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Fungi associated with the pine engraver beetle Ips acuminatus *and their interactions with the host tree*

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DATA CONSEGNA TESI

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July 31st , 2012 Caterina Villari

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Riassunto

Gli scolitidi delle conifere sono insetti spesso associati ad un complesso di funghi con i quali possono instaurare differenti interazioni ecologiche. Alcuni di questi funghi, generalmente non fitopatogeni, sono coinvolti in un'interazione mutualistica diretta e vengono utilizzati come nutrimento per le larve del vettore. Altri funghi associati, invece, sono ritenuti coinvolti nel processo di esaurimento delle difese della pianta, che per l'insetto è uno dei passaggi necessari per superare la resistenza dell'ospite e poterlo quindi colonizzare. In quest'ultimo caso, i funghi associati sono di norma specie patogene appartenenti al gruppo morfologico dei funghi ophiostomatoidi, noti anche come 'funghi di azzurramento'. Nonostante l'interesse che la simbiosi tra scolitidi e funghi ha riscosso nel tempo, molti degli aspetti fondamentali di questa interazione sono ancora discussi, come ad esempio il grado di dipendenza degli insetti vettori dai loro simbionti nelle fasi di colonizzazione della pianta ospite.

In questa tesi è stata presa in considerazione la comunità fungina associata a *Ips acuminatus* (Gyll.), un piccolo scolitide che attacca di preferenza le parti del tronco del pino silvestre (*Pinus sylvestris* L.) con corteccia sottile, e che di recente ha causato numerose infestazioni in varie zone distribuite sull'arco alpino. Una delle specie che fanno parte della comunità fungina associata ad *I. acuminatus* è il simbionte alimentare *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. Fa inoltre parte del complesso anche uno specifico fungo di azzurramento costantemente associato al vettore, ma la cui identità non è ancora ben definita. Le prime segnalazioni lo descrivono come *Ophiostoma clavatum* Math.-Käärik, mentre ricerche successive riportano *O. brunneo-ciliatum* Math. Gli obiettivi che questa tesi si è prefissa sono stati determinare l'effettiva identità del fungo di azzurramento associato ad *I. acuminatus*, ed indagare le interazioni del complesso fungino con la pianta ospite, al fine di definire meglio le relazioni che intercorrono tra scolitidi e fungi associati, e poter quindi contribuire ai tentativi di chiarire l'ecologia e la dinamica di popolazione di questo insetto dannoso.

Nel primo lavoro sono stati descritti l'isolamento e l'identificazione di una specie fungina isolata da individui di *I. acuminatus* raccolti in Italia e in Svezia. L'identificazione della specie è avvenuta sia grazie all'osservazione delle caratteristiche morfologiche sia

mediante un approccio di tipo molecolare. La specie è risultata essere *O. clavatum*, come era stato indicato nelle prime segnalazioni riguardanti i funghi associati ad *I. acuminatus*.

Nel secondo lavoro sono stati descritti la messa punto e l'utilizzo di tre sonde molecolari per loop-mediated isothermal amplification (LAMP), con l'obiettivo di determinare quale delle due specie del genere *Ophiostoma* sopracitate fosse effettivamente il fungo maggiormente associato a *I. acuminatus* nell'arco alpino. I risultati, riguardanti sei popolazioni italiane dell'insetto, hanno confermato che la specie maggiormente presente è *O. clavatum*, mentre *O. brunneo-ciliatum* non è mai stata rilevata. I risultati di questo studio hanno mostrato anche che la frequenza di associazione di *I. acuminatus* con *O. clavatum* varia a seconda della fase epidemica, ed è minore nei nuclei di infestazione rispetto alle popolazioni endemiche.

Nel terzo lavoro sono state caratterizzate le risposte sia locali sia sistemiche del pino silvestre alla colonizzazione da parte del simbionte alimentare e del fungo di azzurramento, identificando e quantificando alcuni metaboliti secondari, come terpeni, fenoli e lignina. I risultati hanno mostrato che il pino silvestre risponde in maniera generica anziché specifica all'induzione. Il fatto inoltre che il simbionte alimentare e il fungo di azzurramento abbiano stimolato una risposta simile di loro suggerisce che anche un fungo non patogeno possa partecipare al processo di esaurimento delle difese della pianta, assistendo quindi l'insetto nelle fasi di colonizzazione dell'ospite. Questo risultato contribuisce quindi allo sviluppo delle attuali teorie sul ruolo dei funghi associati nell'ecologia degli scolitidi.

Nell'ultimo lavoro sono state studiate le correlazioni tra i metaboliti secondari delle difese costitutive del pino silvestre, e le correlazioni tra la concentrazione costitutiva e la variazione indotta dei singoli composti. I risultati hanno messo in evidenza una differenziazione di comportamento tra composti e l'assenza in generale di una correlazione inversa tra i tipi diversi di difese, al contrario di quanto previsto da alcune teorie.

Nel complesso, i quattro contributi di questa tesi suggeriscono la rivalutazione di una delle attuali teorie sul ruolo dei funghi associati nella colonizzazione dell'ospite da parte degli scolitidi, e forniscono degli spunti per la comprensione del ruolo dei funghi associati nella dinamica di popolazione del vettore. Chiariscono inoltre alcuni degli aspetti dei meccanismi di difesa del pino silvestre, mettendo in evidenza la sua competitività.

Summary

Conifer bark beetles are typically associated with complexes of fungi with which they can display different functional relationships. Some of the fungi, generally non phytopathogenic, are known to have a directly mutualistic interaction with the beetles, serving as nourishment to the larvae. Other associated fungi are thought to be involved in the process of exhausting plant defenses, which is a necessary step for the insects to overcome host tree resistance and colonize the plant. In the latter case, bark beetle-associated fungi are often tree pathogenic species belonging to the morphologically homogenous group of the ophiostomatoid fungi, also referred to as 'blue-stain' fungi. In spite of the great interest the bark beetle-fungi symbiosis has gained in time, many fundamental aspects of this relationship are still widely debated, as for instance the degree of dependence of bark beetles on the blue-stain fungi in order to succeed their establishment in the host plant.

In this thesis I addressed the fungal community associated with the pine engraver beetle *Ips acuminatus* (Gyll.), a small bark beetle infesting thin bark of Scots pine (*Pinus sylvestris* L.) and that has been recently reported as pest in many alpine forests. *I. acuminatus* associated fungal community includes the obligate nutritional fungus *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. and a specific blue-stain fungus which is consistently associated with the vector, but which identity is still uncertain. Early reports describe it as *Ophiostoma clavatum* Math.-Käärik, while a later research reported *O. brunneo-ciliatum* Math. instead. Objectives of the thesis were to determine the identity of the blue-stain fungi associated with *I. acuminatus*, and to investigate the fungal community interactions with the host plant, in order to better define the functional relationships occurring between the bark beetle and the associated fungi, and therefore contribute to the attempts in understanding ecology and population dynamics of this damaging species.

In the first work a blue-stain fungus associated with *I. acuminatus* specimens collected in Italy and Sweden was isolated and identified. The identification of the species was achieved with both the support of morphological methods and DNA sequence-based methods, and the species resulted to be *O. clavatum*, in agreement with the first researches focused on *I. acuminatus* associated fungi.

In the second work, to clearly assess which of the previously described *Ophiostoma* species was the main blue-stain fungus associated with the pine engraver beetle, three loop-mediated isothermal amplification (LAMP) assays were developed and employed in a survey which has covered six Italian populations of *I. acuminatus*. The results confirmed that the identity of the blue-stain fungus more consistently associated with *I. acuminatus* in the Italian Alps is *O. clavatum*, while *O. brunneo-ciliatum* was not detected in any of the samples. Results of this study showed also that the occurrence of *O. clavatum* varies accordingly to the population dynamic phase of the vector, and is slightly lower in the outbreak populations.

In the third work, the local and systemic defense responses of Scots pine against both the nutritional and the blue-stain fungi were characterized by identifying and quantifying terpenoids, phenolic compounds, and lignin. Results indicated that Scots pine has a generic, rather than specific, induced response. The fact that the nutritional and the blue-stain fungi triggered comparable induced defense responses suggests that even a non-pathogenic fungus may participate in exhausting host plant defenses, indirectly assisting in the beetle establishment process. This finding contributes to the further development of current theories on the role of associated fungal complexes in bark beetle ecology.

In the last work, correlation patterns within constitutive defense secondary metabolites of Scots pine and potential trade-offs between constitutive concentration and inducible variation of individual chemical compounds were tested. Results revealed that different compounds display different behaviors, but no overall negative associations between defensive traits were found.

On the whole, the four contributions of this thesis provide suggestions for a revaluation of one of the current theories on the role of associated fungi in bark beetles host establishment, and hints to understand the role of associated fungi in the population dynamics of bark beetles. Moreover, they clarify some aspects of Scots pine defense mechanisms, highlighting its competitiveness.

Chapter I

Introduction

The symbiosis between bark beetles and fungi: a still largely debated matter

Bark beetles (Coleoptera: Curculionidae, Scolytinae), which are worldwide represented by a little more than 6,000 described species in at least 225 genera (Knížek and Beaver 2004), are among the more economically and ecologically important insects of the world's forests, especially concerning holartic conifer woodlands (Lieutier et al. 2004, Raffa et al. 2008). Their widespread association with fungi has always captured scientists' attention (Paine et al. 1997, Six 2003, Kirisits 2004, Linnakoski et al. 2012). However, despite its early descriptions date back to the 19th century (Schmidberger 1836, Hartig 1844), many fundamental aspects of this relationship, and in particular the bark beetle degree of dependence on the fungi, are still widely debated.

The majority of bark beetle associated fungi are Ascomycetes belonging to the genera *Ophiostoma*, *Grosmannia*, *Ceratocystis* and *Ceratocystiopsis*, with their related anamorphs *Leptographium*, *Pesotum*, *Hyalorhinocladiella*, *Sporothrix* and *Thielaviopsis*. With a few other genera, they all belong to the morphological homogeneous group of the ophiostomatoid fungi, which is a group that includes fungi sharing a close association with their insect vector and the adaptation to insect dispersal (Wingfield et al. 1993, Kirisits 2004, Linnakoski et al. 2012). Most of the ophiostomatoid fungi are also commonly known as 'blue-stain' fungi, on account of the blue, gray or even black discoloration the species gives to the colonized sapwood (Seifert 1993). Bark beetle associated fungi can however belong also to Hyphomycetes, as for instance *Ambrosiella* genus, to yeasts, as *Ogataea* genus, to Basidiomycetes, as *Entomocorticium* genus, and seldom to Zygomycetes (Kirisits 2004, Davis and Hofstetter 2011).

Symbiosis importance in bark beetle life

Bark beetles can be usually associated with more than one fungal species, which all form a complex that can display a great variation in time and space, depending on several factors (Solheim 1993a, Klepzig and Six 2004, Lieutier et al. 2009). The association of

ophiostomatoid fungi with particular bark beetle species can be either specific or casual, depending on the functional relationship and on the degree of dependence between the organisms (Kirisits 2004, Klepzig and Six 2004, Linnakoski et al. 2012). Functional relationships between bark beetles and associated fungi can indeed correspond to a various array of interactions, including antagonism, commensalism and mutualism, and the outcome of the relationships can be determined by the context, meant as the dynamic interaction of biotic and abiotic conditions (Klepzig and Six 2004). Some of the associated fungi are known to have a directly mutualistic interaction with the beetles, serving as nourishment to the larvae in the form of edible hyphae, spores and exudates (Harrington 2005). Most of bark beetle species, in fact, complete their life cycle in the wood or in the secondary phloem of conifer and deciduous trees (Figure 1). On the basis of their habitat and larval feeding habits, scolytids can be distinguished in three major groups, which show fundamental differences in their association with fungi (Francke-Grosmann 1967, Kirisits 2004). One group is represented by beetles that bore tunnels into the wood, and is termed 'ambrosia beetles' or 'xylomycetophagous bark beetles'. This group also includes the only European Platypodidae, *Platypus cylindrus* (F.) (Kirisits 2004). Since bark beetles are not able to digest lignin, cellulose and hemicelluloses, xylem is a poor substrate for nutrition and ambrosia beetles have overcome this problem through an ectosymbiosis with nutritionally obligate fungi (Francke-Grosmann 1967). These nutritional fungi, mostly Hyphomycetes and Basidiomycetes, and known as 'ambrosia fungi', are typical non-pathogenic species transported by the insects in the newly colonized trees, and larvae of ambrosia beetles feed on them (Paine et al. 1997, Harrington 2005). The second group of bark beetles lives and reproduces in the phloem instead, which is a richer source of nutrition for larvae. In contrast to ambrosia beetle, phloem-feeding bark beetles are most likely not dependent on their fungal associates for nutrition, and are referred to as 'true bark beetles' or 'phloeophagous bark beetles' (Francke-Grosmann 1967, Paine et al. 1997, Kirisits 2004, Harrington 2005). However, they are also commonly associated with various species of fungi, mostly blue-stain ophiostomatoids (Mathiesen-Käärik 1953, Francke-Grosmann 1967, Six 2003, Kirisits 2004), on which they can occasionally feed (Harrington 2005). Nitrogen and phosphorous content of plant tissues, including phloem, is indeed very

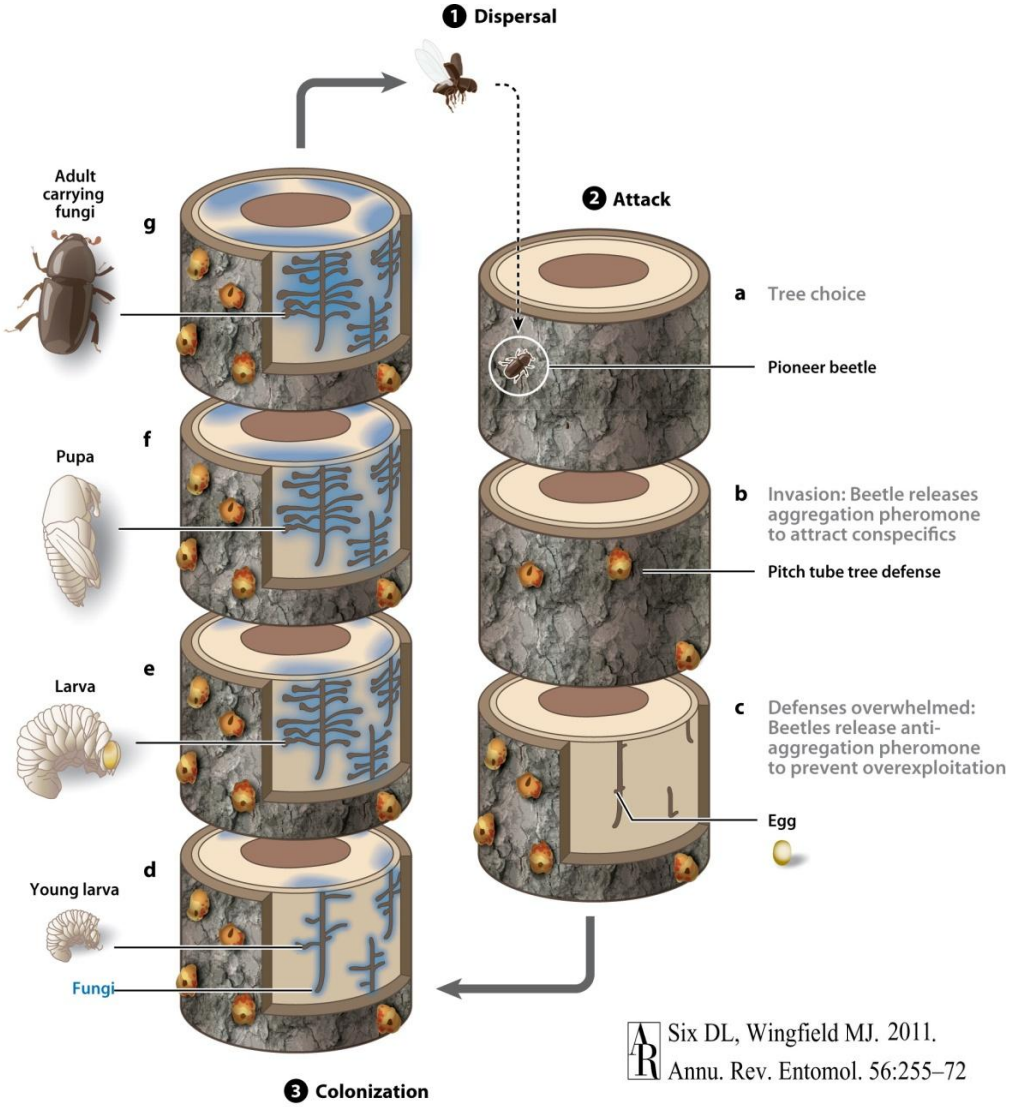

Figure 1. Generalized life cycle of a bark beetle and its associated fungi (from Six and Wingfield 2011). (1) Dispersal of adult beetles carrying fungi. (2) Attack phase: (a) Tree choice by pioneer beetles. (b) Insect entrance into the tree and subsequent release of aggregation pheromones to attract conspecifics. The pheromone-mediated mass attack usually occurs in a relatively short period (2–5 days). Fungi are inoculated in the phloem (c) Tree defenses are overwhelmed and bark beetle can start the colonization of the plant. (3) Colonization phase. (d) Egg-laying and initial larval tunneling. Larvae may feed on the inoculated fungi, which start growing in the bark beetle galleries, in the phloem and in the sapwood. (e) Extensive larval tunneling. (f) Excavation of pupal chambers and pupation. Fungi form spore layers in pupal chambers. (g) Newly emerged adults are contaminated with fungal spores (Figure reproduced with the permission of Annual Reviews, ESA, license Id: 2941251055919).

low, relative to insect metabolic requirements. But associated fungi, including blue-stain fungi, are able to concentrate scarce nutrients and provide the necessary contribution of vitamins, proteins and sterols, which are essential precursors for hormone synthesis and critical factors in the production of viable eggs (Klepzig and Six 2004, Harrington 2005, Bentz and Six 2006). According to Harrington (2005), however, fungal feeding on blue-stain fungi does not seems obligatory for completing the life cycle of phloeophagous bark beetles, but leads to shorter larval galleries and thus reduces intra- and interspecific competition. The third group, known as 'phloeomycetophagous', is represented by bark beetles having an intermediate behavior, feeding both on phloem and on nutritionally obligate fungi (Francke-Grosmann 1967, Kirisits 2004).

Apart from the nutritional aspect, associated blue-stain fungi, which can display different degree of pathogenicity, are considered to be useful for the vectors as a protection against detrimental fungi of the host tissues (Six 2003, Hofstetter et al. 2006), and because they are thought to be involved in the process of exhausting plant defenses, which is a necessary step for the insects to overcome host tree resistance and colonize the plant (Paine et al. 1997, Lieutier et al. 2009). However, the ecological significance of blue-stain fungi for bark beetle establishment on tree is still not fully understood, and will be discussed more in detail later.

Fungal and beetle adaptations

The most important benefits that fungi gain from the association with bark beetle are transport, spread and facilitation in entering the host tissues (Six 2003, Klepzig and Six 2004). Bark beetle associated fungi are indeed completely dependent on their vector for dissemination, and thus adapted to insect dispersal (Malloch and Blackwell 1993). Most species produce sexual fruiting bodies with neck that extrude spores in the insect galleries and pupal chamber, where they are most likely to be encountered by vectors; the spores are sticky and shaped to allow multiple contact points with the vector. Moreover, the adhesive coats of most of the spores disperse in resin but not in water, in order to ensure a release from the vector only in adequate substrates (Six 2003, Klepzig and Six 2004).

On the other hand, also insects have evolved morphological adaptations to ensure maintenance of symbiosis from generation to generation. For storage, transport and transmission of the associated fungi, bark beetles have different tegumental structures, defined mycangia (Batra 1963). The use of the term mycangium can vary from highly restrictive to very broad (Six 2003). In the restrictive sense, mycangia is defined as an invagination of the integument associated with glandular cells, which selectively protects and preserves associated fungi spores from adverse environmental factors, and excludes detrimental or neutral fungi (Batra 1963, Francke-Grosmann 1967). Mycangia can be classified on the basis of their location on the beetles and their structural characteristics (Six 2003). Due to their biologically significance for the bark beetles, nutritional fungi are consistently occurring in the mycangia sensu stricto, and are also referred to as mycangial fungi (Francke-Grosmann 1967, Paine et al. 1997, Six 2003, Harrington 2005). In the more broad sense, however, the term mycangium may refer to any structure that function in the transport of the fungi, regardless of whether is associated with glandular cells or not (Six 2003, Klepzig and Six 2004). This broader definition allows to consider as mycangia also shallow pits and setal brushes occurring on the insect body surface, since they biologically play a similar role to true mycangia (Six 2003). Moreover, fungal spores can also adhere to the median suture and lateral folds of the elytra of some bark beetle species (Harrington 2005).

Contrasting theories

If the evolution of mycangia strongly confirms that bark beetles benefit from the association with nutritional fungi, conversely, the definition of functional relationships between bark beetle and pathogenic blue-stain fungi, which are generally not transported in true mycangia, is still a complex matter. According to Paine et al. (1997), bark beetle species may benefit from the association with phytopathogenic fungi by their contribution to the death of the host, which occurs as a combined consequence of mycelia penetration of the tissues, blocking of the sap conduction, toxin release and beetles tunneling. While Christiansen et al. (1987) even suggested that the association with a pathogenic fungus may

be a prerequisite for scolytids to display an aggressive behavior. The hypothesis that fungi are important components in the ability of bark beetles to kill trees was primary derived from the high level of virulence of some fungal species associated with bark beetles (Berryman 1972, Christiansen et al. 1987, Raffa and Klepzig 1992). This hypothesis was then supported by the observations that beetles are rarely found in the absence of staining fungi, and that some pathogenic blue-stain fungi can kill artificially inoculated trees in the absence of the beetles. Despite the vast number of experiments, however, artificial inoculations with the majority of blue-stain fungi, which are not particularly aggressive, were not resulting in the tree death (Christiansen et al. 1987, Paine et al. 1997, Kirisits 2004, Lieutier et al. 2009, Six and Wingfield 2011). Moreover, some authors have noted that the speed of mortality of bark beetle–attacked pines is too fast to be accounted for by extensive fungal colonization of sapwood (Stephen et al. 1993). In Paine et al. (1997) hypothesis, these controversies were solved by the explanation that trees are killed as a result of simultaneous actions and interactions of both components, as already suggested by Berryman (1972), rather than successive actions of vectors and pathogens. Other controversies, however, were not addressed, as for instance the fact that weakly pathogenic fungi are often associated with aggressive beetles, and on the contrary, highly pathogenic fungi can be associated with beetles that rarely kill trees (Harrington 1993, Stephen et al. 1993); or the fact that aggressive fungi are not consistently associated with their vectors, as in the case of the blue-stain pathogen *C. polonica* (Siem.) C. Moreau and *Ips typographus* (L.) (Solheim 1993a, 1993b). In evaluating the role of fungal pathogenicity, Paine et al. (1997) began thus to point out the importance to distinguish between the early phase of overcoming host defense (initial phloem colonization) and subsequent mortality of the host (sapwood colonization), arguing that the critical question if fungi introduced by colonizing bark beetles are important in killing host trees must be addressed during the early phases of the interaction. According to the authors, fungi may also facilitate tree mortality through the interactions with beetles and trees in ways that are not signaled by sapwood staining or occlusion.

As a prosecution of these latest hinted ideas, and in the attempt to resolve controversies, Lieutier et al. (2009) proposed a more recent theory according to which associated blue-stain fungi play a role in beetle establishment on trees, but based on the stimulation and

subsequent exhaustion of tree defenses by the fungi, resulting in assistance to their insect vectors in overcoming host plant defenses directed at them. In contrast with the previous theories, they argue that several controversies may result from the misinterpretation of the role of fungal pathogenicity in bark beetle establishment process. The opinion of the authors is that since the death of the tree is not a necessary prerequisite for beetle development, fungal pathogenicity is not an important characteristic for the associated species. An important feature for bark beetle populations is instead the ability of their blue-stain associated fungi to stimulate the tree response and thus lower the critical threshold of attack density above which beetle attacks succeed. This hypothesis is based on the fact that pathogenicity is not related to a fungus ability to stimulate tree defenses (Krokene and Solheim 1997, Davis and Hofstetter 2011). Moreover, a highly pathogenic fungus would likely be detrimental, invading the tree too rapidly, outcompeting other mutualistic fungi, and making the tissues unsuitable for beetle brood development, as can occur in the case of *Dendroctonus frontalis* Zimm. and *O. minus* (Hedg.) H. et P. Syd. (Klepzig and Wilkens 1997, Hofstetter et al. 2006). Lieutier et al. (2009) maintain that beetle development in the phloem, fungal invasion in the phloem and external sapwood, and eventual tree death occur only after that tree defenses have been exhausted, and any fungus present in the beetle galleries, even though only saprophytic, may then potentially invade the sapwood. This is, according to the authors, one of the reasons that have lead to a general misunderstanding in identifying the fungal species actually important for bark beetle establishment. To solve controversies concerning the loose association between some tree killing bark beetles and certain blue-stain fungi, Lieutier et al. (2009) also proposed a model according to which bark beetle species using the strategy of overcoming tree resistance may be associated with a fungal complex that can indeed vary in its composition (Solheim 1993a, Klepzig and Six 2004), but in which every component species, even the more casual or loose one, can assume different roles. Roles of the complex components, as described by the authors, corresponds to: (i) stimulate tree reaction in order to overwhelm plant defenses; (ii) grow in the sapwood after tree resistance is overcome, eventually leading to tree death; (iii) control phloem extension of the first other two categories, maintaining the correct balance among functional relationships (i.e., mutualism vs. antagonisms); (iv) serve as nourishment to the larvae. Depending on the composition of the complex, these roles may be played by

different fungal species, or the same species may be involved in several roles (Lieutier et al. 2009). The concept of a 'polysymbiosis', however, had been already formulated by Six (2003), according to which some associated species can be beneficial for the bark beetles, while other can be neutral or even antagonistic. Likewise, fungal associated species may strongly compete with each other in the bark beetle habitat, influencing the outcome of the insect-fungal relationship (Klepzig et al. 2001, Kirisits 2004).

Theories according to which associated blue-stain fungi play a critical role in bark beetle establishment have been recently referred to as Classic Paradigm (CP), and a new model contradicting the CP have been proposed (Six and Wingfield 2011). This model suggests that, rather than playing a supporting role for the host beetle, by both stimulating induced defenses or leading to tree death, phytopathogenicity performs an important role for the fungi, mediating competitive interactions in the fungal community and supporting survival in living and defended trees (Six and Wingfield 2011). According to the authors, the CP is fundamentally flawed, and the arguments that this assumption brings include the lack of consistent association of aggressive fungal associates with tree-killing bark beetles, the lack of correspondence between fungal growth in the host tree and the development of symptoms associated with a successful attack, and the ubiquity of similar associations of fungi with bark beetles that do not kill trees (Six and Wingfield 2011). If this theory clearly contradicts the CP as formulated by Paine et al. (1997), it is not necessary in contrast with Lieutier et al. (2009) model, despite authors assertions (Six and Wingfield 2011). Both theories (Lieutier et al. 2009, Six and Wingfield 2011), indeed, support the idea that fungal pathogenicity is not critical for bark beetle establishment, and the two first arguments brought as flows of the CP theories have been previously addressed in Lieutier et al. (2009) model. Concerning the lack of consistency of association between bark beetles and a single fungus highly efficient in stimulating defenses, which Lieutier et al. (2009) proposed to be compensated by the complex of associated fungi, Six and Wingfield (2011) sustain however that the reliance of bark beetles for a critical function on such an incidental and variable group of fungi seems unlikely.

None of the previous described theories have been completely rejected or accepted yet, and in spite of the overall controversies about functional relationships between bark beetle and blue-stain fungi, several authors agree on the fact that, although much is already known

about the phytopathogenicity of bark beetle associated blue-stain fungi, it would be important to better test the ability of these fungi to stimulate the defense reactions of their host trees (Kirisits 2004, Lieutier 2004). Moreover, there is still a need for further studies with a broader approach, concerning for instance symbioses between fungi and non-tree-killing bark beetle species (Kirisits 2004, Six and Wingfield 2011); or concerning the interactions among the other partners of the community associated with bark beetles, as phoretic mites and nematodes (Lombardero et al. 2003, Klepzig and Six 2004, Hofstetter et al. 2006, Meirmans et al. 2006). Several phoretic mites, for instance, feed as well on beetles associated fungi, and posses specialized structure of the integument that can transport fungal spores (sporothecae) (Bridges and Moser 1983, Moser 1985). Their interaction with the system can therefore arouse strong indirect effects in the bark beetle and associated fungi complex, since pairwise interactions among species may generate reciprocal dynamics that yield endogenous demographic feedbacks, as has been shown in the case of *D. frontalis*, its associated fungi and the phoretic mites of *Tarsonemus* genus (Lombardero et al. 2003, Hofstetter et al. 2006). Finally, still very few data are available about tree responses to non-pathogenic and nutritional fungi (Raffa and Berryman 1982, Hofstetter et al. 2005, Davis and Hofstetter 2011), which, in light of Lieutier et al. (2009) model, could also participate in stimulating tree defenses, and thus assist beetles in their establishment.

The *Ips acuminatus* – associated fungi complex

The pine engraver beetle *I. acuminatus* (Gyll.) is a small bark beetle infesting thin bark of trunk and branches of mature Scots pine (*Pinus sylvestris* L.) (Bakke 1968, Colombari et al. 2012a, 2012b). Spread throughout Europe, it is a polygamous species in which each male can be joined with an average of 2-7 females (Colombari et al. 2012a), and a peculiar trait of the species is the coexistence of both sexual and pseudogamous females (Løyning 2000, Meirmans et al. 2006). Nevertheless, males are known to be highly selective, and prefer sexual over clonal females, even though different clonal strains may differ dramatically in their courtship performance (Løyning and Kirkendall 1996). Male excavates the nuptial chamber under the bark, while egg galleries, which radiate outward from the nuptial chamber, are excavated by females after mating. Eggs are laid on both sides of the galleries and the gallery systems has a distinctive star-shaped pattern (Kirkendall 1989) (Figure 2). Larvae, which have a phloeomycetophagous habit (Francke-Grosmann 1967), create very short galleries, feeding initially on the phloem and completing their later stage development on conidia and mycelium of associated nutritional fungi. Excavated galleries are perpendicular to the maternal ones and characterized by being packed with frass. At the end of their development, larvae move from the phloem to the outer sapwood, where they pupate inside pupal chambers. Before emerging, callow adults need a period of maturation feeding, which takes place in the phloem close to the galleries where the larvae have developed (Faccoli et al. 2010, Colombari et al. 2012b). The life-history traits of *I. acuminatus* vary according to the study sites, since low temperature is a limiting factor (Bakke 1968), and literature reports the species as monovoltine in northern Europe and bivoltine in southern Europe (Colombari et al. 2012a, 2012b). Overwintering occurs in the adult stage, both under the bark of infested trees and probably in the litter (Francke-Grosmann 1963, Colombari et al. 2012a).

Despite is has been considered for many years as a species of minor economic importance (Bakke 1986), in recent decades *I. acuminatus* has been included among the most damaging European wood-boring insects (Grégoire and Evans 2004), and its impact

Figure 2. *Ips acuminatus* subcortical gallery system on *Pinus sylvestris*, Lunsen, Sweden. Maternal galleries radiate outward from the nuptial chamber. Egg niches have been just excavated and eggs have still to hatch. Tissues surrounding the galleries are brownish due to the plant reaction to the insect tunneling and associated fungi colonization. The lower gallery belongs to a separate system (photo: C. Villari).

has gained an important role in the Scots pine decline observed recently in the Alps (Wermelinger et al. 2008). Severe outbreaks of the pine engraver beetle have been reported in several European countries (Romanyk 1977, Legrand 1996, Dobbertin 2005). In Italy, besides several endemic populations, local outbreaks have been reported in central and eastern Alps (Lozzia and Rigamonti 2002, Colombari et al. 2012a, 2012b, Faccoli et al. 2012). As for numerous bark beetle species that have a cooperative attack strategy, *I. acuminatus* infestations are characterized by a very pronounced spatial patterning consisting in groups of killed trees commonly known as 'spots' (Colombari et al., 2012b).

The pine engraver beetle is associated with a complex of symbiotic fungi which includes the obligate nutritional fungus *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. (syn. *Ambrosiella macrospora* Batra) (Batra 1967, Harrington et al. 2010), that serves as nourishment to the larvae (Francke-Grosmann 1963, Batra 1967, Paine et al. 1997). *H. macrospora*, associated uniquely with *I. acuminatus*, is mainly transported in specific mycangia consisting in small paired membranous pouches occurring at the base of the female mandibles (Francke-Grosmann 1952, 1963). The fungus was initially described as *Trichosporium tingens* var. *macrospora*, due to its large conidia (Francke-Grosmann 1952), and is phylogenetically related to *Ophiostoma* genus (Rollins et al. 2001, Harrington et al. 2010). Conidia are formed blastically, and conidiophores aggregate within larval galleries or pupal chambers in dense structures, sporodochia or hymenia, which are an adaptation for grazing by beetles larvae and adults (Batra 1967, Harrington et al. 2010). As with most nutritional fungi, *H. macrospora* is thought to be non-pathogenic (Paine et al. 1997). Tests on its ability to cause disease are however still missing and a better understanding of the species' pathogenicity would be useful.

I. acuminatus is associated also with a complex of blue-stain fungi which are transported in pits and setae occurring on the body surface (Lieutier et al. 1991, 2009, Kirisits 2004) and which, according to Lieutier et al. (2009), benefits the vector by participating in exhausting plant defenses. Furthermore, *I. acuminatus* may occasionally feed on them as well (Francke-Grosmann 1952). Besides some occasional or ubiquist species, as for instance *O. ips* (Rumb.) Nann. (Lieutier et al. 1991, Kirisits 2004), the literature reports a specific fungus which is more consistently associated with the vector (Mathiesen-Käärik 1953, Francke-Grosmann 1963, Harrington 2005). The identity of this

blue-stain fungus is however still uncertain. Initial records described *Ophiostoma clavatum* Math.-Käärik as the main associated species (Mathiesen 1950, 1951, Rennerfelt 1950, Mathiesen-Käärik 1953, Francke-Grosmann 1963), while subsequently - and without confuting the previous records - some authors reported *O. brunneo-ciliatum* Math. (Lieutier et al. 1991, Guérard et al. 2000). A possible explanation for this uncertainty in the published data could be related to the occurrence of a spatial variation in the associated complex composition among different regions, as occurs in other bark beetle species (Klepzig and Six, 2004). Taking into account the fact *I. acuminatus* was found to be associated with the same species (*O. clavatum*) in Sweden, Germany and former Yugoslavia (Francke-Grosmann 1963), however, the possibility of a spatial variation of this complex seems unlikely.

Another possible explanation for the literature uncertainty could be instead related to the high morphological similarity of *O. clavatum* and *O. brunneo-ciliatum* (Mathiesen 1951, Mathiesen-Käärik 1953), which may have led to erroneous species identification. Both of the species are in fact characterized by discrete synnematous conidiophores, *Pesotum*-like, which resemble a flame or a club (hence the specific name 'clavatum', which means club-shaped), although *O. brunneo-ciliatum* conidiophores are twice as big as those of *O. clavatum*, and less regular (Mathiesen-Käärik 1953). The length of the perithecium neck, the shape and number of ostiolar hyphae, and the shape of ascospores and their gelatinous sheath would be also distinctive traits between the two species, but ascocarps are rarely produced, especially concerning *O. clavatum*, hence the distinction of the species may be difficult (Mathiesen 1951, Mathiesen-Käärik 1953). Moreover, the scarce availability of deposited specimens for *O. clavatum* may have also precluded the possibility of species identification using molecular tools (Upadhyay 1981, Zipfel et al. 2006).

Whatever its actual identity, however, the blue-stain fungus more consistently associated with *I. acuminatus* has been shown to be a non aggressive pathogen, since in a field experiment high inoculation densities (above 1000 m^{-2}) were required in order to reach a critical threshold after which trees were killed (Guérard et al. 2000).

Objectives and content of the thesis

Objectives of this thesis are to investigate the fungal community associated with *I. acuminatus* and its interactions with the host plant *P. sylvestris*, in order to better define the functional relationships occurring between the bark beetle and the fungal complex, and therefore contribute to the attempts in understanding ecology and population dynamics of this damaging species. This work complements other investigations that have been conducted within my research group and which were aimed to understand other aspects of *I. acuminatus* behavior, such as life-history traits, dynamic of infestations and the impact of natural enemies on the insect population (Colombari 2011).

This thesis has two main topics. The first mostly concerns the definition of *I. acuminatus* associated fungal complex. From Swedish and Italian populations of the vector I isolated and identified several strains of *O. clavatum*, with the support of both morphological and molecular tools. Results will be included in a taxonomic review of some species of the *Ophiostoma* genus, which will be done in partnership with the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, South Africa (chapter II). Subsequently, to survey which between the two species *O. clavatum* and *O. brunneo-ciliatum* is actually the main blue-stain fungus associated with *I. acuminatus* in the southern Alps, and to verify whether the population dynamic phase of the vector may influence the occurrence of the associated blue-stain fungi, I developed two real-time loop-mediated isothermal amplification assays and I employed them in some Italian populations of the pine engraver beetle. Assays development details and survey results are reported in a manuscript submitted to a scientific journal (chapter III).

The second main topic concerns instead the host plant reaction to the fungi of the complex. In chapter IV, I characterized the defense responses of Scots pine to inoculation with both the nutritional and the blue-stain fungi, by identifying and quantifying terpenoids, phenolic compounds, and lignin. Results, published in Tree Physiology, are discussed according to the current theories about functional relationships occurring between bark beetles and associated fungi. Data from this work have been also used for a broader analysis of Scots pine defensive traits, in which I analyzed the behavior of 35 carbon-based secondary compounds in order to determine correlations patterns within constitutive

defensive traits and to test potential trade-offs between constitutive concentration and inducible variation of individual compounds. Results of this study are incorporated in a manuscript which is still in preparation (chapter V).

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Chapter II

Identification of the blue-stain fungi associated with *Ips acuminatus*

This chapter describes my contribution to a broader project which is nevertheless still not concluded. Consequently the manuscript has not reached the final phase of preparation, but it rather summarizes the work I have done. I have decided however to enclose it in the thesis because I considered it important to understand the following chapters.

I collected most of the data I report, analyzed them and drafted the manuscript.

Abstract

The pine engraver beetle *Ips acuminatus* is a small bark beetle that infests thin bark of Scots pine (*Pinus sylvestris*). Although it has been recently included among the most damaging European wood-boring insects, relatively few studies has focused on its associated mycoflora and literature reports contrasting data concerning its main associated blue-stain fungus. Early studies reports *Ophiostoma clavatum* as the main associated species, while later studies reports *O. brunneo-ciliatum* instead. In this study we isolated and identified five isolates of a blue-stain fungus associated with *I. acuminatus* specimens collected in three Italian and one Swedish sampling sites. The identification of the species was achieved with the support of both morphological methods, comparing the isolates with collection strains, and DNA sequence-based methods, employing in particular the internal transcribed spacer and the β-tubulin genetic data. The species resulted to be *O. clavatum*, in agreement with the first researches focused on the pine engraver beetle associated fungi.

Introduction

Most studies concerning bark beetles associated blue-stain fungi have been focused on economically important species, and this has likely led to a biased view of the fungal biodiversity (Linnakosky et al. 2012). Recently, for instance, broader researches on ophiostomatoid species in northern Europe have shown a high diversity in the association between fungi and bark beetles, more than previously thought, and several new species have been described (e.g., Linnakosky et al. 2010).

In this study we considered the pine engraver beetle *Ips acuminatus* (Gyll.), a small bark beetle spread throughout Europe and infesting thin bark of mature Scots pine (*Pinus sylvestris* L.) (Bakke 1968, Colombari et al. 2012a, 2012b). Although it has been recently included among the most damaging European wood-boring insects (Grégoire and Evans 2004), in the past years was considered a species of minor economic importance (Bakke 1986), hence relatively few studies has focused on its associated mycoflora. *I. acuminatus* larvae have a phloeomycetophagous habit, feeding initially on the phloem and completing

their later stage development on conidia and mycelium of the nutritional obligate fungus *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. (syn. *Ambrosiella macrospora* Batra), which is associated uniquely with *I. acuminatus* (Francke-Grosmann 1963, 1967, Batra 1967, Harrington et al. 2010).

As most bark beetles breeding in conifers, *I. acuminatus* is associated also with a complex of blue-stain fungi transported in pits and setae occurring on the body surface (Kirisits 2004), and which ecological role is still debated (Lieutier et al. 2009, Six and Wingfield 2011). Literature reports numerous species, some of which are occasional or ubiquist species, as for instance *Ophiostoma ips* (Rumb.) Nann. (Lieutier et al. 1991, Kirisits 2004, Linnakoski et al. 2012). Several authors, however, reported a specific fungus consistently associated with *I. acuminatus*. First researches focused on the pine engraver beetle associated fungi, conducted mainly in northern Europe, reported *O. clavatum* Math.-Käärik as the main associated species (Mathiesen 1950, 1951, Rennerfelt 1950, Mathiesen-Käärik 1953, Francke-Grosmann 1963). After its first finding in Sweden (Mathiesen 1950, 1951) and its last report in Germany and former Yugoslavia in 1963 (Francke-Grosmann 1963), *O. clavatum* was however never isolated again, so that some authors even considered it as a doubtful species (Upadhyay 1981). A later research on *I. acuminatus* mycoflora, conducted in France, reported instead *O. brunneo-ciliatum* Math. as the main associated blue-stain species (Lieutier et al. 1991), and a certain number of following works referred to this report when describing the fungal complex associated with the pine engraver beetle (e.g., Guérard et al. 2000, Kirisits 2004). *O. brunneo-ciliatum*, which is morphologically very similar to *O. clavatum* (Mathiesen 1951, Mathiesen-Käärik 1953), is associated also with other vectors, as for instance *I. sexdentatus* (Boern.) (Mathiesen-Käärik 1953). Recent studies has isolated *O. brunneo-ciliatum* in several sites, even though never in association with *I. acuminatus* (e.g., Linnakosky et al. 2010).

This uncertainty in the literature is still not solved, but the recent introduction of DNA sequence-based methods to support cultural methodologies of bark beetle associated species identification may prove to be helpful (e.g., Zipfel et al. 2006, Giordano et al. 2012). The aim of this study was hence to isolate and identify, on the basis of both morphological characteristics and DNA sequence-based methods, the blue-stain fungi associated with *I. acuminatus* specimens collected in Italy and Sweden, which are among

the southern and northern regions of the vector distribution range. Sweden is also the region where *O. clavatum* was isolated the first time (Mathiesen 1950, 1951).

This study is part of a currently undergoing broader project which will involve a taxonomic revision of some species of the genus *Ophiostoma* Syd. & P. Syd., including *O. clavatum* and *O. brunneo-ciliatum*. The project, coordinated by the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria (South Africa), involves also the collaboration of the University of Turku (Finland).

Materials and methods

Fungal isolation and morphological identification

I. acuminatus adults were collected from infested logs of *P. sylvestris* sampled in Val Dogna (UD), Val Venosta (BZ) and Val Camonica (BS), northern Italy, and from Lunsen, Uppsala, in Sweden, and stored singly at 4 °C until analyses. Five individuals per site were analyzed. Samples were vortexed at 40 Hz for 60 s in 200 µl of 1% Tween® 80 (Sigma-Aldrich, St. Louis, MO, USA) (modified from Battisti et al. 1999). The washing solution was then plated on 2 % malt extract agar (MEA, Difco, BD, Franklin Lakes, NJ, USA) containing 200 ppm of cycloheximide and 300 ppm of streptomycin (Sigma-Aldrich), in order to be selective for *Ophiostoma* species (modified from Zhou et al. 2007). Obtained cultures were incubated at 25 °C and purified by transferring mycelium from the edges of single colonies to fresh 2% MEA. Cultures were grouped according to colonies and conidiophores characteristics, and single spore cultures were prepared from germinating conidia of isolates representing morphological groups of different sites (Grobbelaar et al. 2010). After verifying that the isolates had similar morphological characteristics, in an attempt to stimulate ascocarp production and to determine the thallism of the supposedly isolated species, all obtained isolates were mated with each other, following the protocol described in Grobbelaar et al. (2010). Briefly, single spore cultures were transferred to 2% MEA and incubated for 14 days in the dark at 25 °C. Agar blocks plugs (7 mm diameter) covered in mycelium of two different isolates were placed adjacent to each other on plates

containing WA (20 g Difco agar in 1,000 ml distilled water) supplemented with debarked, autoclaved *P. sylvestris* twigs. As a control, all isolates was also paired against themselves. Plates were incubated at 22 °C in the dark for 8 weeks and inspected weekly under a dissecting microscope for the presence of ascomata.

Morphological identification was based on macro- and microscopic characteristics of the isolates (Mathiesen 1951, Mathiesen-Käärik 1953). Specimens were observed both under a stereomicroscope and a light microscope, after anamorph fruiting structures were mounted on glass slides in methylene blue. Anamorph fruiting structures were observed also with a scanning electron microscope (SEM TM-1000, Hitachi, Krefeld, Germany) equipped with a cool stage unit MK3 (Deben, Suffolk, UK), to maintain the temperature below the zero and avoid water evaporation. Measurements of an adequate number of morphological structures in order to compute averages, ranges and standard deviation of the supposedly isolated species characteristics are currently in progress. European strains of *O. brunneo-ciliatum* (type strain CBS 149.54, CBS 117571 and CBS 117591) and *O. clavatum* (type strain CBS 135.51), as well as a herbarium specimen of *O. brunneo-ciliatum* (BPI 595721), were obtained for comparison. A further isolate stated as *O. brunneo-ciliatum* was obtained as a kindly concession of F. Lieutier and A. Yart, Institut National de la Recherche Agronomique (INRA), Orleans (France) (Table 1). No herbarium specimens of *O. clavatum* were available. Preliminary analyses proved that both *O. brunneo-ciliatum* type strain CBS 149.54 and *O. clavatum* type strain CBS 135.51 were contaminated, and consequently not useful for the analyses.

Fungal growth in culture

Two *O. brunneo-ciliatum* strains (CBS 117571, CBS 117591), two Swedish isolates (from Lunsen) and two Italian isolates (from Val Dogna and Val Camonica) of the supposedly isolated species were selected for a preliminary comparative growth study, as described in Grobbelaar et al. (2010). Mycelium-covered agar plugs (7 mm diameter) were transferred from the leading edge of one-week-old actively growing colonies to 90 mm diameter Petri dishes containing 2% MEA. Three plates per isolate were incubated for 6 days in the dark at each temperature, ranging from 5 °C to 35 °C, at 5 °C intervals. In order to measure

colony diameter, two measurements at right angles to each other were made at the end of the experiment. The mean diameter in mm (±SD) was calculated for each group of isolates. To test the effects of group of isolates and temperature on fungal growth we used one-way ANOVA. We initially tested interaction and main effects, simplifying the model if there was no significant interaction. If significant main effects were observed, a Tukey's HSD test was used to compare the different groups of isolates and temperatures. Data were analyzed in R (R Development Core Team 2011).

DNA extraction, PCR amplification, sequencing and phylogenetic analyses

DNA was extracted from the single spore cultures of the isolates representing morphological groups of different sites, as well as from the CBS strains and the French isolate, following a salting out protocol (Patwary et al. 1994). Approximate DNA concentrations were determined at 260 nm using the Nano-drop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA), and extracts were diluted to 10 ng μl^{-1} in double-distilled water (Sigma-Aldrich, St. Louis, MO, USA). Internal transcribed spacer (ITS) regions 1 and 2, including the ribosomal 5.8S gene, were amplified using the primers pairs ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). The reaction mixture contained, in a total volume of 15 µl, 1x PCR Go Taq Flexi buffer (Promega, Milano, Italy), 2 mM $MgCl_2$ (Promega), 0.13 mM dNTPS (Invitrogen, Monza, Italy), 0.2 µM each primer, 4% DMSO (EuroClone, Milano, Italy) 0.5 U of Taq polymerase (Promega) and 2µl of template DNA. The cycling program, modified from White et al. (1990) was carried out in an Eppendorf Mastercycler Gradient and consisted of a first step at 95 °C for 5 min followed by 33 cycles with a denaturation step of 95 °C for 30 s, an annealing step at 55 °C for 30 s and an extension step of 72 °C from 1 min, followed by a final extension at 72 °C for 10 min. The protein coding gene β-tubulin (partial gene) was also amplified using the primers Bt2a and Bt2b (Glass and Donaldson 1995). Amplification was performed in 15 µl of reaction mix (1x PCR Go Taq Flexi buffer, 2 mM $MgCl_2$, 0.09 mM dNTPS, 0.66 µM each primer, 4% DMSO, 0.5 U of Taq polymerase and 1µl of template DNA). Thermal cycling condition were 2 min at 94 °C followed by 15 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s, and further 20 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for

45 s, with a final extension of 72 °C for 5 min. Primers were synthesized by Invitrogen. PCR products of both reactions were checked by 1.0% agarose gel stained with SYBR® Safe (Invitrogen) and purified with the ExoSAP-IT kit (Amersham Biosciences, Glattbrugg, Switzerland) before sequencing.

Sequencing of both strands per amplified product was performed at the BMR Genomic service (Padova, Italy) on automated DNA sequencers, employing the same primers used for PCR amplifications, as well as an additional internal primer for the ITS region (ITSseq2-f 5'-CGAGCCGCCCGAACT-3') and another additional internal primer for the β-tubulin partial region (BTseq1-f 5'-GCGCTCACCGCAAAGAC-3').

A NCBI BLAST (National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov) search was run with the edited sequences for preliminary species identification (Zhang et al. 2000). Sequences were then aligned and visually inspected for the identification of polymorphic loci. A preliminary neighbor-joining tree was obtained with a Kimura 2-parameters substitution model (Kimura 1980), using only the β–tubulin partial gene sequences since they are known to better distinguish among *Ophiostoma* species than ITS region (Lim et al. 2004, Grobbelaar et al. 2010). We added a further sequence of *O. brunneo-ciliatum* (accession no. HM031557) and a sequence of *O. ainoae* H. Solheim as outgroup (accession no. HM031552). No sequence of *O. clavatum* was added since there are no deposited sequences for this species. Estimates of average evolutionary divergences over sequence pairs within and between groups defined by the tree were assessed for both ITS region and β-tubulin partial gene. All analyses were performed with MEGA 5.05 software (Thompson et al. 1994, Tamura et al. 2011).

Broader phylogenetic analyses, including further protein-coding genetic data such as translation elongation factor 1-α (EF1-α) and calmodulin, are currently in progress at FABI. Analyses will involve the comparison of obtained sequences with published sequences of related species from GenBank (NCBI), as well as with new sequences of *O. brunneo-ciliatum* European isolates obtained by R. Linnakosky, University of Turku.

Results

Fungal isolation and morphological identification

The frequency of *I. acuminatus* collected individuals from which blue-stain fungi colonies were isolated was 3/5, 3/5, 2/5 and 3/5 for Val Dogna, Val Venosta, Val Camonica (Italy) and Lunsen (Sweden), respectively. After colonies were grouped according to morphology, five single spore cultures were obtained from the representative isolates: one from each sampling sites in Italy and two from the Swedish sampling site. Morphological identification was based only on the observation of colonies and conidiophores characteristics, since isolates did never produced ascocarps. In fact, none of the mating tests made in the attempt to stimulate ascocarp production was successful. Cultures characteristics resulted similar among isolates: conidiophores were synnematous, *Pesotum*-like and resembled a club. As they were comparable to the French isolate, they were initially identified as *O. brunneo-ciliatum*. However, following molecular analyses (see below) proved that the French isolate was not *O. brunneo-ciliatum*. Preliminary results of the morphological measurements (still in progress) revealed that the French and the obtained isolates conidiophores were actually shorter than true *O. brunneo-ciliatum* ones (Mathiesen-Käärik 1953), and compatible with *O. clavatum* description (Mathiesen 1951) (Figure 1). Obtained isolates have been therefore classified as *O. clavatum*, and together with the French isolate, have been deposited in the FABI culture collection (CMW) (Table 1).

Fungal growth in culture

Results of the preliminary growth study showed a trend where, at all temperatures, Italian and Swedish isolates grew less in comparison to the *O. brunneo-ciliatum* strains. Group of isolates ($F_{2,\ 80} = 7.61$, $P < 0.001$), but not temperature ($F_{1,\ 80} = 3.74$, $P = 0.057$), had a significant effect on the fungal growth, and there was no interaction between the two factors ($F_{2,78} = 0.7$, $P = 0.497$). None of the isolates grew at 35 °C (Figure 2).

DNA sequence data

Amplicons of approximately 610 and 420 bp in length were produced for the ITS and β-tubulin gene regions, respectively. Sequences have been deposited in the GenBank database and will be released to public after the end of the current work.

GenBank similarity BLAST searches with the obtained isolates and the French isolate sequences showed contrasting results. In all the cases, the closest matches for the ITS region had a similarity of 99% with *O. brunneo-ciliatum*, while the ones for the β-tubulin partial gene had a similarity of only 95% with *O. brunneo-ciliatum*. On the contrary, both ITS region and β-tubulin partial gene sequences of the *O. brunneo-ciliatum* CBS strains matched to other *O. brunneo-ciliatum* strain sequences with a similarity that ranged from 99 to 100%.

In the preliminary neighbor-joining tree with the β–tubulin partial gene sequences (Figure 3) all obtained isolates sequences clustered together with the one of the French isolate stated as *O. brunneo-ciliatum*, supported by a bootstrap value of 98%. The sequences of the other *O. brunneo-ciliatum* isolates clustered instead in a separate group, with a bootstrap value of 95%. Estimates of average evolutionary divergence over sequence pairs within groups for ITS region were 0.001 ± 0.001 (SD) and 0.004 ± 0.002 for the obtained isolates plus French isolate group and *O. brunneo-ciliatum* group respectively. The same estimates over sequence pairs for the β–tubulin partial gene were instead 0.002 ± 0.001 and 0.019 ± 0.006 respectively. Finally, estimates of evolutionary divergence over sequence pairs between groups were 0.006 ± 0.003 and 0.049 ± 0.011 for the ITS region and the β–tubulin partial gene respectively.

Discussion

In this study we isolated and identified a blue-stain fungus associated with *I. acuminatus* specimens collected in Italy and Sweden. A total of five isolates were obtained, three from different sites in Italy, and two from the same site in Sweden. Based on morphological, growth and sequence data, isolates resulted to be the same species, which we identified as

O. clavatum, according to the first researches focused on the pine engraver beetle associated fungi (Mathiesen 1950, 1951, Rennerfelt 1950, Mathiesen-Käärik 1953, Francke-Grosmann 1963).

Identification on morphological characteristics has been problematic, since isolates did never produced ascocarps, not even after mating crosses. This may be due to the fact that the species is heterotallic and all isolates were of the same mating type, or just to the fact that *O. clavatum* ascocarps are rarely produced in growing media (Mathiesen 1951). Another problem was that the type strain of *O. clavatum* (CBS 135.51) used for comparison resulted to be contaminated. Since no other isolate of *O. clavatum* is currently available, one of the Swedish isolates should be proposed as a neotype of the species; data collection for this purpose is currently in progress. Lastly, morphological identification has also been hindered by the fact that one of the isolates used as a reference for *O. brunneo-ciliatum*, i.e., the French isolate, has been proved to be *O. clavatum* instead, and this has caused an initial misidentification of the obtained isolates (see Villari et al. 2012, i.e, chapter IV).

With the support of DNA sequence-based methods, Italian and Swedish isolates were grouped together with the French one, and distinguished from true *O. brunneo-ciliatum* isolates, even though the data reported are only preliminary results. The phylogenetic analyses currently in progress will likely strengthen these results and give them the adequate statistical power. As expected, β–tubulin partial gene sequences better distinguished the species in the *Ophiostoma* genus (Lim et al. 2004, Grobbelaar et al. 2010), while the use of the ITS region alone would have brought to an erroneous identification, as shown by the GenBank similarity BLAST searches results. Since there were no deposited sequences for *O. clavatum*, DNA sequence-based methods have not however resolved the identity of the isolates, which was hence based only on the morphological characteristics of colonies and conidiophores (Mathiesen 1951).

Results of the growth study showed that Italian and Swedish isolates of *O. clavatum* were similar to each other, and differentiated from *O. brunneo-ciliatum* CBS strains, which displayed an overall faster grow, also at relatively high temperature. It has to be noted, however, that the growing test was performed with only two replicates per group of isolates, and results are hence to be considered only as preliminary. The low number of replicates is also probably the reason why temperature effect on the fungal growth was not

significant. *O. brunneo-ciliatum* is mainly associated with *I. sexdentatus* (Mathiesen-Käärik 1953), which has a broader geographic distribution in the south of Europe and a wider range of host plants. The outbreaks of *I. sexdentatus* are known only for the southern edge of its distribution range, while *I. acuminatus* outbreaks are restricted to colder areas (Postner 1974). It is therefore likely that different growth rates and ranges of temperature tolerance of the associated fungi may have a part in the differentiation of outbreak development areas between *I. sexdentatus* and *I. acuminatus*, suggesting thus a significant function of these fungi in their vector establishment on the host plant (Lieutier et al. 2009).

In this experiment no other ophiostomatoid species of the ones described as associated with *I. acuminatus*, as for instance *O. ips* (Lieutier et al. 1991, Kirisits 2004), were isolated. But the number of analyzed *I. acuminatus* specimens and sites is too low to generalize this result. For the same reason, we cannot speculate whether or not the true *O. brunneo-ciliatum* may actually be associated with *I. acuminatus*, since spatial variations in the bark beetle associated complex composition among different regions may occur (Klepzig and Six, 2004).

Our results proved again, after almost 50 years since its last report (Francke-Grosmann 1963), the association of *O. clavatum* with *I. acuminatus.* We also found that the French isolate of *O. brunneo-ciliatum* was instead *O. clavatum*. This findings increase thus the likelihood that the report of *O. brunneo-ciliatum* as the main blue-stain fungus associated with *I. acuminatus* (Lieutier et al. 1991) is rather due to an erroneous species identification, instead that to a variation in the insect associated complex composition. However, to clearly assess which of the two species is the main blue-stain fungus associated with the pine engraver beetle, or if *O. brunneo-ciliatum* is actually associated with this vector, a broader survey is needed (see chapter III).

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Table 1. Fungal isolates analyzed in this study.

Species	Host	Vector	Geographic origin	CMW[a] no.	CBS[b] no.
Ophiostoma clavatum	*Pinus sylvestris*	*Ips acuminatus*	Lunsen, Uppsala, SE	37983	
	P. sylvestris	*I. acuminatus*	Lunsen, Uppsala, SE	37984	
	P. sylvestris	*I. acuminatus*	Val Dogna, Udine, IT	37985	
	P. sylvestris	*I. acuminatus*	Val Venosta, Bolzano, IT	37986	
	P. sylvestris	*I. acuminatus*	Val Camonica, Brescia, IT	37987	
	P. sylvestris	*I. acuminatus*	Var, FR	37988[c]	
O. brunneo-ciliatum	*Larix decidua*	*I. cembrae*	Atholl, Scotland, GB		117571
	L. decidua	*I. cembrae*	Kindberg, Styria, AT		117591

[a] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, ZA; [b] Centraalbureau voor Schimmelcultures, Utrecht, NL; [c] previously identified as *O. brunneo-ciliatum*.

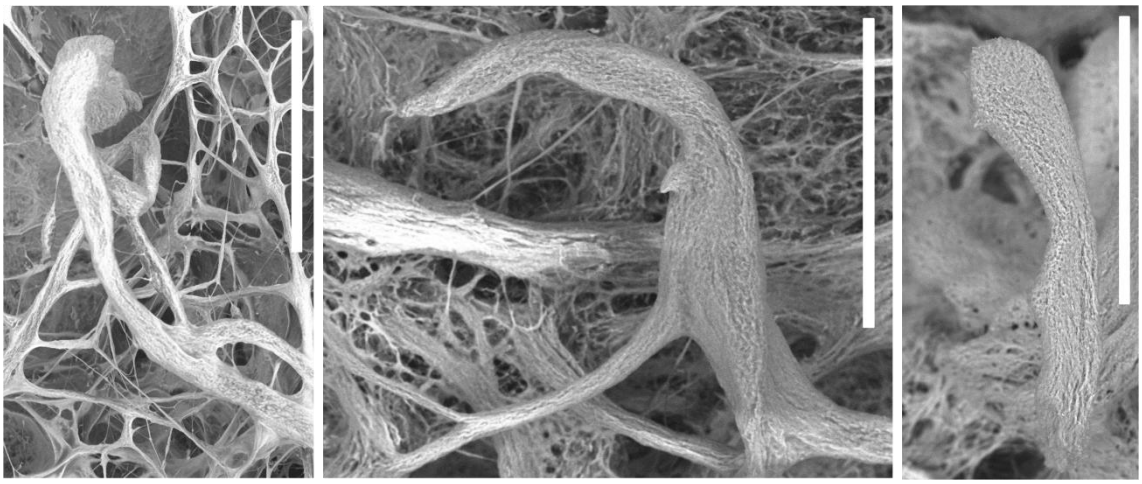

Figure 1: SEM images of the *Pesotum*-like conidiophores of *Ophiostoma clavatum* isolates. One isolate from each region was chosen: from the left, the French isolate (CMW 37988, previously identified as *O. brunneo-ciliatum*), the Italian isolate from Val Camonica (CMW 37987) and one of the two Swedish isolates from Lunsen (CMW 37984). Scale bars = 200 μm.

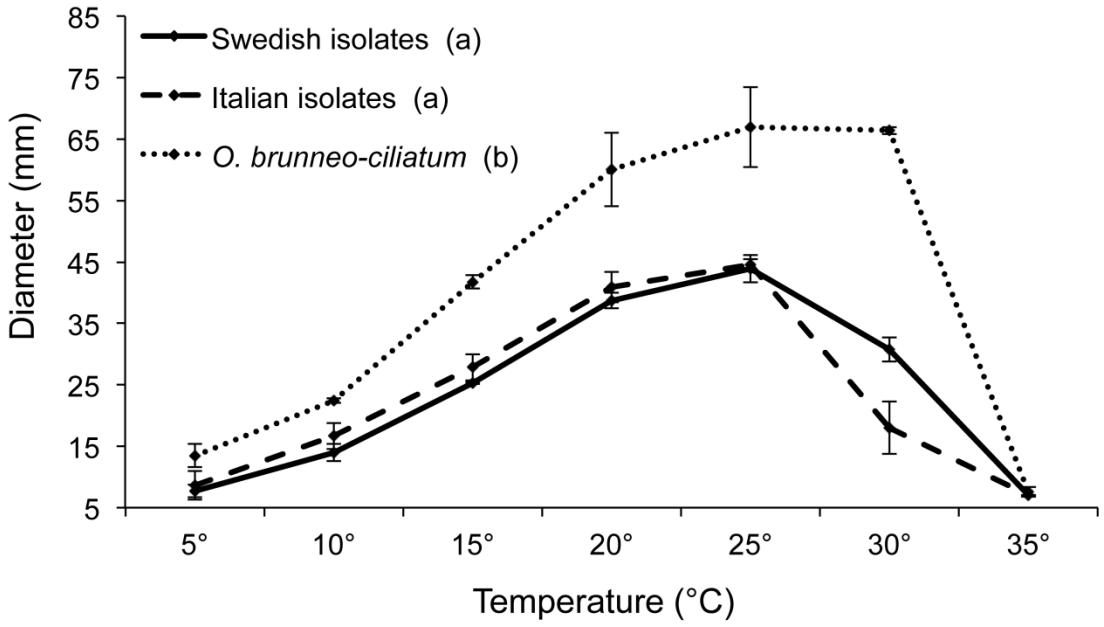

Figure 2. Growth comparison (mean ± standard deviation) of Italian and Swedish isolates and *O. brunneo-ciliatum* strains on MEA after 6 days at different temperatures. Two isolates were tested per each group. Different letters in brackets near to the isolates in the legend represent significant differences in the growth according to the Tukey's HSD test at $\alpha = 0.05$.

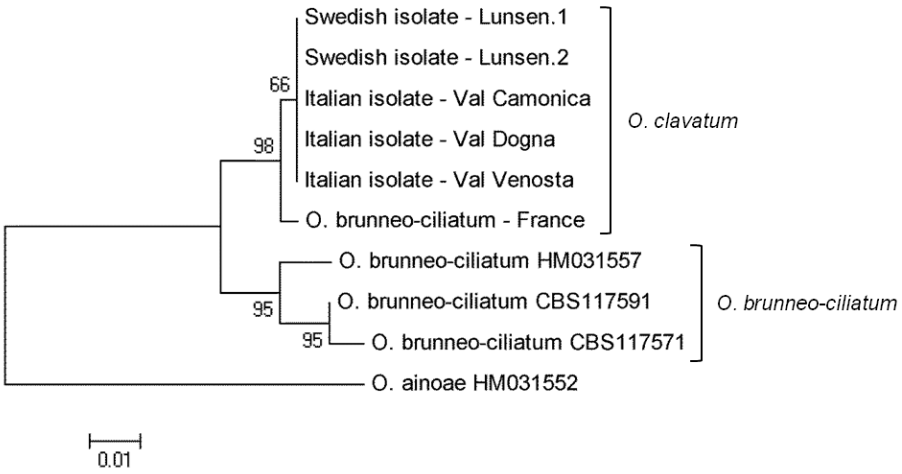

Figure 3. Preliminary neighbor-joining tree constructed upon the alignment of a β-tubulin gene fragment of 350 bp. Nodes report bootstrap values after 500 replications. The right brackets indicates the correct species for each clade. *Ophiostoma brunneo-ciliatum* France isolate is actually included in the *O. clavatum* clade.

Chapter III

Detection of blue-stain fungi associated with *Ips acuminatus* in the Italian Alps using loop-mediated isothermal amplification (LAMP) technology

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I collected most of the data, analyzed them and drafted the manuscript.

Abstract

Ips acuminatus is a bark beetle infesting Scots pine (*Pinus sylvestris*), and is associated with a complex of fungi useful for both insect establishment in the host plant (blue-stain fungi) and larval development (nutritional fungi). While the mycoflora associated to larval development is well characterized, that concerning blue-stain fungi is still uncertain, in particular regarding the identity of the blue-stain fungus more consistently associated with the vector. Moreover, very little is known about the role of the associated blue-stain fungi in the population dynamics of *I. acuminatus*, and if the frequency of association differs under outbreak and non-outbreak conditions. In this study we developed three Loop-mediated isothermal AMPlification (LAMP) assays to survey six Italian populations of *I. acuminatus* sampled from all along the Alps. The results show that the identity of the blue-stain fungus more consistently associated with *I. acuminatus* in the Italian Alps is *Ophiostoma clavatum*, which was detected on more than 50% of the samples. *O. brunneo-ciliatum*, also reported in part of the literature as the main blue-stain fungus associated with *I. acuminatus*, was instead not detected on any of the samples. We found also that the occurrence of *O. clavatum* was lower in the outbreak populations. This study is a further step towards the definition of the importance of associated fungi in the population dynamics of bark beetles, and it provides a molecular tool by which it will be easy to screen populations and derive important data for the ecology of the species.

Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) and their associated symbiotic fungi are among the most economically and ecologically important pests of holarctic conifer forests (Lieutier et al. 2004, Raffa et al. 2008). Furthermore, response of bark beetles to climate change is promoting a progressive forest decline in North America as well as in Europe (Jönsson et al. 2007, Kurz et al. 2008, Marini et al. 2012). As reviewed by Coulson (1979) and Wallner (1987), there are several factors that can lead a bark beetle population

to rise from a latent phase (non-outbreak) to a culmination phase (outbreak); among these factors, host plant resistance is one of the most important (Raffa and Berryman 1983, Raffa et al. 2008, Boone et al. 2011). However, even though ascomycetes fungi associated with bark beetles are known to interact with host plant resistance (Kirisits et al. 2004, Lieutier et al. 2009), very little is known about their role in insect population dynamics (but see Hofstetter et al. 2006 and Raffa et al. 2008). In particular, almost no information is available about the possibility that associated blue-stain fungi may differ in composition and frequency in bark beetle outbreak and non-outbreak conditions (Kirisits 2004). Studying *Ips typographus* (L.), for instance, Solheim suggested that the aggressive blue-stain fungus *Ceratocystis polonica* (Siem.) C. Moreau plays an important role in the initiation and development of *I. typographus* outbreaks, and is therefore predicted to occur at higher frequency than non-outbreak periods, replacing other less aggressive associated fungi (Solheim 1993a, 1993b). This hypothesis, however, needs to be more deeply investigated since Harding (1989) did not find statistical differences in the frequency of *C. polonica* between outbreak and non-outbreak populations of *I. typographus*, and some studies carried out in Austria even recorded an opposite behavior to the one predicted by Solheim (Kirisits 2001). A spatio-temporal variation - even at relatively small scale - in the composition and frequency of blue-stain fungi association is known in several bark beetles (Klepzig and Six, 2004, Six and Wingfield 2011), but almost no study provides clear information about this variation according to the insect population density (e.g., Giordano et al. 2012).

The pine engraver beetle *Ips acuminatus* (Gyll.) is a small bark beetle infesting the thin bark of Scots pine (*Pinus sylvestris* L.) throughout Europe (Bakke 1968, Colombari et al. 2012a, 2012b). Considered for many years as a species of minor economic importance, in recent decades *I. acuminatus* has been included among the most damaging European wood-boring insects (Gregoire and Evans 2004), and its impact has gained an important role in the Scots pine decline observed recently in the Alps (Wermelinger et al. 2008). Severe outbreaks of the pine engraver beetle have been reported in several European countries including France (Legrand 1996), Spain (Romanyk 1977) and Switzerland (Dobbertin 2005, Wermelinger et al. 2008). In Italy, local outbreaks have been reported in the central and eastern Alps (Lozzia and Rigamonti 2002, Colombari et al. 2012a, Faccoli et al. 2012),

and the species occurs in small non-outbreak populations all along the rest of the Alps (Faccoli et al. 2010).

The pine engraver beetle is associated with a complex of symbiotic fungi belonging to the morphologically homogenous group of ophiostomatoid fungi, ascomycetes (Wingfield et al. 1993). These fungi play a role in insect establishment and development (Kirisits et al. 2004, Lieutier et al. 2009, Villari et al. 2012) and are well adapted to dispersal by arthropods due to their sticky spores (Klepzig and Six, 2004). The complex includes the obligate nutritional fungus *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. (syn. *Ambrosiella macrospora* Batra) (Francke-Grosmann 1952, Batra 1967, Harrington et al. 2010), primarily serving as nourishment to the larvae (Francke-Grosmann 1963, Batra 1967, Paine et al. 1997), but also eliciting defense responses in the host trees (Villari et al. 2012). The identity and the role of *H. macrospora*, which is mainly transported in specific mycangia occurring in the female mandibles (Francke-Grosmann 1963), are well established (Batra 1967, Harrington et al. 2010, Villari et al. 2012).

Within small tegumental pits and on setae occurring on the adult body surface of both sexes (Paine et al. 1997, Klepzig and Six 2004), *I. acuminatus* carries also a complex of weakly pathogenic blue-stain fungi involved in the interaction with host defenses (Lieutier et al. 1991, 2009, Guérard et al. 2000, Kirisits 2004, Villari et al. 2012). Among these species, the literature reports a specific fungus which is more consistently associated with the vector (Mathiesen-Käärik 1953, Francke-Grosmann 1963, Harrington 2005), but the identity of this blue-stain fungus is still uncertain. Initial records described *Ophiostoma clavatum* Math.-Käärik as the main associated species (Mathiesen 1950, 1951, Rennerfelt 1950, Mathiesen-Käärik 1953, Francke-Grosmann 1963), while subsequently - and without confuting the previous records - many authors reported *O. brunneo-ciliatum* Math. (Lieutier et al. 1991, Guérard et al. 2000, Villari et al. 2012). Possible explanations for this uncertainty in the published data could be related to the hypothesis of a spatial variation in the associated complex composition among different sites, or to a variation in the insect-fungus association according to the epidemic phase of the insect population, as suggested by Solheim (1993a) for *I. typographus*. Lastly, the high morphological similarity of *O. clavatum* and *O. brunneo-ciliatum* (Mathiesen 1951, Mathiesen-Käärik 1953) may have led to an erroneous species identification. Generally, the correct identification of blue-stain

fungi based on morphological characteristics can be problematic, since species are characterized by simple morphology and many overlapping features (Kirisits 2004). The recent introduction of DNA sequence-based methods has therefore been essential for the correct identification of blue-stain fungi species (e.g., Zipfel et al. 2006), even though it has been shown that cultural methodologies are still important in the study of insect associated fungi (e.g., Giordano et al. 2012).

In recent years, PCR-based methods have been developed to detect fungal species directly from their insect vectors (e.g., Schweigkofler et al. 2005, Roets et al. 2006, Luchi et al. 2011). However, these methods can be limited by several factors, including quantity and quality of DNA preparations and the presence of inhibitors, which have been widely reported to affect PCR sensitivity (Haugland et al., 2002, Ma and Michailides 2007). These methods can also be extremely time consuming. Loop-mediated isothermal AMPlification (LAMP) is a recently developed technology (Notomi et al. 2000) which is highly sensitive, less time consuming than conventional PCR-based methods, and less prone to inhibition from DNA preparations (Tomlinson et al. 2010b).

In light of the importance of the pine engraver beetle and its associated fungi in the Alps (Wermelinger et al. 2008, Colombari et al. 2012a, 2012b), and the lack of information concerning the fungal complex associated with *I. acuminatus* and its variations in outbreak and non-outbreak populations, a better understanding of the system *I. acuminatus* – associated blue-stain fungi has become crucial. The aims of our study were i) to survey which of the previously described species (*O. clavatum* and *O. brunneo-ciliatum*) is the blue-stain fungus more consistently associated with *I. acuminatus* in the southern Alps, and ii) to verify whether outbreak and non-outbreak populations of *I. acuminatus* show significant differences in the occurrence of the associated blue-stain fungi. The aims were pursued by developing two real-time LAMP assays for detection of *O. clavatum* or *O. brunneo-ciliatum* directly in the insect vector, and employing them in Italian populations of the pine engraver beetle. In addition, a third real-time LAMP assay, specific to the insect vector *I. acuminatus,* was developed as an internal control (Tomlinson et al. 2010b, Hodgetts et al. 2011).

Materials and methods

Sample collection and biological material for assay development

Logs (40 cm long) of Scot pine recently infested by *I. acuminatus* were collected in winter 2009 from six sites spread all along the Italian Alps (Figure 1). The sampled sites were chosen on the basis of *I. acuminatus* population characteristics. In the sampling year, Valtellina and Cortina d'Ampezzo were outbreak populations, characterized by strong infestations affecting a large part of the Scots pine forests growing in the sampled regions (Lozzia and Rigamonti 2002, Colombari et al. 2012b). In the other four sites, *I. acuminatus* occurred with non-outbreak populations, killing few isolated trees scattered in the forest (Faccoli et al. 2010). In each site, logs were collected from two different trees, except at Brusson, where only one tree was sampled. After collection, logs were transferred to the University of Padova and kept in individual outdoor rearing cages for insect development. Newly emerged adults were immediately collected, morphologically identified (Faccoli 2004), and stored singly at –80 °C until analyses.

To develop the *O. clavatum* and *O. brunneo-ciliatum* LAMP assays, and test their specificity, 17 isolates of 11 different fungal species were tested (Table 1). Isolates of *O. brunneo-ciliatum*, *Ceratocystis montium* (Rumb.) J. Hunt, and *H. macrospora* were purchased from Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands). Isolates of *O. clavatum*, deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa (CMW), were isolated from *I. acuminatus* samples collected in Italy and Sweden, and identified on morphological and molecular features (Villari et al. in preparation). All other fungal isolates belonged to the collection of the Dipartimento di Biotecnologie Agrarie, University of Florence, Italy (Luchi et al. 2005, Sabbatini Peverieri et al. 2006). Species were chosen for their association with *I. acuminatus* (Kirisits 2004), or for their occurrence on *Pinus* spp. (Kirisits 2004, Luchi et al. 2005, Sabbatini Peverieri et al. 2006).

Adults of *I. acuminatus* and other bark beetle species to be used as a comparison during the *I. acuminatus* LAMP assay development were kindly provided by Dr. E. Petrucco

Toffolo (DAFNAE, University of Padova, Italy). Tested species and provenances are reported in Table 2.

DNA extraction

DNA was extracted from *I. acuminatus* samples following the salting out protocol described by Gilbert et al. (2007), which does not require the homogenization of whole insect tissues. This method was chosen in order to minimize the amount of insect DNA extracted relative to fungal DNA. Total DNA of fungi and insects used for LAMP assay development was instead extracted following a standard salting out protocol (Patwary et al. 1994).

Approximate DNA concentrations were determined at 260 nm using the Nano-drop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA), and extracts were diluted to 10 ng μl^{-1} in double-distilled water (Sigma-Aldrich, St. Louis, MO, USA).

Loop-mediated isothermal amplification assay development

Species-specific LAMP primers for *O. clavatum* and *O. brunneo-ciliatum* were designed manually from the β-tubulin gene antisense sequences (accession no. JX298085 for *O. clavatum*; accession nos. HM031559 and JX298086 for *O. brunneo-ciliatum*) (Linnakoski et al. 2010, Villari et al. in preparation). Species-specific primers for *I. acuminatus* were designed manually from the COX I gene sense sequence (accession nos. U82585, AF113325 and EF115508) (Stauffer et al. 1997, Cognato and Sperling 2000, Cognato and Sun 2007). BLAST analysis of the latter sequences was performed using the National Centre for Biotechnology Information (NCBI) database (Zhang et al. 2000), in order to obtain amplified phylogenic groups and identify suitable regions with high interspecific variability. Sequence alignments were performed using CLUSTALW, in MEGA version 5 software (Thompson et al. 1994, Tamura et al. 2011).

Six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers F-Loop and B-Loop) were designed for each assay (except the *I. acuminatus* assay, which lacked of the F-Loop primer), according to the strategy described by Notomi et al.

(2000) and Nagamine et al. (2002). Primers were designed to exploit interspecific sequence variability. Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and the sequences are reported in Table 3.

Real-time LAMP assays were performed as described by Tomlinson et al. (2010a) and Bekele et al. (2011) with some modifications. Amplifications were carried out on a Genie II instrument (OptiGene, Horsham, UK), using Isothermal MasterMix at 1 × concentration (OptiGene) in a total reaction volume of 25 µl. Primer concentration was as previously described (Bekele et al. 2011). The assays were optimized in terms of reaction time and temperature, and the volume of DNA added per reaction. For the *O. clavatum* assay, 4 µl of template DNA was added per reaction, and reaction was held at 66 °C for 35 min. For the *O. brunneo-ciliatum* assay, 2 µl of template DNA was added per reaction, and reaction was held at 65 °C for 30 min. For the *I. acuminatus* assay, 1 µl of template DNA was added per reaction, and reaction was held at 63 °C for 50 min. After amplification, the nature of the amplification products was confirmed by subjecting the reactions to a slow annealing step (0.05 °C per s) from 95 °C to 75 °C with fluorescence monitoring. An average estimate of annealing temperature for the amplification product of each assay was calculated on the basis of 20 amplifications of target DNA. For initial assay development, LAMP products (5 µl) were run on 2% agarose gels.

To test specificity, assays for *O. clavatum* and *O. brunneo-ciliatum* were initially tested using DNA samples from the isolates described in Table 1, and the assay for *I. acuminatus* was tested on DNA extracted from the species described in Table 2. To evaluate the sensitivity of the three LAMP assays, 10-fold serial dilutions of template DNA were tested. *Ophiostoma clavatum* and *O. brunneo-ciliatum* DNA was diluted in DNA extracted from the insect vector, to account for potential inhibitory effects. Serial dilutions of *I. acuminatus* DNA were prepared in water. For assay optimization and characterization, reactions were carried out in triplicate.

Sample analysis

To survey the occurrence of *O. clavatum* and *O. brunneo-ciliatum* on the collected *I. acuminatus* individuals, ten insects were tested from each tree, for a total of 110 samples

(Table 4). DNA extracted from each sample was tested using both real-time LAMP assays. Reactions were carried out in triplicate, and a sample was considered to be positively associated with each fungus only if at least two of the replicates had a positive response. Testing with the *I. acuminatus* assay was carried out only for those samples for which both the fungal assays had a negative response, in order to confirm that DNA extraction had been successful and the sample supported isothermal amplification, as described in Tomlinson et al. (2010b) and Hodgetts et al. (2011). In this case, reactions were carried out in one replicate. If the *I. acuminatus* assay also had a negative response, samples were replaced with further insects collected from the same site and tree, and tested again with all assays. During sample analysis, each LAMP run contained either *O. clavatum* (isolate CMW 37983), *O. brunneo-ciliatum* (isolate CBS 117591) or *I. acuminatus* (collected in Udine, IT) DNA as a positive control, and water as a negative control.

Statistical analysis

Survey data on the occurrence of *O. clavatum* on the collected *I. acuminatus* were analyzed with a Generalized Linear-Mixed-effect Model (GLMM), using a binomial distribution. To account for the nested nature of the experimental design, we used a model with presence/absence of the species on the vector as the response variable, population characteristic (outbreak or non-outbreak) as fixed effect, and tree within site as random factors. Data were analyzed in R (R Development Core Team 2011) using the lme4 package (Bates et al. 2011). Since the presence of *O. brunneo-ciliatum* was never recorded, statistical analysis on this species was not performed.

Results

Specificity and sensitivity of LAMP assays

Specificity tests for the *O. clavatum* and *I. acuminatus* LAMP assays confirmed that they consistently produced a positive response only in the presence of the target species (Tables 1 and 2). The *O. brunneo-ciliatum* LAMP assay occasionally produced also non-specific responses, which were nevertheless always distinguishable from the specific amplification on the basis of annealing temperature (data not shown). We hence considered also *O. brunneo-ciliatum* LAMP assay as specific (Table 1). Annealing temperature for *O. clavatum*, *O. brunneo-ciliatum*, and *I. acuminatus* LAMP assays were 89.21 ± 0.21 (SD) °C, 89.83 ± 0.05 °C, and 80.73 ± 0.24 °C, respectively.

Sensitivity tests for *O. clavatum* and *O. brunneo-ciliatum* LAMP assays showed that, for both the assays, limit of detection was 0.01 ng μl^{-1}. This dilution produced a positive response in all three replicates for both assays, while further dilution responses were not consistent (Figures 2 and 3). The limit of detection of the *I. acuminatus* assay was 0.1 ng μl^{-1}. At this dilution, two of the three replicates produced a positive response (Figure 4).

Amplification products visualized by gel electrophoresis appeared, as expected, as a ladder-like pattern (Figure 5) (Notomi et al. 2000).

Occurrence of *O. clavatum* and *O. brunneo-ciliatum* on *I. acuminatus*

None of the collected and analyzed *I. acuminatus* was associated with *O. brunneo-ciliatum*. *Ophiostoma clavatum* was detected in all the sampled populations and trees, although with a variable frequency ranging between 30% and 80% (Table 4). After that 19 of the surveyed insects were replaced, all samples for which both the fungal LAMP assays had a negative response (n = 51) were positive for the *I. acuminatus* LAMP assay, confirming that DNA extraction had been successful and that the sample supported isothermal amplification.

The generalized linear-mixed-effect model showed that population characteristic (outbreak or non- outbreak) had a significant influence on the frequency of the fungus-vector association, which was lower in the outbreak populations (Figure 6).

Discussion

This paper illustrates the development of three real-time LAMP assays, which were found to be sensitive and specific for the detection of two fungal pathogens and their insect vector. As reported by Tomlinson et al. (2010a, 2010b), LAMP technology is a robust technique, less prone to inhibition and quicker than real-time PCR; real-time LAMP is also less prone to contamination than nested PCR (Bekele et al. 2011). The two developed fungal assays (specific for *O. clavatum* and *O. brunneo-ciliatum*) had a limit of detection of 0.01 ng μl^{-1}, and the total reaction using the Genie II instrument was less than 40 minutes. The *O. clavatum* assay produced a positive amplification only in the presence of the target DNA, while the *O. brunneo-ciliatum* assay occasionally produced non-specific amplification, that was distinguishable on the basis of annealing temperature. Due to the necessity to exploit the interspecific variability of the COX I gene sequence (Stauffer et al. 1997, Cognato and Sperling 2000, Cognato and Sun 2007), the *Ips acuminatus* LAMP assay lacked the F-Loop primer; as reported previously (Nagamine et al. 2007) this appears to result in this assay being slower than the other assays; however, it was still specific, with a limit of detection of 0.1 ng μl^{-1}.

In this study we show that, among those reported in the literature, the blue-stain fungus more consistently associated with *I. acuminatus* in the Italian Alps is *O. clavatum*, which was detected at all sampling sites and on more than 50% of the samples. On the other hand, *O. brunneo-ciliatum* was never detected. The results of this study confirm what has been previously described by Mathiesen (1950, 1951), Rennerfelt (1950), Mathiesen-Käärik (1953) and Francke-Grosmann (1963) about the identity of the main blue-stain fungus associated with *I. acuminatus*. Subsequent reports of *O. brunneo-ciliatum* as consistently associated with *I. acuminatus* (e.g., Lieutier et al. 1991, Guérard et al. 2000, Villari et al.

2012) are hence probably due to an identification mistake related to the high morphological similarity of the two species (Mathiesen 1951, Mathiesen-Käärik 1953, Villari et al. in preparation). According to Mathiesen-Käärik (1953), *O. brunneo-ciliatum* is instead associated with *I. sexdentatus* (Boern.), a larger species which also may colonize *P. sylvestris*. Both of the blue-stain fungi can therefore be found in the same tree host, but, since they are associated with different vectors which specifically colonize different parts of the stem according to bark thickness, it is unlikely that they can share the same habitat. Moreover, taking into account the fact that *I. acuminatus* has been already found consistently associated with the same species in Sweden, Germany and former Yugoslavia (Francke-Grosmann 1963), and that we also found the same fungus in all the sites samples, in spite of distance and population characteristics, it is also unlikely the occurrence of a spatial variation in the complex composition of blue-stain fungi associated with *I. acuminatus*, at least concerning the main associated species. However, *O. clavatum* and *O. brunneo-ciliatum* are both considered non-aggressive pathogens (Mathiesen 1951, Guérard et al. 2000), and in the system bark beetle − *P. sylvestris* they probably equally stimulate tree response and reduce the critical threshold of attack density above which beetle attacks succeed (Kirisits et al. 2004, Lieutier et al. 2009, Villari et al. 2012).

In this study we found that the occurrence of *O. clavatum* varied significantly between outbreak and non-outbreak populations, and it was lower in the outbreak populations. The variation in the frequency of blue-stain fungus association among sites is not surprising, since it is regulated by a multitude of factors that are context dependent (Klepzig and Six 2004). But the lower occurrence of *O. clavatum* in outbreak populations, according with Kirisits findings in Austrian populations of *I. typographus* (2001), raises an interesting issue. Indeed, since the role of blue-stain fungi is to interact with plant defense (Lieutier et al. 2009), and outbreaks are believed to occur when some factor reduce the ability of the tree to resist beetle attack (Raffa and Berryman 1983, Berryman 1988, Raffa et al. 2008), we would expected an opposite result, as suggested by Solheim (1993b). One possible explanation of this phenomenon is that during the culmination of the outbreak phase, the density of the bark beetle population is so high that the presence of the blue-stain fungus, especially if not aggressive, is no longer critical to overwhelm plant defenses. Indeed, as explained by Raffa et al. (2008), once an eruptive threshold is surpassed, the initial eliciting

factors, such as a specific frequency of association with the fungal complex, may not be needed to sustain the outbreak. Moreover, since symbiotic interaction outcomes can depend on the context (reviewed in Klepzig and Six, 2004), an initially mutualistic relationship may lead to competition for space and nutrients in the culminant phase of the outbreak, characterized by a strong increase in the bark beetle population, and this antagonistic interaction may be the cause of the decrease of *O. clavatum* occurrence. It would be hence interesting to determine the frequency of blue-stain fungi association also in the incipient phase of an outbreak, when the presence of the associated fungi may play an important role in surpassing of the eruptive threshold, in addition to the latent and culmination phases studied here. This would allow us to verify Solheim's hypothesis (1993b) or, on the contrary, to confirm the pattern we found, reconsidering the importance of blue-stain fungi in bark beetle population dynamics (Six and Wingfield 2011).

In conclusion, besides resolving an uncertainty in the literature about the identity of the blue-stain fungus more consistently associated with *I. acuminatus*, our results provide a further assessment of how the fungal associated complex can vary between outbreak and non-outbreak populations. The discussion about the role of associated fungi and their importance in the population dynamics of bark beetles is, however, still open, since none of the suggested hypotheses has been clearly verified so far. It would therefore be helpful to better define this role, in order to understand the mechanisms regulating the bark beetle population dynamics (Raffa et al. 2008). In this paper we used an innovative and recently developed technology, which allowed us to specifically and quickly detect the blue-stain associated fungi directly from the vector insects. The use of LAMP technology could therefore bring a considerable improvement not only in diagnostics but also in ecological and biological studies that need a quick and reliable identification of symbionts, as in the case of bark beetle associated fungi.

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Table 1. Characteristics of the fungal isolates used for development of *Ophiostoma clavatum* and *O. brunneo-ciliatum* LAMP assays.

Fungal species	Geographic origin	Isolate	Collection	LAMP assays assessment	
				O. clavatum	*O. brunneo-ciliatum*
Ophiostoma clavatum	Lunsen, Uppsala, SE	37983	CMW[a]	+	−
	Lunsen, Uppsala, SE	37984	CMW[a]	+	−
	Val Dogna, Udine, IT	37985	CMW[a]	+	−
	Val Venosta, Bolzano, IT	37986	CMW[a]	+	−
	Val Camonica, Brescia, IT	37987	CMW[a]	+	−
O. brunneo-ciliatum	Atholl, Scotland, GB	117571	CBS[b]	−	+
	Kindberg, Styria, AT	117591	CBS[b]	−	+
Ceratocystis montium	Weinviertel, Lower Austria, AT	117580	CBS[b]	−	−
	Oregon	137.36	CBS[b]	−	−
Hyalorhinocladiella macrospora	Sweden	367.53	CBS[b]	−	−
Leptographium serpens	Poggio Valicaia, Firenze, IT	CVLE04	DiBA[c]	−	−
L. procerum	Monte Peglia, Siena, IT	CV05MP01	DiBA[c]	−	−
L. lundbergii	Poggio Valicaia, Firenze, IT	CVLE01	DiBA[c]	−	−
L. wingfieldii	Alberese, Grosseto, IT	CVLE03	DiBA[c]	−	−
L. guttulatum	Alberese, Grosseto, IT	CVLE02	DiBA[c]	−	−
Sphaeropsis sapinea	S. Rossore, Pisa, IT	S10	DiBA[c]	−	−
Dilpodia scrobiculata	Douglas Co.,WI	215	DiBA[c]	−	−

[a] Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, ZA; [b] Centraalbureau voor Schimmelcultures, Utrecht, NL; [c] Dipartimento di Biotecnologie Agrarie, University of Florence, IT.

Table 2. Description of the insects used for *Ips acuminatus* LAMP assay development.

Insect species	Geographic origin	*I. acuminatus* LAMP assay
Ips acuminatus	Lunsen, Uppsala, SE	+
	Lunsen, Uppsala, SE	+
	Cortina d'Ampezzo, Belluno, IT	+
	Val Dogna, Udine, IT	+
I. sexdentatus	Monfalcone, Gorizia, IT	−
I. typographus	Chioggia, Venezia, IT	−
I. cembrae	Chioggia, Venezia, IT	−
Tomicus piniperda	Verzegnis, Udine, IT	−
Orthotomicus erosus	Trieste, IT	−
Crypturgus cinereus	Chioggia, Venezia, IT	−

Table 3. Primers used for the loop-mediated isothermal amplification (LAMP) assays.

LAMP assay target	Primer	Sequence (5' – 3')	Target region length (bp)
Ophiostoma clavatum	F3	CCTCGTTGAAGTAGACGCTC	176
	B3	GATTCCGATCTACGGCTCC	
	FIP	GTTGGACGTTGGACGCCGCGAGG-CGCTCCAGCTGGAGA	
	BIP	GAAGTGGGAGAATACATACACGC-CATGGCTGAACTCAACACTGAC	
	F-Loop	ACCGCAGCTAACGTAATGTCC	
	B-Loop	GCTGTCAAGGCCGTGCTC	
O. brunneo-ciliatum	F3	GACCGAAAGGACCGGCAC	217
	B3	GCTCCACCGCGGCATGA	
	FIP	CTGTCCCTAGGTACAACGGCACAG-ATCGACAAGGACGGCAC	
	BIP	CCACGACCAACATCAGGGGAGACA-GGCAGCAGATTTCCGGC	
	F-Loop	CCTCTGGCAACAAGTACGTG	
	B-Loop	GCTGTCAAGGCCGTGCTC	
Ips acuminatus	F3	CATTTCATGGAGCTCAAATTTC	182
	B3	TCCTGTAAAAAGTGGAAATCATT	
	FIP	TCCGGTTAAACCTCCTAGAGTAAAT-AATCCCTCAAGACTTTGATCT	
	BIP	ATGTAGTTGCCCATTTCCATTATGT-GGACAATTCCTGCAATAATAG	
	B-Loop	ACTTTCAATAGGAGCTGTATTTG	

Table 4. Frequency of association of the blue-stain fungi *Ophiostoma clavatum* and *O. brunneo-ciliatum* with *Ips acuminatus* individuals collected in the Italian Alps (n = 110).

Site number	Site location[a]	Outbreak population	Tree	No. of *I. acuminatus* associated with the fungi (positives /tested samples[b])	
				O. clavatum	*O. brunneo-ciliatum*
1	Brusson, Aosta	−	A	3/10	0/10
2	Val Camonica, Brescia	−	A	7/10	0/10
			B	7/10	0/10
3	Valtellina, Sondrio	+	A	3/10	0/10
			B	4/10	0/10
4	Val Venosta, Bolzano	−	A	5/10	0/10
			B	6/10	0/10
5	Cortina d'Ampezzo, Belluno	+	A	3/10	0/10
			B	5/10	0/10
6	Val Dogna, Udine	−	A	8/10	0/10
			B	8/10	0/10

[a] See Figure 1 for site geographic location.
[b] *Ophiostoma clavatum* and *O. brunneo-ciliatum* were tested on the same samples.

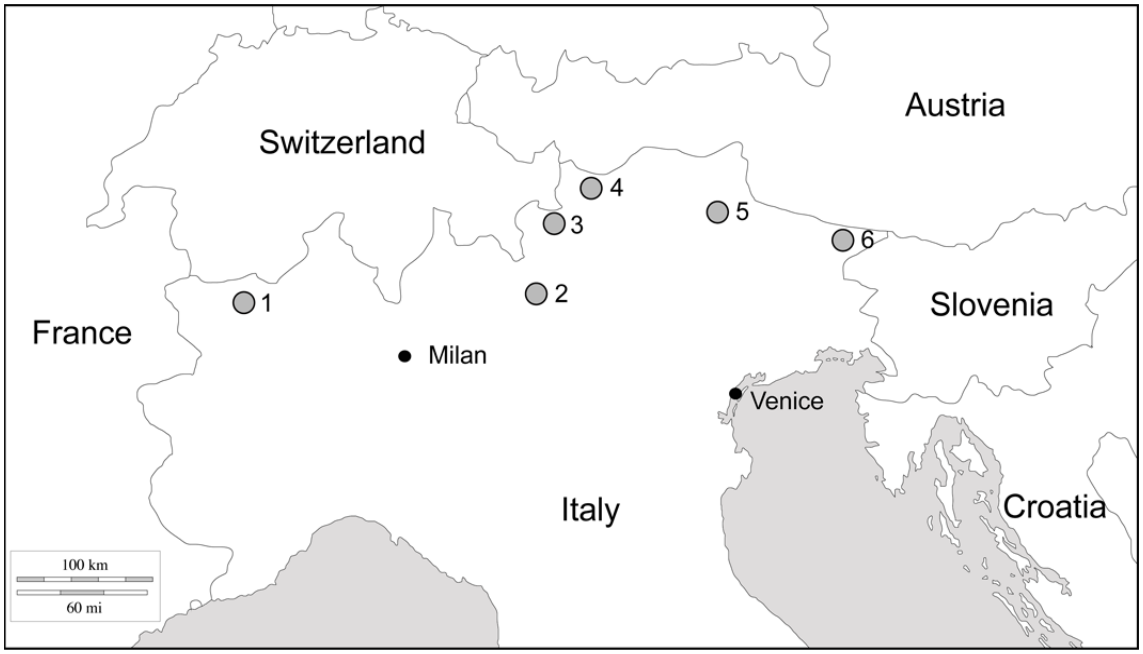

Figure 1. Map of Northern Italy showing survey sampling sites: Brusson, Aosta (1); Val Camonica, Brescia (2); Valtellina, Sondrio (3); Val Venosta, Bolzano (4); Cortina d'Ampezzo, Belluno (5); Val Dogna, Udine (6).

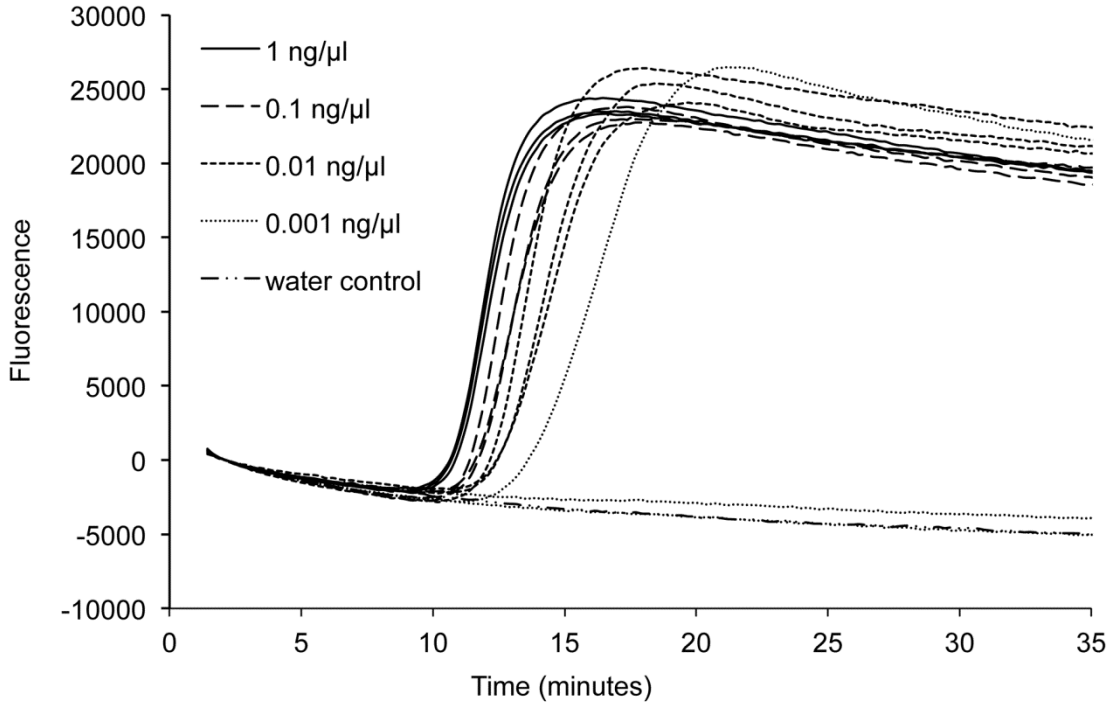

Figure 2. *Ophiostoma clavatum* real-time LAMP assay. Results of the sensitivity test. Each dilution (*O. clavatum* DNA in *Ips acuminatus* DNA) was run in three replicates.

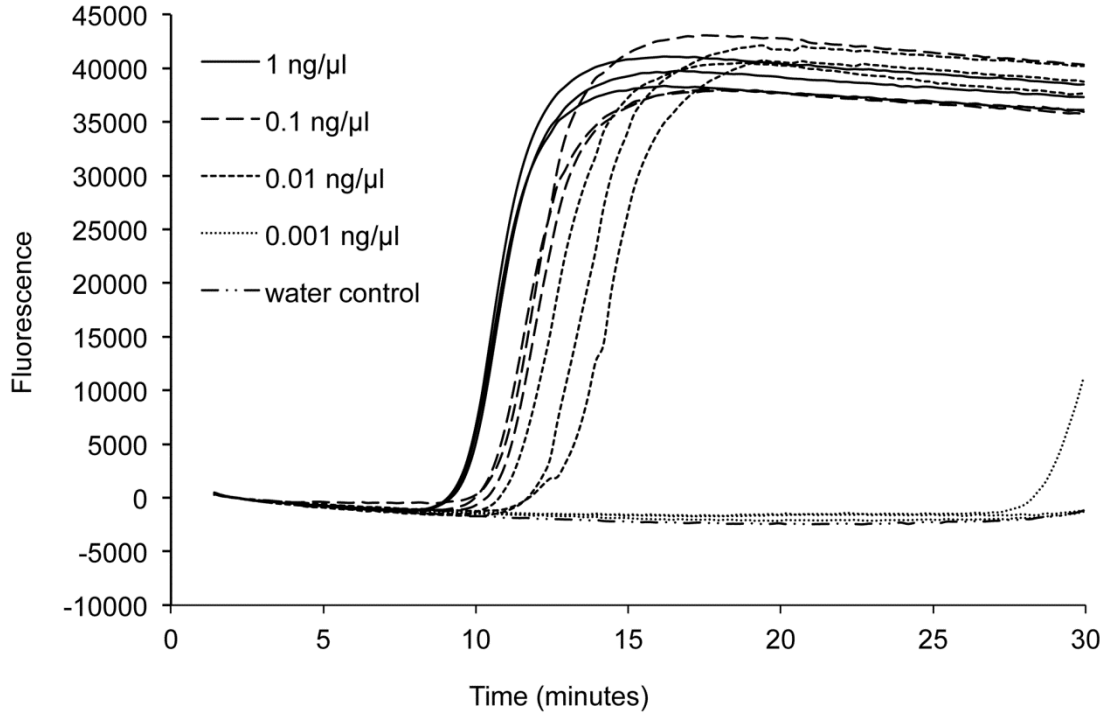

Figure 3. *Ophiostoma brunneo-ciliatum* real-time LAMP assay. Results of the sensitivity test. Each dilution (*O. brunneo-ciliatum* DNA in *Ips acuminatus* DNA) was run in three replicates.

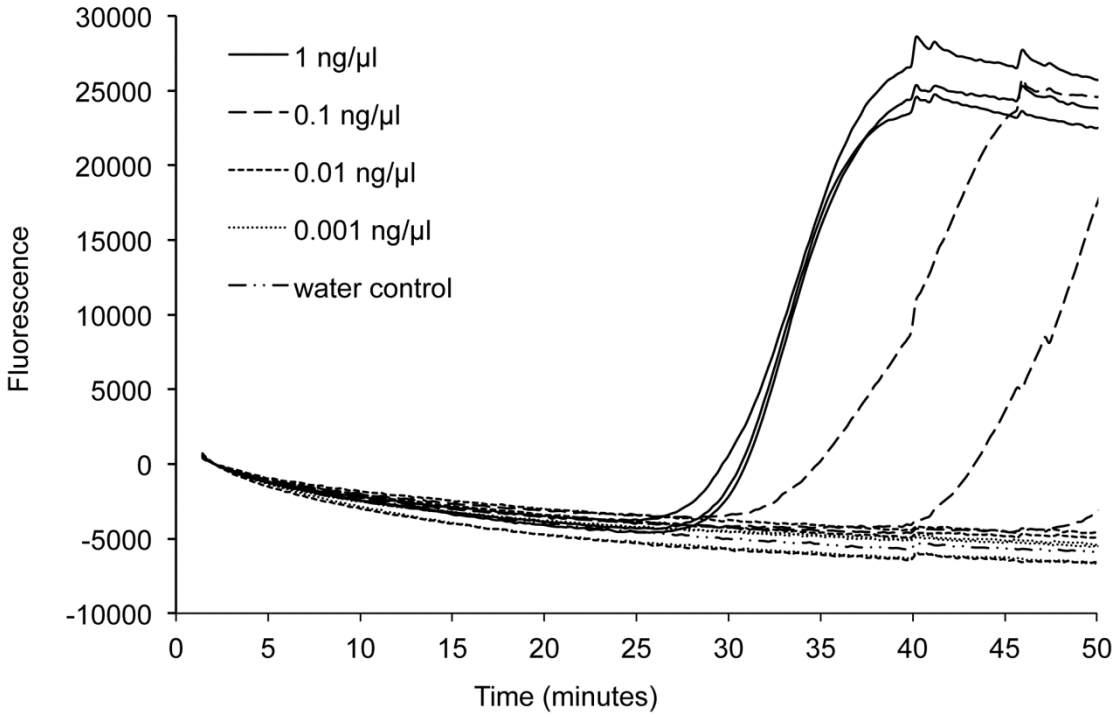

Figure 4. *I. acuminatus* real-time LAMP assay. Results of the sensitivity test. Each dilution (*I. acuminatus* DNA in water) was run in three replicates.

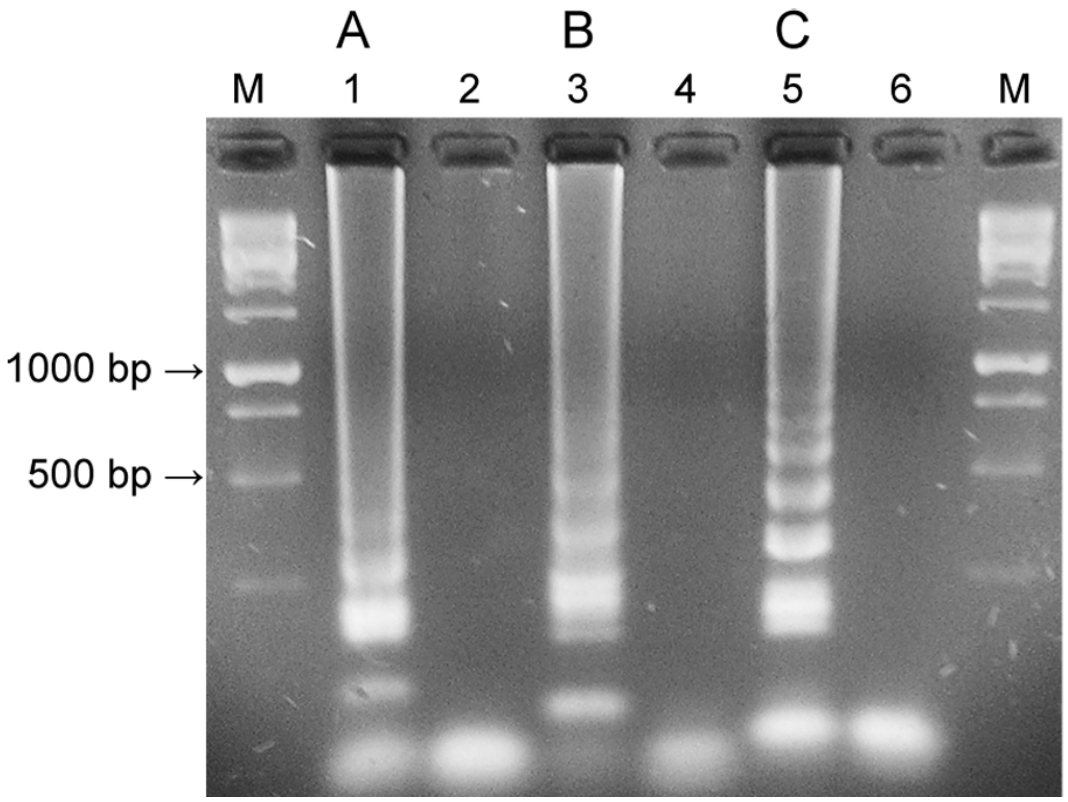

Figure 5. Detection of LAMP results by gel electrophoresis. M: marker (SharpMass 1 kb DNA ladder, EuroClone, Milan, IT); A: *Ophiostoma clavatum* LAMP assay; B *O. brunneo-ciliatum* LAMP assay; C: *Ips acuminatus* LAMP assay; lanes 1 and 4: *O. clavatum* isolate CMW 37983, 10 ng μl^{-1}; lanes 2 and 3: *O. brunneo-ciliatum* isolate CBS 117591, 10 ng μl^{-1}; lane 5: *I. acuminatus*, 10 ng μl^{-1}; lane 6: *I. sexdentatus*, 10 ng μl^{-1}.

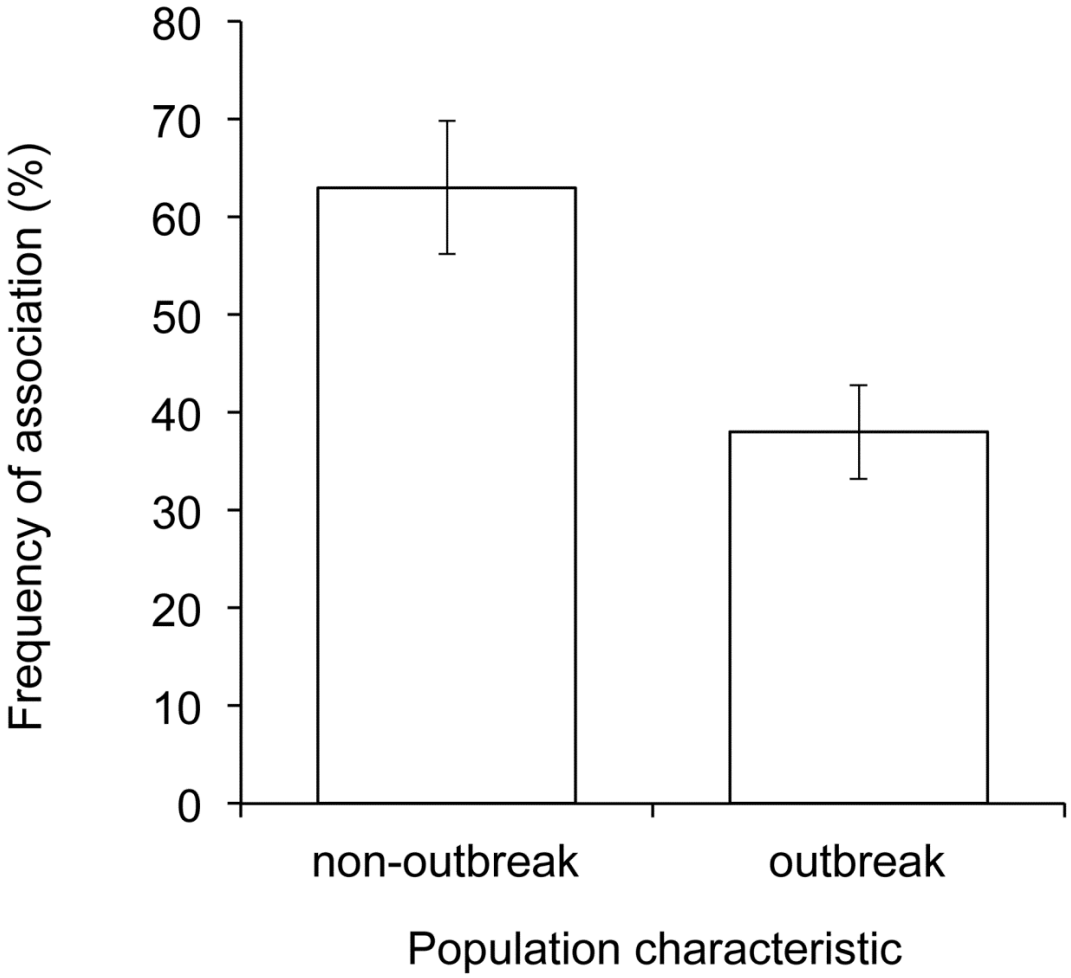

Figure 6. Effects of population characteristics (outbreak or non-outbreak) on the frequency of association of the blue-stain fungus *Ophiostoma clavatum* with *Ips acuminatus* (expressed as mean percentage of insects carrying the fungus ± SE) on the Italian Alps ($z = -2.38$, $Pr = 0.017$).

Chapter IV

Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine

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I collected most of the data, analyzed them and drafted the manuscript

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Preamble

At the time of this manuscript submission, the identity of the blue-stain fungus used in the experiment was not yet correctly defined. In this paper the isolate is referred to as *Ophiostoma brunneo-ciliatum*, but further morphological and molecular analyses proved that its identity was actually *O. clavatum* (see chapter II, isolate CMW 37987). All references to *O. brunneo-ciliatum* in this paper are hence to be intended as to *O. clavatum*.

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Research paper

Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine

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Conifer bark beetles are often associated with fungal complexes whose components have different ecological roles. Some associated species are nutritionally obligate fungi, serving as nourishment to the larvae, whereas others are pathogenic blue-stain fungi known to be involved in the interaction with host defenses. In this study we characterized the local and systemic defense responses of Scots pine (*Pinus sylvestris* L.) against *Ophiostoma brunneo-ciliatum* Math. (a blue-stain pathogen) and *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. (a nutritional fungus). These fungi are the principal associates of the pine engraver beetle, *Ips acuminatus* (Gyll.). Host responses were studied following inoculation with the fungi, singly and as a fungal complex, and by identifying and quantifying terpenoids, phenolic compounds and lignin. Although the length of the necrotic lesions differed between control (wound) and fungal treatments, only two compounds (pinosylvin monomethyl ether and (+)-α-pinene) were significantly affected by the presence of the fungi, indicating that Scots pine has a generic, rather than specific, induced response. The fact that both nutritional and blue-stain fungi triggered comparable induced defense responses suggests that even a non-pathogenic fungus may participate in exhausting host plant defenses, indirectly assisting in the beetle establishment process. Our findings contribute to the further development of current theory on the role of associated fungal complexes in bark beetle ecology.

Keywords: blue-stain, induced response, *Ips acuminatus*, mutualism, phenolics, *Pinus sylvestris*, secondary metabolism, symbiosis, terpenoids.

Introduction

Most bark beetles (Coleoptera: Curculionidae, Scolytinae) that breed in conifers carry complexes of symbiotic fungi in mycangia, pits and setae occurring on the body surface (Francke-Grosmann 1967, Kirisits 2004, Harrington 2005). Some of these associated fungi are known to have a directly mutualistic interaction with the beetles, whereby the insects disseminate the fungus and the fungus serves as nourishment to the larvae in the form of edible hyphae, spores and exudates (Harrington 2005). The nitrogen and phosphorus content of plant tissues is indeed very low, relative to insect metabolic requirements, but associated fungi are able to concentrate scarce nutrients and constitute an important source of sterols, which are essential elements of cellular structure, necessary precursors for hormone synthesis and critical factors in the production of viable eggs (Klepzig and Six 2004, Bentz and Six 2006). In this case the fungi, often referred to as nutritional or mycangial fungi, are typically non-pathogenic to the tree host and are usually transported in mycangia (Paine et al. 1997, Six 2003).

In the majority of cases, however, the effect of the fungi is thought to be indirect, whereby the fungi modify the quality of the host plant tissues in a way that facilitates brood establishment (Paine et al. 1997, Six 2003, Davis and Hofstetter 2011). In this case, bark beetle-associated fungi are often more or less aggressive tree pathogenic species (Kirisits 2004). Nearly

all of them are Ascomycetes belonging to the genera *Ophiostoma*, *Ceratocystis*, *Ceratocystiopsis*, *Grosmannia* and their related anamorphs, which all form—with a few other genera—the morphologically homogenous group of the ophiostomatoid fungi (Wingfield et al. 1993, Kirisits 2004). These fungi are also known as 'blue-stain' fungi, on account of the bluish-grey color most of the species give to colonized sapwood (Harrington 2005).

The role of bark beetle-associated fungi and their importance in vector establishment is still widely debated. One hypothesis, recently called the Classic Paradigm (CP) (Six and Wingfield 2011), postulates that associated fungi have an indirect effect by aiding tree colonization by bark beetles. This hypothesis is divided into two components. The first suggests that tree invasion by aggressive, bark beetle-associated blue-stain fungi leads to tree death, primarily by blocking water conduction in the xylem and facilitating beetle colonization (Paine et al. 1997). The second, more recent concept, proposes that associated fungi play a role in beetle establishment on trees, but based on the stimulation and subsequent exhaustion of tree defenses by the fungi, resulting in assistance to their insect vectors in overcoming host plant defenses directed at them (Lieutier et al. 2009). By combining the views of Paine et al. (1997) and Lieutier et al. (2009), the CP states that beetle establishment proceeds in two successive steps: (i) defeat of tree defense in both the phloem and the external sapwood following pioneer beetle colonization and (ii) mass invasion of tree tissues by beetles (in the phloem) and fungi (in phloem and sapwood). Therefore, rather than fungal pathogenic potential, what is important for bark beetle populations is the ability of their associated fungi to stimulate the tree response and thus lower the critical threshold of attack density above which beetle attacks succeed (Lieutier et al. 2009). Moreover, a highly pathogenic fungus would likely be detrimental, invading the tree too rapidly, outcompeting other mutualistic fungi, and making the tissues unsuitable for beetle brood development, as can occur in the case of *Dendroctonus frontalis* Zimm. (southern pine beetle) and *Ophiostoma minus* (Hedg.) H. et P. Syd. (Klepzig and Wilkens 1997, Hofstetter et al. 2006).

Recently, a new hypothesis contradicting the CP has been proposed, suggesting that, rather than playing a supporting role for the host beetle, phytopathogenicity performs an important role for the fungi, mediating competitive interactions in the fungal community and supporting survival in living and defended trees (Six and Wingfield 2011). Arguments for this hypothesis include the lack of consistent association of aggressive fungal associates with tree-killing bark beetles, the lack of correspondence between fungal growth in the host tree and the development of symptoms associated with a successful attack, and the ubiquity of similar associations of fungi with bark beetles that do not kill trees.

In this work we investigated the fungal complex associated with the pine engraver beetle *Ips acuminatus* (Gyll.), a small bark beetle infesting thin bark of Scots pine (*Pinus sylvestris* L.) that has been recently reported as a pest in many alpine forests (Colombari et al. 2012). *Ips acuminatus* is associated mainly with *Ophiostoma brunneo-ciliatum* Math. and *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. (syn. *Ambrosiella macrospora* Batra). The first species is a blue-stain pathogen thought to be involved in lowering the critical threshold of attack density for successful beetle colonization (Lieutier et al. 1991, Guérard et al. 2000, Kirisits 2004). *Hyalorhinocladiella macrospora*, whose ecological behavior and virulence have been poorly studied, is primarily known as a food source for the larvae. As with most nutritional fungi, this species is thought to be non-pathogenic (Francke-Grosmann 1963, Batra 1967, Paine et al. 1997, Harrington et al. 2010). Thus, the system *I. acuminatus–P. sylvestris* is potentially useful for testing hypotheses on inducible defenses against the fungal complex. The qualitative and quantitative secondary metabolic responses of Scots pine to blue-stain fungi have been studied extensively (e.g., Delorme and Lieutier 1990, Lieutier et al. 1996, Fäldt et al. 2006). However, to our knowledge, no data are available about tree responses to non-pathogenic and nutritional fungi in Scots pine, and only little information is available for other host trees (Raffa and Berryman 1982, Hofstetter et al. 2005, Davis and Hofstetter 2011). Consistent with the finding that pathogenicity is not related to a fungus's ability to stimulate tree defenses (Krokene and Solheim 1997, Davis and Hofstetter 2011), our hypothesis is that *H. macrospora* triggers an inducible defense reaction comparable to the one induced by the blue-stain fungus *O. brunneo-ciliatum*. Moreover, we hypothesize that the fungal species involved in the complex have a synergistic effect on tree defense stimulation compared with the single species. To test our hypotheses, we characterized the defense responses of Scots pine, both locally and systemically, to inoculation with *O. brunneo-ciliatum* and *H. macrospora*, singly and as a fungal complex, by identifying and quantifying terpenoids, including resin acids, phenolic compounds and lignin.

Materials and methods

Plant material, treatments and sampling

In spring 2010, 50 Scots pines of similar size and age (~20 years old) were selected from a young alpine stand in San Vito di Cadore, Belluno Province, Italy (46°29′49″N, 12°10′29″E, 1105 m a.s.l.). The growing conditions and general health of each plant were assessed by measuring plant height and the length of the current year shoot of a randomly chosen branch located in the upper part of the tree crown (Lanner 1976). The experiment was carried out in two trials of 25 trees each, in order to minimize the effects of confounding factors that can

occur in a natural environment. Trees were assigned to five different treatments, with five replicates per treatment, in a completely randomized design in each trial. The first trial started on 27 May, the second on 30 May 2010, and they both lasted 3 weeks. Mean daily temperature during that period was 13.49 ± 4.8 °C (SD). The five treatments consisted of (i) inoculation with *H. macrospora* (certified isolate 367,53, CBS, Utrecht, The Netherlands) and (ii) *O. brunneo-ciliatum* (isolate C_2P, Valcamonica, Italy), (iii) concurrent inoculation with both fungi, (iv) mechanical wounding, and (v) unwounded control. All treatments were performed at low density (50 points m^{-2} of bark), corresponding to four points in a 30 cm band around each tree trunk at 80 cm height (Fäldt et al. 2006). Inoculations were done by removing a plug of outer bark/phloem with a 5 mm cork borer and replacing it with a Scots pine outer bark/phloem plug of the same size previously sterilized and then colonized by the fungi. Full colonization of the bark/phloem plugs was achieved by placing them in a Petri dish containing 1.5% potato dextrose agar (Difco, BD, Franklin Lakes, NJ, USA), alongside plugs of 2% potato dextrose agar taken from fully colonized plates. After 7–10 days of incubation, all the bark/phloem plugs were completely covered with mycelium, homogeneously showing aerial vegetative hyphae, in the case of *H. macrospora*, and numerous synnemata in the case of *O. brunneo-ciliatum*. For treatment (iii), two half plugs, each colonized with the different fungi, were inserted in the same hole. In the wound treatment (iv) nothing was inserted after the plug removal. All of the inoculation sites and lesions were sealed with Scotch duct tape (3M, St Paul, MN, USA) to minimize contamination.

In order to measure constitutive lignin, terpenoids and phenolic compounds, the phloem and outer bark plugs resulting from the inoculation procedures (corresponding to time T_0 of the experiment) were pooled for each plant, immediately frozen in liquid nitrogen and stored at –80 °C until processing.

Three weeks after application of the treatment (corresponding to time T_1 of the experiment), the vertical length of each lesion was measured on the external sapwood. In the three inoculation treatments, phloem tissue was taken from each lesion surrounding the inoculation site to re-isolate the fungi, as described in Bonello et al. (2008).

Samples for the measurement of locally induced lignin, terpenoids and phenolic compounds were taken at T_1, with a 1 cm cork borer. Plugs contained both symptomatic and asymptomatic tissues from all of the four lesion points (position L, local), and were pooled for each plant for a cumulative amount of ~2 g FW. To measure systemic effects, the same number of plugs was taken at 20–30 cm above the inoculation sites, from a 10 cm band around each tree trunk (position S, systemic), and pooled for each plant. At this time, samples were also taken from the five untreated control plants, at the same trunk height of the other treatments. All sample plugs were immediately frozen in liquid nitrogen and stored at –80 °C. All tools were rinsed with 90% ethanol after each use during manipulations in the field. In order to be processed for chemical analysis, all sample plugs were ground to a powder in liquid nitrogen.

Analysis of phenolics and resin acids

All solvents used for sample preparation and analysis were HPLC grade unless otherwise noted. 0.1 g FW of the ground tissue was freeze-dried and extracted twice in 0.5 ml of methanol (Fisher, Pittsburg, PA, USA) for 24 h at 4 °C (Wallis et al. 2008). Five-hundred microliters of combined supernatants were concentrated 2.5 times with a Vacufuge™ Concentrator 5301 (Eppendorf) and stored at –20 °C until analyzed.

Liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization (ESI-MS) (Varian 212-LC pumps and 500-MS; Palo Alto, CA, USA), in parallel with a photodiode array detector (PDA) (Varian ProStar 335), was employed in negative ion mode to identify phenolic compounds and resin acids.

Compounds from the plant extract were then quantified by high-performance liquid chromatography (HPLC-UV), which was performed using a Waters (Milford, MA, USA) Alliance 2690 separation module equipped with a 996 PDA and a 474 scanning fluorescence detector in serial mode. Detailed description of the operating conditions for both the LC-ESI-MS and the HPLC-UV can be found in the Supplementary material.

Authentic external standards of catechin, pinocembrin, pinosylvin and pinosylvin monomethyl ether (Apin Chemicals, Abingdon, UK), procyanidin B2 and taxifolin (Extrasynthese, Genay Cedex, France), vanillic acid, *trans*-coumaric acid, abietic acid, neoabietic acid and ferulic acid (Sigma-Aldrich, St Louis, MO, USA) and pinoresinol (ArboNova, Turku, Finland) were used. Three-point standard calibration curves were used for pinoresinol, catechin, procyanidin B2, vanillic acid, pinosylvin and pinosylvin monomethyl ether; four-point standard curves were used for ferulic acid, pinocembrin, taxifolin and *trans*-coumaric acid; and five-point standard curves were used for neoabietic acid and abietic acid. In all standard curves, three technical replicates were averaged for each concentration. All linear regressions had $R^2 > 0.99$ except for abietic acid which had $R^2 = 0.959$. Additionally, relative standard error, with an upper limit of 5%, was used as a criterion to measure robustness of the curve (Massart et al. 1997). Data are reported as $\mu g\ g^{-1}$ FW. Lack of suitable external standards made it impossible to quantify all compounds; hence, levels of identified compounds not having a direct match with a standard are expressed as standard equivalents, based on different compound classes. The apex-track algorithm (Empower2 software, Waters) was applied for all subsequent peak integrations. Minimum detectable peak area

for PDA was set to 80,000 peak area units, while for fluorescence it was set to 10,000 peak area units. Both values are well above the instrument detection limit. Calibration curves were extrapolated ±30% in both directions (linear range of the detector), and sample responses were quantified only if their peak area fell within the extrapolated range. The instrument calibration was verified periodically by analyzing a known concentration (0.5 mM) of catechin as check standard. To ensure consistency, standards, samples and check standards were run in the same session. A separate standard calibration curve (one replicate per point) was run at the end of the sample analysis. The observed R^2 values for this one replicate-based calibration curve were >0.99 for all standards except for abietic acid ($R^2 = 0.988$).

Lignin analysis

Pellets from the phenolic and resin acids extraction were processed for lignin extraction as described in Bonello and Blodgett (2003), with modifications. Centrifugation in all of the following steps was carried out in an Eppendorf 5415D centrifuge at 16,100 *g* for 5 min. Pellets were washed once each with 1 ml of water and methanol, washed with 0.9 ml of *tert*-butyl methyl ether (Sigma-Aldrich) and dried overnight. Pellets were then hydrolyzed at 40 °C for 21 h on a shaker in 400 µl of 1 N NaOH (Sigma-Aldrich). The reaction mixture was acidified with 200 µl of 1.5 M formic acid (Sigma-Aldrich), followed by 400 µl of methanol. Pellets were then washed once with 1 ml of water and resuspended in 1 ml of 2 N HCl and 0.3 ml of thioglycolic acid (Sigma-Aldrich). Eppendorf tubes containing the solutions were placed in an oven at 86 °C for 4 h and the supernatants were discarded. Pellets were rinsed twice with 1.5 ml of water, resuspended in 1 ml of 0.5 N NaOH, and the tubes were placed on a shaker for 19 h at 200 rpm. The supernatants were saved. The pellets were again suspended in 0.5 ml of 0.5 N NaOH for 19 h and the supernatants were pooled. The solutions were acidified with 0.3 ml of concentrated HCl at room temperature for 4 h and supernatants were discarded. The lignin–thioglycolic acid pellets were dried overnight and then dissolved in 1 ml of 0.5 N NaOH. In all steps, Eppendorf tubes were vortexed after adding solutions, and supernatants and pellets were separated by centrifugation. The solution was diluted 40 times with 0.5 N NaOH and lignin concentration was determined spectrophotometrically at 280 nm, against a five-point standard curve ($R^2 > 0.99$) of spruce lignin (Sigma-Aldrich) (Bonello et al. 1993).

Analysis of terpenoids

For terpenoids analysis, 0.1 g FW of the ground tissue was placed in a 20 ml glass vial and extracted for 24 h at 28 °C in 1.5 ml of *n*-pentane (Sigma-Aldrich) with tridecane 0.05 g l^{-1} (Sigma-Aldrich) as internal standard (Raffa and Smalley 1995). Glass vials were sealed with a Teflon-coated crimped aluminum cap (Perkin-Elmer, Norwalk, CT, USA). One milliliter of extracted solution from each sample was then filtered and transferred to a sealed 2 ml glass vial (Perkin-Elmer). Vials were stored at –20 °C until analyzed. Analyses were performed by gas chromatography–flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC equipped with an automatic sampler for liquid sample injections. The separation of the different enantiomeric monoterpenes was performed on a 30 m Cyclodex-B capillary, 0.25-mm-diameter column (J&W Scientific, Folsom, CA, USA). Cyclodextrins and their derivatives are useful in inducing asymmetric reactions and have been used extensively as stationary phase in chromatographic enantioselective separation of a wide variety of chiral compounds (Allahverdiev et al. 1999). Analysis conditions, modified from Bonello et al. (2008), were as follows: H_2 (carrier gas) at 2 ml min^{-1}; injector temperature 230 °C; flame ionization detector temperature 250 °C. The oven temperature programming started at 40 °C (isothermal, 3 min), and increased linearly to 200 °C at 1 °C min^{-1}; the final temperature of 200 °C was maintained for 10 min (total run time 173 min). For each sample run, 1 µl of extract was injected in the GC. Between each sample run, a blank run (only *n*-pentane) was performed. Data acquisition and subsequent processing were performed using TotalChrom™ 6.2.0.0.0.:B27 chromatography software (Perkin-Elmer). Terpenoids (mono and sesquiterpenes) were identified by comparing their retention times with those of high-purity standards under the same conditions: (–)-α-pinene, (+)-α-pinene, (–)-β-pinene, (+)-β-pinene, (–)-limonene, (+)-limonene, (–)-sabinene, myrcene, *p*-cymene, γ-terpinene and terpinolene (Fluka, Sigma-Aldrich), Δ-3-carene, (+)-camphene, β-caryophyllene and α-humulene (Sigma-Aldrich). The absolute amounts of individual terpenoids were determined by comparison with the internal standard, and expressed as µg g^{-1} FW as tridecane equivalent. The minimum peak area detection limit for quantification was set to 1000 peak area units, which is 10 times above the instrument detection limit.

Statistical analysis

To test if the growing conditions were homogeneously distributed among the treatments we used one-way analysis of variance (ANOVA) on plant height and current year shoot length distribution.

To test the effects of treatment, plant height and shoot length on lesion length we used a general linear model. We initially tested interactions and main effects, simplifying the model if there were no significant interactions. If a significant treatment effect was observed, Tukey's honestly significant difference (HSD) test was used to compare the different treatments.

For metabolites (i.e., lignin, individual phenolics and individual terpenoids), we first compared constitutive concentration among the treatments measured at T_0 and control measured at T_1, using one-way ANOVA. This preliminary analysis showed that constitutive metabolite concentration did not differ between treatments at T_0 (homogeneous starting conditions), and that all treatments at T_0 did not differ from the control at T_1, even though high variability was evident (data not shown). Consequently, we could assume that changes between T_0 and T_1 were due only to the treatment effect, and analyses of inducible variation were based on the difference (Δ) between induced and constitutive metabolite concentration ($T_1 - T_0$), at both sampling positions (L and S). This experimental design, modified from Lombardero et al. (2000), was chosen in order to control for the high variability found in the constitutive metabolite profiles.

Main effects and interaction between treatment and sampling position on inducible variation were analyzed using linear mixed-effect models, incorporating sampling tree as a random factor, in order to account for the nested nature of the experimental design. If not significant, the interaction was removed.

To test if the induction (meant as a generic stress) had produced a significant variation of the constitutive amount of metabolites, irrespective of the treatments, Δ data for all treatment were pooled per sampling position and *t*-test was used to test if the Δ was different from 0, separately for L and S position.

Constitutive amounts and Δ of individual phenolics and individual terpenoids were $\ln(a + x)$ transformed to meet model assumptions, where a was a natural number big enough to avoid negative values. Results were anti-$\ln(x) - a$ back-transformed for graphical representation and discussion, and expressed as mean values and 95% confidence interval (CI), since standard error is not valid after anti-ln transformation (Bland and Altman 1996). Data were analyzed in R (R Development Core Team 2011).

For all the analyses described above, we preliminarily tested whether the results were consistent between the two trials. We therefore tested the interaction between trial (27 May vs. 30 May) and all the other factors in all of the analyses. As this interaction was never significant, the factor trial was excluded from the models, and all data were pooled, resulting in 10 replicates per treatment.

In all analyses we used a conservative significance threshold of $\alpha = 0.01$. No Bonferroni correction was used. The mathematical, logical and practical arguments against applying Bonferroni correction in ecological studies can be found in detail in Moran (2003) and references therein.

One tree was removed from all the analyses, after it was identified as an outlier with Grubb's test ($P < 0.001$). This tree had extremely high amounts of constitutive metabolites (i.e., comparable to inducible levels in other plants) that were probably due to a pre-existing infection or stress condition of that plant.

Results

Tree growth, fungal re-isolation and lesion length

Plant height and shoot length (data not shown) did not vary in relation to the treatment ($F_{4,44} = 0.17$, $P = 0.953$ and $F_{4,44} = 0.94$, $P = 0.449$, respectively), confirming that the growing conditions were homogeneously distributed among the treatments.

The re-isolation rate of the inoculated fungi from the tissues surrounding the inoculum sites was 81.5% for *O. brunneo-ciliatum* and 17.4% for *H. macrospora*.

While relatively small, all lesions produced in response to the fungal inoculations were similar and significantly longer than those produced in response to wounding alone, independently of the inoculated species (Figure 1). There were no associations between plant height and lesion length, and between shoot length and lesion length ($F_{1,33} = 4.56$, $P = 0.040$ and $F_{1,33} = 0.07$, $P = 0.796$, respectively, data not shown). There were no interactions on lesion length among the factors ($P > 0.05$).

Phenolics and resin acids

We analyzed and quantified 20 different phenolic compounds (Table 1 and Supplementary Table S1), all of which have been previously detected in the phloem of Scots or Austrian pine (Lieutier et al. 1996, Sierota et al. 1998, Karonen et al. 2004*a*, 2004*b*, Wallis et al. 2008, 2011), except for unknown compound 1. Unknown compound 2, which had been previously detected also by Wallis et al. (2008), is likely another stilbene

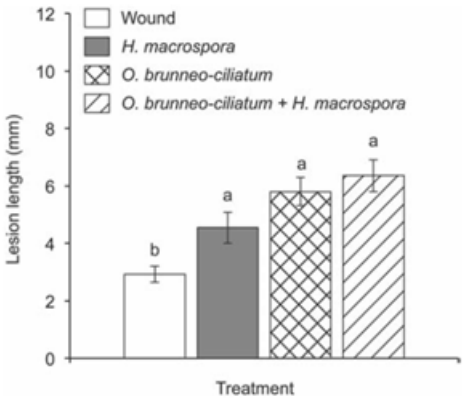

Figure 1. Lesion length (mean ± SE) in *P. sylvestris* measured 3 weeks after the treatment ($F_{3,33} = 13.42$, $P < 0.001$). Different letters represent significant differences according to Tukey's HSD test at $\alpha = 0.05$.

Table 1. Constitutive amounts and inducible variation of identified phenolic compounds, resin acids and lignin in the outer bark and phloem of *P. sylvestris*.

Compound	Constitutive composition T_0 (µg g^{-1} FW)		Inducible variation $T_1 - T_0$ (µg g^{-1} FW)					
	Mean and $CI_{95\%}$		Local mean and $CI_{95\%}$		df, *P* value	Systemic mean and $CI_{95\%}$		df, *P* value
Vanillic acid hexoside	24.5	(18.8 to 30.3)	0.01	(−6.8 5 to 6.83)	38, 0.999	9.9[2]	(2.6 to 17.2)	38, **0.009**
Catechin hexoside	524.4	(447.2 to 602.2)	−173.1[6]	(−247.1 to −98.5)	37, < **0.001**	−157.3[6]	(−239.1 to −74.8)	37, < **0.001**
Procyanidin dimer (B type)	1071.8	(938.7 to 1206.5)	−56.8	(−163.1 to 50.6)	38, 0.291	180.2[2]	(48.5 to 313.6)	38, **0.009**
Epi/Catechin	3378.1	(2922.8 to 3849.6)	−311.1	(−719.6 to 115.3)	38, 0.146	360.2	(−97.6 to 839.1)	38, 0.121
Procyanidin dimer (B2)	1170.4	(950.0 to 1395.4)	−111.9	(−369.4 to 152.5)	38, 0.394	516.9[2]	(187.3 to 857.1)	38, **0.003**
Ferulic acid hexoside	420.9	(362.1 to 480.0)	34.0	(−29.1 to 97.4)	38, 0.283	101.6[2]	(38.1 to 165.4)	38, **0.002**
Procyanidin trimer (C type)	2099.5	(1858.8 to 2345.2)	126.1	(−134.5 to 393.6)	38, 0.337	468.3[2]	(174.0 to 771.0)	38, **0.002**
Hydroxypropiovanillone hexoside	52.1	(39.6 to 64.5)	5.4	(−5.9 to 16.7)	38, 0.332	9.2	(−6.8 to 25.2)	38, 0.252
Unknown 1	124,165[1]	(103,966 to 146,186[1])	−14279[1]	(−42,295 to 23,839[1])	38, 0.410	48,335[1,2]	(23,856 to 76,977[1])	38, < **0.001**
Lignan hexoside	1349.5	(1078.4 to 1627.3)	−111.4	(−424.8 to 212.2)	37, 0.485	303.3	(−19.0 to 635.9)	38, 0.064
Taxifolin hexoside	683.9	(601.6 to 0766.9)	−153.8[6]	(−226.8 to −80.3)	38, < **0.001**	−28.9	(−109.8 to 52.8)	38, 0.478
Taxifolin	982.0	(830.6 to 1135.4)	−292.2[6]	(−437.0 to −145.3)	38, < **0.001**	26.6	(−173.0 to 230.3)	38, 0.790
Procyanidin dimer (A type)	723.5	(585.2 to 863.7)	−56.1	(−108.4 to 223.3)	37, 0.497	456.1[2]	(266.4 to 649.5)	38, < **0.001**
Pinosylvin	0.3	(0.1 to 0.6)	58.8[5]	(46.0 to 71.5)	38, < **0.001**	2.3[3]	(0.7 to 3.9)	38, **0.006**
Pinocembrin	3.4	(1.2 to 5.7)	17.0[3]	(10.7 to 23.3)	38, < **0.001**	4.1	(−0.1 to 8.4)	37, 0.060
Pinosylvin monomethyl ether	1.2	(0.3 to 2.2)	57.3[4]	(44.7 to 70.0)	38, < **0.001**	5.8	(−0.5 to 12.1)	38, 0.068
Unknown 2	35,030[1]	(24,085 to 46,939[1])	68,1625[1,4]	(528,034 to 872,777[1])	38, < **0.001**	8762[1]	(−4596 to 23,990[1])	38, 0.202
Abietic acid	880.6	(346.6 to 1442.2)	23,797.8[4]	(20,146.6 to 27,891.2)	28, < **0.001**	297.3	(−984.3 to 1761.0)	31, 0.656
Lignin	50,005.6	(45,500.3 to 54,510.9)	−13,347,8[6]	(−17,694.6 to −9001.0)	38, < **0.001**	−7333.1[6]	(−11,891.5 to −2774.8)	38, **0.002**

The constitutive amount corresponds to T_0. Inducible variation corresponds to the difference between induced and constitutive metabolite concentrations ($T_1 - T_0$). Since treatment did not have a significant effect on inducible variation for most compounds (see Table 2), data for all treatments were pooled for both sampling positions. Caffeic acid derivative, *trans*-coumaric acid and ferulic acid were below the PDA detection limit for quantification (80,000 peak area units). Neoabietic acid coeluted with an unidentified compound and was not quantified. Anti-ln back-transformed mean values (µg g^{-1} FW) are shown.

P values <0.01 are highlighted in bold.

[1]Peak area.

[2]Induced increase from 10 to 100% of constitutive value.

[3]Induced increase from 100 to 1000% of constitutive value.

[4]Induced increase from 1000 to 10,000% of constitutive value.

[5]Induced increase >10,000% of constitutive value.

[6]Induced decrease from 10 to 100% of constitutive value.

due to its strong autofluorescence and similarity of UV spectrum to those of pinosylvin and pinosylvin monomethyl ether (data not shown).

Treatment had no significant effect on inducible variation of most phenolics (Table 2). The only exception was pinosylvin monomethyl ether, for which the local increase in response to wounding alone was significantly lower than in response to inoculation with both fungi together (Figure 2). A similar but non-significant response was also observed for pinosylvin (Table 2). On the other hand, sampling position (local vs. systemic) had a strong effect on inducible variation of most phenolics, except for vanillic acid hexoside, catechin hexoside, ferulic acid hexoside, procyanidin trimer and hydroxypropiovanillone hexoside (Table 2). However, there was no significant interaction between treatment and sampling position.

After pooling all treatments, *t*-tests on the Δ data, separated by sampling position, showed that almost all phenolics responded significantly to at least one of the sampling positions, except for epi/catechin, hydroxypropiovanillone hexoside and lignan hexoside (Table 1). The stilbenoid pinosylvin, which was present only in trace amounts constitutively (0.3 μg g^{-1} FW, $CI_{95\%}$ = 0.1–0.6), had the strongest response at both sampling positions. The local increase was >10,000% of the constitutive amount, and the one in the systemic position was >600%. Pinosylvin monomethyl ether and unknown 2 showed strong increases as well (up to 5000%), but only locally. Pinocembrin also increased locally, up to 500% over the constitutive

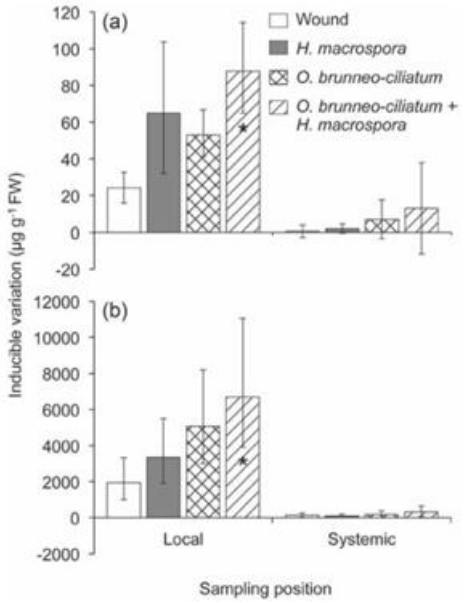

Figure 2. Effects of different treatments on the inducible variation of (a) pinosylvin monomethyl ether ($F_{3,35}$ = 6, P = 0.002) and (b) (+)-α-pinene ($F_{3,34}$ = 5.49, P = 0.003). Inducible variation corresponds to the difference between induced and constitutive concentrations ($T_1 - T_0$), in the outer bark and phloem of *P. sylvestris*. Anti-ln back-transformed mean values (μg g^{-1} FW) are shown. Bars represent $CI_{95\%}$. Asterisks denote significant differences (α = 0.01) with the wound treatment (contrasts from the linear mixed-effect model).

Table 2. Results from the linear mixed model testing the effects of treatment and sampling position on the inducible variation of phenolics, abietic acid and lignin (i.e., difference between induced and constitutive concentrations), in the outer bark and phloem of *P. sylvestris*. *P* values <0.01 are highlighted in bold.

Compound	Treatment			Sampling position		
	df	*F* value	*P* value	df	*F* value	*P* value
Vanillic acid hexoside	3, 35	0	0.899	1, 38	6	0.018
Catechin hexoside	3, 34	1.2	0.333	1, 37	0.3	0.561
Procyanidin dimer (B type)	3, 35	0.71	0.555	1, 38	14.43	< **0.001**
Epi/Catechin	3, 35	0.4	0.754	1, 38	10.82	**0.002**
Procyanidin dimer (B2)	3, 35	0.79	0.510	1, 38	14.12	< **0.001**
Ferulic acid hexoside	3, 35	1.1	0.347	1, 38	6.3	0.016
Procyanidin trimer (C type)	3, 35	0.33	0.807	1, 38	5.08	0.030
Hydroxypropiovanillone hexoside	3, 35	0	0.789	1, 38	0	0.667
Unknown 1	3, 35	0.85	0.478	1, 38	13.00	< **0.001**
Lignan hexoside	3, 35	0.22	0.879	1, 37	21.81	< **0.001**
Taxifolin hexoside	3, 35	2.8	0.055	1, 38	12.6	**0.001**
Taxifolin	3, 35	1.2	0.324	1, 38	9.4	**0.004**
Procyanidin dimer (A type)	3, 35	2.51	0.074	1, 37	27.02	< **0.001**
Pinosylvin	3, 35	3	0.037	1, 38	86	< **0.001**
Pinocembrin	3, 35	3	0.055	1, 37	20	< **0.001**
Pinosylvin monomethyl ether	3, 35	6	**0.002**	1, 38	66	< **0.001**
Unknown 2	3, 35	0.38	0.769	1, 38	239.09	< **0.001**
Abietic acid	3, 31	1.47	0.241	1, 25	184.15	< **0.001**
Lignin	3, 35	0.34	0.798	1, 38	7.64	**0.009**

amount. Catechin hexoside decreased slightly, but significantly, both locally and systemically, whereas taxifolin and taxifolin hexoside decreased slightly, but only locally. Vanillic acid hexoside, ferulic acid hexoside, unknown 1 and all procyanidin derivatives did not vary locally, but showed a slight increase, <70% of the constitutive amount, systemically (Table 1).

Among the resin acids, we identified abietic acid and neoabietic acid (Supplementary Table S1), but the latter coeluted with an unidentified compound, and therefore we were able to quantify only abietic acid (Table 1). Treatment had no significant effect on inducible variation of this resin acid, while sampling position did (Table 2). There was no significant interaction between treatment and sampling position ($F_{3,22} = 0.96$, $P = 0.430$). After pooling all treatments, *t*-tests on the Δ data, separated by sampling position, showed that after induction abietic acid increased very strongly, over 2000%, but only locally (Table 1).

Lignin

The constitutive amount of lignin were 50.0 mg g^{-1} FW ($CI_{95\%}$ = 45.5–54.5). Sampling position, but not treatment, had a significant effect on the inducibility of lignin concentration (Table 2), and there was no interaction between the two factors ($F_{3,35} = 1.2$, $P = 0.325$). After pooling all treatments, *t*-tests on the Δ data, separated by sampling position, showed that after the induction lignin decreased slightly, but significantly, both locally and systemically (Table 1).

Terpenoids

A total of 18 terpenoids were analyzed from the *n*-pentane extract (Table 3), all of which have been detected in previous studies in Scots, Austrian, jack and/or red pine (Delorme and Lieutier 1990, Raffa and Smalley 1995, Sadof and Grant 1997, Fäldt et al. 2006, Wallis et al. 2008), except for three unknown compounds (i.e., 3, 4 and 5)

Similar to lignin, abietic acid and phenolics, treatment had no significant effect on inducible variation of most terpenoids (Table 4), except for (+)-α-pinene, for which the local increase in response to wounding alone was significantly lower than in response to the fungal complex (Figure 2). Similar responses were observed for (+)-β-pinene and *p*-cymene as well, but not significantly (Table 4). Sampling position had again a strong effect on inducible variation of almost all compounds, except for (–)-β-pinene (Table 4), but there was no interaction between sampling position and treatment.

After pooling all treatments, *t*-tests on the Δ data separated by sampling position showed that all terpenoids significantly increased at least in one of the sampling positions ($P < 0.01$) (Table 3). Locally, myrcene had the strongest response, with an increase higher than 100,000% of constitutive amount. (–)-α-Pinene, (+)-α-pinene, (+)-camphene, (–)-sabinene, Δ-3-carene, (–)-limonene, (+)-limonene, *p*-cymene, terpinolene, β-caryophyllene, α-humulene, unknown 4 and unknown 5 increased up to 3500% in the local position, whereas (+)-β-pinene and unknown 3 increased up to 1000%. (–)-β-Pinene was the only compound that did not increase locally ($P = 0.449$), but had a systemic increase higher than 150%. (–)-α-Pinene, (+)-α-pinene, myrcene, *p*-cymene, terpinolene and β-caryophyllene had a significant increase systemically as well, up to 200% of constitutive amount. α-Humulene too had a similar systemical increase, although the significance was marginal ($P = 0.010$) (Table 3).

Discussion

In this paper we show that in the system Scots pine–*I. acuminatus*-associated fungi, the nutritional fungus *H. macrospora* triggers an inducible defense reaction comparable to the one induced by the blue-stain fungus *O. brunneo-ciliatum*, since metabolic responses of the plant, even though strong, showed no differentiation according to the treatment. There was also no overall significant evidence that the fungal species had a synergistic effect on tree defense induction, even though some compounds suggest that behavioral pattern.

Most of the identified and quantified compounds responded locally and/or systemically to the induction. Among the phenolics, pinosylvin, pinosylvin monomethyl ether, unknown 2 and pinocembrin responded most strongly locally, showing an increase of up to 10,000% or more relative to constitutive concentrations. Stilbenes and flavonoids are well known to be widely involved in conifer defense mechanisms against pathogens (Bonello et al. 1993, Lieutier et al. 1996, Bonello and Blodgett 2003, Franceschi et al. 2005), to be associated with disease states (Wallis et al. 2008) and to display strong antifungal activity in in vitro assays (Bonello et al. 1993, Seppanen et al. 2004). Moreover, pathogen-induced phenolics can have indirect effects on the performance of the beetle vectors, e.g., *I. typographus* (L.) on spruce (Faccoli and Schlyter 2007) and *I. cembrae* (Heer) on larch (Rohde et al. 1996). Almost all other phenolic compounds found in this study also appeared involved in the induced response, although their inducible variation was lower than 100% of constitutive amount, perhaps suggesting a limited significance in defense. Lignin decreased after induction both locally and systemically, as found in a previous study (Bonello et al. 2003).

Among the terpenoids, myrcene had the strongest response, with the local increase higher than 100,000% of constitutive amounts. Still, all other terpenoids were also characterized by a strong increase after induction, generally only locally. Furthermore, the enantiomeric composition of β-pinene changed following induction. Locally, only (+)-β-pinene increased, becoming the most prevalent enantiomeric form, whereas (–)-β-pinene was the most prevalent form in the constitutive state. Conversely, only (–)-β-pinene increased

Table 3. Chromatographic data, assigned identities, constitutive amounts and inducible variation of terpenoids isolated from outer bark and phloem of *P. sylvestris*.

Peak number	RT	Assigned identity	Constitutive composition T_0 (µg g^{-1} FW)		Inducible variation $T_1 - T_0$ (µg g^{-1} FW)				
			Mean and $CI_{95\%}$		Local mean and $CI_{95\%}$	df, *P* value	Systemic mean and $CI_{95\%}$		df, *P* value
1	10.57	(–)-α-Pinene	72.0	(48.9 to 95.5)	1381.5[2]	(1071.6 to 1737.9)	37, **<0.001**	87.0[1]	(46.0 to 129.8)	37, **<0.001**
2	10.80	(+)-α-Pinene	160.8	(109.6 to 214.5)	4028.6[2]	(3057.2 to 5232.6)	37, **<0.001**	182.2[1]	(99.2 to 271.5)	37, **<0.001**
3	12.12	(+)-Camphene	3.7	(1.9 to 5.5)	49.3[2]	(37.7 to 61.1)	37, **<0.001**	6.4	(0.1 to 12.8)	37, 0.048
4	12.36	(–)-Sabinene	3.6	(1.9 to 5.4)	49.0[2]	(34.2 to 63.9)	37, **<0.001**	2.3	(–1.7 to 6.3)	37, 0.245
5	14.09	Myrcene	1.7	(0.7 to 2.7)	1915.5[3]	(1247.0 to 2782.8)	37, **<0.001**	3.0[1]	(1.1 to 4.8)	37, **0.002**
6	14.35	(–)-β-Pinene	77.1	(48.3 to 106.6)	32.1	(–50.7 to 122.2)	37, 0.449	126.6[1]	(68.5 to 187.8)	37, **<0.001**
7	15.09	(+)-β-Pinene	27.7	(19.4 to 35.9)	135.2[1]	(105.3 to 165.8)	37, **<0.001**	17.7	(–2.8 to 38.6)	37, 0.088
8	15.80	Δ-3-Carene	40.6	(14.3 to 67.7)	622.9[2]	(332.5 to 976.5)	37, **<0.001**	29.9	(2.6 to 57.9)	37, 0.032
9	17.89	(–)-Limonene	3.9	(1.9 to 6.0)	73.6[2]	(42.9 to 105.2)	37, **<0.001**	7.4	(1.6 to 13.3)	37, 0.014
10	18.01	(+)-Limonene	2.6	(1.4 to 3.7)	35.4[2]	(22.3 to 48.8)	37, **<0.001**	–0.2	(–1.6 to 1.3)	37, 0.826
11	18.63	*p*-Cymene	8.0	(3.9 to 12.0)	149.1[2]	(105.6 to 194.4)	37, **<0.001**	13.2[1]	(4.6 to 21.9)	37, **0.004**
12	20.40	γ-Terpinene	–	–	–	–	–	–	–	–
13	22.83	Terpinolene	3.9	(1.6 to 6.1)	119.1[2]	(74.4 to 165.7)	37, **<0.001**	5.4[1]	(1.7 to 9.1)	37, **0.006**
14	42.75	Unknown 3	6.1	(2.6 to 9.6)	45.6[1]	(27.1 to 64.5)	37, **<0.001**	8.4	(–4.0 to 1.0)	37, 0.175
15	52.28	β-Caryophyllene	1.3	(0.5 to 2.2)	24.1[2]	(13.2 to 35.1)	37, **<0.001**	2.2[1]	(0.7 to 3.6)	37, **0.004**
16	55.79	α-Humulene	1.0	(0.3 to 1.7)	34.4[2]	(12.6 to 56.8)	37, **0.003**	1.9[1]	(0.5 to 3.3)	37, **0.010**
17	58.22	Unknown 4	16.2	(9.7 to 22.9)	310.2[2]	(215.1 to 412.7)	37, **<0.001**	10.6	(1.4 to 20.0)	37, 0.025
18	58.46	Unknown 5	17.2	(10.4 to 24.1)	177.6[2]	(122.0 to 236.0)	37, **<0.001**	2.4	(–6.3 to 11.2)	37, 0.577

Concentrations are expressed as tridecane equivalent. Compounds were identified using standards. The constitutive amount corresponds to T_0. Inducible variation corresponds to the difference between induced and constitutive metabolite concentrations ($T_1 - T_0$). Since treatment did not have a significant effect on inducible variation for most compounds (see Table 4), data for all treatments were pooled for both sampling positions. γ-Terpinene was below detection limit for quantification (1000 peak area units). Anti-ln back-transformed mean values (µg g^{-1} FW) are shown.

P values ≤0.01 are highlighted in bold. RT, retention time.

[1]Induced increase from 100 to 1000% of constitutive value.

[2]Induced increase from 1000 to 10,000% of constitutive value.

[3]Induced increase >100,000% of constitutive value.

Table 4. Results from the linear mixed model testing the effects of treatment and sampling position on the inducible variation of terpenoids (i.e., difference between induced and constitutive concentrations) in the outer bark and phloem of *P. sylvestris*. *P* values <0.01 are highlighted in bold.

Compound	Treatment			Sampling position		
	df	*F* value	*P* value	df	*F* value	*P* value
(–)-α-Pinene	3, 34	1.98	0.135	1, 37	153.52	< **0.001**
(+)-α-Pinene	3, 34	5.49	**0.003**	1, 37	198.73	< **0.001**
(+)-Camphene	3, 34	2.14	0.114	1, 37	47.80	< **0.001**
(–)-Sabinene	3, 34	2.22	0.104	1, 37	41.31	< **0.001**
Myrcene	3, 34	0.99	0.407	1, 37	69.27	< **0.001**
(–)-β-Pinene	3, 34	1.27	0.299	1, 37	3.24	0.079
(+)-β-Pinene	3, 34	2.48	0.078	1, 37	55.10	< **0.001**
Δ-3-Carene	3, 34	0.47	0.706	1, 37	23.86	< **0.001**
(–)-Limonene	3, 34	0.53	0.666	1, 37	24.01	< **0.001**
(+)-Limonene	3, 34	1.75	0.176	1, 37	31.19	< **0.001**
p-Cymene	3, 34	2.55	0.072	1, 37	48.51	< **0.001**
Terpinolene	3, 34	1.46	0.244	1, 37	28.67	< **0.001**
Unknown 3	3, 34	0.31	0.814	1, 37	15.88	< **0.001**
β-Caryophyllene	3, 34	0.50	0.682	1, 37	17.57	< **0.001**
α-Humulene	3, 34	2.77	0.056	1, 37	9.81	**0.003**
Unknown 4	3, 34	0.82	0.493	1, 37	50.14	< **0.001**
Unknown 5	3, 34	0.66	0.578	1, 37	43.44	< **0.001**

systemically, augmenting the difference between the two forms that was already present in the constitutive state. An induced systemic increase of (–)-β-pinene had already been reported in a study by Fäldt et al. (2006), which reports an analysis of the effects of fungal infection and wounding on contents and enantiomeric composition of monoterpenes in Scots pine. The role of terpenoids, including resin acids, in conifer defense against bark beetle-associated fungi is well known (Raffa and Smalley 1995, Franceschi et al. 2005, Zeneli et al. 2006), and several in vitro experiments have shown their toxic effects on fungi (Delorme and Lieutier 1990, Blodgett and Stanosz 1997, Kopper et al. 2005). γ-Terpinene, α-pinene and myrcene, for example, inhibit growth and germination of conifer pathogens (Raffa et al. 1985, Klepzig et al. 1996). Besides affecting associated fungi, many terpenoids are also involved in constitutive and induced defenses against the bark beetles themselves (Faccoli et al. 2005, Seybold et al. 2006). Lastly, the role of terpenoids in conifer defense does not only relate to their direct toxicity, but also, indirectly, to attraction/repellence of the beetles or their enemies (reviewed in Keeling and Bohlmann 2006).

In our experiment, we found a strong effect of sampling position. As expected, most compounds responded more strongly locally than systemically, but some phenolics behaved the opposite way (e.g., procyanidins), suggesting qualitative, besides quantitative, differences between localized and systemic-induced responses (Eyles et al. 2010). On the other hand, we found no defense differentiation according to the invading agent, either as a single species or as a fungal complex. Indeed, average lesion length was similar for all fungal species combinations; however, it was always longer than when resulting from wounding alone. This was true even for *H. macrospora*, in spite of very low re-isolation percentage from the tissues surrounding the lesion site. This suggests that low recovery was not a result of failed inoculation (escape) but was, instead, a reflection of the slow growth rate of *H. macrospora* (Batra 1967). However, induced chemical composition did not vary, not only among fungal species combination, but also between wounding and inoculations. The only exceptions were pinosylvin monomethyl ether and (+)-α-pinene, for which the local increase in response to the fungal complex was higher than in response to wounding alone. Under the conditions of our study, the lack of a specific response for most compounds can be interpreted as Scots pine having a generic, rather than specific, induced response to this bark beetle system. This is in accordance with other studies on Scots pine (Croisé et al. 1998, Krokene et al. 2000, Fäldt et al. 2006), and supports the hypothesis that although plants can specifically recognize the nature of pathogens, the optimal defense strategy is to activate all available defense mechanisms, so that at least some may be effective against a particular pathogen (Katagiri 2004). Furthermore, the inoculated species, even though performing different ecological roles, are taxonomically close (Rollins et al. 2001, Harrington et al. 2010) and could have triggered similar signaling molecules (Eyles et al. 2010). Some authors even maintain that chemical changes are involved in response to wounding rather than in response to the challengers themselves, and that the type of aggressor elicits only variations in rapidity and extension of the response (Mullick 1977, Lieutier 2004). This 'shoot first—ask questions later' strategy is, however, energetically

expensive, and may not be very advantageous for the plant in at least some antagonistic interactions (Keeling and Bohlmann 2006, Bolton 2009).

It must be noted that, working in a natural stand, we found very high levels of variability both in the constitutive and induced metabolite composition, similarly to Sierota et al. (1998) and Fäldt et al. (2006). Results, especially for monoterpenes, stilbenes and flavonoids, seem to follow a pattern in which the inducible response is least for wounding alone, increases with inoculations with single species and is greatest with the fungal complex, suggesting synergism of the two species. But due to high variability, this pattern is not statistically supported for most traits, except for lesion length, pinosylvin monomethyl ether and (+)-α-pinene. In some other cases, such as with pinosylvin, (+)-β-pinene and *p*-cymene, the non-significance is only marginal. The possibility of synergistic effects of the bark beetle fungal associates would give more importance to the complex itself, besides the roles that have been already confirmed for the single species (Lieutier et al. 2009).

In conclusion, our results reveal how even a non-pathogenic, nutritional fungus directly useful for larval development, may also indirectly assist in the beetle establishment process in Scots pine by participating in exhausting host plant defenses. Similar results have been found in other model systems, such as the southern pine beetle-associated complex (Hofstetter et al. 2005), *Scolytus ventralis* LeC. and *Trichosporium symbioticum* Wright (Raffa and Berryman 1982) and *Dendroctonus brevicomis* LeC. and its associated yeast *Ogataea pini* (Holst) Yam., Mat., Maed. et Mik. (Davis and Hofstetter 2011). All these systems show how fungi other than blue-stain fungi can strongly stimulate plant defense. In addition to these, our results therefore provide new implications in the debate on the importance of bark beetle-associated fungi (Lieutier et al. 2009, Six and Wingfield 2011). A new ecological role of non-pathogenic and nutritional fungi, that is the capacity to interact with the host plant in a way that facilitates brood establishment, is hence proposed. This model implies that, in stimulating plant responses to rapidly exhaust tree resistance, the lack of specificity in the host response to beetle-associated fungi can only be an advantage for the insects. Our study provides an initial assessment of the role potentially played by nutritional fungi such as *H. macrospora*. However, a better understanding of the virulence of the nutritional fungus would be helpful to better assess this system. Furthermore, confirmatory work, including more replicates and different associations of tree–bark beetle–fungi, is necessary in order to assess the generality of our findings.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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Supplementary data

Operating conditions for the LC-ESI-MS and the HPLC-UV

Operating conditions for the LC-ESI-MS were modified from Chen et al. (2011). The separation was carried out using a 250 mm Thermo Scientific Hypersil ODS (C18) column (particle size 5 μm, I.D 4.6 mm) connected to a 10 x 4 mm drop-in guard cartridge (C18) (Thermo Fisher Scientific, Waltham, MA, USA). The binary mobile phase consisted of 0.1% acetic acid in water (solvent A), and 0.1% acetic acid in methanol (Solvent B), with a flow rate of 1 ml min^{-1}. The following linear gradient (cumulative run time (min), flow rate (ml min^{-1}), % solvent A) was used: 0.0, 1.0, 95.0; 19.0, 1.0, 65.0; 20.0, 1.0, 45.0; 25.0, 1.0, 40.0; 35.0, 1.0, 4.0; 38.0, 1.0, 0.0; 40.0, 1.0, 100.0; 42.0, 1.0, 100.0 (total run time 42 min). A 10 μl plant extract sample from a pool of concentrates was injected and analyzed by LC-MS. A post-column splitting (approximately 7:3 v/v) device was used to divide the LC effluent through the PDA detector and the electrospray source. Ions in the MS were introduced by employing a negative ion ESI mode with a capillary voltage of −80 V, a needle voltage of −5 kV and a spray shield voltage of 80 V. In the initial screening, survey scan was set to m/z 50 – 1,000. The MS Workstation software (ver 6.9.2, Varian Inc., Palo Alto, CA, USA) allowed data dependent tandem MS generation (Turbo DDS mode). Each sample was also run in full scan mode to capture most of the precursor ions. Data acquisition and subsequent processing were performed using MS Workstation software.

HPLC-UV separation was carried out using an identical column and guard cartridge used in the LC-MS analyses. The Waters Empower2 software was employed for data acquisition and further analysis. Instrument operating conditions were slightly modified from Bonello and Blodgett (2003). In short, a binary eluting solvent system consisting of 2% acetic acid in water (solvent A) and 2% acetic acid in methanol (solvent B) was used for analyte separation. The linear gradient (cumulative run time (min), flow rate (ml min^{-1}), % solvent A) matched the conditions stated in the LC-MS mentioned above.

The auto-sampler and column temperature were held at 4 °C and 40 °C respectively for all analyses. A 10 μl concentrated soluble phenolic and resin acid extract from a single sample was injected each time for analysis. The PDA detector was set to acquire all the

spectra between 210 and 400 nm while the fluorescence detector was set to 300 nm excitation and 400 nm emission wavelengths for stilbene detection (Bonello and Blodgett 2003). Automated chromatographic outputs at 280 and 241 nm absorbance were extracted simultaneously for phenolics and diterpene resin acids respectively (Bonello and Blodgett 2003; Kersten et al. 2006).

Peaks of interest were identified by a combination of strategies. Full scan mass chromatograms were overlaid with Varian PDA chromatogram traces at 280 nm to match the retention times of $[M-H]^-$ parent ions to λ_{max} of individual compounds. PDA data of individual compounds run on the HPLC-ESI-MS-PDA were then compared with the Waters HPLC chromatogram. Phenolic compounds and diterpene resin acids were identified based on negative ion fragmentation pattern, congruence of λ_{max} and retention time based on standards (Table S1).

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Table S1. Chromatographic, UV, and mass-spectral data, and assigned identities, of phenolic compounds and diterpene resin acids isolated from outer bark and phloem of *Pinus sylvestris*.

Peak number	PDA RT	$[M-H]^-$	Main fragments by ESI-MS	λ_{max} (nm)	Assigned identity	Quantification mode	Standard equivalent	References
1	5.97	329	167, 152, 109	250	Vanillic acid hexoside	PDA 280	Vanillic acid	Karonen et al 2004a, Wallis et al 2011
2	7.02	451	289	277	Catechin hexoside	PDA 280	Catechin	Karonen et al 2004a, Wallis et al 2011
3	8.25	577	425, 407, 451, 289, 408	280	Procyanidin dimer (B type)	PDA 280	Procyanidin B2	Karonen et al 2004b
4	9.85	289	245, 205, 203, 227, 161, 206	280	Epi/Catechin	PDA 280	Catechin	Karonen et al 2004b
5	10.92	577	425, 407	280	Procyanidin dimer (B2)	PDA 280	Procyanidin B2	Verified by standard
6	11.75	355	193	291 sh 316	Ferulic acid hexoside	PDA 280	Ferulic acid	Wallis et al 2011
7	12.46	865	695, 739, 575, 543, 287, 449	277	Procyanidin trimer (C type)	PDA 280	Procyanidin B2	Karonen et al 2004b
8	12.67	357	177, 162	278 sh 305	Hydroxypropiovanillone hexoside	FL	Vanillic acid	Karonen et al 2004a, Wallis et al 2011
9	13.63	179	135, 107	290 sh 322	Caffeic acid derivative[a]	PDA 280	-	Clifford et al. 2007
10	14.73	315	300, 297, 269, 251, 121	280	Unknown 1	PDA 280	-	
11	16.47	495	363, 165, 179, 314, 147	278	Lignan hexoside	PDA 280	Pinoresinol	Karonen et al 2004a, Wallis et al 2011
12	17.09	119	93	310	*Trans*-coumaric acid [a]	PDA 280	-	Verified by standard
13	17.47	465	285, 437, 303, 217, 241, 275, 125, 177	290	Taxifolin hexoside	PDA 280	Taxifolin	Karonen et al 2004a, Wallis et al 2011
14	17.68	303	285, 177, 241, 175, 149, 105	290	Taxifolin	PDA 280	Taxifolin	Verified by standard
15	18.87	193	134, 178, 149	324	Ferulic acid [a]	PDA 280		Verified by standard
16	19.07	575	499, 394, 287, 407	281	Procyanidin dimer (A type)	PDA 280	Procyanidin B2	Karonen et al 2004b
17	25.43	211	169	300	Pinosylvin	FL	Pinosylvin	Verified by standard
18	27.4	255	213, 185, 151	291	Pinocembrin	PDA 280	Pinocembrin	Verified by standard
19	29.91	225	210	300	Pinosylvin monomethyl ether	FL	Pinosylvin monomethyl ether	Verified by standard
20	33.39	317	299, 271	299	Unknown 2	FL	-	
21	35.02	301	257, 283	241	Abietic acid	PDA 241	Abietic acid	Verified by standard
22	36.56	301	ND	251	Neoabietic acid [b]	PDA 241	-	Verified by standard

RT = Retention time; PDA 280 / 241 = Photo Diode Array channel 280 nm / 241 nm; FL = Fluorescence; Sh = shoulder; ND = Not detected; [a] Below detection limit (80,000 peak area units); [b] Coeluted with an unidentified compound.

Chapter V

Correlations within and between constitutive and induced defenses in a natural Scots pine population

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I collected most of the data, contribute to analyzed them and drafted the manuscript.

Abstract

Plants protect themselves from pathogens and herbivores by investing resources in constitutive and inducible defenses. Several plant defense theories predict trade-offs between resource investments in various types of defenses, but empirical research, especially concerning conifers, is still lacking. In this study we exploited the intra-population variability of a natural Scots pine stand to investigate the complexity of constitutive and induced defenses. Correlation patterns within constitutive defense secondary metabolites and potential trade-offs between constitutive concentration and inducible variation of individual chemical compounds were tested. Results revealed that different compounds display different behaviors, and no overall negative associations between defensive traits were found. Constitutive metabolites involved in defense mechanisms, such as terpenoids and stilbenes, displayed positive correlations among each other, and their inducible variation was generally not influenced by their constitutive concentration. However, lignin was negatively correlated with other constitutive chemical defenses, while pinocembrin, (+)-camphene and myrcene displayed trade-offs between constitutive concentration and inducible variation. We also found that some of the compounds not expected to be directly involved in the defense reaction display a complex behavior, i.e., their concentration either increased or decreased in trees with respectively low or high constitutive amount. In conclusion, Scots pine appears to possess a varied and complex defense chemistry whose individual components are not functionally redundant, in accordance to observations for several herbaceous plant species.

Introduction

Trees minimize losses due to pathogens and herbivores by investing resources in both constitutive and inducible defenses (Franceschi et al. 2005). In conifers, these can include resin ducts, stone cells and a broad spectrum of secondary metabolites (Franceschi et al. 2005, Mumm and Hilker 2006). Constitutive defenses are general defenses normally

present in the tree before attack and contribute to repel or inhibit invader access. On the other hand, inducible defenses are triggered by microbial or herbivore activity and act to kill, compartmentalize or reduce the fitness of the invading agent (Berryman 1972, Karban and Myers 1989, Zangerl 2003, Bonello et al. 2006). Given that damaging agent loads can vary in space and time, inducible defenses are thought to be a cost-saving adaptation (Karban and Myers 1989, Agrawal and Karban 1999, Heil 2002, Zangerl 2003). However, just as with constitutive defenses, when activated, inducible defenses also come with a fitness cost (Baldwin 1998, Heil 2002, Koricheva 2002, Zangerl 2003, Keeling and Bohlmann, 2006).

Because of their cost, and thus the necessity for trade-offs with plant growth and reproduction (Herms and Mattson 1992), both constitutive and induced defenses are characterized by phenotypic plasticity (Heil 2010), and current hypotheses on the phenotypic variation in plant defenses are based mainly on the resource limitation that occurs in natural environment (Stamp 2003). In a meta-analysis on regulation of woody plant secondary metabolism by resource availability, Koricheva et al. (1998) proposed a hierarchical model of carbon allocation to growth, storage and carbon-based defense secondary compounds. According to this model, at higher hierarchical level carbon is allocated according to resource availability, as predicted by the general theories (Herms and Mattson 1992). But the proportional allocation of available carbon to individual defense secondary compounds, which correspond to the lower hierarchical levels of carbon flow, rather depends on specific evolutionary responses. In this case trade-offs may arise between different defensive pathways as well as between biosynthetically closely related compounds, if they share the same limiting precursor. However, several studies on woody plants have reported positive correlations among some defense secondary metabolites, including compounds within the same pathway (Keinänen et al. 1999, Duncan et al. 2001, Latta et al. 2003, Thoss et al. 2007), which suggests strong genetic control of tree defensive traits (Keinänen et al. 1999, Duncan et al. 2001, Ruuhola et al. 2001, Iason et al. 2006). Further empirical research is therefore still needed to elucidate the behavior of these mechanisms, by investigating more tree species and more defensive pathways.

Induced defenses can also be negatively correlated with their constitutive concentration, i.e., if plants are well defended by constitutive defenses against a particular agent, they are

not expected to allocate resources to induced defenses against that same agent (Karban and Myer 1989). The existence of a trade-off between constitutive and induced defenses has been partially confirmed by Koricheva et al. (2004), even though the variation in constitutive defense levels explained only c. 20% of variation in the inducible response. This suggests that factors other than constitutive defenses may be more important in determining the magnitude of the inducible response. In spite of the growing attention that constitutive/induced defense trade-offs in herbaceous plants has gained in the last several years (Morris et al. 2006, Bingham and Agrawal 2010, Kempel et al. 2011), this topic is still poorly explored in woody plants (Keinänen et al. 1999, Ruuhola et al. 2001), especially in conifers (but see Lombardero et al. 2000). The few studies available have focused only on generic traits, as resin flow or total phenolics, which has led several authors to point out the importance of working with higher chemical resolution, i.e., at the level of the individual compound, since different compounds can display different behaviors (Keinänen et al. 1999, Ruuhola et al. 2001, Latta et al. 2003, Thoss et al. 2007). Analyses on a wide spectrum of compounds are hence necessary to better assess constitutive/induced trade-offs in conifers.

Several studies have reported considerable within-population variability of constitutive and induced tree defenses in conifers, and this has been attributed more to genetic variability than phenotypic plasticity (Forrest 1980, Sierota et al. 1998, Duncan et al. 2001, Latta et al. 2003, Iason et al. 2005, Fäldt et al. 2006, Thoss et al. 2007). In fact, due to slow growth rates and long generation times of trees, the maintenance of a high intra-population genetic diversity of defense secondary metabolites is hypothesized to be an evolutionary advantage, since it may promote a rapid adaptation to environmental stochasticity (Newton et al. 1999, Firn and Jones 2000). This intra-population variability can be suitably exploited to investigate the behavioral complexity of defensive traits, as it seems to be a modulating factor in the biosynthesis and accumulation of both constitutive and induced defense secondary compounds. The general aim of this study was to examine the behavior of phenolic compounds, lignin, and terpenoids in response to an induction by fungal inoculation within a natural population of Scots pine, *Pinus sylvestris* L., by first investigating correlation patterns within constitutive defense secondary metabolites, and

then by verifying potential trade-offs between constitutive concentration and inducible variation of individual chemical compounds.

Materials and methods

This study emanates from a previous investigation of Scot pine defenses, and the biological results related to this work have already been published (Villari et al. 2012). Thus, the experimental design was described in detail in Villari et al. (2012), and will only be summarized briefly here.

Biological system, treatments and sampling

In spring 2010, 50 Scots pines of similar size and age (~ 20 year old, based on the number of whorl branches) were selected from a young alpine stand in San Vito di Cadore, Belluno Province, Italy (46°29'49''N, 12°10'29''E, 1105 m a.s.l.). The stand was the result of natural regeneration under a power line.

The experiment was carried out in two trials of 25 trees each. Trees were assigned to five different treatments, with five replicates per treatment, in a completely randomized design in each trial. The first trial started on 27 May, the second on 30 May 2010, and they both lasted 3 weeks. Preliminary analyses showed that growing conditions, assessed by measuring plant height and the length of the current year shoot of a randomly chosen branch located in the upper part of the tree crown (Lanner 1976), were homogeneously distributed among the treatments, and that experiment results were consistent between the two trials. The factor trial was hence excluded, and all data were pooled, resulting in 10 replicates per treatment (Villari et al. 2012).

Defense induction in trunk tissues was achieved both by wounding alone or by inoculating plants with fungi belonging to the *Ips acuminatus* (Gyll.) associated complex. *I. acuminatus* is a small bark beetle infesting Scots pine (Colombari et al. 2012), and is associated mainly with *Ophiostoma clavatum* Math.-Käärik and *Hyalorhinocladiella macrospora* (Franke-Grosm.) Harr. The first species is a blue-stain pathogen thought to be

involved in lowering the critical threshold of attack density for successful beetle colonization (Mathiesen 1951, Francke-Grosmann 1963, Kirisits 2004, Lieutier et al. 2009). *Hyalorhinocladiella macrospora* is primarily known as a food source for the larvae, but is also able to elicit defense responses in the host tree (Francke-Grosmann 1952, Batra 1967, Paine et al. 1997, Harrington et al. 2010, Villari et al. 2012). The five treatments consisted of inoculation with (i) *H. macrospora* (isolate 367.53, CBS, Utrecht, Netherlands) and (ii) *O. clavatum* (isolate 37987, CMW, Pretoria, South Africa), (iii) concurrent inoculation with both fungi, (iv) mechanical wounding, and (v) unwounded control. It must be noted here that in the previous work the blue-stain fungus used in the experiment was referred to as *O. brunneo-ciliatum* Math. However, further analyses have since revealed that the species used in the experiment was actually *O. clavatum* (Villari et al. in preparation), in accordance with the assessments of Mathiesen (1951) and Francke-Grosmann (1963) with regard to the *I. acuminatus* associated fungi.

Constitutive defense secondary metabolite concentrations were measured for each plant at the beginning of the experiment, corresponding to time zero (T_0), by collecting the plant material resulting from the treatments (i.e., phloem and outer bark plugs). Induced defense secondary metabolite concentrations were then measured in the same plants after three weeks (T_1), by collecting phloem and outer bark plugs from the tissues immediately surrounding the wounding and inoculation sites. At this time, samples were also taken from the unwounded control plants, at the same trunk height of the other treatments. In the previous contribution, Villari et al. (2012) demonstrated that Scots pine has a generic, rather than specific, inducible response to this induction. Different treatments had no significant effect on the induction of most analyzed compounds. Thus, we pooled induction data for all treatments, except for unwounded control. The only compounds for which treatment had a significant effect on local chemical induction were pinosylvin monomethyl ether and (+)-α-pinene. However, their constitutive concentrations were not different between treatments (see trade-off analysis), and thus we included them in the analyses described below.

Chemical analyses

Liquid chromatography – mass spectrometry (LC-MS) with electrospray ionization (ESI-MS) (Varian 212- LC pumps and 500-MS; Palo Alto, CA, USA), in parallel with a photodiode array detector (PDA) (Varian ProStar 335), was employed in negative ion mode to identify phenolic compounds and resin acids. These were then quantified by high performance liquid chromatography (HPLC-UV), which was performed using a Waters (Milford, MA, USA) Alliance 2690 separation module equipped with a 996 photodiode array detector and a 474 scanning fluorescence detector in serial mode. Terpenoid analyses were performed by gas chromatography–flame ionization detection (GC-FID) with a Perkin-Elmer Autosystem XL GC equipped with an automatic sampler for liquid sample injections. The separation of the different enantiomeric monoterpenes was performed on a 30 m Cyclodex-B capillary, 0.25-mm-diameter column (J&W Scientific, Folsom, CA, USA). Lignin concentration was determined spectrophotometrically. Extraction procedures, technical details for the chemical analyses, and individual compound identities and concentrations are reported in Villari et al. (2012). In order to have a broader view of compounds behavior, in the following analyses we considered not only compounds which showed a significant increase after the induction, that are hence compounds directly involved in the defense reaction, but also compounds which decreased or did not shown an overall significant reaction after the induction.

Correlation within constitutive defenses

To investigate the correlation pattern among compounds we used principal component analysis (PCA). We scaled compound concentration to unit variance to reduce the effect of the most abundant ones on the ordination. The PCA was performed using the rda() function of the package vegan (Oksanen et al. 2011) in R (R Development Team 2011).

Trade-offs between constitutive and inducible responses within individual compounds

According to Morris et al. (2006), since constitutive resistance must be measured in the absence of damage and induced resistance in the presence of damage, trade-offs between constitutive concentration and inducible variation are usually tested by comparing family or genotype means for the two types of resistance obtained from groups of replicated plants assigned to control and damage treatments. Herbaceous plants are quite amenable to this type of analysis, but its application to conifer trees requires the use of seedlings, which may display different responses from adult plants, such as those used in our study. Thus, we chose a different approach, exploiting the genetic and phenotypic differences that usually occur within natural populations (Forrest 1980, Newton et al. 1999, Iason et al. 2005, Thoss et al. 2007). Such variability was the only modulating factor for the defensive traits analyzed in this study.

First, we compared compound concentrations among the treatments measured at T_0 and unwounded control measured at T_1, using one-way ANOVA. This preliminary analysis showed that constitutive metabolite concentration did not differ between treatments, and that all treatments at T_0 did not differ from the unwounded control at T_1 ($P > 0.01$). We could thus assume that changes between T_0 and T_1 were due to the induction effect, and analyze the metabolites variation of each tree in relation to the constitutive profile of the same tree, calculating the difference (Δ) between induced and constitutive metabolite concentration ($T_1 - T_0$), called hereafter inducible variation (modified from Lombardero et al. 2000). The main effect of constitutive concentration of each compound on its inducible variation was analyzed with linear-mixed-effect models, incorporating sampling tree as a random factor, in order to account for the repeated measure design. Constitutive concentration and Δ of individual phenolics and individual terpenoids were $\ln(a + x)$ transformed to meet model assumptions, where a was a natural number big enough to avoid negative values. Data were analyzed in R (R Development Core Team 2011).

Compare to Villari et al. (2012), Unknown 1 was excluded from the analyses, including PCA, since its constitutive concentration variability was too low for performing the linear-mixed-model. One tree was removed from all the analyses, after it was identified as an outlier with Grubb's test ($P < 0.001$). This tree had extremely high amounts of constitutive

metabolites (i.e., comparable to inducible levels in other plants) that were probably due to a pre-existing infection or stress condition of that plant.

Results

Correlation within constitutive defenses

The PCA of the constitutive defense secondary metabolites resulted in three clusters of compounds (Figure 1). The first four eigenvalues for the unconstrained axes obtained from the analysis were 8.5, 6.5, 3.3 and 2.4, explaining 24.4, 18.7, 9.5 and 6.8% of the total variation, respectively. The first axis tended to divide the trees with high amount of lignin from trees with high amount of other defense secondary metabolites. Lignin concentration was therefore negatively related to most of the metabolites analyzed. The second axis tended to divide trees with high amount of terpenoids and of the two stilbenes pinosylvin monomethyl ether and unknown 2 from trees with high amount of pinosylvin, taxifolin hexoside, catechin hexoside, and all those secondary metabolites that did not respond significantly to the induction (Villari et al. 2012). The first axis was relatively independent from α-humulene, while the second one from pinocembrin, taxifolin and myrcene.

Trade-offs between constitutive and inducible responses within individual compounds

Different compounds displayed different relationships between their constitutive concentration and their inducible variation (Tables 1 and 2). This analysis resulted in the classification of all compounds into five response types (Tables 1 and 2, Figure 2), based on the slope and significance of the relationship, the sign of the average inducible variation (i.e., increase or decrease relative to the constitutive concentration), and the significance of the response to the induction (Villari et al. 2012).

The first four response types include compounds that responded significantly, positively or negatively, to the induction. Response type A includes compounds that displayed a significant increase after the induction, but whose correlation between constitutive

concentration and inducible variation was not significant. Compounds included in this response type were the three stilbenes pinosylvin, pinosylvin monomethyl ether and unknown 2, and most of the terpenoids. Response type B includes compounds displaying a significant increase after induction, and a significant positive correlation between constitutive concentration and inducible variation. Compounds included in this response type were the terpenoids Δ-3-carene, *p*-cymene and unknown 5. Response type C includes compounds displaying a significant increase after induction, but a significant negative correlation between constitutive concentration and inducible variation, i.e., a trade-off. Compounds included in this response type were pinocembrin and the two terpenoids (+)-camphene and myrcene. Response type D includes compounds displaying a significant decrease after the induction, as well as a significant negative correlation between constitutive concentration and inducible variation. Compounds included in this response type were lignin, the flavonoids taxifolin and taxifolin hexoside, and the flavanol catechin hexoside.

Finally, response type E includes compounds that did not display an overall significant response to the induction, but were characterized by a significant negative relation between constitutive concentration and inducible variation. In this case, which included several phenolic compounds, especially procyanidins, and the terpenoid (−)-β-pinene, metabolite concentration increased or decreased in trees with respectively low or high constitutive amount (Figure 2).

Discussion

In this study we exploited the intra-population variability of a natural stand to investigate the behavioral complexity of Scots pine defenses. Trees exhibited a high variability in the constitutive defense secondary metabolite composition, displaying either high lignin concentration, or high chemical defense. While, with regards to correlations between their constitutive concentration and inducible variation, individual chemical compounds may be grouped in five differential response types.

Constitutive lignin content in the phloem and outer bark of Scots pine stems was negatively correlated with other defense secondary metabolites, particularly soluble phenolics. A recent study showed that the inhibition of enzymes involved in lignin biosynthesis produces a compensatory increase in phenolic glycosides (Meyermans et al. 2000). Thus, the phenomenon observed in our work may reflect a similar compensatory mechanism. This trade-off may have important consequences in defense, depending on the direction of the changes following induction by microbes and herbivores, as it is well known that lignification is an important component of mechanical plant defenses against biotic stressors (Franceschi et al. 2005).

Among other chemical defenses, positive correlations among terpenoids, which are well known to be toxic against many fungi and insects (Delorme and Lieutier 1990, Raffa et al. 1985, Klepzig et al. 1996, Faccoli et al. 2005, Kopper et al. 2005) were observed, and trees with high amounts of terpenoids also had high amounts of stilbenes, e.g., pinosylvin monomethyl ether, which are known to have antifungal properties (e.g., Franceschi et al. 2005). The observed positive correlations in the expression of terpenoids are in accordance with the patterns of inheritance suggested by Hiltunen (1976) and Thoss et al. (2007), which describe how monoterpene biosynthesis in *P. sylvestris* is controlled by few genes.

However, we also found negative correlations among some phenolic compounds, a phenomenon that has been noted before (Koricheva et al. 1998). In particular, the stilbene pinosylvin, which may be directly involved in a defense response (Bonello et al. 1993, Seppanen et al. 2004), appeared to behave in a traded–off with its monomethyl ether and with the other stilbene, unknown 2. Trees with high amount of pinosylvin also had high amounts of flavonoids, flavanols, and procyanidins, which have been shown to have at least indirect defensive effects against bark beetles (Rohde et al. 1996). The trade-offs of the latter compounds with terpenoids may be due to the fact that the synthesis of chalcone (the precursor of the flavanones) requires 40% of carbon via malonyl-CoA, and thus competes for the common substrate, acetyl-CoA, with the terpenoids (Keinänen et al. 1999). Exception made for lignin, our results are therefore in agreement with Koricheva et al. (2004), who argued that there are no overall negative associations between different defensive traits in plants. In fact, plants very seldomly rely on a single defensive mechanism (Schoonhoven 1982), and indeed multiple defensive mechanisms are more

common (Romeo 1998), since they may help to avoid damage from a wider range of attacking organisms (Koricheva et al. 2004). Hence, some authors have even proposed the concept of plant defense syndrome, in which different ecological interactions can result in convergence of suites of co-varying, non functionally redundant defensive traits (Agrawal and Fishbein 2006, Agrawal 2007).

With regards to correlations between constitutive concentration and inducible variation for individual chemical compounds, we observed that compounds can display several different response types. Most of the terpenoids and all stilbenes, all of which are widely involved in conifer induced defense mechanisms (Raffa and Smalley 1995, Lieutier et al. 1996, Bonello and Blodgett 2003, Zeneli et al. 2006, Villari et al. 2012), showed no correlation between constitutive concentration and inducible variation (see also Lombardero et al. 2000). Our results are thus in contrast with the predictions of Karban and Myers (1989) and Herms and Mattson (1992), and support instead theories predicting that the two types of defenses are not functionally redundant and provide different benefits to plants (Raffa and Smalley 1995). In some cases (i.e., the terpenoids Δ-3-carene, *p*-cymene and unknown 5), we even found positive correlations between constitutive concentration and inducible variation. Significant trade-offs between constitutive concentration and inducible variation were however observed for the flavonoid pinocembrin and the two terpenoids (+)-camphene and myrcene, which, in some cases, are directly involved in induced defense mechanism (Raffa et al. 1985, Franceschi et al. 2005, Villari et al. 2012).

The flavonoids taxifolin, taxifolin hexoside, and the flavanol catechin hexoside, which significantly decreased after the induction and are probably indirectly involved in defense (Villari et al. 2012), displayed a strong negative correlation between constitutive concentration and inducible variation. This behavior suggests a rapid metabolic turnover of the latter compounds, which may be involved in catabolic reactions and subsequent reallocation of the products. Indeed, it has been shown that some phenolic compounds may act as a reservoir for the synthesis of other phenolics when phenylpropanoid metabolism is activated, as these reserves would be better defended against herbivory than the ordinary storage carbohydrates (Yao et al. 1995, Keinänen et al. 1999).

A significant decrease after induction and a negative correlation between constitutive concentration and inducible variation have been observed also for lignin, which, however,

due to its complex structure, is very unlikely to be involved in catabolic reactions and components turnover. A possible explanation for this phenomenon may be the fact that the limited availability of some phenolic monomers as a result of induction also reduces the synthesis of polyphenolic compounds, such as lignin, as has been shown by Yao et al. (1995).

The last type of behavior (compound response type E, Figure 2) involves all compounds that did not display an overall significant response to induction, and were thus considered as unlikely contributors to induced defense (Villari et al. 2012). However, these compounds, mostly flavonoids and procyanidins, displayed significant negative correlations between constitutive concentrations and inducible variation. This negative relationship was the result of trees with low levels of these compounds responding to the induction by increasing their accumulation, while trees with high levels responded by decreasing their concentrations. This perspective suggests that these compounds may also be involved in defense, a function that may have been previously masked by the fact that only their final average concentration was taken into consideration, rather than their change (Villari et al. 2012). It would be therefore interesting to better assess the role of these compounds, investigating for instance if they are involved in the synthesis of polyphenolic compounds or in the formation of reservoirs for the synthesis of other soluble phenolics (Yao et al. 1995, Keinänen et al. 1999).

In conclusion, in this study we have shown that in Scots pine there are no overall negative associations between different defensive traits, both considering correlation patterns within constitutive defense secondary metabolites and correlations between constitutive concentration and inducible variation. The few exceptions were lignin, which was negatively correlated with all constitutive phenolics and terpenoids, and pinocembrin, (+)-camphene and myrcene, which displayed a trade-off between constitutive concentration and inducible variation. Therefore, Scots pine appears to possess a complex defense chemistry whose individual components are not functionally redundant, in accordance to observations for several herbaceous plant species (Agrawal and Fishbein 2006, Agrawal 2007). Moreover, thanks to our high chemical resolution, we have discovered that compounds that are not usually considered directly involved in defense, significantly react, suggesting that they may indeed have a role in plant responses to biotic stressors.

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Table 1. Results from the linear mixed model testing the effects of constitutive concentration on the inducible variation (Δ) of phenolic compounds and lignin (i.e., difference between induced and constitutive concentrations) in the outer bark and phloem of *Pinus sylvestris*. *P*-values < 0.05 are highlighted in bold.

Compound	df	*F*-value	*P*-value	Slope	Δ Sign[a]	Response type[b]
Vanillic acid hexoside	1, 37	57	**< 0.001**	−	+	E
Catechin hexoside	1, 37	113.2	**< 0.001**	−	−*	D
Procyanidin dimer (B type)	1 ,37	28.1	**< 0.001**	−	−	E
Epi/Catechin	1, 37	49.1	**< 0.001**	−	−	E
Procyanidin dimer (B2)	1, 37	38.2	**< 0.001**	−	−	E
Ferulic acid hexoside	1, 37	14.2	**< 0.001**	−	+	E
Procyanidin trimer (C type)	1, 37	26.9	**< 0.001**	−	+	E
Hydroxypropiovanillone hexoside	1, 37	13	**0.001**	−	+	E
Lignan hexoside	1, 36	131.7	**< 0.001**	−	−	E
Taxifolin hexoside	1, 37	69.8	**< 0.001**	−	−*	D
Taxifolin	1, 37	19.8	**< 0.001**	−	−*	D
Procyanidin dimer (A type)	1, 36	31.2	**< 0.001**	−	−	E
Pinosylvin	1, 27	1	0.429	NS	+*	A
Pinocembrin	1, 37	6	**0.017**	−	+*	C
Pinosylvin monomethyl ether	1, 37	1	0.315	NS	+*	A
Unknown 2	1, 37	4	0.053	NS	+*	A
Lignin	1, 37	40.69	**< 0.001**	−	−*	D

NS, non significant.

[a] According to results reported in Villari et al. (2012). Asterisks denote significant differences between Δ and zero (*t*-test), which correspond to a significant reaction after the induction.

[b] See Figure 2 for response type classification.

Table 2. Results from the linear mixed model testing the effects of constitutive concentration on the inducible variation (Δ) of terpenoids (i.e., difference between induced and constitutive concentrations) in the outer bark and phloem of *Pinus sylvestris*. *P*-values < 0.05 are highlighted in bold.

Compound	df	*F*-value	*P*-value	Slope	Δ Sign[a]	Response type[b]
(−)-α-Pinene	1, 36	2.2	0.430	NS	+*	A
(+)-α-Pinene	1, 36	1.3	0.256	NS	+*	A
(+)-Camphene	1 ,36	4.9	**0.034**	−	+*	C
(−)-Sabinene	1, 36	0.4	0.553	NS	+*	A
Myrcene	1, 36	6.8	**0.013**	−	+*	C
(−)-β-Pinene	1, 36	4.4	**0.043**	−	+	E
(+)-β-Pinene	1, 36	0.4	0.520	NS	+*	A
Δ-3-Carene	1 ,36	14.4	**< 0.001**	+	+*	B
(−)-Limonene	1, 36	2.4	0.128	NS	+*	A
(+)-Limonene	1, 36	0.2	0.700	NS	+*	A
p-Cymene	1, 36	4.1	**0.049**	+	+*	B
Terpinolene	1, 36	0	1.000	NS	+*	A
Unknown 3	1 ,36	1.2	0.278	NS	+*	A
β-Caryophyllene	1, 36	0.5	0.503	NS	+*	A
α-Humulene	1, 36	1.4	0.244	NS	+*	A
Unknown 4	1, 36	1.1	0.297	NS	+*	A
Unknown 5	1, 36	18.8	**< 0.001**	+	+*	B
Abietic acid	1, 37	0.1	0.811	NS	+*	A

NS, non significant.

[a] According to results reported in Villari et al. (2012). Asterisks denote significant differences between Δ and zero (*t*-test), which correspond to a significant reaction after the induction.

[b] See Figure 2 for response type classification.

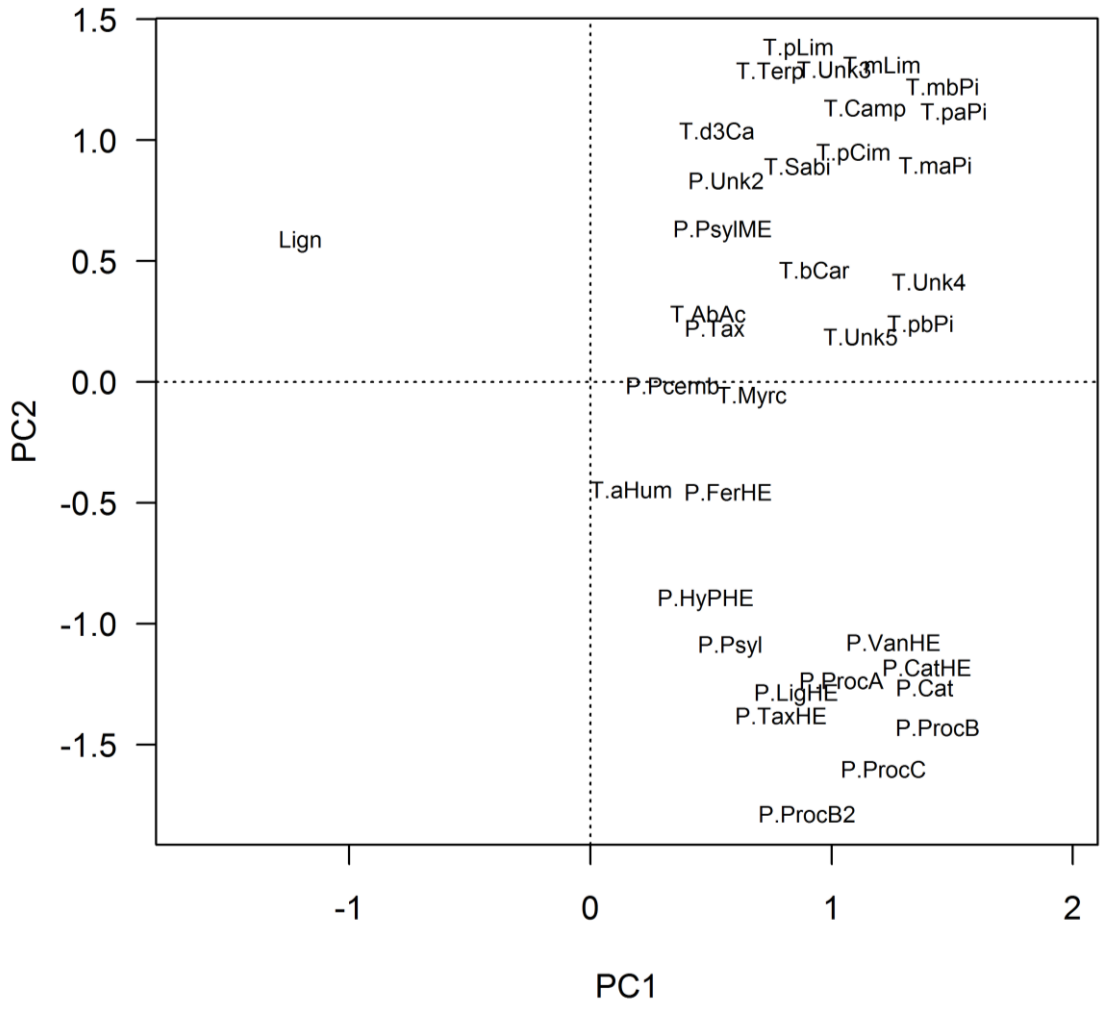

Figure 1. Ordination plot of the constitutive secondary metabolites along the two first axes of PCA. P and T before the abbreviation refer to phenolics and terpenoids, respectively. Abbreviations: Lign, lignin; P.Psyl, pinosylvin; P.PsylME, pinosylvin monomethyl ether; P.Unk2, unknown 2; P.Pcemb, pinocembrin; P.LigHE, lignan hexoside; P.HyPHE, Hydroxypropiovanillone hexoside; P.ProcA, procyanidin A type; P.ProcC, procyanidin C type; P.ProcB, procyanidin B type; P.ProcB2, procyanidin B2; P.VanHE, vanillic acid hexoside; P.TaxHE, taxifolin hexoside; P.FerHE, ferulic acid hexoside; P.CatHE, catechin hexoside; P.Cat, epi/catechin; P.Tax, taxifolin; T.maPi, (−)-α-pinene; T.paPi, (+)-α-pinene; T.Camp, (+)-camphene; T.Sabi, (−)-sabinene; T.Myrc, myrcene; T.mbPi, (−)-β-pinene; T.pbPi, (+)-β-pinene; T.d3Ca, Δ-3-carene; T.mLim, (−)-limonene; T.pLim, (+)-limonene; T.pCim, *p*-cymene; T.Terp, terpinolene; T.Unk3, unknown 3; T.bCar, β-caryophyllene; T.aHum, α-humulene; T.Unk4, unknown 4; T.Unk5, unknown 5; T.AbAc, abietic acid.

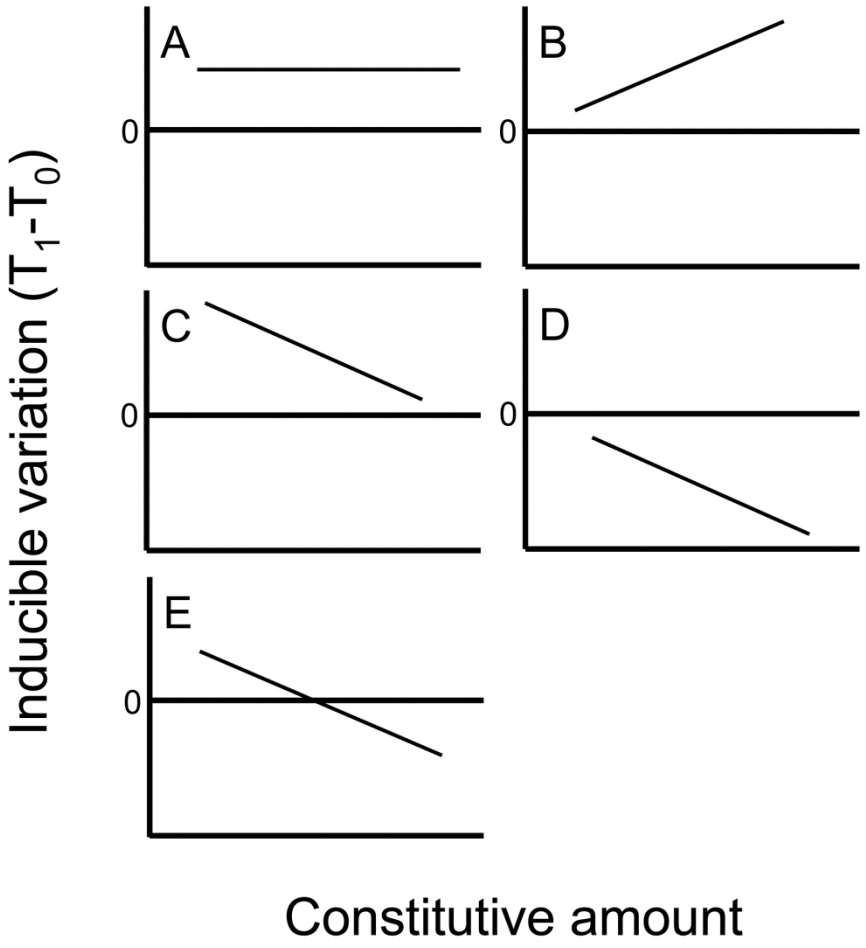

Figure 2. Schematic representation of compound behavior based on the correlation between constitutive concentration and inducible variation (i.e., difference between induced and constitutive concentrations). Response type A represents the case in which there is no correlation between constitutive concentration and inducible positive variation. Response type B represents a positive correlation between constitutive concentration and inducible positive variation. Response type C represents a negative correlation between constitutive concentration and inducible positive variation (i.e., trade-off). Response type D represents a significant decrease of the compound after induction, together with a significant negative correlation between constitutive concentration and inducible variation. Response type E represents compounds that did not display an overall significant reaction to the induction, but were characterized by a significant negative correlation between constitutive concentration and inducible variation.

Conclusions

General conclusions that can be taken from this thesis range in three different topics which go from the widely debated role of associated fungi in bark beetles host establishment (Lieutier et al. 2009, Six and Wingfield 2011), to the body of knowledge known as the 'theory of plant defense' (Stamp 2003), concerning also attempts in understanding ecology and population dynamics of a damaging species as *Ips acuminatus* (Gyll.). In order to enhance clarity, conclusions will be thus discussed separately for each topic.

Functional relationships between bark beetle and associated fungi

Although many fundamental aspects of the bark beetles – fungi symbioses are still to be clarified, the conclusions that can be taken from the results obtained in this thesis may contribute to the further development of the current theories on the role of associated fungal complexes in bark beetle ecology (Lieutier et al. 2009, Six and Wingfield 2011). The recent theory contradicting the Classic Paradigm (CP) is mainly based on three arguments, the first of which is the lack of a consistent association of aggressive fungal associates with tree-killing bark beetles (Six and Wingfield 2011). Results of this thesis have shown though that even a non-pathogenic, nutritional fungus is able to trigger induced defenses comparable to the ones induced by a blue-stain fungus, and may thus participate in exhausting host plant defenses (chapter IV). The carrying out of more than one role by a single species in the fungal community associated with bark beetles has been already predicted by Lieutier et al. (2009), and results of this thesis suggest that besides serving as nourishment to the larvae, nutritional fungi may indirectly assist in the beetle establishment process. Since nutritional obligate fungi are consistently associated with phloeomycetophagous and mycetophagous bark beetles (Kirisits 2004, Harrington 2005), the above-mentioned argument contradicting CP may thus have to be reconsidered.

Another of the arguments contradicting CP is instead the ubiquity of similar associations of fungi with bark beetles that do not kill trees (Six and Wingfield 2011). Results of this thesis have also shown that the frequency of association of a blue-stain fungus with a bark beetle varies accordingly to the population dynamic phase of the vector. In the endemic phases, when the number of beetles is generally too low to overcome the tree defenses, there is a higher frequency of fungal association than outbreak phases

(chapter III). In fact, during the culmination of the outbreak phase, the density of the bark beetle population is so high that the presence of the blue-stain fungus may be no longer critical to overwhelm plant defenses. Moreover, an initially mutualistic relationship may lead to competition for space and nutrients in the culminant phase of the outbreak, resulting in the decrease of the associated fungi (Klepzig and Six 2004). Result of the thesis suggests thus that the occurrence of fungal associations with bark beetles that do not kill trees is not a strong argument to contradict the importance of blue-stain fungi in bark beetle establishment in the hosts. In the light of this consideration, the second mentioned argument contradicting CP may thus have to be reconsidered as well.

However, even though this thesis seems to support the CP as described by Lieutier et al. (2009), the discussion about the role of bark beetle associated fungi remains widely open and there is still a need for further studies. For instance, to clearly assess the importance of blue-stain fungi in bark beetle establishment, it would be useful to determine the frequency of blue-stain fungi association in the incipient phase of an outbreak, when the presence of the associated fungi may be one of the several factors playing a critical role in surpassing of the eruptive threshold (Raffa et al. 2008). Moreover, as only recently has been done for some systems (Lombardero et al. 2003, Hofstetter et al. 2006), it would be useful to consider a broader approach of the matter, analyzing also the interactions that occur among other partners of the community associated with bark beetles.

The *Ips acuminatus* fungal community

In this thesis, the systematic uncertainty in the literature about the identity of the blue-stain fungus more consistently associated with *I. acuminatus* (Mathiesen 1950, 1951, Francke-Grosmann 1963, Lieutier et al. 1991, Kirisits 2004) has been resolved. Even though the work described in chapter II is still not concluded, preliminary results were enough to give an overview of the situation and permitted to proceed with the survey described in chapter III. Overall obtained results confirmed that, between the two species involved in the issue, the blue-stain fungus more consistently associated with *I. acuminatus* is *Ophiostoma clavatum* Math.-Käärik, in agreement with the first researches focused on *I. acuminatus* associated fungi (Mathiesen 1950, 1951, Francke-Grosmann 1963). Characterizing the

actual composition of the fungal community associated with *I. acuminatus* was a fundamental step in the study of this species, complementing other investigations on *I. acuminatus* behavior that have been conducted within my research group (Colombari 2011).

As mentioned above, in this thesis it has been shown that the frequency of association of *O. clavatum* with *I. acuminatus* varies accordingly to the population dynamic phase of the vector (chapter III). Taking into account the preliminary results of *O. clavatum* growing tests at different temperatures (chapter II), we may hypothesize that the influence that climatic changes have been shown to display on bark beetles life traits and outbreaks occurrence (Colombari et al. 2012), may be also related with a variation on the frequency of association with fungal community. In chapter III we described the use of an innovative and recently developed technology (LAMP) to specifically and quickly detect the blue-stain associated fungi directly from their vector. It would be therefore desirable to further employ this technique in order to better follow the variations of *I. acuminatus* associated fungal community, for instance in response to climate change, and therefore contribute to the attempts in understanding the role of *O. clavatum* in the population dynamic of its vector.

Scots pine defense mechanisms behavior

Theory of plant defense (Koricheva et al. 1998, 2004, Stamp 2003, Agrawal 2007) is still a complicated matter, and attempts in understanding its mechanisms, especially in woody plants, are constantly in evolution. In this thesis, different aspects of Scots pine (*Pinus sylvestris* L.) defense mechanisms behavior have been highlighted. In chapter IV it has been shown that Scots pine has a generic, rather than specific, induced response to triggering agents. This result supports the hypothesis that although plants can specifically recognize the nature of pathogens, the optimal defense strategy is to activate all available defense mechanisms, so that at least some may be effective against a particular pathogen (Katagiri 2004). But this 'shoot first—ask questions later' strategy has thought to be energetically expensive and not very advantageous for the plant in at least some antagonistic interactions (Keeling and Bohlmann 2006, Bolton 2009). However, results of this thesis showed also

that in Scots pine there are no overall negative associations between different defensive traits (chapter V), accordingly to Koricheva et al. (2004) findings. Scots pine exhibits therefore multiple and complex defense mechanisms whose individual components are generally not functionally redundant, as predicted for several herbaceous plants (Agrawal and Fishbein 2006, Agrawal 2007), and can be thus defined, borrowing Koricheva et al. (2004) terminology, 'jack-of-all-trades, master of all'. It has however to be noted that in this thesis only the intra-population variability of a natural stand has been considered. Further investigations taking into account also the genetic component are hence needed.

In chapter V, thanks to a high chemical resolution, it has been also shown that compounds that are not usually considered directly involved in defense, significantly react, suggesting that they may indeed have a role in plant responses to biotic stressors. However, the role of these compounds has not been defined yet, and further investigations on their involvement in the defense reaction would be desirable as well.

In conclusion, this thesis provides some new hints for the study of bark beetles – fungi symbioses, pointing out the importance of each member of the associated fungal community in the bark beetle host establishment process, and suggesting the use of new technologies as a support in the attempts to understand fungal role in bark beetle population dynamic. Moreover, it clarifies some aspects of Scots pine defense mechanisms, providing a starting point for further investigations.

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