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Association of entomopathogenic fungi with non-grafted grapevine *Vitis vinifera* does not impact plant growth and increases expression of phylloxera responsive defense genes

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ABSTRACT

Grape phylloxera is a sap-sucking insect that poses a major threat to vineyards worldwide by damaging the roots of *Vitis vinifera* and compromising grape production. Addressing this challenge requires sustainable alternatives to chemical control, and entomopathogenic fungi offer a promising biological approach. In this study, quantitative polymerase chain reaction was used to quantify the expression levels of seven grape phylloxera defense-responsive genes in leaves of Cabernet Sauvignon at 24 h and 120 h after root inoculation with two strains of the entomopathogenic fungus *Metarhizium robertsii* (one from France (EF3.5(2)) and one from Germany (EF047)). 24 h post-treatment with each strain, six genes (VvPr1, VvPr4, VvPr3.2, VvChib, VvG1, VvPr 1-Like) were up-regulated. Also, 120 h after inoculation with the strain EF047, VvChib and VvG1 were down-regulated. Furthermore, the ability of these two fungal strains as well as two other *M. robertsii* strains to colonize the rhizosphere and root-endosphere of non-grafted grapevine was evaluated. Grapevines were inoculated via the "watering" method, and colonization was assessed at 26–28, 47–49, and 68–70 days post-inoculation using a culture based-method. Vegetative growth parameters and leaf pigment parameters were measured throughout the bioassay. All strains persisted in the rhizosphere (60–100 % colonization) and to a lesser extent in the root-endosphere (4.8–33.3 %) up to 70 days post-inoculation. No adverse effects of fungal colonization on assessed parameters were observed during the bioassay. These results suggest that *M. robertsii* strains can durably colonize grapevine rhizosphere and root endosphere without detrimental effects and activate early defense responses against root grape phylloxera. The findings highlight the potential of *M. robertsii* rhizospheric association as a component of integrated pest management strategies in viticulture.

1. Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* (Fitch, 1855)) is a major pest threatening global viticulture. By feeding on roots of grapevine *Vitis vinifera* L., this sap-sucking insect induces gall formation, disrupts nutrient uptake, and ultimately compromises vine vigor and productivity (Powell et al., 2013). Since the late 19th century, when phylloxera devastated European vineyards, management strategies have largely relied on grafting onto resistant rootstocks and, to a lesser extent, chemical control (Granett et al., 2001). However, vineyards in several countries, such as Australia and Argentina, are commonly planted with non-grafted grapevines. Therefore, root infestation by grape phylloxera is prevented through strict quarantine and phytosanitary measures in most of Australian vineyards (Benheim et al., 2012) or by flood irrigation treatment in Argentina, which only partially mitigates pest damage (Arancibia et al., 2018). To that end, alternative crop protection methods are indispensable. Among the promising biocontrol options, entomopathogenic fungi (EPF) such as *Metarhizium robertsii* (Metchnikoff) Sorokin (1883) (Ascomycota: Hypocreales) have attracted growing attention.

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The EPF *Metarhizium robertsii* is a globally recognized biological control agent, used in pest management programs due to its proven effectiveness and broad host range, enabling it to infect more than 200 insect species (Brunner-Mendoza et al., 2019; St. Leger and Wang, 2020). Additionally, it can also grow in the rhizosphere as well as an endophyte without causing apparent disease symptoms (Hu and St. Leger, 2002), colonizing a wide range of plant tissues in both monocotyledons (e.g., wheat *Triticum aestivum* L. and sweet corn *Zea mays* subsp. mays L. (Behie and Bidochka, 2014; Ahmad et al., 2020a)) and dicotyledons (e.g., tomato *Solanum lycopersicum* L., French bean *Phaseolus vulgaris* L., and strawberry *Fragaria x ananassa* Duchesne (Garcia et al., 2011; Barelli et al., 2018; Canassa et al., 2020)).

M. robertsii rhizospheric and endophytic associations provide many benefits to plants such as plant growth stimulation, plant priming, plant disease antagonism, and insect parasitism decrease (Jaber and Ownley, 2018). Its influence on plant growth can occur through two main mechanisms: directly, by modulating hormone balances and plant metabolism (Liao et al., 2017a; Ahmad et al., 2022), thereby affecting plant physiology; and indirectly, by alleviating biotic and abiotic stresses (Dara, 2019). For instance, *Metarhizium* spp. has been shown to secrete siderophores, which can enhance plant nutrient acquisition (Krasnoff et al., 2014; Sánchez-Rodríguez et al., 2016), such as iron in melon *Cucumis melo* L., cucumber *Cucumis sativus* L., and sorghum *Sorghum bicolor* (L.) Moench grown in calcareous soils (Raya-Díaz et al., 2017; García-Espinoza et al., 2023b). It can also transfer nitrogen derived from parasitized insects in a readily metabolizable form to multiple plant species (Behie et al., 2012, 2017).

As a rhizosphere and endophyte colonizer, the EPF *M. robertsii* has also been shown to activate plant defense systems at different stages of its colonization (Ahmad et al., 2020a, 2022; Hu and Bidochka, 2021). This activation establishes a "primed" defense state in the plant, enabling a faster and stronger response to various biotic and abiotic stresses (García-Espinoza et al., 2023b). For instance, elevated salicylic acid concentrations have been quantified in maize roots during *M. robertsii* colonization (Rivas-Franco et al., 2020; Ahmad et al., 2022).

So far, few studies have investigated the potential of EPF as rhizosphere colonizers and endophytes in grapevine *V. vinifera* to control pests and diseases. The EPF *Metarhizium pinghaense* Chen and Guo, 1986 was identified as a natural endophyte of *V. vinifera* var. Carbanate Gernischet in Yunnan, China, based on molecular analysis of whole-plant samples (Jayawardena et al., 2018). Also, *Metarhizium brunneum* Petch, 1931, *M. robertsii* and *Metarhizium guizhouense* Chen and Guo were found to inhabit the rhizosphere of grapevine *Vitis* spp. sampled in the Willamette valley in Oregon (Fisher et al., 2011). *Beauveria bassiana* (Balsamo) Vuillemin, 1912 strains ATCC 74040 and GHA were established endophytically in *V. vinifera* leaves via foliar application, which demonstrated a significant reduction in both downy mildew *Plasmopara viticola* (Berk. & Curt.) Berl. and De Toni, 1888 disease severity and infestation of piercing-sucking insects such as *Planococcus ficus* (Signoret, 1855; Rondot and Reineke, 2018, 2019). Two *M. robertsii* strains, one native to a French vineyard (EF3.5(2)) as well as a laboratory strain expressing a green fluorescent protein (ARSEF-2575-GFP), were shown to be successfully associated in the rhizosphere and root-endosphere of non-grafted grapevines *V. vinifera* and persisted up to 98 days post-inoculation. However, *M. robertsii* strain EF3.5(2), collected from the vineyard soil, had better endophytic capacities toward grapevines than the transformed strain, which was non-native to vineyard soil (Ponchon et al., 2022) . In addition, *M. rbertsii* strain EF3.5(2) associated with the rhizosphere of non-grafted grapevine significantly reduced the infestation and subsequent damages caused by root-feeding grape phylloxera compared to non-inoculated grapevines, emphasizing the antagonistic potential of this strain (Ponchon et al., 2024).

For future use of EPF as biocontrol agents against grape phylloxera or other pests and diseases in grapevine, it is essential to select fungal strains that can persistently associate with the plant and which are adapted to specific local conditions, enhancing their chances of success of permanent establishment in the introduced environment (López Plantey et al., 2019). Therefore, this study assesses four *M. robertsii* strains that were collected during distinct sampling campaigns in vineyard soils in four wine-producing regions with substantially different abiotic conditions (center-west of Argentina, south of Australia, south-west of France, south-west of Germany) (Poidatz et al., 2018; Uzman et al., 2019; Korosi et al., 2019; López Plantey et al., 2019). These four fungal strains were found to inhabit vineyard soils; however, their interaction with the grapevine plant had not yet been characterized. Each of the strains was compared for their association and persistence in the rhizosphere and root-endosphere of non-grafted grapevine *V. vinifera* plants. We also assessed the impact of an endophytic establishment on several grapevine growth features and leaf pigment content. Finally, expression of the gene *Vv*WRKY-75 involved in the grapevine salicylic acid mediated defense response (Welter et al., 2017) and six *PR* genes (*Vv*PR1, *Vv*Pr1-Like, *Vv*Pr3.2, *Vv*Pr4, *Vv*G1, *Vv*Chib), previously reported to be involved in the grapevine defense against radicicole grape phylloxera (Wang et al., 2019), were assessed in leaves 24 h and 120 h after inoculation with two *M. robertsii* strains. The hypotheses tested here were: (i) *M. robertsii* strains native to contrasted environments have a differential potential of association with grapevine; (ii) the inoculation of *M. robertsii* does positively affect vegetative grapevine growth features and leaf pigment content; and (iii) *Vv*WRKY-75 and *Vv*PR1, *Vv*Pr1-Like, *Vv*Pr3.2, *Vv*Pr4, *Vv*G1, *Vv*Chib are up-regulated after *M. robertsii* inoculation in grapevine leaves.

2. Material and methods

2.1. Fungal material

Four native *M. robertsii* strains were used for the experiments: (i) French strain EF3.5(2) (GenBank accession number: PV682335) collected in 2015 from the soil of experimental INRAE vineyard La Grande Ferrade in Villenave-d'Ornon (N 44°47′30.4″ W 0°34′36.9″) (Poidatz et al., 2018), (ii) Australian strain M224B (GenBank accession number: PV682332) sampled in 2014 from vineyard soil in the Yarra Valley, Victoria (Korosi et al., 2019), (iii) Argentinian strain MsoilAR4.3 (GenBank accession number: PV682334) sampled in 2014 from vineyard soil in distrito Las Casitas in Mendoza (S 33°0′5.119″ W 68°0′54.521″) (López Plantey et al., 2019) and (iv) German strain EF047 (GenBank accession number: PV682333) isolated in 2020 from vineyard soil of Kellersgrube in Geisenheim (N50°0′20.16″ E7°58′45.48″). Strains were isolated from samples taken in the inter-row of vineyard plots using the insect bait technique invented by Zimmermann (1986). The strain's genetic identity was established by sequencing a partial sequence of the gene coding for the translation elongation factor 1-alpha (EF-1α). Fungi were grown in a Petri dish with oatmeal agar medium (Fisher Scientific, Waltham, USA) supplemented with 100 mg l^{-1} chloramphenicol (SIGMA Aldrich, Saint-Louis, USA). From each Petri dish, a 5 ml fungal suspension with a concentration of 1 x 10^8 conidia. ml^{-1} was prepared by suspending the conidia in a sterile solution of 1/8 Ringer solution and 0.02 % Tween 80® as wetting agent (Polysorbate 80, SIGMA Aldrich). These conidial suspensions were used to mass multiplicate *M. robertsii* in sweet corn. Under sterile conditions, 80 g of drained organic sweet corn (Bio Village, Marque Repère, Ivry-Sur-Seine, France) was filled in a 75 ml tissue culture flask. The corn was watered with 5 ml of conidial suspension and agitated. Containers were kept in the dark for 14 days at 25 °C. After incubation, corn was immersed in 250 ml of sterile solution of 1/8 Ringer solution and 0.02 % Tween 80® and left for 1 h, after which the fungal suspension was recovered in a 1 l bottle with a sieve and a funnel. The concentration of the conidial suspension was measured and adjusted at a concentration of 1 x 10^7 conidia.ml^{-1} with a hemocytometer.

2.2. Grapevine plants

Grapevine plants *V. vinifera* cv. 'Cabernet Sauvignon' were obtained from hardwood cuttings planted in a mix of 50 % perlite and 50 % standard substrate. Plants were potted in 2 l pots containing clay/white peat substrate ED73 (Patzer, Sinntal, Germany) and placed in a greenhouse chamber at 22–25 °C. 7-week-old grapevine plants were used for trials, and initial measures of the number of leaves and stem length were performed a day before fungal inoculation. All grapevine plants were protected against powdery mildew 5 days before the inoculation with *M. robertsii* by applying the fungicide 2.26 g l^{-1} Luna® Experience (active ingredients: 200 g l^{-1} fluopyram and 200 g l^{-1} tebuconazole). During the trials, preventive treatments against powdery mildew were applied every two weeks, alternating 0.4 g l^{-1} of Vivando® (active ingredient: 500 g l^{-1} metrafenon) or 2.26 g l^{-1} Luna®Experience. During fungicide applications, potting soil was covered with plastic tarpaulin to avoid the accumulation of pesticide residues in the pot substrate.

2.3. Grapevine inoculation

The « Watering » method previously described by Jaber and Araj (2018) at a concentration of 1 x 10^7 conidia.ml^{-1} was employed for the following experiments as it was successful to induce the endophytic association of several EPF strains. Five batches of 63 plants (315 grapevine plants in total) were inoculated with four different treatments consisting of one of the four *M. robertsii* strains (EF3.5(2), M224B, MsoilAR4.3, EF047) and a control treatment. Grapevines were watered with 50 ml of a 1 x 10^7 conidia.ml^{-1} fungal suspension by spreading the suspension on the planting substrate around the base of the plant. The control treatment consisted of watering the plants with the same quantity of sterile water containing 1/8 Ringer solution and 0.02 % Tween 80®. On each assessment event (26–28, 47–49, and 68–70 days post inoculation (dpi)), a random subset of 21 plants per treatment was harvested to assess the rhizospheric and endophytic association. Assessment events were defined based on the results of several pre-trials previously performed. The 21 plants per treatment used for the last assessment day (68–70 dpi) were also used to evaluate their growth and pigment content throughout their growing period, as described below.

2.4. Evaluation of colonization of grapevine rhizosphere and root-endosphere

Potted grapevines were uprooted, and fine, complete root pieces were randomly cut with scissors. The grapevine rhizosphere was defined as complete fine root pieces weighing 0.5 g, which were gently shaken with forceps, and a thin layer of adhered soil was retained. The grapevine root-endosphere was defined as complete fine root pieces weighing 0.5 g, which were disinfected to eliminate microorganisms present on the outer surface of the roots according to the procedure described below.

The sampled thin roots were cut and placed in hermetically sealed tubes (D x H: 27 × 60 mm and 20 ml volume, ZINSSER POLYVIALS® (Zinsser Analytic GmbH, Eschborn, Germany)), filled up with 4 ml of sterile distilled water and 0.02 % Tween 80® as wetting agent. Samples were mixed using the disrupter TissueLyser II (Qiagen, Hilden, Germany). Two aliquots of 100 µl of the obtained homogenate were spread on two plates containing a selective growth medium prepared according to the modified recipe of Fernandes et al. (2010). This growth medium was composed of. 39 g l^{-1} potato dextrose agar, 0.1 g l^{-1} chloramphenicol, 0.002 g l^{-1} thiabendazole, and 0.15 g l^{-1} cycloheximide (SIGMA Aldrich) filled up to 1 l with sterile water. The resulting plates were kept in the dark at 25 °C for 14 days. Fungal growth was visually assessed under the microscope following the morphological criteria of the conidial shape described by Humber (2012). A plant was evaluated to be colonized by *M. robertsii* if at least one colony of the fungus was observed in one of the duplicate plates.

To assess the endophytic potential of *M. robertsii* in grapevines, the 0.5 g of root pieces were disinfected by dipping them twice in 0.5 % NaOCl and 0.02 % Tween 80® for 2 min, followed by 2 min in 70 % ethanol, and finally rinsing thrice in sterile water. Samples were then cut and placed in hermetically sealed tubes filled with 4 ml of sterile distilled water and 0.02 % Tween 80® (as a wetting agent). The samples were then processed using the previous procedure to detect rhizospheric association.

2.5. Grapevine growth assessment

Various growth parameters were assessed in both inoculated and control grapevines to evaluate the potential impact of endophytic association with four strains of *M. robertsii* on grapevine performance. A marking string was used to identify the last newly formed leaf on the day of *M. robertsii* inoculation, and this process was repeated weekly throughout the experimental period. Measurements, including the number and length of new internodes, the number of newly-formed leaves, and the lengths of the main vein and petiole of newly-formed leaves above the marked leaf, were measured using a graduated ruler weekly until 65 dpi. Additionally, at 29, 36, and 43 dpi, various physiological indicators such as nitrogen balance index (NBI), chlorophyll, flavonol, and anthocyanin pigment content were quantified on the last fully expanded leaf by clipping the leaf with the Dualex® leaf clip sensor (Force A, Orsay, France). Grapevine shoots were pruned at a length of 30 cm at 21 dpi to ensure uniform growth, and the cuttings were dried to measure their dry weight. Following pruning, growth features continued to be monitored, including the growth of the highest germinated bud. At 68–70 dpi, the above-ground portion of the plants was harvested and dried in a drying oven at 80 °C for 70 h to determine dry weight. As described above, a subsample of 1 mg of roots per plant was collected for rhizospheric and endophytic detection. Simultaneously, root samples from 10 different plants per treatment were collected and dried in a drying oven at 80 °C for 70 h. The total number of newly formed leaves and internodes throughout the trial period was calculated, and the cumulative length of internodes (from 8 to 65 dpi), main veins (from 8 to 43 dpi), and petioles of newly formed leaves (from 22 to 43 dpi) was calculated.

2.6. Defense gene expression analysis

2.6.1. Experimental setup

For analysis of defense gene expression, a total of 27 seven-week-old potted grapevine plants were inoculated as described above either with *M. robertsii* strain EF3.5(2) or with strain EF047 or were watered with a sterile 1/8 Ringer solution + 0.02 % Tween 80 aqueous solution as a control. These two strains were chosen because they both had demonstrated endophytic potential of association with grapevine in pre-tests (data not shown here). Each treatment involved nine plants. At 24 and 120 h post-treatment (hpt), the sixth leaf of each grapevine plant was collected, promptly flash-frozen in liquid nitrogen, and stored at −80 °C.

2.6.2. RNA isolation

For RNA isolation, three of the nine leaves collected for each treatment from nine separate plants were pooled to obtain three biological replicates per treatment. Leaves were crushed in liquid nitrogen to obtain a total of ca. 100 mg of crushed plant tissue to be used for RNA extraction. Following the manufacturer's protocol, RNA was extracted from each leaf pool using the Spectrum Plant Total RNA kit (Sigma-Aldrich). Intruder DNA was removed by digestion with 0.8 U DNase (Ambion Inc, Carlsbad, USA), followed by lithium chloride precipitation. RNA purity and quantity were assessed based on the absorbance ratio at 260:280 nm of 1.8–2.08 using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, USA).

2.6.3. qPCR analysis

From the ten genes either associated with the SA-mediated signal pathway of grapevine or with defence against radicicol grape phylloxera as indicated by Wang et al. (2019) (*Vv*Wrky-75, *Vv*G1, *Vv*Gh3, *Vv*Chib, *Vv*CHIB1, *Vv*NPr1, *Vv*Pr1, *Vv*Pr3.2, *Vv*Pr4, *Vv*Pr1-like), seven genes (*Vv*Wrky-75, *Vv*G1, *Vv*Chib, *Vv*Pr1, *Vv*Pr3.2, *Vv*Pr4, *Vv*Pr1-Like) that showed adequate amplification performance were analyzed at 24 h post-inoculation and 120 h h post-inoculation using qPCR (primer sequences available in Supplement Table 1). For normalization of expression levels, two housekeeping genes were used, one coding for actin and one coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Timm and Reineke, 2014). Each RNA sample was diluted to 500 ng μL^{-1} for cDNA synthesis and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Quantitative real-time PCRs were performed on the iQ5 Multicolor iCycler qPCR device (Bio-Rad, Hercules, USA) using the Maxima SYBR Green Master Mix (Thermo Fischer Scientific). Each single cDNA sample was diluted to 1:50 before qPCR analysis. The reaction setup for qPCR was performed in a total volume of 25 μL using 10 μL of cDNA sample as a template, 1 μL of each forward and reverse primer at a concentration of 7.5 μM and 12.5 μl of qPCR Master Mix and filled up with 0.5 μl of nuclease-free water. The cycler program was composed of five steps consisting of 95 °C for 10 min, continuing with 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min and 60 °C for 1 min. For each cDNA template, three independent technical replicates were performed for each primer pair. Quantification cycle (Cq) values were calculated by the iQ5 v.2 software (Bio-Rad). Normalized relative expression levels were calculated based on the expression levels of the two housekeeping genes using the "do my qPCR calculation" web tool developed by Tournayre et al. (2019). Statistical differences in average relative fold expression levels between the treated and control groups were determined through pairwise comparisons utilizing a student t-test at a significance level of <0.05.

2.7. Statistical analysis of the rate of colonization and growth parameters

All analyses were carried out by the R Core Team (2018). The percentage of detection of *M. robertsii* as a root-endophyte or as a rhizosphere-associated fungus with grapevine plants at the three detection time points was compared among all strains using the Chi-squared test. The total number of newly formed leaves and internodes throughout the trial period, the cumulative length of internodes (from 8 to 65 dpi), main veins (from 8 to 43 dpi), and petioles of newly formed leaves (from 22 to 43 dpi), the dry above-ground and below-ground biomass weight were analyzed with the ANOVA model. Fungal strains were the main factor tested to characterize significant differences. The Tukey HSD function was used afterward to compare multiple fungal strains. The NBI index, chlorophyll, flavonoid, and anthocyanin content were analyzed separately for each measurement time (28, 36, 43 dpi). After verification of the hypothesis, ANOVA was used to characterize the significant differences for the plants inoculated with the different fungal strains.

3. Results

3.1. M. robertsii association with grapevine rhizosphere and root-endosphere

Regarding rhizospheric colonization, there were no significant differences in colonization rates between the tested strains at 47–49 dpi ($X^2 = 2.625$, df = 3, p = 0.4531) and 68–70 dpi ($X^2 = 3.1613$, df = 3, p = 0.3674), however at 26–28 dpi, the strain EF3.5(2) showed a significantly lower colonization rate of the grapevine rhizosphere than the other strains ($X^2 = 10$, df = 3, p = 0.01857) (Table 1).

In the case of endophytic colonization, there were no significant differences in colonization rates between the tested strains at 26–28 dpi ($X^2 = 1.2222$, df = 3, p = 0.7477) and at 47–49 dpi ($X^2 = 2.4444$, df = 3, p = 0.4854), however at 68–70 dpi, the strain EF3.5(2) showed a significantly lower colonization rate of the grapevine root endosphere ($X^2 = 9$, df = 3, p = 0.02929) (Table 1).

Plates with root extract homogenates of control plants showed no growth of *M. robertsii,* with the exception of rhizosphere samples of three control plants collected at 68–70 dpi, which was attributed to a procedural error during the preparation of the homogenate.

3.2. Impact of endophytic M. robertsii on grapevine growth and leaf pigment content

At 65 dpi, the number of newly formed leaves was significantly lower in grapevines treated with the strain EF047 compared to all other strains and the control (df = 4; f = 3.062; p = 0.02). However, inoculation with the different *M. robertsii* strains did not significantly affect the grapevine shoot growth, the dry weight of the grapevine above-ground part, and the dry weight of the root part measured at the end of the assay. The same was found for the cumulative main vein length growth of newly formed grapevine leaves measured from 8 to 43 dpi and the sum of the main petiole length from 22 to 43 dpi (Supplement Table 2). Regarding the leaf pigment content measures on the last fully formed leaf, only the mean anthocyanin content measured at 43 dpi in grapevines treated with strain M224B was significantly lower than the ones measured in plants treated with the other strains or the control treatment (df = 4; f = 2.842; p = 0.0413). All other leaf pigment parameters (nitrogen balance index, chlorophyll, flavonol and anthocyanin content at other time points) did not differ between grapevines treated with different *M. robertsii* strains and untreated control plants (Supplement Table 3).

3.3. Effect of M. robertsii on the expression of selected grapevine phylloxera-responsive genes

Among the ten tested primers, seven (*Vv*wrky-75, *Vv*PR1, *Vv*Pr1-Like, *Vv*Pr3.2, *Vv*Pr4, *Vv*G1, *Vv*Chib) showed adequate amplification

Table 1
Time-course of detection of four *M. robertsii* strains (Msoil-AR-4.3, M224B, EF3.5(2), EF047) on grapevine roots. The percentage of grapevines colonized at the rhizosphere and the root endosphere was evaluated via the culture-based method, with the evaluation made at 26–28, 47–49, and 68–70 days post inoculation (dpi). Significant differences in colonization between strains at different time points are indicated by different letters and an asterisk (Chi-square test, $p < 0.05$). Control indicates non-inoculated plants.

	26-28 dpi	47-49 dpi	68-70 dpi	26-28 dpi	47-49 dpi	68-70 dpi
	Rhizosphere colonization			Root endosphere colonization		
MsoilAR-4.3	57.1 % a	81.0 % a	60.0 % a	14.3 % a	14.3 % a	14.3 % a
M224B	47.6 % a	52.4 % a	65.0 % a	9.5 % a	33.3 % a	9.5 % a
EF3.5(2)	4.8 % b	76.2 % a	76.2 % a	4.8 % a	23.8 % a	4.8 % b
EF047	62.0 % a	95.2 % a	100.0 % a	14.3 % a	14.3 % a	33.3 % a
Control	0 %	0 %	14.2 %	0 %	0 %	0 %
p=	0.01857*	0.4531	0.3674	0.7477	0.4854	0.02929*

performance and were used subsequently in qPCR analysis. The combination of the two grapevine housekeeping genes (GAPDH and actin) was adequate as a reference to normalize gene expression (M = 0.401, CV = 0.139).

At 24 h post-inoculation of grapevines with *M. robertsii* strain EF3.5 (2), a significant up-regulation of six analyzed SA signaling pathway marker genes (*Vv*Pr1 (p = 0.00026), *Vv*Pr3.2 (p = 4.67 e^{-07}), *Vv*Pr4 (p = 5.05 e^{-09}), *Vv*Pr1-Like (p = 0.035), *Vv*Chib (p = 0.0079), *Vv*G1 (p = 4.52 e^{-08})) compared to the control plants was evident (Fig. 1). At the same time point, inoculation with *M. robertsii* strain EF047 induced gene expression of six of the analyzed SA signaling pathway marker genes (*Vv*Pr1 (p = 0.000065), *Vv*Pr3.2 (p = 0.005), *Vv*Pr4 (p = 0.0052), *Vv*Pr1-Like (p = 0.0033), *Vv*Chib, (p = 0.0029), *Vv*G1 (p = 1.61 e^{-05})).

M. robertsii strain EF047 significantly down-regulated two SA signaling pathway marker genes *Vv*Chib (p = 0.010) and *Vv*G1 (p = 4.40 e^{-05}) at 120 h post-inoculation.

4. Discussion

Our results demonstrate that all four vineyard-native *M. robertsii* strains persist in the rhizosphere of non-grafted grapevines up to 68–70 dpi, indicating a potential adaptation to the specific grapevine root environment. This persistence may be facilitated by compatibility with grapevine root exudates and the ability to compete with the existing root microbiota, which could explain why native strains often outperform non-native ones as demonstrated in a different cropping system (Klingen et al., 2015). The differences in root colonization between strains, such as EF3.5(2) and EF047 at the initial colonization phase, may reflect distinct colonization patterns, potentially linked to conidial germination rates or the capacity to exploit chemical cues in the rhizosphere, as previously observed in other *Metarhizium* spp. strains (Pava-Ripoll et al., 2011). Furthermore, the use of non-sterile plant material in our study may have influenced colonization rates by introducing microbial competition, highlighting that persistence under natural conditions depends on both fungal traits and the resident microbiome. Overall, these findings underscore the importance of selecting vineyard-adapted fungal strains to enhance rhizospheric establishment.

Our findings also show that all four *M. robertsii* strains were able to endophytically colonize grapevine roots, although with differential colonization potential. The French strain EF3.5(2) exhibited the lowest colonization rate (4.8 %), while the German strain EF047 showed the highest (33.3 %) at 68–70 dpi. The significantly lower establishment of the French strain could be the result of a latent or delayed activation of plant immunity that becomes effective at later stages of colonization. Although we observed an early induction of defense genes at 24 h, which had subsided by 160 h, grapevine immunity could mount a second, delayed wave of defense effectors or biochemical responses at mid-to-late colonization stages, thereby restricting fungal persistence. Another study already observed an upregulation of the Pr5 gene in maize around 60 days upon its colonization with *M. robertsii* inoculated as a seed dressing (). A limitation of our study is that the grapevine defense response was assessed only shortly after inoculation; however, this response may exhibit a later rebound, emphasizing the need for future research to investigate not only the early stages but also longer-term dynamics following inoculation.

Finally, the limited colonization observed might also result from methodological constraints, as only a limited portion of the root system was screened, potentially underestimating true colonization levels. Therefore, future studies should employ more sensitive molecular approaches such as qPCR or *dd*PCR to better characterize the association between EPF and plants (García-Espinoza et al., 2023a; Ponchon et al., 2022).

During the studied period of 70 days, the association of *M. robertsii* had neither negative nor positive effects on grapevine growth, thereby rejecting our second hypothesis. According to Rodriguez et al. (2009), established endophytes *in planta* have a commonly neutral effect on plant physiology and growth. Conversely, several studies have shown that *M. robertsii* endophytic colonization has a positive effect on plant performance when the plant is grown in a stressful environment, as it can enhance tolerance to adverse factors such as salt stress (Khan et al.,

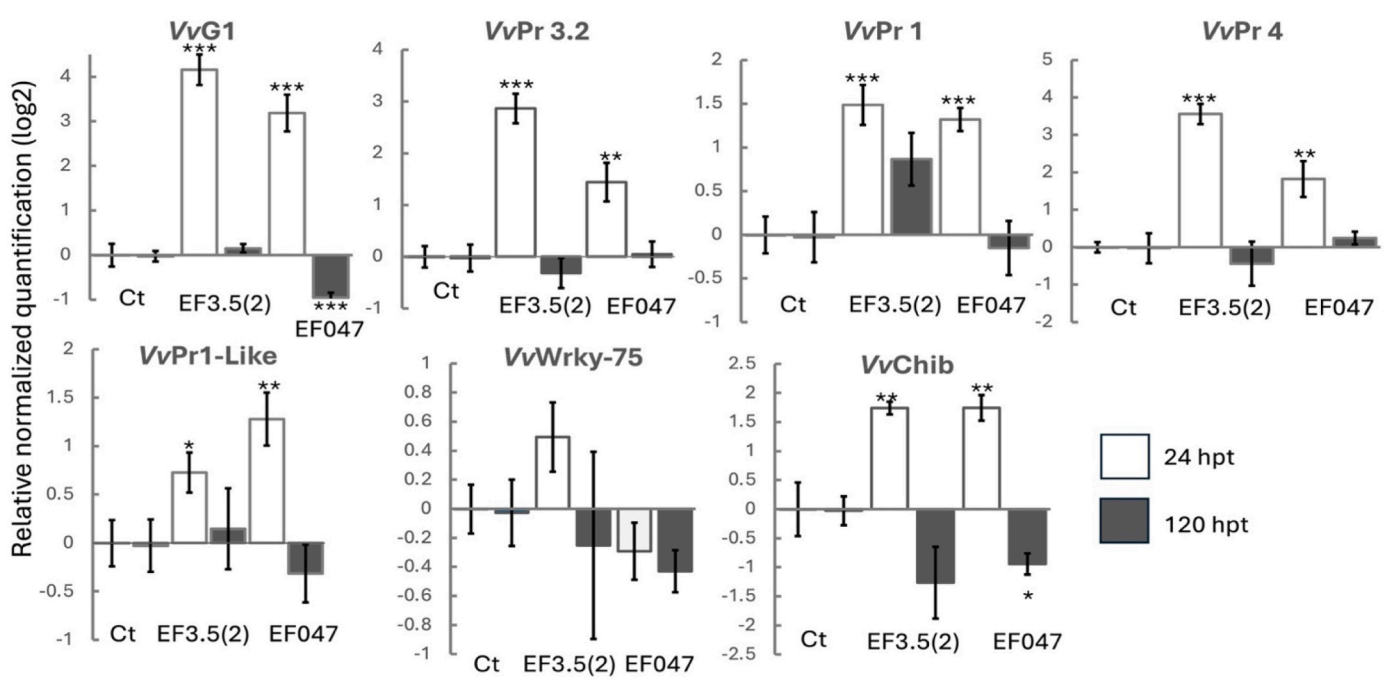

Fig. 1. Relative expression of seven genes involved in grapevine defense response against grape phylloxera analyzed via qPCR and normalized against expression of two housekeeping genes (GAPDH, actin). RNA was extracted at 24 (white bars) and 120 (grey bars) hours post-treatment from grapevine leaves of un-inoculated control plants as well as plants inoculated either with *M. robertsii* strain EF047 or EF3.5(2). Values indicate the mean and the bars indicate the standard error of the mean each calculated from 3 biological replicates per treatment each including leaves from three separate plants. The asterisks above the bars indicate significant differences between the treatment and the control plants (pairwise student t-test; *p < 0.05, **p < 0.01, ***p < 0.001).

2012). In our case, the limited rates of fungal colonization may have been insufficient to provide any measurable benefit to the grapevine plant, or the fungal strains may not have expressed their potential advantages, such as the production of e.g. plant growth-promoting hormones (Liao et al., 2017b). Another plausible explanation is that *M. robertsii* establishment in the rhizosphere did not disrupt the pre-existing microbial community, thereby leaving the recruitment of plant growth-promoting bacteria unaffected (Barelli et al., 2020). Finally, the inoculation method as well as the concentration of fungal spores used could also have limited the successful establishment of the entomopathogenic fungus and therefore its potential growth benefits (Tefera and Vidal, 2009).

Our study showed that inoculation with *M. robertsii* strains EF3.5(2) and EF047 was concomitant with the up-regulation of six defense genes (*Vv*PR1, *Vv*Pr3.2, *Vv*PR4, *Vv*PR1-Like, *Vv*Chib, and *Vv*G1) in grapevine leaves 24 h post-inoculation at the root level. A previous study by Wang et al. (2019) demonstrated a significant up-regulation of the same six genes at the root level of grapevine phylloxera-resistant rootstock 1103 Paulsen '1103P' (*Vitis berlandieri* x *Vitis rupestris*), 24 h after root-feeding phylloxera infestation. These genes are involved in the defense response of 1103P against radicicole grape phylloxera through the salicylic acid-related signaling pathway. Moreover, in their study, the exogenous application of salicylic acid on non-grafted grapevine woody roots resulted in a decreased radicicole grape phylloxera larval survival rate because the larvae failed to establish feeding sites on the roots. Similarly, the endophytic fungus *Trichoderma harzianum* Rifai (1969) primed tomato roots by inducing the overexpression of the marker genes PR1a and PR-P6 at 3 and 6 days post-fungal systemic or local inoculation, leading to an enhanced accumulation of salicylic acid in tomato tissue. Also, after nematode *Meloidogyne incognita* (Kofoid & White (d), 1919) Chitwood, 1949 infection, salycilic acid concentrations in plants pretreated with *T. harzianum* were higher compared with non-pretreated plants. Therefore, the priming effect provided by *T. harzianum* limited the nematodes' invasion. Salicylic acid notably triggers the expression of pathogenesis-related (PR) proteins such as PR-1, PR-2, and PR-5, which can disrupt pathogen activity and make plant tissues less favorable for nematode invasion. At the same time, salicylic acid strengthens the physical and chemical barriers of root cells. It promotes the accumulation of lignin, callose, and phenolic compounds, which thicken cell walls and make it harder for nematodes to penetrate and migrate through the root tissue. Some of these compounds also have toxic or inhibitory effects on nematodes, further reducing their mobility and survival (Martínez-Medina et al., 2017). It would therefore be relevant to investigate whether similar defense mechanisms operate during phylloxera invasion in *M. robertsii*-primed grapevine tissues. Finally, in our study, most genes were overexpressed only 24 h post-inoculation, and PR gene expression declined within 160 h, consistent with the findings of Rondot and Reineke (2019). These results suggest that repeated applications of *M. robertsii* to grapevine may be necessary to protect against subsequent phylloxera attacks and to sustain resistance against the root forms of grape phylloxera.

As all four *M. robertsii* strains collected in different vineyards were persistently associated with the rhizosphere and root-endosphere of young potted grapevines under controlled conditions, it may be necessary to characterize the fungal strains' adaptation to the respective environmental conditions of the different vineyard regions. Thus, the persistence of the association of *M. robertsii* strains with grapevine should be evaluated for a longer time in outdoor conditions on mature grapevines. The vineyard soil texture, temperature, moisture, as well as fungicide applications should be the object of significant considerations that can reduce propagule germination and long-term persistence (Jackson et al., 2010). In addition, the innocuity of *M. robertsii* to beneficial soil invertebrates and herbivores, an important prerequisite for its use as a biocontrol agent, was recently demonstrated for seed-dressed broad beans planted in the field (Novgorodova et al., 2022). If the harmless nature of the four *M. robertsii* strains to non-target macro-organisms in viticulture environment will be proved, their application could represent a new strategy for sustainable grapevine cultivation, offering an environmental-friendly tool for controlling vine soil-borne pests, including grape phylloxera.

Ethical compliance

All procedures performed in studies did not involve human participants or animals.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-4 in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Mathilde Ponchon: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Daciana Papura:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Rodrigo López-Plantey:** Conceptualization, Investigation, Writing – review & editing. **Denis Thiéry:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing. **Annette Reineke:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2025.101222.

Data availability

Data will be made available on request.

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