Effects of *Verticillium nonalfalfae* on *Ailanthus altissima* and associated indigenous and invasive tree species in eastern Austria

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Received: 28 July 2017 / Revised: 6 November 2017 / Accepted: 9 January 2018 / Published online: 26 February 2018

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Abstract

The naturally occurring *Verticillium nonalfalfae* shows promise for biocontrol of the highly invasive Tree of Heaven (*Ailanthus altissima*), but might also bear a risk for non-target tree species. In this study, we conducted inoculations on potted seedlings of *A. altissima* as well as on eight indigenous and two invasive tree species associated with Tree of Heaven in Austria. Although vascular discolourations developed in all inoculated tree species, *V. nonalfalfae* was reisolated from *Ailanthus* and eight of the ten non-target-species, whereas typical disease symptoms and mortality only occurred on *A. altissima*. Results confirmed high susceptibility (*S*) of *A. altissima* to *V. nonalfalfae* but indicated tolerance (*T*) of *Acer campestre*, *Acer pseudoplatanus* and *Quercus robur*, possible resistance (*PR*) of *Fraxinus excelsior*, *Populus nigra*, *Tilia cordata*, *Ulmus laevis* and *Ulmus minor* and resistance (*R*) of *Fraxinus pennsylvanica* and *Robinia pseudoacacia* to this potential biocontrol agent. Results from seedling inoculations were confirmed by cursory field observations in *Ailanthus*-inoculated forest stands, where admixed *A. campestre*, *A. pseudoplatanus*, *F. excelsior*, *Populus alba*, *R. pseudoacacia* and *U. laevis* canopy trees remained asymptomatic, while mortality was induced in *Ailanthus*.

Keywords Tree of Heaven · *Ailanthus altissima* · *Verticillium nonalfalfae* · Host range · Biological control · Non-target-effects

Introduction

According to the European Commission, the annually EU-wide costs caused by invasive alien species (due to the costs for health care, animal health, crop yield losses, damage to infrastructure and the navigability of rivers, and damage to protected species) are estimated to sum up to EUR 12 billion per year (European Commission 2014). For that reason, the European Commission adopted a list that defines restrictions on keeping, importing, selling, breeding and growing of 37 invasive alien species of Union concern (European Commission 2016). Furthermore, measures for early detection, rapid eradication and/or management of these species have to be undertaken that also include the obligation of landowners to actively control and eliminate these species. This is either a costly obligation or—based on the current possibilities of a successful regulatory response—simply impossible. Thus, the current list is a compromise and does not comprise all ecologically relevant species, which are undoubtedly defined as being invasive in Central Europe (Essl and Rabitsch 2002, 2004; Kleinbauer et al. 2010; BfN 2013; Info Flora 2014; LBV 2017).

One of these unlisted species and a major woody pest in Austria is *Ailanthus altissima* (Mill.) Swingle 1916 (Simaroubaceae)—also known as Tree of Heaven and henceforth mentioned as *Ailanthus*. The tree was introduced into Austria in the late eighteenth century (Märter 1796; Borkhausen 1803) and established rapidly as promenade, ornamental and shade tree during the nineteenth century (Neireich 1846, 1866; Wessely 1871). It was further cultivated as pulp wood (Härtel 1955; Schwarz 1955a, b), in windshields (Adamik 1955), in welfare afforestations (Schimitschek 1952) but also as food source for honeybees (Gölles 1955). Widespread
dissemination of *Ailanthus* was further promoted during the twentieth century due to the colonization of sites which were opened by bombs during World War II, as well as the colonization of derelict urban sites and railway areas, or fallow land (Punz et al. 1998, 2004; Adler and Mrkvicka 2003). *Ailanthus* is a fast growing, highly invasive tree species with low requirements on soil quality and climate. Due to its reproduction by root suckers, its fast and copious fructification and its allelopathic properties (Hu 1979; Kowarik and Säumel 2007; Cáceres 2010; Wickert et al. 2017), *Ailanthus* alters natural tree species composition in riparian forests or on dry gravel sites (Kowarik and Böcker 1984; Gutte et al. 1987; Kowarik and Säumel 2007, 2008; Wickert et al. 2017). It has therefore become a major problem in the warmer climate regions of Austria during the last 25 years. Besides *Ailanthus*, there are also other invasive tree species such as box elder (*Acer negundo*), green ash (*Fraxinus pennsylvanica*) and black locust (*Robinia pseudoacacia*) (Essl and Rabitsch 2002, 2004; Kleinbauer et al. 2010) which also invade open-canopy forests, riparian forests, dry gravel sites or river banks in Austria.

In order to repress or eliminate these invasive tree species, different mechanical, chemical or combined control methods have been considered for use (Liefè 2007; Probst 2012; Radtke et al. 2013; ÖBf 2014; Merceron et al. 2016). However, these methods are expensive, often not successful, an undesirable option (e.g. in near natural ecosystems or biosphere reserves) or even prohibited (e.g. in water protection areas or in national parks). Thus, biological control of *Ailanthus* and other invasive tree species using the wilt-inducing fungus *Verticillium nonalfalfae* might become a promising alternative. In particular, because *Acer*, *Ailanthus*, *Fraxinus* and *Robinia* were listed in prior host lists as susceptible genera to *Verticillium* (Spaulding 1958; Pegg and Brady 2002; Sinclair and Lyon 2005 and Butin 2011). *Verticillium* spp. are native to Austria (Cech 1998; Maschek 2011; Maschek and Halmschlager 2016a), and previous surveys and host range studies have confirmed pathogenicity and aggressiveness and support host adaption of *Verticillium nonalfalfae* to *Ailanthus* (Schall and Davis 2009; Kasson et al. 2014, 2015; O’Neal and Davis 2015; Maschek and Halmschlager 2016a, 2017). Furthermore, *V. nonalfalfae* was suggested to be pathogenic to *R. pseudoacacia* (Kasson et al. 2015; Kletzmayr 2016), whereas inoculations on box elder were unsuccessful so far (Kasson et al. 2015).

However, the application of this wilt-inducing pathogen in a forestal environment still bears the risk of non-target-effects on associated tree species in Austria. Previous host range studies referring to *V. nonalfalfae* had been carried out in North America yet and only five of the species defined as susceptible are indigenous (archaeophytes included) in Europe (*Acer platanoides*, *Humulus lupulus*, *Prunus avium*, *Sambucus racemosa* and *Spinacia oleracea*) (Inderbitzin and Subbarao 2014; Kasson et al. 2014, 2015). Thus, the full host range of *V. nonalfalfae*—especially for European species—is still unknown, because after the recent taxonomic changes in the genus *Verticillium* by Inderbitzin et al. (2011) all previous host records of *Verticillium albo-atrum* s.l. cannot be transferred to *V. nonalfalfae*. They may refer to *Verticillium albo-atrum*, *V. nonalfalfae* or the distantly related *V. albo-atrum* s.s., which resemble each other in morphology.

Thus, the aim of this study was to investigate the effects of the Austrian *V. nonalfalfae*-isolate G1/5 on the most common indigenous non-target, as well as on additional invasive tree species through artificial stem inoculations on potted seedlings. The inoculation study was complemented by cursory field observations in *Ailanthus*-inoculated forest stands with admixed *Acer campestre*, *Acer pseudoplatanus*, *Fraxinus excelsior*, *Populus alba*, *R. pseudoacacia* and *Ulmus laevis* canopy trees. The results of the study provided important information on the host adaptation/specificity of the Austrian *V. nonalfalfae* isolate and the potential risk associated using this pathogen as biological control agent.

Materials and methods

The stem inoculation experiment was conducted from May 2013 to October 2014 on three-year-old potted seedlings of *A. campestre*, *A. pseudoplatanus*, *Ailanthus* (control), *F. excelsior*, *F. pennsylvanica*, *Populus nigra*, *Quercus robur*, *R. pseudoacacia*, *Tilia cordata*, *U. laevis* and *U. minor*. Except for *Ailanthus* and *F. pennsylvanica*, seedlings were obtained from the tree nursery Schwanzer (Langenschönbichl, Lower Austria) and the forest nursery Murauer (Hübing, Upper Austria). To cover a possible loss due to transplantation, 35 bare-rooted seedlings of each species (80–120 cm high) were purchased in April 2013 and transplanted the next day into 5.5 l plastic pots filled with a ready mix potting soil for woody and perennial plants (Terra Vita Pfanzsubstrat, T6-Gehölze/Stauden; Franz Kranzinger GmbH, Straßwalchen, Austria). All potted seedlings were kept in the garden of the institute and watered as necessary. In April 2015, seedlings were fertilized applying 20 g Osmocote® Exact Hi.End 8-9 M (15-9-11+2MgO+TE) per plastic pot.

Because seedlings of *Ailanthus* were not available from tree nurseries in 2013, rooted cuttings and excavated root suckers, which were obtained from different trees from two sites (N48°17′22.29″ E16°20′47.249″ and N48°20′21.959″ E16°45′37.35″), were used for inoculation of *Ailanthus*. The same was true for *F. pennsylvanica* seedlings, which were carefully excavated from a natural stand next to the river Danube (N48°19′5.783″ E16°20′26.66″). Cuttings of about 40 cm in length and about 1 cm in diameter and excavated root suckers (80–150 cm in height) from *Ailanthus*,
as well as excavated *F. pennsylvania* seedlings measuring 80–180 cm in height, were transplanted in the same substrate as the other seedlings and kept moist. Due to the physiological stress caused by cutting or excavation and in order to provide enough time to establish roots and to ensure that excavated root suckers/seedlings were uninfected by other pathogens, no inoculations were performed on *Ailanthus* and *F. pennsylvania* in 2013. The same was true for *Q. robur*, which was heavily infested by *Erysiphe alphitoides* and has therefore been treated four times during the vegetation period 2013 with a fungicide (wettable sulphur/COMPO BIO Mehltäufrei Thiovit Jet); thus, inoculation was postponed to 2014. On 23 August 2013, 20 healthy potted seedlings of each tree species (except *Ailanthus*, *F. pennsylvania* and *Q. robur*) were randomly selected and stem-inoculated using the inoculation method described in Maschek and Halmschlager (2016b) using a Stubai® woodcarving tool Sweep 7, size 6 mm (Art. No. 5507 06). If necessary, the inoculation slit was “closed” with the reverse side of the gouge blade. Another six randomly chosen healthy seedlings of each tree species were treated with sterile water (using the same method and tool as for conidial stem inoculations) and served as controls.

Stem inoculations were performed using the *V. nonalfalvae*-isolate G1/5 (GenBank Accession No. KT223526), which was isolated from symptomatic *Ailanthus* in Bad Radkersburg, province of Styria. For production of fungal inoculum, cultures of *V. nonalfalvae* grown on 2% malt extract agar [MEA; 20 g DiaMalt malt extract (Hefe Schweiz AG, Stettfurt, Switzerland), 16 g Becoagar agar (W. Behrens & Co, Hamburg, Germany), 1000 ml tap water, 100 mg streptomycin sulphate (Calbiochem, Merck KGaA, Darmstadt, Germany), added after autoclaving] and incubated at 22–24 °C in the dark for 17 days were flooded with 20 ml of sterile water. A glass spatula was used to release conidia from conidiophores and the resulting conidial suspension of sterile water. A glass spatula was used to release conidia approximately 5 cm above ground level and another on the opposite side of the stem approximately 10 cm above ground level) applying 2 × 0.5 ml conidial suspension or 2 × 0.5 ml sterile water for the controls. Following inoculation, all seedlings were inspected biweekly for the development of disease symptoms until the end of the vegetation period. Disease severity was rated according to a slightly adapted scale used by Bejarano-Alcázar et al. (1996) and Schall and Davis (2009): 0 = no symptoms/healthy leaves, 1 = 1–33% foliage affected/chlorotic leaves, 2 = 34–66% foliage affected/necrotic leaf margins, 3 = 67–99% foliage affected/wilting leaves, 4 = dead or defoliated seedling.

The stem inoculation experiment was repeated on 13 July 2014 with 20 seedlings using the same inoculation method as in 2013, but control comprised only four seedlings for each species in 2014. In order to reduce partial girdling, seedlings were inoculated in 2014 at only one point at the stem base approximately 7.5 cm above ground level, applying 1 × 1 ml conidial suspension. *V. nonalfalvae*-inoculated seedlings comprised 15 randomly chosen seedlings, which were already treated in 2013 but did not develop any symptoms, and five so far untreated seedlings. Again, all seedlings were kept in the garden of the institute and watered as necessary. Since the root cuttings of *Ailanthus*, as well as the *F. pennsylvania* seedlings, developed well and appeared to be vigorous and healthy in 2014, both species were included in the stem inoculation experiment in 2014. The same was true for *Q. robur* in 2014, on which *Erysiphe* infections could be kept to a minimum due to early fungicide treatments. However, due to the mortality of seedlings caused by the massive *Erysiphe* infection in the previous year, only 19 *Q. robur* seedlings could be subjected to *V. nonalfalvae*-stem inoculations in 2014. Again, development of disease symptoms was rated biweekly for all seedlings until the end of the vegetation period applying the same adapted scale as described above.

At the termination of the experiment in November 2015, all plants inoculated in 2014 were cut at ground level and transported to the laboratory. Reisolations were made from three sections of the stem of each seedling: (1) from the inoculation site, (2) 5 cm below the inoculation site and (3) 10 cm above the inoculation site (Table 2). For that purpose, an approximately 4-cm-long sample was taken from each site using sterile pruning shears. Samples were inspected on both cross sections for the occurrence of vascular discolorations that are often associated with *Verticillium* infections (Sinclair and Lyon 2005; Butin 2011). Afterwards, they were surface-sterilized using 96% ethanol for 1 min and then rinsed with sterile water for 30 s. Thereafter, each sample was split longitudinally and tissue samples were excised from vascular discolorations or—if available—from living sapwood tissue at the transition zone to necrotic tissue. Samples were placed (4 samples per Petri dish and stem section) on 9-cm plastic Petri dishes containing 2% MEA medium (as described above). Plates were sealed with ParaFilm®, labelled with a code for the tree species, tree number and section code and incubated at 22–24 °C in the dark for 12 days. Resulting isolates morphologically resembling *V. nonalfalvae* (Domsh et al. 1980; Inderbitzin et al. 2011) were subcultured on the same conditions for another 18 days. Isolated fungal species other than *V. nonalfalvae* were not further examined. A seedling was scored positive for *V. nonalfalvae* if the pathogen was reisolated from at least one of the three sampled sections.
Photographs of vascular/sapwood discolourations were taken from the cross section and longitudinal section (Fig. 1a–j) of representative samples of each species. If possible, photographs were taken from the part opposite of the inoculation site, in order to avoid discolourations caused by embolism, following wound inoculation.

In addition to the inoculation experiment on potted seedlings, three sites within Ailanthus-inoculated or naturally infected forest stands were examined in summer 2015 for wilting symptoms on non-target tree species: (1 + 2) two sites in Lower Austria, where 20 Ailanthus trees had been artificially stem-inoculated in August 2011 and in June 2013, respectively, and (3) a forest plot in Gänserndorf (Lower Austria) that became naturally infected with V. nonalfalfae in summer 2011 (Maschek and Halmschlager 2016a). Observations focused on indigenous woody species, which were listed as susceptible to Verticillium spp. according to Engelhard (1957), Himelick (1969), Pegg and Brady (2002) or Sinclair and Lyon (2005) (Table 1). However, observations were not confirmed by a systematic survey on these sites.

Results

External disease symptoms and mortality on seedlings

Stem inoculations on Ailanthus resulted in a rapid disease progression on 100% (20 of 20 plants) of the trees in 2014: The seedlings exhibited minor disease symptoms (mean disease severity [MDS] of 0.3) already 2 weeks post-inoculations (WPI), progressing rapidly reaching a MDS of 3.2
at the end of the vegetation period in 2014, resulting in 85% (17 of 20 plants) dead Ailanthus trees by November 2015, when the reisolations were carried out. 0% (0 of 4 plants) of the controls developed disease symptoms or died during the evaluation period.

Contrary to these results, stem-inoculated A. campestre, A. pseudoplatanus, U. laevis, U. minor, P. nigra, Q. robur and T. cordata as well as all controls developed no disease symptoms throughout the observation period in 2013 or 2014. No symptoms were also detected on stem-inoculated F. excelsior during the vegetation period in 2013, however, in 2014 20% (4 of 20 plants) of the inoculated seedlings and 25% (1 of 4 plants) of the controls developed chlorosis, necrosis and curled leaves 6 WPI resulting in a MDS of 0.13 for the inoculated seedlings and a MDS rating of 0.25 for the controls at the end of the vegetation period in 2014. Regardless these symptoms, none of these seedlings died until the end of the evaluation period. Similar symptoms were developed on F. pennsylvanica, which was inoculated only in 2014: 15% of these seedlings (3 of 20 plants) developed chlorosis/necrosis 6 WPI and another 10% of the inoculated seedlings (2 of 20 plants) exhibited the same symptoms 8 WPI. The controls of F. pennsylvania remained asymptomatic and no seedling (inoculated or control) died until the end of the evaluation period.

R. pseudoacacia inoculated in 2013 exhibited no symptoms until the end of the vegetation period but did not show any mortality in the following year. Regardless of these results, 20% (4 of 20 plants) of the inoculated seedlings showed symptoms of dieback in spring/early summer in the following year and were dead at the date of the new inoculations in 2014 (13.07.2014). The same was true for 33% (2 of 6 plants) of the controls. However, dieback also occurred on additionally reared untreated seedlings (i.e. seedlings that were neither inoculated with conidial suspension nor with sterile water) in 2013 that served as reserves. Mortality reached 25% (2 of 8 plants) on these untreated seedlings.

Seedlings of R. pseudoacacia inoculated in 2014 again exhibited no symptoms until the end of the vegetation period but did not show any mortality in the following

| Site | Inoculation date | Sea level | Orientation/gradient | Soil type | Stocking level (in tenths) | Composition of tree species (in tenths) | Stem number (N/ha) | Location, province |
|------|------------------|-----------|----------------------|-----------|--------------------------|----------------------------------------|-------------------|-------------------|
| 1    | 30.08.2011       | 145 m     | Plain/0°             | Chernozem (clayey to silty to silty-clayey suspended alluvial material). At 30–100 cm gravel from river Danube | 0.9 | 0.7 Ailanthus altissima 0.1 Acer pseudoplatanus 0.1 Robinia pseudoacacia 0.1 other tree species (Acer campestre, Sambucus nigra, Tilia cordata, Betula pendula) | 2.500 | Niederweiden, Lower Austria |
| 2    | 04.06.2013       | 270 m     | South-east, slight slope/10° | Chernozem/Pararendzina middle-to deep grounded | 0.4 | 0.3 Fagus sylvatica 0.2 Tilia cordata 0.2 Ailanthus altissima 0.2 Quercus cerris 0.1 Carpinus betulus | 2.000 | Schützen am Gebirge, Burgenland |
| 3    | Natural infection in 2010/2011 | 245 m | Plain/0° | Loess, mostly middle- to deep grounded nutritious brown earths | 1 | 0.4 Ailanthus altissima 0.2 Fraxinus excelsior 0.2 Acer campestre 0.2 other tree species (Corylus avellana, Prunus serotina, Sorbus torminalis) | 3.500 | Hohenruppersdorf, Lower Austria |
Vascular discolourations on seedlings

Vascular discolourations in the sapwood varied in colour, appearance and in intensity among the inoculated tree species (Fig. 1a–j). Furthermore, there was also a high variation in the number of seedlings that exhibited such vascular discolourations due to artificial infections between the tested species. In *Ailanthus*, vascular discolourations were yellowish/orange. However, these symptoms could only be observed on seedlings (3 of 3 plants) that still exhibited at least some living cortex chlorenchyma (Table 2). In contrast, dead *Ailanthus* seedlings (85% or 17 of 20 plants) were lacking these typical yellowish/orange discolourations but exhibited brownish, necrotic cortex chlorenchyma (Fig. 2) and sapwood and were additionally characterized by a foul smell. Orange/brownish discolourations within the sapwood (Fig. 1c–e) were typically for *F. excelsior*, *F. pennsylvanica* and *P. nigra* (Fig. 1c–e). However, in contrast to *Ailanthus*, discolourations occurred only on 5% (1 of 20 plants) on *F. excelsior* and *P. nigra* and on 15% (3 of 20 plants) of the inoculated seedlings of *F. pennsylvanica* (Table 2). Brownish discolourations of the sapwood (Fig. 1f, h) were found on 42% (8 of 19 plants) of the inoculated seedlings of *Q. robur* and on 60% (12 of 20 plants) of the examined *T. cordata*-seedlings.
(Table 2). A. campestr and U. minor exhibited brownish/blackish discolorations that were found on 30% (6 of 20 plants) and on 60% (12 of 20 plants), respectively (Table 2; Fig. 1a, j). The most distinctive vascular symptoms were the greenish/blackish discolorations found on A. pseudoplatanus, R. pseudoacacia and U. laevis (Fig. 1b, g, i). While these symptoms were quite rare on A. pseudoplatanus (5% or 1 of 20 plants), they occurred quite commonly on R. pseudoacacia (85% or 17 of 20 plants) and were consistently found on inoculated seedlings of U. laevis (100% or 20 of 20 plants) (Table 2). In contrast, none of the controls exhibited vascular discolorations, which could be related to Verticillium infections (Table 2).

Reisolations of V. nonalfalfa from seedlings

Successful rate of reisolation differed considerably between the tested tree species. It ranged from 95% in A. pseudoplatanus to 0% in F. pennsylvanica and R. pseudoacacia. Furthermore, results revealed that rate of reisolation did not correlate with the intensity of vascular discolorations. Thus, there were species with heavily discoloured sapwood but low rate of successful reisolation (R. pseudoacacia, U. laevis), but there were also species of which V. nonalfalfa could be reisolated quite common although vascular discolorations were rare (A. pseudoplatanus) as well as species that were characterized by both, a low rate of successful reisolation and a low rate of seedlings exhibiting vascular discolorations (Table 2).

Due to the fact that 17 of 20 seedlings of *Ailanthus* were already dead at the time of processing, *V. nonalfalfa* could be reisolated from 66% (2 of 3) of the seedlings that still exhibited at least some living stem or root tissue but showed an advanced stage of dieback and subsequent colonization by saprophytes. *V. nonalfalfa* could not be reisolated from controls of *Ailanthus*.

Besides *Ailanthus*, reisolations were also successful on another 8 of 11 tree species (Table 2): Reisolations were most successful on A. pseudoplatanus (95% or 19 of 20 plants) although vascular discolorations were found on only 5% of these seedlings, followed by A. campestr with 85% (17 of 20 plants) and Q. robur with 63% (12 of 19 plants). *V. nonalfalfa* could also be successfully reisolated from 25% (5 of 20 plants) of the seedlings of *T. cordata* and from 20% (4 of 20 plants) of *F. excelsior*. Low rates of successful reisolations were obtained for *P. nigra, U. laevis* (10% or 2 of 20 plants) and *U. minor* (5% or 1 of 20 plants). *V. nonalfalfa* could not be reisolated at all from *F. pennsylvanica* and *R. pseudoacacia*, although each species was sampled 240 times.

Reisolations were most successful at the point of inoculation (overall rate of reisolation: 20%), and there was only a minor difference between the two other sampled stem sections (14.1% at −5 cm and 15.6% at +10 cm from the point of inoculation). On only two tree species, reisolations were more successful on a stem section, which was not the point of inoculation: on *A. campestr* (65% or 13 of 20 plants) and *U. minor* (5% or 1 of 20 plants), both at +10 cm.

Field observations in mature stands subjected to inoculations/natural infection of *Ailanthus* canopy trees on three forest sites revealed that there was no formation of wilting symptoms on admixed non-target tree species such as *A. campestr*, *A. pseudoplatanus*, *F. excelsior*, *P. alba*, *R. pseudoacacia* and *U. laevis*. However, no isolations were carried out from that symptomless admixed tree species. In contrast, severe wilting symptoms, dieback and mortality were observed on inoculated/naturally infected *Ailanthus* trees as well as on adjacent *Ailanthus* trees that got infected via root grafts (Fig. 4).

Discussion

Knowing the potential host range of *V. nonalfalfa* is of great importance for a hazard and exposure risk assessment. It is also an inevitable prerequisite to avoid undesirable non-target-effects on associated tree species using that fungus for the biological control of *Ailanthus* or other invasive tree species.

However, the descriptions of *V. nonalfalfa* and the morphologically indistinguishable *V. alfalfa*, both resembling the distantly related *V. albo-atrum* in morphology, were provided just a few years ago (Inderbitzin et al. 2011). Thus, previous host range studies of *V. nonalfalfa* are rare and mainly focus on agricultural crops (Inderbitzin and Subbarao 2014), ornamentals (Garibaldi et al. 2016) or woody species native to North America (Schall and Davis 2009; Kasson et al. 2014, 2015). In Europe, *V. nonalfalfa* was confirmed for the first time just recently from a woody host (Maschek and Halmenschlager 2016a). Except for *A. platanoideae*, *Prunus avium* and *Sambucus racemosa*, host range studies on woody plants native to Europe are therefore completely lacking so far.

Although a long list of diverse hosts—including also many tree species—is known for *V. albo-atrum* s.l. (Pegg and Brady 2002; Sinclair and Lyon 2005; Butin 2011), it is not possible to relate the host range information from previous literature on *V. albo-atrum* s.l. to the newly delimited *Verticillium*-species *V. albo-atrum* s.s., *V. alfalfa* or *V. nonalfalfa* (Inderbitzin et al. 2011), as long as molecular data or detailed morphological descriptions or cultures are lacking. In order to expand the information regarding host range of *V. nonalfalfa*, we tested the susceptibility of eight indigenous non-target tree species, often associated with Tree of Heaven, and two invasive exotic tree species.
In our study, *V. nonalfalfa* induced characteristic wilting symptoms and high mortality only on the target host *Ailanthus*, whereas limited or no impact was detected on non-target tree species, thus indicating a high degree of host specificity. In addition, consistent vascular discolourations and the successful reisolation of the applied isolate indicate the high susceptibility (*S*) of *Ailanthus* seedlings to *V. nonalfalfa*. Similar results had been obtained by Schall and Davis (2009) and Kasson et al. (2015), testing the host range for *V. nonalfalfa* in the eastern USA. Wilt, dieback and mortality were also observed on artificially inoculated canopy trees of *Ailanthus* on several sites in eastern Austria (Maschek 2011; Maschek and Halmischlager 2017). The same symptoms were also observed on *Ailanthus* trees adjacent to the inoculated trees, all of which were considered to be connected via root grafts. Intraspecific transmission by root crafts has already been demonstrated by O’Neal and Davis (2015). Extensive dieback and mortality of *Ailanthus* canopy trees additionally confirm the high virulence of the European *V. nonalfalfa*-isolate G1/5 (Fig. 4) found in seeding inoculations and qualify this pathogen as potential biological control agent for this invasive species (Maschek and Halmischlager 2016a).

In contrast to the high susceptibility of *Ailanthus*, *V. nonalfalfa* had no or only limited impact on the tested non-target and the two invasive tree species in this study: Based on disease symptoms, secondary symptoms like vascular discolorations and the rate of successful reisolations, tested tree species were assigned as being tolerant, possibly resistant or resistant.

Inoculated *A. campestre, A. pseudoplatanus* and *Q. robur* exhibited neither primary disease symptoms (chloroses, necroses or wilt) nor mortality; however, *V. nonalfalfa* was frequently reisolated from inoculated seedlings. Recovery of the pathogen from these asymptomatic seedlings indicates a low level of systemic infection that did not result in symptom development. Thus, these three species were considered tolerant (*T*) to European *V. nonalfalfa*-isolate G1/5 (Table 2). Irrespective of the high rate of reisolations, formation of vascular discolorations was only observed to a considerably lesser extent on the examined samples. Similar results concerning tolerance to *V. nonalfalfa* had been obtained in a host range study by Kasson et al. (2015) for red maple (*Acer rubrum*) and sugar maple (*Acer saccharum*), but also for Korean evodia (*Tetrastigma daniellii*) and Tree of Paradise (*Simarouba glauca*). In contrast, inoculated striped maple (*Acer pensylvanicum*) reached similar disease levels as *Ailanthus* in a study carried out in eastern USA by Schall and Davis (2009) and was therefore considered susceptible to *V. nonalfalfa* (isolate PSU140). Susceptibility to this *V. nonalfalfa* isolate was also demonstrated for Norway maple (*A. platanoides*) and Japanese maple (*Acer japonica*) by Kasson et al. (2015). In prior host lists (compiled by Rudolph 1931; Carter 1938; Weiss 1940; Engelhard 1957; Himelick 1969; Pegg and Brady 2002), referring to the former *V. albo-atrum* s.l., also *A. campestre, A. pseudoplatanus*, as well as other *Acer* species, were considered susceptible. Thus, differences concerning susceptibility/tolerance of woody host species between these prior and recent studies indicate a narrower host range of *V. nonalfalfa* compared to *V. albo-atrum* s.l.

In contrast to *Q. robur*, which was considered tolerant in our study, *V. nonalfalfa* could not be reisolated from inoculated northern red oak (*Quercus rubra*) and chestnut oak (*Quercus montana*) and has therefore been classified as resistant by Schall and Davis (2009) and Kasson et al. (2015). According to field observations in *Ailanthus*-inoculated forest stands, resistance was also suggested by the former authors for black oak (*Quercus velutina*). However, no inoculation studies had been performed on this tree species. In addition, vascular discolorations were observed twice as much in *Q. robur* (8 of 19 seedlings) in our study, compared to *Q. rubra* (2 of 10 trees) in the study carried out by Kasson et al. (2015). According to previous papers dealing with *V. albo-atrum* s.l., the genus *Quercus* includes both susceptible and resistant species (Himelick 1969; Pegg and Brady 2002; Sinclair and Lyon 2005). *V. albo-atrum* s.l. has so far been recorded on the surface of *Q. robur* acorns (Urosevic 1987), but is not mentioned in the detailed host index of *V. albo-atrum* s.l. published by Engelhard (1957).

In *F. excelsior, P. nigra, T. cordata, U. laevis* and *U. minor*, rate of reisolations was rather low and did not exceed 15% for each stem section (Table 2). Because these species also did not develop disease symptoms (except for *F. excelsior*) and exhibited no mortality, all five species are considered possibly resistant (*PR*). Disease symptoms on the inoculated seedlings of *F. excelsior* developed simultaneously to the same extent on the controls and comprised chlorotic and necrotic leaves with typical swellings on the acorns. Visual inspections indicated that these swellings could be attributed to the gall-forming midge *Dasineura fraxini*; thus, displayed symptoms were not related to *V. nonalfalfa* infections.

Similar results were obtained by Schall and Davis (2009) for *V. nonalfalfa*-inoculated seedlings and canopy trees of white ash (*Fraxinus americana*), which was considered as tolerant (= resistant according to our classification), because reisolation was unsuccessful in all cases. Susceptibility of *F. excelsior* to *Verticillium* wilt (Sinclair and Lyon 2005) was usually attributed to *V. dahliae* infections in previous reports (Hiemstra 1995; Heffer and Regan 1996; Schuring and van der Schaaf 1999). However, Himelick (1969) also recovered *V. albo-atrum* s.l. from branch specimens of black, blue, European, green and white ash and was able to reisolate the fungus from all inoculated seedlings except black ash, which was not included in his pathogenicity test.
ash was also included in the host list of *V. albo-atrum* s.l. compiled by Engelhard (1957).

Among poplar species, susceptibility to *V. nonalfafae* was previously only tested for White poplar (*P. alba*) and Bigtooth aspen (*Populus grandidentata*) (Kasson et al. 2015). However, the authors did not provide any results for the two species. Assuming that they did not develop symptoms and were resistant, these results would be similar to those in our study, in which reisolations and vascular discolorations (Fig. 1e) were obtained for *P. nigra*, but occurred very rarely. In contrast to the results related to *V. nonalfafae*, the host range of *V. albo-atrum* s.l. comprised susceptible *Populus* species such as *Populus tremula* (Liese 1933; Sinclair and Lyon 2005).

*T. cordata* exhibited neither wilt symptoms nor mortality (Table 2), which is similar to the results obtained by Kasson et al. (2015) for *Tilia americana*—this species was therefore considered resistant. In contrast to this study, *V. nonalfafae* was recovered from a quarter of all inoculated seedlings. Vascular discolorations were found to a lower extent (60%, Fig. 1h) on *T. cordata* when compared to the results of *T. americana* (93%, Kasson et al. 2015). According to Sinclair and Lyon (2005), the genus *Tilia* includes both susceptible and resistant species. In prior papers (Wollenweber 1929) and host lists compiled by Engelhard (1957), Himelick (1969) and Pegg and Brady (2002), *T. cordata* is frequently listed as susceptible host for *V. albo-atrum* s.l. along with other *Tilia*-species from Europe and Northern America.

Both inoculated *Ulmus* species displayed neither wilt symptoms nor mortality (Table 2), but regardless of the absence of these exterior symptoms, vascular discolorations were commonly found in seedlings of *U. laevis* (100%, Fig. 1i) and *U. minor* (60%, Fig. 1j). Nevertheless, *V. nonalfafae* was reisolated only rarely from those vascular discolorations, indicating that the fungus very likely induced these secondary disease symptoms, but could barely survive in both *Ulmus* species until reisolations in Nov. 2015 (i.e. 14 months post-inoculation). Very similar results were obtained by Kasson et al. (2015) for *Ulmus americana* and *U. pumila*: (1) Both species developed neither wilt symptoms nor mortality, (2) both *Ulmus* species exhibited vascular discolorations (*U. americana* 100%, *U. pumila* 40%) and (3) *V. nonalfafae* could not be reisolated from any elm-tree. However, *U. americana, U. glabra* (Syn.: *U. montana*), *U. minor* (Syn.: *U. campestris*), *U. parvifolia, U. procera* and *U. rubra* are mentioned as being susceptible to *V. albo-atrum* s.l. in the host lists compiled by Rudolph (1931), Carter (1938), Engelhard (1957), Himelick (1969) or Pegg and Brady (2002).

Like in *F. excelsior*, some *F. pennsylvanica* seedlings also developed chlorotic and necrotic leaves (Fig. 3). However, according to Heffer and Regan (1996), Hiemstra and Harris (1998) and Sinclair and Lyon (2005), *Verticillium* wilt of *F. pennsylvanica* is generally associated with wilting leaves that remain greenish or is characterized by necroses; thus, symptoms were not specific for *Verticillium* wilt. As there were no typical swellings on the leaf rachises, symptoms on *F. pennsylvanica* could also not be attributed to infestation by *D. fraxini*, as it was the case in *F. excelsior*. In fact, symptoms more resembled to manganese or magnesium deficiencies (Sinclair and Lyon 2005; Hartmann et al. 2007; Butin et al. 2010), but since nutritional status of seedlings was not further analysed, symptoms might alternatively be attributed to other biotic or abiotic agents. Regardless of these unspecified external symptoms, *F. pennsylvanica* also developed less pronounced orange/brownish vascular discolorations (Fig. 1d). However, *V. nonalfafae* could not be reisolated from any of those discolorations; thus, *F. pennsylvanica* was considered resistant (*R*) to *V. nonalfafae*. In contrast, the genus *Fraxinus* is listed as susceptible to *Verticillium* spp. (Himelick 1969; Heffer and Regan 1996; Sinclair and Lyon 2005; Butin et al. 2010) in older publications, taking not into account the recent taxonomic changes (Inderbitzin et al. 2011).

Symptoms on *R. pseudoacacia* already developed in early summer 2014 (before the 2014-inoculation was conducted) and occurred not only on *Verticillium*-infected seedlings, but also on the controls as well as on seedlings that were
kept in 2013 in reserve. Thus, symptoms cannot be related to *Verticillium* infections but were rather related to drought.\(^1\) Although vascular discolorations were found to be second most frequent on *R. pseudoacacia*, *V. nonalfalfae* could not be reisolated from any of the inoculated seedlings (Table 2). Thus, this tree species was also categorized as resistant to the Austrian *V. nonalfalfae* strain. In contrast, artificial inoculations with *V. nonalfalfae* conducted by Kasson et al. (2015) in 2009 and 2010 induced wilt on 20 and 80% of the inoculated plants, but resulted in only 0 and 10% mortality, respectively. Similar to our results, vascular discolorations were consistently observed on trees inoculated in 2010, whereas no discolorations were detected in trees inoculated in 2009 (Kasson et al. 2015). However, sample size in 2009 comprised only five black locust trees. Our results are in contrast to prior publications and host lists referring to *V. albo-atrum* s.l. (Goidanich 1935; Carter 1938; Engelhard 1957; Himelick 1969; Pegg and Brady 2002; Sinclair and Lyon 2005) in which *R. pseudoacacia* is considered susceptible.

Overall, results regarding vascular discolorations and the rate of successful reisolations obtained in our seedling inoculation experiment possibly overestimate the impact of *V. nonalfalfae* under field conditions, because a successful natural infection by this soilborne pathogen might be counteracted or even prevented by root defences (Blanchette and Biggs 1992), which were bypassed by our stem inoculation method (Maschek and Halmschlager 2016b). There are also various effects influencing a natural infection or the establishment of the pathogen within the host: (1) protective effects of mycorrhizal fungi against *Verticillium* infections (Karagiannidis et al. 2002), (2) synergistic effects of root-knot nematodes and *Verticillium* (Santamour 1992) or (3) antagonistic effects of xylem-colonizing bacteria (Hall et al. 1986).

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\(^1\) Symptoms only occurred on seedlings that had been moved—without the knowledge of the authors—to an unfavourable sun-exposed area of the institute’s garden in order to provide space for another experiment. All other *R. pseudoacacia* seedlings remained symptom-free. Seedlings subjected to drought did not recover until the end of the vegetation period 2014 and were classified as loss.
Additionally, the applied sporule concentration for sure exceeds the amount of conidia found in natural infections (Pegg and Brady 2002). Thus, results indicating susceptibility (which was only the case for Ailanthus in our study) in artificial inoculations have to be treated with caution, whereas the absence of wilting symptoms may anyway indicate tolerance or true resistance. Nevertheless, the formation of vascular discolorations confirmed the successful transmission of inoculum into the xylem, resulting in defence reactions such as occlusion of vessels (Tippett and Shigo 1981; Kasson et al. 2015; Maschek and Halsmchlager 2016b).

Results of seedling inoculations are supported for five (A. campestre, A. pseudoplatanus, F. excelsior, R. pseudoacacia and U. laevis) out of the tested species by field observations in Ailanthus-inoculated/naturally infected forest stands, all of which remained asymptomatic, whereas extensive mortality was observed on Ailanthus on the same sites (Maschek 2011, Fig. 4). Similar results (no wilt or mortality related to V. nonalfalafa) had also been obtained for R. pseudoacacia under natural conditions by Kasson et al. (2015), whereas stem-inoculated black locust was classified as susceptible in the same study.

Conclusions

This study expands the comprehensive host range studies for V. nonalfalafa in the USA (Schall and Davis 2009; Kasson et al. 2015) to European tree species often being dominant or admixed in Ailanthus-invaded forest stands. Our results suggest a much narrower host rage and host adaptation of the tested European V. nonalfalafa-isolate G1/S compared to V. albo-atrum s.l., which has been reclassified in the course of recent taxonomic changes (Inderbitzin et al. 2011) into three morphologically indistinguishable species. Since none of the inoculated non-target-species exhibited disease symptoms—except for xylem discolorations in some tree species—or mortality and associated tree species in Ailanthus-inoculated forest stands also remained asymptomatic, V. nonalfalafa is considered to be a potential biocontrol agent to combat the highly invasive Ailanthus. However, further follow-up host range studies are needed to evaluate the impact on other European non-target tree species.

Acknowledgements Open access funding provided by University of Natural Resources and Life Sciences Vienna (BOKU). This research was conducted as part of a project on the biological control of Ailanthus altissima, funded from the Austrian Federal Forests (ÖBF AG), Austrian Federal Railways (ÖBB), Austrian Power Grid AG (APG), the provincial government of Styria, Vienna Lines (Wiener Linien), via donau and Forest Enterprise Esterházy. The authors wish to thank T. Kirisits for his valuable suggestions on the manuscript.

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