

Transmission of *Verticillium nonalfalfae* via root contact from inoculated *Ailanthus altissima* in close-to-nature conditions

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Abstract

Ailanthus altissima is an invasive alien species in Europe. Biological control of this tree species by *Verticillium nonalfalfae* is a potential alternative control approach. This study investigates host specificity, pathogenicity and transmission of *V. nonalfalfae* to neighbouring plants with root contact in mini-ecosystems. *V. nonalfalfae* led to dieback of all inoculated *Ailanthus* trees. Furthermore, *V. nonalfalfae* was transmitted to neighbouring *Ailanthus* trees, causing wilt and dieback, and, in one case, to *Quercus petraea*, which did not display any disease symptoms. Lastly, *V. nonalfalfae* could not be detected in the soil, which suggests transmission via root contact.

1 | INTRODUCTION

Ailanthus altissima (Mill.) Swingle (Simaroubaceae), hereafter referred to as *Ailanthus*, is a deciduous tree originating from Asia (China). Throughout Europe, it is considered an invasive neophyte. It has been shown to have competitive, physical, chemical, structural and indirect impacts on native trees and forest habitats, displaces native vegetation and damages pavement and building foundations in urban areas (Brooks et al., 2020).

A promising biological control method is the treatment with the fungal antagonist *Verticillium nonalfalfae* Inderb., H.W. Platt, R.M. Bostock, R.M. Davis & Subbarao (Kasson et al., 2015). *Ailantex*®, consisting of a *V. nonalfalfae* isolate 'Vert56' spore suspension (1×10^6 spores/ml in autoclaved water), is an approved herbicide in Austria (Pfl. Reg. Nr. 4280-0). *Verticillium nonalfalfae* is a well-known soil-borne, vascular wilt pathogen on various plant species, with *A. altissima* being a very susceptible host (Kasson et al., 2015). It transmits via intraspecific root grafts within *Ailanthus* stands (O'Neal & Davis, 2015). The use of *V. nonalfalfae* for biocontrol of *Ailanthus* has been tested in several studies (Brooks et al., 2020; Maschek & Halmschlager, 2018), although the fungus poses a potential danger for agricultural crops (Kasson et al., 2015). The pathogenicity of the fungus has also been tested on various non-target tree

species co-occurring with *Ailanthus* (Kasson et al., 2015; Maschek & Halmschlager, 2018). This study goes one step further by testing host specificity and transmission of *V. nonalfalfae* from inoculated plants to neighbouring tree species by root contact. It focused on whether (i) Inoculation with *V. nonalfalfae* kills young *Ailanthus*, (ii) Inoculation of young *Ailanthus* causes symptoms (weakening/wilting) on or leads to death of neighbouring trees via root contact, and (iii) *V. nonalfalfae* is transmitted through soil, using a mini-ecosystem approach in a greenhouse.

2 | MATERIAL AND METHODS

Mini-ecosystems (6 replicates and 6 controls) were established (April 2019; Grosscontainer 50 l Jopa, Hortima AG, Hausen, Switzerland; soil for perennials and pot plants, Ökohum GmbH, Herbertingen, Germany) with 5 potted young trees each to simulate a typical southern Swiss forest ecosystem. A central *Ailanthus* was surrounded by four neighbouring trees: *Quercus petraea* (Matt.) Liebl., *Robinia pseudoacacia* L., *Castanea sativa* Mill. (all were 2 years old, Emme-Forstbaumschulen AG, Wilder b. Utzendorf, Switzerland) and a neighbouring *A. altissima* (Pflanzmich GmbH, Pinneberg Schleswig-Holstein, Germany). Roots of the four neighbouring trees were

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intermingled with those of the central *Ailanthus* (Figure 1 A and B). Mini-ecosystems were placed in a greenhouse and watered sufficiently twice a week during the whole vegetation period.

Inoculation of *Ailantex*® (0.2 ml) took place 4 weeks after planting and followed the procedure of Maschek and Halmschlager (2018). For controls, the central *Ailanthus* solely received a cut with the same tool used for inoculation to reproduce wounding.

Disease progression was monitored in categories and intervals of ratings of disease severity were adapted to the observed symptoms in order to adequately record any changes during the phases of rapid disease development. In total, observations covered a time span of 313 days post inoculation (dpi). Plants were sprayed against aphids using a pyrethroid 39 dpi following manufacturer's instructions (Kendo® MAAG/Syngenta, Switzerland). Above-ground parts of the inoculated central *Ailanthus* (stems, twigs and leaves (all dead)) were sampled 55 dpi. The rest of the representative samples were collected 313 dpi (central *Ailanthus*: soil and remaining root material; neighbouring trees: stems, twigs, leaves and roots). Soil samples were taken directly from underneath the rootstock of the central *Ailanthus*. All samples were stored in 1,5 ml tubes (Eppendorf Safe-Lock Tubes, Eppendorf AG, Hamburg, Germany) at -20°C until further analysis.

Samples were lyophilized and milled (soil: 2 × at 30 Hz for 5 min; plant material: 30 Hz for 2 min; MM400 Retsch mill, Retsch GmbH,

Haan, Germany). DNA extraction from 250 mg soil samples used the DNeasy PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) and followed the manufacturer's instructions. DNA extraction from 90 mg plant material was done as described by Schneider et al. (2019). PCR (Veriti thermal cycler; Applied Biosystems, Rotkreuz, Switzerland; 2 min initial activation at 94°C, 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 62°C, 2 min extension at 72°C, 10 min final extension at 72°C) was performed with primers Nof (5'-CCTCGAAAAATCCACCAGCTCTA-3') and NoNur (5'-GTGGTTGAGATCCTCACGCTTC-3') specific for *V. nonalfalfae* designed by Inderbitzin et al. (2013). Reaction volumes of 20 µl contained 2 µl of the 1:10 diluted soil or plant DNA extracts, 1 × JumpStart RED Taq ReadyMix (Sigma-Aldrich, Buchs, Switzerland), 1 mg ml⁻¹ bovine serum albumin (BSA; Sigma-Aldrich) and 0,4 µM of each primer (Microsynth, Balgach, Switzerland). A subsample of soil and plant DNA extracts was checked for DNA quality using universal primers targeting internal transcribed spacer of ribosomal DNA of fungi and plants (White et al., 1990). Samples were checked for amplification by electrophoresis at 90 V for 45 min of the PCR products in 1,5% (w/v) agarose with ethidium bromide staining.

Statistical analyses were performed in R (Version 4.0.5; the R Company for Statistical Computing, Vienna; for graphs: package ggplot2 (Version 3.3.3)) using the Wilcoxon rank-sum test.

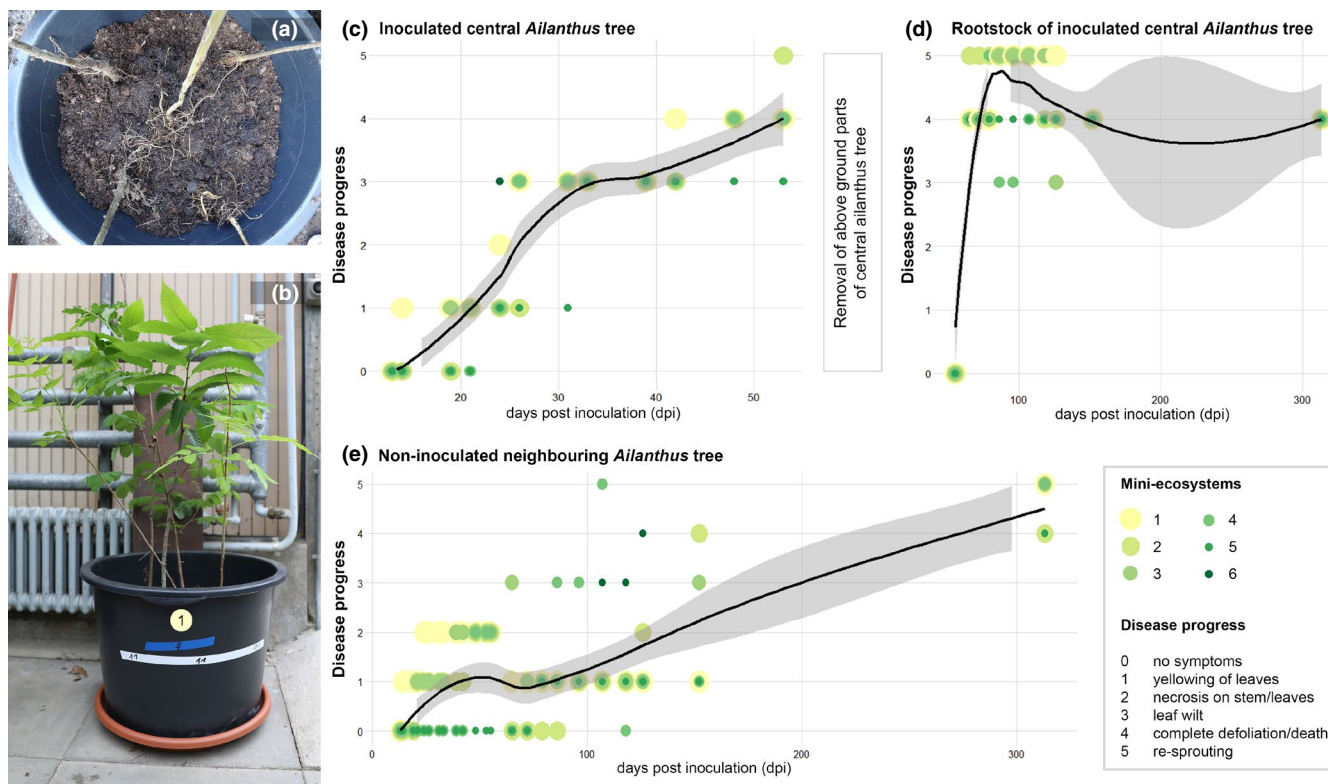


FIGURE 1 (a) Planting of the trees with intermingled roots. (b) Mini-ecosystem. (c) Disease Progress of the 6 inoculated central *Ailanthus* trees from inoculation to harvest (55 dpi). (d) Disease progress of the re-sprouting of the central *Ailanthus* rootstock until the end of experiment (313 dpi) and (e) the non-inoculated neighbouring *Ailanthus*'. Black curve: general pattern of the disease progress across all mini-ecosystems including the standard error in grey shading. Dots in different green colours: different mini-ecosystems. Disease progress was measured in categories from 0 to 5

TABLE 1 Presence of *Verticillium nonalfalfae* in the different tree organs such as roots, stem, shoots and leaves, from the different tree species with corresponding treatment (+ = central tree inoculated with *V. nonalfalfae*; - = non-inoculated neighbouring tree in the same mini-ecosystem)

Tree organ			Roots	Stem	Shoots	Leaves
Mini-ecosystem No	Tree species	Inoculation				
1	<i>A. altissima</i>	+	■		■	
1	<i>A. altissima</i>	-		■		
2	<i>A. altissima</i>	+	■		■	
3	<i>A. altissima</i>	+	■			■
4	<i>A. altissima</i>	+	■			■
4	<i>Q. petraea</i>	-		■		
5	<i>A. altissima</i>	+	■		■	
6	<i>A. altissima</i>	+	■			■
6	<i>A. altissima</i>	-		■		

Note: Only samples with a PCR signal are shown.

3 | RESULTS AND DISCUSSION

The first symptoms on inoculated *Ailanthus* trees (yellowing of leaves) appeared 14 dpi, followed by wilting and leaves with necrosis (24 dpi) and death after 31–53 dpi. When the central *Ailanthus*' were harvested 55 dpi, only one had started to re-sprout already. Later, five of the six trees re-sprouted. Finally, 152 dpi, even the re-sprouted central *Ailanthus*' had died. Therefore, *V. nonalfalfae* led to the complete dieback of all inoculated *Ailanthus*', even if they started to re-sprout (Assumption i; Wilcoxon rank-sum test, $p < .001$; Figure 1c,d). These findings are in accordance with studies conducted in Europe and in the United States (Brooks et al., 2020; Kasson et al., 2015; Maschek & Halmschlager, 2018).

On non-inoculated neighbouring *Ailanthus*', first symptoms (leaf yellowing) appeared 14 dpi in the same mini-ecosystem, which previously showed the earliest symptoms on the central *Ailanthus* tree. 55 dpi, 4 of 6 neighbouring *Ailanthus* trees already showed signs of leaf necrosis. 313 dpi, 3 of the 6 neighbouring *Ailanthus*' had died, while the other three trees had re-sprouted after previous dieback. Hence, we were able to show that inoculation of central trees also leads to symptoms on neighbouring *Ailanthus*' (Assumption ii; Wilcoxon rank-sum test, $p < .001$; Figure 1e). Neither any other neighbouring tree species nor control trees displayed any disease symptoms.

Verticillium nonalfalfae was found in all tree organs examined of inoculated central *Ailanthus*' (Table 1). For all but one inoculated *Ailanthus* *V. nonalfalfae* was detected in at least 2 out of 4 tree organs. In 3 out of 6 mini-ecosystems, *V. nonalfalfae* was transferred from the inoculated central *Ailanthus* to another tree: twice from the central *Ailanthus* to the neighbouring *Ailanthus* and once from *Ailanthus* to *Q. petraea*, the latter not showing any symptoms until 313 dpi (Table 1).

None of the soil samples was tested positive for *V. nonalfalfae* (Assumption iii; Wilcoxon rank-sum test, $p < .001$). These results suggest that the inoculated conidia suspension was not transmitted into the soil and therefore does not pose immediate threat to other potential host species. Considering *V. nonalfalfae*'s character as a soil-borne pathogen which has been detected in

agricultural soils by PCR in other studies (Borza et al., 2019), this result is surprising.

Overall, root contact led to transmission of *V. nonalfalfae* between *Ailanthus* trees. This finding complements the study by O'Neal and Davis (2015) which reports transmission through intra-specific root grafts and clonal root system. Although we suspected that inoculations of *V. nonalfalfae* were not optimal and trees might not have taken in enough spore solution due to a weak transpiration rate, disease progress was as expected and described in the literature (O'Neal & Davis, 2015). Even though symptoms were solely observed on inoculated and neighbouring *Ailanthus*', the effect of multiple *Verticillium* treatments of *Ailanthus* on different neighbouring tree species with root contact remains up to further testing.

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PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/efp.12720>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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