Review

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The complex symbiotic relationships of bark beetles with microorganisms: a potential practical approach for biological control in forestry

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Abstract

Bark beetles, especially *Dendroctonus* species, are considered to be serious pests of the coniferous forests in North America. Bark beetle forest pests undergo population eruptions, causing regionwide economic losses. In order to save forests, finding new and innovative environmentally friendly approaches in wood-boring insect pest management is more important than ever. Several biological control methods have been attempted over time to limit the damage and spreading of bark beetle epidemics. The use of entomopathogenic microorganisms against bark beetle populations is an attractive alternative tool for many biological control programmes in forestry. However, the effectiveness of these biological control agents is strongly affected by environmental factors, as well as by the susceptibility of the insect host. Bark beetle susceptibility to entomopathogens varies greatly between species. According to recent literature, bark beetles are engaged in symbiotic relationships with fungi and bacteria. These types of relationship are very complex and apparently involved in bark beetle defensive mechanisms against pathogens. The latest scientific discoveries in multipartite symbiosis have unravelled unexpected opportunities in bark beetle pest management, which are discussed in this article.
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1   INTRODUCTION

Symbiosis is well known as a 'key driver' force in the evolutionary process of species,[1,2] as the origin of all eukaryotic organisms appears to be the result of serial endosymbiotic relationships with ancient prokaryotic cells.[3] Many scientists have long neglected the importance of symbiosis in the evolution of life on the earth; however, there has been a relatively recent increased interest in this phenomenon, using insects as models. Insects are among the most tolerant organisms with regard to foreign microbial intruders, accepting them both inside and outside their bodies.[1,4] Owing to their symbiotic relationships with microorganisms, insects are able to exploit and occupy ecological niches, otherwise inaccessible to other species, explaining their dominance over many types of ecosystem on earth.[5–7]

The evolutionary success of insects consists mainly in their ability to adapt to a wide variety of diets as a result of symbiosis with microorganisms. For example, feeding behaviour of different insect species can be phytophagous (living plant tissues or sap), detritophagous (decaying plant material), necrophagous (dead animals), coprophagous (excrements), keratinophagous (wool, fur or feathers), mycetophagous (fungi), predatory, blood sucking and microphagous (biofilm).[8]

Symbiosis has been widely used as a model to explain the evolution and diversification of different insect taxa, but very few studies have investigated the potential of using this phenomenon as a tool for biological control of insect pests.

2   PURPOSE OF THE REVIEW

The aim of this review is to shed light on potential new research avenues in forest insect pest management, considering the latest scientific discoveries in insect symbiosis. The focus will be on bark beetles in particular for two reasons: (1) the growing body of literature on the topic of multipartite symbiosis, and (2) the importance of this insect group in forest pest management in North America and other regions.

3   BARK BEETLES AND FOREST ECOSYSTEMS

3.1   Bark beetles and their ecological function in forest ecosystems

Bark beetles (Coleoptera: Curculionidae: Scolytinae), including also ambrosia beetles, especially the coniferous pests, are one

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of the best-studied insect groups in North America owing to their economic importance in the forestry industry.[9] In normal conditions (endemic or low population density), bark beetles perform important ecological functions in forest ecosystems by facilitating the integration of vegetal material in the natural biogeochemical cycle and by providing suitable habitats for other arthropod species, e.g. millipedes, and also for different fungal species.[10] Thus, bark beetles contribute to the decomposition process in forest ecosystems.

3.2 Bark beetle life cycle and forest ecosystems

In order to complete their life cycle, the great majority of bark beetle species attack stressed, weakened or dying trees.[10] Beetle species attacking stressed or dying trees are called secondary bark beetles.[11] These beetles are rarely the source of extensive damage to forest ecosystems and are therefore considered less important from an economic perspective. On the other hand, some bark beetle species using a primary attack strategy, especially those belonging to the genus *Dendroctonus*, are able to kill living trees.[10,12] In boreal forests, these beetles cause extensive damage to coniferous trees and represent a major disturbance factor of the ecosystem.[13] Primary bark beetles initiate pheromone-mediated mass attacks that overcome a tree's defensive system.

Other *Dendroctonus* species do not employ the mass attack strategy, including the lodgepole pine beetle *Dendroctonus murrayanae* Hopkins and the red turpentine beetle *Dendroctonus valens* LeConte, which use a parasitic strategy.[14,15] With these species, few beetle individuals attack living trees, the host defensive system is not exhausted and, consequently, trees are not killed. The parasitic *Dendroctonus* appear to be highly tolerant to toxic monoterpenes secreted by host plants.

Considering the importance of annual economic losses inflicted on the forest industry by primary tree-killer beetles, extensive studies on several aspects of biological control, such as the use of semiochemicals,[16,17] microbial control[18–20] and parasites, parasitoids and predators,[21] have been carried out.

4 MICROORGANISMS ASSOCIATED WITH BARK BEETLES

Bark and ambrosia beetles are insects engaged in symbiotic relationships with mainly fungi and bacteria. Tripartite interactions between the insects, the fungi and the bacteria are constantly modulated by environmental factors, the host phytochemistry and the presence of other organisms naturally occurring in bark beetle galleries. For instance, yeasts,[22,23] mites,[24–27] nematodes[28] and other saproxylic beetles may influence the tripartite relationships between bark beetles, their associated fungi and bacteria. These multiple interactions are always built up around symbiotic fungi and bacteria forming a kind of consortium called the symbiotic complex. A conceptual model of bark beetle multipartite symbiosis is schematically shown in Fig. 1. In this review, only the fungi and bacteria will be discussed, as they represent the central core of the symbiotic complex associated with bark beetles.

4.1 Fungi as symbiotic partners of bark beetles

Most bark beetle species rely on fungi for at least some stages of their development. Bark beetles may potentially ingest fungi (fruiting bodies or mycelium) that naturally colonise their galleries.[29,30] The association existing between bark beetle species and fungi has been intensively studied and reviewed by many authors.[12,30–34]
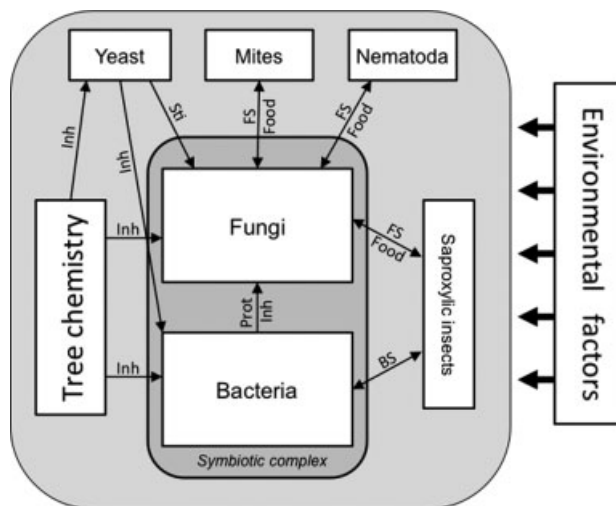

Figure 1. Schematic representation of the symbiotic complex of bark beetles. Bacteria and fungi associated with the insect are represented in frames of the same size to show their equal importance and comprise the central core of the bark beetle symbiotic complex. The symbiotic complex is continually affected by biotic and environmental factors. Arrows mark interactions within the players of the symbiotic complex: Inh, inhibition; Sti, stimulation; Prot, protection; FS, fungal spreading; BS, bacterial spreading.

The association with fungi is not only common in the bark beetle group, but also occurs in other major insect taxa such as Isoptera (termites), Homoptera (cicadas, aphids and scales) and Hymenoptera (Siricidae and Formicidae).[8] Within the Coleoptera order, an association between insects and fungi occurs over a wide range of families: Cerambycidae, Lymexylidae, Anobiidae, Bostrichidae, Scolytidae and Platypodidae.[35] Considering the microenvironment where bark beetle species evolve, the symbiotic association with various fungal species may provide competitive advantages over other xylophagous insects. Generally, plant tissues are recognised as a very poor food resource (nitrogen and phosphorus contents) regarding insect requirements.[36] Subsequently, bark beetles engage in symbiotic associations with microorganisms that enable them to overcome their limited metabolic abilities.[37,38]

4.2 Systematics of fungi

The bark beetle fungal associates, as reviewed in Harrington,[30] belong to the Ascomycota and Basidiomycota phyla. Within the Phylum Ascomycota, fungi belonging to the genus *Ophiostoma* (*sensu lato*), also known as 'blue stain fungi', are the most common bark beetle fungal partners.[33] Actually, ophiostomatoid fungi represent an artificial (polyphyletic) taxonomic group including several teleomorph genera, e.g. *Ophiostoma, Grossmania, Ceratocystiopsis, Ceratocystis, Gondwanamyces* and *Cornuvesica*.[39–42] The anamorph genera closely related to *Ophiostoma* include *Leptographium, Graphium, Pesotum, Hyalorhinocladiella* and *Sporothrix*.[33,43–45] Fungi associated with ambrosia beetles belong especially to the genera *Ambrosiella, Raffaelea* and *Geosmithia*.[46–48] Ophiostomatoid fungi display morphological adaptations such as sticky spores located on the apical region of a stalk-like structure that render them suitable to be vectored by bark beetles and other xylophagous insects.[49]

Within Phylum Basidiomycota, two genera are most commonly recognised as bark beetle associates: *Phlebiopsis* and *Entomocorticium*. The latter appears to be associated with many species of

Dendroctonus, and also with *Ips avulus* (Eichhoff) and *Pityoborus comatus* (Zimmermann).[30,50] These fungi have evolved adaptations, such as sticky basidiospores, that enhance their dissemination by bark beetles.[51] Only one species, in the genus *Phlebiopsis*, namely *P. gigantea* (Fr.) Jülich, is associated with *Dendroctonus approximatus* Dietz.[51]

There are perhaps hundreds or even thousands of fungal species associated with conifer bark beetles, as numbers are likely to be underestimates owing to the biases introduced by research focused almost exclusively on economically important forest pests.[30,32]

4.3 Role of fungi within the symbiotic complex

According to a classical paradigm, the ophiostomatoid fungi confer on aggressive bark beetles the ability to colonise new living or stressed trees by overcoming their defensive system.[12] Bark beetle fungal associates are inoculated in phloem or xylem after tree attacks. Subsequently, the inoculated fungi grow in insect galleries, providing nutritional supply for larvae,[29,52] for callow individuals[53,54] and, especially in the case of ambrosia beetles, for adults.[55] Fungi associated with bark beetles also provide essential sterols, commonly the $\Delta^{5,7}$-sterols, such as ergosterol. These compounds are required for ovogenesis and normal larval development.[37,56] Fungi are also involved in bark beetle pheromone synthesis.[57] Bark beetle symbiotic fungi increase the insect host fitness by significantly reducing the development time of the larvae, as well as the length of their galleries.[58] The fungi associated with the symbiotic complex may, likewise, be compared to a 'root system' or an 'external stomach' of the insect host, because of their ability to concentrate large amounts of phloem nutrients.[59] In several cases, fungi associated with bark beetles also play a role as detoxifiers of hostile chemical compounds secreted by the tree host.[60,61]

4.4 Case studies of bark beetle–fungus interactions

The degree of interaction between a bark beetle and its fungi varies according to the insect feeding habit. Fungal feeding, known as mycophagy, is not widespread among bark beetles. Only four genera are considered mycophagous, namely *Dendroctonus* Erichson, *Ips* De Geer, *Tomicus* (Eichhoff) and *Pityoborus* Blackman.[30] The majority of bark beetle species are phloeophagous (phloem feeders). Among ambrosia beetles, a xylem-boring beetle group, mycophagy is developed to such an extent that the beetles cultivate the fungi upon which they feed (fungus farming).[55,62] Wood[63] includes the members of the tribes Hylesinini, Scolytini, Xyleborini, Xyloterini, Scolytoplatypodini and Corthylini and the members of subfamily Platypodinae in the group of ambrosia beetles. Mycophagy is an outstanding evolutionary achievement for ambrosia beetles, helping them to escape from phloem resource competition, and also giving them the opportunity to exploit other host plants such as angiosperms.[62] The phloeophagous feeding habit might be a primitive character, while xylomycetophagy might have evolved independently from phloeophagy.[62] However, Hulcr and Dunn[59] affirm that the boundary between bark and ambrosia beetles is often unclear, and splitting of the beetles into two distinct ecological groups may no longer be supported by recent scientific evidence.

Mycophagous bark and ambrosia beetles display specialised cuticular structures called mycangia or mycentangia, which shelter symbiotic fungi. The diverse morphology and function of bark beetle mycangia have already been discussed by Six.[32] Among the best studied mycangial fungi are those related to the *Dendroctonus* complex, which includes very aggressive bark beetle species capable of killing living trees and of initiating extensive outbreaks in forest ecosystems.[54,64,65] A tabular synthesis of mycangial fungi associated with mycophagous bark beetles is presented in Harrington.[30] Physiologically, mycangia provide protection and ensure a proper environment for mycelia growth and development owing to secretions produced by mycangial unicellular glands.[32,34]

An interesting debate relates to the importance of mycangial fungi for bark beetle development. For example, the complete removal of fungal partners from the southern pine beetle, *Dendroctonus frontalis* Zimmermann, considerably reduces the number of progeny per gallery and significantly protracts the emergence of adults.[66] Bleiker and Six[52] demonstrated that the absence of fungi could negatively affect the larval development of mountain pine beetle, *Dendroctonus ponderosae* Hopkins. As a consequence, the callow individuals compensate for the lack of fungi by feeding on more phloem. Similar studies on the coffee berry borer, *Hypothenemus hampei* Ferrari, established that removal of mycangial fungi negatively affects moult and larval development.[67] Nonetheless, the benefits for bark beetles and fungi resulting from this mutualistic association are difficult to assess fully and to quantify experimentally.[32] For mycangial bark beetles at least, the association with their fungal partners seems to be critical for their development and maturation.[32] Mycangial fungi have more beneficial effects on their insect hosts than those carried on the exoskeleton.[26,65,68]

Phloeophagous (phloem-feeding) bark beetles are basically non-mycangial, and the association with fungal partners does not appear to be essential for them to complete their life cycle.[32] It is highly likely that, for non-mycangial bark beetles, fungal partners draw more benefits than the insect host. Symbiotic fungi of non-mycangial bark beetles are generally carried on the insect exoskeleton and phoretically spread in galleries.[65] Non-mycangial bark beetles may transport and inoculate a large variety of ophiostomatoid fungi that have no or little beneficial effect for the insect host.[33] Still, some non-mycangial bark beetle species could establish and maintain permanent association with some ophiostomatoid fungi, which increases the host fitness, at least at some stage of its development. For example, the spruce beetle, *Dendroctonus rufipennis* (Kirby), a non-mycangial bark beetle, displays a high degree of association (80–100% of beetle population) with *Leptographium abietinum* (Peck) M.J. Wingf.[69,70] Solheim and Safranyik[71] found *Ceratocystis rufipenni* (Wingfield, Harrington & Solheim) to be a highly phytopathogenic fungus frequently associated with populations of spruce beetle in British Colombia, Canada. Subsequent studies on spruce beetle fungal associates failed to isolate *C. rufipenni*.[69,70,72] Probably, the specificity of fungal association of non-mycangial bark beetles confers some competitive advantages against other saprophytic fungi that naturally grow in bark beetle galleries. The exact mechanism of this specific association is largely unknown. In an extensive study on spruce beetle populations from Alaska, Aukema *et al*.[70] found that the frequency of association of symbiotic fungi with spruce beetle varies within sites and according to spruce beetle population phase. It is likely that this association is density dependent. The observed pattern was not maintained in subsequent years of study. The same variations in fungal associates within sites were observed in the case of pine shoot beetle *T. piniperda* in Europe. For example, the ophiostomatoid fungus *Leptographium wingfieldii* Morelet displays a high frequency of association with the pine shoot beetle, but in some European

studied sites this fungus was not even isolated.[33] The environment could act as a limiting factor of fungal growth and development in coniferous hosts.[73–75]

Some fungal partners associated with non-mycangial bark beetles display both positive and negative effects on the insect host. For instance, in the case of pine engraver, *Ips pini* (Say), and the spruce beetle, *D. rufipennis*, and their fungal associates, *Ophiostoma ips* (Rumbold) Nannf. and *L. abietinum* respectively, both positive and negative effects take place, depending on the beetle's stage of development.[24,76] Some authors interpret bark beetle/fungi symbiosis as 'context dependency'.[2,34] More accurately, the partnership between bark beetles and their fungal associates is extremely dynamic and subject to rapid changes induced principally by environmental pressures.[2] For example, the presence and abundance of fungal partners in bark beetle galleries may be influenced by environmental factors such as temperature[73,74,77] and water availability.[78,79] In some cases, small changes in phloem water potential may increase fungus competitiveness,[80] while variation in phloem oxygen concentration may affect fungal growth and pathogenicity.[81] Recently, using phylogeographic analysis, Roe *et al*.[82] indicated that some bark beetle fungal partners display high genetic variability and rate of recombination among different strains. This genetic flexibility may directly influence the pathogenicity and environmental tolerance of fungi associated with the symbiotic complex. It appears obvious that bark beetles are able to transport several strains of the same fungal species, and these fungal strains may perform different ecological functions.

Furthermore, biotic factors such as tree chemistry may influence bark beetle associated fungi (Fig. 1). For example, fungal growth may be inhibited by monoterpene and diterpene acids and by stilbene phenolics secreted by coniferous hosts.[83–85] In other cases, some components of oleoresins secreted by conifers, e.g. heptanes, could stimulate the growth of bark beetle symbiotic fungi.[86] Recently, Davis and Hofstetter[87] have shown that the growth of nutritional symbiotic fungi associated with some *Dendroctonus* tree-killing beetles may be directly affected by the phytochemistry of the host. Plants displaying phenotypes characterised by high expression of defensive chemical compounds, mostly terpenes, may represent a challenge for these insects. Further studies are needed to test the hypothesis that phytochemical compounds indirectly affect the level of beetle populations by affecting the growth of their symbiotic fungi.

4.5 Discussion

Positive correlation between beetle aggressiveness and the phytopathogenicity of the symbiotic fungi still remains at the centre of scientific debate. Paine *et al*.[12] proposed the hypothesis that aggressive tree-killing bark beetles do not necessarily carry highly phytopathogenic fungi. On the other hand, Solheim and Safranyik[71] and Krokene and Solheim[88] indicated that highly virulent fungal tree pathogens such as *C. rufipenni* and *Ceratocystis polonica* (Siemaszko) may act in partnership with aggressive bark beetles, helping them to kill living trees. Experimental inoculations of ophiostomatoid fungi associated with bark beetles in coniferous trees have shown that some species are highly phytopathogenic.[71,88–90] In contrast, recent studies have demonstrated a low pathogenicity of these fungi against coniferous hosts.[91,92] Fungal pathogenicity is experimentally measured by using the number and length of wounds inflicted on tree host seedlings after inoculation. Nevertheless, this type of experimental approach could introduce serious biases in fungal

pathogenicity measurements, especially in relation to the density and concentration of fungal inocula in the host tree.

Six and Wingfield[93] have recently put forward some interesting arguments that might require the classical paradigm relating to bark beetles and the phytopathogenicity of their associated fungi to be reconsidered: (1) the lack of constancy of the association between tree-killing beetles and highly pathogenic fungi; (2) exhaustion of the defensive system of the host tree by the density of beetles during the attack phase rather than by fungal growth in beetle galleries; (3) the asynchrony between the growth rate of bark beetle symbiotic fungi and the exhaustion point of the tree defensive system. In addition, they indicate that phytopathogenicity is more useful to fungi than to insect beetles. Bark beetles would then only play a role as vectors of highly pathogenic ophiostomatoid fungi. From the evolutionary standpoint, fungi displaying high pathogenic levels would be more privileged in microhabitats dominated by strong competition between different fungal species. In bark beetle galleries, fungi compete for space and resources and may indirectly affect bark beetle population dynamics.[26,94,95]

Another issue that remains poorly understood in the association between saproxylic insects and their symbiotic fungi is the unexpected emergence of phytopathogenicity of fungi associated with alien ambrosia beetles. Normally, fungi carried by ambrosia beetles are known to be innocuous to the host trees. However, in North America, the ambrosia fungus *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, vectored by the ambrosia beetle *Xyleborus glabratus* Eichhoff, causes extensive damage to redbay, *Persea borbonia* (L) Spreng.[96] In a recent review, Hulcr and Dunn[59] proposed some hypotheses that could explain this sudden emergence of pathogenicity in the case of ambrosia fungi occurring outside their original distribution area. These hypotheses point out the importance of the equilibrium that has been established over millions of years between native ambrosia beetles and their hosts. The introduction of bark and ambrosia beetles in new habitats could exert environmental pressure on their symbiotic fungi. Consequently, new virulent and well-adapted fungal genotypes may arise, as recently demonstrated by Lu *et al*.[97] In addition, in a recent literature review, Six *et al*.[98] provide several arguments to support the equilibrium hypothesis and emphasise the importance of the coevolution between bark beetles and their associated microbial complex. This equilibrium is considered to be fragile, and any anthropogenic intrusion could have unpredictable consequences for natural forest ecosystems.

The above arguments demonstrate that current understanding of tritrophic interactions between host tree, bark beetles and symbiotic fungi remains unsubstantial. Furthermore, the overall picture of the relationships existing between bark beetles and their associated fungi still remains unclear. Evidently, not all fungal species found to be associated with bark beetles play a role as symbiotic partners. Six and Wingfield[93] specify that association of bark beetles with fungi ranges from mutualism to antagonism, and only the overall effect on beetle fitness and development determines whether this association should be considered mutualistic, commensal or antagonistic.

By targeting mutualistic fungi, especially those related to aggressive species, novel research avenues for the biological control of bark beetles could be explored. Genetic manipulations of bark and ambrosia beetle mutualistic fungi could potentially disrupt nutrition and normal development of the insect host. In the case of bark beetles, which are less dependent on their

fungal partners, perhaps different control strategies should be considered.

4.6 Bacteria as symbiotic partners of bark beetles

As previously mentioned, fungi associated with bark beetles are major players of the multipartite symbiosis phenomenon. Aside from fungi, several bacterial species are also associated with bark beetles, performing important functions within the symbiotic complex. They may stimulate or inhibit the growth of fungi, as presented in Fig. 1. Some authors suggest that bacteria act as veritable mediators in bark beetle–fungi symbiotic relationships.[49,99]

Bacteria isolated from inside and outside the insect body are referred to as endosymbiotic and ectosymbiotic bacteria respectively. The endosymbiotic bacteria occur in the gut, the body cavity and within cells,[100] whereas ectosymbiotic bacteria are found predominantly on the cuticle and the mouthparts of the insects.[101] The degree of association between endosymbiotic bacteria and their insect host ranges from obligate mutualistic to commensal to parasitic.[102] For example, the obligate mutualistic bacteria that tightly coevolved with their host are also known as primary mutualists and usually occur inside cells in special gut structures called bacteriocytes, often regrouped in bacteriomes.[1,101,103,104] Primary mutualists are strictly vertically transmitted and generally cannot be cultured on microbiological media.[102]

There are also bacteria categorised as facultative mutualists, which are not coevolutionarily linked with their insect host.[1] They occur both inside and outside host cells in a wide range of insect tissues.[102] Facultative mutualistic bacteria are not always vertically transmitted to the next generation of insect host. There is some evidence that facultative mutualistic bacteria could be recurrently acquired from the environment or horizontally transmitted between insect individuals.[100]

4.7 Bacterial species isolated from bark beetles

In bark beetles, bacterial microflora has been characterised in particular from oral secretions,[105,106] the gut[107] and crushed insects.[108] Essentially, species belonging to all major bacterial taxa have been isolated in bark beetles, such as α-proteobacteria: *Methylobacterium, Sphingomonas, Mycoplana, Rhodobacter*; bacteriodetes: *Chryseobacterium*; firmicutes: *Bacillus, Brevibacillus, Staphylococcus, Enterococcus, Lactobacillus, Leuconostoc*; β-proteobacteria: *Burkholderia, Alcaligenes*; γ-proteobacteria: *Raoultella, Serratia, Pantoea, Stenotrophomonas, Acinetobacter, Cedecea, Erwinia, Hafnia, Enterobacter, Klebsiella, Pseudomonas, Rahnella, Salmonella, Yersinia*; and actinobacteria: *Micrococcus, Streptomyces, Microbacterium, Kocuria, Corynebacterium, Mycobacterium, Brevibacterium, Arthrobacter, Aureobacterium*.

The majority of bacterial species isolated in bark beetles also occur in other insect taxa, but their ecological function, as well as their involvement in the bark beetle symbiotic complex, remains less understood. However, species belonging to the class of actinobacteria play an important role within the symbiotic complex.

4.8 Role, functions and interactions of symbiotic bacteria

Insect hosts, especially those displaying phytophagous behaviour, derive a large variety of benefits from their association with bacterial partners. For instance, bacteria of the symbiotic complex may improve nutritional quality of herbivorous insects by providing essential amino acids, B vitamins, nitrogen and sterols.[37,38,109–111] They equally provide ecological opportunity to insects by broadening their food spectrum[112] or ecological specialisation by restraining the food plant range.[113] Those bacteria are also capable of metabolising plant toxic compounds.[109] In addition, bacterial partners may be involved in pheromone synthesis,[114] and they may also provide protection from fungal competitors[115] or fungal pathogens.[116,117] Presumably, symbiotic bacteria and fungi perform similar functions for insect hosts, but Gibson and Hunter[38] argued that, from the nutritional standpoint, fungi rather provide sugars and fats, while bacteria synthesise essential amino acids.

Relatively recent discoveries in the insect microbial-mutualism area indicate that bacteria could play essential functions in insect host resistance against external aggressors. Positive associations between symbiotic bacteria and insect immunity are thoroughly examined by Hurst and Darby.[118] For example, in leaf-cutting ants (subfamily Attinae), filamentous bacteria (actinobacteria) inhibit the growth of an antagonistic fungus (*Escovopsis* sp.) that outcompetes ant beneficial fungi cultivated as food.[119] This inhibitory activity is attributed mainly to the ability of actinobacteria to secret antibiotic secondary metabolites.[120] Actinobacteria, especially *Streptomyces* sp., are carried by ant gynes on the cuticle.[120] Hence, antibiotic secondary metabolites secreted by mutualistic actinobacteria associated with leaf-cutting ants have been recently characterised: candicidin, a macrolide, and dentigerumycin, a cyclic depsipeptide.[99,121]

Similar findings have been reported in bark beetles. For example, Cardoza *et al.*[105] showed that the spruce beetle, *D. rufipennis*, is capable of defending its associate fungus *Leptographium abietinum* against other fungal competitors during the early stage of gallery colonisation. Apparently, bacteria located in the oral secretion of spruce beetle, especially the actinobacterium *Micrococcus luteus* Cohn, are responsible for this defensive mechanism. Further studies on bacterial microflora located in oral secretion have been carried out by Cardoza *et al.*[106] on three bark beetle species: spruce beetle, *D. rufipennis*, mountain pine beetle, *D. ponderosae*, and pine engraver, *I. pini*. They found that the spruce beetle exhibits a higher percentage of actinobacteria (*Streptomyces* sp. and *Micrococcus* sp.) than the pine engraver and mountain pine beetle.

Streptomyces species have also been isolated from galleries and mycangia of the southern pine beetle, *D. frontalis*.[115] In the southern pine beetle, a *Streptomyces* sp. inhibits the growth of *Ophiostoma minus* (Hedgc.) Syd. & Syd., a fungal competitor of the mutualistic fungus *Entomocorticium* sp. Hulcr *et al.*[122] demonstrated that *Streptomyces* species are very abundant and widespread in the majority of bark beetle galleries in North America. *Streptomyces* bacteria have been isolated from all body parts of bark beetles collected in different North American localities. They also found a large variety of *Streptomyces* isolates, but no correlation between a specific isolate and a particular bark beetle species has been established.

Actinobacteria have equally been identified in the gut of various bark beetle species such as red turpentine beetle, *D. valens*,[123] pine engraver, *I. pini*,[124] and other phytophagous forest pests such as gypsy moth, *Lymantria dispar* (L.),[125] and emerald ash borer, *Agrilus planipennis* Fairmaire.[126] According to Morales-Jimenez *et al.*,[123] actinobacteria isolated from the gut of bark beetles may perform cellulolytic activities. In pine engraver, *I. pini*, the actinobacteria community appears to be different in oral secretion versus gut.[106,124] Notably, actinobacteria have not been isolated

in the gut of southern pine beetle, *D. frontalis*,[107] but they occur in their galleries and mycangia.[115]

Other saproxylic beetles, including linden borer, *Saperda vestita* Say, seem to lack actinobacteria in the gut microflora.[127] Grünwald *et al.*[128] found several actinobacterial species in the gut of other Cerambycidae species such as *Rhagium inquisitor* (L.) and *Stictoleptura rubra* (L.). There are other reports concerning the involvement of gut bacteria in protection of insects against pathogens. For example, the gut microbiota of the desert locust, *Schistocerca gregaria* Forsskål, protects the insect host against entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokin by secreting antifungal compounds such as phenols.[117,129]

Actinobacteria isolated from southern pine beetle, *D. frontalis*, produce inhibitory secondary metabolites. Oh *et al.*[130] have purified and isolated mycangimicin, a linear 20-carbon carboxylic acid. Mycangimicin seems to be involved in antimicrobial activity. In addition, the secretion of antimicrobial compounds responsible for protection against pathogens has also been reported in crabronid wasps, *Philanthus triangulum* (Fabricius) and *Trachypus* sp.,[131,132] and in Australian bees.[133] In the pea aphid, *Acyrthosiphon pisum* Harris, the endosymbiotic bacterium *Regiella insecticola* (Moran) protects the insect host against the entomopathogenic fungus *Pandora* (*Erynia*) *neoaphidis* (Remaud. & Hennebert) Humber.[116] The insect gut microflora appears to play a crucial role in survival and normal development of the host. The role and significance of symbiotic gut microbiota for the insect host are discussed and reviewed by Dillon and Dillon.[134] The study of gut microflora in insects is advancing rapidly with the help of new molecular metagenomic, metatranscriptomic and metaproteomic tools.[135]

4.9 Discussion
Symbiotic bacteria associated with insects perform nutritional and protective functions. In a recent review, Feldhaar[103] exposed many other important ecological traits associated with insects and their symbiotic bacteria, such as adaptation to various non-biotic environments, mediation of insect–plant interaction, facilitation of the insect invasiveness, insect population level regulation and insect parasite manipulation. It is quite evident that the obligate or primary mutualistic bacteria represent the first line in the insect–bacteria partnership, and the strong cohesion created after hundreds of millions of years of coevolution between symbiotic partners is almost impossible to disrupt. Accordingly, primary symbiotic bacteria have sustained important genome simplifications, and they are deeply involved in cellular metabolic pathways of the insect host.[136] Primary mutualistic bacteria are absolutely required for insect survival and reproduction.

Facultative mutualistic bacteria could be potentially the second line of the coevolutionary process between insects and bacteria. Facultative mutualistic bacteria are often considered to be potential candidates to replace primary bacteria that have suffered genomic erosion during vertical transmission, and that no longer respond to host metabolic requirements.[7,137,138] A very remarkable evolutionary issue is represented by some environmentally acquired bacteria that are able rapidly to evolve from parasitism to obligate mutualism.[139,140] Well-known examples are *Wolbachia* spp., carried by more than 70% of described insect species living on the earth.[141] Recently, *Wolbachia* have been isolated from the ovarian epithelial tissue of *Pityogenes calcographus* (L.), a bark beetle species.[142] Even though *Wolbachia* cause major reproductive problems to insect hosts, e.g. cytoplasmic incompatibility and sex ratio distortion, the benefits to insects

derived from this relationship seem to exceed the detrimental effects.[104] Feldhaar[103] used the term holobiont to designate an organism whose phenotype arises from combined genes of the host as well as the genes of all symbiotic bacteria. The totality of genes carried by holobionts and their associated microbiota is described as the hologenome. Eventually, the purpose of symbiosis is to create an improved biotic system, such as organisms or consortia better adapted to their environment.

As in the case of fungi, association between bacteria and bark beetles could be influenced by environmental conditions, as Adams *et al.*[143] showed in a study of the gut bacterial microflora of the red turpentine beetle, *D. valens*. The gut bacterial community greatly varies between different sampled sites. Hulcr *et al.*[122] obtained similar findings in the case of *Streptomyces* actinobacteria associated with various *Dendroctonus* species. Tree chemistry could also influence the association between bacteria and bark beetles. Adams *et al.*[144] demonstrated that monoterpenes, such as α-pinene and 3-carene, strongly inhibit the growth of symbiotic bacteria associated with bark beetles.

As mentioned above, actinobacteria play a major role in bark beetle symbiotic relationships and are still a matter of fundamental debate in the literature. It is not yet very clear whether bark beetle-associated actinobacteria are mutualists or whether they are simply acquired from the environment.[49] To date there is no evidence of coevolutionary association between actinobacteria and their insect hosts. Some studies on Attinae ants revealed that their associated actinobacteria are not phylogenetically linked with their host, suggesting an environmental acquisition.[145,146]

In conclusion, it appears that insect hosts exhibit an adaptive or repairing system capable of easily replacing the symbiotic microflora. This observation has profound consequences for insect pest management. Symbiotic bacteria of the bark beetle could theoretically represent a target for physiological disruption or genetic manipulation, but this is presently difficult to achieve, mainly owing to the inadequate level of knowledge on this research topic. Further studies will be needed to clarify the ecological function of bacterial species isolated in the bark beetle group, as well as their role within the symbiotic complex.

5 BIOLOGICAL CONTROL PRINCIPLES USING ENTOMOPATHOGENIC FUNGI TO CONTROL BARK BEETLE FOREST PESTS
An interesting aspect that has never been tackled in the literature is the potential involvement of the bark beetle symbiotic complex in resistance against entomopathogens. Several biological control programmes in forestry attempt to use entomopathogenic fungi as biocontrol agents against bark beetle pests.

5.1 Entomopathogenic fungi encountered in bark beetle galleries
Entomopathogenic fungi naturally occur in the galleries of bark beetles, as reported by several authors. For example, Wegensteiner[147] presents an overview on the entomopathogenic fungi encountered in the European bark beetle galleries. Entomopathogenic fungi such as *Beauveria bassiana* (Bals.-Criv.) Vuill., *B. caledonica* Bissett & Widden, *Isaria farinosa* (Holmsk.) Fr., *I. fumosorosea* Wize, *I. variotii* (Samson) Bainier, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams and *Tolypocaldium cylindrosporum* W. Gams have been isolated from galleries of various European bark

beetle species. Recently, the entomopathogenic fungus *Lecanicillium muscarium* (Petch) Zare & W. Gams has been isolated from galleries of the great spruce bark beetle, *Dendroctonus micans* (Kugelann).[148] The entomopathogenic fungi *B. bassiana* and *B. caledonica* have also been founded in frass and galleries of bark beetles in New Zealand.[149,150] In North America, *B. bassiana* was isolated from western balsam bark beetle galleries, *Dryocoetes confusus* Swaine, in British Columbia, Canada.[151] Some observations on the spruce beetle populations, *D. rufipennis*, in Nova Scotia (Canada) have highlighted that *B. bassiana* occurs predominantly in post-outbreak trees (Guertin C and Lavallée R, unpublished). The presence of entomopathogenic fungi in bark beetle galleries probably originates from collembolans vectoring from forest soil.[152–154] Minute insects such as collembolans or springtails are able to carry entomopathogenic fungi such as *B. bassiana, B. brongniartii* (Sacc.) Petch and *Metarhizium anisopliae*. The fungal carrying capacity is not likely to be related to species but rather to its body size.[154] Mites (Acaria) could also be involved in carrying and spreading entomopathogenic fungal propagules across bark beetle galleries.[155–157]

5.2 Interactions between bark beetles and entomopathogenic fungi

Entomopathogenic fungi isolated from bark beetle galleries are subsequently challenged in the laboratory against their insect host to select an isolate that could be considered as the best candidate for field trials. These assays are also necessary to determine the influence of some environmental factors that could affect fungal growth or pathogenicity. Laboratory tests conducted by several authors with different entomopathogenic fungi are summarised in Table 1.

Several laboratory experiments demonstrated a high efficacy of entomopathogenic fungi against both bark beetle larvae and adults. However, a high mortality rate caused by entomopathogens is seldom achieved in natural forest ecosystems. For instance, in Croatia, studies on populations of two fir bark beetle species, *Pityokteines curvidens* (Germar) and *P. spinidens* (Reitter), showed that the rate of natural mortality caused by *B. bassiana* is maintained under 50% and varies according to the sites studied.[158] The fungus *B. bassiana* induces a high percentage of mortality in coffee berry borer natural populations, *H. hampei*, but the level of natural infection varies geographically.[159] In natural conditions, larvae and callow bark beetle individuals are rarely infected by entomopathogenic fungi.[147]

Field tests with entomopathogenic fungi against various bark beetle species are usually less conclusive than those conducted under controlled laboratory conditions. For example, a field test with *B. bassiana* inoculated to mountain pine beetle, *D. ponderosae*, in British Colombia, Canada, failed to transmit mycosis horizontally in the natural population.[160] Similarly, the use of an isolate of *B. bassiana* in field conditions against the coffee berry borer, *H. hampei,* did not significantly affect the beetle populations in treated plots compared with the control.[161]

In spite of some failures in using entomopathogenic fungi as biological control agents, there are several studies reporting success in infecting a beetle population with a fungus. For example, Hallet et al.[19] demonstrated that *B. bassiana* could be successfully applied to overwintering sites of the European spruce bark beetle, *Ips typographus* (L.), mainly owing to its persistence in the forest soil litter. In an attempt to control the pine shoot beetle, *T. piniperda,* in Europe, Lutyk and Swieczynska[162] used field trap logs sprayed

with *B. bassiana*. After 4 years of survey, the percentage of mycosis in beetle populations ranged between 71 and 100%.

Some researchers have used modified traps combining an insect attractant, a pheromone, with fungal conidia, also known in the scientific literature as the autocontamination and autodissemination process.[18,163,164] Direct contamination with fungal conidia is likely to be more efficient than indirect contamination.[165] Modified traps have been used to inoculate the *I. typographus* populations in Europe. Thus, Vaupel and Zimmermann[164] obtained a reduction in the number of bored holes in field caged logs by 15% in fungus-treated populations, and by 60% in subsequent generations. The modified traps used in the autocontamination and autodissemination process attract insect males, which are inoculated with fungal conidia through a passage into a contamination chamber. Consequently, the insect males acquire the fungal inoculum and then transfer it to females by direct-contact trough mating.

The likelihood of fungal dissemination within bark beetle populations is affected by mating behaviour (monogamy versus polygamy).[18] Lavallée et al.,[163] using a modified trap for beetle autocontamination, obtained a high cumulative mortality in treated spruce beetle, *D. rufipennis*, populations (74% within 4 days) and pine shoot beetle, *T. piniperda*, populations (98–100% within 4 days). Even in the control, some bark beetle individuals developed mycosis, induced principally by other *B. bassiana* isolates that naturally occur in insect galleries.

5.3 Discussion

An interesting outcome of the Lavallée et al.[163] experiments (both in the field and in the laboratory) is the difference in susceptibility to fungal treatment that is observed between the pine shoot beetle, *T. piniperda*, an exotic species in North America, and the spruce beetle, *D. rufipennis*, an indigenous species. It appears that the pine shoot beetles are more susceptible to treatments with entomopathogenic fungi than the spruce beetles.

Mainly because of their cryptic life, it is very difficult to expose wood-boring beetle populations to optimal amounts of fungal inocula.[166] The timeframe when wood-boring insects are vulnerable is very short and occurs during flight and the mating period or during overwintering in soil litter.

Under field conditions, non-biotic and biotic factors can act as a barrier for the growth and development of fungal pathogens. In the context of tritrophic interactions between an entomopathogen, an insect and its host plant, it is highly likely that the plant will influence the effectiveness of fungal pathogens.[167] More research is needed to improve current knowledge about the impact of entomopathogenic fungi on the insect population dynamics.[168] Only the fungal isolates that are able to initiate an epizooty within insect populations, and with limited non-target effects, would add to existing knowledge from the biological control point of view.

All these previous studies validate the potential of entomopathogenic fungi as interesting candidates in biological control of bark beetle forest pests. However, based on promising preliminary results, future studies should focus on improvement of the autocontamination and autodissemination techniques. Further studies on bark beetle microbial control must be able to fill the knowledge gaps in entomopathogenic fungi management, such as molecular tracking of isolates used in treatments, the ecology and fate of fungal isolates in insect natural habitats and their impact on non-target species. Moreover, increasing scientific efforts should be engaged in improvement of fungal formulations that may considerably ameliorate the effectiveness of field trials with microbial control agents. Clearly, the ultimate role of using fungal

Table 1. Laboratory tests conducted with entomopathogenic fungi on different bark beetle species

Bark beetle species	Stage of development[a]	Entomopathogenic fungus tested	Mortality (%)	ATS[b]	Ref.
Scolytus scolytus (F.)	L	*B. bassiana*	90	7–11	172
	A	*B. bassiana*	90	11–26	172
	A	*M. anisopliae*	90	16–30	172
	A	*I. farinose*	90	10	172
	A	*M. anisopliae*	90	9–12	172
	L	*B. bassiana*	85	5	173
	L	*L. lecanii*	100	10	174
Scolytus amygdali Guerin-Men.	A	*B. bassiana*	81.2–100	10	175
Scolytus multistriatus (Marsh.)	A	*B. bassiana*	100	10	176
	A	*M. anisopliae*	100	10	176
	A	*I. fumosorosea*	100	10	176
	A	*C. militaris*	36	10	176
	A	*H. thompsonii*	46	10	176
	A	*N. rylei*	83	10	176
	A	*L. lecanii*	56	10	176
	A	*I. farinosa*	76	10	176
Ips subelongatus Motch.	A	*B. bassiana*	89	n.a.[c]	177
Ips typographus (L.)	A	*B. bassiana*	99–100	6.1	178
	A	*B. bassiana*	70	28	19
	A	*B.bassiana*	100	4	179
	A	*I. farinosa*	90	5	179
	A	*M. ansiopliae*	100	4	179
	A	*L. lecanii*	90	5	179
Ips sexdentatus (Boern.)	A	*B. bassiana*	93.7	8	180
	A	*B.bassiana*	97	4.7	181
	A	*I. farinose*	67	11.7	181
Ips acuminatus (Gyll.)	A	*I. farinosa*	6	n.a.	181
Tomicus piniperda (L.)	A	*B. bassiana*	62.5	4	182
Trypodendron lineatum (Oliv.)	L	*B. bassiana*	100	6–8	183
	A	*B. bassiana*	100	12	183
	A	*B. bassiana*	86.7	1.4	184
Dendroctonus micans (Kugel.)	L	*B. bassiana*	83–100	6	185
	L	*B. bassiana*	90	10	148
	L	*I. fumosorosea*	Weak	n.a.	148
	L	*L. muscarium*	Weak	n.a.	148
	L	*Fusarium* sp.	Weak	n.a.	148
	A	*B. bassiana*	23–100	6	185
	A	*B. bassiana*	100	10	148
	A	*I. fumosorosea*	Weak	n.a.	148
	A	*L. muscarium*	Weak	n.a.	148
	A	*M. anisopliae*	100	6	185
Pityogenes chalcographus (L.)	L and P	*B. bassiana*	100	2.5	186
Polygraphus polygraphus (L.)	A	*B. bassiana*	72–100	1	187
	A	*B. brongniartii*	70–82	0.16	187
Hypothenemus hampei (Ferrari)	A	*B. bassiana*	100	3–4	188
Platypus spp.	L	*B. bassiana*	57–89	3	189
	L	*B. brongniartii*	93	3	189
	L	*M. anisopliae*	50–75	3	189
	A	*B. bassiana*	100	6–8	189
	A	*B. brongniartii*	100	6–8	189
	A	*M. anisopliae*	100	4	189

[a] A – adult bark beetles; L – larvae; P – pupae.
[b] ATS– average time of survival (days).
[c] n.a. – no data available.

entomopathogens in biological control is not to kill the entire pest population but rather to maintain it under an economically acceptable threshold.

Besides the difficulties linked with applying fungal entomopathogens in insect natural habitats, additional challenges occur that relate to variations in susceptibility of bark beetle species to entomopathogenic fungi. One potential explanation arising from the elements presented in this review is that the symbiotic complex associated with bark and ambrosia beetles may be involved in this variation in susceptibility to fungal treatments.

6 CONCLUSIONS

In the light of recent scientific discoveries concerning multipartite symbiosis, it has become evident that novel research avenues could be developed in the field of bark and wood-boring pest management. Multidisciplinary scientific teams involving experts from different areas such as bacteriology, mycology, taxonomy, ecology, biochemistry, genetics and molecular biology should combine their efforts to unravel the complexity of the symbiotic interactions that occur within bark and wood-boring beetles. The most promising research opportunity with respect to bark beetle forest pests concerns the involvement of their symbiotic complex in defensive mechanisms against parasites and pathogens.

Following the arguments put forward in the present review, two main strategies may be potentially used in control of bark beetle pest populations: (1) disruption of the symbiotic association between fungi, bacteria and insect host, and (2) genetic manipulation of bark beetle symbionts in order to impair their ability to secrete metabolites involved in the nutrition and defensive mechanism of the insect host. Biotechnological modern practices such as genetic manipulation of insect symbiotic associates, also known as transgenesis and paratransgenesis, are already applied in medical entomology in order to prevent the transmission of insect-vectored diseases to humans.[169] Manipulation of insect microbial associates by biotechnological practices as a tool for biological control was already predicted a few years ago.[170,171] Perhaps similar strategies should be extrapolated in suppression of bark beetle forest pests. Notwithstanding, the future research opportunities provided by symbiotic relationships in bark beetle pest management should equally consider the ethical problems of genetic manipulation of microorganisms of an insect's symbiotic complex.

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