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Effect of the combined application of copper-tolerant *Trichoderma* spp. and chitosan for sustainable control of fungal grapevine diseases

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Abstract

The ongoing challenge for viticulture is to effectively and sustainably combat diseases like downy mildew and Esca trunk disease. In organic viticulture, the use of copper-based fungicides to control downy mildew is of crucial importance. However, this practice is increasingly criticized due to its ecotoxicological consequences. Furthermore, there is an absence of effective alternatives for the control of Esca pathogens. The development of novel plant protection strategies that incorporate eco-friendly combinations of agents to combat economically relevant pathogens is therefore urgently needed.

The present study aims to develop a sustainable plant protection tool based on a synergistic combination of *Trichoderma*, chitosan and the application of a reduced copper rate for the control of *Plasmopara viticola* and the Esca pathogens *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea* and *Phaeoacremonium minimum*. This tool is intended to reduce the need for copper fungicides. Therefore, *Trichoderma* species isolated from grapevine wood were characterized and tested for copper tolerance. A high degree of compatibility to copper hydroxide was demonstrated. High copper tolerance was particularly shown and increased for *T. koningiopsis* and *T. harzianum*. The copper-tolerant *Trichoderma* isolates were also found to demonstrate a high tolerance to chitosan, confirming the compatibility of *Trichoderma* with chitosan. To provide a detailed description of the agents' effect against *P. viticola*, the effect of *T. koningiopsis*, chitosan, and a reduced copper rate was tested on the upper and lower sides of grapevine leaves inoculated with the pathogen. Both a direct and an indirect effect against downy mildew have been demonstrated. Furthermore, microscopic analysis revealed the successful establishment of *T. koningiopsis* and uniform distribution of chitosan on the grapevine leaves. The indirect effect of the complex was verified for different grapevine cultivars in greenhouse experiments. The present work demonstrated increased pathogen-related gene expression after the simultaneous application of the agents. An increased accumulation of reactive oxygen species was observed in resistant grapevine cultivars following treatment. Furthermore, the agents reduced the number of haustoria, key structures of *P. viticola*. Ensuring the efficacy of the synergistic tool in counteracting Esca pathogens required establishing *Trichoderma* in the grapevine. Grapevine wood colonization capacity was confirmed for all tested *Trichoderma* isolates. Moreover, the direct effect of *Trichoderma* candidates in confrontation tests against Esca pathogens, as well as the indirect effect through released volatile organic compounds, were investigated. A high direct effect on the mycelial growth of *P. chlamydospora* was observed, while an indirect effect on the growth of *P. minimum* could be demonstrated. In addition, the combined field application of tolerant *T. koningiopsis*, chitosan and a reduced copper rate against *P. chlamydospora* resulted in a reduction in pathogen disease severity of infected shoots.

This work shows the potential of a sustainable plant protection tool for controlling relevant grapevine diseases and simultaneously contributes to the reduction of ecotoxic copper fungicides in viticulture.

Kurzfassung

Die effektive und nachhaltige Bekämpfung von Krankheiten wie Falscher Mehltau und die Esca-Holzkrankheit stellt eine kontinuierliche Herausforderung für den Weinbau dar. Im ökologischen Weinbau ist der Einsatz von Kupferfungiziden zur Kontrolle des Falschen Mehltaus unerlässlich, wird aber aufgrund ökotoxikologischer Bedenken zunehmend kritisiert. Darüber hinaus mangelt es an effektiven Alternativen zur Bekämpfung von Esca-Pathogenen. Daher ist die Entwicklung neuartiger Pflanzenschutzstrategien, welche umweltfreundliche Wirkstoffkombinationen zur Bekämpfung wirtschaftlich relevanter Pathogene beinhalten, dringend erforderlich.

Die vorliegende Arbeit befasst sich mit der Entwicklung einer nachhaltigen Pflanzenschutzstrategie, welche auf einer synergistischen Kombination aus *Trichoderma*, Chitosan und dem Einsatz eines reduzierten Kupferaufwands zur Bekämpfung von *Plasmopara viticola* und den Esca-Pathogenen *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea* und *Phaeoacremonium minimum* beruht. Das Ziel dieses Tools besteht darin, den Einsatz Kupferfungizide zu reduzieren. Zu diesem Zweck wurden aus Rebholz isolierte *Trichoderma*-Arten charakterisiert und hinsichtlich ihrer Kupfertoleranz untersucht. Es wurde eine hohe Verträglichkeit gegenüber Kupferhydroxid nachgewiesen. Hierbei zeigten insbesondere *T. koningiopsis* und *T. harzianum* eine hohe Kupfertoleranz, welche im Folgenden weiter gesteigert werden konnte. Für das kupfertolerante *Trichoderma*-Isolat *T. koningiopsis* wurde zudem eine hohe Chitosantoleranz nachgewiesen, sodass ebenfalls die Kompatibilität von *Trichoderma* mit Chitosan sichergestellt wurde. Um die Wirkung der Agentien gegen *P. viticola* näher zu beschreiben, wurde der Effekt von *T. koningiopsis*, Chitosan und einem reduzierten Kupferaufwand auf erregerinokulierten Weinblattober- und -unterseiten getestet. Es wurde sowohl eine direkte als auch eine indirekte Wirkungsweise gegen den Falschen Mehltau demonstriert. Darüber hinaus belegten mikroskopische Analysen die erfolgreiche Etablierung von *T. koningiopsis* sowie eine gleichmäßige Verteilung von Chitosan auf den Rebblättern. Der indirekte Effekt des Wirkkomplexes konnte für unterschiedliche Rebsorten in Gewächshausuntersuchungen verifiziert werden. Hierbei zeigten die durchgeführten Untersuchungen eine gesteigerte pathogenbezogene Genexpression nach gleichzeitiger Applikation der Agentien. Bei resistenten Rebsorten wurde nach Behandlung eine erhöhte Akkumulation reaktiver Sauerstoffspezies beobachtet. Des Weiteren konnte durch die Applikation der Agentien die Anzahl an Haustorien, Schlüsselstrukturen von *P. viticola*, reduziert werden. Um die Wirksamkeit des synergistischen Pflanzenschutztools bei der Bekämpfung von Esca-Pathogenen zu gewährleisten, musste eine Etablierung von *Trichoderma* in der Weinrebe sichergestellt werden. Für alle getesteten *Trichoderma*-Isolate wurde die Fähigkeit zur Besiedlung von Rebholz bestätigt. Außerdem wurde die direkte Wirkung von *Trichoderma*-Kandidaten in Konfrontationstests gegen Esca-Erreger und der indirekte Effekt durch freigesetzte flüchtige organische Verbindungen untersucht. Es wurde eine hohe, direkte Wirksamkeit auf das Myzelwachstum von *P. chlamydospora* beobachtet, während zusätzlich eine indirekte Auswirkung auf das Wachstum von *P. minimum* nachgewiesen wurde. Darüber hinaus führte die Freilandapplikation einer Kombination aus tolerantem *T. koningiopsis*, Chitosan und einem reduzierten Kupferaufwand gegen *P. chlamydospora* zu einer Verringerung des Pathogenbefalls an infizierten Trieben.

Die vorliegende Arbeit demonstriert das Potential eines nachhaltigen Pflanzenschutztools zur Kontrolle bedeutender Rebkrankheiten und leistet zeitgleich einen Beitrag zur Reduzierung ökotoxikologisch bedenklicher Kupferfungizide im Weinbau.

Publications included in this thesis

Chapter 2 has been published:

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List of abbreviations

A. dist.	distilled water
ANOVA	analysis of variance
ATPase	adenosine triphosphatase
BABA	3-aminobutanoic acid
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
BCA	biocontrol agents
BLAST	basic local alignment search tool
BTH	S-methyl benzo[1,2,3]thiadiazole-7-carbothioate
Cabla	Cabernet blanc
CDA	Czapek Dox agar
cDNA	complementary DNA
$C_{16}H_{30}CuO_4$	Copper octanoate
Chi	chitosan
Cu	copper
$Cu(OH)_2$	copper(II) hydroxide
$CuSO_4$	copper(II) sulfate
cv.	cultivar
cvs.	cultivars
DA	degree of N-acetylation
daa	days after spray application
DAB	3,3’-diaminobenzidine
DAMPs	damage-associated molecular patterns
dbi	days before inoculation
dH_2O	distilled water
DNA	deoxyribonucleic acid
DP	degree of polymerization
dpi	days post inoculation
dsDNase	double-strand specific deoxyribonuclease
DüMV	Fertilizer Ordinance/ Düngemittelverordnung
DüngG	Fertilizer Act/ Düngegesetz
EPPO	European Plant Protection Organization
ET	ethylene
$FeSO_4$	iron(II) sulfate
Fmed	*Fomitiporia mediterranea*
gDNA	genomic DNA
GLSD	grapevine leaf stripe disease
GTDs	grapevine trunk diseases
H_2O_2	hydrogen peroxide
hpi	hours post inoculation
HSD	honestly significant difference
ISR	induced systemic resistance
ITS	internal transcribed spacer
JA	jasmonic acid
LS	lower leaf sides
LT	treatment of lower leaf sides
MAMPs	microbe-associated molecular patterns
MEA	malt extract agar
MEB	malt extract broth

mRNA	messenger ribonucleic acid
MTh	Müller-Thurgau
NBT	nitro blue tetrazolium
n. s.	not significant
O_2^-	superoxide radical
qPCR	quantitative polymerase chain reaction
Pch	*Phaeomoniella chlamydospora*
PCR	polymerase chain reaction
PDA	potato dextrose agar
pH	negative decadic logarithm of [H+] (lat. *pondus Hydrogenii*)
Pmi	*Phaeoacremonium minimum*
Pr	pathogen-related
Pv	Plasmopara viticola
qPCR	quantitative polymerase chain reaction
rDNA	riobosomal deoxyribonucleic acid
Reg	Regent
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
Rpv	Resistance to *Plasmopara viticola*
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SA	salicylic acid
SAR	Systemic acquired resistance
SC	suspension concentrate
SD	standard deviation
SEM	scanning electron microscope
sp.	species (single)
spp.	species (plural)
subsp.	subspecies
TBE	tris-borate-EDTA buffer
Tef-1a	translation elongation factor 1-alpha
Tr	*Trichoderma*
US	upper leaf sides
UT	treatment of upper leaf sides
UV	ultraviolet
VDAC3	voltage-dependent anion channel 3
VOC	volatile organic compounds
Vv	Vitis vinifera
v/v	volume per volume
WP	wettable powder
w/v	weight per volume
$ZnSO_4$	zinc sulfate
η^2	partial eta-square

1 Introduction

Considering global environmental changes, increasing population, and the growing demand for resource conservation, efforts are being made to ensure stable agricultural yields. Consequently, the transition to sustainable production methods has emerged as a paramount concern in the contemporary era, a necessity that extends to perennial crops such as viticulture (Cesco et al., 2021). Among the most serious threats to the health and productivity of grapevines are diseases caused by fungal, bacterial, or viral pathogens (Armijo et al., 2016).

The short-term management of pathogens can be accomplished primarily through the precise application of highly effective synthetic chemical pesticides, particularly in conventional or integrated viticulture. In organic viticulture, where the utilization of such substances is strictly forbidden (Vogt and Schruft, 2000), preparations containing copper or sulfur are employed as alternatives in organic systems. However, these products are characterized by limited effectiveness and the copper accumulation in the soil or its harmful effects on non-target organisms resulting in ecological conflicts of interest (Karimi et al., 2021; Furiosi et al., 2022). The increasing social and political pressure to reduce the use of harmful pesticides indicates the need for a reevaluation of current practices. Concurrently, climate change and the increasing occurrence of extreme weather events present additional challenges for plant protection.

In this area of conflict, interest is growing in biological alternatives that enable grapevines to be maintained in a healthy state in an environmentally friendly and effective way. The utilization of biocontrol agents (BCA) and biostimulants, which are, for example, based on beneficial microorganisms, has the potential to serve as a crucial element in the development of future plant protection strategies (Hamza and Suggars, 2001; Gupta and van Staden, 2021). While both approaches, BCA and biostimulants, are being researched and applied separately, their combined use – with the aim of achieving a synergistic effect – remains insufficiently understood.

The present work addresses this need by developing and evaluating a sustainable combined plant protection strategy of BCA and biostimulants against fungal diseases in organic viticulture. In detail, a synergistic sustainable combination of *Trichoderma* sp. and chitosan was selected and its efficacy and applicability were tested with regard to an effective copper-reduced application against downy mildew (*Plasmopara viticola*) as well as for the control of Esca pathogens.

The subsequent chapter aims to provide an introduction to the four studies in chapters 2 to 5. First, the grapevine is introduced and past and future disease challenges are explained. Secondly, this chapter introduces grapevine diseases, focusing on fungal pathogens. A more thorough introduction is given to the epidemiology, symptoms, and control strategies against downy mildew on grapevine and the Esca disease complex. The third part of the introduction is devoted to the utilization of BCA and biostimulants as a sustainable plant protection strategy. BCA and biostimulants are defined, and their regulatory classification is outlined. The importance of these agents for environmentally friendly plant protection is shown. In this context, *Trichoderma* and chitosan are introduced, and their individual mechanisms of action are presented. The synergistic potential of the two agents is also considered. In the final section of the chapter, the objectives of the individual studies are explained in greater detail, and it is shown how an overall contribution to a sustainable plant protection strategy can be made.

1.1 *Vitis vinifera* – a crop facing historical and recent challenges

Grapevines (*Vitis* spp.) are economically important perennial fruit crops that contribute to the production of wine, fresh grapes, dried grapes, and table grapes in many regions worldwide. In 2023, the global grapevine production reached 30.8 million tons of wine grapes (45.7%), 31.9 million tons of table grapes (47.4%), and 4.6 million tons of grapes intended for drying (6.9%). This production was achieved across a total of 7.2 million hectares (ha) of vineyard surface area (International Organisation of Vine and Wine, 2025). The proportion of certified organic vineyards exhibited an average annual increase of 13% between 2005 and 2019, while the conventionally cultivated vineyard area demonstrated an average annual decrease of 0.4% over the same period. In 2019, the global area dedicated to organic viticulture encompassed 454,000 ha across a total of 63 countries (International Organisation of Vine and Wine, 2025).

The grapevine (*Vitis vinifera*) belongs to the family Vitaceae according to taxonomic classification systems. Its genus is subclassified into two subgenera: *Euvitis* and *Muscadinia*. These subgenera are estimated to encompass approximately 60 to 80 species, depending on the specific taxonomic delimitation employed. The subgenus *Euvitis* is further subdivided into three distinct groups: European grapevines (*Vitis vinifera* L.), American grapevines (approximately 30 species), and Asian grapevines (approximately 50 species). The species *Vitis vinifera* L. comprises the predominant cultivars utilized globally, and it is classified into two subspecies: *Vitis vinifera* subsp. *sylvestris*, which is a wild form, and *Vitis vinifera* subsp. *vinifera*, which is employed in winemaking due to its favorable flavor characteristics. While the Asian grapevines are of limited importance for the cultivation of grapes, the American grapevines are highly beneficial for the breeding of rootstocks and resistant grapevine cultivars due to their inherent resistance characteristics (Miedaner, 2018; Rahemi et al., 2022).

American wild species have demonstrated an evolutionary capacity to adapt to various pathogens relevant to viticulture, including phylloxera and fungal pathogens, such as powdery and downy mildew. In contrast, European grapevine cultivars exhibit a lack of sufficient genetic resistance properties. This was particularly evident in the vast decrease in European wine production yields in the middle of the 19th century. Grapevine material was imported from North America to Europe in the mid-nineteenth century through intensive exchange of goods. The pathogen *Erysiphe necator*, the causative agent of powdery mildew, which subsequently spread over large areas, was successfully combated by treating the grapevines with sulfur preparations. The high damage potential of the pathogen led to the importation of rooted North American wild grapevines species to Europe for the purpose of testing their resistance potential to *E. necator*. It is hypothesized that these imports may have also facilitated the introduction of phylloxera (*Daktilosphaira vitifoliae*), the causative agent of grape phylloxera, to Europe. The phylloxera epidemic caused significant destruction to vineyards that were affected, particularly in France. However, the use of resistant American rootstocks, on which European scions were grafted, led to the successful control of the epidemic (Benheim et al., 2012; Vogt and Schruft, 2000; Rahemiet al., 2022). In order to rebuild the vineyards destroyed by phylloxera, American grapevine cuttings were subsequently introduced. With these cuttings came another pathogen, *Plasmopara viticola* (downy mildew on grapevine), which spread rapidly throughout Europe and caused considerable damage to vineyards under favorable weather conditions (Gessler et al., 2011; Vogt and Schruft, 2000; Rahemi et al., 2022).

The introduction of harmful organisms, most notably *E. necator* and *P. viticola*, has led to a substantial paradigm shift in viticulture, as the focus on yield and quality assurance has since been heavily reliant on the implementation of plant protection measures. This is largely attributable to the inherent susceptibility of *V. vinifera* spp. to fungal pathogens (Vogt and Schruft, 2000). Fungicides comprise the predominant category of pesticides utilized in most vineyards (Pertot et al., 2017a). The application of copper-based agents found its way into viticulture after the discovery that the Bordeaux mixture originally used to protect vineyards from grape thieves – a mixture of quicklime and copper sulfate – could also be used to combat downy mildew (Gessler et al., 2011). Copper formulations are among the most prevalent fungicides employed globally to manage downy mildew, while sulfur preparations are utilized to address powdery mildew (Furiosi et al., 2022). However, prolonged and intensive utilization of copper-based fungicides has been observed to result in the entry and subsequent accumulation of copper into the environment. Immobile copper can be fixed, which has the potential to induce ecotoxic effects on soil and its biological quality (Karimi et al., 2021). In order to reduce the negative effects on the environment and human health caused by the extensive application of pesticides, the European Union has set itself the goal of halving the application of chemical pesticides by 2030 with the European Green Deal and the Farm2Fork strategy. For instance, the utilization of copper-based products has been restricted to a maximum of 15 kilograms per hectare over a period of five years, with an average annual quantity of 10 to 12 treatments (European Commission Implementing Regulation 2018/1981 of December 13, 2018; Vereinigung Schweizer Biolandbau-Organisationen, Bio Suisse, 2024). However, up to 16 treatments are required due to unfavorable weather conditions. This demonstrates the critical need for novel sustainable control approaches to ensure the protection of grapevines in viticulture.

However, the challenges posed to viticulture by the introduction of sustainable control mechanisms against the above-mentioned harmful organisms is not the only factor to be considered. Climate change and the resulting environmental changes also offer considerable potential for the spread of grapevine trunk diseases (GTDs), especially in combination with other factors, such as the long-term use of certain cultivation measures like wound-producing cultural practices, such as winter pruning (Mondello et al., 2018a). The Esca disease complex, Eutypa dieback, and Botryospheria dieback are among the most prevalent and significant GTDs in adult vineyards (Gramaje et al., 2018; Mesguida et al., 2023). Of particular concern is the Esca disease complex, which represents a global threat to grapevine viability, with severe consequences for vineyard longevity, berry quality, and wine quality, resulting in substantial yield losses. In order to reduce economic losses caused by the Esca disease complex, it is essential to develop new, effective, and sustainable strategies to advance disease control, since there are currently no effective control treatments (Mondello et al., 2018b; Mesguida et al., 2023).

1.2 Grapevine diseases

Grapevine leaves, shoots, roots, flowers, and berries can be susceptible to a variety of diseases, which can lead to significant impairment to plant development and, in certain cases, even result in the death of entire organs. The causal agents of grapevine diseases encompass fungi, bacteria, phytoplasmas, and viruses, which attack the grapevine and subsist on the resources of their host (Vogt and Schruft, 2000). Fungi represent the most significant group of phytopathogenic microorganisms. Their heterotrophic lifestyle and capacity to degrade complex plant polymers position them as some of the organisms most adapted to plants and plant substrates. The application of exoenzymes enables the dissolution and penetration of plant tissue layers. A taxonomic distinction can be made between biotrophic, hemibiotrophic, and necrotrophic pathogens. Necrotrophic pathogens are capable of rapidly killing the host tissue through the secretion of phytotoxins and lytic enzymes, which facilitate the process of nutrient consumption. In contrast, biotrophic pathogens have evolved to penetrate the host tissue with minimal detection due to their co-evolutionary adaptation. These pathogens form structures that enable them to extract nutrients and assimilate from living host cells for their nutrition. In the initial phase of hemibiotrophic pathogen infection, the infection is biotrophic. This phase subsequently transitions into a necrotrophic phase, resulting in the death of the host at the conclusion of the infection cycle (Armijo et al., 2016; Hallmann and von Tiedemann, 2019). In addition to their close adaptation to the plant, the pathogens' epidemic properties – such as high reproduction rates, short latency periods, high dispersal ability of the spores, and effective persistence – also contribute to their high damage potential (Hallmann and von Tiedemann, 2019). The most notable fungal diseases affecting grapevines include downy mildew (*P. viticola*), powdery mildew (*E. necator*), and gray mold

(*Botrytis cinerea*) (Armijo et al., 2016). Moreover, the Esca disease complex poses a serious and growing threat to viticulture worldwide (Beris et al., 2022).

Since this work aims to develop a sustainable plant protection strategy against downy mildew and the Esca disease complex, these diseases are described in more detail below.

1.2.1 Downy mildew

Plasmopara viticola (Berk. & Curtis) Berl. & De Toni, the causal agent of downy mildew, is a member of the oomycetes and belongs to the family Peronosporaceae. In contrast to true fungi, the cell wall of oomycetes consists primarily of cellulose, whereas that of true fungi is based on chitin. Furthermore, oomycetes possess a diploid nucleus during their vegetative phase. In contrast, the nuclei of ascomycetes are haploid, and those of basidiomycetes are dikaryotic (Gessler et al., 2011). The obligate biotrophic endoparasite *P. viticola* is found exclusively on green organs with stomata of species belonging to the genus *Vitis*. In its ability to undergo asexual and sexual cycles, this pathogen demonstrates a characteristic shared among many other oomycetes (Vogt and Schruft, 2000; Viret and Gindro, 2025).

According to Viret and Gindro (2025), the epidemiological development of the pathogen can be described as follows: During asexual reproduction, flagellated zoospores of *P. viticola* are released from the sporangia upon contact with water. Subsequent to a swarming phase, these organisms attach to the stomata of the plant and undergo a process of flagellar detachment. The zoospores, which were previously only surrounded by a membrane and are only viable in water, also form a cell wall. Subsequent to the attachment to the stomata, a germ tube is formed, which penetrates the substomatal cavity. This germ tube forms a thickening, otherwise known as a substomatal vesicle. A primary hypha forms and the mycelium begins to develop from the substomatal vesicle. The pathogen colonizes the tissue of the host plant intercellularly, spreading within the parenchyma of the plant. Penetration organs are utilized by the hyphae to penetrate the host cells, subsequently producing several intercellular suckers (haustoria). These haustoria are crucial for the ability of the pathogen to take up nutrients, suppress the host's defense mechanisms, and redirect the host's metabolic processes to its own advantage (Armijo et al., 2016; Vogt and Schruft, 2000; Viret and Gindro, 2025). Haustoria form and penetrate the mesophyll cell, invaginating the plasma membrane of the parenchyma cells between 3.5 and 6 hours after penetration (Langcake and Lovell, 1980; Díez-Navajas et al., 2008). The mycelium of the pathogen gradually colonizes the green tissue of the host plant. As the mycelium grows, it typically spreads along the leaf veins and subsequently fills the intercostal spaces of the leaf (Kortekamp et al., 1998; Burruano, 2000; Unger et al., 2007; Armijo et al., 2016). However, studies by Juraschek et al. (2022) demonstrate that *P. viticola* can also form specialized hyphal structures to bridge the vessel cells of the leaf vein of higher order. The intercellular growth of the pathogen occurs in a latent phase, during which no symptoms of the infection are visible (Leroy et al., 2013). The duration of parenchymatous growth can vary. Approximately six to ten days following the initial infection, the symptoms manifest in the form of circular, semi-translucent yellow spots on the adaxial leaf side. These spots are referred to as ”oil spots“, and as the disease progresses, they become necrotic and brown (Fröbel and Zyprian, 2019; Vogt and Schruft, 2000; Koledenkova et al., 2022; Viret and Gindro, 2025). However, a white and cottony growth is observable on the abaxial side of the leaf. Once the pathogen arrives at the substomatal cavity, it forms a thick cushion from which sporangia carriers emerge from the leaf tissue of the grapevine and on which lemon-shaped sporangia develop (Viret and Gindro, 2025). The prerequisites for the process of sporulation are as follows: a period of darkness lasting a minimum of four hours, a high humidity level of at least 98%, mild temperatures ranging from 13 to 19 °C, and a high leaf wetness level exceeding 90% (Blaeser and Weltzien, 1979; Gessler et al., 2011).

Oospores are formed during the sexual phase of the lifecycle. The process of recognition between compatible, heterothallic individuals is facilitated by the action of hormones (Wong et al., 2001; Koledenkova et al., 2022). The hyphae undergo a transformation, becoming reproductive organs. This process gives way to the formation of oogonia and antheridia, where meiosis occurs. The antheridium envelopes the oogonium, forming a fertilization loop that penetrates its interior, thereby enabling fertilization. The cell nuclei undergo fusion, resulting in the development of a diploid oospore from the fertilized oogonium. This oospore possesses the capacity to withstand winter conditions by surviving on the grapevine, within leaf litter, and in the soil, where it maintains its viability as a permanent spore (Rossi et al., 2009, 2013; Vogt and Schruft, 2000; Viret and Gindro, 2025). In certain cases, the pathogen may also persist as mycelium on infected, undead twigs during the winter months (Koledenkova et al., 2022). Late infections during the growing season frequently result in the formation of oospores in late summer or fall. The permanent forms of the pathogen develop in infected leaves, which subsequently fall to the ground. Oospores are capable of maintaining viability in soil for extended periods due to the presence of a thick, multi-layered cell wall that serves as a storage reservoir for reserve substances. In dry conditions and low temperatures, oospores can remain viable for several years (Burruano, 2000; Vogt and Schruft, 2000; Viret and Gindro, 2025). The process of germination in the following spring is influenced by a variety of climatic factors. Primary infection is initiated under conditions of elevated temperature, with a threshold of 8 °C, the accumulation of 10 mm of precipitation within the previous 48 hours, and the emergence of shoots measuring 10 cm in length (Gessler et al., 2011). The germination, thickening, and formation of a macrosporangium (primary sporangium) by the oospore occurs as soon as the conditions are favorable for the pathogen (Viret and Gindro, 2025). Zoospores are formed through the process of mitotic division of the oospore nuclei (Burruano, 2000; Viret and Gindro, 2025). Heavy rainfall and wind have been observed to result in the transfer of developed macrosporangia from the ground to the grapevine’s organs via water splashes (Viret and Gindro, 2025). The release of 40 to 60 infectious zoospores from the primary sporangium is possible under conditions of sufficient moisture, and these zoospores can travel through the water to the stomata of the grapevine, thereby initiating a new infection cycle (Vogt and Schruft, 2000; Viret and Gindro, 2025).

The number of annual infection cycles can vary depending on the prevailing weather conditions. In years with high infection pressure, the number of cycles can reach dozens within the growing season. This can have a significant impact on the quality and quantity of the grapevines. An infestation of the grapevine with downy mildew is particularly severe during the exponential growth phase, which occurs around the time of flowering (Viret and Gindro, 2025).

In regions characterized by a temperate and humid climate, downy mildew poses a significant threat, capable of causing substantial economic damage to wine production. All European grapevines belonging to the species *Vitis vinifera* are susceptible to *P. viticola*, and all green organs of the grapevine can be affected by the pathogen. Leaves showing symptoms of downy mildew exhibit a brown discoloration and undergo premature detachment (Viret and Gindro, 2025). The loss of foliage due to the disease can disrupt the process of assimilate formation, thereby affecting the quality of the berries. A severe infestation of the leaves can also impact the growth of the shoots, potentially reducing the amount of reserve substances stored in the annual shoots. The long-term damage to the foliage by downy mildew has a negative impact on the vigor and performance of the grapevines. In addition, the premature death of the plants is a possible consequence (Vogt and Schruft, 2000). Nevertheless, yield losses are primarily attributable to infestations of shoots and berries. A large number of shoots can be infected by the pathogen, particularly during periods of warm and humid weather in the spring months. Shoots that are infected before or after flowering initially exhibit a light green coloration, which subsequently transitions to a brown hue. This observation can be made on flowers, berry stems, and the entire stem framework. The shoots exhibit a downward growth pattern and adopt a distinctive shape, known as the "shepherd's crook". In the case of infected shoots, death is typically the outcome, although these may persist for an extended duration in a state of withering (Vogt and Schruft, 2000; Viret and Gindro, 2025). In the case of *P. viticola* infection, the development of superficial, longitudinal, purplish-brown lesions is observed in infected shoots. These infected shoots exhibit a tendency to merge in the downward direction and may show sporulation in variable abundance (Viret and Gindro, 2025). The occurrence of infection by the pathogen is possible as long as the stomata of the young berries have not yet undergone corkification. Upon infection of the young berries, sporangia become visible on the surface, which then cover the individual berries, thus indicating that the bunches are infected with grey rot. As the infection progresses, discoloration manifests in the form of brown patches on the stems and peduncles of the grapes and bunches. This initial stage of the disease is accompanied by a change in the color of the berries, which first turn purple and then reddish-brown (brown rot). The berries also develop a leathery, shriveled surface, which is characteristic of grape infestation. An infestation has the capacity to affect a limited number of berries, as well as components of the grapes or entire bunches. Towards the end of the vegetation period, subsequent infections of the leaf apparatus under favorable conditions for downy mildew result in mosaic-like patterns on the leaves, indicating varying degrees of infestation by the pathogen. Juvenile foliage at the tips of shoots or side shoots are particularly susceptible to the disease. An infestation is frequently indicated by the presence of a white fungal turf on the twisted shoot tips and the not yet fully unfolded leaves (Vogt and Schruft, 2000; Viret and Gindro, 2025).

As mentioned before, the majority of European grapevine cultivars exhibit a high degree of susceptibility to downy mildew. In contrast, some American and Asian cultivars demonstrate a range of levels of resistance to this pathogen, ranging from partial to complete protection (Jackson, 2000; Díez-Navajas et al., 2008). This makes the use of their germplasms a sustainable tool for breeding resistant grapevine cultivars. In recent decades, various genetic resistance factors and the associated loci have been identified (Schneider et al., 2019). In the process of backcrossing the susceptible cultivar, *V. vinifera*, with genotypes that carry different loci with resistance to *P. viticola* (*Rpv*), there is an objective to maintain and enhance the desired characteristics of *V. vinifera* (yield, growth, and organoleptic properties) through the supplementation of resistance from the other parent line (Koledenkova et al., 2022; Gouveia et al., 2024). In the meanwhile, 31 resistance loci have been described in association with *P. viticola* (Vezzulli et al., 2022). *Rpv3*, a prominent locus of resistance, has been identified in various cultivars, notably Regent and Cabernet blanc (Wingerter et al., 2021). Nevertheless, research findings indicate that *P. viticola* has already demonstrated an ability to overcome this resistance through *Rpv3* (Paineau et al., 2022). Furthermore, the breeding of resistant varieties is a time-consuming and costly process (Koledenkova et al., 2022).

The effective and economically viable management of the pathogen *P. viticola* is primarily accomplished through the application of fungicides (Koledenkova et al., 2022). Depending on the absorption and distribution of the agents, they can have a contact effect or a systemic or semi-systemic effect (Vogt and Schruft, 2000). In addition to copper-based treatments, a variety of multi-site fungicides (e.g., dithiocarbamates) can also be applied to the plants as a protective barrier prior to pathogen infection. In contrast, single-site fungicides have been shown to have a pre- (preventive) and post-infection (curative) protective effect against the pathogen (e.g., strobilurins) (Koledenkova et al., 2022; Viret and Gindro, 2025). Conventional fungicides primarily impact the sporangia and zoospores of *P. viticola*. Systemic agents, such as phenylamides and aluminum, have also been observed to affect the hyphae within the plant tissue; however, this effect is usually only present within a few days of infection. Recent active substances have also demonstrated an inhibitory effect on the sporulation of the pathogen. This effect is achieved by interfering with the formation of sporangia (Vogt and Schruft, 2000; Koledenkova et al., 2022). However, the efficacy of a fungicide in preventing downy mildew is contingent upon the timing of its application, rather than the specific type of fungicide employed. Control strategies are therefore based on the application of a fungicide coating immediately before possible outbreaks and infections by the pathogen. Decision support systems, which include risk prevention models, allow for adapting the timing of fungicide application and its dosage to the leaf area to be treated or the developmental stage of the grapevine within a vegetation period (Vogt and Schruft, 2000; Viret and Gindro, 2025).

In particular, the utilization of copper agents is a well-established control strategy against downy mildew, and it remains a prevalent practice in both organic and integrated viticulture (Koledenkova et al., 2022). Copper is a crucial fungicide in contemporary organic cultivation systems. It is an active ingredient that is not synthetic, and it has a broad spectrum of activity (Dagostin et al., 2011) and its indispensability is particularly pronounced under conditions of high infestation pressure. However, it is problematic that the long-term intensive use of copper-based fungicides leads to the accumulation of copper in the soil and has negative effects on its fertility and microbiota (Karimi et al., 2021). The development of novel fungicide formulations and safer application techniques have contributed to a reduction in the use of copper fungicides in recent years. However, there is an urgent need for further research to reduce or eliminate the use of synthetic pesticides and copper formulations. Research is also focusing on alternative treatment approaches based on natural extracts, herbal decoctions, or microorganisms (Koledenkova et al., 2022). These approaches may be effective strategies for achieving sustainable and lasting protection.

1.2.2 Esca disease complex

Esca disease is a complex of several diseases, predominantly wood decay and a vascular disease. The following five distinct diseases or syndromes comprise this disease complex: dark wood streaking (caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*), Petri disease in young grapevines (caused by *P. chlamydospora*, *P. minimum*, and *Cadophora luteo-olivacea*), grapevine leaf stripe disease (GLSD) (caused by *P. chlamydospora* and *P. minimum*), white rot (caused by *Fomitiporia* spp. – in Europe *Fomitiporia mediterranea* – and other Basidiomycota), and Esca proper (caused by *P. chlamydospora*, *Phaeoacremonium* spp*.,* and *Fomitiporia* spp.). The expression of the Esca disease complex and the symptom-causing pathogens can vary depending on the age of the grapevine. For instance, the vascular ascomycetes, *P. chlamydospora* and *P. minimum*, have been observed to be associated with the dark wood streaking symptom. This symptom is the result of an infection of the grapevines that has already occurred within the nursery. Petri disease occurs on young grapevines and manifests itself as vascular discoloration, which often occurs together with dark spot-like discolorations within the wood. Affected grapevines exhibit symptoms such as weak growth, withered shoots, and a reduction in the number and size of leaves, which can become chlorotic. The disease may result in the death of the grapevine. In contrast, GLSD has been observed on both young and adult grapevines. The disease manifests itself in the typical tiger stripe pattern on symptomatic leaves. In addition, infected plants frequently exhibit symptoms of partial or complete apoplexy. An orange-brown stripe can also be observed under the bark. Older grapevines frequently exhibit symptoms of white rot and Esca proper. The development of these diseases is partly attributable to the action of basidiomycete decay agents (Mondello et al., 2018b). The white rot is a fungal infection that affects the wood of grapevines, causing symptoms that result in the wood's instability and weakening (Mondello et al., 2018b; Moretti et al., 2021). *F. mediterranea* has the capacity to synthesize particular enzymes that facilitate the decomposition of the primary components of grapevine wood, such as lignin (Schilling et al., 2022). Esca proper is typically observed in adult plants, where both the vascular pathogens previously mentioned and basidomycetes are involved in the disease process. This is typically characterized by a combination of GLSD and white rot (Bertsch et al., 2013; Mondello et al., 2018b). To provide a detailed overview of the various disease symptoms and their causal agents, the Esca disease complex is provided according to Mondello et al. (2018b) in Figure 1. Creating a separate presentation of the complex and the prolonged development of the various Esca diseases exceeds the scope of this work.

Figure 1: Diseases/syndromes and their causal agents contributing to the Esca disease complex according on the age of the grapevine. Pictures: Dark wood streaking, V. Mondello; Petri disease, courtesy of Feuga (Spain); GLSD, courtesy of Plant pathology sector SAF dept., University of Palermo (Italy), DRL Rheinpfalz (Germany), and Maurizio Gily (Italy); white rot, upper picture courtesy of DRL Rheinpfalz (Germany); Esca proper, courtesy of Plant pathology sector SAF dept., University of Palermo (Italy), Feuga (Spain), and ADVID (Portugal). (Mondello et al., 2018b)

The symptoms of Esca-infected grapevines typically become apparent from July onwards and can manifest on individual grapevines or across multiple plants within a defined area. A distinction can be made between an acute, apoplectic form of decline and a chronic, slowly progressing form of decline (Viret and Gindro, 2025). Hot and dry summers tend to lead to apoplexy of the plants (acute form), whereas cool, rainy summers are conducive to the occurrence of GLSD or Esca proper (chronic form) (Surico et al., 2000). In the acute form, the grapevines initially exhibit normal sprouting during the spring season. However, in the event of a prolonged period of precipitation followed by elevated temperatures and subsequent drought, the foliage of plants may exhibit symptoms of wilting, necrosis may be observed, and the entire shoot or plant may undergo desiccation within a relatively brief interval (Viret and Gindro, 2025). As the above-ground parts of the plant undergo death, the grapevine's root system remains intact (Surico et al., 2006; Viret and Gindro, 2025). The chronic form is primarily characterized by the manifestation of conspicuous leaf symptoms. The affected plants exhibit irregular yellowish (in white cultivars) or reddish (in red cultivars) brightening that subsequently leads to desiccation between the veins. However, the main veins maintain a green to yellow-green coloration (Mugnai et al., 1999; Viret and Gindro, 2025). Firstly, symptoms become apparent on the lower leaves, and then they extend to the entire shoots. The berries of the grapevine may also exhibit blue-black spots, which are referred to as ”black measles“. Irrespective of the form of the disease, necroses manifest within the wood, which can exhibit both hard and soft consistencies. These necrotic regions are separated from the healthy wood by a dark border. Typically, soft white rot (“tinder“) is observed, caused by *F. mediterranea* (Viret and Gindro, 2025). Of particular concern after infection with Esca pathogens is that the asymptomatic phase can persist for an extended duration, often spanning years, prior to the manifestation of leaf symptoms (Chacón-Vozmediano et al., 2021). Additionally, symptoms on grapevine leaves typically exhibit inconsistent appearance, which can vary from year to year on individual plants (Mondello et al., 2018b).

The symptoms of Esca can be influenced by a variety of factors, including climate, soil type, grape variety, vineyard management practices, and vineyard physiology (Gramaje et al., 2018; Claverie et al., 2020). Pruning and hygiene measures can significantly influence the occurrence of the disease complex. Contaminated tools or planting material can promote Esca complex infections (Graniti et al., 2000). Climatic conditions, including elevated temperatures and humidity, have been demonstrated to exert a significant influence on the growth of fungal species. For instance, Fischer and Peighami Ashnaei (2019) demonstrated that drought triggers physiological and biochemical alterations in grapevines, thereby promoting the emergence of Esca symptoms. Drought stress has been demonstrated to result in an elevated level of certain metabolites in the xylem of grapevines, which has been shown to favor the growth of pathogens (Lima et al., 2017). A high soil water holding capacity and precipitation in late spring have been associated with a higher incidence of Esca symptoms (Monod et al., 2023). Stress factors for the grapevine, such as a lack of water or nutrients, also increase the risk of an outbreak of symptoms by reducing the plant's defenses (Kuldau and Yates, 2000; Schulz and Boyle, 2005).

It is interesting to note that the mere presence of typical Esca pathogens does not necessarily result in the occurrence of symptoms. A comparison of the microbiome between symptomatic and asymptomatic plants reveals a high degree of similarity between the two groups (Monod 2024). Consequently, the fungal species implicated in Esca disease complex may occur in a symbiotic association within the grapevine or exhibit a pathogenic effect. Depending on the environmental conditions, the type of relationship can alternate between commensalism, mutualism, and pathogenicity (Mishra et al., 2021).

In Southern Europe, the control of Esca pathogens was achieved through the implementation of dormant spray applications of sodium arsenite fungicides until the year 2003. However, these fungicides were subsequently prohibited due to their toxic effects on human health and the environment (Songy et al., 2019). To date, there are no comparably effective control mechanisms. Current measures are thus concentrated on preventive (e.g., use of high-quality mother vineyards, hot-water treatment prior to grafting, correct trunk training, pruning wound protection) and curative (e.g., remedial surgery, trunk renewal) mechanisms. These measures are expected to contribute to the reduction of the risk of infection and the subsequent propagation of the disease (Lecomte et al., 2011; Gramaje et al., 2018). Proper pruning plays a central role in this context, as pruning wounds can serve as entry points for pathogens. It has been demonstrated that large pruning wounds result in an increased formation of dead wood, which, in turn, provides a suitable environment for fungal development (Viret and Gindro, 2025). The Guyot-Poussard principle, a certain pruning method, aims to achieve continuous sap flow and minimize deadwood by means of targeted pruning. Ensuring optimal sap flow is contingent upon concentrating the pruning wounds on the upper part of the grapevine branches and preserving an unaltered part where the sap flow is not interrupted (Mondello et al., 2018a). The timing of pruning, as well as the specific grapevine cultivar, have been shown to play a notable role in determining susceptibility to the complex of pathogens. For instance, grapevines that are pruned at the onset of their growth cycle exhibit an extended period of vulnerability to wounding and a greater propensity to develop dieback symptoms. Sun et al. (2006, 2008) demonstrated that grapevines exhibit a response to injury, characterized by the production of tylose during summer months and the formation of pectin gels during winter. The formation of pectin gels in particular has been shown to serve as a substrate for pathogens. A promising approach to reducing the susceptibility of grapevines to Esca pathogens in nurseries involves the selection of rootstocks with narrow xylem diameters. These rootstocks exhibit a reduced susceptibility to *P. chlamydospora* and *P. minimum* compared to rootstocks with large xylem diameters (Ramsing et al., 2021). Furthermore, thermal processes such as hot water treatment are frequently employed in grapevine nurseries. While this results in alterations to the fungal community within the grapevine wood, it does not entirely eliminate the Esca pathogens (Eichmeier et al., 2018).

Presently, there is a lack of registered chemical active ingredients available for the management of Esca pathogens. Nevertheless, formulations with antagonistic fungi, such as *Trichoderma* spp. or *Fusarium* spp., are commercially available and have demonstrated a certain degree of efficacy in field applications (Mondello et al., 2018a). The utilization of effective, wood-colonizing BCA, which demonstrate a broad spectrum of activity against Esca pathogens over an extended timeframe, has the potential to serve as a successful and sustainable control strategy (Mondello et al., 2018b). *Trichoderma* spp. in particular have shown good effectiveness in protecting pruning wounds in studies (Kotze et al., 2011) and represent a potential tool for reducing mild Esca symptoms and apoplexy in the field (Bigot et al., 2020).

This study focuses on developing a sustainable strategy that incorporates *Trichoderma* spp. and chitosan. Therefore, the subsequent chapters provide more details on biocontrol and biostimulating agents, especially *Trichoderma* and chitosan, as well as regulations and perspectives for plant protection.

1.3 Biocontrol agents and biostimulants for environmentally friendly plant protection

The extensive use of pesticides can lead to various long-term negative effects on human health and the environment. Consequently, restrictions imposed by legislation, as well as consumers' desire for products that are as pesticide-free as possible, have created an urgent need for research into natural alternatives. The utilization of BCA and biostimulants constitutes a sustainable approach that serves as an environmentally friendly alternative to hazardous agrochemicals, fertilizers, and pesticides (Hamza and Suggars, 2001; Gupta and van Staden, 2021). While the modes of action of BCA and biostimulants may exhibit some overlap, differences in the primary mechanisms of action and the regulatory requirements of these biological agents are evident. Du Jardin (2015) offers a definition of plant biostimulants as substances or microorganisms "applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content". Furthermore, du Jardin (2015) adds that biostimulants can also be commercial products containing mixtures of such substances and/or microorganisms. The EU Fertilizer Regulation underscores the distinction between biostimulants and traditional fertilizers, highlighting that biostimulants do not provide nutrients. Rather, they optimize nutrient efficiency and utilization, thereby reducing the need for external nutrient input. The European regulation classifies biostimulants according to the EU Fertilizer Regulation (EU) 2019/1009, which has been in effect since July 16, 2022 (Regulation [EU] 2019/1009). Biostimulants are classified as a distinct product category, designated as product function category 6, which is defined as "plant biostimulants". In addition to the EU regulation, national requirements are applicable in certain instances, as for example outlined in Germany in the Fertilizer Act (DüngG) and the Fertilizer Ordinance (DüMV). Biostimulants can be classified into six non-microbial and three microbial categories: i) Chitosan, ii) humic and fulvic acids, iii) protein hydrolysates, iv) phosphites, v) seaweed extracts, vi) silicon, vii) arbuscular mycorrhizal fungi, viii) plant-growth-promoting rhizobacteria and ix) *Trichoderma* spp. (Cataldo et al., 2022). The application of biostimulants aims to improve plant nutrient use efficiency, tolerance to abiotic stresses, quantity traits, and/or availability of confined nutrients in the soil or rhizosphere (Regulation [EU] 2019/1009).

BCA are usually defined as living organisms that exhibit an antagonistic effect against pests (Jindo et al., 2022). A biological control measure is composed of three components: (1) a BCA, (2) a pest to be controlled, and (3) a farmer or stakeholder who stands to benefit from the implementation of the control measure (Stenberg et al., 2021). A variety of organisms, including but not limited to insects, mites, bacteria, nematodes, and fungi, can be utilized for the biological control of weeds, pests, and/or diseases (Ehlers, 2011). It should be noted that viruses which do not meet the criteria for living organisms but which contain structural biological components (e.g., nucleic acid, proteins) may also be considered BCA (Stenberg et al., 2021). According to Ehlers (2011), semiochemicals and plant extracts that have a direct effect on pathogens or pests can also be considered as BCA. BCA exhibit a diverse array of mechanisms that are employed in the course of their actions against pathogens. For instance, the ability of BCA to compete with pathogens for space, nutrients, water, and/or light has been demonstrated to deplete the pathogens of these essential resources, thereby leading to their suppression (de Almeida et al., 2020). Furthermore, the mechanisms of action of these organisms include the production of antimicrobial diffusible or volatile compounds (e.g., lytic enzymes, antibiotics), direct parasitism, and the induction of plant resistance (Palmieri et al., 2022). The effect of BCA can be influenced by a variety of factors. For instance, climatic factors, reproduction modes, and food availability can exert a substantial influence on the interaction between BCA and the target organism (Jindo et al., 2022). According to EU Regulation 1107/2009, microbial biological control agents that exert a direct effect on harmful organisms are classified as active substances and must be authorized as plant protection products (Regulation [EC] 1107/2009). In addition to an evaluation of the effect, it is imperative to ensure the safety of human health and the environment for the purpose of obtaining approval. A variety of data requirements have been established for microorganisms, as outlined by the European Union in EU Regulation 283/2013 (Commission Regulation [EU] 283/2013).

The properties of BCA and biostimulants, such as the induction of plant resistance and competition for nutrients and space, show a promising opportunity for the reduction of hazardous pesticides and a contribution to sustainable plant protection in viticulture (Cataldo et al., 2022; Jindo et al., 2022). The use of these eco-friendly agents is particularly relevant in the context of increasing regulatory restrictions. For instance, Directive 2009/37/EC of April 23, 2009, established by the EU Commission, mandated the implementation of measures aimed at reducing copper usage across relevant sectors (Commission Directive 2009/37/EC). In addition to the implementation of the copper minimization strategy, copper compounds were authorized as plant protection products on January 1, 2019, and incorporated into Annex I of the EU Regulation (EC) 1107/2009 (Regulation [EC] 1107/2009). However, they were also designated as candidates for substitution, and consequently, their approval was limited to a further seven years for the time being. Consequently, there is an urgent need to develop methods for the targeted reduction or replacement of copper active substances.

The subsequent section thus concentrates on two representatives of BCA and biostimulants: *Trichoderma* spp. and chitosan. These agents have the capacity to contribute to the sustainable control of fungal pathogens in viticulture.

1.3.1 *Trichoderma* species and chitosan – a sustainable tool for plant protection in viticulture

As a perennial crop, grapevines harbor a variety of microorganisms, for example, in their stem tissues or in their phyllosphere and fructosphere (Ranade et al., 2021; Viret and Gindro, 2025). It has been demonstrated that some naturally occurring microorganisms in grapevines exhibit a high degree of biocontrol potential, rendering them a suitable candidate for the development of sustainable plant protection strategies. This includes specific *Trichoderma* spp. (Carro-Huerga et al., 2020).

Trichoderma spp. predominantly occur in their asexual form (the teleomorphic form is Hypocrea) and are classified within the division Ascomycota, order Hypocreales, and family Hypocreaceae. *Trichoderma* spp. are wood-decaying, filamentous fungi that have the capacity to occur across a broad spectrum of environments worldwide, spanning all climatic zones (Chaverri et al., 2003; Kamala et al., 2015; Woo et al., 2023). *Trichoderma* spp. are described as cosmopolitan, saproptrophic fungi, which are able to colonize plants endophytically and communicate with them. These organisms demonstrate rapid growth and can be identified by their distinctive green pigmented conidia (Zin and Badaluddin, 2020). *Trichoderma* spp. are characterized by their capacity for facile isolation from their environment and reproducible cultivation under controlled conditions. Furthermore, the fungi can be sustained for an extended period, spanning multiple months, without any loss in viability or other characteristics (Tyśkiewicz et al., 2022). It is noteworthy that *Trichoderma* spp. possess the capacity to utilize a diverse array of substrates for their growth (Woo et al., 2023). *Trichoderma* spp. demonstrate the capacity to metabolize particular toxins through the action of enzymes, thereby exhibiting a degree of resistance to toxic chemicals, including fungicides, herbicides, and other organic pollutants (Cocaign et al., 2013; Akhtar and Mannan, 2020; de Padua and Dela Cruz, 2021; Escudero-Leyva et al., 2022).

A variety of mechanisms, including parasitism, antibiosis, enzymatic activity, the production of secondary metabolites, competition for nutrients and space, and the production and release of volatile organic compounds (VOC) contribute to the suitability of certain *Trichoderma* spp. as BCA. These mechanisms enable the sustainable reduction of plant diseases (Harman et al., 2004; Collinge et al., 2022). In addition to their capacity for direct biocontrol mechanisms, *Trichoderma* spp. have been shown to function as indirect biocontrol agents. These organisms have been observed to activate the plant's immune system or to stimulate plant growth (Woo et al., 2023). *Trichoderma* spp. thus form an interface between the two categories of biocontrol and biostimulation. For instance, Perazzolli et al. (2008) demonstrated that repeated spray applications of *Trichoderma* led to a substantial decrease in symptoms of downy mildew on grapevines. This effect was attributed to the activation of systemic and local resistance mechanisms. Moreover, Fiorentino et al. (2018) demonstrated a favorable impact on nutrient uptake and growth in diverse salad plants inoculated with *T. virens* and *T. harzianum*.

While numerous antagonistic *Trichoderma* spp. have been identified as promising candidates for biological control due to the aforementioned properties, their biocontrol activity can be significantly influenced by abiotic and biotic environmental parameters. For instance, Kredics et al. (2003) state that low temperatures in winter can influence the biocontrol activity of mesophilic *Trichoderma* strains. Additionally, *Trichoderma* spp. exhibit a limited capacity to withstand arid environmental conditions. The effective biocontrol potential of the fungus is also influenced by the pH value of the environment and the presence of other antagonistic microorganisms (Kredics et al., 2003). For instance, some biocontrol agents exhibit predatory behavior exclusively under nutrient-poor conditions. In this context, *Trichoderma* showed no direct attack on *Rhizoctonia solani* when fresh bark compost was added. The synthesis of chitinase by the *Trichoderma* strain was only observed to be activated under conditions of low cellulose availability. As a result, the production of enzymes exhibiting parasitic activity by the antagonistic fungus was subsequently observed (Pal and Gardener, 2006; Singh et al., 2020). A combination of *Trichoderma* spp. with chemical pesticides and metal ions should also be considered with regard to a sustainable plant protection strategy. The addition of a reduced rate of pesticides can yield an adequate level of support for the agents' effect against plant pathogenic fungi (Monte, 2001). This can contribute significantly to the stability and efficacy of an effective biocontrol and biostimulating complex, especially with regard to the need for copper reduction in the control of downy mildew in the vineyard. It has been demonstrated that specific *Trichoderma* spp. exhibit the capacity to accumulate metal ions, thereby establishing a basis for metal tolerance (Küpper et al., 2022). Accordingly, a combination of suitable *Trichoderma* spp. with a reduced rate of synthetic pesticides or metallic compounds is an essential prerequisite for an environmentally friendly plant protection strategy (Kredics et al., 2003; Küpper et al., 2022).

In order to achieve the most pesticide-reduced and complex protective effect possible from natural agents, it seems reasonable to combine several biological agents with each other. A suitable agent for this purpose is the natural biopolymer chitosan. Chitosan is an N-deacetylated derivative of chitin, a component of exoskeletons, fungal cell walls, and crustacean shells. In addition to its direct antimicrobial effect against pathogens, the biopolymer has been shown to elicit indirect modes of action by promoting various defense genes (e.g., pathogenesis-related genes) and enzymes in the reactive oxygen species scavenging system (e.g., superoxide dismutase and peroxidase) in host plants (Pichyangkura and Chadchawan, 2015; Kappel et al., 2022). Chitosan has been demonstrated to possess biostimulation properties, which include the stimulation of plant growth and an increase in abiotic stress tolerance (Pichyangkura and Chadchawan, 2015). The effect of the active ingredient is contingent upon the size and structure of the molecule, as well as its degree of acetylation (Kauss et al., 1989; Vander et al., 1998). In grapevine, the application of chitosan results in the formation of a semipermeable film surrounding plant tissue. It thereby stimulates the accumulation of phytoalexins, including trans- and cis-resveratrol, as well as the derivatives ε-viniferin and piceid, within grapevine leaves (Aziz et al., 2006; Romanazzi et al., 2017). Studies by de Bona et al. (2021) also highlight the effect of chitosan as an elicitor in grapevines and demonstrate that chitosan was able to induce a systemic resistance response against *Botrytis cinerea* by upregulating the jasmonic acid- and ethylene-mediated response and downregulating salicylic acid, while modulating trans-resveratrol. Chitosan is a promising alternative to conventional plant protection products, such as frequently applied copper products, in organic viticulture. This is due to its biocontrol and biostimulating properties, its natural occurrence, and its potential safe use for humans and the environment (Romanazzi et al., 2019).

The application of a range of BCA, each exhibiting a distinct mode of action, has the potential to ensure the continued effectiveness of biological agents in combating pathogens, even under field conditions (Pertot et al., 2017b). Furthermore, the presence of biological components with biostimulating properties can enhance the plants' resistance to pathogens (Jindo et al., 2022). *Trichoderma* and chitosan, in particular, offer an optimal basis for sustainable pathogen control due to their properties and modes of action. This is particularly crucial in the context of organic viticulture, where the development of effective strategies for reducing copper levels is essential.

In order to address this need and maximize copper-reduced control of *P. viticola*, it is necessary to align and harmonize all agents involved in the sustainable strategy. It has been demonstrated that certain copper- and chitosan-tolerant *Trichoderma* isolates possess the capacity to impede the development of plant pathogenic fungi by releasing a variety of cell wall-degrading enzymes (Saravanakumar et al., 2016). Chitosan has been shown to bind copper through the donation of a free electron pair, thereby forming an effective antifungal complex (Varma et al., 2004; Salama, 2021). A synergistic triple combination of *Trichoderma*, chitosan, and a reduced copper rate may further enhance the individual effects of all agents. The enzymes (chitosanases and cellulases) released by such tolerant *Trichoderma* isolates, on one hand, enzymatically decompose chitosan into its highly bio-effective oligomers and, on the other hand, decompose cell wall components of the pathogen. Consequently, antifungal agents secreted by *Trichoderma* as well as chitosan can act directly on the intracellular components of the pathogen, thereby contributing to its growth inhibition (Bohra, 2018; Malerba and Cerana, 2020). This finding suggests the potential for an effective, copper-reduced, and sustainable crop protection tool.

The tool described here can also be transferred to combat other diseases, such as the Esca disease complex. Against Esca, no chemical control options are currently available, but its importance will continue to increase as climate change progresses.

1.4 Research objectives

Viticulture faces considerable challenges in combating diseases such as downy mildew, caused by *P. viticola*, and wood-decaying diseases, such as the Esca disease complex, both of which must be addressed effectively and sustainably. Organic viticulture, in particular, is subject to a stringent set of guidelines. In the context of organic viticulture, the utilization of copper-based compounds plays a critical role in the management of *P. viticola*. Growing political and social demand for reduction and substitution of these products due to mounting ecotoxicological concerns is increasingly evident. Concurrently and in the context of climate change, there is an absence of effective, sustainable solutions to address Esca. In this regard, the development of novel biological approaches, founded on synergistic combinations of active agents, is of paramount importance. These novel tools will facilitate sustainable, pesticide-reduced, and effective management of economically important pathogens.

The present work addresses this need by developing and evaluating a sustainable plant protection strategy against fungal diseases in viticulture. To this end, compatible combinations of various *Trichoderma* spp. and chitosans were selected, and the efficacy and applicability of the synergistic strategies were evaluated in relation to a copper-reduced application against downy mildew (*P. viticola*) as well as for the control of Esca pathogens.

Since many *Trichoderma*-based products contain strains isolated from soil, which may not be adapted to above-ground plant parts and survive outside their original habitat, first, it was necessary to isolate and characterize *Trichoderma* strains with a high survivability and antagonistic potential which occur naturally in grapevine and are adapted to the "ecosystem" grapevine. In order to achieve a reduction in the use of copper fungicides within a control strategy that incorporates *Trichoderma* as a biological agent to combat downy mildew, it is essential that *Trichoderma* exhibits a certain degree of copper tolerance. Consequently, the previously characterized *Trichoderma* isolates were examined with regard to their compatibility with common copper formulations. A copper formulation was identified that exhibited a high degree of compatibility with *Trichoderma*. Since organic viticulture usually involves regular treatment with copper preparations against *P. viticola* at intervals of around ten days, the next step was to carry out tests to increase the copper compatibility of suitable selected *Trichoderma* strains. The copper tolerance exhibited by two *Trichoderma* candidates, *T. koningiopsis* and *T. harzianum*, should thus be adapted to high concentrations of the selected fungicide. Accordingly, the initial study was conducted with the objective of selecting and characterizing robust *Trichoderma* strains that have adapted to the grapevine ecosystem. These strains were selected for their ability to maintain biological effectiveness under conditions of high, realistic copper stress when the grapevine is treated (chapter 2).

In order to integrate the second agent, chitosan, into the synergistic control strategy, the chitosan tolerance of the previously selected *Trichoderma* isolates was investigated in a subsequent study, building upon the findings of the initial investigations. The objective of this study was to identify optimal combinations of copper-tolerant *Trichoderma* isolates and suitable chitosans for a sustainable and copper-reduced control strategy against *P. viticola.* In addition, the study aimed to verify and characterize their pathogen control effect in more detail. Consequently, the effect of combinations of copper- and chitosan-tolerant *Trichoderma* isolates and chitosan was examined in relation to their efficacy against downy mildew in grapevines. In order to achieve the most effective control of the pathogen, the ideal treatment time was identified as the point at which the agents demonstrated the highest degree of effectiveness against downy mildew. Furthermore, the investigation aimed to enhance understanding of the mechanisms underlying the biocontrol and biostimulating properties of the agents in treating the pathogen-inoculated plant. This objective was approached through more detailed investigations involving the abaxial (direct effect on the pathogen) and adaxial (indirect effect on the pathogen) sides of grapevine leaves. Subsequent to the verification of the direct and indirect effects of the agents on *P. viticola*, the most promising *Trichoderma*-chitosan combinations were evaluated with regard to their efficacy in dual and triple applications, in conjunction with the addition of a reduced copper rate, on grapevines infected with downy mildew. In order to enable uniform protection of the grapevine leaf apparatus when treated with the biocontrol and biostimulating complex, the distribution of chitosan and the establishment of *Trichoderma* after single, dual, or combined treatment with a reduced copper rate were also examined in more detail. These findings contribute substantially to the further development and characterization of a sustainable and effective biocontrol and biostimulating complex consisting of *Trichoderma* and chitosan. This complex could serve as a useful tool to reduce copper doses in the control of downy mildew on grapevines to an ecologically acceptable minimum without compromising disease control (chapter 3).

Based on the previously obtained results, the indirect mechanisms of action of the synergistic complex of a copper- and chitosan-tolerant *T. koningiopsis* isolate and chitosan in interaction with the host plant (*V. vinifera*) and the pathogen *P. viticola* should be characterized in more detail. The work should provide information on whether the complex treatment can modify the plant's defenses and thus address another target (grapevine) in addition to the direct pathogen effect already demonstrated in previous work. Therefore, the study proceeded to analyze plant defense reactions, including the expression of resistance and the accumulation of reactive oxygen species (ROS) following treatment with the active complex and pathogen inoculation. A possible resistance-inducing effect of the complex treatment was investigated on different grapevine genotypes in order to obtain a clear characterization of the effect. Microscopic studies were used to demonstrate the effect on downy mildew at the cellular level of the treated grapevines and to further investigate the effect of the complex on the development of the pathogen's haustoria, an essential structure for nutrient uptake by *P. viticola.* The knowledge gained is of great importance to the further understanding of the tri-trophic interaction between the synergistic active complex, the host plant, and the pathogen. At the same time, it provides a scientific basis for the optimization of the use of environmentally friendly alternatives to copper-based fungicides in viticulture (chapter 4).

In order to develop a multifaceted plant protection strategy that maintains grapevine health and demonstrates broad-spectrum effectiveness against relevant pathogens, the synergistic active complex was investigated for its efficacy against Esca pathogens. In contrast to downy mildew on grapevine (*P. viticola*), which as a leaf-related disease usually has a direct effect on yield and grape quality, Esca pathogens are able to cause wood-destroying diseases that can endanger the vitality of the grapevines in the long-term and cause entire plants to die. The objective of a further study was therefore to investigate the potential of the previously tested synergistic combination of *Trichoderma* and chitosan as an eco-friendly alternative for protection against Esca pathogens, since there are currently no chemical control measures available. The investigative focus was thus directed toward the cultivation of the biological agent *Trichoderma* in grapevine wood and shoots, in addition to the investigation of its direct and indirect biocontrol effects against the important Esca pathogens *P. chlamydospora*, *P. minimum*, and *F. mediterranea*. Following the confirmation of the successful colonization of the grapevine wood by *Trichoderma* spp. and the observation of both direct and indirect control effects, initial field applications of the *Trichoderma*-chitosan complex were conducted in conjunction with a reduced copper application. This approach was adopted to obtain information regarding the effectiveness of the tool under challenging conditions. The findings yielded insights into the application potential of the synergistic complex for Esca control (chapter 5).

In summary, the present work is intended to contribute to the development of a sustainable and effective strategy against the economically important pathogens *P. viticola* and different Esca pathogens and to address central requirements of sustainable viticulture.

2 *Trichoderma* species isolated from grapevine with tolerance towards common copper fungicides used in viticulture for plant protection

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2.1 Abstract

Background:

Copper-containing fungicides are broadly applied in organic viticulture against downy mildew caused by *Plasmopara viticola.* Although long-term application of copper-based fungicides is associated with ecotoxic effects on the environment, their use in viticulture is still required until sustainable alternatives are available. *Trichoderma* spp. might be a promising approach to fungicide reduction while promoting plant growth and development and displaying biocontrol activity. The study aims to examine the tolerance and compatibility of *Trichoderma* spp. to copper fungicides. This work contributes to the development of a spray application consisting of a copper-tolerant *Trichoderma* sp. combined with a downscaled copper fungicide rate against *P. viticola.*

Results:

Trichoderma spp. isolated from grapevine wood in vineyards were identified and used for tolerance screening in various concentrations of copper fungicides. Copper hydroxide was identified as being highly compatible with *Trichoderma.* Two *Trichoderma* candidates, *T. koningiopsis* and *T. harzianum*, showed high copper tolerance in mycelial growth and germination tests, and were adapted to 2.85 g Cu $L^{-1}$of the selected fungicide. Microscopic investigations showed the attachment of copper compounds to fungal cell walls and copper uptake within the cytoplasm. In the case of high tolerance, large-scale copper uptake was prevented.

Conclusion:

Our findings identified two highly copper-tolerant *Trichoderma* isolates with natural adaptation to the vineyard ecosystem, which could be further tested as biostimulants and biocontrol agents, combined with a reduced fungicide rate for sustainable plant protection.

Keywords: *Plasmopara viticola*, copper tolerance, biostimulants, downy mildew

2.2 Introduction

Since the end of the 19th century, copper-containing fungicides have been applied broadly in vineyards worldwide to protect grapevine against diseases, mainly downy mildew caused by *Plasmopara viticola.*[1] As a consequence, the widespread and constant use of copper fungicides has led to various negative impacts for the environment.[2] To control *P. viticola* in Europe, copper amounts of about 3–4 kg ha^{-1} over a period of up to ten treatments are applied.[3] Copper use in Europe is regulated by EU Commission Regulation 2018/1981 of 13 December 2018.[4] Repeated long-term application of copper-based fungicides and the subsequent run-off from treated plants may lead to the contamination of surface water and abundant copper accumulation in soils.[2] Depending on the amount and the bioavailability of copper in soils, plant growth and development are negatively affected.[5] In particular, the growth of young grapevines and cover plants with shallow root systems may be affected by high copper topsoil concentrations resulting from former applications. However, the use of copper-based fungicides in viticulture and other crops is still tolerated and is required until effective alternatives are available.

Biostimulants may prove a favorable tool with which to reduce hazardous agrochemicals, fertilizers and pesticides.[6] European regulation establishes biostimulant marketing as either plant protection products or fertilisers. Plant protection products are defined as influencing life processes in a way that differs from nutrients, for example microorganisms. Certain biostimulants, such as chelating agents, are classified as fertilisers because they primary improve plant nutrition and growth.[7] The properties of biostimulants, such as biocontrol capacity[8] and plant growth enhancement[9], present a promising opportunity to contribute to sustainable plant protection. *Trichoderma* spp. show an array of antagonistic and biostimulating modes of action. Perazzolli *et al.*[10] found a significant reduction in downy mildew symptoms upon repeated spray treatment with *Trichoderma* on grapevine, which they attributed to the activation of systemic and local resistance. Fiorentino *et al.*[11] reported a positive effect on nutrient uptake and the growth of different salad plants inoculated with *T. virens* and *T. harzianum*. The combination of suitable *Trichoderma* spp. with synthetic pesticides or metallic compounds constitutes an essential prerequisite for an environmentally friendly plant protection strategy.[12] Because certain filamentous fungi show the ability to accumulate metal ions[13-15] a basis for metal tolerance is formed. Although effective copper reduction strategies are required in organic viticulture, there is less information about the effects of copper fungicides and their associated copper salts and other ingredients, such as solvents and detergents, on *Trichoderma* species.

This study contributes to a sustainable plant protection strategy against *P. viticola* consisting of *Trichoderma* combined with a reduced amount of copper fungicide. Therefore, *Trichoderma* spp. with biocontrol potential and high copper tolerance need to be identified. To select *Trichoderma* isolates showing antagonistic potential, candidates were isolated from grapevine wood that had never been in contact with *Trichoderma*-based products. Although several *Trichoderma*-based products are sold, their benefits are often limited because growth, propagation and biological activity depend on environmental conditions. Many products contain strains isolated from soil, which may not be adapted to above-ground plant parts and survive outside their original habitat. Therefore, strains naturally occurring in grapevine were isolated and characterized. Furthermore, copper fungicides at various concentrations were tested for their compatibility with *Trichoderma* in mycelial growth and germination tests. To ensure fungal survivability in the selected copper fungicide, the copper tolerance of the most suitable candidates was improved by fungicide adaptation using increased concentrations. The effects of copper on *Trichoderma* were investigated microscopically.

2.3 Materials and Methods

2.3.1 Sample collection and isolation of wood-colonizing *Trichoderma* spp.

Wood samples from grapevines growing in Rhineland-Palatinate (Germany) were taken in the years 2010 to 2012 from cordon branches, trunks and wooden shoots. Only wood that has not come into contact with *Trichoderma*-based products was used. All samples were decorticated, sterilized and pieces were transferred to malt extract agar (MEA, 2% malt extract, Arche Naturküche, 2% agar, Roth; 0.01% tetracycline, Roth) for incubation at 21 °C and 70% relative humidity (RH). The recovered colonies were purified on MEA 2% according to Siddiquee[16]. In total, 130 isolates were collected, identified and screened for their copper tolerance.

2.3.2 Molecular methods for identification of *Trichoderma* spp.

2.3.2.1 DNA extraction

The DNA extraction method followed the user manual for the REDExtract-N-Amp Plant PCR Kit (Sigma Aldrich). Deviating from the instructions, high-molecular mass DNA was extracted from a spore suspension. Spore suspensions were adjusted to 10^6 spores per ml using a haemocytometer. One ml of spore suspension was transferred to 2.0-ml microfuge tube, and 0.5 ml of glass beats (diameter 1.0 mm, Roth) and extraction solution were added. Spores were homogenised for 3 x 1 min at 30 000 rpm in a ball mill.

2.3.2.2 PCR and sequencing

The nuclear rDNA internal transcribed spacer region (*ITS*) and the translation elongation factor 1-alpha (*Tef-1a*) gene were amplified by polymerase chain reaction (PCR) using the universal primer pairs ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3')[17] and EF1-728F (5'-CAR CGA GAA GTT CGAGAA GG-3') and EF1-986R (5'-TAC TTG AAG GAA CCC TTA CC-3')[18], respectively. Genomic DNA was amplified in accordance with the user manual. PCR was programmed with an initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 54,5 °C (*ITS*) or 57 °C (*Tef-1a*) for 30 s, extensions at 72 °C for 60 s and the final extension at 72 °C for 10 min. The amplified product was visualized by electrophoresis (1,5% agarose gel, 1x TBE buffer), stained with Midori Green (Nippon Genetics Europe).

PCR products were purified using GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd.) in accordance with the user manual. Sequences were edited and assembled at MWG Biotech. Using BioEdit software, the obtained nucleotide sequences were proven in consideration of the respective chromatograms.[19] Species identification was carried out via NCBI Nucleotide BLAST, TrichOKEY second version[20] and TrichoMARK.[21] In addition, all strains were grown on different media (MEA, Czapek Dox agar [CDA] and potato dextrose agar [PDA]) for two weeks (21 °C, 70% RH,12:12 h light/dark cycle) allowing macroscopical characterisation, for example colour, staining of the medium and arrangement of sporophores according to Gams and Bissett[22].

2.3.3 Copper tolerance analysis

2.3.3.1 Selection of *Trichoderma* candidates and suitable copper fungicide agents

Copper tolerance tests were performed to both, identify a suitable fungicide for dual applications and simultaneously select *Trichoderma* isolates showing natural tolerance. Therefore, mycelium growth of all 130 isolates was measured on CDA minimal medium amended with copper fungicides and compared with colonies grown on medium without copper. The copper fungicides Cuproxat® (tribasic copper sulfate, Biofa AG; suspension concentrate [SC]), Cueva® (copper octanoate, W. Neudorff; SC), Cuprozin® progress (copper hydroxide, Biofa AG; SC) and Funguran® progress (copper hydroxide, Biofa AG; wettable powder [WP]) were used in various concentrations (Table 1). The used concentrations are linked to field rate and depend on leaf surface and fruit development. Fungicide effort required at budburst and the beginning of leaf growth (until Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie [BBCH] 16) is given as (1x) the field rate (declared by the producer in kg ha^{-1}) and is adapted to higher rates (up to 4x the field rate) depending on grapevine development. Instead of commercial names, active ingredients are used in subsequent sections. Because the active ingredients are the same in Cuprozin® progress and Funguran® progress (copper hydroxide), different types of formulations are added for differentiation.

Fungal isolates were preincubated on MEA (2%) at 21 °C and 70% RH for 7 days. Discs (6 mm diameter) were cut out from the margin area and placed on the centre of the plates. Samples were incubated (21 °C, 70% RH) for 14 days. Meanwhile, fungal colony development was measured six times and the average growth was calculated (horizontal and vertical mycelial growth). In a second step, isolates with high copper tolerance were identified. Isolates showing growth rates of a minimum 50% compared with the control were selected for further investigations. Growth of all candidates was also equally tested on nutrient-rich MEA (2%) medium.

2.3.3.2 Fitness assay

Spore germination capacity was analysed in copper-amended liquid malt extract broth (MEB, 2%), using 0.5x and 1x the field rate of Funguran® progress and control medium. Cultures grown in media described in 2.3.3.1 were used for conidia collection. One millilitre of spore suspension (10^6 spores per ml Aqua distilled) was transferred to a microfuge tube and centrifuged (11 900 x g, 10 min). Spore pellets were solved in liquid medium and 50 µl spore medium mixture was incubated (23 °C for 18 h). Using a light microscope, 100 randomly selected spores were characterized as germinated or non-germinated. Samples not treated immediately were stored at 4 °C until examination. Spores were considered to have germinated if a germ tube was apparent. A selection limit of 50% germinated spores was determined to identify copper-tolerant isolates. To discriminate the most tolerant isolates, mycelial growth was examined on MEA (2%) amended with Funguran® progress representing 0.5x and 1x the field rate. The experimental setup was the same as the procedure described above.

2.3.3.3 Copper tolerance adaptation

To enhance copper tolerance, preselected isolates were cultivated in flasks containing MEB (2%) amended with different fungicide concentrations according to the adaptation procedure of Becher *et al.*[23] Starting with 2.5-fold field rate of Funguran® progress (0.03125 g L^{-1} MEB), the fungicide rate was incrementally increased to tenfold (0.125 g L^{-1} MEB). To maintain tolerance, isolates were cultivated on copper-free medium for three generations followed by a transfer back to MEA (2%) containing tenfold field rate. Tolerance to Funguran® progress was expressed by a comparison of radial growth and conidial germination in wild-type and adapted isolates on MEA (2%) or MEB (2%) containing different fungicide concentrations (2.5x, 5x, 7.5x, 10x the field rate). Mycelial growth was measured daily over a 10-day period. Each distinct combination of isolate and fungicide concentration was tested. A spore germination assay was conducted as described in 2.3.3.2.

2.3.3.4 Microscopic analysis

Rhodanine staining was used to describe effects of copper salts on four selected isolates (two highly tolerant and two less tolerant) according to Lindquist.[24] Seven-days-old conidia were adjusted to 10^6 spores per ml Aqua distilled. Ten microlitres of each suspension were cultivated in 1 ml MEB (1%). The medium was supplemented with Funguran® progress (field rate, 0.0125 g L^{-1}), $Cu(OH)_2$ (0.0044 g L^{-1} [, 0.00045 mol]), $CuS0_4$ (0.0029 g L^{-1}), $ZnS0_4$ (0.0029 g L^{-1}), $FeS0_4$ (0.0025 g L^{-1}) and $MnS0_4$ (0.0025 g L^{-1}). Each metal salt concentration equals the amount of $Cu(OH)_2$ in Funguran® progress. MEB without additional salts was used as control. Non-copper salts were used to ensure the copper specificity of the staining procedure. After incubation (14 days, 1 x g), fungal mycelia and metals salts were separated by centrifugation (5700 x g, 10 min). One millilitre of rhodanine staining solution was added to each sample. All samples were embedded in glycerine (80% v/v) and examined microscopically.

2.3.4 Data analysis

For statistical analysis, IBM SPSS Statistics version 28 was used. Data were interpreted by one-way analysis of variance (ANOVA) and non-parametric tests (Kruskal-Wallis-H).

Tukey's test or pairwise comparison ($p \leq 0.05$) was used to determine differences among means. Different letters in the figures indicate significant differences between variants.

2.4 Results

2.4.1 Identification of *Trichoderma* spp. naturally occurring in grapevines

To identify *Trichoderma* spp. for a successful use in organic plant protection, promising isolates have to show a high survivability and antagonistic potential within the "ecosystem" grapevine. In total, of 97 of 130 isolates naturally occurring in Rhineland-Palatinate were successfully identified as belonging to 14 different species. The species *T. harzianum* and *T. atroviride* were represented most frequently (Table S1). Thirty-three isolates could not be assigned to any species and need to be characterized in more detail. The inspected locations showed various *Trichoderma* species, which reflects the high survivability and assertiveness towards other organisms in grapevine-growing regions.

2.4.2 *Trichoderma* isolates exhibit copper tolerance during mycelial growth and spore germination in fungicide media

To evaluate differences in the compatibility of the tested copper fungicides to *Trichoderma*, mycelial growth of *Trichoderma* isolates was analyzed in different field rates of copper fungicides (Table 1). Highest growth rates reflected the most suitable copper fungicide formulation. The results revealed best biocompatibility and various suitable candidates for copper hydroxide (WP), in which significantly higher growth rates were observed compared with the other fungicides tested (Figure 2). For copper hydroxide (WP) an average mycelial growth inhibition of 34% compared with control was reported, whereas growth inhibition with control for copper octanoate (45%), copper hydroxide (SC) (44%) and tribasic copper sulfate (40%) was approximately 5%–10% higher. Thus, copper hydroxide (WP) was selected for use in further tolerance assays.

Table 1: Conversion values of the applied field rates of different copper fungicides added to Czapek Dox agar and malt extract agar (2%)

Applied field rate[a]	**Unit**[b]	**Copper fungicide**			
		Cuproxat®	**Cuproxin® progress**	**Cueva®**	**Funguran® progress**
		Active ingredient: $CuSO_4$	Active ingredient: $Cu(OH)_2$	Active ingredient: $C_{16}H_{30}CuO_4$	Active ingredient: $Cu(OH)_2$
0.25x field rate	**mg Cu L^{-1}**	59,00	40,71	45,40	71,24
	mmol Cu L^{-1}	0,93	0,64	0,71	1,12
0.5x field rate	**mg Cu L^{-1}**	118,01	81,42	90,79	142,48
	mmol Cu L^{-1}	1,86	1,28	1,43	2,24
0.75x field rate	**mg Cu L^{-1}**	177,01	122,13	136,19	213,72
	mmol Cu L^{-1}	2,79	1,92	2,14	3,36
1x field rate	**mg Cu L^{-1}**	236,02	162,84	181,58	284,96
	mmol Cu L^{-1}	3,71	2,56	2,86	4,48
2x field rate	**mg Cu L^{-1}**	472,03	325,67	363,16	569,93
	mmol Cu L^{-1}	7,43	5,12	5,71	8,97

[a] Copper concentrations were calculated with regard to the field rates used in viticulture and adjusted to concentrations that equal 0.25x, 0.5x, 0.75x, 1x and 2x the field rates.

[b] Associated copper concentrations in mg Cu L^{-1} and mmol Cu L^{-1} are given as standard values.

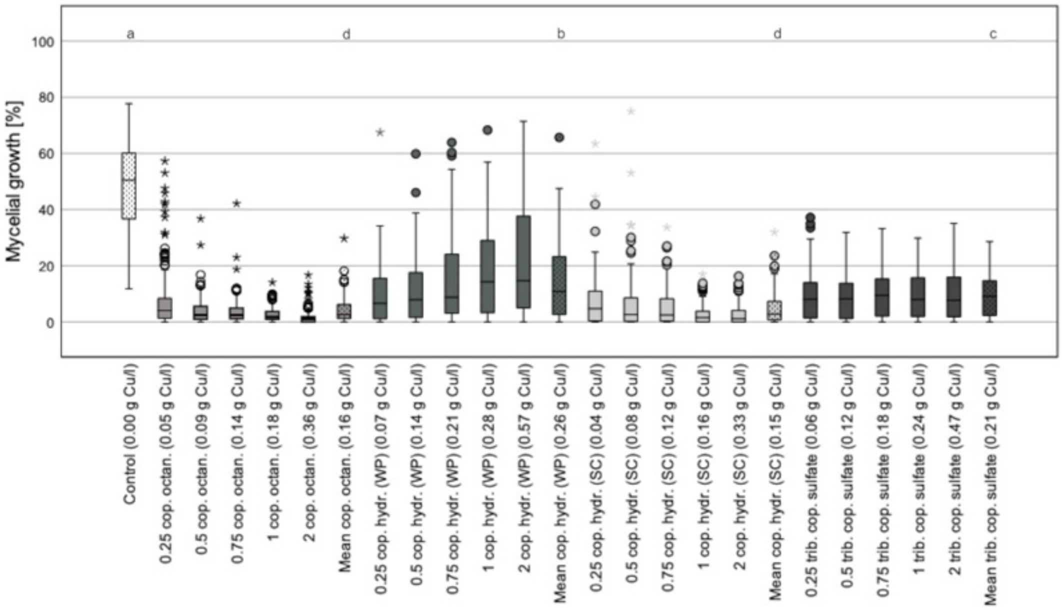

Figure 2: Effect of different copper fungicides on mycelial growth rates of *Trichoderma* species. Mycelial growth was measured on Czapek Dox agar amended with different concentrations (0.25x, 0.5x, 0.75x, 1x and 2x the field rate) of the copper fungicides with the active ingredients copper octanoate, copper hydroxide (WP), copper hydroxide (SC) and tribasic copper sulfate. Concentration factor refers the field rate specified for each fungicide. Within the control, no copper was used. Boxplots represents the average of two independent experiments. In each experiment, mycelial growth was measured for 130 isolates with 6 replicates per isolate, fungicide and concentration. Values are shown as means ± SD. "Mean" of each fungicide represents the average of all respective concentrations. Kruskal-Wallis-H was used to determine the effects between the concentration means of different fungicide formulations. Means were compared by pairwise comparison. Means with different letters (a, b, c, d) are significantly different ($p \leq 0.05$).

To identify copper-tolerant *Trichoderma* candidates showing high growth performance in copper hydroxide (WP), growth rates were calculated with regard to the growth of the corresponding controls. If growth rate exceeded a limit value of 50% mycelial growth, the respective *Trichoderma* isolates were selected for further investigations (Figure 3). A total of 28 isolates showed low mycelial growth inhibition. *Tr80* and *Tr56* showed the lowest mycelial growth inhibition compared with control of 4% and 23%.

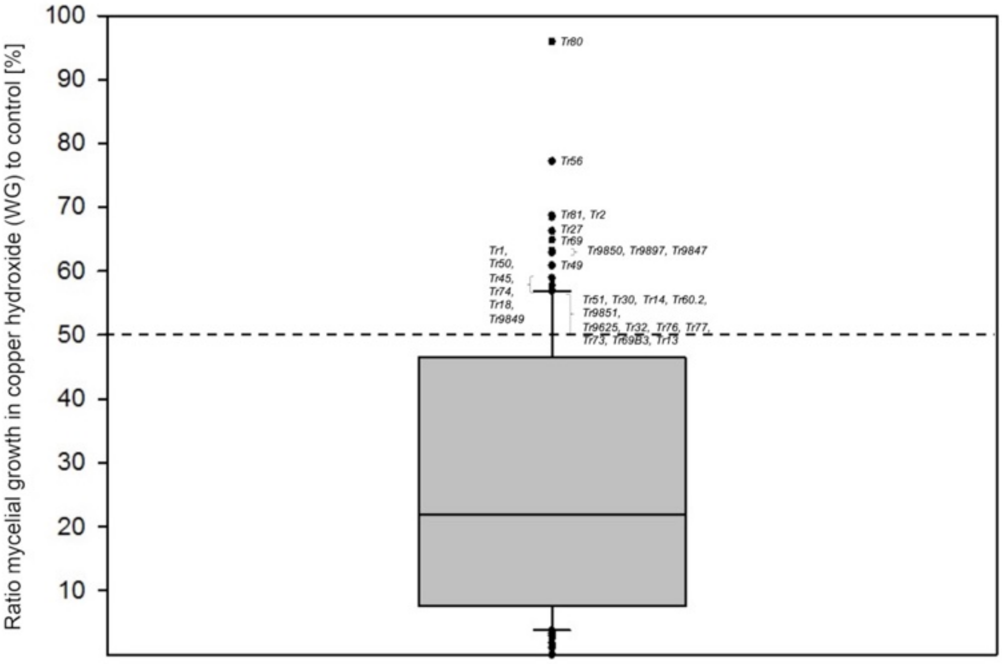

Figure 3: Selection of *Trichoderma* candidates showing high mycelial growth rates over 50% in copper hydroxide (WP). Ratio of the average mycelial growth rates of isolates to the corresponding controls is shown for Czapek Dox agar medium amended with copper hydroxide (WP). Boxplot represents the average of two independent experiments. Each experiment represents the average of 130 isolates replicated 6 times. Isolates were tested in five different concentrations of copper hydroxide (WP) (0.25x, 0.5x, 0.75x, 1x and 2x the field rate), the average mycelial growth of all concentrations is shown ± SD. Isolates exceeding the selection limit value of 50% mycelial growth (dotted line) are indicated with isolate designation regarding their achieved growth rate.

In addition, the growth of the 28 selected isolates was tested in complex medium to verify a similar growth performance as in minimal medium. Figure 4 shows significantly higher mycelial growth rates within the concentration means of copper hydroxide (WP) compared with copper octanoate and tribasic copper sulfate. For copper hydroxide (WP and SC) an average mycelial growth inhibition of 58% and 60% compared with control was reported, whereas growth inhibition compared with control for copper octanoate (72%) and tribasic copper sulfate (78%) was approximately 15–20% higher. The compatibility of copper hydroxide (WP) to the selected candidates was confirmed and further analyzed in complex medium. Complex medium allowed higher growth rates and an improved discrimination of copper-tolerant strains with the different fungicide concentrations (Figure 4). Growth inhibition correlated with copper dose for copper octanoate, copper hydroxide (SC) and tribasic copper sulfate. However, copper hydroxide (WP) was tolerated by the selected candidates at concentrations up to double the field rate.

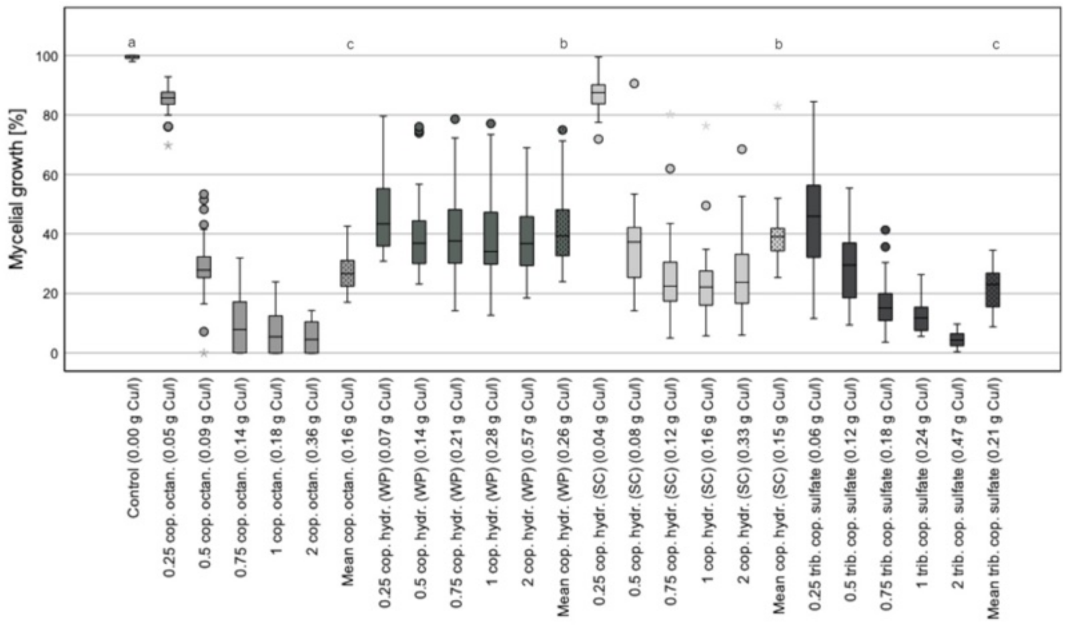

Figure 4: Effect of different copper fungicides on mycelial growth rates of 28 *Trichoderma* candidates in complex medium. Mycelial growth was measured in malt extract agar amended with different concentrations (0.25x, 0.5x, 0.75x, 1x and 2x the field rate) of the copper fungicides with the active ingredients copper octanoate, copper hydroxide (WP), copper hydroxide (SC) and tribasic copper sulfate. Concentration factor relates to the field rate specified for each fungicide. Within the control, no copper was used. Boxplots represent the average of two independent experiments. In each experiment, mycelial growth was measured for 28 isolates with 6 replications per isolate, fungicide and concentration. Values are means ± SD. "Mean" of each fungicide represents the average of all respective concentrations. Kruskal-Wallis-H was used to determine the effects between the concentration means of different fungicide formulations. Means were compared by pairwise comparison. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

Because field applications are performed with spore suspensions, spore germination rates of preselected isolates were analysed and defined as sufficiently fit once a 50% limit was exceeded in copper medium compared with control. Eight isolates were successfully selected when exposed to copper hydroxide (WP) (Figure 5).

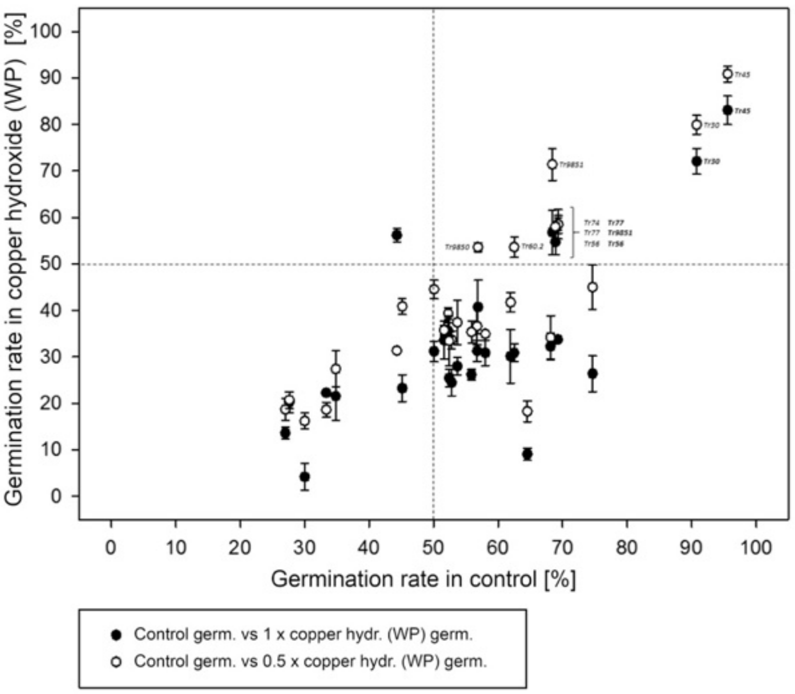

Figure 5: Selection of *Trichoderma* candidates showing spore germination rates over 50% in copper hydroxide (WP). Ratio of the germination rates of 28 isolates to the corresponding controls are shown within malt extract liquid medium amended with different concentrations of copper hydroxide (WP). Dots represent the average of three independent experiments. Each spore germination experiment was conducted with five replicates per isolate. Error bars show standard deviation. Isolates exceeding the selection limit value of 50% mycelial growth (dotted lines) are indicated with isolate designation regarding their achieved growth rate.

To evaluate differences in growth fitness of the eight candidates, mycelial growth was investigated under the influence of 0.5x and 1x the field rate of copper hydroxide (WP). *T. koningiopsis* (*Tr30*) and *T. harzianum* (*Tr56*) showed the highest mycelial growth rates in comparison with the others (Figure 6). These isolates were able to overgrow the copper medium within the trial period and showed no inhibition of mycelial growth compared with control in either copper concentration (17 dpi). Mycelial growth inhibition compared with control for the other candidates varied from 23% to 43% (0.5x the field rate) and 21% to 46% (1x the field rate). Based on their high growth rates and because they were less affected, *T. koningiopsis* (*Tr30*) and *T. harzianum* (*Tr56*) were selected for further investigations.

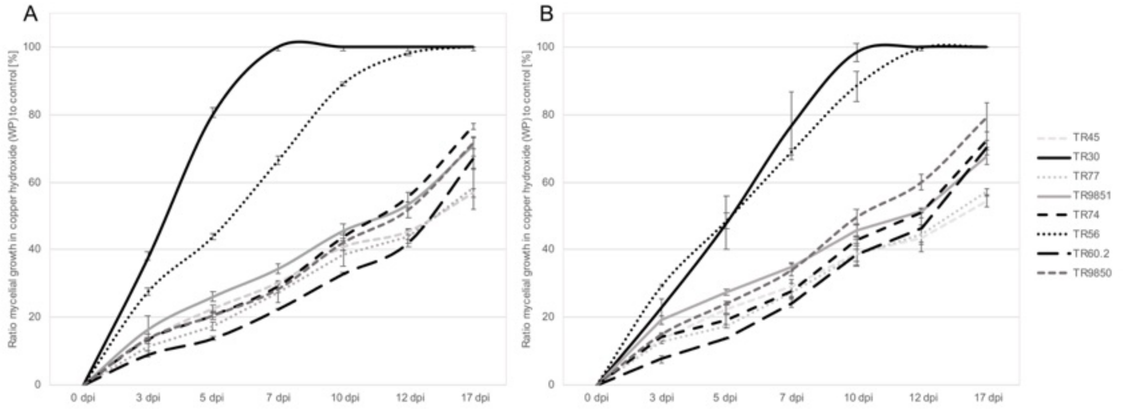

Figure 6: Effect of copper hydroxide (WP) on mycelial growth rates of *Trichoderma* species. Ratio of the mycelial growth of eight isolates to the corresponding controls is given as growth curves in malt extract agar amended with copper hydroxide (WP) until 17 days post inoculation (dpi). Mycelial growth curves are shown for 0.5x (A) and 1x (B) the field rate of copper hydroxide (WP). Curves represent the average of three independent experiments. Each experiment was conducted with five repetitions per isolate. Error bars show standard deviation.

2.4.3 Adaptation of *T. koningiopsis* and *T. harzianum* to tenfold field rate of copper fungicide

To enhance copper tolerance and ensure the viability of the two preselected candidates, isolates were incrementally adapted to the tenfold field rate of copper hydroxide (WP) (2,85 g Cu L^{-1}). Mycelial growth rates and germination capacity of initial isolates and adapted strains were compared by using copper and control media (Figure 7). Both copper-tolerant isolates showed higher growth rates in copper medium than the corresponding initial isolates. The copper-tolerant isolate of *T. koningiopsis* (*TrF830*) at 10 dpi showed mycelial growth enhancement of near to 30% the growth rate compared with the initial isolate (*Tr30*) and mycelial growth was inhibited only 4% compared with control. In case of initial isolate, growth inhibition of 31% compared with control was observed (Figure 7A). *T. harzianum* (*TrF856*) showed greater growth inhibition than *TrF830* (36% growth inhibition compared with control). However, adapted strains showed 19% less growth inhibition than initial isolates of *T. harzianum* (*Tr56*) (Figure 7B). Controls of *T. koningiopsis* and *T. harzianum* in copper-free medium showed no noticeable differences, which indicated no fitness effect through the adaptation procedure.

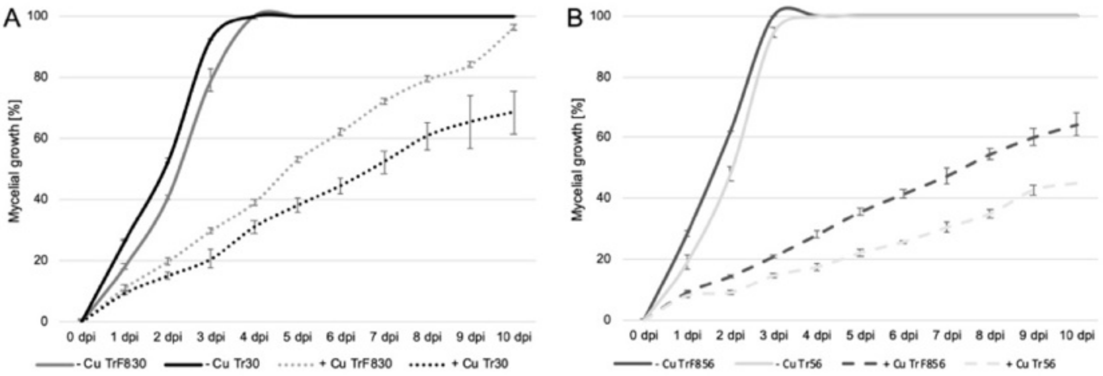

Figure 7: Effect of copper tolerance enhancement on mycelial growth of *Trichoderma* spp. using copper hydroxide (WP). Mycelial growth rates of initial isolates and copper-adapted isolates in malt extract agar amended with copper hydroxide (WP) are given as growth curves until 10 days post inoculation (dpi). Mycelial growth curves are shown for: (A) *T. koningiopsis* as the initial isolate (*Tr30*) and copper-tolerant isolate (*TrF830*); and (B) *T. harzianum* as the initial isolate (*Tr56*) and copper-tolerant isolate (*TrF856*). Mycelial growth rates are shown as means of applied copper concentrations (2.5x, 5x, 7.5x 10x the field rate; dotted lines) and as control without copper (continuous lines). Curves represent the average of two independent experiments. Each experiment was conducted with 12 repetitions per isolate, concentration and time point. Error bars show standard deviation.

Furthermore, germination capacity of copper-tolerant and initial isolates was compared within copper medium (Figure 8). Germination rates were significantly higher for *T. koningiopsis* and *T. harzianum* after the copper adaptation procedure. In addition to a higher general fitness level of *T. koningiopsis* (*Tr30*) compared with *T. harzianum* (*Tr56*), the copper-tolerant isolate of *T. koningiopsis* (*TrF830*) again showed an approximately 15% higher germination rate than the adapted isolate of *T. harzianum* (*TrF856*).

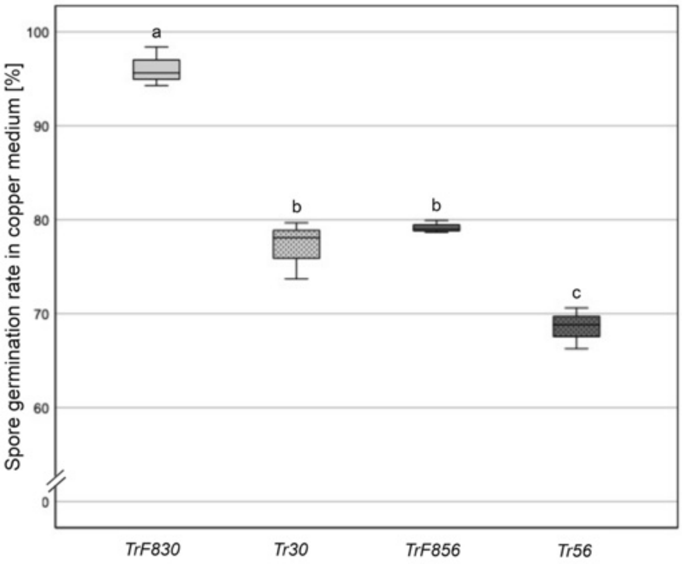

Figure 8: Effect of copper tolerance enhancement on spore germination of *Trichoderma* spp. using copper hydroxide (WP). Spore germination rates of initial isolates and copper-adapted isolates in malt extract agar amended with copper hydroxide (WP). Spore germination is shown for *T. koningiopsis* as the initial isolate (*Tr30*) and copper-tolerant isolate (*TrF830*) and *T. harzianum* as the initial isolate (*Tr56*) and copper-tolerant isolate

(*TrF856*). Spore germination rates are given as means of applied copper concentrations (2.5x, 5x, 7.5x, 10x the field rate). Boxplots represent the average of three independent experiments. Each experiment contains five repetitions per isolate and concentration. Error bars show standard deviation. Analysis of variance was used to determine the effects between means of initial and tolerant germination rates. Means were compared by Tukey's Honestly Significant Difference (HSD) test. Means denoted with different letters (a, b, c) are significantly different ($p \leq 0.05$).

2.4.4 Copper effect on *Trichoderma* spp. identified via rhodanine staining

For closer examination of the copper effect on *Trichoderma*, highly copper-tolerant to moderately tolerant *Trichoderma* isolates were studied using rhodanine staining after incubation with or without metal salts. The copper specificity of the staining technique was confirmed by fungal incubation in media amended with different metal salts (not shown). A red to red-brown precipitation after rhodanine addition was only observed in the presence of copper compounds ($Cu(OH)_2$, $CuSO_4$, copper hydroxide (WP)) (Figure 9A). When added to the medium, copper-specific staining also occurred to an isolate-specific extend in hyphae of *Trichoderma* (Figure 9B). Microscopical analysis revealed copper uptake into the fungal cells. In particular, the moderately tolerant isolate of *T. priscilae* (*Tr45*) showed a copper uptake into the fungal cytoplasm ("3c" in Figure 9B). However, copper compounds seemed also to be attached to the fungal cell walls of *T. harzianum* (*TrF856*) and *T. priscilae* (*Tr45*) ("2a", "3a" in Figure 9B). The highly tolerant *T. koningiopsis* (*TrF830*) showed a reduced staining intensity compared with the other isolates examined ("1a-c" in Figure 9B). However, the increased copper uptake of *T. harzianum* (*TrF856*, *Tr77*) and *T. priscilae* (*Tr45*) led to bloated and pearl cord-like cell deformations ("2b", "4b" in Figure 9B). Rhodanine staining indicates that the isolate of *T. koningiopsis* (*TrF830*) tolerates higher amounts of copper than the remaining isolates. The effect of copper ingredients on *Trichoderma* spp. is related to the attachment of copper compounds to the fungal cell walls or the uptake of copper in the fungal cytoplasm. In the case of *T. koningiopsis* (*TrF830*), large-scale copper uptake and thus a strong staining intensity in the cells was prevented.

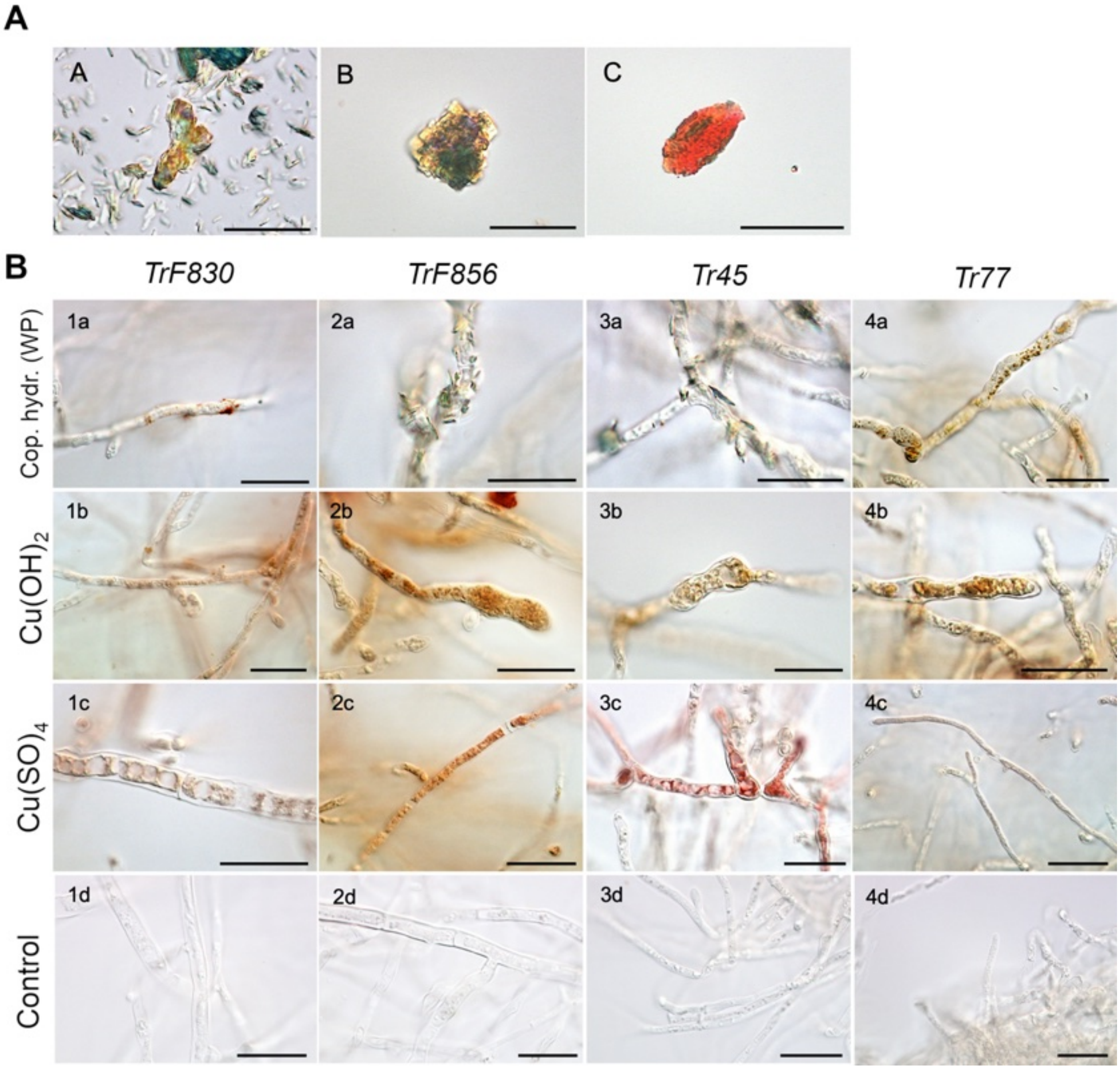

Figure 9: Effect of various copper ingredients on hyphae of *Trichoderma* spp. with different copper tolerance levels. (A) Staining intensity of pure copper salts or fungicide, respectively. Three copper ingredients were added to the medium: (a) fungicide formulation based on copper hydroxide (WP), (b) copper hydroxide and (c) copper sulfate. (B) Detection of different copper ingredients was evaluated using the rhodanine staining technique. Effects of copper on *Trichoderma* spp. are shown for isolates with different copper compatibility: *TrF830* and *TrF856* (highly tolerant) and *Tr45* and *Tr77* (moderately tolerant). Three copper ingredients were added to the medium: fungicide formulation based on copper hydroxide (WP) (1a–4a); copper hydroxide ($Cu(OH)_2$; 1b–4b); and copper sulfate ($CuSO_4$; 1c–4c). A control without metal compounds is shown (1d–4d). Images represent the average of two independent experiments. Each experiment contains three repetitions. Scale bars = 20 μm.

2.5 Discussion

In this study, isolates of *T. koningiopsis* and *T. harzianum* were successfully identified. These isolates occur naturally in grapevine and showed high copper tolerance towards commonly used copper fungicide. An antagonistic capability plus a detected copper tolerance are important prerequisites for the application as biocontrol agents within a sustainable and copper-reduced plant protection strategy, especially in organic viticulture.

All classified isolates were assigned to various *Trichoderma* clades and species, which represents the biodiversity of *Trichoderma* population on grapevine (Table S1). Using copper tolerance analysis, two different isolates were selected belonging to the *Trichoderma* clade Harzianum – *T. harzianum* (*Tr56*) and the clade Viride – *T. koningiopsis* (*Tr30*).[25] These isolates show a high degree of survivability within grapevines as well as copper tolerance. Because the candidates were isolated from grapevines in a vineyard, adaption to copper fungicides possibly ensued via a regular copper contact through plant protection procedures. Natural isolate-specific copper tolerance facilitates combination with selected copper fungicides used in viticulture.

Besides copper tolerance, the biocontrol activity of the *Trichoderma* candidates can contribute to an effective reduction in fungicides.[6] A large number of non-taxonomic studies of *Trichoderma* spp. are associated with the clades Viride and Harzianum[25], which also underlines use of the identified isolates as biocontrol agents in viticulture. Moreno *et al.*[25] showed a resistance-inducing effect of *T. koningiopsis*-inoculated tomato plants against *Fusarium oxysporum* f. sp. *radicis-lycopersici* and a plant growth stimulating effect after isolate application in seed priming. Different studies have demonstrated the antagonistic potential of *T. harzianum* against plant pathogens as well as a beneficial effect on plant growth, nutrient uptake and stress tolerance.[13,14] Thus, the copper-tolerant isolates of the species *T. harzianum* and *T. koningiopsis* could be suitable biocontrol candidates for an environmentally friendly plant protection strategy in organic viticulture.

For all examined *Trichoderma* isolates the highest mycelial growth rates were detected with copper hydroxide (WP) (Figures 2 and 4). General growth inhibition was observed upon addition of any of the tested copper fungicides. The effect of copper is based on the absorption and accumulation of copper ions in fungal cells, which inactivates essential enzymes[27] and also explains the general growth reduction of *Trichoderma* spp. subjected to different copper fungicides (Figures 2 and 4). Our results show that the level of growth reduction depends on both dose and formulation, as seen for copper octanoate used at low field rates. This deviates from the work of Jovičić-Petrović *et al.*,[28] who demonstrated growth reduction for lower copper concentrations and complete growth inhibition of *Trichoderma* strains for similar copper amounts used in this study. Anand *et al.*[29] showed maximum copper accumulation in *T. viride* at copper concentrations comparable with the minimum (tribasic copper sulfate) or medium (copper hydroxide [SC]) level used in this study, followed by a stationary growth phase of the fungus. By contrast, the mycelial growth of most isolates in this study was affected only slightly by copper hydroxide (WP). At rates up to double the field concentration of copper hydroxide (WP), a large number of isolates showed high growth rates. Iskandar *et al.*[30] demonstrated high copper tolerance in *T. asperellum*, but the growth rate decreased at a copper concentration comparable with the amount of copper in our copper hydroxide (WP). This may indicate higher natural adaption to copper in the isolates tested in this study, which potentially results from the isolation of *Trichoderma* strains from grapevines regularly treated with copper in the vineyard.

The growth rates of *Trichoderma* isolates depend not only on copper concentrations, but on fungicide formulation, as seen for copper hydroxide applied as wettable powder and suspension concentrate. This indicates that the compatibility with the respective formulation is also a crucial factor for the successful development of *Trichoderma*. Besides copper dihydroxide, both fungicides contain further components such as naphthalene and propan-2-ol in case of powder formulation (https://www.biofa-profi.de/de/f/funguran-progress.html). In addition to tribasic copper sulfate, suspension concentrate also contains 1.2-benziothiazolin-3-one (https://www.biofa-profi.de/de/c/cuproxat.html). Jangir *et al.*[31] demonstrated that the derivatives pyran, naphthalene and morpholines excreted by *Bacillus subtilis* showed an antifungal effect against *Fusarium oxysporum* f. sp. *lycopersici*. Tahir *et al.*[32] revealed an inhibitory effect of 1.2-benziothiazol-2(2H)-one on the development and vitality of *Ralstonia solanacearum*. However, the ingredients and the active compound copper hydroxide in the wettable powder formulation seem to be better detoxified by *Trichoderma* compared with those present in the other fungicides. There are only few reports about the compatibility of different copper fungicides with *Trichoderma*. In accordance with Valarmathi *et al.*,[33] the results of our study also show a high compatibility of *Trichoderma* spp. with a copper hydroxide-based fungicide, whereas other studies revealed a compatibility with various fungicides (for example, see refs[34] and [35]). However, because the exact composition of a given formulation is normally kept secret by the producer, the influence of emulsifiers and solvents in particular on *Trichoderma* spp. remains rather unclear.

Our results indicate that, as expected, germination of *Trichoderma* spores decreased with increasing copper concentration. Spore germination in several isolates was reduced only slightly by the copper fungicide (Figure 5). Considering a combined field application of *Trichoderma* spores with a copper fungicide, distinct resistance of spores to copper is important for an effective plant protection procedure. Dłużniewska[36] demonstrated an inhibitory effect of a copper-based fertilizer on spore germination of *T. harzianum* and explained the inhibition as being due to the fungistatic effect of copper. In accordance with our results, Anand *et al.*[29] were able to detect sporulation of *Trichoderma* sp. with the addition of copper. However, the lag phase of the fungus was extended.[29] The minor effect of copper compounds on spore germination may be explained by a general capability to detoxify metal salts and by increased copper tolerance achieved after repeated field application. Akhtar and Mannan[37] demonstrated secretion by *T. ghanense* of ligninolytic enzymes for heavy metal biodegradation and deposition of antioxidant enzymes for minimization of oxidative stress through metal influence. Isolate-specific binding characteristics may explain the different fitness levels of the isolates tested in this study, which also becomes apparent in growth test studies (Figure 6). In particular, *T. koningiopsis* and *T. harzianum* reached the stationary growth phase with copper, unlike the other selected isolates. Anand *et al.*[29] demonstrated achievement of a growth plateau for *T. viride* in copper after five days which is comparable with the growth maximum of *T. koningiopsis* with half the field rate of copper hydroxide (WP) after 7 days. However, *T. koningiopsis* and *T. harzianum* were able to tolerate higher copper concentrations than described for *T. viride*. Even at the field rate of copper hydroxide (WP), the two isolates showed less-affected mycelial growth, whereas Anand *et al.*[29] demonstrated high growth reduction at lower copper concentrations for *T. viride*. These results are almost in accordance with the growth reduction seen with the other selected isolates tested in growth fitness assay. Other from that, it was possible to increase the copper tolerance of *T. koningiopsis* and *T. harzianum* without a loss of fitness (Figures 7 and 8). Accordingly, we were able to increase copper tolerance of *T. koningiopsis* and *T. harzianum* to a sufficient extent for field use. Moreover, the high fitness level of the copper-tolerant isolates guarantees uninhibited development. This is an important prerequisite and allows reduced copper fungicide application strategy in viticulture.

Our microscopic results indicate copper uptake in the fungal cells of *Trichoderma* spp., as well as its attachment to the cell wall surface (Figure 9A). Anand *et al.*[29] also demonstrated copper absorption to cell wall surface and cytosol. Biosorption of metal ions through filamentous fungi can be explained by extracellular accumulation, intracellular accumulation absorption and cell surface precipitation.[37,38] Unique cell wall characteristics of *Trichoderma* spp. (composed mainly of glucan, polymers and chitin) enable high metal biosorption of this species.[7,37] In particular, chitin, containing chitosan, improves the uptake and binding of heavy metals.[15] Furthermore, Hassett and Kosman[39] showed that *Saccaromyces cerevisiae* was able to reduce copper by metalloreductases located at the cell wall surface. The reduced copper was then transported through the plasma membrane using high-affinity membrane-associated transporters. A similar approach is also conceivable for copper-tolerant *Trichoderma* strains. Copper uptake in cytosol was also shown by El-Kassas and El-Taher[40] for *T. viride*. In this context, the authors pointed out that metabolism-independent absorption processes include, in particular, superficial attachment via ionic and chemical interactions, whereas metabolic processes show both surface binding of substances and their intercellular accumulation. Our results are in accordance with El-Kassas and El-Taher[40], who observed that copper accumulation in the fungal tissue also led to changes in the mycelium structures. We noted a slight swelling of the hyphae in all isolates of *T. harzianum* regardless of their level of tolerance (“2b”, “3b”, “4b” in Figure 9B). The highly tolerant isolate of *T. koningiopsis* showed no deformations (“1a-c” in Figure 9B). Based on the weakly stained mycelium of *T. koningiopsis*, it could be assumed that less copper was incorporated into the fungal cells than it was the case for *T. harzianum*. Puig and Thiele[41] described a copper transport mechanism in yeast based on copper chaperones and ATPases that can lead copper into a secretory pathway. Once trapped, metal ingredients can be transported into the extracellular environment via plasma membrane and finally remain harmless to the cell. This could also be possible with *T. koningiopsis* and may provide an explanation for low copper levels in fungal cells.

2.6 Conclusion

In conclusion, two *Trichoderma* isolates of *T. koningiopsis* and *T. harzianum* showed high copper tolerance to a common copper fungicide used primary to control *P. viticola* in viticulture. Owing to their isolation from grapevine in the field, these isolates possess a natural adaptation to grapevine and even to the ecosystem of the vineyard. This selection process may also include a natural antagonistic potential towards other microorganisms such as pathogens and may also induce resistance mechanisms in grapevines. Moreover, copper tolerance of *Trichoderma* spp. seems to be associated with surface binding capacities and copper excretion. These properties suggest use as biostimulants and biocontrol agents combined with reduced fungicide rate, allowing sustainable plant protection especially in organic viticulture. Based on the experimental setup presented above, further investigations in terms of plant-pathogen-*Trichoderma* interactions will provide insights into the biocontrol potential of the selected isolates against downy mildew and other diseases.

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2.8 Supplementary Materials

Table S1: Location, extraction information and species affiliation of selected *Trichoderma* isolates

Isolate number	**Location**	**Scion variety**	**Rootstock variety**	**Isolation from**	**Species**
Tr1	Lachen-Speyerdorf	Seyval blanc	SO4	Middle trunk	*T. harzianum*
Tr2	Lachen-Speyerdorf	Chardonnay	Fercal	Middle trunk	*T. harzianum*
Tr3	Lachen-Speyerdorf	Regent	Binova	Basal trunk	*T. harzianum*
Tr4	Freinsheim	Dornfelder	-	Basal trunk	*T. koningiopsis*
Tr4.2	-	-	-	-	*T. asperellum*
Tr5	Bad Dürkheim/Ungstein	Bacchus	12AA	Middle trunk	*T. citrinoviride*
Tr6	Sommerhausen	Cabernet Cortis	5BB	Basal trunk	*T. viride*
Tr6.2	-	-	-	-	*Trichoderma* sp.
Tr7	Bad Dürkheim/Ungstein	Muskat	SO4	Basal trunk	*T. citrinoviride*
Tr8	Sommerhausen	Gr. Silvaner	Rici	Middle trunk	*T. harzianum*
Tr8.2	-	-	-	-	*Trichoderma* sp.
Tr9	Sommerhausen	Silvaner	Rici	Basal trunk	*T. viride*
Tr9.2	-	-	-	-	*Trichoderma* sp.
Tr10	Sommerhausen	Sauvignon blanc	SO4	Trunk head	*T. hamatum*
Tr10.2	-	-	-	-	*Trichoderma* sp.
Tr11	Sommerhausen	Sauvignon blanc	SO4	Basal trunk	*T. virens*
Tr12	Sommerhausen	Helios	SO4	Trunk head	*T. virens*
Tr13	Sommerhausen	Riesling	SO4	Trunk head	*T. harzianum*
Tr14	Sommerhausen	Chardonnay	Binova	Middle trunk	*T. harzianum*
Tr15	Obersülzen	Acolon 2	5BB	Middle trunk	*T. citrinoviride*
Tr16	Obersülzen	Acolon 2	5BB	Middle trunk	*T. harzianum*
Tr17	Jechtlingen	Nobling	5BB	Basal trunk	*T. harzianum*
Tr18	Jechtlingen	Gewürztraminer	SO4	Basal trunk	*T. harzianum*
Tr19	Jechtlingen	Weißburgunder	124AA	Basal trunk	*T. hamatum*
Tr19.2	-	-	-	-	*Trichoderma* sp.
Tr20	Jechtlingen	Solaris	SO4	Basal trunk	*T. harzianum*
Tr21	Jechtlingen	Solaris	SO4	Basal trunk	*T. harzianum*
Tr22	Maikammer	Weißburgunder	SO4	Trunk head	*T. citrinoviride*
Tr22.2	-	-	-	-	*Trichoderma* sp.

Tr24	Neustadt/Mußbach	-	-	-	*T.* cf. *priscilae*
Tr25	Nahe	Weißburgunder	-	-	*T. koningiopsis*
Tr25.2	-	-	-	-	*Trichoderma* sp.
Tr26	Neustadt/Mußbach		-	-	*T. sinuosum*/ *T. cremea*
Tr26.2	-	-	-	-	*Trichoderma* sp.
Tr27	Bad Dürkheim/Ungstein	Polöskei-Muskat	-	Basal trunk	*T. harzianum*
Tr28	Neustadt/Mußbach	Riesling	N90/5C	Trunk head	*T. gamsii*
Tr29	Neustadt/Mußbach	Riesling	N90/5C	Trunk head	*T. atroviride*
Tr29.2	-	-	-	-	*Trichoderma* sp.
Tr30	Neustadt/Mußbach	Riesling	N90/5C	Basal trunk	*T. koningiopsis*
Tr30.2	-	-	-	-	*Trichoderma* sp.
Tr31	Neustadt/Mußbach	Riesling	N90/5C	Middle trunk	*T. atroviride*
Tr32	Lachen-Speyerdorf	Chardonnay	SO4	Middle trunk	*T. harzianum*
Tr33	Essingen Roßberg	Müller-Thurgau	-	Scion	*T. atroviride*
Tr33.2	-	-	-	-	*Trichoderma* sp.
Tr34	Neustadt/Mußbach	Dornfelder	-	Scion	*T. atroviride*
Tr34.2	-	-	-	-	*Trichoderma* sp.
Tr35	Lachen-Speyerdorf	Bacchus	SO4	Trunk head	*T. harzianum*
Tr36	Lachen-Speyerdorf	Regent	Binova	Basal trunk	*T. velutinum*
Tr36.2	-	-	-	-	*Trichoderma* sp.
Tr37	Neustadt/Mußbach	Riesling	N90/5C	Basal trunk	*T. koningiopsis*
Tr37.2	-	-	-	-	*Trichoderma* sp.
Tr38	Neustadt/Mußbach	-	-	-	*T. harzianum*
Tr39	Neustadt/Mußbach	Dornfelder	-	Scion	*T. atroviride*
Tr39.2	-	-	-	-	*Trichoderma* sp.
Tr40	Neustadt/Mußbach	-	-	-	*T. harzianum*
Tr40.2	Neustadt/Mußbach	Dornfelder	-	Scion	*T. atroviride*
Tr41	Neustadt/Mußbach	-	-	-	*T. harzianum*
Tr42	Neustadt/Mußbach	Dornfelder	-	Scion	*T. atroviride*
Tr44	Weiher	Portugieser	-	-	*T. atroviride*
Tr44.2	-	-	-	-	*Trichoderma* sp.
Tr45	Neustadt/Mußbach	-	-	-	*T.* cf. *priscilae*

Tr45.2	-	-	-	-	*T. agressivum f. europaeum*
Tr46	Neustadt/Mußbach	-	-	-	*T.* cf. *priscilae*
Tr49	Haardt	Muskateller	3309/161-49	Basal trunk	*T. harzianum*
Tr50	Haardt	Muskateller	3309/161-49	Basal trunk	*T. harzianum*
Tr51	Obersülzen	Muskaris	SO4	Basal trunk	*T. harzianum*
Tr52	Minheim	Spätburgunder	SO4	Trunk head	*T. citrinoviride*
Tr53	Obersülzen	Ruländer	125AA	Basal trunk	*T. hamatum*
Tr54	Obersülzen	Ruländer	125AA	Basal trunk	*T. atroviride*
Tr55	Obersülzen	Dakapo	SO4	Middle trunk	*T. citrinoviride*
Tr56	Obersülzen	Spätburgunder	125AA	Basal trunk	*T. harzianum*
Tr57	-	-	-	Middle trunk	*T. harzianum*
Tr58	-	-	-	Middle trunk	*T. harzianum*
Tr59	-	-	-	Middle trunk	*T. citrinoviride*
Tr60	Endingen-Kirchlingsbergen	Spätburgunder	-	Scion	*T. harzianum*
Tr60.2	Neustadt/Mußbach	Johanniter	-	Scion	*T. harzianum*
Tr61	Endingen-Kirchlingsbergen	Spätburgunder	-	Scion	*T. virens*
Tr61.2	Endingen-Kirchlingsbergen	Spätburgunder	-	Scion	*Trichoderma* sp.
Tr61.3	-	-	-	-	*Trichoderma* sp.
Tr62	LTT Mervill (FRA)	-	-	Scion	*Trichoderma* sp.
Tr62.2	Neustadt/Mußbach	Johanniter	-	Scion	*Trichoderma* sp.
Tr63	LTT Mervill (FRA)	-	-	Scion	*T. atroviride*
Tr64 B3	LTT Mervill (FRA)	-	-	Scion	*Trichoderma* sp.
Tr65	LTT Mervill (FRA)	-	-	Scion	*Trichoderma* sp.
Tr65 B3	-	-	-	-	*Trichoderma* sp.
Tr66	LTT Mervill (FRA)	-	-	Scion	*Trichoderma* sp.
Tr67	LTT Mervill (FRA)	-	-	Scion	*Trichoderma* sp.
Tr68 B3	-	-	-	-	*Trichoderma* sp.
Tr69	-	-	-	-	*Trichoderma* sp.
Tr69 B3	-	-	-	-	*Trichoderma* sp.
Tr71	Laubheim, Johannisberg	Silvaner	SO4	Middle trunk	*T. harzianum*
Tr73	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr74	Laubheim, Johannisberg	Silvaner	SO4	-	*T. harzianum*

Tr76	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr77	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr78	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr79	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. gamsii*
Tr80	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr81	Laubheim, Johannisberg	Silvaner	SO4	Middle trunk	*T. harzianum*
Tr82	Laubheim, Johannisberg	Silvaner	SO4	-	*Trichoderma* sp.
Tr83	Laubheim, Johannisberg	Silvaner	SO4	-	*Trichoderma* sp.
Tr84	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. atroviride*
Tr85	Laubheim, Johannisberg	Silvaner	SO4	Middle trunk	*T. harzianum*
Tr86	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr87	Laubheim, Johannisberg	Silvaner	SO4	Middle trunk	*T. harzianum*
Tr88	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. atroviride*
Tr89	Laubheim, Johannisberg	Silvaner	SO4	-	*Trichoderma* sp.
Tr90	Laubheim, Johannisberg	Silvaner	SO4	Trunk head	*T. harzianum*
Tr116	-	-	-	-	*Trichoderma* sp.
Tr117	-	-	-	-	*Trichoderma* sp.
Tr9625	Bad Dürkheim/ Ungstein	Polöskei-Muskat	-	Basal trunk	*T. harzianum*
Tr9668	Neustadt/Mußbach	Riesling	N90/5C	Basal trunk	*T. koningiopsis*
Tr9669	Neustadt/Mußbach	Riesling	N90/5C	Trunk head	*T. atroviride*
Tr9670	Neustadt/Mußbach	Riesling	N90/5C	Trunk head	*T. gamsii*
Tr9671	Neustadt/Mußbach	Riesling	N90/5C	Middle trunk	*T. atroviride*
Tr9689	Neustadt/Mußbach	Dornfelder	-	Scion	*T. atroviride*
Tr9800	Nahe	Weißburgunder	-	-	*T. koningiopsis*
Tr9839	Essingen Roßberg	Müller-Thurgau	-	-	*T. atroviride*
Tr9847	Neustadt/Mußbach	Neustadt W.	-	-	*T.* cf. *priscilae*
Tr9848	Neustadt/Mußbach	Neustadt W.	-	-	*T. harzianum*
Tr9849	Neustadt/Mußbach	Neustadt W.	-	-	*T. harzianum*
Tr9850	Neustadt/Mußbach	Neustadt W.	-	-	*T. harzianum*
Tr9851	Neustadt/Mußbach	Neustadt W.	-	-	*T. rufobrunneum*
Tr9852	Neustadt/Mußbach	Neustadt W.	-	-	*T.* cf. *priscilae*
Tr9853	Neustadt/Mußbach	Neustadt W.	-	-	*T. sinuosum/ T. cremea*
Tr9878	Maikammer	Weißburgunder	SO4	Trunk head	*T. citrinoviride*

Tr9894	Lachen-Speyerdorf	Regent	Binova	Basal trunk	*T. velutinum*
Tr9895	Lachen- Speyerdorf	Seyval blanc	SO4	Trunk head	*T. citrinoviride*
Tr9896	Lachen-Speyerdorf	Chardonnay	Fercal	Middle trunk	*T. harzianum*
Tr9897	Lachen-Speyerdorf	Bacchus	SO4	Trunk head	*T. harzianum*

3 Combining *Trichoderma koningiopsis* and chitosan as a synergistic biocontrol and biostimulating complex to reduce copper rates for downy mildew control on grapevine

Chapter 3 has been published:

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3.1 Abstract

Plasmopara viticola, causal agent of downy mildew and one of the most severe grapevine diseases, is commonly controlled by multiple fungicide applications, primarily based on copper compounds in organic viticulture. Biostimulants and biocontrol agents could make a major contribution to pesticide reduction. A synergistic biocontrol and biostimulating complex consisting of *Trichoderma* sp. and chitosan was investigated to achieve copper fungicide reduction in the control of *P. viticola*. To ensure complex efficacy, the interactions and effects of all agents need to be investigated at the tri-trophic level of grapevine, downy mildew, and biocontrol and biostimulating complex. To guarantee the compatibility of *Trichoderma* sp. and chitosan, the chitosan tolerance of different copper-tolerant *Trichoderma* isolates was investigated in mycelial growth and germination tests. A *T. koningiopsis* isolate proved to be highly chitosan-tolerant was identified for further studies. The timing of spray application with *T. koningiopsis* and various chitosans was evaluated for efficacy against *P. viticola*. In the period 0 to 1 day before inoculation with the pathogen, the formation of the sporangia number was significantly reduced by the tested agents. To further describe the mode of action and efficacy of *T. koningiopsis* and chitosan against *P. viticola*, the application of single agents on upper and lower grapevine leaves inoculated with the pathogen in comparison to resistance inducers and copper fungicide were tested. Moreover, the combination of *T. koningiopsis* with different chitosans and a reduced copper rate was investigated on the respective leaf sides of greenhouse plants. Both, a direct and indirect mode of action against downy mildew was demonstrated. Regardless of the treated leaf side, sporulation of downy mildew was clearly inhibited compared to the untreated control. Moreover, microscopic images showed successful establishment of *T. koningiopsis* and uniform distribution of chitosan on leaves. The tested biocontrol and biostimulating complex might show a promising combatting tool against *P. viticola* while simultaneously reducing copper rate.

Keywords: *Plasmopara viticola*, biostimulants, copper reduction, chitosan tolerance, organic viticulture, *Vitis vinifera*

3.2 Introduction

The obligate biotrophic endoparasite *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni is the causal agent of downy mildew, one of the most severe diseases in worldwide grape-growing areas. By entering through the stomata, *P. viticola* is able to infect all green plant parts which can lead to significant reductions in berry yield and quality (Gessler et al., 2011). In organic viticulture, disease management of downy mildew highly depends on the regular sprays of copper fungicides (Dagostin et al., 2011; Gessler et al., 2011). Due to the intensive use of copper and the potentially resulting ecotoxic effects on the soil and the organisms living in it, as well as the effects on the environment directly associated with it (e.g., surface water), copper application has been reduced by the European Commission to 15 kg per hectare over a five year period with approximately of 10 to 12 treatments per year (Dagostin et al., 2011; European Commission Implementing Regulation 2018/1981 of 13 December 2018). In addition, copper compounds were classified as substitution candidates, further limiting their application (European Commission Implementing Regulation 2015/408 of 11 March 2015). This is in contrast to the European farm-to-fork strategy which includes the aim to increase the area used for organic production from 10 to 25%. In addition, there is the urgent need as well as the consumer demand for effective alternatives that contribute to copper reduction or substitution.

Opportunistic and avirulant *Trichoderma* spp. show promising plant-protective, biostimulating, and biofertilizing properties as well as high decomposition and bioremediation activity contributing to environmentally friendly agricultural practices (Fiorentino et al., 2018; Jaroszuk-Ściseł et al., 2019). Biocontrol mechanisms include direct pathogen impact with the production of cell wall degrading enzymes and antibiotics, competition for nutrients and space, and mycoparasitism (Jaroszuk-Ściseł et al., 2019). Many *Trichoderma* spp. are able to improve nutrient uptake and plant growth and increase plant tolerance to abiotic stress through biostimulation (Fiorentino et al., 2018). The beneficial extent of the biostimulating effect is related to molecular and hormonal crosstalk between *Trichoderma* and its host plant (Manganiello et al., 2018).

Another environmental-friendly approach for fungicide reduction is the application of chitosans (ß-1,4-linked-D-glucosamine), polysaccharides obtained from the deacetylation of chitin, which can be used to control pathogens through their antimicrobial properties and defense-enhancing effects on plants (Aziz et al., 2006; Malbera and Cerana, 2020) depending on size and structure, and furthermore, on the degree of acetylation of the molecule (Kauss et al., 1989; Vander et al., 1998). In grapevine, applied chitosan forms a semipermeable film around plant tissue and stimulates the accumulation of phytoalexins, such as trans- and *cis*-resveratrol as well as the derivatives ε-viniferin and piceid in grapevine leaves (Aziz et al., 2006; Romanazzi et al., 2017).

However, under field conditions, treatments with a single biocontrol agent or biostimulant are often not sufficient to ensure an effective crop protection (Monte, 2001; Kishore et al., 2005), which may also be the case when both are applied in combination. Shukla et al. (2021) showed that neither *Trichoderma* nor chitosan were effective in pathogen control when used as single treatments and concluded that the addition of a reduced copper rate may sufficiently support the effect of the tested agents. Thus, the addition of a reduced copper fungicide rate can adequately support the agents' effect against plant pathogenic fungi (Monte, 2001) and contributes to the stability and efficacy of a biocontrol and biostimulating complex, especially in the vineyard. To maximize copper-reduced plant pathogen control, it is necessary to align all agents. It was shown that selected copper- and chitosan-tolerant *Trichoderma* isolates can inhibit the development of plant pathogenic fungi by releasing various cell wall degrading enzymes (Saravanakumar et al., 2016). Chitosan binds copper through a free electron pair, forming an effective antifungal complex (Varma et al., 2004; Salama, 2021). A synergistic triple combination of *Trichoderma*, chitosan and a reduced copper rate may further enhance the individual effects of all agents. The enzymes (chitosanases and cellulases) released by such tolerant *Trichoderma* isolates, on one hand, enzymatically decompose chitosan into its highly bio-effective oligomers and, on the other hand, decompose cell wall components of the pathogen. As a result, antifungal agents also released by *Trichoderma* can act directly on the intracellular components of the pathogen and contribute to growth inhibition (Bohra, 2018; Malerba and Cerana, 2020), so that an effective, copper-reduced and sustainable crop protection concept can be used.

The present study investigates a synergistic biocontrol and biostimulating complex consisting of *Trichoderma* sp. and chitosan that may contribute to a copper-reduced fungicide application for downy mildew control in organic viticulture (Fig. 10). To enable an effective and simultaneous application, high copper and chitosan tolerance of suitable *Trichoderma* candidates are fundamental prerequisites. For this purpose, *Trichoderma* spp. with biocontrol potential and high copper tolerance, already reported in previous work (Küpper et al., 2022), need to be investigated with respect to their chitosan tolerance (Attjioui et al., 2021). Therefore, mycelial growth and spore germination experiments were used to screen the most compatible combination of the tested *Trichoderma* isolates and also chitosans for the composition of a synergistic biocontrol and biostimulating complex. Leaf disc tests were used to select the time period in which single agent applications show the highest efficacy. This was investigated for all biocontrol and biostimulating agents in more detail by treating either the abaxial or the adaxial leaf side of grapevine leaves in order to draw conclusions about a direct or indirect mode of action against the pathogen. Furthermore, the most promising *Trichoderma*-chitosan combinations were tested in dual and triple applications with the addition of reduced copper rate on greenhouse plants infected with downy mildew. Different microscopical techniques were used to illustrate both the distribution of chitosan on leaves and the establishment of *Trichoderma* sp. when applied solely, in combination with chitosan, and together with a reduced rate of copper.

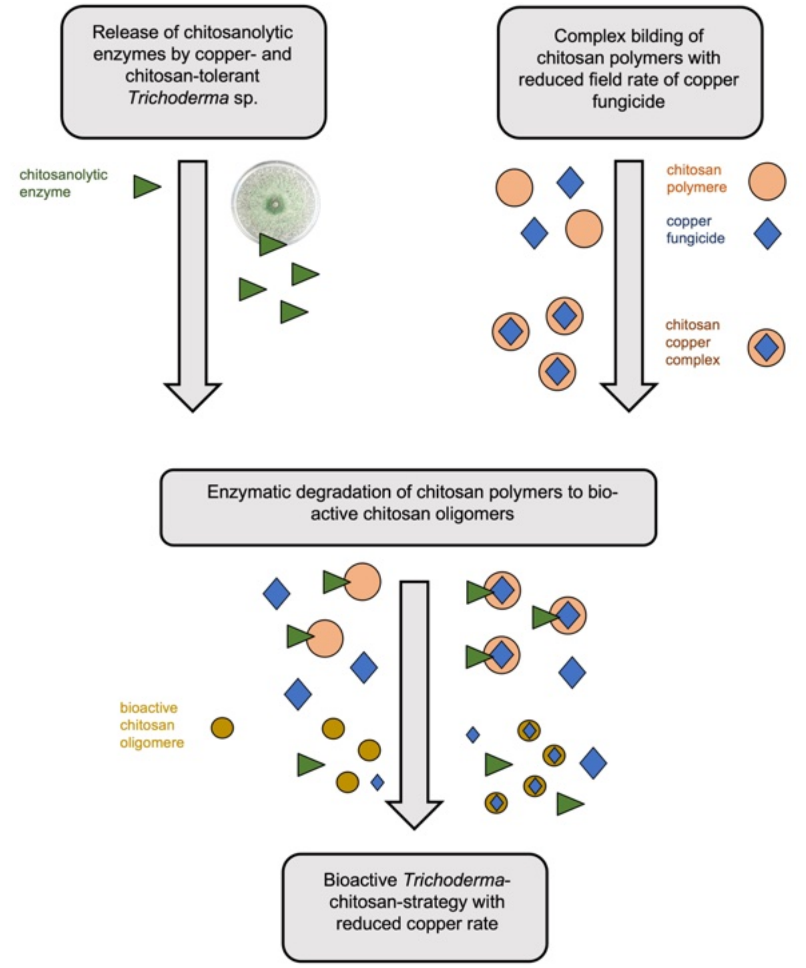

Figure 10: Interaction of *Trichoderma* sp. and chitosan within a biocontrol and biostimulating complex for copper fungicide reduction.

3.3 Materials and Methods

3.3.1 *Trichoderma* isolates and chitosans

Eight copper-tolerant *Trichoderma* isolates were used in this study. *Tr30*, *Tr74*, *Tr9850*, *Tr56*, *Tr77*, *Tr9851*, *Tr45* and *Tr60.2,* belonging to the species *T. koningiopsis* Samuels, Carm. Suárez & H.C. Evans, *T. harzianum* Rifai*,* and *T. rufobrunneum* Z.X. Zhu & W.Y. Zhuang, were isolated from wood of cordon arms, trunks and wooden shoots from grapevines growing in Rhineland-Palatinate (Germany). Plants had never been treated with any *Trichoderma*-based products before. The copper tolerance of the isolates was already shown in previous investigations (Küpper et al., 2022). All purified isolates were grown on malt extract agar plates (MEA, 2 % malt extract, Arche Naturküche, 2 % agar, Roth, Karlsruhe, Germany; 0.01 % Tetracycline, Roth, Karlsruhe, Germany) at 21 °C and 70 % relative humidity (RH) in a climate chamber and subcultured monthly.

In this study, four types of chitosan were used that differed in molecular structure, average degree of N-acetylation (DA), and average degree of polymerization (DP): The chitosans 651 (polymer-mixture, 1 % w/v, DA 20 %, average degree of polymerization DP 800), 661 Ac (polymer-oligomer-mixture, 10 % w/v, DA 19 %, DP 250), 661 HCl (polymer-oligomer-mixture, 10 % w/v, DA 17 %, DP 350) and 671-TvChi (polymer-oligomer-mixture, 1 % w/v, DA 16 %, DP 200) were kindly provided by the Institute of Plant Biology and Biotechnology, University of Münster, Germany, and will be described in detail elsewhere (Richter, Cord-Landwehr, Moerschbacher, personal communication).

3.3.2 Plants and pathogen

The *Vitis vinifera* cv. 'Müller-Thurgau' with high susceptibility to *P. viticola* was used for laboratory and greenhouse trials. Bud cuttings were collected from mature shoots obtained from vineyards of the State Education and Research Center of Viticulture, Horticulture and Rural Development (Neustadt a. d. Weinstraße, Germany). Cuttings were soaked in water overnight, disinfected for 8 h with 0.5 % Chinoplant® (FMC Cheminova, Germany) and stored at 4 °C and 95 % RH in a cold chamber until further use. For cultivation, cuttings were freshly cut on endings and apical sides were dipped in antifungal and plant growth promoting wax (80 °C). Cuttings were cultivated in Perligran® Extra (Knauf AQUAPANEL, Germany) for 7 weeks in greenhouse (∅ 24°C) with daily irrigation until sufficient root development was apparent. Grapevine soil mixture (90.1 % Floradur®, multiplication substrate (Floragard, Germany), 9.0 % Perligran® Extra, 0.5 % carbonic magnesium-lime (Hufgard, Germany), 0.4 % MANNA® COTE 6 M (Hauert MANNA, Switzerland) was used for potting. Potted cuttings were grown under greenhouse conditions (22 °C/ day, 18 °C night, 50 % RH) until young grapevine plants displayed six to eight leaves and were used for laboratory and greenhouse trials.

The *P. viticola* isolate used in this study was provided by the State Education and Research Center of Viticulture, Horticulture and Rural Development. The isolate is a mixture of different field isolates collected from grapevines susceptible to downy mildew. These isolates have not been selected for host and fungicide resistance. Isolate of *P. viticola* was propagated according to Malacarne et al. (2011).

3.3.3 Chitosan tolerance analysis

3.3.3.1 Selection of a *Trichoderma* candidate and suitable chitosan agents

Chitosan tolerance tests were performed to identify suitable types of chitosan for application with *Trichoderma* and copper fungicide and to select a *Trichoderma* isolate showing natural tolerance to chitosan. Therefore, mycelium growth of eight copper-tolerant isolates was measured on MEA (2 %) amended with 0.1 % chitosan concentration (651, 661 Ac, 661 HCl or 671) and compared to colonies grown on control MEA without chitosan.

Fungal isolates were preincubated on MEA at 21 °C, 70 % RH for seven days. Six mm diameter discs were cut off from the margin area of each isolate and placed on the centre of the chitosan-enriched and the control plates. Samples were incubated (21 °C, 70 % RH) for 14 days. Meanwhile, the fungal colony development was measured daily after 3 days post inoculation (dpi) and the average growth was calculated (measurement of horizontal and vertical mycelial growth).

Additionally, spore germination capacity was analysed in liquid malt extract broth (MEB, 1 %) amended with respective chitosan (0.01 %). The experimental setup was conducted according to Küpper et al. (2022).

3.3.4 Application of *T. koningiopsis* and chitosan

3.3.4.1 Leaf disc assay

A leaf disc infection assay was used to describe the effects of *T. koningiopsis* and chitosans on sporangia development of *P. viticola* according to Eisenmann et al. (2019). A 40 µl droplet of zoospore suspension (50,000 sporangia ml^{-1}) or sterile dH$_2$O (mock variant) was placed on the abaxial surface of each leaf disc. Leaves were sampled from greenhouse grapevines which were treated with spray applications of *T. koningiopsis* (10^6 spores ml^{-1} MEB 1 %), 661 Ac, 661 HCl and 671 (0.01 % in dH$_2$O), respectively. Spray applications were performed until application run-off and leaf discs were excised with a cork borer after complete drying. The resistance inducers BABA (3-aminobutanoic acid, Sigma-Aldrich, USA, 50 µg ml^{-1}) and BTH (S-methyl benzo[1,2,3]thiadiazole-7-carbothioate, Bion®, Syngenta, Switzerland, 0.5 g l^{-1}) and a reduced field rate (0.5-times field rate) of the copper fungicide Funguran® progress (active ingredient: copper hydroxide, Biofa, Germany, 0.625 g l^{-1}) were used to verify the effect of the tested agents. The fungicide concentration used is linked to the field rate, which is dependent on leaf area and fruit development. The amount of fungicide needed at bud burst and the start of leaf growth (up to BBCH 16) is equivalent to 1 time the field rate (specified by the producer in kg/ha) and will be adjusted to higher rates (up to 4 times the field rate) depending on the development of the grapevine. To gain knowledge of a direct biocontrol or indirect biostimulating mode of action towards *P. viticola* and *V. vinifera,* grapevines were treated either on the abaxial or adaxial leaf surface. Additionally, spray applications were performed 7, 3, 1 day/s before pathogen inoculation (dbi) as well as at the inoculation day (0 dbi) and 1 day after visible infection of *P. viticola* (appearance of conidiophores) to enclose most effective application time. At 1 day after visible infection spray application was directly carried out on leaf discs. In accordance to Merz et al. (2015), the degree of infection was quantified by counting the sporangia per leaf disc produced by *P. viticola* 7 days after pathogen inoculation.

3.3.4.2 Greenhouse plant assay

Infection assay on whole greenhouse plants was used to describe the effects of biocontrol and biostimulating complex (*T. koningiopsis* and chitosan) and a reduced rate of Funguran® progress (0.5-times field rate) on leaf infestation of *P. viticola*. Spray application with the individual agents and dual (*Trichoderma* and chitosan) and triple mixtures (*Trichoderma*, chitosan and reduced copper rate) was performed as described above in leaf disc infection assay. dH_2O was used as control. Application treatment was conducted one day before inoculation with *P. viticola*. Greenhouse grapevines were inoculated by spraying abaxial leaf surface with *P. viticola* zoospore suspension (50,000 sporangia ml^{-1}) or sterile dH_2O (mock variant). Plants were incubated overnight in separated wet chambers, creating an optimal micro-climate for the infection process. Six days after pathogen inoculation, disease development was assessed according to the disease severity scheme of the European Plant Protection Organization (EPPO, 2000) with some modifications: Contrary to the EPPO disease severity scheme, only seven infection grades (0 % = no symptoms; <5 %; <10 %; < 25%; < 50%; < 75% and ≤ 100%) were used. The night before assessment, grapevines were again incubated in wet chambers to induce sporulation of *P. viticola* and facilitate infection assessment.

3.3.5 Microscopical investigations

3.3.5.1 Scanning electron microscopy

To assess *T. koningiopsis* establishment and chitosan distribution on grapevine leaves, abaxial and adaxial leaf surfaces of treated grapevines were investigated via scanning electron microscope (SEM). Leaf discs were excised with a cork borer (Ø 2 mm) and mounted on aluminium stubs (0.5” Aluminium Specimen stubs, Agar Scientific Ltd., Stansted, UK) with double sided adhesive carbon tape (Plano Leit-Tabs, Plano GmbH, Wetzler). Leaf samples were dried for seven days and then coated with gold (ca. 4 nm) in a sputter coater (Automatic Sputter Coater, Ingenieurbüro Peter Liebscher, Wetzler). Coating of chitosans and development *T. koningiopsis* on grapevine leaves were documented using a SEM (Phenom-World BV, Eindhoven, Netherlands).

3.3.5.2 Light microscopy

To investigate the development of *T. koningiopsis* on grapevine leaves within triple combination (*Trichoderma* sp. + 661 Ac/ 661 HCl/ 671 + 0.5-times field rate of Funguran® progress) leaf discs (Ø 1 cm) from treated grapevine plants were analyzed via light microscopy. Six and 13 days after application, leaf discs were clarified in saturated chloral hydrate solution (250 g/ 100 ml dH_2O) added with 0.01 % formaldehyde, to reduce conidia wash off, for 7 days at room temperature until green chlorophyll colour was vanished. Cleared leaf samples were immersed in a staining solution according to Bruzzese and Hasan

(1983) (300 ml 95 % EtOH, 150 ml $CHCl_3$, 125 ml 90 % $C_3H_6O_3$, 450 g $C_2H_3Cl_3O_2$ and 0.6 g aniline blue) for 1 day or they were stained within a mixture of Pelikan ink (10 %, v/v) and 5 % acetic acid (90 %, v/v) for 15 min. Bruzzese and Hasan (1983) solution allows staining of fungal structures on surface and within plant tissue while ink solution only enables colouring on leaf disc surface. Samples were mounted in 70 % glycerine. Fungal development was analysed and documented using an interference contrast filter of a Leitz DMRB microscope (Leica Microsystems GmbH, Wetzlar, Germany). Pictures were taken with an appropriate video camera (3 CCD-HV-C20A video camera, Hitachi Ltd., Tokio, Japan) and saved using the image analysis system Diskus (Technisches Büro Hilgers, Koenigswinter, Germany).

3.3.6 Statistical analysis

The statistical data was analysed with the statistic program IBM SPSS Statistics Version 28 (IBM Corporation, Armonk, USA). ANOVA was used to compare means of treatments. If conditions of normal distribution (Kolmogorov-Smirnov test) and homoscedasticity (Levene test) were complied, Tukey HSD was used ($p \leq 0.05$) to determine differences among the means. If no homoscedasticity was given Welch-ANOVA (comparison of means) and Games-Howell (Post-hoc test) were used ($p \leq 0.05$). Data that did not meet normal distribution and homoscedasticity were analysed using Kruskal-Wallis-H to determine effects between treatments and means were compared by pairwise comparison ($p \leq 0.05$).

3.4 Results

3.4.1 Selection of *Trichoderma*-compatible chitosans and highly chitosan- and copper-tolerant *Trichoderma* species

To evaluate differences within the compatibility of the different chitosans to *Trichoderma* spp., average mycelial growth of copper-tolerant *Trichoderma* isolates was analyzed within several chitosan media. Highest growth rates reflected most suitable chitosan candidate for a combined application. The results revealed best biocompatibility to copper-tolerant *Trichoderma* candidates for 661 Ac chitosan, in which significantly higher mycelial growth rates were observed compared to the other chitosans tested (Fig. 11A). Mycelial growth inhibition of 8 % was detected for 661 Ac compared to the control, whereas growth inhibition to control of the other chitosans was approximately 14 % (661 HCl), 33 % (671), and 49 % (651).

Since field application of the envisioned biocontrol and biostimulating complex will include copper- and chitosan-tolerant *Trichoderma* spore suspension, spore germination rates of copper-tolerant *Trichoderma* isolates were investigated additionally in different chitosan media. The significantly highest spore germination rate was observed within chitosan 661 Ac

(Fig. 11B). Here, the average germination rate exceeded that of the control. A germination inhibition of about 5 % compared to control was observed for 671, whereas higher inhibition rates were detected for the other chitosans tested (24 % in case for 661 HCl and 36 % when 651 was used).

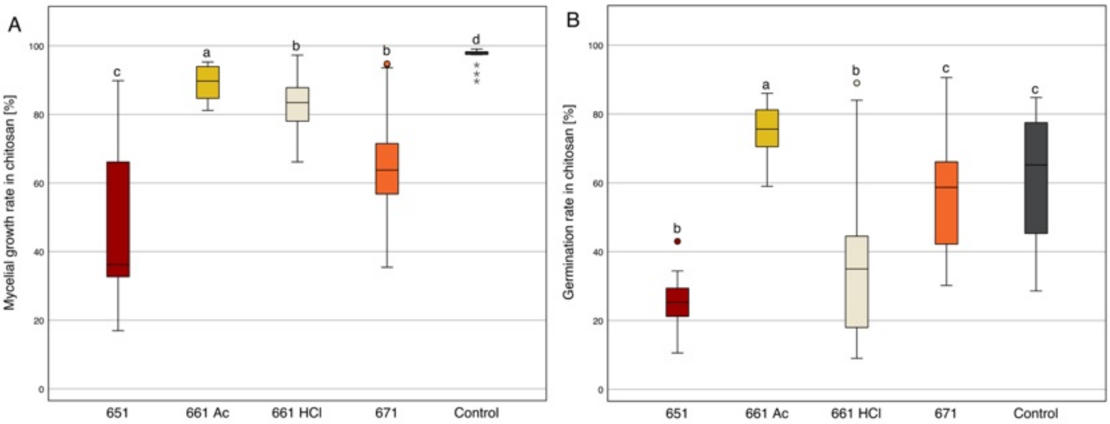

Figure 11: Compatibility of *Trichoderma* isolates to different chitosans. A) Average mycelial growth of eight *Trichoderma* isolates in chitosan 651, 661 Ac, 661 HCl or 671. Mycelial growth was measured in malt extract medium amended with 0.1 % chitosan. Boxplots represent the average of three independent experiments with seven repetitions for each of the eight *Trichoderma* isolates. B) Average spore germination of eight *Trichoderma* isolates in different chitosans. Spore germination was measured in malt extract medium amended with 0.01 % chitosan. Boxplots represent the average of four independent experiments with five repetitions for each of the eight *Trichoderma* isolates. *Trichoderma* isolates used belong to three different species (*T. koningiopsis*, *T. harzianum* and *T. rufobrunneum*). Within control variant no chitosan was used. Values are means ± SD. Welch-ANOVA was used to determine the effects between fungal mycelial growth or spore germination and different chitosans used. Means were compared by Dunnett-T3 test. Means with various letters (A: a, b, c, d or B: a, b, c) are significantly different ($p \leq 0.05$).

Due to low mycelial growth and spore germination rates of *Trichoderma* isolates within the 651 medium, the 651 chitosan was excluded from further experiments.

Mycelial growth and spore germination rates of eight copper-tolerant *Trichoderma* isolates were compared to select the most suitable *Trichoderma* isolate with a high fitness level in presence of chitosan. *T. koningiopsis* (*Tr30*) showed the highest growth and germination rates in comparison to the other tested isolates (Fig. 12) and compared to the control. Other test isolates just reached growth and germination rates between 55 and 64 %, whereas *T. koningiopsis* was able to achieve an average of 84 %.

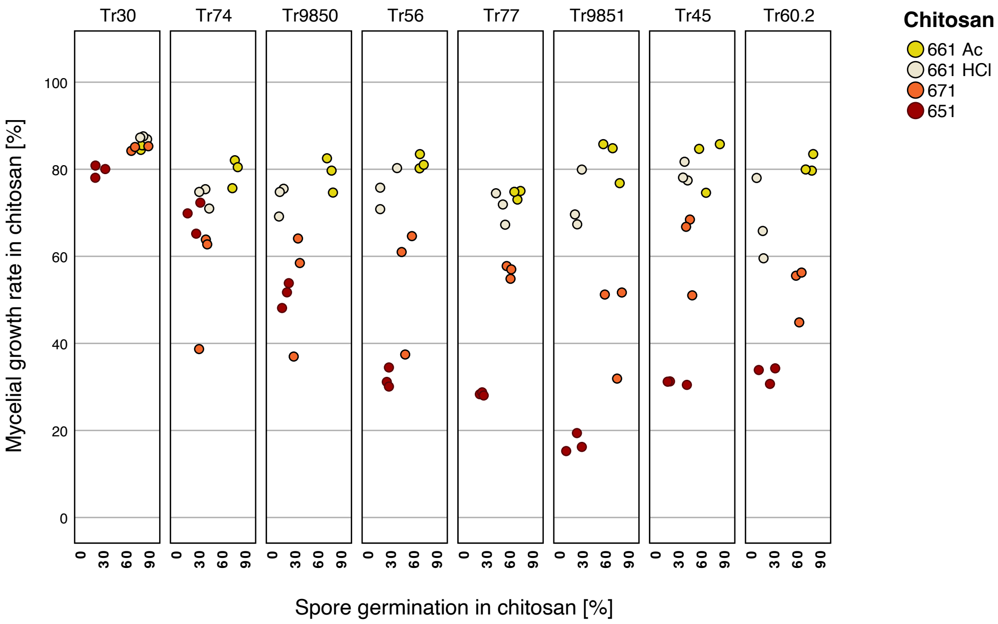

Figure 12: Fitness level of chitosan-tolerant *Trichoderma* candidates in chitosan. Ratio of mycelial growth rates to controls to the corresponding germination rates to controls of eight *Trichoderma* isolates are shown within malt extract medium amended with 661 Ac, 661 HCl, 671 or 651 chitosan in a final concentration of 0.1 %. Spore germination was measured in the same medium but using a lower concentration (0.01 % for each chitosan). Icons represent four independent experiments with three repetitions for each of the eight *Trichoderma* isolates.

Based on the high growth and germination rates as well as due to a high copper tolerance and since less affected by chitosan, *T. koningiopsis* was selected for further investigations.

3.4.2 Effect of application time of *Trichoderma* sp. and chitosan against *P. viticola*

To determine a suitable application period, spore production of the pathogen was analyzed after spraying the selected biocontrol agents and biostimulants at five different application times. Protective as well as a curative treatment/s were investigated. At 1 dbi (application one day before inoculation with downy mildew) to 0 dbi (application and inoculation on the same day), the sporulation of downy mildew was lowest (Fig. 13). Number of sporangia was reduced by 62 % (1 dbi) up to 68 % (0 dbi) compared to the untreated control. Therefore, this time period was considered in more detail for subsequent efficacy studies.

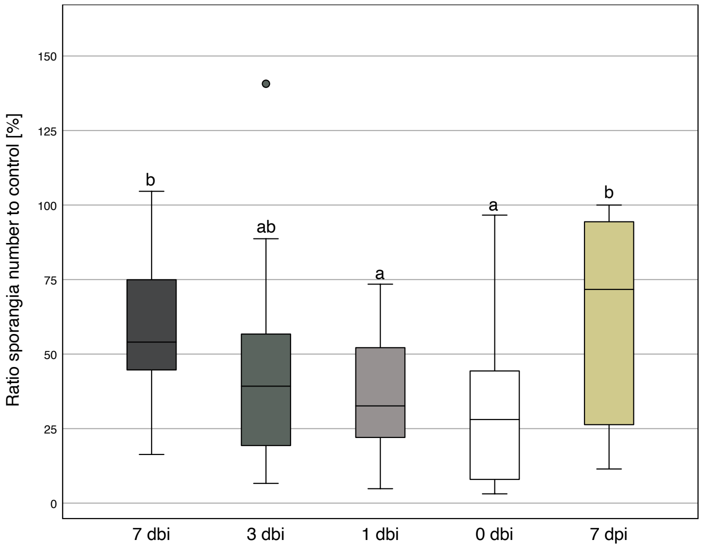

Figure 13: Sporangia production after *T. koningiopsis* and chitosan treatments against *P. viticola* at different time points. Ratio of sporulation on infected grapevines in comparison to untreated controls is shown in case for applications before and after inoculation with the pathogen (7 dbi, 3 dbi, 1 dbi, 0 dbi and 7 dpi). Spray applications of *T. koningiopsis* and different chitosans (661 Ac, 661 HCl and 671) were conducted with 10^6 *Trichoderma* spores ml^{-1} MEB 1 % and 0.01 % chitosan in dH_2O, respectively. Controls remain untreated. Sporulation was quantified seven days after pathogen inoculation. Boxplots represent the average of all biocontrol and biostimulant variants for which three independent experiments with 160 repetitions were performed. Values are means ± SD. Kruskal-Wallis-H was used to determine the effects between treatment time points. Means were compared by pairwise comparison. Means with various letters (a, b) are significantly different ($p \leq 0.05$).

3.4.3 Effect of combined treatments using a reduced copper rate against *P. viticola* on greenhouse grapevines

To investigate the effect of the selected *Trichoderma* strain and chitosan in combination with a reduced copper rate against *P. viticola*, single, dual and triple combinations were tested. For this purpose, greenhouse plants were first treated, then inoculated with *P. viticola*. In addition, upper and lower leaf sides were treated to conclude an indirect or direct mode of action. Regardless of the treated leaf side, sporulation of downy mildew was clearly inhibited compared to the untreated control (Fig. 14) and markedly reduced (average by 45 %) when the lower leaf side was treated. To our surprise, treatments of the upper leaf side also reduced spore production to a high extend (average by 42 %) compared to the untreated control. When treating the lower leaf sides, best results were achieved with the triple combinations of *T. koningiopsis*, 661 HCl or 661 Ac and a reduced copper rate, leading to significant spore reductions of about 48 %. For treatment of the upper leaf sides, best results were obtained with the combination of 661 HCl, 671 chitosan or *T. koningiopsis* and a reduced copper rate, and with the triple combination of *T. koningiopsis*, 661 HCl and copper (significant spore reduction of about 46 %). However, reduction of pathogen sporulation behaved similarly for all dual and triple agent combinations when the upper leaf sides were treated.

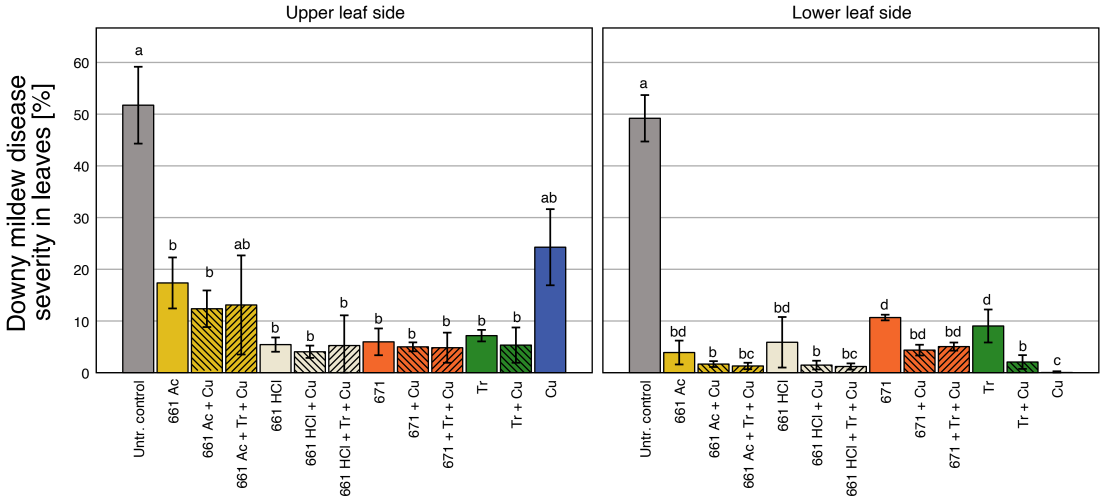

Figure 14: Direct and induced effects of single and combined applications of *T. koningiopsis* and chitosan together with a reduced copper rate on greenhouse grapevines inoculated with *P. viticola.* Severity of downy mildew disease [%] is shown in case of application 1 day before pathogen inoculation (1 dbi). Spray applications of *T. koningiopsis* and different chitosans (661 Ac, 661 HCl, and 671) to either the upper or lower leaf sides were made using 10^6 *Trichoderma* spores ml^{-1} MEB 1 % or 0.01 % chitosan in dH_2O. Half the field rate of the regular copper rate applied at the beginning of grapevine vegetation was used in the experiment (active ingredient: copper hydroxide, 0.625 g l^{-1}). Controls remain untreated. Sporulation of each treatment was quantified seven days after pathogen inoculation. Treatment of the upper leaf sides represents indirect effect of the treatments, whereas direct effect is shown by treatment of the lower leaf sides. Bars represent the average of three independent experiments with 10 repetitions for each of the treatment variants. Values are means ± SD. ANOVA (lower leaf side) or Welch-ANOVA (upper leaf side) was used to determine the effects between the treatments of each leaf side. Means were compared by Tukey HSD (lower leaf side) or by Games-Howell (upper leaf side) test. Means with various letters (a, b, c, d) are significantly different ($p \leq 0.05$).

3.4.4 Mode of action of biostimulants against *P. viticola*

To reveal the effect of each agent as a solo application and applied as a mixture, upper and lower sides of leaf discs were first treated followed by an inoculation with downy mildew. A low sporulation indicates high efficacy of the agents against *P. viticola*. Resistance inducers (Bion, BABA) were included in the study as additional controls. Compared to the untreated control, a reduced sporulation was observed with all biocontrol agents and biostimulants regardless which leaf side was treated (Fig. 15). Application of chitosan (661 HCl, 661 Ac and 671) led to the lowest sporangia production at both time points. Compared to the untreated control, lower leaf side treatment resulted in a reduced sporangia production of about 71,000 sporangia/ml (671), 80,000 sporangia/ml (661 Ac), and up to 85,000 sporangia/ml (661 HCl) at 0 dbi, which was slightly lower at 1 dbi (63,000 sporangia/ml, 671; 69,000 sporangia/ml, 661 Ac; 78,000 sporangia/ml, 661 HCl). Chitosans 661 HCl and 671 exhibited an indirect effect against *P. viticola* that partially exceeded the efficacy of the resistance inducers Bion and BABA. Spore production was reduced compared to the control by 47,000 sporangia/ml (661 HCl) to 49,000 sporangia/ml (671) at 1 dbi. *T. koningiopsis* was able to reduce sporangia numbers by 27,000 sporangia/ml (1 dbi) and 54,000 sporangia/ml (0 dbi) compared to the untreated control when abaxial leaf sides were treated. A similar indirect effect was observed (upper leaf side treatment). Treatment with *T. koningiopsis* was found to cause similar sporangia reduction compared to BABA and Bion.

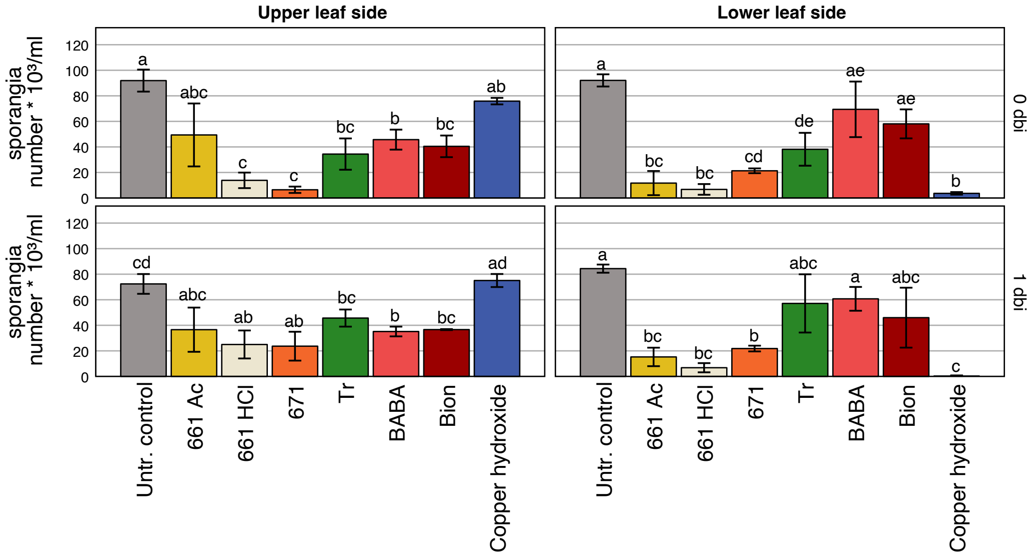

Figure 15: Direct and induced effects of *T. koningiopsis*, chitosan, and resistance inducers (BABA and Bion) on the number of *P. viticola* sporangia on inoculated grapevine leaf discs. The number of *P. viticola* sporangia is shown in the case of biocontrol and biostimulant treatment before inoculation with the pathogen (0 and 1 dbi). Spray applications of *T. koningiopsis* and different chitosans (661 Ac, 661 HCl, and 671) to either the upper or lower leaf disc sides were made using 10^6 *Trichoderma* spores ml^{-1} MEB 1 % or 0.01 % chitosan in dH_2O. Controls remain untreated. Resistance inducers (Bion, 0.5 $g\ l^{-1}$; BABA, 50 $\mu g\ ml^{-1}$) and a reduced field rate of copper fungicide (half the field rate of the regular copper rate applied at the beginning of grapevine vegetation, 0.625 $g\ l^{-1}$, active ingredient: copper hydroxide) were included as additional controls. Sporulation was quantified seven days after pathogen inoculation. Treatment of the upper leaf sides (UT) represents indirect effect of the treatments, whereas direct effect is shown by treatment of the lower leaf sides (LT). Bars represent the average of three independent experiments with 20 repetitions for each of the treatment agents. Values are means ± SD. Welch-ANOVA (UT) or Kruskal-Wallis-H (LT) was used to determine the effects between the agents for the respective times of treatment and corresponding treatment leaf side. Means were compared by Games-Howell (UT) or by pairwise comparison (LT) test. Means with various letters (a, b, c, d, e) are significantly different ($p \leq 0.05$).

3.4.5 Colonization of *Trichoderma* and distribution of chitosan on grapevine leaves

In order to ensure the efficacy of biocontrol agents and biostimulants against downy mildew, both successful development of *Trichoderma* spores and establishment of the fungus and uniform distribution of chitosan on abaxial and adaxial grapevine leaf sides are a basic requirement. To provide evidence, electron microscopic observations were performed 6 days and 16 days after spray application (daa) of *Trichoderma* spores and chitosan, respectively, to abaxial and adaxial leaf sides of grapevines. After 6 daa, mycelium formation and sporulation of *T. koningiopsis* was observed on abaxial leaf sides (Fig. 16B). The colonization of the fungus on grapevine leaves was also evident for adaxial leaf sides at 16 daa (Fig. 16A). Chitosan distribution was found as a platelet-like structure evenly distributed on leaf upper (Fig. 16C) and lower sides (Fig. 16D) at both time points of investigation.

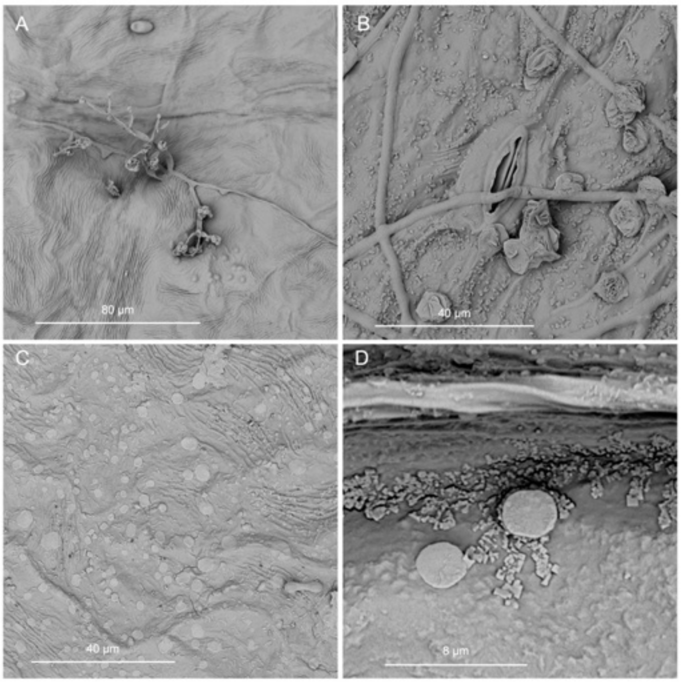

Figure 16: Colonization of grapevine leaves by *T. koningiopsis* and chitosan coating of leaves after spray application to upper and lower leaf sides. The development of *T. koningiopsis* and the distribution of chitosan on abaxial and adaxial leaf discs of grapevine were examined 6 and 16 days after spray application (daa) by scanning electron microscopy. Spray applications were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1 % and 0.01 % chitosan (661 Ac, 661 HCl and 671) in dH_2O, respectively. No *Trichoderma* and chitosan were applied in the control (not shown). (A) Leaf colonization and conidiophores formation of *T. koningiopsis* at 16 daa of the upper leaf side (US). (B) Mycelium formation and sporulation of *T. koningiopsis* at 6 daa of the lower leaf side (LS). (C) Overview of platelet-like structure of 671 chitosan evenly distributed on US at 16 daa. (D) Close-up of the platelet-like structure of 661 HCl chitosan at 6 daa of LS. Images are representative of two independent experiments with ten replicates for each variant.

3.4.6 Colonization of grapevine leaves by *Trichoderma* after simultaneous application of chitosan and copper

To verify a successful development of the selected *T. koningiopsis* isolate in combination with chitosan and copper on grapevine leaves, light microscopic examinations were carried out 6 and 13 days post treatment (dpi) (Fig. 17). At 6 dpi, an enlarged mycelium was observed in all samples of each variant, regardless of leaf side treated (Fig. 17, 6 dpi). In addition, the formation of conidiophores of *T. koningiopsis* was observed in in case for 661 Ac (Fig. 17, 2b) and 661 HCl plus copper (Fig. 17, 3a, b). This was not observed when chitosan 671 and copper was used (Fig. 17, 4a, b). Compared to the control, mycelial growth decreased significantly at 13 dpi (Fig. 17, 13 dpi) or was absent (Fig. 17, 2d). However, mycelial growth seemed not to be affected in presence of 671 and copper (Fig. 17, 4d) compared to the previous time point (Fig. 17, 4b). In contrast to the control, the formation of conidiophores and associated conidia was not observed at 13 dpi in any sample treated with chitosan and copper (Fig. 17, 1-4c, d).

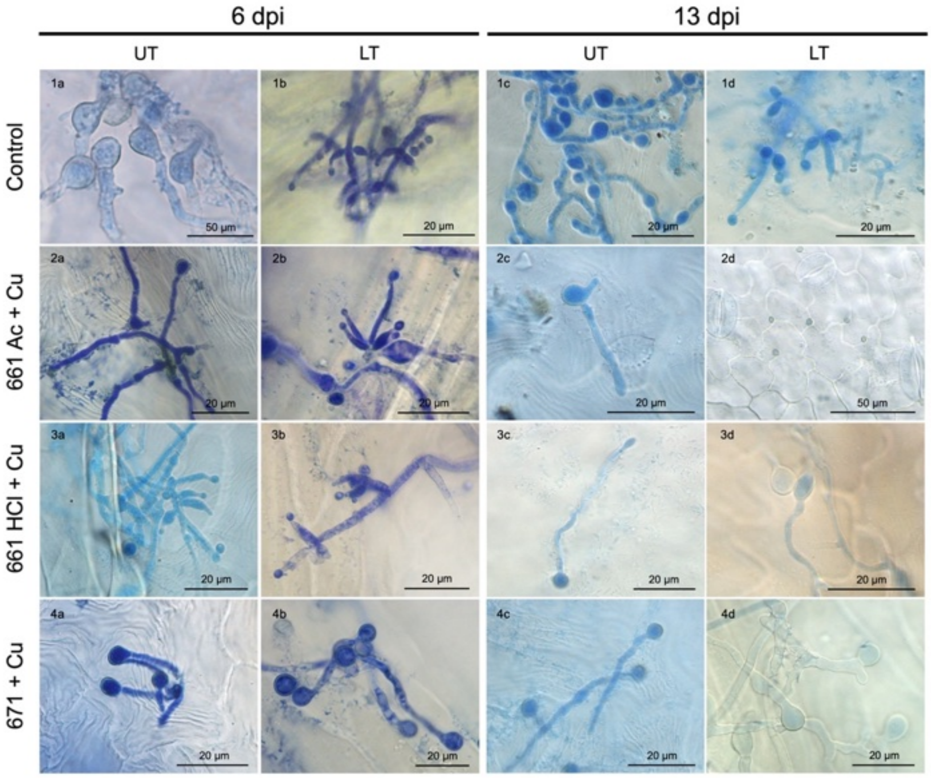

Figure 17: Leaf colonization of *T. koningiopsis* after a simultaneous application of chitosan and copper. Germinating spores, mycelium formation and sporulation of *T. koningiopsis* is shown in different chitosans (661 Ac, 661 HCl or 671) and copper. Spray applications were conducted with 10^6 *Trichoderma* spores ml^{-1} MEB 1 % and 0.01 % chitosan in dH_2O, respectively. Within control variant no chitosan and copper were applicated. Colonization of *T. koningiopsis* was examined for upper leaf side treatment (UT) and lower leaf side treatment (LT) at 6 days post application (6 dpi) and 13 dpi. Images represent the average of two independent experiments with 15 repetitions for each variant.

3.5 Discussion

In this study, a copper- and chitosan-tolerant isolate of *T. koningiopsis* and three chitosans compatible to *Trichoderma* were identified as suitable candidates for a combined application to develop a copper-reduced plant protection strategy against downy mildew on grapevine. To contribute to an environmentally friendly crop protection strategy, the field rate of the copper fungicide used at the beginning of the vegetation period was reduced by half. However, the tested compatibility of all agents is a prerequisite to allow better understanding of the complex, thus contributing to its optimization and to copper reduction in organic viticulture.

The investigation of mycelial growth and spore germination revealed a high biocompatibility of chitosan polymer-oligomer-mixtures (661 Ac, 661 HCl, and 671) together with *Trichoderma*, especially *T. koningiopsis*, in contrast to the chitosan-polymer-mixture (651) (Fig. 11). The low mycelial growth and spore germination rates of *Trichoderma* spp. observed with the chitosan polymer-mixtures could be explained by a higher direct antifungal effect of chitosan polymers compared to oligomers due to their polycationic structure, which destabilizes the fungal plasma membrane (Wattjes et al. 2020). The long chain structure of the polymers allows them to bind to a larger cell surface of the fungus than chitosan oligomers (Krauss et al., 1989). Consequently, the initial development of *Trichoderma* isolates is inhibited more strongly than with chitosan oligomers, unless there is a high tolerance to the active ingredient (Fig. 12, Tr30). *Trichoderma* species, including *T. koningiopsis* can be shown to have a high and innate resistance to fungicides (McLean et al., 2001; Singh et al., 2002; Küpper et al., 2022) and also to other antifungal molecules such as chitosan (Chittenden and Singh 2009; Zavala-González et al., 2016). In accordance to the conclusions of Zavala-González et al. (2016), the high mycelial growth and spore germination capacity of *T. koningiopsis* in chitosan medium demonstrated in this study could be explained by the ability of the isolate to degrade chitosan enzymatically. In this regard, chitosanolytic activity of various *Trichoderma* isolates has also been confirmed in other studies (e.g., da Silva et al., 2016). Chitosan interacts with the fungal cell membrane by increasing its permeability (Lopez-Moya et al., 2019). Lopez-Moya et al. (2019) showed that fungal chitosan resistance is associated with an enrichment of saturated free fatty acids in the plasma membrane plus a high glucan/ chitin ratio in the cell wall. In this context, Zavala-González et al. (2016) concluded a relationship between lower membrane fluidity due to a low content of linolenic acid and the resistance of *T. koningiopsis* to chitosan.

Different chitosans and a highly compatible isolate of *T. koningiopsis* were tested as single and combined applications, also together with a reduced copper rate to control *P. viticola*. Spore production as well as disease severity was already reduced by a single treatment of the biocontrol and biostimulating agent under controlled conditions (Figs. 14 and 15). In particular, chitosan showed a high antifungal effect against *P. viticola*. However, there are molecule-specific differences. Rahman et al. (2015) assigned higher antifungal activity towards the grey mold pathogen *Botrytis cinerea* when degree of acetylation was low and degree of polymerisation was high. Both, spore germination and germ tube elongation were in that case effectively suppressed. This is in accordance to the observed indirect effects when upper leaf sides were treated, since 661 Ac and 661 HCl have a similar degree of polymerisation and N-acetylation. However, it still remains unclear why a lower antifungal effect against *P. viticola* was observed when treating the lower leaf sides with 671, which also shows a high degree of polymerisation and only slightly exceeds the degree of acetylation of the other chitosans used. In this context, a difference in chitosan deposits on the leaves could offer a potential explanation for the divergent direct effect of the two chitosans. In addition to the (chemical) antifungal properties, the dispersion of chitosan leading to platelet-like elevations that may represent a direct physical barrier for the pathogen, can impede the penetration of stomata and thus the subsequent leaf invasion. Adamuchio-Oliveira et al. (2020) demonstrated thickening of the cell walls of tomato leaf epidermis after a chitosan-copper treatment, that provide another effective barrier to pathogens as shown for powdery mildew.

Even though an application of biocontrol and biostimulating agents is an environmental-friendly and consumer-safe approach, single treatments are often not effective to control diseases under field conditions. However, by combining *T. koningiopsis* and chitosan and adding a reduced field rate of a common copper fungicide, spore production of *P. viticola* was significantly reduced compared to single treatments (Fig. 14). Monte (2001) confirmed that single *Trichoderma* treatments in the field have not yet achieved sufficient success against pathogens. Similarly, Shukla et al. (2021) showed that neither *Trichoderma* nor chitosan were effective in combating late blight infection when applied as single treatments and concluded that the addition of reduced copper rate might sufficiently support the effect of the tested agents. This is in accordance with the results obtained in this study, since it was also shown that the addition of copper, even at a reduced rate, enhanced the antifungal effect of the respective biocontrol and biostimulating agent. Chitosan is able to bind copper through a free electron pair, forming an effective chitosan-copper complex (Varma et al., 2004). Thus, the antifungal effect of chitosan can be increased due to a synergistic effect of copper. Copper-tolerant *Trichoderma* isolates are able to bind copper to their cell surface in order to prevent uptake of the active ingredient (Küpper et al., 2022). Apart from that, the antifungal effect of *Trichoderma* is attributed to the production of cell wall degrading enzymes (e.g., endochitinase, exochitinase, exo-β-D-N-acetylglucosaminidase), for which inhibition of spore germination as well as hyphal elongation of pathogens were demonstrated in literature (e. g., Saravanakumar et al., 2016).

The greenhouse experiments conducted in this study showed a more effective reduction in sporulation of *P. viticola* by combining all agents compared to single or dual application (Fig. 14). Investigations of Bohra (2018) also addressed a compatible triple combination of copper, chitosan, and *Trichoderma* and explain the interaction of the three agents and the mode of action for potato late blight management as a process of subsequent events. First, suitable *Trichoderma* isolates are able to produce and release chitosanases and cellulases, then chitosan is enzymatically degraded into its highly effective antifungal oligomers; in addition, cellulases degrade simultaneously the cell wall of the pathogen. An enzymatically modified cell wall allows the copper active ingredients to enter and act directly on the intracellular components. Thus, the pathogen's growth can be inhibited. This approach is also conceivable to the *Trichoderma*-chitosan-copper combination used here. In this context, it has to be ensured that, even applied as triple combination, the biological agent can successfully colonize the plant and remain in the pathogen's living environment, even if this environment does not correspond to the original habitat of the agent. Our microscopic results demonstrated successful mycelial growth and sporulation of *T. koningiopsis* when applied in combination with chitosan and copper on upper and lower sides of grapevine leaves (Fig. 17). However, since leaves are not the original environment, the presence of living mycelium seems to decline with time, as mycelial growth was significantly reduced after 13 dpi. These findings are in accordance to Samuelian (2016) who also demonstrated successful establishment and development of *Trichoderma* on field bananas. A decrease in colonization in banana was observed after six days when spores were applied in aqueous solution, but after 12 days when a *Trichoderma* molasses mixture (5 %) was applied (Samuelian, 2016). Therefore, weekly applications seem to be necessary to guarantee high vitality of the biological agent.

Besides a direct antifungal effect when treating the lower leaf sides, our results also indicate an indirect resistance-inducing effect against *P. viticola* when treating the upper leaf sides with *T. koningiopsis* and chitosan by spray application. All individual treatments of the upper leaf sides led to a reduced sporulation or disease severity of downy mildew in an agent-specific manner (Figs. 14 and 15). This is in accordance to the literature and was also been demonstrated for *T. harzianum* strain T39 that induces systemic resistance in various horticultural and field crops (Elad, 2000; de Meyer et al., 1998) including grapevine (Perazzolli et al., 2008). *Trichoderma* increases the activity of resistance-related enzymes such as glucanases, chitinases, cellulases and peroxidases by releasing elicitors and increasing plant defense (Yedidia et al., 2000). Since chitosan acts itself as a potential elicitor molecule de Bona et al. (2021) demonstrated a chitosan-induced upregulation of JA- and ET-mediated defense responses, salicylic acid downregulation and *trans*-resveratrol modulation in grapevine inoculated with *B. cinerea*. Lucini et al. (2018) used a similar low chitosan concentration (0.03 %) as in our investigations for spray treatment of grape bunches and demonstrated increased chitosan-induced accumulation of phytoalexins and alteration of oxidative stress response enzymes. Such resistance-increasing effects triggered by *Trichoderma* and chitosan in grapevine could explain the inhibitory effect against *P. viticola* when upper leaf sides were treated and both, *Trichoderma* and chitosan, do not interfere directly with the pathogen. This hypothesis is also supported by the similar inhibiting effect induced by resistance inducers (Fig. 15). Da Silva et al. (2018) showed in greenhouse experiments a significant reduction in disease severity in chickpea leaves after an inoculation with *Colletotrichum lindemuthianum* if different abiotic resistance inducers (Bion, BABA, chitosan and SA) or *T. aureoviride* were applied prior inoculation.

Interestingly, disease-inhibiting effect against *P. viticola* induced by chitosan was higher compared to *T. koningiopsis* at both selected time points (Fig. 15), maybe due to a faster induction of resistance mechanisms. Aziz et al. (2006) demonstrated an increase in phytoalexin production within two days after chitosan application to grapevines, whereas an expression of several resistance genes induced by volatile organic compounds of *Trichoderma* occurred in grapevines after six days (Lazazzara et al., 2021). However, different resistance genes were already expressed one day after an inoculation with *P. viticola* and *Trichoderma* (Perazzolli et al., 2011), which is in accordance to the reduced spore production in the present study. It seems that the resistance-inducing effect and its dynamic depends on the isolate of *Trichoderma* and the type of chitosan used. Thus, to achieve a high efficacy after applying a *Trichoderma*-chitosan combination a better knowledge about the underlying mechanisms and their activity in a distinct time frame is important and has to be considered in further studies and may lead to a preventive and sustainable crop protection.

3.6 Conclusion

In conclusion, this study identified an effective and synergistic combination of *Trichoderma*, chitosan and a reduced copper rate to control *P. viticola*. The investigations allow a better understanding of the biocontrol and biostimulating complex, thus contributing to its optimization and the development of a stable synergistic plant protection strategy. A strain of *T. koningiopsis* was proven to exhibit a high tolerance towards copper and chitosan, even when applied in combination. This allows a reduction of the rate of copper-based fungicides especially in organic viticulture due to 1) direct antifungal and resistance-inducing properties of *Trichoderma* and chitosan, 2) a successful establishment of *T. koningiopsis*, and 3) the uniform and stable distribution of chitosan on grapevine leaves. Further studies on the direct and resistance-inducing mode of action of the agents tested could help to better understand the tri-trophic interplay between grapevine, downy mildew and the applied biocontrol and biostimulating complex. Furthermore, trials have to be performed in vineyards and under field conditions to confirm the results obtained in this study. However, sufficient amounts of chitosans are an essential prerequisite.

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4 *Trichoderma koningiopsis* and chitosan acting in a complex as resistance inducer against *Plasmopara viticola*

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4.1 Abstract

Downy mildew, caused by *Plasmopara viticola*, leads to significant reductions in grape yield and quality. Copper-based compounds are used for control, which can have ecotoxicological impact on the environment. Biocontrol agents and biostimulants show an environmentally-friendly alternative for pesticide reduction. However, understanding of the modes of action is needed to achieve maximum efficacy against pathogens. Previous work has demonstrated the successful application of a synergistic complex of a copper- and chitosan-tolerant *Trichoderma* isolate and chitosan, resulting in the reduction of downy mildew on grapevine. For a better understanding of the tri-trophic interaction between the synergistic complex, grapevine and *P. viticola*, the induced resistance by *T. koningiopsis* and chitosan has to be further characterized. The indirect mode of action of the complex was verified in greenhouse experiments. Moreover, pathogen-related gene expression analysis showed an increased expression in different grapevine cultivars when both agents were applied simultaneously. Similarly, studies of reactive oxygen species accumulation after treatment with agents showed an oxidative burst in resistant cultivars. Since haustoria are a key structure of *P. viticola*, haustoria number after agent treatment was studied. An inhibitory effect on haustoria formation was observed. This study contributes to a better understanding of the multipurpose properties of the synergistic complex against *P. viticola* and improves the use of a promising tool with simultaneous copper reduction.

Keywords: *Plasmopara viticola*, biostimulants, induced resistance, copper reduction, organic viticulture, *Vitis vinifera*

4.2 Introduction

Downy mildew, caused by the obligate biotrophic oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni, can cause significant yield and quality reductions in grapevines (Gessler et al. 2011). Regular copper-based fungicide applications are used in organic viticulture to control the disease (Gessler et al. 2011). However, long-term intensive use of these fungicides leads to copper entering and accumulation. Immobile copper can be fixed, with potential ecotoxic effects on soil and its biological quality (Karimi et al. 2021). Therefore, copper active substances have been classified as candidates for substitution by the European Commission (European Commission Implementing Regulation 2015/408 of March 11, 2015) and their application has been limited to 15 kg per hectare over a 5-year period with an average of 10 to 12 treatments per year (European Commission Implementing Regulation 2018/1981 of December 13, 2018; Vereinigung Schweizer Biolandbau-Organisationen, Bio Suisse, 2024). However, up to 16 treatments are required due to unfavorable weather conditions. Unfortunately, a higher copper application rate in one year leads to a reduced flexibility in another year due to the 15 kg limitation in a 5-year period.

This highlights the need for effective, sustainable crop protection strategies to reduce copper application and to replace it sustainably.

Using biocontrol agents and/or biostimulants is a promising alternative to reduce pesticide use (Galli et al. 2024). These agents control pathogens in two ways: directly, e.g., through production of inhibitory compounds or mycoparasitism; and by inducing plant immune system, which also contributes to sustainable control of pathogens (Kamble et al. 2021; Galli et al. 2024). Pathogen attack is recognized by plants based on microbe-associated molecular patterns (MAMPs) or host-derived damage-associated molecular patterns (DAMPs) using specific plant receptors (plant plasma membrane pattern recognition receptors). Microbial compounds such as fungal chitin, β-glucans, or released toxins and enzymes act as MAMPs, while DAMPs represent endogenous warning signals (Boutrot and Zipfel 2017). These structures trigger a series of signaling events that induce defense-related genes, leading to the synthesis of pathogen-related proteins and antimicrobial compounds, cell wall reinforcement, or hypersensitive response in the plant (Garcia-Brugger et al. 2006; Héloir et al. 2019).

Trichoderma species impair microorganism in various ways, making them a promising and eco-friendly alternative to pesticides. In addition to five major direct control mechanisms (parasitism, antibiosis, enzymatic activity, competition, and production and release of volatile organic compounds), the induction of local and systemic plant defenses has been demonstrated (Woo et al. 2023). *Trichoderma* is able to activate different types of plant resistance: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Salwan et al. 2022). Kamble et al. (2021) showed increased enzymatic activity and an induced defense response against *P. viticola* after treating field-grown grapevines with *T. harzianum*. They attributed this to a biopriming effect.

Chitosan, an N-deacetylated derivative of chitin, exhibits antimicrobial properties against pathogens as well as indirect modes of action through resistance induction in host plants (Kappel et al. 2022). Chitosan acts as an elicitor, inducing systemic resistance to *Botrytis cinerea* in grapevine by upregulating the jasmonic acid and ethylene-mediated response and downregulating salicylic acid and modulating trans-resveratrol (De Bona et al. 2021).

However, single biocontrol or biostimulant treatments often prove to be insufficient for pathogen control (Shukla et al. 2021). Combining agents with a reduced pesticide rate may enhance stability of the control effect (Shukla et al. 2021; Küpper et al. 2023). Achieving the most beneficial effect of a biocontrol and biostimulating complex against pathogens depends on filling knowledge gaps about agents' modes of action. Our previous work demonstrated the efficacy of a synergistic biocontrol and biostimulating complex consisting of *Trichoderma* and chitosan, against grapevine downy mildew (Küpper et al. 2023).

To understand the tri-trophic interaction between the synergistic complex, grapevine, and *P. viticola*, this study examines the resistance mechanisms of grapevine induced by copper-and chitosan-tolerant *T. koningiopsis* and chitosan under the impact of downy mildew. A possible resistance-inducing effect of the complex treatment was investigated on different grapevine genotypes in order to obtain a clear characterization of the effect. The work should also provide information on whether the complex treatment can modify the plant's defenses and thus addresses another target (grapevine), in addition to the direct effect on the pathogen already demonstrated. For this purpose, the effect of an indirect mode of action of agents against *P. viticola* on susceptible grapevines was demonstrated in greenhouse. Since the expression of pathogen-related (*Pr*) genes encoding for proteins or enzymes is involved in plant defense responses to pathogens, *Pr10* gene expression analysis was performed on the susceptible cultivar (cv.) Müller-Thurgau and two resistant cvs., Regent and Cabernet blanc, previously treated with *T. koningiopsis* and chitosan and infected with *P. viticola*. *Pr10* is associated with grapevine resistance to *P. viticola* by inducing the production of reactive oxygen species (ROS) and a subsequent cell death defense (Ma et al. 2018). ROS accumulation is linked to defense responses of grapevine against *P. viticola* (Wingerter et al. 2022). The effects of single or combined treatments on susceptible and resistant cultivars, and *P. viticola* infection, were studied by monitoring the accumulation of superoxide ions and hydrogen peroxide.

Microscopic studies were used to demonstrate the effect on downy mildew at the cellular level of the treated grapevines and to further investigate the effect of the complex on the development of the pathogen's haustoria, an essential structure for nutrient uptake by *P. viticola*.

4.3 Materials and Methods

4.3.1 *Trichoderma* isolate and chitosan

The copper- and chitosan-tolerant *Trichoderma* isolate of *T. koningiopsis* Samuels, Carm. Suárez & H.C. Evans was used for the investigations, which has been characterized in previous studies (Küpper et al. 2022, 2023). *T. koningiopsis* was isolated from grapevines (wood from cordon arms, stems, and wooden shoots) growing in Rhineland-Palatinate (Germany) as several other *Trichoderma* spp. indicating grapevine wood as a natural habitat for this genus. Previously, *Trichoderma*-based products were never applied to the used grapevines. The purified isolate was grown on malt extract agar (MEA, 2% malt extract, Arche Naturküche, 2% agar, Roth, Karlsruhe, Germany; 0.01% Tetracycline, Roth, Karlsruhe, Germany) plates in a climate chamber (21 °C, 70% relative humidity [RH]) and was subcultured every month.

Chitosan 661 HCl (polymer-oligomer-mixture, 10% w/v, average degree of N-acetylation [DA] 17%, average degree of polymerization [DP] 350) was used for laboratory and greenhouse experiments. In addition, chitosan 671 (polymer-oligomer-mixture, 1% w/v, DA 16%, DP 200) was used for greenhouse trials. The chitosans were kindly provided by the

Institute of Plant Biology and Biotechnology, University of Münster (Germany) and are described in detail by Richter et al. (2025).

4.3.2 Plants and pathogen

The highly to *P. viticola* susceptible *Vitis vinifera* cv. Müller-Thurgau and two resistant cvs. Regent (Fischer et al. 2004; Welter et al. 2007) and Cabernet blanc (Eisenmann et al. 2019) were cultivated from canes of grapevines of the State Education and Research Center of Viticulture, Horticulture and Rural Development (Neustadt/ Weinstr., Germany) as already described in previous work (Küpper et al. 2022).

The *P. viticola* isolate was provided by the State Education and Research Center of Viticulture, Horticulture and Rural Development and was propagated according to Malacarne et al. (2011).

4.3.3 Greenhouse trials

An infection assay on greenhouse plants was used to describe the effects of the complex (*T. koningiopsis* and chitosan) on leaf infection by *P. viticola*. In order to obtain information on the mode of action against *P. viticola*, grapevines were treated with the biocontrol and biostimulating spray application of *T. koningiopsis* (10^6 spores ml^{-1} MEB 1%), chitosan (661 HCl or 671, 0.01% in dH_2O) or a combination (until run-off) either on the abaxial or adaxial leaf surface, one day before pathogen inoculation. dH_2O served as control. Infection with *P. viticola* by spaying abaxial leaf surface, treatment with the single and mixed agents one day prior to pathogen inoculation, and disease assessment were performed as described previously (Küpper et al. 2023).

4.3.4 RNA extraction and quantitative real time PCR expression analysis

Four individual plant replicates of cvs. Müller-Thurgau, Regent and Cabernet blanc were sprayed with the agents (*T. koningiopsis*, chitosan 661 HCl) as described previously. At 0, 9 and 15 hpi, ten leaf discs per replicate and treatment were sampled, pooled and used for RNA extraction. Sampling of leaf discs and pathogen inoculation or dH_2O treatment (control) was performed according to Eisenmann et al. (2019). For RNA isolation, Spectrum Plant Total RNA Kit (Sigma Aldrich) was used according to the manufacturer's instructions. A Nano-drop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) was used to determine RNA purity (A_{260}/A_{280} nm) and quantification. Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific Inc., Wilmington, DE, USA) was used for reverse transcription of 500 ng of grapevine total RNA and removal of gDNA contamination according to the manufacturer's instructions. qPCR was conducted with the SYBR Green method on a Rotor-Gene Q (Qiagen). Quantification of cDNA, normalization with the reference genes *VvUbiquitin*, *VvEF1a* and *VvGAPDH* (Reid et al. 2006), analysis of melt curves, and measurement of primer efficiency were carried out as already described by Eisenmann et al. (2019) and Pfaffl et al. (2002).

4.3.5 Histochemical studies

To investigate the effect of *T. koningiopsis* and chitosan on grapevine resistance to *P. viticola*, individual plants of each cultivar were treated with spray application of *T. koningiopsis* (10^6 spores ml^{-1} MEB 1%), 661 HCl (0.01% in dH_2O), or a combination of both (until run-off) one day before pathogen inoculation. dH_2O served as control. After one hour, leaf discs (Ø 1 cm) were cut with a cork borer from the fourth or fifth fully expanded leaf below shoot apex. For all staining studies, 12 leaf discs from three individual plants of each cultivar were inoculated with *P. viticola* in three independent experiments and incubated according to Eisenmann et al. (2019). A 40 µl droplet of zoospore suspension (50,000 sporangia ml^{-1}) or sterile dH_2O (mock variant) was placed on the abaxial surface of each leaf disc. For all histochemical studies Leitz DMRB microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with an appropriate video camera (3 CCD-HV-C20A video camera, Hitachi Ltd., Tokio, Japan) was used. Taken pictures were saved via image analysis system 'Diskus' (Technisches Büro Hilgers, Koenigswinter, Germany).

4.3.6 DAB and NBT staining

To access hydrogen peroxide (H_2O_2) accumulation in infected leaf discs, diaminobenzidine staining with 3,3'-diaminobenzidine (DAB; Merck) was performed according to Wingerter et al. (2021). Localization of superoxide radical (O_2^-) accumulation was performed by staining with nitro blue tetrazolium (NBT; Merck) according to Shinogi et al. (2003). Oxidation of DAB by H_2O_2 results in a visible brown precipitate in plant tissue, whereas O_2^- forms a bluish complex when oxidizing NBT. Reactive oxygen species (ROS) were detected at 6, 12, 18, 24, 30, and 36 hpi. Quantification of the respective ROS levels was based on coloured accumulation areas measured with Image J (Version 1.53a, National Institute of Health, USA). Three images of different leaf discs were taken at 20× magnification, in which the colored areas were manually outlined for area calculation by the program. To verify ROS accumulation, sections of the stained leaf surfaces were viewed and photographed at 100× magnification. Appropriate images were stacked using Helicon Focus software (Version 3.20.3, Helicon Soft Ltd., Ukraine) to determine the contact points of the pathogen with the cell areas.

4.3.6.1 Investigations of haustoria

Stained leaf discs were also examined for differences in pathogen haustoria number after previous biocontrol and biostimulating agent treatment. Deviating from the previous procedure, the number of haustoria at ten infection sites of different leaf discs was counted at 100× magnification for each application variant and grapevine variety. Data were collected at 12, 18 and 24 hpi.

4.3.7 Statistics

Data were statistically analysed using IBM SPSS Statistics Version 28 (IBM Corporation, Armonk, USA). Means of treatments were compared with ANOVA. When normal distribution (Kolmogorov-Smirnov test) and homoscedasticity (Levene test) were met, Tukey HSD was used ($p \leq 0.05$) to detect differences between means. When homoscedasticity was not demonstrated, Welch-ANOVA (comparison of means) and Dunnett-T3 (Post-hoc test) were applied ($p \leq 0.05$). Data that were not normally distributed and lacked homoscedasticity were analysed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison ($p \leq 0.05$).

For gene expression studies, treatment and cultivar were compared using a two-way ANOVA. Tukey HSD or Bonferroni were used for multiple comparisons. Partial eta-square (η^2) was calculated to assess the effect sizes of the factors and their interaction.

4.4 Results

4.4.1 Effects of *Trichoderma koningiopsis* and chitosan against *Plasmopara viticola* on greenhouse grapevines

The effect of single and combined treatments with *T. koningiopsis* and chitosan on downy mildew disease reduction was evaluated following treatment of the lower or upper leaf surfaces. Both *T. koningiopsis* and chitosan treatments showed a reduction in disease severity on grapevine leaves infected with *P. viticola* (compared to untreated control) (Fig. 18). This effect was observed on both sides of the leaves. Treatment with all biocontrol and biostimulant variants reduced disease severity compared to the untreated variant, especially when a combined treatment was used. When treating the upper leaf sides, the induced effect of each biostimulation treatment showed a reduction in downy mildew disease severity from 78% (Tr) to 92% (661 HCl + Tr). A significant reduction in upper leaf side disease severity was observed after application of the single and combined agents compared to copper fungicide application. When treating the lower leaf sides, a treatment with the reduced copper rate showed the highest disease reduction (100%). A significantly lower effect on pathogen was observed for the individual treatments compared to the 100% effective copper variant, while the combination variants of *T. koningiopsis* and chitosan showed no significant difference in their efficacy compared to the copper variant. The treated biocontrol and biostimulating agents reduced disease severity by 78% (Tr) to 90% (661 HCl + Tr) on lower leaf sides, similar to treatment on upper leaf sides. Combining *T. koningiopsis* with chitosans increased the effect for both upper and lower leaf treatments compared to the treatment with only one agent.

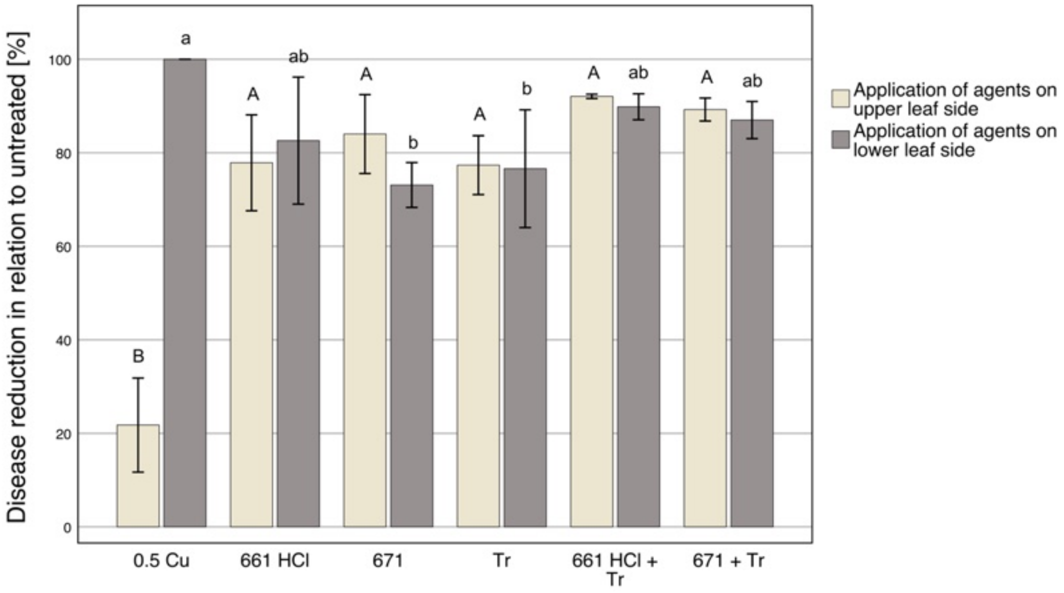

Figure 18: Disease reduction of downy mildew by single and combined application of *T. koningiopsis* and chitosan to the upper or lower leaf sides of greenhouse grapevines infected with *P. viticola*. Disease reduction is shown in relation to untreated controls (= 100% disease severity). Leaves of cv. Müller-Thurgau were used for quantification. Spray applications of *T. koningiopsis* (Tr) and different chitosans (661 HCl, 671) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH_2O, respectively. Half the field rate of the regular copper rate applied at the beginning of grapevine vegetation was used in the experiment (0.5 Cu; active ingredient: copper hydroxide, 0.625 g l^{-1}). Plants were inoculated by spraying abaxial leaf surface with *P. viticola* zoospore suspension (50,000 sporangia ml^{-1}) one day after treatment of the agents. Sporulation was quantified seven days after *P. viticola* inoculation. Bars represent the average of three independent experiments with 10 replicates for each treatment. Values are means ± SD. ANOVA was used to determine the effects between treatments. Means were compared by Tukey HSD test. Means with different letters (a, b or A, B) are significantly different ($p \leq 0.05$).

4.4.2 Effect of biostimulation treatment on the gene expression of *Pr10* of different grapevine cultivars inoculated with *Plasmopara viticola*

The relative gene expression of the defense gene *Pr10* from different grapevine cultivars was analyzed by quantitative real-time RT-PCR. Gene expression of *Pr10* after *P. viticola* infection was observed in the resistant cvs. Regent and Cabernet blanc (Fig. 19, with Pv). In the absence of pathogen treatment, no increase in gene expression was detected among the different grapevine cultivars (without Pv). The cv. Cabernet blanc significantly increased gene expression at 0 hpi compared to the other two cultivars. The relative gene expression increased of approximately 19-fold compared to the non-infected Cabernt blanc control (Fig. 19, 0 hpi). At 9 hpi, expression further increased to 28-fold compared to the uninfected control. Furthermore, cv. Cabernt blanc showed significantly higher *Pr10* gene expression compared to the other two pathogen-infected varieties. However, a higher expression level

(10-fold) of the cv. Regent was also detected at 9 hpi compared to the uninfected control. This expression level was found to be significantly different from the expression level of the sensitive cv. Müller-Thurgau (0.5-fold). At 15 hpi, the relative gene expression of the cv. Regent (1.3-fold) was still significantly different from that of the cvs. Müller-Thurgau (0-fold) and Cabernet blanc (0.5-fold), but no major increases in gene expression were observed compared to the respective uninfected controls.

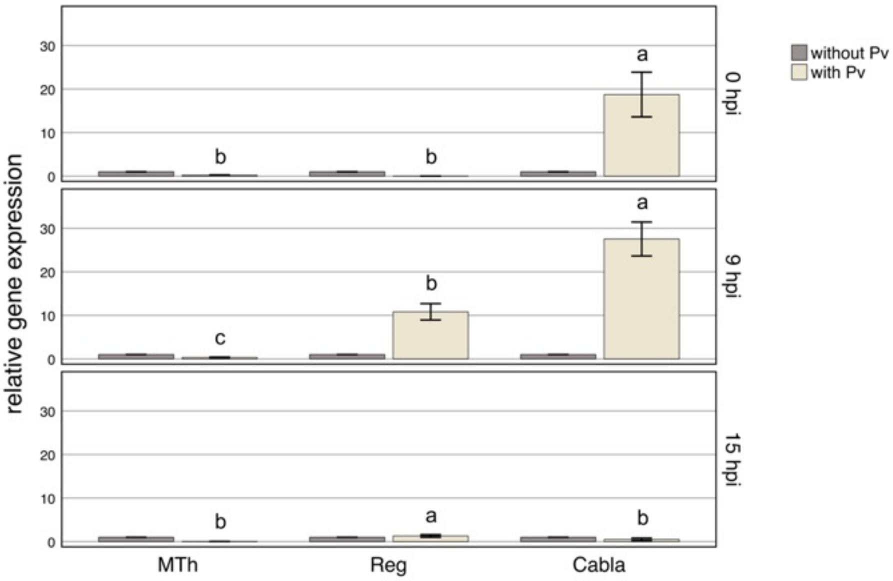

Figure 19: Effect of *P. viticola* infection on gene expression of the defense-related *Pr10* gene in susceptible cv. Müller-Thurgau, and resistant cvs. Regent and Cabernet blanc. Relative effect on gene expression to untreated controls is shown (with Pv). The expression levels at 0, 9, and 15 hours post inoculation (hpi) with *P. viticola* (with Pv) and without pathogen inoculation (without Pv) are shown. The expression levels were determined by qPCR and normalized against the housekeeping genes *VvGADPH*, *VvEF1a*, and *VvUBI*. Bars represent the mean of three independent measurements of triplicates of four pooled biological replicates. Error bars indicate standard deviation. Since the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means for *P. viticola*-infected variants per time point. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

The effect of *T. koningiopsis* and chitosan (individually and in combination) on the expression with and without the pathogen was investigated. Gene expression of *Pr10* was significantly higher induced in cv. Müller-Thurgau at 15 hpi after infection with *P. viticola* compared to the expression after treatment and pathogen inoculation at 0 and 9 hpi (Fig. 20). This was especially the case for the combined treatment with *T. koningiopsis* and chitosan. The *Pr10* level was increased 19-fold (15 hpi, Tr + Chi) compared to the untreated control (untreated control = 1). Strikingly, combined treatment with both agents enhanced the effects of single treatment with *T. koningiopsis* and chitosan on gene expression at 15 hpi after pathogen infection. Even without pathogen inoculation, a slight increase in gene expression was observed at 9 hpi, especially with chitosan treatment and the combined treatment of *T. koningiopsis* and chitosan. Here, cv. Müller-Thurgau showed a 3-fold increase in expression at 9 hpi after treatment with the respective agents compared to the respective untreated variants. However, this elevated level of expression decreased by 15 hpi, when expression resembled that of untreated variants. Furthermore, a significant interaction effect between the two factors time point and treatment was found in both *P. viticola* infected and uninfected grapevines (partial eta-squared [η^2] of factor interaction in uninfected plants: 0.537; η^2 of factor interaction in infected plants: 0.966). Between the factors time point and treatment exists a disordinal interaction, so that the effect of one factor depends on the other factor.

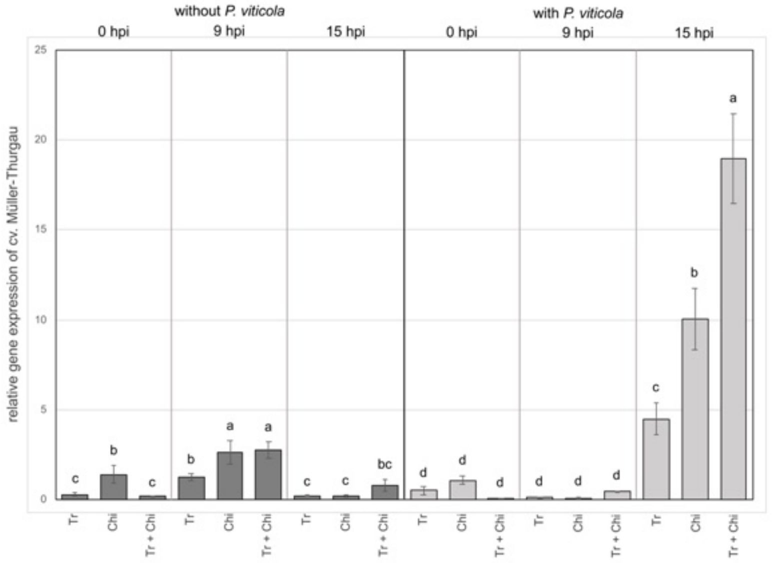

Figure 20: Effect of *T. koningiopsis* and chitosan treatment on gene expression of the defense-related *Pr10* gene in cv. Müller-Thurgau inoculated with *P. viticola*. Relative effect on gene expression to untreated controls are shown. Spray applications of *T. koningiopsis* (Tr) and chitosan (661 HCl) were made with 10^6 Trichoderma spores ml^{-1} MEB 1% and 0.01% chitosan in dH$_2$O, respectively. The expression levels at 0, 9, and 15 hours post inoculation (hpi) with *P. viticola* (right side of the figure) or at the same time points without pathogen inoculation (left side of the figure) are shown. The expression levels determined by qPCR and normalized against the housekeeping genes *VvGADPH*, *VvEF1a* and *VvUBI*. Bars represent the mean of three independent measurements of triplicates of four pooled biological replicates. Error bars indicate standard deviation. Since the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means for treatments at the different time points. Analysis was conducted separately for no infection (without *P. viticola*) and infection (with *P. viticola*) with the pathogen. Means with different letters (without *P. viticola*: a, b, c; with *P. viticola*: a, b, c, d) are significantly different ($p \leq 0.05$).

Cv. Regent showed increased expression levels of *Pr10* at 15 hpi with prior treatment with both single and combined biostimulating application and pathogen inoculation (Fig. 21). Here, the *Pr10* level at 15 hpi after *P. viticola* infection was increased especially by the combined treatment with *T. koningiopsis* and chitosan compared to the untreated variant (130-fold higher than untreated variant). Single treatments of *T. koningiopsis* and chitosan were able to increase the expression level of *Pr10* 99-fold (Tr) and 117-fold (Chi) after *P. viticola* infection compared to untreated variants. In the absence of pathogen infection, a 2-fold increase in the gene expression level of *Pr10* was detected at 9 hpi after combined treatment

(Tr + Chi) and at 15 hpi after treatment with *T. koningiopsis*, chitosan or the combination of both agents compared to the untreated variant. A significant interaction effect between the two factors time point and treatment was found in both *P. viticola* infected and uninfected grapevines (η^2 of factor interaction in uninfected plants: 0.941; η^2 of factor interaction in infected plants: 0.435). Between the factors time point and treatment exists a disordinal interaction, so that the effect of one factor depends on the other factor.

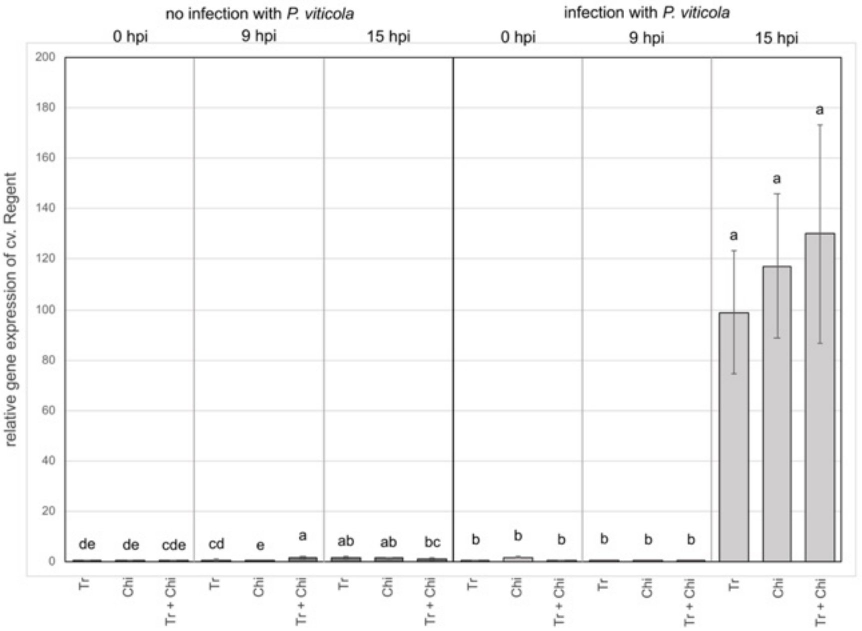

Figure 21: Effect of *T. koningiopsis* and chitosan treatment on gene expression of the defense-related *Pr10* gene in cv. Regent inoculated with *P. viticola*. Relative effect on gene expression to untreated controls are shown. Spray applications of *T. koningiopsis* (Tr) and chitosan (661 HCl) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH_2O, respectively. The expression levels at 0, 9, and 15 hours post inoculation (hpi) with *P. viticola* (right side of the figure) or at the same time points without pathogen inoculation (left side of the figure) are shown. The expression levels determined by qPCR and normalized against the housekeeping genes *VvGADPH*, *VvEF1a* and *VvUBI*. Bars represent the mean of three independent measurements of triplicates of four pooled biological replicates. Error bars indicate standard deviation. Since the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means for treatments at the different time points. Analysis was conducted separately for no infection (without *P. viticola*) and infection (with *P. viticola*) with the pathogen. Means with different letters (without *P. viticola*: a, b, c, d, e; with *P. viticola*: a, b) are significantly different ($p \leq 0.05$).

Single treatment with *T. koningiopsis* and chitosan, as well as combined treatment with both agents, increased *Pr10* gene expression in cv. Cabernet blanc by 11-fold (Tr), and 12-fold (Chi; Tr + Chi), respectively, at 9 hpi after *P. viticola* infection compared to the untreated variant (Fig. 22). An increased expression of *Pr10* was observed from 0 to 9 hpi in the absence of pathogen inoculation. In particular, the *Pr10* level at 9 hpi was increased by single treatment with chitosan and combined treatment of *T. koningiopsis* and chitosan compared to the untreated variant (5.5-fold [Chi] and 5-fold [Tr + Chi] higher than the untreated variant). However, higher gene expression was observed after inoculation with the pathogen. A significant interaction effect between the two factors time point and treatment was found in both *P. viticola* infected and uninfected grapevines (η^2 of factor interaction in uninfected plants: 0.673; η^2 of factor interaction in infected plants: 0.788). Between the factors time point and treatment exists a disordinal interaction, so that the effect of one factor depends on the other factor.

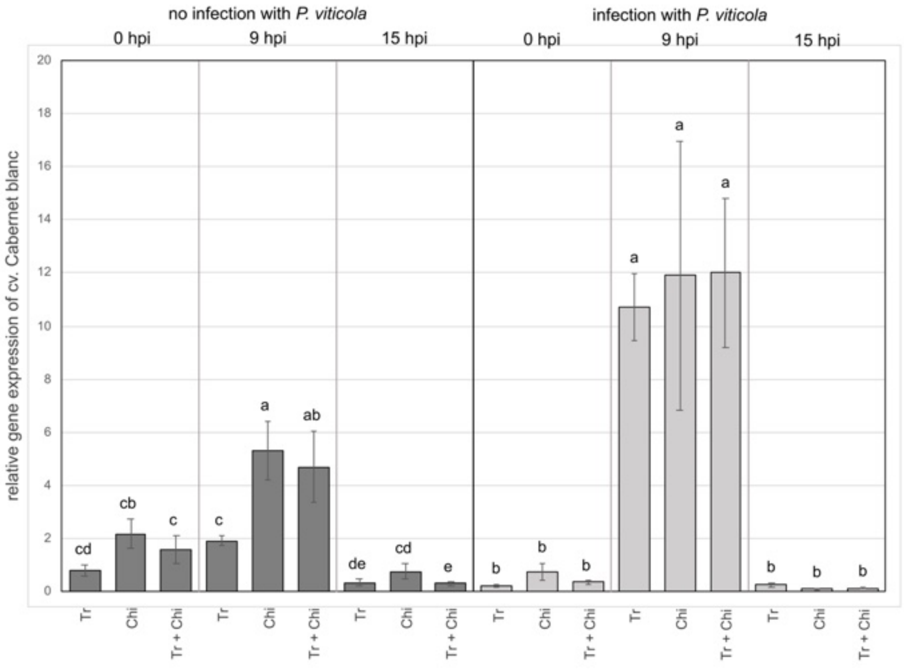

Figure 22: Effect of *T. koningiopsis* and chitosan treatment on gene expression of the defense-related *Pr10* gene in cv. Cabernet blanc inoculated with *P. viticola*. Relative effect on gene expression to untreated controls are shown. Spray applications of *T. koningiopsis* (Tr) and chitosan (661 HCl) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH$_2$O, respectively. The expression levels at 0, 9, and 15 hours post inoculation (hpi) with *P. viticola* (right side of the figure) or at the same time points without pathogen inoculation (left side of the figure) are shown. The expression levels determined by qPCR and normalized against the housekeeping genes *VvGADPH*, *VvEF1a* and *VvUBI*. Bars represent the mean of three independent measurements of triplicates of four pooled biological replicates. Error bars indicate standard deviation. Since the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means for treatments at the different time points. Analysis was conducted separately for no infection (without *P. viticola*) and infection (with *P. viticola*) with the pathogen. Means with different letters (without *P. viticola*: a, b, c, d, e; with *P. viticola*: a, b) are significantly different ($p \leq 0.05$).

4.4.3 Effect of biostimulation treatment on the accumulation of reactive oxygen species (ROS) of different grapevine cultivars inoculated with *Plasmopara viticola*

Since the accumulation of ROS is associated with the hypersensitive response of grapevine to *P. viticola*, the accumulation of superoxide ions (O_2^-) in grapevine leaves of different cultivars was analyzed. Compared to the untreated variants, an increased accumulation of O_2^- was detected in cv. Regent at 12, 18 and 30 hpi and in cv. Cabernet blanc at 12 and 18 hpi with *P. viticola* (Fig. 23). In particular, when cv. Regent was treated with chitosan, two and three times the accumulation areas of colored O_2^- ions were detected at 12 and 30 hpi (70,851 μm^2 and 58,280 μm^2, respectively) compared to the untreated control (35,346 μm^2 and 18,199 μm^2, respectively). The combined treatment of cv. Regent with *T. koningiopsis* and chitosan at 18 hpi showed an 8-fold increase in stained leaf area (68,076 μm^2) compared to the untreated variant (8,441 μm^2). In cv. Cabernet blanc, after combined treatment with *T. koningiopsis* and chitosan, an increased accumulation of O_2^- was detected at 12 and 18 hpi, which at 18 hpi significantly exceeded that of the untreated variety by 12 times the stained leaf area. No significant accumulation of O_2^- was detected for cv. Müller-Thurgau.

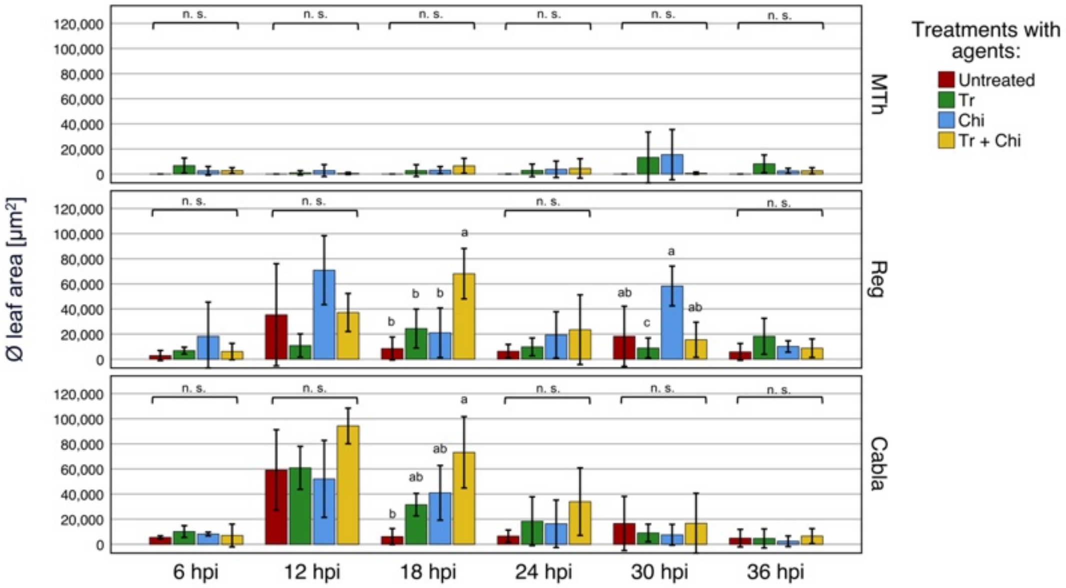

Figure 23: Effect of *T. koningiopsis* and chitosan treatment on superoxide ion (O^{2-}) accumulation in grapevines inoculated with *P. viticola*. Spray applications of *T. koningiopsis* (Tr) and chitosan (661 HCl) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH_2O, respectively. Controls were left untreated. Quantification of the area stained with Nitro blue tetrazolium was carried out for the grapevine cvs. Müller-Thurgau (MTh), Regent (Reg), and Cabernet blanc (Cabla) at 6, 12, 18, 24, 30, and 36 hours post inoculation (hpi) with *P. viticola*. Bars represent the average of three independent experiments with three replicates for each treatment. Values are means ± SD. When the conditions of normal distribution and homoscedasticity were met, Tukey HSD was used to detect differences between means. When homoscedasticity was not demonstrated, Welch-ANOVA (comparison of means) and Dunnett-T3 (post-hoc test) were used. Data that were not normally distributed and lacked homoscedasticity were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. Differences between means were detected separately for each time point and cultivar. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

To further investigate the previously observed induced defense response, the accumulation of hydrogen peroxide (H_2O_2) was analyzed in grapevine leaves of different cultivars. Compared to the untreated variants, an increased accumulation of H_2O_2 was detected in the resistant grapevines (cvs. Regent and Cabernet blanc) at 12 and 18 hpi (Fig. 24). In particular, treatment of cv. Regent with chitosan showed a significant 8.5-fold increase in H_2O_2 accumulation area (53,623 µm^2) at 12 hpi compared to the untreated control (6,305 µm^2). At 18 hpi, all treatments of cv. Regent with biostimulating agents showed a similar high accumulation area of H_2O_2, which exceeded that of the control by 3-fold (Tr + Chi) to 4-fold (Tr). In cv. Cabernet blanc, after treatment with chitosan as well as after combined treatment with *T. koningiopsis* and chitosan, a 5-fold increased accumulation of H_2O_2 (Chi: 38,393 µm^2 and Tr + Chi: 32,402 µm^2, respectively) was detected at 12 hpi compared to the untreated variant (6,912 µm^2). No significant accumulation of H_2O_2 was detected in cv. Müller-Thurgau.

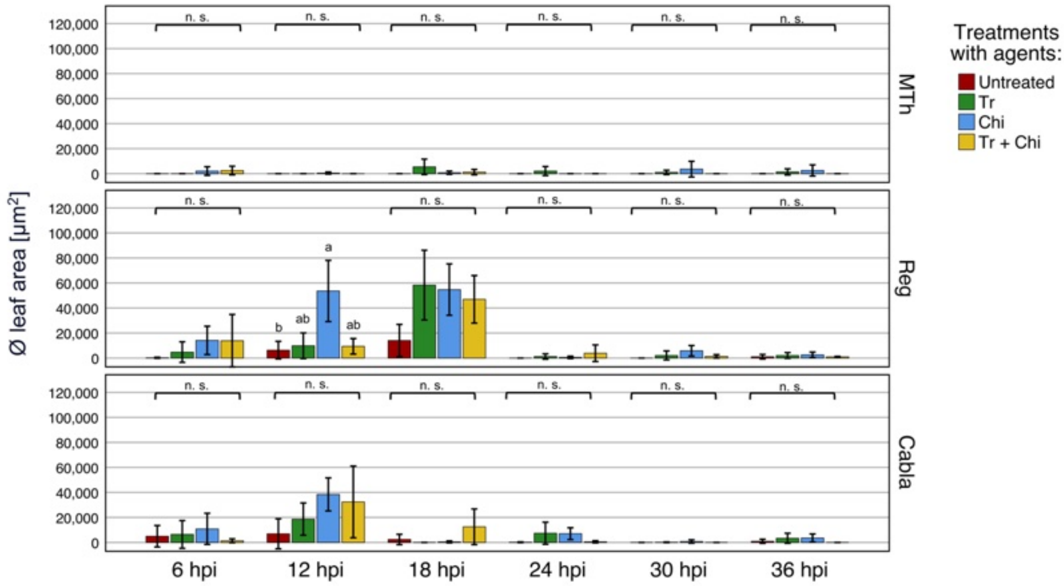

Figure 24: Effect of *T. koningiopsis* and chitosan treatment on hydrogen peroxide (H_2O_2) accumulation in grapevines inoculated with *P. viticola*. Spray applications of *T. koningiopsis* (Tr) and chitosan (661 HCl) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH$_2$O, respectively. Controls were left untreated. Quantification of the area stained with 3,3´-diaminobenzidine was carried out for the grapevine cvs. Müller-Thurgau (MTh), Regent (Reg), and Cabernet blanc (Cabla) at 6, 12, 18, 24, 30, and 36 hours post inoculation (hpi) with *P. viticola*. Bars represent the average of three independent experiments with three replicates for each treatment. Values are means ± SD. When the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means. Data that were not normally distributed and lacked homoscedasticity were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. Differences between means were detected separately for each time point and cultivar. Means with different letters (a, b) are significantly different ($p \leq 0.05$).

To determine the accumulation of ROS after *T. koningiopsis* and chitosan treatment of grapevines infected with *P. viticola*, microscopic analysis was used to visualize the leaf area of the grapevine defense response and the points of contact between the pathogen and the cell. Microscopic images after NBT and DAB staining at times of high ROS accumulation (18 hpi) of the cvs. Cabernet blanc (a), Regent (b) and Müller-Thurgau (c) are shown in

Fig. 25 after treatment with *T. koningiopsis* and chitosan. It was shown that the ROS accumulation observed here was induced by the contact of the pathogen with the cell walls of the respective grapevines (Fig. 25, d-f). Cells of cvs. Cabernet blanc and Regent showed cell discoloration only when the grapevine cell was in direct hyphal contact with *P. viticola*. This effect was not observed with cv. Müller-Thurgau.

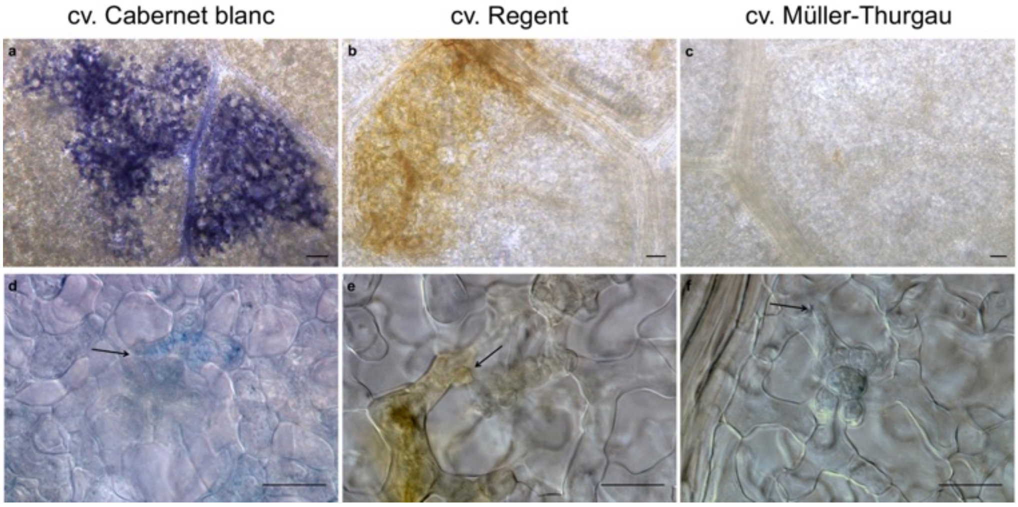

Figure 25: ROS staining of grapevine cells of different cultivars induced by *P. viticola*. **a** Effect of *T. koningiopsis* and chitosan treatment on superoxide (O_2^-) accumulation in cv. Cabernet blanc after nitrotetrazolium blue (NBT) staining at 18 hours post inoculation (hpi) with *P. viticola*. **b** Effect of *T. koningiopsis* and chitosan treatment on hydrogen peroxide (H_2O_2) accumulation in cv. Regent after diaminobenzidine (DAB) staining at 18 hpi with the pathogen. **c** Effect of *T. koningiopsis* and chitosan treatment on H_2O_2 accumulation in cv. Müller-Thurgau after DAB staining at 18 hpi with *P. viticola*. **d** O_2^- accumulation after NBT staining of plant cells in contact with the pathogen on cv. Cabernet blanc; **e** H_2O_2 accumulation after DAB staining of plant cells in contact with the pathogen on cv. Regent; **f** No ROS staining of plant cells in contact with the pathogen on cv. Müller-Thurgau. Arrows indicate the site of contact between *P. viticola* and the plant cell. Images are representative of three independent experiments with three replicates. Scale bars correspond to 20 µm.

4.4.4 Effect of biostimulation treatment on the number of *Plasmopara viticola* haustoria of different grapevine cultivars

To further determine the indirect effect of *T. koningiopsis* and chitosan treatment on pathogen development, the haustoria of *P. viticola* were considered in more detail as key structures for nutrient uptake. For this purpose, the number of haustoria per infection site of *P. viticola* was analyzed in grapevine leaves of cultivars with different levels of resistance to the pathogen. In general, the number of haustoria of downy mildew increased with time, regardless of treatment and grapevine cultivar. However, the resistant cvs. Regent and Cabernet blanc showed a lower number of haustoria per infection site than the susceptible cv. Müller-Thurgau (Fig. 26). In particular, cv. Cabernet blanc generally showed a lower number of haustoria than the susceptible cv. Müller-Thurgau at all investigation time points, regardless of treatment. Over time (12 to 24 hpi), the number of haustoria in the untreated variant almost doubled in cv. Müller-Thurgau, while the smallest overall increase in haustoria number was observed in cv. Cabernet blanc.

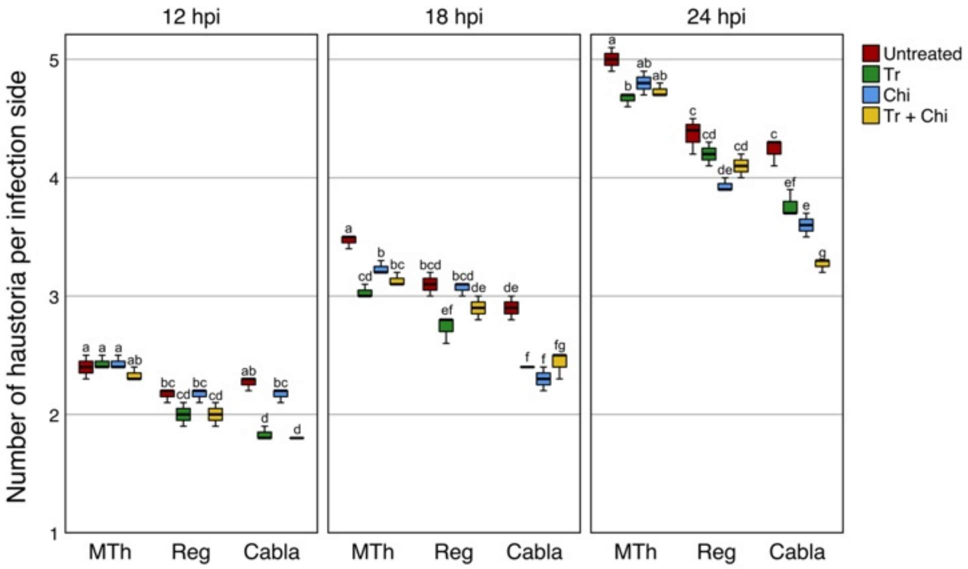

Figure 26: Effect of *T. koningiopsis* and chitosan treatment on the number of *P. viticola* haustoria per infection site in different grapevine cultivars at 12, 18 and 24 hours post pathogen inoculation (hpi). Spray applications of *T. koningiopsis* (Tr) and chitosan 661 HCl (Chi) were made with 10^6 *Trichoderma* spores ml^{-1} MEB 1% and 0.01% chitosan in dH$_2$O, respectively. Controls were left untreated. Quantification of haustoria number was performed for the grapevine cvs. Müller-Thurgau (MTh), Regent (Reg), and Cabernet blanc (Cab) at the time points 12, 18, and 24 hpi with *P. viticola*. Boxplots represent the average of three independent experiments with ten replicates for each treatment. Since the conditions of normal distribution and homoscedasticity were met, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means per time point. Means with different letters (a, b, c, d, e, f, g) are significantly different ($p \leq 0.05$).

4.5 Discussion

By treating different grapevine varieties with a synergistic *Trichoderma*-chitosan-complex, this study demonstrates an induced effect on grapevines against downy mildew. This is in contrast to the application of copper, which only shows a contact effect. In order to achieve high efficacy of the biocontrol and biostimulation complex, a profound knowledge of the modes of action of the combined agents is required. For this reason, the induced effect of the complex and the modes of action on grapevines were studied in detail. Only a reliable and versatile action of the synergistic agents can contribute to an effective protection against *P. viticola* and thus reduce the use of copper fungicides for a sustainable plant protection.

Treatment of greenhouse grapevines susceptible to *P. viticola* with *T. koningiopsis* and chitosan resulted in an induced effect on downy mildew disease severity. Single biostimulation treatment with *T. koningiopsis* or chitosan reduced pathogen infestation. Combined treatment was found to be more effective than single treatment (Fig. 18), suggesting a synergistic effect against the pathogen. It is possible that a combined treatment leads to an increase in stability within the complex. The synergistic effect of *T. koningiopsis* and chitosan against *P. viticola* observed in the greenhouse experiments was similar to the findings of El-Sharkawy et al. (2023), who demonstrated a higher effect against downy mildew on field-grown grapevines by combining *Trichoderma* with chemical inducers. El-Sharkawy et al. (2023) attributed the effect to the increased potential of *T. harzianum* induced by combining agents. Furthermore, combining resistance inducers with copper compounds can enhance downy mildew reduction and provide a suitable plant protection strategy to control *P. viticola* (Taibi et al. 2022). Moreover, a direct biocontrol activity of *Trichoderma* spp. is attributed to five different mechanisms as described in the introduction. The antifungal bioactivity of chitosan is attributed to the interaction of the positively charged chitosan with the negatively charged phospholipid components of the pathogen. This interaction increases the permeability and leakage of the pathogen's cellular contents. These chelating properties cause the pathogen to lose essential components required for normal development. In addition, binding to the DNA of the pathogen has a negative effect on mRNA synthesis and thus on the production of proteins and enzymes. This effect can be enhanced by bioactive compounds as a possible component of secondary metabolites of natural compounds (Ruano-Rosa et al. 2022). A possible explanation for the observed synergistic effect of both agents could be the release of lytic enzymes by *Trichoderma* in combination with the antifungal effect of chitosan, since *Trichoderma* spp. are able to produce lytic enzymes (e.g., endochitinase, exochitinase, exo-β-D-N-acetylglucosaminidase) which degrade cell walls of the pathogen (Woo et al. 2023).

Greenhouse investigations demonstrated a resistance-inducing effect by treating grapevine leaves with *T. koningiopsis* and chitosan (Fig. 18). *Trichoderma* spp. are able to exert an indirect effect on pathogens by inducing local or systemic plant defense (Salwan et al. 2022; Woo et al. 2023). Moreover, chitosans can activate plant defense mechanisms as effective elicitors (De Bona et al. 2021). Kappel et al. (2022) demonstrated an induced synergistic effect against *Cercospora beticola* by spraying sugar beet with *T. atroviride* and chitosan. In addition to a biocidal effect, chitosan induced systemic resistance in beets, while *Trichoderma* mainly affected the expression of defense genes, suggesting that both agents act via different signaling pathways (Kappel et al. 2022). The analyses performed in this study confirmed the modulation of grapevine gene expression against *P. viticola* by the treatment with the agents, especially after the combined application of *T. koningiopsis* and chitosan (Figs. 20-22). In contrast, the expression levels of the grapevine cultivars after pathogen infection and without agent treatment were markedly lower and showed partially different expression time points, especially for the cvs. Müller-Thurgau and Regent (Fig. 19). The obtained resistance effect after treatment with the agents was demonstrated both in the *P. viticola*-sensitive cultivar and in resistant cultivars, although it differed in timing and strength of expression (Figs. 20-22). In particular, a strong expression of *Pr10* in the resistant cv. Regent was observed in the study carried out after treatment with biostimulants and inoculation with the pathogen (Fig. 21). Merz et al. (2015) also showed a subsequent upregulation of *Pr10* after inoculation of cv. Regent with *P. viticola*, and furthermore, an early induction of the WRKY33 gene. In addition, resistant and susceptible grapevine cultivars were shown to have a similar downstream signaling pathway of WRKY33. Therefore, there must be differences in the upstream level of WRKY33 gene expression in the respective defense response of the grapevine (Merz et al. 2015), which could possibly explain the differences in the timing and strength of expression of the different grapevine cultivars demonstrated here. In the present study, higher gene expression of *Pr10* was mainly observed in the resistant cultivars. An effect on gene expression was also shown when the susceptible cultivar was treated with a combination of *T. koningiopsis* and chitosan (Fig. 20). The observed effect was not demonstrated in the absence of *T. koningiopsis* and chitosan treatment (Fig. 19). However, this effect was noticeably smaller than the effect demonstrated on the cv. Regent. Nevertheless, the biostimulants show an effect on the resistance of susceptible cv. Müller-Thurgau, especially when used in combination. This was particularly evident in the greenhouse studies (Fig. 18). It is possible that also other forms of resistance against downy mildew are increasingly activated by the biostimulants, leading to a reduction in pathogen infestation. Furthermore, treatment with chitosan resulted in higher gene expression in grapevines inoculated with *P. viticola* compared to those treated with *T. koningiopsis*. However, the extent of the chitosan effect varied within each cultivar, as did the resistance effect of *T. koningiopsis*. Banani et al. (2014) state that both the resistance inducer and the plant defense signal could be associated with the different treatment effects. In addition, different grapevine cultivars show different molecular responses to pathogens and also to resistance inducers. This finding was also confirmed in the present study (Figs. 19-22). The respective plant defense response could possibly be due to differences in receptors or cellular components of the signaling network (Banani et al. 2014). However, after high gene expression in resistant cv. Cabernet blanc, there was a noticeable drop in expression at the following time point. Therefore, additional time points or closer time intervals should be used to best map the kinetics of this variety.

Since *Pr10* has been associated with the induction of cell death and thus may contribute to the generation of ROS as a plant defense mechanism (Ma et al. 2018), the accumulation of superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2) in biostimulant-treated grapevines of different cultivars was examined. Increased accumulation of O_2^- and H_2O_2 was observed in the treated resistant cvs. Regent and Cabernet blanc, whereas the susceptible cv. Müller-Thurgau showed less ROS formation (Figs. 23-25). *Rpv3-1*-mediated resistance has been described in cvs. Regent and Cabernet blanc, which is not found in susceptible cv. Müller-Thurgau (Eisenmann et al. 2019). Eisenmann et al. (2019) showed that the *Rpv3* locus is associated with the synthesis of fungitoxic stilbenes and programmed cell death, thus reducing downy mildew growth and development. Therefore, *Rpv3-1*-mediated resistance may be responsible for the accumulation of ROS in the resistant cultivars observed in this study. Although late gene expression of *Pr10* in susceptible cv. Müller-Thurgau was observed after treatment with both biostimulants (Fig. 20), no significant ROS formation could be detected. It is possible that the effect of the biostimulants on the susceptible cultivar was not strong enough to show corresponding impacts on later ROS formation. This could also indicate that another form of resistance is activated in the susceptible cultivar, as mentioned above. Furthermore, over the time period studied, the results show a much more dispersed formation of O_2^- compared to H_2O_2 formation (Figs. 23 and 24). In the mitochondrial electron transport chain, ROS play an important signaling role and are constantly produced to a certain extent (Palma et al. 2024). Palma et al. (2024) suggested in their work that ROS production should be considered as a primary mitochondrial function. Furthermore, O_2^- is highly reactive and not as stable as H_2O_2 (Kuźniak and Urbanek 2000). Possibly, there was not enough O_2^- available for conversion to H_2O_2 at the time of investigation, resulting in less visible H_2O_2 detection. Moreover, Wingerter et al. (2022) described no accumulation of H_2O_2 before 24 hpi in grapevine cultivars with *Rpv3*-mediated defense. This is in contrast to the results obtained here, where earlier ROS accumulation was observed in biostimulant-treated grapevine cultivars, which may be related to an accelerated resistance response of grapevine to downy mildew caused by *T. koningiopsis* and chitosan. Besides *Trichoderma*, chitosans are also associated with the accumulation of ROS as elicitors of defense mechanisms (Galli et al. 2024). Mian et al. (2023) demonstrated a significant reduction in downy mildew infection and an increased abundance of transcripts, including *Pr10*, by treating field-grown grapevines with chitosan. *Pr10* stimulates a cell death-like defense in downy mildew-resistant grapevines, where ROS production is accelerated via interaction with a voltage-dependent anion channel 3 (VDAC3) protein (Ma et al. 2018), which may be critical for the increased ROS accumulation observed here.

The haustoria formed by *P. viticola* during pathogenesis are essential structures for nutrient uptake and play a critical role in host-pathogen interaction compatibility via host-pathogen recognition and/or effector production and resistance suppression (Gessler et al. 2011; Juraschek et al. 2022). Therefore, the impairments in the formation of these structures caused by grapevine defense mechanisms can significantly affect successful pathogen spread. The results obtained in this study showed a reducing effect on haustoria formation by grapevine treatment with *T. koningiopsis* and chitosan, with a stronger effect observed in resistant than in susceptible cultivars (Fig. 26). This is in agreement with Juraschek et al. (2022), who also demonstrated differences in haustoria formation on grapevine cultivars with different levels of resistance. Fewer and smaller haustoria were formed by *P. viticola* in resistant grapevine cultivars, which could also be associated with callose deposition (Juraschek et al. 2022). The studies conducted showed an enhanced defense response against *P. viticola* by combined agent treatment. Jacquens et al. (2022) also showed a reduction in the haustoria number by combined treatment of grapevines with a biostimulant and a defense elicitor, noting a fluorescent effect around the pathogenic hyphae indicating the accumulation of callose and laminarin. Jacquens et al. (2022) concluded a priming effect of the biostimulant on the defense elicitor associated with induced resistance to downy mildew. A priming effect of *T. koningiopsis* on chitosan or of chitosan on *T. koningiopsis* cannot be excluded in the investigations conducted here either, although the combination treatment generally did not achieve the most effective haustoria reduction. Nevertheless, the results obtained indicate a harmful effect on pathogenic haustoria formation induced by *T. koningiopsis* and/or chitosan. *Trichoderma* spp. are known to produce secondary metabolites (Lazazzara et al. 2021). Such production of secondary metabolites by *T. koningiopsis* may be associated with reduced haustoria formation by the pathogen after biostimulant treatment and may be synergistically supported by the defense-inducing effect of chitosan.

Since a significant effect of the biostimulants against downy mildew could be demonstrated in the laboratory and greenhouse experiments, it seems reasonable to test the complex under high infestation pressure in the vineyard, where conditions are more difficult (e.g., due to UV radiation and temperature). In this context, Løvschall et al. (2024) have developed an innovative encapsulation method for *Trichoderma* spores based on a layer-by-layer technology using bio-based lignin derivatives to protect *Trichoderma* from damage caused by physical stimuli or prolonged storage. The efficacy of this technology was demonstrated by its ability to preserve the spore stability of *Trichoderma* under extreme temperatures and exposure to ultraviolet radiation. This technology offers a promising opportunity to improve the stability of the complex as a sustainable tool to control *P. viticola*.

4.6 Conclusion

In conclusion, this study verifies the resistance inducing-effect of the biocontrol and biostimulating complex consisting of *T. koningiopsis* and chitosan and allows a better understanding of its modes of action. The combined application of both agents modulates the gene expression of different grapevine cultivars, affected the accumulation of ROS and inhibited the development of *P. viticola* haustoria. The combination of the multipurpose properties of *Trichoderma* and chitosan allows the reduction of copper-based fungicides, especially in organic viticulture, and shows an effective and sustainable tool for grapevine downy mildew management. Particularly in resistant grapevines, a strong effect was induced by the complex. In addition to an induced effect through the plant, a direct effect against the pathogen was also demonstrated. This dual effect can be an important contributor to plant resistance protection. Therefore, a well thought out and appropriate selection of grapevine cultivars in combination with *T. koningiopsis* and chitosan application can contribute to an effective reduction of pathogen infestation, while at the same time reducing the use of copper fungicides and strengthening the plant's defenses.

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5 Tri-trophic complex of *Trichoderma koningiopsis*, chitosan and reduced copper rate open up an eco-friendly tool for sustainable control of Esca disease in grapevines

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Author Contributions

Verena Küpper: project conceptualization, experimentation, methodology, data analysis, visualization, writing – original draft. Andreas Kortekamp: project conceptualization, funding acquisition, methodology, project administration, writing – review and editing. Ulrike Steiner: project conceptualization, methodology, visualization, writing – review and editing.

5.1 Abstract

Esca, a disease complex caused by various fungal pathogens, is one of the most important diseases of the grapevine trunk, leading to a wood decay and the death of the entire plant. Since sodium arsenite application has been banned due to its harmful effects, there are no comparably effective control mechanisms. Biocontrol agents and biostimulants such as *Trichoderma* species and chitosan provide an eco-friendly alternative to control fungal diseases. Previous work demonstrated the successful application of a synergistic complex consisting of a copper- and chitosan-tolerant *Trichoderma* isolate and chitosan for downy mildew reduction on grapevine. To successfully apply the complex against Esca pathogens and provide lasting protection, it is necessary to ensure establishment of the biological agent in grapevine. Therefore, different *Trichoderma* spp. were tested for their ability to colonize both wood and grapevine shoots. Colonization was confirmed for all isolates. Moreover, the direct biocontrol activity of *Trichoderma* candidates against Esca pathogens was investigated in confrontation tests and the indirect effect through released volatile organic compounds in sandwich plate tests. A direct effect on mycelial growth of *Phaeomoniella chlamydospora* was detected, whereas hardly any effect on the growth of *Phaeoacremonium minimum* was shown. However, *T. koningiopsis* showed a significant indirect effect on *P. minimum*. Field application of *T. koningiopsis* and chitosan against *P. chlamydospora* showed a reduction in pathogen infestation of infected shoots. The effect was improved by combining a reduced copper rate. The tested biocontrol and biostimulation complex may represent a promising eco-friendly tool for protecting grapevines from Esca.

Keywords: *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum*, *Fomitiporia mediterranea*, trunk disease, biocontrol, biostimulants, copper reduction, organic viticulture, *Vitis vinifera*

5.2 Introduction

In recent decades, grapevine trunk diseases have become increasingly important and are a major challenge in grapevine-growing areas worldwide due to the damage they cause and the resulting financial losses (Mondello et al., 2018a).

Esca disease, caused by fungal Ascomycetes and Basidiomycetes, is one of the most important trunk diseases of mature grapevines and consists of several diseases, depending on grapevine age, wood type and pathogens involved. The Esca complex is divided into different diseases, in particular wood decay and a vascular disease: dark wood streaking (caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*), Petri disease in young vines (caused by *P. chlamydospora*, *P. minimum*, and *Cadophora luteo-olivacea*), grapevine leaf stripe disease (caused by *P. chlamydospora* and *P. minimum*), white rot (caused by *Fomitiporia* spp. – in Europe *Fomitiporia mediterranea* – and other Basidiomycota), and Esca proper (caused by *P. chlamydospora*, *Phaeoacremonium* spp., and *Fomitiporia* spp.) (Mondello et al., 2018b). The main points of entry for these pathogenic fungi are pruning wounds, but infection with Esca pathogens can also occur during the propagation process in nurseries (Gramaje et al., 2018). The colonization of grapevine trunk and cordon tissues leads to a large number of discolorations, necroses and vascular infections in grapevine wood, older plants also show white rot (Mondello et al., 2018b; Bruez et al., 2021). Leaf symptoms appear as tiger stripe pattern in chronic form, although symptoms may be more widespread on individual leaves and shoots and are caused by interveinal necrosis and yellow or red chlorosis (Mugnai et al., 1999). In contrast to the chronic form, external symptoms can also occur in an acute apoplectic form or apoplexy, which leads to the death of the entire plant in short time (Letousey et al., 2010).

Of particular concern after infection with Esca pathogens is the asymptomatic phase, which can last for years before leaf symptoms become visible (Chacón-Vozmediano et al., 2021). In addition, there is usually an inconsistent appearance of symptoms on grapevine leaves, which can vary from year to year on individual plants (Mondello et al., 2018b).

Esca was controlled in Southern Europe by dormant spray applications of sodium arsenite fungicides until 2003. However, these fungicides were banned due to its toxic effects on health and the environment (Songy et al., 2019). So far, there are no comparably effective control mechanisms. Current mechanisms mostly focus on preventive (e.g., use of high-quality mother vineyards, hot-water treatment prior to grafting, correct trunk training, pruning wound protection) and curative methods (e.g., remedial surgery, trunk renewal), with wound protection being among the most effective disease prevention strategies (Gramaje et al., 2018). Therefore, the development of new, effective and sustainable strategies to protect pruning wounds is essential to advance disease control (Mesguida et al., 2023).

Biocontrol and biostimulant agents represent a promising and eco-friendly alternative for the control of Esca diseases (Mesguida et al., 2023). Suitable agents can contribute to the sustainable control of pathogens both through direct pathogen control mechanisms (e.g., nutrient competition, antibiosis and mycoparasitism) and indirectly through the induction of plant defense mechanisms (Galli et al., 2024). The colonization of woody tissues by suitable candidates, such as *Trichoderma* spp., and the potential range of control mechanisms through agents may constitute a sustainable strategy for wound protection, particularly in grapevines (Mondello et al., 2018b).

Apart from direct mechanisms, indirect mechanisms have also been demonstrated in *Trichoderma* spp., which induce local and systemic plant defense (Woo et al., 2023). In this context, Di Marco et al. (2022) demonstrated high direct mycoparasitizing activity as well as indirect activity against *P. chlamydospora* and *F. mediterranea* in *in vitro* studies with *Trichoderma* spp. against grapevine trunk disease pathogens. Long-term *in vivo* studies in commercial vineyards also demonstrated a high protective effect of pruning wound treatment with *Trichoderma* spp. against grapevine trunk disease (Di Marco et al., 2022).

Chitosan is a N-deacetylated derivative of chitin and exhibits direct antimicrobial properties against pathogens as well as indirect modes of action through induction of plant resistance (Singh et al., 2022). Studies by Cobos et al. (2015) demonstrated an antifungal effect of chitosan oligomers against *P. chlamydospora* and showed that infection of the pathogen by pruning wounds could be significantly reduced by the application of chitosan. Furthermore, Martín et al. (2023) demonstrated a fungistatic effect of chitosan application against *P. chlamydospora* by showing a reduction in vascular necrosis and pathogen recovery. The induction of plant defense responses against Esca disease by chitosan treatment was also demonstrated (Martín et al., 2023).

However, effective and sustainable control of Esca disease should not rely solely on single control measures. The suitable and synergistic combination of biocontrol agents enables to use different mechanisms of action to control the pathogen and prevent the development of resistance (Del Pilar Martínez-Diz et al., 2021; Mesguida et al., 2023). The biocontrol agents employed must demonstrate tolerance to pesticides, as well as resistance to antimicrobials and toxins, to ensure viability in the grapevine (Mesguida et al., 2023). Cobos et al. (2015) demonstrated that a mixture of chitosan with other effective natural antifungal agents exhibited superior efficacy compared to the application of the tested agents alone.

The present study investigates a complex consisting of *Trichoderma* sp., chitosan and a reduced copper rate to sustainably control Esca pathogens on grapevines. Previous studies have already confirmed the compatibility of the agents involved, as well as the copper and chitosan tolerance of the *Trichoderma* spp. used, and demonstrated the efficacy of the complex against downy mildew on grapevine (Küpper et al., 2022; Küpper et al., 2023). To successfully apply the complex against Esca pathogens and ensure sustainable protection, it is necessary to confirm agent establishment and availability in the grapevine. For this purpose, several *Trichoderma* spp. were screened with regard to wood and shoots colonization. Candidates with high biocontrol activity were used to study their direct and indirect effects against P. *chlamydospora*, *P. minimum* and *F. mediterranea* in plate tests. In addition, the efficacy of *T. koningiopsis* and chitosan in combination with copper applied in a reduced rate against *P. chlamydospora* was tested on Riesling grapevines in the field.

5.3 Materials and Methods

5.3.1 *Trichoderma* isolate and chitosan

Isolates of *T. koningiopsis* Samuels, Carm. Suárez & H.C. Evans, *T. harzianum* Rifai, and *T. rufobrunneum* Z.X. Zhu & W.Y. Zhuang were isolated from grapevines (wood from cordon arms, stems, and wooden shoots) growing in Rhineland-Palatinate (Germany). These *Trichoderma* spp. represent strains of natural origin, since *Trichoderma*-based products had never been applied before to the grapevines used. Tolerance to copper and chitosan has been demonstrated in previous studies (Küpper et al., 2022; Küpper et al., 2023). Malt extract agar plates (MEA, 2% malt extract, Arche Naturküche, 2% agar, Roth, Karlsruhe, Germany; 0.01% Tetracycline, Roth, Karlsruhe, Germany) were used to culture purified isolates at 21°C and 70% relative humidity. Isolates were subcultured monthly.

Chitosan 661 HCl (polymer-oligomer-mixture, 10% w/v, average degree of N-acetylation [DA] 17%, average degree of polymerization [DP] 350) and chitosan 671-TvChi (polymer-oligomer-mixture, 1% w/v, DA 16%, DP 200) were used. The chitosans were kindly provided by the Institute of Plant Biology and Biotechnology, University of Münster (Germany) and are described in detail by Richter et al. (2025).

5.3.2 Plants and pathogen

The *Vitis vinifera* cultivar Müller-Thurgau was cultivated from canes of grapevines of the State Education and Research Center of Viticulture, Horticulture and Rural Development (DLR Rheinpfalz) (Neustadt/ Weinstr., Germany) as already described in previous work (Küpper et al., 2023). Cuttings were grown in perlite under greenhouse conditions for four weeks until roots and first leaves developed.

Isolates of *Fomitiporia mediterranea* M. Fisch. (Fmed), *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams (Pch) and *Phaeoacremonium minimum* (Tul. & C. Tul.) D. Gramaje, L. Mostert & Crous (Pmi) were used for the studies. All pathogens were provided by DLR Rheinpfalz and were cultivated as previously described (5.3.1).

5.3.3 Colonization assays

To investigate the *Trichoderma* isolates' ability of colonize grapevine wood and shoots, freshly cut bud cuttings were placed in perlite and inoculated with 50 µl spore suspension of the respective isolate (10^6 spores/ml in A. dist.). The cuttings were greenhouse-incubated for four weeks.

5.3.3.1 Microbial analysis of wood

The treated cuttings were decorticated and cut into 0.5 to 1.0 cm thick wood discs at three positions: 1.0 cm above the grafting point (diaphragm), 1.0 cm below the diaphragm, and 1.0 cm above the roots. Under sterile conditions, wooden discs were surface sterilized by passing them through a flame. Each disc was cut into four pieces and the pieces were transferred to Petri dishes filled with MEA. Wood pieces were incubated in a climate chamber at 21°C with 70% humidity for one to two weeks to assess *Trichoderma* growth.

5.3.3.2 Microbial analysis of shoots

To study the shoots' colonization by *Trichoderma* spp., 0.5 cm pieces from two points on the shoot were used: 0.5 cm directly from wood and 0.5 cm directly after the first shoot piece. Each shoot piece was immersed in ethanol (70%, v/v) under sterile conditions for surface disinfection, cut in half, and placed on MEA. The shoot pieces were incubated as described above until *Trichoderma* growth was evaluated.

5.3.4 Biocontrol assays

5.3.4.1 Direct confrontation test

The direct biocontrol effect of *Trichoderma* candidates (*T. koningiopsis* and *T. harzianum*) on the Esca pathogens *F. mediterranea, P. minimum* and *P. chlamydospora* was investigated using confrontation tests according to Patel and Brown (1969) and Bendahmane et al. (2012). However, *Trichoderma* and pathogenic agar plugs were transferred to MEA, with each plug placed 4 mm from the edge of the Petri dish. Plates were dark-incubated at 23°C. Controls received a plug on one side only. After two days, vertical and horizontal mycelial growth was measured. Additional measurements were taken after three, four, eight, nine, 15 and 22 days. The growth of the variants was related to the control as described in Bararkat et al. (2014) to quantify the inhibitory effect of *Trichoderma* spp. on the respective pathogen.

5.3.4.2 Indirect confrontation test

The effect of the potential volatile compounds released by *T. koningiopsis* and *T. harzianum* was evaluated according to Bararkat et al. (2014). To avoid interference, antagonistic and pathogenic isolates were placed at the center of two MEA plates with inner sides separated by a growth barrier (filter paper). The plates were sealed with four layers of Parafilm to prevent exudate leakage. Controls received an isolate plug on one side only. After five days of 23°C dark incubation, vertical and horizontal mycelial growth of each variant was measured, and growth inhibition was quantified.

5.3.5 Field trials

The potential of *T. koningiopsis* and chitosans to control *P. chlamydospora* was evaluated in a vineyard planted with 'Riesling' grapevines in Neustadt/Weinstr. (Germany). The shoots were freshly pruned after bleeding and treated with 40 µl of dual and triple combinations of *T. koningiopsis* (10^6 spores/ml), chitosan (661 HCl or 671, concentration: 0.01%), and reduced copper rate (Funguran® progress; active ingredient: copper hydroxide, Biofa, Germany, half field rate: 0.625 g l^{-1}). After one day, the inoculation with the pathogen (40 µl, 200.000 spores/ml) was conducted. Controls remained untreated. To incubate effectively, all cut surfaces were sealed with rubber caps overnight. The treated shoots were pruned in the fall of the same year and processed as described above. The three sampling points for the wooden discs were distinguished by their distance from the inoculation site. Starting at a distance of 0.5 cm, followed by 1.0 and 2.0 cm. *P. chlamydospora* disease severity and incidence was assessed using a binocular after seven days, then again after 14 days. Photos of Petri dishes and wooden disks were taken.

5.3.6 Statistics

Data were statistically analyzed using IBM SPSS Statistics version 29 (IBM Corporation, Armonk, USA). Normality and homoscedasticity were assessed using the Kolmogorov-Smirnov and Levene test. A t-test was used to compare the means of normally distributed and homoscedastic variables of two groups ($p \leq 0.05$). Variables that were not normally distributed or homoscedastic were analyzed using the Mann-Whitney-U test ($p \leq 0.05$). Variables with two or more groups, with normally distributed and homoscedastic data, were analyzed using ANOVA for a comparison of means and Tukey HSD post-hoc test to detect differences between means. Kruskal-Wallis H was used since the data was not normally distributed and heteroscedastic. Means were compared by pairwise comparison ($p \leq 0.05$).

5.4 Results

5.4.1 Colonization ability of *Trichoderma* spp.

The ability of different *Trichoderma* spp. to colonize grapevines was characterized by incubating previously treated cuttings followed by an identification of the respective isolates in different wood positions as well as on shoots that grew out from the wood during the test period. All tested *Trichoderma* isolates were able to colonize grapevine and could be reisolated from different parts of the wood (Fig. 27) and also from different areas of the outgrown shoot (Fig. 28). Successful reisolation of each isolate used decreased with increasing distance from the inoculation site during wood testing (Fig. 27). Tr60.2 demonstrated a notably high wood colonization ability, ranging from 61% (above roots) to 79% (above diaphragm), as compared to the other isolates tested. Tr74 and Tr77 exhibited similarly elevated colonization rates, particularly within the wood area above the diaphragm (51% and 47%, respectively). With increasing distance from the inoculation site, colonization decreased considerably from 14% (Tr74) to 34% (Tr77). Tr30 showed a constant colonization rate of 40-45% in all tested wood areas.

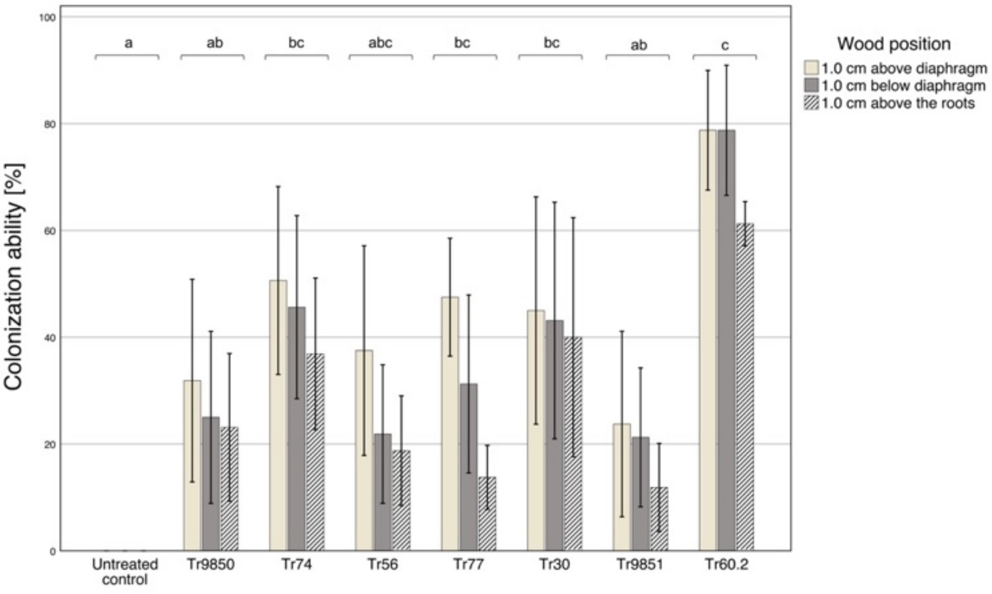

Figure 27: Wood colonization ability of different *Trichoderma* isolates. Controls remained non-inoculated. Quantification of colonization was carried out for different wood sections: 1.0 cm above diaphragm, 1.0 cm below diaphragm and 1.0 cm above the roots of the tested grapevine cuttings. Bars represent the average of four independent experiments with ten replicates for each variant. Values are means ± SD. Since data were not normally distributed and lacked homoscedasticity, data were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

The wood colonization rate averaged 33% across all sites, while the shoot colonization rate averaged 12% (Fig. 28). In particular, Tr77 exhibited a high colonization ability in both shoot areas, ranging from 29% (farther from wood) to 31% (closer to wood). Notably, isolates previously exhibiting higher levels of colonization in different wood areas did not necessarily show increased levels of colonization within the shoot tissue.

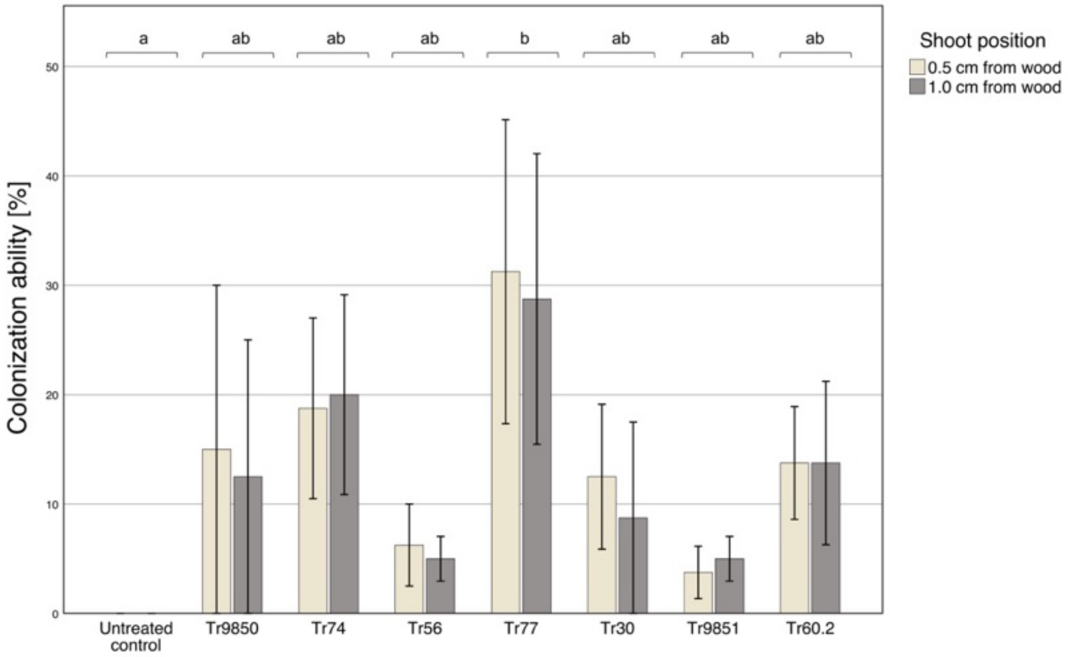

Figure 28: Shoot colonization ability of different *Trichoderma* isolates. Controls remained non-inoculated. Quantification of colonization was carried out for different shoot sections: 0.5 cm and 1.0 cm removed from wood of the tested grapevine cuttings. Bars represent the average of four independent experiments with ten replicates for each variant. Values are means ± SD. Since data were not normally distributed and lacked homoscedasticity, data were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. Means with different letters (a, b) are significantly different ($p \leq 0.05$).

5.4.2 Biocontrol potential of *Trichoderma* spp.

5.4.2.1 Direct biocontrol ability of *Trichoderma koningiopsis* and *Trichoderma harzianum*

Tests were conducted to investigate the direct effect of selected *Trichoderma* spp. on Esca pathogens. The mycelial growth of pathogens *F. mediterranea, P. minimum* and *P. chlamydospora* was investigated in direct confrontation tests.

Both *T. koningiopsis* (Fig. 29) and *T. harzianum* (Fig. 30) showed a similar growth inhibitory effect on the respective pathogens and significantly inhibited their mycelial growth over time. *P. chlamydospora* mycelial growth was most strongly inhibited by *Trichoderma* spp., reducing pathogen growth by up to 63% when *P. chlamydospora* was confronted with *T. koningiopsis* (Fig. 29, Pch, 22 dpi) and by up to 62% when confronted with *T. harzianum* (Fig. 30, Pch, 22 dpi). Notably, *Trichoderma* spp. suppressed the growth of pathogens, even in the early stages of these developments (Figs. 29 and 30, Pch, 2-3 dpi). In both studies, *F. mediterranea* showed delayed initial growth compared to the other pathogens (Figs. 29 and 30, Fmed, 1-3 dpi). The pathogen's growth was strongly inhibited by the direct effect of *T. koningiopsis* and *T. harzianum* starting at 15 dpi (Figs. 29 and 30, Fmed, 15 dpi). Both *Trichoderma* spp. subsequently reduced the mycelial growth of *F. mediterranea* by 34% (Figs. 29 and 30, Fmed, 22 dpi). *Trichoderma* spp. had the lowest overall effect when confronted with *P. minimum*.

T. koningiopsis exhibited maximum growth inhibition of 1% (Fig. 29, Pmi, 22 dpi) over time, while *T. harzianum* displayed a similar level of 2% (Fig. 30, Pmi, 22 dpi). *T. koningiopsis* and *T. harzianum* showed no noticeable difference in growth compared to the *Trichoderma* control without pathogen influence (Figs. 29 and 30, Tr in confrontation vs. Tr without confrontation). Both *Trichoderma* isolates showed high growth rates, often higher than the respective pathogen control variant, at all test times. This effect was noticeable when comparing the pathogen controls of *F. mediterranea* and *P. minimum* with the respective *Trichoderma* controls (Figs. 29 and 30, Fmed and Pmi, pathogen without confrontation vs. Tr without confrontation).

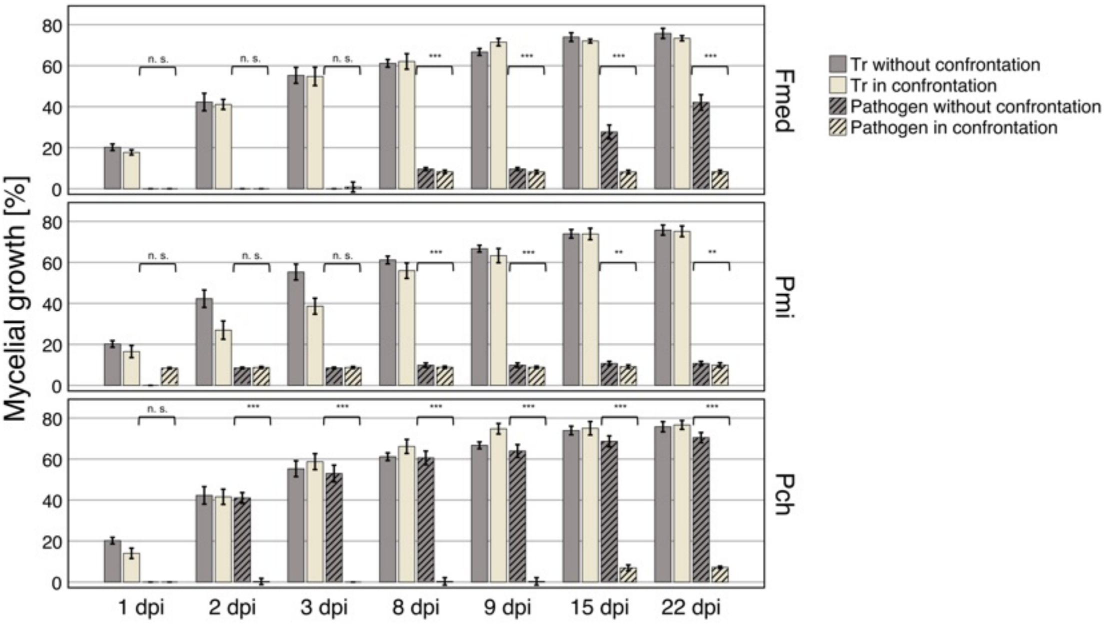

Figure 29: Direct effect of *T. koningiopsis* (Tr30) on the mycelial growth of the Esca pathogens *Fomitiporia mediterranea* (Fmed), *Phaeoacremonium minimum* (Pmi) and *Phaeomoniella chlamydospora* (Pch). Pathogen mycelial growth was quantified 1, 2, 3, 8, 9, 15 and 22 days after inoculation and confrontation with *T. koningiopsis* (dpi). Controls were not exposed to confrontation (Tr/ pathogen without confrontation). Bars represent the average of three independent experiments with 10 replicates for each variant and time point. Values are means ± SD. For data with a normal distribution and homoscedasticity, t-test was used to compare means. Data that did not show normal distribution and homoscedasticity were analyzed using the Mann-Whitney-U test. The number of asterisks indicates the p-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data not statistically different are indicated by n. s.

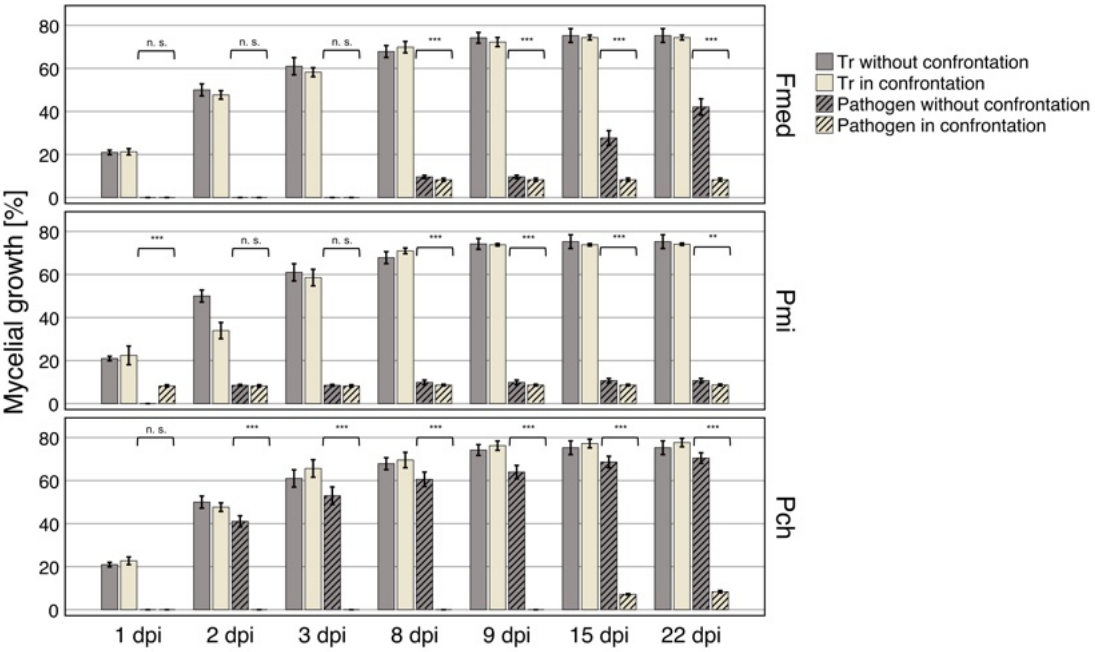

Figure 30: Direct effect of *T. harzianum* (Tr56) on the mycelial growth of the Esca pathogens *Fomitiporia mediterranea* (Fmed), *Phaeoacremonium minimum* (Pmi) and *Phaeomoniella chlamydospora* (Pch). Pathogen mycelial growth was quantified 1, 2, 3, 8, 9, 15 and 22 days after inoculation and confrontation with T. koningiopsis (dpi). Controls were not exposed to confrontation (Tr/ pathogen without confrontation). Bars represent the average of three independent experiments with 10 replicates for each variant and time point. Values are means ± SD. For data with a normal distribution and homoscedasticity, t-test was used to compare means. Data that did not show normal distribution and homoscedasticity were analyzed using the Mann-Whitney-U test. The number of asterisks indicates the p-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data not statistically different are indicated by n. s.

5.4.2.2 Indirect biocontrol ability of *Trichoderma koningiopsis* and *Trichoderma harzianum*

Indirect confrontation tests investigated the effect of volatiles from *T. koningiopsis* and *T. harzianum* spp. on Esca pathogens *F. mediterranea, P. minimum* and *P. chlamydospora* (Fig. 31).

Remarkably, both *Trichoderma* isolates had a similar indirect inhibitory effect on the growth of *F. mediterranea* and *P. chlamydospora* mycelia. *T. koningiopsis* and *T. harzianum* significantly reduced *F. mediterranea* growth by 22% and *P. chlamydospora* growth by 10% compared to the control without *Trichoderma*. In treating *P. minimum*, *T. harzianum* showed a significant higher reduction effect on pathogen's mycelial growth (15% inhibition) than *T. koningiopsis* (13.5% inhibition) compared to the untreated pathogen.

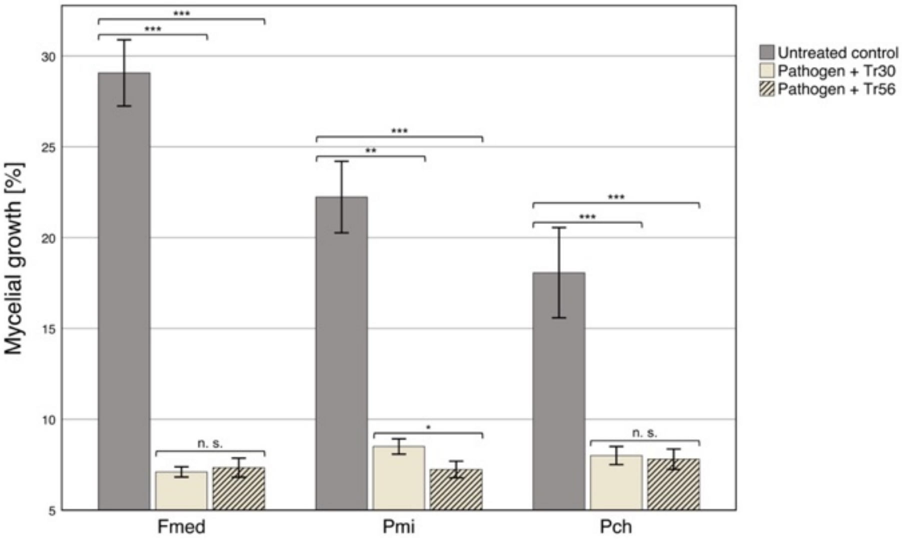

Figure 31: Indirect effect of *Trichoderma* spp. on the mycelial growth of the Esca pathogens *Fomitiporia mediterranea* (Fmed), *Phaeoacremonium minimum* (Pmi) and *Phaeomoniella chlamydospora* (Pch). Indirect effect on pathogens of the *Trichoderma* spp. *T. koningiopsis* (Tr30) and *T. harzianum* (Tr56) is shown. Controls were not indirectly exposed to *Trichoderma* spp. Bars represent the average of three independent experiments with 10 replicates for each variant. Values are means ± SD. Since data were not normally distributed and lacked homoscedasticity, data were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. The number of asterisks indicates the p-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data not statistically different are indicated by n. s.

5.4.3 Biocontrol potential of *Trichoderma koningiopsis*, chitosan and copper against *Phaeomoniella chlamydospora* in the vineyard

To investigate the effect of *Trichoderma* on Esca pathogens under field conditions, grapevines were treated with a combination of *T. koningiopsis*, chitosan (661 HCl or 671), and reduced copper rate, followed by an inoculation with *P. chlamydospora*. After several months, various parts of the shoot were removed and examined for pathogens (Figs. 32 and 33). Treatment of grapevines with *T. koningiopsis* and chitosan significantly reduced *P. chlamydospora* severity in every part of the wood compared to the pathogen control (Pch) (Fig. 32). As illustrated by the photographic images, the pathogen demonstrated an increased growth in the absence of agents' treatment (Fig. 33 A-C). When treated with *T. koningiopsis* and chitosan (plus a reduced copper rate), however, a strong overgrowth of the pathogen by *Trichoderma* was observed (Fig. 33 D-F). Overall, the pathogen disease severity became less severe from the treated area (0.5 cm) to the trunk (2 cm). This was also observed with *P. chlamydospora* alone. Untreated and non-inoculated grapevines showed a low disease severity of 7% (shoot, untreated, and non-inoculated), with the pathogen's disease severity decreasing with wood depth. The effect of each dual treatment was increased by an average of 3% (shoot, Tr30 + 661 HCl + Cu) and 4% (shoot, Tr30 + 671 + Cu), respectively, by adding a reduced copper rate. The triple treatment inhibited most in the area near the application site (43% at 0.5 cm, Tr30 + 661 HCl + Cu and 20% at 0.5 cm, Tr30 + 671 + Cu) compared to the pathogen treatment (Pch). The treatment of the grapevines with a combination including chitosan 661 HCl led to higher reduction in disease severity of *P. chlamydospora* by an average of 17% (shoot, no Cu) and 16% (shoot, with Cu), compared to treatment with chitosan 671. Furthermore, the triple treatment with *T. koningiopsis*, 661 HCl, and the reduced copper rate resulted in a significant reduction in pathogen disease severity by an average of 31% (shoot, Tr30 + 661 HCl + Cu) in all wood areas.

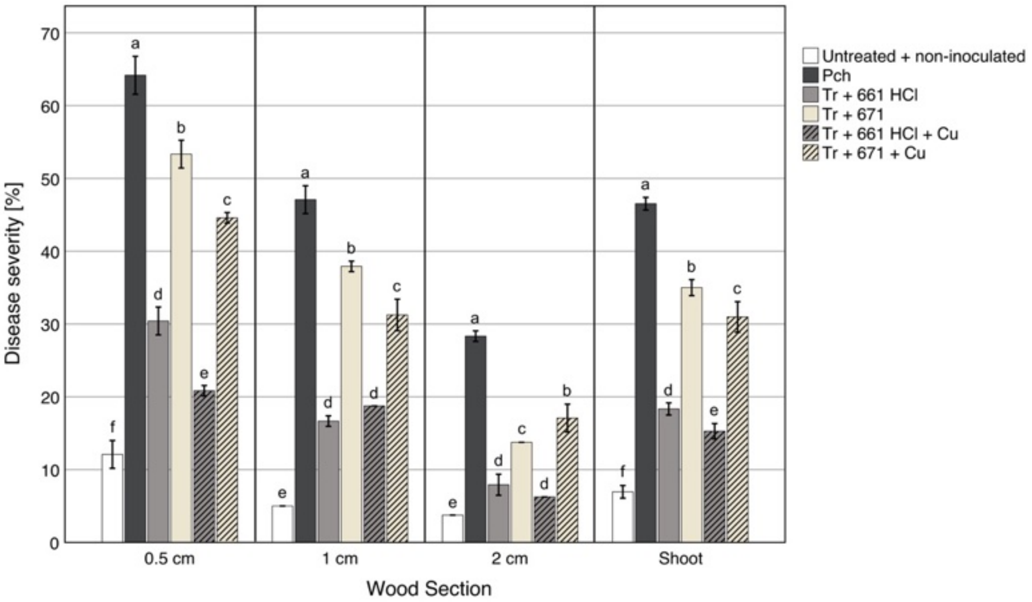

Figure 32: Effect of *T. koningiopsis* and chitosan application in combination with reduced copper rate against *Phaeomoniella chlamydospora* (Pch) in vineyard. Spray applications of *T. koningiopsis* (Tr30) and different chitosans (661HCl and 671) to pruning wounds of grapevines were made using 10^6 *Trichoderma* spores ml^{-1} and 0.01% chitosan in dH_2O after bleeding of grapevines. Half the field rate of the regular copper rate was used in the experiment (Cu; active ingredient: copper hydroxide, half field rate: 0.625 g l^{-1}). After one-day incubation of treatments Pch inoculation was carried out (40 µl, 200.000 spores/ ml). Pch disease severity is shown for different wood sections (0.5 cm above diaphragm, 1.0 cm below diaphragm and 2.0 cm below diaphragm of the tested grapevine cuttings and averaged for the whole shoot) of Riesling grapevines. Controls remained untreated und non-inoculated. Bars represent the average of three independent experiments with 20 replicates for each variant. Values are means ± SD. Since data was normally distributed, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means. Means with different letters (a, b, c, d, e, f) are significantly different ($p \leq 0.05$). The indicated letters compare the values of the different treatments separately per factor level of the factor wood section (statistical comparison separately per wood section).

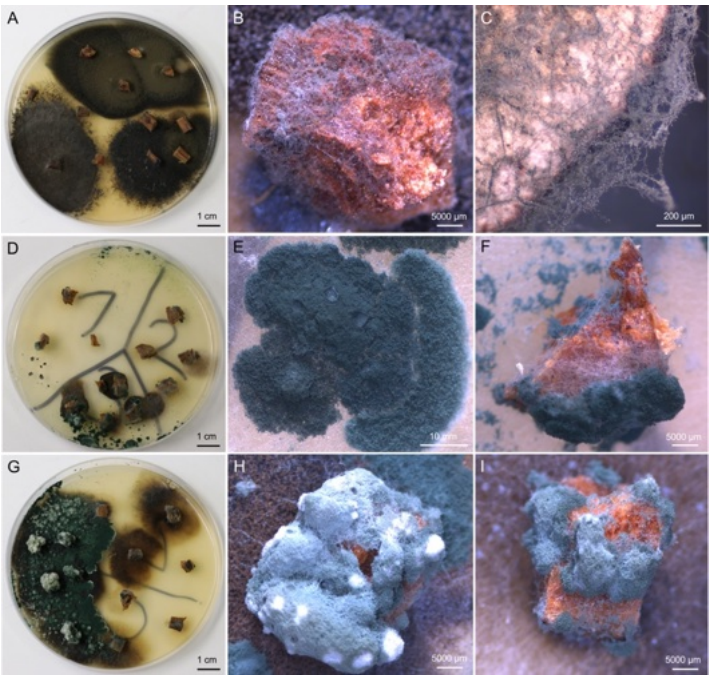

Figure 33: Effect of treatment with *T. koningiopsis* and chitosan, combined and not combined with reduced copper rate, on *Phaeomoniella chlamydospora* (Pch) development in field grapevines. The growth of Pch and *T. koningiopsis* from treated, isolated pieces of wood is shown. Reduced Pch growth indicates an effective treatment with *T. koningiopsis* and chitosan. A-C) Pch mycelium spread without treatment with an agent (pathogen control). D, G) Overview of the reduction in Pch growth compared to the untreated control (= A). E, F, H, I) Close-up of the reduction in Pch growth of treated wood pieces. D-E) Reduced mycelial growth of Pch by *T. koningiopsis* and 661 HCl chitosan treatment. F) Reduced mycelial growth of Pch by *T. koningiopsis* and 671 chitosan treatment. G-H) Reduced mycelial growth of Pch by *T. koningiopsis*, 661 HCl chitosan plus a reduced copper rate. I) Reduced mycelial growth of Pch by *T. koningiopsis*, 671 chitosan plus a reduced copper rate.

In addition to the effect of the treatments to pathogen infestation, *Trichoderma* colonization rate of the treated subareas was examined (Fig. 34). *Trichoderma* colonization of treated grapevines was observed in all treatments and wood areas. The average colonization rate ranged from 65% (shoot, Tr30 + 671) to 80% (shoot, Tr30 + 661 HCl + Cu). *Trichoderma* colonization rates showed an average 7% higher colonization in shoots with reduced copper than in Tr30 and 661 HCl or 671 treatments. Furthermore, highest colonization rates for each treatment were identified in the area close to the application site. *T. koningiopsis* colonization rates were highest in all subareas treated with Tr30 + 661 HCl + Cu. Furthermore, this treatment showed a significantly higher colonization rate than the 671 chitosan treatments.

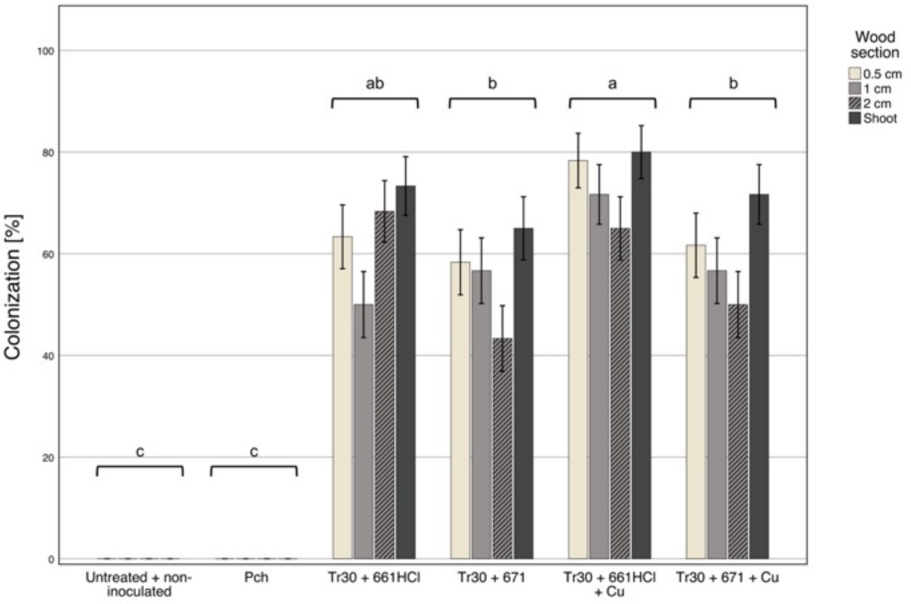

Figure 34: Wood colonization ability of *T. koningiopsis* after vineyard treatment against *Phaeomoniella chlamydospora* (Pch). Spray applications of *T. koningiopsis* (Tr30) and different chitosans (661HCl and 671) to pruning wounds of grapevines were made using 10^6 *Trichoderma* spores ml^{-1} and 0.01% chitosan in dH_2O after bleeding of grapevines. Half the field rate of the regular copper rate was used in the experiment (Cu; active ingredient: copper hydroxide, half field rate: 0.625 $g\ l^{-1}$). After one-day incubation of treatments Pch inoculation was carried out (40 µl, 200.000 spores/ ml). Tr30 colonization is shown for different wood sections (0.5 cm above diaphragm, 1.0 cm below diaphragm and 2.0 cm below diaphragm of the tested grapevine cuttings and averaged for the whole shoot) of Riesling grapevines. Controls remained untreated and non-inoculated. Bars represent the average of three independent experiments with 20 replicates for each variant. Values are means ± SD. Since data was normally distributed, ANOVA (comparison of means) and Tukey HSD (post-hoc test) were used to detect differences between means. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

The observed effect of agent combinations on *P. chlamydospora* also affects the effectiveness of treatments against Esca (Fig. 35). Treating grapevines with a combination of *T. koningiopsis* and 661 HCl chitosan was 35% more effective against *P. chlamydospora* than treating them with *T. koningiopsis* and 671 chitosan. Copper addition slightly increased the effect of both combinations. Treating the grapevines with *T. koningiopsis*, 661 HCl, and a reduced copper rate was 67% effective against *P. chlamydospora*, significantly more than with the 671 chitosan treatment.

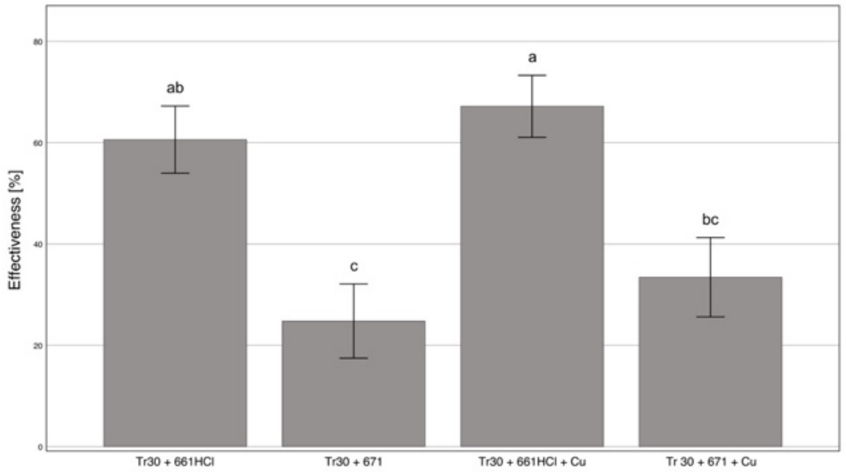

Figure 35: Effectiveness of *T. koningiopsis* and chitosan application in combination with reduced copper rate against *Phaeomoniella chlamydospora* (Pch) in vineyard. Spray applications of *T. koningiopsis* (Tr30) and different chitosans (661HCl and 671) to pruning wounds of grapevines were made using 10^6 *Trichoderma* spores ml^{-1} and 0.01 % chitosan in dH2O after bleeding of Riesling grapevines. Half the field rate of the regular copper rate was used in the experiment (Cu; active ingredient: copper hydroxide, half field rate: 0.625 g l^{-1}). After one-day incubation of treatments Pch inoculation was carried out (40 µl, 200.000 spores/ ml). The effect of each treatment against Pch is shown relative to the untreated pathogen variant. Bars represent the average of three independent experiments with 60 replicates for each variant. Values are means ± SD. Since data were not normally distributed and lacked homoscedasticity, data were analyzed using Kruskal-Wallis-H to define effects between treatments, and means were compared by pairwise comparison. Means with different letters (a, b, c) are significantly different ($p \leq 0.05$).

5.5 Discussion

The present study identified *T. koningiopsis* and *T. harzianum* as effective biocontrol agents for reducing the spread of Esca pathogens on grapevine. The shown antagonistic activity and grapevine colonization are crucial prerequisites for a sustainable, fungicide-reduced crop protection strategy. Initial field studies demonstrated that a combination of *T. koningiopsis*, chitosan, and a reduced copper rate effectively contributes to the development of a tool controlling *P. chlamydospora* infestation.

All examined *Trichoderma* isolates were successfully re-isolated from the grapevine tissue after the incubation period (Figs. 27 and 28). However, the colonization ability of the different isolates varied in the wood and shoot grapevine tissue, respectively. Carro-Huerga et al. (2021) demonstrated differences in the growth of various *Trichoderma* clades depending on temperature, sporulation, colonization and survival of *Trichoderma* spp. in pruning wounds. These differences were also observed within a clade/section, attributable to differing optimal colonization conditions for various *Trichoderma* spp. (Carro-Huerga et al., 2021). Consistent with the results obtained here, John et al. (2008) showed that the whole tissue of the canes was colonized. The frequency of recovery decreases with distance from the inoculation point (John et al., 2008). However, it is principally important to avoid wound colonization by Esca pathogens. Since *Trichoderma* seems to be able to create a bio-barricade and thus blocks the penetration into the wood, its application offers a suitable measure to protect pruning wounds. A high colonization rate of *Trichoderma* is therefore a significant prerequisite to achieve a high protection level (Carro-Huerga et al., 2021).

The spread of all *Trichoderma* isolates applied to wounds was less in green shoots (Fig. 28) compared to the wood (Fig. 27). Bailey et al. (2008) showed that *Trichoderma* spp. (including *T. harzianum* and *T. koningiopsis*) are not only able to survive in the root system and woody parts of plants, but can also colonize aboveground tissues in an endophytic association regardless of the species or strain used. The results of Bailey et al. (2008) and our own finding revealed that different *Trichoderma* spp. can remain viable in the aboveground plant tissue, which is a critical factor for an enduring and sustainable control strategy.

Both *Trichoderma* spp., *T. koningiopsis* (Fig. 29) and *T. harzianum* (Fig. 30), showed a similar inhibitory effect on the pathogens and significantly inhibited mycelial growth over the test duration. A variety of studies showed various *Trichoderma* species and commercial *Trichoderma* products effective in controlling Esca pathogens (e. g., Carro-Huerga et al., 2021; Mesguida et al., 2023). In particular, *T. koningiopsis* and *T. harzianum* have been demonstrated to provide a high biocontrol capacity against grapevine trunk pathogens based on antibiosis, an enhancement of defense response, and a competition for space and nutrients (Mesguida et al., 2023). The studies demonstrated that *T. koningiopsis* and *T. harzianum* exhibited direct biocontrol of all pathogens, however, with varying degrees of growth inhibition (Figs. 29 and 30).

P. chlamydospora's growth was most strongly inhibited by direct confrontation with *T. koningiopsis* (Fig. 29) or *T. harzianum* (Fig. 30). This is in agreement with findings of Mesguida et al. (2023), who described high biocontrol efficacy against the Esca pathogen *P. chlamydospora*, which was caused by overgrowth, competition for nutrients, and direct antagonistic properties. The confrontation studies demonstrated that *Trichoderma* spp. suppressed pathogen growth even at early stages. Simultaneously, a rapid mycelial growth of *Trichoderma* was observed (Figs. 29 and 30, Pch). Wallis (2021) demonstrated that *T. atroviride* and *T. harzianum* exhibited superior efficiency in utilizing the provided carbon and nitrogen sources compared to *P. chlamydospora*. The enhanced utilization of resources by *Trichoderma* in comparison to the pathogen may provide a plausible explanation for the observed growth inhibition, particularly in *P. chlamydospora*, and the uninhibited growth of *T. koningiopsis* and *T. harzianum*.

Delayed initial growth was also observed for *F. mediterranea*, although this was also evident in the growth of the pathogen without *Trichoderma* influence (Figs. 29 and 30, Fmed, 1-3 dpi, without confrontation). *Trichoderma*'s superior nutrient utilization and resulting initial developmental advantages, combined with the antagonist's competitiveness, may explain again the growth rates (Figs. 29 and 30, Fmed, 1-3 dpi). Wallis (2021) described outcompeting other organisms as the most significant mechanism for successful *Trichoderma* colonization.

This may offer protection against Esca pathogens within a brief time window after application to pruning wounds or cuttings and may counteract the pathogens' establishment in plant tissue.

Trichoderma overgrowth of the pathogen has also been described as an effective biocontrol mechanism against Esca pathogens (Silva-Valderrama et al., 2021). As demonstrated by Silva-Valderrama et al. (2021), an antagonistic *Trichoderma* sp. exhibited overgrowth in dual culture studies with *P. chlamydospora*. In the present study, *P. chlamydospora* and *Trichoderma* showed that their individual growth was only influenced by time and nutrients in the medium (Figs. 29 and 30, Pch, 2 dpi). Following the interaction between *P. chlamydospora* and *Trichoderma*, pathogen mycelial growth was found to be markedly restricted by *Trichoderma* (Figs. 29 and 30, Pch, 2-22 dpi). These findings suggest that overgrowth mechanism may be important in suppressing pathogenic growth. Furthermore, research has shown that *Trichoderma*'s antagonistic activity against *F. mediterranea* leads to complete overgrowth in dual culture tests (Mannerucci et al., 2023). The *Trichoderma* isolates in this study exhibited high antagonistic activity against *F. mediterranea* (Figs. 29 and 30, 8 to 22 hpi). There was a slight increase in pathogenic growth following the overlap in growth between the two species (Figs. 29 and 30, Fmed, 8 dpi). This growth inhibition was evident in the presence of the *Trichoderma* isolate (Figs. 29 and 30, Fmed, 8-22 dpi), suggesting overgrowth by *Trichoderma*.

In contrast, the inhibitory effect on the pathogen growth of *P. minimum* was markedly lower for both *Trichoderma* spp. in the present studies compared to the other pathogens investigated (Figs. 29 and 30, Pmi). In his investigations, Wallis (2021) indicated a moderate utilization rate of the carbon and nitrogen source by *P. minimum*. It can be hypothesized that *P. minimum* and the *Trichoderma* isolates utilized in this study exhibit a comparable utilization rate of these resources, thereby resulting in a limited influence on the growth behavior of the pathogen by *Trichoderma*.

In addition to a direct influence of *Trichoderma* on the pathogen's development, the release of volatile substances also suppresses the growth of the Esca pathogens. A variety of volatile secondary *Trichoderma* metabolites, including 6-pentyl-alpha-pyrones, hydrocarbons, terpenes, contribute to pathogen control (Salwan et al., 2019).

Our studies show that both *T. koningiopsis* and *T. harzianum* were able to significantly reduce the growth of *F. mediterranea, P. minimum* and *P. chlamydospora* compared to the respective control without the influence of *Trichoderma* (Fig. 31). Bararkat et al. (2014) observed that *Trichoderma* spp. can inhibit pathogen development and sporulation without direct contact, due to the ability to produce volatile compounds that diffuse into the medium and cause hyphal and spore lysis. These findings offer a potential explanation for the inhibitory effect observed on mycelial formation of the pathogens studied here by indirect confrontation with *T. koningiopsis* or *T. harzianum*.

The effect caused by *T. harzianum* is predominantly similar to that caused by *T. koningiopsis*, although the inhibition effect against *P. minimum* is significantly higher (Fig. 31). As Meena et al. (2017) demonstrated, there is a discrepancy in the number of compounds produced by different *Trichoderma* spp. Specifically, *T. harzianum* produced a greater quantity of compounds than *T. viride* (Meena et al., 2017). This suggests a possible explanation for the significantly enhanced inhibitory effect of *T. harzianum* compared to *T. koningiopsis* in the present study (Fig. 31). However, the extent of the effect of the volatile substances released depends on the *Trichoderma* isolate and the type of pathogen, as well as the pathogen's ability to reduce the effect of the substances released.

The biocontrol effect of *Trichoderma*, as observed in the *in vitro* studies conducted here, was also demonstrated in field studies. Treating grapevines with a mixture of *Trichoderma*, chitosan, and a reduced copper rate significantly lowered *P. chlamydospora* severity compared to the untreated *P. chlamydospora* variant (Fig. 32).

Del Pilar Martínez-Diz et al. (2021) conducted field trials to investigate the efficacy of *Trichoderma* against *P. chlamydospora*. In contrast to the results of this study, del Pilar Martínez-Diz et al. (2021) demonstrated that two different *T. atroviride* products did not reduce pathogen infection in two-year field trials. Del Pilar Martínez-Diz et al. (2021) ascribed these findings to a low pruning wound colonization of *Trichoderma* strains and observed that pruning at a specific time, followed by the application of biocontrol agents at mild temperatures, can enhance their development and effectiveness. The dual and triple combinations utilized in the present study during the late winter/early spring period resulted in adequate *T. koningiopsis* colonization of the grapevine wood, as evidenced by the findings (Fig. 34). Furthermore, these combinations demonstrated a substantial decrease in pathogen severity within the combination treatments (Fig. 32).

Bigot et al. (2020) also investigated the effect of seasonal treatment of grapevines with *Trichoderma* spp. against Esca pathogens in field trials. The study revealed that *T. asperellum* and *T. gamsii* exhibited efficacy in reducing mild tiger stripe symptoms and apoplexy. However, no significant effects were found on reducing severe symptoms. In summary, they demonstrated a 22% decrease in the disease incidence during the trial period after *Trichoderma* treatment. The investigations conducted here demonstrate a significant reduction of up to 43% in infestation intensity compared to the *P. chlamydospora*-inoculated variant without biocontrol treatment (Fig. 6, 0.5 cm, Pch vs. Tr + 661 HCl + Cu), especially with *T. koningiopsis*, the 661 HCl chitosan, and a reduced copper rate. Furthermore, the stimulation through secondary metabolite production by *Trichoderma*, as observed in this study, could be a contributing factor to the noted reduction effect.

The enhanced reduction effect evident in the studies conducted here may be attributable to the combination of the *Trichoderma* isolate with the antifungal agents, namely, chitosan and copper. These agents have been demonstrated to be highly efficacious against various pathogens, including downy mildew in grapevines (Küpper et al., 2023). The treatment of grapevines' pruning wounds with a combination of agents – including the application of chitosan – in the present study, resulted in a significant reduction of the pathogen (Fig. 32). These findings are consistent with the results reported by Cobos et al. (2015), who observed a high level of protection against *P. chlamydospora* after treating pruning wounds of field-grown grapevines with chitosan oligiosaccharides. Chitosan has been demonstrated to enhance the permeability of fungal cell walls and destabilize the plasma membrane of the pathogen. This may result in a loss of cellular content and, consequently, an inhibitory effect, provided that the fungus exhibits insufficient resistance to the active ingredient (Lopez-Moya et al., 2019). Our findings indicate that the efficacy of chitosan against pathogens can vary by formulation. For example, *T. koningiopsis* with 661 HCl chitosan is more effective against *P. chlamydospora* than with 671 chitosan (Fig. 35). Consequently, biocontrol agent effectiveness depends on the isolate used and the agent composition, plus the application technique.

The combination of *T. koningiopsis* and chitosan with a reduced copper rate has been shown to result in a significant reduction in the infestation intensity of *P. chlamydospora* (Fig. 32). Moreover, this combination treatment has been observed to exhibit higher efficacy against the pathogen than dual agent treatment (Fig. 35). These findings suggest that the enhanced effectiveness in treating *P. chlamydospora* may be attributed to copper rate when utilized with other agents. Di Marco et al. (2011) investigated an experimental formulation based on copper oxychloride and gluconates in wood treatments (field trials) against fungi causing Esca disease. Their findings revealed a significant reduction in the incidence of leaf stripe symptoms with the copper formulation tested (1.5 kg Cu/ha). The findings align with previous results of the present study showing the lowest *P. chlamydospora* severity levels were attained involving a reduced copper rate (Fig. 32). Investigations by Di Marco et al. (2011) demonstrated that the copper formulation inhibited *P. chlamydospora* germination and mycelial growth. This finding may provide a possible explanation for the increased efficacy observed with the triple combination compared to the dual combination in the present study. Furthermore, the colonization rate of *Trichoderma* could be further increased in the experiments conducted here by adding a reduced copper rate (Fig. 34). Since a copper-tolerant *Trichoderma* isolate was used in the biocontrol complex tested here, it can be hypothesized that *T. koningiopsis* shows a growth advantage in a copper environment compared to other fungal organisms that lack such tolerance, resulting in better initial development and higher pathogen control. This indicates that the effect can be increased by adding a reduced copper rate, but also that an effect is highly dependent on the choice of a suitable *Trichoderma* isolate with high compatibility (copper tolerance).

The results presented here demonstrate the efficacy of *Trichoderma* and chitosan to suppress or decelerate growth of Esca pathogens. Furthermore, the findings indicate that the control efficacy of a combination of both agents can be augmented by the addition of copper in a reduced rate. To ensure a sustainable effect, it is essential to improve the efficacy of the biocontrol agent and to confirm stability in further studies. In this regard, Løvschall et al.

(2024) have developed an encapsulation method for *Trichoderma* spores based on a layer-by-layer technology using bio-based lignin derivatives to protect the biocontrol agent from damage caused by physical stimuli or prolonged storage. The efficacy of this technology has been demonstrated through its ability to preserve the spore stability of *Trichoderma* under extreme temperatures and exposure to ultraviolet radiation, comparable to that of naked spores. This plays an important role in pathogen control, especially when used externally. Our previous work has shown that the active complex used here is also effective against downy mildew (Küpper et al., 2023). This technology could therefore contribute to the high stability of the complex in the control of different pathogens. The implementation of above-mentioned encapsulation technique appears to be a sustainable approach, which has the potential to contribute to the further development and enhancement of the tool tested here, based on *T. koningiopsis*, chitosan and a reduced copper rate.

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6 Discussion

6.1 Development of a sustainable plant protection tool for effective control of downy mildew and Esca in organic viticulture: necessity and prospects

The ability to combat plant diseases is of particular importance in order to ensure adequate crop yields and quality for a growing global population. In the context of viticulture, downy mildew, a disease that affects grapevines, is considered to be among the most devastating. The disease is caused by the pathogen *Plasmopara viticola* and causes significant damage to European grapevines, especially in humid years with regular rainfall. In order to protect quality and yield, pesticides are applied to control pathogens. The regular application of copper preparations is an effective measure of combating downy mildew, which is frequently used, especially in organic viticulture, due to a lack of alternatives (Gessler et al., 2011; Pertot et al., 2017a). However, regular application of these agents has been demonstrated to cause copper to accumulate in vineyards, which has a significant negative impact on the environment and soil (Karimi et al., 2021). The implementation of political restrictions by the European Union (Commission Implementing Regulation [EU] 2018/1981) and an increasing social demand for the reduction of harmful copper preparations are exerting additional pressure on winegrowers. Years such as 2016 and 2021, with high *P. viticola* infection pressure, led to considerable damage in Germany's organic wine industry – especially after the possibility of applying potassium phosphonate in organic vineyards was restricted in 2013 (Bleyer et al., 2020; Schumacher et al., 2022). Moreover, climatic changes triggered by climate change may also increasingly lead to the development of downy mildew in the future. Models demonstrate that warmer and more humid climates are likely to result in more severe and early epidemics, and that an increase in milder winters could significantly favor the pathogen's chances of survival (Bove et al., 2020).

However, viticulture is not only threatened by diseases such as downy mildew, which have the capacity to cause extensive damage to the leaves, shoots, and berries of grapevines under suitable climatic conditions (Gessler et al., 2011; Bove et al., 2020), but also by the infection of grapevines with the complex wood disease Esca. The high damage potential resulting from Esca disease is favored by changing precipitation and temperature patterns (e.g., more frequent warm and humid phases) (Beris et al., 2022). Following the restriction of the chemical control measure sodium arsenite due to its toxic effects on health and the environment (Songy et al., 2019), preventive measures and cultural practices to protect grapevines have been primarily implemented to reduce the risk of infection and further spread of the disease (Lecomte et al., 2011; Gramaje et al., 2018).

The heightened threat of damage to viticulture resulting from climate change, in conjunction with the lack of treatment alternatives, particularly within the context of organic viticulture, underscores the urgent need for eco-friendly and effective solutions. The utilization of biocontrol agents (BCA) and biostimulants holds considerable potential in ensuring the sustainability of crucial plant protection measures. The utilization of effective BCA and biostimulants offers a multitude of advantages compared to conventional pesticides, including low toxicity, minimal residue left in food, and the capacity for both preventive and curative measures (Llorens and Agustí-Brisach, 2022). In particular, the combined application of these agents has been shown to enhance the effectiveness of protection for the plant (Pertot et al., 2017b). *Trichoderma* and chitosan have been shown to achieve a synergistic and effective effect against pathogens by exhibiting different, additive modes of action (Kappel et al., 2022). In this context, an increase in fungal enzyme activity was demonstrated to be associated with the amount of added chitosan, thereby further enhancing the biocontrol potential of the synergistic complex (Rautela et al., 2019). In addition to the application of the agents as a spray on plant surfaces, the combination can also be used to combat diseases within the wood. While chitosan provides surface protection, *Trichoderma*'s capacity to penetrate the wood ensures the efficacy of its action at deeper levels in the wood (Singh and Chittenden, 2021).

The objective of this study was to develop a sustainable plant protection tool based on a synergistic combination of *Trichoderma* sp., equipped with a high copper tolerance, and chitosan to assess its efficacy against relevant pathogens in viticulture. This environmentally friendly tool is intended to make an effective contribution to the copper-reduced control of downy mildew on grapevines (*P. viticola*) and to open up a new control strategy against Esca pathogens. As demonstrated in this thesis, the developed complex of copper-tolerant *Trichoderma* sp. and chitosan achieved a control effect against downy mildew on grapevines, thereby enabling a 50% reduction in copper fungicide use. Furthermore, the efficacy of the tool in controlling Esca pathogens was also demonstrated. In this overarching discussion, the step-by-step development of the tool is presented, and the main statements of the chapters 2 to 5 are discussed and linked together.

6.2 Sustainability through reducing copper: substitute the copper effect with a tolerant biological alternative

In order to reduce the use of copper in viticulture to combat downy mildew, an effective plant protection tool must at least replace the effectiveness of the copper agent. The efficacy of this tool depends on the compatibility of the biological agent, *Trichoderma*, with a suitable copper fungicide. In chapter 2, as a first step in developing the tool, a large number of *Trichoderma* isolates were isolated from grapevine wood that had never previously been in contact with *Trichoderma* products. These isolates were characterized and examined with regard to their natural tolerance to various common copper fungicides. The objective of this chapter was to identify a suitable *Trichoderma*-copper combination for utilization in a plant protection tool appropriate for grapevines. In order to ensure the viability of *Trichoderma* in the vineyard, the copper tolerance of the most suitable *Trichoderma* candidates was further increased, and the effects of copper on the various *Trichoderma* isolates were examined in more detail in order to describe the copper tolerance of the isolates.

6.2.1 Isolation and selection of copper-tolerant *Trichoderma* isolates naturally occurring in grapevines

The initial step in selecting suitable *Trichoderma* candidates in terms of their copper tolerance was to isolate *Trichoderma* strains that are highly adapted to the grapevine ecosystem. Although several *Trichoderma*-based products are commercially available, their benefits are often significantly limited, as the growth, reproduction, and biological activity of the fungus are highly dependent on environmental conditions (Dutta et al., 2022). A variety of products have been found to contain *Trichoderma* strains, which were initially isolated from soil samples. These isolates may not be adapted to above-ground plant parts and must remain viable outside their original habitat. Consequently, endophytes originating from a particular environmental context demonstrate optimal efficacy within their native or analogous agricultural environments (Murphy et al., 2018). For this reason, it seemed appropriate to develop an effective strategy by isolating strains that occur naturally in grapevines and exhibit an antagonistic effect in the ecosystem, thereby remaining viable and competitive. Although *Trichoderma* spp. are typically found in wood, soil, and soil litter (Kubicek et al., 2019) and not on leaves – this becomes important when treating grapevine leaves to control downy mildew – a naturally given adaptation to grapevines increases the potential survival and effectiveness of *Trichoderma* (antagonistic potential) on leaves (discussed in detail in chapter 3). This approach appears to be particularly advantageous in combating wood diseases caused by Esca pathogens (a topic discussed in detail in chapter 5).

6.2.2 Considering the influence of copper formulations on *Trichoderma* spp.

In order to select *Trichoderma* isolates for the control strategy compatible with copper, the isolated *Trichoderma* spp. were tested for their copper tolerance to various copper fungicides used to control *P. viticola* in vineyards. The preparations utilized are based on the active ingredients copper octanoate, copper hydroxide, and tribasic copper sulfate, which were added to the growth medium of the fungus in varying doses (ranging from a quarter to twice the field rate). The copper tolerance tests demonstrated that the growth level of *Trichoderma* was contingent on both the dosage of the used fungicide and its formulation. In principle, the addition of copper active ingredients to *Trichoderma* spp. investigated exhibited a tendency to inhibit general growth, with this inhibition increasing in accordance with the increasing fungicide rate. The effect of copper is based on the absorption and accumulation of copper ions in the fungal cell, which causes the inactivation of essential enzymes and explains the observed growth inhibition (Gisi and Sierotzki, 2008). The results of chapter 2 are consistent with those of other investigations that also examined the growth of *Trichoderma* in copper

(Anand et al., 2006; Iskandar et al., 2011; Jovičić-Petrović et al., 2014; Mitiohlo et al., 2022). Mitiohlo et al. (2022) demonstrated that a minimal amount of copper (less than 1 mg per 100 cm^3) can also elicit a growth-promoting effect on *Trichoderma*.

The studies conducted in chapter 2 also demonstrated that when two different fungicides based on copper hydroxide were used – a wettable powder and a suspension concentrate – significant differences in the mycelial growth of the fungi could be observed. This finding indicates that, in addition to the active ingredient, the selected formulation components of the plant protection product can also exert an effect on the growth of *Trichoderma* spp.

A study by Mitiohlo et al. (2022) demonstrated that copper compounds in chelated form, particularly a mixed ligand complex, exhibited a more pronounced effect on the growth of *T. viride* compared to the mineral form of copper sulfate. In the studies conducted here, copper hydroxide, in the form of a wettable powder, was found to be more efficacious in the detoxification process when exposed to the various *Trichoderma* spp. in comparison to the other active ingredients that were examined. As the precise composition of formulations is often kept confidential by manufacturers, the potential impact of emulsifiers and solvents on *Trichoderma* spp. remains unclear. This underscores the potential challenges associated with the combination of *Trichoderma* spp. with copper fungicides. Ideally, the active agent, *Trichoderma*, should exhibit high compatibility with all components of the fungicide to ensure optimal field application and maximum efficacy. Consequently, the utilization of other, less compatible fungicide formulations may diminish the biocontrol efficacy by impeding the agent's development. Furthermore, modifications in the formulation of the compatible fungicide may result in growth and performance issues in the intended control strategy. This underscores the sensitivity of systems that incorporate biological components. In this context, it appears crucial to further enhance the stability of the tool by ensuring its compatibility with the active ingredient, on the one hand, and by increasing the tolerance of the biological agent *Trichoderma* to harmful influences such as copper, on the other.

6.2.3 Increasing copper tolerance of suitable *Trichoderma* candidates and detecting the tolerance mechanisms

The investigations conducted in this study enabled the selection of two *Trichoderma* isolates that exhibited a high degree of survivability within grapevines and a high natural copper tolerance. These isolates were identified as *Trichoderma harzianum* and *Trichoderma koningiopsis*. *T. harzianum* and *T. koningiopsis* demonstrated elevated mycelial growth and a high spore germination rate when exposed to field rate. As previously indicated, this tolerance was particularly evident in conjunction with the fungicide in the form of a wettable powder based on the active ingredient copper hydroxide. It was possible to increase the copper tolerance to a tenfold field rate (2.85 g Cu l^{-1}) without compromising the fitness of *Trichoderma* spp., which is an important prerequisite for the unhindered development of the isolates in combination with copper. This is of particular importance at the field level, as it is only through a high level of agent efficacy that the common rate of copper fungicide can be reduced.

Microscopic examinations conducted in chapter 2 revealed that the copper active ingredient is absorbed into the cells by the selected *Trichoderma* isolates and deposited on the cell walls. These observations are consistent with the findings of Anand et al. (2006), who also demonstrated copper absorption by the cell wall surface and cytosol of *T. viride*. The biosorption of metal ions by filamentous fungi can be explained by various processes, including extracellular accumulation, intracellular accumulation/ absorption, and surface precipitation (Akhtar and Mannan, 2020; Dusengemungu et al., 2020). The cell wall of *Trichoderma* spp., which consists primarily of glucan, polymers, and chitin, facilitates the species' high biosorption capacity for metals (du Jardin, 2015; Akhtar and Mannan, 2020). Chitin, which contains chitosan, has been shown to enhance the absorption and binding of heavy metals (Latha et al., 2012). This is particularly advantageous in the context of a planned combination of suitable *Trichoderma* isolates with the additional active agent, chitosan. The binding properties of the additional chitosan could protect the biological agent, *Trichoderma*, from excessive copper contamination even though *Trichoderma* already exhibits a certain degree of copper tolerance. As a result, *Trichoderma*'s fitness and control potential remain preserved. Regardless of the tolerance level of the tested *Trichoderma* spp., discrepancies between the respective isolates were observed in the conducted studies. This finding suggests the presence of isolate-specific binding characteristics. Maldaner et al. (2020) also indicate that isolate-specific differences in the tolerance of *Trichoderma* isolates to heavy metals have been observed in their studies, emphasizing the necessity of evaluating the tolerance level at the isolate level. In the studies conducted in chapter 2, the accumulation of copper led to changes in the mycelial structure, which was observed in all *T. harzianum* isolates with varying tolerance to the active ingredient. In contrast, *T. koningiopsis* showed no deformations. It can be hypothesized that a more effective tolerance mechanism exists, which, due to a less pronounced color detection of the copper active ingredient, led to reduced copper uptake into the cells and/ or resulted in more effective removal of the active ingredient. The efficacy of such transport mechanisms has already been described in the context of yeast, wherein copper chaperones and ATPases have been shown to facilitate the transportation of copper into the secretory pathway. The metal ingredients are transported across the plasma membrane into the extracellular environment and remain harmless to the cell (Puig and Thiele, 2002). The capacity for detoxification and the resulting compatibility of pesticides and biocontrol agents are crucial factors in determining their effectiveness, which in turn affects the management of pathogens (Stark et al., 2007).

6.2.4 The necessity of *Trichoderma*'s detoxification capacity as essential feature for practical application

The high compatibility of *Trichoderma* with a copper active ingredient or the copper tolerance detected in chapter 2, as well as the associated high detoxification capacity of the heavy metal, contribute significantly to the stability of the effectiveness of the BCA. Consequently, these factors also contribute to the overall effectiveness of the desired plant protection tool. This property is of great importance for maintaining the vitality of *Trichoderma* and may also make it possible to maintain the fitness and effectiveness of the agent under the influence of other externally supplied components in organic viticulture, such as sulfur treatments against powdery mildew. In viticulture, sulfur treatments are the primary method employed for the management of powdery mildew, analogous to the utilization of copper for the control of downy mildew (Pertot et al., 2017b). In practice, sulfur- and copper-based fungicides are often applied in combination, often as a tank mixture (Meissner et al., 2019). In order to enable the most practical application of a sustainable plant protection strategy with *Trichoderma*, it is essential to consider the agent's high detoxification capacity. This capacity offers the opportunity to remain viable and effective even when other additives are present.

The observed copper tolerance in the isolates could be attributed to a natural adaptation to this active ingredient, developed through consistent exposure to copper in vineyards under practical conditions (Küpper et al., 2022). This aspect is of interest not only with regard to copper-based agents, which are frequently utilized in organic viticulture to combat downy mildew. It is also possible that the isolates may already have adapted to other agents, such as regularly applied sulfur preparations mentioned above. The copper tolerance studies address a fundamental aspect that is critical for the development of a copper reduction tool. A proven and subsequently increased fungicide tolerance of *Trichoderma* can be an important indicator of high fitness and increased biocontrol potential even under stressful conditions. This should be considered an important selection criterion for the development of effective biocontrol strains (Kovács et al., 2021). However, it is important to consider that when utilized in field environments, *Trichoderma* is subjected to a range of external factors that have the potential to influence the efficacy of the agent (Yao et al., 2023). This underscores the necessity to expand the proposed plant protection tool with at least one additional supporting pillar. In an ideal scenario, the implementation of an additional component would facilitate the proliferation of *Trichoderma*, thereby introducing an additional compatible mechanism of action for the control of pathogens.

6.3 Implementation of chitosan: copper-tolerant *Trichoderma* spp. and chitosan work together to control downy mildew

Chapter 3 examines the expansion of the tool to include an additional control pillar: the agent chitosan. Here, the primary objective was to assess the compatibility of *Trichoderma* spp., chitosan, and the selected copper fungicide. A comprehensive understanding of the synergistic effects of these agents is essential for expanding the knowledge base regarding the complex mechanism of action. This, in turn, facilitates the optimization of the system. The compatibility of the components and an understanding of the tool are therefore essential prerequisites for effective pathogen control with simultaneous copper reduction, especially in organic viticulture.

6.3.1 Ensure the compatibility of copper-tolerant *Trichoderma* spp. and chitosan for expanding the plant protection tool

Four distinct chitosans, three chitosan polymer-oligomer mixtures, and one chitosan polymer-polymer mixture, were examined for their compatibility with various copper-tolerant *Trichoderma* isolates selected in chapter 2.

The mycelium growth and spore germination tests conducted for this purpose confirmed the high biocompatibility of the polymer-oligomer mixtures with the selected *Trichoderma* spp. In particular, a high compatibility with the copper-tolerant *T. koningiopsis* isolate was demonstrated. The structure of the chitosans affects the compatibility of the active ingredient. The long chain structure of polymers, in comparison to oligomers, results in an increased binding surface on the fungal cell surface (Kauss et al., 1989). Furthermore, the polycationic structure of the polymer contributes to a higher direct antifungal effect and the destabilization of the fungal plasma membrane (Wattjes et al., 2020). Consequently, initial growth is inhibited in all chitosan examined, particularly in the polymer-polymer mixture, unless there is a high tolerance to the active ingredient, as was observed here for *T. koningiopsis*. Similar to copper, the biological agent *Trichoderma* must exhibit a high level of tolerance to chitosan to achieve optimal effectiveness within the combination of all agents. The combination of two antifungal agents requires the fungus to have a high level of fitness and a pronounced detoxification capacity in order to demonstrate a control effect against pathogens in a following step.

6.3.2 Mechanism of chitosan tolerance of *Trichoderma* spp. and combined agent's use

The high level of tolerance exhibited by the *T. koningiopsis* isolate to chitosan, as evident in the mycelial growth and spore germination tests, suggests the presence of a pronounced ability to enzymatically degrade chitosan. This ability could serve to mitigate the antifungal effect of chitosan on *Trichoderma* sp. The ability of *Trichoderma* spp. to demonstrate chitosanolytic activity is well documented and has been previously shown in other studies (Witkowska and Maj, 2002; Zavala-González et al., 2016; da Silva et al., 2016). Research by Zavala-González et al. (2016) also demonstrated that the increased tolerance of *T. koningiopsis* to chitosan is directly related to the lower membrane fluidity of the fungus. This property renders the fungal membrane less mobile, thereby impeding chitosan to enter the cells

(Zavala-González et al., 2016). The capacity to secrete lytic enzymes enables certain *Trichoderma* spp. to not only withstand the antifungal effects of chitosan, but also to disrupt the cell walls of pathogens (Witkowska and Maj, 2002). This biocontrol effect is further enhanced by the fungistatic effect of chitosan, which contributes to morphological changes in the cell wall and cell membrane of pathogens (Vesentini et al., 2007). When suitable candidates are selected, a synergistic effect of the agents results, which can contribute effectively to pathogen control (Singh and Chittenden, 2021).

6.3.3 Demonstration of a direct and an indirect effect of *T. koningiopsis* and chitosan on *P. viticola*

Once compatible components had been selected for the development of an eco-friendly tool, another key aspect of the work was to examine and describe in more detail the effect and mode of action of the components *Trichoderma* and chitosan against pathogens. To this end, the effect of the previously selected *Trichoderma*-compatible chitosan candidates with the tolerant *T. koningiopsis* isolate was investigated in single and combined applications, also together with a reduced copper rate, on downy mildew on grapevines. At the same time, the effect of the agents on the pathogen was examined more closely by analyzing either the underside of the leaf (direct effect on *P. viticola*) or the upper side of the leaf (indirect effect on *P. viticola*) in greater detail. The results of chapter 3 demonstrated a direct and indirect effect on the number of sporangia in leaf discs inoculated with the pathogen, as well as on downy mildew infestation on whole leaves of greenhouse grapevines through spray treatments with the agents. The findings demonstrated that the application of the respective agents resulted in a reduction in spore production and in the disease severity of *P. viticola*, in comparison to the untreated control.

Chitosan demonstrated a high antifungal effect. However, the efficacy of different chitosan molecules varied, indicating that the molecular structure of the polymer influences its capacity to interact with and affect the pathogen. The antifungal bioactivity is attributed to the interaction of positively charged chitosan with the negatively charged phospholipid components of the pathogen. This interaction has been shown to lead to increased permeability and leakage of the pathogen's cellular contents. Furthermore, the chelating properties of the substance result in the loss of essential components required for normal development of the pathogen. Additionally, the ability of chitosan to bind to the DNA of the pathogen has been demonstrated, thereby potentially affecting the synthesis of mRNA and, consequently, the production of proteins and enzymes (Ruano-Rosa et al., 2022). The observed variability in the effectiveness of the tested chitosans can be attributed to their size, structure, and, in particular, their degree of acetylation and polymerization (Kauss et al., 1989; Vander et al., 1998; Rahman et al., 2015).

Furthermore, variations in the deposition of chitosan on the leaves, in conjunction with the chemical properties of the chitosans, may have resulted in divergent efficacies against the pathogen. Microscopic examinations revealed that the dispersion of the polysaccharide led to the formation of platelet-like structures on the grapevine leaves. These platelet-like formations potentially hindered the penetration of the pathogen and, consequently, the subsequent invasion of the leaves (Küpper et al., 2023). Furthermore, the results obtained in chapter 3 suggest a predominantly uniform distribution of chitosan on the leaf surface. An uniform distribution of the agent is particularly advantageous for a direct antifungal contact effect, as well as for the absorption of the agent by the plant cell (Birr et al., 2023), thereby enabling the indirect induction of resistance by chitosan (Kappel et al., 2022). A direct impact on the pathogen originating from *T. koningiopsis* was also detected in the conducted investigations, although it was weaker than that caused by chitosan. The underlying direct biocontrol mechanisms of many *Trichoderma* spp. can be attributed to the production of cell wall-degrading enzymes and antibiotics, competition for nutrients and space, and mycoparasitism (Jaroszuk-Ściseł et al., 2019).

6.3.4 Combining agents enhances effectiveness and stability of the tool

The greenhouse studies demonstrated that a combination of all agents (tolerant *T. koningiopsis*, chitosan, and a reduced copper rate) resulted in a more effective reduction of the pathogen compared to single or dual application. This finding aligns with the observations reported by Monte (2001) and Shukla et al. (2021), who demonstrated that the treatment with a single agent often results in insufficient efficacy against pathogens. Particularly under field conditions, the single treatment effect lacks in stability (Pertot et al., 2017b). Consequently, the addition of a reduced amount of copper as an additional component appears to be a reasonable approach for sufficiently supporting the effect of *Trichoderma* and chitosan (Shukla et al., 2021). Chitosan is capable of binding copper via a free electron pair and forming an effective complex (Varma et al., 2004). Accordingly, the antifungal effect of chitosan is enhanced by a synergistic effect of copper. Furthermore, copper-tolerant *Trichoderma* isolates have been shown to bind copper to their cell surface, thereby preventing excessive uptake of the active substance and maintaining viability (Küpper et al., 2022). Moreover, suitable *Trichoderma* isolates have been shown to produce cell wall-degrading enzymes, which contribute to the suppression of spore germination and hyphal elongation of pathogens (Saravanakumar et al., 2016). Research by Bohra (2018) also examined a compatible triple combination of copper, *Trichoderma*, and chitosan. Bohra (2018) attributed the interaction of the three agents and the mode of action to a process of subsequent events: Suitable *Trichoderma* isolates have been shown to produce chitosanases and cellulases, which enzymatically break down chitosan into highly effective antifungal oligomers. Furthermore, the cellulase secreted by *Trichoderma* facilitates the degradation of the pathogen's cell wall. This allows the copper active ingredient to penetrate the cells and act on the intercellular components. Consequently, the pathogen is inhibited. A similar explanation for the synergistic effect of the components is also conceivable for the combination of copper- and chitosan-tolerant *T. koningiopsis*, chitosan, and copper used in this work.

The findings of this work demonstrate that, under suitable conditions, the strategic selection of coordinated agents can lead to a significant increase in the antifungal biocontrol and biostimulating properties of the agents, even with a minimal amount of copper. This is of crucial importance for achieving the objectives of the tool. The distinct modes of action exhibited by the agents, namely direct biocontrol effect and indirect inducing effect, facilitate the synergetic action of the compatible duo of copper- and chitosan-tolerant *T. koningiopsis* isolate and chitosan, enabling their action on multiple targets. This stabilizes the overall effect of the tool. Furthermore, the efficacy of this effect can be enhanced by the presence of copper, thereby ensuring a robust antifungal outcome, even under field conditions (Pertot et al., 2017b). Since one central objective of the work here is to ensure the compatibility of the agents with a reduced rate of copper, it is feasible to concurrently pursue the goals of stable and effective pathogen control using *Trichoderma* and chitosan, as well as the objective of reducing the use of fungicides, without compromising the effectiveness of the agents.

6.3.5 Required persistence and establishment of tolerant *Trichoderma* spp. on grapevine leaves

Maintaining the synergistic effect requires the biological agent *Trichoderma* to remain viable in the pathogen's living environment in combination with the other agents, even if this environment does not correspond to the original habitat of the agent. Accordingly, insufficient persistence of agents can be a significant disadvantage for the desired effect if the applied agent cannot develop its effect sufficiently within a short time after spray application (Hommen et al., 2024). The microscopic results obtained in chapter 3 demonstrated that, even when applied as a triple combination, tolerant *T. koningiopsis* was capable of successfully forming mycelium and sporulating on the upper and lower surfaces of the grapevine leaves. As expected, the presence of living mycelium decreased significantly after 13 dpi. These results are consistent with those reported by Samuelian (2016), who investigated the establishment and development of *Trichoderma* on field banana leaves. The results obtained in the conducted work indicate that, due to the pronounced growth of *T. koningiopsis* on the grapevine leaves after six days, the tool should be applied approximately once a week to ensure the biological agent remains highly viable. Since copper fungicides are typically applied at ten-day intervals in organic viticulture (Berkelmann-Löhnertz et al., 2008), a mixture of copper- and chitosan-tolerant *T. koningiopsis*, chitosan, and a reduced copper rate appears to be a feasible option at the standard treatment times in viticulture against *P. viticola* without requiring a significant increase in spraying effort.

6.4 Understanding the induced defense mechanisms of tolerant *T. koningiopsis* and chitosan in grapevine to control *P. viticola*

The findings presented in chapter 3 demonstrate that the effects of *Trichoderma* and chitosan on downy mildew in grapevines can be ascribed to both a direct and an indirect effect. Consequently, the present work investigated the inducing effect of the agents with the objective of achieving a comprehensive understanding of the processes involved. In order to achieve high efficacy of the biocontrol and biostimulating complex, it is essential to possess a profound knowledge of the modes of action of the combined agents. Consistent with the findings reported in the previous chapter, the greenhouse experiments outlined in chapter 4 further supported the induced defense effect of the plant against downy mildew following prior treatment with the agents. The induction of plant defense mechanisms by *Trichoderma* spp. and chitosan is well documented and has been previously described in the literature. For instance, the induction of systemic resistance in grapevine has been demonstrated for *T. harzianum* strain T39 (Perazzolli et al., 2008). Suitable *Trichoderma* spp. have been shown to enhance the activity of resistance-related enzymes (e.g., glucanases, chitinases, cellulases, and peroxidases) through the release of elicitors, thereby positively affecting plant resistance (Yedidia et al., 2000). In contrast, chitosan itself functions as a potential elicitor molecule, possessing the capacity to enhance defense responses in grapevines (de Bona et al., 2021). For instance, following the application of chitosan to grapevines, an enhancement in the accumulation of phytoalexins induced by the polymer, along with a modulation in the enzymes contributing to the oxidative stress response, was observed (Lucini et al., 2018).

The enhanced resistance observed in grapevines, triggered by the combined action of *Trichoderma* and chitosan, could provide a plausible explanation for the previously documented inhibitory effect against *P. viticola* (see chapter 3). Notably, *Trichoderma* and chitosan do not directly interfere with the pathogen, suggesting alternative mechanisms through which they elicit an immune response in grapevines. This hypothesis is further supported by the observation that resistance inducers (Bion, BABA) showed a similar inhibitory effect.

6.4.1 Tolerant *T. koningiopsis* and chitosan affect gene expression and defense proteins in different grapevine cultivars

The findings presented in chapter 4 demonstrated that treatment with the agents exhibited a modulating effect on grapevine gene expression in different grapevine cultivars, exhibiting varying degrees of resistance to downy mildew. This effect differed significantly from the expression levels of the grapevine cultivars after pathogen inoculation and without agent treatment. In particular, the cvs. Müller-Thurgau (sensitive) and Regent (resistant) exhibited increased *Pr10* expression following treatment with the agent. The observed effect on *Pr10* gene expression was particularly evident after combined treatment with tolerant *T. koningiopsis* and chitosan and was apparent in both sensitive and *P. viticola*-resistant cultivars, although the timing and intensity of gene expression differed. The upregulation of pathogen-related proteins, which play a central role in the recognition and defense against pathogens, by biostimulants enables the plant to respond more quickly and effectively to pathogens, thereby strengthening the plant's immune defense (Monteiro et al., 2022). The *Pr10* family constitutes a critical component of the defense response against fungal invasion, as well as biotic and abiotic stress factors (Liu and Ekramoddoullah, 2006). Monteiro et al. (2013) demonstrated in their studies that *Pr10* was more strongly upregulated after infection in resistant grapevine cultivars than in susceptible ones, attributing a central role to *Pr10* in the defense against *P. viticola*. Consequently, increased upregulation of the gene can be associated with higher resistance to the pathogen. In the work conducted here, the resistant cv. Regent also exhibited strong expression of *Pr10* after treatment with biostimulants and pathogen inoculation. This is consistent with the observations reported by Merz et al. (2015), who also demonstrated an upregulation of *Pr10* after inoculation of the cv. Regent with *P. viticola*. In addition, Merz et al. (2015) demonstrated an early induction of the WRKY33 gene and showed that resistant and susceptible grapevine cultivars exhibited a similar downstream signaling pathway of WRKY33. Consequently, there are differences in the upstream level of WRKY33 gene expression in the respective defense response (Merz et al., 2015). This offers a potential explanation for the observed variations in the timing and strength of gene expression among the different cultivars examined in this work.

It is particularly noteworthy that, following combined treatment of susceptible grapevines cultivar with *T. koningiopsis* and chitosan, an effect on gene expression was also observed – though less pronounced than in resistant cultivars. The results obtained indicate that this effect can be demonstrably induced by *T. koningiopsis* and chitosan, as no upregulation of *Pr10* could be observed in the absence of the agents. Although this effect was less pronounced than in the resistant cultivars, targeted defense through the induction of *Pr10* using biostimulants contributes to reducing disease pressure, especially in sensitive cultivars. Jacquens et al. (2022) demonstrated that biostimulants considerably increase the effectiveness of defense elicitors and can thus reduce pathogen infestation. This additional target of resistance induction highly contributes to reducing the need for pesticides by improving the plant's own defenses (Jacquens et al., 2022; Monteiro et al., 2022). Therefore, particularly in the case of susceptible grapevine cultivars, the ability to activate *Pr10* is an important step toward environmentally friendly plant protection and securing stable yields under increasing disease stress.

6.4.2 Reactive oxygen species accumulation induced by tolerant *T. koningiopsis* and chitosan as part of grapevines' resistance response

The production of reactive oxygen species (ROS) and the expression of *Pr10* genes are closely related. As Ma et al. (2018) demonstrated in their research, the expression of the *Pr10* protein *VpPR10* of a wild grapevine resistant to *P. viticola* in conjunction with a mitochondrial channel protein (voltage-dependent anion channel 3) led to the triggering of a cell death-mediated defense response associated with ROS accumulation. This response has been shown to help the plant in its defense against downy mildew infection. The results obtained in this work demonstrated an increased accumulation of O_2^- and H_2O_2 in the resistant grapevine cvs. Regent and Cabernet blanc treated with biostimulants. The ROS accumulation observed in this work could be associated with the *Rpv3-1*-mediated resistance exhibited by the grapevines. The *Rpv3* locus has been demonstrated to be associated with the synthesis of fungitoxic stilbenes and programmed cell death, which in turn leads to a reduction in downy mildew growth and development. Furthermore, *Rpv3-1*-mediated resistance has already been demonstrated for the resistant grapevine cvs. Regent and Cabernet blanc, whereas it could not be described for the susceptible grapevine cv. Müller-Thurgau (Eisenmann et al., 2019). Although late gene expression of *Pr10* was observed in the susceptible cv. Müller-Thurgau in the work conducted here, no significant ROS accumulation could be demonstrated. It is plausible that the effect of the biostimulants was insufficient to demonstrate an influence on subsequent ROS formation. Alternatively, a different form of resistance could be responsible for the defense reaction.

Furthermore, the observation revealed that the treatment of biostimulants to resistant grapevine cultivars exhibited divergent temporal patterns of ROS accumulation compared to previously documented findings. In contrast to the findings of Wingerter et al. (2022), who observed no accumulation of H_2O_2 before 24 hpi in grapevine cultivars with *Rpv3*-mediated defense, the work conducted in chapter 4 demonstrated earlier ROS accumulation in grapevines previously treated with biostimulants. This could be attributed to an accelerated resistance response of grapevine to downy mildew caused by tolerant *T. koningiopsis* and chitosan. The treatment of grapevines with *Trichoderma* has been demonstrated to result in increased ROS production in leaf tissue following pathogen infestation (Palmieri et al., 2012). Moreover, treatment with chitosan, functioning as an elicitor of defense mechanisms, has been associated with the accumulation of ROS (Galli et al., 2024).

It is advantageous if the biostimulants *T. koningiopsis* and chitosan integrated into the tool stimulate the production of ROS in grapevines, as this can enhance the plant's intrinsic defense mechanisms against pathogens such as *P. viticola.* In this context, the observed dependence of the immune response effect on the respective grapevine cultivar must be taken into account. The induction of ROS is an early and locally effective defense reaction against *P. viticola* (Kortekamp and Zyprian, 2003). In particular, the combined application of *T. koningiopsis* and chitosan had a reinforcing effect on the plant's immune response. Kappel et al. (2022) also confirmed the influence of *Trichoderma* and chitosan on the production of ROS in their work. Consequently, the plant's resilience to biotic stress is enhanced, thereby facilitating a reduction in the use of pesticides.

6.4.3 Tolerant *T. koningiopsis* and chitosan reduce *P. viticola* haustorium formation

To provide a more detailed description of the effect of the plant protection tool on *P. viticola*, the impact of a biostimulant treatment on the haustorium formation of the pathogen was examined in more detail. The formation of haustoria plays a critical role in the pathogen's nutrient uptake, thereby constituting a fundamental initial step in the infection process. Disruption or inhibition of haustoria formation can directly impact the pathogen's colonization success (Gessler et al., 2011; Juraschek et al., 2022). The results obtained in chapter 4 demonstrated a reducing effect on haustorium formation through the treatment of grapevines with tolerant *T. koningiopsis* and chitosan, with the strongest effect observed in resistant cultivars. These results are consistent with those reported by Juraschek et al. (2022), who also demonstrated differences in haustorium formation in grapevine cultivars with different levels of resistance and linked this defense reaction to callose deposits. The findings of chapter 4 demonstrated that a combined treatment of tolerant *T. koningiopsis* and chitosan resulted in an enhanced defense response against *P. viticola*. Similarly, Jacquens et al. (2022) also demonstrated a reduction in haustoria after treating grapevines with a combination of a biostimulant and a defense elicitor.

The obtained findings on the harmful effect on pathogenic haustoria formation induced by tolerant *T. koningiopsis* and chitosan contribute significantly to further expanding the potential of this plant protection tool against *P. viticola*. A comprehensive understanding of the influence of *Trichoderma* and chitosan on the pathogen's haustoria formation facilitates the development of a precise plant protection strategy that disrupts the infection cycle of *P. viticola* at a critical phase in the early stages of infection. This contributes substantially to the efficacy of the strategy against *P. viticola*.

6.4.4 Importance of the induced effect of the tool for grapevine protection

The induction of indirect defense mechanisms by the plant protection tool, as demonstrated in the work conducted in chapter 4, ensures the precise activation of the plant's own immune defense. In addition to the direct defense effect of the tool described in chapter 3, tolerant *T. koningiopsis* and chitosan can thus trigger another target, providing an additional pillar of support for effective pathogen defense. The proven indirect defense reactions induced by tolerant *T. koningiopsis* and chitosan make the tool's protection more comprehensive and eco-friendlier and can help to further reduce the need for high-risk plant protection products such as copper-based agents used against downy mildew.

6.5 Expanding the tool's application range: from combating downy mildew on leaves to controlling Esca pathogens in grapevine wood

To contribute to holistic plant protection and maintain the health of grapevines, the synergistic plant protection complex should ideally be effective against a wide range of pathogens relevant to viticulture. In addition to *P. viticola*, which causes downy mildew on grapevines, Esca disease can cause significant damage in viticulture worldwide. Downy mildew, a leaf-related disease, typically exerts a direct impact on yield and grape quality. In contrast, Esca pathogens may lead to wood-destroying diseases, potentially threatening the long-term vitality of grapevines and resulting in the death of entire plants. Fungicides are utilized in particular to protect the foliage and grapes of the grapevines from downy mildew. In contrast, classic control of Esca pathogens is not possible; preventive measures are recommended to avoid infestation.

As demonstrated in chapters 2 to 4, the copper and chitosan tolerance of *Trichoderma* spp. has been confirmed, as well as the compatibility of *T. koningiopsis*, chitosan, and copper. Furthermore, the efficacy of the tool against downy mildew was demonstrated.

Another objective was to expand the potential of the previously tested synergistic combination of *Trichoderma* and chitosan as an eco-friendly alternative for plant protection. In doing so, the combination was tested for protection against Esca pathogens. Such a combination could provide a possible tool for effectively combating Esca pathogens in addition to control downy mildew. The broad effectiveness of the tool against various relevant pathogens makes it possible to develop a holistic plant protection approach for maintaining the health of the grapevine in a sustainable way.

6.5.1 Necessary establishment of the biological agent *Trichoderma* in grapevine wood

In order to successfully apply the synergistic complex against wood-destroying Esca pathogens, it is particularly important to ensure the establishment and availability of the biological agent *Trichoderma* within the grapevine wood. Consequently, a component of the study entailed the screening of various *Trichoderma* spp. for their capacity to colonize the wood and shoots of grapevines. The investigations conducted indicated that all *Trichoderma* isolates examined exhibited successful reisolation from the wood and shoots of the grapevines following an adequate inoculation period. The colonization ability of the respective tissue was found to be isolate-dependent, which can be explained by different optimal colonization conditions for different *Trichoderma* spp. (Carro-Huerga et al., 2021). Consistent with the findings of John et al. (2008), the conducted work revealed that the fungal agent colonized the entire tissue of the canes, with the frequency of recovery decreasing with increasing distance from the inoculation point. A crucial aspect of Esca prevention involves the protection of grapevine wounds to impede the entry of Esca pathogens through such potential entry points. Consequently, the presence of high levels of *Trichoderma* colonization in proximity to such entry points offers a promising approach for enhancing wound protection, since *Trichoderma* shows the ability to form a biological barrier, thereby impeding the entry of pathogens into the wood. The highest possible colonization rate is therefore an important prerequisite for a high level of wood protection (Carro-Huerga et al., 2021) and can be particularly advantageous at the aforementioned entry points in order to control pathogens before they become established.

In addition to the colonization of wood, the studies demonstrated colonization of shoots, although to a lesser extent than that of wood. Bailey et al. (2008) also demonstrated the ability of *Trichoderma* spp. to colonize above-ground tissues in addition to wood. This finding indicates that, depending on the *Trichoderma* isolate selected, the fungus possesses the capacity to maintain viability in green above-ground tissue. In alignment with the findings presented in chapter 3, which demonstrated the capacity of *Trichoderma* spp. to persist on leaf surfaces, further expansion of the fungal agent's viability radius can be attributed to the survivability in shoots exhibited in chapter 5. These findings contribute substantially to the endurance and sustainability of the plant protection tool.

A plant protection tool that is capable of influencing a variety of habitats of pathogens enables the control of multiple infection pathways. *Trichoderma*'s capacity to persist in both green and woody plant components enables its efficacy in combating primary leaf diseases, such as downy mildew on grapevines, as well as wood diseases, including Esca. Since the *Trichoderma* isolates utilized in this study were initially obtained from vineyard grapevine wood, which corresponds to their natural habitat, there is a high probability that they will establish themselves in the grapevine wood used in the investigations, depending on the optimal growth conditions of the selected isolate. A prolonged existence of the organism within the plant may contribute to a long-term protective effect in the trunk of the perennial grapevine. The treatment of the leaves and wood of the plant with the agents contained in the tool could result in a generally stronger defense of the grapevine through an accumulative effect. This, in turn, could strengthen overall plant health and the defense against chronic wood diseases as well as acute leaf infections.

6.5.2 Demonstration of a direct and an indirect biocontrol effect of *Trichoderma* spp. against Esca pathogens

In order to achieve high biocontrol activity against selected Esca pathogens in addition to the high biocontrol activity against *P. viticola*, a possible direct and indirect effect was investigated through the release of potential volatile organic compounds from *Trichoderma* spp. against relevant Esca pathogens. As demonstrated in the chapters 2 to 4, the *Trichoderma* isolates *T. koningiopsis* and *T. harzianum* have exhibited both high fitness and high biocontrol potential. Furthermore, chapter 5 confirmed that these isolates possess a high colonization capacity in grapevine wood. Consequently, the isolates *T. koningiopsis* and *T. harzianum* exhibited the potential to control the Esca pathogens *Phaeomoniella chlamydospora*, *Fomitiporia mediterranea* and *Phaeoacremonium minimum*.

The confrontation tests carried out for this purpose demonstrated a significant inhibitory effect of *T. koningiopsis* and *T. harzianum* on pathogen mycelial growth, thus confirming the high biocontrol capacity of the selected *Trichoderma* isolates. The two *Trichoderma* isolates exhibited a direct biocontrol effect against all the pathogens tested, although the degree of growth inhibition varied. Studies by Mesguida et al. (2023) also demonstrated that *T. koningiopsis* and *T. harzianum* exhibit a high biological control capacity against grapevine pathogens. The authors attributed this capacity to three factors: antibiosis, a strengthening of the defense response, and competition for space and nutrients. In particular, the work conducted here demonstrated that *T. koningiopsis* and *T. harzianum* strongly suppressed the growth of the Esca pathogens *P. chlamydospora* and *F. mediterranea*. Mesguida et al. (2023) also demonstrated a high biocontrol efficacy of *T. koningiopsis* and *T. harzianum* against *P. chlamydospora* and attributed this to overgrowth, competition for nutrients, and direct antagonistic properties of *Trichoderma*.

Wallis (2021) showed that suitable *Trichoderma* spp. can demonstrate superior efficiency in utilizing the provided carbon and nitrogen sources compared to the respective pathogen. Furthermore, Wallis (2021) described outcompeting other organisms as the most significant mechanism for successful *Trichoderma* colonization. The enhanced utilization of available resources by *Trichoderma* compared to the pathogen may have contributed to the observed reduction in the growth of *P. chlamydospora* and *F. mediterranea*. This may offer protection against Esca pathogens within a brief time window after application to pruning wounds or cuttings and may counteract the pathogen's establishment in plant tissue. The overgrowth of the pathogen by *Trichoderma* is also described as an effective biocontrol strategy against Esca pathogens, among other antagonistic mechanisms (Silva-Valderrama et al., 2021). The findings of the work conducted in chapter 5 demonstrated that the individual growth of the pathogen and *Trichoderma* was solely influenced by time and nutrients in the medium. In the dual confrontation between *P. chlamydospora* and *Trichoderma*, the mycelial growth of the pathogen was highly suppressed by *Trichoderma*, with the mycelium of *Trichoderma* occupying a markedly larger area of the medium than was the case with *P. chlamydospora*. These findings suggest that overgrowth mechanisms may play an important role in the suppression of pathogenic growth.

While this effect was clearly evident in *P. chlamydospora* and *F. mediterranea*, *P. minimum* revealed a lesser inhibitory effect. Wallis (2021) attributed moderate resource utilization to *P. minimum*. A comparable level of resource utilization by *Trichoderma* and the pathogen does not guarantee a direct growth advantage for the biocontrol agent, resulting in a limited effect on the growth behavior of *P. minimum*. Consequently, this could explain the reduced inhibitory effect on *P. minimum* observed in comparison to other pathogens.

To provide a more comprehensive characterization of the inhibitory effect of *Trichoderma* spp. on *F. mediterranea, P. minimum* and, *P. chlamydospora*, the indirect biocontrol potential of the selected *Trichoderma* isolates against the pathogens was also examined. It has been demonstrated that the release of volatile substances, such as 6-pentyl-alpha-pyrones, hydrocarbons, or terpenes, from suitable *Trichoderma* spp. can contribute to the suppression of the growth of Esca pathogens (Salwan et al., 2019). In the sandwich plate tests conducted here, the growth of all three Esca pathogens was also significantly reduced by an indirect influence of *Trichoderma*. This finding aligns with the findings of studies conducted by Bararkat et al. (2014), which also demonstrated a control effect on pathogens without direct contact with *Trichoderma*. The observed effect on development and sporulation was attributed to the capacity of *Trichoderma* to produce volatile compounds that diffuse into the medium and induce hyphal and spore lysis (Bararkat et al., 2014). Research on *T. koningiopsis* and *T. harzianum* has demonstrated that both species are capable of producing volatile organic compounds (Meena et al., 2017; You et al., 2022). A total of 24 volatile organic compounds with abiotic properties were identified for *T. koningiopsis* (You et al., 2022). The identified compounds were primarily alkenes, alkanes, and esters. Investigations into *T. harzianum* have demonstrated that its high inhibitory effects are attributable to its mechanisms of mycoparasitism and the synthesis of antibiotic substances. In this case, volatile compounds such as glacial acetic acid and propylbenzene were detected for *T. harzianum* (Meena et al., 2017). It can be hypothesized that the production of the aforementioned volatile organic compounds may have contributed to the observed growth inhibition of the pathogens in the studies conducted here. However, the extent of the effect of the volatile substances released is contingent upon the *Trichoderma* isolate selected and the type of pathogen, as well as the pathogen's capacity to reduce the effect of the compounds released.

The results obtained in chapter 5 demonstrate that both a direct and an indirect antifungal effect can be achieved by suitable *Trichoderma* spp. on Esca pathogens. The plant protection tool, which has previously been successfully tested against *P. viticola*, thus also offers the possibility of exerting an effect on other pathogens in a different environment (in the grapevine wood instead of on the grapevine leaves). Consequently, these findings significantly expand the range of applications for the tool. As with the effects of the tool on *P. viticola* previously described, direct and indirect effects on the pathogens *P. chlamydospora F. mediterranea*, and *P. minimum* can also be demonstrated. It is noteworthy that the indirect effect characterized for the Esca pathogens in the work presented in chapter 5 is attributable to *Trichoderma*. Contrary to the studies examining the effect on *P. viticola*, the stimulation of the plant was not the primary objective in these investigations. Nevertheless, the obvious production and release of volatile organic compounds by *Trichoderma* can be described as an additional mechanism of the biocontrol agent for controlling pathogens. This contributes to a multi-layered and effective control strategy. The biocontrol agent *Trichoderma* has been demonstrated to exert an antifungal effect in direct contact with pathogens (e.g., through competition for nutrients and overgrowth). Additionally, it has been observed to cover a larger area of the medium and reduce the development and growth of pathogens by releasing volatile organic compounds. Although no studies on the induction of plant defenses could be conducted within the scope of Esca, there are indications in literature that the release of volatile organic compounds by *Trichoderma* can contribute to plant stimulation and pathogen defense (Jiménez-Bremont et al., 2024). Furthermore, studies by Lazazzara et al. (2021) demonstrate that the release of volatile organic compounds by *Trichoderma* spp. can induce resistance mechanisms in grapevines, thereby contributing to the defense against powdery mildew. Specifically, 6-pentyl-2H-pyran-2-one and 2-pentylfuran exhibited notable efficacy against *P. viticola* by increasing the accumulation of callose and the expression of defense genes following infection with *P. viticola*. These multifaceted defense mechanisms of *Trichoderma*, which demonstrate efficacy at both the leaf level and in the wood, can contribute to a high degree of efficiency against pathogens when suitable *Trichoderma* isolates are selected, as the overall effect can be composed of a pool of defense mechanisms of various kinds. The addition of supplementary components, such as chitosan and copper, when necessary, can further enhance this efficiency and can contribute to the stability of the effect.

6.5.3 Tool shows high efficacy against *P. chlamydospora* in the vineyard

To verify the effect of the eco-friendly plant protection tool against *P. chlamydospora* under practical conditions, field studies were conducted on grapevines. The experimental vineyard studies demonstrated that grapevines previously treated with a combination of tolerant *T. koningiopsis*, chitosan, and a reduced copper rate exhibited significantly lower *P. chlamydospora* severity compared to the untreated variant inoculated with *P. chlamydospora*. The studies demonstrated that the colonization of the wood by *T. koningiopsis* was adequately achieved through treatment of the grapevines in late winter/ early spring. The application of the biocontrol agent at mild temperatures is of particular importance for the development of *Trichoderma*, thereby also influencing the effectiveness of the plant protection measure on the plant (Martínez-Diz et al., 2021).

In particular, the combination of tolerant *T. koningiopsis*, 661 HCl chitosan, and reduced copper application exhibited a significant reduction in infestation intensity compared to the *P. chlamydospora*-inoculated variant without biocontrol treatment. The findings indicate that the reduction effect is enhanced by the combination of copper- and chitosan-tolerant *Trichoderma* isolate and additional antifungal agents, namely chitosan and copper. The treatment of grapevine pruning wounds with a combination of tolerant *Trichoderma* and chitosan resulted in a significant reduction of the pathogen. An antifungal effect of chitosan oligosaccharides against *P. chlamydospora* was also demonstrated by Cobos et al. (2015) under field conditions. The mechanism underlying this effect involves the ability of chitosan to increase fungal cell wall permeability and destabilize the plasma membrane, resulting in an inhibitory effect on fungal growth, since no chitosan resistance is present (Lopez-Moya et al., 2019). It is important to acknowledge that the efficacy of chitosan in combating pathogens is contingent upon the formulation of the active ingredient (Kauss et al., 1989; Vander et al., 1998). The efficacy of biological control agents is contingent upon the isolate utilized, the composition of the active ingredient, and the application technique.

The addition of a reduced copper rate further enhanced the effect against *P. chlamydospora*. Di Marco et al. (2011) demonstrated the efficacy of an experimental formulation, composed of copper oxychloride and gluconate, in reducing the incidence of leaf stripe symptoms in the vineyard. They also exhibited the ability to inhibit the germination and mycelial growth of *P. chlamydospora* by copper formulation. The results obtained in this study demonstrated that the lowest *P. chlamydospora* severity could be achieved by adding a reduced amount of copper. Since a copper-tolerant *Trichoderma* isolate was utilized in the tested complex, it can be hypothesized that tolerant *T. koningiopsis* has a growth advantage in a copper-containing environment compared to other fungal organisms that lack this tolerance. This leads to an enhanced initial development and an increased capacity to combat pathogens. However, this finding also underscores the critical importance of selecting an optimal *Trichoderma* isolate, as it directly influences the efficacy of the plant protection tool. The findings of the conducted work demonstrate that, particularly under field conditions, a combination of suitable biocontrol agents can enhance the efficacy against Esca pathogens.

6.6 Synergistic plant protection tool with tolerant *T. koningiopsis*, chitosan and reduced copper: a multi-target approach against downy mildew and Esca in viticulture

The investigations conducted in chapters 2 to 5 of this thesis indicate that the integration of a copper- and chitosan-tolerant *T. koningiopsis* isolate, a compatible chitosan, and a reduced rate of a suitable copper agent constitutes a sustainable plant protection strategy against downy mildew (*P. viticola*) as well as for the control of Esca pathogens, particularly in organic viticulture, which synergistically addresses several current challenges facing the sector.

In order to establish an environmentally friendly plant protection strategy for copper reduction with *Trichoderma* against downy mildew on grapevines, it is essential to employ *Trichoderma* agents that have adapted to the grapevine ecosystem and exhibit increased copper tolerance. The efficacy of this tool depends on the compatibility of the biological agent, *Trichoderma*, with a suitable copper fungicide. Therefore, *Trichoderma* isolates from the grapevine were examined for compatibility with common copper preparations, and the copper tolerance of suitable candidates was enhanced. The demonstrated high compatibility of *Trichoderma* with the copper active ingredient as well as *Trichoderma*'s copper tolerance and its associated high detoxification capacity of the heavy metal, contribute substantially to the stability of the effectiveness of the BCA. Consequently, these factors also enhance the overall effectiveness of the desired plant protection tool. This contributes to the development of an effective plant protection tool that can replace the effectiveness of the copper agent, which will be reduced.

In a further step, the agent chitosan was incorporated into the tool to provide an additional control pillar. Therefore, the compatibility of *Trichoderma* spp., chitosan, and copper was examined and optimal combinations of copper- and chitosan-tolerant *Trichoderma* isolates with compatible chitosans against *P. viticola* were identified. Similar to copper, the biological agent *Trichoderma* must exhibit a high level of tolerance to chitosan in order to be optimally effective in combination with the other agents. For the combination of two antifungal agents to be effective against pathogens, the fungus must show a high level of fitness and a pronounced detoxification capacity. Therefore, the compatibility of the components and an understanding of the tool are essential prerequisites for the effective control of pathogens with simultaneous copper reduction, particularly in organic viticulture.

A comprehensive understanding of the synergistic effects of all agents is essential to expand the knowledge base on the complex mechanisms of action. This, in turn, facilitates system optimization. In this context, the effect and mode of action of *Trichoderma* and chitosan against pathogens were examined and described in more detail, demonstrating direct and indirect effects against the pathogen. As demonstrated in this work, the induction of indirect defense mechanisms by the plant protection tool ensures the precise activation of the plant's own immune defense. In addition to the tool's direct defense effect, tolerant *T. koningiopsis* and chitosan can trigger another target, providing an additional pillar of support for effective pathogen defense. This makes the tool's protection more comprehensive and eco-friendlier, which can help reduce the need for high-risk plant protection products. Furthermore, the work shows that, under suitable conditions, the strategic selection and combining of coordinated agents can lead to a significant increase in the antifungal biocontrol and biostimulating properties of the agents, even with a minimal amount of copper. This is of crucial importance for achieving the objectives of the tool. The distinct modes of action exhibited by the agents – namely, a direct biocontrol effect and an indirect inducing effect – facilitate the synergistic action of the compatible combination of the copper- and chitosan-tolerant *T. koningiopsis* isolate and chitosan. This allows the tool to act on multiple targets, which stabilizes its overall effect. Furthermore, the efficacy of this effect can be enhanced by the presence of copper, thereby ensuring a robust antifungal outcome, even under field conditions (Pertot et al., 2017b). Since reducing copper rate was a primary goal of this work, this multiple-target approach enables to pursue the concurrent goals of stable and effective pathogen control using *Trichoderma* and chitosan, as well as reducing fungicide use, without compromising the agents' effectiveness.

To develop an effective plant protection strategy, the synergistic complex was also investigated for its effect against wood-destroying Esca pathogens, demonstrating biocontrol effects against relevant Esca pathogens. A plant protection tool capable of influencing various habitats of pathogens enables the control of multiple infection pathways.

Trichoderma's ability to persist in both green and woody plant components enables the fungus to combat primary leaf diseases, such as downy mildew on grapevines, as well as wood diseases, including Esca. Since the *Trichoderma* isolates used in this work were initially obtained from grapevine wood, which corresponds to their natural habitat, there is a high probability that they will establish themselves again in the grapevine wood, depending on the optimal growth conditions of the selected isolate. The prolonged existence of the organism within the plant may contribute to a long-term protective effect in the trunk of the perennial grapevine. Treating the leaves and wood of the plant with the agents contained in the tool could strengthen the grapevine's defense through a cumulative effect. This could strengthen the plant's overall health and the defense against chronic wood diseases and acute leaf infections.

7 Conclusion and outlook

This work demonstrates a sustainable plant protection tool consisting of *Trichoderma koningiopsis* and chitosan, which shows promising potential for controlling relevant grapevine diseases and thus opens up the possibility of significantly reducing the use ecotoxic plant protection products such as copper preparations. The control strategy developed here is aimed at combating pathogens that differ significantly in ecological terms: *Plasmopara viticola* as a foliar-transmitted, obligate biotrophic pathogen, and relevant Esca-associated pathogens (*Phaeomoniella chlamydospora*, *Fomitiporia mediterranea* and *Phaeoacremonium minimum*), which cause wood-decay and latent diseases. The eco-friendly plant protection strategy addresses two distinct infection environments: leaf tissue and woody tissue. This underscores its potential for broad effectiveness and strategic relevance in plant protection.

A key aspect of the plant protection tool developed are the high tolerance properties of the selected *T. koningiopsis* isolate. *T. koningiopsis* has demonstrated a high degree of compatibility with commercially available copper-based formulations, which play a central role in organic viticulture. The enhanced copper tolerance exhibited by the selected *Trichoderma* isolate offers a distinct advantage for its application in the management of downy mildew on grapevines, particularly in conjunction with other strategies or as a supplementary measure. The studies also confirmed the tolerance of *T. koningiopsis* to chitosan, which forms the basis for the development of a synergistic approach.

The tested combination strategy of this work had an effect on multiple levels: A direct, synergistic control effect against downy mildew on grapevines and Esca pathogens was demonstrated. Furthermore, the plant protection tool enabled the induction of defense mechanisms against *P. viticola* in the grapevine (*Pr10* expression, reactive oxygen species accumulation). This multifaceted approach is of particular importance because it enhances the resilience and reliability of the tool in variable conditions, thereby mitigating the risk of pathogen resistance emergence. Furthermore, it establishes a broad spectrum of potential applications in conjunction with other measures. A particularly notable aspect of this approach is its capacity to substantially reduce the reliance on copper-based plant protection products without compromising the efficacy of the treatment. Consequently, the tool makes a substantial contribution to sustainable plant protection, particularly in the context of organic viticulture.

The results obtained provide a wide range of prospects for further research and development work as well as practical application. Subsequent basic research will concentrate on further elucidating the mechanisms of action – both the direct antagonistic effect and the plant defense induced by *T. koningiopsis* and chitosan. For instance, the role of plant defense mechanisms against Esca pathogens, which may be induced by *Trichoderma* and chitosan, has not been sufficiently investigated. The capacity of these agents to function as classic biostimulants, thereby enhancing grapevine vitality (e.g., nutrient efficiency, growth promotion, etc.), remains unaddressed in the present work. The strategic implementation of multifunctional biocontrol strategies, which simultaneously inhibit pathogens and strengthen plants, demonstrates considerable promise for the development of sustainable plant protection systems. Furthermore, subsequent field testing in vineyards is essential for evaluating the efficacy of the tool, particularly in the context of *P. viticola*. In order to achieve reliable results, it is necessary to conduct field trials under realistic conditions and with sufficiently high infection pressure. Integration of the tool into current viticultural practices would seem to be a rational subsequent step, contingent upon the successful outcome of preliminary testing. In this regard, it is crucial to consider the simultaneous utilization of sulfur preparations or other measures to ensure a practical application approach.

In a subsequent step, the transformation of the tool into a stable and application-ready formulation poses a challenge that needs to be addressed. The formulation of the product must meet several criteria for successful approval and market launch, including effectiveness, stability, storability, and facile application. In this context, it is necessary to consider the technological, regulatory, and economic aspects equally. Furthermore, potential side effects on non-target organisms (e.g., beneficial organisms in viticulture, pollinating insects) must be systematically investigated. The utilization of the tool in an ecologically sustainable manner is contingent upon the ability to guarantee high selectivity against pathogens while ensuring the protection of the ecosystem.

A further aspect that has received insufficient consideration to date is the potential influence of the application on the sensory quality of the wine. It is possible that both *Trichoderma* and chitosan, either directly or via plant reactions, influence secondary metabolic processes in the grapevine, which have an effect on the aroma, taste, or stability of the wine. Consequently, future research efforts should include the implementation of targeted sensory tests and the analysis of grape and wine quality.

Taking the aforementioned approaches into account, the synergistic plant protection tool developed in this work could, in the long term, become an integrated component of a sustainable grapevine protection system that is highly environmentally friendly, reduces dependence on copper formulations, and has the potential to effectively address complex grapevine diseases through multimodal mechanisms of action. The combination of a broad spectrum of activity and high compatibility contributes to the innovative potential of this tool in modern plant protection.

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