

**HOCHSCHULE GEISENHEIM UNIVERSITY**

and

**BORDEAUX UNIVERSITY**

**Doctoral School Sciences et Environnement**

**The entomopathogenic fungus *Metarhizium robertsii* and its  
endophytic potential in grapevine to regulate radicolle grape  
phylloxera populations**

Submitted by

**Mathilde Ponchon, M. Sc.**

Born: 01.01.1996

Paris, France

In fulfillment of the requirements of a Doctorate under international joint supervision  
leading to the award of a double degree

**Doktorin der Naturwissenschaften (Dr. rer. nat.)**

and

**Docteur de l'Université de Bordeaux**

**Spécialité Écologie évolutive, fonctionnelle et des communautés**

Submitted in Geisenheim (Germany) on the May 8, 2023

This thesis was accepted on October 27, 2023, as a doctoral dissertation in fulfillment of the requirements for the degree Doktorin der Naturwissenschaften (Dr. rer. nat.) by Hochschule Geisenheim University and Université Bordeaux.

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I declare that this thesis has been composed solely by myself, without the unpermitted aid of others, except where explicitly stated in the text. All text passages taken in letter or in spirit from published writings and all information based on oral statements are clearly referenced as such.

I have adhered to the principles of good scientific practice as laid down in the Statutes of Hochschule Geisenheim University and the University of Bordeaux to ensure good scientific practice in the studies I have carried out, and which are referred to as such in the doctoral thesis.

Place: Geisenheim

Signature: Mathilde Ponchon

Examination Committee:

Chair :	Prof. Dr. Simone Loose, Hochschule Geisenheim University, Germany
Reviewer/Supervisor:	Prof. Dr. Annette Reineke, Hochschule Geisenheim University, Germany
Reviewer:	Prof. Dr. FORNECK Astrid, Universität für Bodenkunde Wien, Austria
Reviewer:	Prof. Dr. REY Patrice, Université de Pau et des Pays de l'Adour, France
Examiner:	Prof. Dr. BIDOCHKA Michael, Brock University, Canada
Examiner:	Dir. u. Dr. MALAUSA Thibaut, Université Côte d'Azur, France
Supervisor:	Dir. u. Dr. Denis Thiéry, Université de Bordeaux, France
Guest:	Dr. Daciana Papura, , Bordeaux Sciences Agro, France

**Title : The entomopathogenic fungus *Metarhizium robertsii* and its endophytic potential in grapevine to regulate radicolle grape phylloxera populations**

**Abstract:**

**Abstract:**

Grape phylloxera *Daktulosphaira vitifoliae* destroyed 30% of the European vineyard by the end of the 19<sup>th</sup> century, attacking the woody roots of the grapevine *Vitis vinifera* (L.). To date, grafting grapevine on American resistant rootstocks is the leading solution to control the pest. However, grape phylloxera is present in most wine-growing soils and remains a threat in regions planted with non-grafted vines. Thus, alternative control solutions are investigated. The entomopathogenic fungus *Metarhizium robertsii* is known for its capacity to parasitize insects. Besides, the fungus can colonize plant rhizosphere and establish as an endophyte (*i.e.*, within plant tissues) in many plant species. The thesis aimed to investigate if *M. robertsii* could persistently associate with the rhizosphere and as an endophyte in non-grafted grapevine *V. vinifera* without harming its growth while preventing the development of the grape phylloxera radicolle forms.

Four *M. robertsii* strains native to four different vineyards were used and came from: i) the center-West Argentina (MsoilAR4.3), ii) the south of Australia (M224B), iii) the south-west of France (EF3.5(2)) and iv) the west of Germany (EF047). Greenhouse experiments were conducted to assess the association between *M. robertsii* and potted non-grafted grapevines. First, the comparison of the kinetic of grapevine rhizosphere and endosphere colonization was made, comparing the strain EF3.5(2) with a laboratory strain non-native to the vineyard (ARSEF-2575-GFP) to evaluate if a vineyard-native strain had a more durable colonization. Droplet Digital PCR (ddPCR), a culture-based method, and confocal imaging of root segments were used to characterize the associations. The strain EF3.5(2) had a higher establishment in the rhizosphere and root-endosphere of non-grafted grapevine than the strain ARSEF-2575-GFP. Both strains were established for up to 96-98 days post-inoculation (dpi) in both compartments. Then, the potential of association with the non-grafted grapevine of all four-vineyard native *M. robertsii* strains was compared. The rates of rhizospheric colonization at 68-70 dpi ranged from 60% for the Argentinian MsoilAR4.3 strain, 65% for the Australian M224B strain, 76% for the French EF3.5(2) strain, to 100% for the German EF047 strain. The rates of root endosphere colonization were 14%, 10%, 5%, and 33% at 68-70 dpi, respectively. No significant differences of rhizosphere and root-endosphere colonization between tested strains were recorded. Also, the fungal association unaffected the grapevine's growth and leaf pigment content during the experimental period. Finally, the effect of direct parasitism of the strain EF3.5(2) on the survival and development of radicolle grape phylloxera clone 'Pcr7' was evaluated in the woody root section bioassay. Depending on the method used to infect the insect, a significant reduction of 90 %, 70 %, and 86% in survival probability was found, and 92%, 89%, and 93 % in reduction of adults' development compared with control treatments. Also, the symptoms induced by grape phylloxera on non-grafted grapevines co-inoculated with *M. robertsii* were reduced by 91% (mean of  $4.8 \pm 8.4$  nodosities) compared with the phylloxera infested-plants (mean of  $52.7 \pm 50.7$  nodosities). The qPCR analysis performed on grapevine inoculated with strain EF3.5(2) showed after 24h, the up-regulation of the *VvWRKY-75*, *VvPR3.2*, *VvPR4*, and *VvG1*, which are phylloxera-responsive defense genes involved in the resistance against phylloxera.

In conclusion, *M. robertsii*'s persistent association with non-grafted grapevine represents an alternative protection strategy against radicolle grape phylloxera, especially for wine-growing regions planted with non-grafted vines. Further field experiments should be carried out to determine the environmental factors affecting the effectiveness and persistence of *M. robertsii* associated with grapevine.

**Keywords :** Biological Control, non-grafted *Vitis Vinifera*, *Metarhizium robertsii*, grape phylloxera, endophytism

**Titre : Le champignon entomopathogène *Metarhizium robertsii* et son potentiel endophyte sur vigne pour réguler les populations de phylloxéra radicicole**

**Résumé :**

Le phylloxéra *Daktulosphaira vitifoliae* a détruit 30 % du vignoble européen à la fin du 19e siècle, en s'attaquant aux racines ligneuses de la vigne *Vitis vinifera* (L.). À ce jour, le greffage des vignes sur des porte-greffes américains résistants est la principale solution de protection contre ce ravageur. Pourtant, le phylloxéra est présent dans la plupart des sols viticoles et reste une menace dans les régions où les vignes non greffées sont cultivées. C'est pourquoi d'autres solutions de lutte sont étudiées. Le champignon entomopathogène *Metarhizium robertsii* est connu pour sa capacité à parasiter les insectes. En outre, il peut coloniser la rhizosphère de plusieurs espèces végétales et s'établir en tant qu'endophyte (*i.e.*, à l'intérieur des tissus végétaux). Cette thèse visait à étudier si *M. robertsii* pouvait s'associer durablement à la rhizosphère et en tant qu'endophyte à la vigne non greffée sans nuire à sa croissance, tout en empêchant le développement des formes radicicoles du phylloxéra.

Quatre souches de *M. robertsii* originaires de différents vignobles ont été étudiées et provenaient du: i) centre-ouest de l'Argentine (MsoilAR4.3), ii) sud de l'Australie (M224B), iii) sud-ouest de la France (EF3.5(2)), iv) ouest de l'Allemagne (EF047). Des essais en serre ont été menés pour évaluer leur association à des vignes en pot non greffées après inoculation. La comparaison des cinétiques de colonisation de la rhizosphère et de l'endosphère de la vigne a été réalisée entre la souche EF3.5(2) et une souche de laboratoire, non originaire du vignoble (ARSEF-2575-GFP). Le potentiel de colonisation par la souche EF3.5(2) était supposé supérieur et plus durable dû à son origine. La Droplet Digital PCR (ddPCR), la mise en culture de tissus végétaux, et l'imagerie confocale de segments racinaires ont permis de caractériser ces associations. La souche EF3.5(2) a été plus détectée dans la rhizosphère et l'endosphère racinaire de la vigne non greffée que la souche ARSEF-2575-GFP. Les deux souches se sont établies jusqu'à 96-98 jours après inoculation (dpi) dans les deux compartiments. Le potentiel d'association avec la vigne non greffée des souches originaires des quatre vignobles a ensuite été comparé. La colonisation rhizosphérique à 68-70 dpi était de 60% des plants testés pour la souche argentine MsoilAR4.3, 65% pour la souche australienne M224B, 76% pour la souche française EF3.5(2), à 100% pour la souche allemande EF047. De même, les taux de colonisation de l'endosphère racinaire étaient respectivement de 14 %, 10 %, 5 % et 33 % à 68-70 dpi. Les souches testées se sont établies sans différences significatives dans les deux compartiments. De plus, l'inoculation n'a pas affecté la croissance de la vigne et la teneur en pigments des feuilles pendant l'essai. Enfin, l'effet du parasitisme direct de la souche EF3.5(2) sur la survie et le développement du phylloxéra radicicole clone 'Pcr7' a été évalué sur tronçons de racines ligneuses. En fonction de la méthode utilisée pour infecter l'insecte, une réduction de 90 %, 70 % et 86 % de la probabilité de survie a été quantifiée et de 92 %, 89 % et 93 % de la réduction du développement des adultes par rapport aux 3 traitements contrôles. De même, les symptômes induits par le phylloxéra sur les vignes non greffées co-inoculées avec *M. robertsii* ont été réduits de 91% (moyenne de 4,8 ± 8,4 nodosités) par rapport aux vignes contrôles (moyenne de 52,7 ± 50,7 nodosités). De plus, l'analyse qPCR réalisée sur la vigne inoculée avec la souche EF3.5(2) montre, après 24h, l'induction des gènes VvWRKY-75, VvPR3.2, VvPR4, et VvG1, qui sont impliqués dans la résistance contre phylloxéra.

En conclusion, l'association durable de *M. robertsii* avec des vignes non greffées représente une stratégie alternative de protection contre le phylloxéra radicicole, notamment pour les régions viticoles où la vigne est cultivée non-greffée. Des futures expériences en vignoble devraient être réalisées afin de déterminer les facteurs environnementaux affectant la persistance et l'efficacité de *M. robertsii* associé à la vigne.

**Mots-clefs:** Lutte Biologique, vigne non-greffée *Vitis Vinifera*, *Metarhizium robertsii*, phylloxera, endophytisme

### **Titel : Der entomopathogene Pilz *Metarhizium robertsii* und sein endophytisches Potential in der Weinrebe zur Regulierung der radicolen Reblauspopulation**

#### **Zusammenfassung:**

Die Reblaus *Daktulosphaira vitifoliae* zerstörte bis zum Ende des 19. Jahrhunderts 30 % der europäischen Weinberge, indem sie die älteren, holzigen Wurzeln von wurzelechten, nicht gepfropften Weinreben (*Vitis vinifera* (L.)) angriff. Bis heute ist das Aufpfropfen von Weinreben auf amerikanische, Reblaus-resistente Unterlagsreben die verbreitetste Lösung zur Bekämpfung dieses Schädlings. Die Reblaus ist jedoch in Böden fast aller Weinbaugebiete vorhanden und stellt in Regionen, in denen nicht gepfropfte Reben angebaut werden, weiterhin eine Gefahr dar. Daher werden alternative Bekämpfungsmöglichkeiten untersucht. Der entomopathogene Pilz *Metarhizium robertsii* ist für seine Fähigkeit bekannt, Insekten zu parasitieren. Neben seiner entomopathogenen Eigenschaft kann der Pilz die Rhizosphäre von Pflanzen besiedeln und sich als Endophyt (d. h. im Pflanzengewebe) in einer Vielzahl von Pflanzenarten etablieren. Im Rahmen der vorliegenden Dissertation sollte untersucht werden, ob sich *M. robertsii* dauerhaft in der Rhizosphäre und als Endophyt auf nicht gepfropften, wurzelechten Weinreben ansiedeln kann, und die Entwicklung der radicolen Form der Reblaus verhindert wird, ohne das Wachstum der Weinrebe zu beeinträchtigen.

Es wurde eine Auswahl von vier *M. robertsii*-Stämmen verwendet, die in vier verschiedenen Weinbaugebieten beheimatet sind: i) im mittleren Westen Argentiniens (Stamm MsoilAR4.3), ii) im Süden Australiens (Stamm M224B), iii) im Südwesten Frankreichs (Stamm EF3.5(2)) oder iv) im westen Deutschlands (Stamm EF047). Mit Hilfe künstlicher Inokulationsmethoden wurde in Gewächshaus-Experimenten die Assoziation zwischen *M. robertsii* und nicht veredelten Topfreben (Cv Cabernet Sauvignon) untersucht. Zunächst wurde die Besiedlung der Reben-Rhizosphäre und -Endosphäre durch den Stamms EF3.5(2) mit der durch einen nicht im Weinberg heimischen Laborstamm (ARSEF-2575-GFP), verglichen, um festzustellen, ob ein im Weinberg heimischer Stamm zu einer dauerhafteren Besiedlung der Rebe führt. Mit Hilfe von Droplet Digital PCR (ddPCR), einer kulturbasierten Methode und konfokaler Bildgebung von Wurzelsegmenten zur Bewertung der Assoziationen wurde festgestellt, dass der im Weinberg heimische Stamm eine bessere Etablierung in der Rhizosphäre und Wurzel-Endosphäre aufwies als der Stamm ARSEF-2575-GFP. Beide Stämme waren für bis zu 96-98 Tage nach der Inokulation (days post-inoculation, dpi) noch in beiden Kompartimenten etabliert. Anschließend wurden die aus vier unterschiedlichen Weinbauregionen stammenden *M. robertsii*-Stämme hinsichtlich ihres Assoziationspotenzials mit nicht veredelten Weinreben verglichen. Es zeigte sich, dass der Anteil an Pflanzen mit erfolgreich besiedelter Rhizosphäre nach 68-70 dpi zwischen 60 % für den argentinischen Stamm MsoilAR4.3, 65 % für den australischen Stamm M224B, 76,2 % für den französischen Stamm EF3.5(2) und 100 % für den deutschen Stamm EF047 lag. Die Besiedlungsrate der Wurzel-Endosphäre betrug 14,3 %, 9,5 %, 4,8 % bzw. 33,3 % nach 68-70 dpi. Zwischen den getesteten Stämmen wurden keine signifikanten Unterschiede bei der Besiedlung der Rhizosphäre und der Wurzel-Endosphäre festgestellt. Die Pilzassoziation hatte auch keinen Einfluss auf das Wachstum der Rebe und den Pigmentgehalt der Blätter während des Versuchszeitraums. Schließlich wurde der Einfluss von direktem Parasitismus des Stammes EF3.5(2) auf das Überleben und die Entwicklung des radikalen Reblausklons 'Pcr7' in einem Bioassay mit holzigen Wurzelabschnitten untersucht. Abhängig von der Methode, mit der das Insekt infiziert worden war, wurde eine signifikante Verringerung der Überlebenswahrscheinlichkeit um 90 %, 70% und 86,1 % sowie eine Verringerung der Entwicklung von adulten Tieren um 92 %, 89 % und 93 % im Vergleich zur Kontrollbehandlung festgestellt. Außerdem wurden die durch die Reblaus induzierten Symptome bei nicht gepfropften Reben, die mit *M. robertsii* infiziert waren, um 91 % reduziert (Mittelwert von  $4,8 \pm 8,4$  Nodositäten) im Vergleich zu den nur mit der Reblaus infizierten Reben (Mittelwert von  $52,7 \pm 50,7$  Nodositäten). Die qPCR-Analyse, die an der mit dem Stamm EF3.5(2) inokulierten Rebe durchgeführt wurde, zeigte nach 24 Stunden eine Hochregulierung von VvWRKY-75, VvPR3.2, VvPR4 und VvG1, bei denen es sich um Abwehrgene der Weinrebe handelt, die auf die Reblaus reagieren und vermutlich am Kontrollmechanismus beteiligt sind.

Zusammenfassend lässt sich sagen, dass die Assoziation von *M. robertsii* mit nicht veredelten *V. vinifera*-Reben eine nachhaltige Pflanzenschutzstrategie gegen die Reblaus darstellt, insbesondere in Weinbauregionen, in denen Reben nicht veredelt angebaut werden, wie z. B. in Australien und Argentinien. In Zukunft sollten Feldversuche durchgeführt werden, um die Umweltfaktoren zu ermitteln, die die Wirksamkeit und Persistenz der *M. robertsii*-Stämme beeinflussen.

**Schlüsselwörter : Biologischer Pflanzenschutz, nicht veredelte *Vitis vinifera*, *Metarhizium robertsii*, Reblaus, Endophytismus**

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This ph.D. project was funded within the Bordeaux-Adelaide-Geisenheim (BAG) international project alliance and partly through the French-German Doctoral College CDFA-03-18.

The ph.D. was prepared for 18 months in the UMR INRAE 1065 Santé et Agroécologie du Vignoble, Centre INRAE Bordeaux Nouvelle-Aquitaine, Villenave d'Ornon and for 18 months in the Department of Phytomedicine of the Hochschule Geisenheim University.



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## Acknowledgments, Remerciements, Danksagung

Firstly, I thank the rapporteurs of the present Ph.D. thesis who agreed to evaluate the manuscript. I also acknowledge the examiners of my Ph.D. thesis.

My Ph.D. journey started on the 2<sup>nd</sup> of March 2020 while navigating rougher waters. Only one week after I arrived at the Department of Santé et Agroécologie du Vignoble, the COVID-19 pandemic gained momentum in France, closing the laboratory for around three months. I was starting literature research and thinking about how I was about to mass-multiply the fungal strains of *Metarhizium robertsii* to inoculate them to cuttings of grapevines, and the experimental design abruptly stopped. This pandemic restrained our access to laboratory infrastructure, delivery of various research materials, and possibilities of face-to-face contact with colleagues. The uncertainty of the situation was substantial. As a Ph.D. project is considered a 3-year marathon, the premature break of my project has generated a lot of questioning and rethinking. I guess this is how I understood that a Ph.D. project is challenging because it raises an unsuspecting number of questions about the future. Only at the last minute does one know if one can carry it out. To continue, the experimental work of this project began timidly in June 2020 with the help of a very passionate Msc. Student Angélique André. I completed 16 months of experiments at the UMR Santé et Agroécologie du Vignoble (SAVE) in Villenave-d'Ornon in the pandemic context. In November 2021, I started a new life and moved to Germany in the small city of Geisenheim. I pursued my research there in the Department of Phytomedicine of the Hochschule Geisenheim University. I am unsure if I'm entirely French or if the German culture has imprinted itself on me.

My first acknowledgments go to my three excellent supervisors, Annette, Daciana, and Denis, for their helpful guidance and support during these three years (and a half) of the Ph.D. project. Thank you for believing in me as a Ph.D. candidate for your project. It grew on me significantly. Thank you to Directeur de Recherche INRAE Dr. Denis Thiéry for the continuous encouragement you gave me every step of the way, in person and during online meetings. You always reassured me during my project's crucial phases, such as the beginning of our experiments on ddPCR and grape phylloxera bioassays, the mid-term of my ph.D. when we anticipated the experimental planning to perform in the Department of Phytomedicine of Geisenheim. You have also generously supported me in asking for a Ph.D. extension and finally for publishing the first article of the Ph.D. Secondly, I thank Dr. Daciana Papura for her unwavering dedication to my Ph.D. project. It was the first Ph.D. project you initiated and supervised, and you succeeded with Brio. Starting a new project on native strains of *M. robertsii* sampled in the vineyard to associate them with grapevine in their rhizosphere is an original and ambitious project offering enormous prospects for biocontrol use. You maintain the dynamics of the laboratory around entomopathogenic fungi for biocontrol, and I hope that it will continue for many years. You



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generously supported me, especially for the first year and a half I have been investigating in the UMR Santé et Agroécologie du Vignoble. You helped me to design and perform the experimental work of the first part of my project and supported every step of the process. You gave me significant training in microbiology, molecular biology, and grape phylloxera rearing and manipulation, allowing me to pursue my project. You have supported me morally in all the critical steps of this project. You also helped me during the isolation period of covid 19 and even invited me for lunch. Finally, all the corrections you made to my thesis manuscript gave a lot of structure to the writing; I am very grateful for this. Vă mulțumesc foarte mult! Last but not least, I thank Prof. Dr. Annette Reineke. I have felt fulfilled in your Department and enjoyed conducting my research under your supervision. The Department of Phytomedicine is a great place to work. You were able to reassure me and give me very sound advice during the crucial stages of the project. I imagine that the deadlines and regulations of a Cotutelle thesis project were also new for you. You accompanied me very well for the serene finalization of my Ph.D. project. You gave me a lot of freedom in my research, allowing me to develop confidence in my abilities. I appreciate your always having a measured and coherent response to stressful questions. Liebe Annette, ich möchte dir ganz herzlich für deine wertvolle Unterstützung und deine sehr klugen Ratschläge danken. Thank you to all three because your enthusiasm and scientific passion led to excellent results. I am sure that our collaboration is not over yet!

Secondly, I would like to acknowledge all the supervising committee members. Thank you Prof. Dr. Isabelle Masneuf-Pomarede, Dr. Rana Haidar, Dr. Rodrigo López-Plantey and Dr. Yvonne Rondot. You each took the time to read my thesis committee reports, immerse yourself in the thesis project, and give valuable advice. I also thank you for taking the time to listen to me.

I will first acknowledge the whole Santé et Agroécologie du vignoble research unit. I don't know if you remember me because I left almost two years ago! I thank Directeur de Recherche and Dr. François Delmotte for recruiting me to his research unit and for listening to me. My memory of SAVE is of a very dynamic research environment with new people and projects coming every month. I appreciate the research themes and the scientific direction of the laboratory. I think these projects align with the actual and future challenges viticulture has to face. Moreover, the diversity of the research profiles created a form of scientific emulation in me, which I liked. More socially, it is a laboratory where you will always find a nice person to share a coffee with ☺. Secondly, I thank Directeur de Recherche Dr. Adrien Rusch, I greatly appreciated your kindness, your listening skills, and the dynamics you brought to the Biodiversity Group "GT BIODIV." It's a little tricky for me to thank everyone, and I'm sure I'll forget some people because I think I've solicited or asked questions to everyone in the unit, and of course, I've received many solutions. So, thank you to all the post-doctoral researchers: Charlotte Poeydebat, Juliette Poidatz, Benoît Laurent, Thomas Perrot, Léa Beaumelle, Célia Bordier, Léa Tison, Sebastian Ortiz-Martinez, Severine Lopez, Lionel Delbac, I am sure I ask all of you many questions on how to succeed and manage my ph.D. project. I also thank

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all the scientists with special thanks to Dr. Agnès Calonnec, Dr. Marie-France Corio-Costet, Dr. Marc Fermaud, and Dr. Jessica Vallance, who took the time to answer my questions.

Thank you to the fantastic technician team of this laboratory. Thank you, Isabelle Demeaux, for always autoclaving my material even when the request was a bit timely and for ordering goods for my experiments. Thanks to Sylvie Bastien, Natalie Ferrer, and Gwenaëlle Comont for the technical advice on experimental microbiology. Thank you to our fantastic greenhouse growers: Sebastian Gambier, Jérôme Jolivet, Lionel Druelle, and Gilles Taris, for preparing my plant cuttings. A special thank you to Cyril Hévin for helping me prepare the *vitro* plants used in my experiments. Thank you to Gilles and Lionel for putting together some furniture and shelves for me. Finally, thank Marie-Christine Médalin and Nadège Cialti for their adequate administrative support.

Kindly acknowledge all Ph.D. students completing their projects during my stay. Ninon Dell'Acqua, Marie Konan, Manon Paineau, Noémie Ostandie, Mathilde Lacombrade, Giovanni Bortolami, Paola Fournier and Enora Bodin. We all helped each other in one way or another. Finally, I thank everyone with whom I shared an excellent time: Marie Laurens, Louisiane Burkart, Eva Courchinoux, Suzy Flamé, Jessie Beslay, Julie Bourg, Melissa Anne... Sorry for those I forgot; my memory fails me.

I secondly acknowledge the Department of Phytomedicine of the Hochschule Geisenheim University. Again, thank you, Prof. Dr. Annette Reineke, for also leading the Department. Thank you to the researchers Dr. Christine Becker, Dr. Yvonne Rondot, and Dr. Moustafa Selim for their generous listening and thoughtful advice. A special thank you to Dr. Maria Schulze-Sylvester for reading the draft of my first publication and giving some determinant pieces of advice. Thank you to Mirjam Hauck and Hubertus Fehres for maintaining the microbiology and entomology laboratory and material with such a good organization. It is always a pleasure to work in these laboratories. I thank Christine Nickel for her organization and kindness; coming to your office is always a pleasure. I thank Harald Schmidt and Winfried Schönbach for several months preparing and maintaining my fast-growing vines. Also, thank you, Winfried, for taking lovely pictures of my Petri dishes! Thank you to my office colleagues for the lovely chat during lunch at the mensa: Clara Böniger, Sophie Wenz, Lena Enderic, Maria Schulze-Sylvester, and Krýstina Korotchova!

I want to acknowledge everyone who helped me with my project's significant steps. Thank you, Dr. Rodrigo López-Plantey, for your unique ideas and advice. Without you, this project may not exist. I am amazed by your published work and that you sampled entomopathogenic fungi throughout Argentina. Your Ph.D. thesis and papers significantly helped me build my investigation. Thank you for taking the time to read my emails and to meet me online several times. You also gave me significant training in microbiology by answering several questions and reading my protocols at the beginning of my project. I hope that the scientific collaboration will continue between the investigators of this project and that the research incomes will be significant.

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I acknowledge Ing. Dr. Lysiane Brocard. Thank you for insisting on giving me training in confocal microscopy imaging. Thanks to you, we have the first empiric proof of the rhizosphere association of *M. robertsii* with grapevine! Also, thank you for your support and listening to me when my mood was very low.

I acknowledge Ing. Marie Massot and Olivier Fabreguette for giving me significant training in molecular biology, especially in ddPCR. Thanks to all the scientific discussions and pre-testing we performed, we developed a method to quantify *M. robertsii* as a root endophyte in the vine. Thank you, Marie, for performing the PCR analysis. Thank you, Olivier, for your massive support during the first part of my Ph.D. You listened to my complaints a lot. You cheered me up at times when my spirits were very low.

I acknowledge Dr. Jessica Vallance for taking the time to discuss some of the scientific results we obtained and giving numerous pieces of advice on how to plan my microbiology experiments. I acknowledge Benjamin Joubard for maintaining the grape phylloxera breeding and helping in the investigations to evaluate grape phylloxera parasitism.

I acknowledge Olivia Herczynski for her precious advice in molecular biology. I appreciate working with you. Thank you for your organization, your method, and your rigor, as well as your pedagogy, in explaining RNA extraction, the CTAB method, and qPCR analysis. Without your help, molecular biology and qPCR would have remained a somewhat mysterious world to me. As I told you, you inspire me with everything in the best German rigor ☺. We work efficiently and pleasurefully in the molecular biology laboratory thanks to you.

I acknowledge all the Master's students that helped me complete my Ph.D. project. Thank you to Angélique André for being present at the first steps of my work to get the first proof of the endophytic capacities of *M. robertsii* with grapevine. Thank you to Manmeet Singh for his great job on grape phylloxera parasitism. Your careful handling of the insects allowed us to carry out these experiments. Thank you, Karima Rahmani, for the impressive Master thesis that you have completed with Daciana and Denis. Thank you to Dea Caca and her professor Dr. Evangelos Berris for coming to Germany and performing your first bioassays in a laboratory with entomopathogenic fungi.

I acknowledge the Bordeaux-Adélaïde-Geisenheim (BAG) international project alliance for financing the Ph.D. project and the French-German Doctoral College CDFA-03-18.

Lastly, thank my parents and sister Alex for their outstanding support and listening. Thank you to Roé and Alessandro for reading my Ph.D. introduction. Thank you to all my best friends for listening to all my complaints via vocal messages on Whatsapp: Morgane, Sarah, Eva, Marine... Thank you to all the international students of Geisenheim for inviting me to drink wine, and coffee, to teach me German, and to party. I found a huge family here.

These acknowledgments are lengthy. I hope you still have the energy and focus on reading the rest manuscript.

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## List of Abbreviations

BCAs = Biological Control Agents  
CI=Confidence Interval  
CTC = Chloramphenicol Thiabendazole Cycloheximide  
Cv.= Cultivar  
DNA= Desoxyribonucleic Acid  
Dpi = Days post-inoculation  
Dpt = Days post-treatment  
DTX = Destruxins  
EPF = Entomopathogenic fungi  
Et= Etylene  
EU = European Union  
FD = Favescence Dorée  
GDD = Growing Degree Day  
GTDs = Grapevine Trunk Diseases  
Ha = hectare  
Hpi= Hours post-inoculation  
ISR= Induced Systemic resistance  
JA=Jasmonic acid  
Mio = Million.s  
Mrd = Billion  
OAC = Oat Agar Chloramphenicol  
OIV = Organisation Internationale de la Vigne et du Vin  
PAMPS= Pathogens associated molecular pattern  
PDA = Potatoe Dextrose Agar  
qPCR = quantitative Polymerase Chain Reaction  
RH= Relative Humidity  
RNA = Ribonucleic Acids  
SA=Salycilic Acid  
SAR= Systemic Acquired resistance  
Spp. = All the species of the genus  
T = Tonne  
USA = United States of America  
UV= Ultra Violet

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# Introduction



## 1-Preamble

In current agrosystems, especially in viticulture, the management of pest populations is highly dependent on synthetic pesticides. The consequences of these chemicals on human health, the environment, and food webs have become critical issues for grape production (Desneux et al., 2007; Geiger et al., 2010). Additionally, the intensive use of synthetic pesticides is responsible for the emergence of resistance to different active ingredients in pest populations (Whalon et al., 2008). Consequently, future crop protection methods must limit their dependence on synthetic pesticides to prevent their detrimental impacts (Barzman et al., 2015).

The European grapevine *Vitis vinifera* (L.) is a susceptible crop to many pests and diseases and therefore requires a high level of pesticide treatments to meet qualitative and quantitative production standards (Fermaud et al., 2016). For instance, 15% of the total chemical products consumed in France are dedicated to grapevine protection, whereas the French vineyard only accounts for 4% of the French agricultural area. Amongst the products used, 80% are fungicide treatments and 12% are insecticides (Mézière et al., 2009; Butault et al., 2010). Therefore, the grapevine is one of the most demanding crops for pesticide consumption. The European project PURE ([www.pure-ipm.eu](http://www.pure-ipm.eu)) demonstrates that biocontrol is an important protection tool that could substitute part of the chemical pesticides currently used in viticulture (Pertot et al., 2017). Through regulatory incentives, the European regulatory framework strongly encourages the development and commercialization of biocontrol products (Lefebvre et al., 2015). As arthropod pests can be responsible for significant vine damage and threaten grape yield and quality, many of the chemical products used in viticulture are insecticides (Pertot et al., 2017). Among the most significant pests, the Grape phylloxera *Daktulosphaira vitifoliae* (Hemiptera: Phylloxeridae) is one of the most emblematic invasive species viticulture has ever encountered. It was first introduced in France in 1861 by the trade of vegetal material with North America and rapidly spread across France and Europe. Grape phylloxera decimated the worldwide wine industry, destroying almost a third of the worldwide vineyard by the late 19th

century and eroding a whole segment of the economy (Pouget, 1990; Thiéry et al., 2013). Following thirty years of research to save the wine industry, the most efficient solution to suppress the damage caused by grape phylloxera was grafting *Vitis vinifera* cultivars on phylloxera-resistant American rootstocks or phylloxera-resistant hybrids between American and European *Vitis* species (Huglin and Schneider 1998). Nevertheless, grape phylloxera is ubiquitous in most wine-growing regions worldwide (Tello et al., 2019); multiple strains classified as ‘biotypes’ were identified (Forneck et al., 2016).

Grape phylloxera is still a serious pest, and significant risks of its resurgence are identified worldwide and include:

- Possible bypass of phylloxera-resistant rootstocks by genetically-evolved virulent grape phylloxera strains. Such as the case of Napa Valley in 1980, the resistance of the hybrid rootstock AxR #1 was surpassed, consequently destroying a large part of the Californian commercial vineyards (Granett et al., 1996). In addition, some other rootstocks (e.g., 5C, 101-14 Mgt, C3309) presented a high level of phylloxera infestations symptoms under laboratory and field conditions (Forneck et al., 2001; King and Rilling 1985; Kocsis et al., 2002)
- Occurrence of gallicole phylloxera as a seasonal pest of grapevines in European commercial vineyards (Forneck et al., 2019)
- Establishment of significant grape phylloxera populations that could have an epidemic impact in non-grafted commercial vineyards of several vine-growing regions worldwide, including Australia and Argentina (Powell et al., 2013; Arancibia et al., 2018).

Management options to prevent these risks are scarce and costly. They mostly revolve around the grafting method, which is not affordable for all winegrowers (Waite et al., 2015). Also, the current pesticides used to control grape phylloxera are non-specific and ineffective (Powell 2008). Therefore, alternative control solutions are needed that regulate grape phylloxera populations in the vineyard. Entomopathogenic fungi (EPF) are naturally ubiquitous in the soil and, in some cases native to vineyard environments (Fisher et al., 2011; Sharma et al.,

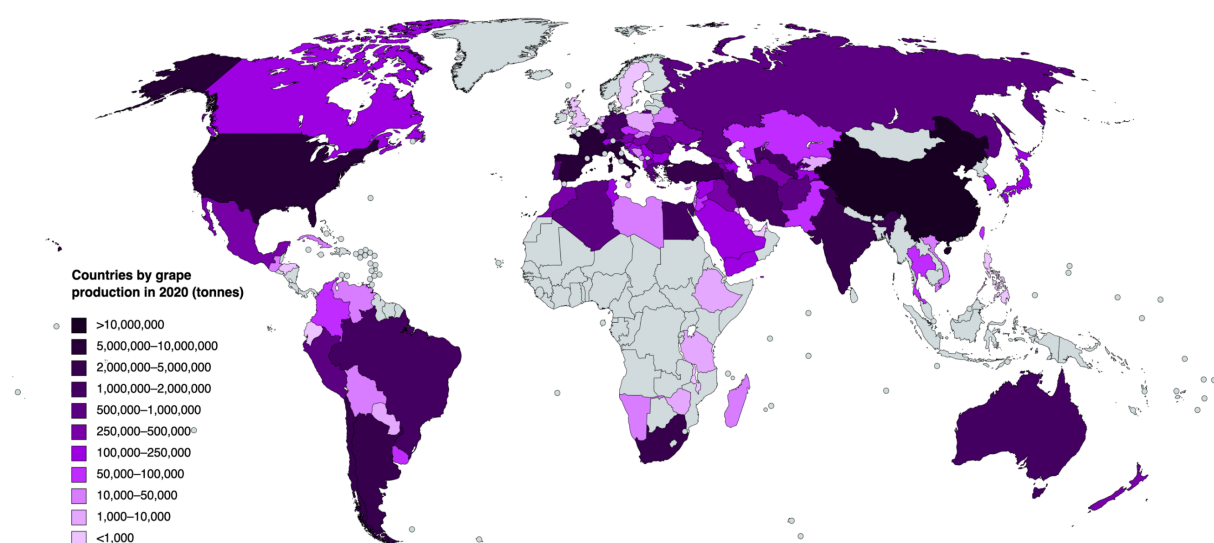
2018; Poidatz et al., 2018; Korosi et al., 2019; López Plantey et al., 2019; Uzman et al., 2019; Moloinyane et al., 2020; Mantzoukas et al., 2020). They could be promising biocontrol agents to control grapevine root pests, as preliminary studies demonstrated a significant control potential toward radicolle grape phylloxera (Kirchmair et al., 2004a, 2004b, 2009). EPF are pathogenic to arthropods, feeding upon them as a nutrient source and usually causing death (Samson et al., 1988). Some EPF strains are specialist to a narrow range of hosts, can persistently establish in the environment, and reportedly do not cause resistance in pest populations compared to synthetic insecticides (Butt et al., 2016). Today, the species belonging to the genera *Beauveria* and *Metarhizium* are the entomopathogenic fungi most widely used for biological control because of their pathogenic effect on arthropods (Faria and Wraight, 2007). However, the efficiency of their utilization in the field has not been systematically demonstrated because of the significant influence of abiotic and biotic parameters. An understanding of the ecology of these fungi is necessary to ensure optimal use conditions and to define the limits of their application for biological control (Lacey et al., 2015).

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## 2-General Introduction

### 2.1. Viticulture worldwide

Viticulture and winemaking represent a vital economic sector for several countries worldwide and affect the sustainability of many local communities (Fraga et al., 2012). The world vineyard area was estimated at 7.3 Mio. hectares (ha) in 2021, including 3.120 Mio of ha located in Europe, according to Eurostat 2022. The total world wine volume produced (exempted from juice and must) is estimated at 260 Mio. hectoliters (hl). The value of worldwide exports is 34.5 Mrd€. In France, the wine sector is the 2<sup>nd</sup> net trade surplus after the aeronautic sector (FranceAgriMer 2022 synthesizing OIV 2022). Therefore, the grapevine is a global commodity plant producing 75 Mio. T. of grapes per year (FAO 2022). It is one of the most economically significant perennial crops worldwide (Figure 1).



**Figure 1** : Map of the world grape production in tonnes (t) per country based on the 2020 data of the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) from [https://upload.wikimedia.org/wikipedia/commons/1/1f/Countries\\_by\\_grape\\_production\\_in\\_2020.png](https://upload.wikimedia.org/wikipedia/commons/1/1f/Countries_by_grape_production_in_2020.png)

### 2.2. Grapevine taxonomy

The European grapevine *Vitis vinifera* subsp. *vinifera* L. domestication started 6000-8000 years ago. It was one of the major fruit crops grown for raisin and wine production that represented two of the most emblematic products of the Near East and Mediterranean agriculture (Zohary et al., 2012).

Grapevine is a dicotyledonous angiosperm plant of the Vitacea grape family composed of 16 genera and around 950 species (Wen et al., 2018). Most of the plants belonging to this family are perennial, herbaceous, or lianescent. They are mainly found in tropical regions such as Asia, Africa, Australia, the Neotropics, and the Pacific islands (Ren et al., 2011). The grapevine (*V. vinifera*) belongs to the *Vitis* genus, composed of approximately 70 species which comprises two sub-genera: *Vitis* (2n= 38 chromosomes) with ca. 65 species and *Muscadinia* (2n= 40 chromosomes) with two species (Wen et al., 2018). The European grapevine is a single species of *V. vinifera* sub-divided into two sub-species: (i) *V. vinifera* subsp. *vinifera*, also known as subsp. *sativa* is considered by the scientific community to be domesticated, and (ii) *V. vinifera* subsp. *sylvestris*, the wild one (Arroyo-García et al., 2006). The domesticated European grapevine is today the most cultivated *Vitis* species, with more than 10000 existing cultivars. The rich cultivar composition is characterized by a high morphological and genetic diversity, and flexibility regarding asexual propagation. This plant is appreciated especially for the attractiveness and the excellent quality of its fruits (Alleweldt and Possingham 1988), mainly because of their attractive color, the size of its berries, their thin skin, and their high sugar content (15-25% per volume) (Zohary et al., 2012).

### 2.3. Grapevine development cycle

*V. vinifera* thrives in a large set of climatic conditions, from a Mediterranean-type climate to a cool and humid climate. Therefore, its natural distribution area is located on the northern fringe of the Mediterranean region (Zohary et al., 2012). *V. vinifera* is cultivated in many climate types, such as oceanic, warm oceanic, transition temperate, continental, cold continental, Mediterranean, subtropical,

attenuated tropical, arid, and hyper-arid climates (Schultz 2016). It is cultivated in regions with mild to hot summers and cool, wet winters with annual rainfall not exceeding 900 mm and being well distributed during the year (Alleweldt and Possingham 1988). The specific thermal and hydrological requirements critically influence the plant development, productivity, yield, and berry composition (Droulia and Charalampopoulos 2021).

The annual development cycle of domesticated grapevine is built around two distinct phases: the vegetative phase, the period of development of grapevine shoots and foliage; the reproductive phase, the period of formation of the reproductive organs (*i.e.*, inflorescence and fruits). The vegetative phase starts during the winter chilling when the buds break dormancy at 50 to 60 Growing Degree Days (GDD) for most grape varieties. This phase takes place optimally at a temperature ranging from 50 to 60 GDD and with a tolerance range of 40 to 92 GDD for some grape varieties. The reproductive phase starts with inflorescence and continues with flower buds' formation at 321 to 414 GDD. After the fertilization of the flower buds, the grapes start forming until the Veraison phase from 908 to 1250 GDD. Finally, the berries ripen when they reach a suitable color and size. The early ripening grape varieties, such as Chardonnay or Pinot Noir, ripen at a range of 1204 to 1317 GDD, and the late-ripening ones, such as Grenache or Syrah, at a range of 1672 to 1940 GDD (cycle detailed in *Supplement 1 – Figure 1*) (Huglin and Schneider 1998; Van Leeuwen et al., 2008; Mosedale et al., 2016; Droulia and Charalampopoulos 2021).

### 2.4. Grapevine pests and diseases management

Grapevine is highly vulnerable to many pests and pathogens, reducing both grape yield and quality and impairing the lifespan of commercial vineyard plots (*i.e.*, Esca complex) (Bettiga 2013). Commercial vineyards suffered from multiple biological invasions consequential of plant material trading, especially from North America (Powell 2008; Sforza 2008; Fontaine et al., 2021). The major grapevine diseases and the most difficult to manage, according to European winegrowers, are:

- (i) the downy mildew (causal agent: *Plasmopora viticola* ((Berk. & M.A. Curtis) Berl. & De Toni)))
- (ii) the powdery mildew (causal agent: *Erysiphe necator* (Schwein.))
- (iii) the grey mold, also called Botrytis (*Botrytis cinerea* (Pers.)) (EIP-AGRI Focus Group 2019).

Because of their high responsibility for grapevine decline, two other grapevine diseases are considered the most destructive:

- (i) the Flavescence Dorée (FD), a severe disease caused by the Flavescence dorée phytoplasma directly transmitted by the leafhopper *Scaphoideus titanus*, reduces the plant's long-term productivity and quickens its decline (Chuche and Thiéry 2014). It is present in restricted area in Croatia, Czech Republic, France, Hungary, Italy, Portugal, Romania, Slovenia and Switzerland. The disease is also widespread in Serbia (EPPO 2023).
- (ii) the Grape Trunk Diseases (GTDs), which includes a pallet of diseases: Eutypa, Esca, and Black Dead Arm dieback, all attacking the perennial organs of the vine and accelerating its death and generating high replantation costs (estimate of 1.132 Mrd€ per year) (Hofstetter et al., 2012). The GTDs were detected and monitored in Croatia, Czech Republic, France, Germany, Greece, Hungaria, Italy, Montenegro, Portugal, Romania, Slovakia, Slovenia, Spain, Switzerland and United Kingdom (Guerin-Dubrana et al., 2019).

The main arthropod grape pests in European commercial vineyards are the following:

- (i) the two grapevine moths, the European grapevine moth (*Lobesia botrana*) and Cochilis grapevine moth (*Eupocilia ambiguella*)
- (ii) several mite species, such as *Calepitrimerus vitis*, *Eriophyes vitis*, *Eotetranychus pruni*, *Panonychus ulmi*
- (iii) the smaller green leafhopper (*Empoasca vitis*)
- (iv) the grape mealybug (*Planococcus ficus*)

- (v) the gallicole grape phylloxera *Daktulosphaira vitifoliae* (all the pests and diseases photographs are presented in *Figure 2* and *Supplement 2 – Table 1*).

Several protection strategies can be complemented to control grape pests and diseases, such as:

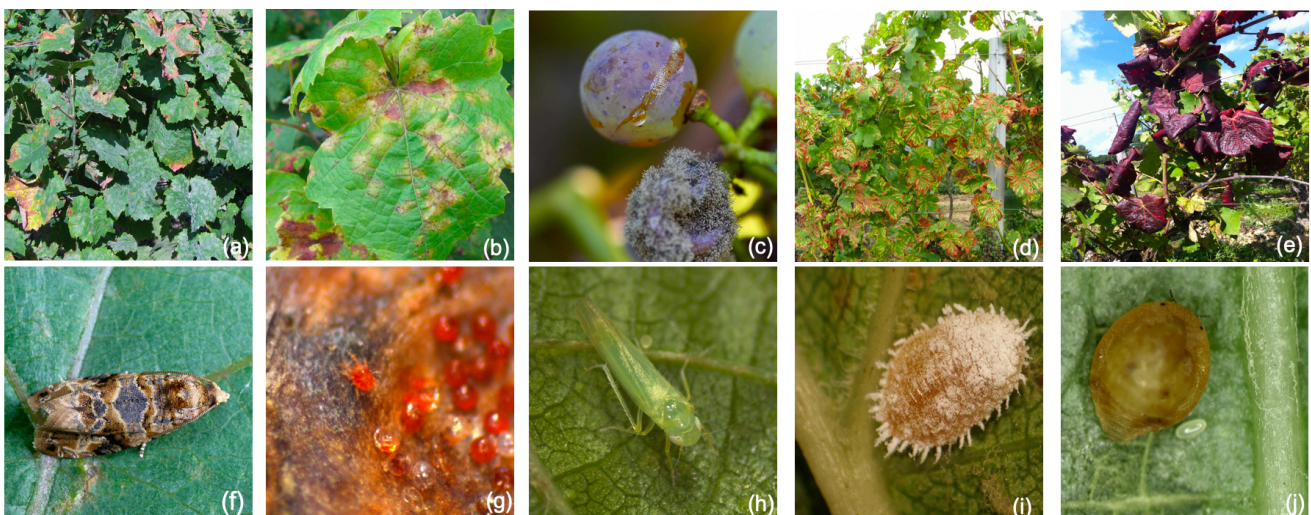
- **Prevention practices:** the creation of ecological infrastructures to favor the natural regulation of pests by native natural enemies of the ecosystem (e.g., maintenance of flower strips in the vine inter-row), use of fungal diseases tolerant varieties and rootstocks (e.g., Artaban, a red variety resistant to downy mildew), cultural practices (e.g., the soil management, the pruning techniques), sanitation measures (e.g., removal of diseased plants from the vineyard; planting of certified healthy material)
- **Piloting tools:** forecasting tools to characterize the epidemiology of infectious diseases, monitoring tools to give an estimate of pest or disease density of population in the vineyard (e.g., insect traps), decision support systems to guide farmers on their phytosanitary treatment calendar
- **Direct control methods:** mechanical control methods (e.g., mechanical weeding of the inter-row) and biological control methods (e.g., mating disruption with pheromone dispensers to control *L. botrana*). Finally, applying pesticides based on natural products (e.g., mineral products, BCAs, resistance inducers) and synthetic pesticides (summarized from [EIP-AGRI Focus Group, 2019](#)).

Using synthetic pesticides is still a prevalent measure to protect grape against pests and diseases. It is perceived to be efficient by winegrowers and shows the most effective short-term controlling effect to ensure grapevine yield and limit the risk of grape loss ([Aka et al., 2018](#)). Consequently, synthetic pesticides (herbicides, fungicides, and insecticides) are extensively applied in commercial vineyards with high frequency and concentration during the growing season, making grapevine one of the most reliant crops on synthetic pesticides ([Chen et al., 2022](#)). Fungicides are synthetic pesticides with the most important usage in commercial vineyards to control downy and powdery mildew. The European



average of applications is 12 to 15 per year, increasing to 25 to 30 in the most susceptible vine-growing regions (most recent statistics of [Pertot et al., 2017](#)). Accordingly, 71% of all fungicide treatments applied in Europe from 1992 to 2003 were devoted to grapevine crops, representing only 4.6% of the European agricultural area (most recent statistics from [Eurostat 2007](#)). On the other hand, insecticides are moderately used in Europe, with an average of 1 to 4 insecticides applied per year ([Pertot et al., 2017](#)).

As the negative impacts of synthetic pesticides on the environment and human health are significantly referenced and acknowledged by the scientific community ([Rani et al., 2021](#)), it justifies the research and development of alternative solutions to these products. Likewise, the production and application of synthetic pesticides induce significant carbon emissions ([Cech et al., 2022](#)). Therefore, wine growers are pressured by essential stakeholders such as consumers, retailers, and politicians into reducing their dependence on synthetic pesticides ([Villemaine et al., 2021](#)). Finally, the cost of pesticide use in viticulture is high, with an estimation of 398€/ha in 2002, further motivating a change of protection measures ([Réseau d'informations comptables agricoles 2002](#) ; [Aubertot et al., 2005](#); [Bourguet and Guillemaud, 2016](#)).



**Figure 2 :** Most common and injurious pests and diseases of grapevine in Europe (a) powdery mildew *Erysiphe necator* ; (b) downy mildew *Plasmopara viticola* ; (c) botrytis or Grey mould *Botrytis cinerea* ; (d) esca disease complex ; (e) flavescence dorée disease ; (f) grapevine moth *Lobesia botrana* ; (g) mite *Panonychus ulmi* ; (h) grapevine leafhopper *Empoasca vitis* ; (i) grapevine mealybug *Planococcus ficus* ; (j) gallicole grape phylloxera *Daktulosphaira vitifoliae* (credit : Hochschule Geisenheim University photo bank and D. Blancard INRAE-SAVE)

## 3-The grape phylloxera – a major pest of European grapevine

### 3.1 Taxonomy of the grape phylloxera

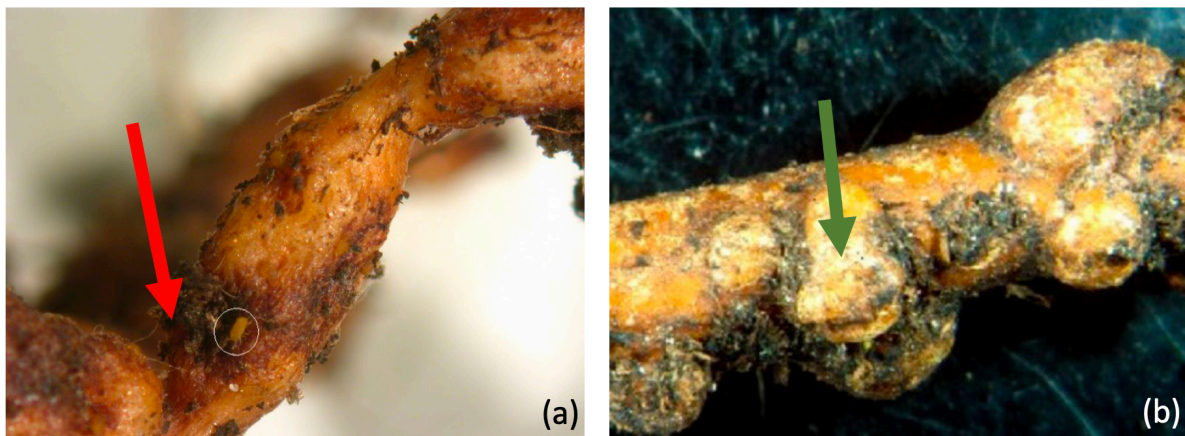
Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, 1855) (Hemiptera, Phylloxeridae) belongs to the Phylloxeridae family, which includes ca. 83 species and 8 genera characterized by female oviparous reproduction. The Phylloxeridea belongs to the Aphidoidea superfamily, comprising the Adelgidae and Aphididae. Thus, phylloxerids share a lot of biological and ecological features with aphids. Half of the Phylloxeridae species are cecidogenic, they induce galls on trees of diverse botanical families such as *Juglans* spp. (Juglandaceae), *Quercus* spp. (Fagaceae), *Pyrus* spp. (Rosaceae), *Ulmus* spp. (Ulmaceae). Only one North American genus containing one species - Grape phylloxera - is associated with *Vitis* spp. (taxonomy presented in [Supplement 3 – Figure 2](#)) (Podsiadlowski 2016; [website 1](#)).

### 3.2. Ecology of the grape phylloxera

Grape phylloxera is an obligatory parasite and a monophagous insect that only feeds on *Vitis* spp., excluding other Vitaceae members as host plants. It is a sap-sucking insect meaning that the larval instars penetrate their stylet into the plant tissue, from where they start sucking parenchymal plant sap ([Wilmink et al., 2021b](#)). The feeding act is responsible for modifying the plant growth hormone balance causing the plant to develop foliage or root deformities called galls ([Warick and Hildebrandt 1966](#)).

The principal hosts of grape phylloxera are *Vitis* spp. including American *Vitis* species *V. aestivalis*, *V. cinerea*, *V. labrusca*, *V. riparia*, *V. vulpina*. The European grapevine *V. vinifera* is its major host. In their native area, American wild *Vitis* plants are commonly found on climbing trees and other vegetation types such as forest gaps and edges. These plants also spontaneously colonize roadsides, riparian

habitats, and field borders (Downie et al., 2000). Under its native range, grape phylloxera is highly tolerated by American *Vitis* species. Indeed, it induces many leaf galls in American *Vitis* species, but these infestations are not lethal to the plant. It also causes root galls, called ‘nodosities’ to the young rootlets but does not produce root-galls on mature woody roots older than one year called tuberosities, consequently preserving the most structuring roots. The immunity of mature roots of American *Vitis* species to severe root-galling preserves them from the most harmful damages of grape phylloxera (Figure 3 b). Consequently, the resistance of mature roots against root-galling damages confers resistance to grapevine against the grape phylloxera and is the most important characteristic of a resistant rootstock (Granett et al., 2013). On European grapevines, *Vitis vinifera*, grape phylloxera marginally infests the above-ground part of the plant by leaf-galling. Still, it heavily damages the root system by inducing nodosities on the young rootlets and tuberosities on mature roots (Figure 3 a) (Powell et al., 2013).



**Figure 3** : Tuberosities induced on mature roots by the grape phylloxera : (a) European grapevine *V. vinifera* mature root section where pest damages have created a hole-like depression penetrating far into the interior of the root marked by the red arrow ; (b) American *Vitis* species (species not specified) mature root section where the tuberosities have been sealed off by the formation of cork tissue (marked by the green arrow) thus preserving the original root structure (Credit : Joachim Schmid, Hochschule Geisenheim University)

### 3.3. *Biology and life cycle of the grape phylloxera*

Phylloxerids have complex life cycles that include sexual and asexual reproductions and involve multiple distinctive phenotypes. Several phenotypes possessing the same genotype can coexist due to environmental stimuli complexifying the comprehension of their biology. The grape phylloxera life cycle is very complex because it involves several individual phenotypes (*i.e.*, winged (alate) and wingless (apterous) phenotypes), two distinct reproduction modes (*i.e.*, sexual and parthenogenetic reproduction forms) and occurs in two separate plant compartments where distinct phases of the cycles unfold (*i.e.*, the hypogea phase occurs underground in the grapevine root system, and the epigeal phase occurs above ground in the grapevine canopy) (Podsiadlowski 2016).

Grape phylloxera multiplies via cyclic parthenogenesis involving several consecutive rounds of asexual reproduction followed by a sexual event. The life cycle of grape phylloxera was first described in the mid to late nineteenth century simultaneously in North America and Europe by separate research teams (Forneck and Huber 2009).

In *Vitis vinifera*, grape phylloxera overwinters on roots as first-instar larvae called ‘hibernants.’ When the soil temperature increases in spring, the hibernants start feeding, gain maturity, and lay eggs. The maturity takes less than a month, depending on the quality of the root host and soil temperature. The adult female multiplies parthenogenetically and can produce up to 300 eggs on healthy susceptible rootstocks and favorable soil temperature. Five or more generations of female larvae and parthenogenetic female adults can overlap from summer to autumn. Some phylloxera larvae are called crawlers as they move and emerge from the soil. The phylloxera crawlers and adults are called “radicole” (or root-feeding phylloxera) as their feeding habits are associated with roots. They are one of the most volatile phylloxera forms, as the wind can convey them to other feeding sites. Some first-instar larvae begin to hibernate in September, and in November, all larvae go through hibernation. In warmer climates, the hibernation phase does not

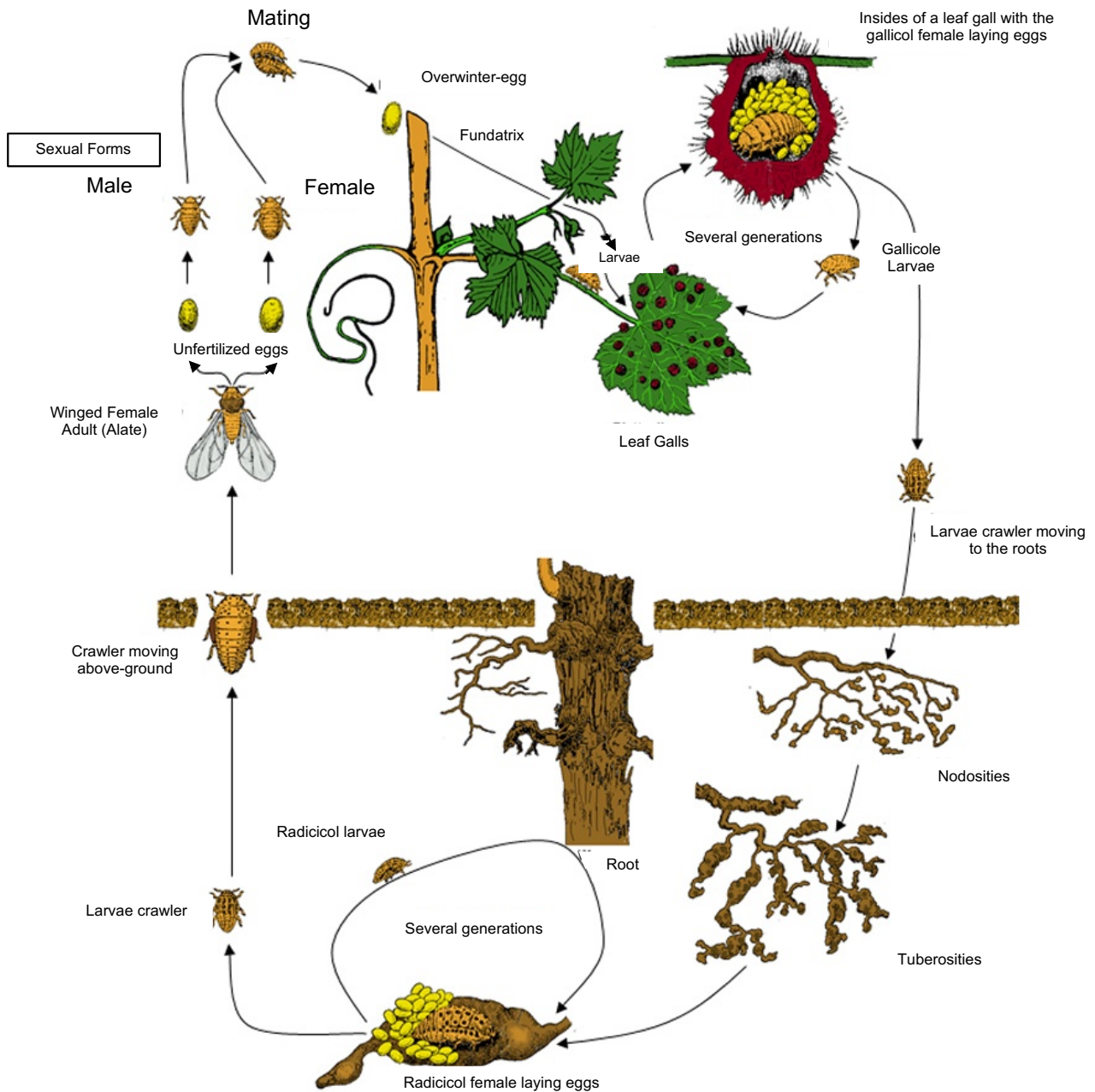
occur, and the reproducing and feeding phylloxera forms are observable during the winter period, especially on immature roots.

During the end of summer and autumn, winged female adults (alates) frequently appear from severely damaged roots. The alates do not feed after their emergence. These forms are more mobile and can move to the grapevine canopy. They significantly disperse from their initial source to lay eggs. They can lay a few eggs that can emerge into males or females. These emerging forms are called sexual or reproductive forms because they can mate together and do not harbor functional mouthparts enabling feeding. The sexual forms breed together, and the fertilized females produce one single overwintering egg commonly laid only under the bark of American *Vitis* species or on the poorly-cut rootstock suckers of grafted European grapevines.

During spring, the overwintering eggs hatch, and the first emerging larvae females, called “fundatrix,” feed on the upper surface of a young leaf of American *Vitis* species. The feeding induces a gall that overhangs on the underside of the young leaf. Several asexual phylloxera generations overlap on the foliage and are called “gallicole” or “leaf-feeding” phylloxera as adults initiate galling on the foliage. Fundatrix females evolve into wingless adults that lay eggs parthenogenetically in the leaf gall. The emerging instars are female crawlers that crawl out of the gall and look for new feeding on leaves, and some directly move to the soil to feed on roots. After some generations, some gallicole crawlers go belowground to the roots and cohabit with the radicolle forms. From spring to the end of summer, the ratio of radicolle forms over gallicole ones increases in favor of the radicolle form, which predominates in the soil, whereas the gallicole form disappears (cycle description is synthesized from [Granett et al., 2001](#); [Forneck and Huber 2009](#); [Bettiga 2013](#)); (cycle is illustrated in [Figure 4](#) and the grape phylloxera stages photographs are presented in [Figure 5](#)).

European grapevine *V. vinifera* leaves are tolerant to phylloxera leaf galling development, thus reducing the occurrence of gallicole phylloxera in commercial vineyards ([Powell 2008](#)). Consequently, the asexual phase of the grape phylloxera cycle is predominantly observed in European commercial vineyards. Different

variations of the grape phylloxera life cycle exist worldwide; for instance, in California, the phylloxera was reported to only parasite the roots of non-grafted European grapevine *V. vinifera*. This cycle variation mainly depends on the host plants of the *Vitis* species (Forneck and Huber 2009).



**Figure 4 :** Classical life-cycle of the grape phylloxera (*Daktulosphaira vitifoliae* Fitch, 1855) modified from the original figure of Weber & Weidner 1974

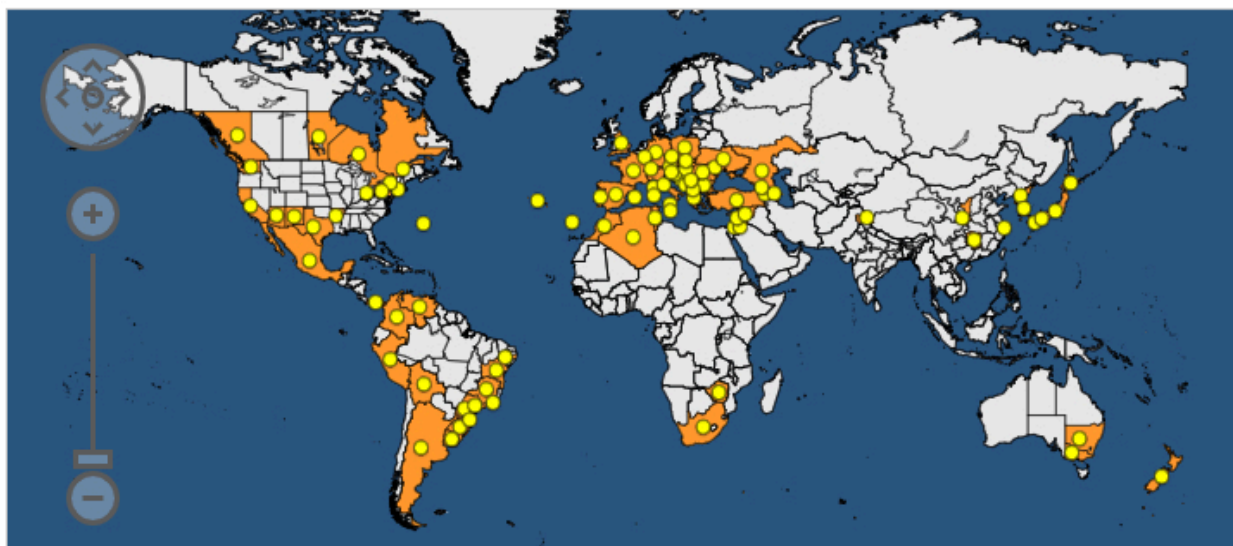


**Figure 5:** Grape phylloxera (*Daktulosphaira vitifoliae* Fitch, 1855) life-stages (a) eggs laid by a gallicole female on leaf gall ; (b) larvae crawling underneath the grapevine leaf ; (c) parthenogenetic female phylloxera located underneath the grapevine leaf ; (d) dissected leaf-gall containing a multitude of eggs with the parthenogenetic female ; (e) eggs laid by a radicole parthenogenetic female on the root surface ; (f) larvae crawling on the root surface ; (g) parthenogenetic females feeding on the root surface ; (h) grape phylloxera winged-female ; credit: Hochschule Geisenheim University photo bank and Dominique Blancard (INRAE-SAVE)

#### 3.4. Grape phylloxera global geographical distribution

Grape phylloxera is today ubiquitous in most wine-growing regions worldwide (*Figure 6*). Over the past 150 years, the pest spread rapidly due to human activity to most grapevine-planted areas worldwide, encompassing South America, New Zealand, Australia, South Africa, and China. Indeed, grape phylloxera is tolerated by the commercial rootstocks used to graft European grapevine *Vitis vinifera*. Consequently, they represent a habitat and food source for the pest, maintaining native grape phylloxera populations. Additionally, grape phylloxera spreads rapidly between vineyard plots and is mainly conveyed by human activities (e.g., shoes, clothing, machinery, and plant material). Its natural vector is the wind that blows the crawler instars and the winged adults (Ji et al., 2021). Some

grape-growing countries, such as China, Australia, Russia, and Armenia, are only partly colonized by grape phylloxera, where the pest is contained to precise areas. Also, it was not yet observed in Chile, Cyprus, Denmark, Estonia, Finland, Ireland, Latvia, Lithuania, Norway, Sweden, and the Netherlands (Powell and Clarke 2018) (Figure 6).



**Figure 6** : Cartography of worldwide grape phylloxera distribution based on the data aggregated by the European and Mediterranean Plant Protection Organization (EPPO) (<https://gd.eppo.int/taxon/VITEVI/distribution> with last update: 13/10/2022)

#### 3.5. Grape phylloxera damages

Grape phylloxera has the status of a serious economic pest since the late nineteenth century when it destroyed a third of European commercial vineyards. Previously, European vineyards were planted entirely with non-grafted grapevines *V. vinifera*, which are highly susceptible to the radicle forms of the grape phylloxera (Pouget 1990). As the European grapevine *V. vinifera* is not vulnerable to the grape phylloxera gallicole form (photographs Figure 7 e, f, g, h), this form does not pose serious economic losses to the growers. It is often observed in the foliage of rootstocks with American *Vitis* parentage resistant to radicle grape phylloxera. These vines are usually found in abandoned natural habitats, nursery



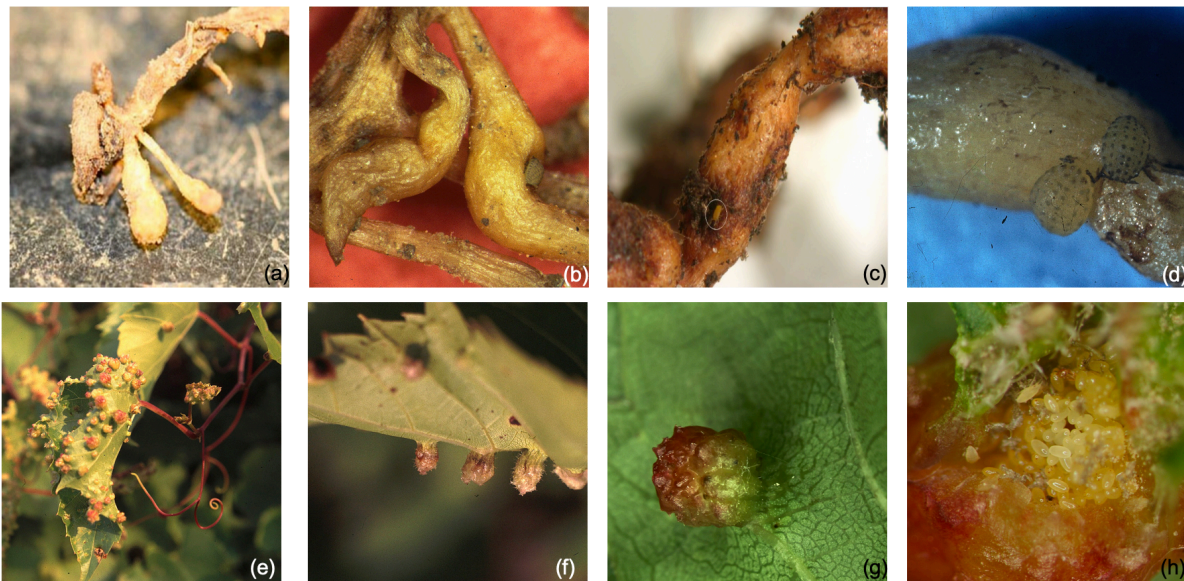
plantings, and poorly-cut rootstock suckers of grafted grapevines (Powell and Clarke 2018).

The first field evaluation of the impact of gallicole grape phylloxera on *V. vinifera* scion grafted on American rootstock was conducted in three German commercial vineyards of the Baden region. No effect was characterized on grapevine leaf carbon acquisition capacities, pruning weight, internode length, grapevine productivity, and wine sensory analysis when vineyard plots were infected by gallicole grape phylloxera. In the most infested vineyards planted with *V. vinifera* scions, grapes had significantly decreased sugar content (10%), and vines had reduced perennial wood starch reserves (11%). The short-term effects of the infestation of gallicole grape phylloxera in European vineyards were marginal, according to the study (Wilmink et al., 2022b). In another grape-growing country, in Australia, Only 15% of the commercial vineyards are grafted on resistant rootstocks. The rest is planted with non-grafted European grapevines *V. vinifera*, consequently limiting the occurrence of gallicole forms (Powell and Clarke 2018).

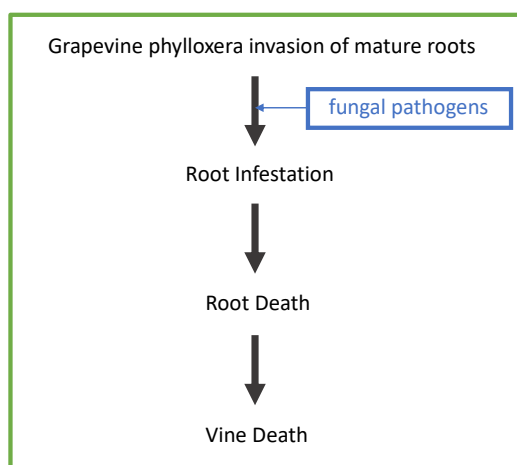
Radicicole grape phylloxera are the deadliest for the European grapevine *V. vinifera*. Indeed, radicicoles attack young plant rootlets inducing the formation of nodosities and also feed on more mature woody roots (older than one year), where they induce severe deformations named tuberosities. The nodosities darken and necrose, causing a characteristic ‘crooked root’ deformation or “club” that curves around the insect (*figure 7 a, b*). The most harmful deformations for the grapevine are the tuberosities characterized by the hypertrophy of the cortical tissues, consequently creating a capsule form (*figure 7 c*). After deformation, root fissures develop a pathway of entry for opportunist pathogenic fungi native to the soil (i.e., *Fusarium* sp., *Trichoderma* sp., *Rhizoctonia* sp., *Alternaria* sp., *Pythium* sp., *Mucor* sp. and *Penicillium* sp. species (Omer et al., 1995)). These fungi aggravate the root fissures and cause significant lesions that extend to the cambium and phloem vessels. These lesions interrupt the sap flow and destroy the distal root part (explicative diagram *figure 8*) (Granett et al., 2013). An infested European grapevine with radicolle grape phylloxera may exhibit decreased shoot growth and foliage reduced lengthening, shortened internodes, and reduced plant biomass

and fruit production as visual symptoms of plant decline (Powell 2008). Grapevines are also significantly more susceptible to water stress and nutrient deficiency (Granett et al., 2001, 2013; Bettiga 2013). Vines damaged at the main structuring root die 3 to 10 years post-infestation (Korosi 2019).

The severity of the grapevine damage depends on the rootstock cultivar, plant age, plant vigor, soil type, soil temperature, soil drainage, and vineyard soil microbiota (Bettiga 2013).



**Figure 7** : Grape phylloxera damages observed on European grapevine *Vitis vinifera* (a) young rootlet of grapevine deformed with 2 nodosities ; (b) young grapevine rootlets deformed with nodosities which consequently evolved into crooked-root ; (c) and (d) mature grapevine deformed with tuberosities evolved into a hole-like depression penetrating far into the interior of the root ; (e) and (f) leaf galls observed on the underneath of a grapevine leaf ; (g) close zoom on a red leaf-gall ; (h) inside of a cut leaf gall containing a multitude of gallicole grape phylloxera eggs (Credit : Hochschule Geisenheim University Photo Bank & Prof. Dr. Joachim Schmid)



**Figure 8** : Steps leading from radicle grape phylloxera invasion of mature roots of grapevine *Vitis vinifera* to grapevine death (modified from Granett et al., 2013)

#### *3.6. Abiotic factors favoring grape phylloxera infestation of grapevine*

The presence of grape phylloxera in the vineyard is mainly influenced by temperature, rainfall, and soil type where grapevines are planted.

- The range of neighboring soil temperatures allowing suitable development of radicle grape phylloxera is 16 to 32°C. Outside of this range, the insect does either not develop at all, or the number of parthenogenic generations is significantly reduced, ultimately reducing the grape phylloxera soil populations
- Spring and summer rains can significantly reduce radicle grape phylloxera populations. The rains can wash off the gallicole populations established in the grapevine foliage, consequently reducing the number of grape phylloxera going down to the roots
- Conversely, irrigation and rainfall may stimulate the development of rootlets, allowing radicle grape phylloxera populations to thrive because of the development of multiple new feeding-sites

### 3- The Grape Phylloxera – a major pest of European grapevine

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- Winter flooding of vineyard soil kills radicolle grape phylloxera overwintering populations
- Radicolle grape phylloxera tend to prefer heavy clay soils compared to sandy soil
- Radicolle phylloxera populations can survive for up to 6 years in fallow vineyard plots where remaining root pieces still exist in the soil, making the eradication of grapevine phylloxera from a vineyard impossible (synthesized from Bettiga 2013).

#### 3.7. Grafting of European grapevines on resistant rootstock of American *Vitis* parentage

The grafting of European grapevine *Vitis vinifera* cultivars on phylloxera-resistant rootstock is the primary controlling measure to limit the severity of radicolle grape phylloxera damage to the plant. It is a systematic management method used worldwide to control this pest (Granett et al., 2001). The biological control protection method was established in France during the grape phylloxera crisis from 1868 to 1895 due to a scientific partnership between American and French entomologists (Carton et al., 2007).

The phylloxera-resistant rootstocks are clone-selected from a single American *Vitis* species or hybrids between two American *Vitis* or American *Vitis* and European *Vitis* species, with 100 species currently used for crop protection (Walker et al., 2014). The mainly used species are *V. rupestris*, *V. riparia*, and *V. berlandieri* for rootstock selection programs (Bettiga, 2013). These are also effective resistance means to multiple soil biotic stressors such as root nematodes and soil-borne pathogens causing root diseases, besides abiotic stressors such as drought or waterlogging, soil pH, salinity, and soil type (*i.e.*, soil rich of limestones with consequent plant chlorosis risks) (the main characteristics of the most famous rootstocks are presented in the *Supplement 4 - Table 2*). Rootstocks help mediate nutrient uptake influencing juice and wine composition. They can also improve or

reduce vigor and fruit yield depending on viticultural planting conditions of nutrients and water availability (Jones et al., 2009).

As multiple grape phylloxera strains exist, bypassing rootstock resistance cannot be excluded. Consequently, the durability of resistance is a key feature when choosing the appropriate rootstock. Indeed, clone-selected from a single American *Vitis* species have shown durable resistance against radicle grape phylloxera since the 1870s compared to hybrids with *V. vinifera* parentage from which resistance may break (Granett et al., 1983). Also, as phylloxera can spread rapidly from different vineyards and stay long-term in the colonized vineyard plots, the risks of new rootstock-resistant strains emerging and colonizing the vineyard are substantial (Walker et al., 2014). Consequent care should be paid for rotating the rootstocks with different parentage groups to avoid selecting aggressive radicle strains (Bettiga 2013).

Rootstocks are usually resistant to radicle grape phylloxera through three distinct mechanisms:

- Antibiosis (reduction of pest fitness)
  - Antixenosis (reduction of host attractiveness to the pest)
- Tolerance (the ability of the host to tolerate pest pressure)

Antibiosis may be a consequence of the chemical root composition (phenols, tannins, amino acids, plant hormones) affecting the capacities of the radicle phylloxera to digest proteins. In addition, a possible root toxin secretion by *V. berlandieri* parentage rootstocks may severely affect radicle grape phylloxera survival. Rootstock with antixenosis component provokes nutrient deficiency or inhibition which may lengthen development time and suppress fecundity of radicle phylloxera, which is classical in the rootstock of *V. rupestris* parentage. Finally, several species, such as *V. riparia* and *V. rupestris*, create a cork envelope around the radicle phylloxera feeding site, trapping the pest and preventing further tuberosity development and root fissure. These mechanisms are distinct between rootstock species, complex, and need complementary research (synthesized from Bettiga 2013; Granett et al., 2001). In addition, the gene defense

response against radicle grape phylloxera in resistant rootstock was investigated in two studies. Comparative microarray analysis performed on roots with nodosities of non-grafted grapevine cv. ‘Crimson Seedless’ and rootstock ‘140Ru’ (*V. berlandieri* x *V. rupestris*) show a higher transcription level in the resistant rootstock. Transcripts were significantly up-regulated only in ‘140Ru’, correspondent to proteins involved in secondary metabolite biosynthesis, which compounds may cause radicle phylloxera antibiosis or antixenosis (Du et al., 2014). Also, the WRKY46 transcription factor gene was significantly more up-regulated in rootstock ‘1103 Paulsen’ of the same parentage as ‘140Ru’. In addition, sur-transcription of this gene in composite grapevine root reduced radicle grape phylloxera attacks and delayed larvae development (Wang et al., 2019). In parallel to rootstock breeding and selection programs, several management strategies are developed to better control grape phylloxera, especially in non-grafted vineyards.

#### 3.8. Management methods of grape phylloxera

Several management methods can be implemented in the vineyard to control grape phylloxera:

- **Monitoring techniques:** the grape phylloxera radicle populations are primarily observable in the spring or late summer and are challenging to monitor in the grapevine vineyards as they are hardly visible. Their observations necessitate laborious soil digging. Therefore, researchers first focus on the visible grapevine above-ground symptoms associated with early-plant decline. The spatial distribution of rapidly declining concentric patches of grapevines in the field can signify a grape phylloxera infestation. A sampling of the first soil layers of infested grapevines allows the observation of nodosities associated with young rootlets. The most damaged roots are black-colored with poorly-adherent bark of dry and spongy consistency (Bettiga 2013).

**Eradication method via vine-uprooting:** as radicolle grape phylloxera populations can be supported on both American *Vitis* species and *V. vinifera* parentage, grapevine uprooting removes an existing population of grape phylloxera in soil. Nevertheless, it is almost impossible to eradicate the pest once it colonizes a vineyard plot, especially through uprooting as populations maintain themselves on deeply buried roots from previous plantations (Bettiga, 2013). However, some countries, such as Australia, extensively use this measure to contain radicolle grape phylloxera expansion through the vineyards (Benheim et al., 2012).

- **Limiting contamination methods:** by avoiding viticultural work (e.g., cultivation, ripping, row plowing, mechanical harvesting) in grape phylloxera-infested vineyard plots, the risk of grape phylloxera vectorization via machinery tires with adhering soil, tools, workers' footwear, and clothes is limited. Also, cleaning and disinfecting the tools and clothes may reduce the risks (Benheim et al., 2012).
- **Sanitation of nursery vines:** using plant material certified pest-free because of insecticide or hot water dip treatments (Powell, 2012)
- **Cultural practice methods to slow grapevine decline:** curing an infected grapevine with radicolle grape phylloxera is difficult. Plant general health can be sensibly improved by favoring root health and plant vigor, primarily through discontinuing disking and broader and recurrent irrigation. Winter 40 to 50-day-long flooding of vineyards could suppress the radicolle grape phylloxera population (Bettiga, 2013).
- **Insecticide control method:** eliminating grape phylloxera radicolle populations with insecticides is tough. First, the penetration of synthetic chemicals is modest in clay soils where phylloxera has an installation preference and can also colonize deep in the ground. Likewise, the product applied via drip irrigation may not homogeneously reach the grapevine root

system as radicolle phylloxera has a rapid population growth in grapevine roots, with 3 to 10 generations per year observed in the vineyard. Therefore, they are challenging to stem via single insecticide application. Finally, the plant's decline after radicolle grape phylloxera damage is an irreversible process, and radicolle population suppression may not help the vine to recover once damages are done to roots as other root pathogens are involved in the declining process (synthesized from Bettiga 2013; Granett et al., 2001).

- **Biological control methods:** using natural enemies of the grape phylloxera, such as predators and parasitoids, did not achieve promising results (Benheim et al., 2012). Since the 19<sup>th</sup> century, entomopathogenic fungi have been envisioned as effective biological control agents against grape phylloxera. Still, the discovery of the grafting method slowed the flow of investigations on the topic. Since 2000, research on entomopathogenic fungi has gained impulses, and their use for the control of grape phylloxera is still under investigation (Kirchmair et al., 2009).

To date, several emblematic vine-growing regions worldwide decided to maintain their vines non-grafted. For instance, 15% of the vineyard area in Australia is grafted on resistant rootstocks. Hence, grape phylloxera is contained predominantly through the adherence of strict quarantine regulations and National Phylloxera Management protocols (Powell and Clarke 2018). Grape phylloxera is found in several Australian vineyard regions (e.g., King Valley in 1991, Upton in 2000, Buckland Valley in 2003, Yarra Valley in 2006, and Macedon in 2008), which are labeled as 'PIZ' (Phylloxera Infested Zones) in which monitoring and controlling methods are systematically applied (Powell 2008 ; Benheim et al., 2012). However, all these measures are partially efficient in stemming the phylloxera invasion front (Powell 2008). In Argentina, 90% of the vineyard area is planted with non-grafted grapevines *V. vinifera*, and grape phylloxera does not pose serious economic



problems. The Argentinian vineyard's tolerance to grape phylloxera could stem from vineyard soil-type parameters because most soils are sandy to loam-sandy or irrigation as it is performed by flooding. However, Argentinian vineyard irrigation methods are switching to drip irrigation to limit water consumption. Future increases in phylloxera populations are expected to happen because of this practice change (Arancibia et al., 2019). Threats of invasion of grape phylloxera in non-grafted vineyards worldwide justify developing biological control strategies to control the pest.

## 4- Entomopathogenic fungi and their biological control potential against radicolle grape phylloxera

### *4.1. Biological control definition*

Biological control is a pest control strategy used along with pesticide application to regulate pests and diseases damaging crops. The definition and scope of this discipline were specified by [Stenberg et al., 2021](#) :

*“Biological control is the exploitation of living agents (including viruses) to combat pestilential organisms (pests and pathogens), directly or indirectly, for human good.*

*Biological control must always involve the following three separate players:*

*1) a biocontrol agent,*

*2) a pest, and*

*3) a human stakeholder benefitting from the pest control service provided by the biocontrol agent.”*

[Stenberg et al., 2021](#)

Adding to this definition [Eilenberg et al., 2001](#) said, “The goal of this plant protection strategy is to maintain pest populations below a certain threshold without its eradication in order to achieve a sustainable and economically-viable agricultural production.” The mean used for crop protection has to be a living organism such as predators, parasitoids, nematodes, fungi, or bacteria. Also, it includes viruses, although there are not considered living organisms, consequently excluding the metabolites produced from insect or weed pathogens that do not involve the living causal agent ([Eilenberg et al., 2001](#)). All these controlling living agents imply a large variety of controlling mechanisms. This discipline has generated much interest and support from policymakers, retailers, consumers, and growers because of its potential for substitute chemical control.

The main assets of biological control are:

- Safety of use for the environment, applicators, and food supply (absence of toxic residues remaining in the environment after application compared to synthetic pesticides)
- Absence of toxic residue contamination on the food itself
- Absence of development of resistance against biological control agent
- High selectivity of the products (leaving at rest beneficial non-target organisms)
- Cost of production (lower compared to synthetic pesticides) ([van Lenteren et al., 2018](#))

The demand for new biological control solutions has grown for the last few years. However, the product offer is limited. For instance, microbial pesticides represent 1-2% of all pesticides traded worldwide, according to the most recent 2015 evaluation (Lacey et al., 2015). Though, the European legislation sets ambitious goals to restrict residue levels of most synthetic chemical pesticides and to take off the market a significant number of synthetic active compounds stimulating the emulation around biological control products (van Lenteren et al., 2018). Finally, these products imply using living organisms with complex biology, ecology, and control mechanisms. Thus, their efficacy of action is not consistent for every specific case of pest control. The variability of pest-controlling results limits the growers' trust in the efficiency of these products ([Bardin and Nicot 2022](#)).

## 4.2. Biology of Entomopathogenic fungi (EPF)

Entomopathogenic fungi constitute a group of various individuals which are phylogenetically diverse, heterotrophic, eukaryotic unicellular or multicellular (filamentous fungi), some EPF having the capacity to reproduce via sexual spores and others via asexual reproduction (Esparza Mora et al., 2018). Diversity among EPF is immense and reflects the diversity of taxonomic groups represented. An EPF was defined as the following:

“Entomopathogenic fungi live in the soil and infect their hosts through the external cuticle by penetrating their body to kill them and feed upon them eventually. They are pathogenic to soft- and hard-bodied insects and other arthropods, including Acari (ticks, mites).”

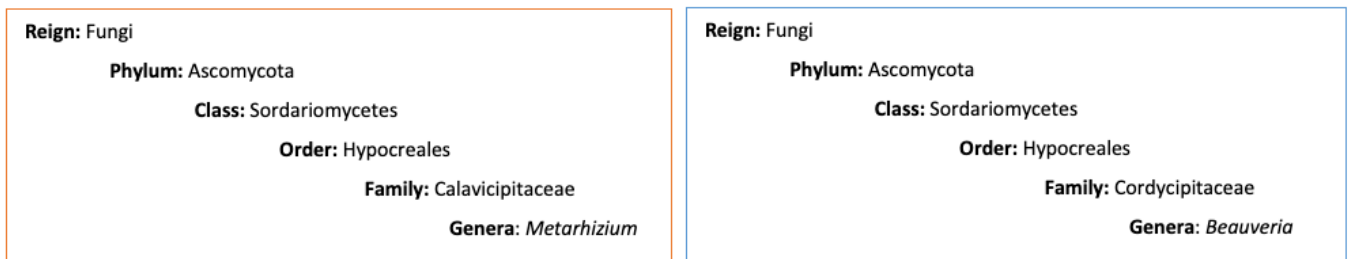
*Summarized definition of Lacey et al., 2015 and Dara, 2019*

### 4.2.1. Phylogeny of the most emblematic EPF

Most of the famous EPF belong to the Ascomycota phylum and Pezizomycotina subphylum, from which members exhibit the most morphologic and ecological complexity. Indeed, their life cycle implies sexual and asexual reproductive forms coexisting as distinct phenotypes in the environment. The sexual state is the rarest in the environment (Vega et al., 2012). Ascomycota comprises ca. 64000 described species that possess unicelled or pluricelled filamentous septate talli. Their sexual reproductive phase is characterized by the fusion of the nucleus of two asci (*i.e.*, distinct reproductive cells) that, after karyogamy and meiosis, produce new asexual spores called ascospores (Bennett and Turgeon 2016).

The class Sordariomycetes, from which the order Hypocreales branch out, is very famous and most of its members belong to the monophyletic Clavicipitaceae family. This family includes the renowned grass endophytes (*Balensia*, *Claviceps*,

*Epichloë*) and the well-known entomopathogens genera *Aschersonia*, *Hypocrella*, *Regiocrella*, and *Metarhizium*. The important species of *Beauveria* genera belong to the other monophyletic family, the Cordycipacea (*Figure 9*) (Vega et al., 2012). In particular, both notable species *Beauveria bassiana* and *Metarhizium robertsii*, are ubiquitous soil-dwellers of worldwide ecosystems in diverse geographic, climatic, and agroecological contexts (Lacey et al., 2015).



**Figure 9** : Systematic of the two most famous genera of EPF used for microbial biological control: *Metarhizium* and *Beauveria*.

#### 4.2.2. Biology of EPF and general life-cycle

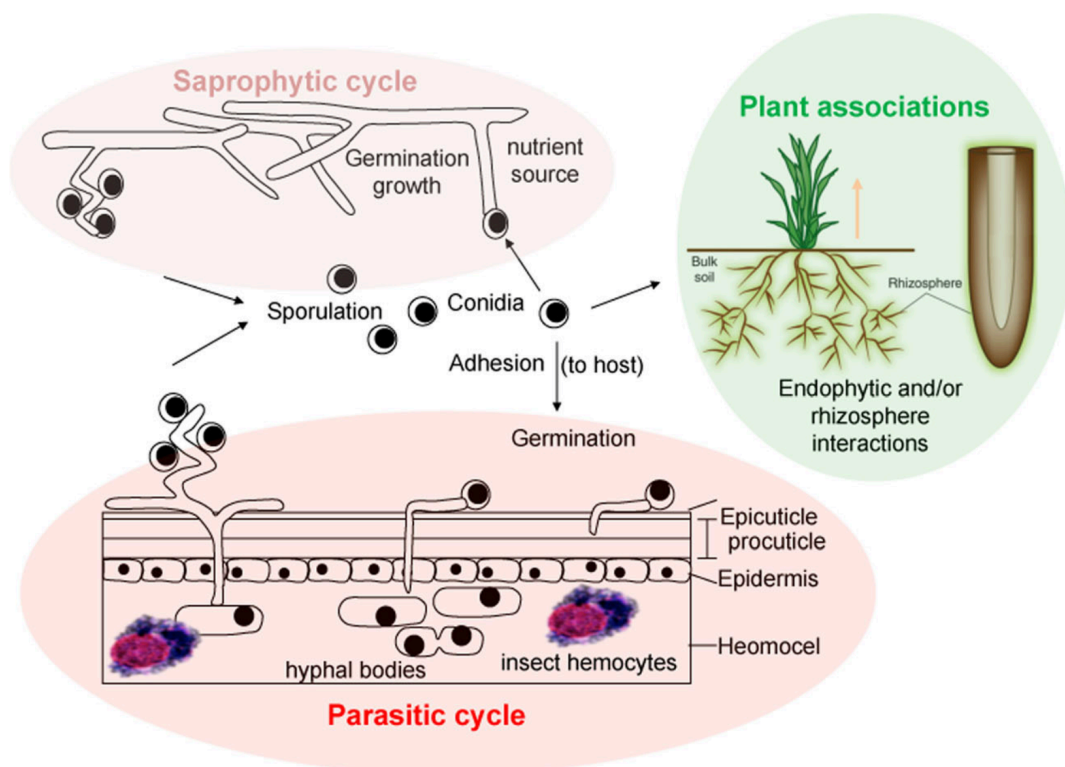
Fungi are absorbotroph microorganisms. Thus they feed through the transmembrane absorption of trace elements, mineral salts, and organic molecules through active transport or passive diffusion (Dix and Webster 2013).

The different phases of EPF's lifecycle are:

- **Saprophytic phase:** as heterotroph organisms, EPF live in soil as saprobes feeding upon dead decaying materials or organic matter in the ground. They absorb nutritive elements pre-digested by secretion of extracellular digesting enzymes
- **Parasitic phase:** EPF can take away nutrients from other organisms, and the consequences of the association are detrimental to the host. Consequently, EPF kill insects by attaching to their cuticular surface

- **Mutualistic phase:** EPF associate with the plant as plant symbionts, a durable association that benefits both partners. This association can grow even closer to becoming endophytic (all phases are illustrated in *Figure 10*).

They do not require systematic interaction with insects or plants to complete their lifecycle. Instead, their life cycle can be completed in the saprophytic or parasitic phases (Dix and Webster 2013; Ortiz-Urquiza et al., 2015). The EPF exhibit a ‘vegetative form,’ also known as the growth form, characterized by multiple tubular compartmentalized branched structures called hyphae, which agglomerate to form the mycelium. The EPF can also be present in the ground attached to soil particles as dormant propagules waiting for susceptible hosts. Some species harbor specialized forms of resistance to adverse abiotic conditions in the environment called sclerotium that enable them to withstand harsh environments (Vega et al., 2012).



**Figure 10:** Illustration of the main phases of the life cycles of the two most emblematic entomopathogenic fungi *B. bassiana* and *M. robertsii* (from Ortiz-Urquiza et al. 2015)

### 4.2.3. The parasitic phase of EPF

EPF have the specificity to infect insects externally via contact with the cuticle, not involving ingestion by the host insect, which is not the case with bacteria and viruses. They infect soft- and hard-bodied insects, with all orders of *Insecta* class susceptible to being parasitized by them (Lacey et al., 2015).

The infection process of insects by EPF unfolds in distinct phases:

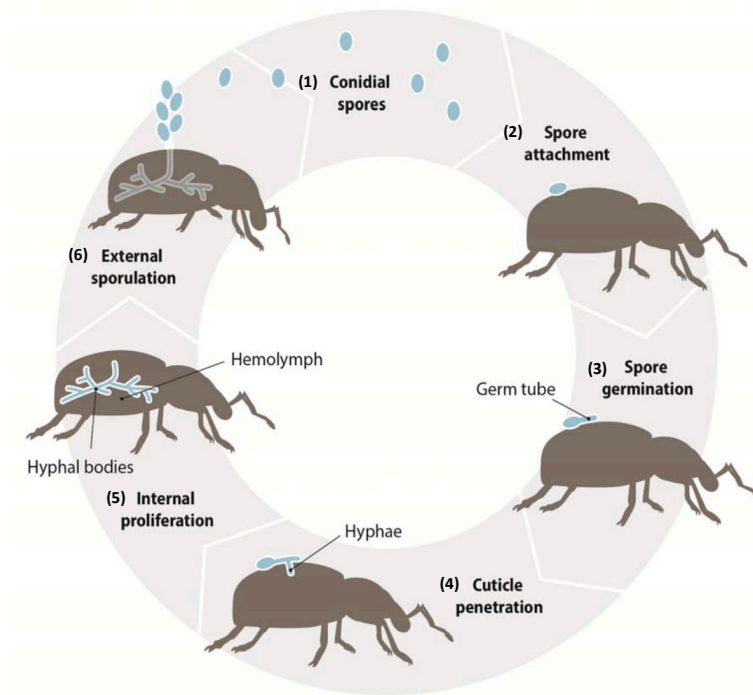
- 1- **Conidia adhesion:** conidia have hydrophobic properties; consequently, they passively adhere to the insect cuticle as a result of hydrophobic interactions and the action of adhesion proteins (e.g., adhesin-like protein MAD1 of species *M. anisopliae*)
- 2- **Conidia germination:** after adhesion to the insect's cuticle, conidia start germinating by forming a germ tube that can create an appressorium (*i.e.*, a fungal structure penetrating the insect insides). The germination process is triggered by many factors, such as abiotic parameters (*i.e.*, temperature, humidity), and biotic parameters (*i.e.*, nutrition, chemistry, and physics)
- 3- **Cuticle penetration:** to penetrate the cuticle, EPF use mechanical pressure and fungal cuticle-degrading enzymes (the composition of the cuticle is 70% of chitin). Through this process, EPF can also gain nutrition. Secretion of secondary metabolites, also named mycotoxins by EPF during cuticle penetration, can also be responsible for insect death. Moreover, death can be the result of the colonization of insect organs, the physical obstruction of insect body parts, and the breaking of the insect's external envelopes (*i.e.*, the cuticle)
- 4- **Hemocoel penetration:** once EPF reaches the hemocoel, they feed upon the nutrients contained in the hemolymph. For some species, transforming into specific cells called 'blastospores,' hyphal bodies, is necessary to bypass the

insect immune response system. Finally, this nutrition phase allows both reproduction and growth of EPF that produce hyphae and aerial conidia inside the insect. This phenomenon is, in part, responsible for host death.

- 5- **Saprophytic re-emergence:** EPF grows out of the insect's body by breaching its cuticle again, then externally colonizes it by producing mycelium. Finally, EPF sporulates again on the insect body to disperse and start new infections (synthesized from the review of Vega et al., 2012 and illustrated in *Figure 11*).

The fungal conidia are naturally present in the soil, and they opportunely get in contact with host insects, as some have a long-term persistence in the environment and persistent infective capacities. The EPF with a broad host range, also called 'generalist,' are reported to have significant infective abilities because they possess numerous enzymes and toxins. Both emblematic EPF species are generalist, *B. bassiana* having more than 700 arthropods classified as host species and *M. anisopliae* with around 200 species (Vega et al., 2012). The insect infection process by EPF depends on abiotic factors such as temperature, relative humidity, and UV radiations, but also biotic ones such as competition with other infective microorganisms and insect immunity response to infection (Vega et al., 2012). Substantial variations of infection patterns are observed between strains of the same species with different genetic backgrounds, geographic origins, and morphologic features, which impacts their virulence and pathogenicity (Castro et al., 2016). Finally, EPF are important contributors to microbial biological control as fungi represent the second most trade biopesticides after *Bacillus thuringiensis* (Lacey et al., 2015). Most mycoinsecticides are based on the formulation of the latest species: *Beauveria* spp., *Metarhizium* spp., *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*), and *Lecanicillium* spp. (Faria and Wraight 2007).





**Figure 11:** Illustration of arthropod infection process by EPF *B. bassiana*. (1) The asexual spore (i.e. conidium here) adheres to the host insect and (2) germinates on its surface; (4) germination continues with the formation of a germ tube and its penetration into insect cuticle with the appressorium in some case; (5) then, EPF colonizes the hemocoel through hyphal growth or blastospores and utilize hemolymph nutrients. (6) After the arthropod death, EPF breaches insect cuticle again and sporulates on the cadaver – Courtesy: Yvonne Rondot

### 4.3. Biology of entomopathogenic fungus *Metarhizium robertsii*

#### 4.3.1. *Metarhizium* genus overview

*Metarhizium* is a genus forming a monophyletic clade of numerous fungi able to parasitize insect hosts from many arthropod taxa (St. Leger and Wang 2020). The phylogenetic background of this genus reveals that these species are more closely related to the fungal grass endophytes *Claviceps* and *Epichloë* than to insect parasites. Barelli et al., 2016 suggest that species from the *Metarhizium* genus originally belonged to a plant symbiont lineage and evolved by acquiring the capacity to infect and kill insects. *Metarhizium anisopliae* (Metschn.) Sorokin (1883) is one of the pioneering EPF used for biological control against agricultural

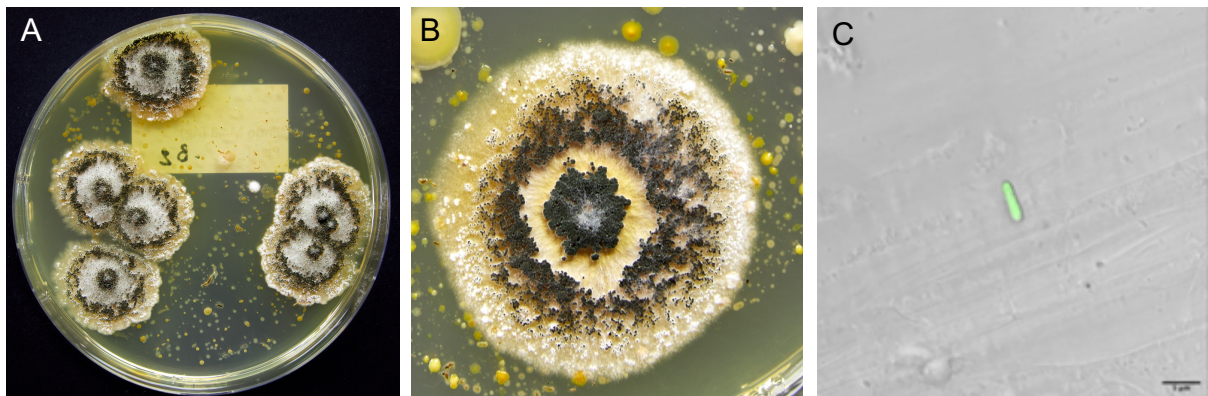
pests (Lord 2005). The fungus is still currently employed under biological programs and significantly contributes to plant protection with the example of *M. anisopliae* strain ESALQ1604 yearly applied as a biopesticide to 2 Mio. ha of sugar cane fields to control spittlebugs (Iwanicki et al., 2019).

#### 4.3.2. *Metarhizium robertsii* taxonomy and morphology

Metschnikoff first described *Metarhizium* spp. by observing a parasitized scarab beetle, *Anisopliae austriaca*, naming the fungus *Entomophthora anisopliae*. The EPF was renamed *Isaria destructor* in 1880. Sorokin harmonized the genus nomenclature with the name *Metarhizium* as an independent genus. The main morphological criteria of classification were the size and shape of the asexual spores, also called conidia, which are very characteristic features to discriminate between *Metarhizium* species and strains. The first three main species, *M. album*, *M. anisopliae*, and *M. flavoviride*, were differentiated (Brunner-Mendoza et al., 2019). Later, the molecular analysis allowed the resolution of the *M. anisopliae* species complex with the 5'EF-1 $\alpha$  DNA sequence variation as the primary classifier. It is now considered as nine separate monophyletic terminal taxa *M. anisopliae*, *M. robertsii*, *M. pingshaense*, *M. brunneum*, *M. majus*, *M. guizhouense*, *M. lepididotae*, *M. acridum*, *M. globosum* and recently added *M. indigotica* (Bischoff et al., 2006, 2009; Kepler et al., 2014). The species *Metarhizium robertsii* (Metchnikoff) Sorokin (1883) (Ascomycota: Hypocreales) is one of the species that causes a disease named 'green muscadine disease' in insects. The disease is named after the green color of the fungal pellicle that covers the killed insect's body. The *M. robertsii* host range includes 200 species of insects from the orders Symphyla, Orthoptera, Dermaptera, Isoptera, Homoptera, Heteroptera, Diptera, Hymenoptera, Siphonaptera, Lepidoptera and other arthropods (Kernasa et al., 2016).

Mature colonies of *M. robertsii* observed in the growth medium are characterized by their dark to light green color and white or brownish center, surrounded by a white edge. On the reverse side of the Petri dish, the colonies exhibit characteristic brownish, orange, and yellow or white colors (Brunner-Mendoza et al., 2019) (Figure 12 A & B).

The fungal species mainly multiplies via asexual propagation and disseminates via conidia (St. Leger and Wang 2020). The dissemination structures called conidia (i.e., the fungal asexual spores) are characterized by their hyaline green color, their cylindrical shape, and their size of 5.0–7.5 X 2.5–3.5  $\mu\text{m}$  (length x width) according to the measures of Bischoff et al., 2009. They are aseptate and generally form chain patterns. The asexual spores are formed in structures called conidiophores. The formation of conidia (i.e., conidiogenesis) is a phialidic conidiogenesis because specific bottle-shaped cells (phialides) are attached to the conidiophores that produce conidia and give them these specific shapes (Humber 2012; Samson et al., 1988). Notably, the conidia shape is the most critical feature in discriminating several species of the *Metarhizium* genus (Figure 12 C) (Bischoff et al., 2009).



**Figure 12:** (a),(b) Colonies of *M. robertsii* observed on potato dextrose agar (PDA) growth medium (photo courtesy of Winfried Schönbach), (c) Conidia of *Metarhizium robertsii* on the surface of grapevine root tissues observed via confocal microscopy (Ponchon et al. 2022)

#### 4.3.3. *Metarhizium robertsii* habitat association and climatic requirements

*M. anisopliae* complex species are mesophilic fungi because they can grow between 10°C and 40°C and optimally germinate between 25 and 30°C. Consequently, they are located in geographical areas with temperate climates (Ment et al., 2011). Nonetheless, they have been discovered worldwide in tropical,

hot semiarid climates, with remarkable records near the Antarctic Circle (St. Leger et al., 1992; Roberts and St. Leger 2004). Therefore, they are highly resilient to diverse habitats. So, it was found that some *M. anisopliae* strains native to fields of Ontario (Canada) are thermophiles with heat tolerance of up to 35°C and others are psychrophiles showing active growth at 8°C (Bidochka et al., 2001, 2005). Such temperature tolerance could stem from their adaptation to specific habitats where strains were selected for their ability to maintain populations under extreme conditions (Brunner-Mendoza et al., 2019).

*M. robertsii* was found to establish preferentially in cultivated soils such as farmland compared to soils in natural habitats (Vega et al., 2012). Another study confirmed the prevalence of *M. robertsii* in agricultural and open-field habitats compared to *M. brunneum* and *M. guizhouense*, which predominate in forested areas (Wyrebek et al., 2011). *M. robertsii* has been collected from many agrosystems worldwide (Zimmermann 2007; Wyrebek et al., 2011; Steinwender et al., 2014, 2015; Clifton et al., 2015; Castro et al., 2016; Iwanicki et al., 2019; Fernández-Bravo et al., 2021). In comparison, *B. bassiana* and *I. fumosorosea* were sampled more often in natural soils than in agricultural soils (reviewed by Vega et al., 2012). Therefore, habitat type is an important selection factor shaping the populations' genetic structure of *M. anisopliae* complex species. By comparison, host insect species seem to be a less important factor in genetic variation/specialization. For instance, a field sampling study conducted in Ontario demonstrates that two distinct *M. anisopliae* genotypes: Ontario Group 1 (OG1) and Ontario Group 2 (OG2), inhabit two different habitats (*i.e.*, OG1 inhabited agricultural soils and open-field and OG2 inhabited forest soils) (Bidochka et al., 2001), and further molecular analysis confirmed that these strains represent different cryptic species (Bidochka et al., 2005).

In soil, the abundance of fungal propagules of *M. robertsii* is dependent upon abiotic factors such as soil temperature, soil texture, soil moisture, organic matter content, soil pH and soil salinity, and general fertility but also biotic ones such as plant presence, host insects and microorganisms interactions (Randhawa et al., 2018; Jaronski 2007 for an extensive review).

#### 4.3.4. *Metarhizium robertsii* plant rhizosphere interaction

For the definition of the rhizosphere, we will refer to the following:

"The narrow zone of soil that surrounds and is influenced by plant roots and is home to an overwhelming number of microorganisms and invertebrates, and is considered to be one of the most dynamic interfaces on Earth."

*Philippot et al., 2013*

The plant rhizosphere is a thin layer of soil with significant dynamic interactions between plants and microorganisms. The plant metabolism itself mainly directs this area. Indeed, plants release oxygen and excrete very complex molecules: the root exudates (main compounds being sugars, amino acids, organic acids, and polysaccharides). Microorganisms use these molecules to gain energy, but they can also have antimicrobial activity. Recent research suggests that plants' microorganism recruitment in the rhizosphere significantly relies on increasing microbial functional capacities (Vandenkoornhuysen et al., 2015). Plant root systems provide nutrients and a physical habitat for microorganisms in exchange. Microorganisms recruited in the rhizosphere are commonly quantified more significantly in this area than in the bulk soil because of the recruitment mechanisms (Hu and Bidochka 2019).

Hu and St. Leger 2002 discovered the rhizosphere competence of *Metarhizium anisopliae* strain ARSEF-1080. Their research aimed to study the field dispersion of a genetically modified *M. anisopliae* strain in a cabbage *Brassica oleracea* var. *capitata* L. field plot. They found that after 10 months of a trial where soil had been inoculated with propagules via hydraulic spraying, the propagules concentration in the inner rhizosphere was close to the initial sprayed concentration ( $10^5$  propagules/g soil). In contrast, the concentration decreased in the outer rhizosphere and the bulk soil ( $10^3$  propagules/g soil). Also, Bruck 2005 found that the same *M. anisopliae* strain propagules persistently remained in two types of potting mix where Norway Spruces *Picea abies* L. plantlets were planted (i.e., peat and bark-based bulk potting media) at 342 days post inoculation (dpi).

Propagules were also quantified in the rhizosphere of *P. abies* until 342 dpi, and their concentration was significantly higher compared to the bulk soil where it was planted.

A field study conducted in Canada found a natural association between *M. robertsii* and other *Metarhizium* spp with the rhizosphere of grass, wildflower, shrub, and trees sampled from the same site composed of an open field and an adjacent forest (Wyrebek et al., 2011). Also, Behie et al., 2015 found that among the 82 flower species randomly sampled in Canadian grassland, 47 (57.3%) of these were colonized at the root level by *Metarhizium* sp. Strains of *Metarhizium* spp. were also isolated from roots of plant species: oat *Avena sativa* L., rye *Secale cereale* L. and cabbage *B. oleracea* L. in several field plots in Denmark. The culturing of root tissues on selective growth medium showed the preponderance of three species naturally associated at the rhizosphere level (*i.e.*, *M. brunneum* was the most common, followed by *M. robertsii* and *M. majus*) without preference of the species strains for specific plant species. Plants are important recruiters that might be associated with the most ubiquitous *Metarhizium* spp. strains present in the soil and select the ones having the best colonizing capacities to predominate in the rhizosphere and bulk soil (Steinwender et al., 2015).

Finally, from a laboratory perspective, a survey conducted on several strains of six *Metarhizium* spp.: *M. robertsii*, *M. acridum*, *M. flavoviride*, *M. brunneum*, *M. pingshaense*, *M. guizhouense* showed that all isolates were rhizosphere competent for barley *Hordeum vulgare* L., corn *Zea mays* L. subsp. *mays*, French beans, and peas *Pisum sativum* L. when planted in vermiculite 10 and 20 days after artificial inoculation via root drenching (Moonjely and Bidochka 2019). Subsequent microscopic observations with confocal scanning electron microscopy were performed on root pieces of several plants. For instance, switchgrass *Panicum virgatum* L. roots were observed after artificial inoculation with a green fluorescence protein (GFP) tagged *M. robertsii* strain. Ten days after inoculation, a significant fluorescent mycelial mass surrounding scanned root sections showed empiric proof of rhizosphere association (Sasan and Bidochka 2012).

#### 4.3.5. *Metarhizium robertsii* molecular basis of rhizosphere interaction

The molecular cross-talk between *Metarhizium* spp. and the plant in the rhizosphere is an essential step for establishing these associations. Amongst *M. robertsii*, *M. brunneum*, *M. acridum*, and *M. flavoviride*, the first produced the highest number of secondary metabolites destruxins (*i.e.*, 25 in total) while in coculture with corn and French bean plantlets (Barelli et al., 2022). It suggests many communication signals between *M. robertsii* and the two plants tested. *M. robertsii* strain ARSEF-2575-GFP conidial germination after 12, 24, and 48h was significantly more important when the conidia were sown in switchgrass root exudates compared to without, while *M. brunneum* and *M. guizhouense* had very low germination rate (Wyrebek et al., 2011). Propagules of the same *M. robertsii* strain germinated more importantly in French black pea *Vigna unguiculata* subsp. *unguiculata* root exudates at concentrations ranging from 1 mg.ml<sup>-1</sup> to 20 mg.ml<sup>-1</sup> compared to *M. acridum*, *M. brunneum*, *Trichoderma harzianum*, *Aspergillus niger*, and *B. bassiana*. The most significant discrepancies between *M. robertsii*, *T. harzianum*, and *A. niger* were found at concentrations ranging from 0.01 mg.ml<sup>-1</sup> to 1 mg.ml<sup>-1</sup>. Thus, the rhizosphere competence of *M. robertsii* stems from its ability to germinate at low concentrations of root exudates excreted (Pava-Ripoll et al., 2011). The rhizosphere competence of *M. robertsii* is also related to its capacity to utilize secreted plant metabolites, such as sucrose (Liao et al., 2013). From the transcriptomic side, this study shows the up-regulation of specific genes by *M. robertsii* while in contact with root exudates, a subset of these also being expressed during insect infection (Pava-Ripoll et al., 2011). Finally, the *Metarhizium* adhesin gene *Mad2* was upregulated when *M. robertsii* was in contact with root exudates of black beans (Pava-Ripoll et al., 2011). This gene encodes an adhesion protein that plays a key role in the association of *M. robertsii* with plants through adhesion to plant epidermis (Wang and St Leger 2007; Wyrebek and Bidochka 2013) and root colonization (Liao et al., 2014).

4.3.6. *Metarhizium robertsii* endophytic potential in plant

The bifunctional lifestyle of *M. robertsii* is supported by its possession of two adhesin-like proteins, *Mad1* and *Mad2*, conferring capacities to adhere to two different types of surface (*i.e.*, *Mad1* enabling it to attach to insect cuticles, and *Mad2* enabling to bind to plant epidermis) (Wang and St. Leger 2007). More profound associations than root adhesion and rhizosphere competence were likewise demonstrated. Indeed, *M. robertsii* can be associated with plants through a close symbiotic association called “endophytism.” Anton De Bary used the term “endophyte” to define a plant microorganism colonizer with asymptomatic infection. We rely on the definition:

“[Endophytes] include all organisms inhabiting plant organs that, at some time in their life, can colonize internal plant tissues without causing apparent harm to their host.”  
*Petrini 1991*

Endophytes are mutualistic plant microorganisms. Therefore, the results of their association are beneficial or non-detrimental for the host plant (Carroll 1986 ; Wilson 1995). Consequently, to be considered endophytes, plant colonizer microorganisms must not induce symptoms in the plant due to their installation. As most of these microorganisms switch between different lifestyles, such as saprophytism and parasitism of host insects, the endophytic lifestyle can represent only a temporary phase in the fungal endophyte lifecycle and establish for a short period. The endophytes encompass a diverse polyphyletic group of microorganisms with various relationships with plants, such as true symbionts or latent pathogens (González and Tello 2011; Jayawardena et al., 2018).

Fungal endophytes are ubiquitous in terrestrial and agricultural environments (Schulz and Boyle 2005). Vega 2018 provided a first overview of the subset of EPF naturally colonizing plants endophytically (Table 2 of Vega 2018). Natural EPF endophytic installations occur through two principal modes of transmission:



- **Vertical transmission:** “passage of an endophytic fungus from a plant [host] to its progeny through seeds” (Saikkonen 2004), thus EPF mycelium can colonize the ovaries and the developing ovules to be passed to the second generation (Vega 2018)
- **Horizontal transmission:** “passage of an endophytic fungus to a new plant without the involvement of a primary host plant.” Asexual spores spontaneously get in contact with a plant, and in case of hyphal germination, they colonize the new host plant through plant stomata or by direct penetration through the epidermis (Daley et al., 2017).

Similar passive modes of transmission are assumed to occur in soil. For example, *M. robertsii* is hypothesized to have two survival strategies: multiply in soil and eventually encounter a host insect or plant. Otherwise, *M. robertsii* may produce a mass of conidia dormant in the soil that may, in turn, get passively in contact with a host, or they may produce a vast web of mycelium that moves through the soil to encounter a host (Angelone et al., 2018). Therefore, *Metarhizium* spp. natural presence as endophyte was also characterized on several plants: *Glycine max* L. (soy), *Quercus robur* L. (peduncle oak), *Taxus chinensis* (Rehder & E.H.Wilson) Rehder (Chinese yew) (Vega 2018). Furthermore, endophytic *M. robertsii* was found in the root tissues of 82 herbaceous plants collected from 3 sites in Ontario (Canada) (Behie et al., 2015). Finally, phylogenetic evidence of species of *Metarhizium* genus relatedness to fungal grass endosymbionts *Claviceps* and *Epichloë* suggests a closer parentage between these genera and endophytes and plant pathogens compared to insect pathogens (Barelli et al., 2016).

#### 4.3.7. Endophytic potential through artificial inoculation

Artificial inoculation of endophytes also successfully induced endophytic installation of EPF in plants. Endophytic associations are classically caused by a fungal treatment with conidia, which can be done through different application means, such as foliar application of conidia (Batta 2013; Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017), stem injection of conidia (Cherry et al., 2004), soil application of conidia via soil drenching or fungal soil contact (Behie et al., 2015; Kaushik and Dutta 2016; Greenfield et al., 2016) and seed immersion in a suspension of conidia (Ahmad et al., 2020a, 2020b). Through these modes of inoculation, different patterns of colonization can be observed inside plants:

- **Transient endophytic colonization:** the fungus is detected in plants for a limited time. It was indeed recorded in several studies that *M. robertsii* propagules detection decreased during the evaluation time in leaves and stems of screened plants, primarily upon seed-inoculation or foliar application (Garrido-Jurado et al., 2017; Siqueira et al., 2020)
- **Localized colonization:** the fungus is detected in plants in a single organ or tissue, sometimes close to its inoculation site. For instance, many studies record that after soil drenching of roots with fungal conidia, *M. robertsii* is only detected as an endophyte in the root system (Garcia et al., 2011; Akello and Sikora 2012; Sasan and Bidochka 2012; Behie et al., 2015; Greenfield et al., 2016; Canassa et al., 2019). Colonization can also be limited to a very restricted area of the plant organ itself. For example, GFP-tagged *B. bassiana* leaf colonization was observed via confocal imaging. It was bound to the first epidermal layers of leaves but did not extend to the inner tissues a few days after leaf-spraying to opium poppy *Papaver somniferum* L. (Landa et al., 2013).
- **Movement inside the plant:** the fungus is detected farther from its inoculation site in different plant parts. For example, *B. bassiana* was inoculated to grapevine *V. vinifera* grafted at the root level via drenching

of conidial suspension and was found to occupy 50% of screened grapevine leaf tissues 28 days post-inoculation (Moloinyane and Nchu 2019).

Consequently, *M. robertsii* can endophytically colonize an important variety of plants from diverse botanic families after artificial inoculation, including monocots: *Zea mays* subsp. *mays* (sweet corn), bread wheat *Triticum aestivum* L., *Panicum vergatum* L. (switchgrass), *Glycine max* L. (soy), *Sorghum bicolor* L. (sweet sorghum); and also dicots: *Phaseolus vulgaris* L. (bean), *Solanum lycopersicum* L. (tomato), *Vicia faba* L. (bean), *Brassica napus* L. (wild shuttle), *Brassica oleracea* L. (cabbage), *Cucumis sativus* L. (cucumber), peanut *Arachis hypogaea* L., strawberries *Fragaria x ananassa*, *Vigna unguiculata* L. (Cowpea), *Manihot esculenta* Crantz (cassava), *Camellia sinensis* (L.) Kuntze (tea plant), *Quercus robur* L. (peduncle oak), and many more plants (non-exhaustive review from Table 1 of Vega, 2018).

#### 4.3.8. Consequence of *M. robertsii* endophytic colonization on plant immune response and related signaling hormones

In response to plant colonization by EPF, the plant immune system activates. During the first steps of colonization, EPF are recognized by protein receptors located at the surface of plant cells called microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs) (Jones and Dangl 2006; Mengistu 2020; Nishad et al., 2020). This recognition induces the plant defense system responsible for the initial PAMP/Pattern-triggered immunity, an effective defense response mechanism in the plant (Chisholm et al., 2006). In parallel, the salicylic acid (SA) signaling pathway activates together with the accumulation of several phytohormones in plant tissues (*i.e.*, SA, jasmonic acid (JA), ethylene (ET)), which play a signaling role in guiding downstream defense responses (González-Guzmán et al., 2022). The salicylic acid (SA) signaling pathway is usually involved in response to biotroph fungi colonization, including EPF (Glazebrook 2005). The SA activates the plant defense response and SA biosynthesis. Systemic acquired resistance (SAR) activates with the production and

concentration of salicylic acid (SA) and pathogenesis-related proteins in plant tissues. Accumulation of these components triggers the cascade of SAR gene expression responsible for priming (Dara 2019). Consequently, SA starts a continuous systemic response against many biotic plant stressors (e.g., microbial pathogens, nematodes, and some herbivore insects) (Wang et al., 2019). Priming is essential for the plant to defend against stressors as primed plants can defend stronger and quicker against bioaggressors and external stressors perturbation (Dara, 2019). Endophytic colonization of *M. anisopliae* and *M. robertsii* was reported in several studies to induce the production of SA in plants compared to the un-inoculated control (Rivas-Franco et al., 2020; Ahmad et al., 2022). However, low concentrations of SA and JA were quantified in peanut root tissues 4 days after *M. anisopliae* inoculation (Hao et al., 2017). The gene defense expression study in maize leaves from plants inoculated via seed-dressing with *M. robertsii* reveals the induction of genes from the JA biosynthesis pathway (*lipoxygenase 1* (*lox1*) and *12-oxo-phytodienoate reductase 7*(*opr7*)) and two genes encoding proteins of the SA-response pathway (*pathogenesis-related protein 5* (*Pr5*) and chitinase gene *endochitinase A*) compared with controls (Ahmad et al., 2020a). Hu and Bidochka, 2021 quantified down-regulations of genes encoding *pathogenesis-related proteins Pr1, Pr2, Pr3, and Pr4*, the *Ethylene-Responsive Transcription Factor 1* (*ERF1*) genes and *ERF5* and *Hydroperoxide lyase* (*HPL*) from the JA pathway in common bean leaf tissues 14 days post-root drenching of *M. robertsii*. These down-regulated genes were shown to be the fate of a decreased abscisic acid synthesis secondary to *M. robertsii* colonization of plant tissues. It is no surprise that studies are contradictory as the endophytic colonization is known to result in the upregulation of some defense-related pathways with a particular dynamic in time, implying a phytohormone cross-talk with positive and negative cross-talk non-investigated in the case of *M. robertsii* inoculation effect (Ahmad et al., 2022; González-Guzmán et al., 2022).

#### 4.3.9. *Metarhizium* association benefits for plants

The association between *Metarhizium* spp. and plants was demonstrated to provide numerous benefits. It was proven to stimulate plant growth in many ways.

Stimulation of root features growth was found in many studies. Root length and total root's wet and dry weight were increased compared to control in tomato after seed inoculation with *M. robertsii* (Siqueira et al., 2020). The entire root surface measured was increased for bread wheat at 56 dpi with *M. brunneum* (González-Guzmán et al., 2022). Post root-drenching with *M. anisopliae*, a higher taproot length and an increase in the number of lateral roots of peanut was measured (Liu et al., 2017). Also, an increase in root hair density in switchgrass roots was observed at 10 days post root-drenching with *M. robertsii* (Sasan and Bidochka 2012). Finally, stimulation of lateral root growth and root hair development of *Arabidopsis thaliana* L. was demonstrated 10 days upon inoculation with *M. robertsii* (Liao et al., 2017).

Growth stimulation of the aerial part of plants by *Metarhizium* spp. was demonstrated for many crops. For example, increased stem length, number of leaf pairs, and fresh shoot weight compared to controls were observed post-treatment with *M. brunneum* on broad beans early post-inoculation (Jaber and Enkerli 2016). Siqueira et al., 2020 found a higher number of flowers per plant and an increase in fruit fresh and dry weight compared to controls in *M. robertsii* seed-inoculated tomato plants. Canassa et al., 2020a found an increase in fruit mass yield and dry weight of leaves compared to controls for strawberries inoculated in field conditions with *M. robertsii* and *Metarhizium* spp. strains. *M. brunneum*, *M. anisopliae*, and *M. robertsii* seed-dressed in sweet corn were demonstrated to increase leaf collar formation, stalk length, and foliage biomass (Liao et al., 2014). In addition, the total chlorophyll content was significantly higher in the leaves of *A. thaliana* 7 days post-contact inoculation with *M. anisopliae* strains (González-Guzmán et al., 2022).

Others benefits from *Metarhizium* spp. associations with plants were recorded, such as the alleviation of biotic stress. It was found that *A. thaliana* planted in a highly concentrated cadmium (Cd) substrate had less Cd accumulation

in the shoots and roots when associated at the root level with *M. robertsii* and that the plants were more stress-resilient to Cd-rich environments because of a global stimulation of their growth (Jiang et al., 2022). Also, *M. brunneum* inoculated to sorghum roots improved the availability of iron for the plant during the trial (Raya-Díaz et al., 2017), similar to the findings of Sánchez-Rodríguez et al., 2016. *M. anisopliae* was demonstrated to help soybean plants resist salinity stress (Khan et al., 2012).

Finally, *M. robertsii* was shown to induce disease-suppressive soil potential because common beans planted in soil amended with *M. robertsii* propagules and a root pathogen *Fusarium solani* f. sp. *phaseoli* had fewer disease symptoms compared to plants planted in sterile soil containing the pathogen (Barelli et al., 2020). A similar result was also obtained when common bean seedlings infected with the same root pathogen were planted in a potting mix containing *M. robertsii* propagules. After 31 dpi, *M. robertsii* prevented root necrosis and reduced root rot's area by 40% compared to control plants without *F. solani* f. sp. *phaseoli*, or *M. robertsii*. It is suggested here that the EPF established as an endophyte in the plants may have antagonistic potential against plant pathogens colonizing the plant tissues (Sasan and Bidochka 2013).

#### 4.3.10. *Metarhizium robertsii* plant association for pest biological control

The use of *M. robertsii* as a biopesticide has been done for several decades and applied in the same way as a synthetic pesticide, as a spraying treatment on the crop. They have been judged inconsistent and poorly persistent in the environment because several abiotic factors such as temperature, UV rays, light intensity, humidity, and rainfall influence the stability and performance in the field. Also, they necessitate a significant quantity of fungal propagules application for soil pest biological control, as a substantial soil volume must be covered (Jackson et al., 2010). Therefore, many researchers state that EPF are not well employed in biological control, and a better understanding of their general ecology should be

completed in the future (Bruck 2010; Vega et al., 2012; Moonjely et al., 2016; Vega 2018). As effective biological control relies on the use of controlling agents persistently establishing in the environment and also able to multiply, EPF lifestyles must be better understood for their optimal use and long-term protection against telluric pests (Bruck 2010).

The association between EPF and roots can induce regulation of telluric pests as EPF may act as a “plant bodyguard” (Bruck 2005, 2010). Theoretically, plants would (i) maintain EPF in their rhizosphere to (ii) increase the contact rate between phytophagous insects, EPF which would, in turn, (iii) increase pest susceptibility to EPF (Elliot et al., 2000; Bruck 2010). This theory is supported by the increase of *M. robertsii* conidia germination in contact with root exudates of several plant species and evidence of an increased propagule concentration of *M. robertsii* in the rhizosphere of plants compared to the surrounding bulk soil after homogeneous inoculation to both of them (see above 4.3.4.). Evidence also shows that, in some cases, endophytic associations benefit both EPF and plants. EPF can translocate nitrogen from infected insect cadavers to colonized plants in exchange for photosynthates. This cooperation between host plants and EPF benefits both partners: insects’ bodies source for nitrogen is a very specialized niche that would be otherwise impossible to access for plants, and EPF benefits from photosynthates as carbon source provided by the plants (Behie et al., 2012; Behie and Bidochka, 2014a, 2014b; Behie et al., 2017; Barelli et al., 2019). In addition, by endophytically colonizing plants and especially roots, EPF might shield against abiotic factors affecting their vitality (Canassa et al., 2020b), and they may gain mobility, increasing the chance of encountering new host insects (Keyser et al., 2014).

The regulation of telluric pests by rhizosphere-associated EPF was demonstrated in several studies, such as the root colonization of Norway spruce by *M. anisopliae*, which eliminated almost 80% of black vine weevil *Otiorhynchus sulcatus* F. larvae population in proximity to the inoculated roots after 14 days of larval root feeding (Bruck 2005). The larvae *Tenebrio molitor* feeding on roots of wheat treated via seed-dressing with *Metarhizium* spp. were efficiently controlled after 14 and 31 dpi while in contact with the potting soil or the root-piece itself,

with a control ranging from 16 to 54% at 14 dpi (Keyser et al., 2014). Kabaluk and Ericsson 2007 observed dead cadavers of wireworm larvae *Agriotes obscurus* that harbored fungal propagules of *Metarhizium* in a field sown with seed-dress treated corn seeds.

#### 4.3.11. Endophyte *Metarhizium robertsii* as biocontrol agent against Grape phylloxera

Grapevine above-ground endophytic fungal communities are under critical investigation, primarily because of their presumed role in preventing fungal diseases such as the GTDs, but to date, their diversity and distribution are still poorly referenced (González and Tello, 2011; Jayawardena et al., 2018; Gramaje et al., 2022; Fournier et al., 2022). One study characterizing the total endophytic microbiota of *V. vinifera* found that *M. pinghaense* was a natural grapevine endophyte of *V. vinifera* cv. Cabarnate Gernischet isolated from the pooled homogenate of all above-ground tissue samples (Jayawardena et al., 2018). This species could therefore be soil-borne and have colonized grapevine tissues as an endophyte through time, as was already speculated by a previous study for other plant species (Steinwender et al., 2015). Fisher et al., 2011 found a natural association of *M. robertsii* and other *Metarhizium* spp. in the grapevine rhizosphere from vine roots collected in the field. Hence, *M. robertsii* could have a natural endophytic potential for the establishment within grapevine as this species was also found native to vineyard soils all around the world in the United States, Portugal, France, Australia, Argentina, Germany, Greece, South Africa (Fisher et al., 2011; Sharma et al., 2018; Poidatz et al., 2018; Korosi et al., 2019; López Plantey et al., 2019; Uzman et al., 2019; Moloinyane et al., 2020; Mantzoukas et al., 2020). In most of these studies, *B. bassiana* and *Metarhizium* spp. predominated as the main EPF species inhabiting vineyard soil. The EPF preference for establishment in vineyard soil could be related to its characteristics. Higher frequencies of EPF, including *Metarhizium* spp. were recorded in vineyard soil with a high carbon: nitrogen ratio and increased copper content according to a comparative field study of 30 German vineyards (Uzman et al., 2019).



Artificial inoculation of *B. bassiana* was previously performed several times in grafted and non-grafted grapevines; also a successful endophytic establishment in the roots was found in both (Mantzoukas et al., 2021), also evidence of systemic colonization of the foliage upon root-drenching was obtained in one study (Moloinyane and Nchu 2019). Upon foliage application, *B. bassiana* persisted in the aerial part of the grapevines for several weeks and had a bio-insecticidal effect on vine mealybug (*Planococcus ficus*) and green leafhopper (*Empoasca vitis*) (Rondot and Reineke 2018). In two studies, the disease antagonism of *Plasmopara viticola* by *B. bassiana* was observed with a subsequent reduction in the severity of plant symptoms (Jaber 2015; Rondot and Reineke 2019).

Radicicole grape phylloxera biological control was investigated by Kirchmair et al., 2004a. Researchers introduced grape phylloxera radicolle to non-grafted vines *V. vinifera* which had been potted in substrate mixed with barley kernels coated with *M. anisopliae* conidia. A month after co-inoculation, eight out of ten *Metarhizium*-treated grapevines exhibited no new phylloxera infections on the roots compared to the untreated grapevine roots, which had a significant number of fresh nodosities and single or multiple phylloxera infestation. Field experimentation also confirmed these results: inoculation of coated barley kernels in non-grafted vineyard interrow with *M. anisopliae* reduced grape phylloxera infestations in the vineyard plot (Kirchmair et al., 2004b). However, the study did not investigate the rhizospheric and endophytic associations between *M. anisopliae* and grapevine.

# Objectives

## 5-Objectives

EPF are ubiquitous soil fungi and important natural antagonists of arthropod pests investigated for two centuries as biocontrol agents. Increasing interest was raised in their potential to associate as endophytes with a wide range of plant species. *Metarhizium robertsii* can associate with the rhizosphere and root-endosphere of numerous plant species and offer them multiple benefits. Consequently, the association between *M. robertsii* and plants opens excellent prospects for alternative crop protection methods. *M. robertsii* was found native to vineyard soils worldwide but so far, *M. robertsii* endophytic colonization of non-grafted grapevine *Vitis vinifera* and its association with the rhizosphere was never investigated. Within the framework of the study of these associations, characterizing the grapevine response to colonization by *M. robertsii* is a primary step before understanding its functional role in the plant, as adverse effects of endophytic colonization on plant physiology must be prevented. In addition, several studies have shown significant potential for *Metarhizium* to control soil-borne pests associated with grapevine roots, especially radicolle grape phylloxera *Daktulosphaira vitifoliae*, without deeper characterization of underlying regulatory mechanisms and the association between grapevine and *Metarhizium*.

The objective of the present thesis is to explore the rhizosphere-association and endophytic potential of several *M. robertsii* strains native to contrasted vineyard environments with non-grafted grapevines. The study focuses on the interaction between *M. robertsii*, non-grafted grapevine, and radicolle grape phylloxera to better characterize the tri-trophic interactions in the context of biological control strategy in viticulture. Two main hypotheses are tested:

- (i) *M. robertsii* has rhizospheric and endophytic potential for association with non-grafted grapevine roots without harming grapevine's growth
- (ii) Grapevine root-associated *M. robertsii* can prevent the development of radicolle forms of grape phylloxera

The following research questions are addressed:

- 1- Can *M. robertsii* association with non-grafted grapevine rhizosphere and root endosphere be artificially induced *in vitro*?
- 2- What is the dynamic of the endophytic establishment of *M. robertsii* inside non-grafted grapevine?
- 3- Is grapevine endophytic colonization more successful for *M. robertsii* strains native to the vineyard soil than a non-vineyard-native GFP-transformed strain?
- 4- Does the rhizosphere association and endophytic establishment of *M. robertsii* induce modifications of grapevine growth?
- 5- Does the rhizosphere association and endophytic establishment of *M. robertsii* induce a grapevine grape phylloxera-responsive defense gene expression?
- 6- Does *M. robertsii* have entomopathogenic potential to parasitize radicicole grape phylloxera directly?
- 7- Do the rhizosphere association and endophytic establishment of *M. robertsii* induce a pathogenic effect against radicicole grape phylloxera?

This thesis is based on three manuscripts included in the presented in three separate chapters. The question 1, 2 and 3 will be addressed in the Chapter 1 of the thesis :

- (i) Mathilde Ponchon, Annette Reineke, Marie Massot, Michael J. Bidochka, Denis Thiéry, and Daciana Papura. **Three methods assessing the association of the endophytic entomopathogenic fungus *Metarhizium robertsii* with non-grafted grapevine *Vitis vinifera*. *Microorganisms* 10 (2437), 1-16**

The question 4 and 5 will be addressed in the second Chapter 2 of the thesis:

- (ii) Mathilde Ponchon, Daciana Papura, Denis Thiéry, and Annette Reineke. **Association of four *Metarhizium robertsii* strains native to vineyards with non-grafted grapevine *Vitis vinifera* and their impact on growth and expression of phylloxera responsive defense genes.** *In preparation*

The question 6 and 7 will be addressed in the third manuscript Chapter 3 of the thesis.

- (iii) Mathilde Ponchon, Daciana Papura, Manmeet Singh, Karima Rahmani, Annette Reineke, and Denis Thiéry, ***Metarhizium robertsii* associated with non-grafted grapevine *Vitis vinifera*, a biological control candidate against the radicolle grape phylloxera (*Daktulosphaira vitifoliae*).** *In preparation*

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# Chapter 1

# 6-Three Methods Assessing the Association of the Endophytic Entomopathogenic Fungus *Metarhizium robertsii* with Non-Grafted Grapevine *Vitis vinifera*

Mathilde Ponchon <sup>1,2</sup>, Annette Reineke <sup>1</sup>, Marie Massot <sup>3</sup>, Michael J. Bidochka <sup>4</sup>, Denis Thiéry <sup>2,\*</sup> and Daciana Papura <sup>2</sup>

- 1- Department of Crop Protection, Hochschule Geisenheim University, 65366 Geisenheim, Germany
  - 2- INRAE, Bordeaux Sciences Agro, ISVV, UMR SAVE, 33140 Villenave d'Ornon, France
  - 3- INRAE, Univ. Bordeaux, UMR BIOGECO, 33610 Cestas, France
  - 4- Department of Biological Sciences, Brock University, St. Catharines, ON L2S 3A1, Canada
- Correspondence: denis.thiery@inrae.fr; Tel.: +33-557-122-618

Type of authorship: First Author

Type of article: Research article

Contribution to the article: Conceptualization and design of the experiments; Development of the methodology; Planning of the experiments; Performance of most experimental work; Evaluation and statistical analysis of the data; Preparation of all figures and tables; Writing of the manuscript

Contribution of other authors: D.P, D.T., and A.R. contributed to the conceptualization and the experimental design, data analysis, and writing the manuscript; D.P. contributed to the microscopy and microbiology experimental work; M.M. helped design the ddPCR methodology and performed the ddPCR analysis; M.J.B. contributed to writing the manuscript

Journal: *Microorganisms*

Date of submission: 7 November 2022

Date of publication: 9 December 2022

DOI: <https://doi.org/10.3390/microorganisms10122437>



Article

# Three Methods Assessing the Association of the Endophytic Entomopathogenic Fungus *Metarhizium robertsii* with Non-Grafted Grapevine *Vitis vinifera*

Mathilde Ponchon <sup>1,2</sup>, Annette Reineke <sup>1</sup>, Marie Massot <sup>3</sup>, Michael J. Bidochka <sup>4</sup>, Denis Thiéry <sup>2,\*</sup> and Daciana Papura <sup>2</sup>

<sup>1</sup> Department of Crop Protection, Hochschule Geisenheim University, 65366 Geisenheim, Germany

<sup>2</sup> INRAE, Bordeaux Sciences Agro, ISVV, UMR SAVE, 33140 Villenave d'Ornon, France

<sup>3</sup> INRAE, Univ. Bordeaux, UMR BIOGECO, 33610 Cestas, France

<sup>4</sup> Department of Biological Sciences, Brock University, St. Catharines, ON L2S 3A1, Canada

\* Correspondence: denis.thiery@inrae.fr; Tel.: +33-557-122-618

**Abstract:** Characterizing the association of endophytic insect pathogenic fungi (EIPF) with plants is an important step in order to understand their ecology before using them in biological control programs. Since several methods are available, it is challenging to identify the most appropriate for such investigations. Here, we used two strains of *Metarhizium robertsii*: EF3.5(2) native to the French vineyard environment and ARSEF-2575-GFP a laboratory strain expressing a green fluorescent protein, to compare their potential of association with non-grafted grapevine *Vitis vinifera*. Three methods were used to evaluate the kinetics of rhizosphere and grapevine endospheric colonization: (i) Droplet Digital (ddPCR), a sensitive molecular method of *M. robertsii* DNA quantification in different plant parts, (ii) culture-based method to detect the live fungal propagules from plant tissues that grew on the medium, (iii) confocal imaging to observe roots segments. Both strains showed evidence of establishment in the rhizosphere of grapevines according to the culture-based and ddPCR methods, with a significantly higher establishment of strain EF3.5(2) (40% positive plants and quantified median of  $\exp(4.61)$  c/μL) compared to strain ARSEF-2575-GFP (13% positive plants and quantified median of  $\exp(2.25)$  c/μL) at 96–98 days post-inoculation. A low incidence of association of both strains in the grapevine root endosphere was found with no significant differences between strains and evaluation methods (15% positive plants inoculated with strain EF3.5(2) and 5% with strain ARSEF-2575-GFP according to culture-based method). ddPCR should be used more extensively to investigate the association between plants and EIPF but always accompanied with at least one method such as culture-based method or confocal microscopy.

**Keywords:** endophytes; rhizosphere; ddPCR; fungal entomopathogens



**Citation:** Ponchon, M.; Reineke, A.; Massot, M.; Bidochka, M.J.; Thiéry, D.; Papura, D. Three Methods Assessing the Association of the Endophytic Entomopathogenic Fungus *Metarhizium robertsii* with Non-Grafted Grapevine *Vitis vinifera*. *Microorganisms* **2022**, *10*, 2437. <https://doi.org/10.3390/microorganisms10122437>

Academic Editors: Vadim Kryukov and Victor Glupov

Received: 7 November 2022

Accepted: 7 December 2022

Published: 9 December 2022

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## 1. Introduction

Endophytic insect pathogenic fungi (EIPF) support plant health in multiple ways and are known as plant biological control agents against pests [1], plant growth stimulators [2] and plant vaccines [3]. Besides their pathogenic nature, most of these attributes stem from their rhizosphere competence and endophytic potential with diverse plant species even if the underlying mechanisms are not well elucidated [4]. Indeed, EIPF are able to establish inside root tissues without causing significant symptoms of infection in plants [5,6] while simultaneously surrounding the root surface [7]. *Metarhizium robertsii* (Metchnikoff) Sorokin (1883) is an important species used as a commercial microbial biological control agent because of its direct parasitism toward insect pests [8], its potential to colonize the plant rhizosphere [9,10] as well as its ability to establish as an endophyte [11–13]. Its root colonization potential is an important feature in order to optimize its capacity as a biological control agent and as a stimulator of plant health.



Efficient and practicable modes of inoculation are a prerequisite to successfully use EIPF as endophytes in crop plants. Previous studies have evaluated and compared techniques for EIPF endophytic inoculation in various plants, such as stem injection [14], leaf spraying [15], root drenching [16], root dipping [17,18] and seed dressing [19,20]. These techniques allowed the characterization of EIPF inoculation success, the respective plant organs colonized, and the extent of colonization [2,6]. However, the dynamics of EIPF establishment inside inoculated plants needs further investigation as very little is known about fungal behavior inside the respective plant and its propensity to colonize within the plant. Moreover, important research gaps still exist regarding the process of endophytic colonization by EIPF, including insights in the endophytic path of entry into the plant tissue, the precise cartography of colonized areas of plants organs as well as distinct tissue colonization.

A panel of methods is available to evaluate endophytic colonization of EIPF. The most common is the culture-based method, which consists of plating excised plant pieces or a homogenate of surface-sterilized plant tissues from inoculated plants on a growing medium containing antibiotics and to morphologically characterize the microorganism growing on the plate after incubation [21]. This method has the advantage of being affordable, easy to handle, and rapid if the fungus grows relatively quickly on respective plates [22]. However, quantification of fungal propagules via culture-based method has a high degree of imprecision [23]. Moreover, the magnitude of endophytic colonization of plants can be overestimated by the culture-based method. Indeed, it allows quantification of the rate of colonization of the sampled plant area, and, by sampling several plant areas, to characterize the total percentage of colonized plant surfaces [16]. However, these measurements are only partially reliable as the mean percentage of plant area colonization can be overestimated by a single plant or a single organ being totally colonized.

Microscopic techniques, including confocal imaging, are highly appropriate for such studies as they constitute an empirical method available to characterize the endophytic colonization of the plant and the rhizosphere [24]. They provide visual proof of colonization with the advantage of giving clear information of the proportion of samples and tissues being colonized by respective fungal structures [6]. However, microscopic methods are labor intensive and require exhaustive screening of respective plant organs and tissues [7]. As the extent of endophytic colonization also depends on the respective fungal strain, the host plant [12] and the environment from which the strain was sampled [25], applying a more reliable method for calculating the percentage of EIPF colonization of plants would improve identification of suitable EIPF strains.

Droplet digital PCR (ddPCR<sup>TM</sup>) is a molecular PCR-based method allowing absolute quantification of targeted DNA even in very minimal amounts with better resolution than quantitative PCR (qPCR) [26]. Its specificity and sensitivity are key features of this method, which in turn is important for targeting endophytes as they are present in small quantities in plant organs and other microbial taxa could, as well, be amplified in standard qPCR in case of poor sensitivity [21,24,27]. As an example, ddPCR was proven efficient and more precise than qPCR to quantify inoculum of *Ilyonectira liriodentri* in samples of soil, rhizosphere and grapevine rhizoendosphere [28]. However, ddPCR quantifies DNA from viable and non-viable cells and thus cannot assess the viability of the quantified inoculum. As a consequence, this method should be used together with a method that allows an estimation of the viability of the fungal propagules quantified such as the culture-based method.

Proof of endophytic colonization of *M. robertsii* was found in diverse plants from various botanical families such as tomato *Solanum lycopersicum* L., soy *Glycine max* L., wheat *Triticum aestivum* L., the broad bean *Vicia faba* L., cabbage *Brassica oleracea* L., and the French bean *Phaseolus vulgaris* L. [24,29–33]. However, it was never investigated in domesticated grapevine *Vitis vinifera*, which is a perennial dicot plant grown grafted in most commercial vineyards worldwide [34]. Grafting stands as the most efficient biological control solution against one of the main soil-borne pests, the grapevine phylloxera (*Daktulosphaira vitifoliae*). However, some global regions still grow grapevines non-grafted, such as Argentina and

Australia [35,36] necessitating costly quarantine measures to protect the plants. For this reason, protecting own-rooted grapevine through endophytic association with *M. robertsii* would represent a major advantage for sustainable crop protection.

Grapevine above-ground endophytic fungal communities are of critical importance, especially because of their putative role in preventing fungal diseases, however their diversity and distribution are poorly investigated [37,38]. A close relative of *M. robertsii*, *M. pinghaense* was once assessed as a natural grapevine endophyte of *V. vinifera* cv. Cabarnate Gernischet from a pooled homogenate of all above-ground tissue samples, with speculation that this species could be native to the soil and had colonized grapevine tissues as an endophyte through time [38]. Thus, *M. robertsii* could have an endophytic potential of establishment within grapevine as this species was also found native to vineyard soil all around the world [39–45]. Some studies investigated the potential of artificially inoculated endophytes in grapevine. The EIPF *Beauveria bassiana* has been shown to colonize grapevine as an endophyte [46], with antagonistic activity against downy mildew *Plasmopara viticola* [47,48], grapevine mealybugs *Planococcus ficus* and leafhopper *Empoasca vitis* [48,49].

The aim of this study was to demonstrate the rhizospheric and endophytic potential of *M. robertsii* with non-grafted grapevine *V. vinifera* after artificial inoculation. Accordingly, we screened the presence of endophytic *M. robertsii* in different parts of the grapevine (root, stem and leaf pieces) using in vitro plants. For this purpose, we used three methods: (i) ddPCR for absolute quantification of minimal amounts of *M. robertsii* DNA inside grapevine tissues, (ii) culture-based method to detect live propagules of *M. robertsii* extracted from plant tissues, (iii) confocal imaging to visually track endophytic *M. robertsii* inside the colonized plant. In this study ddPCR was used for the first time to characterize the association between EIPF and plant. Another aim of this study was to compare the kinetics of grapevine colonization by a *M. robertsii* strain native to a French vineyard environment (EF3.5(2)) and a *M. robertsii* transformant strain (ARSEF-2575-GFP) expressing green fluorescence protein (GFP), which is a laboratory strain originally non-native to the vineyard environment [50]. Accordingly, the goal was to assess whether a strain collected in the same environment as grapevine that we wish to protect, has a better and more durable colonization potential. We hypothesized that (i) *M. robertsii* colonization of grapevine is limited to the roots and occurs more extensively in the rhizosphere compared to the root endosphere, (ii) the strain EF3.5(2) native to the vineyard has higher potential to persistently associate with grapevine rhizosphere and root endosphere compared to the ARSEF-2575-GFP strain.

## 2. Materials and Methods

### 2.1. Fungal Cultures

Two *M. robertsii* strains were used in this study. *M. robertsii* strain EF3.5(2) was collected in the INRAE experimental vineyard soil in spring 2015 (Villenave d'Ornon, South West of France, 44°47'30.4" N 0°34'36.9" W) via insect bait technique [51], and was kept as a laboratory culture since then. A previous study has shown its potential as a biocontrol agent against the Asian hornet *Vespa velutina* [39]. The fungal identification was confirmed by morphological analysis and by genetic sequencing of the translation elongation factor 1-a [52]. A transformant of *M. robertsii* (ARSEF-2575-GFP) expressing green fluorescence protein (GFP), originally collected from a coleopteran host insect (*Curculio caryae* [Coleoptera: Curculionidae]) in the United States [53] and maintained after transformation in the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA) [54] was also used for the experiment (courtesy of Prof. Dr. Michael Bidochka and Dr. Shasha Hu). Stock cultures were grown on oat agar chloramphenicol media (40 g organic oat flour, (Moulin Des Moines, Krautwiller, France)), 20 g agar (SIGMA Aldrich, St. Louis, MO, USA), 50 mg chloramphenicol (SIGMA Aldrich), and 1 L of water. Conidia were dislodged from the surface into a sterile suspension solution

(1/8 Ringer + 0.02% Tween 80<sup>®</sup> (SIGMA Aldrich). The conidial suspension was adjusted to  $1 \times 10^7$  conidia·mL<sup>-1</sup> using a hemocytometer.

## 2.2. Plant Material

Grapevines *Vitis vinifera* cultivar Cabernet Sauvignon were micro propagated in vitro and acclimated in humid plastic containers for 44 days, as described in [55]. Grapevines harboring an average of 8 newly formed leaves were planted in pots (volume 0.3 L) filled with vineyard soil taken from the same vineyard where *M. robertsii* strain EF3.5(2) was originally isolated, however in a different vineyard plot. The soil was previously sterilized by autoclaving twice. Plants were grown and watered every third day and maintained for 60 days in a growth chamber with a 16:8 photoperiod, 23 °C and 60% relative humidity in the facilities of the INRAE UMR 1065—SAVE.

## 2.3. Fungal Inoculation of Grapevines

Forty-four potted grapevine plants were inoculated with either *M. robertsii* strain ARSEF-2575-GFP or EF3.5(2) by watering the roots with 50 mL of a  $1 \times 10^7$  conidia·mL<sup>-1</sup> suspension split into two doses of 20 mL on the first day and 30 mL on the 6th day. The concentration was judged optimal to successfully establish several strains of EIPF including *Metarhizium* spp. as endophyte with root drenching treatment according to [56]. Twenty-eight control plants were treated with the same volume of a sterile suspension solution.

## 2.4. Rhizospheric Detection and Endophytic Association Assessments of Roots, Leaves and Stems Using Culture-Based Method

After 14-, 35-, 63-, 96–98- days post-inoculation (dpi), grapevines were uprooted for analysis. For rhizosphere association assessment, grapevines were gently removed from their pots and roots were shaken with forceps to remove adhering soil particles. Roots were not disinfected and soil particles were adherent to these roots, hence we considered these root samples representative of the grapevine rhizosphere. From each plant, an average of 1 g of randomly picked root pieces was cut and placed in a tube (height × width: 60 mm × 27 mm and 20 mL volume, ZINSSER POLYVIALS<sup>®</sup>) with 4 mL of sterile distilled water and 0.02% Tween 80<sup>®</sup> and mixed with 2 inox balls of 8 mm diameter using the disrupter TissueLyser II (Qiagen, Hilden, Germany). A 100 µL of root homogenate as well as a 10-fold diluted homogenate was spread in duplicates onto a chloramphenicol, thiabendazole, cycloheximide (CTC) medium according to a modified recipe from [57] (39 g·L<sup>-1</sup> potato dextrose agar, 0.1 g·L<sup>-1</sup> chloramphenicol diluted in 96% ethanol, 0.002 g·L<sup>-1</sup> thiabendazole and 0.15 g·L<sup>-1</sup> cycloheximide each diluted in sterile water (SIGMA Aldrich) and filled up to 1 L with sterile water). The plates were incubated at 25 °C for 14 days. *M. robertsii* colonies were visually identified according to morphological features according to criteria described by [54]. The remaining root homogenate was immediately lyophilized for extraction of DNA.

For endophytic analysis, 1 g of root pieces was sampled in the same way as described above, as well as the third and the terminal leaf and a 3 cm part of the upper stem from each grapevine plant. All root samples were surface-disinfected by dipping them twice in two different solutions of 0.5% NaOCl and 0.02% Tween 80<sup>®</sup>, followed by 2 min in 70% ethanol and rinsing thrice in sterile water. Leaves and stems were disinfected in the same manner, except that they were dipped only once in 0.5% NaOCl and 0.02% Tween 80<sup>®</sup> for 2 min. Samples were then processed as described above, except for stem pieces which were entirely lyophilized after sampling.

## 2.5. Quantification of Rhizospheric and Endophytic Association Using Droplet Digital PCR

DNA from inoculated grapevine samples was extracted with the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) following the manufacturer's directions. To carry out the molecular absolute quantification, the device QX200 DROPLET DIGITAL PCR (ddPCR<sup>™</sup>) System (Bio-Rad, Hercules, CA, USA) installed at the Genome Transcriptome

Platform of Bordeaux (BIOGECO, Bordeaux, France) was used. The primers used for the PCR reactions were Ma 1763 (5'-CCAACTCCCAACCCCTGTGAAT-3') and Ma 2097 (5'-AAAACCAGCCTCGCCGAT-3') designed by [58] positioned in the regions of ITS 1 and ITS 2 of the nuclear ribosomal RNA gene cluster, respectively, which were shown to be specific to *Metarhizium* clade 1 which includes *M. robertsii*. The 22  $\mu$ L PCR reaction mix per sample was composed of 2  $\mu$ L DNA, 11  $\mu$ L of QX200™ ddPCR™ EvaGreen Supermix (containing a dsDNA-binding dye) (Bio-Rad, USA), 2.2  $\mu$ L of each primer at 150 nM, plus 4.6  $\mu$ L of pure water. Samples were run as single replicates with a volume of 20  $\mu$ L of mix per sample. A negative control with 2  $\mu$ L of ultrapure water, as well as one positive control with fungal DNA of *M. robertsii* strain EF3.5(2) extracted from a pure culture was included on every ddPCR plate. Each 20  $\mu$ L sampling mix was divided into droplets with the QX200 Droplet Generator (Bio-Rad) and then transferred to a 96-well PCR plate. The thermocycling program was set as [95 °C  $\times$  5 min; 40 cycles of (95 °C  $\times$  30 s, 61 °C  $\times$  1 min), 4 °C  $\times$  5 min and 90 °C  $\times$  5 min] in the Bio-Rad C1000 (Bio-Rad). The device QX200 droplets reader screened each droplet solely for fluorescent signal. The absolute number of copies of targeted fungal DNA sequence per  $\mu$ L of the sample was calculated with a Poisson model, processing the number of positive droplets out of 20,000 droplets (QuantaSoft™ version 1.7, Bio-Rad software). The threshold defining the detection of the positive droplets was adjusted manually at the value of 5000 of fluorescence amplitude. Finally, the dilution factor of the DNA extract in the reaction mix (2  $\mu$ L in 22  $\mu$ L) was used to calculate the ultimate absolute concentration of each sample. When analyzing the data, the final values of quantification that were inferior to 1 copies/ $\mu$ L were considered as null for the analysis.

#### 2.6. Observations of Rhizospheric and Endophytic Association Using Confocal Microscopy

Confocal imaging was completed at the Bordeaux Imaging Center (BIC) (Bordeaux University, Bordeaux, France) on a Zeiss LSM 880 confocal laser scanning microscope equipped with fast Airy Scan using Zeiss C PL APO  $\times$  63 oil-immersion objective. For GFP, excitation was achieved with a 488 nm laser power and fluorescence emission collected at 505–550 nm. Grapevines used for microscopic observation were produced as described above (see Section 2.2) and 12 grapevines in vitro were inoculated by drenching with 50 mL of fungal suspension of  $1 \times 10^7$  conidia·mL<sup>-1</sup> of *M. robertsii* strain ARSEF-2575-GFP. After 14-, 31-, 63- and 98- dpi, respectively, 3 plants were uprooted and analyzed through laser scanning confocal microscopy.

#### 2.7. Statistical Analysis

All analysis were carried out in the R software updated version RStudio 2022.07.2 + 576 (2022 RStudio©, PBC. All Rights Reserved). The non-parametric Kruskal–Wallis test was used to determine significant differences ( $p < 0.05$ ) between the two tested strains and the control treatment for the number of DNA copies/ $\mu$ L quantified in the rhizosphere and root endosphere via ddPCR. Finally, the percentage of detection of *M. robertsii* as established in the rhizosphere and root-endosphere was analyzed separately for each strain by comparing results from both ddPCR and culture-based method with the Chi<sup>2</sup> test.

### 3. Results

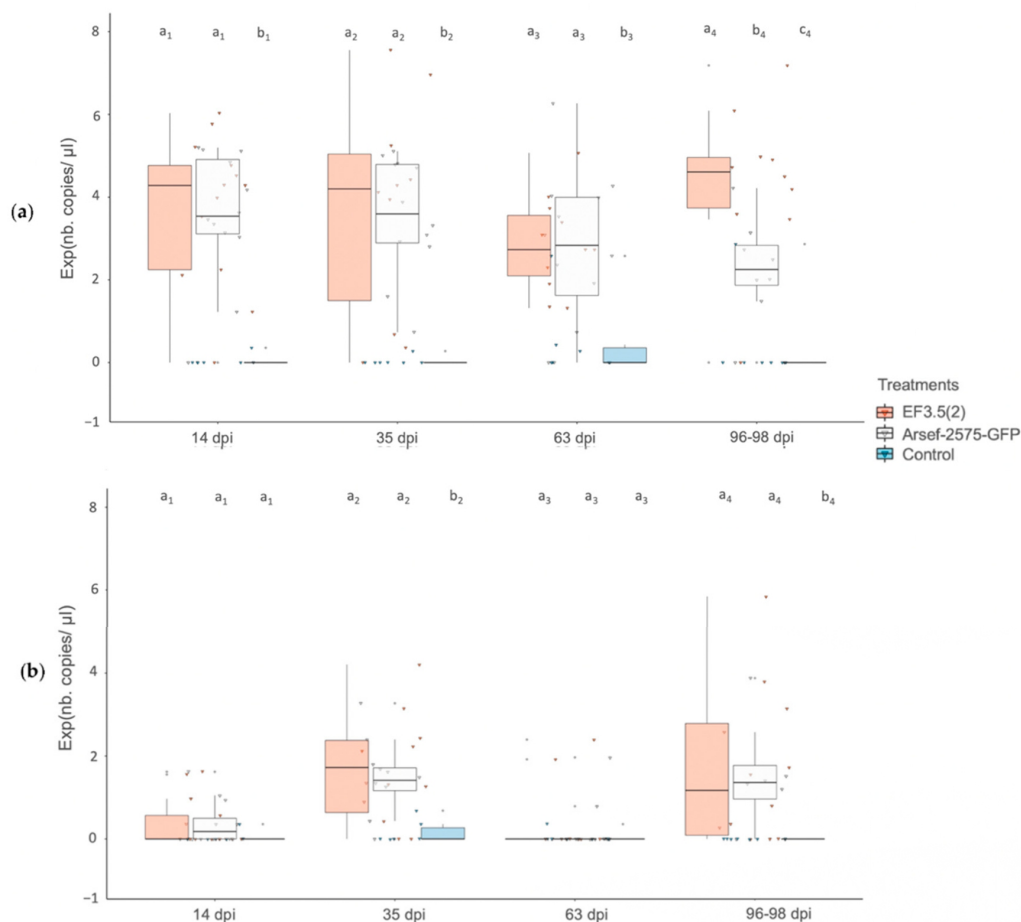
#### 3.1. Quantification of Association of Two *M. robertsii* Strains with Grapevines via ddPCR

##### 3.1.1. Quantification of Rhizospheric Potential of Two *M. robertsii* Strains

For the rhizosphere, ddPCR detected *Metarhizium robertsii* DNA copies (c) in 87 of the 117 (74.4%) tested root samples. In detail, 41 of 44 (82.2%) roots inoculated with EF3.5(2) or ARSEF-2575-GFP, respectively, were tested positive. However, we also detected *M. robertsii* DNA in the roots of the control plants with 6 out of 28 (21.4%) plants being positively amplified by ddPCR.

A median of exp(4.30) c/ $\mu$ L (range 0–exp(6.04)) at 14 dpi, exp(4.20) c/ $\mu$ L (range 0–exp(7.60)) at 35 dpi, exp(2.74) c/ $\mu$ L (range exp(1.32)–exp(5.08)) at 63 dpi and exp(4.61) c/ $\mu$ L (range 0–exp(7.20)) at 96–98 dpi, respectively, was quantified in DNA extracted of root

samples inoculated with the strain EF3.5(2). Meanwhile, *M. robertsii* DNA copies from roots of the ARSEF-2575-GFP treatment reached median concentrations of  $\exp(3.55)$  c/ $\mu\text{L}$  (range 0– $\exp(5.20)$ ) at 14 dpi,  $\exp(3.60)$  c/ $\mu\text{L}$  (range  $\exp(0.74)$ – $\exp(5.11)$ ) at 35 dpi,  $\exp(2.84)$  c/ $\mu\text{L}$  (range 0– $\exp(6.30)$ ) at 63 dpi and  $\exp(2.25)$  c/ $\mu\text{L}$  (range 0– $\exp(4.22)$ ) at 96–98 dpi. Of the tested control plants, 6 positive amplifications were obtained, yet with very low copy numbers ranging from  $\exp(0.3)$  to  $\exp(2.9)$  c/ $\mu\text{L}$ . While significant differences between the two EIPF treatments occurred only at the end of the experiment at 96–98 dpi (pairwise Wilcoxon test,  $p = 0.0136$ ), both treatments had higher EIPF concentrations than the control at all times (Kruskal–Wallis test,  $\chi^2 = 13.496$ ,  $df = 2$ ,  $p = 0.001173$ ;  $\chi^2 = 14.044$ ,  $df = 2$ ,  $p = 0.0008922$ ;  $\chi^2 = 9.6779$ ,  $df = 2$ ,  $p = 0.007915$  for 14, 35, 63 dpi, respectively) (Figure 1a).



**Figure 1.** (a) Time-course of quantification of rhizospheric *V. vinifera* association of two *M. robertsii* strains (EF3.5(2), red boxes; GFP transformed strain ARSEF-2575-GFP, white boxes) and a control treatment (blue boxes) using ddPCR. The boxplots represent the logarithm of the number (nb) of DNA copies (of non-null values) of *M. robertsii* per microliter of DNA extracted of mixed non-disinfected grapevine roots with adhering soil (rhizosphere) 14-, 35-, 63- and 96–98 days post inoculation (dpi). Significant differences are indicated by small letters above to boxes (Kruskal–Wallis test,  $p < 0.05$ ); (b) Time-course of quantification of endophytic *V. vinifera* root association of two *M. robertsii* strains using ddPCR. The boxplots represent the logarithm of the number (nb) of DNA copies (of non-null values) of *M. robertsii* per microliter of DNA extracted of mixed disinfected grapevine roots (root endosphere) 14-, 35-, 63- and 96–98 days post inoculation (dpi).

### 3.1.2. Quantification of the Root Endophytic Potential of Two *M. robertsii* Strains

Considering the endophytic potential of *M. robertsii*, 51 of 115 tested root samples (44.4%), amplified positively in the ddPCR. *Metarhizium robertsii* DNA copies (c) were detected in 21 of 44 (47.8%) root samples treated with *M. robertsii* strain EF3.5(2), while the transformed strain ARSEF-2575-GFP was detected in 26 of 43 (60.5%) root samples. Among the control root samples, 4 out of 28 (14.3%) tested positive in the ddPCR.

The endophytic concentration of DNA from the root samples treated with either strain EF3.5(2) (median of  $\exp(1.72)$  c/ $\mu\text{L}$  (range 0– $\exp(4.20)$ )) or strain ARSEF-2575-GFP (median of  $\exp(1.41)$  c/ $\mu\text{L}$  (range 0– $\exp(3.27)$ )) was significantly higher compared to the controls at 35 dpi (Kruskal–Wallis test,  $X^2 = 8.6207$ ,  $df = 2$ ,  $p = 0.01343$ ), respectively). Similarly, endophytic root concentrations of *M. robertsii* differed between EIPF treatments and the control at 96–98 dpi (Kruskal–Wallis test,  $X^2 = 10.784$ ,  $df = 2$ ,  $p = 0.004554$ ), with a median  $\exp(1.17)$  c/ $\mu\text{L}$  (range 0– $\exp(5.84)$ ) for samples treated with the strain EF3.5(2) and  $\exp(1.36)$  c/ $\mu\text{L}$  (range 0– $\exp(3.88)$ ) for samples treated with the strain ARSEF-2575-GFP. However, no significant differences were detected at 14 dpi (Kruskal–Wallis test,  $X^2 = 2.4277$ ,  $df = 2$ ,  $p = 0.2971$ ) and 63 dpi (Kruskal–Wallis test,  $X^2 = 0.15558$ ,  $df = 2$ ,  $p = 0.9252$ ) (Figure 1b).

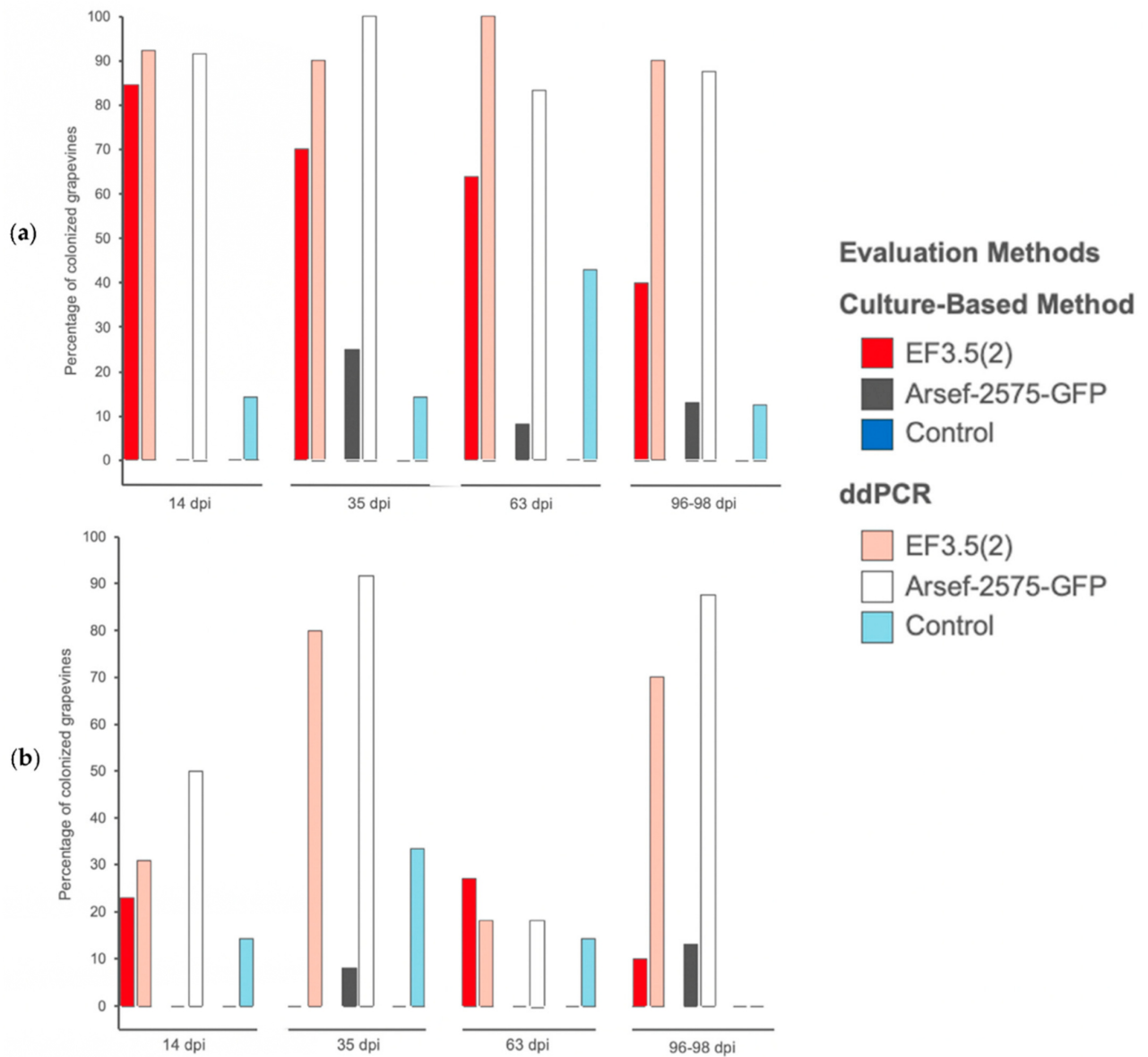
### 3.1.3. Quantification of Endophytic Potential of Two *M. robertsii* Strains in *V. vinifera* Leaves and Stem

Regarding the potential of systemic colonization of grapevine by *M. robertsii* strain EF3.5(2), DNA was positively quantified in 12.2% (5/41) of the third leaves sampled, 9.3% (4/43) of the stem pieces, and 7.5% (3/40) of the terminal leaves, albeit with an overall low concentration range from  $\exp(0.28)$  to  $\exp(1.01)$  c/ $\mu\text{L}$ . In control plants, 3.6% (1/28) of the third leaves, 3.2% (1/31) of the stem pieces, and 9.6% (3/31) of the terminal leaves had a positive signal for *M. robertsii* DNA with a range of  $[0.28\text{--}1.5] \exp(\text{c}/\mu\text{L})$ . In grapevines inoculated with the transformed *M. robertsii* strain ARSEF-2575-GFP, 26.3% (10/38) of the third leaves, 2.6% (1/39) of the stem pieces, and 28.2% (11/39) of the terminal leaves showed positive signals. Positive third and terminal leaf samples ranged from  $\exp(0.36)$  to  $\exp(3.35)$  c/ $\mu\text{L}$  with 5 plants scoring higher than  $\exp(2.36)$  c/ $\mu\text{L}$  which is judged as a high value indicating a potential systemic colonization of the transformed strain ARSEF-2575-GFP. Results are summarized in Appendix A—Table A1.

### 3.2. Association of *M. robertsii* with *V. vinifera* Assessed via Culture-Based Method

Using the culture-based method *M. robertsii* strain EF3.5(2) and the transformed strain ARSEF-2575-GFP were classified as being associated with the rhizosphere in 65.9% (29/44) and 11.4% (5/44) of the grapevine plants, respectively, when all four post-inoculation periods were considered (Figure 2a). An association to the root endosphere was detected in 13.6% (6/44) of the tested grapevines for strain EF3.5(2) and 4.5% (2/44) for the transformed strain ARSEF-2575-GFP (Figure 2b). The strain EF3.5(2) was significantly associated with the rhizosphere of more grapevine plants than the transformed strain ARSEF-2575-GFP (Chisq.test:  $X^2 = 184.94$ ,  $df = 7$ ,  $p < 2.2 \times 10^{-16}$ ) relative to overall post-inoculation periods. However, there was no significant difference in the percentage of grapevines being endophytically associated with both strains (Chisq.test:  $X^2 = 23.882$ ,  $df = 7$ ,  $p = 0.001195$ ) according to the culture-based method. Additionally, the culture-based method did not reveal an evidence of systemic colonization of the plants as none of the tested leaves grown on the culture medium showed evidence of *M. robertsii* colonies (data not shown here).

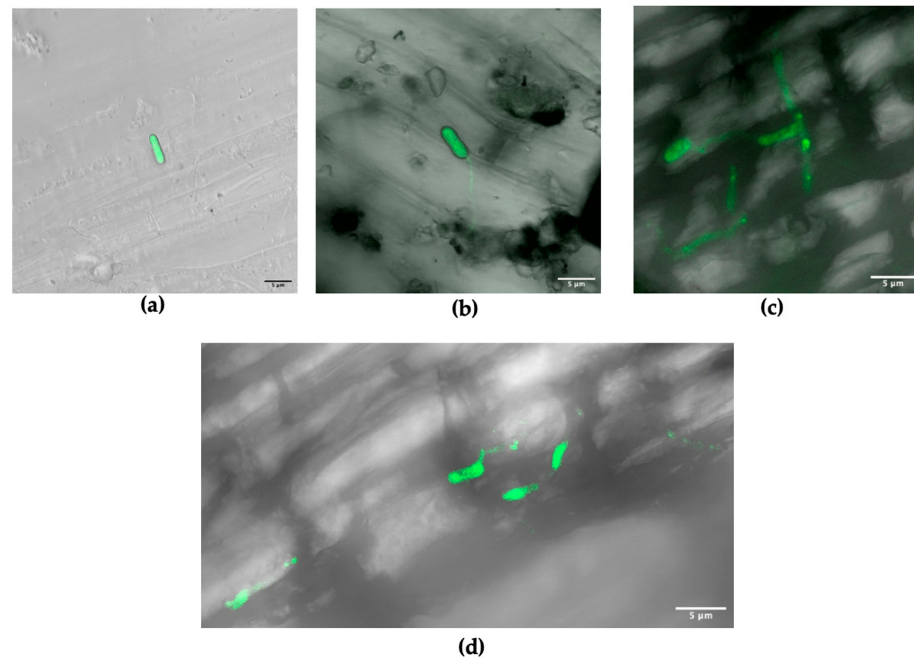
The association of *M. robertsii* to the grapevine rhizosphere was significantly more often detected by ddPCR compared to the culture-based method for the transformed strain ARSEF-2575-GFP (Chisq.test:  $X^2 = 30.556$ ,  $df = 7$ ,  $p = 7.507 \times 10^{-5}$ ) but not for the strain EF3.5(2) (Chisq.test:  $X^2 = 5.6571$ ,  $df = 7$ ,  $p = 0.5803$ ). Same was true for the association of *M. robertsii* to the root endosphere which was higher when assessed by ddPCR compared to the culture-based method (strain EF3.5(2): Chisq.test:  $X^2 = 15.429$ ,  $df = 7$ ,  $p = 0.03088$ ; strain ARSEF-2575-GFP: Chisq.test:  $X^2 = 32.571$ ,  $df = 7$ ,  $p = 3.181 \times 10^{-5}$ ).



**Figure 2.** Time-course of detection of two *M. robertsii* strains (EF3.5(2); GFP-transformed strain ARSEF-2575-GFP) and a control treatment on *V. vinifera* roots. The bars represent the percentage of *M. robertsii* colonized grapevines at (a) the rhizosphere and (b) the root endosphere evaluated with two methods: culture-based method (EF3.5(2): red bars, ARSEF-2575-GFP: grey bars, controls: dark blue bars) and ddPCR (EF3.5(2): pink bars, ARSEF-2575-GFP: white bars, controls: light blue bars). Evaluation was made 14-, 35-, 63- and 96–98 days post inoculation (dpi).

### 3.3. Observation of the *M. robertsii* Transformed Strain ARSEF-2575-GFP Association with Grapevine Roots

The microscopic results of grapevine roots inoculated with the transformed *M. robertsii* ARSEF-2575-GFP strain showed the successful adhesion of the fungus to the root surface as indicated by positive fluorescent signals at 14 dpi (Figure 3a). At 31 dpi spore germination was observed at the root surface (Figure 3b–d) with a few hyphae emerging from the spores attached to the root surface, which indicated the rhizospheric competence of this strain.



**Figure 3.** Confocal images of grapevine root association with *M. robertsii* ARSEF-2575-GFP expressing green fluorescent protein (GFP) observed at (a) 14 dpi, and at (b–d) 31 dpi. All scale bars are 5 µm and total magnification was  $\times 63$  using an oil-immersion objective.

#### 4. Discussion

*Metarhizium* spp. strains were previously demonstrated to be rhizosphere and root endosphere colonizers of diverse plant species from annual to wild flowers, grasses, annual to perennial crops, shrubs and trees [6,9,16,24,29,59–62] with subsequent stimulation of the root growth in some cases [7,63]. The potential for association of *M. robertsii* with roots has been supported by studies of *Metarhizium* spp. demonstrating its natural association with the rhizosphere and root-endosphere of several plant species from diverse botanical families [6,30,59,64–66]. It was reinforced by culturing above and below ground tissue of 82 flower species randomly sampled in Canadian grassland, with 47 (57%) of the samples being colonized at the root level by *Metarhizium* spp. [6]. Based on results obtained via culture-based method we found that 85% of the inoculated grapevines were colonized at the rhizosphere level at 14 dpi by the strain EF3.5(2), dropping to 40% at 96–98 dpi compared to the respective 0% and 13% at both assessment dates for the strain ARSEF-2575-GFP. For the root endosphere, the colonization level ranked from 23% at 14 dpi to 10% at 96–98 dpi for the strain EF3.5(2) and from 0 to 13% for the strain ARSEF-2575-GFP. Previously, *M. robertsii* was found to endophytically establish in the roots of tomatoes with colonization levels of 100% to 95% from 10 to 30 dpi after seed inoculation [67]; in maize roots the level of endophyte colonization was 82% after seed-dressing [60]. A high level of endophytic colonization of cassava *Manihot esculenta* Crantz with three *M. anisopliae* strains was observed from 100% to 50% after 7–9 dpi and 100% to 20% after 47–49 dpi with root drenching inoculation [16]. The endophytic root colonization of two strawberry *Fragaria*  $\times$  *ananassa* Duch varieties ranked from 20 to 100% at 180 dpi after plantlet dipping also depending on the tested strain [68]. Additionally, *M. anisopliae* succeeded high percentage of root colonization (83%) of *Vicia faba* L. 30 dpi after its seed-inoculation [32]. In comparison, the colonization of French bean roots was judged low by the authors with 30% of endophytic root colonization by *M. robertsii* at 35 dpi after seed immersion [69] compared to their previous results. [70] recorded no endophytic association of *M. anisopliae* with both common bean and French bean, 14 days post seed-dressing, and [71] found the same results using the same *M. anisopliae* strain inoculated to maize using seed-treatment. Thus, the endophytic root-colonization of



grapevines in our trial by the two *M. robertsii* was minimal, which could be attributed to the use of a sterile soil substrate, as [72] found that *B. bassiana* endophytically colonized less leaves, stems and roots of sorghum *Sorghum bicolor* L. planted in sterile and non-sterile soil compared to vermiculite. Additionally, Ref. [19] found low endophytic root-colonization levels of *M. anisopliae* in the common bean planted in sterile and non-sterile soil, and concluded that sterility of the substrate had impaired the colonization capacity.

A previous study showed successful colonization of the EIPF *Beauveria bassiana* strain H2S32 with grapevine *V. vinifera* var. Sideritis both grafted on R110 rootstock and self-rooted after drenching with this fungus. The respective percentage of endophytic root colonization after 53 dpi ranked from 82.5% for the self-rooted plants to 80% for the grafted ones and the colonization had a subsequential enhancement of grapevine growth [46]. Additionally, it was recorded that 50% of tested grafted grapevines *V. vinifera* cv. Pinotage were endophytically colonized by *B. bassiana* at leaf level 21 days after root drenching [49]. Thus, EIPF have the potential of endophytic association with both grafted and un-grafted grapevines which can in turn evolve into systemic colonization.

Remarkably, evidence of systemic colonization of grapevine plants by *M. robertsii* strain ARSEF-2575-GFP was demonstrated via ddPCR but not via the culture-based method, with 6.5% (5/77) of tested leaves classified as endophytically colonized. Yet, a lot of studies focusing on *Metarhizium* spp. endophytic colonization did not record proof of colonization in the above-ground part of the trialed plant species, but only in the roots [6,7,16,29,32,69]. In one study, French bean *Phaseolus vulgaris* was drenched with a fungal suspension of the strain ARSEF-2575-GFP, resulting in 10% of stems and leaves being endophytically colonized with EIPF at 60 dpi [24]. However, a systemic colonization of the EIPF *Metarhizium* spp. is commonly observed when employing other methods of inoculation than root-drenching or direct contact with the soil [73]. The seed-treatment method usually induced high rates of systemic colonization by *Metarhizium* spp. during the initial phase of the trial. For example, 28.9% of maize leaf sections were observed to be endophytically colonized with *M. robertsii* strain after seed inoculation [60]. When tomato seeds were inoculated with *M. robertsii*, 60% of the sampled leaves were endophytically colonized at 10 dpi, decreasing to 20% at 30 dpi and from 20% to 5% in stems [67]. The foliar application of conidia of one leaf of rapeseed *Brassica napus* L. with *M. anisopliae* induced systemic colonization of the plants ranking from 50% to 80% for sampled leaves, 35% to 75% for the petioles, and finally 15% to 35% for the stems after 14 to 35 dpi [74]. Additionally, tomato, melon *Cucumis melo* L. var. *reticulatus* Naud and Alfalfa *Medicago sativa* L. were endophytically colonized by *Metarhizium* spp. with a rank of colonization from 65% to 35% in the leaves and 70 to 35% in the stems after 24 to 96 h post treatment [75].

In this study, the ddPCR revealed that both strains colonized the grapevine root rhizosphere to the same extent at 63 dpi. However, the culture-based method showed a higher percentage of rhizosphere colonization by the strain EF3.5(2) native to vineyard compared to the transformed strain ARSEF-2575-GFP during the time course of the experiment. This result was corroborated by quantification using ddPCR at the end of the experiment, which showed a higher persistence of association of the native strain. The reasons for this higher establishment in the grapevine rhizosphere could be related to the propensity of the strain EF3.5(2) to exploit the photosynthetic products secreted by grapevine in the soil [10]. Indeed, *M. robertsii* rhizosphere-competence genes are up-regulated when in contact with high concentration of root exudates secreted by the surrounding plant. Additionally, the success of one fungal strain to colonize the rhizosphere is directly related to its capacity to utilize secreted plants metabolites, such as sucrose [76]. It was demonstrated that some species from the *M. anisopliae* complex, including *M. robertsii* strain ARSEF-2575-GFP have a better ability to grow and germinate at high concentrations of root exudates [10]. As the strain ARSEF-2575-GFP is non-native to the vineyard soil, it might lack specialization of specific grapevine excreted root exudates compared to the strain EF3.5(2), thus impairing its ability to multiply in the rhizosphere and colonize the roots. Thus, the vineyard native

strain seems to be the best candidate as a biocontrol agent to protect grapevine roots against soil pests because it shows a high persistence of association with grapevine roots.

Both strains were found to endophytically establish inside grapevine roots with the same pattern according to ddPCR and culture-based methods, yet with quite a low incidence of colonization. The endophytic *M. robertsii* DNA quantification in grapevine roots inoculated either with *M. robertsii* EF3.5(2) or the strain ARSEF-2575-GFP did not differ significantly from the quantification in the control plants at 14 and 63 dpi. This could be explained by the fact that the establishment of *M. robertsii* inside roots happened independently of the experimental timing. As a result, more plants from the pool of replicates screened at 35 dpi were endophytically colonized compared to the ones at 63 dpi. Similar results were obtained when quantifying endophytic French bean root colonization by *M. robertsii* strain ARSEF-2575-GFP [24]. The amount of *M. robertsii* DNA quantified decreased from 3 to 10 dpi and then increased from 10 to 14 dpi. These quantifications variations could be explained by the number of plants colonized at the respective time of detection. Additionally, when quantifying an artificially inoculated endophyte, *Serendipita herbamans* associated with the knotweed *Reynoutria* ssp. roots, high variations of quantification were found particularly under suitable conditions of establishment [77].

Comparing the different methods used in this study, ddPCR is a sensitive method which can precisely detect and quantify microorganisms from various environments even if they are present at very low concentrations [78], with more precision and less technical preparation than qPCR or nested qPCR [26–28,79,80]. It has the best quantification performance compared to other DNA quantification methods for samples taken from environments with complex matrix prone to PCR inhibition and rich of non-target DNAs like soil or plant tissue [81]. These attributes of ddPCR make it an insightful tool for DNA quantification of endophytes in plants allowing better characterization of endophyte' association with plants and the kinetics of establishment. However, the high sensitivity of the method may consequently influence its specificity by detecting very low levels of DNA. In our study, the presence of *M. robertsii* was detected in roots, leaves and stems of control plants which might originate from other microorganisms with similar DNA sequences [82]. Thus, ddPCR is also prone to detect false positives, depending on primer specificity.

To characterize the endophytic potential of *M. robertsii* in grapevine plants, we also employed a more traditional culture-based method in our study. This method is most commonly used as it is affordable, easy to handle with standard microbiology instruments, and is rapid with on average 10 days necessary to observe the fungal growth on the plates [22]. In contrast, ddPCR is a costly molecular tool which requires sophisticated PCR equipment, some pre-testing for preparation of samples and significantly extra work with DNA extraction. The culture-based method has the main advantage to characterize the microorganism viability as only the live propagules grow on the medium, as opposed to ddPCR, which quantifies DNA from viable and non-viable cells complicating scientific interpretations on the quantified inoculum [83]. In addition, the culture-based method is not suitable for fungal propagules quantification because the plating does not assure homogeneous distribution of fungal spores or the respective inoculum has a low vitality [27]. Additionally, the competitiveness of other endophytic species that grow abundantly on the respective growth medium because of its lack of selectivity can bias the quantification of individuals which are over or under represented [23]. The ddPCR is more sensitive than the culture-based method used to detect fungal endophytes, which was apparent in our study when detecting both *M. robertsii* strains in the root endosphere and in the aboveground parts of grapevine. ddPCR characterized several leaf and stem tissue samples as being endophytically colonized with both *M. robertsii* strains, while culture-based method showed no evidence of systemic colonization of grapevine by the respective strains.

Confocal microscopy was used to complete the range of methods employed in this study. Compared to the two other methods discussed so far, it gives the most irrefutable proof of endophytic and rhizosphere colonization of the plant [24]. It is the only method that characterizes the fungal distribution inside the plant tissues as well as the fungal structures

colonizing the plant [6,7]. Additionally, a transformed strain with green fluorescent protein is usually inoculated for microscopic tracking, making it an undisputable proof of the colonization potential of the fungus [53]. However, a significant amount of time is necessary to create the transformant strain, to screen a large number of plant replicates and tissues of a single plant, and to prepare a significant number of microsections of different tissues. Additionally, the chances of detecting endophytic colonization with confocal microscopy are low compared to the ddPCR or the culture-based method, making the use of these more sensible methods mandatory for endophytic detection surveys [84]. The results of our study are in line with this finding, we found no evidence of endophytic colonization of the transformed strain ARSEF-2575-GFP by confocal microscopy observation while ddPCR and culture-based method detected endophytic colonization. However, further confocal microscopy observations with more screened root segments should be made to confirm the obtained results.

## 5. Conclusions

The present study is the first to demonstrate the power of a combination of methods used to investigate endophytic establishment of *M. robertsii* in grapevine. We found a significantly greater establishment of the vineyard native *M. robertsii* EF3.5(2) strain compared to the GFP-transformed strain ARSEF-2575-GFP in the rhizosphere of grapevine. This could potentially be explained by the specific affinity of the native strain to the root exudates of grapevine enhancing its multiplication in the rhizosphere.

ddPCR is the most sensitive detection method and we recommend to include it in studies aiming to characterize the kinetics of endophytic fungal establishment and their systemic colonization. Nonetheless, the method is not a stand-alone technique, but should be accompanied by culture-based and/or confocal microscopy that provides additional information on the viability of the fungus.

**Author Contributions:** Conceptualization, M.P., D.P., D.T. and A.R.; methodology, M.P., D.P., D.T. and A.R.; validation, M.P., D.P., D.T. and A.R.; formal analysis, M.P., D.P., D.T. and A.R.; investigation, M.P., D.P., D.T. and A.R.; resources D.P., M.J.B., D.T., M.M. and A.R.; writing—original draft preparation, M.P., D.P., D.T. and A.R.; writing—review and editing, D.P., D.T., A.R., M.J.B.; visualization, M.P., D.P., D.T. and A.R.; supervision, D.P., D.T. and A.R.; project administration, D.P., D.T. and A.R.; funding acquisition, D.P., D.T. and A.R. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded within the framework of the Bordeaux-Adelaide-Geisenheim (BAG) international project alliance and in part through the French-German Doctoral College CDFa-03-18.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We would like to thank the PGTB as part of the experiments of ddPCR analysis were performed at the PGTB (doi:10.15454/1.5572396583599417E12). The microscopy was done in the Bordeaux Imaging Center a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging supported by the French National Research Agency (ANR-10-INBS-04) under the supervision of the engineer Lysiane Brocard. Authors would like to thank Olivier Fabreguette for the technical support in molecular analysis and Manmeet Singh for its help with data collection, Shasha Hu for shipping the transformed strain ARSEF-2575-GFP, Marie Schulze-Sylvester for her helpful comments on the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Appendix A

**Table A1.** Detection of *M. robertsii* DNA copies in aboveground grapevine tissue (stem, third leaf, terminal leaf) 14-, 35-, 63- and 96–98 days post inoculation (dpi) after watering roots with a conidial suspension of two *M. robertsii* strains (EF3.5(2) and ARSEF-2575-GFP). Presented are the number of positive plants in relation to the total number of plants (or percentage positive plants) and the copy numbers/ $\mu$ L sample (or range of copy numbers) determined via ddPCR. Control plants were treated with the same volume of a sterile suspension solution.

	14 dpi			35 dpi			63 dpi			96–98 dpi		
	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Controls
STEM	1/13 (7.7%)	0/11 (0%)	0/7 (0%)	0/10 (0%)	1/10 (10.0%)	0/7 (0%)	0/11 (0%)	0/11 (0%)	0/7 (0%)	3/9 (33.3%)	0/7 (0%)	1/7 (14.3%)
	1.43 c/ $\mu$ L				1.54 c/ $\mu$ L					1.98–2.75 c/ $\mu$ L		4.51 c/ $\mu$ L
THIRD LEAF	1/13 (7.7%)	3/12 (25.0%)	0/7 (0%)	0/9 (0%)	1/9 (11.1%)	0/7 (0%)	1/10 (10.0%)	4/10 (40.0%)	0/7 (0%)	3/9 (33.3%)	2/7 (28.6%)	1/7 (14.3%)
	1.32 c/ $\mu$ L	1.43–3.41 c/ $\mu$ L			2.20 c/ $\mu$ L		1.32 c/ $\mu$ L	1.43–17.6 c/ $\mu$ L		1.32–2.20 c/ $\mu$ L	1.76–11 c/ $\mu$ L	1.43 c/ $\mu$ L
TERMINAL LEAF	0/12 (0%)	3/12 (25.0%)	0/7 (0%)	0/9 (0%)	2/11 (18.2%)	2/7 (28.7%)	2/10 (20.0%)	5/10 (50.0%)	0/7 (0%)	1/9 (11.1%)	1/6 (16.7%)	0/7 (0%)
		1.43–28.6 c/ $\mu$ L			2.97– 10.67 c/ $\mu$ L	1.32–3.52 c/ $\mu$ L	1.32–1.65 c/ $\mu$ L	1.98–24.2 c/ $\mu$ L		1.43 c/ $\mu$ L	2.86 c/ $\mu$ L	

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# Chapter 2



## 7- Association of four *Metarhizium robertsii* strains native to vineyards with non-grafted *grapevine Vitis vinifera* and their impact on growth and expression of phylloxera responsive defense genes

Mathilde Ponchon <sup>1,2</sup>, Daciana Papura <sup>1</sup>, Denis Thiéry <sup>1</sup>, and Annette Reineke <sup>2</sup>

1- INRAE, Bordeaux Sciences Agro, ISVV, UMR SAVE, 33140 Villenave d'Ornon, France

2- Department of Crop Protection, Hochschule Geisenheim University, 65366 Geisenheim, Germany

Type of authorship : First Author

Type of article : Research article

Contribution to the article : Conceptualization and design of the experiments ; Development of the methodology; Planning of the experiments ; Performance of the experimental work ; Evaluation and statistical analysis of the data ; Preparation of all figures and tables ; writing of the manuscript

Contribution of other authors : D.P, D.T. and A.R. contributed to the conceptualization and the experimental design, data analysis and writing the manuscript

Status : *In prep. for submission*

## Abstract

Persistently associating entomopathogenic fungi as endophytes with plant root-system is an important perspective for biological control of pests that avoids repeated-spraying, potentially damaging the natural enemies. The objective of the present study is to evaluate and compare the ability to colonize the rhizosphere and root-endosphere of non-grafted grapevine *Vitis vinifera* of four entomopathogenic fungal strains of *Metarhizium robertsii* native to four contrasting vineyard environments such as: i) the center-west of Argentina (strain MsoilAR4.3), ii) the south of Australia (strain M224B) iii) the south-west of France (strain EF3.5(2)) and iv) the center-west of Germany (strain EF047). The root-drenching inoculation method was used, and the fungal association was detected after 26-28, 47-49, and 68-70 days post-inoculation (dpi). All four *M. robertsii* strains were found to be associated with grapevine rhizosphere with rates of colonization of 60%, 65%, 76.2%, and 100% by MsoilAR4.3, M224B, EF3.5(2), EF047 at 68-70 dpi. They also colonized the grapevine root-endosphere with respective rates of 14.3%, 9.5%, 4.8%, and 33.3% at 68-70 dpi. The fungal strains inoculation during the experimental period unaffected the grapevine's growth and leaf pigment content. In addition, qPCR was used to quantify the expression level of seven grape phylloxera-responsive genes in grapevine roots 24h and 120h after the inoculation with two *M. robertsii* strains, EF3.5(2) and EF047. Five of the seven grape phylloxera-responsive genes, *VtPR1*, *VtPR3.2*, *VtPR4*, *VtChib* and *VtG1*, were up-regulated 24h after inoculation of the two strains. After 120h, *VtChib* was principally down-regulated after inoculation of the two strains. Overall, our study proved that all four *M. robertsii* vineyard-native strains were able to persistently associate with *V. vinifera* roots without harming plant growth and physiology, thus opening new doors for biological control.

## 7.1. Introduction

The insect pathogenic fungus *Metarhizium robertsii* (Metchnikoff) Sorokin (1883) (Ascomycota: Hypocreales) is a microbial control agent commercially used worldwide because of its success in several biological control programs (Faria and Wraight, 2007; St. Leger and Wang, 2020). Besides being able parasitize more than 200 insect species (Brunner-Mendoza et al., 2019), *M. robertsii* also displays a second lifestyle living both as a rhizosphere colonizer (Hu and St. Leger, 2002; Wyrebek et al., 2011) and as a plant endophyte (e.g., living within the plant tissues for at least part of its life, without causing apparent disease symptoms (definition of Petrini, 1991)). These specific features provide several benefits for plants endophytically colonized by *M. robertsii*, such as (i) plant growth stimulation; (ii) plant vaccination; (iii) plant disease antagonism; (iv) insect parasitism decrease (Jaber and Ownley, 2018).

For future perspectives on designing integrative pest management (IPM) strategies, using *M. robertsii* as an endophytic colonizer of crop plants is of particular interest if its utilization can be field-optimized to increase its efficiency. *M. robertsii* as a plant symbiont was shown to preferentially establish in the roots targeting both the rhizosphere and the root-endosphere when artificially inoculated in laboratory and field studies (Behie et al., 2015; Vega, 2018). It can endophytically colonize diverse plants belonging to several botanical families, such as monocots, with the example of soy *Glycine max* (L.) Merr., wheat *Triticum aestivum*, and sweet corn *Zea mays* subsp. *mays* L. (Khan et al., 2012; Behie and Bidochka, 2014; Ahmad et al., 2020a) and dicots: tomato *Solanum lycopersicum* L., the French bean *Phaseolus vulgaris* L., strawberry *Fragaria x ananassa* Duchesne (Garcia et al., 2011; Barelli et al., 2018; Canassa et al., 2020b). In some cases, the endophytic colonization resulted in successful pest biological control. For instance, *M. robertsii* as endophyte in spring wheat showed significant control of soil populations of two wireworm species, *Limonius californicus* and *Hypnoidus bicolor* (Reddy et al., 2014).

Domesticated grapevine, *Vitis vinifera* (L.), is a perennial dicot plant grown as a grafted plant in most countries worldwide. Grafting on resistant rootstocks is a successful control strategy against its major soil-borne pest, grape phylloxera (*Daktulosphaira vitifoliae*). In addition, it contributes to grapevine adaptation to respective soil biotic and abiotic conditions (Rubio et al., 2020). However, vineyards in Australia and Argentina are commonly planted with non-grafted grapevines, therefore grape phylloxera still poses a significant risk for growers. Root infestation is restricted through resolute quarantine phytosanitary measures Champ (Benheim et al., 2012) and flood irrigation, respectively (Arancibia et al., 2018). Consequently, developing alternative crop protection methods has become necessary for grapevine pest and disease management, especially for non-grafted grapevines. Only a few studies have already investigated the potential of entomopathogenic fungi as an endophyte in grapevine *V. vinifera* to control pests and diseases. Artificial inoculation of *Beauveria bassiana* strain H3S32 by root drenching was performed both with grafted grapevines *V. vinifera* on R110 rootstock and non-grafted *V. vinifera*, and the fungus persistently established as an endophyte on both grapevine root system with subsequent benefit for the grapevine growth (Mantzoukas et al., 2021). Another study found the endophytic establishment of *B. bassiana* strain SM3 in grapevine leaves after root inoculation of grafted grapevines (Moloinyane and Nchu, 2019). Rondot and Reineke (2018, 2019) and Jaber (2015) have shown that *B. bassiana* strains ATCC 74040 and GHA originating from the commercial product Naturalis were able to establish endophytically in *V. vinifera* leaves via foliar application, reducing downy mildew *Plasmopara viticola* disease severity and infestation with piercing-sucking insects such as *Planococcus ficus*. Two strains of *M. robertsii* (strain EF3.5(2) native to the French vineyard and ARSEF-2575-GFP, a laboratory strain expressing a green fluorescent protein) were for the first time associated via root-drenching in the rhizosphere and the root-endosphere of non-grafted grapevines *V. vinifera* and persisted up to 98 days post-inoculation (Ponchon et al., 2022). In addition, Kirchmair et al., 2004 showed the potential of *M. anisopliae* var. *anisopliae* (now *M. robertsii*) to control grape phylloxera, characterized by a reduction of grapevine root damage induced by

phylloxera one month after planting in soil inoculated with barley kernels coated with fungal propagules. Accordingly, the fungus is a relevant biological control candidate to protect grapevine roots against soil-borne pests.

The defense of non-grafted grapevine (*V. vinifera*) against radicolle grape phylloxera has yet been poorly investigated. [Du et al., 2014](#) found a significant up-regulation of a set of defense genes involved in the biosynthesis of secondary metabolites in the roots of the resistant rootstock '140Ru' (*V. berlandieri* x *V. rupestris*) infested with radicolle grape phylloxera compared to non-grafted grapevine *V. vinifera* cv. 'Crimson Seedless.' Notably, the gene *VWRKY-46* involved in the salicylic acid (SA) mediated defense response was significantly up-regulated in roots of another resistant rootstock studied with the same parentage, the '1103 Paulsen', compared to non-grafted grapevine 'Crimson Seedless.' The up-regulation of *VWRKY-46* was accompanied by up-regulation of *VWRKY-46* downstream target genes *VIG1*, *VICHIB*, *VICHIB1*, *VPR1*, *VPR4*, and *VPR3.2* in roots of '1103 Paulsen'. The sur-expression of the *VWRKY-46* gene or the exogenous injection of SA to vine roots conferred resistance against radicolle grape phylloxera attack and delayed larvae development in composite *V. vinifera* roots ([Wang et al., 2019](#)). Moreover, the SA-response pathway commonly activates in response to biotrophic pathogens' invasion inside plant tissues ([Glazebrook, 2005](#)). As an endophyte, *M. robertsii* was previously demonstrated to activate plant defense systems during endophytic colonization of plants ([Ahmad et al., 2020a, 2022](#); [Hu and Bidochka, 2021](#)). This activation of defense genes may consequently turn the plant into a primed state by which it may be able to achieve both faster and stronger activation of a high level of resistance to various biotic and abiotic stressors ([Dara 2019](#)). For instance, increased SA concentrations in maize roots were also quantified during the endophytic colonization of *M. robertsii* ([Rivas-Franco et al., 2020](#) ; [Ahmad et al., 2022](#)). Thus, we expect that *M. robertsii* could induce up-regulation of *VWRKY-46* and its downstream target genes in non-grafted grapevines, contributing to its global resistance to radicolle grape phylloxera.

For future protection of grapevines against grape phylloxera, selecting EPF strains that could adapt to various environments and host plants and evaluating their effect on plant growth and performance is essential. It was previously demonstrated that *M. robertsii* strain EF3.5(2) collected in the vineyard soil of Bordeaux has better endophytic capacities toward grapevines compared to a strain non-native to vineyard soil (Ponchon et al., 2022). For this purpose, *M. robertsii* strains collected from different vineyard environments all around the globe could represent a reservoir of strains with adaptations to specific local conditions and could be more successful biological agent candidates (Fisher et al., 2011; Uzman et al., 2019; Korosi et al., 2019; López Plantey et al., 2019).

The goal of this study was to compare the potential of rhizospheric and endophytic potential of association with non-grafted grapevine *V. vinifera* of four *M. robertsii* strains collected from vineyard soils in four wine-producing regions with substantially different abiotic conditions: the center-west of Argentina in Mendoza region, southern Australia in Victoria region, south-west of France and the center-west of Germany. We also assessed the impact of an endophytic establishment on several grapevine growth features as well as leaf pigment content. Finally, the expression of gene *VvWRKY-75* involved in the grapevine salicylic acid (SA) mediated defense response and its downstream target genes were assessed in leaves 24h and 120h after the inoculation of *M. robertsii*. We hypothesize that (i) *M. robertsii* strains native to contrasted environments have the differential potential of association with grapevine; (ii) the inoculation of *M. robertsii* does not affect plant growth and leaf pigment content; and (iii) *VvWRKY-75* and its downstream target defense genes are up-regulated 24h post-*M. robertsii* inoculation in grapevine leaves.

## 7.2. Material and Methods

### 7.2.1. Fungal material

Four native *M. robertsii* strains were used for the experiments: (i) French strain EF3.5(2) 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 sampled in 2014 from vineyard soil in the Yarra Valley, Victoria (Korosi et al., 2019), (iii) Argentinian strain MsoilAR4.3 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), (iv) German strain EF047 isolated in 2020 from vineyard soil of Kellersgrube in Geisenheim (N50°0'20.16" E7°58'45.48"). Strains were sampled in the inter-row of vineyard plots with an insect bait technique. Their genetic identity was confirmed by sequencing the translation elongation factor 1-a gene (Bischoff et al., 2009). Fungi were grown in a Petri dish with oatmeal agar medium (Fisher Scientific, Waltham, USA) supplemented with 100 mg.l<sup>-1</sup> chloramphenicol (SIGMA Aldrich, Saint-Louis, USA). From each Petri dish, a 5 ml fungal suspension with a concentration of 1 x 10<sup>8</sup> conidia.ml<sup>-1</sup> was prepared by dislocating the conidia with a sterile suspension 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 multiply *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 cm<sup>3</sup> 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 the sterile suspension solution and left for one hour, 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 with a hemocytometer.

### 7.2.2. 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 treated with a fungicide to prevent powdery mildew infestations (2,26 g.l<sup>-1</sup> Luna®Experience, active ingredients: 200 g.l<sup>-1</sup> fluopyram and 200 g.l<sup>-1</sup> tebuconazole) 5 days before the inoculation with *M. robertsii*. During the trials, preventive treatments against powdery mildew were applied every two weeks, alternating 0,4 g.l<sup>-1</sup> of Vivando® ; active ingredient: 500 g.l<sup>-1</sup> metrafenon) or 2,26 g.l<sup>-1</sup> Luna®Experience. During fungicide applications, potting soil was covered with plastic tarpaulin to avoid the accumulation of pesticide residues in the soil.

### 7.2.3. Screening experiments

#### 7.2.3.1. Grapevine inoculation

The « Watering » method previously used in another study was employed for the following experiments. Indeed, the method was demonstrated to be an efficient and user-friendly inoculation method to induce the rhizospheric and endophytic association of *M. robertsii* with non-grafted *V. vinifera* at concentration 1 x 10<sup>7</sup> conidia.ml<sup>-1</sup> of fungal suspension (Ponchon et al., 2022). 21 grapevine plants were inoculated with one of the four *M. robertsii* strains (EF3.5(2), M224B, MsoilAR4.3, EF047) by watering the plants with a 50 ml of a 1 x 10<sup>7</sup> conidia.ml<sup>-1</sup> fungal suspension. Control plants were watered with the same quantity of sterile water with 1/8 Ringer solution and 0.02% Tween 80®. Grapevines were uprooted 26-28, 47-49, and 68-70 days post-inoculation (dpi) to assess the endophytic and rhizospheric association of *M. robertsii* strains. For the grapevines used to evaluate *M. robertsii* association at 68-70 dpi, their growth and pigment content were monitored as described in part 7.2.4.



### 7.2.3.2. Evaluation of rhizospheric and endophytic colonization

To demonstrate the association of *M. robertsii* and grapevine at the rhizospheric level, grapevines were uprooted and gently shaken with forceps to separate the adhered soil. A thin layer of soil that stayed adhered to the roots was kept and considered part of the rhizosphere. An average mass of 0.5 g of randomly sampled thin roots was cut and placed in hermetically sealed tubes (D x H: 27 x 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 subsets of 100 µl of the obtained homogenate were spread in two Petri dishes plates composed of cycloheximide, thiabendazole, chloramphenicol (CTC) growth medium prepared with the modified recipe of [Fernandes et al., 2010](#) (39 g·L<sup>-1</sup> potato dextrose agar, 0.1 g·L<sup>-1</sup> chloramphenicol, 0.002 g·L<sup>-1</sup> thiabendazole, and 0.15 g·L<sup>-1</sup> 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 with morphological criteria and microscopic observations of conidial shape ([Humber, 2012](#)). A plant was considered as colonized by *M. robertsii* if at least one colony of the fungus was observed in one of the duplicate plates.

Root pieces and third and terminal leaves were collected to demonstrate the endophytic potential of *M. robertsii* to the grapevine. Their surface disinfection was done by dipping them in 0.5% NaOCl and 0.02 % Tween 80® for 2 min (once for leaves, twice for roots), 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 same procedure described before for detection of rhizospheric association.

#### 7.2.4. Grapevine growth assessment

To assess if endophytic association with the four *M. robertsii* strains affected grapevine performance, several growth measurements were obtained from inoculated and control grapevines. The last newly-formed leaf on the day of inoculation was marked with a string, and this step was repeated each week during the experimental period. Measurements above the last newly-formed leaf (i.e., number and length of new internodes, number of newly-formed leaves, main vein, and petiole length of newly-formed leaves) were taken every week until 65 dpi. At 29, 36, and 43 dpi, the nitrogen balance index, chlorophyll, flavonol, and anthocyanin pigment content were measured on the last fully expanded leaf using the Dualex® leaf clip sensor (CONTACT ForceA, Orsay, France) by including an average of three measurements along the lamina of the leaf. Grapevine shoots were cut at 30 cm at 21 dpi to ensure harmonious growth, and the cuttings were dried to measure their dry weight, as described below. After cutting, the same growth features continued to be monitored from the shoot growth of the highest bud germinated. At 68-70 dpi, the above-ground part of the plant was harvested and dried for 70 h at 80°C in a heat chamber to obtain the dry weight. At the same time, 10 root samples of 10 different plants per treatment were taken. One root sample was constituted of all the roots of a single grapevine. The samples were dried at a temperature of 80°C for 70 h for dry weight measurements.

#### 7.2.5. Gene defense expression study

##### 7.2.5.1. Experimental setup

Nine 7-week-old potted grapevines plants were inoculated each with *M. robertsii* strains EF3.5(2) and EF047 were inoculated to 7-week-old potted grapevines as described in 7.2.2. and a control treatment was made by watering grapevines with a sterile ringer solution and 0.02% Tween 80. At 24 and 120 hours post-treatment (hpt), the sixth leaf of each grapevine was sampled, immediately flash-frozen in liquid nitrogen, and stored at -80°C. For each treatment, 9 plants

inoculated with the same treatment were sampled at the sixth leaf. The nine leaves were distributed in 3 pools, each comprising 3 leaves.

#### 7.2.5.2. RNA isolation

RNA was extracted from each leaf pool using the Spectrum Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's protocol. Leaves were crushed in liquid nitrogen to obtain a total of ca. 100 mg of crushed plant tissues ready to be used for RNA extraction. Intruder DNA was removed by digestion with 0.8 U DNase (Ambion Inc, Carlsbad, USA) followed by lithium chloride precipitation. RNA purity and quantity was 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).

#### 7.2.5.3. qPCR analysis

Gene expression levels of ten genes associated with the SA-mediated signal pathway of grapevine to defend against radicle grape phylloxera were evaluated using qPCR (Wang et al., 2019). The evaluated genes were: *Vvwrky-75*, *VvG1*, *VvGH3*, *VvCHIB*, *VvCHIB1*, *VvNPR1*, *VvPR1*, *VvPR3.2*, *VvPR4*, *VvPR1*-like described in Wang et al., 2019. Two housekeeping genes were used, one coding for the protein actin and one coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Timm and Reineke, 2014). For cDNA synthesis, each RNA sample was diluted to  $100 \text{ ng} \cdot \mu\text{L}^{-1}$ , 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 (Bio-Rad, Hercules, USA) qPCR device using the Maxima SYBR Green Master Mix (ThermoFischer 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  $\mu\text{L}$  using 10  $\mu\text{L}$  of cDNA sample as a template, 1  $\mu\text{L}$  of each forward and reverse primer at a concentration of 7.5  $\mu\text{M}$  and 12.5  $\mu\text{L}$  of qPCR Master Mix and filled up with 0.5  $\mu\text{L}$  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, finally 60 °C for 10 s. For each cDNA template, three independent technical replicates were performed for each primer pair. Quantification cycle (C<sub>q</sub>) 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 “do my qPCR calculation” webtool 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, and significance was defined by a p-value below 0.05.

#### 7.2.6. Statistical analysis of data

All analyses were carried out by R Core Team (2018). The percentage of detection of *M. robertsii* as a root-endophyte or as a rhizosphere-associated fungus with grapevine plants was compared between all the tested strains using the Fisher exact test followed by a pairwise multi comparison test with Bonferroni correction. The same test was used to compare these percentages at different detection times. The total number of newly formed leaves and internodes during the trial was counted concerning the growth measurements. The sum of the length of all the newly formed internodes from 8 dpi to 65 dpi was calculated. The sum of the length of all the main veins of the newly-formed leaves from 8 dpi to 43 dpi was calculated, as well as the sum of the length of petioles of the newly-formed leaves from 29 dpi to 43 dpi. All listed quantitative variables and the dry above-ground and below-ground biomass weight were analyzed separately with the ANOVA model after verification of the hypothesis, including the strains inoculated as explicative factors. The Tukey HSD function was used afterward for multiple comparisons of the different strains used as treatment. The NBI index, chlorophyll content, flavonoid content, and anthocyanin content were analyzed separately for each measurement time (28, 36, 43 dpi) in the same way described above.

## 7.3. Results

### 7.3.1. The potential of *M. robertsii* strains to associate with the grapevine rhizosphere and root endosphere

At 26-28 dpi, the percentage of grapevine *V. vinifera* associated at the rhizosphere with *M. robertsii* was: 57.1% of treated grapevines with the strain MsoilAR-4.3, 47.6% treated with the strain M224B, 4.8% treated with strain EF3.5(2) and 62.0% treated with the strain EF047 (Fig. 16-A). At 47-49 dpi, the colonization rates were 81.0%, 52.4%, 76.2%, and 95.2%, respectively. At the termination of the assessment period at 68-70 dpi, 60.0% of the grapevine that received a treatment with *M. robertsii* strain MsoilAR-4.3 were colonized by the strain at the rhizosphere, compared to 65.0% of the plants treated with M224B, 76.2% treated with EF3.5(2) and 100.0% of the plants treated with EF047. Colonization rates were not significantly different between the tested strains for each detection time. Additionally, the strain colonization rates did not increase during the experiment for all the tested strains (Fisher exact test:  $p=0.05534$ ).

At 26-28 dpi, 14.3% of the root endosphere of grapevine plants treated with *M. robertsii* strain MsoilAR-4.3 were colonized compared to 9.5% of the plants treated with strain M224B, 4.8% treated with strain EF3.5(2) and 14.3% treated with strain EF047 (Fig. 16-B). At 47-49 dpi, colonization rates were 14.3%, 33.3%, 23.8%, and 14.3%, respectively; and at the termination of the measurement of the growth features at 68-70 dpi, 14.3%, 9.5%, 4.8%, 33.3% respectively. There was no significant difference in colonization rates among tested strains. Also, for all tested strains, colonization rates did not significantly vary across time (Fisher exact test:  $p=0.3069$ ). The analysis of the number of colonies counted on the plate in the rhizosphere and the root endosphere is presented in Supplement 6,7,8.

In the third and terminal leaves pooled together, endophytic *M. robertsii* was not detected as no plate with leaf extracts homogenate showed fungal colonies in the 68-70 dpi trial. Consequently, their colonization was not further investigated in the 26-28 dpi and 47-49 dpi trials.

*M. robertsii* was not found on the plates with root extract homogenates of control plants, except for the 68-70 dpi trial. At this time point, three control plants showed proof of rhizosphere colonization by *M. robertsii*, which we believe is a mistake made during the experimental preparation of the homogenate plating.

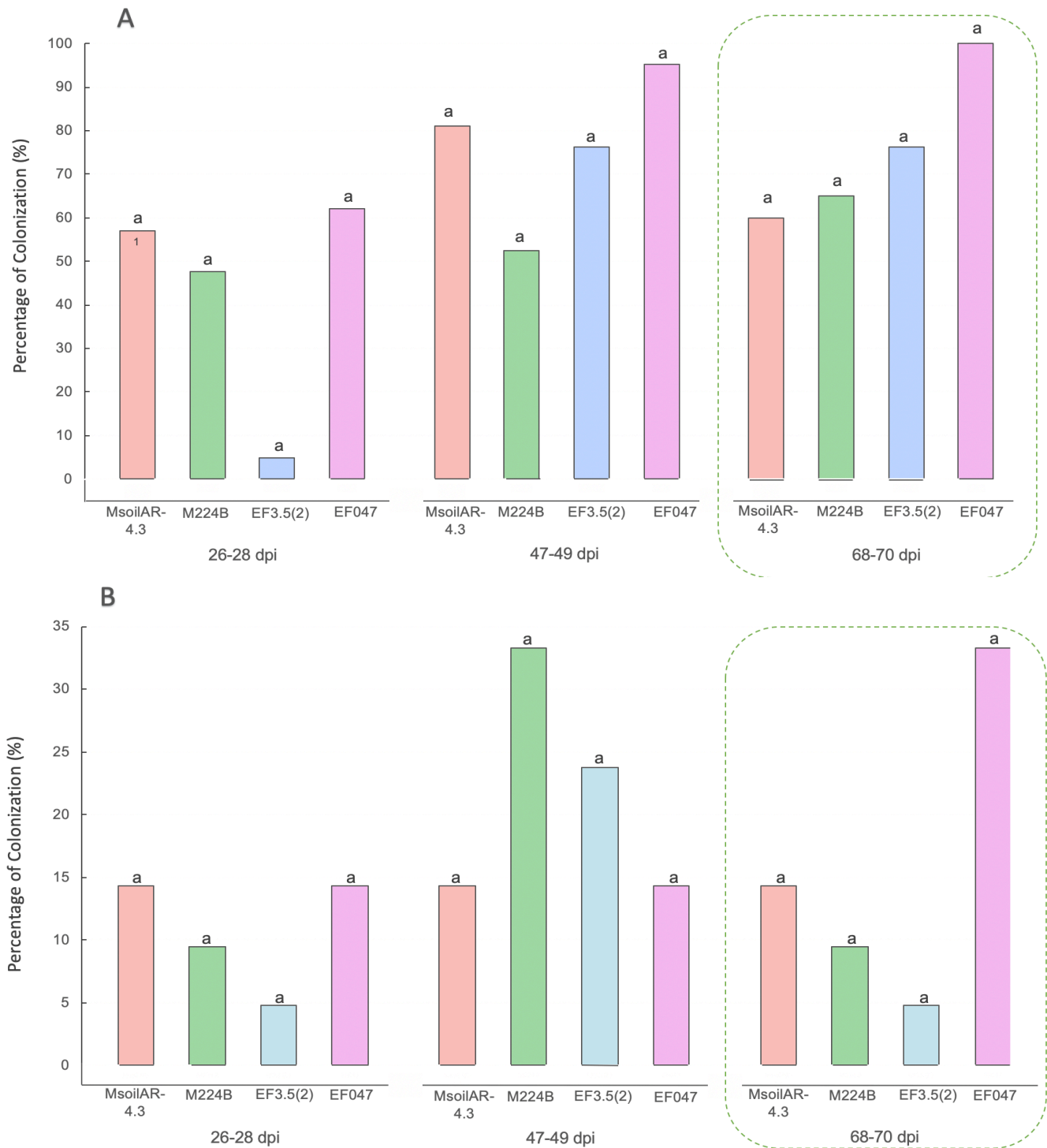


Figure 16 : Time-course of detection of four *M. robertsii* strains (Msoil-AR-4.3 (red bars) ; M224B (green bars) ; EF3.5(2) (blue bars) ; EF047 (pink bars)) on grapevine roots. The bars represent the percentage of colonized grapevines at (A) the rhizosphere, (B) the root endosphere evaluated via culture-based method with evaluation made at 26-28, 47-49, 68-70 days post inoculation (dpi). The green frame highlights the results obtained on vines at termination of measurements of their growth features. Significant differences are indicated by small letters above the bars (Fisher Exact test,  $p < 0.05$  followed by Bonferroni correction for multi comparisons).

### 7.3.2. Impact of endophytic *M. robertsii* on grapevine growth

Regarding the growth features measured at 21 dpi with *M. robertsii* (Table 1), the cut shoot dry weight was significantly higher in grapevines treated with the strain EF047 compared to all other treatments (Df=4; F-value=13.83; P-value=  $6.31 \times 10^{-9}$ ). The sum of the main vein length of newly formed grapevine leaves measured from 7 to 43 dpi did not vary significantly between all treatments with *M. robertsii* strains (Df=4; F-value=1.703; P-value=0.155), and the same was found for the sum of main petiole length of newly formed leaves measured from 21 to 35 dpi (Df=4; F-value=0.949; P-value=0.439).

At 65 dpi, the total number of newly formed leaves during the trial was calculated. The mean number was significantly lower in grapevines treated with the strain EF047 compared to all other strains and the control (Df=4, F-value=3.062, P-value=0.02). However, no significant difference was observed between grapevines treated with the respective four tested strains and the control treatment regarding the features: total number of newly formed grapevine internodes (Df=4; F-value=0.403; P-value=0.806) and total length of the grapevine newly formed shoots at 65 dpi (Df=4; F-value=0.403; P-value=0.806). The dry weight of the grapevine above-ground part taken at 68-70 dpi did not differ significantly between the plants inoculated with either of the four strains and the control treatment (Df=4; F-value=1.402; P-value=0.239), and this was also the case for the dry weight of grapevine total roots (Df=4; F-value=1.619; P-value=0.186).

### 7.3.3. Impact of endophytic *M. robertsii* on grapevine pigment content

The nitrogen balance index (NBI) of the youngest fully-formed leaf at 29, 36, and 43 dpi was not affected in plants treated with the respective strains compared with the control treatment (Df=4, F-value=0.927, P-value=0.452; Df=4, F-value=0.182, P-value=0.947; Df=4, F-value=0.949, P-value=0.448) (Table 2). The same was found for the chlorophyll content of the youngest fully-formed leaf measured at 29, 36, and 43 dpi (Df=4, F-value=1.959, P-value=0.107; Df=4, F-



value=0.326, P-value=0.86; Df=4, F-value=0.43, P-value=0.786) as well as for the flavonoid content of the youngest fully-formed leaf measured at 29, 36 and 43 dpi (Df=4, F-value=0.408, P-value=0.802; Df=4, F-value=0.439, P-value=0.78; Df=4, F-value=1.33, P-value=0.279). The anthocyanin content of the youngest fully-formed leaf did not differ significantly between plants treated with respective strains and control treatment measured at 29, 36 dpi (Df=4, F-value=1.469, P-value=0.218; Df=4, F-value=0.229, P-value=0.922). However, 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-value=2.842, P-value=0.0413) (Table 2).

**Table 1:** Effect of 4 *Metarhizium robertsii* strains and control on 8 growth parameters of grapevine plants evaluated until 65-68 days post inoculation (dpi). Values indicate the mean value  $\pm$  CI95%, each calculated from 21 replicates. The letters next to the values represent the significant differences obtained with the Anova test by using different letters.

<i>Growth Parameters</i>								
Inoculated <i>M. robertsii</i> strains	Dry-weight cut shoot at 35 dpi (g)	Sum of the length of the main vein of newly-formed leaves measured at 7, 14, 21, 28, 35 dpi (cm)	Sum of the length of the petiole of newly- formed leaves measured at 21, 28, 35 dpi (cm)	Total number of new leaves at 65 dpi	Total number of new internodes at 65 dpi	Shoot length at 65 dpi (cm)	Dry-weight grapevine total above- ground part at 68-70 dpi (g)	Dry-weight grapevine total root system at 68-70 dpi (g)
MsoilAR-4.3	1.75 $\pm$ 0.37b	52.5 $\pm$ 3.5a	14.4 $\pm$ 1.6a	27.9 $\pm$ 1.5a	26.0 $\pm$ 1.5a	146.5 $\pm$ 10.3a	10.1 $\pm$ 0.9a	0.32 $\pm$ 0.08a
M224B	2.25 $\pm$ 0.46b	51.5 $\pm$ 3.5a	14.2 $\pm$ 2.5a	27.3 $\pm$ 1.7a	25.7 $\pm$ 1.6a	141.5 $\pm$ 13.0a	10.1 $\pm$ 1.0a	0.44 $\pm$ 0.14a
EF3.5(2)	2.74 $\pm$ 0.64b	52.4 $\pm$ 3.8a	12.8 $\pm$ 2.2a	27.1 $\pm$ 1.3a	25.2 $\pm$ 1.3a	143.6 $\pm$ 10.9a	10.4 $\pm$ 1.2a	0.41 $\pm$ 0.11a
EF047	4.29 $\pm$ 0.41a	58.1 $\pm$ 5.3a	14.6 $\pm$ 2.3a	24.2 $\pm$ 1.6b	27.3 $\pm$ 1.8a	152.7 $\pm$ 13.3a	11.6 $\pm$ 1.3a	0.53 $\pm$ 0.12a
Control	2.68 $\pm$ 0.50b	58.2 $\pm$ 5.8a	16.0 $\pm$ 2.6a	28.0 $\pm$ 2.2a	26.1 $\pm$ 2.1a	147.7 $\pm$ 16.2a	11.2 $\pm$ 1.3a	0.47 $\pm$ 0.11a
<i>P</i> =	6.31 $\times$ 10 <sup>-9</sup> ***	0.155	0.439	0.02*	0.806	0.806	0.239	0.186

**Table 2:** Effect of 4 *Metarhizium robertsii* strains and a control on the nitrogen balance index (NBI), the chlorophyll content, the flavonol content and the anthocyanin content of the grapevine youngest fully-formed leaf measured at 28 (n=21), 35 (n=21), 42 (n=8) days post inoculation (dpi). Values indicate the mean  $\pm$  CI95% each calculated from 21 replicates, and the letters next to the values present the significant differences obtained with the Anova test by using different letters.

Time post inoculation		Pigment contents		
Inoculated Strains	Nitrogen Balance Index (NBI)	Chlorophyll content	Flavonol content	Anthocyanin content
29 dpi				
MsoilAR-4.3	22.1 $\pm$ 2.9a <sub>1</sub>	15.9 $\pm$ 0.9a <sub>1</sub>	0.77 $\pm$ 0.07a <sub>1</sub>	0.51 $\pm$ 0.03a <sub>1</sub>
M224B	14.9 $\pm$ 2.7a <sub>1</sub>	16.8 $\pm$ 1.4a <sub>1</sub>	0.78 $\pm$ 0.10a <sub>1</sub>	0.51 $\pm$ 0.04a <sub>1</sub>
EF3.5(2)	24.8 $\pm$ 3.6a <sub>1</sub>	20.8 $\pm$ 6.3a <sub>1</sub>	0.74 $\pm$ 0.06a <sub>1</sub>	0.51 $\pm$ 0.03a <sub>1</sub>
EF047	21.8 $\pm$ 2.8a <sub>1</sub>	16.6 $\pm$ 1.3a <sub>1</sub>	0.80 $\pm$ 0.06a <sub>1</sub>	0.54 $\pm$ 0.04a <sub>1</sub>
Control	20.5 $\pm$ 2.8a <sub>1</sub>	15.5 $\pm$ 0.9a <sub>1</sub>	0.80 $\pm$ 0.07a <sub>1</sub>	0.56 $\pm$ 0.04a <sub>1</sub>
P=	0.452	0.107	0.802	0.218
36 dpi				
MsoilAR-4.3	14.6 $\pm$ 1.4a <sub>2</sub>	11.8 $\pm$ 0.7a <sub>2</sub>	0.83 $\pm$ 0.05a <sub>2</sub>	0.58 $\pm$ 0.04a <sub>2</sub>
M224B	14.3 $\pm$ 1.9a <sub>2</sub>	11.7 $\pm$ 0.6a <sub>2</sub>	0.89 $\pm$ 0.10a <sub>2</sub>	0.60 $\pm$ 0.05a <sub>2</sub>
EF3.5(2)	13.7 $\pm$ 1.8a <sub>2</sub>	11.9 $\pm$ 0.8a <sub>2</sub>	0.91 $\pm$ 0.10a <sub>2</sub>	0.60 $\pm$ 0.06a <sub>2</sub>
EF047	15.0 $\pm$ 2.4a <sub>2</sub>	12.4 $\pm$ 1.1a <sub>2</sub>	0.88 $\pm$ 0.07a <sub>2</sub>	0.62 $\pm$ 0.04a <sub>2</sub>
Control	14.5 $\pm$ 2.5a <sub>2</sub>	11.9 $\pm$ 1.1a <sub>2</sub>	0.90 $\pm$ 0.10a <sub>2</sub>	0.61 $\pm$ 0.06a <sub>2</sub>
P=	0.947	0.86	0.78	0.922
43 dpi				
MsoilAR-4.3	11.8 $\pm$ 2.1a <sub>3</sub>	9.7 $\pm$ 1.3a <sub>3</sub>	0.89 $\pm$ 0.10a <sub>3</sub>	0.61 $\pm$ 0.05a <sub>3</sub>
M224B	13.5 $\pm$ 2.1a <sub>3</sub>	10.6 $\pm$ 0.8a <sub>3</sub>	0.80 $\pm$ 0.09a <sub>3</sub>	0.56 $\pm$ 0.04b <sub>3</sub>
EF3.5(2)	12.0 $\pm$ 2.5a <sub>3</sub>	10.6 $\pm$ 1.2a <sub>3</sub>	0.93 $\pm$ 0.12a <sub>3</sub>	0.63 $\pm$ 0.06a <sub>3</sub>
EF047	10.9 $\pm$ 2.4a <sub>3</sub>	10.0 $\pm$ 1.3a <sub>3</sub>	0.96 $\pm$ 0.12a <sub>3</sub>	0.64 $\pm$ 0.05a <sub>3</sub>
Control	10.6 $\pm$ 1.8a <sub>3</sub>	9.9 $\pm$ 1.0a <sub>3</sub>	0.96 $\pm$ 0.12a <sub>3</sub>	0.70 $\pm$ 0.06a <sub>3</sub>
P=	0.448	0.786	0.279	0.0413*

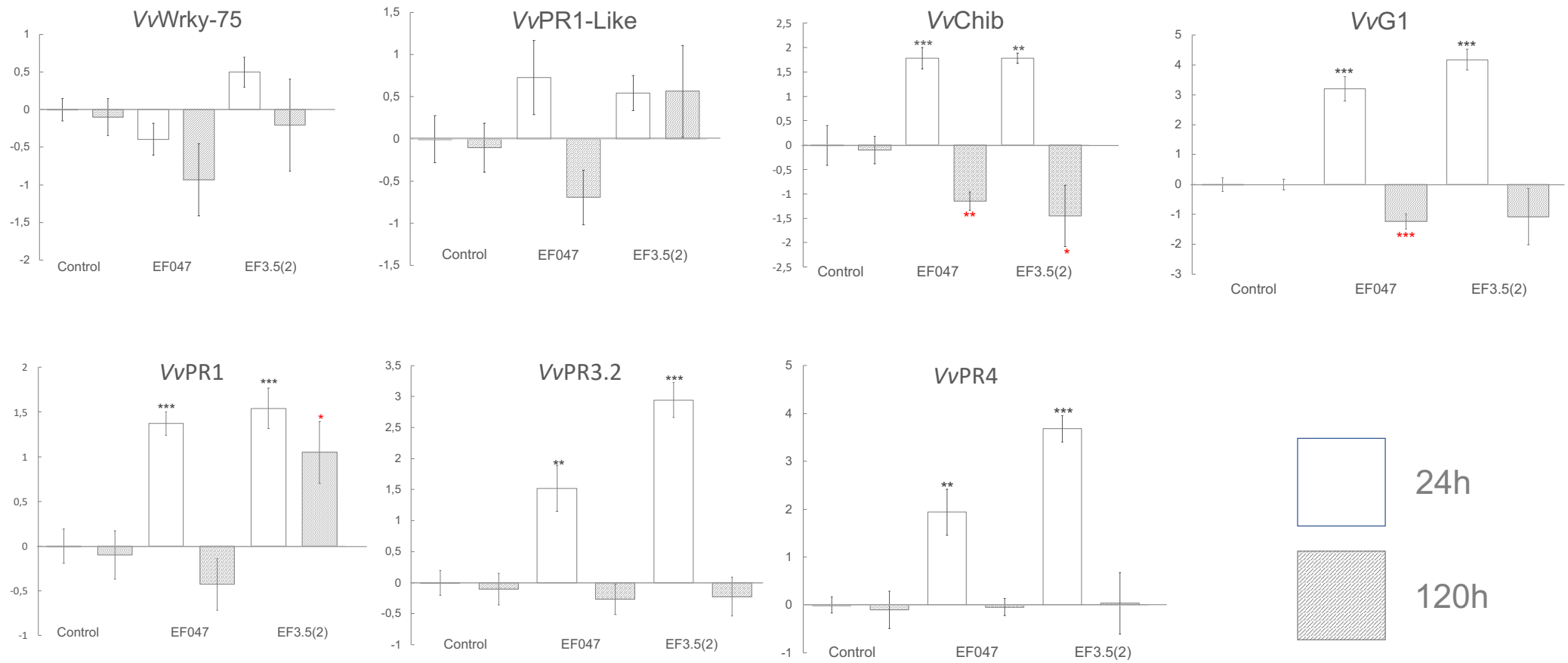
### 7.3.5. Effect of *M. robertsii* on the expression of selected grapevine phylloxera-responsive genes

The assessment of variations in the gene expression levels of ten defense-related genes, known to play a role in the grapevine's defense response to radicle grape phylloxera, was investigated for the strain EF3.5(2) having the lowest rates of endophytic establishment, and the strain EF047 having the highest (Figure 17). Among the ten tested primers, seven (*VvWrky-75*, *VvPR1*, *VvPE1-Like*, *VvPR3.2*, *VvPR4*, *VvG1*, *VvChib*) showed adequate amplification 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 ( $M = 0.401$ ,  $CV = 0.139$ ).

Gene expression analysis at 24 hpt of grapevines (Figure 17) with *M. robertsii* strain EF3.5(2) showed significant up-regulation of five analyzed SA signaling pathway marker genes (*VvPR1* ( $p = 9.75e^{-05}$ ), *VvPR3.2* ( $p = 1.27e^{-07}$ ), *VvPR4* ( $p = 1.80e^{-09}$ ), *VvChib*, ( $p = 0.0023$ ), *VvG1* ( $p = 1.009e^{-08}$ )) compared with the controls. Besides, the strain inoculation downregulated none of the seven evaluated genes. *M. robertsii* strain EF047 inoculation positively induced five of the analyzed SA signaling pathway marker genes (*VvPR1* ( $p = 2.21e^{-05}$ ), *VvPR3.2* ( $p = 0.0024$ ), *VvPR4* ( $p = 0.0040$ ), *VvChib*, ( $p = 0.0014155$ ), *VvG1* ( $p = 2.03e^{-06}$ )). Moreover, the strain EF047 caused a minor decrease in the expression of the *VvWrky-75* gene after 24 hours, whereas EF3.5(2) yielded the opposite outcome.

Gene expression analysis results at 120 hpt of grapevines with *M. robertsii* strain EF3.5(2) inoculation demonstrated a significant up-regulation of one SA signaling pathway marker gene: *VvPR1* ( $p = 0.020$ ) and a significant down-regulation of the gene *VvChib* ( $p = 0.051336$ ). Besides, *M. robertsii* strain EF047 significantly down-regulated two SA signaling pathway marker genes, *VvChib* ( $p = 0.0085381$ ) and *VvG1* ( $p = 6.1834e^{-05}$ ), and none of the screened genes yielded up-regulations.

**Figure 17:** Relative expression of seven genes involved in plant immune response in grapevine leaf against radicle grape phylloxera analyzed via qPCR. The RNA was extracted at 24- (white bars) and 120-hours (grey bars) post-inoculation (hpi) in grapevine leaves from un-inoculated control, inoculated with strain EF3.5(2), inoculated with strain EF047. Values indicate the mean  $\pm$  CI95% each calculated from 3 technical replicates. Statistical differences are shown by asterisk; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 and were obtained with a Student t-test. Asterisk alone indicates significant difference in gene expression in the tested group when compared to that in the un-inoculated control at the time post-treatment (24h: dark grey asterisk, 120h: red asterisk).



## 7.4. Discussion

All *M. robertsii* tested strains native to vineyard environments demonstrated rhizosphere association with non-grafted grapevines that increased over time. The significant installation prevalence of the four *M. robertsii* strains was found. The levels of rhizosphere colonization ranged from 4.8 to 76.2% for the less established strain EF3.5(2) from 26-28 dpi to 68-70 dpi and 62 to 100% for the highest established strain EF047. In our previous study, using the same strain EF3.5(2) inoculated by drenching to *in vitro* produced grapevines, the rates of rhizosphere colonization ranged from 80 to 40% from 14 to 96-98 dpi (Ponchon et al., 2022). In addition, the laboratory strain expressing a green fluorescent protein ARSEF-2575-GFP had a significantly lower rate of rhizosphere association with *in vitro* grapevine rankings from 0 to 13%; compared with all the strains of our study (Ponchon et al., 2022). As this strain does not originate from a vineyard environment, it may poorly associate with grapevine rhizosphere compared with the vineyard native strains. Most likely, the *M. robertsii* strains native to the vineyard could grow and germinate significantly in contact with grapevine root exudates, explaining the increased rates of colonization over time (Moonjely and Bidochka, 2019).

*Metarhizium* was previously demonstrated to establish in the rhizosphere of various plants (e.g., strawberry; cabbage; common bean; sweet corn) and also to significantly multiply in the region close to the roots in comparison with the surrounding bulk soil (Hu and St. Leger, 2002; Klingen et al., 2015; Lahey et al., 2020). Also, Fisher et al., 2011 demonstrated a natural association of several species of *Metarhizium*, including *M. robertsii*, with grape *Vitis* spp. roots sampled in the Willamette Valley fields in Oregon. However, the association was less frequent than other plant species, such as strawberries and Christmas trees. In our study, all the *Metarhizium* strains colonized the rhizosphere without significant differences between tested strains. As they all originate from the same environment (*i.e.*, vineyard environment), they may have significantly more ability to colonize the grapevine rhizosphere. Similarly, two native Brazilian strains, *M. robertsii* ESALQ1426 and *M. anisopliae* ESALQ1037, significantly persisted in the strawberry field and associated with plant rhizosphere for up to 12 months after watering in

the field (Castro et al., 2016). A previous study demonstrated that these strains originated from infected insects found in sugarcane fields, and both seemed to be adapted to Brazilian agrosystem soils (Rezende et al., 2015).

All *M. robertsii* tested strains native to vineyard environments demonstrated endophytic potential to be associated with grapevines. Based on the results obtained via the culture-based method, we found that the French strain EF3.5(2) had the lowest potential to establish as a root endophyte (4.8%), followed by the Australian strain M224B (9.5%), the Argentinian strain MsoilAR4.3 (14.3%) and the German strain EF047 (33.3%) at the endpoint of evaluation (i.e., 68-70 dpi). There was no significant difference in establishment between strains. Our previous study demonstrated low rates of endophytic colonization by the laboratory strain *M. robertsii* ARSEF-2575-GFP of non-grafted grapevine *V. vinifera* var. Cabernet Sauvignon after using artificial root-drenching. The colonization rates ranged from 0 to 13% within 14 to 96 dpi. In comparison, the strain EF3.5(2) native to the Bordeaux vineyard environment and sampled close to vine roots demonstrated significantly more important endophytic potential toward grapevine, with an initial ranking of 23% dropping to 10% (Ponchon et al., 2022). In another study, *B. bassiana* strain H2S32, native to the Greek vineyard environment, endophytically colonized the roots of 82.5% of non-grafted grapevines *V. vinifera* var. Sideritis and 80% of grafted ones with R110 rootstock after 53 dpi (Mantzoukas et al., 2021). Several studies aimed at evaluating the endophytic potential of strains collected in the environment or *in planta* of the crop that had to be protected against biotic and abiotic stressors (Khan et al., 2012; Gan et al., 2017; Farhat et al., 2023). This approach is particularly relevant as EPF are challenged by many biotic and abiotic stressors that impede their persistence in the environment where they are desired to be established (Jaronski, 2010). Therefore, using strains already native to the environment of the crop wished to be protected offers an increased chance of finding ecologically-competent and persistent fungal strains that ultimately have more important endophytic potential toward native crops (Lacey et al., 2015). For instance, the re-inoculation of fungal endophyte strains primarily isolated from the tall fescue *Festuca arundinacea* Schreb was successfully established as endophytes

in the same plant species; and had a positive effect on the plant resistance against herbivores in *in vitro* bioassay (Gan et al., 2017).

Previous studies demonstrated high rates of endophytic colonization of *Metarhizium* spp. towards a range of annual and perennial crops of distinct botanical families (Mantzoukas et al., 2015; Ahmad et al., 2020a; Lahey et al., 2020; Shaalan et al., 2021). Based on the culture-based method evaluation, *M. anisopliae* was established as a root endophyte in 100% of the tested cucumber *Cucumis sativus* L. planted outdoors at 31 dpi upon seed-dressing (Shaalan et al., 2021). Using the same inoculation method, *M. robertsii* endophytically colonized tomato roots *Solanum lycopersicum* L. with a range of 95 to 100% from 10 to 30 dpi (Siqueira et al., 2020). Also, using a hydroponic culturing system, *M. anisopliae* was found to endophytically colonize the roots of 100% of tested maize plants 31 dpi post-drenching (Liu et al., 2022). The low root-endosphere colonization levels in our trials could be explained by the fact that we screened for *M. robertsii* in a very minimal part of the root system; thus, we may have assessed partial endophytic colonization levels. Also, as a non-sterile substrate was used for experiments, other microorganisms naturally comprised in the substrate may have competed with *M. robertsii* for nutrients or directly antagonized its growth, therefore, impeding its potential to colonize the rhizosphere and root endosphere as it was previously demonstrated for *B. bassiana* endophytic colonization of sorghum *Sorghum bicolor* L. (Tefera and Vidal, 2009). In addition, competition with other microorganisms may have adversely affected the growing potential of *M. robertsii* on plates in the trial (Jaber and Enkerli, 2017).

All *M. robertsii* strains persisted in the rhizosphere and the root-endosphere of the grapevine until 68-70 dpi. Similarly, previous studies recorded a persistent association of *M. anisopliae* in the rhizosphere of *Picea abies*(L) up to 342 days after its inoculation via incorporation into the soil substrate (Bruck, 2005). The long-term association of biological control agents with plant is a critical feature as some failed to establish in the long term in the rhizosphere or as an endophyte of grapevine (Andreolli et al., 2021). *Metarhizium* spp. propagules are subjected to various biotic and abiotic stressors impairing their viability and persistence after



their application in large quantities in the soil. Therefore, the repeated application of *Metarhizium* in the ground is partially effective to control soil-borne pests (Jaronski, 2010). The association between *Metarhizium* and plants in the rhizosphere is significant for biological control as it could maintain a high concentration of fungal propagules in this region. Therefore, root herbivores may readily get in contact with *Metarhizium* by direct feeding (Bruck, 2005). The area of origin of *Metarhizium* fungal strains could also contribute to their persistence of installation. For instance, Klingen et al., 2015 found that Norwegian fungal strains persistently associated with the strawberry rhizosphere for up to 471 dpi after application with coated barley kernel in Norwegian field soil. The strawberry rhizosphere was also significantly more colonized with fungal propagules from the native strains of *M. brunneum* and *B. pseudobassiana* than the exotic strain *M. brunneum* F52. Hence, the relevance of using fungal strains native to vineyard environments in our study may explain their persistence in installation with grapevine.

We found that *M. robertsii* strains used in this study did not colonize the grapevines above-ground, with no post-inoculation recovery of *M. robertsii* strains from the cultivation of grapevine leaf and stem tissue homogenate. These results are in line with several previous studies demonstrating the sole endophytic establishment of *M. robertsii* in the roots of several plants (Sasan and Bidochka, 2012; Behie et al., 2015; Greenfield et al., 2016; Garcia et al., 2011; Akello and Sikora, 2012; Canassa et al., 2019). However, *Metarhizium* was also found to be a plant systemic colonizer in many studies employing diverse inoculation methods (Batta, 2013; Garrido-Jurado et al., 2017; Barelli et al., 2018; Ahmad et al., 2020a; Siqueira et al., 2020; Ahmad et al., 2020b). Three studies recorded proof of movements of *Metarhizium* spp. from roots to the foliage, with one investigating *M. robertsii* and non-grafted grapevine (Barelli et al., 2018; Ahmad et al., 2020b; Ponchon et al., 2022; Ahmad et al., 2022). However, the different factors controlling *M. robertsii* localization and *in-planta* movements are not identified, and its systemic colonization is hardly predictable (Ahmad et al., 2020b). Also, as *M. robertsii* may scarcely colonize the leaves, thus molecular methods may be more

sensitive to detect this low level of colonization compared with culture-based methods (Ponchon et al., 2022).

During the period studied of 70 days, the association of *M. robertsii* had neither negative effects on the vine growth nor growth-stimulant effect. The only negative impact recorded on plant development was that the mean number of newly formed leaves in grapevines inoculated with the *M. robertsii* German strain EF047 was lower than the ones inoculated with the control treatment. Previously, an evaluation of sorghum growth features 37 days post-*M. robertsii* or *B. bassiana* or *I. fumosorosea* inoculation via leaf spray showed that the number of leaves, the plant height, dry leaf weight, stem dry weight, and above and below ground part dry weight were unchanged by endophyte inoculation under no stress conditions Mantzoukas et al., 2015. According to Rodriguez et al., 2009, established endophytes *in planta* have a commonly neutral effect on plant physiology and growth.

On the other hand, when planted in a stressful environment, a positive impact of *M. robertsii* establishment as endophyte on plant growth was observed in multiple cases because it may enhance plant resistance against adverse environmental factors, for example, salt stress. For instance, two salt concentrations were tested (*i.e.*, 70 mM and 140 mM). It was demonstrated that rice plants inoculated with *M. anisopliae* had higher shoot length, chlorophyll content, shoot dry weight, and shoot fresh weight under increased salt concentration in the substrate compared with control plants (Khan et al., 2012). The review of Rodriguez et al., 2009 reported only one plant physiologic change induced by endophytes associated with the rhizosphere, which is the enhancement of root hairs and the increased exudation of phenolic-like compounds into the rhizosphere. Therefore, many studies recorded root growth-enhancing effects of *M. robertsii* and *Metarhizium* spp. such as root length, total root system surface, length of the tap root, number of lateral roots, and root hair density in many crops such as tomato *S. lycopersicum* L., bread wheat *Triticum aestivum* L., peanut *Arachis hypogaea* L., switchgrass *Panicum virgatum* L., or French bean *Phaseolus vulgaris* (Sasan and Bidochka, 2012; Liao et al., 2014; Liu et al., 2017; Siqueira et al., 2020;

González-Guzmán et al., 2022; Liu et al., 2022). We did not evaluate the morphological features of vine roots in our study. Still, we believe that it will be relevant to study the long-term effect of *M. robertsii* in vine root morphological structure under controlled conditions and challenged by drought or nutrient deficiency as this structure plays a determinant role in its potential response to nutrient and water stresses in outdoor conditions (Krzyzaniak et al., 2021).

Our study recorded no effect of endophytic colonization on the chlorophyll, flavonol, and anthocyanin pigment content of grapevine. These results are consistent with previous studies on bread wheat, maize, cucumber, sorghum, French beans, Austrian winter pea *Pisum sativum* L., cereal rye *Secale cereal* L. and winter canola *Brassica napus* L.. The inoculation via several application methods of *M. robertsii* or *Metarhizium* spp. inoculation did not affect the leaf chlorophyll content (Liao et al., 2013, 2014; Mantzoukas et al., 2015; Ahmad et al., 2020a, 2020b; Shaalan et al., 2021). The leaf chlorophyll content increased significantly in studies evaluating the effect of inoculation of *Metarhizium* spp. in plants challenged by environmental stresses such as culture in calcareous and Cadmium-rich soil (Raya-Díaz et al., 2017; Jiang et al., 2022). Mantzoukas et al., 2015 states that endophytes established *in planta* can increase photosynthetic rates, transpiration, or stomata behavior under environmental stresses. As grapevines are susceptible to multiple abiotic stressors such as water stress or nutrient deficiency, it will be particularly relevant to evaluate the same features we did and similar ones of Mantzoukas et al., 2015 on vines challenged by different abiotic stressors.

Our study showed that *M. robertsii* strain EF3.5(2) was responsible for the induction of *VvPr3.2*, *VvPR4*, and *VvG1* defense genes in grapevine leaves after 24 hours post inoculation. *Vvwrky-75* was not significantly induced compared to the control, but its mean expression level was higher. Plants commonly harbor on their surface receptors called microbial-/pathogen-associated molecular patterns (MAMPs/PAMPs) activated by microorganisms attached to the plant surface, therefore triggering the first response of plant immunity called (PAMP)-triggered-immunity (PTI) (Mengistu, 2020; Nishad et al., 2020). In addition, the salicylic acid (SA) signaling pathway activates in response to biotrophic and hemibiotrophic

pathogens *in planta* invasion. The accumulation of SA hormone in plant tissues initiates systemic immune response against intruders by mediating the plant's immune regulatory networks, including WRKY transcription factor regulation, which is involved in the SA biosynthesis. Wang et al., 2019 previously found a significant induction of *VvG1*, *VvCHIB*, *VvCHIB1*, *VvPR1*, *VvPR4*, and *VvPR3.2* genes characteristic of a strong response of the SA-signalling pathway in vine-resistant rootstock 1103P tissues sampled from plant infested 16 hours before with radicolle grape phylloxera. Therefore, in our trial, *M. robertsii* strain EF3.5(2) must have been sensed by vine root MAMPs/PAMPs receptors, which initiated the SA-signaling pathway in response to biotroph invasion via up-regulation of *VvPr3.2*, *VvPR4*, and *VvG1* defense genes.

All the four *M. robertsii* strains that were collected in different vineyards, persistently associated with the rhizosphere and the root-endosphere of young potted grapevines under controlled conditions. All these strains are thus, relevant biological control candidates for integrated grapevine pest management against grape phylloxera. They may also be adapted to the environmental conditions of the important vineyard regions studied here: European, Argentinean, and Australian. For instance, the Argentinian *M. robertsii* strain conidia could germinate at 35°C, which is the temperature of the warm summer of regions of North and Central Argentina (López Plantey et al., 2019). The *M. robertsii* strains persistence of association with grapevine should be evaluated at longer time under similar laboratory conditions and followed by their evaluation in outdoor conditions on the mature grapevine. As *M. robertsii* is intended to be applied in the soil of various wine-growing regions, significant considerations on the vineyard soil texture, temperature, moisture, and fungicide applications should be studied in the future, as they represent major abiotic factors affecting propagule germination and long-term persistence (Jackson et al., 2010). In addition, their innocuity to beneficial soil invertebrates and herbivores was recently demonstrated for seed-dressed broad beans planted in the field (Novgorodova et al., 2022). While they do not seem to cause harm to non-target macro-organisms, they represent a significant

environmental-friendly tool for controlling vine soil-borne pests, including grape phylloxera. They will be new sustainable plant protection agents.

### Conclusions:

In the study, all four *M. robertsii* isolates, native to contrasted wine-growing regions, successfully colonized grapevines after artificial inoculation, with no adverse effect on plant physiology or growth after their establishment. The potential priming effect of one *M. robertsii* isolate of the *VWRKY46* gene and several of its down-stream targets suggests the promising potential of this fungus to control grapevine pests such as grape phylloxera attacking non-grafted grapevines. Further studies are needed to assess whether the association of these *M. robertsii* strains with non-grafted grapevine could limit phylloxera damage in roots and to further explore grapevine gene expression following inoculation of the Argentinean and Australian strains.



# Chapter 3

## 8- *Metarhizium robertsii* associated with non-grafted grapevine *Vitis vinifera*, a biological control candidate against the radicolle grape phylloxera (*Daktulosphaira vitifoliae*)

Mathilde Ponchon <sup>1,2</sup>, Daciana Papura <sup>1</sup>, Rodrigo López-Plantey <sup>3</sup>, Manmeet Singh<sup>4</sup>, Karima Rahmani <sup>1</sup>, Annette Reineke <sup>2</sup>, Denis Thiéry <sup>1</sup>

1- INRAE, Bordeaux Sciences Agro, ISVV, UMR SAVE, 33140 Villenave d'Ornon, France

2- Department of Crop Protection, Hochschule Geisenheim University, 65366 Geisenheim, Germany

3- Laboratorio de Fitopatología, IBAM, Universidad Nacional de Cuyo, CONICET, Facultad de Ciencias Agrarias, Almirante Brown 500, Chacras de Coria M5528AHB, Argentina

4- Center for Genomics and System Biology, Department of Biology, New York University, New York, NY, 10003

Type of authorship: First Author

Type of article: Research article

Contribution to the article: Conceptualization and design of the experiments; Development of the methodology; Planning of the experiments; Performance of part of the experimental work; Evaluation and statistical analysis of the data; Preparation of all figures and tables; writing of the manuscript

Contribution of other authors: D.P, D.T., R.L.P. and A.R. contributed to the conceptualization and the experimental design, data analysis, and writing the manuscript; M.S. and K.R. participated in the experimental design and performed part of the experimental work

Status: *In prep. For submission*



## Abstract

The potential of *M. robertsii* to protect non-grafted grapevine *Vitis vinifera* against radicolle grape phylloxera *Daktulosphaira vitifoliae* was studied in laboratory conditions. Firstly, we evaluated the effect of the direct parasitism of *M. robertsii* strain EF3.5(2), collected from a French vineyard, on the survival and development of grape phylloxera single lineage clonal ‘Pcr7’ in woody root section bioassay. We used three methods to infect grape phylloxera eggs: (i) direct spraying of eggs with a conidial suspension of *M. robertsii* and laying on the woody root section, (ii) spraying of the root section and laying of the eggs, (iii) spraying of both root section and eggs separately before laying onto the root section. We then monitored the grape phylloxera development for up to 28 days. At the end of the trial, we observed a significant reduction of 90.0%, 70.0%, and 86.1% in survival probability of grape phylloxera for “Egg-treat,” “Root-treat,” and “Both-Treat,” respectively, compared with the three associated control treatments, and 92.0%, 89.0% and 93.0% in reduction of adults’ development compared with control treatments. Secondly, we evaluated the impact of *M. robertsii* association with non-grafted grapevine *V. vinifera* roots on the radicolle grape phylloxera establishment and induction of symptoms, in pot experiment. After 76 days post-phylloxera inoculation, a reduction of damage by 91% from plants co-inoculated with *M. robertsii* and grape phylloxera (mean of  $4.8 \pm 8.4$  nodosities) compared with the ones infected only with phylloxera (mean of  $52.7 \pm 50.7$  nodosities) was observed. 100% of *M. robertsii*-inoculated plants and 92.0% of grape phylloxera-*M. robertsii* co-inoculated plants were endophytically colonized by the fungus. The grapevine growth was not significantly affected by *M. robertsii* or phylloxera infestation during the trial. Investigations should continue under field conditions to determine the potential of *M. robertsii* for durable association and phylloxera biocontrol of non-grafted grapevine.

## 8.1. Introduction

Grape phylloxera, *Daktulosphaira vitifoliae* Fitch (Hemiptera: Phylloxeridae), is one of the most emblematic invasive species viticulture has ever encountered (Carton et al., 2007; Thiéry et al., 2013). It was first unintentionally introduced in the French territory in 1861 after trading American plant material. It rapidly spread across France and through European vineyards planted with domesticated grapevine *Vitis vinifera* L. (Powell, 2008). Consequently, it jeopardized the wine industry by destroying almost a third of the worldwide vineyard by the late 19th century, eroding a whole segment of the economy (Pouget, 1990). Grape phylloxera is originally native to the eastern part of the United States of America and tolerated by its natural hosts, the American wild *Vitis* species, from which it feeds only on young leaves and root tips (Granett et al., 2001). However, in its invasion area, it severely damages the non-grafted *V. vinifera* at the root level. Indeed, radicolle phylloxera forms induce hooked galls of fine vine root tips (nodosities) and large swollen galls on mature woody roots (tuberosities), which can deform and fissure. The second root deformation is usually significant and accompanied by disease development caused by soil-borne pathogens that amplify the root damage (Powell, 2008; Granett et al., 2013). Aerial vine symptoms of radicolle infestation are characterized by reduced leaf surface area and shoot growth, yield loss, vine decline, and plant death is estimated to occur within 2 to 5 years post-infestation (Granett et al., 2013).

The most effective measure used worldwide to control the pest is the grafting of European grapevine *V. vinifera* cultivars onto grape phylloxera-resistant rootstocks of American *Vitis* species or hybrids of American and European *Vitis* species (Rubio et al., 2020). Nevertheless, grape phylloxera is still spreading in most wine-growing regions worldwide due to human activity (*e.g.*, shoes, clothing, machinery, and plant material). The crawler instars, and the winged adults can also disperse passively by the wind. In addition, the tolerance of commercial rootstocks for the pest helps maintain a steady phylloxera population by providing habitat and food sources (Ji et al., 2021). Therefore, grape phylloxera is ubiquitous today in most wine-growing regions worldwide, with observations made in the entire

American continent, Europe, New Zealand, and South and North-West Africa. In China, Australia, Russia, and Armenia, the pest is contained only to precise areas by quarantine measures (Powell and Clarke, 2018; Tello et al., 2019; Jiang et al., 2022).

The phylloxerid pest is difficult to manage because of its high genetic plasticity, which amplifies its potential to bypass rootstock resistance (Forneck et al., 2016). Therefore, the risks of a grape phylloxera resurgence as a significant grapevine pest are identified because of (i) possible bypass of rootstock resistance by new emerging virulent grape phylloxera biotypes (Forneck et al., 2016), (ii) leaf-feeding grape phylloxera increased damages to European vineyards (as is the case in some Italian and Bulgarian vineyards (Wilmink et al., 2021b)), (iii) grape phylloxera invasion of new non-grafted vine-growing regions, as has been the case in Australia and Argentina (Powell et al., 2013; Arancibia et al., 2018).

Management options to cure grape phylloxera-infested vineyards are limited and partially efficient in preserving non-grafted grapevines. Grapevine uprooting is used to reduce existing grape phylloxera populations in the soil. Still, it is almost impossible to suppress them, as they can remain on deeply buried roots of previous plantations for several years. Grape phylloxera can be contained by limiting the risk of vectorization by human activity and planting grapevines labeled certified pest-free (Bettiga, 2013). Insecticides are inconsistently suppressing radicolle grape phylloxera populations because: firstly, there are difficult to reach because of poor penetration capacities of products inside soils, especially in clay soils that are preferred by grape phylloxera; secondly, grape phylloxera has a rapid population growth and regeneration potential of 3 to 10 generations per year, which can be decreased only by repeating the treatment or using long-residual chemicals; third, once the decline process of grapevine is initiated, it becomes challenging to stem it by suppressing radicolle phylloxera populations (Granett et al., 2001).

In some wine-growing regions, such as Australia, representing 2% of the world vineyard surface area (OIV 2023), the grafting is not systematically employed and therefore, it is substituted by quarantine rules applied to avoid the spread of the pest. However, they are costly, partially effective, and did not prevent grape

phylloxera from spreading in the Australian territory. The consequences of grape phylloxera invasion in these non-grafted commercial vineyards are significant because of high replanting costs and quarantine measures, thus endangering the viticulture industry's long-term success and economic sustainability (Benheim et al., 2012).

Biological control has been poorly investigated, but it is a promising way to control radicolle grape phylloxera instead of insecticides. Research on the natural enemies of grape phylloxera, such as predators and parasitoids, is not abundantly documented (Benheim et al., 2012; Papura et al., 2020). Entomopathogenic nematodes were also examined, and the biotype of *Heterohabditis bacteriophora* Poinar (Hb Oswego) had an efficient reduction of 80% grape phylloxera larvae compared to control in the Petri-dish trial. The same biotype had significant controlling potential in soil-cup trials. However, it did not perennially establish in vineyard soil (English-Loeb et al., 1999).

Entomopathogenic fungi (EPF) are ubiquitous pathogens of arthropods naturally living in the soil and have important biocontrol potential to control grapevine root pests (Lacey et al., 2015). EPF are phylogenetically diverse and more than 700 species and 90 different genera were established to parasitize insects (Vega et al., 2012). EPF parasitize their host via their asexual spores, which can germinate in contact with the insect cuticula depending on environmental factors (*i.e.*, ultraviolet light, temperature, and humidity) and penetrate the inside of the insect body to utilize its nutrient resources, which causes the host's death (Shukla and Afzal, 2021). Besides their potential to infect multiple arthropod hosts, a subset of EPF display a bifunctional lifestyle, characterized by their capacity to colonize the rhizosphere and the root-endosphere of various plant species (Sasan and Bidochka, 2012; Hu and Bidochka, 2019). Indeed, EPF asexual spores can adhere and grow on the external root surface and asymptotically colonize several host plant tissues for extended period and build a mutualistic relationship (Vega, 2008). Their colonization potential offers several benefits for plants, such as nutrition, alleviation of nutrient stresses, stimulation of plant growth and immune defense system, and protection against pests and diseases (Sasan and Bidochka, 2012; Khan

et al., 2012; Raya-Díaz et al., 2017; Jaber and Ownley, 2018; Ahmad et al., 2022). *Metarhizium robertsii* (Metchnikoff) Sorokin is a famous species as it is one of the most commercially used EPF as a biopesticide worldwide (Brunner-Mendoza et al., 2019). Some studies found that it inhabited vineyard soils in the United States, Portugal, France, Australia, Argentina, Germany, Greece, South Africa (Fisher et al., 2011; Sharma et al., 2018; Poidatz et al., 2018; Korosi et al., 2019; López Plantey et al., 2019; Uzman et al., 2019; Moloinyane et al., 2020; Mantzoukas et al., 2020). One first study demonstrated a natural association of *M. robertsii* in the rhizosphere of *Vitis* spp. after field sampling of vine roots in the Valleys of Oregon (Fisher et al., 2011). Also, the two strains of *M. robertsii*: strain EF3.5(2) native to a French vineyard and the laboratory strain ARSEF-2575-GFP expressing a green fluorescence protein, were artificially associated with the rhizosphere and root-endosphere of non-grafted *V. vinifera* for up to 96-98 dpi (Ponchon et al., 2022). Another experiment artificially inoculated *Metarhizium* with potted non-grafted *V. vinifera*. It was demonstrated that barley kernels soaked in a conidial suspension of *M. anisopliae* var. *anisopliae* (now *M. robertsii*), incorporated into a planting substrate could reduce by 80% the number of grapevines infested with grape phylloxera compared with controls (Kirchmair et al., 2004a). However, the association between this EPF and the grapevine could not be established. Two other field trials confirmed the efficacy of *M. anisopliae* as a soil treatment to control grape phylloxera abundance in the field over two years, highlighting the important potential of *Metarhizium* to control radicicole grape phylloxera (Kirchmair et al., 2004b, 2007, 2009).

Here, we evaluate the potential of *M. robertsii* to protect non-grafted grapevines against radicicole grape phylloxera. We hypothesized that: i) *M. robertsii* can reduce the development and the survival of radicicole grape phylloxera by conidia direct infection via mechanisms of conidia adhesion to the insect shell, germination, and grape phylloxera parasitism and ii) *M. robertsii* association with *V. vinifera* roots (rhizosphere) can reduce radicicole phylloxera' establishment and the development of symptoms on fine roots (nodosities). For the first bioassay, *M. robertsii* was applied as conidia to radicicole grape phylloxera

eggs by three means: (i) direct spraying of eggs and laying onto root section; (ii) spraying of conidia on root section before laying the eggs; (iii) spraying of conidia on both root section and eggs separately before laying the eggs. For the second bioassay, young grapevines obtained from wood cuttings inoculated via root-dipping with *M. robertsii* were inoculated with radicolle grape phylloxera eggs. After 76 days post-inoculation, the root-symptoms of phylloxera infestation (i.e., nodosities) were recorded on plants after uprooting. We expected a reduction in root symptoms induced by radicolle grape phylloxera in plants that received co-inoculation of *M. robertsii* and the pest compared to those that did not.

## 8.2. Material and Methods

### 8.2.1. Insect material

All grape phylloxera (*D. vitifoliae*) used in this study were obtained by multiplying the gallicole isofemale clone Pcf7. This clone was established from grape phylloxera individuals sampled in 2010 at Pineuilh (Gironde, France) on *V. vinifera* cv. 'Cabernet franc' scions grafted on SO4 rootstock (*V. berlandieri* x *V. riparia*). It was maintained in rearing collection at INRAE-Bordeaux through parthenogenetic reproduction on leaves of young grapevines planted in pots of American grape variety 'Harmony,' a susceptible hybrid between Dog-Ridge (*V. champinii*) and accession 1613C (*V. labrusca* x *V. riparia* x *V. vinifera*). Plants were installed in a controlled chamber at 22 °C, L:16/D:8, and 60% of humidity (Rispe et al., 2020; Rubio et al., 2020). Grape phylloxera radicle form was used for experiments. Their multiplication was performed as described: 50 grape phylloxera eggs from the gallicole breeding were reared for three generations by transferring them on freshly collected healthy woody root sections measuring 5-6 cm of susceptible cultivar *V. vinifera* cv. 'Cabernet Sauvignon' (see 8.2.2. Plant Material). Root sections were previously disinfected in calcium hypochlorite 50 g.L<sup>-1</sup> for 15 minutes and rinsed three times in sterile water. They were then placed on moistened filter paper disks in Petri dishes and kept in the dark boxes at 22°C and 60% humidity (Rispe et al., 2016). Then, roots were observed weekly to remove necrotic ones and replace them with healthy ones while ensuring the filter moisture.

### 8.2.2. Plant Material

For Bioassay 1, healthy woody 5-6 cm root sections of *V. vinifera* cv. Cabernet Sauvignon were collected from potted grapevines and used for experiments. The vines were grown in semi-field conditions, planted in pots containing vineyard soil, and kept outside for several years in the experimental facilities of INRAE-Bordeaux-Aquitaine.

For Bioassay 2, 40 grapevine seedlings were used for experimentation and obtained from cuttings of *V. vinifera* cv. Cabernet Sauvignon. These young vines were planted in vineyard soil previously sterilized by autoclaving twice to avoid native phylloxera or *M. robertsii* contaminations. Plants were maintained in a growth chamber with controlled conditions (temperature 22°C, 60% relative humidity, 16:8 photoperiod) and watered every third day with water and once a week with nutrient solution.

### 8.2.3. Fungal Material

The *Metarhizium robertsii* strain EF3.5(2) was used for all the bioassays. This strain was collected in 2015 from vineyard soil in the INRAE experimental vineyards (Villenave d'Ornon, South West of France, 44°47'30.4" N 0°34'36.9" W) and then isolated by insect baits method (Poidatz et al., 2018). *M. robertsii* stock cultures were maintained on oat agar chloramphenicol media (40 g organic oat flour (Moulin Des Moines, Krautwiller, France)), 20 g agar (SIGMA Aldrich, St. Louis, MO, USA), 50 mg chloramphenicol (SIGMA Aldrich), and 1 L of water. To prepare *M. robertsii* conidial suspension, conidia were removed from the Petri dish surface and suspended into a sterile water 1/8 Ringer + 0.02% Tween 80® (Polysorbate 80, SIGMA Aldrich). Conidial suspensions were adjusted to  $1 \times 10^7$  conidia·mL<sup>-1</sup> using a hemocytometer.

### 8.2.4. Bioassay 1: assessment of the direct parasitism effect of *M. robertsii* on the development and survivorship of radicolle grape phylloxera

A total of 20 grape phylloxera eggs obtained from the radicolle breeding were dropped with a paintbrush onto an 80 mm diameter sterile disc of filter paper gently moistened. The disc was then lodged directly onto the root section that was previously disinfected as described in 8.2.1. Insect Material. Three methods were applied to infect the grape phylloxera eggs with *M. robertsii*. The 'Egg-Treat' method consisted of directly spraying phylloxera eggs onto the paper disc with a  $1 \times 10^7$  conidia·mL<sup>-1</sup> suspension using a micro-atomizer before laying the disc on the



root section. The 'Root-Treat' method consisted of directly spraying only the root section with the same conidia suspension before depositing phylloxera eggs onto the disc. The last method trialed was the 'Both-Treat' consisting of spraying the root section and phylloxera eggs separately with the same conidia suspension before depositing the disc on the root section. Three control treatments were performed: 'Egg-Cont,' 'Root-Cont,' and 'Both-Cont' with the same methodology as for the three methods described above by replacing conidial suspension with sterile water 1/8 Ringer + 0.02% Tween 80®. For each treatment, 10 replicates were made. Petri dishes were then sealed using Parafilm and cautiously placed in the dark at a temperature of 25°C.

The Petri dishes were first opened at 5 days post-treatment (dpt). The number of live and dead larvae and viable eggs were recorded by observation with a binocular magnifier. The procedure was repeated at 8, 11, 15, 18, 21, and 28 days to count the number of live and dead larvae and the number of emerging adults. During every observation, the filter paper was moistened with sterile water, and dishes were closed with Parafilm. All eggs, dead larvae, and adults were removed from the Petri dishes and pricked on a PDA medium under sterile conditions. Presumed *M. robertsii* outgrowths in the medium were recorded to assess the number of phylloxera individuals harboring external or internal infections by the fungus.

#### 8.2.5. Bioassay 2: Evaluation of the impact of *M. robertsii* associated with the *V. vinifera* roots on the symptom severity induced by grape phylloxera and on the grapevine growth

*V. vinifera* potted seedlings planted in vineyard soil were removed from the pot after 2.5 months of plantation. The plants had been produced as described in 8.2.2. *Plant material*. Twenty grapevines were dipped for 12 h (overnight) in a  $1 \times 10^7$  conidia·mL<sup>-1</sup> of *M. robertsii* strain EF3.5(2) suspension with sterile water 1/8 Ringer + 0.02% Tween 80®. For the control treatment, 20 other plants were dipped only in sterile water 1/8 Ringer + 0.02% Tween 80® in the same way. Plants were replanted in sterile soil the next day and maintained as described in 8.2.2. *Plant*

*Material.* Four treatments were made, and 10 plants were prepared for each treatment. 'Meta' treatment consisted of maintaining during 107 days in growth chamber conditions 10 plants previously inoculated with *M. robertsii*. 'Meta-Phyllo' treatment consisted of inoculating 10 other plants with 50 eggs of radicicole phylloxera 31 days after their inoculation with *M. robertsii* and maintaining them in growth chamber conditions for 76 days. The phylloxera inoculation was performed by depositing 50 phylloxera eggs on an 80 mm diameter moistened paper disc and then placing them near grapevine roots at ca. 1-2 cm soil depth. Control treatment 'Cont' consisted of maintaining plants for 107 days in growth chamber conditions after their watering with sterile water containing Ringer solution. 'Cont-Phyllo' treatment consisted of inoculating 50 eggs of radicicole grape phylloxera to vines, in the way as described above. 31 days after watering sterile water containing Ringer solution. Afterwards plants were maintained 76 days in growth chamber conditions.

Grapevines were maintained in the same cultural conditions described in 8.2.2.. Furthermore, an insect-proof transparent plastic bell was placed on each grapevine to avoid grape phylloxera larvae movements between plants and cross-contaminations.

107 days after the inoculation (with *M. robertsii* suspension or control solution), each vine was carefully dug up. The soil was gently removed from the vines' root system, and the nodosity number was counted in vines treated with 'Meta-Phyllo' or 'Cont-Phyllo' treatments to characterize the level of phylloxera attack. Roots of plants inoculated only with the control solution or *M. robertsii* were observed in the same way to verify the absence of phylloxera contamination. Also, 1 g of pooled root pieces were sampled on each vine, and the rhizospheric *M. robertsii* was processed as described in 8.2.6. Fresh and dry weight of total roots, and aerial parts were measured after symptom characterization to evaluate the effect of the different treatments on plant growth. For this, freshly sampled roots and aerial parts were separately weighed with a scale, placed in paper bags, dried at 50°C for 48h, and weighed again.

### 8.2.6. Assessment of rhizospheric association of *M. robertsii* with grapevine roots by the culture-based method

For each of the 40 grapevines tested, 1 g of pooled root pieces were sampled to evaluate grapevine association with *M. robertsii*. The roots were only removed from the coarsest soil particles by gentle shaking and the remaining adherent soil was considered as rhizospheric soil. From each plant, an amount of 1 g of root pieces was placed in a plastic tube (height × width: 60 mm × 27 mm and 20 mL volume, ZINSSER POLYVIALS® (Zinsser Analytic GmbH, Eschborn, Germany)) and put together with 4 ml of sterile distilled water and 0.02% Tween 80® to which was added 2 inox balls of 8 mm. The root tissues were mixed to create a root homogenate using a disrupter (Qiagen, Hilden, Germany). A 100 µL of the homogenate was plated in duplicate onto chloramphenicol, thiabendazole, cycloheximide (CTC) medium. Its composition was based on a modified recipe of [Fernandes et al., 2010](#) consisting of 39 g·L<sup>-1</sup> potato dextrose agar filled up to 1 L with sterile water and additioned with 0.1 g·L<sup>-1</sup> chloramphenicol diluted in 96% ethanol, 0.002 g·L<sup>-1</sup> thiabendazole and 0.15 g·L<sup>-1</sup> cycloheximide (SIGMA Aldrich) both diluted in sterile water. After 14 days of incubation in the dark at 25°C, each plate was visually analysed for *M. robertsii* colonies by characterization of morphological features ([Humber, 2012](#)). A plant was considered as colonized in the rhizosphere level by *M. robertsii* if a one of its colony was found in one of the duplicate plates.

### 8.2.7. Statistical analysis

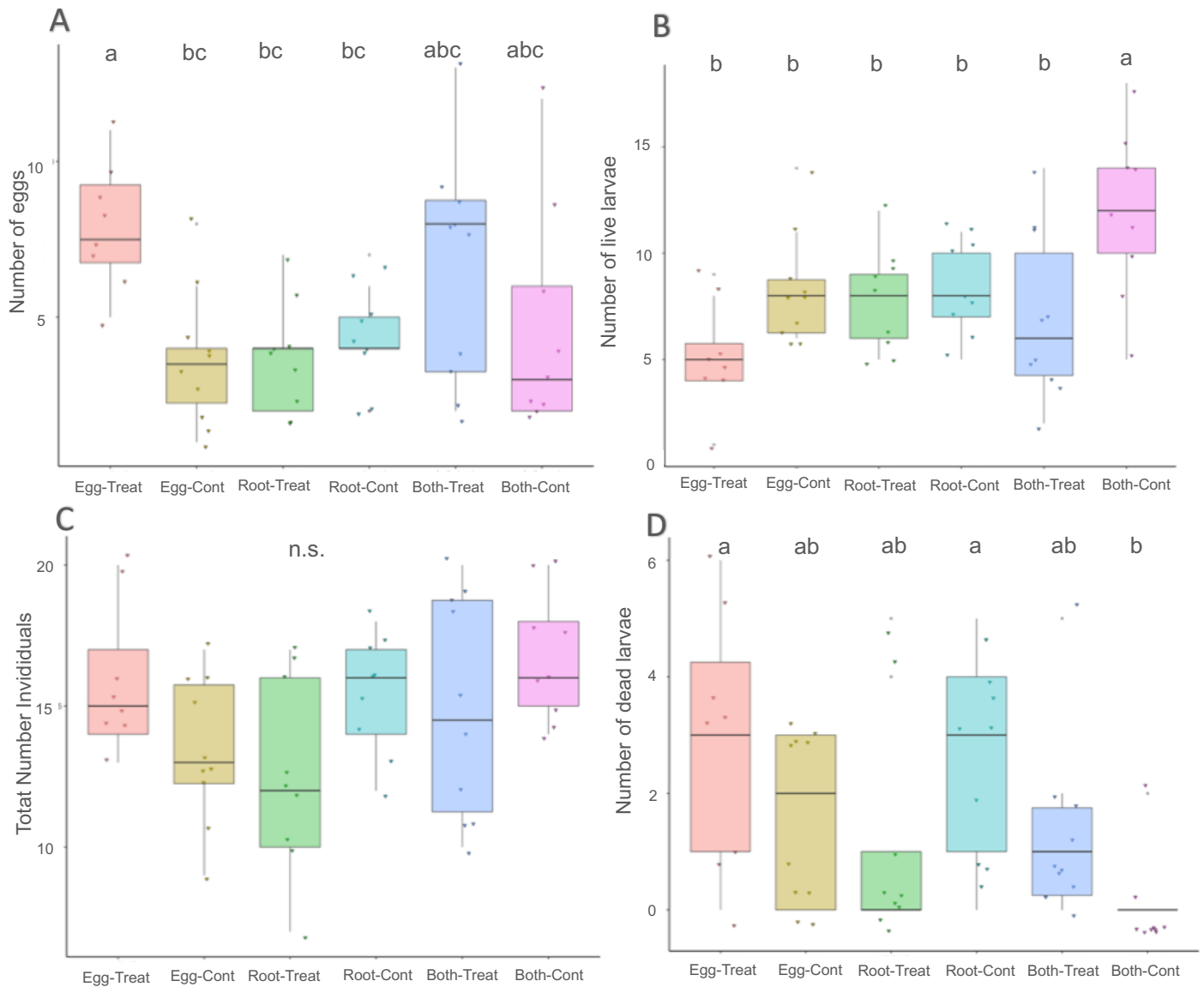
Statistical analysis was processed in the R software's most recent version RStudio 2022.07.2 + 576 (2022 RStudio©, PBC. All Rights Reserved), and figures were made with the ggplot2 package of the software. For the data analysis of bioassay 1, the non-parametric Kruskal–Wallis test and then multiple pairwise comparisons posthoc tests were used to determine significant differences ( $p < 0.05$ ) between all treatments performed for initial counting at 5 days post-treatment (dpt) of number of viable phylloxera eggs, number of live larvae, the total number of individuals, and number of dead larvae. Kaplan-Meier curve analysis was performed to analyze the patterns for each applied treatment of (i) survival probability of individuals, (ii) cumulative probability of adults' emergence using the survival, survminer, and ggsurvfit R packages. Significant differences between treatments were obtained by using the Log-Rank test ( $p < 0.05$ ), followed by multiple pairwise comparisons (post-hoc) of treatments with the Bonferroni test. The statistical methodology was described and validated by Tripathi and Pandey, 2017. Finally, the percentages of contaminated grape phylloxera with *M. robertsii* for treatment 'Egg-Treat,' 'Root-Treat,' and 'Both-Treat' were compared with the Chi-2 test ( $p < 0.05$ ).

For bioassay 2, the number of nodosities for plants treated with 'Meta-Phyll' and 'Phyll' was compared with the non-parametric Kruskal–Wallis test ( $p < 0.05$ ). Finally, the wet above-ground biomass weight, wet root system biomass weight, dry above-ground biomass weight, and dry root system biomass were analyzed for the four treatments 'Cont-Phyllo,' 'Meta-Phyllo,' 'Cont,' 'Meta' with the Kruskal-Wallis test followed by multiple pairwise comparisons posthoc tests ( $p < 0.05$ ).

## 8.3. Results

### 8.3.1. Grape phylloxera development and survival after 5 days post-treatment (dpt) with *M. robertsii* conidial suspension

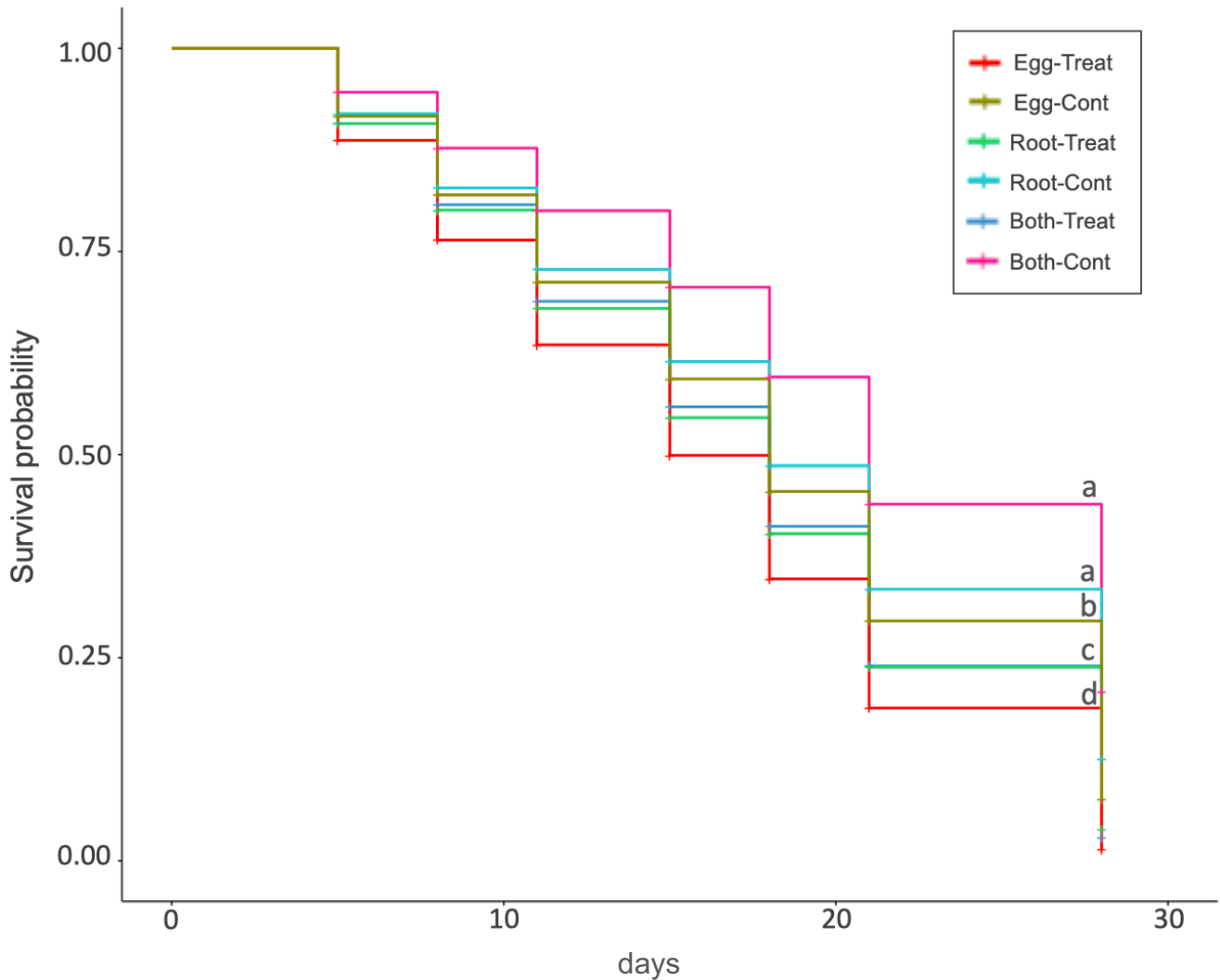
The number of viable grape phylloxera eggs (Fig. 18 A) counted at 5 days post-treatment (dpt) in the Bioassay 1 differed significantly between the six treatments ( $X^2=14.253$ ,  $df= 5$ ,  $p=0.01408$ ). Indeed, the number of viable eggs in the ‘Egg-Treat’ (median (med) of 7.5 Range [5;11]) was significantly higher than in the ones of the ‘Egg-Cont’ (med of 3.5 [1;8]), ‘Root-Treat’ (med of 4 [2;7]) and the ‘Root-Cont’ (med of 4 [2;7]) treatments. The ‘Egg-Cont’ had the lowest mean number of viable eggs recorded. Thus, direct egg spraying with suspension without *M. robertsii* conidia significantly impacted egg mortality (Fig. 18 A). Also, the number of live grape phylloxera larvae counted at 5 dpt varied significantly between treatments ( $X^2=16.27$ ,  $df= 5$ ,  $p=0.006115$ ). Indeed, the number of live larvae in ‘Both-Cont’ (med of 12 [5;18]) was significantly higher compared to all other treatments (Fig. 18 B). The total number of grape phylloxera individuals (eggs and larvae) counted at 5 dpt did not differ significantly between treatments ( $X^2= 9.6719$ ,  $df= 5$ ,  $p= 0.08508$ ) (Fig. 18 C). Also, the number of dead grape phylloxera larvae counted at 5 dpt differed significantly among treatments ( $X^2= 15.447$ ,  $df= 5$ ,  $p= 0.008615$ ), and was significantly higher in the ‘Egg-Treat’ (med of 3 [0;6]) and the ‘Root-Cont’ (med of 3 [0;5]) compared to the ‘Both-Cont’ (med of 0 [0;2]) (Fig. 18 D).



**Figure 18 :** A) Number of viable phylloxera eggs 5 days post-inoculation with *M. robertsii* conidial suspension, B) Number of live larvae, C) Total number of individuals, D) Number of dead larvae counted at 5 days post treatment (dpt) represented as box plots. *M. robertsii* strain EF3.5(2) was applied as conidial suspension via three treatments to grape phylloxera eggs laid on *V. vinifera* root sections: 'Egg-Treat' (red boxes), 'Root-Treat' (green boxes) and 'Both-Treat' (dark blue boxes) and Control treatments: 'Egg-Cont' (yellow boxes), 'Root-Cont' (light blue boxes), 'Both-Cont' (pink boxes). Significant differences are indicated by small letters above the boxes (Kruskal–Wallis test,  $p < 0.05$ ).

### 8.3.2. Grape phylloxera development and survival after 8 to 28 days post-treatment (dpt) with *M. robertsii* conidial suspension

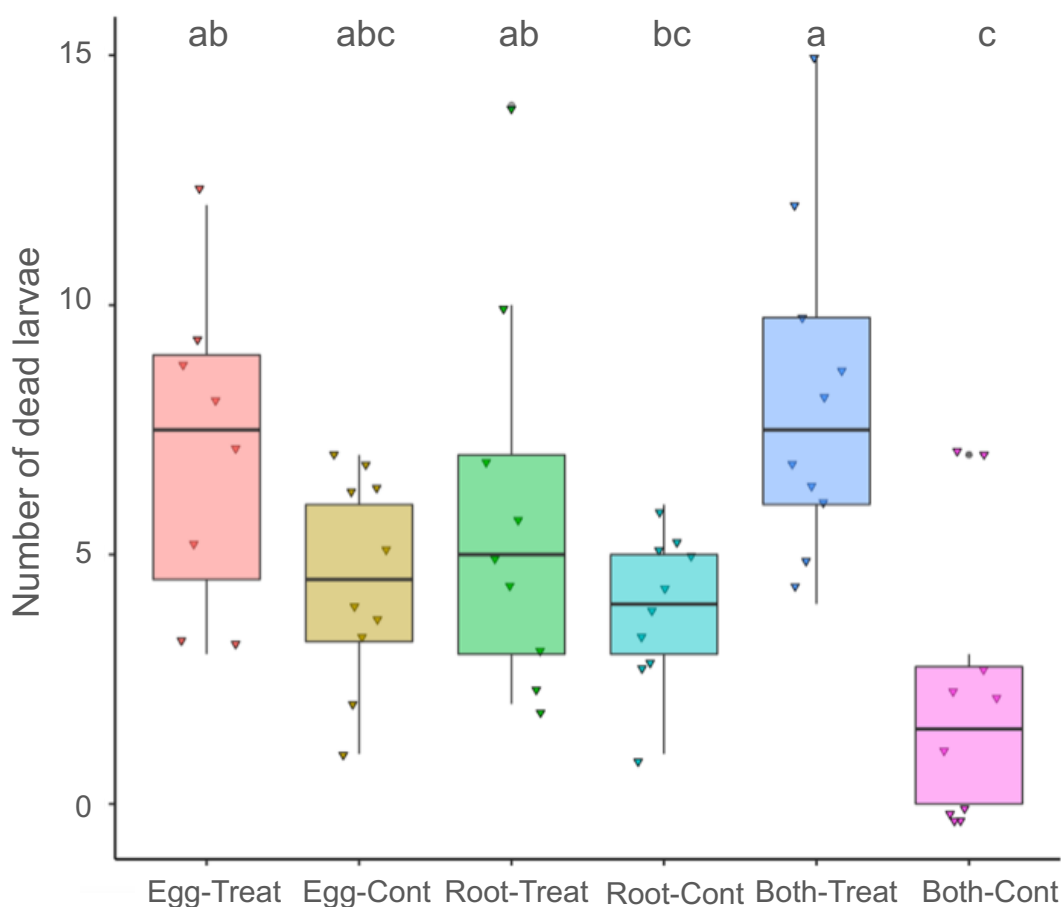
Kaplan-Meier survival curves (Fig. 19) reveal a highly significant difference in the survival of grape phylloxera larvae feeding on control root sections vs. *M. robertsii*-treated root sections (Log-Rank test:  $\text{chisq} = 282$ ,  $\text{df} = 5$ ,  $p < 2e^{-16}$ ). Pairwise comparisons highlighted that individuals treated via ‘Egg-Treat’ treatment had the lowest survival probability (at 28 dpt  $0.00141 \pm 0.00361$ ), followed by ‘Root-treat’ (at 28 dpt  $0.0382 \pm 0.0273$ ) and ‘Both-Treat’ (at 28 dpt  $0.0288 \pm 0.0057$ ) with significant differences between all treatments. In comparison, control individuals treated via ‘Both-Cont’ had the highest survival probability (at 28 dpt  $0.208 \pm 0.017$ ) followed by ‘Root-Cont’ (at 28 dpt  $0.125 \pm 0.0128$ ) and was significantly higher than all other treatments, including ‘Egg-Cont’ (at 28 dpt  $0.0141 \pm 0.0036$ ) (Fig. 19).



**Figure 19** : Survival probability of grape phylloxera individuals (live larvae and adults) over the course of 28 days post-treatment (dpt) with *M. robertsii* conidial suspension represented as line plots. Lines represent Kaplan-Meier analysis of cumulative survivorship of phylloxera individuals. *M. robertsii* strain EF3.5(2) conidia were applied by three treatments to grape phylloxera eggs laid on *V. vinifera* root sections: 'Egg-Treat' (red line), 'Root-Treat' (green line) and 'Both-Treat' (blue line) and Control treatments: 'Egg-Cont' (yellow line), 'Root-Cont' (light blue line), 'Both-Cont' (pink line). Confidence Interval (IC) 95% were omitted for more readability. Significant differences are indicated by small letters above the end of the survival curves (Log-Rank test and pairwise comparison with Bonferroni correction, considering  $p < 0.05$ ).

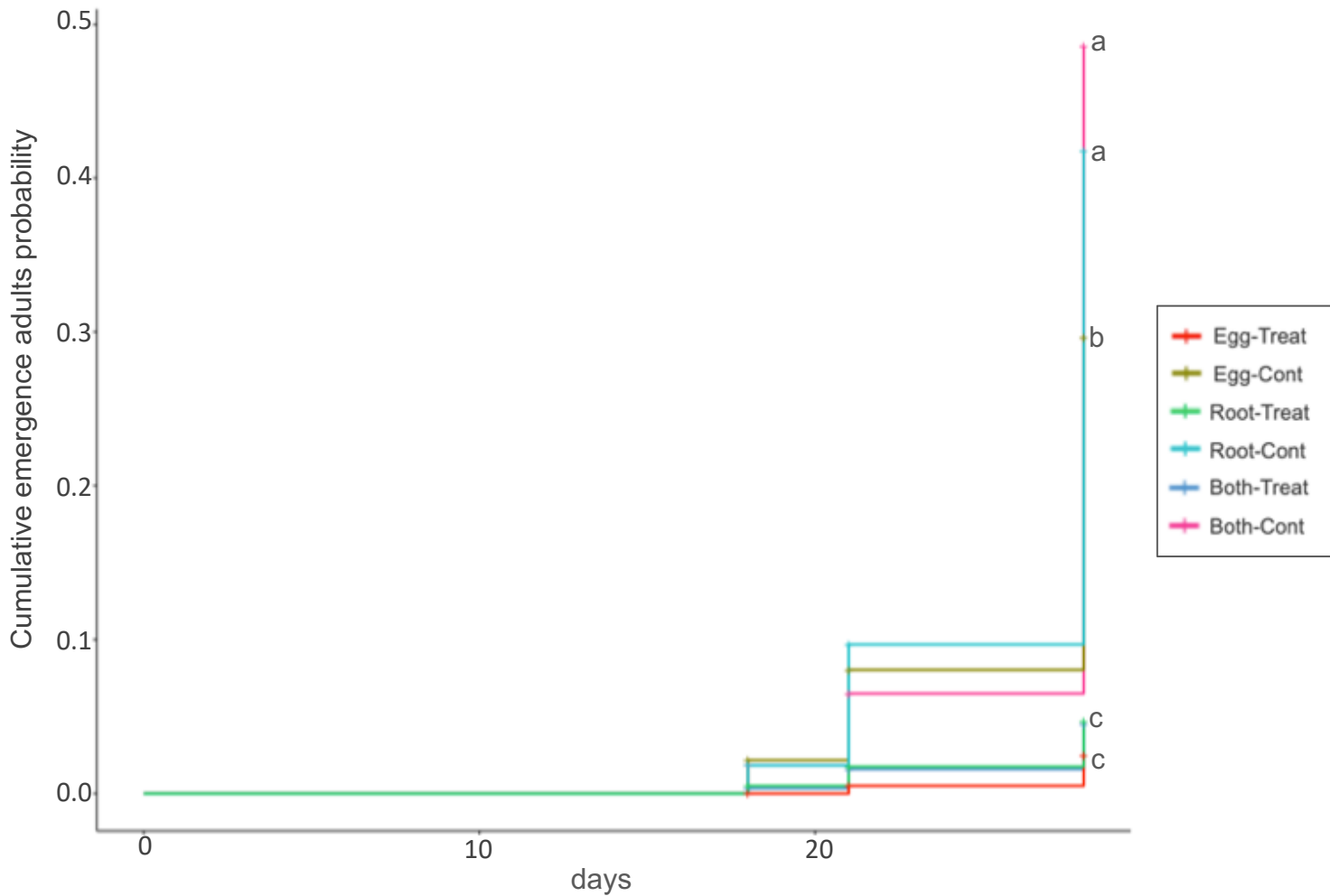


Also, the total number of dead larvae counted (Fig. 20) at the end of the trial was significantly higher in the treatment ‘Egg-Treat’(med of 7.5 [3;12]), ‘Root-Treat’ (med. of 5 [2;12]) and ‘Both-Treat’ (med. of 7.5 [4;15]) compared to the control treatment ‘Both-Cont’ (med. of 1.5 [0;7]) ( $X^2=20.002$ ,  $df=5$ ,  $p=0.001249$ ). However, the number of dead larvae during the trial did not differ significantly between the ‘Egg-Treat’ and the ‘Egg-Cont’ (med. of 4.5 [1;7]) treatments and also between the ‘Root-Treat’ and the ‘Root-Cont’ (med. of 4 [1;6]) treatments (Fig. 20).



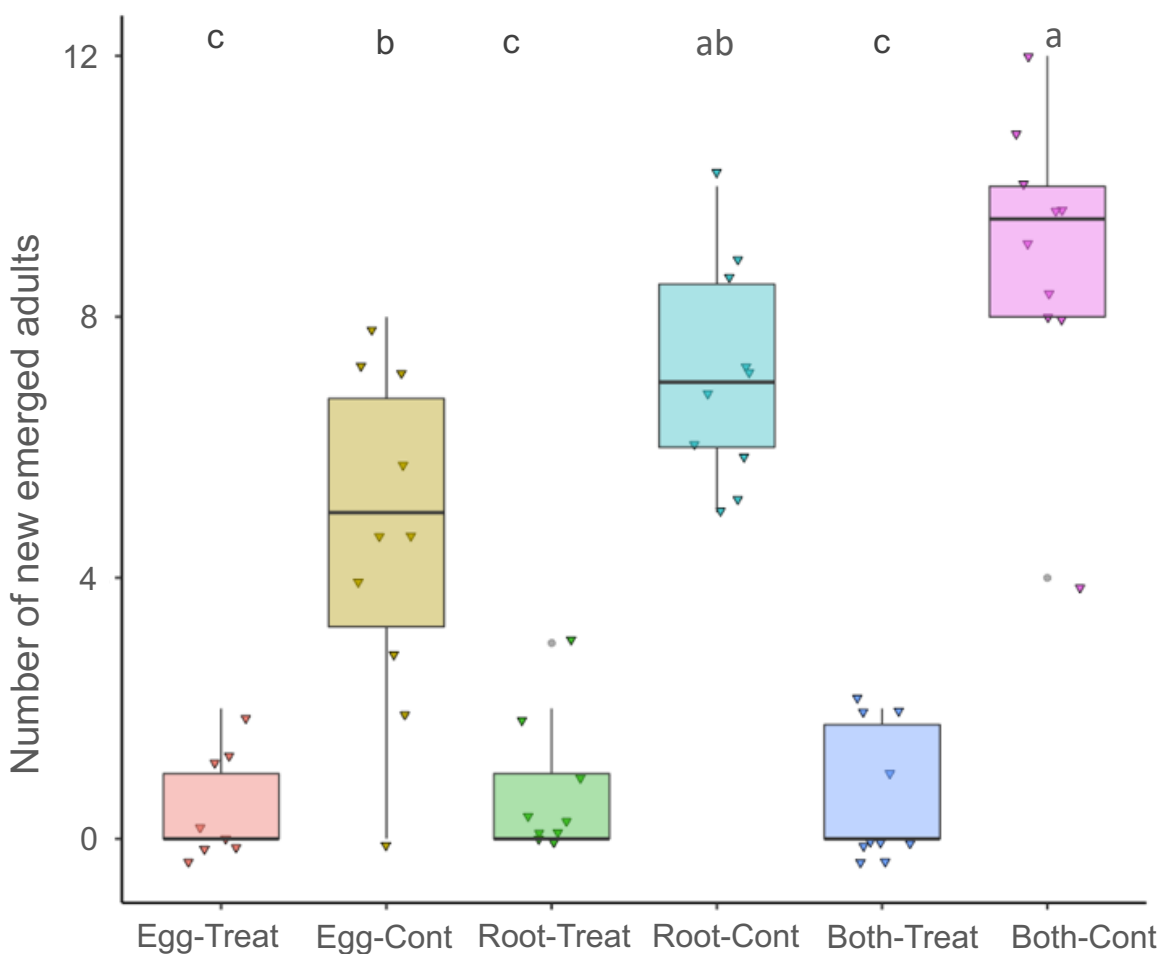
**Figure 20** : Total number of dead grape phylloxera larvae after 28 dpt with *M. robertsii* conidial suspension represented as box plots. *M. robertsii* strain EF3.5(2) was applied as conidial suspension by three treatments to grape phylloxera eggs laid on *V. vinifera* root sections: ‘Egg-Treat’ (red box), ‘Root-Treat’ (green box) and ‘Both-Treat’ (dark blue box) and Control treatments: ‘Egg-Cont’ (yellow box), ‘Root-Cont’ (light blue box), ‘Both-Cont’ (pink box). Significant differences are indicated by small letters above the boxes (Kruskal–Wallis test,  $p < 0.05$ ).

Kaplan-Meier survival curves (Fig. 21) showed a significant difference in the adults' emergence of grape phylloxera feeding on control root sections vs. *M. robertsii* treated root sections (Log-Rank test:  $\text{chisq} = 281$ ,  $\text{df} = 5$ ,  $p < 2e^{-16}$ ). Cumulative grape phylloxera adults' emergence was significantly lower for individuals treated via 'Egg-Treat' (at 28 dpt, probability of  $0.025 \pm 0.010$ ), 'Root-Treat' (at 28 dpt  $0.047 \pm 0.013$ ), and 'Both-Treat' (at 28 dpt  $0.045 \pm 0.014$ ) with no difference amongst treatments (Fig. 21). In comparison, adults' emergence was higher for individuals treated via 'Both-Cont' (at 28 dpt  $0.416 \pm 0.030$ ), or via 'Root-Cont' (at 28 dpt  $0.417 \pm 0.031$ ) and were significantly higher than all other treatments including 'Egg-Cont' (at 28 dpt  $0.300 \pm 0.030$ ). Significant differences between treatments were the most discriminant at 28 dpt at the end of the trial (Fig. 21).



**Figure 21 :** Cumulative adults emergence probability of grape phylloxera over the course of 28 days post-treatment (dpt) with conidial suspension represented as line plots. Lines represent Kaplan-Meier analysis of cumulative emergence of grape phylloxera adults. *M. robertsii* strain EF3.5(2) conidia were applied by three treatments to grape phylloxera eggs laid on *V. vinifera* root sections: 'Egg-Treat' (red line), 'Root-Treat' (green line) and 'Both-Treat' (blue line) and Control treatments: 'Egg-Cont' (yellow line), 'Root-Cont' (light blue line), 'Both-Cont' (pink line). Confidence Interval (IC) 95% were omitted for more readability. Significant differences are indicated by small letters above the end of the survival curves (Log-Rank test and pairwise comparison with Bonferroni correction, considering  $p < 0.05$ ).

Also, the number of emerged adults counted in total during the trial (Fig. 22) was significantly higher in the treatment ‘Both-Cont’ (med of 9.5 [4;12]) compared with the ‘Egg-Cont’ (med of 5 [0;8]) ( $X^2=43.643$ ,  $df=5$ ,  $p=2.737e^{-08}$ ). However, there was no significant difference between the ‘Root-Cont’ (med of 7 [5;10]) and the ‘Both-Cont’ and ‘Egg-Cont’. The number of emerged adults counted in total was significantly higher in the treatments ‘Both-Cont’, ‘Egg-Cont’, ‘Root-Cont’ compared with the ‘Egg-Treat’ (med of 0 [0;2]), ‘Root-Treat’ (med of 0 [0;3]), and ‘Both-Treat’ (med of 0 [0;2]) treatments (Fig. 22).



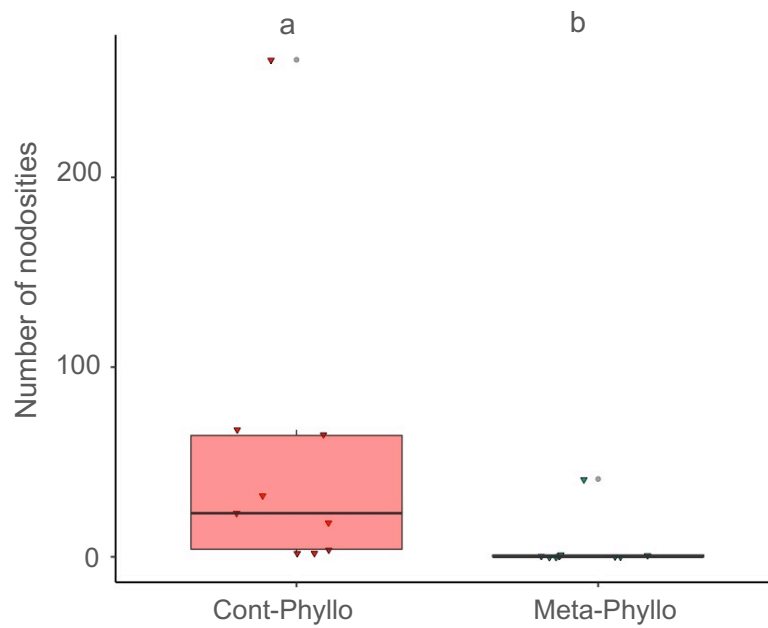
**Figure 22** : Total number of emerged grape phylloxera adults after 28 dpt represented as box plots. *M. robertsii* strain EF3.5(2) was applied as conidial suspension by three treatments to grape phylloxera eggs laid on *V. vinifera* root sections: ‘Egg-Treat’ (red box), ‘Root-Treat’ (green box) and ‘Both-Treat’ (dark blue box) and Control treatments: ‘Egg-Cont’ (yellow box), ‘Root-Cont’ (light blue box), ‘Both-Cont’ (pink box). Significant differences are indicated by small letters above the boxes (Kruskal–Wallis test,  $p < 0.05$ ).

### 8.3.3. Grape Phylloxera individuals transporting inoculum of *M. robertsii*

In bioassay 1, 48% of dead phylloxera individuals (*i.e.*, eggs, larvae, adults) cultured on PDA medium had carried *M. robertsii* propagules or mycelium after receiving the ‘Egg-Treat,’ 49% after receiving the ‘Root-Treat’ and 47% for ‘Both-Treat.’ None of the Control treatments of both bioassays (*i.e.*, ‘Egg-Treat,’ ‘Root-Treat,’ ‘Both-Treat’) had dead grape phylloxera individuals contaminated with *M. robertsii* after culturing on PDA medium.

### 8.3.4. Impact of *M. robertsii*, associated with *V. vinifera* roots, on the symptom severity induced by grape phylloxera

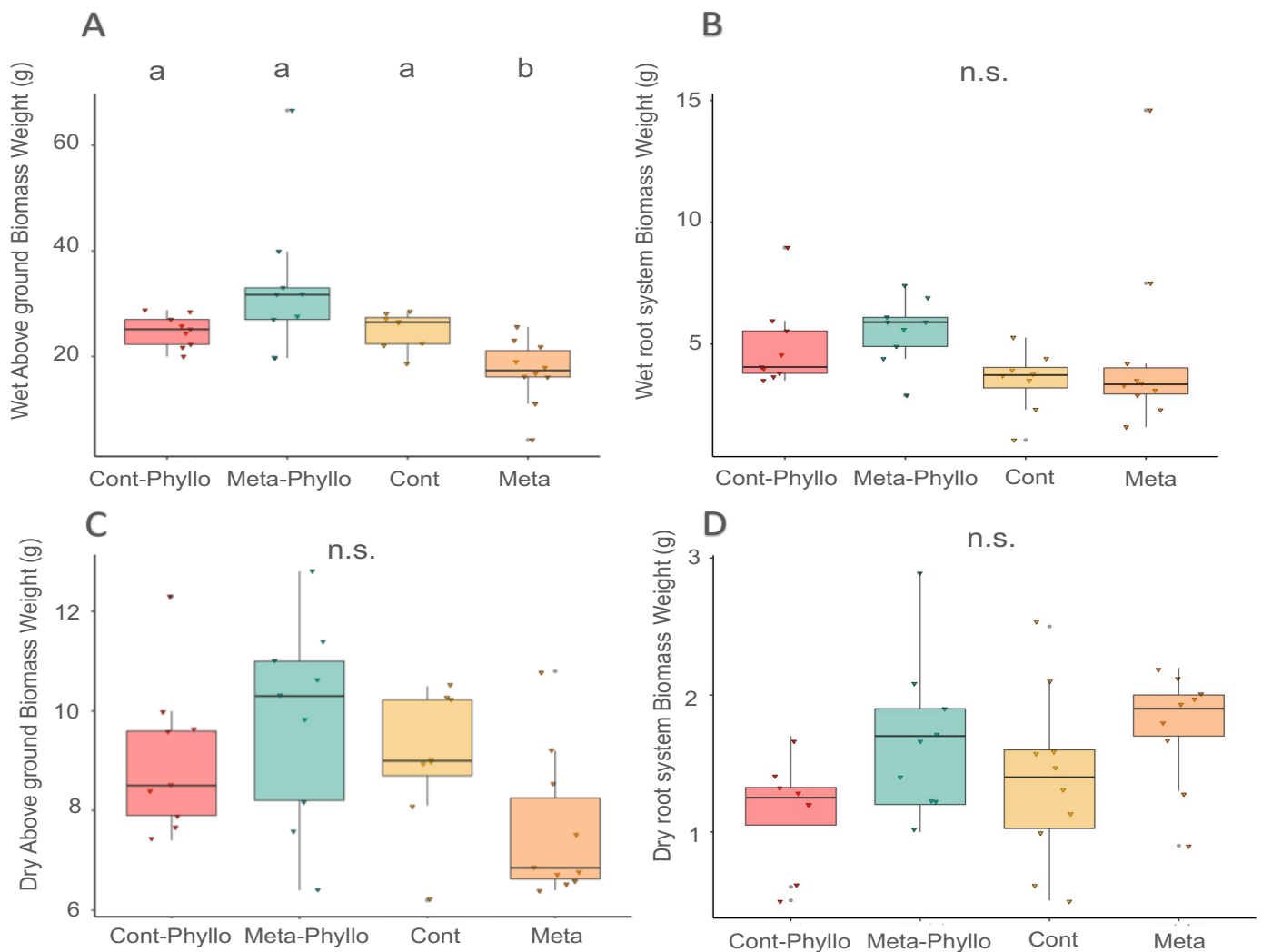
In bioassay 2, the number of nodosities counted on grapevine root (med. of 23 range [2; 262]) (Fig. 23) from plants inoculated only with grape phylloxera (‘Cont-Phyll’ treatment) was significantly higher than the ones that were previously inoculated with *M. robertsii* and with grape phylloxera, the ‘Meta-Phyll’ treatment (med. of 0 range [0; 41]) ( $X^2= 9.6491$ ,  $df= 1$ ,  $p = 0.001894$ ). From grapevine receiving the ‘Meta-Phyll’ treatment, only two plants had nodosities (41 and 1 counted, respectively) induced by radicolle grape phylloxera. No nodosities were observed in the grapevines that had not been inoculated with grape phylloxera and respectively had received control solution (‘Cont’) and *M. robertsii* (‘Meta’) treatments confirming the absence of cross-contamination between grapevines during the trial (data not presented in Fig. 23).



**Figure 23:** Number of nodosities induced by radicicole grape phylloxera on non-grafted grapevine *V. vinifera* represented as box plots. Treatment 'Cont-Phyllo' (red box) consisted of non-grafted grapevines planted in pot that did not receive fungal inoculation and later on radicicole grape phylloxera eggs inoculation. Treatment 'Meta-phyllo' (blue box) consisted of plants received fungal inoculation of *M. robertsii* strain EF3.5(2) and later on radicicole grape phylloxera eggs inoculation. Significant differences are indicated by small letters above the boxes (Kruskal–Wallis test,  $p < 0.05$ ).

### 8.3.5. Effect of co-inoculation of *M. robertsii* and grape phylloxera on grapevine growth

In bioassay 2, the measures of wet above-ground biomass weight (Fig. 24 A) revealed that grapevines that were co-inoculated with *M. robertsii* and grape phylloxeras (‘Meta-Phyllo’ treatment) with a median weight of 31.7 [19.7; 66.6] were significantly heavier than the ones that were inoculated only with *M. robertsii* ‘Meta’ treatments (med of 17.4 [4.2; 25.6]) ( $X^2 = 16.106$ ,  $df= 3$ ,  $p= 0.001079$ ). Measures of wet root system biomass weight, dry above-ground biomass weight, and dry root system biomass weight revealed no significant difference between all four treatments: ‘Meta-Phyllo,’ ‘Cont-Phyllo,’ ‘Cont,’ ‘Meta’ ( $X^2 = 8.7376$ ,  $df= 3$ ,  $p= 0.03299$ ;  $X^2 = 7.2559$ ,  $df= 3$ ,  $p= 0.05413$ ;  $X^2 = 7.0707$ ,  $df= 3$ ,  $p= 0.06968$ ) (Fig. 24 B, C, D).



**Figure 24 :** A) Wet above-ground biomass weight, B) Wet root system biomass weight, C) Dry above-ground biomass weight, D) Dry root system biomass weight measured of non-grafted grapevine *V. vinifera* represented as box plots. Treatment 'Cont-Phyllo' (red boxes) consisted of non-grafted grapevines planted in pot that did not receive fungal inoculation and later on radicicole grape phylloxera eggs inoculation. Treatment 'Meta-phyllo' (blue boxes) plants received fungal inoculation of *M. robertsii* strain EF3.5(2) and later on radicicole grape phylloxera eggs inoculation. Treatment 'Cont' (yellow boxes) plants received no fungal inoculation and later on no radicicole grape phylloxera inoculation. Treatment 'Meta' (orange boxes) plants received inoculation of *M. robertsii* and later on no radicicole grape phylloxera inoculation. Significant differences are indicated by small letters above the boxes (Kruskal–Wallis test,  $p < 0.05$ ).



#### 8.3.6. Rhizosphere and endophytic colonization of grapevines by *M. robertsii*

In bioassay 2, the level of rhizosphere colonization by *M. robertsii*, 107 days after being inoculated, reached 100% of tested plants for the ones that had received the ‘Meta’ treatment and 92% for the ones that had received the ‘Meta-Phyllo’. In contrast, no association was detected in the grapevine rhizosphere of plants receiving ‘Phyllo’ or ‘Control’ treatments where no conidial suspension of *M. robertsii* had been applied.

## 8.4. Discussion

In our study *M. robertsii* French strain EF3.5(2) infected radicol grape phylloxera eggs of single clonal lineage ‘Pcf7’ and showed an efficient reduction of the pest development and survivorship in non-grafted grapevine *V. vinifera* root section bioassay. A low survival rate was generally observed at the beginning of the trial (*i.e.*, 5 days post-treatment), both for the phylloxera eggs inoculated with the *M. robertsii* conidial suspension and for those treated only with the control solution. The viability of grape phylloxera eggs could have been affected negatively by egg manipulations with tools and probably by contact with an aqueous solution. There is limited scientific literature on the effect of humidity on phylloxera, but some general trends in phylloxera survival have been observed (Korosi et al., 2012).

Amongst treatments, ‘Both-Treat’ was the most efficient in killing grape phylloxera larvae. This treatment involved spraying the root section and eggs with a conidial suspension of *M. robertsii*. It is possible that root sections externally covered with *M. robertsii* propagules and presumed secondary metabolites produced by the fungus increased grape phylloxera larvae mortality through antibiosis mechanisms. For instance, more than 39 secondary metabolites Destruxins (DTXs) A, B, and E were extracted from *M. anisopliae*, and their insecticidal properties were demonstrated (Pedras et al., 2002; Wang et al., 2019). *M. anisopliae* was also demonstrated to express these secondary metabolites during the resource acquisition inside the hemolymph of larvae of *Manduca sexta* (Vega et al., 2012). Therefore, applying ‘Both-Treat,’ phylloxera larvae probing on the sprayed root section had significantly more chances of receiving a lethal dose of the conidial suspension. This effect was additional to the one resulting from the egg-spraying. In addition, the grape phylloxera crawlers were in contact with the inoculated-root section for an extended period in opposition to the ‘Egg-Treat’ treatment. Also, another study investigating the potential of *M. anisopliae* to increase aphid adults mortality on detached leaves suggests that the time of contact between *M. anisopliae* and aphids must be long to observe an effect of the fungal spraying (Jandricic et al., 2014). Higher larvae survival was recorded for eggs

that had received the ‘Egg-Treat’ and the ‘Root-Treat’ compared with the ‘Both-Treat.’ This lower efficiency of *M. robertsii* could result from the grape phylloxera larval molting process, as several successive larval life stages take turns through molting (Forneck et al., 2001b). Jandricic et al., 2014 mentioned that some aphid species have high molting frequency (ca. 1-2 days), making them increasingly difficult to control with single EPF spraying applications, primarily if the treatment occurs shortly after molting.

The adults’ developmental rates of grape phylloxera were likewise reduced in the ‘Both-Treat’ and ‘Root-Treat’ compared with respective ‘Both-Cont’ and ‘Root-Cont.’ It has been shown that grape phylloxera adults’ development is commonly reduced due to nutritional deficiency or inhibition in root section bioassays (Granett et al., 2001; Makee, 2004). The difficulty of larvae to establish new feeding sites on non-grafted grapevine root sections covered with propagules of *M. robertsii* could be a consequence of the antifeedant properties of *M. robertsii*. For instance, direct feeding of mycelium of *M. anisopliae* grown in liquid medium by larvae of corn earworm *Helicoverpa zea* induced high larval mortality and delayed their development (Leckie et al., 2008). Also, presumed secondary metabolites secretion by *M. anisopliae* were questioned to intervene in the development time of adults, as they were demonstrated to have antifeedant and repelling properties (Golo et al., 2014) but also to cause growth-delay effects and suppression of immunity to insects (Wang et al., 2019). However, some insect species seem to be able to detect these fungal secondary metabolites and avoid them (Gurulingappa et al., 2011).

Several studies have already characterized the control potential of *Metarhizium* spp. by direct spraying on economically-important aphid pest species (Vu et al., 2007; Jandricic et al., 2014). For instance, Vu et al., 2007 showed that *M. anisopliae* propagules directly sprayed on detached leaves of Chinese cabbage *Brassica rapa* subsp. *chinensis* L. controlled adults of peach aphid *Myzus persicae* that had been deposited on the leaf. The survival rate of adults was 30% after 8 dpt in the treated insect population. Our study showed an efficient regulation of grape

phylloxera by reducing larvae survival and adults' development which constitutes the first results of the direct effect of *M. robertsii* to control this telluric pest.

Amongst treatments, 50% of dead grape phylloxera eggs and larvae were assessed to transport *M. robertsii* propagules or mycelium after culturing on a growth medium. Since the grape phylloxera larval stages are tiny and fragile, we could not visualize if these individuals were infected by *M. robertsii*. But using scanning electron microscopy, Kirchmair et al., 2004a could observe conidiophores with conidial chains of *M. anisopliae* var. *anisopliae* (now *M. robertsii*) attached to the abdomen of grape phylloxera. These phylloxera were collected from grapevine roots planted in pots treated with barley kernel covered with fungal propagules. However, several arguments suggest that phylloxera parasitism by *M. robertsii* is difficult to observe and characterize: (i) the high turn-over of molts of grape phylloxera larvae may remove adhered propagules (Korosi, Pers. communications), the small size of the youngest larvae may reduce their chance of contamination (Jandricic et al., 2014).

As both 'Root-Treat' and 'Both-Treat' significantly controlled radicle grape phylloxera by both reducing larvae survival and adults' development in root section bioassay, we evaluated *M. robertsii* capacity to reduce grape phylloxera' symptom severity on susceptible *V. vinifera* by inoculating roots with a *M. robertsii* conidial suspension. In our potted grapevine bioassay, the mean number of nodosities (4.8) induced by radicle grape phylloxera single clonal lineage 'Pcf7' in non-grafted *V. vinifera* with a rhizospheric association of *M. robertsii* was significantly lower than without *M. robertsii* association (52.7). Only two of the ten *M. robertsii*-inoculated grapevines had nodosities induced by radicle grape phylloxera, and 41 and 1 nodosities were counted, respectively. According to the classification of Kellow et al., 2002, non-grafted *V. vinifera* cv. Cabernet Sauvignon associated with *M. robertsii* can be asserted as resistant to grape phylloxera, whereas the absence of *M. robertsii* renders *V. vinifera* susceptible. These results align with the previous study by Kirchmair et al., 2004a. Authors observed two out of ten grapevines having few radicle grape phylloxera infestations and almost no freshly-formed nodosities damaging young vines. These grapevines were potted

for 32 days in potting substrate containing barley kernel surfaced with propagules of *M. anisopliae* var. *anisopliae* strain Ma 500. The eight other grapevines were poorly infested and had only old nodosities. Conversely, 100% of the non-inoculated plants with *M. anisopliae* were significantly damaged by radicle grape phylloxera. According to the results of bioassay 1, the rhizosphere association of *M. robertsii* may deter grape phylloxera from feeding on the roots, which may prevent them from establishing and inducing symptoms in susceptible *Vitis vinifera*.

In our bioassay, we did not record a significant impact of the co-inoculation of *M. robertsii* and grape phylloxera on the dry above-ground biomass weight, and the root system dry and wet biomass weight of grapevines compared with *M. robertsii*-inoculated grapevines, grape phylloxera-inoculated grapevines, and non-inoculated grapevines. The wet above-ground biomass was lower when plants were only inoculated with *M. robertsii* compared with the other treatments. Kirchmair et al., 2004 also reported no significant differences between all these treatments during the growth evaluation phase of 32 days on non-grafted grapevines. The relatively short observation time of these grapevines (76 days), following *M. robertsii* and then phylloxera inoculations, may explain these non-significant effects on plant growth.

The establishment of *M. robertsii* strain EF3.5(2) was demonstrated in the rhizosphere of 100% of tested non-grafted grapevine pre-inoculated with *M. robertsii* via dipping method after 107 dpi. These colonization rates are significantly higher than previously recorded for the same strain associated with non-grafted *V. vinifera*, ranging from 85% to 40% from 14 to 96-98 dpi (Ponchon et al., 2022). This may be a consequence of the efficiency of root-dipping to induce rhizosphere association compared with root-drenching. Propagules directly drenched in a soil substrate are sensitive to fungistatic effects caused by antagonistic microorganisms, and they also disperse significantly in the substrate after application (Jaronski, 2010; Bamisile et al., 2018). Additional studies verified dipping-method efficiency to establish root association of *M. anisopliae* and *B. bassiana* to banana *Musa* spp. (Akello et al., 2007); to tomatoes *Solanum lycopersicum* L. (Qayyum et al., 2015; Mwamburi, 2021) and field-grown

strawberries *Fragaria* × *ananassa* Duchesne (Ansari and Butt, 2013; Canassa et al., 2020). This method is performant, presumably because of the high surface of roots in contact with fungal propagules, which enhances the chance of colonization (Qayyum et al., 2015). The dipping method could be used to inoculate young cuttings of *V. vinifera* during the standard process of grapevine nursery propagation, as performed by Andreolli et al., 2021. For instance, the standard procedure for grapevine grafting implies fungicides or biological control treatment applied via dipping to protect the grapevines before the phase of cold storage, after disbudding, after the grafting step, and after callusing step to protect freshly-grafted grapevines against trunk disease pathogens (Gramaje et al., 2018). Future innovative inoculation methods of *M. robertsii* could rely on dipping *V. vinifera* grapevine plantlets in conidial suspension during the hydration phase and before plantation in vineyards. In this way, pre-inoculating these susceptible grapevines with *M. robertsii* could allow the fungus establishment in their rhizosphere to potentially protect it against soil-borne pests.

Our study sheds light on the importance of the rhizospheric association of *M. robertsii* to control non-grafted grapevines against soil-borne pests. It also suggests that *M. robertsii* can provide an efficient control of radicolle phylloxera populations. Although it is essential to focus on inoculating *M. robertsii* in the habitat in which it interacts with radicolle grape phylloxera, mainly the rhizosphere where *M. robertsii* is active and thrives (Bruck, 2005). Our study also indicates the potential for *M. robertsii* to be established up to 107 days in grapevine rhizosphere after artificial inoculation, which is longer than what we found in our previous study where the same strain established up to 98 dpi (Ponchon et al., 2022). Moreover, we previously demonstrated that native strains from wine-growing regions with very contrasted climatic and environmental conditions could persistently associated with the rhizosphere and root-endosphere of grapevine for up to 70 days under controlled conditions (Ponchon et al., *in prep.*). The significant persistent potential of association of the strain EF3.5(2) may be related to the area of origin of the fungus, as it was collected from vineyard soil in France and from the soil layer near the grapevine roots. For instance, Klingen et al., 2015 demonstrated

that two strains, *M. brunneum* and *B. pseudobassiana*, native to Norwegian soils, were more significantly associated with the rhizosphere of strawberries planted in a Norwegian field compared with a commercial strain of *M. brunneum* F52; after their artificial inoculation. They also demonstrated that more propagules of the two native strains were quantified in the rhizosphere up to 471 days of persistence. Similarly, two Brazilian strains of *M. robertsii* were persistently associated with the strawberry rhizosphere for up to 12 months from plants planted in Brazilian fields and artificially drenched (Castro et al., 2016). For this reason, it is essential to rely on fungal strains that originate from the same area where they are desired to be persistently established, as they may better withstand the abiotic and biotic stressors of the environment. For instance, the isolates of *M. brunneum* and *B. pseudobassiana* native Norwegian strains were significantly active in parasitizing larvae of *O. sulcatus* at a temperature of 12°C, which is the soil temperature for part of the strawberry growing season in Norway (Klingen et al., 2015). Therefore, the potential of *M. robertsii* establishment with grapevine and their biocidal effect on radicle grape phylloxera should be assessed in contrasted environmental conditions. Interestingly, some *M. robertsii* strains native to wine-growing regions of Northern and Central Argentina were demonstrated to grow at high temperatures of 35°C, making them able to endure warm summer conditions, such as in the vineyards of their area of origin (López Plantey et al., 2019). This feature makes them particularly relevant strains for controlling telluric grapevine pests.

**Conclusions :**

The rhizospheric association of *M. robertsii* in non-grafted grapevine plants represents an innovative and eco-friendly plant protection strategy. Further studies should evaluate: i) the potential association of the *M. robertsii* strains native to different wine-growing environments with diverse *V. vinifera* cultivars emblematic of several wine-growing regions, ii) their long-term persistence beyond 107 days under contrasted field conditions, and iii) their potential to protect grapevine roots against an extensive range of radicicole grape phylloxera biotypes.



# General discussion

## 9- General discussion

Entomopathogenic fungi (EFPs) are raising increasing interest as major natural enemies of arthropod populations (Vega, 2018; Sharma et al., 2023). Recently, they were broadly investigated for biological control of various economically serious pests (Lacey et al., 2015). Their usage is often reported to pose low risks against beneficial non-target organisms such as pollinators, microfauna involved in organic matter recycling, and natural enemies of pests (*i.e.*, predators and parasitoids), making them attractive biopesticides to control economically significant pests while preserving the native fauna (Lacey et al., 2015). Other roles of these fungi were recently emphasized, such as plant endophytes, rhizosphere colonizers, plant growth promoters, and plant disease antagonists (Jaber and Ownley, 2018). Therefore, their endophytic lifestyle has been praised for developing environmentally-friendly crop protection tools. However, this lifestyle is poorly understood, and the mechanisms involved in establishing this symbiosis are not well characterized. Minimal research was implemented on the tri-trophic interaction of *M. robertsii*, the grapevine plants, and their target pests. Therefore, the objectives of the present thesis were to investigate the potential association of the fungus *M. robertsii* with the rhizosphere and root-endosphere of non-grafted grapevine *V. vinifera* and the effect of this association on the regulation of radicle grape phylloxera *D. vitifoliae* populations. Additionally, this thesis is intended to study the plant response to *M. robertsii* endophytic colonization in plant growth and grape phylloxera-responsive defense gene expression. These investigations were carried out to develop innovative grapevine biological control protection strategies as an alternative to grafting.

We previously discussed our main results in the respective manuscripts. We will put into perspective the main results of our study concerning the objectives of this research. Furthermore, we will discuss the limitations of our research and highlight the applications and implications of the analysis in the present research context. The Discussion will focus on the following:

- (1) *M. robertsii* potential of rhizospheric and endophytic association with non-grafted grapevine
- (2) Challenges in characterizing mechanisms involved in the antagonistic potential of *M. robertsii* on root-feeding grape phylloxera
- (3) Potential Applications and practical implementations of the experimental results

### 9.1. *M. robertsii* potential of rhizospheric and endophytic association with non-grafted grapevine

One of the principal objectives of the present thesis was to establish several *M. robertsii* strains native to vineyard environments in the rhizosphere and the root-endosphere of non-grafted grapevine via an artificial application.

#### 9.1.1. Choice of *M. robertsii* strains for the enhanced potential of endophytic capacities

Previous studies have shown the potential of *M. anisopliae* var. *anisopliae* to control radicle grape phylloxera under controlled conditions and field studies (Kirchmair et al., 2004a, 2004b, 2007; Huber and Kirchmair, 2007). More recently, several EPF strains of *Beauveria* and *Metarhizium* genera native to Australian phylloxera-infested vineyard soils were evaluated for their control potential against radicle grape phylloxera, to protect Australian non-grafted commercial vineyards (PhD thesis of Korosi, 2019). These studies highlighted EPF's natural occurrence in the vineyards and underlined the difficulties of performing an exhaustive EPF sampling covering the entire wine-growing area. They also pointed out the challenge of finding EPF from infected grape phylloxera native to vineyard soil as their small body mineralizes fast or becomes infested with other competitive soil microorganisms (Huber and Kirchmair, 2007).

All the *M. robertsii* used in our study were native to vineyard environments (collected from European, Australian, and Argentinian vineyard soils). We hypothesized that they might be ecologically competent to persist in the vineyard

and have the potential to be associated with grapevine. Fisher et al., 2011 found the rhizosphere association of *M. robertsii* and other *Metarhizium* spp. with Grape *Vitis* spp. highlighting the potential of this species to be naturally associated with grapevine. Previous studies also found *M. robertsii* as a natural endophyte of several plant species and the simultaneous presence of *M. robertsii* fungal strains in the soil (Wyrebek et al., 2011; Behie et al., 2015; Steinwender et al., 2015; Vega, 2018; Tyurin et al., 2021). It justified the investigation and the sampling of *Metarhizium*-strains from the vineyard soil near the grapevine roots. Therefore, we found that all the strains native to vineyard environments had the potential to be associated with non-grafted grapevines at the rhizosphere and root-endosphere level (Chap. 1, Chap. 2). Yet, the comparison of one strain native to the vineyard environment and one non-native showed a higher establishment of the native strain to vineyard environment in the root-endosphere of grapevine. We believe it could be a potential adaptation of vineyard native strains to root exudation of grapevines favoring their germination and growth potential in the vine rhizosphere (Pava-Ripoll et al., 2011; Moonjely and Bidochka, 2019) (Chap. 1).

Furthermore, Bidochka et al., 2001 suggested that the habitat preferences of the *Metarhizium* strains (forest or agricultural environments) may be related to their temperature requirements. And it has also been found that the temperature is a significant abiotic parameter for the differentiation of cryptic species from *Metarhizium* species complex (Bidochka et al., 2005). The phenotypic response (*i.e.*, germination and growth potential) of *Metarhizium* at differential temperatures may be a biological marker of strain adaptation to specific environments (López Plantey et al., 2019). Future research should verify if the fungal strains used in our study can germinate and grow at high temperatures such as 35°C, the summer temperature of some Argentinian vineyards.

Finally, it should be considered that the vineyard environment is an anthropized environment where large quantities of fungicides are applied. As multiple stressors may impair the potential of EPF establishment in this environment, it is important to use native fungal strains that function under these specific conditions (López Plantey et al., 2019; Uzman et al., 2019). Uzman et al.,

2019 reported that the high intensity of fungicide treatments and high copper content in the soil did not impair the presence and density of *Metarhizium* spp. propagules in vineyard soil of the wine-growing region of Rhinehessen (Germany). Another study also found a high tolerance of *M. anisopliae* for copper as they observed growth of the fungus on malt agar medium plates containing a high concentration of copper (Cu) (i.e., 200 mg Cu.l<sup>-1</sup>) (Bååth 1991). Also, a previous Ph.D. thesis evaluated *Metarhizium* native to Argentinian vineyard response to four principal fungicides used in Argentinian region of Mendoza: Quinoxifen 25% SC (active ingredient: quinoline), Mancozeb 80% WP (active ingredient: zinc and manganese), Copper oxychloride 84% WP and Sulfur 80% WG. The author found high toxicity of Mancozeb 80% and moderate of Quinoxifen 25% SC but tolerance of Sulphur and copper by *Metarhizium* (López-Plantey, 2019). Therefore, testing strains from contrasted vineyard environments may help choose the most adapted ones for the vineyard abiotic conditions to propose them as biocontrol candidates against grapevine pests.

#### 9.1.2. Importance of experimental framework for the enhanced potential of *M. robertsii* endophytic capacities

Since the establishment of *M. robertsii* as a grapevine endophyte has never been tested before, we found that the methodology used and the controlled conditions significantly impacted the success of *M. robertsii*'s establishment in grapevine roots. We chose to rely only on one principal *Vitis vinifera* cultivar 'Cabernet Sauvignon' as it is classified as an 'international cultivar' as the second most planted cultivar worldwide, representing 4% of the vine-growing area (341 000 ha estimated in 2015). This cultivar, mostly grown in France, China, Chile, United-States, Australia, Spain, Argentina, Italy, and South Africa (Organisation Internationale de la Vigne et du Vin, 2017), encompasses the native regions of the *M. robertsii* strains of our study. For most of our experiments, grapevines were obtained from hardwood cuttings pruned in vineyard plots (Chap. 2, Chap. 3). It was essential to ensure that *M. robertsii* was not pre-established in grapevines

before its artificial inoculation, as one previous study identified a close relative species *M. pinghaense* as a natural endophyte of *V. vinifera* cv. ‘Cabarnate Gernischet’ in China after DNA sequencing analysis in vine tissues (Jayawardena et al., 2018). To address this problem, we used *in vitro*-produced grapevines obtained from successive sub-cultures of shoots of young plantlets achieved in sterile conditions (Chap. 1). This type of grapevine production reduces the eventual and unexpected contamination with exogenous *M. robertsii* native to the vineyard environment.

We chose first to investigate the artificial inoculation of *M. robertsii* to grapevine in greenhouse conditions to precisely control the abiotic environmental conditions. For instance, vines were grown at optimal temperatures (*i.e.*, 25-30°C) for *M. robertsii* propagules germination (Ekesi et al., 1999; Jaronski, 2010). They were planted shortly after rooting in two substrates: clay/white peat and sterilized vineyard ground. Sterilization was proven efficient in avoiding any fungistatic effect inhibiting *M. robertsii* propagule germination caused by other soil microorganisms that could have a natural competitive potential (Kessler et al., 2003; Jaronski, 2010; Jaber and Enkerli, 2017).

Important care was given to selecting a suitable inoculation method to establish *M. robertsii* as a rhizosphere-associated and an endophyte fungus in non-grafted grapevine (Bamisile et al., 2018). The specific characteristics of grape-plant production procedures make it impossible to use seed treatment methods. Therefore, two inoculation methods were implemented (Chap. 1,2,3): root drenching method: watering plants at their collar using a significant volume of fungal suspension. This method is user-friendly for the artificial inoculation of potted plants. Yet, it was judged partially efficient in inducing high rates of endophytic colonization, supposedly because of the important propagules dispersal in the soil (Jaronski, 2010; Bamisile et al., 2018). The dipping method, consisting of dipping the root system of young seedlings into conidial suspensions for 24 hours before planting in a pot, was also implemented to limit fungal propagule dispersion (Tefera and Vidal, 2009). From the perspective of implementing both inoculation methods in the vineyard, we suggest that the

drenching of well-established mature grapevines with fungal suspension could be possible using the existing irrigation system. The dipping method could be used in the nurseries to associate the fungus preventively to the grapevine seedling roots before planting in the vineyard. For instance, [Andreolli et al., 2021](#) used a dipping-like method for inoculation of the bacterial endophyte *Pseudomonas protegens* MP12 to plantlets of grapevine. During the grafting procedure, the scion and rootstock were dipped in bacterial suspension for a day during the hydration step before grafting and then assemblage. Alternatively, grafted vines were immersed in bacterial suspension after their forcing period for seven days before plantation. It may be ideal for inoculating young grafted or non-grafted grapevines with *M. robertsii* as plants may be readily inoculated with *M. robertsii* before planting in the vineyard.

### 9.1.3. Evaluation and characterization of *M. robertsii* association with grapevine

Characterizing the association of EPF with plants is important to understand their ecology before using them in biological control programs. Indeed, characterizing their association with roots could help persistently establish the EPF with the grapevine. The long-term persistence and increased propagule concentration of *M. robertsii* in the grapevine rhizosphere could be a significant feature for its effective biocontrol potential against grapevine root-feeding pests.

#### 9.1.3.1. Finding the correct methodology to study the interaction between plants and endophytes

Among all the conditions we assayed (*i.e.*, inoculation method, type of substrate, type of grapevine production), we demonstrated that the association of *M. robertsii* in the rhizosphere and root-endosphere with non-grafted grapevines was established after artificial inoculation.

This Ph.D. thesis reflects complex challenges in characterizing the EPF colonization perimeter inside plants and the fungal colonization mechanisms of plant tissues. Several methods are commonly used to study *M. robertsii* endophytic colonization, but as there are costly and challenging to implement ([Landa et al.](#),

2013; Barelli et al., 2018; Cai et al., 2019), we present a procedure to follow for characterization of EPF endophytic association to the host plant (Figure 25) which is based on three methods: (i) the molecular Droplet Digital PCR (ddPCR), (ii) the culture-based method and (iii) the confocal imaging of roots segments (Figure 25). We primarily relied on the culture-based method to assess *M. robertsii* association with non-grafted grapevine. This procedure is relevant for the first evaluation of EPF association with a plant species as it is accessible to implement, does not require expensive material, and can detect the live fungal propagules from plant tissues that grow on the culturing medium. ddPCR is the most sensitive detection and quantification method of the *M. robertsii* DNA that we recommend being included in studies aiming to characterize the kinetics of endophytic fungal establishment and their systemic colonization.

Nonetheless, each method does not stand-alone of its own and should be complemented by at least another one (*Supplement 9*). For instance, DNA quantification of endophytes should be complemented by culture-based and/or confocal microscopy that provides additional information on the viability of the fungus. An elegant methodology was presented by Barelli et al., 2018 that aimed at correlating the number of colonies of *M. robertsii* counted on culture plates from root homogenate of inoculated French beans with the quantity of DNA copies of *M. robertsii* quantified via qPCR in the same sample. Future investigations should employ this methodology to evaluate if the DNA quantified came from viable or non-viable fungal cells of *M. robertsii*.

Future research should perform microscopic observations of *M. robertsii* on the grape roots after a long period of association. To our knowledge, no study achieved microscopic observations of plants with a long-term root association with *M. robertsii*. A more comprehensive visualization of the association between *M. robertsii* and vine in the long term would support the characterization of the fungal structures having antagonistic effects on soil-borne pests.



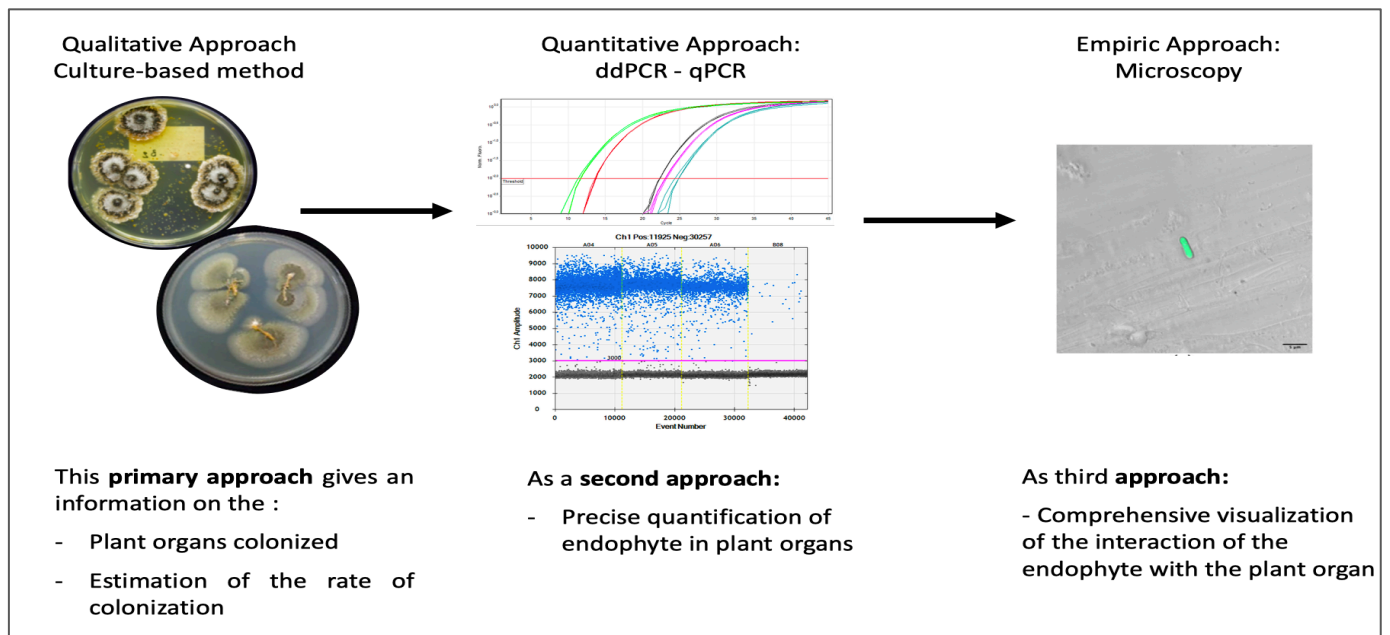


Figure 25 : Procedure to follow for the characterization of endophytic association composed with complementary approaches

### 9.1.3.2. *M. robertsii* is associated with the rhizosphere and root-endosphere of non-grafted grapevine

Our study demonstrated the association of *M. robertsii* with the rhizosphere and root-endosphere of non-grafted grapevine up to 107 days post-inoculation (dpi) (Chap. 1, 2, 3). In another study, the persistence of rhizosphere association of *M. robertsii* Brazilian strain ESALQ1426 with the strawberry roots after root-drenching was demonstrated for up to a year post-inoculation in a field in Brazil (Castro et al., 2016). It has also been shown that *M. robertsii* propagules can persist in the soil environment after exogenous application and multiply in the plant rhizosphere (Hu and St. Leger, 2002; Bruck, 2005; Jaronski, 2010). In addition, the rhizosphere association of *M. robertsii* was previously demonstrated to convey a persistent shield to plants against soil-borne pests (Bruck, 2005). Indeed, *M. robertsii* association with grapevine may maintain a population of EPF that would play a bodyguard role on the root surface, consequently increasing the chances of contact between this EPF and the plant root-pests (Elliot et al., 2000). The persistent

association of *M. robertsii* with grapevine is thus an important key for the biological control of vine soil-borne pests.

## 9.2. Challenges in characterizing mechanisms involved in the antagonistic potential of *M. robertsii* on radicicole grape phylloxera

The common pathway for insect infection by *M. robertsii* is via fungal propagule adhesion, cuticular penetration by propagule germination, acquisition of insect inside resources, and re-multiplication outside of the host insect inducing insect mycosis (Vega, 2018). Previous studies have reported dead infected larvae of black vine weevil *Otiorhynchus sulcatus* that had fed on roots of *Picea abies* pre-inoculated with *M. anisopliae* (Bruck, 2005). Additionally, Keyser et al., 2014 recorded dead infected larvae of *Tenebrio molitor* that had fed on root sections of wheat plants that were seed-dressed with *Metarhizium* spp. and grown on sterile filter paper or potted with soil. The number of dead infected larvae increased for root sections of wheat inoculated with higher fungal concentration; or when plants had been inoculated with *Metarhizium* spp. for a long time. Therefore, both studies suggest a pathogenic effect induced by the direct feeding of insect pests on colonized roots (Bruck, 2005). Our research (Chap. 3) found a low percentage of dead infected grape phylloxera larvae that fed on vine woody root sections of *V. vinifera* cv. Cabernet Sauvignon inoculated with *M. robertsii* fungal suspension, according to the results of larvae cultivation on growth medium. We could not demonstrate if grape phylloxera individuals were directly parasitized and/or infected by *M. robertsii* because of feeding on these inoculated roots. Nevertheless, we observed increased mortality of grape phylloxera feeding on woody root sections inoculated with fungal suspension at the end of the trial (Chap. 3), and we hypothesized that it mainly resulted from possible fungal secondary metabolites produced at the root endosphere and/or the rhizosphere level that was responsible for feeding deterrence and/or antibiosis (Vega et al., 2008; McKinnon et al., 2017; Vega, 2018; Jaber and Ownley, 2018).

In the review of Vega 2018, several indirect mechanisms of host plant response to EPF endophyte colonization were suggested to negatively affect

herbivores' feeding capacities, such as the alteration of chemical signals produced by plants (*i.e.*, kairomones), the potential expression of herbivore-induced plant volatiles and the plant defense systemic response induced by the endophyte. In the Chap. 2, we demonstrated the up-regulation in grape leaf tissues of four grape phylloxera responsive defense genes 24h after *M. robertsii* strain EF3.5(2) inoculation via root-drenching to the grapevine. Our investigations focused on the gene *VWRKY46*, one WRKY transcription factor involved in response to grape phylloxera attack, and several downstream target genes. Previously, '1103 Paulsen' resistant rootstock (*V. berlandieri* x *V. rupestris*) defense response was characterized by significant expression of transcripts of *VWRKY46* and downstream target genes compared with susceptible cultivar *V. vinifera* "Crimson Seedless" under the attack of radicle grape phylloxera. Additionally, the over-expression of the *VWRKY46* gene in transgenic roots of *V. vinifera* cv. "Crimson Seedless" significantly reduced grape phylloxera larvae attacks and delayed their development (Wang et al., 2019). The involvement of *VWRKY46* in the SA-mediated grapevine defense network was also demonstrated. In our study (Chap. 2), we quantify the significantly increased expression of *VWRKY46* in *Vitis vinifera* cv. "Cabernet Sauvignon" leaves 24h post-*M. robertsii* strain EF3.5(2) inoculation and three other genes involved in the resistance response against radicle grape phylloxera. Consequently, it will be relevant to continue the evaluation of the phylloxera-responsive genes in non-grafted grapevine leaves in three cases: i) of inoculation with *M. robertsii* only, ii) with grape phylloxera only, or iii) with *M. robertsii* and grape phylloxera. It will allow us to compare and distinguish the defense reaction induced in the grapevine by each of the invaders, grape phylloxera and *M. robertsii*.

Additionally, the application of exogenous salicylic acid (SA) inside roots of *Vitis vinifera* cv. "Crimson Seedless" induced reduced grape phylloxera larvae survival because of feeding deterrence. It was also previously demonstrated that rootlets with galls of grape phylloxera induced on the rootstock Telekis 5C (*V. berlandieri* x *V. riparia*) contained a significant concentration of SA that intervened in the rootstock defense response (Eitle et al., 2019). Also, two studies

demonstrated increased SA concentration inside the roots of seed-coated maize plants with *M. anisopliae* or *M. robertsii* plants co-inoculated with the root pest *Costelytra giveni* (Rivas-Franco et al., 2020) or the root disease *Cochliobolus heterostrophus* (Ahmad et al., 2022). The increased SA concentration was also quantified in maize plant tissues when plants were only inoculated only with *M. robertsii* or *M. anisopliae*. Since both radicolous phylloxera and *M. robertsii* may be perceived by the colonized plant as biotrophic invaders, the grapevine response could similarly be characterized by the stimulation of the SA biosynthesis pathway, which may result in the increase of SA in the grapevine roots. Therefore, it will be important to quantify the SA concentration in the root tissues of the grapevine after inoculation of (i) *M. robertsii* only, ii) grape phylloxera only, or iii) *M. robertsii* and grape phylloxera. It will be important to distinguish the defense reaction induced in the grapevine by each of the invaders, grape phylloxera and *M. robertsii*.

In Chap. 3, we found significantly fewer symptoms characteristic of radicolous grape phylloxera infestation (*i.e.*, nodosities) in non-grafted grapevine *V. vinifera* previously inoculated with *M. robertsii* via root-dipping, two months before infesting plants with grape phylloxera radicolous eggs. We suggest a reduced possibility of the larvae establishing feeding sites on the infected roots due to feeding repellence caused by *M. robertsii* mycelial presence and its possible production of secondary metabolites in the grapevine rhizosphere. Additionally, some larvae that would succeed in establishing feeding sites may get in contact with secondary metabolites produced by *M. robertsii* in the grapevine root tissues (as endophyte), causing antibiosis. The grapevine amplified defense response caused by the priming effect of *M. robertsii* may also reduce their survival. However, further tests are needed, with a more significant number of replicates (in laboratory conditions), to confirm the ability of *M. robertsii* to reduce the symptoms produced by phylloxera.

### 9.3. Potential Applications and practical implementations of our experimental results

*Innovative biocontrol agent delivery system:* the repeated-spraying application of EPF in the field is still judged inconsistent when shifting to field implementation. The previous paradigm stated that control of soil pests relied on applying a significant number of propagules (ranging from  $10^5$  to  $10^{15}$  propagules.  $\text{ha}^{-1}$ ) to prevent high asexual spore dispersion in the soil (Jaronski, 2010). But these large quantities of fungal inoculum are expensive to produce, and large volumes are wasted because the volume of applied propagules is usually higher than the pest-preferred area. Additionally, the propagules' persistence capacity without impaired germination is not well characterized as there is an important lack of understanding of their ecology and biology. Thus, soil-borne pests could be challenging to control as EPF applications in the soil are hardly optimizable. Our study opens new doors for using colonized plants at the rhizosphere level with *M. robertsii* as an optimal and innovative delivery system for biological control of vine root pests. This system may remain valid for an extended period as the association between grapevine roots and *M. robertsii* seems persistent. Further studies are needed to find the abiotic factors critically influencing the persistence association of *M. robertsii* with non-grafted grapevine in the laboratory and under field conditions. The persistence of association should also be characterized at longer time post-inoculation.

*M. robertsii treatment as a potential alternative to grafting:* *M. robertsii* associated with non-grafted grapevine roots represents an effective alternative to grafting or using synthetic pesticides to control radicle grape phylloxera populations. The investigations should be continued using different grape phylloxera single clonal biotypes obtained from several locations in Europe, Australia, and Argentina, as they may respond differently to *M. robertsii* infection in single-root bioassay. Similarly, various *V. vinifera* cultivars should be tested in root section bioassay and/or potted-grapevine bioassay in the same way as done in Chap. 3 to better cover the range of grape varieties used in viticulture worldwide.

*M. robertsii* usage in the context of viticulture: To continue investigations, the next step of this work is to pursue a trial in an experimental vineyard (i.e., in field conditions) to associate *M. robertsii* with already established field non-grafted grapevines. First, the potential of germination and growth of *M. robertsii* should be investigated at the high-temperature representative of the wine regions where it could be implemented (e.g., Australia, Argentina), because the temperature can critically influence the capacity of establishment of *M. robertsii* in the vineyard environment. Additionally, possible adverse outcomes may result from combining *M. robertsii* and other plant protection products or their residues used for vine protection. Thus, careful testing should be performed to evaluate their potential antagonistic effects. Also, the mass production of fungal suspension of *M. robertsii* is expensive and energy-consuming, and using existing irrigation systems of vineyard for asexual spore applications may not be possible. Therefore, new innovative methods of inoculation of fungal propagules to grapevines, especially in the early stages of the vine development (i.e., at nursery propagation), should be developed as root-drenching may be challenging to implement at the vineyard scale. Alternatively, *M. robertsii* could be applied to the vineyard soil through a cover-cropping. For instance, some common species employed for cover-cropping such as the cereal rye, Winter canola, or Austrian winter pea, were demonstrated to be endophytically colonized after seed-dressing inoculation and direct-planting in the field (Ahmad et al., 2020b). This cover-cropping could bring a new source of fungal propagules in the vine interrow near grapevine roots. These propagules may readily be in contact with grapevine roots, and the cover cropping would act as a donor plant. Also, it was demonstrated that the arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae* inoculated beneath the grapevine and accompanied by a cover-cropping increased the symbiosis between the grapevine roots and the AMF species (Nogales et al., 2021). Therefore, cover-cropping may favor *M. robertsii*'s persistence of association with grapevine rhizosphere and contribute to the reduction of radicle grape phylloxera damages. Also, one study demonstrated that the incorporation of seed-coated barley kernel in the interrow close to the vine roots in a field planted with grafted vines on *V. berlandieri* x *V.*

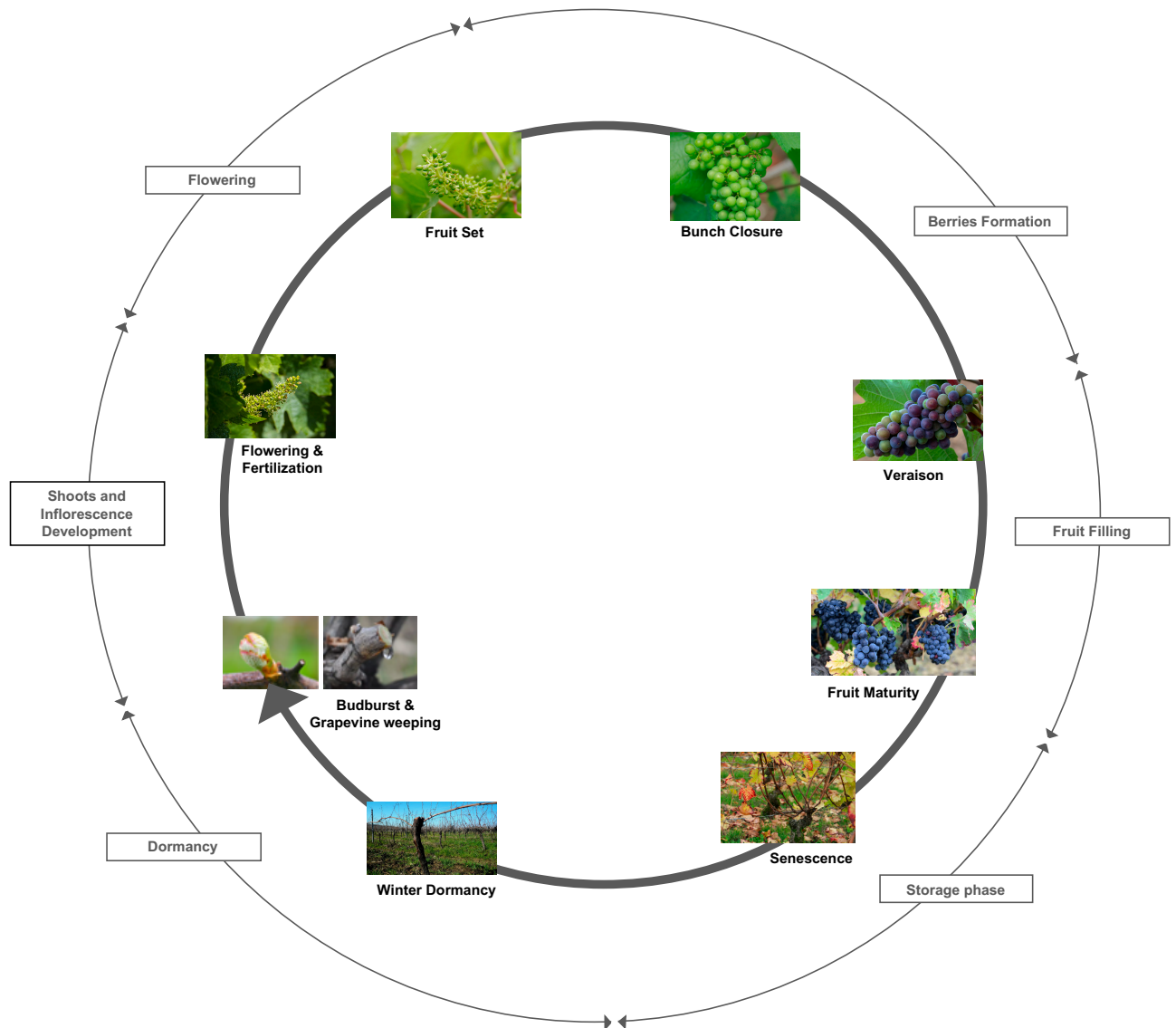
*riparia* resulted in a reduction of grape phylloxera populations in the interrow (Kirchmair et al., 2004a, 2007).

*M. robertsii* intrinsic effect on the grapevine: as stated by Mantzoukas et al., 2015, three major conditions should be satisfied to ascertain the potential of entomopathogenic fungi as endophytes to control grapevine soil-borne pests: (1) success of colonization of grapevine by *M. robertsii*, (2) absence of symptoms on physiology and slowdown in the growth of vine consequent to colonization, (3) effective control of the targeted pest. We were able to satisfy the first two conditions fully. However, as grapevine is a perennial crop, it is relevant to evaluate the long-term impact of *M. robertsii* inoculation on plant growth and physiology in the case of potted vine trials or mature grapevine planted in the field. It is especially important for a crop such as the grapevine as the quality and sensory attributes of must and wine must be maintained while applying external inputs on the plant. Thus, evaluating the quality of grape sensory attributes and valuable compound expression after artificial inoculation of *M. robertsii* to the plant will be a significant research prospect. As several studies found the co-occurrence of mycotoxins secondary metabolites *in planta* and endophytic colonization of *M. robertsii*, it is important to localize them inside the grapevine and to consider the health risks they may pose to humans and other non-target microorganisms when consumed (Resquín-Romero et al., 2016; Ríos-Moreno et al., 2016; Garrido-Jurado et al., 2017). For example, Golo et al., 2014 quantified the secondary metabolites: Destruxin A, B, and E in the cowpea plant from plant seed-dressed with the laboratory strain ARSEF-2575-GFP after 14 dpi. Also, the detrimental effects on health of mycotoxins might be concentration dependant, and the concentration recorded in planta may be low (Zimmerman 2007).





# Supplements



**Supplement 1 – Figure 1:** Annual development cycle of Grapevine with the main phenological stages shown as photographs and the main plant physiological process of the cycle indicated in the boxes, adapted from Guilpart 2014

Supplements – 2 – General Introduction – 2.3. Grapevine pests and diseases management

**Supplement 2 - Table 1:** Most common and injurious grapevine pest and diseases in Europe classified by the EIP-AGRI Focus Group in 2017 and 2019 as major threat for grapevine production performance.

Informations of the European distribution were taken from the EIP-Agri reports and the visible symptoms and consequence for the grapevines were taken from the book of Bettiga 2013

Pest Name	Causal Agent/ Species Name	European Distribution	Visible Symptoms	Impact on Grapevine
<b>Downy Mildew</b>	<i>Plasmopara viticola</i>	All Countries	Necrosis of the stem or shoot, discoloration including brown spotting and yellowish-green tips of the leaves	Reduction in grape yield and grape quality
<b>Powdery Mildew</b>	<i>Erysiphe necator</i>	All Countries	Gray-white, dusty formation on the upper sides of the leaves, it can also infect the bottom sides, buds, flowers, young fruit, and young stems	Reduction in grape yield and grape quality
<b>Grey Mould / Botrytis</b>	<i>Botrytis cinerea</i>	All Countries	Young shoots, flowers and leaves attacked with patches of brown necrotic tissues, berries turning brown for white varieties and reddish for red and black berries finally turning characteristic gray velvety color	Reduction in grape yield and grape quality
<b>Flavescence Dorée</b>	<i>Candidatus Phytoplasma vitis</i> transmitted by the vector <i>Scaphoideus titanus</i>	See Introduction	Reduction of shoot growth, no lignification of shoots, rolled leaves red or yellow colored, drying of berries and inflorescences	Reduction in grape yield and grape quality Acceleration of grapevine decline
<b>GTDs*</b>	Different species of one fungus genus or by different geni	See Introduction	Stunted growth, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, dieback and death	Reduction in grape yield and grape quality Acceleration of grapevine decline
<b>Grapevine moths</b>	European grapevine moth ( <i>Lobesia botrana</i> ) and Cochilis grape moth ( <i>Eupoecilia ambiguella</i> )	All Countries	Round holes in pre-blooming flowers & webbing from larvae feeding, feeding of berries with dark spots. Brown to purple shriveled berries attached to the cluster.	Reduction in grape yield and grape quality
<b>Mites</b>	Several species : <i>Calepitrimerus vitis</i> , <i>Eriophyes vitis</i> , <i>Eotetranychus pruni</i> , <i>Panonychus ulmi</i>	Hungary, Spain, Bulgaria and Romania	Small yellow spots on upper leaf surfaces. Sometimes complete yellowing of the leaf or bronze discolorations Later through the growing season, dry brown leaves and brittle leaves.	Reduction in grape yield and grape quality
<b>Smaller green leafhopper</b>	<i>Empoasca vitis</i>	Spain, Hungary, Greece	Feeding damages on foliage of young newly-formed leaves. Ruffled, misshapen leaves, with green patches on the blade. Shoot internodes are shortened and zigzag, with bumpy, slightly purple discoloration. With high infestation, many distorted leaves	Reduction in grape yield and grape quality
<b>Grape mealybug</b>	<i>Planococcus ficus</i>	Italy, Greece, and northern Spain	Feeding on trunk, roots, cordon, shoots, leaves and fruits. Defoliation and infested fruit clusters. Sticky honeydew accumulates in all the vine parts that can form white to brown crystalline layers, favoring the building of sooty molds.	Reduction in grape yield and grape quality

**Classe:** Insecta  
**Order:** Hemiptera  
**Suborder:** Sternorrhyncha  
**Superfamily:** Phylloxeroidea  
**Family:** Phylloxeridae  
**Genus:** *Daktulosphaira*  
**Species:** *Daktulosphaira vitifoliae*

**Supplement 3 - Figure 2:** Taxonomy of the Grape Phylloxera (*Daktulosphaira vitifoliae* Fitch, 1855)  
transcribed from [https://inpn.mnhn.fr/espece/cd\\_nom/854038/tab/taxo](https://inpn.mnhn.fr/espece/cd_nom/854038/tab/taxo) accessed on 11/11/22

**Supplement 4 - Table 2:** Most common rootstocks used in California and their main characteristics: (i) type of rootstock, (ii) main adaptation and sensitivity, (iii) physiology properties, information synthesized from Bettiga et al. 2013

Common name	Species	Types of Rootstock	Main Adaptations/sensitivity	Plant characteristics
Rupestris St. George or Rupestris du Lot	<i>Vitis rupestris</i> Scheele	American grapevine species	<ul style="list-style-type: none"> <li>- Adapted for non-irrigated soils or dry and shallow soils</li> <li>- Susceptibility for nematodes, oak root fungi, Phytophthora root rot</li> <li>- Insensitive to virus jeopardizing the grafting compatibility</li> </ul>	<ul style="list-style-type: none"> <li>• Shrubby vines, Abundant laterals and short shoots</li> <li>• Easy to root, bud and graft</li> </ul>
Riparia Gloire or Riparia Gloire de Montpellier	<i>Vitis riparia</i> Michaux	American grapevine species	<ul style="list-style-type: none"> <li>- Adapted to alluvial soil</li> <li>- Sensitive to drought</li> <li>- Susceptible for lime, prone to chlorosis symptoms, jeopardizing grafting</li> <li>- Moderate resistance to root-knot nematodes</li> </ul>	<ul style="list-style-type: none"> <li>• Low vigor of the plant</li> <li>• Shallow root system helping control vigor</li> </ul>
Couderc 3309 (3309C)	<i>V. Riparia</i> x <i>V. rupestris</i>	Hybrid of American grapevine species	<ul style="list-style-type: none"> <li>- Sensitive to drought</li> <li>- Susceptible for lime</li> <li>- Susceptible to nematodes</li> <li>- Highly susceptible to virus jeopardizing grafting compatibility</li> </ul>	<ul style="list-style-type: none"> <li>• Low to moderate vigor of the plant</li> <li>• Easy to root, bud and graft</li> </ul>
Teleki 5C	<i>V. Berlandieri</i> Planch. x <i>V. riparia</i>	Hybrid of American grapevine species	<ul style="list-style-type: none"> <li>- Susceptible for drought and water deficit</li> <li>- Lime tolerance</li> <li>- Moderate resistance to root knots and dagger nematodes</li> </ul>	<ul style="list-style-type: none"> <li>• Moderate vigor</li> <li>• Architecture with long internodes and canes and a few laterals</li> <li>• Medium to root, bud and graft</li> </ul>
Richter	<i>V. Berlandieri</i> x <i>V. rupestris</i>	Hybrid of American grapevine species	<ul style="list-style-type: none"> <li>- Relative tolerance to drought</li> <li>- Relative tolerance to lime</li> <li>- Susceptibility for nematodes</li> <li>- Susceptible for virus jeopardizing grafting compatibility</li> </ul>	<ul style="list-style-type: none"> <li>• High vigor in deep fertile soil</li> <li>• Architecture with long canes with few laterals</li> <li>• Easy to root, bud and graft</li> </ul>

**Supplement 5 - Table 3:** Detection of *M. robertsii* DNA copies in aboveground grapevine tissue (stem, third leaf, terminal leaf) 14-, 35-, 63- and 96-98 days post inoculation (dpi) after watering roots with a conidial suspension of two *M. robertsii* strains (EF3.5(2) and ARSEF-2575-GFP). Presented are the number of positive plants in relation to the total number of plants (or percentage positive plants) and the copie number/ $\mu$ L sample (or range of copy numbers) determined via ddPCR. Control plants were treated with the same volume of a sterile suspension solution.

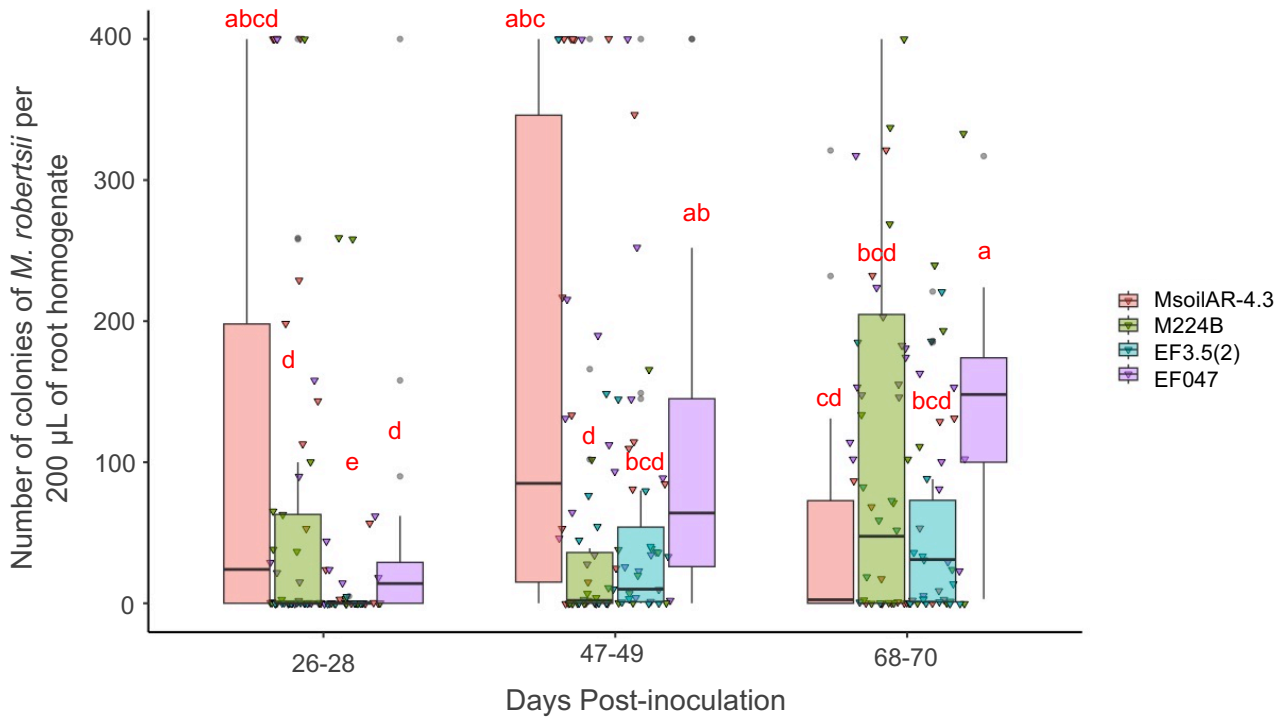
## Appendix A

**Table A1.** Detection of *M. robertsii* DNA copies in aboveground grapevine tissue (stem, third leaf, terminal leaf) 14-, 35-, 63- and 96–98 days post inoculation (dpi) after watering roots with a conidial suspension of two *M. robertsii* strains (EF3.5(2) and ARSEF-2575-GFP). Presented are the number of positive plants in relation to the total number of plants (or percentage positive plants) and the copy numbers/ $\mu$ L sample (or range of copy numbers) determined via ddPCR. Control plants were treated with the same volume of a sterile suspension solution.

	14 dpi			35 dpi			63 dpi			96–98 dpi		
	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Control	EF3.5(2)	ARSEF-2575-GFP	Controls
STEM	1/13 (7.7%) 1.43 c/ $\mu$ L	0/11 (0%)	0/7 (0%)	0/10 (0%)	1/10 (10.0%) 1.54 c/ $\mu$ L	0/7 (0%)	0/11 (0%)	0/11 (0%)	0/7 (0%)	3/9 (33.3%) 1.98–2.75 c/ $\mu$ L	0/7 (0%)	1/7 (14.3%) 4.51 c/ $\mu$ L
THIRD LEAF	1/13 (7.7%), 1.32 c/ $\mu$ L	3/12 (25.0%) 1.43–3.41 c/ $\mu$ L	0/7 (0%)	0/9 (0%)	1/9 (11.1%) 2.20 c/ $\mu$ L	0/7 (0%)	1/10 (10.0%) 1.32 c/ $\mu$ L	4/10 (40.0%) 1.43–17.6 c/ $\mu$ L	0/7 (0%)	3/9 (33.3%) 1.32–2.20 c/ $\mu$ L	2/7 (28.6%) 1.76–11 c/ $\mu$ L	1/7 (14.3%) 1.43 c/ $\mu$ L
TERMINAL LEAF	0/12 (0%)	3/12 (25.0%) 1.43–28.6 c/ $\mu$ L	0/7 (0%)	0/9 (0%)	2/11 (18.2%) 2.97– 10.67 c/ $\mu$ L	2/7 (28.7%) 1.32–3.52 c/ $\mu$ L	2/10 (20.0%) 1.32–1.65 c/ $\mu$ L	5/10 (50.0%) 1.98–24.2 c/ $\mu$ L	0/7 (0%)	1/9 (11.1%) 1.43 c/ $\mu$ L	1/6 (16.7%) 2.86 c/ $\mu$ L	0/7 (0%)

**Supplement 6 - Table 4:** Number of colonies of *M. robertsii* strains MsoilAR-4.3, M224B, EF3.5(2), EF047 counted in total from duplicate plates of root extracts homogenized of non-disinfected roots representative of the root-rhizosphere. Values indicate mean value  $\pm$  IC95% and range [min., max.] correspond to the minimum and maximum numbers counted on medium plates. Roots were harvested from vines at 26-28 days post-inoculation with *M. robertsii* (dpi), 47-49 dpi and 68-70 dpi. Colonies were counted with a colony counter but the degree of imprecision of the measures is recognized high because the plates representative from the rhizosphere were contaminated with other microorganisms that complicated the counts.

Number of <i>M. robertsii</i> colonies counted/200 $\mu$ L of mixed root tissues			
Mean $\pm$ IC95%			
Range [min., max.]			
Inoculated strains	26-28 dpi	47-49 dpi	68-70 dpi
MsoilAR-4.3	115.2 $\pm$ 65.5 [0 ; 400]	153.6 $\pm$ 68.1 [0 ; 400]	54.0 $\pm$ 37.2 [0 ; 321]
M224B	58.4 $\pm$ 46.0 [0 ; 400]	39.1 $\pm$ 38.6 [0 ; 400]	109.0 $\pm$ 56.9 [0 ; 400]
EF3.5(2)	0.2 $\pm$ 0.5 [0 ; 5]	50.1 $\pm$ 38.5 [0 ; 400]	51.6 $\pm$ 28.2 [0 ; 221]
EF047	42.0 $\pm$ 38.0 [0 ; 400]	109.0 $\pm$ 50.6 [0 ; 400]	131.4 $\pm$ 32.1 [3 ; 317]





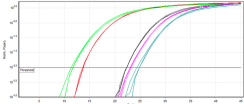
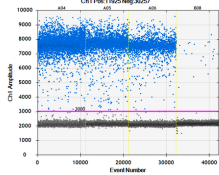
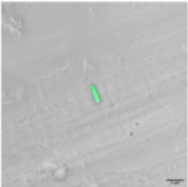
**Supplement 7 - Figure 3:** Number of colonies of *M. robertsii* represented as boxplots of strains MsoilAR-4.3 (red boxes), M224B (green boxes), EF3.5(2) (blue boxes), EF047 (purple boxes) counted in total from duplicates plates of root extracts homogenized of non-disinfected roots representative of the root-rhizosphere. Roots were harvested from vines at 26-28 days post-inoculation with *M. robertsii* (dpi), 47-49 dpi and 68-70 dpi. Colonies were counted with a colony counter but the degree of imprecision of the measures is recognized high because the plates representative from the rhizosphere were contaminated with other microorganisms that complicated the counts. Significant differences are indicated by different letters above the boxes and were obtained by the Kruskal-Wallis test with  $p < 0.05$  followed by post-hoc pairwise multiple comparisons.



**Supplement 8 - Table 5:** Number of colonies of *M. robertsii* strains MsoilAR-4.3, M224B, EF3.5(2), EF047 counted from root extracts homogenized and spread in duplicate plates. Values indicates the total number of colonies counted on medium plates and added for the whole bioassay. Samples of disinfected roots representative of the root-endosphere are presented, roots being harvested from vines at 26-28 days post-inoculation with *M. robertsii* (dpi), 47-49 dpi and 68-70 dpi. Fisher test and post-hoc multi-comparison indicates no significant differences between the strains and also between times of detection.

Total number of <i>M. robertsii</i> colonies counted for the global bioassay			
Inoculated strains	26-28 dpi	47-49 dpi	68-70 dpi
MsoilAR-4.3	9	17	3
M224B	3	43	3
EF3.5(2)	6	20	8
EF047	1	9	1

**Supplement 9 - Table 6:** Comparative table of the methods existent to evaluate the rhizospheric and endophytic association between entomopathogenic fungi and plants

Method	Main Principle	Value Quantified	Pros	Cons
 <p><b>Culture-based method</b> <b>Counting of colonies</b></p>	Plating of homogenate of surface-sterilized plant tissues from inoculated plants on a growing medium	Number of colonies per $\mu\text{L}$ of homogenate	<ul style="list-style-type: none"> <li>- Easy to implement with classical microbiology techniques</li> <li>- Rapid method</li> <li>- Quantification of live inoculum</li> </ul>	<ul style="list-style-type: none"> <li>- Imprecise quantifications due to competition with other microorganisms on growth medium and uneven distribution of fungal nuclei on plate limiting optimal germination</li> <li>- Limiting selectivity of the medium complicating identification of the targeted endophyte</li> <li>- Gives synthetic and general information on the colonization status of a given tissue</li> </ul>
 <p><b>Culture-based method</b> <b>Percentage of plant segments colonized</b></p>	Plating of plant segments of surface-sterilized plant tissues from inoculated plants on a growing medium	Number of plant segments colonized	- See above	<ul style="list-style-type: none"> <li>- Time consuming (i.e. cutting and laying of plant segments)</li> <li>- Gives synthetic and general information on the colonization status of a given tissue (e.g. : a plant segment is considered colonized if there is fungal growth on medium however this segment could be very poorly colonized, also a plant is considered colonized if only on plant segment is colonized by the fungus)</li> </ul>
 <p><b>Quantitative real-time polymerase chain reaction</b> <b>qPCR</b></p>	PCR-based method allowing quantification of targeted DNA with respect to a standard value; from DNA extracted of plant tissues from inoculated plants	$\mu\text{gDNA}$ of targeted DNA/ $\mu\text{L}$ DNA of extracted	<ul style="list-style-type: none"> <li>- Precise quantification of the targeted endophyte inside the plant tissue</li> </ul>	<ul style="list-style-type: none"> <li>- No information on the viability of the quantified targeted endophyte</li> <li>- Unprecise spatial information on the colonization of targeted endophyte</li> </ul>
 <p><b>ddPCR</b></p>	PCR-based method allowing absolute quantification of targeted DNA even in very minimal amounts from DNA extracted of plant tissues from inoculated plants	Absolute Number of copies of targeted DNA/ $\mu\text{L}$ DNA of extracted	<ul style="list-style-type: none"> <li>- Very Precise quantification of the targeted endophyte inside the plant tissue</li> <li>- High sensitivity of quantification</li> </ul>	<ul style="list-style-type: none"> <li>- No information on the viability of the quantified targeted endophyte</li> <li>- Unprecise spatial information on the colonization of targeted endophyte</li> </ul>
 <p><b>Confocal Imaging</b></p>	Observation via confocal microscope of plant segments of plants taken from inoculated plants with a strain tagged with Green fluorescence protein (GFP)	Number of plant segments colonized	<ul style="list-style-type: none"> <li>- Provide visual proof of colonization</li> <li>- Precise information on the amount of plant segment colonized, the tissues colonized by the respective fungal structures</li> </ul>	<ul style="list-style-type: none"> <li>- Very labor intensive, require and exhaustive screening of the numerous plant segments</li> </ul>

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**Sitography :**

- website 1 (consulted on the 25<sup>th</sup> of November 2022) :  
<https://influentialpoints.com/aphid/Phylloxeridae.htm>