf area wilted, 2 = 21 to 40% leaf area wilted, 3 = 41 to 60% leaf area wilted, 4 = 61 to 80% leaf area wilted, and 5 = 81 to 100% leaf area wilted). [b] mpi, months post inoculation

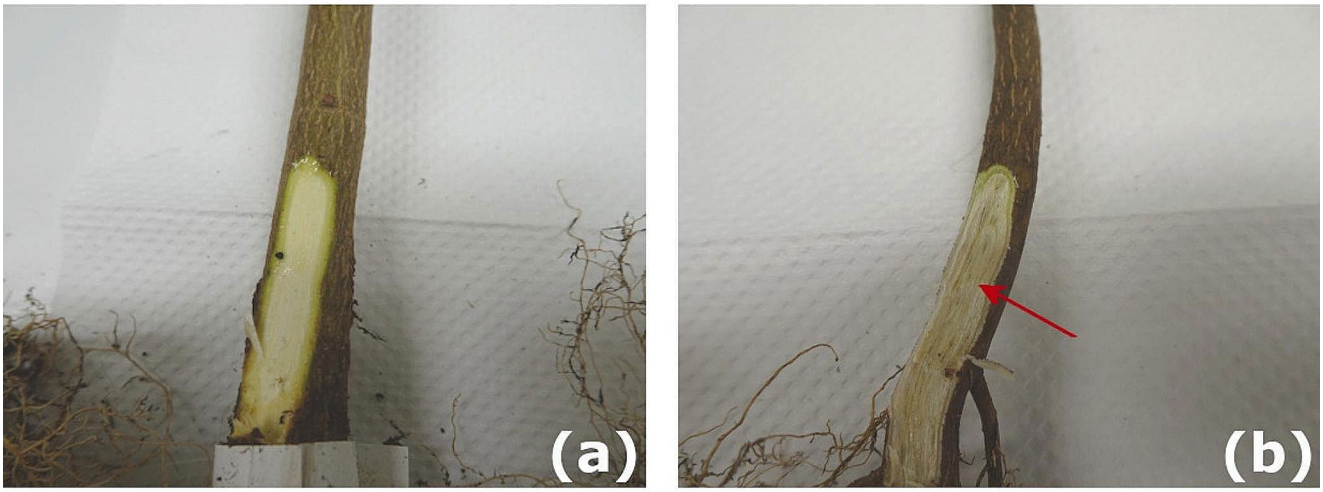

Fig. 1 Sycamore maple control (**a**) and inoculated sapling (isolate PAP-19) with evident vascular discoloration (**b**)– red arrow

Table 2 Re-isolation success (%) of different *V. dahliae* isolates on three maple species

Maple species	Sycamore maple		Norway maple		Field maple	
Isolate	RS 1[a]	RS 2[b]	RS 1[a]	RS 2[b]	RS 1[a]	RS 2[b]
ZLVG 774	37.5	50.0	50.0	66.7	25.0	33.3
ZLVG 909	50.0	57.1	50.0	57.1	50.0	50.0
PAP-19	87.5	100.0	87.5	87.5	87.5	87.5

[a] RS 1– Overall re-isolation success. [b] RS 2– Re-isolation success in connection with vascular discoloration. Only plants with vascular discoloration were included in the calculation of re-isolation success

We found a highly significant ($p < 0.001$) positive correlation between re-isolation success and vascular discoloration in the inoculated plants ($r = 0.54$).

Of the inoculated saplings, seven (two sycamore maples inoculated with ZLVG 774, one sycamore maple inoculated with ZLVG 909, one Norway maple inoculated with ZLVG 774, one Norway maple inoculated with ZLVG 909, and two field maples inoculated with ZLVG 774) showed no vascular discoloration at the end of the pathogenicity test and were thus excluded from further DSI and AUDPC analysis. The same approach was applied to the controls.

The mean DSI of the inoculated plants was similar across all three maple species when they were inoculated with the same isolate (Table 3). However, the most striking differences in DSI values were observed when the plants were inoculated with isolate PAP-19, with Norway maple showing the highest average DSI values.

The rAUDPC values were highest when the plants were inoculated with isolate PAP-19. Consistent with average DSI values, rAUDPC values decreased from Norway to sycamore and field maple. The values for ZLVG 774 and ZLVG 909 were statistically similar. The mean rAUDPC values differed significantly ($p < 0.001$) between isolate PAP-19 on Norway maple and isolates ZLVG 774 and ZLVG 909 on field maple, and isolate ZLVG 744 on Norway maple ($p < 0.05$) (Fig. 2).

Differences in height and weight of the saplings were significantly different between treated and control plants (Figs. 3 and 4).

Discussion

Bark cracks, necrosis, exudate, and wilting have been observed in a partially artificially established sycamore maple stand in central Slovenia. Isolations from the wood and fruiting bodies revealed six different fungal species, which are known endophytes and saprophytes (*Biatriospora* sp., *Cladosporium* sp., *E. maura*, *P. scoparia*) (Unterseher et al. 2005; Vasilyeva and Stephenson 2006; Bensch et al. 2012; de Errasti et al. 2014; Kolarik et al. 2017) or pathogens (*F. sambucinum*, *V. dahliae*) (Phillips and Burdekin 1992; Halász 2002; Yikilmazsoy and Tosun 2021). *Biatriospora* is a genus of endophytes of terrestrial and marine-associated plants in temperate and tropical forest (Kolarik et al. 2017). *Cladosporium* is a genus of cosmopolitan fungi commonly found on senescent and dead plant material, with some species being plant-specific pathogens (Bensch et al. 2012). *Eutypa maura* is a typical saprophytic fungus commonly found on the dead twigs and branches of sycamore maple (Unterseher et al. 2005). *Peroneutypa scoparia* is associated with the dead branches of various tree species

Table 3 The mean DSI values of the inoculated and control plants after 60, 101, and 140 days post inoculation

Isolate[a]	ZLVG 774			ZLVG 909			PAP-19			Control		
Maple species/ DPI[b]	60	101	140	60	101	140	60	101	140	60	101	140
Sycamore maple	0.00 (0.00)	1.00 (0.00)	1.83 (0.75)	0.00 (0.00)	0.86 (0.69)	2.00 (1.00)	0.75 (1.07)	1.50 (0.49)	2.50 (0.90)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Norway maple	0.14 (0.38)	0.86 (0.69)	1.57 (0.53)	0.00 (0.00)	1.00 (0.58)	2.14 (0.90)	1.50 (1.20)	2.63 (0.74)	3.50 (1.20)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Field maple	0.00 (0.00)	0.33 (0.52)	1.17 (0.41)	0.00 (0.00)	0.63 (0.52)	1.00 (0.76)	0.75 (0.71)	0.88 (0.83)	2.00 (0.53)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Average	0.05 (0.08)	0.73 (0.35)	1.52 (0.34)	0.00 (0.00)	0.83 (0.19)	1.71 (0.62)	1.00 (0.43)	1.67 (0.89)	2.67 (0.76)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

[a] Average values of DSI with standard deviation in brackets. [b] DPI– days post inoculation

(e.g., elm, black locust, rowan) (Vasilyeva and Stephenson 2006) and is also found as an endophyte in healthy tissues (de Errasti et al. 2014). *Fusarium sambucinum* and *V. dahliae* are the primary pathogens of various plant species and are also suspected to be involved in the symptoms observed in our forest stand case. *Fusarium sambucinum* is a known plant pathogen of potato (Yikilmazsoy and Tosun 2021) and woody hosts (Phillips and Burdekin 1992; Halász 2002). However, we found no information on the pathogenicity of *F. sambucinum* on sycamore maple during a literature search. Testing *F. sambucinum* was out of the scope of this paper but should be conducted in another study as it is possible that the fungus could cause disease despite the lack of literature on the subject. *Verticillium dahliae*, the causal agent of Verticillium wilt, on the other hand, infects numerous herbaceous and woody plant species worldwide, with maple, elm, beech, horse chestnut, and linden being particularly sensitive tree species (Hiemstra 1998). Observed symptoms (wilting and colour change of the wood) are also frequently reported as typical symptoms of Verticillium wilt (Hiemstra 1998), but *V. dahliae* infections can also predispose infected maples to frost damages (May 1961), resulting in bark cracks and oozing sap. The observed symptoms had been known at this forest stand for a longer time and are most likely a result of various damaging agent involved (e.g., frost, saprophytic and endophytic fungi).

In Slovenia, Verticillium wilt is frequent in nurseries (Maček 2008), but has not been reported to cause damages in forest stands.

Fungus *Verticillium dahliae* is known for its high aggressiveness and potential transmission from agricultural land. The studied forest stand, which borders agricultural land, raised concerns about potential biosecurity risks due to the past deposition of material from the fields, including damaged or low-quality potatoes, which could represent a possible transmission pathway from agricultural land into the studied forest stand (Hiemstra and Harris 1998).

In our study, three isolates of *V. dahliae* were assessed for their pathogenicity and virulence on the three most widespread European maple species: sycamore, Norway, and field maple. The study showed that all three *V. dahliae* isolates of different origins (sycamore maple and bell pepper) could cause Verticillium wilt symptoms in the saplings of different maple species. However, there was significant variability in pathogenicity between the tested isolates of *V. dahliae*. Isolate PAP-19 proved to be highly aggressive on all three maple species, while isolates ZLVG 774 and ZLVG 909 were less aggressive. These two isolates were isolated from the same stand at different times and likely represent the same population of *V. dahliae*. Variability among *V. dahliae* isolates has also been reported in other

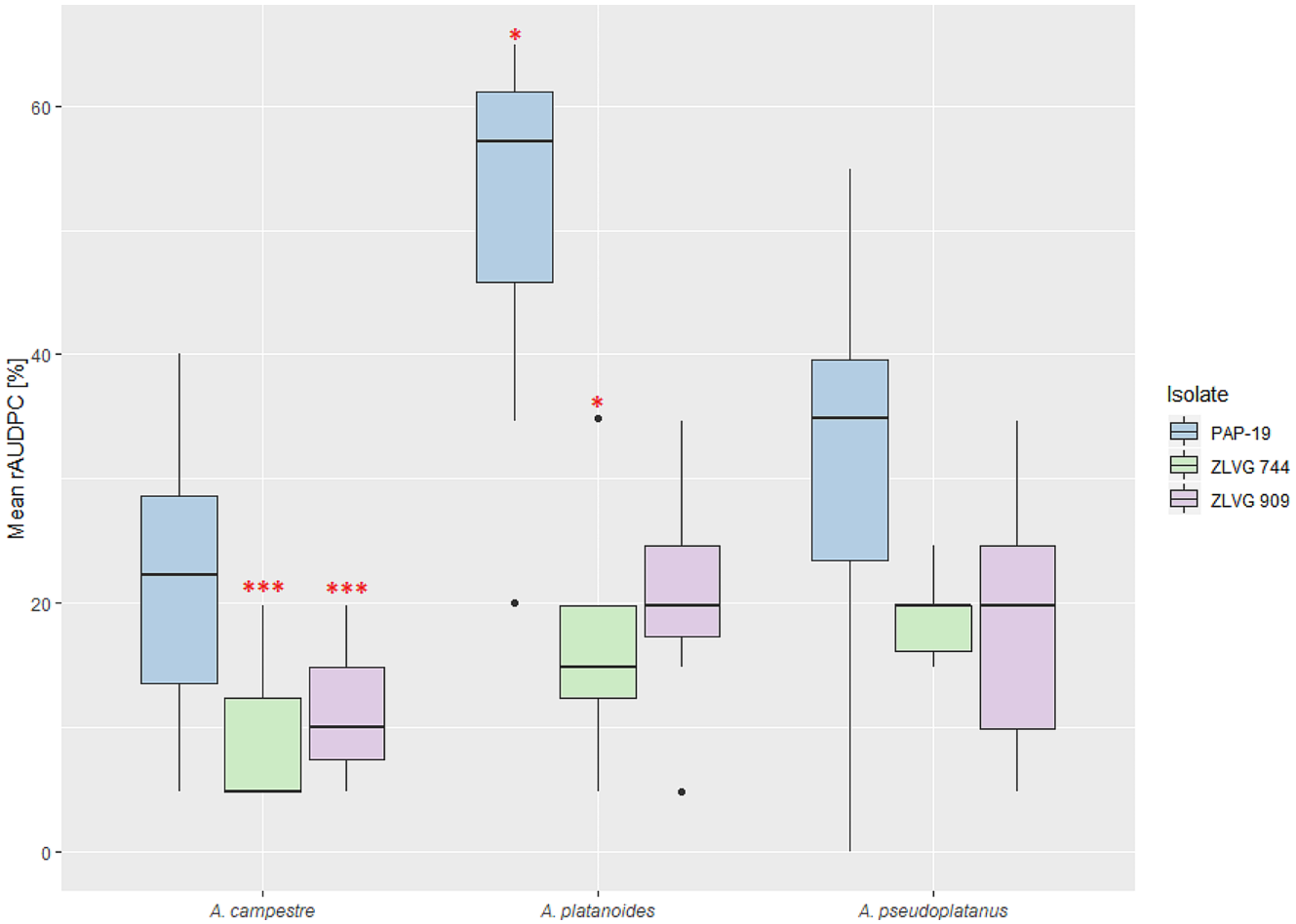

Fig. 2 Mean rAUDPC values of three *V. dahliae* isolates on three maple species. Boxplot represents minimum, first quartile (Q1), median, third quartile (Q3), and maximum value. Asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

studies (Goud and Termorshuizen 2002; Chandelier et al. 2003; Neubauer et al. 2009).

Verticillium dahliae was successfully re-isolated from all three artificially inoculated maple species four months after inoculation. Our results align with other reports indicating the ability of various isolates of *V. dahliae* to cause Verticillium wilt in *Acer* species (Goud and Termorshuizen 2002). Many isolates of the fungus have a broad host range but may differ in their ability to cause disease in specific hosts (Barbara et al. 1998). Typically, isolates are more pathogenic to the host from which they were isolated than to other hosts (Adams and Tattar 1976; Barbara et al. 1998; Goud and Termorshuizen 2002), but surprisingly this was not confirmed in our study. Wilt symptoms were most strongly expressed on Norway maple saplings (not on sycamore maples, where they were primarily isolated from), and the most aggressive isolate in our pathogenicity test proved to be the isolate obtained primarily from pepper. Differences in susceptibility of maples may be related to differences in nutrient availability and the presence of inhibitors in the sap (Regulski and Peterson 1983).

The highest disease severity index (DSI) value recorded in the study was 3.5 for the *V. dahliae* isolate PAP-19 on Norway maple, which is much lower than the DSI values reported by Goud and Termorshuizen (2002) for field maple after 200 days post inoculation with *V. dahliae* isolates derived from different origins (potato, ash, Norway maple, red currant, blackberry, and lilac). Although, disease developed slowly in field, Norway, and sycamore maple of a referenced study, high disease scores (of nearly 5) were eventually observed for the most virulent isolates (Goud and Termorshuizen 2002). In our experiment, disease symptoms were assessed only three times, making it difficult to determine the exact course of disease development. On the other hand, Goud et al. (2011) reported much lower average DSI values (1.6) of *V. dahliae* (isolated from potato) on Norway maple after one growing season, but a direct comparison of both studies is not appropriate because of differences in the methodology of the pathogenicity test (different isolates, duration, weather conditions, etc.).

To evaluate the quantitative disease resistance of *Acer* species to *V. dahliae*, we used the rAUDPC. As explained

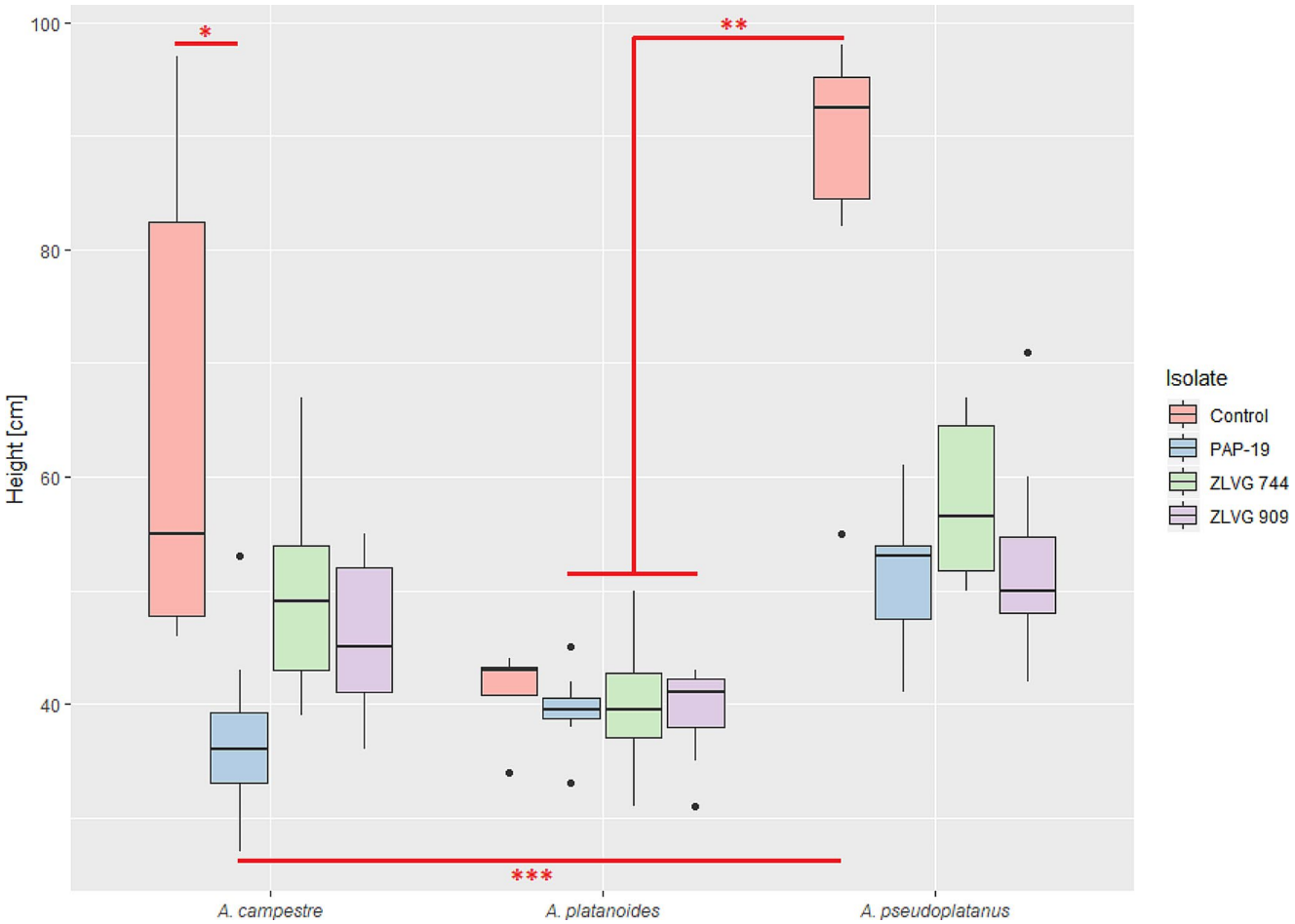

Fig. 3 Assessed height of the saplings inoculated with three *V. dahliae* isolates on three maple species at the end of the experiment. Boxplot represents minimum, first quartile (Q1), median, third quartile (Q3), and maximum value. Asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

by Jeger et al. (2018), estimating the AUDPC from two data points provides an equivalent amount of information as from repeated assessments. Consistent with the DSI values, the highest rAUDPC values were observed in Norway maple (51%) and sycamore maple (31%) inoculated with isolate PAP-19.

The calculation of DSI and rAUDPC, as well as the success of re-isolation, indicate that in our study Norway maple was the most susceptible species to inoculation with *V. dahliae*, while sycamore and field maple were less susceptible. In agreement with Goud and Termorshuizen (2002), acute wilt, necrosis, and defoliation of inoculated maple saplings were noted. However, it is challenging to compare our results with those of other studies as most focus exclusively on individual maple species (Valentine et al. 1981; Chambers and Harris 1997; Chandelier et al. 2003; Keykhasaber et al. 2018a). Overall, the calculated values and physical observations indicate that *V. dahliae* has the potential to cause significant damage on the selected maple species.

At the end of the pathogenicity test, there were significant differences in height and weight between inoculated and control plants. In our case, the DSI values did not reach high numbers, indicating that the symptoms on the leaves were not as pronounced as the lower biomass growth. It would be interesting to observe the inoculated plants in the following months or years to possibly detect the recovery of the saplings, since Goud et al. (2011) found that 67% of Norway maple trees recovered from Verticillium wilt disease in the following season. But, again, recovered trees showed higher probability of becoming diseased again (Goud et al. 2011).

In our study, the "agricultural" isolate (isolated from pepper) was found to be significantly more virulent than two "forestry" isolates (isolated from sycamore maple). However, it should be noted that the origin of these two "forestry" *V. dahliae* isolates is unknown, and it is possible that they could have originated from surrounding agricultural land. *Verticillium dahliae* is easily spread through infected planting material, associated soil, and field harvest waste (EPPO 2020), making it essential to be mindful of potential

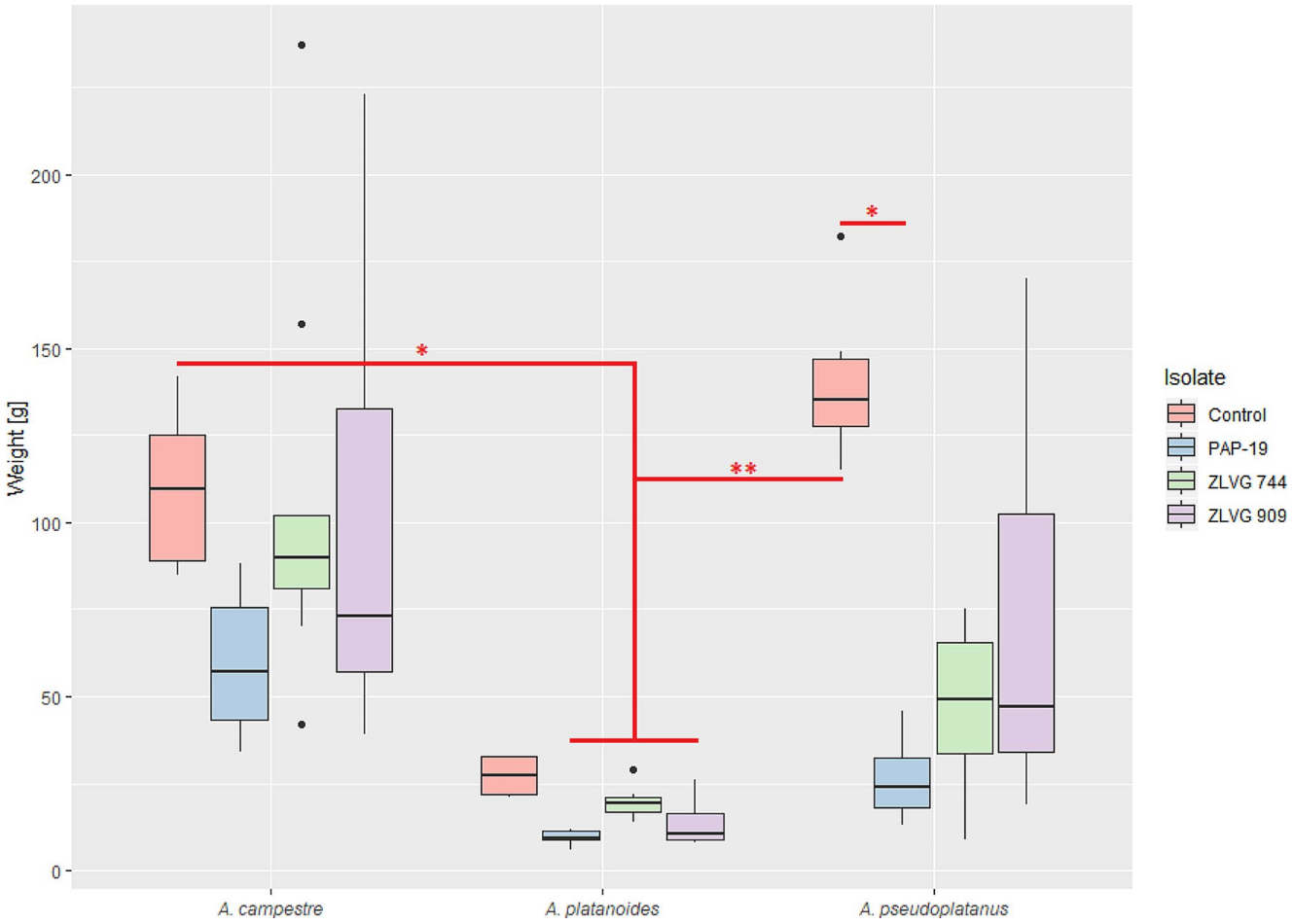

Fig. 4 Assessed weight of the saplings inoculated with three *V. dahliae* isolates on three maple species at the end of the experiment. Boxplot represents minimum, first quartile (Q1), median, third quartile (Q3), and maximum value. Asterisks indicate statistically significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

spread mechanisms and to avoid high-risk practices. It is crucial to emphasise the importance of properly disposing of potentially infected green waste and soil, and disinfecting vehicles, tools, and footwear after visiting sites that may be contaminated with *V. dahliae* or any other harmful organisms (FAO 2011; Hwang et al. 2017). To prevent or limit their spread at the local level, raising awareness of basic biosecurity measures among professional services, landowners, and all visitors to natural areas is crucial. Biosecurity refers to a range of measures aimed at reducing or eliminating the transfer of harmful organisms from one place to another (Forestry Commission 2012). For example, the United Kingdom has launched a comprehensive *Keep it Clean* campaign (https://www.gov.uk/guidance/prevent-the-introduction-and-spread-of-tree-pests-and-diseases) to raise awareness about biosecurity, which could be adapted and implemented in Slovenia. Given the unrestricted access to Slovenian forests allowed by national law, the proximity to agricultural land, the extensive network of forest roads, and the recent increase in forest visits particularly during the COVID-19 pandemic (Japelj 2020), the potential for the rapid spread of harmful organisms is increasing. It is also important to note that stands of trees artificially established on former agricultural land are at a higher risk of developing Verticillium wilt. Since *V. dahliae* is not regulated on hosts other than hops (European Commission 2019), there is no systematic monitoring to detect its presence in woody hosts in Slovenia. As *Verticillium* species are extremely polyphagous, they could pose a threat to many native species in our forests if they go unnoticed.

Wilt control is challenging, and the disease is best avoided by planting in soils with little or no wilt risk (Chambers and Harris 1997). Soils can be tested in advance to determine the level of risk (Harris 1998b). Utilising host resistance is considered the most effective approach to wilt control (Chambers and Harris 1997; Harris 1998b). Certain species, such as *Fagus*, *Quercus*, *Pinus*, *Abies*, and *Picea* species, are naturally resistant to Verticillium wilt (McCain et al. 1981). Additionally, resistance to *V. dahliae* has also been found in saplings of some maples, including Norway (Valentine

et al. 1981), boxelder (*A. negundo* L.) (Regulski and Peterson 1983), and red maple (*A. rubrum* L.) (Townsend and Hock 1973), nevertheless in the light of differences among different *V. dahliae* isolates as shown also in our study, the debate about the resistance of maples remains open. It is also crucial to emphasise the importance of proper forest management and forest regeneration in case of *V. dahliae* or other harmful organism outbreaks. Diseased trees should be promptly removed from forests to prevent further spread of the disease. Forest management should focus on promoting diverse stands and planting healthy seedlings. Diverse stands are more stable and resilient to pathogens such as *V. dahliae* and better able to withstand future disturbances (Guyot et al. 2016; Roberts et al. 2020). Artificial forest regeneration should begin with tested and healthy seedlings from nurseries that follow good biosecurity practices. Furthermore, to keep our forests healthy and functional, it is crucial to educate forest and agricultural owners that forests should not be used for disposing of agricultural and other waste.

Conclusion

A case study of an outbreak and pathogenicity on maple species revealed the relative susceptibility of *Acer* spp. to Verticillium wilt caused by *V. dahliae*. Of the maple species tested, Norway maple was found to be the most susceptible to infection and development of the disease. Statistically significant differences were observed between the pathogenicity of the three isolates of *V. dahliae* used in the study, with agricultural isolate proving to be the most aggressive on all three maple species, indicating that poor agricultural practices may also pose a threat to forest health. Furthermore, significant differences in the biomass of saplings were observed between treated and control plants, implying that maples in forest stands are at risk of infection with *V. dahliae*. These findings underscore the importance of implementing simple biosecurity measures to prevent or reduce further spread of the disease in forests.

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Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no conflict of interest (financial or non-financial).

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