THESIS

PHENOTYPIC AND EXPERIMENTAL VARIATION IN *BEAUVERIA BASSIANA* ISOLATES FROM THE ROCKY MOUNTAIN REGION

Submitted by

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ABSTRACT

PHENOTYPIC AND EXPERIMENTAL VARIATION IN *BEAUVERIA BASSIANA* ISOLATES FROM THE ROCKY MOUNTAIN REGION

The ubiquitous insect-killing fungus *Beauveria bassiana* is widely used as a biological control agent to treat a variety of arthropods ranging from mites to beetles. *Dendroctonus rufipennis* has been responsible for the death of 17 million *Picea engelmannii* trees over the past two decades and is currently considered to be one of the major forest pests in western North America. Despite the promise that *B. bassiana* brings as a form of augmentative biological control against *D. rufipennis*, a recent laboratory evaluation did not lead to successful field application likely due to a lack of cohesion between environmental conditions that *D. rufipennis* and *B. bassiana* prefer. Chapter 1 describes the previous literature on *B. bassiana* as a biological control agent of Hylesinini bark beetles. In 32 studies to date, not one has studied the pathogenicity of potential strains against a range of abiotic and biotic conditions representative of bark beetle habitats. Therefore, I summarize findings of how *B. bassiana* might respond to abiotic and biotic factors representative of Hylesinini beetle systems extrapolating from findings in other systems. There is a particular dearth of literature in how *B. bassiana* responds to competition with other microorganisms and plant secondary metabolites.

In chapter 2, I tested 14 *B. bassiana* isolates from the Rocky Mountain region for their growth, pathogenicity, and virulence in a series of environmental assays representative of the *D. rufipennis* study system such as a range of temperatures, competition with the spruce beetle symbiotic fungus *Leptographium abietinum,* constitutive and induced concentrations of five

Picea engelmannii monoterpenes, varying levels of osmotic potentials, a nutrient limited environment, and sunlight. Three major findings emerged from this chapter: (1) genetically related *B. bassiana* isolates from similar habitats and sources exhibit considerable variation in their growth response to environmental conditions; (2) low temperatures and monoterpenes are highly inhibitory to *B. bassiana* growth, pathogenicity, and virulence; and (3) the interpretation of isolate virulence and pathogenicity can differ substantially depending on bioassay design. These collective findings have implications for the field application of *B. bassiana* as a bark beetle control agent and could help explain discrepancies between laboratory field assessments.

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INTRODUCTION

Insect-infecting fungi are ubiquitous natural enemies of many arthropod populations ranging from the tropics to the arctic. Coevolution between fungi and the most diverse class of animals (insects) has produced an estimated 700 species of fungi in approximately 90 genera that attack and kill insects in 20 of the 30 orders, in addition to several arthropods such as ticks and mites (Araújo and Hughes 2016). The entomopathogenic fungi most commonly employed as biological control agents are Ascomycetes from the genera *Beauveria*, *Isaria*, *Lecanicillium*, and *Metarhizium* due to their ease of commercial-scale production, wide geographic host range, and their ability to infect many target organisms (de Faria and Wraight 2007; Araújo and Hughes 2016). In particular, a considerable literature has developed around the application of the genus *Beauveria* as an arthropod killer and plant endophyte. Since 2015, approximately one paper has been published each day on the genus. This, coupled with predictions that the mycoinsecticide market is expected to greatly increase in the near future (Arthurs and Dara 2018), indicates that direction may be warranted to ensure that redundant studies on entomopathogenic fungi are not taking place.

Bark beetles (Coleoptera: Curculionidae: Scolytinae) of the tribe Hylesinini are "landscape engineers;" pests that cause substantial destruction of forests by attacking vulnerable trees from the family Pinaceae in temperate regions around the world (Raffa et al. 2015). In general, Hylesinini beetles emerge as adults from phloem galleries during the late spring or early

summer to search for a mate and a new host tree with the help of aggregation and anti-aggregation semiochemicals (Saint-Germain et al. 2007). Their bodies are often home to phoretic mites (Hofstetter et al. 2013) and mycangial structures which aid in transportation of symbiotic fungi (Harrington 2005; Six 2012). Hylesinini beetles are generally monogamous (Bleiker et al. 2013) though some polygamous activity does occur through re-emergence and re-mating (Six and Bracewell 2015). Females tunnel into trees during the summer and are met by a combination of resin and induced levels of chemical defenses in the form of monoterpenes and phenolics (Raffa 2014). After overcoming plant defenses, females form species-specific galleries within the phloem to lay their eggs (Wood 1982).

Beauveria bassiana (Balsamo-Crivelli) Vuillemin ('white muscardine') is a promising biological control agent for population management of bark beetles, with little or no adverse effects on plants or vertebrates (including human applicators) (Zimmermann 2007), though host specificity against arthropods is unclear (Devi et al. 2008; Table 1.1). A cosmopolitan species, *B. bassiana* has been isolated from interior and exterior plant surfaces (St. Leger et al. 1992; Bidochka et al. 2002; Meyling and Eilenberg 2006a; Monzón et al. 2008; Yao et al. 2012), soil and water (Bing and Lewis 1992; St. Leger et al. 1992; Bidochka et al. 2002; Rehner and Buckley 2005; Quesada-Moraga et al. 2007; Yao et al. 2012; Popowska-Nowak et al. 2016), air samples (Basilico et al. 2007), insect cadavers (St. Leger et al. 1992; Bidochka et al. 2002; Rehner and Buckley 2005; Michalková et al. 2012; Schebeck et al. 2016), forest environments (St. Leger et al. 1992; Bidochka et al. 2002; Ormond et al. 2010; Davydenko et al. 2014; Popowska-Nowak et al. 2016), agricultural fields, (Bidochka et al. 2002; Meyling and Eilenberg 2006b; Pérez-González et al. 2014), and urban areas (St. Leger et al. 1992).

Insects are the primary host of *B. bassiana*, but the fungus can also saprophytically or endophytically colonize secondary hosts, which may lead to multiple pathways for dispersal. *Beauveria bassiana* is the anamorph, or asexual stage, of *Cordyceps* spp. (Rehner and Buckley 2005), and produces conidial spores which disperse, attach to, swell, and form a germ tube on suitable soft- and hard-bodied arthropod hosts (Samson et al. 1988; Shah and Pell 2003; Lacey et al. 2015). Following attachment, spore elongation increases exponentially until approximately 24 hours after germination, to a size of ~230 μm, followed by linear growth (Liu et al. 2015). The functional enzymes chitinase and protease then allow the fungus to penetrate the cuticle and infect the arthropod body and circulatory system, usually resulting in death of the host within five to fifteen days (Samson et al. 1988; St. Leger 1995; Ortiz-Urquiza and Keyhani 2016).

Following death of the arthropod host, the fungus releases infective spores within and around the cadaver or produces chlamydospores, which can tolerate long periods of dormancy that may or may not be obligate (Shahid et al. 2012). Once developed, conidia and chlamydospores are then transported passively via wind or actively via phoresy on arthropod species (Hemmati et al. 2001; Roy et al. 2001). The ability of fungi to infect arthropod hosts by direct penetration of the exoskeleton gives entomopathogenic fungi an advantage over other entomopathogenic organisms such as bacteria and viruses which often need to be ingested before killing the host.

There are substantial differences in pathogenicity and biology among isolates of *B. bassiana* (Shah and Pell 2003), which, along with genetic testing, has led to the recognition of *B. bassiana* as a species complex (Rehner and Buckley 2005). The ecological consequences of this genetic diversity remain unknown, but phenotypic variability in *B. bassiana* is well-described (Hajek and St. Leger 1994; Baverstock et al. 2010; Jackson et al. 2010). Numerous studies have

tested the ability of specific strains to sporulate, germinate, grow, and infect insects in response to various environmental factors (Doberski 1981; Morley-Davies et al. 1995; Fargues et al. 1996; Fargues et al. 1997; Ekesi et al. 1999; Bidochka et al. 2002; Devi et al. 2005; Fernandes et al. 2007; Bugeme et al. 2008; Huang and Feng 2009; Ortiz-Urquiza et al. 2016, for example). However, it has remained a challenge to broadly adopt *B. bassiana* as a reliable biological control agent across diverse environments, potentially due to an inconsistent phenotypic response and slow rate of kill (Lacey et al. 2015). As a result, commercial products have had trouble developing and often have not thrived in the market despite their appeal as potential alternatives to traditional chemical control methods (de Faria and Wraight 2001; Vega et al. 2009).

Despite more than a century of research on entomopathogenic fungi as biological control agents of insects, a gap in our knowledge remains about why promising laboratory evaluations do not always lead to successful field application in several systems (de Faria and Wraight 2001; Vega et al. 2012; Lacey et al. 2015) including with Hylesinini beetles (Davis et al. 2018). Consequently, an improved understanding of the biotic and abiotic factors that contribute to the persistence and efficacy of entomopathogenic fungi could promote more efficient and cost-effective applications for target pests, especially in the complex microhabitat that bark beetles inhabit (de Faria and Wraight 2001; de Faria and Wraight 2007; Vega et al. 2009; Hesketh et al. 2010; Jaronski 2010; Vega 2018).

In this literature review I discuss the previous research regarding the use of *Beauveria* as a biological control agent to limit bark beetle populations. I will focus on the Scolytinae tribe Hylesinini because of its unique behavior and managerial needs in temperate coniferous forest ecosystems. A vast amount of literature exists about the potential use of entomopathogenic fungi as biological control agents of other Scolytinae beetles such as *Ips* (Colepotera: Curculionidae:

Scolytinae: Tribe Ipini; Lipták et al. 2013) which are primarily secondary-colonizers of previously attacked trees, ambrosia beetles (Coleoptera: Curculionidae: Scolytinae: Tribe Xyleborini and Coleoptera: Curculionidae: Platypodinae: Tribe Platypodini; Popa et al. 2012) which are fungus farmers rather than phloem feeders, and *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae: Scolytinae: Tribe Cryphalini; Infante 2018), which is a pest of a tropical angiosperm (*Coffea*, Gentianales: Rubiaceae) rather than temperate and subtropical conifers.

This review will expand upon a review by Popa et al. (2012), which assessed the current state of biological control as a management tool for Scolytinae and complement similar reviews on biological control of *Ips* (Lipták et al. 2013) and *H. hampei* (Coleoptera: Curculionidae: Scolytinae; Infante 2018). Since system-specific environmental factors are largely lacking from the current literature (Tables 1.1, 1.2, 1.3), I will (1) examine how *Beauveria* has responded to Hylesinini-relevant biotic and abiotic factors in other systems, and (2) discuss a framework for progressing the study of *Beauveria* spp. as a biological control agent of bark beetles from the tribe Hylesinini.

ENVIRONMENTAL LIMITATIONS ON PERSISTENCE AND ENTOMOPATHOGENICITY OF *BEAUVERIA BASSIANA* IN THE BARK BEETLE STUDY SYSTEM

Beauveria bassiana is a predominantly subterranean species that requires some degree of physical protection from adverse environmental conditions, and primarily relies on arthropods for medium- to long-distance spore dispersal (Villani et al. 1999; Bidochka et al. 2001; Vega et al. 2009; Lacey et al. 2015), as opposed to mycelial growth or wind dispersal. Since Hylesinini

beetles occupy complex above-ground habitats, there is the potential that *Beauveria* will have difficulty tolerating environmental factors that are unique to these habitats. Accordingly, a variety of abiotic and biotic factors most relevant to the Hylesinini system such as (1) temperature, (2) exposure to ultraviolet light, (3) moisture conditions, (4) competition with other microbes, and (5) plant secondary metabolites can inhibit or promote *B. bassiana* performance. Each of these factors are explored in detail below.

Temperature. Ambient temperature strongly affects the efficacy of entomopathogenic fungi, and, due to the considerable differences observed between isolates at certain temperatures, is often argued to be the most important environmental factor when selecting isolates to develop as mycoinsecticides (Fargues et al. 1996; Fargues et al. 1997; Inglis et al. 1997; Blanford and Thomas 1999; Ekesi et al. 1999; Dimbi et al. 2004; Kiewnick 2006; Bugeme et al. 2008; Jaronski 2010). Partially due to ease of study and acknowledged importance, several hundred studies have recorded *Beauveria* response to temperature stresses through sporulation, germination, growth, and virulence over the previous four decades making it the most frequently tested environmental factor in *Beauveria* ecology. Despite differences between isolates at certain temperatures, overall radial or biomass growth rates are typically maximized at or near 25 °C (Walstad et al. 1970; Hallsworth and Magan 1996; Fargues et al. 1997; Ekesi et al. 1999; Yeo et al. 2003; Bugeme et al. 2008), with sporulation rates highest between 25 – 30 °C (Walstad et al. 1970; Ekesi et al. 1999; Yeo et al. 2003; Bugeme et al. 2008). Optimal temperatures for virulence generally range from 15 – 35 °C, with the majority of studies reporting maximum virulence results between 20 – 25 °C (Doberski 1981; Ekesi et al. 1999; Bidochka et al. 2002; Yeo et al. 2003; Kikankie et al. 2010). Intolerance of temperature extremes is attributed to stress

caused by osmotic imbalance (Ortiz-Urquiza and Keyhani 2015) and tissue damage resulting from the production of reactive oxidative species (Lovett and St. Leger 2018).

Despite a wide range of arthropod taxa tested for susceptibility to *B. bassiana*, including Coleoptera (Doberski 1981), Acarina (Bugeme et al. 2008), Thysanoptera (Ekesi et al. 1999), Orthoptera (Inglis et al. 1996), Diptera (Kikankie et al. 2010), Heteroptera (Leland et al. 2005), and Hemiptera (Yeo et al. 2003), minimal differences between optimal temperatures for virulence have been observed, indicating that thermal thresholds for virulence are similar across isolates. Additionally, geographic range many not be a reliable predictor of isolate growth, germination, or virulence (Fargues et al. 1997; Ekesi et al. 1999; Bidochka et al. 2002; Yeo et al. 2003; Devi et al. 2005; Bugeme et al. 2008). For instance, Ekesi et al. (1999) examined one isolate from Kenya and another from Maine, USA. The isolate from Maine experienced faster radial growth and was more virulent at the highest temperature tested, 30 °C, than the isolate from Kenya, where mean annual temperatures are typically much higher.

Isolate response to diurnal temperature fluctuations (as opposed to multiple stable temperatures typical of chamber studies) is a characteristic of natural habitats that has been understudied. Such studies are particularly important as Hylesinini systems are characterized by extreme low and variable temperatures (Six and Bracewell 2015). Inglis et al. (1999) measured *B. bassiana* growth rates and virulence against *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) nymphs in temperatures ranging from a constant 25 °C to fluctuating temperatures of 20 to 30 °C, 15 to 35 °C, and 10 to 40 °C. Growth and virulence rates tended to decrease as temperature variation increased, indicating again that *B. bassiana* is most virulent near a constant temperature of 25 °C. Devi et al. (2005) performed a similar *in vitro* test comparing fluctuating temperatures of 25 to 32 °C, 25 to 35 °C, and 25 to 38 °C against constant 25 °C. Similarly,

isolates grew and germinated best at a constant 25 °C and performed incrementally worse as temperature variability increased. This study identified an upper growth threshold of 38 °C for most isolates. Better modeling of how *B. bassiana* responds to fluctuating temperatures would enhance *B. bassiana* application in Hylesinini systems worldwide.

Ultraviolet light. Ultraviolet light, particularly UV-A and UV-B, is another limiting factor responsible for the short persistence of fungal entomopathogens in natural and managed environments (Jaronski 2010). Agostino Bassi, for which the species is named, recommended using exposure to sunlight as one way to disinfect silkworms exposed to *B. bassiana* during the Beauveriosis crisis of the 19th century (Steinhaus 1956). *Beauveria bassiana*, which lacks melanin, is not well-adapted to resist UV exposure. Like with temperature stress, UV radiation leads to oxidative stress in *B. bassiana* (Lovett and St. Leger 2018). Irradiance from the UV-A component of solar light (320 – 400 nm) can lead to conidial death and delayed sporulation (Braga et al. 2001). Additionally, the UV-B component (280 – 320 nm), while only accounting for around 5% of total solar irradiance, typically causes more tissue damage to fungal entomopathogens than UV-A (Moore et al. 1993; Inglis et al. 2001). Gardner et al. (1977) demonstrated that entomopathogenic fungi can become inactive within just hours of exposure to sunlight.

UV exposure inhibits fungal growth, sporulation, germination, and pathogenicity for practically every isolate examined and is often considered the most limiting environmental factor in field applications of *B. bassiana* (Fernandes et al. 2015). In *H. hampei* systems, for example, application of *B. bassiana* as a biological control agent is extremely successful when the beetles are protected from sunlight by shade. However, once the beetles exit the shade of *Coffea* plants and enter into direct sunlight, they are able to avoid or limit infection by *B. bassiana* through UV

inactivation (Edgington et al. 2000). While Hylesinini beetles mainly occupy the phloem layer of trees and are thus safe from the damaging effects of UV, this presents a difficulty in the mechanism of exposing beetles during their summer flight.

Since isolates perform poorly in practically any amount of UV exposure, it is advisable to apply *B. bassiana* during times of indirect solar irradiation. Some authors have even concluded that despite cases where shade or dark environments has prolonged conidial survival, *B. bassiana* will eventually die as a result of indirect irradiation (Inglis et al. 2001). In a field-based study on *B. bassiana* response to UV radiation, Inglis et al. (1997) determined that high infection rates and a more rapid development of disease symptoms were observed in *M. sanguinipes* grasshoppers placed in shaded cages than in cages exposed to full sunlight or protected from UV-B radiation. Similarly, Zhang et al. (2011), examined entomopathogenicty of 12 Coleopteran-derived *B. bassiana* isolates on a Hylesinini species, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae), and their results corroborated Inglis et al. (1997): no tested isolates performed well under conditions of UV exposure and minimal insect infection was observed. Among the isolates characterized by Zhang et al. (2011) for biological control of *D. valens*, those which grew faster and germinated more quickly were generally more virulent, possibly because they were more tolerant of UV light. Additionally, Fernandes et al. (2007) concluded that tropical isolates can tolerate more light exposure than non-tropical isolates, indicating that converse to thermal tolerances, geographic range does potentially correlate to tolerance of solar radiation.

Despite spending most of their time below the surface of a tree, application of *Beauveria* as a biological control agent of bark beetles still needs to take the effects of sunlight into account because most application methods rely on beetles becoming exposed while flying around outside

a tree. Thus, several additional methods have been explored for optimizing applications of *B. bassiana* in settings exposed to sunlight. The first is selecting genotypes, however rare, which are most resistant to UV damage. Genetic modification is another viable option for prolonging UV resistance or increasing virulence, which decreases the amount of time the fungus needs to tolerate environmental conditions (Lovett and St. Leger 2018). For example, utilizing photolyases (DNA repair enzymes that function in the repair of UV damage) from *Halobacterium salinarum* (Kingdom Euryarchaeota) improved entomopathogen spore germination rates by 32 times under UV conditions (Fang and St. Leger 2012).

Another approach considers location within the application microhabitat. Studies have attempted to apply mycoinsecticides within plant canopies (Inglis et al. 1993), which serves as a natural barrier to direct sunlight exposure and is the primary habitat for many insect pests. This approach also includes application of *B. bassiana* to the abaxial surface of leaves; though has had mixed success attributed to the difficulty of execution involved with this application method (Wraight and Carruthers 1999).

Oil-soluble sunscreens provide a promising technique for remediating detrimental effects of UV exposure, but have not been largely successful outside of controlled environments (Moore et al. 1993; Inglis et al. 1995; Bernhard et al. 1998; Inglis et al. 2001; Fernandes et al. 2015). Edgington et al. (2000) tested 22 cost-effective substances for protection of *B. bassiana* from sunlight in a laboratory setting. The authors concluded that neither egg albumen nor skimmed milk powder improved control of *H. hampei* in the field. Clay-based solar blocks, however, have proven to significantly increase fungal survival compared to liquid-based control (Inglis et al. 1995). Additionally, using *B. bassiana* as a plant endophyte has gained traction in recent years

and provides a possibility of alleviation from the UV damage problem especially if plants are grown in the shade (Vega 2018).

Relative humidity and moisture content of substrate. Relative humidity (RH) and substrate water potential also limit *B. bassiana* survival because high water availability is required for germination (Hallsworth and Magan 1996; Devi et al. 2005), and, again, this form of abiotic stress contributes to production of reactive oxygen species (Ortiz-Urquiza and Keyhani 2016). Additionally, water availability regulates conidiogenesis on cadavers which have suffered from mycosis (Inglis et al. 2001). A study by Devi et al. (2005) observed decreasing growth rates as water potentials decreased (from 0 to -1.76 MPa). Results varied from complete growth inhibition to just a 4% decrease when compared to the control under an osmotic potential of -1.76 MPa for the 29 isolates. Results also varied when both temperature and water availability were implemented in conjunction, indicating that these environmental factors interact to drive growth responses in *B. bassiana*. Temperature proved to be more limiting than water potentials for the isolates overall, and nearly half of the isolates showed complete inhibition when temperatures were increased while water potential was brought to less stressful conditions.

Two conclusions regarding *B. bassiana* response to RH have pervaded the literature. The first is that relatively low virulence is likely in environments with a relatively low RH ranging from 46 – 53% (Akbar et al. 2004; Lord 2011). In contrast, other authors have concluded that *B. bassiana* germination, growth and virulence is maximized in RH conditions ranging from 95 – 100% (Walstad et al. 1970; Doberski 1981; Marcandier and Khachatourians 1987; Wraight et al. 2000; Shipp et al. 2003). An example of *B. bassiana* preferring low humidity is the study by Akbar et al. (2004) who tested one *B. bassiana* isolate of unknown geographic and host source for the effects of differing RH on virulence of *Tribolium castaneum* Herbst (Coleoptera:

Tenebrionidae). This isolate was most virulent at 56% RH (LC_{50} of 248.9 mg/kg) compared to 75% RH where the LC_{50} was 298.3 mg/kg. Doberski (1981) examined one isolate of unknown geographical origin and host source for its virulence against a bark beetle, *Scolytus scolytus* Fabricius (Coleoptera: Scolytinae), in relative humidity values ranging from 51 – 100%. While the isolate was able to infect and kill insects at all tested RH's, it was most virulent at RH ranging from 95 – 100%. Similarly, Walstad et al. (1970) concluded that isolates generally prefer RH above 92.5% for spore germination, mycelial growth, and sporulation.

Some studies have even shown that ambient humidity may not always be relevant to *B. bassiana* (Marcandier and Khachatourians 1987; Fargues et al. 1997; Inglis et al. 2001). As with thermoregulation in insects and shade provided by plants to reduce UV harm, boundary layers containing high moisture surround vegetation and arthropod exoskeletons which can allow for *B. bassiana* to persist in arid environments. Similarly, rainfall plays a role in conidial dispersal and the ability of entomopathogenic fungi to survive in varying environments, though it can also aid in dislodging *B. bassiana* from its host in many microhabitats (Inglis et al. 1995; Inglis et al. 2000). The apparent discrepancy among these published results complicate the interpretation of effects of RH on *B. bassiana* life history traits and does not indicate any general conclusions. Hylesinini beetles, which spent the majority of their time in the phloem, an extremely humid environment, must tolerate gradually drier conditions as trees die. More research in this area, particularly emulating conditions representative of Hylesinini systems (e.g. Klepzig et al. 2004), could benefit our understanding of factors impacting field applications of *Beauveria* against Hylesinini.

Competition with other microorganisms. Studies on *B. bassiana* competition with other microbes are uncommon but extremely important to understanding the role that *B. bassiana*

plays within the Hylesinini microhabitat. In a field study in the *M. sanguinipes* system, Inglis et al. (1999) examined competition between two entomopathogenic fungi, *B. bassiana* and *Metarhizium flavoviride* Gams & Roszypal (Hypocreales: Clavicipitaceae). The authors found that *M. flavoviride* was a better competitor due to its ability to tolerate heat stress. Field studies on fungal competition can be difficult to interpret but can be very informative if done properly (Shearer 1994); yet, Petri dish studies are generally necessary for testing direct interactions related to primary (acquiring space) and secondary (maintaining space) resource capture mechanisms. Jaber and Alananbeh (2018) tested inhabitation between *B. bassiana* and several species of the plant pathogen *Fusarium* (Hypocreales: Nectriaceae) in *in vitro* tests and as an endophytic plant pathogen antagonist in *Capsicum annuum* L. (Solanales: Solanaceae). *Beauveria bassiana* was able to significantly inhibit growth of every *Fusarium* species in the Petri dish studies and limit *Fusarium* establishment *in planta*.

Many bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae Latreille) are associated with a consistent community of symbiotic fungi (Harrington 2005). These fungi function as plant pathogens or nutritional mutualists; thus, in bark and ambrosia beetle systems *B. bassiana* is mainly competing for nutrients and space within a shared area. Using the bark beetle symbiotic fungus *Leptographium abietinum* (Peck) Wingfield (Ophiostomatales: Ophiostomataceae), Davis et al. (2019) showed that *L. abietinum* was a slightly better competitor than *B. bassiana* in assays testing acquisition of primary growth space, but both fungi were able to maintain captured space. Castrillo et al. (2016) observed the same deadlock in assays testing secondary resource capture behavior between *B. bassiana* and the ambrosia fungi *Ambrosiella roeperi* Harrington & McNew (Microascales: Ceratocystidaceae) and *Ambrosiella grosmanniae* McNew, Mayers, & Harrington when *B. bassiana* and the ambrosia fungi were allowed to start

competition at the same time. However, when the ambrosia fungi were given a head start, to mimic delayed entrance of *B. bassiana* into the system, the ambrosia fungi were able to capture primary resource space and grow over *B. bassiana*, suggesting that priority effects may occur among fungal species *in situ*. Zhou et al. (2018) examined *B. bassiana* competition with different ambrosia fungi (*Raffaelea lauricola* Harrington, Fraedrich, & Aghayeva; Ascomycota: Ophiostomatales), but also observed slower growth rates in *B. bassiana* than ambrosia fungi. Another study exposed volatile compounds produced by the yeast *Ogataea pini* Holst (Saccharomycetales: Saccharomycetaceae) to *B. bassiana* and the *Dendroctonus brevicomis* LeConte symbiotic fungus *Entomocorticium* sp. (Agaricomycetes: Peniophoraceae; Davis et al. 2011) to examine how *O. pini* indirectly affects growth of each fungus individually. Growth was inhibited in *B. bassiana*, while growth of the Hylesinini-symbiotic fungus was significantly enhanced. Though *B. bassiana* appears to be a fairly poor competitor overall, an understanding of the complex multitrophic interactions between *B. bassiana* and beetle-symbiotic fungi is vital to addressing pathogenicity of *B. bassiana* against bark beetles.

Plant secondary metabolites. As plants are attacked by herbivores, they generally produce secondary metabolites which often function as their main line of defense; this is especially apparent in Hylesini systems. Most bark beetles and their fungal symbionts are exposed to significant quantities of monoterpene hydrocarbons during the process of tree colonization, and they are highly tolerant of these compounds. Since *B. bassiana* attacks insects to complete its life cycle, it is inevitable that *B. bassiana* will come into contact with plant secondary chemicals, especially monoterpenes, and that their response could have dramatic effects on the tri-trophic interaction between plants, insects, and fungi. Tomato plants upregulate monoterpene production in response to the presence of beneficial fungi such as *B. bassiana*

(Shrivastava et al. 2015). However, one study indicates that when looking at the interaction from the perspective of *B. bassiana*, induced levels of monoterpenes produced by conifers could have detrimental effects on *B. bassiana* growth (Davis et al. 2018). Another study found that *B. bassiana* was very tolerant of *Pelargonium graveolens* L'Hér (Geraniales: Geraniaceae) plant secondary metabolites in the form of essential oils (Nardoni et al. 2018). A lack of literature on this subject is problematic, especially from a Hylesinini management perspective. As research on the application *B. bassiana* as a plant endophyte increases, it will be necessary to understand tolerance of the fungus to plant secondary compounds which might limit colonization or virulence. The few available studies that examine *B. bassiana* growth responses to plant secondary metabolites indicates that few isolates are likely to be tolerant of acute exposure to plant defensive chemicals.

INSECT BEHAVIOR AS A LIMITATION TO ENTOMOPATHOGENICITY

Insects explore and evaluate their environment for both abiotic and biotic dangers when obtaining food, mates, oviposition sites, nesting locations, and refuge (Bell 1990). In nature there is often a combination of external abiotic and biotic factors that influence insect avoidance, attraction, or 'non-avoidance' behavior (Baverstock et al. 2010). Pathogenic and non-pathogenic *B. bassiana* isolates produce different volatile blends, which are likely able to be detected by insects to influence behavior (Mburu et al. 2013). Understanding how insects are able to respond to the presence of their pathogens is critical to the use of *B. bassiana* as a biological control agent.

Most *Beauveria*-Hylesinini studies have included some type of material from the host tree in their bioassays to serve as nutrients for the beetles (Table 1.3), a first step in addressing

how bark beetle behavior is affected by *Beauveria*. Kreutz et al. (2004b) tested the ability of *B. bassiana* to kill *Ips typographus* L. (Coleoptera: Curculionidae: Scolytinae) in bioassays which included three different substrates – (1) filter paper, (2) spruce bark, and (3) an artificial diet. While the beetles died fastest on filter paper, treatment of *B. bassiana* on filter paper only killed the beetles 0.8 days faster than the filter paper control on average; versus a 5 or 6 day spread created by the artificial diet and spruce bark material, respectively. These Hylesinini system-specific bioassay mediums have been presented in the form of sawdust (Krams et al. 2012), pieces of bark (Moore 1970; Moore 1973; Burjanadze 2010; Sevim et al. 2010; Tanyeli et al. 2010; Kocacevik et al. 2015; Kerchev et al. 2017), branches (Srei et al. 2017; Khanday and Buhroo 2018), and phloem material (Pabst and Sikorowski 1980; Whitney et al. 1984; Zhang et al. 2011; Xu et al. 2018) held in either dark plastic cages (Moore 1970; Moore 1973; Burjanadze 2010; Sevim et al. 2010; Tanyeli et al. 2010; Zhang et al. 2011; Kocacevik et al. 2015), translucent containers (Krams et al. 2012), or in glass 'sandwiches' (Whitney et al. 1984). Ideally, future bioassays on Hylesinini species would include a representative medium, such as phloem, in an arena that the investigators could view the behavior of bark beetles as *Beauveria* is introduced into the system (e.g. Aflitto et al. 2014).

The only *Beauveria*-Hylesinini study to truly take insect behavior into account was designed to observe the 'walling off' activity that reportedly occurs after beetles are infected by an entomopathogenic fungus so that remaining brood or other colonizing beetles do not become contaminated (Whitney et al. 1984). The 'walling off' behavior is an example of bark beetles being able to detect and avoid an entomopathogenic fungus, something that could have severe management implications in future application of the fungus. Despite the altered behavior observed by Whitney et al. (1984) when bark beetles were in the presence of *B. bassiana*, there

have not been any Hylesinini studies to elaborate upon this finding in the behavioral sense; however the potential for vertical (Kocacevik et al. 2015) and horizontal transmission (Kreutz et al. 2004a) is gaining increased attention in bark beetle studies. Due to the lack of Hylesinini-specific studies regarding bark beetle behavioral response to entomopathogenic fungi, I summarize findings from other systems.

Insect attraction to entomopathogenic fungi. Collembolans (Order: Collembola) have been shown not only to tolerate *B. bassiana* and other entomopathogenic fungi, but through behavioral assays have shown an attraction towards entomopathogens at low levels of fungal concentration (Dromph and Vestergaard 2002). Similarly, above-ground insects such as females of the mosquito *Anopheles stephensi* Liston (Diptera: Culicidae) have also shown attraction towards entomopathogenic fungi (George et al. 2013). The authors of both of these studies concluded that since *B. bassiana* is ubiquitous, it is probable that many insect species will be exposed to the fungus and that attraction towards the fungus is likely due to vector manipulation mechanisms that facilitate pathogen dispersal (*sensu* Eigenbrode et al. 2018).

Another hypothesis regarding insect attraction towards entomopathogenic fungi is that insects prefer the same environmental conditions as their pathogens. Brütsch et al. (2014) demonstrate this with ant queens of the species *Formica selysi* Bondroit (Hymenoptera: Formicidae). They found that queens preferred to settle in nest sites containing fungal pathogens and despite *B. bassiana* being the most virulent of the three entomopathogenic fungi species examined, the ant queens most frequently settled in *B. bassiana*-infected nest sites. As discussed in earlier sections, several studies have shown that entomopathogenic fungi exhibit increased growth, germination, and virulence when exposed to certain environmental factors such as a moderate temperature, low ultraviolet light, high relative humidity, and ample nutrient

availability--the same conditions that are generally favorable to soil-dwelling insects (Morley-Davies et al. 1995; Doberski 1981; Fargues et al. 1996; Fargues et al. 1997; Ekesi et al. 1999; Bidochka et al. 2002; Bugeme et al. 2008; Devi et al. 2005; Fernandes et al. 2007; Huang and Feng 2009; Ortiz-Urquiza et al. 2016).

Pontieri et al. (2014) provide another potential hypothesis to explain insect attraction towards pathogens; in social insects, entomopathogenic fungi may serve as a tool to "immunize" the colony. This hypothesis derives from an example where *Monomorium pharaonis* L. (Hymenoptera: Formicidae) colonies detected and preferred nests infected with the *Metarhizium brunneum* Petch, significantly more than noninfected nests in the same area. The authors speculated that this behavior may serve to expose the colony to non-lethal levels of pathogens and consequently enhance immunocompetence. Since practitioners usually take an augmentative approach to biological control when applying entomopathogenic fungi in an effort to overwhelm pest populations with pathogens, the ability of insects to tolerate low levels of entomopathogenic fungi might not be informative to pest managers.

Insect detection and avoidance of entomopathogenic fungi. A sizeable literature indicates that the most prominent arthropod behavior exhibited in relation to *B. bassiana* is avoidance of the pathogen, often as detected through chemoreception. The ability of insects to detect and avoid pathogens affects fungal virulence and, while largely ignored by practitioners, could play a significant role in the application of entomopathogenic fungi as biological control agents in many systems (Baverstock et al. 2010).

Soil-dwelling and subterranean arthropods appear to be especially capable of avoiding entomopathogenic fungi based on chemical cues, potentially due to co-evolution with *B. bassiana* as a predominantly soil-inhabiting fungus. Thompson and Brandenburg (2005)

examined how two species of mole cricket, *Scapteriscus borellii* Giglio-Tos (Orthroptera: Gryllotalpidae) and *S. vicinus* Scudder reacted to the presence of *B. bassiana* in soil. They found that the crickets produced significantly less new surface tunnels and fewer tunnels descending into the soil when *B. bassiana* was present, resulting in reduced exposure to harmful conidia. Mburu et al. (2009) concluded that subterranean *Macrotermes michaelseni* Sjöstedt (Isoptera: Termitidae) were able to detect and avoid virulent isolates of either *M. anisopliae* or *B. bassiana* from a distance, before pathogenicity was possible. Similarly, the same authors concluded in a later publication that *M. michaelseni* was able to detect harmful levels of *B. bassiana* and, thus, were repelled by the lethal concentrations via volatile signals (Mburu et al. 2013).

Similar patterns of avoidance have been documented in terrestrial arthropods. Meyling and Pell (2006) found that when *Anthocoris nemorum* L. (Heteroptera: Anthocoridae) were presented with infected exoskeletons, they were able to detect and avoid *B. bassiana* while foraging on host plants. Harmless paper ball dummies created to mimic *B. bassiana* were, however, ignored, indicating the behavioral cues were non-visual. Ormond et al. (2011) also studied virulence of *B. bassiana* endophytic in leaves against *Coccinella septempunctata* L. (Coleoptera: Coccinellidae). Both male and female *C. septempunctata* detected and avoided infected leaf surfaces and soils inoculated with *B. bassiana*.

Natural enemy populations can also detect and avoid entomopathogenic fungi. For instance, Seiedy et al. (2013) examined whether the predatory mite *Phytoseiulus persimilis* Evans (Mesostigmata: Phytoseiidae) altered behavior to avoid *B. bassiana* used to treat *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae). Their results indicated that *P. persimilis* can detect *B. bassiana* and that the predator avoids the fungus, suggesting that behavioral avoidance may benefit natural enemies and could enhance pest control applications.

In a subsequent study, Seiedy (2015) found similar results with a predatory mite, *Amblyseius swirskii* Athias-Henriot (Mesostigmata: Phytoseiidae) and the pest herbivore *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae). The predator in this scenario was, again, able to recognize the presence of *B. bassiana* and avoid infected *T. vaporariorum*. In a third study, *Hippodamia variegata* Goeze (Coleoptera: Coccinellidae) avoided *Aphis fabae* Scopoli (Hemiptera: Aphididae) infected with *B. bassiana* conidia on plants (Seiedy et al. 2015).

Insect behavior may also be responsive to changes in entomopathogenic fungi application techniques. For example, in a study that applied *M. anisopliae* to abaxial leaf surfaces, the pest insects *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Phaedon cochleariae* Fabricius (Coleoptera: Chrysomelidae), altered their feeding behavior and larvae began feeding on adaxial leaf surfaces (Amiri et al. 1999).

Despite the dearth of literature directly addressing Hylesinini mechanisms that may explain the 'walling off' behavior observed by Whitney et al. (1984), previous studies examining how bark beetles respond to microbial volatile organic compounds allow for some prediction. For example, hexanol (a typical 'green leaf volatile') is a prominent component of both pathogenic and non-pathogenic isolates of *B. bassiana* (Mburu et al. 2013) and also acts as a repellent in *Dendroctonus ponderosae* Hopkins and *D. frontalis* Zimmermann (Kandasamy et al. 2016). The processes and mechanisms behind insect detection and attraction or avoidance behaviors in response to entomopathogenic fungi are generally unclear, but a stronger understanding of these effects could substantially improve application of entomopathogenic fungi as biological control agents of bark beetles and other pests.

Arthropod resistance mechanisms. Grooming behavior by insects can reduce infection rates of *B. bassiana* (Villani et al. 1999). Boucias et al. (1996) studied how *B. bassiana* affected

behavior of *Reticulitermes flavipes* Kollar (Blattodea: Rhinotermitidae) in Florida, concluding that under low levels of exposure worker-caste *R. flavipes* could, through grooming, suppress *B. bassiana* from reproducing and consequently reduce transmission rates. However, when the chemical pesticide imidacloprid was added, less grooming behavior was observed, indicating that addition of biological agents to traditional chemical methods could be synergistic and useful for insect control.

Similarly, Yanagawa et al. (2008) studied how colonies of *Coptotermes formosanus* Shiraki (Blattodea: Rhinotermitidae) defended themselves against three species of fungal pathogens including *Isaria fumosorosea* Wize (Hypocreales: Clavicipitaceae), *Beauveria brongniartii* (Saccardo) Petch, and *Metarhizium anisopliae* (Metchnikoff) Sorokin. When reared in groups, colonies were highly resistant to the entomopathogenic fungi. However, when individuals were challenged with fungal inoculum, they were not as effective at defending themselves against the fungi. These results suggest that, when exposed to harmful pathogens, self-grooming is less effective at removing conidia than mutual grooming of nest-mates in *C. formosanus* colonies. Yanagawa et al. (2011a) compared *C. formosanus* grooming behavior following exposure to entomopathogenic fungi of varying virulence. Workers groomed infected nestmates more frequently than uninoculated workers, but differing amounts of virulence had no effect on grooming behaviors. In a follow-up study, Yanagawa et al. (2011b) determined that fungal odors triggered the behavioral response to groom infected individuals.

In addition to grooming, basking and thermoregulation behaviors may serve to limit infection by *B. bassiana*; these effects are well-described on the migratory grasshopper *M. sanguinipes* in a series of studies by Inglis et al. (1996; 1997; 1999). Nymphs reduced *B. bassiana* infection rates by 98% by basking for at least 6 hours per day. Infected individuals also

chose to go to the areas with the highest temperatures, suggesting that thermoregulation is taking place within the body and that *M. sanguinipes* responds to *B. bassiana* infection with a 'behavioral fever.' Collectively, these studies indicate that matching fungal traits to environmental conditions alone may not be sufficient as a means of pest control of Hylesinini species, as a suite of insect behaviors can also strongly impact the efficacy of *B. bassiana* and other mycological agents in the field and much is still unknown about how Hylesinini beetles respond to *B. bassiana*.

CURRENT STATE AND FUTURE DIRECTIONS FOR BIOLOGICAL CONTROL OF HYLESININI BEETLES WITH *BEAUVERIA BASSIANA*

Hylesinini population control is complicated because the beetles have a cryptic lifecycle, live in a complex environment, and mainly attack chemically well-defended hosts across large natural landscapes. As a whole, literature documenting that *Beauveria* exists in natural systems is vast; with isolations coming from 11 of the 15 studied Hylesinini species (Tables 1.1 and 1.S1). This is an important first step, especially when marketing *B. bassiana* as an augmentative biological control for a pathogen that is already present in that pest's natural environment. Isolating and identifying strains from a certain environment is also beneficial to understanding isolate to isolate variation and answering basic biological questions such as "how host specific is *Beauveria* and what determines host specificity?"; and "how do strains from certain geographical locations and hosts differ in their response to certain environments?" Future studies should focus on gathering information on the amount of *B. bassiana* present in Hylesinini systems by both measuring colony forming units (see Reay et al. 2008 and Yao et al. 2012) and by documenting

the amount of samples that contain *Beauveria* (as in Brownbridge et al. 2012; Takov et al. 2012; and Schebeck et al. 2016).

Screening potential *B. bassiana* isolates for tolerance in system-specific environmental conditions should be the next step when selecting a strain for potential Hylesinini field application. Evaluations of environmental tolerance have only been conducted on 6 of the 15 studied Hylesinini species (Table 1.1), which could explain laboratory to field discrepancies and is the inspiration for this literature review. Future research should address how potential biological control isolates respond to fluctuating temperatures, the mechanisms behind *B. bassiana* competition with other microorganisms, and if *B. bassiana* is able to tolerate a variety of plant secondary metabolites. Additionally, studies should address the ability of isolates to tolerate multiple stressful environmental conditions simultaneously, as every growth, germination, and tolerance test was done under 'room conditions,' or at around 20 – 25 °C with presumed humidity levels between 30 and 60%. Hylesini beetles live in complex environments and a multivariate approach should be taken when evaluating potential strains of *B. bassiana* for biological control to enhance our understanding of which traits can be matched with habitat conditions.

Laboratory bioassays under representative environmental conditions which take insect behavior into account are the next step to evaluating potential *B. bassiana* strains for control of Hylesini beetles. Bioassays have been done on the majority of Hylesinini beetles that have been studied in association with *B. bassiana* (Table 1.1). Gaps in the literature likely exist due to difficulty capturing sufficient supply of individuals (as in Reay et al. 2010 and Glare et al. 2008), controlling for insect age and life history, or a lack of interest in developing management strategies to control that Hylesinini species. Every bioassay conducted on Hylesinini species, so

far, has taken place under ‘room conditions’ (Table 1.3) apart from Moore (1973) which examined pathogenicity at a variety of temperatures and humidity levels. The rate of mortality increased as temperature increased, though a mycosis test was not done post-mortality to confirm that *B. bassiana* was the mortality agent. Nonetheless, this study points out the potential impacts of variable environments on interpretation of isolate virulence.

The use of tree-based bioassay media has been extremely common throughout the Hylesinini-*Beauveria* literature (Table 1.3). A tree-based medium containing the phloem is an extremely important step when evaluating potential Hylesinini biological controls as a spore solution filled filter paper can have misleading results (Kreutz et al. 2004a). A proper medium also provides shelter and nutrients for beetles while exposing the beetles and *B. bassiana* to other microorganisms and tree secondary metabolites. Phloem-based bioassays also allow investigators to observe beetles and to take insect behaviors into account when screening potential isolates. Very little is presently known about potential ‘detection and avoidance’ behaviors or even horizontal and vertical transmission that may occur inside Hylesinini galleries when *B. bassiana* is introduced.

Surface sterilization to remove any incidental *B. bassiana* contamination on the Hylesinini cuticle only occurred in four of the 18 Hylesinini-*Beauveria* studies that conducted bioassays. I do not advise surface sterilization when attempting to create the most representative bioassay conditions because it removes all of the symbiotic microorganisms that are representative of the bark beetle microhabitat, can be damaging to adult beetles and larvae, and may allow pathogens to penetrate the exoskeleton more easily. However, it is necessary in certain studies, such as those that isolate a specific aspect of the Hylesinini-*B. bassiana* interaction (as in Xu et al. 2018). Future studies involving *B. bassiana* strain selection for

biological control of Hylesinini beetles should use surface sterilization sparingly in laboratory bioassays to reproduce conditions representative of bark beetle microhabitats.

The Hylesinini-*B. bassiana* studies also varied in their method of inoculation. It is important to match the method of inoculation in a laboratory setting with the method that practitioners plan to use in the field. Nine of the studies used a direct method of inoculation such as dipping the insects or applying drops of spore solution directly to the insect. Direct application is desirable when standardizing for spore concentration is important, such as an initial pathogenicity evaluation. It can also be beneficial if the planned method of field inoculation is by releasing infected individuals with the goal of horizontal or vertical transmission through populations. An indirect inoculation method, such as spraying a surface before insects were added to the arena, was used in ten of the studies. These techniques may be a more accurate representation of future field application, especially with Hylesinini beetles who can only become exposed to a mycoinsecticide during their summer flight period and, thus, need to come into contact with spores that have been added to the environment likely either by spraying or through an auto contamination trap.

Formulations that kill both adults and larvae will be the most effective for bark beetle management applications. Once the adult enters the tree and reproduces, it has already succeeded and a method that targets solely adults will not effectively reduce the size of Hylesinini populations if adults do not suffer mortality prior to reproduction. Killing larvae is also dependent on effective vertical transmission of *B. bassiana* from adults to eggs or larvae during maturation feeding because larvae only feed in uncontaminated areas of the phloem (Wegensteiner et al. 2015).

The method of treatment is likely a limiting factor in *Beauveria* success in Hylesinini field settings and should be refined in 'semi-field' environments similar to Kreutz et al. (2004b). Davis et al. (2018) conducted the only field trial on the ability of *Beauveria* to act as a microbial control agent of a Hylesinini species. The researchers used a spraying method to coat the surfaces of trees with *B. bassiana* conidia during a *Dendroctonus rufipennis* Kirby outbreak, though the amount of beetles that emerged from treated trees did not differ from the amount of beetles that emerged from the control. Many practitioners and scientists now recommend the use of 'assisted auto-dissemination' systems for introducing entomopathogenic fungi into field environments (Baverstock et al. 2010) which attract an insect into an inoculation device to become contaminated with the entomopathogen before returning to its host environment to infect other pests either horizontally or vertically. These can be particularly valuable for bark beetle systems where spraying an entire forest is not feasible and beetle aggregations are easily incited with the deployment of sex pheromones (Gillette et al. 2012). Additionally, a dissemination chamber can protect *Beauveria* from UV and rainfall which may be particularly limiting for *B. bassiana* foliar and bark application. This type of technology is currently being developed in the emerald ash borer system (*Agrilus planipennis* Fairmaire Coleoptera: Buprestidae) system by coating multifunnel traps (Lyons et al. 2012) and has been adapted for use against *Dendroctonus* species by creating a fungal-coated pouch inoculated with *B. bassiana* on a pheromone-baited Lindgren funnel trap (Srei et al. 2017). These technologies, and matching isolate selection, are still in the early stages of development as a recent field trial on *Beauveria* versus *I. typographus* had unsuccessful results Grodzky and Kosibowicz (2015).

Another alternative to the potentially ineffective bark and foliar sprays is the use of *Beauveria* as an endophyte. *Beauveria* has been isolated from pines (Ganley and Newcombe

2006) and used as an endophyte to control *Hylastes ater* Paykull (Coleoptera: Curculionidae: Scolytinae) and *Hylurgus ligniperda* Fabricius (Coleoptera: Curculionidae: Scolytinae), both of which are invasive species in New Zealand (Reay et al. 2010; Brownbridge et al. 2012). Studying the effects of *B. bassiana* as an endophyte is difficult due to the fact that young trees are not susceptible to bark beetle attacks and mature trees may be difficult to inoculate (Vega 2018). Regardless, future techniques are surely going to be safer than methods that involve spreading conidial powder via the use of explosives, as has been done in the past to treat bark beetles with *B. bassiana* (Li et al. 2010).

Finally, I recommend studying the potential adverse effects of applying *B. bassiana* to a largely unmanaged natural environment. Studies should evaluate how a strain of *B. bassiana* changes as it passes through the environment over many generations, similar to Valero-Jimenez et al. (2016). There are many insects that occupy trees, understory vegetation, and soil in forest environments that may be susceptible to *B. bassiana* (Makino et al. 2006; Lacey et al. 2015). None of the Hylesinini-*B. bassiana* studies have addressed the potential for off-target effects, and *Beauveria*'s effects on various arthropods and its degree of host specificity are unclear (Devi et al. 2008; Imoulan et al. 2017). Albeit under 'room conditions', one potentially promising prospect has arisen, though, in a case where *B. bassiana* was highly virulent against *Ips sexdentatus* Boerner (Coleoptera: Curculionidae: Scolytinae) but not pathogenic against its predator *Thanasimus formicarius* L. (Coleoptera: Cleridae; Steinwender et al. 2010).

CONCLUSION

This chapter explores the potential abiotic and biotic limitations on *B. bassiana* as a biological control agent of Hylesinini pests. Bark beetles in the Hylesinini tribe inhabit complex

systems with variable temperatures, low levels of ultraviolet light, increasingly drier conditions as trees die, a series of symbiotic microorganisms, and significant quantities of tree phytochemicals. There is also the potential that beetles can avoid or are attracted to entomopathogenic fungi which would play a role in the efficacy of *B. bassiana* in Hylesinini habitats. Overall, the previous literature on pest management of Hylesinini using mycologically-based pesticides, such as *B. bassiana*, has neglected evaluations of how *B. bassiana* responds to environmental conditions representative of Hylesinini habitats. Future studies should match fungal traits to the environment; paying particular attention to how strains of *B. bassiana* may respond to the presence of bark beetle symbiotic fungi and monoterpenes found in the phloem of trees that Hylesinini beetles attack. These environmental factors have not been thoroughly covered in other systems but play particularly important roles in Hylesinini habitats. Finally, future isolate screening processes and bioassay methods should be standardized to minimize discrepancies between laboratory evaluations and field application.

TABLES

Table 1.1. *Beauveria bassiana* studies in Hylesinini systems.

Hylesinini species	Presence of *B. bassiana* in the system	*B. bassiana* growth, germination, or sporulation	Lab bioassay	Field trial
Dendroctonus brevicomis	-	Growth[1]	-	-
Dendroctonus frontalis	Larvae[2,4], Pupae[4], Adults[3,4]	Tolerance[3]	Larvae[6], Adults[3,5]	-
Dendroctonus micans	Adults[9]	-	Larvae[7,8,9], Adults[7,8,9]	-
Dendroctonus ponderosae	Oral secretions[10]	Germination[11,12]	-	-
Dendroctonus rufipennis	Oral secretions[10]	Growth[13,14]	Adults[13]	X[13]
Dendroctonus simplex	-	-	Adults[15]	-
Dendroctonus valens	Soil[17], Trees[17], Adults[17], Frass[17]	Growth[18], germination[18], and tolerance[18]	Larvae[16,18]	-
Dryocoetes confusus	Larvae[19], Pupae[19], Adults[19]	-	Adults[19]	-
Hylastes ater	Soil[22], Trees[20,22,23], Adults[21,22], Frass[22]	-	Adults[22]	-
Hylurgops palliatus	Adults[24]	-	-	-
Hylurgus ligniperda	Soil[22], Trees[20,22,23], Adults[21,22,25], Frass[22]	-	Adults[21,22,23]	-
Polygraphus major	-	-	Adults[26]	-
Polygraphus proximus	-	-	Adults[27]	-
Tomicus minor	Adults[28,29]	Conidial length and width[29], growth[29]	-	-
Tomicus piniperda	Adults[24,32]	-	Adults[30,31]	-

Citations: Davis et al. 2011[1], Harrar and Martland 1940[2], Moore 1970[3], Moore 1971[4], Moore 1973[5], Pabst and Sikorowski 1980[6], Kocacevik et al. 2015[7], Sevim et al. 2010[8], Tanyeli et al. 2010[9], Cardoza et al. 2009[10], Hunt et al. 1984[11], Hunt 1986[12], Davis et al. 2018[13], Davis et al. 2019[14], Srei et al. 2017[15], Xu et al. 2018[16], Yao et al. 2012[17], Zhang et al. 2011[18], Whitney et al. 1984[19], Brownbridge et al. 2012[20], Glare et al. 2008[21], Reay et al. 2008[22], Reay et al. 2010[23], Takov et al. 2012[24], Davydenko et al. 2014[25], Khanday and Buhroo 2018[26], Kerchev et al. 2017[27], Jankevica 2004[28], Schebeck et al. 2016[29], Burjanadze 2010[30], Krams et al. 2012[31], Silva et al. 2015[32]

Table 1.2. Measurements of *Beauveria bassiana* growth, germination, and sporulation in Hylesinini systems.

Hylesinini species	Growth	Germination	Tolerance
Dendroctonus brevicomis	With yeast[1]	-	-
Dendroctonus frontalis	-	-	UV and heat[3]
Dendroctonus ponderosae	-	On cuticle[11,12]	-
Dendroctonus rufipennis	Range of temperatures[13], monoterpenes[13], UV[13], with other fungi[13,14]	-	-
Dendroctonus valens	On media[18]	On media[18]	UV[18]
Tomicus minor	Conidial length and width[29], growth on media[29]	-	-

Citations: Davis et al. 2011[1], Moore 1970[3], Hunt et al. 1984[11], Hunt 1986[12], Davis et al. 2018[13], Davis et al. 2019[14], Zhang et al. 2011[18], Schebeck et al. 2016[29]

Table 1.3. Bioassay methods for *Beauveria bassiana* studies in Hylesinini systems.

Hylesinini species	Lab bioassay	Bioassay conditions	Bioassay medium	Bioassay inoculation method
Dendroctonus frontalis	Larvae[6], Adults[3,5]	Room conditions[3,5,6], representative of system[5]	Tree-based[5,6]	Direct[6,], Indirect[3]
Dendroctonus micans	Larvae[7,8,9], Adults[7,8,9]	Room conditions[7,8,9]	Tree-based[7,8,9]	Direct[7,8,9]
Dendroctonus rufipennis	Adults[13]	Room conditions[13]	Tree-based[13]	Direct[13], Indirect[13]
Dendroctonus simplex	Adults[15]	Room conditions[15]	Tree-based[15]	Direct[15], Indirect[15]
Dendroctonus valens	Larvae[16,18]	Room conditions[16,18]	Tree-based[16,18]	Direct[18], Indirect[16]
Dryocoetes confusus	Adults[19]	Room conditions[19]	Tree-based[19], Filter paper[19]	Direct[19]
Hylastes ater	Adults[22]	Room conditions[22]	Filter paper[22]	Indirect[22]
Hylurgus ligniperda	Adults[21,22,23]	Room conditions[21,22,23]	Filter paper[21,22,23]	Indirect[21,22,23]
Polygraphus major	Adults[26]	Room conditions[26]	Tree-based[26]	Indirect[26]
Polygraphus proximus	Adults[27]	Room conditions[27]	Tree-based[27]	Direct[27]
Tomicus piniperda	Adults[30,31]	Room conditions[30,31]	Tree-based[30,31]	Direct[31], Indirect[30]

Citations: Moore 1970[3], Moore 1973[5], Pabst and Sikorowski 1980[6], Kocacevik et al. 2015[7], Sevim et al. 2010[8], Tanyeli et al. 2010[9], Davis et al. 2018[13], Srei et al. 2017[15], Xu et al. 2018[16], Zhang et al. 2011[18], Whitney et al. 1984[19], Reay et al. 2008[22], Reay et al. 2010[23], Khanday and Buhroo 2018[26], Kerchev et al. 2017[27], Burjanadze 2010[30], Krams et al. 2012[31]

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CHAPTER 2: PHENOTYPIC VARIATION IN GROWTH, PATHOGENICITY, AND VIRULENCE OF *BEAUVERIA BASSIANA* (ASCOMYCOTA: HYPOCREALES) ISOLATES FROM THE ROCKY MOUNTAIN REGION

INTRODUCTION

The ubiquitous insect-killing fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) is isolated from an incredible diversity of sources including soil, phylloplane habitats, and a wide variety of insect species (St. Leger et al. 1992; Bidochka 2002; Rehner and Buckley 2005). Accordingly, a great deal of phenotypic variation exists among isolates of this species. Commercial development of nontoxic biological controls has coincided with public recognition of the safety issues associated with traditional chemical control methods; *B. bassiana* has been widely tested as a biological control agent for arthropod pests in recent years as an alternative to these methods (de Faria and Wraight 2007). Additionally, *B. bassiana* is a desirable biological control agent because it can penetrate the insect exoskeleton and does not need to be ingested by its host (Rustiguel et al. 2018). However, promising laboratory assessments often result in unsuccessful field applications of *B. bassiana* for insect population control (Edgington et al. 2000; de Faria and Wraight 2001; Hajek and Goettel 2000; Lacey et al. 2015; Vega et al. 2012; Davis et al. 2018b). This discrepancy could exist as a result of poor isolate selection due to a mismatch between laboratory and field conditions during evaluation; for instance, highly virulent strains may not tolerate a wide range of environmental conditions representative of a target pest's habitat.

The spruce beetle, *Dendroctonus rufipennis* Kirby (Coleoptera: Curculionidae: Scolytinae), colonizes Engelmann spruce (*Picea engelmannii* Parry ex Engelm.; Pinales:

Pinaceae) and is one of the most significant forest pests in western North America (Jenkins et al. 2014; O'Connor et al. 2014; Colorado State Forest Service 2017); beetle population activity is associated with mortality of at least 17 million *P. engelmannii* over the past two decades. Due to these impacts, recent work has focused on assessing the potential for *B. bassiana* to control *D. rufipennis* population growth, but field tests have had limited success due in part to the challenging environmental conditions that occur in high elevation forests characteristic of *D. rufipennis* habitat (Davis et al. 2018b). This is despite the apparent ubiquity of *B. bassiana* in forest soil (Niemczyk et al. 2019; Reay et al. 2008; Yao et al. 2012), bark (Yao et al. 2012), bark beetle oral secretions (Cardoza et al. 2009), bodies (Yao et al. 2012), frass (Reay et al. 2008; Takov et al. 2012; Yao et al. 2012), and as an endophyte of coniferous trees (Reay et al. 2010; Brownbridge et al. 2012). Accordingly, a better understanding of how spruce forest conditions impact growth and virulence of *B. bassiana* can improve its use as a biological control agent of spruce beetle.

Spruce forests within the southern Rocky Mountain region are typically characterized by average summer maximum temperatures ranging between 10 – 20 °C (Dell 2018) and dense overstory vegetation with little sun penetration to the forest floor (Johnson et al. 2004). The spruce beetle life cycle is cryptic and primarily spent in subcortical phloem environments that are rich with tree secondary compounds, especially monoterpene hydrocarbons (Davis et al. 2018a). During the process of tree death following bark beetle attack, coniferous tree water potentials decline over time and generally range between -0.5 and -2.0 MPa (Klepzig et al. 2004), which may limit fungal growth. In addition, *D. rufipennis* is associated with a symbiotic fungus, *Leptographium abietinum* (Peck) Wingfield (Ophiostomatales: Ophiostomataceae; Six and Bentz 2003), which inhibits growth of antagonistic microorganisms (Davis et al. 2019). Consequently,

B. bassiana parasitizing *D. rufipennis* must contend with a range of conditions including cool temperatures, competing microbial species, exposure to tree secondary compounds, significant water deficits and low-intensity ultraviolet light. Despite this habitat complexity, most studies evaluate entomopathogen virulence to target *Dendroctonus* beetles under simplified laboratory conditions that may not represent factors limiting growth in natural environments (Chapter 1).

The goal of this study was to characterize the phenotypic variation in growth response, pathogenicity, and virulence to *D. rufipennis* among isolates of *B. bassiana* collected across the Rocky Mountain region of the western United States. Specific objectives included (1) an evaluation of the impacts of environmental conditions characteristic of the *D. rufipennis* habitat on *B. bassiana* growth and (2) to describe the pathogenicity and virulence of regional *B. bassiana* isolates to *D. rufipennis* across a range of experimental conditions. These studies contribute new insights into the factors driving *B. bassiana* success in spruce forest habitats and can help to inform subsequent strain selection processes. My results indicate that experimental conditions greatly impact virulence in entomopathogenic fungi, with consequences for development and application of mycologically-based biocontrol agents in coniferous forests.

MATERIALS AND METHODS

Isolation and molecular identification of fungus strains

Isolation of the *B. bassiana* strains used in this study occurred from a range of forest habitats across the Rocky Mountain region (see Table 2.1). Strains were initially isolated on selective media and experiments were performed using spore powders produced through industrial-scale solid substrate culture methods as described in Davis et al. (2018b), and a total of fourteen isolates were chosen for use in the present study. In addition, six isolates of *L. abietinum* were

collected from spruce forests in Colorado for evaluation of *B. bassiana* competition with *L. abietinum* as described in Davis et al. (2019).

For species identification and comparison of genetic diversity among *B. bassiana* isolates, DNA was extracted from 100 mg of fresh mycelia using ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research Corporation, Irvine, CA, USA). Concentration of extracted DNA was measured using a nanodrop (ThermoScientific, Waltham, MA) to ensure a 260 nm/280 nm ratio of ~1.8 before sequencing of Internal Transcribed Spacer (ITS) and Elongation Factor 1-α (EF1-α) regions following the methods of Rehner and Buckley (2005). ITS region was amplified and sequenced using primers ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). EF1-α region was amplified and sequenced using primers EF1T (5′-ATGGGTAAGGARGACAAGAC-3′) and 1567R (5′-ACHGTRCCRATACCACCSATC-3′). Polymerase chain reaction mixtures (total 25 µl) contained 10 ng of template DNA (or no DNA template for negative control) and used the following conditions for 36 amplification cycles: 30 second denaturation at 94 °C, 30 second annealing at 56 °C, 1 minute extension at 72 °C, and 10 minute incubation at 72 °C.

Sequence data for each isolate was aligned in Geneious Software (Biomatters, Auckland, New Zealand) and compared to the NCBI BLAST database (Altschul et al. 1990) for putative species identification. Sequencing of the ITS region, the universal DNA barcode for fungi (Schoch et al. 2012), yielded a sequence length of 596 base pairs and EF1-α sequences yielded 1175 base pairs. All sequences were matched to sequences of *Beauveria bassiana* (Genbank accession numbers: ITS ≥99% match with EU272501.1 and KX901310.1; EF1-α 79-98% match with FJ177453.1) with 99% coverage and an e-value of 0.0. The ITS and EF1-α regions were

concatenated and a maximum likelihood phylogeny was created in MEGA7 using 200 bootstraps (Kumar et al. 2016). Isolates were all one haplotype based on ≥70% node support.

Beauveria bassiana *growth in response to different environmental conditions representative of* **Dendroctonus rufipennis** *habitat*

I tested the hypothesis that there is phenotypic variation among isolates to tolerate and grow in environmental conditions representative of spruce beetle habitats. Mycelial radial growth rate strongly positively correlates with biomass production in fungal species (Ogidi et al. 2016) and is an indicator of environmental tolerance in entomopathogenic fungi (Jaronski 2010). Accordingly, this trait is analyzed as the primary response variable in studies evaluating fungal growth in response to environmental conditions. *Beauveria bassiana* growth was tested in response to six environmental factors. These environmental factors included (1) a range of temperatures, (2) competition with the spruce beetle symbiont *L. abietinum*, (3) a range of concentrations of *P. engelmannii* phloem monoterpenes, (4) a range of concentrations of chitin (as a sole nutrient source), (5) a range of osmotic water potentials, and (6) effects of sunlight exposure.

Except where noted below, the following is true for all tests: each test was simultaneously replicated 3-6 times for each isolate in 60 × 15 mm Petri dishes (VWR International, Radnor, Pennsylvania) containing 2% malt extract agar (MEA, pH 5.3, Sigma-Aldrich, St. Louis, MO, Davis et al. 2019). Petri dishes were inoculated with ~0.1 mg *B. bassiana* conidial powder (22499 ± 4748 colony forming units, CFU) by aseptic transfer using a sterile probe dipped into a surfactant solution (0.01% Silwet L77, Helena Agri-Enterprises, Collierville, TN). Dishes were inverted after 48 hours and tests occurred in dark growth

chambers (Thermo Fisher Scientific, Waltham, MA) at a constant 23 °C. Mycelial growth was traced and measured every 24-48 hours for 10–13 days. Growth rate (mm/d) was determined by diving the total distance of radial growth (measured in two places on each dish and averaged together) by the total period of the assay. Mean radial growth rate at 23 °C on 2% MEA served as the control in tests evaluating *B. bassiana* growth responses to environmental conditions because growth rates were generally maximized at this temperature and there was no evidence of isolate-to-isolate variation in growth at this temperature.

Beauveria bassiana response to temperature. *Dendroctonus* beetles live in complex systems driven by overcoming extremely cold and fluctuating temperatures (Six and Bracewell 2015). Three simultaneous replicates of all isolates took place in growth chambers maintained at 5, 10, 15, 20, 25, 30, and 35 °C (N=292 total experimental units). Growth and comparison to the control was measured as described above.

Beauveria bassiana competition with Leptographium abietinum. All *B. bassiana* isolates were placed into competition against one replication of six unique *L. abietinum* isolates (CF4, CF6, CF9, CF11, CF17, and CF22 described in Davis et al. 2019) for a total of 84 combinations to test the hypothesis that a bark beetle symbiont, *L. abietinum*, inhibits growth of *B. bassiana*. Each fungus was inoculated 8 mm from the edge of 95 x 15 mm Petri dishes (Fisherbrand, Waltham, MA). After 20 d of growth, plates were scanned (Epson V600, Suwa, Japan) and these images were analyzed using ImageJ software (National Institutes of Health, Washington, D.C). Total area (% of dish) occupied by *B. bassiana* for each replicate was treated as the response variable.

Beauveria bassiana growth response to spruce tree defense chemicals. The phloem environment colonized by bark beetles is rich in monoterpenes, which can impact fungal growth.

Petri dishes were amended with one of five monoterpenes found in the phloem of all Engelmann spruce trees in Colorado (Davis et al. 2018a) including (+)-α-pinene (98% purity, Sigma-Aldrich), (-)-β-pinene (99% purity, Sigma-Aldrich), (+)-3-carene (>90% purity, Sigma-Aldrich), terpinolene (>90% purity, Sigma-Aldrich), and myrcene (>95% purity, Sigma-Aldrich) at three concentrations including 0.1, 1.0, and 5.0% (v/v), consistent with constitutive (0.1 and 1%) and induced (5%) monoterpene concentrations (N=907 total experimental units). Growth and comparison to the control was measured as described above.

Beauveria bassiana response to media containing chitin as a nutrient. To evaluate the ability of isolates to grow in minimal media containing only arthropod exoskeleton contents, a potential virulence factor, Petri dishes containing water agar (Sigma-Aldrich) were amended with either 0.1, 1.0, or 5.0% (v/v) shrimp chitin (Sigma-Aldrich) (N=127 total experimental units) to represent potential growth on the bark beetle exoskeleton. Growth and comparison to the control was measured as described above.

Beauveria bassiana growth on media with limited water availability. The subcortical gallery environment gradually desiccates following colonization by spruce beetle, which may limit fungal growth. To assess effects of water limitation on entomopathogen growth, *B. bassiana* isolates were transferred to Petri dishes containing 1% MEA amended with KCl (EMD Chemicals Gibbstown, NJ) and sucrose (Sigma-Aldrich) to generate osmotic potentials of -0.5 MPa, -1.0 MPa, -2.0 MPa (N=160 total experimental units) using ratios described in Whiting and Rizzo (1999); these levels are representative of phloem water potential in drought-stressed conifers or trees in decline following bark beetle attack (methods described in Klepzig et al. 2004). Growth and comparison to the control was measured as described above.

Beauveria bassiana response to ultraviolet light. Deployment of *B. bassiana* for spruce beetle control in forested environments requires that isolates tolerate some degree of exposure to low-intensity light during summer months. Exposure to UV light is reported to be highly damaging to *B. bassiana* tissues. To assess the response of *B. bassiana* to low-intensity light conditions, Petri dishes containing replicated *B. bassiana* isolates were placed in a windowsill and exposed to 13 d of indirect sunlight at an intensity of 4.8 ± 0.2 μmol/m^2/sec for 12 h 58 min ± 9 min per day. Growth and comparison to the control was measured as described above.

Beauveria bassiana *pathogenicity and virulence to* Dendroctonus rufipennis

General procedure. Four experiments were performed to test the hypothesis that Rocky Mountain isolates of *B. bassiana* vary in their pathogenicity and virulence to *D. rufipennis* across a range of conditions. Experiments used sterile 95 × 15 mm Petri dishes as the arena, and replicates consisted of six adult beetles; each isolate was replicated ten times in each experiment (N=140 experimental units and 840 beetles per experiment). Pathogenicity (the ability of *B. bassiana* to cause spruce beetle mortality) of isolates in each experiment was determined by comparison against a sham treatment. The sham treatments (N=10 experimental units and 60 beetles per experiment) were treated with distilled water containing 0.01% Silwet L77 and no *B. bassiana*. The concentration of CFU was standardized using serial dilution to 10^6 CFU/mL suspension for each test isolate, and spore suspensions were administered to surfaces contacted by beetles rather than directly to beetle integuments.

Experiments spanned a range of conditions, varying test temperatures, substrates, and beetle source (Table 2.2). Experiments were designed to allow for beetle attraction, avoidance, and grooming behaviors by allowing test individuals space to move around within test arenas.

Insect behavior is an important factor to consider in bioassays as detection and avoidance of *B. bassiana* by target insect species is previously reported (Meyling and Pell 2006, Mburu et al. 2013) and may be associated with host resistance. Additionally, beetles were not surface sterilized in an effort to include potential interactions with the microbial symbionts associated with *D. rufipennis* in bioassays.

To evaluate infectivity following beetle mortality in bioassays, a mycosis test was performed on all dead beetles according to methods of Bugeme et al. (2008). A 1 mL aliquot of distilled water was added to the arena substrate and maintained at a constant 30° C for 48 hours in the dark; beetles colonized by *B. bassiana* readily sporulate under these conditions and appear to 'mummify,' confirming which adults were infected by *B. bassiana* in each replicate.

Experimental conditions. Experiment 1 took place at 23 °C with *D. rufipennis* reared from logs collected from five infested *P. engelmannii* trees at Cameron Pass, Colorado (coordinates: 40.52058 N, 105.89283 W, elevation: 3100 meters). To incite colonization of selected trees, trees were baited with an attractant pheromone lure containing Frontalin and MCOL (Synergy Semiochemicals Corporation, Burnaby, BC) during May 2017. During September and October 2017 following *D. rufipennis* colonization, baited trees were felled, cut into billets of ~0.6-meter length, and placed into rearing containers ventilated with a 1 x 1mm mesh in a laboratory at 23 °C with a relative humidity of ~30%. Billets in rearing chambers accumulated approximately 800-degree days in the laboratory, after which point new adult beetles were harvested from logs for testing. *Dendroctonus rufipennis* has individuals within the same population that exhibit either a 1- or 2-year life cycle (Holsten et al. 1999), so this experiment was intended to control for beetle age by ensuring that all beetles were new adults and not a mix of 1- and 2- year beetles. An aliquot of 1 mL of standardized spore suspension (10^6

CFU/mL) was applied to filter papers contacted by beetles (Whatman Grade 2, 4.25 cm diameter, Maidstone, United Kingdom), as described in Davis et al. (2018b).

Subsequent experiments (experiments 2-4) used beetles actively responding to pheromones collected during their dispersal period, as actively flying beetle populations are likely the most vulnerable to *B. bassiana* applications. To capture dispersing beetles, a total of 10 Lindgren funnel traps (Synergy Semiochemicals, Burnaby, Canada) baited with the spruce beetle enhanced pheromone lure (Synergy Semiochemicals) were deployed to collect beetles during peak flight season (Dell 2018) at Monarch Pass, Colorado (coordinates: 38.49666 N, 106.32558 W, elevation: 3448 m). Moist single ply paper towels were placed in collection cups to provide a surface for beetles to adhere to, and beetles were collected and returned to the lab within 48 hours. Collections were made twice weekly from mid-June through July 2018 until all experiments were complete. Prior to use in experiments, beetles were subjected to a simple fitness test using the approach described in Chiu et al. (2017).

Experiments 2 and 3 were identical to experiment 1 in all parameters, with the condition that the source of beetles differed (experiment 1 tested effects of *B. bassiana* on lab-reared beetles) and experiment 3 was performed at a constant 10 °C. This temperature was chosen to reflect mean temperatures in the field during dispersal (Dell 2018). Experiment 4 was an *ex vivo* test that supplied beetles with phloem substrate, which could affect survival of *D. rufipennis*. Phloem also contains monoterpenes that may reduce efficacy of *B. bassiana*. Methods for creating 'phloem sandwiches' followed Aflitto et al. (2014) with slight modifications: 150 8×8 cm pieces of phloem were excised from standing *P. engelmannii* with outer bark still intact and later cut into circles to fit firmly on the bottom of 6 cm diameter Petri dishes. As above, a 1 mL

aliquot of *B. bassiana* spore suspension was applied to filter papers at a cell density of 10^6 CFU/mL; however, in experiment 4 spore suspension was applied to the bark surface.

Replicates were checked regularly and all beetles in each dish were scored as 'alive' or 'dead' at each recording. Experiments 1-3 were scored daily in this way until all beetles had died. In experiment 4, replicates were scored for survival and mycosis every three days for 90 d, which is the approximate length of the spruce beetle flight season at Monarch Pass, Colorado (Dell 2018).

Data analysis

All analyses were performed using the R statistical programming language (R Core Team 2017). To test the hypothesis that isolates vary in their radial growth in response to environmental conditions, I converted test growth rate to a percentage (%) of control growth rate for each isolate; each test was analyzed using ANOVA procedures appropriate to the experimental design. A one-way ANOVA was used to analyze the fixed effects of *L. abietinum* presence (*L. abietinum* present or *L. abietinum* absent) on the response variable of percent of the Petri dish (%) occupied by *B. bassiana* isolates. Similarly, one-way ANOVA was used to analyze the fixed effect of sunlight exposure (exposed to sunlight or not exposed to sunlight) on the response of *B. bassiana* growth rate. Isolate growth rates across a range of temperatures (5, 10, 15, 20, 25, 30, and 35 °C), concentrations of chitin in media (0.1, 1.0, and 5.0%), and osmotic water potentials (-0.5, -1.0, and -2.0 MPa) were analyzed using two-way ANOVA to test the fixed effects of treatment, isolate, and the treatment × isolate interactions on response of mean *B. bassiana* growth rate. Mean *B. bassiana* growth rate in response to different monoterpene concentrations (0.1, 1.0, and 5.0%) and identities (alpha-pinene, beta-pinene, 3-carene, terpinolene, and

myrcene) were analyzed using a three-way ANOVA to test the fixed effects of isolate, monoterpene concentration, and monoterpene identity and all two- and three-way interactions on the response of mean *B. bassiana* growth rate. Pairwise comparisons among all treatment means were made post-hoc in each test using the Tukey-Kramer HSD test.

In experiments testing pathogenicity and virulence of *B. bassiana* isolate to spruce beetle, the median survival time (MST) of test beetles was the primary response variable analyzed. MST was analyzed using Kaplan-Meier survival analysis and a log-rank test implemented using R packages 'ggplot2' (Wickham 2009), 'survminer' (Kassambara and Kosinski 2018), and 'survival' (Therneau and Grambsch 2000) for calculations and 'ggpubr' (Kassambara 2018), 'gridExtra' (Auguie 2017), and 'cowplot' (Wilke 2019) for visualization. Every isolate was compared to the sham treatment through log-rank tests to evaluate differences in pathogenicity and virulence across isolates and experiments. In all analyses, a Type I error rate of $\alpha=0.05$ was used for assigning statistical significance.

RESULTS

Beauveria bassiana *growth in response to different environmental conditions representative of the* **Dendroctonus rufipennis** *habitat*

There was considerable isolate-to-isolate variability in growth as a response to the six environmental conditions. Isolates exhibited statistically significant phenotypic variation in every environmental condition tested except competition against *L. abietinum* (Figure 2.1, Table 2.S1).

Beauveria bassiana response to temperature. There was significant variation in the mean growth rate of isolates due to the effects of temperature ($F_{6, 194}=76.560$, $P<0.001$), isolate ($F_{13, 194}=29.741$, $P<0.001$), and an isolate × temperature interaction ($F_{78, 194}=4.529$, $P<0.001$).

No growth of any *B. bassiana* isolate occurred at 5 °C and 35 °C; maximum growth occurred between 20 and 30 °C. Growth was reduced by 88% at 15 °C and by 97% at 10 °C relative to growth radial growth rate at 23 °C.

Beauveria bassiana competition with Leptographium abietinum. Both *B. bassiana* and *L. abietinum* grew until touching, showing no signs of an inhibition zone, and maintained the captured space in competition assays for at least 20 d. On average, *B. bassiana* captured only 44% of the available space and proved to be a slightly weaker competitor than *L. abietinum* (56%) on average ($t_{167, 166} = 3.010$, $P=0.003$). *Beauveria bassiana* isolates did not differ in their ability to compete with *L. abietinum* ($F_{83, 70}=0.664$, $P=0.790$).

Beauveria bassiana growth response to spruce tree defense chemicals. Mean radial growth of *B. bassiana* isolates varied due to the effects of isolate ($F_{13, 697}=65.546$, $P<0.001$), monoterpene identity ($F_{4, 697}=20.947$, $P<0.001$, monoterpene concentration ($F_{2, 697}=322.025$, $P<0.001$), isolate × monoterpene identity interaction ($F_{52, 697}=2.547$, $P<0.001$), isolate × monoterpene concentration interaction ($F_{26, 697}=11.933$, $P<0.001$), monoterpene identity × concentration interaction ($F_{8, 697}=14.261$, $P<0.001$), and the three-way isolate × monoterpene identity × concentration interaction ($F_{104, 697}=1.493$, $P=0.002$). Monoterpenes were the most inhibitory environmental factor for *B. bassiana* growth across all tests. Mean isolate growth rate was reduced by 86% in constitutive levels (0.1 – 1.0%) and by 98% on average when exposed to induced (5.0%) concentrations. Terpinolene was the most inhibitory monoterpene overall. Every isolate grew in 5% myrcene but the rate of growth was reduced by 96% on average compared to control growth rates in the absence of monoterpenes.

Beauveria bassiana response to media containing chitin as a nutrient. There was significant variation in the growth of *B. bassiana* isolates due to the effect of isolate ($F_{13,}$

$_{85}$=257.543, P<0.001), chitin concentration ($F_{2, 85}$=11.415, P<0.001, and isolate × chitin concentration in growth media ($F_{26, 85}$=4.633, P<0.001). The 14 isolates experienced an 85 – 87% reduction in growth rate in experiments where *B. bassiana* was exposed to low concentrations of chitin as the only nutrient source, but growth rates were near to or exceeded control growth rates under conditions of high chitin concentration.

Beauveria bassiana growth on media with limited water availability. There was significant variation in the mean growth rates of *B. bassiana* isolates due to the effects of substrate water potential ($F_{2, 118}$=166.890, P<0.001), isolate ($F_{13, 118}$=15.530, P<0.001), and the isolate × water potential interaction ($F_{26, 118}$=3.246, P<0.001). Growth rate was enhanced in tests containing media amended with -0.5 MPa (103.63 ± 3.21%) and -1.0 MPa (122.06 ± 2.42%) levels of osmotic water potential. The *B. bassiana* isolates had a moderate (19%) reduction in mean growth rates in the -2.0 MPa condition.

Beauveria bassiana response to ultraviolet light. The effects of exposure to ultraviolet light significantly affected mean growth rate of *B. bassiana* isolates ($F_{41, 28}$=73.764, P<0.001). Mean colony growth rate was reduced by 78.7% on average when *B. bassiana* was exposed to ultraviolet light during exponential growth, though some isolates (e.g., 429DA) were relatively tolerant of exposure.

Beauveria bassiana *pathogenicity and virulence to* Dendroctonus rufipennis

The range of MST differed considerably among experiments and ranged from 6-10 days in experiment 1 (Figure 2.2), 5 days for every isolate in experiment 2 (Figure 2.3), 8-11 days in experiment 3 (Figure 2.4), and 19-62 days in experiment 4 (Figure 2.5). Phenotypic variation in virulence among isolates was significant according to log-rank tests based on Kaplan-Meier

assumptions in experiments 1 ($P<0.001$), 2 ($P<0.001$), and 4 ($P<0.001$), though not in experiment 3 ($P=0.660$). A group of isolates (429BTF, GHA, D900, L429, L447, and 90(1)MPB) all had the lowest MST times in experiment 1, but Isolate 50C caused the lowest MST (consistent with most rapid spruce beetle mortality) in Experiments 3 and 4.

The relative pathogenicity of isolates differed from experiment to experiment based on MST relative to sham treatments ($\chi^2 = 58.300$, df = 3, $P<0.001$). Log-rank tests indicate that every isolate was pathogenic in experiments 1 (Figure 2.2) and 2 (Figure 2.3), but only isolate 50C was pathogenic in experiment 3 (Figure 2.4), and only isolates 50C and 14B were pathogenic in Experiment 4 (Figure 2.5).

DISCUSSION

Beauveria bassiana strains isolated from various sources throughout the Rocky Mountain region expressed considerable phenotypic variation in terms of the environmental factors that affected growth rates and their relative ability to reduce survival of *D. rufipennis* under a range of experimental conditions. Forest systems introduce new factors to overcome in the application of *B. bassiana* as a biological control agent (Hesketh et al. 2010), and aspects of habitat complexity are often not accounted for in agricultural study systems or laboratory evaluations of fungal virulence (e.g. Jaronski 2010). However, the ability of isolates to grow under a range of environmental conditions, as well as their relative ability to impact insect populations across those conditions, are key for the development of successful mycologically-based biocontrol technologies. Here, my results demonstrate several important issues related to this point: (1) *B. bassiana* isolates vary widely in their growth response to environmental conditions, even when isolated from similar habitats and sources; (2) tree phytochemicals were highly inhibitory to *B.*

bassiana growth, especially at high concentrations, though low temperatures also strongly reduced growth; (3) the interpretation of isolate pathogenicity and virulence differs substantially depending on experimental conditions—with many isolates exhibiting pathogenicity under laboratory conditions, but few isolates exhibiting pathogenicity when tests are performed on actual beetle substrate. These collective findings have implications for the application of *B. bassiana* as a biological control agent of bark beetles.

My results confirm that *B. bassiana* is highly affected by temperature and, consistent with the literature, nearly every isolate maximizes radial growth rates at or near 25 °C (Yeo et al. 2003; Bugeme et al. 2008). The thermal growth threshold of 5 °C may be a potential problem for *B. bassiana* application in the *D. rufipennis* habitat; though the low temperatures did not cause the fungus to die, but rather freeze as growth resumed when Petri dishes were brought into room temperature. The fungal competition results corroborate recent studies indicating that both *B. bassiana* and *L. abietinum* are able to capture and maintain space (Davis et al. 2018b, 2019), and that the fungi apparently compete with one another for growth resources, but neither fungus is able to overtake its competitor. The concentration of chitin in growth media also had a significant overall effect on the growth of *B. bassiana*, though radial growth of isolates was lowest in media with the highest concentration of chitin—potentially indicating a reduced need for radial growth under high nutrient conditions. This also indicates that contact with host insects may be more likely in low-chitin conditions, as *B. bassiana* will rapidly expand surface area.

Isolates exhibited enhanced growth in media containing osmotic water potentials of -0.5 and -1.0 MPa along with only moderate reductions in overall growth in the highest level of osmotic potential. *Beauveria bassiana* varies greatly in its ability to tolerate dry environments. In an earlier study, an osmotic potential of -1.76 MPa caused complete growth inhibition for some

isolates while just a 4% decrease in others (Devi et al. 2005). *Dendroctonus* beetles must also contend with arid conditions as trees die but spend most of their lives in the phloem of conifer trees – a humid environment. My results suggest that practitioners should prioritize environmental factors other than osmotic potential during *B. bassiana* strain selection for control of bark beetles, especially when choosing among this group of isolates. For instance, exposure to sunlight can completely inhibit fungal growth, likely due to a lack of melanin in mycelial tissues (Fernandes et al. 2015). I found significant phenotypic variation between isolates in their ability to grow in an environment with low-intensity sunlight exposure, though no isolates were completely inhibited by exposure to sunlight. Like osmotic potential, sunlight may not be particularly important in bark beetle habitats because bark beetles spend most of their lives below the surface of the tree. Multifunnel traps that contain *B. bassiana* in dissemination chambers have been tested for the control of emerald ash borer (*Agrilus planipennis* Fairmaire Coleoptera: Cuprestidae; Lyons et al. 2012), *D. simplex* LeConte (Srei et al. 2017), and *Ips typographus* Linnaeus (Coleoptera: Curculionidae: Scolytinae; Grodsky and Kosibowicz 2015) and may further alleviate the need for a chosen *B. bassiana* isolate to tolerate sunlight in application against bark woodboring beetles, as *B. bassiana* isolates can be inoculated into a relatively protected habitat.

Conifer secondary metabolites, including monoterpenes, are a central aspect of tree defense in response to bark beetles and other herbivores (Raffa et al. 2014). While monoterpenes are always produced by conifer trees at low (constitutive) levels, the composition and concentration of these monoterpenes is often upregulated (i.e., induced) when trees are challenged by pest organisms (Litvak and Monson 1998). Inoculation with *B. bassiana* can also induce a plant defense response (Shrivastava et al. 2015) which suggests that induced trees may

inhibit *B. bassiana* growth even though the presence of entomopathogens might benefit tree survival of a bark beetle challenge (Hay et al. 2004). Monoterpenes were extremely inhibitory in this study and aside from low temperature, are likely the singly most limiting factor to successful biological control of *Dendroctonus* species in forest environments. Chemical identity played a critical role in limiting *B. bassiana* growth; especially terpinolene and 3-carene, which both reduced growth by over 92% compared to the control. These compounds are present in virtually all Engelmann spruce trees in the southern Rocky Mountains (Davis et al. 2018a). As concentrations of monoterpenes increased, so did growth inhibition. Growth was reduced by over 90% in media amended with 1% monoterpenes and over 98% in media containing 5% monoterpenes. The induced level (5% v/v concentration) in this study is actually a quite conservative treatment, as monoterpene concentrations can increase by over 30 times when conifers are challenged by bark beetles and their symbiotic fungi (Raffa and Smalley 1995). Inhibition of fungal growth caused by monoterpenes may functionally eliminate the possibility of *B. bassiana* entomopathogenicity towards bark beetles colonizing host tree tissues and should be one of the primary environmental factors that practitioners consider in future field applications.

My results also show that the interpretation of isolate pathogenicity can differ substantially depending on the experimental design, which is problematic as most studies rely on short tests under unrealistic laboratory conditions to assign isolate virulence. While every isolate was pathogenic against *D. rufipennis* in filter paper bioassays under room temperature (a favorable condition for *B. bassiana*), only two isolates were pathogenic against *D. rufipennis* in bioassays that took place on actual plant substrate and at a temperature representative of a Colorado spruce forest (a favorable condition for *D. rufipennis*). If tests of *B. bassiana* pathogenicity and virulence are not done *in planta* or *ex vivo*, they are likely to be misleading

and misrepresent the efficacy of isolates at reducing insect population densities under field conditions. Interestingly, neither of the pathogenic isolates in the realistic phloem experiment were among the most virulent at 23 °C. Hence, a key reason why so many promising laboratory studies lead to ineffective field application may be the lack of consideration for system-specific environmental conditions during strain evaluation. The results in this study support findings by Kreutz et al. (2004) where filter paper was deemed an unsuitable bioassay substrate for *I. typographus* because it did not provide nutrients to the beetles. Furthermore, neither of the pathogenic isolates in the phloem bioassay were top growers in 10 °C or when exposed to monoterpenes. Thus, eliminating isolates from the screening process based solely on growth rate could also lead to misleading results as positive correlations between pathogenicity, virulence, and growth rate do not always occur with *B. bassiana*.

The studies reported here have several implications for the future development and application of *B. bassiana* as a mycologically-based method of pest control. First, future studies on the biology and potential application of *B. bassiana* should take a multivariate approach and include complex environmental factors unique to the desired application habitat. Second, *in planta* bioassays under representative conditions are vital; bioassays performed under simplified conditions are misleading because *B. bassiana* isolates are highly phenotypically variable and growth responses do not necessarily translate to the expression of pathogenecity in the presence of host plant material. Finally, understanding off-target effects is necessary when applying any method of pest management. Host specificity in *B. bassiana* is complex (Devi et al. 2008), but apparently does occur with bark beetles and their predators (Clerid beetles; Steinwender et al. 2010), suggesting that targetted applications are possible. Building a stronger understanding of these collective effects will enhance our ability to understand the basic mechansims of

entomopathogenecity, and can help to inform more realistic studies that accurately evaluate virulence factors for biocontrol applications.

TABLES

Table 2.1. Geographic and host origin of *Beauveria bassiana* isolates used in this study.

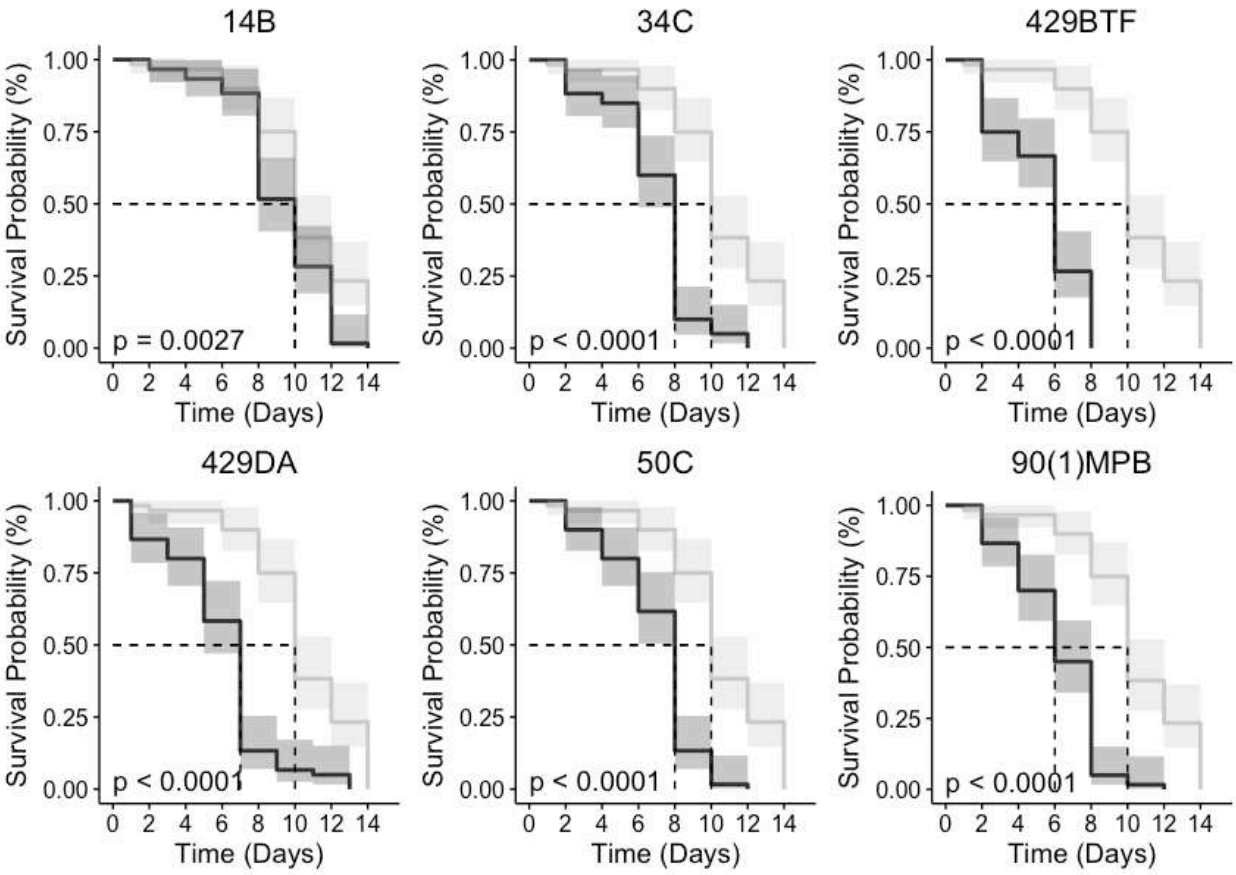
Isolate Name	Geographic Origin	Source of Isolate
14B	Montana	*Pinus ponderosa*
34C	Montana	*Pinus ponderosa*
429BTF	Wyoming	*Picea engelmannii*
429DA	Wyoming	*Dendroctonus rufipennis* adult
50C	Montana	*Pinus ponderosa*
90(1)MPB	Montana	*Dendroctonus ponderosae* adult
AZ5	Arizona	*Pinus ponderosa* forest soil
AZ6	Arizona	*Pinus ponderosa* forest soil
D900	Montana	*Pinus ponderosa* forest soil
ES12(1)	Montana/Idaho	*Picea engelmannii* forest soil
GHA		Registered strain of *B. bassiana*
L429	Wyoming	*Picea engelmannii*
L447	Utah	*Dendroctonus rufipennis* larva
SPRUCE1	Utah	*Picea engelmannii*

Table 2.2. Conditions for bioassays testing *Beauveria bassiana* isolate pathogenicity and virulence to *Dendroctonus rufipennis*, arranged from least representative to most representative of the *Dendroctonus rufipennis* habitat.

Experiment number	Beetle source	Temperature	Location of Beetle Collection	Test Substrate
1	Reared from logs	23 °C	40.52058 N, 105.89283 W	Filter paper
2	Flight capture	23 °C	38.49666 N, 106.32558 W	Filter paper
3	Flight capture	10 °C	38.49666 N, 106.32558 W	Filter paper
4	Flight capture	10 °C	38.49666 N, 106.32558 W	Phloem

FIGURES

Figure 2.1. Growth rate of 14 *Beauveria bassiana* isolates in six different environmental conditions representative of the *Dendroctonus rufipennis* habitat. Growth was measured as a percent of the control and is represented by the size of the circle. The control was average growth rate on 2% MEA at 23 °C for each isolate. In this figure, larger circles represent faster growth rates. Values greater than 100% grew faster than the control.


14B
34C
429BTF
429DA
50C
90(1)MPB
Survival Probability (%)
1.00
0.75
0.50
0.25
0.00
0 2 4 6 8 10 12 14
Time (Days)
p = 0.0027
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001

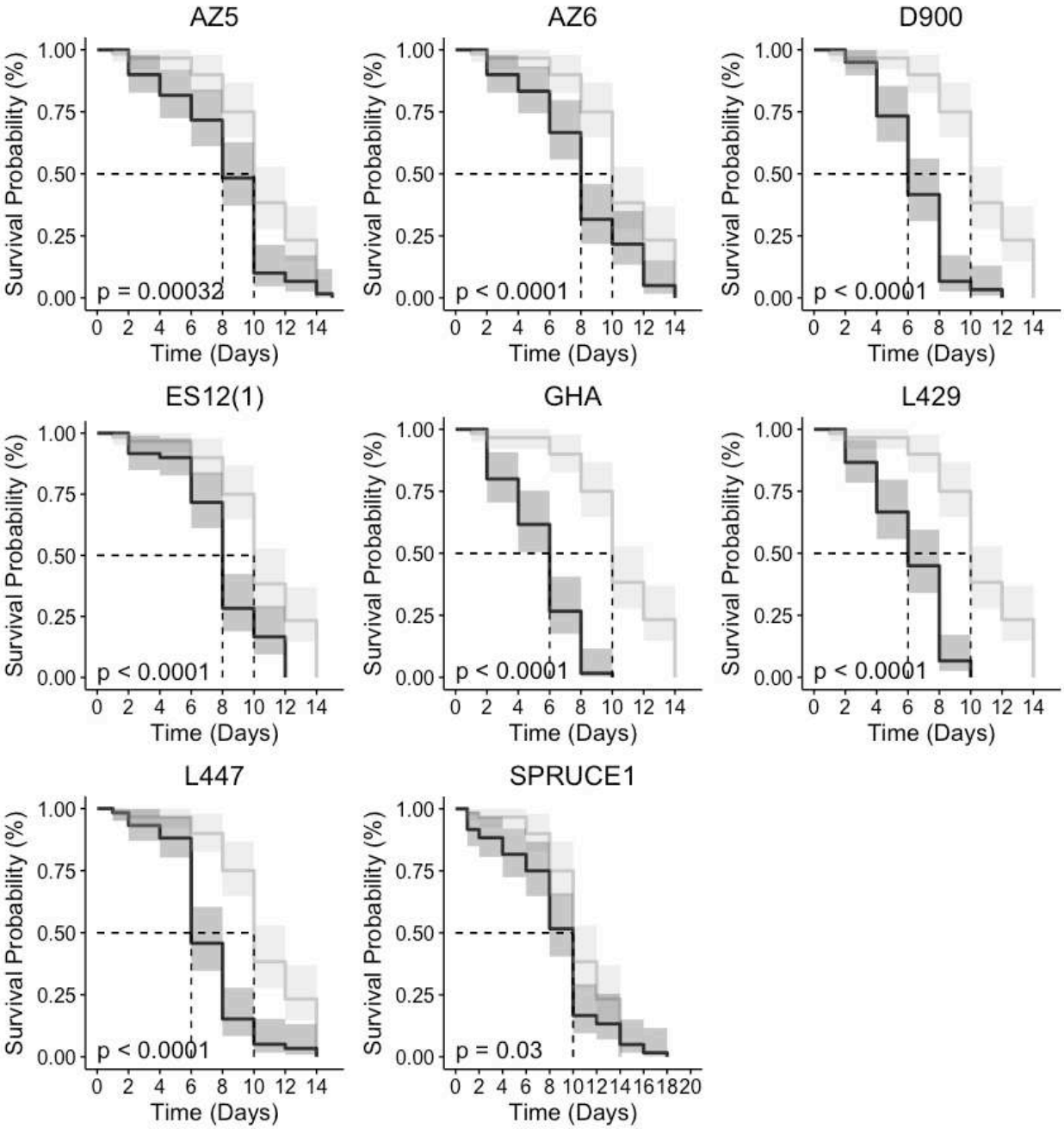
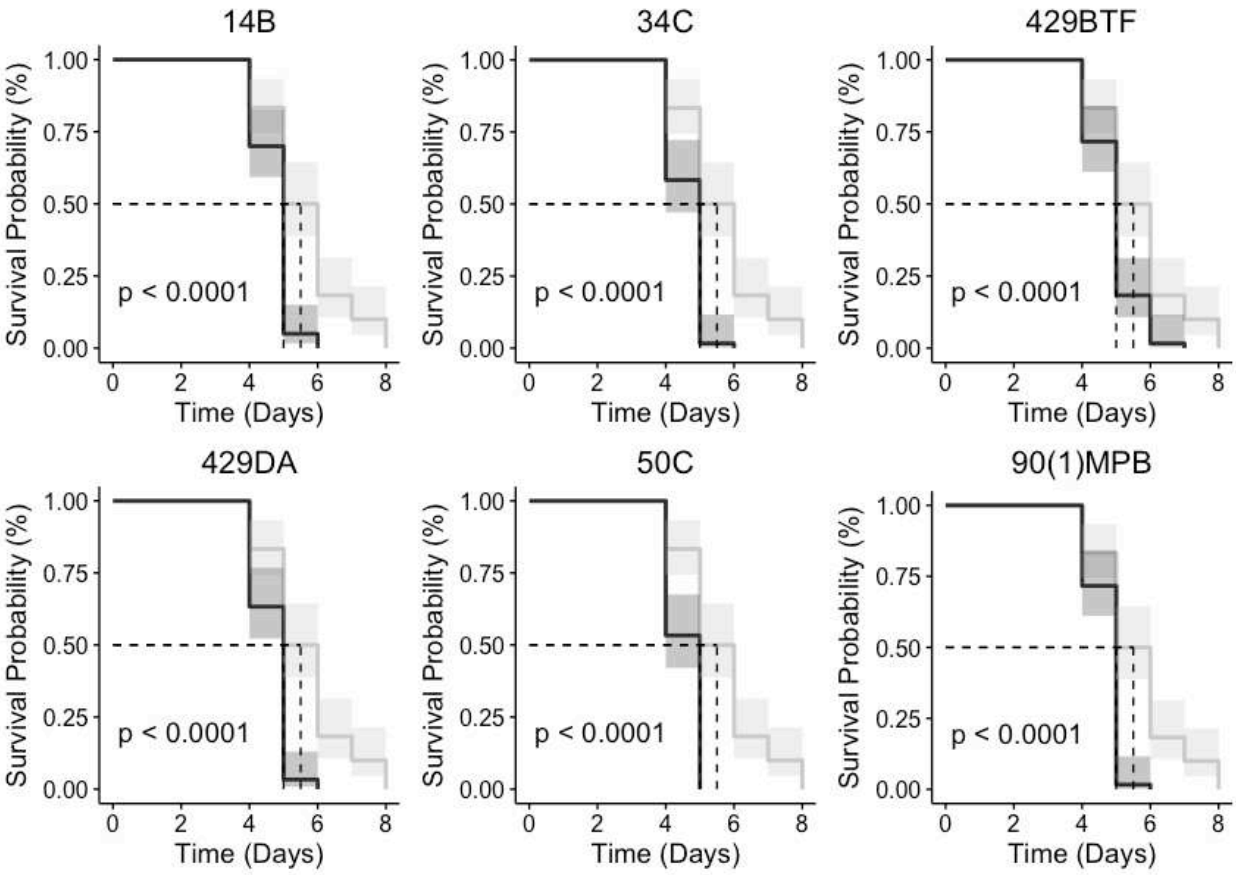


Figure 2.2. Kaplan-Meier survivorship curves for experiment 1. Solid black lines indicate survival time of *Dendroctonus rufipennis* after exposure to the indicated isolate of *Beauveria bassiana*. Solid grey lines show sham treatments (no *B. bassiana*), solid color fills denote plus or minus standard error, and dashed black lines show MST (50% mortality) for each treatment. *P*-value is based on a log-rank test comparing the median beetle survival time when exposed to sham treatment to median beetle survival time when exposed to each isolate.


14B
34C
429BTF
429DA
50C
90(1)MPB
Survival Probability (%)
1.00
0.75
0.50
0.25
0.00
p < 0.0001
0
2
4
6
8
Time (Days)

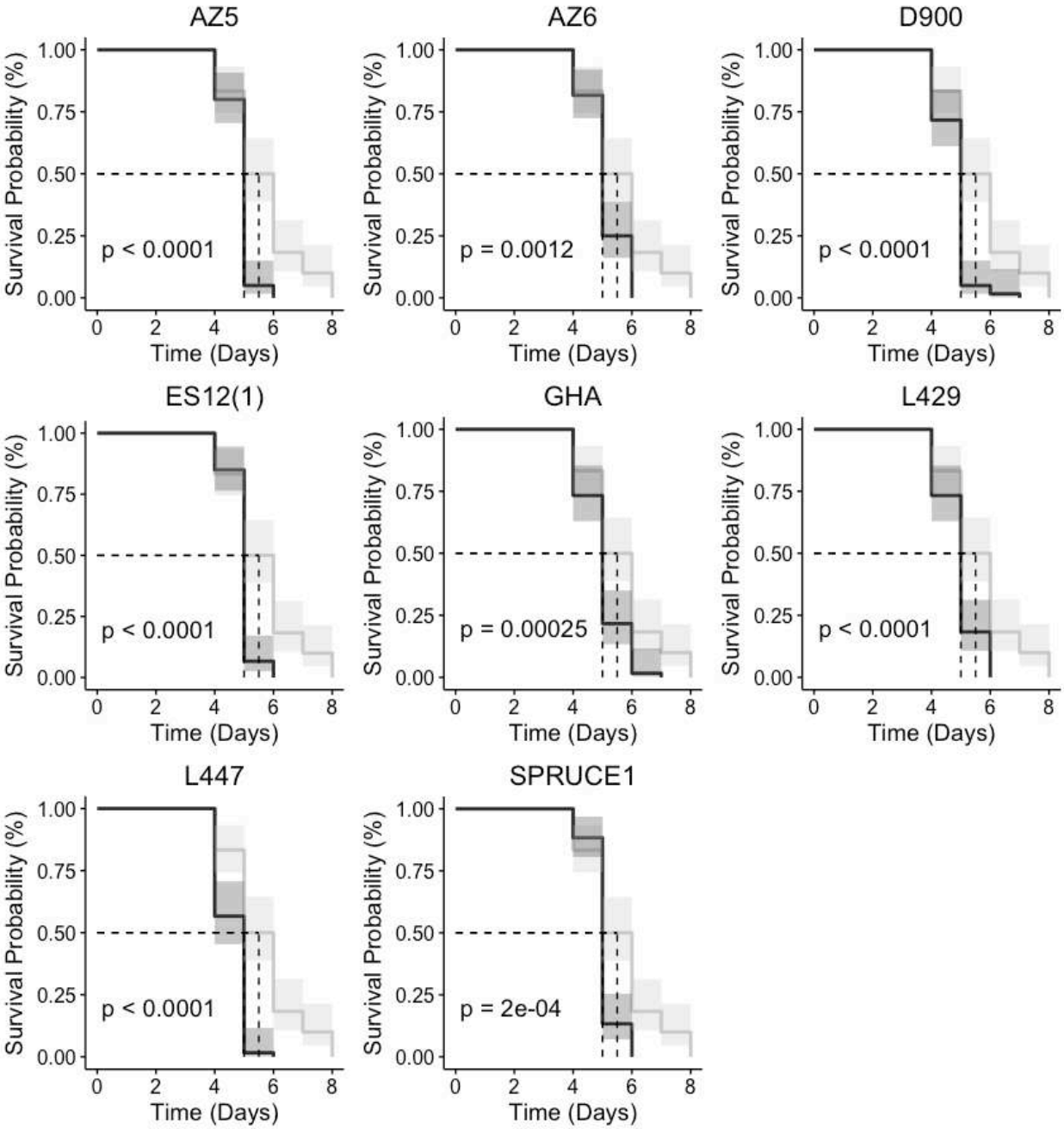
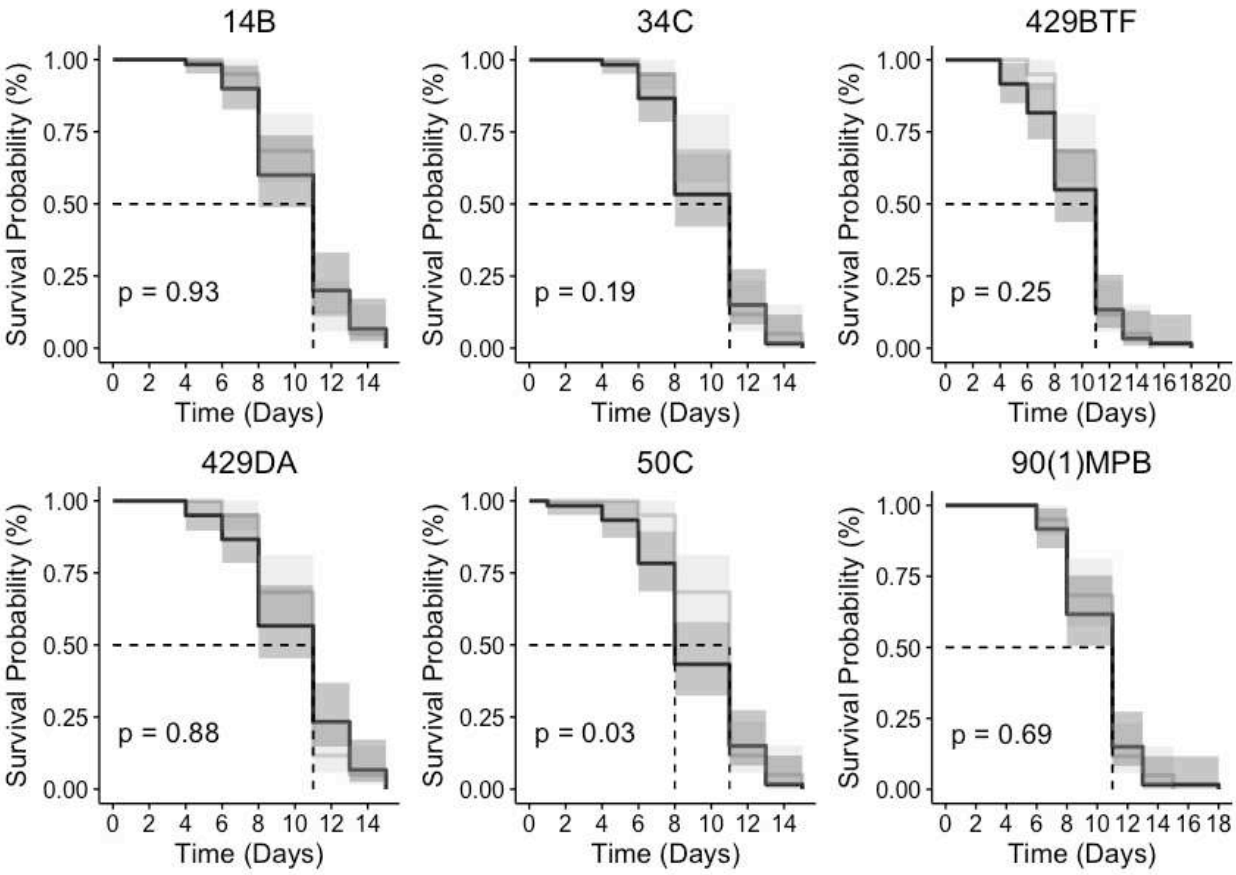


Figure 2.3. Kaplan-Meier survivorship curves for experiment 2. Solid black lines indicate survival time of *Dendroctonus rufipennis* after exposure to the indicated isolate of *Beauveria bassiana*. Solid grey lines show sham treatments (no *B. bassiana*), solid color fills denote plus or minus standard error, and dashed black lines show MST for each treatment and control. *P*-value is based on a log-rank test comparing the median beetle survival time when exposed to sham treatment to median beetle survival time when exposed to each isolate.


14B
34C
429BTF
429DA
50C
90(1)MPB
Survival Probability (%)
Time (Days)
p = 0.93
p = 0.19
p = 0.25
p = 0.88
p = 0.03
p = 0.69

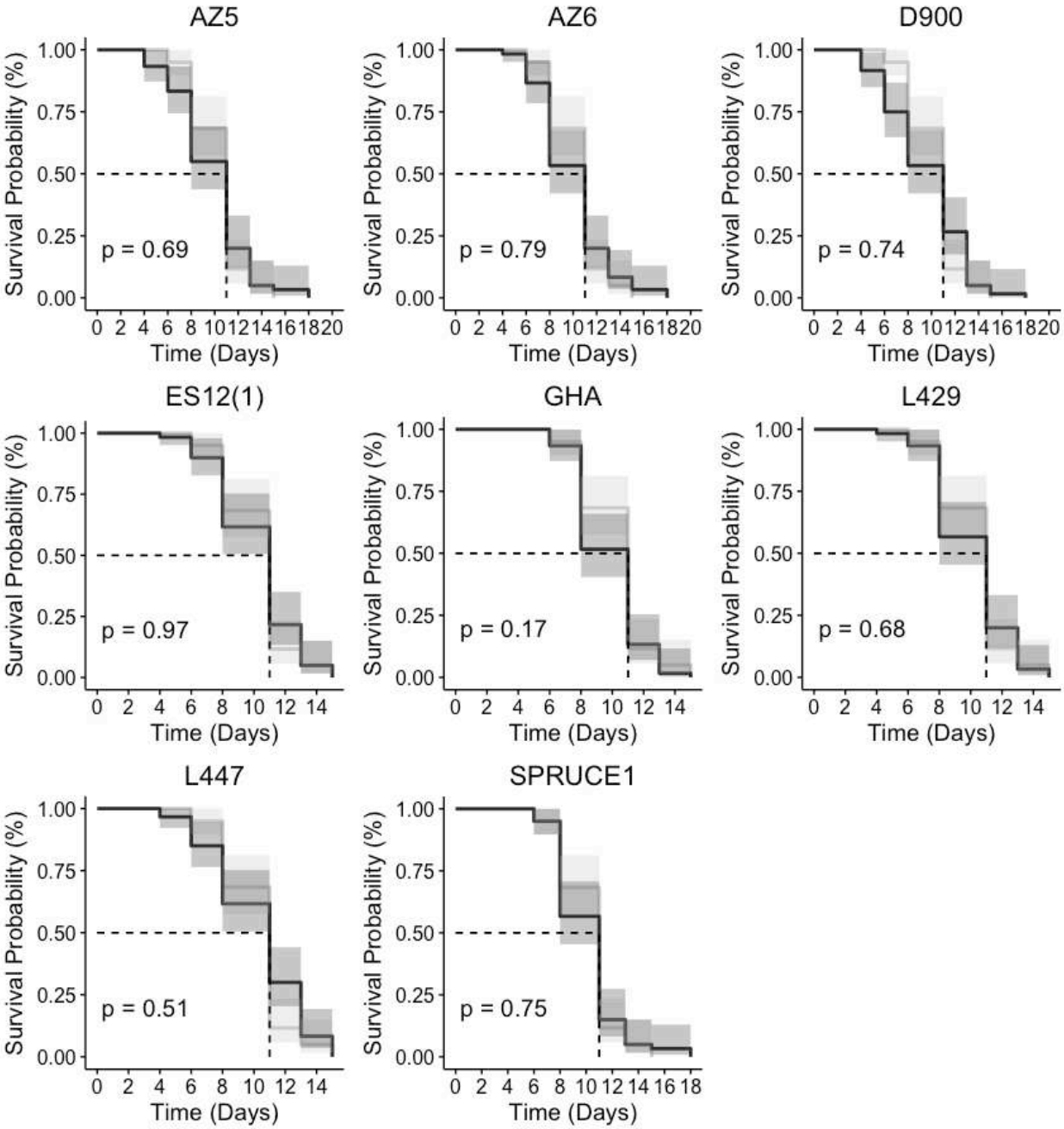
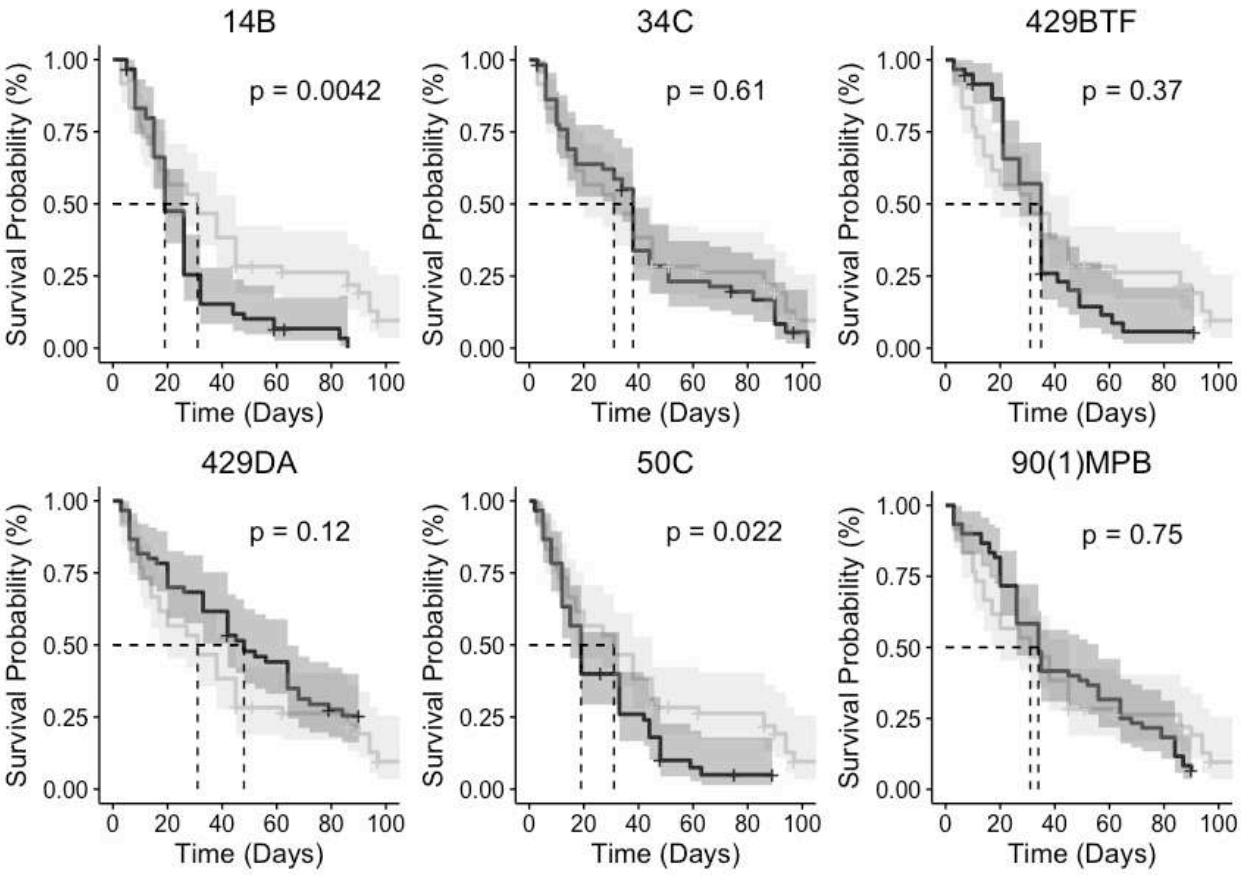


Figure 2.4. Kaplan-Meier survivorship curves for experiment 3. Solid black lines indicate survival probability of *Dendroctonus rufipennis* after exposure to the indicated isolate of *Beauveria bassiana*. Solid grey lines show sham treatments (no *B. bassiana*), solid color fills denote plus or minus standard error, and dashed black lines show MST for each isolate and sham treatment. *P*-value is based on a log-rank test comparing the median beetle survival time when exposed to sham treatment to median beetle survival time when exposed to each isolate.


14B
p = 0.0042
34C
p = 0.61
429BTF
p = 0.37
429DA
p = 0.12
50C
p = 0.022
90(1)MPB
p = 0.75
Survival Probability (%)
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
Time (Days)

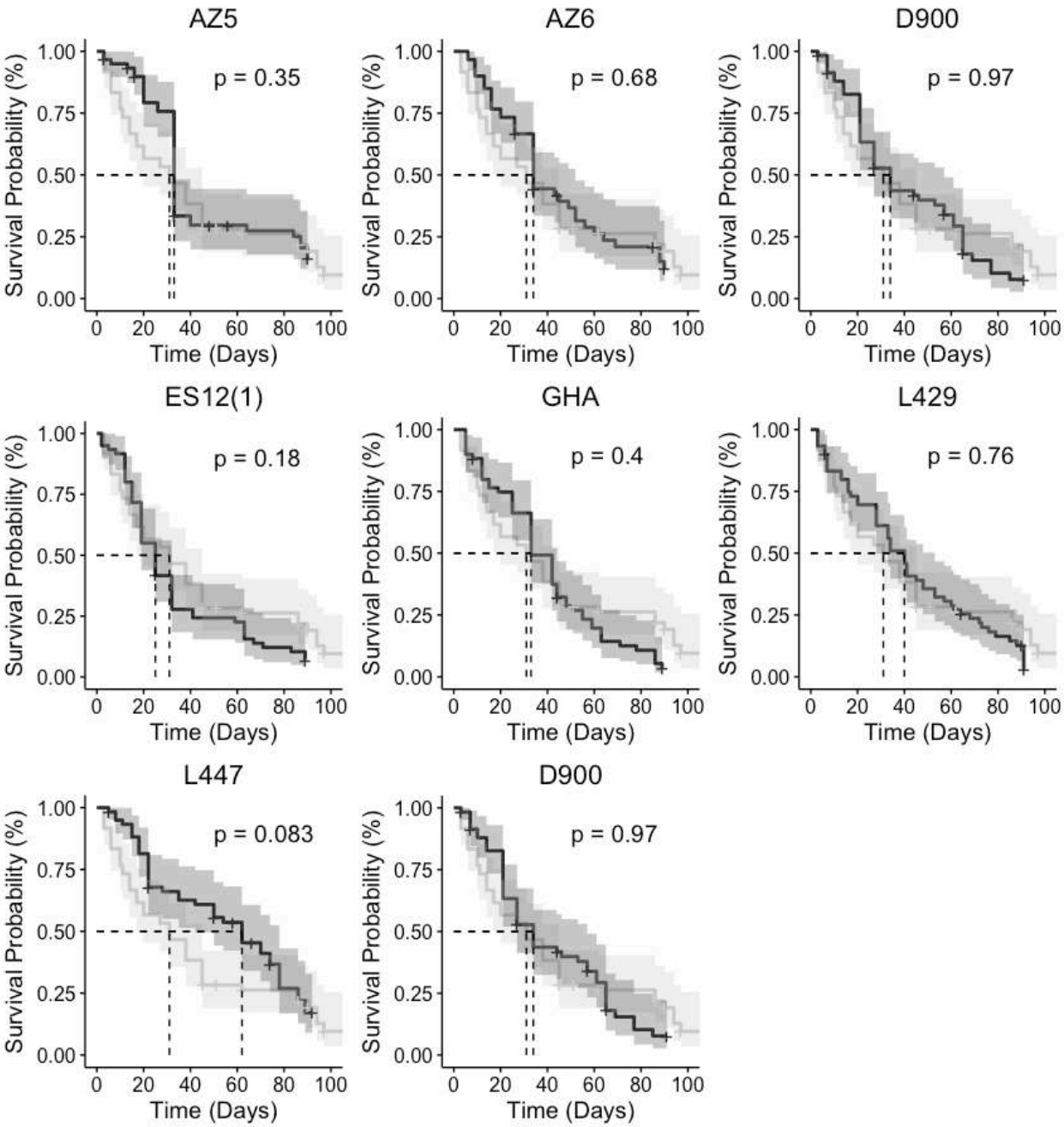


Figure 2.5. Kaplan-Meier survivorship curves for experiment 4. Solid black lines indicate survival time of *Dendroctonus rufipennis* after exposure to the indicated isolate of *Beauveria bassiana*. Solid grey lines show control treatments (no *B. bassiana*), solid color fills denote plus or minus standard error, and dashed black lines show MST for each treatment and control. P-value is based on a log-rank test comparing the control to each treatment.

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APPENDIX

Table 1.S1. Hylesinini subtribes and locations of studies involving *Beauveria bassiana* and Hylesinini.

Hylesinini species	Subtribe	Location
Dendroctonus brevicomis	Tomicina	Western United States[1]
Dendroctonus frontalis	Tomicina	Southeastern United States[1,2,3,4,5,6]
Dendroctonus micans	Tomicina	Turkey[7,8,9]
Dendroctonus ponderosae	Tomicina	Western North America[10,11,12]
Dendroctonus rufipennis	Tomicina	Western North America[10,13,14]
Dendroctonus simplex	Tomicina	Quebec, Canada[15]
Dendroctonus valens	Tomicina	China[16,17,18]
Dryocoetes confusus	Tomicina	British Columbia, Canada[19]
Hylastes ater	Hylesinina	New Zealand[20,21,22,23]
Hylurgops palliatus	Hylastina	Bulgaria[24]
Hylurgus ligniperda	Tomicina	New Zealand[1,20,21,22,23,25]
Polygraphus major	Polygraphina	India[26]
Polygraphus proximus	Polygraphina	Siberia[27]
Tomicus minor	Tomicina	Europe[28,29]
Tomicus piniperda	Tomicina	Eastern Europe[24,30,31,32]

Citations: Davis et al. 2011[1], Harrar and Martland 1940[2], Moore 1970[3], Moore 1971[4], Moore 1973[5], Pabst and Sikorowski 1980[6], Kocacevik et al. 2015[7], Sevim et al. 2010[8], Tanyeli et al. 2010[9], Cardoza et al. 2009[10], Hunt et al. 1984[11], Hunt 1986[12], Davis et al. 2018[13], Davis et al. 2019[14], Srei et al. 2017[15], Xu et al. 2018[16], Yao et al. 2012[17], Zhang et al. 2011[18], Whitney et al. 1984[19], Brownbridge et al. 2012[20], Glare et al. 2008[21], Reay et al. 2008[22], Reay et al. 2010[23], Takov et al. 2012[24], Davydenko et al. 2014[25], Khanday and Buhroo 2018[26], Kerchev et al. 2017[27], Jankevica 2004[28], Schebeck et al. 2016[29], Burjanadze 2010[30], Krams et al. 2012[31], Silva et al. 2015[32]

Table 2.S1. *Beauveria bassiana* growth rate response to environmental conditions representative of the *Dendroctonus rufipennis* study system. Mean responses are shown as percent of control ± SE. Values over 100% indicate increased growth under these conditions compared to the control. Significant values ($P<0.05$) are indicated in bold. Capital letters are Tukey HSD values relative to each experiment and 'n.g.' indicates no growth.

Isolate Name	5 °C	10 °C	15 °C
14B	n.g. J	2.36 ± 0.00 IJ	15.04 ± 1.58 DEFGHIJ
34C	n.g. J	2.38 ± 0.71 IJ	3.75 ± 0.67 HIJ
429BTF	n.g. J	1.59 ± 0.12 IJ	7.92 ± 0.90 FGHIJ
429DA	n.g. J	9.86 ± 1.38 EFGHIJ	36.00 ± 4.97 CD
50C	n.g. J	1.45 ± 0.10 IJ	9.80 ± 0.97 EFGHIJ
90(1)MPB	n.g. J	1.84 ± 0.45 IJ	7.93 ± 0.54 FGHIJ
AZ5	n.g. J	1.78 ± 0.37 IJ	6.91 ± 0.39 FGHIJ
AZ6	n.g. J	3.33 ± 0.23 HIJ	15.06 ± 2.18 DEFGHIJ
D900	n.g. J	1.44 ± 0.15 IJ	8.05 ± 1.14 FGHIJ
ES12(1)	n.g. J	1.31 ± 0.38 IJ	6.35 ± 1.85 EFGHIJ
GHA	n.g. J	3.49 ± 1.22 HIJ	16.97 ± 1.90 DEFGHIJ
L429	n.g. J	4.22 ± 1.74 HIJ	5.59 ± 0.21 GHIJ
L447	n.g. J	2.11 ± 0.37 IJ	11.63 ± 0.96 DEFGHIJ
SPRUCE1	n.g. J	2.24 ± 0.26 IJ	13.84 ± 0.74 DEFGHIJ

Table 2.S1. Continued.

Isolate Name	20 °C	25 °C	30 °C
14B	26.32 ± 3.40 CDEFGHI	32.44 ± 10.17 CDEF	28.40 ± 2.65 CDEFGH
34C	9.11 ± 1.60 FGHIJ	5.04 ± 2.34 HIJ	8.68 ± 0.49 FGHIJ
429BTF	9.86 ± 4.57 EFGHIJ	12.23 ± 5.25 DEFGHIJ	14.86 ± 5.17 DEFGHIJ
429DA	93.86 ± 16.15 A	51.00 ± 25.61 BC	75.86 ± 4.78 AB
50C	11.94 ± 0.89 DEFGHIJ	8.69 ± 2.98 FGHIJ	17.30 ± 1.19 DEFGHIJ
90(1)MPB	14.91 ± 1.78 DEFGHIJ	11.29 ± 4.89 DEFGHIJ	14.06 ± 2.43 DEFGHIJ
AZ5	11.93 ± 1.66 DEFGHIJ	8.57 ± 4.32 FGHIJ	6.98 ± 2.60 FGHIJ
AZ6	20.19 ± 5.52 DEFGHIJ	16.36 ± 7.90 DEFGHIJ	26.00 ± 1.93 CDEFGHI
D900	12.61 ± 0.38 DEFGHIJ	11.94 ± 4.61 DEFGHIJ	17.01 ± 0.82 DEFGHIJ
ES12(1)	11.02 ± 1.89 DEFGHIJ	18.01 ± 7.44 DEFGHIJ	14.17 ± 1.60 DEFGHIJ
GHA	30.95 ± 3.64 CDEFG	23.82 ± 13.55 DEFGHIJ	35.03 ± 1.65 CDE
L429	12.62 ± 3.11 DEFGHIJ	9.22 ± 3.78 FGHIJ	12.22 ± 2.72 DEFGHIJ
L447	15.29 ± 10.59 DEFGHIJ	15.33 ± 4.32 DEFGHIJ	17.04 ± 1.42 DEFGHIJ
SPRUCE1	17.51 ± 2.17 DEFGHIJ	18.06 ± 0.47 DEFGHIJ	19.75 ± 0.44 DEFGHIJ

Table 2.S1. Continued.

Isolate Name	35 °C	Temperature Statistics	Competition	Competition Statistics
14B	n.g. J		49.00 ± 12.77	
34C	n.g. J		45.50 ± 12.58	
429BTF	n.g. J		46.83 ± 9.95	
429DA	n.g. J		54.67 ± 10.65	
50C	n.g. J		34.00 ± 9.22	
90(1)MPB	n.g. J	F: 29.742	55.50 ± 13.41	F: 0.664
AZ5	n.g. J	**P<0.001**	42.83 ± 10.02	P: 0.790
AZ6	n.g. J	DF: 78	30.50 ± 5.33	DF: 83
D900	n.g. J	N: 292	27.67 ± 6.29	N: 84
ES12(1)	n.g. J		36.00 ± 12.81	
GHA	n.g. J		45.83 ± 11.58	
L429	n.g. J		44.83 ± 10.29	
L447	n.g. J		55.83 ± 12.93	
SPRUCE1	n.g. J		47.33 ± 11.50	

Table 2.S1. Continued.

Isolate Name	0.1% Alpha-pinene	1.0% Alpha-pinene	5.0% Alpha-pinene
14B	30.67 ± 0.50 DEFGHIK	27.10 ± 1.54 EFGHIJKLM	n.g. Q
34C	8.23 ± 0.97 LMNOPQ	0.37 ± 0.37 Q	2.86 ± 2.86 KLMNOPQ
429BTF	13.10 ± 1.14 EFGHIJKLMNOPQ	4.77 ± 1.95 NOPQ	n.g. Q
429DA	71.49 ± 15.01 AB	32.83 ± 10.42 DEFG	n.g. Q
50C	14.33 ± 4.10 EFGHIJKLMNOPQ	3.97 ± 3.97 NOPQ	n.g. Q
90(1)MPB	11.38 ± 1.78 GHIJKLMNOPQ	6.70 ± 3.20 MNOPQ	n.g. Q
AZ5	10.16 ± 3.17 HIJKLMNOPQ	1.88 ± 1.88 OPQ	n.g. Q
AZ6	18.38 ± 5.02 EFGHIJKLMNOPQ	8.22 ± 5.87 LMNOPQ	n.g. Q
D900	13.66 ± 4.59 EFGHIJKLMNOPQ	3.24 ± 3.24 OPQ	n.g. Q
ES12(1)	6.53 ± 1.11 MNOPQ	0.22 ± 0.22 Q	1.20 ± 0.60 Q
GHA	33.65 ± 6.12 DEF	6.95 ± 6.95 MNOPQ	3.19 ± 3.19 OPQ
L429	11.85 ± 2.64 GHIJKLMNOPQ	3.32 ± 1.18 OPQ	0.79 ± 0.79 Q
L447	17.60 ± 4.14 EFGHIJKLMNOPQ	6.23 ± 3.66 MNOPQ	0.57 ± 0.57 Q
SPRUCE1	13.49 ± 2.02 EFGHIJKLMNOPQ	7.24 ± 4.09 MNOPQ	0.23 ± 0.23 Q

Table 2.S1. Continued.

Isolate Name	0.1% Beta-pinene	1.0% Beta-pinene	5% Beta-pinene
14B	12.29 ± 4.39 EFGHIJKLMNOPQ	15.94 ± 8.95 EFGHIJKLMNOPQ	1.74 ± 1.74 NOPQ
34C	5.30 ± 0.29 LMNOPQ	6.75 ± 3.40 HIJKLMNOPQ	1.80 ± 1.80 NOPQ
429BTF	12.95 ± 1.66 EFGHIJKLMNOPQ	15.44 ± 2.25 EFGHIJKLMNOPQ	2.04 ± 1.34 MNOPQ
429DA	54.57 ± 4.68 ABCD	75.71 ± 10.11 A	37.50 ± 37.50 BCDEFGHIJ
50C	13.66 ± 0.69 EFGHIJKLMNOPQ	19.44 ± 2.90 EFGHIJKLMNOPQ	0.96 ± 0.96 NOPQ
90(1)MPB	10.18 ± 1.32 EFGHIJKLMNOPQ	14.99 ± 1.00 EFGHIJKLMNOPQ	n.g. Q
AZ5	9.67 ± 0.13 FGHIJKLMNOPQ	12.31 ± 1.56 EFGHIJKLMNOPQ	0.94 ± 0.94 NOPQ
AZ6	20.14 ± 1.02 EFGHIJKLMNOPQ	22.22 ± 5.41 EFGHIJKLMNOPQ	1.92 ± 1.92 NOPQ
D900	10.15 ± 1.80 EFGHIJKLMNOPQ	12.40 ± 4.17 EFGHIJKLMNOPQ	n.g. Q
ES12(1)	7.57 ± 1.18 HIJKLMNOPQ	4.13 ± 4.13 LMNOPQ	4.29 ± 2.92 LMNOPQ
GHA	18.06 ± 2.36 EFGHIJKLMNOPQ	14.57 ± 7.30 EFGHIJKLMNOPQ	9.32 ± 5.13 FGHIJKLMNOPQ
L429	11.44 ± 0.85 EFGHIJKLMNOPQ	16.85 ± 2.51 EFGHIJKLMNOPQ	2.86 ± 1.58 MNOPQ
L447	15.67 ± 1.14 EFGHIJKLMNOPQ	12.19 ± 3.95 EFGHIJKLMNOPQ	0.60 ± 0.60 NOPQ
SPRUCE1	18.27 ± 2.35 EFGHIJKLMNOPQ	13.29 ± 4.58 EFGHIJKLMNOPQ	1.60 ± 1.60 NOPQ

Table 2.S1. Continued.

Isolate Name	0.1% 3-Carene	1.0% 3-Carene	5.0% 3-Carene
14B	27.10 ± 4.37 EFGHIJKLM	25.39 ± 6.29 EFGHIJKLMN	6.67 ± 6.67 MNOPQ
34C	5.97 ± 1.78 MNOPQ	0.81 ± 0.50 Q	n.g. Q
429BTF	10.16 ± 1.44 HIJKLMNOPQ	1.68 ± 1.04 OPQ	n.g. Q
429DA	51.69 ± 15.49 ABCD	21.09 ± 8.74 EFGHIJKLMNOPQ	n.g. Q
50C	8.82 ± 2.13 JLMNOPQ	8.61 ± 5.20 HIJKLMNOPQ	1.72 ± 1.72 OPQ
90(1)MPB	8.31 ± 1.96 LMNOPQ	1.40 ± 1.40 Q	n.g. Q
AZ5	10.68 ± 2.69 FGHIJKLMNOPQ	1.40 ± 0.87 Q	n.g. Q
AZ6	12.15 ± 3.43 FGHIJKLMNOPQ	9.54 ± 3.41 HIJKLMNOPQ	n.g. Q
D900	8.00 ± 3.06 LMNOPQ	2.44 ± 0.69 OPQ	0.48 ± 0.48 Q
ES12(1)	8.11 ± 1.78 LMNOPQ	2.63 ± 1.29 OPQ	n.g. Q
GHA	15.82 ± 4.02 EFGHIJKLMNOPQ	14.60 ± 3.77 EFGHIJKLMNOPQ	n.g. Q
L429	7.06 ± 1.53 MNOPQ	2.92 ± 0.86 OPQ	2.83 ± 1.77 OPQ
L447	11.62 ± 2.53 GHIJKLMNOPQ	1.40 ± 0.95 Q	1.56 ± 1.56 Q
SPRUCE1	14.56 ± 1.12 EFGHIJKLMNOPQ	6.84 ± 1.66 MNOPQ	2.23 ± 1.39 OPQ

Table 2.S1. Continued.

Isolate Name	0.1% Myrcene	1.0% Myrcene	5.0% Myrcene
14B	25.35 ± 0.48 EFGHIJKLMN	14.14 ± 3.76 EFGHIJKLMNOPQ	6.43 ± 3.75 MNOPQ
34C	8.26 ± 0.31 LMNOPQ	4.73 ± 1.35 MNOPQ	2.23 ± 1.05 OPQ
429BTF	13.63 ± 0.69 EFGHIJKLMNOPQ	10.86 ± 1.66 HIJKLMNOPQ	2.88 ± 1.65 OPQ
429DA	58.63 ± 3.67 ABC	30.32 ± 6.98 DEFGHIJKL	12.60 ± 7.49 FGHIJKLMNOPQ
50C	11.02 ± 3.63 HIJKLMNOPQ	9.35 ± 3.32 IJKLMNOPQ	3.74 ± 2.35 NOPQ
90(1)MPB	12.09 ± 1.53 FGHIJKLMNOPQ	9.33 ± 3.07 IJKLMNOPQ	4.83 ± 1.94 NOPQ
AZ5	12.19 ± 0.48 EFGHIJKLMNOPQ	7.58 ± 3.43 LMNOPQ	3.81 ± 1.98 NOPQ
AZ6	22.22 ± 2.51 EFGHIJKLMNOPQ	18.98 ± 3.53 EFGHIJKLMNOPQ	3.35 ± 1.38 OPQ
D900	14.00 ± 0.82 EFGHIJKLMNOPQ	9.20 ± 3.85 IJKLMNOPQ	1.76 ± 0.97 OPQ
ES12(1)	13.22 ± 1.35 EFGHIJKLMNOPQ	8.27 ± 3.07 LMNOPQ	1.28 ± 1.07 Q
GHA	36.00 ± 1.38 CDE	17.39 ± 8.24 EFGHIJKLMNOPQ	7.25 ± 4.88 MNOPQ
L429	15.10 ± 2.26 EFGHIJKLMNOPQ	14.44 ± 2.60 EFGHIJKLMNOPQ	5.45 ± 4.25 MNOPQ
L447	18.96 ± 1.17 EFGHIJKLMNOPQ	11.85 ± 2.90 GHIJKLMNOPQ	2.83 ± 1.77 OPQ
SPRUCE1	18.25 ± 1.03 EFGHIJKLMNOPQ	15.75 ± 1.85 EFGHIJKLMNOPQ	4.05 ± 2.03 NOPQ

Table 2.S1. Continued.

Isolate Name	0.1% terpinolene	1.0% terpinolene	5.0% terpinolene	Monoterpene Statistics
14B	26.71 ± 0.73 DEFGHIJKLMNOP	3.50 ± 1.70 OPQ	n.g. Q	
34C	7.60 ± 0.48 HIJKLMNOPQ	n.g. Q	n.g. Q	
429BTF	15.90 ± 0.71 EFGHIJKLMNOPQ	2.53 ± 0.91 NOPQ	0.08 ± 0.08 Q	
429DA	68.86 ± 3.87 AB	1.11 ± 1.11 Q	n.g. Q	
50C	14.24 ± 2.52 EFGHIJKLMNOPQ	n.g. Q	n.g. Q	
90(1)MPB	11.96 ± 0.92 EFGHIJKLMNOPQ	2.52 ± 2.52 OPQ	n.g. Q	F: 1.437
AZ5	11.01 ± 0.49 EFGHIJKLMNOPQ	1.11 ± 0.69 OPQ	n.g. Q	**P: 0.005**
AZ6	26.79 ± 0.95 DEFGHIJKLMNO	1.62 ± 1.62 PQ	n.g. Q	DF: 104
D900	10.19 ± 1.15 EFGHIJKLMNOPQ	0.95 ± 0.59 OPQ	n.g. Q	N: 913
ES12(1)	11.36 ± 1.74 EFGHIJKLMNOPQ	n.g. Q	n.g. Q	
GHA	34.60 ± 0.63 CDEFGH	4.81 ± 2.89 MNOPQ	n.g. Q	
L429	11.35 ± 2.04 EFGHIJKLMNOPQ	0.87 ± 0.55 Q	n.g. Q	
L447	19.86 ± 0.82 EFGHIJKLMNOPQ	0.25 ± 0.25 Q	n.g. Q	
SPRUCE1	18.06 ± 1.67 EFGHIJKLMNOPQ	3.09 ± 2.81 OPQ	n.g. Q	

Table 2.S1. Continued.

Isolate Name	0.1% Chitin	1.0% Chitin	5.0% Chitin	Chitin Statistics
14B	15.57 ± 0.28 CDEFGHI	16.89 ± 1.38 CDEF	14.37 ± 1.05 CDEFGHIJK	
34C	4.41 ± 0.18 N	4.97 ± 0.47 MN	4.32 ± 0.16 N	
429BTF	6.88 ± 0.03 LMN	9.18 ± 0.68 GHIJKLMN	8.96 ± 0.10 IJKLMN	
429DA	57.71 ± 2.54 A	55.14 ± 1.36 A	40.43 ± 3.47 B	
50C	9.76 ± 0.48 GHIJKLMN	9.95 ± 0.49 FGHIJKLMN	9.03 ± 0.08 HIJKLMN	
90(1)MPB	7.47 ± 0.13 KLMN	8.87 ± 0.80 JKLMN	7.38 ± 1.06 KLMN	F: 4.633
AZ5	6.76 ± 0.18 LMN	10.08 ± 1.65 FGHIJKLMN	6.56 ± 0.16 LMN	**P<0.001**
AZ6	15.51 ± 1.98 CDEFGHIJ	13.65 ± 0.44 DEFGHIJKL	15.57 ± 0.68 CDEFGHIJ	DF: 26
D900	10.15 ± 0.55 FGHIJKLMN	9.59 ± 0.72 GHIJKLMN	9.77 ± 0.53 GHIJKLMN	N: 127
ES12(1)	8.10 ± 0.41 KLMN	8.18 ± 0.27 KLMN	7.05 ± 0.18 LMN	
GHA	21.05 ± 2.04 C	16.24 ± 4.36 CDEFG	17.33 ± 0.51 CDE	
L429	16.12 ± 0.20 CDEFGH	18.62 ± 0.76 CD	16.26 ± 1.50 CDEFG	
L447	9.06 ± 0.57 HIJKLMN	12.08 ± 1.08 DEFGHIJKL	8.69 ± 0.68 JKLMN	
SPRUCE1	11.56 ± 0.75 DEFGHIJKLM	11.31 ± 0.60 EFGHIJKLMN	10.00 ± 0.70 FGHIJKLMN	

Table 2.S1. Continued.

Isolate Name	-0.5 MPa	-1.0 MPa	-2.0 MPa	Osmotic Potential Statistics
14B	93.26 ± 9.37 EFGHIJKLM	112.31 ± 1.81 CDEFGH	71.31 ± 1.12 LM	
34C	103.20 ± 3.43 EFGHIJKL	124.31 ± 1.98 BCDE	74.77 ± 2.22 JKLM	
429BTF	112.82 ± 2.29 CDEFGH	118.49 ± 1.72 BCDEFG	77.94 ± 0.93 IJKL	
429DA	122.15 ± 2.92 BCDE	127.85 ± 0.91 ABCDE	92.47 ± 2.25 EFGHIJKLM	
50C	85.96 ± 10.94 FGHIJKLM	109.82 ± 0.87 DEFGHIJ	67.82 ± 5.93 M	
90(1)MPB	99.07 ± 9.16 EFGHIJKLM	108.75 ± 3.85 DEFGHIJK	73.53 ± 2.55 LM	F: 3.246
AZ5	92.31 ± 4.08 EFG	100.27 ± 2.03 EFGHIJKLM	66.73 ± 0.34 M	**P<0.001**
AZ6	86.55 ± 13.46 GHIJKLM	114.56 ± 2.78 CDEFGH	72.85 ± 5.07 LM	DF: 26
D900	77.38 ± 4.94 JKLM	143.85 ± 3.65 ABC	95.98 ± 3.40 EFGHIJKLM	N: 160
ES12(1)	140.01 ± 10.86 ABCD	150.45 ± 2.49 AB	98.34 ± 1.27 EFGHIJKLM	
GHA	124.34 ± 19.39 BCDE	122.87 ± 6.14 BCDEF	81.57 ± 0.76 HIJKLM	
L429	106.72 ± 0.69 DEFGHIJKL	158.87 ± 2.52 A	116.91 ± 2.42 BCDEFG	
L447	104.18 ± 10.01 EFGHIJKL	110.84 ± 2.32 DEFGHI	75.98 ± 0.85 KLM	
SPRUCE1	96.74 ± 10.69 EFGHIJKLM	108.88 ± 1.78 DEFGHIJK	68.73 ± 0.81 M	

Table 2.S1. Continued.

Isolate Name	Sunlight	Sunlight Statistics
14B	26.04 ± 2.33 BC	
34C	7.49 ± 0.45 F	
429BTF	13.98 ± 0.65 EF	
429DA	72.14 ± 5.02 A	
50C	16.26 ± 0.10 DEF	
90(1)MPB	14.58 ± 0.81 DEF	F: 73.764
AZ5	12.79 ± 0.40 EF	**P<0.001**
AZ6	24.14 ± 2.00 BCD	DF: 41
D900	13.20 ± 1.16 EF	N: 42
ES12(1)	12.28 ± 0.58 EF	
GHA	31.39 ± 1.39 B	
L429	15.17 ± 0.81 DEF	
L447	19.63 ± 0.86 CDE	
SPRUCE1	19.11 ± 2.64 CDE	