Endophytic establishment of *Beauveria bassiana* in grapevine plants as a sustainable pest management strategy

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Gartenbauwissenschaften

(Dr. rer. hort.)

genehmigte Dissertation

von

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2019

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Tag der Promotion:	30. August 2019

Abstract

Fungal entomopathogens like *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) are known as antagonists of insects with multiple functional and ecological roles and have attracted increased attention as biocontrol agents in integrated pest management programs. Besides its entomopathogenic habit, evidence has accumulated that the fungus can also establish as an endophyte in a wide array of plant species. However, only limited information is currently available on the endophytic colonization of grapevine, *Vitis vinifera* (L.), plants with *B. bassiana.* In addition, the functional role of the fungus *in planta,* and/or the plant's response to colonization by *B. bassiana* as well as the mechanisms underlying these responses and putative protection effects, still require elucidation. Therefore, the objectives of this thesis were to investigate whether the fungus *B. bassiana* is able to colonize grapevine plants, still maintains its entomopathogenic potential against insect pests, and can provide additional protection against plant fungal pathogens or limit their damaging effects. The investigation focused on the interaction between *B. bassiana,* grapevine plants, and potential target insect pests as well as fungal pathogens to gain more knowledge on this particular tritrophic interaction with regard to potential biological control strategies.

In the present thesis, greenhouse and field experiments were conducted to optimize endophytic establishment of the entomopathogenic fungus *B. bassiana* in potted and mature grapevine plants. Two different commercialized *B. bassiana* strains (ATCC 74040/product Naturalis® and GHA) were applied on the leaf surfaces of grapevine plants. To determine if endophytic colonization of grapevine leaves by *B. bassiana* was successful, a culture dependent approach was used and the assessment was verified by the amplification of strain-specific microsatellite markers. Endophytic survival of *B. bassiana* inside leaf tissues was evident for at least 21 days after inoculation in potted grapevine plants and up to five weeks after the last application in mature grapevine plants in the vineyard. The antagonistic activity of endophytic *B. bassiana* against putative target pest insects like the vine mealybug *Planococcus ficus* was assessed in a bioassay using surface sterilized leaves. Infestation rate and growth of *P. ficus* were significantly reduced. Possible effects of endophytic *B. bassiana* on the host choice preference of adult black vine weevil *Otiorhynchus sulcatus* choosing between control and *B. bassiana* inoculated plants were examined through choice assays. Adult *O. sulcatus* chose significantly more often the control plants as a host plant compared to grapevine plants treated with Naturalis®, where *B. bassiana* putatively had established as an endophyte. These results suggest that adult black vine weevils are able to detect and subsequently avoid plants treated with *B. bassiana* and indicate a new mode of action of plant-associated entomopathogenic fungi. Furthermore, the protective potential of endophytic *B. bassiana* against grapevine downy mildew *Plasmopara viticola* was investigated in greenhouse experiments. A significant effect on the disease severity and disease incidence of downy mildew on grapevine leaves was observed if plants were treated with *B. bassiana* 3 and 7 days before inoculation with *P. viticola.* To work out fundamental aspects of genes involved in the interaction between grapevine and the endophytic fungus *B. bassiana,* a microarray and an RT-qPCR analysis were performed. The results indicate an up-regulation of diverse defense-related genes in grapevine as a response to the endophytic establishment of *B. bassiana.*

In conclusion, the results of this thesis indicate that endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants can represent an alternative and sustainable plant protection strategy, with the potential for reducing pesticide applications in viticulture.

Keywords: *Beauveria bassiana*, grapevine, endophyte, biological control

Zusammenfassung

Im integrierten und ökologischen Pflanzenschutz stellen entomopathogene Pilze bei der Bekämpfung verschiedener Arthropoden eine gute Alternative zu chemischen Pflanzenschutzmitteln dar. Dieses Potential wird allerdings bislang noch unzureichend ausgeschöpft. Insbesondere ist über die Fähigkeit dieser Pilze, sich endophytisch in Pflanzen zu etablieren nur wenig bekannt. Durch eine endophytische Etablierung könnten entomopathogene Pilze wie *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) zum einen eine Infektionsquelle für Schädlinge darstellen oder zum anderen über Mechanismen der induzierten Resistenz Abwehrrektionen gegen Schaderreger in der Pflanze aktivieren. Ein verbessertes Wissen über diese Interaktionen könnte eine vermehrte und effizientere Nutzung entomopathogener Pilze in biologischen Pflanzenschutzstrategien unterstützen.

Im Rahmen der vorliegenden Dissertation wurde ein Verfahren für die endophytische Etablierung des entomopathogenen Pilzes *B. bassiana* in Reben *Vitis vinifera* (L.) entwickelt. Dazu wurden zwei Stämme des Pilzes (ATCC 74040/Präparat Naturalis® und GHA) verwendet. Die Behandlung von Topfreben im Gewächshaus und von ausgewachsenen Reben im Weinberg erfolgte mittels Sprühapplikation auf die Blattober- sowie Blattunterseiten. Der Nachweis einer endophytischen Besiedelung der Blätter wurde durch Blattscheibentests auf Selektivmedium mit anschließender Verifizierung durch Amplifikation mittels stamm-spezifischer Mikrosatelliten erbracht. Es konnte gezeigt werden, dass sich *B. bassiana* in Topfreben über einen Zeitraum von mindestens drei Wochen endophytisch etablieren konnte und auch in Weinbergsreben fünf Wochen nach der letzten Applikation nachweisbar war. Das antagonistische Potential von endophytisch etabliertem *B. bassiana* gegenüber Schmierläusen (*Planococccus ficus*) wurde unter Verwendung von oberflächensterilisierten Blättern von behandelten Topfreben in einem Bioassay bewertet. Der endophytische *B. bassiana* hatte einen signifikanten Einfluss auf die Mortalität und das Wachstum von *P. ficus* in der ersten Woche nach der anfänglichen Festsetzungsphase. Mögliche Auswirkungen des endophytischen *B. bassiana* auf die Wirtspflanzenwahl von adulten Rüsselkäfern *Otiorhynchus sulcatus* wurden durch Olfaktometer-Tests mit Kontrollpflanzen und mit *B. bassiana* inokulierten Pflanzen untersucht. Adulte *O. sulcatus* wählten signifikant häufiger Kontrollpflanzen als Wirtspflanze verglichen mit Naturalis® behandelte Reben, bei denen sich *B. bassiana* mutmaßlich als Endophyt etabliert hatte. Diese Ergebnisse legen nahe, dass der Gefurchte Dickmaulrüssler in der Lage ist, mit *B. bassiana* behandelte Pflanzen zu erkennen und aufgrund dessen zu meiden und deuten auf einen neuen Wirkmechanismus pflanzenassoziierter entomopathogener Pilze hin. Zusätzlich wurde das protektive Potential von *B. bassiana* gegenüber dem Erreger des Falschen Rebenmehltaus *Plasmopara viticola* an Topfreben untersucht. Bei einer protektiven Behandlung von Reben mit *B. bassiana* 3 und 7 Tage vor einer Inokulation mit *P. viticola* konnte eine signifikante Reduktion der Befallsstärke und -häufigkeit von Falschem Mehltau an Topfreben beobachtet werden. Um grundlegende Aspekte der Wechselwirkung zwischen Weinrebe und endophytischem *B. bassiana* auf Genebene aufzudecken, wurden ein Microarray und eine RT-qPCR-Analyse durchgeführt. Die Ergebnisse zeigen nach der Behandlung mit *B. bassiana* eine erhöhte Expression verschiedener Gene der Weinrebe, welche in Zusammenhang mit der Abwehrreaktion von Pflanzen stehen.

Zusammenfassend lässt sich festhalten, dass die endophytische Etablierung eines entomopathogenen Pilzes wie *B. bassiana* in Weinreben eine alternative und nachhaltige Pflanzenschutzstrategie darstellen kann, mit dem Potenzial, den synthetisch-chemischen Wirkstoffeinsatz im Weinbau zu reduzieren.

Schlüsselwörter: *Beauveria bassiana*, Weinrebe, Endophyt, biologischer Pflanzenschutz

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List of Abbreviations

ANOVA	analysis of variance
ATCC	American Type Culture Collection
BSM	Beauveria selective medium
CI	confidence intervall
CNRQs	calibrated normalized relative quantities
cv	cultivar
DAI	days after inoculation
DBU	Deutsche Bundesstiftung Umwelt
DNA	deoxyribonucleic acid
EC	European Commission
EPF	entomopathogenic fungi
ET	ethylene
EtOH	ethanol
EU	European Union
GEP	good experimental practice
GLRaV	grapevine leafroll associated virus
hpt	hours post treatment
HR	hypersensitive response
ISR	induced systemic resistance
JA	jasmonic acid
MAMPs	microbe-associated molecular patterns
NaOCl	sodium hypochlorite or active chlorine
OD	oil dispersion
OIV	International Organisation of Vine and Wine
P bzw p	propability
PDA	potato dextrose agar
PGPR	plant growth promoting rhizobacteria
PR	pathogenesis-related
RH	relative humidity
(RT-q)PCR	(quantitative reverse transcription) polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SD/SE	standard deviation/error
SSR	simple sequence repeats
UV	ultra violet
VOCs	volatile organic compounds

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1 General Introduction

1.1 Plant protection in viticulture

Grapevine (*Vitis vinifera* L.) is an economically important fruit crop, which is mostly cultivated in the temperate climatic belt (between 40°N and 50°N and between 30°S and 40°S) with the area dedicated to viticulture exceeding 7.5 million ha worldwide (OIV 2017). However, most cultivars of *V. vinifera* commonly used are highly susceptible to a considerable number of pests and pathogens, which has a significant effect on both yield and quality of the must and wine (Flaherty 1992). The application of chemical control agents is still the most effective and predominantly used method to control these pests and pathogens. Accordingly, viticulture is considered to be very input intense, both in terms of frequency and intensity of pesticide (herbicides, fungicides, and insecticides) applications during the growing season (Roßberg 2007). Indeed, a report on the use of plant protection products in the European Union over the period 1992–2003 (EUROSTAT EC 2007) indicated that on average 71% of all fungicides applied to crops in the EU were applied to grapevines in European vineyards while viticulture only accounted for 4.6% of the cultivated area in that period. Since fungal and oomycete infections are one of the primary reasons for losses in grape quality and yield, most pesticides applied in viticulture are fungicides, with an average of 12-15, in some years up to 25-30 applications in the most problematic conditions (Pertot et al. 2017). They are predominantly used to control downy mildew (causal agent: *Plasmopara viticola* (Berk. and Curt) Berl. and de Toni), powdery mildew (causal agent: *Erysiphe necator* Schw., formerly *Uncinula necator* (Schw.) Burr.) and grey mould (causal agent: *Botrytis cinerea* Pers.: Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel)). However, the inadequate use of pesticides in viticulture can cause increased concentrations of their residues in vineyard soils, other environmental compartments and the wine (Romić et al. 2014; Hildebrandt et al. 2008; Cabras and Angioni 2000), raising public concerns (Jacobson et al. 2005). Furthermore, especially in organic viticulture, copper is the most widely used fungicide, because of its natural origin and wide-spectrum activity (Dagostin et al. 2011; Gessler et al. 2011). The long-term use of copper-containing fungicides in vineyards resulted in their persistence and accumulation in the soil, with putative detrimental effects on soil microorganisms or microbial activity (Komárek et al. 2010; Jacobson et al. 2005). Therefore, one of the major goals of sustainable viticulture is the reduction of pesticide and copper input in vineyards.

Although grapevines are hosts of various arthropod pests, pesticide use against them is usually low to moderate with one to four insecticide applications on average per year (Pertot et al. 2017). Damage by insect pests occurs at different parts of the plant such as roots, buds,

berries or leaves and is caused either directly due to feeding activities or indirect via the transmission of pathogens such as bacteria or viruses. Pests that threaten grapevine include phytophagous mites, leafhoppers, piercing-sucking insects and leaf-eating or cluster- feeding Lepidoptera.

Current predictions on the possible effects of climate change on disease and pest pressure suggest that even more pesticide applications will be necessary in the future (Salinari et al. 2006; Caffarra et al. 2012; Reineke and Thiéry 2016). Recent findings suggest that the impact of some pest insects will increase with increasing temperatures, but the implications of climate change on plant diseases and arthropod development in global viticulture seem to be more complex than expected (Caffarra et al. 2012; Reineke and Thiéry 2016; Gregory et al. 2009). They are either affected directly through impacts on their life history and epidemiology or indirectly by changes of grapevine physiology and phenology. Even if precise predictions are not yet possible, imaginable changes include a) an increase of incidence of pests and diseases in viticulture; b) a shift in species causing problematic situations; c) a change in pests and diseases biological cycles that will make their control more difficult; d) and increased difficulty in forecasting due to extreme variation in climatic conditions and, consequently, in the vine growth and in pests and diseases development.

Although viticulture has been pioneering in terms of the adoption of several alternatives to synthetic chemical pesticides, it is still regularly depending on multiple applications of synthetic pesticides for pest and disease management. Therefore, grape growers face increasing pressure by politicians, retailers, and consumers to reduce their reliance on conventional chemical pesticides (Jacobson et al. 2005; Komárek et al. 2010). Hence, there is an increasing interest to identify alternative treatments and more sustainable methods of pest management (Dagostin et al. 2011; Gessler et al. 2011).

1.2 Biological control of pests and plant diseases

1.2.1 Microbial control agents

The use of microorganisms for biological control of plant pests and diseases is a promising alternative to the use of chemical pesticides. Microbial biological control agents consist of bacteria, fungi, or viruses (and sometimes include the metabolites that bacteria or fungi produce as well) and are used as active substances to control different kinds of crop pests (Montessinos and Bonaterra 2009). Microbial pesticides are often considered to have a low risk to the environment and generate little or no toxic residues when compared to chemical pesticides. They can also have a high level of selectivity as well as lower production costs compared to conventional pesticides. Due to these positive characteristics, biological control is

currently receiving a lot of attention and support by politicians, policy makers, retailers, consumers, growers, and grower organizations (van Lenteren et al. 2018). With its Sustainable Use of Pesticides Directive, the European Union (EU) has been recommending the use of biological control since 2009 (EC 2009). Worldwide, biological control is currently applied on more than 30 million ha. However, the global market of biological control agents represents merely less than 2% of the pesticide market (van Lenteren et al. 2018). Although various studies showed promising results about the use of microbial agents, only rather few antagonistic microorganisms were registered as biological control agents (Fravel 2005). The most limiting factor is their inconsistent efficiency, which was particularly observed in studies done under field conditions (Alabouvette et al. 2006; Fravel 2005; Butt and Copping 2000; Lacey et al. 2015). Biotic and abiotic factors in the environment can greatly influence and alter the growth, survival, and pathogenicity of microorganism and thus, bring variability and uncertainty in their activity and efficiency as biological control agents.

Microbial biological control agents can be characterized by their modes of action or the mechanisms underlying their protection. Potential modes of action, which might be involved in the control of plant diseases, range from antibiosis, mycoparasitism, and competition to induced resistance (Alabouvette et al. 2006; Jaber and Ownley 2018; Pal and McSpadden Gardener 2006).

Antibiosis is a form of direct interaction resulting from the production of secondary metabolites by one microorganism, which inhibits other microorganisms. Secondary metabolites involved in the mechanism are reported to be antibiotics, bioactive volatile organic compounds (VOCs), or enzymes (Ownley et al. 2010). Antibiosis is a well-described phenomenon responsible for the activity of a range of biological control agents (Alabouvette et al. 2006). Mycoparasitism represents another mechanism of direct antagonism. It involves specific recognition between the antagonist and its target pathogen. Due to the production of lytic enzymes, which break down cell wall components, the parasite can penetrate the cell wall and enter the hyphae of the pathogen. Enzymes involved in mycoparasitism of plant pathogens are distinctly different from those involved in antibiosis. A prerequisite for the third direct interaction, competition for space and nutrition between an antagonist and a pathogen, is that both share the same ecological niche while the resources are limited. A more rapid colonization of plant tissues by the antagonist will reduce the amount of available nutrients as well as the available space for the pathogen, resulting in reduced spore germination and reduced growth of the pathogen. Therefore, successful competition is often a matter of timing.

An indirect mechanism of biological control is the activation of the plant's inherent defense system, known as induced resistance, whereby the biocontrol agent and the phytopathogen do

not have direct physical contact with one another. Induced resistance can be either local (hypersensitive response, HR) or systemic throughout the plant and is defined as the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (Kloepper et al. 1992). It can be activated by microbial pathogens or insect herbivores, but also by beneficial microbes, abiotic stresses or chemical applications. Forms of induced resistance that have so far been described are systemic acquired resistance (SAR), induced systemic resistance (ISR) and herbivore-induced direct defense and indirect defense (Van Loon et al. 1998; Pieterse and Dicke 2007).

SAR is a form of induced resistance that is activated throughout a plant typically following infection by a pathogen that causes localized necrotic lesions induced by a pathogenic disease or as a result of a hypersensitive response (HR) (Ryals et al. 1996). This causes a local accumulation of salicylic acid (SA) that stimulates a signal to the rest of the plant, and the plant becomes resistant to pathogens in areas distant from the original infection. Therefore, SAR often involves the signal molecule SA and is accompanied by the accumulation of genes encoding pathogenesis-related (PR) proteins and their protein products (Durrant and Dong 2004). These PR proteins include, amongst others, the antifungal chitinases, β-(1,3)-glucanases, peroxidases, as well as PR-1 and PR-5 proteins that have anti-oomycete activity (Verberne et al. 2000; Van Loon et al. 2006). The development of SAR takes several days, but it is persistent for weeks to months and protects the plant against secondary infections by a broad spectrum of microorganisms including bacteria, true fungi, oomycetes, and viruses.

ISR develops in response to the interaction with certain plant growth promoting rhizobacteria (PGPR) that do not induce a necrotic response or cause visible damage. The term was coined by Kloepper et al. (1992) to distinguish resistance induced by PGPR from SAR, which has different underlying mechanisms. Unlike SAR, ISR does not result in the systemic expression of PR genes, but its induction is dependent on signaling pathways of the plant hormones ethylene (ET) and jasmonic acid (JA) (Van Loon et al. 1998). Although SAR and ISR work through different pathways, they can act antagonistically, complementary, or synergistically depending on the intensity and duration of the signals provided to the host plant (Mur et al. 2006).

Many microbial biological control agents do not exclusively feature one single mode of action. Strong evidence suggests that a combination of these mechanisms is involved in biological control of pests and pathogens (Ownley et al. 2010; Elad 2000; Hubbard et al. 2014).

1.2.2 Entomopathogens

The term "entomopathogenic fungi" (EPF) refers to a polyphyletic group of fungi, which are natural pathogens of a wide variety of insects and other arthropods (Hegedus and

Khachatourians 1995). Since EPF are found ubiquitously in the soil throughout the world and are effective against a wide variety of insect pests, they have attracted increased attention as environmentally friendly biological control agents (Hajek 1994). According to Keller (2008), with approximately 800 fungal taxa, only a fraction of the existing entomopathogenic fungi are described so far. The best-known among them were assigned to the anamorphic fungi (Deuteromycota), the Clavicipetales (Ascomycetes), or the Entomophthorales (Zygomycota) by the long-term valid taxonomic nomenclature (Keller 2008).

The infection of the host by EPF takes place mainly via the cuticle, unlike to most other insect-pathogenic organisms. Accordingly, for a successful infection, only the contact of the pathogen with the insect is necessary and therefore, consumption by feeding is not obligatory. The basic steps of the infection process, illustrated in Figure 1, are summarized in four steps by Schmutterer und Huber (2005):

- adhesion of the fungal spores and subsequent germination on the insect’s cuticle
- mechanical and enzymatical penetration of the cuticle layers
- interaction with the host's immune system, colonization of the hemolymph and destruction of the host due to several factors (release of fungal toxins, invasion of organs, water and nutrients depletion, and physical obstruction)
- saprophytic re-emergence of the fungus from the host with the characteristic outgrowth of fungal mycelia on the cadaver followed by sporulation

A more detailed version is given by Hegedus and Khachatourians (1995) and a comprehensive description of the infection steps can be found in Mora et al. (2017). Hence, entomopathogenic fungi also have a saprophytic phase in addition to the pathogenic one. Both phases are influenced by different abiotic and biotic factors such as temperature, relative humidity, or UV-portion of the solar radiation as well as other microorganisms present in the soil or inside or outside of the host insect (Schmutterer and Huber 2005; Wraight et al. 2007; Vega 2018). They are also dependent on the pathogenicity and virulence of the fungal isolate. In this context, the pathogenicity describes the basic ability of the pathogen to infect a host and cause disease symptoms, whereas the virulence defines the magnitude of this ability. Both parameters are in turn influenced by the genetic constitution of the fungus as well as the physiology and the developmental stage of the host.

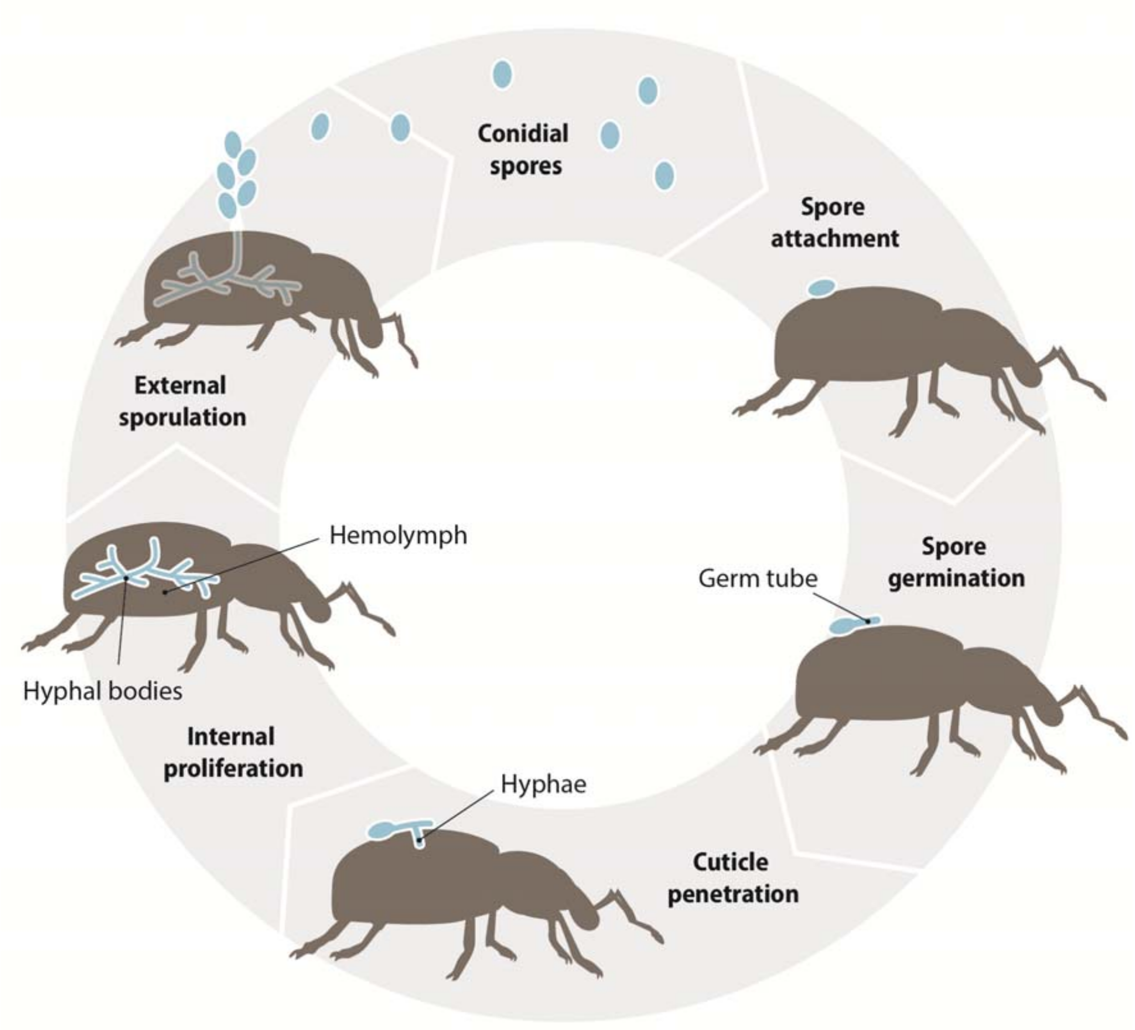


Figure 1: Illustration of how entomopathogenic fungi (on the example of *B. bassiana*) infect arthropod hosts by spores, proliferate, and disperse. A conidium adheres to the insect host to induce germination (step 1). This is followed by germination and production of a germ tube (step 2). Mechanical pressure and secretion of enzymes are employed to breach the cuticle (step 3). The fungus colonizes the host hemocoel through hyphal growth or blastospores, where it feeds on sugars in the hemolymph (step 4). The secretion of toxins facilitates the death of the host. After the host has died, the fungus breaches the cuticle again from the inside and sporulates on the cadaver (step 5).

A prerequisite for the use of EPF as a biological insecticide (mycoinsecticide) is that highly virulent strains of the fungus are available through sufficient selection. Also, mass-production methods and application techniques must have been developed and reviewed for product registration. Schmutterer and Huber (2005) explained that resistance of host insects to EPF is improbable to develop due to the multifactorial virulence, but it cannot be excluded with absolute certainty (Shelton et al. 2007). It is also possible to combine EPF with chemical insecticides and, in some cases, this can even lead to increased efficacy of entomopathogenic fungi (Butt and Ansari 2011; Lacey et al. 2015). The combination with chemical fungicides, however, can lead to a reduction in efficacy. Shah and Pell (2003) explained that

entomopathogenic fungi are best used when total elimination of a pest is not required, but instead, insect populations should stay below an economic threshold.

In recent years, various biological insecticides have been developed, which are mainly based on various species of the entomopathogenic fungi *Beauveria*, *Metarhizium*, *Lecanicillium,* and *Isaria*. Despite the commercial availability of approximately 150 mycoinsecticides (Jaronski 2010) and the recent growth of biological control programs (van Lenteren et al. 2018), their potential application as biological control of insect pests is, according to Zimmermann (2007), still not fully exploited. Reasons for this limited use are costs, product quality, efficacy, and the handling of the products as well as aspects of regulatory restrictions on the environmental impact such as effects on non-target organisms (Jackson et al. 2010; Jaronski 2010). However, Roy (2010) emphasizes that limited success is also caused by a lack of some basic understanding of the ecology and evolution of entomopathogens. Within the terrestrial ecosystem, EPF have diverse functions, but their different roles have rarely been studied (Vega et al. 2009). However, studying their ecology is a prerequisite for developing efficient plant protection strategies basing on them (Vega et al. 2009).

1.3 *Beauveria bassiana*

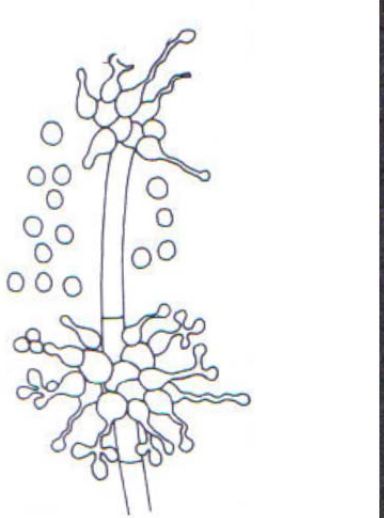
1.3.1 Taxonomy and morphology

Beauveria bassiana (Balsamo-Crivelli), Vuillemin (Ascomycota: Hypocreales) is a well-known and worldwide distributed entomopathogenic fungus, which normally occurs in the soil. The fungus was first described in 1835 by the Italian scientist Agostino Bassi as a cause of the "White Muscardine" in silkworms and received different taxonomic names before Vuillemin (1912) named the genus with *Beauveria* as an independent genus.

Taxonomically, the fungus was assigned to the Deuteromycota (Fungi imperfecti) due to its anamorphic developmental cycle. Deuteromycetes classified fungi, which have only asexual reproduction or whose sexual propagation stages are not known so far. According to Rehner (2005), this classification is no longer common, and most Deuteromycetes can be classified between their sexual relatives with the help of new molecular biology methods. *B. bassiana* is presently assigned to the Cordycipitaceae family in the order of Hypocreales and therefore belongs to the Ascomycota. The telomeric form of *B. bassiana*, *Cordyceps bassiana*, has only been discovered and proved in Asia (Li et al. 2001).

The Ascomycetes are dominated by asexual propagation, which is mainly responsible for the rapid spreading of the fungus (Raven et al. 2006). The dissemination structures used are mononuclear conidiospores, called conidia, which are usually formed by special conidiogenic cells (Figure 2). These usually sit at the tip of specialized hyphae, the conidiophores. The dense

clusters of short-globose to flask-shaped conidiogenic cells result in the characteristic white, woolly colonies of *B. bassiana* (Figure 3). The hyaline (colorless) conidia themselves are single cellular, haploid, and hydrophobic and sit on a zigzag-shaped rhachis (Rehner 2005). Although the conidiophores tend to become finer and less dense after longer periods of artificial culture, the fungus is morphologically relatively easy to determine. Rehner (2005) explains that a morphological differentiation of the individual species within the genus *Beauveria*, however, is only possible based on the exact shape and size of the conidia. Since these characteristics vary according to the culture conditions, a routine determination of the species is problematic and questionable. Therefore, molecular methods to identify the species are essential. With microsatellite markers, even a strain-specific determination (Rehner and Buckley 2003) and detection (Reineke et al. 2014) of *B. bassiana* in different habitats are possible.



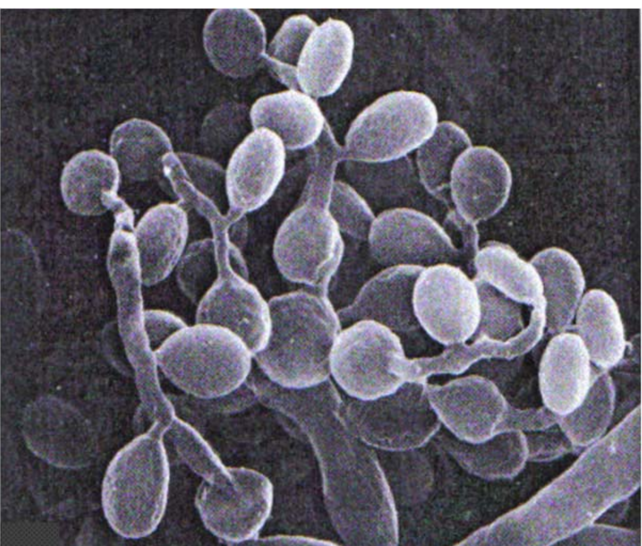


Figure 2: Left: Conidiophores with conidiogenic cells of *B. bassiana*, Right: Picture of the conidiospores of *B. bassiana* by electron microscopy (x 4000) (pictures taken from Domsch et al. (1980))

Figure 3: Characteristic white, woolly colonies of *B. bassiana* on a solid culture medium (photo courtesy of Winfried Schönbach)

1.3.2 *Beauveria bassiana* as an entomopathogen

Beauveria bassiana has a broad host range and infects Coleoptera, Lepidoptera, Diptera, and other insect orders in both temperate and tropical climates (Domsch et al. 1980). As an entomopathogenic fungus, it is capable of entering the insect directly through the cuticle instead of depending on being eaten by the insect or on the opportunity to enter through a natural opening like other entomopathogens (e.g., nematodes or viruses). The spores can be transported by wind or just be picked up by the insect as it moves through its environment.

B. bassiana has a dimorphic mode of growth and passes through an asexual vegetative life cycle in the absence of an insect host. On contact with a susceptible host, *Beauveria* switches to the pathogenic life cycle. After germination the hyphal tube uses mechanical pressure by specialized physical structures, such as appressoria, to penetrate the insect host cuticle directly. Toxic metabolites are secreted by the fungus, which may assist in the infection process. When having entered the hemocoel, the fungus alters its growth morphology to a yeast-like phase with the production of blastospores and/or hyphal bodies, circulating in the hemolymph and multiplying by budding. The death of the host is caused by the proliferation of the fungus and due to dehydration or depletion of nutrients (Ladurner et al. 2008). During the infection process, toxic secondary metabolites can be secreted by the fungus, that assist in parasitism of insects but are not required (Griffin 2007). Following the death of the host, *B. bassiana* re-emerges from the cadaver and produces new conidia in the form of the characteristic white covering called “white muscardine” (Figure 4).

Figure 4: Left: *B. bassiana* re-emerges from the cadaver and produces the characteristic white covering called “white muscardine”. Right: Endophytic *B. bassiana* re-emerges from leaves discs of grapevine plants (photos courtesy of Winfried Schönbach)

1.3.3 Plant colonization by *Beauveria bassiana*

Besides being important natural enemies of many insects, recent studies reported various additional roles of EPF in nature, including endophytism and rhizosphere colonization as well as plant disease antagonism or plant growth promotion (Vega et al. 2009). The importance and

complexity of these ecological roles are not yet fully understood but can provide opportunities for the use as alternative pest management strategies (Jaber and Enkerli 2017). In the present thesis, the role as endophyte will be subject of closer examination.

The term endophyte was first introduced by the German scientist Anton de Bary (1884). Many other definitions have been used ever since, changing in accordance with the increased understanding of endophytic lifestyle (Wilson 1995). While initially, the term refers to all microorganisms living inside plants, it was subsequently restricted to organisms living asymptomatic within the plant by Carroll (1986). As further studies revealed that the same organism could switch between different lifestyles, the definition was expanded to the following and still most commonly used one:

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

(Petrini 1991)

So defined, endophytes cover a diverse polyphyletic group of microorganisms that can exhibit more than one type of life stages, including true symbionts as well as latent pathogens (Arnold and Lewis 2005).

Fungal endophytes are ubiquitous amongst terrestrial and agricultural plants and are reported to protect host plants against pathogens, plant parasitic nematodes or herbivores (Arnold and Lewis 2005; Vidal and Jaber 2015; West et al. 1988; Schulz and Boyle 2005). Vega et al. (2008) provided an overview of the diversity of fungi traditionally known as insect pathogens, which have been isolated as endophytes. Both, naturally occurring and artificially introduced entomopathogenic fungi are mentioned in the literature, including publications on endophytic colonization by *Beauveria bassiana.*

The first observation of endophytically growing *B. bassiana* was made by Lewis and Cossentine (1986) and Bing and Lewis (1992a) in corn plants *Zea mays* L. (Poaceae). These authors not only proved the endophytic colonization of corn plant tissues by this fungus but also found antagonistic potential against the European corn borer (*Ostrinia nublialis* Hbn.) (Lepidoptera: Pyralidae). Besides higher mortality when feeding on the plants endophytically colonized by this fungus (Bing and Lewis 1993), a season-long suppression of larvae – measured as reduced tunneling by the corn borer – was reported (Lewis and Cossentine 1986). Subsequent work by Lewis and colleagues examined the *in planta* growth of the fungus and is reviewed in Arnold and Lewis (2005). Movement of *B. bassiana* has been detected, but the

mechanism of movement is poorly understood. Wagner and Lewis (2000) observed in their studies on maize plants using light and electron microscopy that *B. bassiana* can colonize the plant systemically. In addition to the observation of hyphal growth between the parenchyma cells in the apoplast, hyphae could also be detected in the xylem vessels of the plants, where they may move passively. Quesada-Moraga et al. (2006) confirmed these observations, as they, too, could not detect any intracellular colonization by *B. bassiana* on opium poppy, but the fungus did indeed spread with hyphae in the xylem.

In addition to corn plants, a wide variety of plants have also been shown to host *B. bassiana* as an endophyte. Table 1 summarizes the literature about colonized plant species, the reported materials including used strain, inoculated and analyzed tissue type as well as (if available) investigations concerning the antagonistic potential against pests and pathogens. In most of the studies published so far on endophytic *B. bassiana* or other entomopathogenic fungi, mycosis of insects has either not been tested or was not observed (see Table 3 in Vidal and Jaber (2015)). The traditional mode of infection by fungal entomopathogens takes place via direct contact with the cuticula. Therefore, Arnold and Lewis (2005) do not regard the hyphal state of endophytes in planta and the consumption of infected plant tissues as a significant source of entomopathogenic infections. In addition, the conidia of *B. bassiana,* which are usually the infective propagule, have not yet been observed inside plant tissues or the vascular system (Vega 2008). However, some investigations suggest that infections of chewing or sucking pest insects by endophytic *B. bassiana* can occur (Gurulingappa et al. 2010; Quesada-Moraga et al. 2009; Reddy et al. 2009). The described lack of mycosis and the lack of conidia inside the plants suggest other modes of action against insects than direct fungal infections. It is speculated that the protective effects are mediated by secondary metabolites, produced by the fungus and causing feeding deterrence or antibiosis (Cherry et al. 2004; Akello et al. 2008b; Vega 2008; Gurulingappa et al. 2010). Despite accumulating evidence on the potential of endophytic *B. bassiana,* the mechanisms underpinning the protective effects remain little understood. The colonization with *B. bassiana* showed to induce proteins related to plant defense and stress response (Gómez-Vidal et al. 2009) suggesting that endophytic colonization by entomopathogenic fungi induces plant defense responses, probably by activating the plant immune system.

In addition to its biocontrol activity against insect pests, there is substantial evidence that endophytic *B. bassiana* may also demonstrate antagonistic activity against plant pathogens and therefore effectively suppresses plant diseases (Goettel et al. 2008; Ownley et al. 2008). Research on the control for plant pathogens by *B. bassiana* as has been mostly limited to *in vitro* studies with an array of soilborne and foliar plant pathogens and were summarized in Table 1 in Ownley et al. (2010). Only a few studies investigated the antagonistic potential by

using soil-borne pathogens and seed treatments in greenhouse trials. Seed treatment with *B. bassiana* strain 11–98 resulted in suppression of damping-off caused by the soil-borne pathogens *Rhizoctonia solani* Kuhn (Basidiomycota: Cantharellales) and *Pythium myriotylum* Drechsler (Oomycota: Pythiales) in tomato (Ownley et al. 2004; Clark et al. 2006) and cotton seedlings (Griffin 2007; Ownley et al. 2008). The treatment of cotton seedlings with the same *B. bassiana* strain has also been reported to reduce the severity of bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* (Xam) (Griffin et al. 2006; Ownley et al. 2008). Following foliar inoculation of plants with *B. bassiana,* a reduced incidence and severity of Zucchini yellow mosaic virus in squash (Jaber and Salem 2014) and downy mildew in grapevines (Jaber 2015) was recently reported. These findings provide promising potential for the multiple uses of fungal entomopathogens as biopesticides against both insects and pests in integrated plant protection strategies when used as endophytes. The precise mechanisms underlying such protection mediated by (endophytic) *B. bassiana* remain at an early stage. It is assumed that the mechanisms of plant disease antagonism involve competition for space, induced systemic resistance, and the production of various secondary metabolites (Ownley et al. 2008; Griffin et al. 2006; Ownley et al. 2010; Vega et al. 2009).

1.3.4 The product Naturalis®

Beauveria bassiana is one of the most frequently commercialized fungal mycoinsecticides (Faria and Wraight 2007). In the present thesis, *B. bassiana* strain ATCC 74040 was also used in the form of the commercial product Naturalis® (CBC (Europe) S.r.l. – BIOGARD Division). Naturalis® is formulated as oil dispersion (OD) and contains approximately 2.3×10^7 colony forming units/ml of *B. bassiana* strain ATCC 74040 as active ingredient. The strain ATCC 74040 has been isolated from the cotton boll weevil *Anthonomus grandis* (Boheman), in the Lower Rio Grande Valley, Texas, USA, and does not produce any toxins (Copping 2004). In 2005, Intrachem Bio International S.A. (Geneva, Switzerland), now CBC Europe S.r.l. (Italien), acquired production and marketing rights for Naturalis® from Troy Biosciences Inc. (Phoenix, USA). Manufacturing takes now place under the control of CBC Europe.

Initially, in Germany, the product was only allowed to be used from the 1st of April 2008 for a period of 120 days in order to combat wireworms on potatoes (including *Limonius* spp. and *Agriotes* spp.) as part of a temporary approval according to former § 11 (2) now Art. 53 PflSchG ("Gefahr im Verzug"). Since January 2009, the strains ATCC 74040 and GHA of the fungus *B. bassiana* have been listed in Annex 2 (positive list for active substances) of EC Regulation No. 1107/2009 (formerly Annex I of EU Directive 91/414) (European Commission 2011). On this basis, national approvals already existed in several EU member states, including Spain, France, Italy, Sweden, and the Netherlands. Furthermore, products containing the same strain are also registered in the USA. Since 2015 the product is registered in Germany, and

other EU member states, e.g. for control of whiteflies in tomato, herbs, and ornamentals in greenhouses.

The identification of an appropriate fungal pathogen or strain for development as mycoinsecticide can be complex and expensive till market launch (Jackson et al. 2010). Therefore the present thesis focused on the already registered and formulated strain ATCC 74040 and the product Naturalis® in order to speed up the implementation in and the development of alternative plant protection strategies in viticulture.

Table 1: Studies reporting natural and artificial establishment of endophytic entomopathogenic *B. bassiana* and effects on herbivorous insect or plant pathogens

Reference	Plant species	Strain	Plants parts treated – sampled	Insect/pathogen observed
Akello et al. (2007), (2009)	Banana *Musa* spp.	G41	Roots, rhizomes –pseudostems	
Akello et al. (2008a), (2008b)	Banana *Musa* spp.	G41	Roots, rhizomes –pseudostems	*Cosmopolites sordidus*
Akello and Sikora (2012)	Bean *Vicia faba*	G1LU3; S4SU1	Seeds – roots	*Acyrthosiphon pisum*, *Aphis fabae*
Akutse et al. (2014)	Bean *V. faba*	G1LU3, S4SU1 and ICIPE 279,	Seeds – leaves, stems, roots	*Phaedrotoma cabriventris*, *Diglyphus isaea*
Akutse et al. (2013)	Bean *V. faba* and *Phaseolus vulgaris*	Three different isolates	Seeds – leaves, stems, roots	*Liriomyza huidobrensis*
Amin et al. (2014)	Cocoa *Theobroma cacoa*		Pods – pods	*Conopomorpha cramerella*
Barta (2018)	Horse-chestnut trees (*Aesculus hippocastanum* L.)	AM_EF0111, AM_EP0715	Leaves – leaves	*Cameraria ohridella*
Behie et al. (2015)	Bean *P. vulgaris*	ARSEF 252	Roots – stems, leaves, hypocotyl, roots	
Bing and Lewis (1991), (1992a), (1992b), (1993) Lewis and Bing 1991, Lewis et al. (1996, 2001, 2002)	Corn *Zea mays*	ARSEF 3113	Leaves – stems, whorl stage parts, pith	*Ostrinia nubilalis*
Bills and Polishook (1991)	Ironwood *Carpinus caroliniana*		Isolation from natural habitat – Bark	
Biswas et al. (2012)	Jute *Corchorus olitorius*	ITTC 4796	Seeds – roots, leaf, stem, capsule	
Biswas et al. (2013)	White jute *Corchorus capsularis*	Nine different strains	Seeds, roots – leaves and nonspecified segments	*Apion corchori*
Brownbridge et al. (2012)	Pine *Pinus radiata*	F647 (Genbank GU237004), F668 (Genbank GU237005)	Seeds, roots – roots, needles	
Canassa et al. (2019)	Bean *P. vulgaris*	ESALQ 3375	Seeds – leaves, stems, roots	*Tetranychus urticae*, *Phytoseiulus persimilis*
Castillo Lopez et al. (2014)	Cotton *Gossypium* sp.	Strain from Botanigard®	Seeds – leaves, stems, roots	*Aphis gossypii*

Table 1: continued

Castillo Lopez and Sword (2015)	Cotton *Gossypium* sp.	Strain from Botanigard®	Seeds	*Helicoverpa zea*
Cherry et al. (1999)	Corn *Z. mays*	Five different isolates	Injection – stems	*Sesamia calamistis*
Cherry et al. (2004)	Corn *Z. mays*	Six different isolates	Seeds, leaves, stems – stems	*Sesamia calamistis*
Clifton et al. (2018)	Soybean *Glycine max* L.	GHA	Seeds – stems, leaves	*Aphis glycines*
Dara et al. (2013)	Strawberry *Fragaria* X *ananassa*		Roots – roots, foliage	
Dash et al. (2018)	Bean *P. vulgaris*	B12, B13, B16	Seeds – root, stem, leaves	*Tetranychus urticae*
El-Deeb et al. (2012)	Tomato *Solanum lycopersicum*		Injection – non specified	Tomato leaf curl virus, *Bemisia tabaci*
Evans et al. (2003)	Cocoa *Theobroma gileri*			
Ganley and Newcombe (2006)	Western white pine *Pinus monticola*		Natural habitant – Seeds, needles	
Gomez-Vidal et al. (2006), (2009)	Date palm *Phoenix dactylifera*		Petioles – petioles	
Greenfield et al. (2016)	Cassava *Manihot esculenta*	Five isolates	Soil – stems, leaves, roots	
Guesmi-Jouini et al. (2014)	Artichoke *Cynara scolymus* L.		Leaves – leaves	
Gurulingappa et al. (2010), (2011)	Cotton *Gossypium sp.*, wheat *Triticum aestivum*, bean *P. vulgaris*, corn *Z. mays*, tomato *Lycopersicum esculentum*, pumpkin *Cucurbita maxima*	GenBank AN GU953211, AN GU953212	Leaves – leaves	*Aphis gossypii*; *Chortoicetes terminifera*
Jaber (2015)	Grapevine *Vitis vinifera*	ATP01, ATP05, EABb04/01-Tip and ATCC 74040	Leaves – leaves	*Plasmopara viticola*
Jaber und Araj (2018)	Sweet pepper *Capsicum annum*	ATCC 74040	Soil – roots, stems, leaves	*Myzus persicae, Aphidius colemani*
Jaber et al. (2018)	*Brassica oleracea*	ATCC 74040	Leaves - leaves, stems, roots	*Bremisia tabaci*
Jaber (2018)	Wheat *Triticum aestivum* L.	ATCC 74040	Seeds – shoots, roots	*Fusarium culmorum*
Jaber and Alananbeh (2018)	Sweet pepper *C. annuum* L.	ATCC 74040	Roots – roots, stems, leaves	*Fusarium* spp.
Jaber and Enkerli (2016)	Broad bean *V. faba*	ATCC 74040	Seed – root, leaf, and stem	
Jaber and Salem (2014)	Squash *Cucurbita pepo*	ATCC 74040	Leaves – leaves	Zucchini yellow mosaic virus

Table 1: continued

Jia et al. (2013)	Rice *Oryza sp.*	Bb0062 (Bb-4 and Bb-7)	Leaves – leaves, stems, roots, seeds	
Klieber and Reineke (2016)	Tomato *S. lycopersicon*	ATTC 74040	Leaves – leaves	*Tuta absoluta*
Landa et al. (2013)	Opium poppy *Papaver somniferum*	EABb 04/01-Tip	Leaves – leaves	
Lewis and Bing (1991), Lewis et al. (1996), (2001)	Corn *Zea mays*	ARSEF 3113	Leaves – stems, whorl stage parts, pith	*Ostrinia nubilalis*
Mantzoukas et al. (2015)	Sweet sorghum *Sorghum bicolor*	IGE3	Leaves – leaves, stems	*Sesamia nonagrioides*
McKinnon et al. (2018)	Corn *Z. mays*	BG11, FRh2 and J18)	Roots – roots	
Moloinyane and Nchu (2019)	Grapevine *V. vinifera*	SM3	Roots – leaves	*Planococcus ficus*
Ownley et al. (2008)	Tomato *S. lycopersicon*; cotton *Gossypium sp.*	11-98	Seeds – seedlings	*Rhizoctonia solani, Pythium myriotylum*
Parsa et al. (2013)	Bean *P. vulgaris*	GHA	Leaves, soil – leaves, stems, roots	
Parsa et al. (2018)	Bean *P. vulgaris*	11 isolates	Seeds – leaves, stems, roots	
Pelizza et al. (2017)	Corn *Z. mays*	LPSc 1067 (accession number KF500409)	Leaves – leaves	*Dichroplus maculipennis*
Posada et al. (2007)	Coffee *Coffea arabica*	ARSEF 5486, ARSEF 2687, ARSEF 1480	Leaves, stems, roots – leaves, stems, roots	
Posada and Vega (2005)	Cocoa *T. cacoa*	IC-5486, CS16-1	Topical – leaves, stems, roots	
Posada and Vega (2006)	Coffee *C. arabica*	IC-5486, CS16-1	Roots – leaves, stems, roots	
Posada et al. (2010)	Cocoa *T. cacao*	Bb04005	Flowers – pods	
Powell et al. (2009)	Tomato *S. lycopersicum*	11 - 98	Seeds – leaves, stems, roots	
Qayyum et al. (2015)	Tomato *S. lycopersicum*	WG-40, WG-14, WG-1	Roots, stems, leaves – leaves	*Helicoverpa armigera*
Quesada-Moraga et al. (2006)	Opium poppy *P. somniferum*	EABb 04/01-Tip	Leaves – leaves	
Quesada-Moraga et al. (2009)	Opium poppy *P. somniferum*	EABb 04/01-Tip	Seeds, soil, leaves – leaves	*Iraella luteipes*
Quesada-Moraga et al. (2014)	Opium poppy *P. somniferum*	EABb 04/01-Tip	Seeds – leaves	
Razinger et al. (2014)	Cauliflower *Brassica oleracea*	Isolate 1174, ATCC 74040	Soil – roots, stems	
Reay et al. (2010)	New Zealand pine *P. radiata*		Isolation from natural habitat	
Reddy et al. (2009)	Sorghum *Sorghum bicolor*	ITCC 4688	Leaves – stems	*Chilo partellus*
Renuka et al. (2016)	Corn *Z. mays*	NBAII-Bb-5a, 7, 14, 19, 23, 45	Leaves – leaves, stems	

Table 1: continued

Resquín-Romero et al. (2016)	Alfalfa *Medicago sativa*, tomato *L. esculentum*, melon *Cucumis melo*	EABb 01/33-Su	Leaves – roots, stems, leaves	*Spodoptera littoralis*
Rondot and Reineke (2017)	Grapevine *V. vinifera*	ATTC 74040	Leaves	*Otiorhynchus sulcatus*
Rondot and Reineke (2018)	Grapevine *V. vinifera*	ATCC 74040, GHA	Leaves – leaves	*Planococcus ficus, Empoasca vitis*
Russo et al. (2015)	Tobacco *Nicotiana tabacum*; corn *Z. mays*, wheat *T. aestivum*; soybean *Glycine max*	LPSC 1067	Seeds, roots, leaves – leaves	
Russo et al. (2019)	Corn *Z. mays*,	LPSc 1098 (GenBank KT16325)	Leaves – Leaves, seeds	*Rachiplusia nu*
Sánchez-Rodríguez et al (2018)	Bread wheat *Triticum aestivum*, durum wheat *T. durum*	EABb 04/01-Tip	Soil, seed, leaf - grains	*Spodoptera littoralis*
Tefera and Vidal (2009)	Sorghum *S. bicolor*	Bb-04	Roots – leaves, stems, roots	
Vidal and Jaber (2015)	Bean *V. faba*; Oilseed rape *Brassica napus*	Eight different isolates	Leaves – leaves	*Helicoverpa armigera*
Wagner and Lewis (2000)	Corn *Z. mays*	ARSEF 3113	Leaves – leaves	

2 Objectives

Fungal entomopathogens are important antagonists of arthropod pests and have attracted attention as biocontrol agents. In addition to colonizing arthropods, evidence has accumulated that some entomopathogenic fungi like *Beauveria bassiana* can endophytically colonize a wide array of plant species. However, only limited information is currently available on the endophytic colonization of grapevine plants with *B. bassiana.* In addition, the functional role of the fungus *in planta,* and/or the plant response to colonization by *B. bassiana* and the mechanisms underlying these responses, still require elucidation. The objectives of the present study were to investigate whether the fungus *B. bassiana* is able to colonize grapevine plants, still maintains its entomopathogenic potential against insect pests, and has additional antagonistic potential against other fungal pathogens. The investigation focused on the interaction between *B. bassiana,* grapevine plants, and potential target insect pests as well as fungal pathogens to gain more knowledge of this particular tritrophic interaction with regard to potential biological control strategies in viticulture.

The specific objectives were:

- to optimize the endophytic establishment of *B. bassiana* in grapevine plants via artificial application in the greenhouse and under field conditions (chapter 3)
- to characterize the entomopathogenic potential of endophytic *B. bassiana* against selected insects pests attacking grapevine (chapter 3 and 4)
- to evaluate the antagonistic potential of endophytic *B. bassiana* against a key-fungal pathogen in viticulture, the causal agent of downy mildew (chapter 5)
- to study the effects of the endophytic establishment of *B. bassiana* in grapevine plants on gene level (chapter 5)

This thesis is based on three (peer-reviewed) manuscripts included in the following chapters as follows:

I. **Yvonne Rondot and Annette Reineke (2018): Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects.** *Biological Control, 116, 82-89*

II. **Yvonne Rondot and Annette Reineke (2017): Endophytic *Beauveria bassiana* in grapevine plants influences host plant selection of adult black vine weevils, *Otiorhynchus sulcatus.*** *Biocontrol Science and Technology, 27(7), 811-820*

III. **Yvonne Rondot and Annette Reineke: Endophytic *Beauveria bassiana* activates expression of defence genes in grapevine and prevents infections by grapevine downy mildew *Plasmopara viticola*.** *Chapter 5 under revision*

The contribution of Yvonne Rondot to the manuscripts listed above was as follows:

I. Planned the experiments together with the co-author. Performed most of the experimental work in the greenhouse and the laboratory as well as some of the experimental work in the field. Supervised a student in performing some parts of the experimental work in the laboratory. Evaluated and statistically analyzed all data. Prepared all figures and tables. Wrote the manuscript together with the co-author.

II. Planned the experiments together with the co-author. Performed most of the experimental work and supervised a student in performing some parts of the experimental work. Evaluated and statistically analyzed all data. Prepared all result figures and tables. Wrote the manuscript together with the co-author.

III. Planned the experiments together with the co-author. Performed all of the experimental work in the greenhouse as well as parts of the experimental work in the laboratory. Evaluated and statistically analyzed the data. Prepared all figures and tables. Wrote the manuscript together with the co-author.

3 Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects

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Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned and performed most of the experiments Evaluated and statistically analyzed all data Prepared all figures and tables Wrote the manuscript
Contribution of other authors:	Annette Reineke contributed to experimental design and writing the paper
Journal:	Biological Control, 116, 82-89
Impact Factor:	2.112
Date of manuscript submission:	27. April 2016
DOI:	https://doi.org/10.1016/j.biocontrol.2016.10.006

This publication included a graphical abstract (see Figure 16, supplementary material)

Highlights

- Successful endophytic establishment of the entomopathogen *B. bassiana* in grapevine plants.
- In potted plants, endophytic survival of *B. bassiana* was evident for at least 21 days after inoculation.
- Endophytic *B. bassiana* reduces infestation rate and growth of vine mealybugs.
- In the vineyard, *B. bassiana* was detected as an endophyte up to five weeks after last application.
- *B. bassiana* reduces infestation with grape leafhopper in the vineyard.

Abstract

Fungal entomopathogens are important antagonists of arthropod pests and have attracted increased attention as biocontrol agents. In addition to colonizing arthropods, evidence has accumulated that some entomopathogenic fungi like *Beauveria bassiana* can endophytically colonize a wide array of plant species. However, only limited information is currently available on the endophytic colonization of grapevines with *B. bassiana* and whether the fungus still maintains its antagonistic potential against insect pests.

Greenhouse and field experiments were conducted to optimize endophytic establishment of the entomopathogenic fungus *B. bassiana* in potted and mature grapevine plants. We used two different commercialized *B. bassiana* strains and applied them as conidial suspensions or as the formulated product Naturalis® on grapevine leaves. The antagonistic activity of endophytic *B. bassiana* against putative target pest insects like the vine mealybug *Planococcus ficus* was assessed in a bioassay using surface sterilized leaves. Endophytic survival of *B. bassiana* inside leaf tissues of potted plants was evident for at least 21 days after inoculation, irrespective of the inoculum used. Endophytic *B. bassiana* reduces infestation rate and growth of *P. ficus*. In the vineyard, *B. bassiana* was detected as an endophyte in mature grapevine plants up to five weeks after last application with a significant impact on infestation with grape leafhopper, *Empoasca vitis*.

Keywords:

Beauveria bassiana; endophyte; entomopathogenic fungi; *Vitis vinifera; Planococcus ficus*; interactions

3.1 Introduction

The entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is a well-known microbial antagonist of a diverse range of arthropod species. Hence, this species has attracted increased attention as a potential microbial biocontrol agent for integrated pest management of arthropod pests with a couple of *B. bassiana* based commercial products being available on the market (Zimmermann 2007; Jackson et al. 2010). Generally, for control of target species, preparations of blastospores or aerial conidia formulated in oil or other adjuvants are sprayed onto the plant's phylloplane. Besides its entomopathogenic habit of life style, this fungus has also been shown to be able to thrive saprophytically in the soil, to colonize the rhizosphere of plants, to have antagonistic activities against plant pathogens, as well as to grow endophytically inside plants (Vega et al. 2009). As far as the latter is concerned, a few studies have shown that *B. bassiana* is occurring as part of the natural endophytic community of certain plant species (Ormond et al. 2010; Reay et al. 2010; Vega et al. 2008). Moreover, endophytic establishment of *B. bassiana* has been achieved via an artificial application of this fungus on the plant's tissue following a subsequent colonization of the entire host plant. Using such an approach, successful endophytic establishment of *B. bassiana* has been proved for a variety of crop plant species including cocoa (Posada and Vega 2005) and pine seedlings (Brownbridge et al. 2012), corn (Wagner and Lewis 2000), coffee (Posada et al. 2007), sorghum (Reddy et al. 2009; Tefera and Vidal 2009), tomato(Klieber and Reineke 2016), banana (Akello et al. 2009), and jute (Biswas et al. 2012; Biswas et al. 2013). So far, no negative effects of the presence of endophytic *B. bassiana* on performance of the colonized host plant were evident in a range of studies (Akello et al. 2009; Tefera and Vidal 2009; Wagner and Lewis 2000; Klieber and Reineke 2016). Endophytic *B. bassiana* has been reported to provide systemic protection against several insect pests or to inhibit insect development and establishment (Quesada-Moraga et al. 2009; Reddy et al. 2009; Gurulingappa et al. 2010; Biswas et al. 2013). At the same time, presence of endophytic *B. bassiana* has been shown to reduce disease symptoms caused by a variety of fungal pathogens (Griffin et al. 2005; Ownley et al. 2010; Ownley et al. 2008; Jaber 2015) Therefore, defining means of ensuring an endophytic establishment of *B. bassiana* strains in target crop plants is currently receiving increased attention, as this would represent a dual biocontrol strategy both against insect pests and plant pathogens.

Grapevine (*Vitis vinifera* L.) is an important global commodity crop which is planted throughout temperate regions worldwide. A substantial number of different insect pests and pathogens are associated with grapevine and are significant factors influencing both the quantity of the yield as well as the quality of must and wine (Flaherty 1992). Accordingly, grapevine cultivation is regarded as being quite input intensive, in particular regarding the

frequency and intensity of fungicide and insecticide applications throughout the year (Roßberg 2007). Insects with a piercing-sucking mode of feeding are frequently attacking grapevines and cause damage either by extracting sap fluids or feeding in mesophyll cells or by transmitting grapevine pathogens. The grape leafhopper *Empoasca vitis* (Goethe) (Homoptera: Cicadellidae, Typhlocybinae) feeds on mesophyll cells or on phloem sap and is recognized as a major insect pest in many European grapevine growing areas (Olivier et al. 2012). Moreover, the vine mealybug *Planococcus ficus* (Signoret) (Homoptera: Pseudococcidae) is regarded as a key pest in many countries around the world with grapevine cultivation (Daane et al. 2012). *Planoccocus ficus* causes direct damage to grapevine due to phloem-feeding on leaves and fruit and excretion of honeydew. Additionally, *P. ficus* acts as a vector for grapevine leafroll associated virus (GLRaV), one of the most economically destructive grapevine viruses that occur in all the major grape-growing regions of the world (Almeida et al. 2013). Accordingly, a combination of methods including insecticide applications, biological control via antagonists or mating disruption is usually applied by growers to control *P. ficus* (Almeida et al. 2013). The system grapevine (as an input intensive crop) - *P. ficus* and *E. vitis* (as phloem-feeding pest insects) - *B. bassiana* (as a commercially available biopesticide) is thus ideal for studying tritrophic interactions between plants, insects and entomopathogenic endophytic fungi. Endophytic establishment of an entomopathogenic fungus like *B. bassiana* still having antagonistic activity against insect pests and fungal pathogens would therefore represent a novel and sustainable plant protection strategy in viticulture, with the potential to reduce frequency of pesticide applications.

Here we demonstrate for the first time, that endophytic establishment of commercially available *B. bassiana* strains in grapevine displays antagonistic activity against insects with a piercing-sucking mode of feeding. Moreover, we proved that an endophytic colonization of *B. bassiana* is possible, both in greenhouse potted grapevine plants as well as in mature and lignified plants grown in the field.

3.2 Materials and methods

3.2.1 Fungal material

Beauveria bassiana strains ATCC 74040 and GHA were isolated from the commercial products Naturalis® (CBC (Europe) S.r.l., Italy) and Botanigard® 22WP (BioWorks, Inc., USA), respectively. Naturalis® is formulated as an oily fluid and contains approximately 2.3 x 10^7 colony forming units/ml of *B. bassiana* strain ATCC 74040 as active ingredient. The isolates were maintained on a *Beauveria* medium at 24 °C in the dark. The medium consisted of 10 g soy peptone (AppliChem, Germany), 20 g glucose (Sigma-Aldrich, Germany) and 18 g

Bacto™Agar (BD Difco, USA) dissolved in 1000 ml ultrapure water and was subsequently autoclaved for 20 min at 120 °C.

To obtain spore suspensions, conidia were harvested by gently scraping the surface of Petri dishes containing 8-day-old *B. bassiana* cultures and suspending them in 20 ml sterile 1/8 concentrated Ringer's solution containing 0.02% Tween 80. The conidia concentration was determined using a Thoma haemocytometer and adjusted to 2×10^7 conidia/ml for strain GHA and to 1×10^7 and 2×10^7 conidia/ml for strain ATCC 74040. Both, the freshly collected conidia suspensions and the formulated product Naturalis® (at concentrations of 3% and 5%), were used in the experiments. Aliquots of 50 µl of spore suspensions were plated on *Beauveria* medium using the Spiralplater WASP 2 (Meintrup DWS Laborgeräte GmbH, Germany). Concentrations of viable conidial spores were calculated using the colony forming unit's method. Germination rate was 100% for conidial spores present in Naturalis® and around 70% for the spore suspensions of isolates ATCC 74040 and GHA. Accordingly, concentrations of viable conidia applied onto plants were 1.4×10^7 conidia/ml for strain GHA and 7×10^6 (conc. 1) and 1.4×10^7 (conc. 2) conidia/ml for strain ATCC 74040.

3.2.2 Endophytic establishment in potted grapevine plants

Grapevine plants, *Vitis vinifera* (L.) cv. 'Riesling', were obtained from hardwood cuttings. After root development, the plants were potted and grown in a greenhouse chamber at 22-25 °C. Seven-week-old grapevine plants with 4-7 fully expanded leaves were used for inoculation with either *B. bassiana* conidial suspensions or the commercial product Naturalis® (3% and 5%). For each treatment, 10 replicate plants were inoculated by spraying the adaxial and the abaxial surfaces of all fully expanded leaves until run-off using a 1 l one-hand pressure sprayer. The control plants were sprayed with sterile 1/8 concentrated Ringer's solution containing 0.02% Tween 80. Position of the last fully expanded leaf used for inoculation was labeled using a tapener (Max tapener HT-B, Max Staple, Japan). Inoculated and non-inoculated plants were kept in a greenhouse chamber for three weeks (daily mean temperature 23-25° C, daily mean relative humidity 50-70%) and were watered regularly.

3.2.3 Re-isolation of *B. bassiana*

Endophytic colonization of plants by *B. bassiana* was assessed 7, 14, and 21 days after inoculation (DAI) by re-isolation following surface sterilization. No newly developed leaves were included in the present study. At each sampling period one leaf from each of the 10 replicate plants was excised and transported to the laboratory on ice. The leaves were individually surface sterilized under sterile conditions by dipping them in 0.5% NaOCl (active chlorine) containing 0.05% Tween 80 for 2 min, followed by 70% EtOH for 2 min and rinsed twice with sterile distilled water according to Akello et al. (2009). The success of this

disinfection process was assessed by plating three replicates of 200 µl of the residual rinse water on PDA (potato dextrose agar). No fungal growth was recorded in any of the rinse water samples after 21 days of incubation. After surface sterilization, six leaf discs (d = 1.2 cm) were obtained with a sterile cork borer from each leaf. Leaf discs were placed on *Beauveria* selective medium (BSM), the same medium as indicated above (2.1) but supplemented with 0.1 g/l streptomycin (Sigma-Aldrich, Germany), 0.05 g/l tetracycline (Sigma-Aldrich, Germany), 0.1 g/l dodine (as aliquot of the product Syllit®, Spiess-Urania Chemicals, Germany) and 0.05 g/l cyclohexamide (Sigma-Aldrich, Germany). This medium is based on a medium initially described by Strasser et al. (1996) for the isolation of *Beauveria brongniartii* and adapted by Meyling and Eilenberg (2006) for isolation of *B. bassiana*. Plates were incubated at room temperature with a 12:12 h light:dark photoperiod (mean light intensity of 11.2 µmol m^{-1} s^{-1}).

After 7 and 14 days leaf discs were examined visually for the presence of any fungal growth. Fungal tissue was characterized as endophytic *B. bassiana* if characteristic white dense mycelia, becoming creamy at the edge (Humber 1997) grew from internal plant tissues of surface sterilized leaf discs. Final assessment of the presence of endophytic *B. bassiana* was recorded after 14 days and was expressed as percentage colonization by dividing the number of leaf discs exhibiting *B. bassiana* outgrowth by the number of total leaf discs and multiplying the obtained value with 100. If one of the six leaf discs obtained from a single plant showed fungal outgrowth the total leaf was classified as being endophytically colonized. Differences in percentage colonization of plant tissues at the different sampling dates were analyzed for statistical significance with a Kruskal-Wallis-ANOVA using Dell Statistica data analysis software system (Dell Inc., version 13, software.dell.com).

3.2.4 Strain-specific detection of *B. bassiana*

To ensure that fungal tissue present at the edges of grapevine leaf discs originated from the respective inoculated *B. bassiana* strain (ATCC 74040 or GHA), now internally colonizing plant tissues as an endophyte, a subset of mycelia samples was further analyzed with molecular techniques. DNA was extracted from fungal tissues using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions with an additional step for 30 min on ice after the recovering step with isopropanol. Accordingly, extracted fungal DNAs were subjected to strain-specific PCR analysis using three *B. bassiana* microsatellite (simple sequence repeats, SSR) primers, namely Ba01, Ba12 and Ba13 (Rehner and Buckley 2003). In previous studies, these primers have proved to allow a confident discrimination among different *B. bassiana* isolates (Reineke et al. 2014).

For fluorescent labelling of the generated PCR products, a M13(-21) tail was placed at the 5'-end of each forward primer and a CY5 labelled universal primer M13(-21) was added to the

PCRs according to the method described by Schuelke (2000). PCR amplifications were set up in a total volume of 15 µl consisting of 90 ng DNA, 10x reaction buffer, 5 pmol of forward primer, 10 pmol of reverse primer, 2.25 mM $MgCl_2$, 3 mM dNTPs and 0.5 U of Dream Taq Polymerase (Fermentas, St. Leon-Rot, Germany). PCRs were performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60.2 °C for 45 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. An aliquot of each PCR product was checked for successful amplification on a 1% agarose gel. PCR products were analyzed for SSR sizes via capillary electrophoresis on a Beckman GenomeLab GeXP DNA Genetic Analysis System (Beckman Coulter, Inc., CA, USA). As different fluorescent primers were used for labeling the obtained PCR products (DY-751 for Ba01, BMN5 for Ba12 and DY-681 for Ba13) reactions were loaded as a multiplex analysis with 1 µl of each PCR product, mixed with 36.7 µl sample loading solution (Beckman Coulter, Inc., CA, USA) and 0.3 µl of a 400 bp size standard. Allele sizes were determined using GenomeLab GeXP Version 10.2 (Beckman Coulter, Inc., CA, USA).

3.2.5 Mealybug bioassay

The antagonistic activity of endophytic *B. bassiana* against piercing-sucking insects was tested with a detached leaf assay and vine mealybugs, *P. ficus*. 60 potted grapevine plants cv. 'Riesling' were inoculated with Naturalis® (3%) or water as control as described above. The whole experiment was repeated twice. Two weeks after inoculation two leaves per plant were obtained, with one leaf used for the bioassay and the other leaf to verify endophytic establishment by re-isolation as described above. To ensure that mealybugs were only influenced by endophytic and not by epiphytic fungal propagules, all grapevine leaves were surface disinfected before the bioassay according to the procedure described above. With a pretest (data not shown) we verified that any leftovers of NaOCl still present on the leaves did not harm the mealybugs.

Vine mealybugs were grown on potato sprouts in a growth room with 23 ± 1 °C, 60–65% RH and 16:8 h light:dark period. In all experiments, first instar *P. ficus* individuals were used, which were removed from potato tubers by irritation with a paintbrush until their stylets were withdrawn. Ten *P. ficus* larvae each were carefully transferred with a paintbrush to the surface sterilized leaves. Leaves with mealybugs were maintained in enclosed transparent plastic containers (height 10 cm, diameter 13.5 cm) with water provided for the leaf and were placed in a growth chamber under the conditions mentioned above.

After two days infestation rate was calculated as the number of remaining larvae on the leaf in relation to the initially used ten individuals. This procedure was repeated once a week over a period of three weeks (7, 14, and 21 days after initial settlement) and was supplemented by

determination of the size of all individual mealybug larvae with a binocular microscope and measurement software (Leica Microsystems, Application Suite, Switzerland). A total of 300 mealybugs werw assessed for each, the endophytic and the control leaves. Size and infestation rate were analyzed for statistical significance between endophytic and control leaves with a Mann-Whitney-U-Test ($\alpha = 0.05$) using Dell Statistica data analysis software system (Dell Inc., version 13, software.dell.com).

3.2.6 Field trial

In addition to greenhouse and laboratory experiments we conducted a field trial as proof of principle to get preliminary evidence of efficacy of endophytic establishment of *B. bassiana* and its antagonistic potential against insect pests in the field. The field trial was realized in the framework of GEP (good experimental practice) certified efficacy tests of plant protection products. The experimental vineyard was located in the Rheingau region, Germany (49°58'N, 7°57'E) and included 0.3 ha of grapevine plants, *Vitis vinifera* (L.) cv. 'Riesling' planted in 1999. The experiment was conducted in a completely randomized block design with 4 plots (replicates) of 114 m2 size and 14 vines per plot.

Naturalis® (1%) was applied in the vertical canopy by a tunnel sprayer with 8 Teejet® flat spray nozzles and driving speed of 0.7 m/s. Control plots were treated with water. Application was carried out in step with other plant protection measures (fungicide applications against powdery mildew, *Erysiphe necator,* using the products Vivando®, Talius®, Luna® Experience and Topas® in rotation). Interval between applications was approximately 10 days depending on weather and disease pressure of other grapevine pathogens with a first application on 15 May 2014. In two of the four Naturalis® treated plots treatment included nine applications during the season. The other two plots were treated twice in the beginning of the season to determine how long the fungus can be detected endophytically (15 and 26 May 2014).

Endophytic establishment in grapevine leaves was evaluated at 4 dates (22 May, 12 June, 2 July, and 23 July 2014) in 10 leaves per plot according to the method described above. In addition we assessed the infestation with grape leafhopper, *Empoasca vitis,* at 5 dates (15 July, 23 July, 31 July, 07 Aug and 12 Aug 2014) in treated plots and control plots by counting *E. vitis* larvae on 25 leaves per plot. Infestation data was analyzed for statistically significant differences with nonparametric Wilcoxon signed-rank test (McDonald 2014).

3.3 Results

3.3.1 Endophytic colonization of potted grapevine plants

During the assessment period of 7, 14, and 21 DAI *B. bassiana* was successfully re-isolated from 46%, 40%, and 46% of all inoculated grapevine plants, respectively. None of the leaf discs obtained from control plants showed signs of fungal outgrowth, thus none of the control plants were colonized by the fungus. Not all leaf discs from colonized plants showed fungal outgrowth, causing a high variance in percentage colonization in all treatments (Figure 5). In some instances contaminating fungi and bacteria were occasionally found growing from leaf discs of both inoculated and control plants (data not shown).

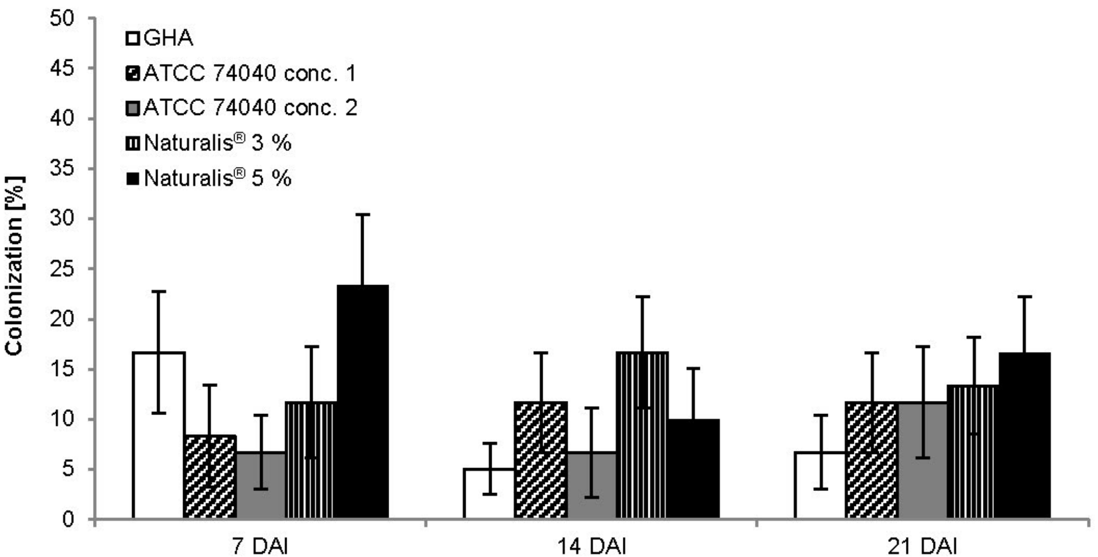

Figure 5: Mean (±SE) percentage colonization of *Vitis vinifera* leaf discs 7, 14 and 21 days after inoculation (DAI) with a conidial suspension of *Beauveria bassiana* strains GHA (1.4×10^7 conidia/ml) or ATCC 74040 (conc. 1: 7×10^6 conidia/ml; conc. 2: 1.4×10^7 conidia/ml) or with the formulated product Naturalis® (3% and 5%). Differences between treatments were not statistically significant ($p<0.05$). In control leaves (treated with Ringer's solution) no *B. bassiana* was present (not shown).

If applied as a conidial suspension on foliage of grapevine plants, both *B. bassiana* strains (GHA and ATCC 74040) were able to establish as an endophyte, with no significant differences in percentage colonization being evident between the different spore concentrations and the strains applied (Figure 5). The same was obvious if *B. bassiana* strain ATCC 74040 was applied as the formulated product Naturalis®, with colonization rates being not significantly different for both concentrations (3% and 5%) applied (Figure 5). During the assessment period, no significant decline or increase in percentage colonization by endophytic *B. bassiana* was observed 7, 14, and 21 DAI.

3.3.2 Strain-specific detection of endophytic B. bassiana

In capillary electrophoresis, DNA from all analyzed mycelia samples obtained from endophytic fungal tissues showed the respective strain-specific peaks after amplification with three *B. bassiana* microsatellite primers (Table 2). Amplicons of primer pairs Ba01, Ba12 and Ba13 showed peaks at 117 bp, 231 bp and 216 bp for strain ATCC 74040 and 117 bp, 222 bp and 168 bp for strain GHA, respectively. These results indicate that endophytic *B. bassiana* re-isolated from inoculated leaf discs originated from the previously applied strains.

Table 2: Amplification of *B. bassiana* strain GHA or ATCC 74040 specific SSR markers in a subset of eight obtained mycelia samples from leaf discs of the different treatments 14 and 21 days after inoculation (DAI) with *B. bassiana*.

Treatment	DAI	No. of leaf discs used and screened positive with three SSR markers			
		n total	Ba01	Ba12	Ba13
ATCC 74040 (1.4×10^7 conidia/ml)	14	2	2	2	2
Naturalis® 3%	14	2	2	2	2
GHA (1.4×10^7 conidia/ml)	21	1	1	1	1
ATCC 74040 (7×10^6 conidia/ml)	21	2	2	2	2
ATCC 74040 (1.4×10^7 conidia/ml)	21	1	1	1	1

3.3.3 Mealybug bioassay

Antagonistic potential of endophytic *B. bassiana* against vine mealybug larvae was assessed on detached and surface sterilized grapevine leaves of Naturalis® treated and control grapevine plants. Endophytic establishment in the repective grapevine plants was 30% in the first and 60% in the second experimental replicate (data not shown). Because of this difference in endophytic establishment results of the two replicates were not combined but were analyzed separately.

Mealybug larvae were smaller when feeding for a period of 3 weeks on Naturalis® treated leaves compared to those feeding on control leaves (Figure 6). In the first experimental replicate this difference was not statistically significant (Mann-Whitney-U-Test: 1 week $p = 0.057$; 2 weeks $p = 0.159$; 3 weeks $p = 0.286$), while in the second replicate the mean size of the mealybugs on treated leaves was significant smaller over the whole assessment period of three weeks (Mann-Whitney-U-Test: 1 week $p<0.001$; 2 weeks $p<0.005$; 3 weeks $p<0.05$).

In the first experimental replicate significantly less mealybug larvae stayed alive on leaves with endophytic *B. bassiana* (Naturalis®) compared to control leaves ($p<0.05$) over the period of the bioassay (Figure 7). In the second replicate this effect was only observed at the beginning of the assessment period (1 week of feeding).

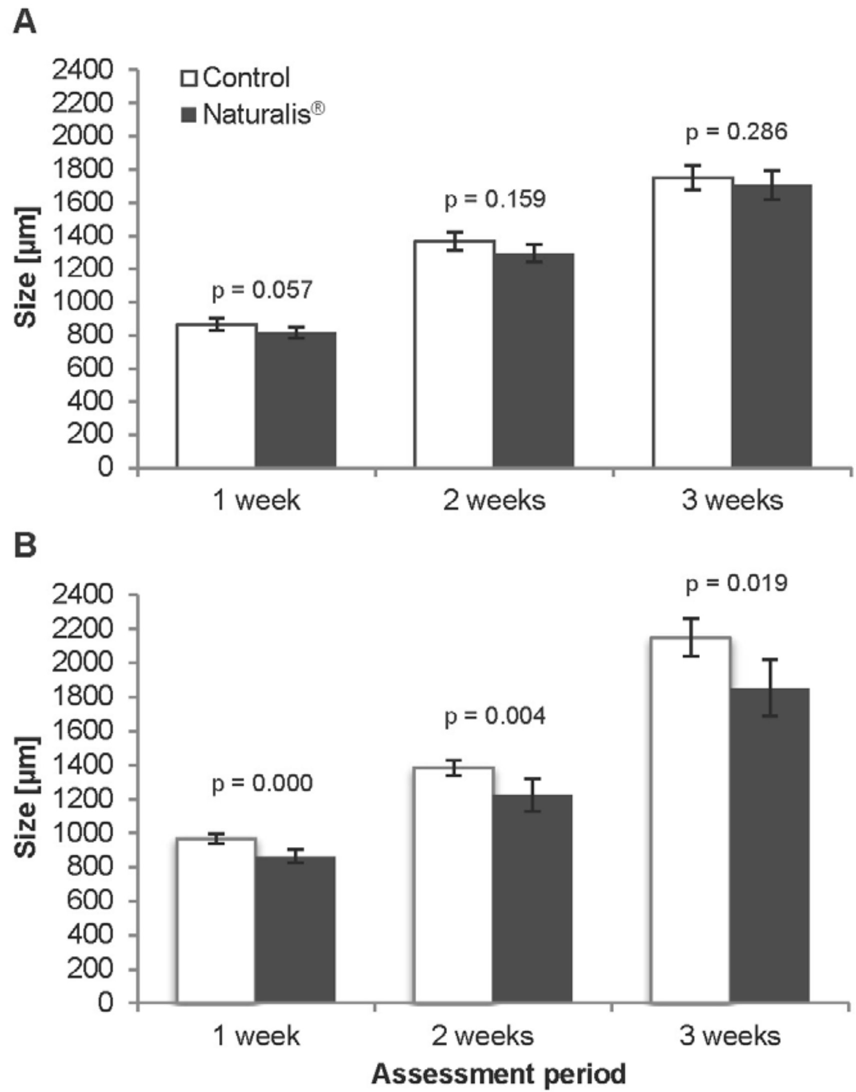

Figure 6: Mean (± 95% CI) size of vine mealybug larvae (*P. ficus*) after feeding for three weeks on detached grapevine leaves of control plants and plants with endophytic *B. bassiana* (Naturalis®) in two replicates (A and B). Statistical significant differences between treatments were analyzed with a Mann-Whitney-U-Test (α=0.05).

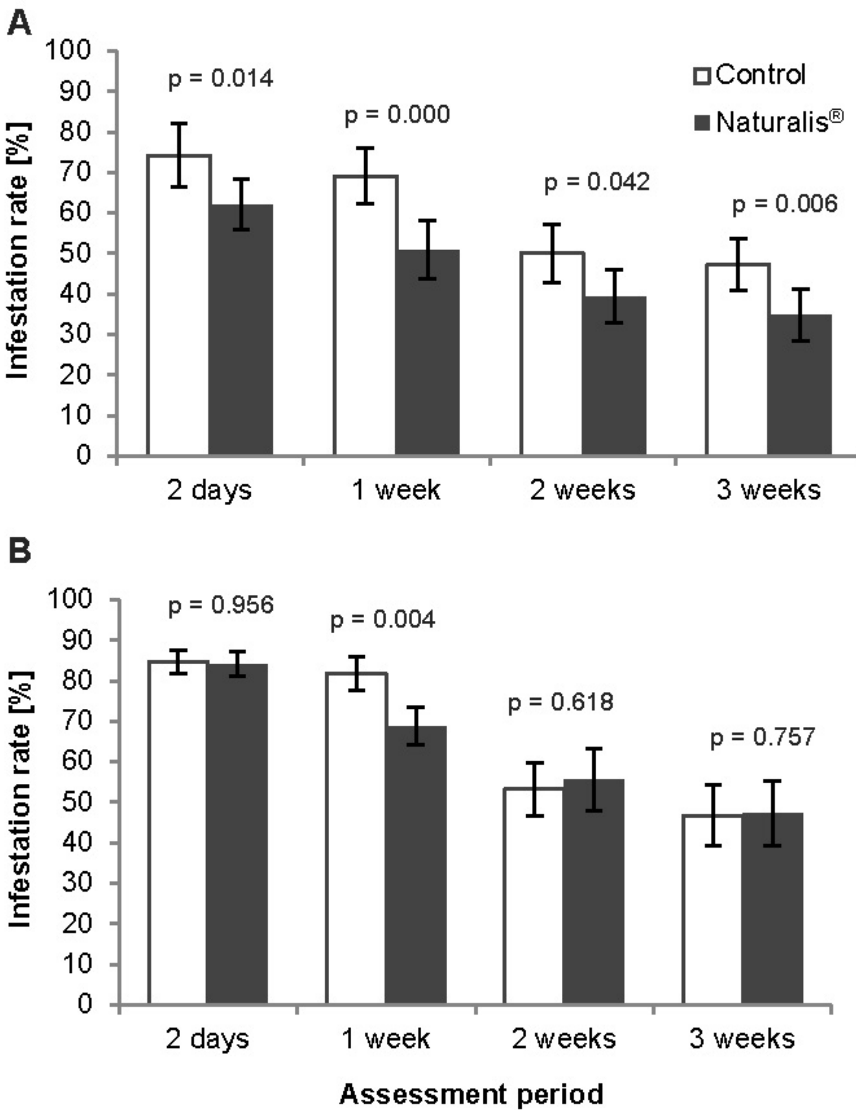

Figure 7: Mean (± 95% CI) infestation rate of vine mealybug larvae (*P. ficus*) after feeding for three weeks on detached grapevine leaves of control plants and plants with endophytic *B. bassiana* (Naturalis®) in two replicates (A and B). Statistical significant differences between treatments were analyzed with a Mann-Whitney-U-Test (α=0.05).

3.3.4 Field trial

Re-isolation of *B. bassiana* after application in the field showed that the fungus was able to establish as an endophyte in perennial and lignified grapevine plants in the vineyard (Table 3). In the plots treated several times with Naturalis® the fungus could be detected at all sampling dates (22 May, 12 June, 02 July and 23 July 2014). Detection rate declined over the season. In plots treated only at the beginning of the season *B. bassiana* was successfully re-isolated up to five weeks after the last application of Naturalis®. In control plots no *B. bassiana* was re-isolated from the leaves.

Table 3: Number of leaf discs assessed (n) and showing *B. bassiana* outgrowth collected from Naturalis® treated and control plots of a grapevine field trial in 2014.

Treatment	**n**	**No. of leaf discs with endophytic *B. bassiana***			
		22 May	**12 June**	**02 July**	**23 July**
Control	80	0	0	0	0
Naturalis® (2 applications)	160	16	8	8	0
Naturalis® (9 applications)	160	47	19	10	13

Infestation rate with grape leafhopper *E. vitis* in the vineyard was overall low in the year 2014. At all five monitoring dates the mean number of larvae was lower in Naturalis® treated plots than in control plots (Table 4). Over the whole assessment period the total number of *E. vitis* larvae was higher in control than in Naturalis® treated plots (246 vs. 183 individuals). Wilcoxon signed-rank test showed that the median difference between the mean number of grape leafhopper larvae per monitoring date in control plots vs. Naturalis® treated plots was significantly greater than zero ($W=0$, $P<0.001$)

Table 4: Mean number (±SE) of grape leafhopper *E. vitis* larvae in four control and four Naturalis® treated plots (25 leaves/plot) assessed at five observation dates in a grapevine field trial in 2014. Mean number of grape leafhopper larvae per monitoring date in control plots vs. Naturalis® treated plots were statistically different (Wilcoxon signed-rank test; $W=0$, $P<0.001$).

Date 2014	**Mean number (± SD) of *E. vitis* larvae**	
	Control	**Naturalis®**
15 July	15,50 (± 5,07)	12,75 (± 2,22)
23 July	18,75 (± 9,71)	12,75 (± 6,60)
31 July	14,00 (± 5,48)	13,50 (± 5,20)
07 Aug	6,00 (± 2,94)	4,50 (± 3,11)
12 Aug	4,75 (± 2,06)	2,25 (± 0,96)

3.4 Discussion

Successful endophytic colonization of both potted grapevine plants in the greenhouse as well as mature plants in the field with two different commercially available *B. bassiana* strains was achieved via artificial spray inoculation. Analysis of fungal mycelia obtained after re-isolation with strain-specific molecular markers confirmed our initial assessment based on morphology of endophytic fungal mycelia obtained from colonized grapevine plants. In greenhouse experiments, no significant difference in percentage colonization by endophytic *B. bassiana* was observed during the assessment period of 21 DAI. This suggests that endophytic colonization of grapevine by *B. bassiana* was evident as early as 7 DAI and did not decline

during the period of screening for presence of endophytic *B. bassiana* of 21 DAI. Moreover, percentage colonization of grapevine plants did not vary significantly among the different strains or inoculum doses used. This may be a consequence of the relatively small number of positive samples identified and the apparent variability in isolation success.

In the present study, mean colonization rates of potted grapevine plants by *B. bassiana* were between 5% and 23% and were thus rather low compared to colonization rates of leaves of other plant species like corn (Wagner and Lewis 2000), tomato(Klieber and Reineke 2016), sorghum (Tefera and Vidal 2009), and jute (Biswas et al. 2013). In contrast to these plants grapevines are deciduous, woody perennial plants, and plants used for our greenhouse trials were cultivated from hardwood cuttings. Seed treatment as an alternative inoculation method as it has been successfully shown for tomato, cotton (Ownley et al. 2008), opium poppy (Quesada-Moraga et al. 2009) and sorghum (Tefera and Vidal 2009) is therefore not possible. Previous grapevine inoculation trials via root dipping or soil inoculation resulted in no colonization at all (data not shown). Root dipping or soil inoculation has been used for endophytic establishment of *B. bassiana* in banana (Akello et al. 2007), sorghum (Tefera and Vidal 2009) and pine seedlings (Brownbridge et al. 2012). Therefore, inoculation via spray application is apparently the only option for endophytic inoculation of grapevine plants and we have shown here that such an application is compatible with viticultural practice.

Jaber (2015) reported slightly higher colonization rates of up to 50% of grapevine plants by *B. bassiana* after artificial spray inoculation. Endophytic establishment of entomopathogenic fungi is known to be dependent on plant cultivar, fungal strain and many other environmental conditions (Vidal and Jaber 2015). However, to the best of our knowledge it was not yet possible to prove systemic establishment of *B. bassiana* in grapevine plants.

Here, we used molecular SSR markers to prove that the re-isolated *B. bassiana* strain was the one previously applied. Direct detection with PCR-based techniques of endophytic *B. bassiana* after spray application is difficult because of the likelihood of contamination with epiphytic propagules and is thus only applicable for systemic establishment. In addition, surface sterilization is regarded as an insufficient technique for subsequent molecular assessment of endophytic establishment (McKinnon et al. 2014). Evidence has accumulated that for culture-based techniques surface sterilization can result in underestimated colonization rates, due to diffusion of the chemicals used for sterilization into the leaves (Lohse et al. 2015; Ownley et al. 2008). In consequence and in line with other reports only a combination of different detection methods will result in sound qualitative and quantitative data about endophytic colonization of plants (Lohse et al. 2015). In this context methods must be adapted for every plant species and different plant material.

Despite the comparatively low endophytic colonization rates of *B. bassiana* in grapevine we observed significant antagonistic effects of endophytic *B. bassiana* on infestation and size of vine mealybug larvae in bioassays. Moreover, grape leafhopper *E. vitis* larvae were significantly more abundant on control than on endophytic *B. bassiana* grapevine plants in the field. Usually, fungal entomopathogens infect their insect hosts via cuticular penetration by germinating propagules (Arnold and Lewis 2005). Infection by endophytic entomopathogens via consumption of infected plant tissue or ingestion of hyphae or spores seems to be unlikely and rare (Vidal and Jaber 2015). Existing reports about mycosis due to endophytic entomopathogens are so far restricted to insects living inside plant tissues like stem-borers or leafmining larvae (Akello et al. 2009; Klieber and Reineke 2016), where a direct contact of insects feeding inside plant tissues and endophytic fungal propagules can be envisaged. In contrast to stem-borers or leaf-miners mealybug larvae live on the plant surface and have a piercing-sucking feeding habit with the consequence that a direct mode of action due to direct contact is not likely to occur. On surface disinfected leaves previously treated with *B. bassiana*, mealybug larvae were smaller and mortality rates were higher than on control leaves, but none of the dead larvae exhibited symptoms of mycosis. These results suggest a mode of action involving feeding deterrence, antibiosis or changes in metabolism of the host plant and thus host plant quality rather than a direct fungal infection of the insects. Colonization of grapevine plants was different in the two replicates of the bioassay. At a higher *B. bassiana* colonization rate, size of vine mealybug larvae was significantly smaller after feeding on endophytic leaves compared to control leaves. Vice versa, at a lower colonization rate, we detected significant differences in vine mealybug infestation rates. Accordingly, two different modes of action of endophytic entomopathogens might account for these observations, depending on rate of tissue colonization by *B. bassiana.* In any case, we have shown that the presence of entomopathogens as endophytes negatively influences insect performance, yet further investigations are required to determine the mechanisms underlying these effects. Results presented here point to the importance to also study sublethal effects of endophytic entomopathogens on insects in order to understand tritrophic interactions between plants, endophytes, and insects.

In the present study we have shown for the first time that an endophytic establishment of *B. bassiana* in mature grapevine plants under field conditions is possible. Our results also indicate the potential for a long term establishment of the fungus in grapevine plants and that endophytic establishment does apparently not interfere with common viticultural management practices. In this regard, a couple of studies have shown that *B. bassiana* is sensitive against various pesticides (Todorova et al. 1998; Sapieha-Waszkiewicz et al. 2004; Kos and Celar 2013). However, even though synthetic fungicides were simultaneously applied in our experimental vineyard, an endophytic establishment of *B. bassiana* in the mature plats

was successful. Moreover, *B. bassiana* conidia are known to be extremely sensitive to ultraviolet radiation and consequently persistence as well as germination of conidial suspensions applied on the foliage is limited. Lohse et al. (2015) emphasized the importance of an adequate formulation for endophytic establishment of entomopathogenic fungi. Here, we used a commercially available fungal-based product (Naturalis®, active ingredient *B. bassiana* isolate ATCC 74040) formulated as an oily dispersion, which may provide a benefit for the colonization process of *B. bassiana* on grapevine plants. This product is registered in some EU member states i.e. for control of whiteflies in tomato thus having the perspective of a rapid registration for other applications.

Overall, grapevine plants seemed not to be negatively affected by the presence of endophytic *B. bassiana*, as growth and performance of the respective inoculated plants was visually similar to control plants during the period of observation (data not shown). This is in accordance with previous studies on plant performance after endophytic establishment of entomopathogenic fungi (Akello et al. 2009; Tefera and Vidal 2009; Wagner and Lewis 2000; Klieber and Reineke 2016). However, whether presence of endophytic *B. bassiana* in grapevine plants has an effect on quality and sensory attributes of must and wine still remains to be tested with fruit-bearing grapevine plants.

Endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants represents a new and sustainable plant protection strategy. The implementation of the indirect effects (endophyte) in combination with direct effects (epiphyte) of entomopathogens on both plant and insect herbivores will show their full potential value in insect pest management. Further research should also include an in-depth study on the mode of action of endophytic entomopathogens against insects as well as identifying possible effects on induced resistance mechanisms against both grapevine pathogens and insect pests.

Acknowledgements

This work was supported by Deutsche Bundesstiftung Umwelt (DBU). We thank Edith Ladurna from CBC (Europe) S.r.l., Italy, for providing the product Naturalis® and Ottmar Baus for support during the field trial. Gordan Behaderovic is kindly acknowledged for help in mealybug bioassays.

4 Association of *Beauveria bassiana* with grapevine plants deters adult black vine weevils, *Otiorhynchus sulcatus*

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Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned all experiments Performed most of the experimental work Evaluated and statistically analyzed all data Prepared all figures and tables Wrote the manuscript
Contribution of other authors:	Annette Reineke contributed to experimental design and writing the paper
Journal:	Biocontrol Science and Technology
Impact Factor:	0.918
Date of manuscript submission:	22. Feb 2017
DOI:	https://doi.org/10.1080/09583157.2017.1347604

Abstract

Fungal entomopathogens are known as microbial pathogens of insects, colonizing multiple habitats and ecosystems. Besides being an entomopathogen, the fungus *Beauveria bassiana* can also establish as an endophyte in plants. Limited knowledge is so far available on the ability of plant-associated *B. bassiana* to influence plant-feeding insects. Here, we assessed the capability of adult black vine weevils *Otiorhynchus sulcatus* to select grapevine as a host plant in the presence of plant-associated *B. bassiana* after foliar application of a commercially available mycoinsecticide (product Naturalis®) on young potted grapevine plants. Three pairwise comparisons of weevil behaviour were conducted when weevils were released in a two-choice olfactometer and were given the choice between (i) control plants and plants treated with Naturalis®, (ii) control plants and plants treated with the formulation of Naturalis® without fungal propagules, and (iii) plants treated with Naturalis® and plants treated with the formulation. Adult *O. sulcatus* were significantly deterred by plants treated with Naturalis® or the formulation in comparison to control plants. In a direct comparison between plants treated either with Naturalis® or the formulation weevils significantly preferred plants treated with the formulation and avoided Naturalis® treated plants, where *B. bassiana* putatively had established as an endophyte. These results suggest that adult black vine weevils are able to detect and subsequently avoid plants treated with *B. bassiana* and indicate a new mode of action of plant-associated entomopathogenic fungi when integrated in pest management programmes.

Keywords

Beauveria bassiana, endophyte, entomopathogen, *Otiorhynchus sulcatus*, choice assay, olfactometer

4.1 Introduction

Endophytes, a term first defined by De Bary (1884), are fungi or bacteria occurring within plant tissues without causing visible disease symptoms in the colonized plant. Even though their presence does not seem to negatively influence the plant, some endophytes have profound impacts on plant communities or have the ability to influence interactions between plants and their natural enemies. For example, certain endophytes can enhance overall plant fitness (Rodriguez et al. 2009) or increase resistance of plants against herbivores or pathogens as well as limit their spread and damage (Arnold and Lewis 2005; Ownley et al. 2010; Backman and Sikora 2008; Vega 2008). Although endophytes are present in most, if not all, plants in natural as well as in agricultural ecosystems, their function in shaping plant-insect interactions is yet not fully understood and their potentially beneficial role in sustainable

plant production is not exploited so far. However, the ability of endophytes to colonize internal host tissues could be used to improve crop performance or pest management strategies. Reduced herbivory on endophyte hosting plants can be a direct result from decreased survival rates of herbivorous insects, which is often attributed to the production of defensive compounds or toxins (Clay 1993). In addition, alterations in the plant's nutritional quality as well as changes in plant volatile profiles or secondary plant metabolites of endophyte-associated plants may influence developmental time, fecundity, host location, or oviposition behaviour of herbivorous insects (Jallow et al. 2008; Vega 2008).

Host plant selection by herbivorous insects includes a series of behavioural and decision events. The ability to detect the presence of natural enemies or pathogens in the respective host plant's environment and to react accordingly would be advantageous for any insect during foraging or oviposition site selection. Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales), are important mortality factors of insect pests. Some potential pest insects are able to detect entomopathogenic fungi and to avoid contact with them. The common flower bug *Anthocoris nemorum* for example can recognise its natural enemy *B. bassiana* and has been shown to avoid fungus infected leaves (Meyling and Pell 2006). Another example of such a prevention strategy in the presence of the entomopathogenic fungus *B. bassiana* has been proved for the seven-spot ladybird (*Coccinella septempunctata*), which is able to avoid lethal densities of *B. bassiana* conidia in soil or on leaves (Ormond et al. 2011). Other insects, such as the termite *Coptotermes lacteus*, are capable of recognising the presence of the entomopathogenic fungus *Metarhizium anisopliae* and were shown to avoid direct contact with this fungus (Staples and Milner 2000). In contrast, Kepler and Bruck (2006) described a significant attraction of black vine weevil *Otiorhynchus sulcatus* larvae to pots containing plants and the fungus *M. anisopliae,* which is likely due to changes in volatile profiles when roots are colonized by this entomopathogenic fungus. Pivotal for these reactions are active detection mechanisms by the insects. However, the exact processes are not yet fully understood. In this context, Elliot et al. (2000) extended the herbivory-bodyguard hypothesis describing tritrophic interactions among plants, herbivores and their predators or parasitoids also to entomopathogens and their plant association.

The black vine weevil, *O. sulcatus* F. (Coleoptera: Curculionidae), is a serious insect pest of economic importance in nursery, ornamental and soft fruit production worldwide (Moorhouse et al. 1992). While adult weevils are nocturnal and cause mostly cosmetic damage by feeding on the leaves, larvae are ground dwelling and feed on root systems, which may result in high levels of plant damage and subsequently kill the plant (Shah et al. 2007). Because *O. sulcatus* has a parthenogenetic mode of reproduction, a single weevil left uncontrolled can lay up to

900 eggs, resulting in the infestation of an entire nursery (Bruck 2007). Keeping the insect out of nurseries is one main issue in its control.

Infestation by larval stages of *O. sulcatus* can be limited by the incorporation of synthetic insecticides into the potting media (Kepler and Bruck 2006). An alternative biological control strategy is the application of entomopathogenic nematodes. However, practical use of this group of biological control agents is limited due to insufficient efficacy at low temperatures, a short shelf life, and high application costs (Johnson and Rasmann 2015; Lu et al. 2016). Moreover, the management of adult *O. sulcatus* includes foliar applications of pesticides, however, adult weevils are active at night, which necessitates and complicates an application at the right site and right time. Entomopathogenic fungi showed considerable potential as biological control agents against adults and larvae of the black vine weevil (Bruck 2007; Shah et al. 2007; Ansari et al. 2008; Hirsch and Reineke 2014). Accordingly, the simultaneous use of endophytic entomopathogens as plant bodyguards as defined by Elliot et al. (2000) in addition to the already proved direct effect of entomopathogenic fungi against *O. sulcatus* would represent a dual mode of action of entomopathogens against this pest insect. For example, the presence of endophytic entomopathogenic fungi might influence host choice behaviour of adult weevils, resulting in an avoidance of the colonized plant.

In the present study we assessed the behaviour of adult black vine weevils when given the choice between grapevine plants treated several weeks before with *B. bassiana* containing mycosinsecticide and control plants. We hypothesised that weevils are able to detect and avoid *B. bassiana* when actively searching for a host plant. The results presented here will provide information on the potential of endophytic fungi to influence herbivore host choice behaviour, and promote the development of improved management strategies for insect pests.

4.2 Materials and methods

4.2.1 Source of fungus, insects, and plants

Beauveria bassiana strain ATCC 74040 was used in the form of the commercial product Naturalis® (CBC (Europe) S.r.l. – BIOGARD Division). Naturalis® is formulated as an oil dispersion (OD) and contains approximately 2.3 x 10^7 colony forming units/ml of *B. bassiana* strain ATCC 74040 as active ingredient. The product is registered in some EU member states, e.g. for the control of whiteflies in tomato. In addition, the pure formulation of this product without conidia of *B. bassiana* was used as a control in our experiments (CBC (Europe) S.r.l. – BIOGARD Division).

A population of black vine weevil, *O. sulcatus,* was kept at 22° C and fed with grapevine leaves. Egg and larval development was completed in boxes (h = 9 cm, w = 22 cm, l = 34 cm)

filled with 8 cm soil and using *Impatiens walleriana* plants and carrots as food source. In all assays, we used adult weevils that had emerged from pupae in the soil boxes at least 4weeks but not more than 12 weeks before the start of experiments. At this age, weevils are in the period of maturation feeding; accordingly foliar feeding on the respective host plant is required for egg production. Prior infection experiments carried out with the same *O. sulcatus* population and the *B. bassiana* strain via direct inoculation resulted in 48 to 65% mortality of adult weevils within 28 days (Hirsch and Reineke 2014).

Grapevine plants, *Vitis vinifera* (L.) cv. 'Riesling', were propagated from hardwood cuttings. After root development, the plants were potted and grown in a greenhouse chamber at 22-25 °C. Seven-week-old grapevine plants with four to six fully expanded leaves were used for treatment either with the commercial product Naturalis® (3%), the Naturalis® formulation without conidia (3%) or water. For each treatment, 40 plant replicates were inoculated separately by spraying the adaxial and abaxial surfaces of all fully expanded leaves using a 2-l handheld pressure sprayer. Approximately 10 ml were applied at each plant. Prior to the experiments, treated plants were retained in a greenhouse chamber for 1-3 weeks (mean temperature 23-25° C, mean relative humidity 50-70%) and were watered as required. The rate of endophytic establishment of *B. bassiana* in grapevine plants was tested under the same conditions and using plants of the same origin and age in parallel experiments. As reported by Rondot and Reineke (2018), between 30-60% of plants could be detected as having *B. bassiana* as an endophyte. In this study, endophytic colonization of 7-week-old potted grapevine plants by *B. bassiana* was assessed 7, 14, and 21 days after inoculation (DAI) by re-isolation from leaf tissues after surface sterilization. For the experiments described here, we excluded additional re-isolations of *B. bassiana* or other leaf sampling analysis as described by Rondot and Reineke (2018), in order to prevent activation of plant defense reactions by mechanical damage of leaves. Since treatment and cultivation conditions were similar in both experiments, we expect identical colonization rates.

4.2.2 Design and validation of the two-choice olfactometer

In order to assess host choice behaviour of adult black vine weevils we constructed a two-choice still-air olfactometer (Figure 8). Transparent plastic cylinders (h = 30 cm, d = 13.5 cm) were modified by drilling a hole (d = 2.5 cm) into the side of each cylinder (6 cm from the bottom) and fitting a horizontal connection tube (l = 6.5 cm, d = 2.3 cm) into the hole. Two of these tubes were connected by a T-shaped piece of PVC pipe (d = 2.5 cm). The middle section of the T-shaped piece was plugged with a small petri dish lid which served as a release point for the weevils. Each cylinder was placed on the soil surface of a potted grapevine plant and was sealed with gauze to allow sufficient air flow within the olfactometer and prevent excessive moisture. Prior to the experiments, the newly designed olfactometer was validated

by releasing weevils in the T-shaped middle section and giving them the choice between a control plant placed inside a cylinder and an empty cylinder in order to observe if black vine weevils were generally able to choose the host plant in this test system. The design of the olfactometer permitted the weevils to change sides after an initial selection.

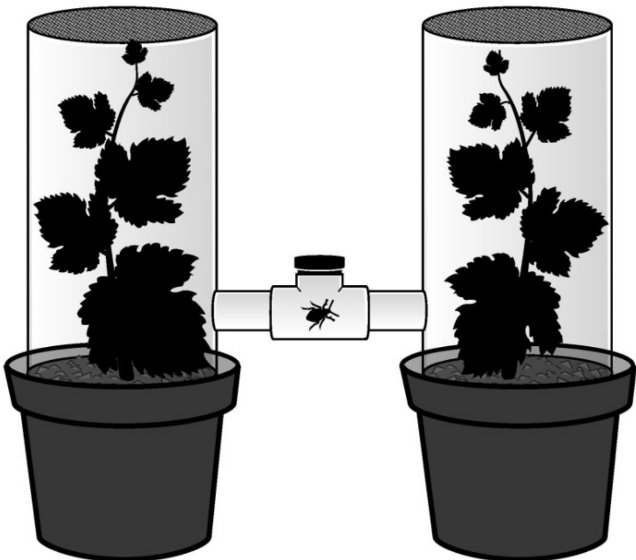

Figure 8: Design of the two-choice still-air olfactometer used in the experiments. Adult black vine weevils were placed inside the lid of the T-shaped piece connecting the two cylinders and were allowed 1 h to choose between plants in the cylinders.

4.2.3 Experimental design

With the olfactometer described earlier, three different pairwise comparisons of weevil host choice behaviour were performed. Adult black vine weevils were allowed to choose between (i) control plants and grapevine plants treated 7-21 days before with Naturalis®, (ii) control plants and plants treated 7-21 days before with the Naturalis® formulation, and (iii) plants treated with Naturalis® and plants treated with the Naturalis® formulation. Plants were not surface sterilized before use in the olfactometer trials.

Because *O. sulcatus* has a nocturnal lifestyle and trials should be performed in the active period of adults, the natural daily rhythm of adult *O. sulcatus* was switched by 12 h with the help of artificial lighting. Additionally, weevils were deprived of food for 24-36 h prior to testing. Each test lasted 1 h, starting when weevils were in the active period for food searching (2-4 h after "sunset"). Pretests indicated that most of the weevils had made their decision within 1 h with no significant changes occurring compared to their initial selection even if they were given more time (data not shown).

Moreover, we decided to assess host choice behaviour of single adult *O. sulcatus* instead of releasing several weevils at the same time to avoid aggregation behavioural effects. All trials

were performed in a dark room (24 ± 2 °C, 55 ± 8 % RH). Nine olfactometers were used simultaneously, three for each pairwise comparison. Orientation of the olfactometer in the room was changed for every replicate. Adult *O. sulcatus* were used only once and plants were replaced every second day or when feedings sites were visible on the leaves. Cylinders and connecting tubes were thoroughly washed before each experimental day. Trials were repeated 3 times a day for 12 days, so that 108 decisions were realised and documented for every pairwise comparison. The whole experimental set-up was repeated twice in two subsequent years (2013 and 2014).

The preference of adult *O. sulcatus* was compared relative to each other. Weevils that remained in the connecting T-tube were categorised as unresponsive. Each pairwise comparison as well as the validation experiment were analysed separately. Number of decisions for each side was counted and the proportion out of the total number of responsive adult *O. sulcatus* in the trial was analysed with an exact binominal test (McDonald 2014). The number of responsive weevils and the number of unresponsive weevils in all comparisons throughout the study were compared using a two sample t-test (McDonald 2014).

4.3 Results

The usefulness of the designed olfactometer was validated by the black vine weevils' ability to select a cylinder with a grapevine plant over an empty cylinder. In both experimental replicates (years 2013 and 2014), the majority of adult *O. sulcatus* was recovered from cylinders containing a plant (2013: $p = .03$; 2014: $p < .001$; Figure 2). Throughout all comparisons, the percentage of responsive weevils was generally high (mean = 82%) and was significantly greater than the percentage of unresponsive weevils ($p < .0001$). The weevils in the first experimental replicate in 2013 were less decisive (70% responsive) than in the second experimental replicate in 2014 (94% responsive).

When black vine weevils were allowed to choose between control grapevine plants and plants treated 7-21 days before with Naturalis®, significantly more weevils decided for the cylinders with a control plant (Figure 9). In the first replicate (2013), we recovered 67 weevils from the cylinders with a control plant, 9 weevils from the cylinders with plants treated with Naturalis® ($p < .0001$), and 32 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 62 versus 41 recovered weevils, respectively ($p = .048$), and 5 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

When weevils were given a choice between control plants and plants treated 7-21 days before with the Naturalis® formulation, significantly more weevils decided for the cylinders with a control plant (Figure 9). In the first replicate (2013), we recovered 64 weevils from the

cylinders with a control plant, 14 weevils from cylinders with plants treated with formulation ($p < .0001$), and 30 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 69 versus 35 recovered weevils, respectively ($p = .001$), and 4 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

When weevils were allowed a choice between plants treated 7 to 21 days before with the Naturalis® formulation without *B. bassiana* conidia or plants treated with Naturalis®, significantly more weevils decided for the cylinders with plants treated with the formulation (Figure 9). In the first replicate (2013), we recovered 64 weevils from the cylinders with a formulation-treated plant and 3 weevils from cylinders with plants treated with Naturalis® ($p < .0001$), and 41 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 62 versus 38 recovered weevils, respectively ($p = .02$), and 8 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

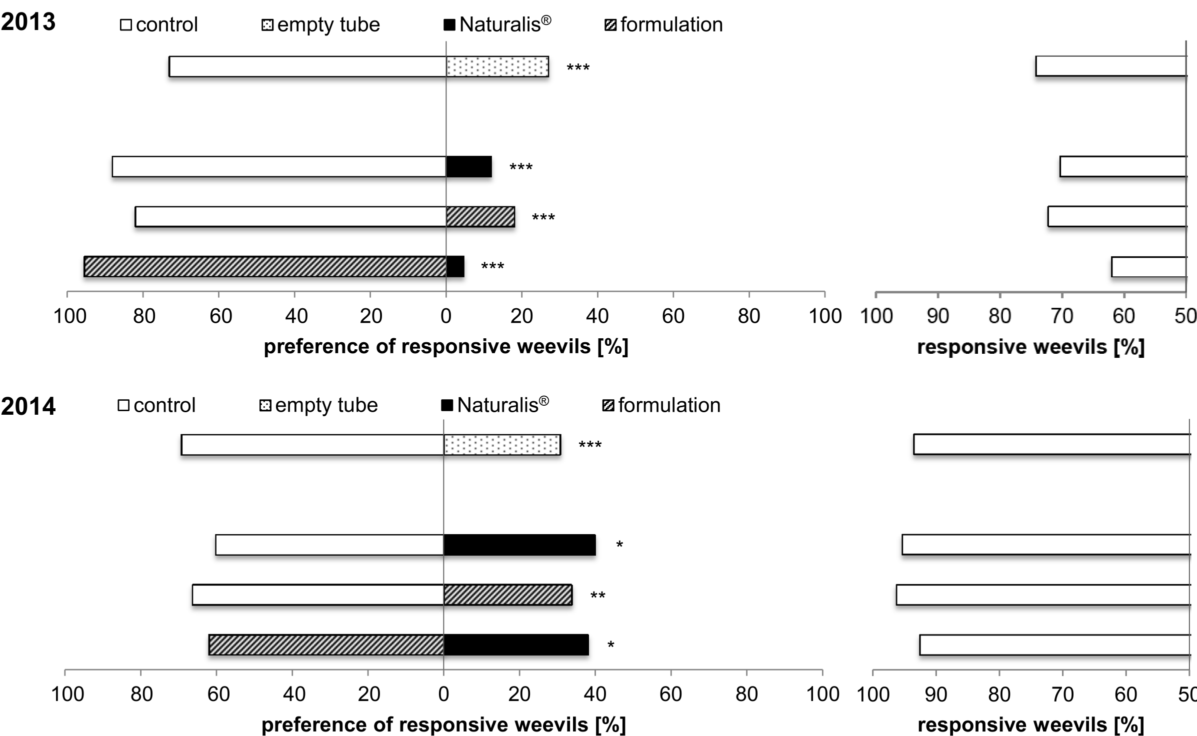

Figure 9: Percentage of adult black vine weevils *O. sulcatus* recovered when released in an olfactometer containing (i) control plants and no plant (validation assay), (ii) control plants and plants treated with Naturalis®, (iii) control plants and plants treated with the formulation, and (iv) plants treated with Naturalis® and plants treated with the formulation. Results from two independent replicates (2013 and 2014) are shown. Asterisks indicate significant differences from even distribution with $p \leq .05$ (*), $p \leq .01$ (**) or $p \leq .001$ (***). Right side of the graph depicts percentage of responsive weevils in the respective experiments.

4.4 Discussion

In this study, we proved that adult black vine weevils are able to identify grapevine plants that have been treated with a *B. bassiana* containing mycoinsecticide when actively searching for a host plant. In choice tests carried out in our newly developed plant olfactometer, weevils avoided grapevine plants treated 1-3 weeks earlier with the *B. bassiana* containing product Naturalis® as well as plants treated with the formulation of the same product. Since in a direct comparison, weevils significantly preferred plants treated with the formulation over Naturalis®-treated plants, we suppose that the presence of plant-associated *B. bassiana* is the chief factor influencing host choice behaviour. In parallel experiments using the same conditions as reported here, we have shown that the endophytic establishment of *B. bassiana* in grapevine plants can be achieved via spray inoculation of the product Naturalis®, with 30 – 60% of the plants being colonized between 7 and 21 DAI (Rondot and Reineke 2018). However, fungal inoculum or other foliar residues still present on the leaf surface could be another factor contributing to adult *O. sulcatus* host plant choice behaviour, because the plants were not cleansed of any residual Naturalis® or formulation carrier before being used. Taken together, these results suggest that adult black vine weevils are able to discriminate between plants previously treated and not treated with *B. bassiana* and subsequently avoid treated plants, where *B. bassiana* is present or has established as an endophyte. Although black vine weevils are polyphagous herbivores, known to feed and reproduce on over 140 different plant species (Bruck 2007), it has been previously shown that adults are able to discriminate between different plant species and are attracted to the odour of some but not all host plants (Van Tol et al. 2002). Moreover, in the same study weevils were attracted to volatiles of weevil-damaged foliage of certain host plants (Van Tol et al. 2002). Visual as well as chemical cues (volatiles, aggregation pheromones, or leaf surface chemicals) are involved in the attraction of insect herbivores towards feeding or oviposition sites (Bernays and Chapman 1994). In the context of fungal endophytes, the biochemical cues thereto can be altered directly by the growth of the fungus or indirectly mediated by the response of the plant to the fungal infection. Plants can detect the mere presence of microbes on their cuticle via microbe-associated molecular patterns (MAMPs) and respond with a number of biochemical changes (Newman et al. 2013). We ascribe the mechanism underlying this tritrophic interaction between the grapevine plant, *B. bassiana* and the insect *O. sulcatus* to a complex process, mediated, e.g. through the combination of metabolic and hormonal changes in the colonized plant.

The mechanisms involved in the detection of endophytic *B. bassiana* by adult *O. sulcatus* were not examined in this study. Preliminary studies have, however, indicated that the volatile profile of endophytic *B. bassiana* grapevine plants is different compared to non-endophytic

plants (Peiter 2013). A quantitative or qualitative change in plant volatile profiles may thus play a key role for *O. sulcatus* to discriminate between endophytically colonized and endophyte-free plants. In this regard, Jallow et al. (2008) have detected significant quantitative differences in certain volatiles of tomato plants when roots were colonized by the endophytic fungus *Acremonium strictum*, which accordingly influenced host selection by adult *Helicoverpa armigera* moths. In a similar way, the colonization of perennial ryegrass plants (*Lolium perenne*) by an endophytic fungus altered the composition of volatile compounds, which significantly influenced attraction of plants to adult African black beetles (*Heteronychus arator*) (Qawasmeh et al. 2015). Yet in our study, it is also possible that the establishment of endophytic *B. bassiana* altered visual, contact chemoreception and mechanoreception cues, or changed the leaf surface itself. Since weevils were able to freely move around in the plant olfactometer and behavioural assays were carried out in the dark without observing weevils during the 1 h period of the choice assays, it might as well be possible that weevils decided to leave a plant after initial contact.

Assessing putative behavioural responses of insect pests including recognition and avoidance of fungal entomopathogens present as an epiphyte or endophyte on or inside the respective host plant is pivotal for designing successful biological control strategies. The observed effects on the behaviour of *O. sulcatus* in the presence of the entomopathogen *B. bassiana* are contributing to our increased understanding of the function of entomopathogens as bodyguards of plants. Endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants might thus represent a new and sustainable plant protection strategy. Moreover, the combination of indirect effects (endophyte) and direct effects (epiphyte) of entomopathogens on insect herbivores represents a dual-control strategy of entomopathogenic fungi when integrated in pest management programmes. In this regard, future experiments should also simulate field conditions, where usually all plants are treated in the same way and *O. sulcatus* would have no choice between *B. bassiana* associated and non-associated plants. Under these conditions, we speculate an overall reduction in feeding rates and/or an increase in unresponsive weevils. Further research should also focus on the mode of action of endophytic entomopathogens as plant bodyguards against insect pests as well as on the identification of possible effects on induced resistance mechanisms in the host plant itself targeting both pathogens and insect pests.

Acknowledgements

This work was supported by Deutsche Bundesstiftung Umwelt (DBU). We thank Edith Ladurner from CBC (Europe) S.r.l. – BIOGARD Division for providing the product Naturalis® and the formulation without conidia. Roman Peiter is kindly acknowledged for help in choice assays and Sandra Linck for graphical illustration of the olfactometer.

5 Endophytic *Beauveria bassiana* activates expression of defense genes in grapevine and prevents infections by grapevine downy mildew *Plasmopara viticola*

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Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned all experiments Performed most of the experimental work Evaluated and statistically analyzed the data Prepared all figures and tables Wrote the manuscript
Contribution of other authors:	Annette Reineke contributed to experimental design, data analysis and writing the paper
Journal:	Plant Pathology
Impact Factor:	2.303
Date of manuscript submission:	9. June 2019
DOI:	https://doi.org/10.1111/ppa.13089

Abstract

Fungal entomopathogens like *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) are known as antagonist of insects with multiple functional and ecological roles and have attracted increased attention as biocontrol agents in integrated pest management programs. For some crop plants, it has been proven that endophytic *B. bassiana,* besides its entomopathogenic habit, can provide protection against plant pathogens or limit their damaging effects. For grapevine, limited knowledge is however available on the influence of endophytic *B. bassiana* on fungal pathogens and about the mechanisms underlying putative protection effects.

Here, we assessed the protective potential of endophytic *B. bassiana* against grapevine downy mildew *Plasmopara viticola* in greenhouse experiments. Three and seven days after a *B. bassiana* treatment, respectively, potted grapevine plants were inoculated with *P. viticola* and the evolving disease severity was assessed. Disease severity was significantly reduced in *B. bassiana*-treated plants compared to control plants depending on the age of leaves. Furthermore, a microarray and an RT-qPCR analysis were performed to work out fundamental aspects of genes involved in the interaction between grapevine and the endophytic fungus *B. bassiana.* The results indicate an up-regulation of diverse defense-related genes in grapevine as a response to endophytic establishment of *B. bassiana.* Thus, endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants would represent an alternative and sustainable plant protection strategy, with the potential for reducing pesticide applications in viticulture.

Keywords

Beauveria bassiana, endophyte, *Plasmopara viticola*, gene expression, microarray analysis, biological control, *Vitis vinifera*

5.1 Introduction

Plasmopara viticola (Berk. and Curt.) Berl. and de Toni, the causal agent of grapevine downy mildew, is one of the most destructive fungal diseases of European grapevine (*Vitis vinifera* L.) plants. As an obligate biotrophic oomycete, it attacks all green parts of the vine, negatively influencing both the quantity of the yield as well as the quality of must and wine. In consequence, the repeated application of fungicides each vegetation period is practically inevitable to limit the pathogen infections. Under optimal weather conditions for the fungus (moist and moderately warm) and high disease pressure, an average of 12-15 fungicide applications may be necessary for cool climate viticulture to keep the infection level under

control (Pertot et al. 2017). Current predictions on the effects of climate change on downy mildew disease pressure suggest that even more fungicide applications will be necessary in the future (Salinari et al. 2006). Concerns about environmental safety, the appearance of resistant pathogen strains, and the economic costs associated with these applications require alternative strategies for disease management. In organic viticulture, even shorter application intervals are necessary due to the lower persistence of copper-containing products (Dagostin et al. 2011). Given the poor soil-ecological properties of copper (persistence and accumulation in the soil) and the limited availability of other organic fungicides, the exploration and provision of alternative treatment strategies are thus becoming increasingly important.

Biological control agents suitable for use in organic as well as integrated viticulture originate from many different sources (e.g., plant, microbial or mineral) and exhibit different modes of action (e.g., antibiosis, competition or hyperparasitism). Besides the production of preinfectional defense substances (e.g., stilbenes, saponins), plants have evolved inherent effective defense mechanisms against phytopathogenic fungi, herbivorous insects or abiotic stressors (Kaplan et al. 2008; Pieterse and Dicke 2007). In order to successfully prevent infection or infestation, these mechanisms must be already activated or the defense reactions initiated prior to infection. In the first case, a so-called acquired resistance can occur in the plant against a later infection due to an initial infection by apathogenic microorganisms. In the second case, the plant may be put into a state by which it can react more rapidly and intensively to an attack by treatment with certain microorganisms or substances (Conrath et al. 2006). This phenomenon is called "priming".

Fungal entomopathogens are traditionally known as microbial pathogens of insects but have recently shown to play additional roles in nature and colonize multiple habitats and ecosystems. These newly emerging ecological roles, including endophytism, plant disease antagonism, plant growth promotion, and rhizosphere colonization, provide opportunities for the multiple uses of fungal entomopathogens in integrated pest management (IPM) strategies (Vega et al. 2009; Lacey et al. 2015). Among the group of entomopathogenic fungi, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is the most widely researched as an endophyte (Parsa et al. 2013) and is commercialized in the form of mycopesticides (Faria and Wraight 2007). Several studies have demonstrated that endophytic *B. bassiana* can protect its host plant against plant pathogens (Griffin et al. 2005; Ownley et al. 2010; Ownley et al. 2008). However, so far, only limited knowledge is available about the mechanisms underlying such protection. Jaber and Ownley (2018) suggest that a combination of mechanisms might be used by endophytic fungal entomopathogens against plant pathogens, rather than a single mechanism. The colonization of date palm with *B. bassiana* showed to induce proteins related to plant defense and stress response (Gómez-Vidal et al. 2009). This suggests that endophytic

colonization by entomopathogenic fungi induces plant defense responses, probably by activating the plant immune system.

The objective of the present study was to examine the biocontrol potential of endophytic *B. bassiana* against *P. viticola* by pre-infection application of *B. bassiana* inoculum on grapevine leaves. In addition, we investigated the effects of an inoculation with endophytic *B. bassiana* on the innate plants' defense reactions of grapevine by gene-based analyses (microarray and RT-qPCR) of gene expression levels. Analyses of expression of respective defense-related genes can expand our understanding of interactions between an endophytic fungus and host plant and may provide a future basis for novel pest management approaches with beneficial microorganisms.

5.2 Materials and methods

5.2.1 Plant and fungal material

Two-eye hardwood cuttings of *Vitis vinifera* cv. Riesling were obtained from mature shoots from a vineyard in the Rheingau region, Germany (49.58°N, 7.57°E) after the first frost. After disinfection (0.5% Chinoplant® solution for 12 h) they were stored at 4°C and 95% rel. humidity until use. For rooting the lower eye was removed, and the cuttings were put in boxes filled with a mixture of 50% perlite and 50% standard substrate. Thereafter, plants were potted in 2 l containers with standard substrate (ED 73) and cultivated in a greenhouse chamber at an average temperature of 24:22°C day:night with a photoperiod of 12 hours. Seven-week-old grapevine plants with four to seven fully expanded leaves were used in all experiments.

Beauveria bassiana strain ATCC 74040 was isolated from the commercial product Naturalis® (CBC (Europe) S.r.l. – BIOGARD Division, Italy). Naturalis® is formulated as an oily dispersion and contains 69.1 g/l of *B. bassiana* strain ATCC 74040 as active ingredient with a concentration of at least 2.3 x 10^7/ml viable spores. The isolate was maintained on a solid medium at 24°C in the dark. The medium consisted of 10 g soy peptone (AppliChem, Germany), 20 g glucose (Sigma-Aldrich, Germany) and 18 g Bacto™Agar (BD Difco, USA) dissolved in 1000 ml ultrapure water and subsequently autoclaved for 20 min at 120°C.

To obtain spore suspensions, the conidia were harvested by gently scraping the surface of Petri dishes containing 8-day-old *B. bassiana* cultures and suspending them in 20 ml sterile 1/8 concentrated Ringer's solution containing 0.02% Tween 80. The conidia concentration was determined using a Thoma haemocytometer and was adjusted to 2 × 10^7 conidia/ml. Both, the freshly collected conidia suspensions and the formulated product Naturalis® (1%) were used in the experiments. Aliquots of 50 µl of spore suspensions were plated on *Beauveria* medium using the Spiralplater WASP 2 (Meintrup DWS Laborgeräte GmbH). Concentrations of viable

conidial spores were calculated using the colony forming unit's method. Germination rate was 100% for conidial spores present in Naturalis® and around 70% for the spore suspensions of isolate ATCC 74040. Accordingly, the concentration of viable conidia applied onto plants was 1.4×10^7 conidia/ml.

Plasmopara viticola was maintained on potted grapevine plants (*in vivo*) and infected leaves with visible sporangia on the abaxial side were collected and stored at -20°C. For inoculation of grapevine plants, these leaves were used to prepare a suspension containing approximately 1×10^5 sporangia/ml. One week before inoculation of the plants used for the experiments, one infection cycle was carried out on living plants to get fresh sporangial material.

5.2.2 Treatment of plants with *B. bassiana*

Seven-week-old grapevine plants with four to seven fully expanded leaves were used for treatment with either *B. bassiana* conidial suspensions or the commercial product Naturalis® (1%). For each treatment, 40 replicate plants were sprayed at the adaxial and the abaxial surfaces of all fully expanded leaves using a 2 l one-hand pressure sprayer. Control plants were sprayed with tap water. Position of the last fully expanded leaf was labeled using a tapener (Max tapener HT-B, Max Staple, Japan). Treated plants were kept in a greenhouse chamber (daily mean temperature 23-25 °C, daily mean relative humidity 50-70%) and were watered regularly. This procedure has been shown to allow the successful endophytic establishment of *B. bassiana* in grapevine plants (Rondot and Reineke 2018).

For analyzing effects of endophytic *B. bassiana* on grapevine gene expression levels, 30 potted grapevine plants were treated with a *B. bassiana* conidia suspension or sterile 1/8 concentrated Ringer's solution containing 0.02% Tween 80 as a control as described above. 24, 72 and 168 hours post treatment (hpt) one leaf of each grapevine plant was carefully cut at its base from the plant and was immediately shock-frozen in liquid nitrogen before storage at -80°C prior to RNA extraction.

5.2.3 Inoculation of plants with *P. viticola*

For assessment of the preventive activity of endophytic *B. bassiana* against downy mildew grapevine plants with endophytic *B. bassiana* were inoculated with *P. viticola* three and seven days after treatment (dat) with *B. bassiana*, respectively. To obtain fresh *P. viticola* sporangia containing zoospores infected leaves were carefully washed by spraying tap water at the abaxial side. The concentration of the sporangial solution was adjusted to 10^5-10^6 sporangia ml^{-1} using a Thoma haemocytometer.

Ten plants per treatment were inoculated in the first replicate experiment. For the second and third replicate experiment the number of plants was increased to 15 per treatment.

Sporangia suspension was sprayed, using a handheld sprayer, on the abaxial leaf surface. After inoculation, grapevine plants were immediately covered with a dark plastic wrap, previously moistened with tap water, for 24 h to create an ideal microclimate for the infection process and disease development. After 24 h the plastic wrap was removed. In order to induce sporulation, plants were wrapped again for twelve hours overnight at the end of the incubation period on day seven after inoculation. The whole experimental setup was replicated individually in July 2013, autumn 2013, and July 2014.

5.2.4 Disease assessment

Disease severity (percentage of leaf surface covered by sporulation) was visually estimated on ten leaves of each plant using the disease severity scheme from guideline EPPO/OEPP PP 1/31 (3) by the European Plant Protection Organization (EPPO 2001). Example leaves of each disease severity group can be found in Figure 17 (supplementary material). Leaves were selected according to the labeling conducted before treatment and as indicated in Figure 10 and assigned to one of the twelve grades of disease severity (Figure 17, supplementary material). Based on the disease severity found in the treatment and in the control, the efficacy of the treatments with endophytic *B. bassiana* was determined according to Abbott's formula (Abbott 1925). Disease incidence was calculated as the number of leaves with visible sporulation divided by the total number of leaves and was expressed as percentage. Differences between treatments in mean disease severity of each leaf level (Figure 10) were analyzed with Kruskal-Wallis test using Dell Statistica data analysis software system (Dell Inc., version 13, software.dell.com). Disease incidences were compared using a Kruskal-Wallis rank sum test followed by multiple comparisons by Dunn (1964) with p-values adjusted by the Benjamini-Hochberg method. These analyses were calculated using the R-programming language (R Core Team 2019) and graphs were produced with the R-Package ggplot2 (Wickham 2016).

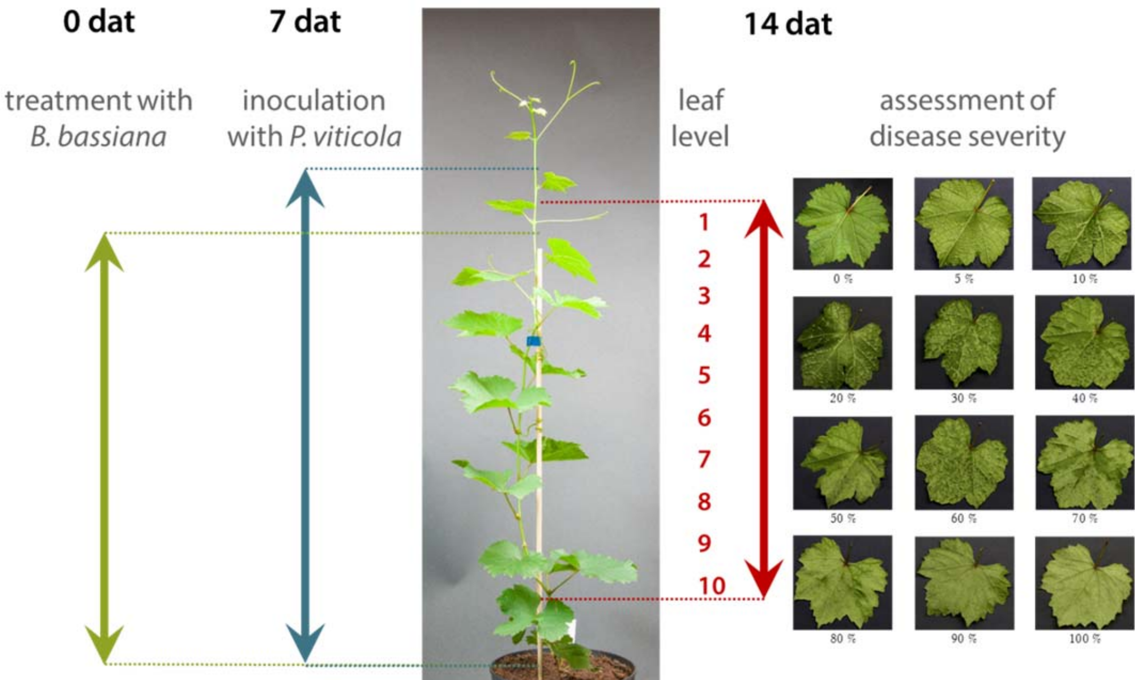

Figure 10: Time schedule of experiments and assignment of leaf levels of potted grapevine plants for the disease severity assessment. As an example, assessment of disease severity 14 days after treatment with *B. bassiana* and 7 days after inoculation with *P. viticola* is shown. Disease severity assessment was conducted based on a scheme with twelve grades of disease severity, according to EPPO (2001) and Figure 17.

5.2.5 Assessment of endophytic colonization

Ten plants of each treatment were used for confirmation of endophytic colonization of *B. bassiana* by re-isolation of the fungus following surface sterilization of the leaves. At each day of inoculation and at the end of the experiment (3, 7 and 14 dat) one leaf from each of the 10 replicate plants per treatment was excised and individually surface sterilized under sterile conditions by dipping in 0.5% NaOCl (active chlorine) containing 0.05% Tween 80 for 2 min, followed by 70% EtOH for 2 min. Finally, the leaves were dipped twice in sterile water each for 1 min and additionally rinsed with sterile distilled water. The success of this disinfection process was assessed by plating three replicates of 200 µl of the residual rinse water on PDA (potato dextrose agar). No fungal growth was recorded in any of the rinse water samples after 21 days of incubation. After surface sterilization, eight leaf discs (d = 0.8 cm) were obtained with a sterile cork borer from each leaf. The leaf discs were placed on *Beauveria* selective medium (BSM), the same solid medium as indicated above but supplemented with 0.1 g/l streptomycin (Sigma-Aldrich, Germany), 0.05 g/l tetracycline (Sigma-Aldrich, Germany), 0.1 g/l dodine (as aliquot of the product Syllit, Spiess-Urania Chemicals, Germany) and 0.05 g/l cycloheximide (Sigma-Aldrich, Germany). This medium is based on a medium initially described by Strasser et al. (1996) for the isolation of *B. brongniartii* and adapted by Meyling

and Eilenberg (2006) for isolation of *B. bassiana*. The plates were incubated at 24° C in the dark.

After 7 and 14 days of incubation the leaf discs were examined visually for the presence of any fungal growth. Fungal tissue was characterized as endophytic *B. bassiana,* if characteristic white dense mycelia, becoming creamy at the edge (Humber 1997) grew from internal plant tissues of surface sterilized leaf discs. A final assessment of the presence of endophytic *B. bassiana* was recorded after 14 days and was expressed as percentage colonization by dividing the number of leaf discs exhibiting *B. bassiana* outgrowth by the number of total leaf discs and multiplying the obtained value with 100. If one of the eight leaf discs obtained from a single plant showed fungal outgrowth the total leaf was classified as being endophytically colonized with *B. bassiana*.

5.2.6 RNA isolation

RNA was extracted from individual leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Steinheim, Germany) following the manufacturer's protocol. Leaves were crushed using liquid nitrogen and a total of up to 100 mg leaf tissue was used for RNA extraction. Contaminating DNA was removed by digestion with 0.8 U DNase (Ambion, Heidelberg, Germany) followed by lithium chloride precipitation. RNA purity and quantity were assessed based on the absorbance ratio of 1.8 to 2.0 at 260/280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

5.2.7 Microarray analysis

For microarray analysis, twelve independent pools of RNA samples were constructed: For both time points (24 h and 168 h) and *B. bassiana* treated or control plants, respectively, three RNA pools each were generated. Each RNA pool contained an individual leaf from 9 biological replicates. In this study, the Affymetrix GeneChip® *Vitis vinifera* Genome Array was used. Sample preparation for microarray hybridization was carried out as described in the Affymetrix GeneChip 3' IVT Express Kit User Manual (Affymetrix, Inc., Santa Clara, CA, USA). In brief, 250 ng of total RNA were reverse transcribed into double-stranded copy DNA (cDNA) followed by an in vitro transcription generating biotin-labeled amplified RNA (aRNA). The length of the purified aRNA products was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, USA). Following fragmentation, 12 µg aRNA were hybridized to Affymetrix GeneChip Vitis vinifera Genome Arrays for 16 h at 45° C and 60 rpm in a GeneChip hybridization oven 640. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000 7G. Fluidics and scan functions were controlled by the Affymetrix GeneChip Command Console v4.1.3 software. Sample processing and Affymetrix

microarray hybridization were performed at an Affymetrix Service Provider and Core Facility (KFB - Center of Excellence for Fluorescent Bioanalytics; KFB, University of Regensburg, Germany; www.kfb-regensburg.de).

Summarized probe set signals in log2 scale were background-adjusted, quantile normalized and log-transformed by using the robust multi-chip average (RMA) algorithm (Irizarry et al. 2003) with the Affymetrix GeneChip Expression Console v1.4 Software. After exporting into Microsoft Excel, average signal values, comparison fold changes, and significance p-values (student's t test) were calculated. Genes were regarded as being significantly up- or down-regulated when the log ratio of the change in expression between a *B. bassiana* treated and a control sample was >= 1 or <= -1 and the adjusted p-value was <= 0.05, with a log ratio of 1 representing a two-fold change in expression. Affymetrix probesets were annotated using the NetAffx Annotation Files. Sequence information included public content from GenBank and dbEST and was used to retrieve Gene Ontology (GO) annotations. To group similar classes into wider groups, GO categories were associated to related biological processes using the owltool map2slim (https://github.com/owlcollab/owltools/wiki/Map2Slim) on the basis of GO-BASIC.obo und the PLANT-subset (http://geneontology.org/docs/download-ontology/). Additionally, some terms were manually associated to related biological processes (TAIR). GO terms were reconstructed using the R-programming language (R Core Team 2019) and the GO.db-package (Carlson 2018). The complete microarray data set has been deposited in NCBI's Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) (Edgar et al. 2002) and are accessible through GEO Series accession number GSE132311.

5.2.8 RT-qPCR

Gene expression levels of three genes known to be associated with defense responses to herbivore or pathogen attack (Table 5) were additionally assessed using RT-qPCR in eight *B. bassiana* treated or control grapevine plants 24, 72 and 168 hpt, respectively. Gene-specific primers were designed using the software Geneious® 6.1.7 (Biomatters, New Zealand). For primer design, a stringent set of criteria was used, which included a predicted melting temperature of 60-65°C, primer lengths of 18-24 nucleotides, GC contents of 40-60 % and PCR amplicon lengths of 80-200 nucleotides. Grapevine genes coding for actin and GAPDH were used as a stable set of reference genes for endogenous quantification controls of gene expression data. Primer sequences for reference genes were used as designed by Timm and Reineke (2014). Melt-curve analysis was performed to check the specificity of each primer pair. Furthermore, the efficiency and amplification performance of each primer pair was evaluated using a tenfold-dilution series of a known template, analyzed with a minimum of three independent technical replicates.

Individual RNAs from single leaves of each treated and control plants for each of the three time points (24, 72 and 168 hpt) were used for RT-qPCR. The single RNA samples were diluted to 100 ng/µl before cDNA synthesis. First strand cDNA was synthesized using 1 µg RNA with the DyNAmo M-MuLV reverse transcription system (Finnzymes) with an oligo (dT)15 primer. Quantitative real-time PCR reactions were performed on an iQ5 Multicolor iCycler (Bio-Rad) using the DyNAmo™ ColorFlash SYBR® Green Kit (Finnzymes) according to the manufacturer's instructions. The single cDNA samples were diluted 1:40 before qPCR analysis. Amplifications were performed in a total volume of 25 µl using 2 µl cDNA as template, 10 pmol of each primer and 12.5 µl DyNAmo master mix. As control reactions, nuclease-free water replaced the cDNA template. For standard template reactions, a two-step cycling program was used consisting of 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. A minimum of three independent technical replicates was performed for each cDNA template with each primer pair.

Table 5: Primer sequences and PCR characteristics of two grapevine reference genes (Actin, GADPH) and three defense-related genes used in RT-qPCR experiments.

Gene	Identification	Primer	Sequence 5'-3'	Amp. Length (bp)	PCR efficiency (%)
Actin	AY847627	for	GCCTGATGGGCAAGTCAT	244	92.3
		rev	TGGGAGCAAGAGCAGTG		
GAPDH	EF192466	for	TCAAGGTCAAGGACTCTAACACC	226	97.0
		rev	CCAACAACGAACATAGGAGCA		
ATPase	LOC100251261	for	TTTCGCCCATCAGGTACAGC	146	95.1
		rev	TGAAACGCCTTGAGCTGGAA		
PR-1-like	LOC100256515	for	GTCACAAACAACCCGAGCAC	168	94.3
		rev	AACGGCGATACATGGACTCC		
beta-1,3-Glucanase	LOC100233076	for	GACAGGACGCCACTCTTGAA	148	124.4
		rev	TTGTTCTCCCTGCCATGCAA		

Quantification cycle (C_q) values were calculated using the iQ5 version 2 software (BioRad). Reference genes were evaluated based on expression stability (M values) and coefficients of variation (CV) using qBasePlus software (Biogazelle, Zulte, Belgium). Target sample expression levels were normalized based on three independent technical replicates with relation to mean C_q values of the two reference genes. Quantification of gene expression was calculated using the method implemented in qBase software (Hellemans et al., 2007), which allows the inclusion of multiple reference genes for normalization and corrects for different amplification efficiencies. Statistical differences between expression levels of treated and control leaves at the three time points were calculated on the basis of the calibrated normalized relative quantities (CNRQs) using the Mann-Whitney-U test, with a p-value of < 0.05 considered to be significant.

5.3 Results

5.3.1 Endophytic colonization

Plant colonization with *B. bassiana* was determined 3, 7, and 14 days after treatment with a conidia suspension or the product Naturalis® by culture-based re-isolation of the fungus. Depending on sampling date and experimental replicate between 10 and 100% of the ten tested leaves per plant were categorized as being endophytically colonized (Table 6). Throughout the experiments, a higher re-isolation rate was achieved after treatment with a conidia suspension in comparison with a Naturalis® treatment. The highest colonization percentage was recorded 7 dat in plants inoculated with conidia suspension in July 2014 (100%). We could not detect any decline in colonization percentage over time but recorded a high variability between the three experimental replicates. No fungal colonization was observed in any of the control plants.

Table 6: Percentage of colonized leaves per plant 3, 7 and 14 dat with a *B. bassiana* conidia suspension or the product Naturalis® in three different experimental replicates (July 2013, autumn 2013 and July 2014). None of the control plants showed fungal outgrowth (not shown).

		Colonized leaves per plant (%)		
		3 dat	**7 dat**	**14 dat**
Conidia susp.	**July 2013**	30%	20%	30%
	Autum 2013	80%	80%	60%
	July 2014	90%	100%	80%
Naturalis®	**July 2013**	10%	10%	20%
	Autum 2013	10%	10%	10%
	July 2014	40%	80%	60%

5.3.2 Preventive activity against *Plasmopara viticola*

A treatment of potted grapevine plants with *B. bassiana* as conidia suspension or the product Naturalis® three and seven days before inoculation with *P. viticola* resulted in a reduction in disease severity (percentage leaf area infected) of downy mildew in all experimental replicates. The results of the experimental replicates in July 2013 (Figure 11), autumn 2013 (Figure 12) and July 2014 (Figure 13) are presented as mean disease severity for the ten assessed leaves per plant and treatment. The varying disease severity on the ten leaves of the control plants reflects the different susceptibility of the leaves depending on their leaf level (1-10) and thus age. Basically, the susceptibility of grapevine leaves to downy mildew decreases with increasing leaf age. Despite of this, the uppermost leaves (leaf level 1-3) usually showed a lower disease severity level, since they were not yet unfolded or still very small at the time of

inoculation. This effect was less pronounced with a longer period (7 dat) between the treatment with *B. bassiana* and the inoculation with *P. viticola.* Both in July 2013 and in July 2014, therefore, a significant reduction ($p<0.05$) in disease severity was only evident starting at leaf level 3 and subjacent. Although the disease pressure of *P. viticola* was lower in 2014, the antagonistic effect of an application of *B. bassiana* conidia suspension or the product Naturalis® was visible. A significant reduction ($p<0.05$) of mean disease severity of both treatments was examined on all leaf levels except leaf level 1 and 2 with a *P. viticola* inoculation 3 dat with *B. bassiana.*

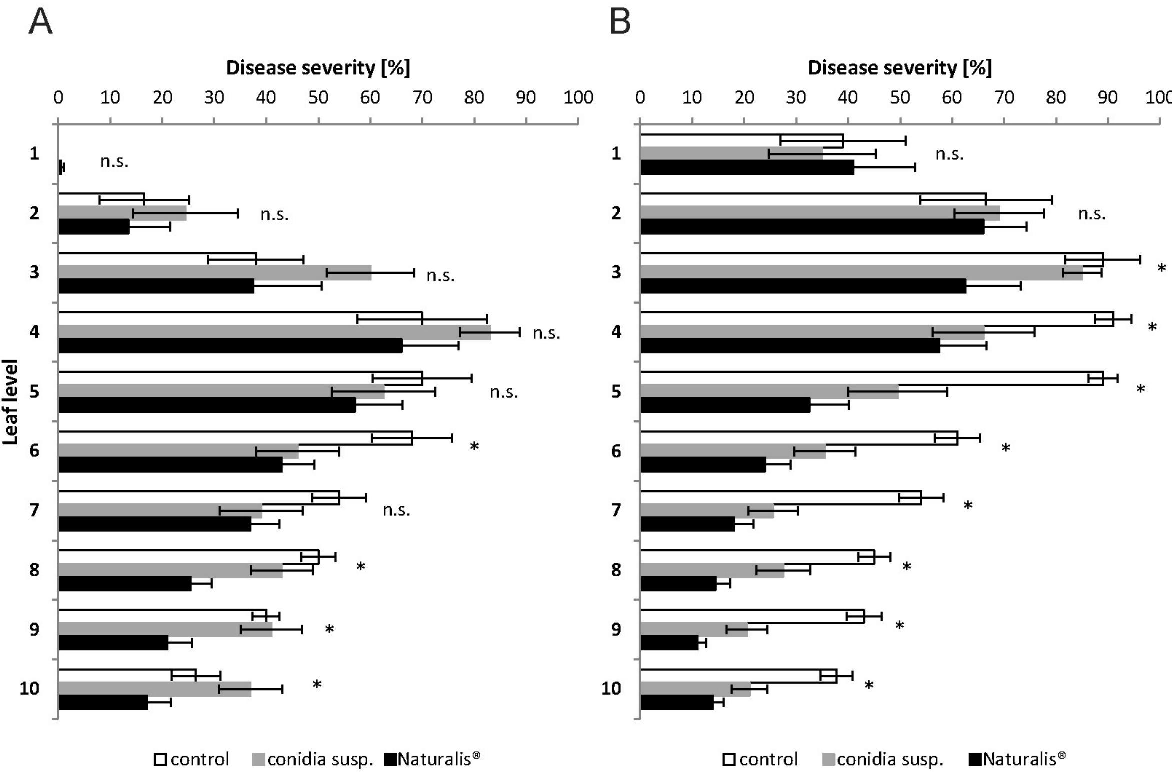

Figure 11: Mean percentage of downy mildew disease severity (+/-SE) of ten leaves of grapevine plants treated with *B. bassiana* (conidia suspension or Naturalis®) A) 3 und B) 7 days before the inoculation with *P. viticola*. Leaves were examined from the upper (1) to the lower (10) leaf level of the plants (see Figure 10). Asterisks indicate significant differences between the treatments with $p \leq 0.05$ (*). Experimental replicate of July 2013.

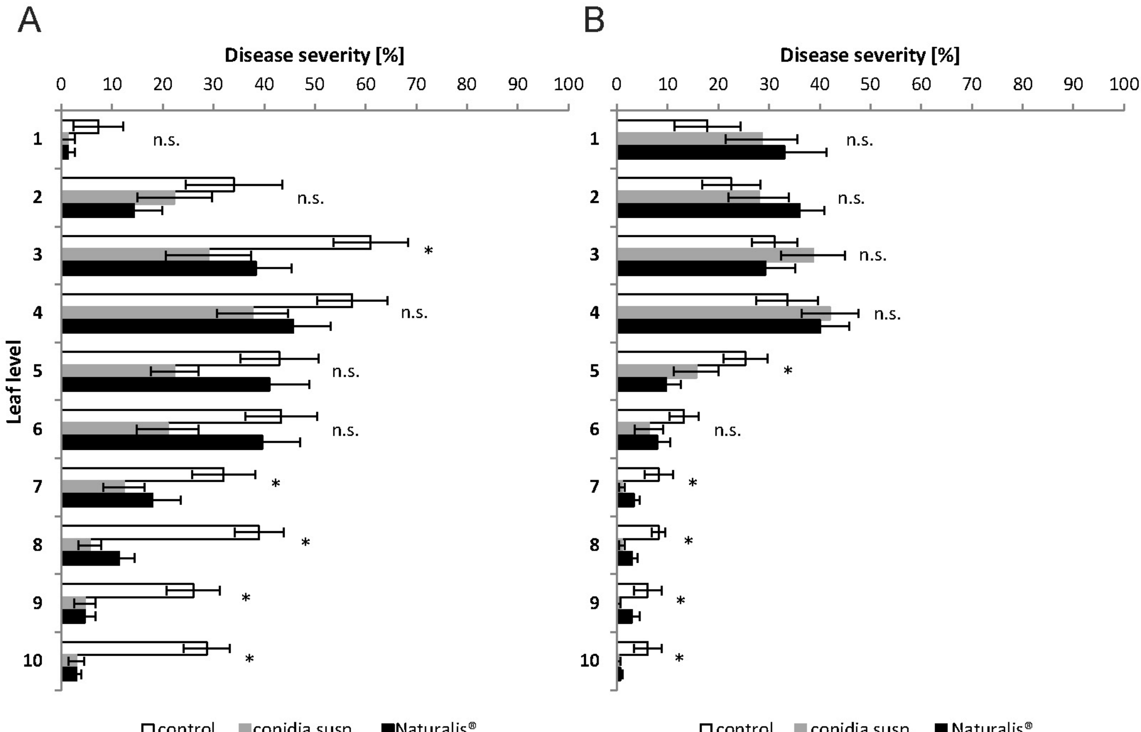

Figure 12: Mean percentage of downy mildew disease severity (+/-SE) of ten leaves of grapevine plants treated with *B. bassiana* (conidia suspension or Naturalis®) A) 3 und B) 7 days before the inoculation with *P. viticola*. Leaves were examined from the upper (1) to the lower (10) leaf level of the plants (see Figure 10). Asterisks indicate significant differences between the treatments with $p \leq 0.05$ (*). Experimental replicate of autumn 2013.

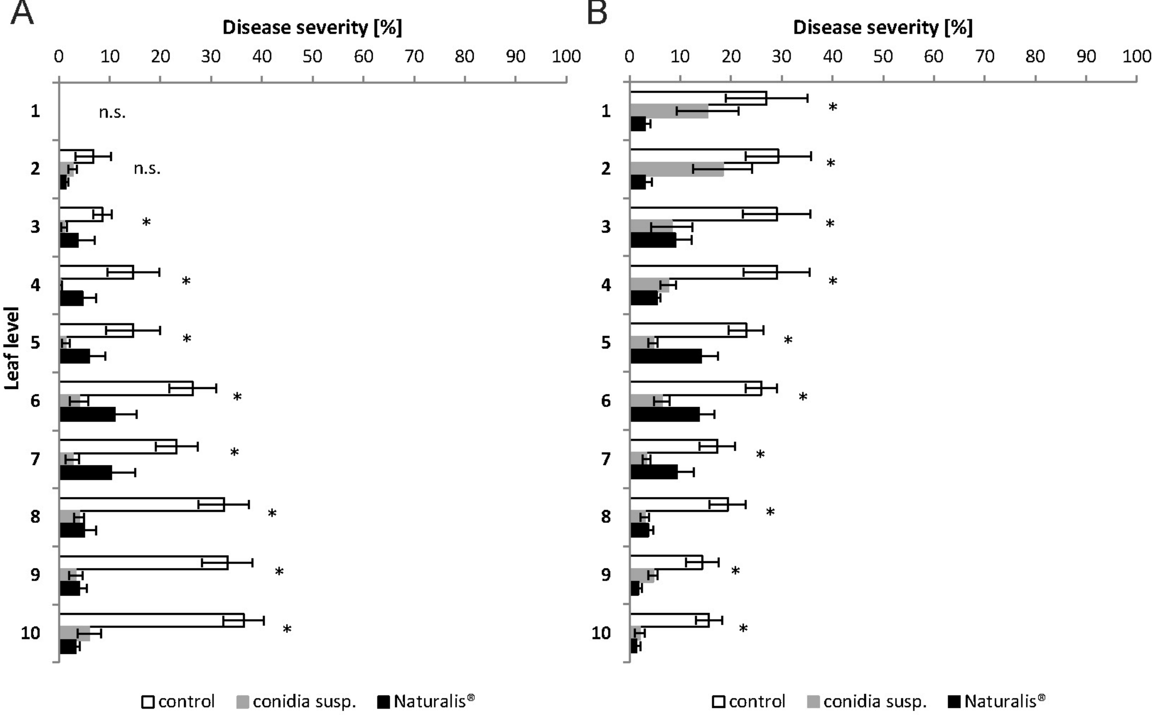

Figure 13: Mean percentage of downy mildew disease severity (+/-SE) of ten leaves of grapevine plants treated with *B. bassiana* (conidia suspension or Naturalis®) A) 3 und B) 7 days before the inoculation with *P. viticola*. Leaves were examined from the upper (1) to the lower (10) leaf level of the plants (see Figure 10). Asterisks indicate significant differences between the treatments with $p \leq 0.05$ (*). Experimental replicate of July 2014.

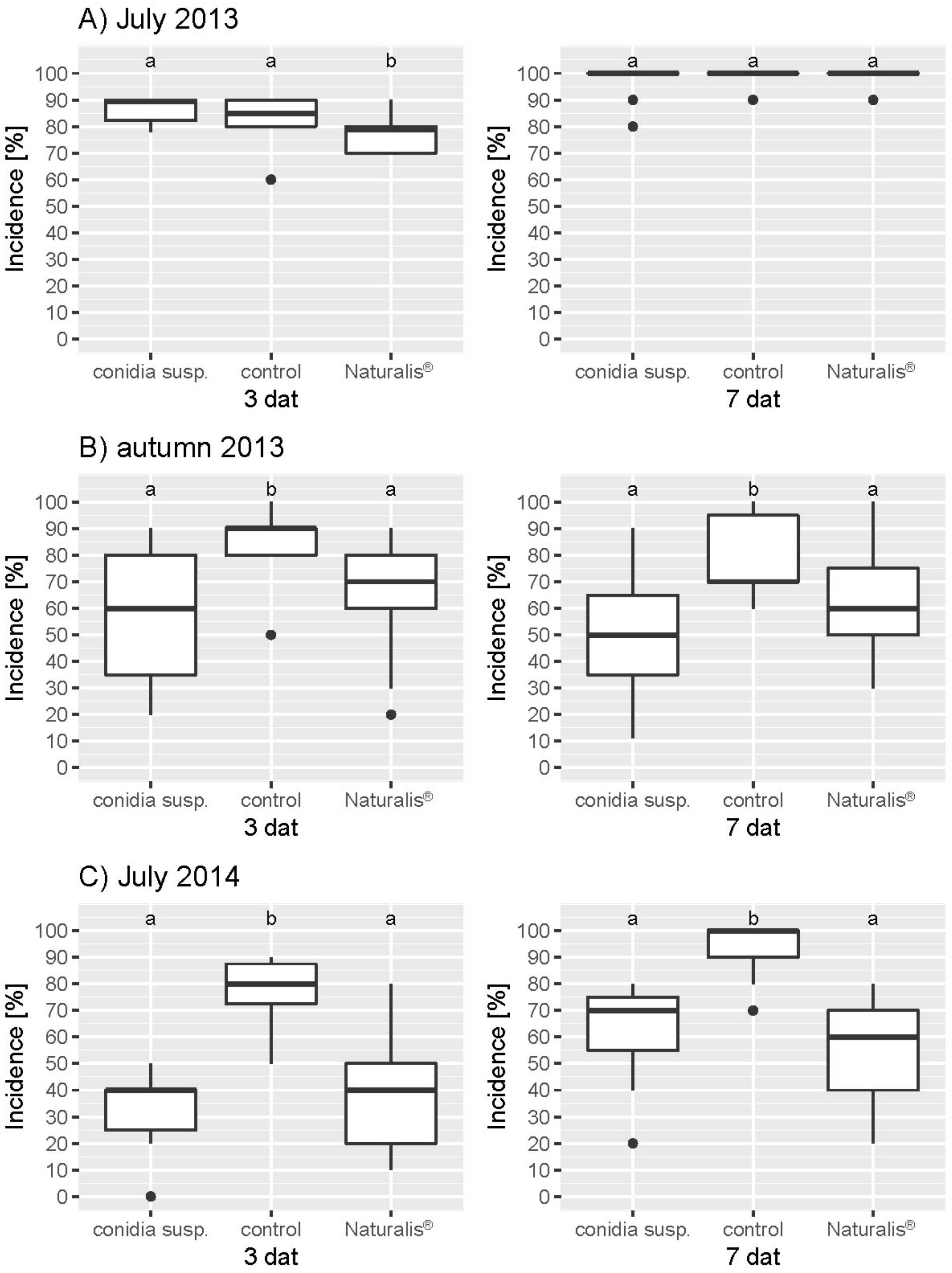

Figure 14: Boxplots of percentage disease incidence of grapevine plants inoculated with *P. viticola* 3 und 7 days after a treatment with endophytic *B. bassiana* (conidia suspension or Naturalis®). Control plants were only inoculated with *P. viticola*. Different letters indicate significant differences between the treatments at $p<0.05$ (Dunn's multiple comparison after Kruskal-Wallis rank sum test). Experimental replicates of A) July 2013, B) autumn 2013, and C) July 2014.

Incidence of downy mildew (percentage of leaves with visible sporulation) was significantly reduced following a treatment with endophytic *B. bassiana*. In experimental replicate of July 2014 (Figure 14 C) disease incidence of grapevine plants after treatments with Naturalis® or conidia suspension differed significantly from control plants for both inoculation time points (3 dat: $p = 6.58 \times 10^{-05}$ and $p = 6.57 \times 10^{-06}$, 7 dat: $p = 1.78 \times 10^{-06}$ and $p = 1.31 \times 10^{-04}$, respectively). Also in experimental replicate of autumn 2013 (Figure 14 B) disease incidence of grapevine plants after treatments with Naturalis® or conidia suspension differed significantly from control plants for both inoculation time points (3 dat: $p = 0.00781$ and $p = 0.00368$, 7 dat: $p = 0.0214$ and $p = 0.000840$, respectively). In experimental replicate of July 2013 (Figure 14 A) disease incidence of grapevine plants only differed significantly from control plants after treatment with Naturalis® for inoculation time point 3 dat ($p = 0.0484$).

Table 7: Mean efficiency according to Abbott against downy mildew on grapevine leaves by a treatment with *B. bassiana* (conidia suspension or Naturalis®) 3 und 7 days before the inoculation with *P. viticola* in three different experimental replicates (July 2013, autumn 2013 and July 2014).

	Mean efficiency according to Abbott			
	3 dat		**7 dat**	
	conidia susp.	**Naturalis®**	**conidia susp.**	**Naturalis®**
July 2013	3.45	32.13	43.15	61.82
autumn 2013	64.63	45.68	28.31	26.43
July 2014	89.03	72.06	78.41	67.89

The calculated mean efficiencies according to Abbott (Table 7) confirm the observations of a reduction in downy mildew disease severity and disease incidence due to treatment with endophytic *B. bassiana.* For data obtained in the last experimental run in July 2014, the efficiencies differed between 67 and almost 90%, despite a low disease pressure of *P. viticola.* In the previous runs carried out in 2013, the efficiencies were significantly lower, usually below 50%. The efficiencies were averaged over all leaves, thus including also the upper leaves which were probably not colonized with *B. bassiana.*

5.3.3 Changes in expression patterns after treatment with *B. bassiana*

The Affymetrix GeneChip® *V. vinifera* genome array represents comprehensive parts of the 30,344 genes predicted in *V. vinifera.* It consists of 16,436 probesets: 14,496 derived from *V. vinifera* transcripts and 1940 derived from other *Vitis* species or hybrids transcripts. The design of the *Vitis* GeneChip® is based on sequences selected from GenBank, dbEST, and NCBI Reference Sequences (RefSeq).

With microarray analysis changes in global gene expression of *B. bassiana* treated grapevine plants were found both 24 and 168 h after treatment. Differences in gene expression between *B. bassiana* treated and control leaves allowed the identification of differentially expressed genes which were up-regulated or down-regulated as a response to endophytic *B. bassiana.* Overall, the transcriptional response of grapevine plants due to treatment with *B. bassiana* was higher 24 hpt and declined 168 hpt. At 24 hpt 65 transcripts of the 16,436 analyzed transcripts were significantly up-regulated (fold change in gene expression levels >2), and 25 transcripts were significantly down-regulated (fold change <-2) compared to the control plants. Whereas 168 hpt with *B. bassiana* only 14 genes were significantly induced (fold change >2) and 14 genes were repressed (fold change <-2) in response to endophytic *B. bassiana.* The strongest transcriptional response of grapevine plants was evident at an early stage of the colonization process (24 hpt) with *B. bassiana* with 16 transcripts being significantly up-regulated with a fold change greater than 3 and two transcripts being significantly down-regulated with a fold change lower than -3 compared to the control plants. One transcript with unknown gene ontology was up-regulated more than six-fold as a response to *B. bassiana* treatment. 168 hpt, no transcript showed higher changes than three in the expression level due to *B. bassiana* treatment. The significantly regulated genes (factor > 2 and < -2 against the control) 24 and 168 hpt with *B. bassiana* are reported in Table 9 and Table 10 (supplementary material).

Most of the genes associated to biological processes like defense response or response to biotic stimulus had similar expression patterns as a result of treatment with *B. bassiana* 24 and 168 hpt. However, some genes like the pathogenesis-related protein 10.3 (RefSeq Protein ID XP_002274483), major allergen Pru ar 1-like (RefSeq Protein ID XP_002274785) and disease resistance response protein 206-like (RefSeq Protein ID XP_002266825) were induced 24 hpt with endophytic *B. bassiana.* In addition we found that the expression level of genes encoding pathogenesis-related proteins PR-2 (b-1,3-glucanases; RefSeq Protein ID XP_002277446), PR-3 (chitinases; e.g. RefSeq Protein ID XP_002266583), and PR-5 (thaumatin-like proteins; e.g. RefSeq Protein ID XP_002274443) increased upon treatment with *B. bassiana* within 24 h. However, expression of protein PR-1-like (RefSeq Protein ID XP_002276867) was repressed in grapevine plants 24 hpt with endophytic *B. bassiana.* While expression of all four genes was not significantly affected after 168 hpt with *B. bassiana*, pathogenesis-related protein PR-1 (RefSeq Protein ID XP_002273788) was significantly down-regulated in grapevine plants as a response to treatment with endophytic *B. bassiana.* Also, various genes involved in stilbene synthesis and related genes (e.g., RefSeq Protein ID XP_002269293 or XP_003634066), which are predominantly categorized to the GO response to stress, were up-regulated 24 hpt with endophytic *B. bassiana.*

Gene Ontology slim tools were used to identify major groups of biological processes affected by treatment with endophytic *B. bassiana*. Main groups of GO slim classes associated to significantly regulated genes are represented as a bar chart in Figure 15. 168 hpt only nine (4 induced, 7 repressed) GO slim categories were influenced by treatment with *B. bassiana*, whereas 24 hpt all 14 categories were influenced (9 induced, 11 repressed). Categories involved in anatomical structure morphogenesis, cell death, flower development, and signal transduction were only represented by inhibited genes 24 hpt. As highlighted in Figure 15, some GO slim categories were mostly represented by induced genes 24 hpt with *B. bassiana*. In particular, more genes categorized to metabolic process (GO:0008152), response to stimulus (GO:0050896) and response to stress (GO:0006950) were up-regulated than down-regulated. These were also the three processes, which were most strongly affected by treatment with endophytic *B. bassiana*, with each process representing around 20% of all significantly regulated and assigned transcripts.

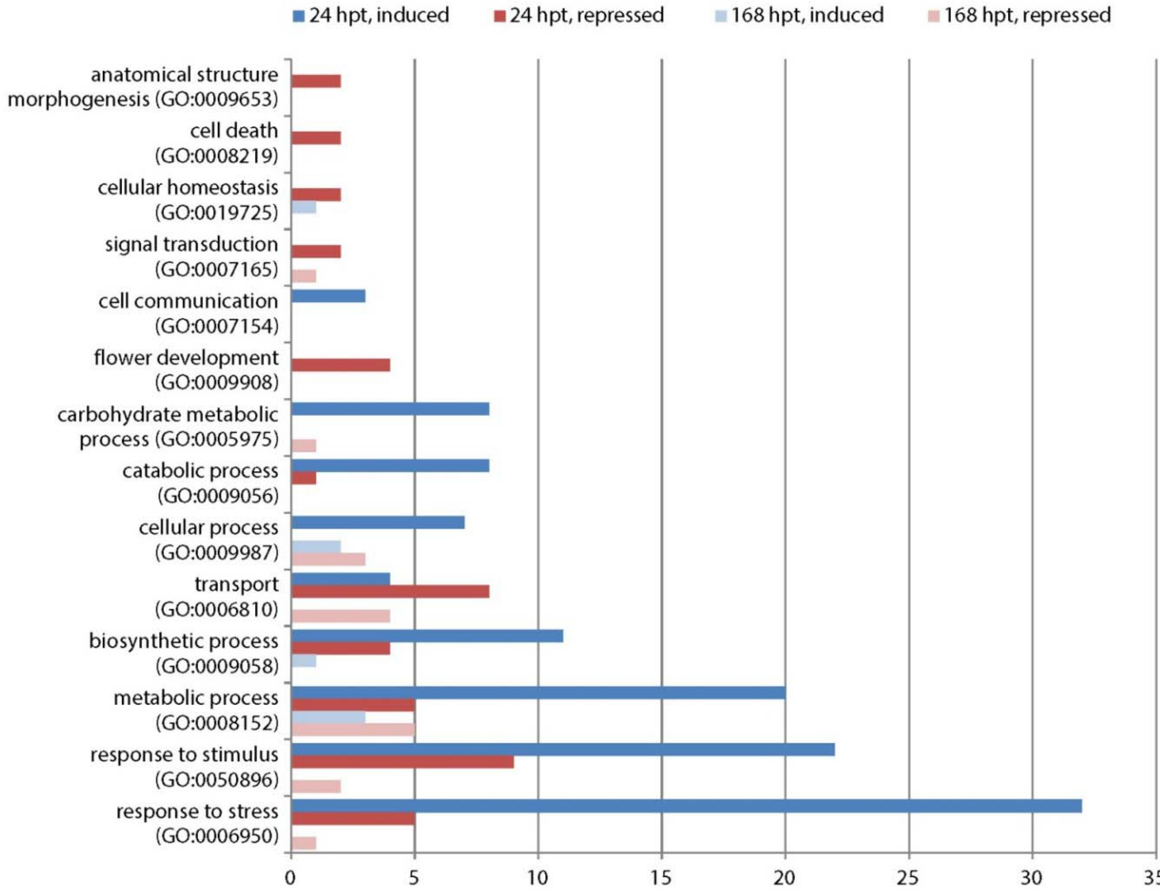

Figure 15: Main groups of GO slim classes (y-axis labels, ordered regarding their impact level) concerning the biological processes affected in grapevine plants after treatment with *B. bassiana*. The figure shows the number of transcripts assigned to terms of Gene Ontology biological processes induced or repressed by *B. bassiana* treatment 24 hpt and 168 hpt. GO slim classes with N=1 are not shown.

In a second experimental approach, differences in expression levels of three selected defense-related genes were assessed by RT-qPCR in grapevine plants after treatment with *B. bassiana* (24, 72 and 168 hpt). The three designed primer pairs showed adequate performance in amplification, in melt curve analysis and in investigation of the efficiency by means of standard curves. A combination of the two grapevine housekeeping genes (GADPH and actin), was found to be suitable as reference for normalization of gene expression ($M = 0.850$, $CV = 0.299$ and $M = 0.850$, $CV = 0.293$, respectively). After normalization and correction of the differences between the individual reaction plates, only the PR1-like gene showed a significant difference in the expression levels between *B. bassiana* treated and control plants 24 and 168 hpt (Table 8). These findings confirm the result of the microarray analysis, in which the transcript of this gene was also down-regulated by a factor of three 24 hpt.

Table 8: Mean [95% CI] expression levels of three genes 24, 72 and 168 hpt of grapevine with *B. bassiana* analyzed with RT-qPCR. Asterisks indicate a significant difference between treated and control plants with $p \leq 0.05$ (*)

	24 hpt			72 hpt			168 hpt		
	conidia susp.	**control**	**p-value**	**conidia susp.**	**control**	**p-value**	**conidia susp.**	**control**	**p-value**
PR-1-like	0.151 [0.102, 0.225]	0.54 [0.263, 1.109]	0.01119*	0.531 [0.114, 2.477]	1.555 [1.007, 2.402]	0.3618	9.021 [6.283,12.95]	2.590 [0.915, 7.335]	0.04545*
beta-1,3-Glucanase	1.589 [1.09, 2.317]	0.667 [0.364, 1.258]	0.06014	0.924 [0.409, 2.087]	1.015 [0.562, 1.835]	0.9551	1.341 [1.047, 1.719]	0.963 [0.545, 1.700]	0.2695
ATPase	0.851 [0.573, 1.263]	1.259 [0.867, 1.829]	0.152	0.937 [0.578, 1.52]	0.896 [0.505, 1.591]	0.9551	1.431 [0.711, 2.879]	0.985 [0.407, 2.384]	0.3939

5.4 Discussion

Here, we showed that endophytic *B. bassiana* is able to reduce downy mildew disease severity and incidence on grapevine plants and that its colonization triggers the plant's inherent defense system. The results of this study add to the increasing evidence of supplementary positive effects of entomopathogenic fungi when present as an endophyte in crop plants as already described by Vega et al. (2009) and Vidal (2011). In addition to the recently proven antagonistic effect of endophytic *B. bassiana* against grapevine insect pests (Rondot and Reineke 2017, 2018), a protective effect of endophytic *B. bassiana* against the causal agent of grapevine downy mildew, *P. viticola,* was evident. In line with results reported by Jaber (2015), both the disease incidence and severity on grapevine plants were reduced following treatment with *B. bassiana*. Grapevine plants were treated with *B. bassiana* before the infection with *P. viticola*. Therefore, our experimental set-up only allows an assessment of the protective potential of *B. bassiana*. As explained by Gessler et al. (2011), it is hard to control *P. viticola* with biocontrol antagonists after infection because the fungus quickly penetrates and develops inside host tissues.

The observed positive effect of a treatment with *B. bassiana* against downy mildew was particularly noticeable in older leaves and after a longer establishing period of seven compared to three days. A period of seven days between the treatment with *B. bassiana* and the inoculation with *P. viticola* also showed substantial reductions in disease severity in the study by Jaber (2015). These observations, therefore, indicate the necessary time period the fungus *B. bassiana* needs for endophytic establishment. Accordingly, the state of plant development and leaf growth must be taken into account, when considering the protective effects of a treatment with *B. bassiana* against any fungal pathogen. To our knowledge, leave susceptibility and timing of the treatment were not yet considered in previous experiments regarding the tritrophic interaction between grapevine plants, endophytes, and phytopathogens. However, on leaves developed after the treatment with *B. bassiana*, downy mildew disease severity was not significantly reduced, supporting previous studies, where systemic colonization of grapevine plants by *B. bassiana* could not be detected (Rondot and Reineke 2018).

With a reduction of 3 to 89 % of *P. viticola* disease severity by colonization with endophytic *B. bassiana*, efficiencies are lower than those reported and expected for a treatment with synthetic fungicides. Our findings showed a higher variation in downy mildew disease reduction of *B. bassiana* strain ATCC 74040 than observed by Jaber (2015) but are in line with research results of other potential microorganisms against *P. viticola* reviewed by Gessler et al. (2011). Despite an overall good activity of the respective microorganisms, they were not

capable of completely controlling downy mildew disease. As already mentioned above, one reason for this can be found in the nature of *P. viticola*, which penetrates the leaves very rapidly through the stomata. Moreover, most of the microorganisms tested so far are insufficient to control this disease, because they have a low persistence with only short periods of activity after application or are easily washed off by precipitation (Pertot et al. 2017). The endophytic lifestyle of *B. bassiana* in grapevine plants may accordingly lead to longer persistence and thus longer periods of activity. However, this assumption warrants further studies.

In the present constellation of an endophytic entomopathogenic fungus, grapevine and downy mildew, the mechanisms underlying the protective potential of the endophyte against the pathogen are not yet known. In general, the following mechanisms are possible: antibiosis, competition for space and nutrients, parasitism, and induction of plant defense. Since it has been shown that the fungus colonizes the plants, competition for space or resources might be involved in the protection mechanism. Successful competition depends on both timing and magnitude of colonization as resources and nutrients are supposed to go to the initial and best plant colonizer. In the present study, plant colonization with *B. bassiana* was confirmed by the time of plant inoculation with *P. viticola* (3 dat and 7 dat) as well as the latest date of disease assessment (14 dat). Although percent colonization of plants differed among the replicates and the tested treatments, *B. bassiana* was able to provide protection against downy mildew in all experiments. A direct linkage between colonization rate and disease severity was not possible due to methodical aspects. However, this supports the hypothesis of Vega et al. (2009) and Ownley et al. (2010) that multiple mechanisms of biocontrol might be operating in *B. bassiana*-colonized plants.

Activation of plant-mediated systemic resistance could be another possible mechanism of suppression of *P. viticola* in *B. bassiana*-colonized grapevines. Griffin et al. (2006) and Ownley et al. (2008) suspect this mechanism to operate against *Xanthomonas* spp. in cotton seedlings after treatment with endophytic *B. bassiana*. A similar ability to induce systemic resistance in grapevine plants against grapevine downy mildew, the disease investigated here, has already been demonstrated for other non-pathogenic fungi such as *Trichoderma harzianum*, which caused a direct modulation of defense-related genes and the activation of priming (Perazzolli et al. 2008). However, in contrast to our findings, where we could not detect a systemic effect of *B. bassiana* against downy mildew, in the aforementioned study, homogeneous disease resistance was observed, independent of leaf position.

In the present study, we observed that a set of genes in grapevine leaves were up- or down-regulated following the treatment with endophytic *B. bassiana*. Genes associated to biological processes like defense response or response to biotic stimulus are of particular interest for

understanding the tritrophic interaction between grapevine and endophytic *B. bassiana* and its antagonistic potential against insects or pathogens. We observed that the expression level of genes encoding PR-2 (b-1,3-glucanases), PR-3 (chitinases), PR-5 (thaumatin-like proteins), and the PR-protein 10.3 were increased upon the treatment with *B. bassiana* within 24 h after treatment. These results confirm previous reports that these genes are involved in the defense response of vines to infestation with pathogens (Enoki and Suzuki 2016; Fung et al. 2008; Albertazzi et al. 2009; Jacobs et al. 1999; Ferreira et al. 2004; Kortekamp 2006; Adrian et al. 2012) and are also related in response to colonization by biotrophic fungi (Perazzolli et al. 2008). In addition, other defense-related genes like genes associated to the stilbene synthesis and related genes were found to be regulated. Stilbenes are also known to be involved in the plant-pathogen interaction of grapevine (Schnee et al. 2008; Olivier et al. 2018; Adrian et al. 2012), therefore providing indications of the interaction between endophytic fungus and grapevine plant. Induced expression of key defense genes strongly suggested that a defense response was activated in grapevine plants due to treatment with endophytic *B. bassiana*. However, most of the genes were only regulated 24 hpt and expression of defense-related genes, including PR genes and genes associated to the stilbene synthesis, declined within 168 hpt. Similar results are reported for the grapevine reaction to powdery mildew by Fung et al. (2008), who observed the expression of defense-related genes and secondary metabolite biosynthesis genes to reach a maximum level at 12 hours post inoculation and then declined. Such a decline in expression of defense-related genes suggests that the plant and *B. bassiana* may establish a symbiotic relationship. Perazzolli (2008) emphasizes the importance of repeated applications of *T. harzianum* T39 to significantly induce plant resistance against downy mildew in grapevine plants. A similar approach with *B. bassiana* remains to be investigated. We already found significant reductions of downy mildew with a single protective treatment of endophytic *B. bassiana*. Yet, we did not analyze the expression level of defense-related genes after a post treatment inoculation with *P. viticola*. So we can only hypothesize if grapevine enters a ‘‘primed state’’ that results in broad-spectrum resistance to pathogens, insects, or abiotic stress as described by Conrath et al. (2006).

Successful plant protection strategies against *P. viticola* based on microorganisms will need to target *P. viticola* at multiple sites and multiple stages of its life cycle (Vecchione et al. 2007). *B. bassiana,* with its diverse roles and multiple modes of action, could represent one component in such a strategy with the potential of reducing frequencies of chemical pesticide applications. Its ability to act both as an epiphyte and endophyte with effects against both insect pests and pathogens might be the answer to overcome the poor persistence and efficacy of other microorganisms used as a biocontrol control agents, which are applied on the leaf surface. Nevertheless, for optimal exploitation of *B. bassiana* as part of a new and sustainable

plant protection strategy in viticulture, an identification of the relevant operating mechanisms is required.

Acknowledgments

This work was financially supported by Deutsche Bundesstiftung Umwelt (DBU). We thank Edith Ladurna from CBC Europe S.r.l. (Italy) for providing the product Naturalis® and Dustin Kulanek for help in the performance of the RT-qPCRs. We kindly acknowledged Dominik Schmidt for support with the GO slim tools and Matthias Olberz for assistance in statistical analyses in 'R'.

6 General discussion

The increased awareness of additional roles that entomopathogenic Hypocreales may play in the ecosystem led to numerous research projects during the last years. Particularly, the endophytic lifestyle is viewed as a promising function with enormous potential in the development of novel integrated crop protection tools and as a component of environmentally friendly pest management strategies. However, despite the newly recognized importance of this additional role much remains unknown about the ecology and environmental interaction of endophytic entomopathogens. For instance, only limited information is available on the tritrophic interaction between entomopathogenic endophytes, grapevine plants, and potential target insect pests as well as fungal pathogens. Therefore, the objective of the present thesis was to investigate whether the fungus *B. bassiana* is able to colonize grapevine plants, still maintains its entomopathogenic habit against insect pests, and has additional antagonistic potential against fungal pathogens. Additionally, this thesis focused on the plant response to endophytic colonization on gene level. The investigations were carried out with regard to the development of potential biological control strategies.

The results of the three conducted studies were already discussed in the respective manuscripts. Here, the main findings and conclusions will be summarized again by referring back to the objectives (see chapter 2). Furthermore, additional aspects will be emphasized based on limitations as well as applications and implications of the studies to associate the experiments to the context of current research. Therefore, this supplementary discussion focuses on three aspects: (1) alternative strategies for and restrictions in the endophytic establishment of *B. bassiana* in grapevine plants; (2) challenges in characterization of the antagonistic potential of endophytic *B. bassiana*; (3) potential applications of the experimental results in viticultural practice. Finally, future prospects for research objectives are given.

Alternative strategies for and restrictions in the endophytic establishment of *B. bassiana* in grapevine plants

The aim of this thesis was to optimize the endophytic establishment of *B. bassiana* in grapevine plants via artificial application. Amongst others, the endophytic establishment of entomopathogenic fungi is known to be dependent on plant cultivar and fungal strain (Vidal and Jaber 2015). For both experimental sites, the greenhouse and the field, we used *Vitis vinifera* cv. 'Riesling' as these plants are most frequently planted in the German Rheingau region where the experiments were carried out. Since the identification of strains suitable for

endophytic establishment is time-consuming, we decided to focus on the already registered and formulated strains ATCC 74040 (product Naturalis®) and GHA (product Botanigard®). Strain ATCC 74040 was also reported to be the strain with the highest colonization rates by Jaber (2015), who compared the endophytic establishment in grapevine plants of four different *B. bassiana* strains. Another possibility to achieve a successful endophytic establishment is to select a suitable inoculation method. Since plants used for our greenhouse trials were cultivated from hardwood cuttings according to common practice for cultivation and grafting of grapevine plants in nurseries, seed treatment as an alternative inoculation method as it has been successfully shown for tomato, cotton (Ownley et al. 2008), opium poppy (Quesada-Moraga et al. 2009) and sorghum (Tefera and Vidal 2009) was not possible. Inoculation trials via root dipping or soil inoculation by drenching or mixing conidia containing material in the planting substrate resulted in no colonization of the leaves (own unpublished results). Therefore, inoculation via spray application is apparently the only option for endophytic inoculation of grapevine plants/leaves and in addition simple to implement in viticultural practice due to already existing spray equipment for other pesticides.

Greenhouse and field experiments showed that endophytic establishment by the entomopathogenic fungus *B. bassiana* in potted and mature grapevine plants after artificial spay inoculation is possible. Although *B. bassiana* is reported to colonize some plants, i.e. opium poppy and corn, systemically (Quesada-Moraga et al. 2006; Quesada-Moraga et al. 2009; Landa et al. 2013; Wagner and Lewis 2000), in all of our studies we could not detect systemic colonization by *B. bassiana* in the grapevine plants. In contrast to the plants referred to in the aforementioned studies, grapevines are deciduous, woody perennial plants, which might be the reason for the different behavior of *B. bassiana* inside this crop plant. As already pointed out by several authors, many endophytes of leaves or other plant tissues are host, host genus or host family specific (Arnold 2007; Hyde and Soytong 2008). Further tests regarding the optimization of endophytic establishment of *B. bassiana* in grapevine, therefore, should consider other grape varieties as well as different fungal strains. Although, the literature indicates a frequent association of *B. bassiana* with multiple plant species, with reports of isolation from different plant organs (see Table 1), the genotypic basis – of the fungus as well as of the host – for endophytism and the biological mechanism involved in proliferation within a host plant have not been elucidated. As soon as the plant recognizes the presence of a potential fungal invader, defense pathways are activated, leading to the suppression or death of the fungus (Dangl and Jones 2001). Therefore, fungal endophytes must somehow be able to indicate that they are not pathogens (Redman et al. 2001; Rodriguez et al. 2009). The endophyte may be restricted in terms of distribution and metabolic activity within plant tissues; remain localized in a nearly dormant phase or proliferate systemically throughout multiple tissues of the host (Rodriguez et al. 2009). Studies reporting the exact location of the

fungi within plant tissues are extremely rare. So far, colonization by endophytic *B. bassiana* is reported to occur through intercellular spaces and vascular xylem elements directly after the fungus has penetrated the plant epidermis (Wagner and Lewis 2000; Quesada-Moraga et al. 2006; Landa et al. 2013). The attempt to visualize the endophytic colonization of *B. bassiana* in *Z. mays* by light and electron microscopy demonstrated fungal colonization of inoculated plant tissue (Wagner and Lewis 2000; Gómez-Vidal et al. 2006). However, potential other resident fungal endophytes were neither identified nor differentiated from the inoculum. More recently, *B. bassiana* isolate EABb-04/01-Tip was transformed with a green fluorescent protein (GFP) to visualize association with opium poppy using a confocal microscope (Landa et al. 2013). During a stay at Rusty Rodriguez' company Adaptive Symbiotic Technologies, Seattle (USA) within a short term scientific mission (STSM) of the Cost Action Fa1103 'Endophytes in Biotechnology and Agriculture' we also tried a GFP-transformation of *B. bassiana* strain ATCC 74040 according to a method described by Maciá-Vicente et al. (2009) to visualize the fungus *in planta* (own unpublished results). The transformation by electroporation of the *Agrobacterium tumefaciens* AGL-1 strain was successful. However, it was not possible to select the transformed colonies from untransformed ones with the method of choice because of an existing tolerance of the used *B. bassiana* strain against hygromycin – the agent chosen for the selection. Due to time restriction, an adaption of the method could not be realized during the stay. Although, Quesada-Moraga et al. (2006) and Landa et al. (2013) predominantly observed hyphal growth on or near epidermal cells and not into stomata, Jaber (2015) hypothesizes the survival of *B. bassiana* in substomatal chambers of the leaves as was described for a strain of *Burkholderia* sp when colonizing grapevines (Compant et al. 2005; Compant et al. 2008).

Summarized over all conducted experiments, we found plant colonization by endophytic *B. bassiana* to be highly inconsistent even with high inoculum loads. According to Wagner and Lewis (2000), who observed the mode of penetration of *B. bassiana* conidia into the leaves of maize, approximately 3% of the applied conidia germinate and less than 1% succeeded to penetrate the leaf surface. Also, Quesada-Moraga et al. (2006) rarely observed germinating and penetrating conidia on the leaves of opium poppy, *Papaver somniferum.* With a Color 3D Laser Scanning Microscope, we observed conidia on the abaxial and axial surface of grapevine leaves after application, hypothesizing that they belong to *B. bassiana* (own unpublished results). In addition some hyphae, but no germinating conidia were detected. However, it was not possible to definitively differentiate between other fungal residues and the inoculum fungus. The endophytic growth and occurrence of *B. bassiana* in the plant that was detected in our studies as well as in many other reports appears to be opportunistic (random) irrespective of the used inoculation method (Wagner and Lewis 2000; Quesada-Moraga et al. 2006; Behie et al. 2015; Landa et al. 2013; McKinnon et al. 2017). Despite the comparatively low endophytic

colonization rates of *B. bassiana* in grapevine, we observed antagonistic effects of endophytic *B. bassiana* on vine mealybug larvae, black vine weevil, and downy mildew. As will be further addressed in the next section of the discussion, not all of these effects can be assigned exclusively to the endophytic lifestyle of *B. bassiana*.

Challenges in characterizing the antagonistic potential of endophytic *B. bassiana*

The study of endophytes is generally regarded as method-dependent, thus problematic and accompanied by some flaws (Hyde and Soytong 2008). The main challenge of endophyte research is to differentiate endophytes from epiphytes and to assign observed effects to each lifestyle. The widely used surface sterilization is shown to be inadequate or ineffectively to remove epiphytic DNA or inoculum depending on the type of experiment (McKinnon et al. 2017). Therefore it is advised by McKinnon et al. (2017) to consider the fungus present on the plant surface as an epiphyte as an additional contribution to observed effects on plants, plant-associated insects or the plant's microbiome. In the experiments with *P. viticola*, the microarray experiments as well as in the choice assays with *O. sulcatus* we used whole potted grapevine plants, accordingly it was not possible to surface disinfect these plants before usage. Therefore, it is stated that not all effects after spay application of *B. bassiana* reported in this thesis could be assigned to the endophytic lifestyle of the fungus. The possibility that residual *B. bassiana* surviving on the leaf surface as an epiphyte could also have contributed to the observed effects against insects and pathogens cannot be ruled out completely (Rondot and Reineke 2017). However, the persistence of *B. bassiana* conidia in the phyllosphere is very poor due to the high sensitivity of the fungus to ultraviolet radiation (Inglis et al. 1993). A decrease in persistence and viability up to a complete loss of *B. bassiana* conidia in the phyllosphere within few days after application is reported by several authors (Daoust and Pereira 1986; Gardner et al. 1977; Inglis et al. 1993, 1995). Vega (2018) pointed out that amongst others, the susceptibility to ultraviolet (UV) light might have been an impulse to use fungal entomopathogens as endophytes to overcome this characteristic. However, most of our experiments were carried out in a greenhouse roofed with float glass, which absorbs most of the detrimental UV-B waves. Therefore, we cannot assume that residues on the phyllosphere of grapevine plants were limited due to UV-susceptibility, as was hypothesized by Jaber (2015), and shelf life of *B. bassiana* conidia on grapevine stays to be tested under different conditions. As a conclusion, residues on the plant surfaces may provide a greater contribution to observed effects in plants such as growth promotion, indirect effects to insect herbivores or the induction of plants defense system than realized so far. Nevertheless, the combination of an epiphytic and (temporary) endophytic lifestyle, when using *B. bassiana* as an antagonist to pests and pathogens, may optimize overall effectiveness and its full potential value may be

retrieved. We conclude that the present thesis gives hints that *B. bassiana* can be endophytic in grapevine plants and its presence – endophytic as well as epiphytic – negatively influences insect pests and pathogens.

The common infection pathway of the insects by fungal entomopathogens is via cuticular penetration by germinating propagules (Arnold and Lewis 2005). Most of the reports about mycosis due to endophytic entomopathogens are so far restricted to insects living inside plant tissues (Akello et al. 2008b), where direct contact between insects and endophytic fungal propagules are imaginable (Klieber and Reineke 2016). As explained by Vega (2018), there are no studies elucidating why endophyte sporulation should be inhibited inside plants. However, reports about conidia of *B. bassiana* and other entomopathogenic fungi inside plants lack of additional information to give sufficient evidence on infecting propagules and therefore, it remains elusive what mechanism led to reported mycosis in association with endophytes, if no infecting propagules were present (Vega 2018). McKinnon (2017) emphasized that clear verifications are missing in most of the studies, that the endophytic form of the entomopathogens and no residual plant surface inocula caused the infection. This again points to the already mentioned challenge of assigning observed effects to the endophytic lifestyle. Consumption of infected plant material or ingestion of hyphae of endophytic entomopathogens seems to be unlikely to cause an infection (Vidal and Jaber 2015). However, negative effects on insect herbivores performance due to these incidences might be possible, but not well investigated (Vega 2018). Additional effects of endophytic entomopathogenic fungi on insects and possible operating mechanisms are reviewed and summarized amongst others by Vega (2008, 2018) and McKinnon et al. (2017). Whereas Jaber and Ownley (2018) and Ownley et al. (2010) also discuss mechanisms of plant disease suppression. Summarizing, fungal metabolites, produced *in planta* and causing feeding deterrence or antibiosis, are suggested to cause negative effects against herbivorous insect. In addition induced systemic plant resistance is also considered as the mode of action. However, potential effects against plant pathogens are attributed to mycoparasitism, competition and antibiosis directly caused by the endophytic entomopathogenic fungi or those mechanisms mediated through the host plant, like induction of systemic plant resistance or stimulation of plant secondary metabolites. Only recently, a meta-analysis about entomopathogenic endophytes was performed with the aim to identify reasons within the analyzed studies (e.g. experimental conditions, used methods) for the inconsistency of reported effects against herbivores insects (Gange et al. 2019).

The results presented in this thesis suggest that different modes of action accounted for the diverse observed effects on insect performance and behavior as well as on plant pathogens including feeding deterrence, antibiosis or changes in metabolism of the host plant and thus

host plant quality or induction of the plant defense system. Indeed, the results of the microarray analysis indicated that also the host plant transcriptome reacts to the inoculation with endophytic *B. bassiana*, for instance, by the up-regulation of various genes involved in plant defense signaling pathways such as genes encoding for several PR-proteins (b-1,3-glucanases, chitinases, thaumatin-like proteins and PR-protein 10.3) and genes associated to the stilbene synthesis (Chapter 5.3.3). However, the response of the plant to a subsequent inoculation with downy mildew with the aim to detect possible priming mechanism was not analyzed.

Potential applications and practical implementations of the experimental results

The inundative application of EPF in the field still suffers from inconsistency and provides only limited disease control (Vega 2018), presumably because of a lack of understanding their ecology and biology (Roy et al. 2010). In addition, Jackson et al. (2010) pointed out the importance to link the new insight to ecology and biology to production and formulation aspects as well as to consider environmental conditions. Also Lohse et al. (2015) emphasized the importance of an adequate formulation for endophytic establishment of entomopathogenic fungi. To provide a benefit for the colonization process of *B. bassiana* on grapevine plants, we used a commercially available fungal-based product (Naturalis®, active ingredient *B. bassiana* isolate ATCC 74040) formulated as oil dispersion.

However, already more than twenty years ago Waage (1998) explained, why the main mistakes regarding biological control agents are to apply the “chemical model”, to create false expectations of chemical-like efficacy and to under evaluate their properties. Therefore, we suggest that entomopathogenic fungi should be considered as an additional option within integrated crop management strategies rather than to directly replace synthetic pesticides. In this regard, a couple of studies have shown that *B. bassiana* is sensitive against various pesticides (Todorova et al. 1998; Sapieha-Waszkiewicz et al. 2004; Kos and Celar 2013), but combinable/compatible with a range of insecticides (Faraji et al. 2016; Alizadeh et al. 2007) and acaricides (Oliveira and Neves 2004). Due to the high frequencies in the application of fungicides, compatibility with fungicides seems to be most important in viticulture, but also most challenging and depending on the spectrum (range of controlled pathogens) of the used fungicide. Differences in compatibility between broad-spectrum multisite, site-specific systemic and specific action based fungicides depending on the timing of the application would be expected. Application of contact fungicides after the endophytic establishment of *B. bassiana* certainly appears to be conceivable. In our experimental vineyard, an endophytic establishment of *B. bassiana* in the mature plants under field conditions was successful, even though synthetic fungicides against powdery mildew (*Uncinula necator*) were simultaneously

applied (Rondot and Reineke 2018). Possible limitations or synergies of a combination between *B. bassiana* and a diverse range of other plant protection products used in viticulture under laboratory as well as under field conditions remain to be tested. Our results also indicate the potential for a long term establishment of the fungus in grapevine plants and that endophytic establishment does apparently not interfere with common viticultural management (Rondot and Reineke 2018), as inoculation via spray application is simple to implement in viticultural practice due to already existing spray equipment for other pesticides.

A temporal colonization of grapevine plants, activation of the plants defense system or a long term establishment of (endophytic) *B. bassiana* in the ecosystems of the vineyards could improve the overall effect of the treatment as biological control agent and overcome some of their limitations addressed by several authors (Butt and Copping 2000; Copping and Menn 2000; Lacey et al. 2015). Our results showed a limited, but significant effect of endophytic *B. bassiana* on the performance of vine mealybug *P. ficus* and the leafhopper *E. vitis* as well as an impact on host choice behavior of *O. sulcatus* (Rondot and Reineke 2017, 2018). In addition, we observed a reduction in disease incidence and severity of downy mildew *P. viticola* due to a protective treatment with *B. bassiana.* (see chapter 5) However, in a preliminary field trial with repeated curative applications, we could not confirm the suppressive effect on *P. viticola* (own unpublished results) and point to the still existing potential for optimization under field conditions. Even though results from our studies allow tantalizing glimpses on the potential of *B. bassiana* in viticulture with various effects on different trophic levels, the long term impact on grapevine, the surrounding agroecosystem and associated (micro)organisms remains elusive. However, due to its multi-layer effects, the utilization of *B. bassiana* in vineyards as well as vine nurseries is imaginable with the restriction of conducting further investigations.

Finally, whether the presence of endophytic *B. bassiana* in grapevine plants has an effect on the quality and sensory attributes of must and wine still remains to be investigated with fruit-bearing grapevine plants (Rondot and Reineke 2018). The need to consider these and further “unusual impacts” was also addressed by Vega (2018) in his review. To our knowledge, sensory attributes and valuable/secondary compounds as important parameters of the inner plant quality are issues not yet attributed in connection with *B. bassiana* as an endophyte in plants giving first contents for future endophyte research in addition to those prospects addressed in the next section.

Future prospects of endophyte research

In this thesis, different dimensions of the multitrophic interaction between endophyte, plant, and pest/pathogen were addressed, however much remains elusive. Some research gaps,

particularly those regarding the endophytic establishment of *B. bassiana* in grapevine plants and its consequences, were already indicated in the previous sections and should be integrated into future studies. Furthermore, additional, more general prospects in endophyte research remain to be elucidated and are described hereafter. As already pointed out previously, endophyte research is challenging, complex, and method-depending. Supporting evidence for method dependency of reported results and the influence of experimental design comes from the meta-analysis conducted by Gange et al. (2019). Therefore, first of all, methods and protocols for determining endophytism, the ecology of endophytes and their effects must be validated and adapted to prevent ambiguity of reported results. The need for stringent protocols is also emphasized by McKinnon et al. (2017), who reviewed current methods and elucidated associated pitfalls.

Although many studies on the endophytic establishment of *B. bassiana* were conducted in the past years (see Table 1, chapter 1), further studies are certainly warranted to explore the impact of endophytic entomopathogens on multitrophic levels. Previous studies primarily aimed at introducing those fungi into a wider array of plants or focused on their potential activity against insects and plant pathogens when *in planta*. However, evidence has accumulated that there is an increasing relevance to understand their ecology and complete life history in association with plants (Vega et al. 2009; Roy et al. 2010; Lacey et al. 2015). Thus the understanding and the optimization of conditions and mechanisms underlying fungal endophytism as well as the response of the plant, herbivorous insects and plant pathogens need to be the focus of future research efforts (Vega 2018). Summarizing, the following issues, amongst others, within the different multitrophic levels should be further addressed:

- Understanding the conditions that facilitate, as well as those that impede **endophytic colonization** by fungal entomopathogens. This includes monitoring fungal habits and the extent and persistence of endophytic fungal colonization within the plant. Exploring the reasons for variation in plant colonization ability of fungal entomopathogens could contribute to reproducible introductions into crops and to precise predictions of their outcome.
- Characterization of the range of **host plant responses** to the endophytic establishment, including the production of secondary metabolites, shifts in volatile profile, and induction of transcriptional changes. To provide more detailed data on gene expression by the plant, studies using next-generation sequencing technology (RNAseq) could be conducted.
- Modifications in the **fungal biology** by changes in gene expression of the fungus during colonization should also be taken into account. However, these investigations require a more and improved annotation of the fungal genomes. First, but still quite

limited information is available on *B. bassiana* genome sequences and transcriptional responses of the fungus to insect cuticles, insect hemocoel, and plant root exudates (Xiao et al. 2012).

- Hypothesis about the mechanisms underlying the antagonistic effects on **insect pests** and **plant pathogens** must be verified.
- Analysis of the interactions between entomopathogenic fungi and other plant-associated microorganisms (internal and external) apart from pathogens. Thus, increased attention must be paid to the impact on or by the **functional microbiome** due to changes in microbial community diversity, density, and activity. Here, too, next-generation sequencing technology could contribute to more detailed data on, e.g. the microbial diversity.
- **Compatibility** with other (biological and chemical) control measures must be evaluated to improve the incorporation of fungal entomopathogen-based biological control agents within IPM programs.
- In this context, **higher trophic levels** like parasitoids and predators must be considered and shifts in communities of (non-target) insects monitored.
- Finally, more evaluation of entomopathogenic endophytes under field conditions is required to ascertain long term effects and impacts on natural habitats.

All issues mentioned above are individual pieces, but contributing to the complete picture, and not supposed to be addressed merely separated. The need for a holistic approach is also espoused by Vega (2018), who stated: “The more we hunker down and focus on simple things, (…) the more likely we are to miss the big picture”. Hence, collaboration among insect pathologists, plant biologists, endophyte specialists, chemists, system biologists, and scientists of other disciplines, as also advised by McKinnon at al. (2017) and Vega (2018), is needed to shed light on the whole system of endophytes and understand the complexity of the multitrophic interaction. Therefore, combining methods from different disciplines like entomology, mycology, and botany will be necessary and utilization of new technologies such as transcriptomics and proteomics helpful to elucidate the genuine biocontrol potential of entomopathogenic endophytes like *B. bassiana* and make them work as a pest management strategy. Although limited, the results of the studies conducted in this thesis contributed to our understanding of the ecology of *B. bassiana.* To conclude, endophyte research, in particular the exploration of entomopathogenic endophytes, is challenging and exhibits a number of knowledge gaps. Though, the knowledge about the negative impacts of chemical control and the intention to further promote biological control is worth the extensive efforts.

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A Supplementary Material

A1 Figures

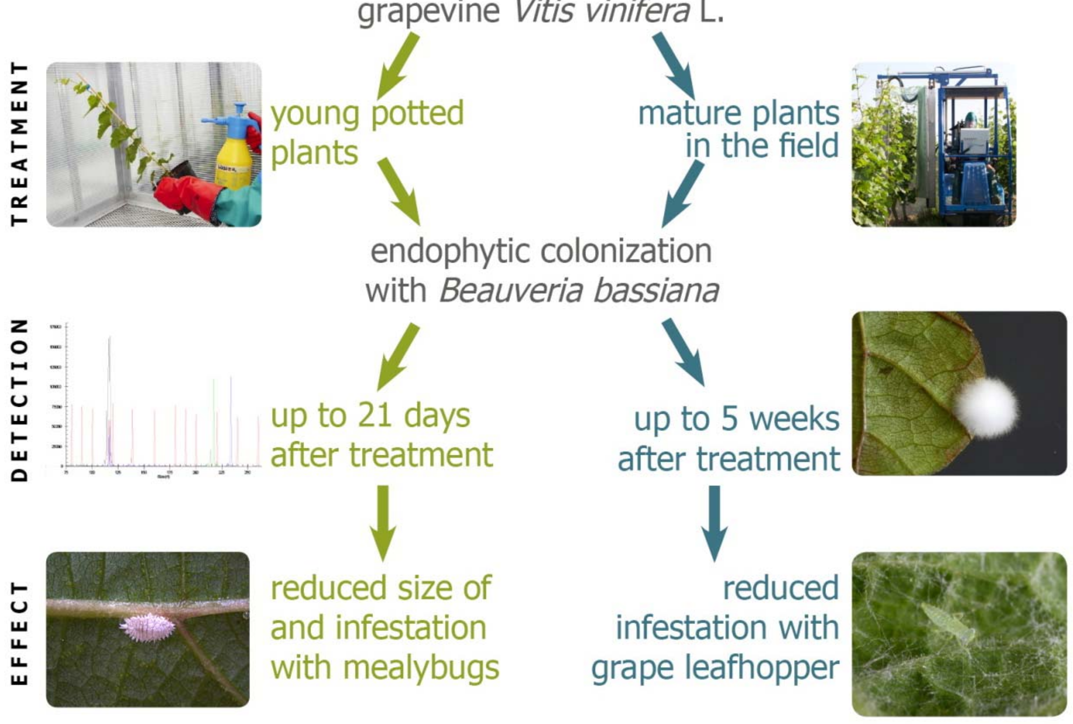

Figure 16: Graphical abstract of manuscript I

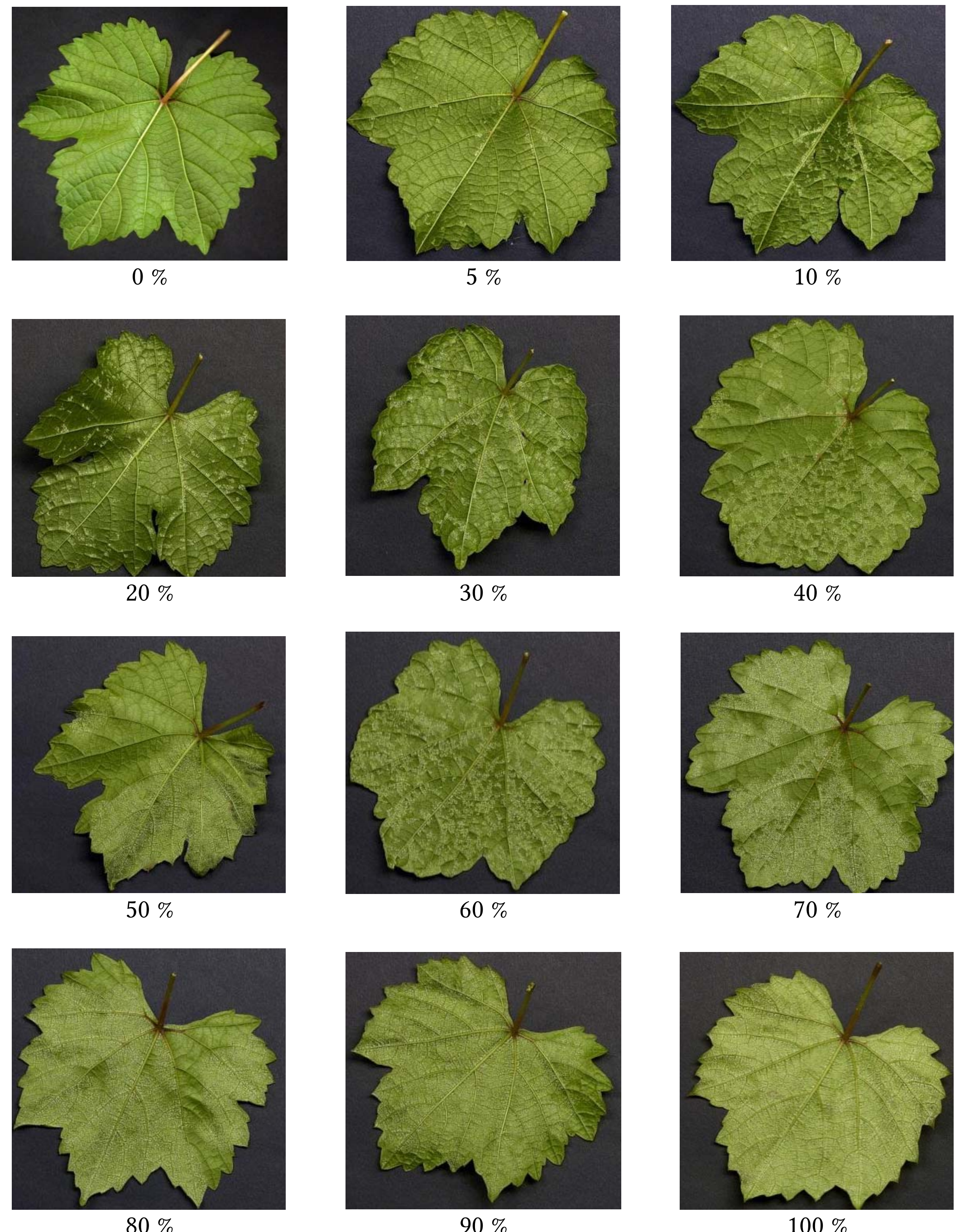

Figure 17: Assessment of disease severity of grapevine leaves with *P. viticola*

A2 Tables

Table 9: Induced and repressed genes in grapevine plants 24 hpt with *B. bassiana*. The List includes genes that are significantly regulated more than twofold in response to *B. bassiana* treatment in comparison to control plants ($p < 0.05$).

Probe Set ID	Fold Change	p-value	Representative Public ID	UniGene ID	Gene Title
1608304_at	6.32	0.0253	CD011601	Vvi.3800	---
1610704_at	5.92	0.0002	CA809376	Vvi.24460	major allergen Pru ar 1-like
1618568_s_at	5.66	0.0002	CF205010.1	---	major allergen Pru ar 1-like
1617963_at	4.97	0.0149	CF074697	Vvi.8718	---
1610850_at	4.64	0.0041	S63225.1	Vvi.27488	stilbene synthase 1-like
1609696_x_at	4.55	0.0034	S63225.1	Vvi.27488	stilbene synthase 1-like
1615118_at	4.38	0.0000	CF373171	Vvi.15301	3-ketoacyl-CoA synthase 12-like
1618663_s_at	4.16	0.0007	BM436446	Vvi.9447	uncharacterized LOC100263839
1610824_s_at	3.66	0.0002	AY059639.1	Vvi.25322	stilbene synthase 2-like
1609697_at	3.62	0.0002	CF207058.1	Vvi.27363	Stilbene synthase 4
1612804_at	3.58	0.0001	X76892.1	Vvi.25322	stilbene synthase 2-like
1620964_s_at	3.57	0.0014	S63225.1	Vvi.27488	resveratrol synthase
1622638_x_at	3.45	0.0004	X76892.1	Vvi.25322	stilbene synthase 2-like
1608009_s_at	3.33	0.0001	S63221.1	Vvi.25322	stilbene synthase 2-like
1611190_s_at	3.24	0.0010	AF274281.1	Vvi.27488	stilbene synthase
1619034_at	3.17	0.0006	CK136955.1	---	cytochrome P450 87A3-like
1620792_at	2.83	0.0005	CF605390	---	uncharacterized LOC100242276
1621102_at	2.75	0.0370	CB971771	Vvi.5498	acidic endochitinase-like
1614436_at	2.72	0.0011	CF415505	Vvi.13239	uncharacterized LOC100855161
1618260_s_at	2.67	0.0018	CD799434	Vvi.9106	myb-related protein Myb4-like
1613999_x_at	2.63	0.0014	CF202364.1	---	chitinase
1610800_at	2.59	0.0122	CF204250.1	---	---
1619517_at	2.58	0.0071	CF202125.1	Vvi.25952	resveratrol O-methyltransferase
1616788_at	2.58	0.0002	CF202817.1	Vvi.20128	high affinity nitrate transporter 2.5-like
1619986_s_at	2.51	0.0099	CD800813	Vvi.8580	anthocyanidin 5,3-O-glucosyltransferase-like
1611117_at	2.43	0.0012	CF201368.1	---	---
1615967_at	2.42	0.0010	CF211449	Vvi.14598	peroxidase 73-like
1618373_at	2.40	0.0026	Z68123.1	Vvi.18	acidic chitinase
1622455_at	2.39	0.0006	CF207039.1	Vvi.674	peptide transporter PTR3-A-like
1622633_at	2.39	0.0014	CF201563.1	---	---
1614487_at	2.37	0.0000	CF403809	Vvi.15496	3-hydroxy-3-methylglutaryl-coenzyme A reductase 1-like
1607619_s_at	2.36	0.0003	CA814423	Vvi.5009	uncharacterized LOC100243642
1613871_at	2.35	0.0010	CF207387	Vvi.8893	endochitinase PR4-like
1608864_s_at	2.35	0.0026	CF202364.1	Vvi.18	acidic chitinase
1613006_at	2.34	0.0022	CF204981.1	---	---
1614769_at	2.33	0.0001	CF207048.1	---	---
1610011_s_at	2.33	0.0027	CF200913.1	---	---
1614404_x_at	2.32	0.0011	CF201563.1	---	---
1622745_at	2.32	0.0001	BQ796736	---	flavoprotein wrbA-like
1620245_at	2.26	0.0018	CF202722.1	---	cytochrome P450 71A1-like
1608538_at	2.26	0.0021	CA813555	Vvi.5021	uncharacterized LOC100855082
1613141_at	2.25	0.0061	CF518362	Vvi.11367	NAC domain-containing protein 42-like
1616575_at	2.22	0.0010	AF418567.1	---	---

Probe Set ID	Fold Change	p-value	Representative Public ID	UniGene ID	Gene Title
1621553_at	2.21	0.0188	CB002757	Vvi.9919	---
1610914_at	2.19	0.0089	CF203251.1	Vvi.25984	copper transporter
1613344_at	2.16	0.0002	CF202628.1	---	---
1613811_a_at	2.15	0.0103	CB920849	Vvi.9248	VVTL1
1614803_at	2.14	0.0029	AY046416.1	Vvi.161	proline-rich protein 1
1621371_at	2.14	0.0006	CF202171.1	Vvi.19808	disease resistance response protein 206-like
1616413_at	2.13	0.0066	AF003007.1	Vvi.9248	VVTL1
1615401_at	2.11	0.0008	CB342555	Vvi.1393	putative UDP-glucose flavonoid 3-O-glucosyltransferase 3-like
1620063_at	2.10	0.0042	CB921343	Vvi.644	beta-1,3-glucanase
1607193_at	2.10	0.0000	BQ796845	Vvi.683	alternative oxidase 3, mitochondrial-like
1615458_at	2.10	0.0020	CB969727	Vvi.5161	uncharacterized LOC100255664
1621970_at	2.09	0.0007	CD713131	Vvi.5009	uncharacterized LOC100243642
1616822_at	2.08	0.0042	AF220196.1	Vvi.161	proline-rich protein 1
1620390_s_at	2.07	0.0318	AF532965.1	Vvi.8525	thaumatin-like protein
1606453_x_at	2.07	0.0216	CF203408.1	Vvi.24387	pathogenesis-related protein 10.3
1609653_at	2.06	0.0005	BQ797078	---	---
1609156_at	2.05	0.0130	CF211313	---	valencene synthase-like
1615789_at	2.04	0.0161	BQ795769	Vvi.553	extensin-3-like
1622369_at	2.02	0.0009	CB342790	Vvi.7017	germin-like protein subfamily T member 2-like
1610243_at	2.02	0.0006	BM437744	Vvi.9617	probable glutathione S-transferase-like
1622550_at	2.02	0.0006	AY427148.1	---	---
1618589_s_at	-2.01	0.0051	CF206361.1	Vvi.27529	uncharacterized LOC100260620
1610488_at	-2.02	0.0054	CK138238.1	---	---
1609901_at	-2.03	0.0172	CF212785	Vvi.14816	monothiol glutaredoxin-S1-like
1612562_at	-2.04	0.0024	CA808714	Vvi.7197	uncharacterized LOC100264675
1609749_at	-2.08	0.0071	CD716155	Vvi.5707	gibberellin 3-beta-dioxygenase 4-like
1611996_at	-2.10	0.0161	CF373384	Vvi.15425	---
1607561_at	-2.14	0.0086	CF209184	Vvi.5178	ABC transporter G family member 5-like
1613022_s_at	-2.15	0.0032	CF569215.1	Vvi.7621	non-specific lipid-transfer protein P5-like
1613301_at	-2.20	0.0141	CF372159	Vvi.27809	stem-specific protein TSJT1-like
1617940_at	-2.33	0.0035	CA809342	Vvi.2349	nuclease S1-like
1610299_at	-2.40	0.0114	CF373165	Vvi.5632	MLP-like protein 423
1618921_at	-2.43	0.0209	CK138176.1	---	---
1613442_at	-2.43	0.0085	CF415231	Vvi.21002	glutaredoxin-C1-like
1617400_at	-2.45	0.0078	BQ798101	Vvi.6741	sulfate transporter 3.1-like
1607541_at	-2.46	0.0049	CF208308	---	uncharacterized LOC100257913
1622416_at	-2.60	0.0051	CF518913	Vvi.7621	non-specific lipid-transfer protein P5-like
1615985_at	-2.63	0.0035	CF516133	Vvi.13054	auxin-induced protein 22D-like
1608268_at	-2.67	0.0028	CB970701	Vvi.5372	thebaine 6-O-demethylase-like
1615445_at	-2.70	0.0296	BQ794327	Vvi.4693	metallothionein-like protein type 2-like
1608175_at	-2.73	0.0168	CF404148	Vvi.15636	non-specific lipid-transfer protein-like
1617786_at	-2.80	0.0081	CB972580	Vvi.5653	uncharacterized LOC100263887
1619751_at	-2.85	0.0076	CB341549	Vvi.13054	auxin-induced protein 22D-like
1613471_at	-2.92	0.0327	CF215857	Vvi.14794	pathogenesis-related protein PR-1-like
1615971_a_at	-3.89	0.0046	CB980630	---	uncharacterized LOC100262468
1615109_at	-3.90	0.0293	CK138176.1	---	---

Table 10: Induced and repressed genes in grapevine plants 168 hpt with *B. bassiana*. The List includes genes that are significantly regulated more than twofold in response to *B. bassiana* treatment in comparison to control plants ($p < 0.05$).

Probe Set ID	Fold Change	p-value	Representative Public ID	UniGene ID	Gene Title
1622767_at	2.17	0.0013	CB982859	Vvi.7837	hypothetical protein
1621587_at	2.11	0.0115	CF214129	Vvi.5464	adenosine 5'-phosphosulfate reductase
1617074_s_at	2.19	0.0210	CF204027.1	---	---
1622010_at	2.14	0.0213	CF207538	Vvi.9839	---
1621593_s_at	2.07	0.0032	AF347624.1	---	---
1613911_s_at	2.53	0.0310	CF201679.1	---	---
1613371_s_at	2.09	0.0088	CB340927	---	---
1609916_s_at	2.24	0.0085	CD005933	Vvi.3813	---
1609373_at	2.23	0.0052	CD800734	Vvi.11248	30S ribosomal protein S7, chloroplastic-like
1616528_s_at	2.94	0.0353	CD801342	---	uncharacterized LOC100242887
1607341_at	3.09	0.0441	CB970018	---	uncharacterized LOC100242887
1613853_s_at	2.03	0.0057	CF203997.1	---	---
1615527_at	2.01	0.0326	CF372317	Vvi.10595	uncharacterized LOC100246222
1619204_at	2.14	0.0409	CF373337	Vvi.15313	dnaJ homolog subfamily C member 1-like
1622374_at	-2.03	0.0254	CB920589	Vvi.11581	osmotin-like protein
1610638_at	-2.11	0.0035	CD719790	Vvi.10998	uncharacterized LOC100855409
1607353_at	-2.08	0.0032	CF206245.1	---	uncharacterized LOC100249186
1608852_at	-2.36	0.0418	CF516023	Vvi.3488	probable sulfate transporter 3.4-like
1615169_at	-2.10	0.0353	BQ792970	Vvi.1874	uncharacterized LOC100258290
1608692_s_at	-2.14	0.0213	CF074673	Vvi.8923	pathogenesis-related protein
1609391_s_at	-2.05	0.0101	CF404650	Vvi.13978	blue copper protein-like
1617047_at	-2.14	0.0299	BQ792281	Vvi.7462	peptidyl-prolyl cis-trans isomerase-like
1619479_a_at	-2.14	0.0014	CB980068	Vvi.7570	lanC-like protein 2
1608229_s_at	-2.03	0.0086	CD003870	Vvi.9783	glycogenin-2-like
1620065_at	-2.23	0.0415	CD798903	Vvi.888	probable sulfate transporter 3.5-like
1621688_at	-2.01	0.0393	CF415096	Vvi.3163	glycerophosphodiester phosphodiesterase GDE1-like
1612443_at	-2.65	0.0026	CF211151	Vvi.2313	CBL-interacting protein kinase 16
1621592_s_at	-2.70	0.0424	BQ792954	Vvi.4682	dehydrin

Biological Control 116 (2018) 82–89

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects

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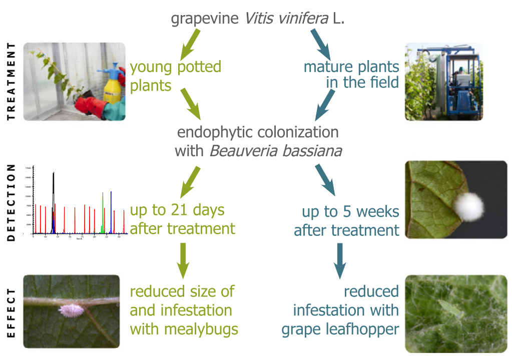
HIGHLIGHTS

- Successful endophytic establishment of the entomopathogen *B. bassiana* in grapevine plants.
- In potted plants endophytic survival of *B. bassiana* was evident for at least 21 days after inoculation.
- Endophytic *B. bassiana* reduces infestation rate and growth of vine mealybugs.
- In the vineyard *B. bassiana* was detected as an endophyte up to five weeks after last application.
- *B. bassiana* reduces infestation with grape leafhopper in the vineyard.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 27 April 2016
Revised 11 October 2016
Accepted 21 October 2016
Available online 22 October 2016

Keywords:
Beauveria bassiana
Endophyte
Entomopathogenic fungi
Vitis vinifera
Planococcus ficus
Empoasca vitis
Interactions

ABSTRACT

Fungi are important natural pathogens of arthropod pests and are successfully used as biocontrol agents in various crops. In addition to colonizing arthropods, evidence has accumulated that some entomopathogenic fungi like *Beauveria bassiana* can endophytically colonize a wide array of plant species. However, only limited information is currently available on the endophytic colonization of grapevines with *B. bassiana* and whether the fungus still maintains its pathogenic habit against insect pests.

Greenhouse and field experiments were conducted to optimize endophytic establishment of the entomopathogenic fungus *B. bassiana* in younger, potted plants and mature grapevine plants in the vineyard. We used two different commercialized *B. bassiana* strains, applied either as conidial suspensions (ATCC 74040 and GHA) or as a formulated product (Naturalis®, strain ATCC 74040) on grapevine leaves. The potential of endophytic *B. bassiana* to provide protection against putative target pest insects like the vine mealybug *Planococcus ficus* was assessed in a bioassay using surface sterilized leaves. Endophytic survival of *B. bassiana* inside leaf tissues of seven-week-old potted plants was evident for at least 21 days after inoculation, irrespective of the inoculum used. Endophytic *B. bassiana* reduces infestation rate and growth of *P. ficus*. In the vineyard *B. bassiana* was detected as an endophyte in mature grapevine plants up to five weeks after last application with significant reduction of infestation with grape leafhopper, *Empoasca vitis*.

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1. Introduction

The hypocrealean fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) is a well-known microbial entomopathogen of a diverse range of arthropod species. Hence, this species is successfully used as a microbial biocontrol agent for integrated pest management of arthropod pests with many *B. bassiana* based commercial products being available on the market (Jackson et al., 2010; Zimmermann, 2007). Generally, for control of target species, preparations of blastospores or aerial conidia formulated in oil or other adjuvants are sprayed onto the plant's phylloplane.

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http://dx.doi.org/10.1016/j.biocontrol.2016.10.006
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Besides its entomopathogenic habit of life style, this fungus has also been shown to be able to thrive saprophytically in the soil, to colonize the rhizosphere of plants, to have antagonistic activities against plant pathogens, as well as to grow endophytically inside plants (Vega et al., 2009). As far as the latter is concerned, a few studies have shown that *B. bassiana* is occurring as part of the natural endophytic community of certain plant species (Ormond et al., 2010; Reay et al., 2010; Vega et al., 2008). Moreover, endophytic establishment of *B. bassiana* has been achieved via an artificial application of this fungus on the plant's tissue following a subsequent colonization of specific parts of the plant or the entire host plant. Using such an approach, successful endophytic establishment of *B. bassiana* has been proved for a variety of crop plant species including cocoa (Posada and Vega, 2005) and pine seedlings (Brownbridge et al., 2012), corn (Wagner and Lewis, 2000), coffee (Posada et al., 2007), sorghum (Reddy et al., 2009; Tefera and Vidal, 2009), tomato (Klieber and Reineke, 2016), banana (Akello et al., 2009), and jute (Biswas et al., 2012, 2013). So far, no negative effects of the presence of endophytic *B. bassiana* on performance of the colonized host plant have been reported in a range of studies (Akello et al., 2009; Klieber and Reineke, 2016; Tefera and Vidal, 2009; Wagner and Lewis, 2000). Endophytic *B. bassiana* has been reported to provide systemic protection against several insect pests or to inhibit insect development and establishment (Biswas et al., 2013; Gurulingappa et al., 2010; Quesada-Moraga et al., 2009; Reddy et al., 2009). At the same time, presence of endophytic *B. bassiana* has been shown to reduce disease symptoms caused by a variety of fungal pathogens (Griffin et al., 2005; Jaber, 2015; Ownley et al., 2010, 2008) Therefore, defining means of ensuring an endophytic establishment of *B. bassiana* strains in target crop plants is currently the focus of several studies, as this would represent a dual biocontrol strategy both against insect pests and plant pathogens. Thus, the use of endophytes for the purpose of pest and disease control is of particular interest for perennial crops like grapevine, which regularly require frequent and intensive applications of pesticides.

Grapevine (*Vitis vinifera* L.) is an important global commodity crop which is planted throughout temperate regions worldwide. A substantial number of different insect pests and pathogens are associated with grapevine and are significant factors influencing both the quantity of the yield as well as the quality of must and vine (Flaherty, 1992). As a result, grapevine cultivation is regarded as being input intensive, in particular regarding the frequency and intensity of fungicide and insecticide applications throughout the year (Roßberg, 2007). Insects with a piercing-sucking mode of feeding frequently attack grapevines and cause damage either by extracting sap fluids or feeding in mesophyll cells or by transmitting grapevine pathogens. The grape leafhopper *Empoasca vitis* (Goethe) (Homoptera: Cicadellidae, Typhlocybinae) feeds on mesophyll cells or on phloem sap and is recognized as a major insect pest in many European grapevine growing areas (Olivier et al., 2012). Moreover, the vine mealybug *Planococcus ficus* (Signoret) (Homoptera: Pseudococcidae) is regarded as a key pest in many countries around the world (Daane et al., 2012). *Planococcus ficus* causes direct damage to grapevine due to phloem-feeding on leaves and fruit and excretion of honeydew. Additionally, *P. ficus* acts as a vector for grapevine leafroll associated virus (GLRaV), one of the most economically destructive grapevine viruses that occur in all the major grape-growing regions of the world (Almeida et al., 2013). Accordingly, a combination of methods including insecticide applications, biological control via predators and parasitoids or mating disruption is usually applied by growers to control *P. ficus* (Almeida et al., 2013). The system grapevine (as an input intensive crop) - *P. ficus* and *E. vitis* (as phloem-feeding pest insects) - *B. bassiana* (as a commercially available biopesticide) is thus ideal for studying tritrophic interactions between plants, insects and entomopathogenic endophytic fungi. Endophytic establishment of an entomopathogenic fungus like *B. bassiana* still having antagonistic activity against insect pests and fungal pathogens would therefore represent a novel and sustainable plant protection strategy in viticulture, with the potential to reduce frequency of pesticide applications.

In the present study we have addressed the following main questions (1) Is an endophytic establishment of commercially available *B. bassiana* strains possible both in young potted greenhouse grapevine plants as well as in mature and lignified plants grown in the field? (2) Does an endophytic *B. bassiana* strain present in grapevine have negative impacts on insects with a piercing-sucking mode of feeding?

2. Materials and methods

2.1. Fungal material

Beauveria bassiana strains ATCC 74040 and GHA were isolated from the commercial products Naturalis® (CBC (Europe) S.r.l. – BIOGARD Division, Italy) and Botanigard® 22WP (BioWorks, Inc., USA), respectively. Naturalis® is formulated as an oily fluid and contains approximately 2.3×10^7 colony forming units/ml of *B. bassiana* strain ATCC 74040 as active ingredient. The isolates were maintained on a solid medium at 24 °C in the dark. The medium consisted of 10 g soy peptone (AppliChem, Germany), 20 g glucose (Sigma-Aldrich, Germany) and 18 g Bacto™Agar (BD Difco, USA) dissolved in 1000 ml ultrapure water and was subsequently autoclaved for 20 min at 120 °C.

To obtain spore suspensions, conidia were harvested by gently scraping the surface of Petri dishes containing 8-day-old *B. bassiana* cultures and suspending them in 20 ml sterile 1/8 concentrated Ringer's solution containing 0.02% Tween 80. The conidia concentration was determined using a Thoma haemocytometer and adjusted to 2×10^7 conidia/ml for strain GHA and to 1×10^7 and 2×10^7 conidia/ml for strain ATCC 74040. Both, the freshly collected conidia suspensions and the formulated product Naturalis® (at concentrations of 3% and 5%), were used in the experiments. Aliquots of 50 µl of serially diluted spore suspensions were plated on solid medium mentioned above using the Spiralplater WASP 2 (Meintrup DWS Laborgeräte GmbH, Germany). Germination rates were thereafter assessed by plate counts of viable conidial spores and were calculated using the colony forming unit's (CFU) method (Goldman and Green, 2008). Germination rate was 100% for conidial spores present in Naturalis® and around 70% for the spore suspensions of isolates ATCC 74040 and GHA. Accordingly, concentrations of viable conidia applied onto plants were 1.4×10^7 conidia/ml for strain GHA and 7×10^6 (conc. 1) or 1.4×10^7 (conc. 2) conidia/ml for strain ATCC 74040.

2.2. Endophtic establishment in potted grapevine plants

Grapevine plants, *Vitis vinifera* (L.) cv. 'Riesling', were obtained from hardwood cuttings. After root development the plants were potted in a clay/white peat substrate ED73 (Patzer, Sinntal, Germany) and grown in a greenhouse chamber at 22–25 °C. Seven-week-old grapevine plants with 4–7 fully expanded leaves were used for inoculation with either *B. bassiana* conidial suspensions or the commercial product Naturalis® (3% and 5%). For each treatment, 10 replicate plants were inoculated by spraying the adaxial and the abaxial surfaces of all fully expanded leaves using a 1 L one-hand pressure sprayer. During application, pots with plants were held in an almost horizontal position so that any run-off was not contaminating the soil. Control plants were sprayed with sterile 1/8 concentrated Ringer's solution containing

0.02% Tween 80. Position of the last fully expanded leaf used for inoculation was labeled using a tapener (Max tapener HT-B, Max Staple, Japan). Inoculated and non-inoculated plants were randomized in blocks and were kept in a greenhouse chamber for three weeks (daily mean temperature 23–25° C, daily mean relative humidity 50–70%). Plants were watered as needed.

2.3. Re-isolation of *B. bassiana*

Endophytic colonization of plants by *B. bassiana* was assessed 7, 14, and 21 days after inoculation (DAI) by re-isolation following surface sterilization. No newly developed leaves were included in the present study. At each sampling period one leaf from each of the 10 replicate plants was excised and transported to the laboratory on ice. The leaves were individually surface sterilized under sterile conditions by dipping them in 0.5% NaOCl (active chlorine) containing 0.05% Tween 80 for 2 min, followed by 70% EtOH for 2 min and rinsed twice with sterile distilled water according to Akello et al. (2009). The success of this disinfection process was assessed by plating three replicates of 200 µl of the residual rinse water on PDA (potato dextrose agar). No fungal growth was recorded in any of the rinse water samples after 21 days of incubation. After surface sterilization, six leaf discs (d = 1.2 cm) were obtained with a sterile cork borer from each leaf. Leaf discs were placed on *Beauveria* selective medium, the same solid medium as indicated above (2.1) but supplemented with 0.1 g/l streptomycin (Sigma-Aldrich, Germany), 0.05 g/l tetracycline (Sigma-Aldrich, Germany), 0.1 g/l dodine (as aliquot of the product Syllit®, Spiess-Urania Chemicals, Germany) and 0.05 g/l cycloheximide (Sigma-Aldrich, Germany). This medium is based on a medium initially described by Strasser et al. (1996) for the isolation of *B. brongniartii* and adapted by Meyling and Eilenberg (2006) for isolation of *B. bassiana*. Plates were incubated at room temperature with a 12:12 h light:dark photoperiod (mean light intensity of 11.2 $\mu mol\ m^{-1}\ s^{-1}$).

After 7 and 14 days leaf discs were examined visually for the presence of any fungal growth. Fungal tissue was characterized as endophytic *B. bassiana* if characteristic white dense mycelia, becoming creamy at the edge (Humber, 1997) grew from internal plant tissues of surface sterilized leaf discs. Final assessment of the presence of endophytic *B. bassiana* was recorded after 14 days and was expressed as percentage colonization by dividing the number of leaf discs exhibiting *B. bassiana* outgrowth by the number of total leaf discs and multiplying the obtained value with 100. If one of the six leaf discs obtained from a single plant showed fungal outgrowth the total leaf was classified as being endophytically colonized. Differences in percentage colonization of plant tissues at the different sampling dates were analyzed for statistical significance with a Kruskal-Wallis-ANOVA using Dell Statistica data analysis software system (Dell Inc., version 13, software.dell.com).

2.4. Strain-specific detection of *B. bassiana*

To ensure that fungal tissue present at the edges of grapevine leaf discs originated from the respective inoculated *B. bassiana* strain (ATCC 74040 or GHA), now internally colonizing plant tissues as an endophyte, a subset of mycelia samples was further analyzed with molecular techniques. DNA was extracted from fungal tissues using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions with an additional step for 30 min on ice after the recovering step with isopropanol. Accordingly, extracted fungal DNAs were subjected to strain-specific PCR analysis using three *B. bassiana* microsatellite (simple sequence repeats, SSR) primers, namely Ba01, Ba12 and Ba13 (Rehner and Buckley, 2003). In previous studies, these primers have proved to allow a confident discrimination among different *B. bassiana* isolates (Reineke et al., 2014).

For fluorescent labeling of the generated PCR products, a M13 (21) tail was placed at the 5′-end of each forward primer and a CY5 labeled universal primer M13(−21) was added to the PCRs according to the method described by Schuelke (2000). PCR amplifications were set up in a total volume of 15 µl consisting of 90 ng DNA, 10x reaction buffer, 5 pmol of forward primer, 10 pmol of reverse primer, 2.25 mM $MgCl_2$, 3 mM dNTPs and 0. U of Dream Taq Polymerase (Fermentas, St. Leon-Rot, Germany). PCRs were performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60.2 °C for 45 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. An aliquot of each PCR product was checked for successful amplification on a 1% agarose gel. PCR products were analyzed for SSR sizes via capillary electrophoresis on a Beckman GenomeLab GeXP DNA Genetic Analysis System (Beckman Coulter, Inc., CA, USA). As different fluorescent primers were used for labeling the obtained PCR products (DY-751 for Ba01, BMN5 for Ba12 and DY-681 for Ba13) reactions were loaded as a multiplex analysis with 1 µl of each PCR product, mixed with 36.7 µl sample loading solution (Beckman Coulter, Inc., CA, USA) and 0.3 µl of a 400 bp size standard. Allele sizes were determined using GenomeLab GeXP Version 10.2 (Beckman Coulter, Inc., CA, USA).

2.5. Mealybug bioassay

The potential of endophytic *B. bassiana* to provide protection against piercing-sucking insects was tested with a detached leaf assay and vine mealybugs, *P. ficus*. Sixty-seven weeks old potted grapevine plants cv. 'Riesling' were inoculated with Naturalis® (3%) or water as control as described above. The experiment was repeated twice. Two weeks after inoculation two leaves per plant were obtained, with one leaf used for the bioassay and the other leaf to verify endophytic establishment by re-isolation as described above. To ensure that mealybugs were only influenced by endophytic and not by epiphytic fungal propagules, all grapevine leaves were surface disinfected before the bioassay according to the procedure described above. With a pretest (data not shown) we verified that any leftovers of NaOCl still present on the leaves did not harm the mealybugs.

Vine mealybugs were grown on potato sprouts in a growth room with 23 ± 1 °C, 60–65% RH and 16:8 h light:dark period. In all experiments, first instar *P. ficus* individuals were used, which were removed from potato tubers by irritation with a paintbrush until their stylets were withdrawn. Ten *P. ficus* larvae each were carefully transferred with a paintbrush to the surface sterilized leaves. Leaves with mealybugs were maintained in enclosed transparent plastic containers (height 10 cm, diameter 13.5 cm) with water provided for the leaf and were placed in a growth chamber under the conditions mentioned above.

After two days infestation rate was calculated as the number of remaining larvae on the leaf in relation to the initially used ten individuals. This procedure was repeated once a week over a period of three weeks (7, 14, and 21 days after initial settlement) and was supplemented by determination of the size of all individual mealybug larvae with a binocular microscope and measurement software (Leica Microsystems, Application Suite, Switzerland). A total of 300 mealybugs were assessed for each, the endophytic and control leaves. Size and infestation rate were analyzed for statistical significance between endophytic and control leaves with a Mann-Whitney-U-Test ($\alpha = 0.05$) using Dell Statistica data analysis software system (Dell Inc., version 13, software.dell.com).

2.6. Field trial

In addition to greenhouse and laboratory experiments we conducted a field trial as proof of principle to get preliminary evidence of efficacy of endophytic establishment of *B. bassiana* and its potential to control insect pests in the field. The field trial was realized in the framework of GEP (good experimental practice) certified efficacy tests of plant protection products (EPPO, 2012). The experimental vineyard was located in the Rheingau region, Germany (49°58′N, 7°57′E, 95 masl) and included 0.3 ha of grapevine plantscv. 'Riesling' planted in 1999. The experiment was conducted in a completely randomized block design with 4 plots (replicates) of 114 m2 size and 14 vines per plot.

Naturalis® (1%) was applied in the vertical canopy by a tunnel sprayer with 8 Teejet® flat spray nozzles and driving speed of 0.7 m/s. Control plots were treated with water. Applications were carried out at the same time with other plant protection measures (fungicide applications against powdery mildew, *Erysiphe necator,* using the products Vivando®, Talius®, Luna® Experience and Topas® in rotation). Interval between applications was approximately 10 days depending on weather and disease pressure of other grapevine pathogens with a first application on 15 May 2014. In two of the four Naturalis® treated plots, treatment included nine applications during the season. The other two plots were treated twice in the beginning of the season to determine how long the fungus can be detected endophytically (15 and 26 May 2014).

Endophytic establishment in grapevine leaves was evaluated at four dates (22 May, 12 June, 2 July, and 23 July 2014) in 10 leaves per plot according to the method described above. In addition we assessed the infestation with grape leafhopper, *Empoasca vitis,* at five dates (15 July, 23 July, 31 July, 07 Aug and 12 Aug 2014) in treated plots and control plots by counting *E. vitis* larvae on 25 leaves per plot. Infestation data was analyzed for statistically significant differences with nonparametric Wilcoxon signed-rank test (McDonald, 2014).

3. Results

3.1. Endophytic colonization of potted grapevine plants

During the assessment period of 7, 14, and 21 DAI *B. bassiana* was successfully re-isolated from 46%, 40%, and 46% of all inoculated grapevine plants, respectively. None of the leaf discs obtained from control plants showed signs of fungal outgrowth, thus none of the control plants were colonized by the fungus. Not all leaf discs from colonized plants showed fungal outgrowth, causing a high variance in percentage colonization in all treatments (Fig. 1). In some instances contaminating fungi and bacteria were occasionally found growing from leaf discs of both inoculated and control plants (data not shown).

If applied as a conidial suspension on foliage of grapevine plants, both *B. bassiana* strains (GHA and ATCC 74040) were able to establish as an endophyte, with no significant differences in percentage colonization being evident between the different spore concentrations and the strains applied (Fig. 1). The same was obvious if *B. bassiana* strain ATCC 74040 was applied as the formulated product Naturalis®, with colonization rates being not significantly different for both concentrations (3% and 5%) applied (Fig. 1). During the assessment period, no significant decline or increase in percentage colonization by endophytic *B. bassiana* was observed 7, 14, and 21 DAI.

3.2. Strain-specific detection of endophytic B. bassiana

In capillary electrophoresis, DNA from all analyzed mycelia samples obtained from endophytic fungal tissues showed the respective strain-specific peaks after amplification with three *B. bassiana* microsatellite primers (Table 1). Amplicons of primer pairs Ba01, Ba12 and Ba13 showed peaks at 117 bp, 231 bp and 216 bp for strain ATCC 74040 and 117 bp, 222 bp and 168 bp for strain GHA, respectively. These results indicate that endophytic *B. bassiana* re-isolated from inoculated leaf discs originated from the previously applied strains.

3.3. Mealybug bioassay

Negative potential of endophytic *B. bassiana* against vine mealybug larvae was assessed on detached and surface sterilized grapevine leaves of Naturalis® treated and control grapevine plants.

Table 1
Amplification of *B. bassiana* strain GHA or ATCC 74040 specific SSR markers in a subset of eight obtained mycelia samples from leaf discs of the different treatments 14 and 21 days after inoculation (DAI) with *B. bassiana*.

Treatment	DAI	No. of leaf discs used and screened positive with three SSR markers			
		n total	Ba01	Ba12	Ba13
ATCC 74040 (1.4×10^7 conidia/ml)	14	2	2	2	2
Naturalis® 3%	14	2	2	2	2
GHA (1.4×10^7 conidia/ml)	21	1	1	1	1
ATCC 74040 (7×10^6 conidia/ml)	21	2	2	2	2
ATCC 74040 (1.4×10^7 conidia/ml)	21	1	1	1	1

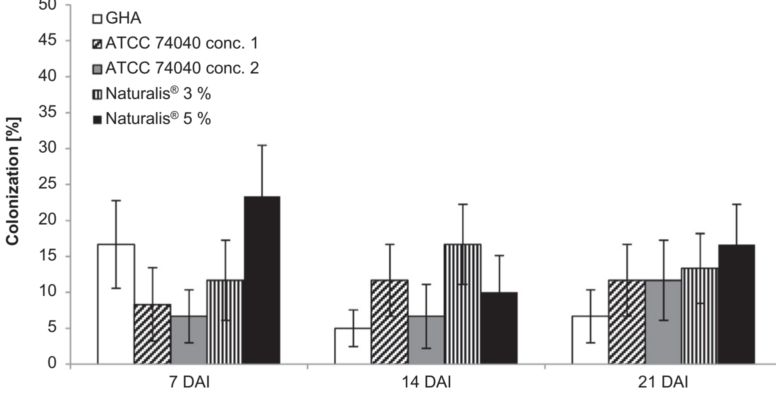

Fig. 1. Mean (±SE) percentage colonization of *Vitis vinifera* leaf discs 7, 14 and 21 days after inoculation (DAI) with a conidial suspension of *Beauveria bassiana* strains GHA (1.4×10^7 conidia/ml) or ATCC 74040 (conc. 1: 7×10^6 conidia/ml; conc. 2: 1.4×10^7 conidia/ml) or with the formulated product Naturalis® (3% and 5%). Differences between treatments were not statistically significant ($p < 0.05$). In control leaves (treated with Ringer's solution) no *B. bassiana* was present (not shown).

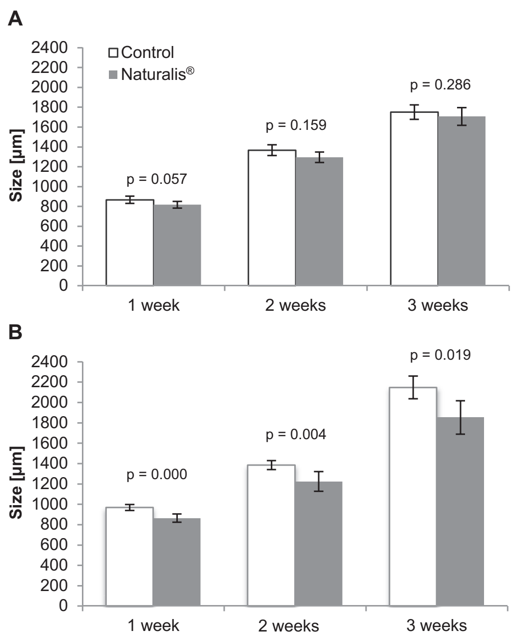

Fig. 2. Mean (±95% CI) size of vine mealybug larvae (*P. ficus*) after feeding for three weeks on detached grapevine leaves of control plants and plants with endophytic *B. bassiana* (Naturalis®) in two replicates (A and B). Statistical significant differences between treatments were analyzed with a Mann-Whitney-*U*-Test (α = 0.05).

Endophytic establishment in the respective grapevine plants was 30% in the first and 60% in the second experimental replicate (data not shown). Because of this difference in endophytic establishment results of the two replicates were not combined but were analyzed separately. Data on success of endophytic establishment were

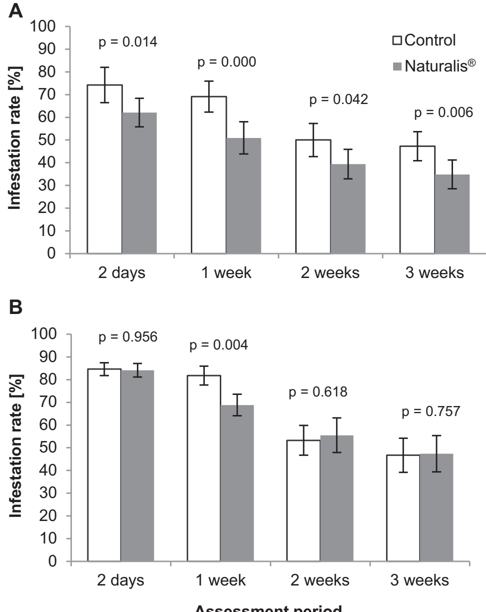

Fig. 3. Mean (±95% CI) infestation rate of vine mealybug larvae (*P. ficus*) after feeding for three weeks on detached grapevine leaves of control plants and plants with endophytic *B. bassiana* (Naturalis®) in two replicates (A and B). Statistical significant differences between treatments were analyzed with a Mann-Whitney-*U*-Test (α = 0.05).

Table 2
Number of leaf discs assessed (n) and showing *B. bassiana* outgrowth collected from Naturalis® treated and control plots of a grapevine field trial in 2014.

Treatment	n	No. of leaf discs with endophytic *B. bassiana*			
		22 May	12 June	02 July	23 July
Control	80	0	0	0	0
Naturalis® (2 applications)	160	16	8	8	0
Naturalis® (9 applications)	160	47	19	10	13

assessed on a separate leaf obtained from the same plant as the one used in the mealybug bioassay.

Mealybug larvae were smaller when feeding for a period of 3 weeks on Naturalis® treated leaves compared to those feeding on control leaves (Fig. 2). In the first experimental replicate this difference was not statistically significant (Mann-Whitney-U-Test: 1 week $p = 0.057$; 2 weeks $p = 0.159$; 3 weeks $p = 0.286$), while in the second replicate the mean size of the mealybugs on treated leaves was significant smaller over the whole assessment period of three weeks (Mann-Whitney-U-Test: 1 week $p < 0.001$; 2 weeks $p < 0.005$; 3 weeks $p < 0.05$).

In the first experimental replicate significantly less mealybug larvae stayed alive on leaves with endophytic *B. bassiana* (Naturalis®) compared to control leaves ($p < 0.05$) over the period of the bioassay. In the second replicate this effect was only observed at the beginning of the assessment period (1 week of feeding) (see fig. 3).

3.4. Field trial

re-isolation of *B. bassiana* after application in the field showed that the fungus was able to establish as an endophyte in perennial and lignified grapevine plants in the vineyard (Table 2). In the plots treated several times with Naturalis® the fungus could be detected at all sampling dates (22 May, 12 June, 02 July and 23 July 2014). Detection rate declined over the season. In plots treated only at the beginning of the season *B. bassiana* was successfully re-isolated up to five weeks after the last application of Naturalis®. In control plots no *B. bassiana* was re-isolated from the leaves.

Infestation rate with grape leafhopper *E. vitis* in the vineyard was overall low in the year 2014. At all five monitoring dates the mean number of larvae was lower in Naturalis® treated plots than in control plots (Table 3). Over the whole assessment period the total number of *E. vitis* larvae was higher in control than in Naturalis® treated plots (236 vs. 183 individuals). Wilcoxon signed-rank test showed that the median difference between the mean number of grape leafhopper larvae per monitoring date in control plots vs. Naturalis® treated plots was significantly greater than zero ($W = 0$, $P < 0.001$)

Table 3
Mean number of grape leafhopper *E. vitis* larvae in four control and four Naturalis® treated plots (25 leaves/plot) assessed at five observation dates in a grapevine field trial in 2014. Mean number of grape leafhopper larvae per monitoring date in control plots vs. Naturalis® treated plots were statistically different (Wilcoxon signed-rank test; $W = 0$, $P < 0.001$).

Date 2014	Mean number (± SD) of *E. vitis* larvae	
	Control	Naturalis®
15 July	15.50 (±5.07)	12.75 (±2.22)
23 July	18.75 (±9.71)	12.75 (±6.60)
31 July	14.00 (±5.48)	13.50 (±5.20)
07 Aug	6.00 (±2.94)	4.50 (±3.11)
12 Aug	4.75 (±2.06)	2.25 (±0.96)

4. Discussion

Successful endophytic colonization of both young potted grapevine plants in the greenhouse as well as mature plants in the field with two different commercially available *B. bassiana* strains was achieved via artificial spray inoculation. Analysis of fungal mycelia obtained after re-isolation with strain-specific molecular markers confirmed our initial assessment based on morphology of endophytic fungal mycelia obtained from colonized grapevine plants. In greenhouse experiments, no significant difference in percentage colonization by endophytic *B. bassiana* was observed during the assessment period of 21 DAI. This suggests that endophytic colonization of grapevine by *B. bassiana* was evident as early as 7 DAI and did not decline during the period of screening for presence of endophytic *B. bassiana* of 21 DAI. Moreover, percentage colonization of grapevine plants did not vary significantly among the different strains or inoculum doses used. This may be a consequence of the relatively small number of positive samples identified and the apparent variability in isolation success.

Mean colonization rates of potted grapevine plants by *B. bassiana* were between 5% and 23% and were thus rather low compared to colonization rates of leaves of other plant species like corn (Wagner and Lewis, 2000), tomato (Klieber and Reineke, 2016), sorghum (Tefera and Vidal, 2009), and jute (Biswas et al., 2013). In contrast to these plants, grapevines are deciduous woody perennial plants and plants used for our greenhouse trials were cultivated from hardwood cuttings. Seed treatment as an alternative inoculation method as it has been successfully shown for tomato, cotton (Ownley et al., 2008), opium poppy (Quesada-Moraga et al., 2009) and sorghum (Tefera and Vidal, 2009) is therefore not possible. Previous grapevine inoculation trials via root dipping or soil inoculation resulted in no colonization at all (data not shown). Root dipping or soil inoculation has been used for endophytic establishment of *B. bassiana* in banana (Akello et al., 2007), sorghum (Tefera and Vidal, 2009), pine seedlings (Brownbridge et al., 2012) and for cassava (Greenfield et al., 2016). Therefore, inoculation via spray application is apparently the only option for endophytic inoculation of grapevine plants. Moreover we have shown here that such an application is also compatible with viticultural practice in the field.

Jaber (2015) reported slightly higher colonization rates of up to 50% of young grapevine plants by *B. bassiana* after artificial spray inoculation. In these experiments as well as in the present study no systemic establishment of *B. bassiana* in grapevine plants was proved. Endophytic establishment of entomopathogenic fungi is known to be dependent on plant cultivar, fungal strain and many other environmental conditions (Vidal and Jaber, 2015).

Here, we used molecular SSR markers to prove that the re-isolated *B. bassiana* strain was the one previously applied. In general, direct detection of endophytic *B. bassiana* after spray application using PCR-based techniques is difficult because of the likelihood of contamination with epiphytic propagules. In addition, surface sterilization is regarded as an insufficient technique for subsequent molecular assessment of endophytic establishment (McKinnon et al., 2014). Evidence has accumulated that for culture-based techniques surface sterilization can result in underestimated colonization rates, due to diffusion of the chemicals used for sterilization into the leaves (Lohse et al., 2015; Ownley et al., 2008). In consequence and in line with other reports only a combination of different detection methods will result in sound qualitative and quantitative data about endophytic colonization of plants (Lohse et al., 2015). In this context methods must be adapted for every plant species and different plant material.

Despite the comparatively low endophytic colonization rates of *B. bassiana* in grapevine we observed significant negative effects of endophytic *B. bassiana* on infestation and size of vine mealybug larvae in bioassays. Moreover, grape leafhopper *E. vitis* larvae were significantly more abundant on control than on endophytic *B. bassiana* grapevine plants in the field. Usually, fungal entomopathogens infect their insect hosts via cuticular penetration by germinating propagules (Boomsma et al., 2014) (Arnold and Lewis, 2005). Infection by endophytic entomopathogens via consumption of infected plant tissue or ingestion of hyphae or spores seems to be unlikely and rare (Vidal and Jaber, 2015). Existing reports about mycosis due to endophytic entomopathogens are so far restricted to insects living inside plant tissues like stem-borers or leafmining larvae (Akello et al., 2009; Klieber and Reineke, 2016), where a direct contact of insects feeding inside plant tissues and endophytic fungal propagules can be envisaged. In contrast to stem-borers or leaf-miners mealybug larvae live on the plant surface and have a piercing-sucking feeding habit with the consequence that a direct mode of action due to direct contact is not likely to occur. On surface disinfected leaves previously treated with *B. bassiana*, mealybug larvae were smaller and mortality rates were higher than on control leaves, but none of the dead larvae exhibited symptoms of mycosis. These results suggest a mode of action involving feeding deterrence, antibiosis or changes in metabolism of the host plant and thus host plant quality rather than a direct fungal infection of the insects (Vega, 2008; Vega et al., 2008). Colonization of grapevine plants was different in the two replicates of the bioassay. At a higher *B. bassiana* colonization rate, size of vine mealybug larvae was significantly smaller after feeding on endophytic leaves compared to control leaves. Vice versa, at a lower colonization rate, we detected significant differences in vine mealybug infestation rates. Accordingly, two different modes of action of endophytic entomopathogens might account for these observations, depending on rate of tissue colonization by *B. bassiana*. In any case, we have shown that the presence of entomopathogens as endophytes negatively influences insect performance, yet further investigations are required to determine the mechanisms underlying these effects. Results presented here point to the importance to also study sublethal effects of endophytic entomopathogens on insects in order to understand tritrophic interactions between plants, endophytes, and insects.

We have shown for the first time that an endophytic establishment of *B. bassiana* in mature grapevine plants under field conditions is possible. Our results also indicate the potential for a long term establishment of the fungus in grapevine plants and that endophytic establishment does apparently not interfere with common viticultural management practices. In this regard, a couple of studies have shown that *B. bassiana* is sensitive against various pesticides (Kos and Celar, 2013; Sapieha-Waszkiewicz et al., 2004; Todorova et al., 1998). However, even though synthetic fungicides were simultaneously applied in our experimental vineyard, an endophytic establishment of *B. bassiana* in the mature plats was successful. Moreover, *B. bassiana* conidia are known to be extremely sensitive to ultraviolet radiation and consequently persistence as well as germination of conidial suspensions applied on the foliage is limited. Lohse et al. (2015) emphasized the importance of an adequate formulation for endophytic establishment of entomopathogenic fungi. Here, we used a commercially available fungal-based product (Naturalis®, active ingredient *B. bassiana* isolate ATCC 74040) formulated as an oily dispersion, which may provide a benefit for the colonization process of *B. bassiana* on grapevine plants. This product is registered in some EU member states i.e. for control of whiteflies in tomato thus having the perspective of a rapid registration for other applications.

Overall, grapevine plants seemed not to be negatively affected by the presence of endophytic *B. bassiana*, as growth and performance of the respective inoculated plants was visually similar to

control plants during the period of observation (data not shown). This is in accordance with previous studies on plant performance after endophytic establishment of entomopathogenic fungi (Akello et al., 2009; Klieber and Reineke, 2016; Tefera and Vidal, 2009; Wagner and Lewis, 2000). However, whether presence of endophytic *B. bassiana* in grapevine plants has an effect on quality and sensory attributes of must and wine still remains to be tested with fruit-bearing grapevine plants.

Endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants represents a new and sustainable plant protection strategy. The implementation of the indirect effects (endophyte) in combination with direct effects (epiphyte) of entomopathogens on both plant and insect herbivores will show their full potential value in insect pest management. Further research should also include an in-depth study on the mode of action of endophytic entomopathogens against insects as well as identifying possible effects on induced resistance mechanisms against both grapevine pathogens and insect pests.

Acknowledgments

This work was supported by Deutsche Bundesstiftung Umwelt (DBU). We thank Edith Ladurner from CBC (Europe) S.r.l. – BIOGARD Division, Italy, for providing the product Naturalis® and Ottmar Baus and Mirjam Hauck for support during the field trial. Gordan Behaderovic is kindly acknowledged for help in mealybug bioassays.

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BIOCONTROL SCIENCE AND TECHNOLOGY, 2017
https://doi.org/10.1080/09583157.2017.1347604

RESEARCH ARTICLE

Association of *Beauveria bassiana* with grapevine plants deters adult black vine weevils, *Otiorhynchus sulcatus*

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ABSTRACT

Fungal entomopathogens are known as microbial pathogens of insects, colonising multiple habitats and ecosystems. Besides being an entomopathogen, the fungus *Beauveria bassiana* can also establish as an endophyte in plants. Limited knowledge is so far available on the ability of plant-associated *B. bassiana* to influence plant-feeding insects. Here, we assessed the capability of adult black vine weevils *Otiorhynchus sulcatus* to select grapevine as a host plant in the presence of plant-associated *B. bassiana* after foliar application of a commercially available mycoinsecticide (product Naturalis®) on young potted grapevine plants. Three pairwise comparisons of weevil behaviour were conducted when weevils were released in a two-choice olfactometer and were given the choice between (i) control plants and plants treated with Naturalis®, (ii) control plants and plants treated with the formulation of Naturalis® without fungal propagules, and (iii) plants treated with Naturalis® and plants treated with the formulation. Adult *O. sulcatus* were significantly deterred by plants treated with Naturalis® or the formulation in comparison to control plants. In a direct comparison between plants treated either with Naturalis® or the formulation weevils significantly preferred plants treated with the formulation and avoided Naturalis® treated plants, where *B. bassiana* putatively had established as an endophyte. These results suggest that adult black vine weevils are able to detect and subsequently avoid plants treated with *B. bassiana* and indicate a new mode of action of plant-associated entomopathogenic fungi when integrated in pest management programmes.

ARTICLE HISTORY
Received 22 February 2017
Returned 8 June 2017
Accepted 15 June 2017

KEYWORDS
Beauveria bassiana; endophyte; entomopathogen; *Otiorhynchus sulcatus*; choice assay; olfactometer

Introduction

Endophytes, a term first defined by De Bary (1884), are fungi or bacteria occurring within plant tissues without causing visible disease symptoms in the colonised plant. Even though their presence does not seem to negatively influence the plant, some endophytes have profound impacts on plant communities or have the ability to influence interactions between plants and their natural enemies. For example, certain endophytes can enhance overall plant fitness (Rodriguez, White, Arnold, & Redman, 2009) or increase resistance of plants against herbivores or pathogens as well as limit their spread and damage (Arnold

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& Lewis, 2005; Backman & Sikora, 2008; Ownley, Gwinn, & Vega, 2010; Vega, 2008). Although endophytes are present in most, if not all, plants in natural as well as in agricultural ecosystems, their function in shaping plant–insect interactions is yet not fully understood and their potentially beneficial role in sustainable plant production is not exploited so far. However, the ability of endophytes to colonise internal host tissues could be used to improve crop performance or pest management strategies. Reduced herbivory on endophyte hosting plants can be a direct result from decreased survival rates of herbivorous insects, which is often attributed to the production of defensive compounds or toxins (Clay, 1993). In addition, alterations in the plant's nutritional quality as well as changes in plant volatile profiles or secondary plant metabolites of endophyte-associated plants may influence developmental time, fecundity, host location, or oviposition behaviour of herbivorous insects (Jallow, Dugassa-Gobena, & Vidal, 2008; Vega, 2008).

Host plant selection by herbivorous insects includes a series of behavioural and decision events. The ability to detect the presence of natural enemies or pathogens in the respective host plant's environment and to react accordingly would be advantageous for any insect during foraging or oviposition site selection. Entomopathogenic fungi, such as *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales), are important mortality factors of insect pests. Some potential pest insects are able to detect entomopathogenic fungi and to avoid contact with them. The common flower bug *Anthocoris nemorum* for example can recognise its natural enemy *B. bassiana* and has been shown to avoid fungus infected leaves (Meyling & Pell, 2006). Another example of such a prevention strategy in the presence of the entomopathogenic fungus *B. bassiana* has been proved for the seven-spot ladybird (*Coccinella septempunctata*), which is able to avoid lethal densities of *B. bassiana* conidia in soil or on leaves (Ormond, Thomas, Pell, Freeman, & Roy, 2011). Other insects, such as the termite *Coptotermes lacteus*, are capable of recognising the presence of the entomopathogenic fungus *Metarhizium anisopliae* and were shown to avoid direct contact with this fungus (Staples & Milner, 2000). In contrast, Kepler and Bruck (2006) described a significant attraction of black vine weevil *Otiorhynchus sulcatus* larvae to pots containing plants and the fungus *M. anisopliae*, which is likely due to changes in volatile profiles when roots are colonised by this entomopathogenic fungus. Pivotal for these reactions are active detection mechanisms by the insects. However, the exact processes are not yet fully understood. In this context, Elliot et al. (2000) extended the herbivory-bodyguard hypothesis describing tritrophic interactions among plants, herbivores, and their predators or parasitoids also to entomopathogens and their plant association.

The black vine weevil, *O. sulcatus* F. (Coleoptera: Curculionidae), is a serious insect pest of economic importance in nursery, ornamental and soft fruit production worldwide (Moorhouse, Charnley, & Gillespie, 1992). While adult weevils are nocturnal and cause mostly cosmetic damage by feeding on the leaves, larvae are ground dwelling and feed on root systems, which may result in high levels of plant damage and subsequently kill the plant (Shah, Ansari, Prasad, & Butt, 2007). Because *O. sulcatus* has a parthenogenetic mode of reproduction, a single weevil left uncontrolled can lay up to 900 eggs, resulting in the infestation of an entire nursery (Bruck, 2007). Keeping the insect out of nurseries is one main issue in its control.

Infestation by larval stages of *O. sulcatus* can be limited by the incorporation of synthetic insecticides into the potting media (Kepler & Bruck, 2006). An alternative biological

control strategy is the application of entomopathogenic nematodes. However, practical use of this group of biological control agents is limited due to insufficient efficacy at low temperatures, a short shelf life, and high application costs (Johnson & Rasmann, 2015; Lu, Baiocchi, & Dillman, 2016). Moreover, the management of adult *O. sulcatus* includes foliar applications of pesticides, however, adult weevils are active at night, which necessitates and complicates an application at the right site and right time. Entomopathogenic fungi showed considerable potential as biological control agents against adults and larvae of the black vine weevil (Ansari, Shah, & Butt, 2008; Bruck, 2007; Hirsch & Reineke, 2014; Shah et al., 2007). Accordingly, the simultaneous use of endophytic entomopathogens as plant bodyguards as defined by Elliot et al. (2000) in addition to the already proved direct effect of entomopathogenic fungi against *O. sulcatus* would represent a dual mode of action of entomopathogens against this pest insect. For example, the presence of endophytic entomopathogenic fungi might influence host choice behaviour of adult weevils, resulting in an avoidance of the colonised plant.

In the present study, we assessed the behaviour of adult black vine weevils when given the choice between grapevine plants treated several weeks before with *B. bassiana* containing mycosinsecticide and control plants. We hypothesised that weevils are able to detect and avoid *B. bassiana* when actively searching for a host plant. The results presented here will provide information on the potential of endophytic fungi to influence herbivore host choice behaviour, and promote the development of improved management strategies for insect pests.

Materials and methods

Source of fungus, insects, and plants

Beauveria bassiana strain ATCC 74040 was used in the form of the commercial product Naturalis® (CBC (Europe) S.r.l. – BIOGARD Division). Naturalis® is formulated as an oil dispersion (OD) and contains approximately 2.3×10^7 colony forming units/ml of *B. bassiana* strain ATCC 74040 as active ingredient. The product is registered in some EU member states, e.g. for the control of whiteflies in tomato. In addition, the pure formulation of this product without conidia of *B. bassiana* was used as a control in our experiments (CBC (Europe) S.r.l. – BIOGARD Division).

A population of black vine weevil, *O. sulcatus*, was kept at 22°C and fed with grapevine leaves. Egg and larval development was completed in boxes ($h = 9$ cm, $w = 22$ cm, $l = 34$ cm) filled with 8 cm soil and using *Impatiens walleriana* plants and carrots as food source. In all assays, we used adult weevils that had emerged from pupae in the soil boxes at least 4 weeks but not more than 12 weeks before the start of experiments. At this age, weevils are in the period of maturation feeding; accordingly foliar feeding on the respective host plant is required for egg production. Prior infection experiments carried out with the same *O. sulcatus* population and the *B. bassiana* strain via direct inoculation resulted in 48–65% mortality of adult weevils within 28 days (Hirsch & Reineke, 2014).

Grapevine plants, *Vitis vinifera* (L.) cv. 'Riesling', were propagated from hardwood cuttings. After root development, the plants were potted and grown in a greenhouse chamber at 22–25°C. Seven-week-old grapevine plants with four to six fully expanded leaves were used

for treatment either with the commercial product Naturalis® (3%), the Naturalis® formulation without conidia (3%) or water. For each treatment, 40 plant replicates were inoculated separately by spraying the adaxial and abaxial surfaces of all fully expanded leaves using a 2-l handheld pressure sprayer. Approximately 10 ml were applied at each plant. Prior to the experiments, treated plants were retained in a greenhouse chamber for 1–3 weeks (mean temperature 23–25°C, mean relative humidity 50–70%) and were watered as required. The rate of endophytic establishment of *B. bassiana* in grapevine plants was tested under the same conditions and using plants of the same origin and age in parallel experiments. As reported by Rondot and Reineke (2016), 30–60% of plants could be detected as having *B. bassiana* as an endophyte. In this study, endophytic colonisation of 7-week-old potted grapevine plants by *B. bassiana* was assessed 7, 14, and 21 days after inoculation (DAI) by re-isolation from leaf tissues after surface sterilisation. For the experiments described here, we excluded additional re-isolations of *B. bassiana* or other leaf sampling analysis as described by Rondot and Reineke (2016), in order to prevent activation of plant defence reactions by mechanical damage of leaves. Since treatment and cultivation conditions were similar in both experiments, we expect identical colonisation rates.

Design and validation of the two-choice olfactometer

In order to assess host choice behaviour of adult black vine weevils, we constructed a two-choice still-air olfactometer (Figure 1). Transparent plastic cylinders (h = 30 cm, d = 13.5 cm) were modified by drilling a hole (d = 2.5 cm) into the side of each cylinder (6 cm from the bottom) and fitting a horizontal connection tube (l = 6.5 cm, d = 2.3 cm) into the hole. Two of these tubes were connected by a T-shaped piece of PVC pipe (d = 2.5 cm). The middle section of the T-shaped piece was plugged with a small petri dish lid which

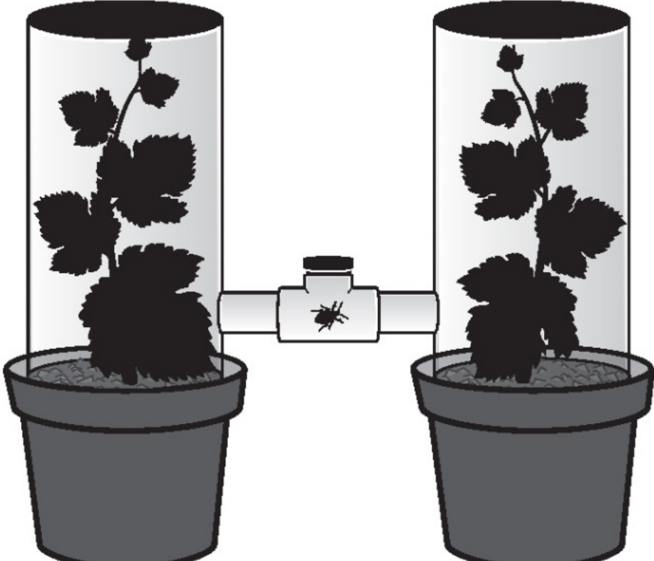

Figure 1. Design of the two-choice still-air olfactometer used in the experiments. Adult black vine weevils were placed inside the lid of the T-shaped piece connecting the two cylinders and were allowed 1 h to choose between plants in the cylinders.

served as a release point for the weevils. Each cylinder was placed on the soil surface of a potted grapevine plant and was sealed with gauze to allow sufficient air flow within the olfactometer and prevent excessive moisture. Prior to the experiments, the newly designed olfactometer was validated by releasing weevils in the T-shaped middle section and giving them the choice between a control plant placed inside a cylinder and an empty cylinder in order to observe if black vine weevils were generally able to choose the host plant in this test system. The design of the olfactometer permitted the weevils to change sides after an initial selection.

Experimental design

With the olfactometer described earlier, three different pairwise comparisons of weevil host choice behaviour were performed. Adult black vine weevils were allowed to choose between (i) control plants and grapevine plants treated 7–21 days before with Naturalis®, (ii) control plants and plants treated 7–21 days before with the Naturalis® formulation, and (iii) plants treated with Naturalis® and plants treated with the Naturalis® formulation. Plants were not surface sterilised before use in the olfactometer trials.

Because *O. sulcatus* has a nocturnal lifestyle and trials should be performed in the active period of adults, the natural daily rhythm of adult *O. sulcatus* was switched by 12 h with the help of artificial lighting. Additionally, weevils were deprived of food for 24–36 h prior to testing. Each test lasted 1 h, starting when weevils were in the active period for food searching (2–4 h after 'sunset'). Pretests indicated that most of the weevils had made their decision within 1 h with no significant changes occurring compared to their initial selection even if they were given more time (data not shown).

Moreover, we decided to assess host choice behaviour of single adult *O. sulcatus* instead of releasing several weevils at the same time to avoid aggregation behavioural effects. All trials were performed in a dark room (24 ± 2°C, 55 ± 8% RH). Nine olfactometers were used simultaneously, three for each pairwise comparison. Orientation of the olfactometer in the room was changed for every replicate. Adult *O. sulcatus* were used only once and plants were replaced every second day or when feedings sites were visible on the leaves. Cylinders and connecting tubes were thoroughly washed before each experimental day. Trials were repeated 3 times a day for 12 days, so that 108 decisions were realised and documented for every pairwise comparison. The whole experimental set-up was repeated twice in two subsequent years (2013 and 2014).

The preference of adult *O. sulcatus* was compared relative to each other. Weevils that remained in the connecting T-tube were categorised as unresponsive. Each pairwise comparison as well as the validation experiment were analysed separately. Number of decisions for each side was counted and the proportion out of the total number of responsive adult *O. sulcatus* in the trial was analysed with an exact binominal test (McDonald, 2014). The number of responsive weevils and the number of unresponsive weevils in all comparisons throughout the study were compared using a two sample *t*-test (McDonald, 2014).

Results

The usefulness of the designed olfactometer was validated by the black vine weevils' ability to select a cylinder with a grapevine plant over an empty cylinder. In both experimental replicates (years 2013 and 2014), the majority of adult *O. sulcatus* was recovered from

cylinders containing a plant (2013: $P = .03$; 2014: $P < .001$; Figure 2). Throughout all comparisons, the percentage of responsive weevils was generally high (mean = 82%) and was significantly greater than the percentage of unresponsive weevils ($P < .0001$). The weevils in the first experimental replicate in 2013 were less decisive (70% responsive) than in the second experimental replicate in 2014 (94% responsive).

When black vine weevils were allowed to choose between control grapevine plants and plants treated 7–21 days before with Naturalis®, significantly more weevils decided for the cylinders with a control plant (Figure 2). In the first replicate (2013), we recovered 67 weevils from the cylinders with a control plant, 9 weevils from the cylinders with plants treated with Naturalis® ($P < .0001$), and 32 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 62 versus 41 recovered weevils, respectively ($P = .048$), and 5 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

When weevils were given a choice between control plants and plants treated 7–21 days before with the Naturalis® formulation, significantly more weevils decided for the cylinders with a control plant (Figure 2). In the first replicate (2013), we recovered 64 weevils from the cylinders with a control plant, 14 weevils from cylinders with plants treated with formulation ($P < .0001$), and 30 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 69 versus 35 recovered weevils, respectively ($P = .001$), and 4 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

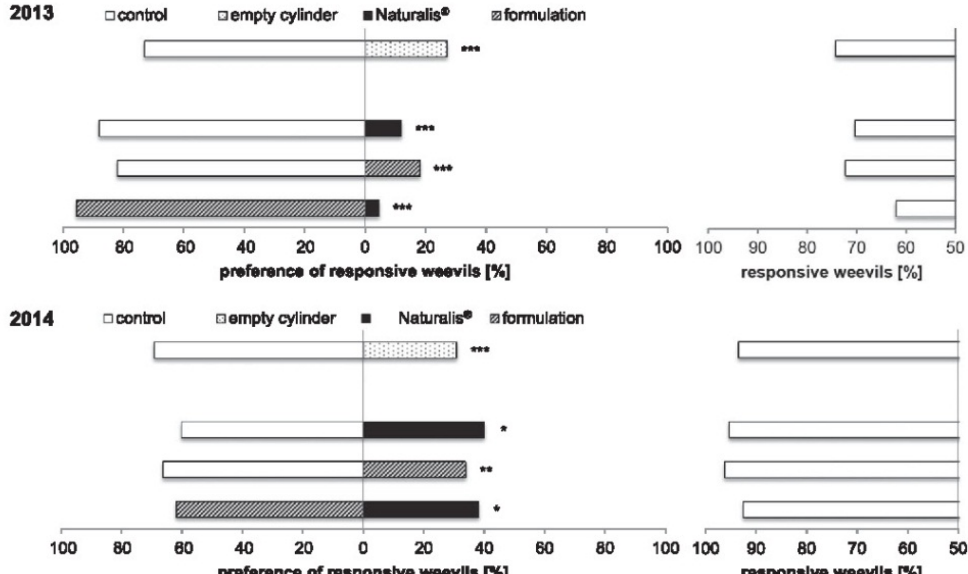

Figure 2. Percentage of adult black vine weevils *O. sulcatus* recovered when released in an olfactometer containing (i) control plants and no plant (validation assay), (ii) control plants and plants treated with Naturalis®, (iii) control plants and plants treated with the formulation, and (iv) plants treated with Naturalis® and plants treated with the formulation. Results from two independent replicates (2013 and 2014) are shown. Asterisks indicate significant differences from even distribution with $P \leq .05$ (*), $P \leq .01$ (**) or $P \leq .001$ (***). Right side of the graph depicts percentage of responsive weevils in the respective experiments.

When weevils were allowed a choice between plants treated 7–21 days before with the Naturalis® formulation without *B. bassiana* conidia or plants treated with Naturalis®, significantly more weevils decided for the cylinders with plants treated with the formulation (Figure 2). In the first replicate (2013), we recovered 64 weevils from the cylinders with a formulation-treated plant and 3 weevils from cylinders with plants treated with Naturalis® ($P < .0001$), and 41 weevils were categorised as unresponsive. In the second replicate (2014), the proportion was 62 versus 38 recovered weevils, respectively ($P = .02$), and 8 weevils were categorised as unresponsive. In both replicates, the distribution of weevils significantly differed from random.

Discussion

In this study, we proved that adult black vine weevils are able to identify grapevine plants that have been treated with a *B. bassiana* containing mycoinsecticide when actively searching for a host plant. In choice tests carried out in our newly developed plant olfactometer, weevils avoided grapevine plants treated 1–3 weeks earlier with the *B. bassiana* containing product Naturalis® as well as plants treated with the formulation of the same product. Since in a direct comparison, weevils significantly preferred plants treated with the formulation over Naturalis®-treated plants, we suppose that the presence of plant-associated *B. bassiana* is the chief factor influencing host choice behaviour. In parallel experiments using the same conditions as reported here, we have shown that the endophytic establishment of *B. bassiana* in grapevine plants can be achieved via spray inoculation of the product Naturalis®, with 30–60% of the plants being colonised between 7 and 21 DAI (Rondot & Reineke, 2016). However, fungal inoculum or other foliar residues still present on the leaf surface could be another factor contributing to adult *O. sulcatus* host plant choice behaviour, because the plants were not cleansed of any residual Naturalis® or formulation carrier before being used. Taken together, these results suggest that adult black vine weevils are able to discriminate between plants previously treated and not treated with *B. bassiana* and subsequently avoid treated plants, where *B. bassiana* is present or has established as an endophyte. Although black vine weevils are polyphagous herbivores, known to feed and reproduce on over 140 different plant species (Bruck, 2007), it has been previously shown that adults are able to discriminate between different plant species and are attracted to the odour of some but not all host plants (Van Tol, Visser, & Sabelis, 2002). Moreover, in the same study weevils were attracted to volatiles of weevil-damaged foliage of certain host plants (Van Tol et al., 2002). Visual as well as chemical cues (volatiles, aggregation pheromones, or leaf surface chemicals) are involved in the attraction of insect herbivores towards feeding or oviposition sites (Bernays & Chapman, 1994). In the context of fungal endophytes, the biochemical cues thereto can be altered directly by the growth of the fungus or indirectly mediated by the response of the plant to the fungal infection. Plants can detect the mere presence of microbes on their cuticle via microbe-associated molecular patterns (MAMPs) and respond with a number of biochemical changes (Newman, Sundelin, Nielsen, & Erbs, 2013). We ascribe the mechanism underlying this tritrophic interaction between the grapevine plant *B. bassiana* and the insect *O. sulcatus* to a complex process, mediated, e.g. through the combination of metabolic and hormonal changes in the colonised plant.

The mechanisms involved in the detection of endophytic *B. bassiana* by adult *O. sulcatus* were not examined in this study. Preliminary studies have, however, indicated that the volatile profile of endophytic *B. bassiana* grapevine plants is different compared to non-endophytic plants (Peiter, 2013). A quantitative or qualitative change in plant volatile profiles may thus play a key role for *O. sulcatus* to discriminate between endophytically colonised and endophyte-free plants. In this regard, Jallow et al. (2008) have detected significant quantitative differences in certain volatiles of tomato plants when roots were colonised by the endophytic fungus *Acremonium strictum*, which accordingly influenced host selection by adult *Helicoverpa armigera* moths. In a similar way, the colonisation of perennial ryegrass plants (*Lolium perenne*) by an endophytic fungus altered the composition of volatile compounds, which significantly influenced attraction of plants to adult African black beetles (*Heteronychus arator*) (Qawasmeh, Raman, & Wheatley, 2015). Yet in our study, it is also possible that the establishment of endophytic *B. bassiana* altered visual, contact chemoreception and mechanoreception cues, or changed the leaf surface itself. Since weevils were able to freely move around in the plant olfactometer and behavioural assays were carried out in the dark without observing weevils during the 1 h period of the choice assays, it might as well be possible that weevils decided to leave a plant after initial contact.

Assessing putative behavioural responses of insect pests including recognition and avoidance of fungal entomopathogens present as an epiphyte or endophyte on or inside the respective host plant is pivotal for designing successful biological control strategies. The observed effects on the behaviour of *O. sulcatus* in the presence of the entomopathogen *B. bassiana* are contributing to our increased understanding of the function of entomopathogens as bodyguards of plants. Endophytic establishment of an entomopathogenic fungus such as *B. bassiana* in grapevine plants might thus represent a new and sustainable plant protection strategy. Moreover, the combination of indirect effects (endophyte) and direct effects (epiphyte) of entomopathogens on insect herbivores represents a dual-control strategy of entomopathogenic fungi when integrated in pest management programmes. In this regard, future experiments should also simulate field conditions, where usually all plants are treated in the same way and *O. sulcatus* would have no choice between *B. bassiana* associated and non-associated plants. Under these conditions, we speculate an overall reduction in feeding rates and/or an increase in unresponsive weevils. Further research should also focus on the mode of action of endophytic entomopathogens as plant bodyguards against insect pests as well as on the identification of possible effects on induced resistance mechanisms in the host plant itself targeting both pathogens and insect pests.

Acknowledgements

We thank Edith Ladurner from CBC (Europe) S.r.l. – BIOGARD Division for providing the product Naturalis® and the formulation without conidia. Roman Peiter is kindly acknowledged for help in choice assays and Sandra Linck for graphical illustration of the olfactometer.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Deutsche Bundesstiftung Umwelt (DBU).

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B Danksagung/Acknowledgements

An dieser Stelle möchte ich allen herzlich danken, die mich während der (langen) Phase der Promotion begleitet und durch ihre fachliche oder persönliche Unterstützung zum Gelingen dieser Arbeit beigetragen haben.

Liebe Annette, dir möchte ich ganz herzlich danken für die Unterstützung bei der Bewerbung um das Stipendium sowie für die Offenheit, die du der Umsetzung meiner persönlichen Pläne entgegengebracht hast und dass du dennoch stets an den Abschluss der Arbeit geglaubt hast. Während der Promotion hast du mir die Möglichkeit gegeben, Erfahrungen auf internationalen Tagungen zu sammeln, mich zur Selbständigkeit im wissenschaftlichen Arbeiten ermutigt und trotzdem immer eine offene Tür für mich gehabt. Danke dafür!

Herr Poehling, der diese Arbeit ebenfalls mitbetreut hat und mir immer sein vollstes Vertrauen entgegengebracht hat, Ihnen möchte ich danken für die bereitwillige Übernahme der Betreuung seitens der Leibniz Universität, Ihre Ideen und die umgehenden Antworten auf meine Nachrichten unabhängig von Ihrem Aufenthaltsort.

Frau Winkelmann und Herrn Maiß danke ich für die spontane Bereitschaft zur Mitwirkung an der Promotionskommission.

Der Deutschen Bundesstiftung Umwelt (DBU) für die finanzielle Unterstützung und die Möglichkeit, auf Seminaren viele tolle Menschen kennenzulernen und den Ansporn immer wieder über den Tellerrand zu blicken. Frau Dr. Schlegel-Starmann für die Betreuung von Seiten der DBU und die besonderen Einblicke in die Stiftungsarbeit durch mein Amt als Sprecherin.

Many thanks to Rusty Rodriguez and his team of Adaptive Symbiotic Technologies for being my host during the short term scientific mission, for providing insights in their work and for the unforgettable stay in rainy Seattle.

Ein weiterer Dank geht an das gesamte Institut für Phytomedizin der Hochschule Geisenheim „Allen Phytos“, die mich herzlich in ihr Team aufgenommen und mich mit Rat und Tat unterstützt haben. Danke für die stete Hilfsbereitschaft und die hervorragende Arbeitsatmosphäre, die zum Gelingen dieser Arbeit beigetragen haben. Dabei bedanke ich mich besonders bei:

Winfried für die tollen Fotos von allen Arbeitsbereichen, die unermüdliche Bereitstellung von Topfreben sowie sein offenes Ohr und kreativen Lösungsvorschlägen bei Fragen zur Versuchsumsetzung; Mirjam und Dustin für die Unterstützung im Gewächshaus und im Labor; Ottmar für die Umsetzung und Unterstützung bei dem Freilandversuch; Helga für die Weitergabe ihrer Erfahrung mit Pero.

Justine, Elizabeth und Jacqui, ihr habt dafür gesorgt, dass mir das Ankommen in Geisenheim, der Phyto und der Promotion leicht fiel und viel Spaß gemacht hat. Ich wollte nicht mehr weg und bin deswegen geblieben. Danke für die unvergessliche gemeinsame Zeit! Auch bei Holger, Deniz und Ginger möchte ich mich für die unendlich vielen tollen und lustigen Stunden im Büro und im Labor bedanken. Holger, danke für die Aufmunterung und den Ansporn bis zu Letzt „Do it!“. Tina für die vielen lustigen Stunden in der WG und das Abschalten nach dem Arbeitstag. Sandra für die Anfertigung wundervoller Graphiken zum besseren Verständnis meiner Arbeit.

Besonders möchte ich meiner Familie danken, die immer hinter mir steht und mich bei meinem Lebensweg unterstützt. Ein großer Dank geht an meine Eltern, die uns in den letzten Jahren immer wieder in ihr Haus aufgenommen haben, um mir das Schreiben am Stück zu ermöglichen und meinen beiden Kindern gleichzeitig eine unvergessliche Zeit geschenkt haben. Danke für die viele Mühe, die uneingeschränkte Rückendeckung, das Vertrauen.

Lieber Michael, lieber Johann, lieber Anton, danke, dass es euch gibt!

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Auszeichnung der Rudolf-Hermanns-Stiftung für eine herausragende Masterarbeit im Studiengang Gartenbauwissenschaften

List of Publications

Peer-reviewed

Rondot Y. & A. Reineke (2019): Endophytic *Beauveria bassiana* activates expression of defence genes in grapevine and prevents infections by grapevine downy mildew *Plasmopara viticola*. Plant Pathology 68, 1719-31.

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Oral Presentations

Rondot, Y. & A. Reineke (2014): Potential of endophytic *Beauveria bassiana* in grapevine against insects. 47th Annual Meeting of the Society for Invertebrate Pathology and International Congress on Invertebrate Pathology. Berichte aus dem Julius-Kühn-Institut 174:68

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Rondot, Y. & A. Reineke (2013): Potential of the entomopathogenic fungus *Beauveria bassiana* as an endophyte in grapevine *Vitis vinifera* plants. 14th Meeting of the IOBC/wprs Working Group "Integrated Protection and Production in Viticulture", 13-17 October 2013, Ascona.

Rondot, Y. & A. Reineke (2013): The entomopathogenic fungus *Beauveria bassiana* as an endophyte in garpevine *Vitis vinifera* (L.) plants. Proceedings of the International Symposium on Plant Protection and Plant Health in Europe, „Endophytes for plant protection: the state of the art". Berlin, Germany 26-29 May 2013

Poster Presentations

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