**SHORT COMMUNICATION**

**Investigating physiological effects due to artificial infections of grapevine with Verticillium nonalfalfae**

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**ABSTRACT**

*Ailanthus altissima* is among the most invasive woody species worldwide, outcompeting native trees. The fungus *Verticillium nonalfalfae* (VN) is promising for *A. altissima* biocontrol, and its effects on the host have been studied via visual assessment in a range of host species. However, little research was performed to address fungal effects on the physiological processes of non-target woody plants. We investigated the occurrence of visual and non-Visually recognisable perturbations of VN infection on potted vines to evaluate the potential risks of the biocontrol pathogen on viticulture. Eighteen four-years-old *Vitis vinifera* (cultivar Grüner Veltliner grafted on Kober 5BB) potted plants were inoculated with VN conidial suspension of the fungus (F), while nine plants were treated with sterile water (C, control). Disease symptoms and physiological parameters were monitored throughout the experiment (seven evaluation dates), while leaf water potential, leaf mass per area (LMA) and biomass were measured at the end of the study when plant tissue was sampled for re-isolation of the fungus. In our trial, inoculations with VN induced characteristic wilting symptoms only in *Ailanthus* (used as side control of the inoculum), while vines remained asymptomatic, thus indicating a high degree of host specificity of VN. Limited or no impact was detected on the physiology of the non-target *V. vinifera*. Furthermore, the LMA and biomass measured in the two experimental groups were not different. Although fungal colonisation induced vascular discolouration in both species, the fungus could only be re-isolated from dying *Ailanthus* but not from vine tissue. Results suggest that *V. vinifera* cv Grüner Veltliner is resistant to the applied VN isolate. However, the susceptibility and physiology of additional grapevine cultivars, as well as other native woody species to VN, should be studied before promoting large-scale use of the biocontrol agent.

**KEYWORDS:** non-target effects, wilt disease, biological control, *Ailanthus altissima*, fungal pathogen, invasive species
INTRODUCTION

The invasion of natural habitats by alien species threatens ecosystem conservation. *Ailanthus altissima* (Mill.) Swingle (tree of haven, family Simaroubaceae, order Sapindales, henceforth mentioned as *Ailanthus*) is one of the most invasive and globally widespread trees. The species, native to Southeast Asia, was intentionally introduced worldwide starting from the 18th century and due to its fast growth, vegetative propagation, high seed production, high phenotypic plasticity, and competitiveness, it caused significant ecological, economic, and social negative impacts (Sladonja et al., 2015; Petruzelli et al., 2019; Martí-Garrido et al., 2020). In particular, the massive spread of *Ailanthus* recorded in the last 25 years suppressed the regeneration of indigenous species, altered natural tree composition and disrupted many native forest ecosystems (Kowarik and Säumel, 2007; Sladonja et al., 2015). *Ailanthus* has also been recorded in perennial cropping systems as vineyards, where its control poses significant challenges, particularly in the case of organic vineyards, where herbicides are not allowed (EPPO, 2020).

Various mechanical, chemical or combined strategies have been tested to stop the spread of *Ailanthus*, eradicate it, prevent further diffusion of the pest and restore the natural cover (Ließ and Drescher, 2008; Sladonja et al., 2015). However, the applied strategies are frequently laborious, expensive, and often not efficient and effective due to the high re-sprouting capacity of *Ailanthus* (EPPO, 2020). The wilt-inducing fungus *Verticillium nonalfalfae* (family Plectosphaerellaceae, order Hypocreales, henceforth VN) has been proposed as a biological method against *Ailanthus* and has given promising results. VN is a soilborne fungus which grows as an endophyte in the xylem of a range of host plants (Robb, 2007) but can turn pathogenic in some host plants (Robb, 2007) and can turn pathogenic in some of them, causing *Verticillium* wilt (Schall and Davis, 2009; Kasson et al., 2014; Kasson et al., 2015; Maschek and Halsmchlager, 2018; Brooks et al., 2020). After infection, the spores diffuse in the vascular tissue favoured by the transpiration stream, where they germinate and form local mycelium aggregations. The first symptoms of leaf wilting and necrosis are usually seen after a few weeks to a month after infection, depending on the tree species and size (Kasson et al., 2015). Vulnerable species may succumb to the pathogen, showing extensive dieback of leaves and branches, which is frequently followed by death.

Many VN inoculation studies on natural stands and potted saplings of *Ailanthus* were performed worldwide (Kasson et al., 2014; Maschek and Halsmchlager, 2017; Maschek and Halsmchlager, 2018; Brooks et al., 2020; Moragrega et al., 2021) to confirm its pathogenicity. In particular, Kasson et al. (2014) inoculated with *V. nonalfalfae* conidial suspension 100 *Ailanthus* trees in different natural stands. After five years, thousands of canopy trees and sprouts of this species desiccated, thanks to the spread of the pathogen from infected individuals to non-infected ones via root grafts (O’Neal and Davis, 2015; Dubach et al., 2021).

Potential risks that the pathogen may bear for non-target native trees and agricultural crops should be deeply addressed before promoting its broad use in ecosystems (Dauth et al., 2022). The majority of recent studies have addressed visible symptoms of VN infection in native species co-occurring with *Ailanthus*, like leaf wilting, chlorosis, and wood discoloration (Kasson et al., 2014; Kasson et al., 2015; O’Neal and Davis, 2015; Maschek and Halsmchlager, 2018; Brooks et al., 2020; Dauth et al., 2022; Lechner et al., 2023). Brownish vascular discolorations were observed in almost all studied genotypes and indicated successful transmission of inoculum into the xylem, resulting in defence reactions (Kasson et al., 2015; Maschek and Halsmchlager, 2018). Since extensive leaf wilting and mortality occurred mainly in *Ailanthus*, and other physiological parameters were not addressed, native species were denoted as tolerant or resistant to the pathogen (Kasson et al., 2015; Maschek and Halsmchlager, 2018; Dauth et al., 2022; Lechner et al., 2023). To the best of our knowledge, no trials were performed to explore whether plant processes on a deep physiological level (eventual shifts in photosynthesis, water relations, biomass allocation) are somehow affected by the infection with VN. These remain largely unknown and need further investigation to elucidate the vulnerability/resistance responses of different plant species to the biocontrol agent.

To make a step forward in this direction, the present study addressed visible and invisible physiological (gas exchange, leaf temperature, water status) perturbations of VN on a non-target woody species, i.e., *Vitis vinifera*, through artificial stem inoculations on potted plants. Biomass allocation under the biotic stressor or in the absence of it was also investigated, and the resistance degree of the specific cultivar-rootstock combination to the biocontrol agent was characterised. We hypothesised that gas exchange and photosynthesis decrease due to initial fungal spread in the xylem and consequent plant defence mechanisms but with no significant changes in biomass in the medium term.

MATERIALS AND METHODS

The experiment was carried out in the greenhouse of the University of Natural Resources and Life Sciences Vienna (Austria). In total, 27 four-year-old grapevines of the cultivar Grüner Veltliner grafted on Kober 5BB were used as plant material. We selected *V. vinifera* because it is an economically important species with vineyards often invaded by the alien tree *Ailanthus* (EPPO, 2020). The vines were planted in 20 l pots filled with a mixture of commercial potting media and perlite (70:30 v/v). In late winter, the pots were randomly disposed on a greenhouse bench and connected to automatic drip irrigation. To each pot, 10 g of a slow-release fertiliser was added (ENTERC vino, EuroChem Agro GmbH). Besides the three basic plant macronutrients, i.e., potassium (20 %), nitrogen (15 %), and phosphorous (5 %), the fertiliser also contains sulphur (8 %) and magnesium (2 %). The temperature and relative humidity of the greenhouse chamber were set to 22 °C and 50 %, respectively.
The plants grew at 12 h day and 12 h night period cycles and were maintained well-watered throughout the experiment by providing 300–600 mL of water per pot and per day (substrate near to field capacity). After budbreak, two shoots per vine were allowed to develop for two months (establishment phase). All plants were thus standardised to eight leaves per shoot (about 45 cm long at that time) by trimming the apex to uniform the leaf area across treatments.

One week after canopy standardisation (DPI 0, days post inoculation), the vines were subdivided into two groups with random selection. Plants of the first group were inoculated with the bioherbicde “Ailantex”, containing a conidial suspension (10⁶ spores/ml) of the isolate Vert56 strain G1/5 of VN (F, n = 18), whereas those of the second group were inoculated with deionised sterile water (C, control, n = 9). The inoculation was performed at noon on the stem about 5 cm above the root collar (below the grafting point) using a woodcarving gouge and following the protocol described in Maschek and Halmschlager (2016). The spores diffused in the plant’s vascular system with the transpiration stream. To confirm the viability of the used inoculum, four potted Ailanthus altissima plants were also inoculated with the V. nonalfalfae conidial suspension and placed among the grapevines. To avoid any influence with the VN treatment, plant protection was performed by spraying only copper and sulphur on the leaves surface, avoiding systemic fungicides.

A picture of each plant was taken at the beginning (DPI 0) and at the end (DPI 100) of the experiment to visually evaluate their health status. Moreover, plants were carefully examined for foliar symptoms to track eventually occurring symptoms of wilt disease during the whole experiment.

To investigate the possible occurrence of non-visual perturbations of the fungus on deep physiological processes involved in plant metabolism, physiological traits were monitored throughout the experiment. In particular, leaf temperature (Tₙₐₜ), sub-stomatal CO₂ (ci), stomatal conductance to water vapour (g) and net photosynthesis (A) were regularly measured. The measurements were performed on one to three mature (depending on data variability within and among treatments) healthy leaves per plant using a portable gas-exchange system (LCpro-SD, ADC BioScientific Ltd., Hertfordshire, UK) with a light source set at 1000 µmol m⁻² s⁻¹ and ambient CO₂ (about 430 ppm) and humidity. On seven dates (DPI 7, 13, 20, 32, 48, 63, 73), all nine control plants (C) and 15 randomly selected inoculated plants (F) were measured between 12 AM and 2 PM (see Figure 3). The photosynthetic photon flux density (PPFD) in the greenhouse chamber during the measurements ranged between 200 and 700 µmol m⁻² s⁻¹. A first measurement of physiological parameters was also performed before the inoculation (DPI 0) on 12 randomly selected plants.

To evaluate the water status of plants, at DPI 73, the minimum leaf water potential (Ψₑₑₑₑₑₑₑₑₑₑₑₑₑₑ) was measured on nine vines per experimental treatment. At 2 PM, one mature leaf per plant (used for physiological measurements) was sampled from the terminal end of the shoot, immediately wrapped in plastic film and inserted in a sealed plastic bag containing wet paper. The leaves were transported to the laboratory using a cool bag, and leaf water potential was measured with a Scholander pressure chamber (3000 Series Plant Water Status Consoles, Soilmoisure, Santa Barbara, CA) within 1 hour after sampling.

To verify the eventual effects of the fungus on biomass allocation, leaf mass per area (LMA) and aboveground plant biomass were measured at the end of the experiment (DPI 100). Two leaves per plant were detached from at least nine plants per experimental group, and their leaf area was measured with a scanner (A₁ₑₑₑₑₑₑₑₑₑₑₑₑₑₑ). The leaf dry biomass (DMₑₑₑₑₑₑₑₑₑₑₑₑₑₑ) was recorded after overnight drying at 70 °C, and LMA was calculated as DMₑₑₑₑₑₑₑₑₑₑₑₑₑₑ/A₁ₑₑₑₑₑₑₑₑₑₑₑₑₑₑ. After the collection of samples for fungal re-isolation (see below), the rest of the aboveground portion of each plant was dried in the oven (70 °C), and biomass was measured after 96 h.

At DPI 100, three about 2 cm long sections of the stem were cut from each grapevine using sterile pruning shears. The vascular discoloration was visually evaluated, and samples were then cultured for re-isolation of the pathogen according to the protocol described in Maschek and Halmschlager (2017). In particular, a piece of rootstock, a piece of the shoot at the inoculation site, and a piece of young non-lignified shoot developed after inoculation were sampled. To confirm the pathogen colonisation of Ailanthus, the stems of four plants were also sampled for fungal re-isolation.

Statistical analyses were performed with R (v 3.6.3) software. The effect of fungal inoculation was tested separately for each parameter. The normality of residuals and homogeneity of variances assumptions were verified using the ‘qqPlot’ and ‘leveneTest’ functions (‘CAR’ package), respectively. The General Linear Model was used to determine whether the means of the two groups of data (F and C) differ (the ‘glm’ function in the ‘lme4’ package). Each trait was considered as a response variable, while the treatments (C and F) were treated as explanatory variables (factors). Results were considered statistically significant at P ≤ 0.05. All values are given as mean ± standard error of the mean (SEM).

**RESULTS AND DISCUSSION**

To the best of our knowledge, this is the first trial performed to investigate, besides visible symptoms, plant processes on a deep physiological level triggered by the infection with VN in a non-target woody species. The results are promising and add value to the European VN strain Vert56 as a biocontrol agent of Ailanthus for future nature conservation actions.

Ten days after infection with VN conidial solution, the lower leaves of Ailanthus plants that were placed between the experimental vines started to wilt. Two to three weeks after inoculation, extensive leaf wilting followed by chlorosis was exhibited by 100 % of Ailanthus seedlings (Figure 1), confirming the efficacy of the inoculum. After about one month, the leaves of Ailanthus were dropped, and the main stem showed necrotic lesions. Subsequently, some buds on
the lower part of the stem resprouted, but as expected, the small newly developed leaves also wilted soon. In contrast, the infected grapevines did not develop visually recognisable symptoms of Verticillium wilt disease but maintained green and exhibited apparently healthy foliage until the end of the experiment (Figure 1), suggesting resistance of the species/cultivar used to the pathogen. Regardless of these results, the sapwood of inoculated *Ailanthus* exhibited typical yellowish/orange vascular discoulourations or was already brownish/necrotic, as described in Maschek and Halmschlager (2018). In contrast, the vine showed dark brownish vascular discoulourations that were only confined to the section near the point of inoculation but were also found on the controls and are, therefore, considered as a reaction to wounding (Figure 2). Re-isolation of VN was successful in the stems sampled from dying *Ailanthus*, but the pathogen could not be cultured from grapevine tissue. Similarly, in a recent study, the pathogen could also not be re-isolated from samples of *Fraxinus pennsylvanica* and *Robinia pseudoacacia*, i.e., two non-target species (Maschek and Halmschlager, 2018). These results suggest that in the grapevines, the inoculated fungus was likely neutralised and killed in the short to medium term. It was not the aim of our study to verify whether the defence mechanisms of the vines played a significant role in this process since the production of phenolic compounds and other secondary metabolites involved in plant response against biotic stress (Morris et al., 2020) was not measured. However, some fungi (e.g., *Aureobasidium* sp.) which are known to produce antifungal substances were found in the culture of sampled tissue, indicating a possible role of the plant-endogenous fungi in the vine’s defence against VN (Dimakopoulou et al., 2008; Zeilinger et al., 2016). The latest results suggest again the resistance of the tested grapevine cultivar to VN.

**FIGURE 1.** *A. altissima* potted plant with chlorotic leaves three weeks after inoculation (a). One experimental potted grapevine at the end of experiment (DPI 100) showing green and healthy foliage (b).

During physiological measurements, the leaf temperature (T$_{leaf}$) ranged between 25 °C and 33 °C, with the highest peaks recorded on spring days characterised by higher sun irradiance penetrating the greenhouse. T$_{leaf}$ in the two treatments was almost the same in each of the seven days of measurements, and it differed by at most 0.9 °C (P > 0.05, data not shown). In comparison to other similar greenhouse studies, the T$_{leaf}$ recorded was moderate (Savi et al., 2019; Savi et al., 2021) as a likely consequence of the well-watered status in which the plants were maintained throughout the experiment to exclude eventual effects of drought, which could influence the fungal spread, the plants’ gas-exchange, and their response to the pathogen. Hence, as expected, at DPI 73, the favourable water status was reflected in an average Ψ$_{leaf}$ of –0.60 ± 0.04 MPa (Girona et al., 2006, data not shown). No differences were observed between the C and F.

**FIGURE 2.** Discolorations of the sapwood of a grapevine plant inoculated with *V. nonalfalfae*. Sampling points where wood tissue was removed for fungus re-isolation can be observed (white arrows).

**FIGURE 3.** Transpiration rate (E$_L$) and net photosynthesis (A) measured in the C and F groups throughout the experiment. The values confirmed a favourable water status of plants and ranged between 2.2 ± 0.2 mol m$^{-2}$ s$^{-1}$ and 4.2 ± 0.4 mol m$^{-2}$ s$^{-1}$, and 8.5 ± 0.6 and 11.8 ± 0.4 µmol m$^{-2}$ s$^{-1}$ for E$_L$ and A, respectively (Herrera et al., 2017; Zufferey et al., 2017). The two treatments were apparently performing differently in terms of E$_L$ on DPI 20 and DPI 63, and in terms of A on DPI 73 (Figure 3). On these specific dates, the inoculated plants showed slightly lower values of physiological parameters (by about 20 %) compared to control ones, but in this respect, the high error of data has to be considered. In particular, the difference detected on DPI 20 and DPI 73 (P = 0.047) was due to one low value recorded in the F group (i.e., 0.96 mol m$^{-2}$ s$^{-1}$ and 3.0 µmol m$^{-2}$ s$^{-1}$ for E$_L$ and A, respectively), which could not be considered outlier, but tip the balance toward statistical significance. Overall, a clear trend towards generally lower values of E$_L$ and A in F plants over the entire duration of the experiment could not be observed. The stomatal conductance to water vapour (g$_{st}$) and sub-stomatal CO$_2$ (ci), which are intrinsically linked to E$_L$ and A and usually show similar trends, also confirmed no significative differences (P > 0.05) between control and
infected vines on none of the measurement days. Hence, we suppose that the patchy greenhouse environment in terms of light availability, the plants’ position on greenhouse tables, which inexorably led to some canopy shading, and the relatively low number of replicates might have partially affected the $E_L$ and $A$ measurements on DPI 20, 63 and 73.

Table 1 summarises the results of biomass and leaf mass per area (LMA) measurements. The LMA is often used to study the biomass allocation and productivity in the shoot under stressors since it is positively correlated with carbon investments in secondary compounds such as cell wall constituents (Savi et al., 2016; Baird et al., 2017; Petruzelli et al., 2019; Savì et al., 2019). The LMA of infected plants showed slightly lower values compared to those of the control group (3.8 ± 0.1 and 4.1 ± 0.2 mg cm$^{-2}$, respectively), while the aboveground plant biomass was slightly higher in F than in the C group (77.6 ± 5.7 and 65.5 ± 9.2 g vine$^{-1}$, respectively). However, our second hypothesis was confirmed since none of these parameters was statistically different between treatments, suggesting that the inoculation of the fungus did not cause medium-term effects on biomass allocation.

<table>
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<tr>
<th>Control</th>
<th>Fungus inoculated</th>
<th>P-value</th>
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<tr>
<td>Aboveground biomass (g vine$^{-1}$)</td>
<td>65.5 ± 9.2</td>
<td>77.6 ± 5.7</td>
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<tr>
<td>Leaf mass per area (mg cm$^{-2}$)</td>
<td>4.1 ± 0.2</td>
<td>3.8 ± 0.1</td>
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CONCLUSION

Besides the lack of visually recognisable symptoms of wilt disease, the results of this study show for the first time the absence of clear disturbance in gas-exchange functions in a non-target woody species infected with *Verticillium nonalfalfae*. Furthermore, the unsuccessful re-isolation of the pathogen from any tissue advocates for resistance to VN of the 5BB rootstock and/or the *V. vinifera* Grüner Veltliner cultivar while confirming the high susceptibility of *Ailanthus* (necrotic wood, successful re-isolation). The output of this study, although limited to one season and one cv/rootstock combination, contributes to our knowledge of potential risks associated with VN in nature conservation actions and gives further importance to the pathogen as a valuable biocontrol tool against *Ailanthus*.

Invisible, hidden symptoms of additional grapevine cultivars and rootstocks, as well as other native woody species, after VN infection, should be addressed before promoting large-scale application of the biocontrol agent. The use of a higher number of replicates or measurement days and the check of multi-season response should be promoted in future testing to achieve low variability of data and clear statistical testing. Moreover, the effects of climatic parameters (water shortage, high temperature) on different dyads (host plant species-VN) have to be investigated to verify if fungal pathogenicity and the ability of the plants to cope with it, change under stressful conditions (Zeilinger et al., 2016).

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REFERENCES


