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Pilar Fernandez-Conradi

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COMMUNAUTÉS

Par Pilar Fernandez-Conradi

**Diversité des arbres et résistance des forêts aux invasions
biologiques** : Application au châtaigner et son complexe de
bioagresseurs exotiques, chancre (*Cryphonectria parasitica*) et cynips
(*Dryocosmus kuriphilus*)

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Diversité des arbres et résistance des forêts aux invasions biologiques : Application au châtaigner et son complexe de bioagresseurs exotiques, chancre (*Cryphonectria parasitica*) et cynips (*Dryocosmus kuriphilus*)

Résumé :

Les plantes sont au centre d'une grande diversité d'interactions biotiques entre organismes plus ou moins proches qui les exploitent en tant que ressources. L'objectif de cette thèse a été de comprendre comment les infections fongiques de la plante et la diversité des arbres en forêt modifient les interactions arbres-insectes. Nous avons tout d'abord effectué une méta-analyse pour poser le cadre théorique des effets indirects des infections fongiques sur les insectes herbivores associés aux mêmes plantes hôtes. L'effet de l'infection préalable des plantes par les champignons sur les préférences et performances des insectes s'avère généralement négatif. Cependant, la magnitude de cet effet délétère varie selon le mode de vie du champignon, la guildes trophique de l'insecte et la spatialité des interactions (interactions locales vs distantes). Nous avons ensuite analysé de façon empirique les interactions tripartites entre le châtaignier européen (*Castanea sativa*) et deux de ses bioagresseurs exotiques: le cynips (*Dryocosmus kuriphilus*), insecte galligène, et *Cryphonectria parasitica*, champignon pathogène responsable de la maladie du chancre. L'effet sur les taux d'infestation par le cynips de la composition spécifique en essences forestières des forêts de châtaigniers atteintes de chancre a été également étudié. Afin d'identifier les mécanismes sous-jacents aux effets de la diversité des forêts sur cet insecte invasif, les communautés d'insectes parasitoïdes et de champignons endophytes présents dans les galles ont été décrites. Les taux d'infection par le cynips étaient plus faibles dans les mélanges de châtaignier avec du chêne et du frêne que dans des parcelles de châtaignier monospécifiques ou dans les mélanges avec du pin. La composition des forêts influence aussi la composition des communautés de parasitoïdes associés aux galles du cynips mais pas leur abondance, richesse ou diversité. Les communautés de champignons endophytes des galles, étudiées par des méthodes de séquençage de nouvelle génération, sont indépendantes de la composition forestière. Par contre, celles présentes dans les galles diffèrent fortement de celles des tissus foliaires adjacents. Nous avons ainsi apporté de nouvelles preuves que la diversité des plantes et les champignons pathogènes sont des facteurs clés déterminant les interactions plantes-insectes. Etudier comment les plantes interagissent avec leurs insectes et champignons associés, et les mécanismes sous-jacents à l'effet de la diversité des plantes sur ces interactions, doit permettre de mieux comprendre les relations entre diversité et fonctionnement des écosystèmes et de proposer des applications pour la gestion des bio-agresseurs forestiers natifs et exotiques.

Mots clés : diversité, interactions plante-insecte, espèces invasives

Title :

Tree biodiversity and forest resistance to biological invasions: Application on chestnut and its exotic pest complex, chestnut blight (*Cryphonectria parasitica*) and Asian chestnut gall wasp (*Dryocosmus Kuriphilus*)

Abstract :

Plants are the playground of a large diversity of biotic interactions between related and unrelated organisms exploiting them as common resources. The aim of this thesis was to understand how plant-insect interactions vary with fungal infection of their host plant and plant diversity. I first performed a meta-analysis to provide a theoretical background for plant-mediated effects of fungal infection on herbivorous insects. Overall, I found a negative plant-mediated effect of fungi on both insect preference and performance. However, this effect varied according to fungus lifestyle, insect feeding guild and spatial location of the interactions (local *vs* distant). Then I experimentally tested plant-fungus-insect tripartite interactions in the particular case of exotic bio-aggressors of the European chestnut (*Castanea sativa*): the Asian chestnut Gall Wasp (ACGW, *Dryocosmus kuriphilus*), and the fungal pathogen *Cryphonectria parasitica*, the causal agent of chestnut blight. I performed an observational study, in natural chestnut forest stands in Italy, where I tested how ACGW infestation rates vary with the tree species composition. I also investigated the mechanisms underlying plant diversity effects on the invasive pest, with a particular focus on its natural enemies such as insect parasitoids and endophytic fungi. ACGW infestation rates was lower in oak and ash chestnut mixtures compared to monocultures or pine-chestnut mixtures. Plot composition also influenced ACGW parasitoid community composition but not their abundances, diversity or richness. Endophytic communities of galls, described by using next generation sequencing methods, did not vary with plot composition. However, they strongly differed from surrounding leaf tissues. We thus provided evidence that plant diversity and fungal pathogens are key drivers of plant-insect interactions. Understanding how plants interact with associated insects and fungi, and mechanisms underlying plant diversity effect on these interactions, will improve our knowledge on diversity-ecosystem functioning relationships and will have practical applications for the management of native and exotic forest pests.

Keywords : diversity, plant-insect interactions, invasive species

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TABLE OF CONTENTS

1. INTRODUCTION	13
1.1 The host plant: a shared resource for heterotrophic organisms	15
1.1.1 Different ways to exploit plants	17
1.1.2 Plant defenses against fungi and insects	20
1.2 Direct and plant-mediated interactions between fungi and insects	23
1.2.1 Effects of fungi on insect herbivores	23
1.2.2 Effects of insects on plant fungi.....	23
1.2.3 Effect of dual attack of the same host plant by fungal pathogens and herbivore insects 24	
1.3 Plant diversity effects on associated organisms	27
1.3.1 Plant diversity effects on insect herbivore	27
1.3.2 Plant diversity effects on fungal pathogens	28
1.3.3 The case of invasive insect and fungus in forests	30
1.4 Model system	33
1.4.1 The European chestnut, <i>Castanea sativa</i>	33
1.4.2 Chestnut blight, <i>Cryphonectria parasitica</i>	34
1.4.3 Asian chestnut gall wasp, <i>Dryocosmus kuriphilus</i>	37
1.5 Research questions and manuscript structure	40
1.6 References	42
2. CHAPTER I	51
2.1. Introduction	55
2.2. Materials and methods	59
2.3. Results	61
2.4. Discussion	66
2.5. References	72
2.6. Appendix S1 – Supplementary figures and tables	77
2.7. Appendix S2 – List of studies included in the quantitative synthesis	82
2.8. Appendix S3 – Effect size calculation	90
2.9. Appendix S4 – Endophyte groups	91
3. CHAPTER II	92
3.1. Introduction	96
3.2. Materials and Methods	98
3.3. Results	104
3.4. Discussion	108
3.5. References	111
4. CHAPTER III	114
4.1. Introduction	118
4.2. Materials and Methods	121
4.3. Results	125
4.4. Discussion	130
4.5. Conclusion	132
4.6. References	134

5. CHAPITRE IV	150
5.1. Introduction	154
5.2. Materials and Methods	156
5.3. Results	159
5.4. Discussion	165
5.5. Conclusion	167
5.6. References	169
5.7. Supplementary material	174
5.7.1. APPENDIX A: Endophytic fungi OTUs differed between chestnut leaves and tree neighbor species in our plots.....	174
5.7.2. APPENDIX B: List of fungal endophytes found in ACGW winter galls.....	175
6. DISCUSSION	178
6.1. Tripartite interactions: How do fungal pathogens affect co-occurring insects on host plants and vice versa?	180
6.1.1. Temporal variability of interactions	181
6.1.2. Spatial scale of interactions	182
6.1.3. Mechanisms of tri-partite interactions	183
6.1.4. How could we predict the outcome of plant- fungi-insect tripartite interactions? Knowledge gaps and research perspectives	184
6.2. Multipartite interactions: plant-fungus-herbivore interactions from a community wide perspective	185
6.2.1. From tripartite to multipartite interactions	185
6.2.2. The mediating effect of plant community diversity and structure.....	188
6.2.3. The particular case of non-native pests and pathogens.....	189
6.3. Some avenues for future research	191
6.4. References	193
GENERAL SUMMARY IN FRENCH	199
7. SUPPLEMENTARY MATERIAL	207
7.1. APPENDIX 1	209
7.2. APPENDIX 2	241

1. INTRODUCTION

1.1 The host plant: a shared resource for heterotrophic organisms

Plants are the main primary producers in terrestrial ecosystems and many diverse organisms depend on them for living. Evolution selected different types of positive interactions between plants and closely associated organisms, such as mycorrhizal fungi, growth enhancing rhizobacteria, pollinators or enemies of plants bio-agressors (Pieterse and Dicke 2007, Pineda et al. 2013). In contrast, plants may also suffer the simultaneous or sequential attacks of multiple exploiters, like diverse herbivores, that range from large mammals to microscopic arthropods, and plant pathogens, such as pathogenic fungi, oomycetes, bacteria and viruses.

Plant is the playground of a large diversity of interactions between related and unrelated organisms exploiting the same resource (Pieterse and Dicke 2007; figure 1). The degree to which these different plant exploiters influence each other is therefore a key issue to plant survival (Moran 1998, Hauser et al. 2013). Moreover, in the top of plant-exploiters interactions, plant-plant interactions may also play a key role in plant fitness, either directly (Cardinale et al. 2011), either indirectly through the mediation of plant-exploiters interactions (Schuldt et al. 2017).

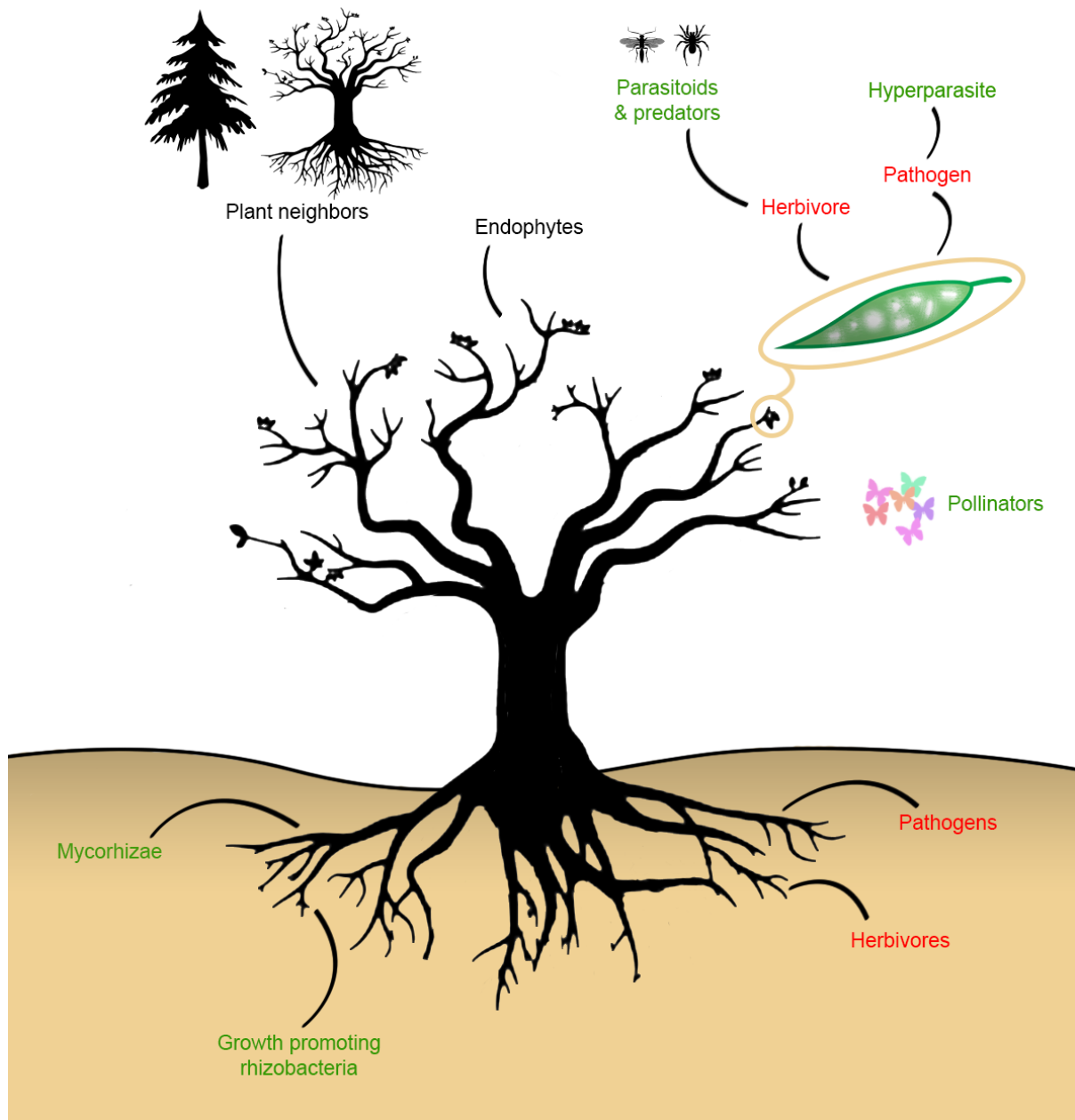


Figure 1. Plants as playground of complex interactions. Plants interact with different aggressors like pathogens and herbivores (in red) and beneficial organisms (in green) such as growth promoting rhizobacteria and enemies of aggressors. In addition, some of these organisms (i.e., plant endophytes) may be benefic or detrimental in function of their biotic and abiotic environment. Plant interactions with associated organisms may vary with their plant neighbourhood.

However, despite decades of research on interactions between plants and associated organisms, we still lack a clear understanding of tripartite interactions between plants, fungal pathogens and insect herbivores (Hatcher 1995, Stout et al. 2006, Hauser et al. 2013), which are the focus of this work. In addition, even less is known about how these interactions depend on plant diversity and which mechanisms are involved or shape these interactions.

1.1.1 Different ways to exploit plants

Different lifestyles of plant-associated fungi

All fungi are heterotrophic organisms that require organic nutrients for their energy source.

Fungi associated to living plants may be considered as plant symbionts (Box 1). Between them, we can distinguish fungal pathogens, mutualists (*e.g.* mycorrhizas) and commensals (*e.g.* epiphytes).

Hereafter and along this thesis we have focused on fungal pathogens and endophytes (Figure 2).

Plant pathogens differ in how they obtain their resources from the host plant:

Necrotrophic pathogens secrete enzymes and toxins that degrade and kill host cells and then live and feed on the dead plant tissues (Mengiste 2012, García-Guzmán and Heil 2014). Generally, they are able to produce a great variety of phytotoxins, cell-wall degrading enzymes and reactive oxygen species (ROS), which induce cell necrosis allowing penetration and leakage of nutrients (Mengiste 2012). For example, the necrotrophic pathogen *Armillaria ostoyae*, produces root rot in maritime pine (*Pinus pinaster*) inducing significant mortality on pine forest in Southwestern France (Labbé et al. 2017).

Biotrophic pathogens are those which develop and extract their nutrients from living plant tissues without killing cells (Delaye et al. 2013, García-Guzmán and Heil 2014). They develop specialized organs to draw nutrients from the plant cells. As they exploit living tissues, they usually establish long-term relationship with their host in order to complete their lifecycle (García-Guzmán and Heil 2014). In contrast to necrotrophs, they do not generally produce toxins but low quantities of cell-degrading enzymes (Mengiste 2012). For example, the powdery mildew, *Erysiphe*

Box 1. Glossary

Symbiosis: the relationship between two intimately interacting organisms or populations, commonly used to describe all relationships (including mutualism, commensalism and parasitism) between members of two different species, and also to include intraspecific associations. The members are called symbionts (Desprez-Lousteau et al. 2007).

Parasite: species that draw their food resources in the living bodies of another species (Price 1986).

Pathogen: a microorganism that causes phenotypically visible disease symptoms, thereby negatively affecting plant fitness (Partida-Martinez and Heil 2012).

Mutualism: an interaction between species that is beneficial to both (Bronstein 1994).

Commensalism: symbiotic relationship in which one species derives benefit and the other is unharmed (Casadevall and Pirofski 2000).

Plant microbiome : collective genomes of microorganisms living in association with plants (Hardoim et al. 2015).

Plant microbiota : microbial communities associated with a particular plant (Hardoim et al. 2015).

alphitoides, is a biotrophic pathogen causing one of the most common diseases in oaks (Glawe 2008).

However, this distinction between biotrophic and necrotrophic pathogens is not always stable in time. Some pathogens starts as biotrophs but in a determined stage of their infection cycle they can switch to a necrotrophic phase (Horbach et al. 2011). These fungi are usually named **hemi-biotrophs** (García-Guzmán and Heil 2014). For example, *Moniliophthora perniciosa*, the causal agent of witches broom disease of cacao plants, first lives as a biotrophic pathogen intercellular to plant tissues but this is followed by intracellular mycelium growth which is associated with the subsequent accumulation of ROS and cell death (de Oliveira Ceita et al. 2007).

Fungal **endophytes** are defined as commensal fungi that live at least one part of their lifecycle inside living plant tissues (in the apoplast) without causing disease symptoms (Parida-Martinez & Heil 2011; Figure 2). In several cases they behave as mutualists, conferring plant resistance to abiotic (*e.g.*, Redman et al. 2002) and biotic stressors (Clay 1996, Saikkonen et al. 2010). However, a fungus that behaves as an endophyte in a given host and under a specific set of environmental conditions can also develop as a necrotrophic pathogen in another host or shift from one life style to the other with changing environments (Delaye et al. 2013). The continuum between the endophyte and necrotrophic lifestyle is still little understood and seems evolutionarily and ecologically instable (Delaye et al. 2013). For example *Sphaeropsis sapinea* (syn. *Diplodia pinea*) is usually considered as an endophyte, as it is present in virtually all plant part of pines without producing disease symptoms (reviewed in Decourcelle et al. 2015). However, this fungus can also acts as a necrotrophic, inducing shoot blight, steam and branch canker disease, tip dieback, cone infection, blue stain and rot disease, often killing trees of susceptible *Pinus sp.* worldwide (Sinclair and Lyon 2005, Desprez-Loustau et al. 2006).

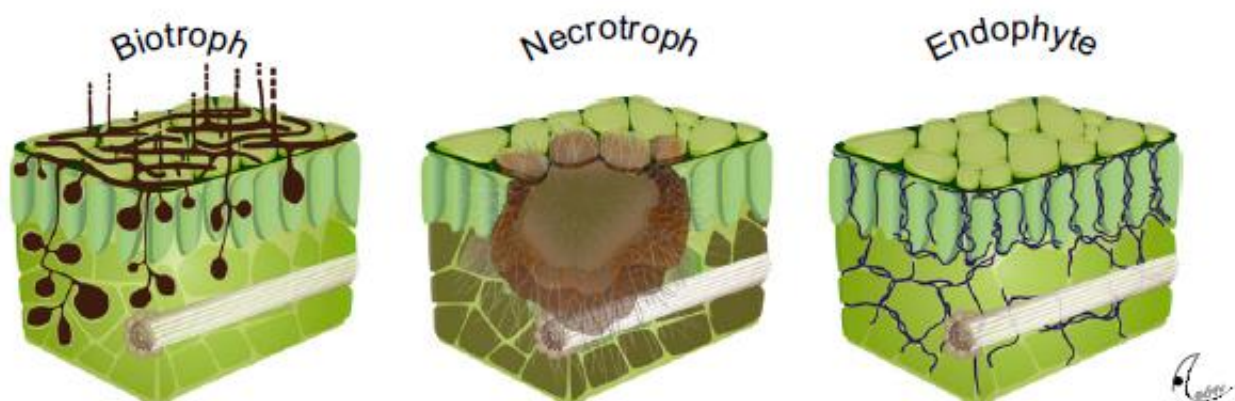


Figure 2. Different lifestyles of fungus infecting plants. From Garcia-Guzman and Heil (2014).

As stated by Partida-Martiez & Heil (2011): “A plant that is completely free of microorganisms represents an exotic exception, rather than the – biologically relevant –

rule". Fungal endophytes are a part of **plant microbiota** which also includes bacterial endophytes, mycorrhizal fungi, rhizobia, plant-growth promoting rhizobacteria and microbes living in the phyllosphere or epiphytes (Partida-Martínez and Heil 2011a, Vacher et al. 2016a). The recent development of next generation high-throughput sequencing methods have led to an increasing knowledge of the microbiome (Turner et al. 2013) and the interactions between these complex communities, their host plants and other organisms such as insect herbivores (Vacher et al. 2016b, Shikano et al. 2017).

Different insect feeding guilds

Similarly to fungi, insect herbivores can consume their host plant in different ways. They can target all plants organs from roots to flowers. Some insects feed externally on plant tissues (ectophagous) while others feed internally (endophagous). Herbivores can also have different feeding strategies, generally classified in two categories: chewing and piercing-sucking (Schoonhoven et al. 2005). A feeding guild is a group of species that exploit the same class of environmental resources in a similar way and that overlap significantly in niche requirements (Root 1967, Blaum et al. 2011). One can distinguish several insect feeding guilds depending on their feeding strategy, their location in plant organs (*i.e.* leaves, shoots, trunk, roots...) and plant tissues (*i.e.* ecto or endophagous). Based on these criteria, Novotni et al. (2010) proposed a classification in 24 feeding guilds, but this classification is not universally used. Some examples of these feeding guilds are given in Figure 3. Although the concept of guild went through several criticisms in the last decades, recent studies on plant defenses confirms that it is still an operationally efficient way to describe herbivores from the functional point of view (Blaum et al. 2011).



Figure 3. Different insect feeding guilds associated to oak leaves in France. Provided by B. Castagneyrol

1.1.2 Plant defenses against fungi and insects

Plants are under the continuous threat of a wide diversity of bio-aggressors that can attack them in many different ways. As plants cannot just move to escape from their attackers, they have evolved a wide diversity of defensive mechanisms. There is several ways, not conveniently mutually exclusive, to classify plant defenses: physical *vs.* chemical, primary *vs.* secondary, and induced *vs.* constitutive.

Constitutive defenses are the first line of plant defenses against an insect or a pathogenic fungus. Plant physical and mechanical defenses include structures like trichomes, which may deter herbivores: some of them may reject chemicals compounds as terpenoids and alkaloids that are toxic for the insects and pathogenic microorganisms. Also, various tissues such as the sclerenchyma that surrounds vascular bundles in young stems, may reduce water loss and provide protection against fungal infection.

When these first lines of defenses are overwhelmed **induced defenses (IR, induced resistance)** are triggered. For the purposes of this manuscript, we divided these defense mechanisms in five categories following Eyles et al. (2010):

- **Inducible chemical defenses:** include secondary metabolites such as phenolic compounds, terpenoids and alkaloids. These compounds have toxic, antimicrobial, anti-nutritive or anti-digestive activity against biotic aggressors.

- **Inducible protein-based defenses:** also have toxic, anti-microbial, anti-nutritive and anti-digestive activity but via proteins and peptides. This includes families of pathogenesis-related proteins (PR proteins) such as PR-3 proteins which affects fungal cell wall or membrane integrity, or PR-6 proteins, which reduces digestive enzyme activity of insects and pathogens. It also includes wound induced proteins such as proteinase inhibitors, cysteine protease, lectins, lipoxygenases and polyphenols oxidases, triggered by and accumulated after insect attack.

Box 2. Glossary

Defense induction : the novo synthesis of defenses in an organ in the absence of local attack.

Defense priming: pre-activation of mechanisms that make plants able to better or more rapidly mount defense responses against attackers

- **Inducible ecological or indirect defenses:** they protect the plant via tritrophic interactions. They usually result in plant emission of volatile organic compounds (VOCs) that can attract herbivore enemies such as parasitoids or predators. These VOCs can also be used as plant-plant communication priming defensive responses in neighbor plants or undamaged parts of the same plant. These VOCs have been mainly studied in the case of herbivorous insects but they can be also elicited by pathogenic nematodes (Dicke et al. 2009), endophytic fungi (e.g., Rostás et al. 2015) and fungal pathogens (reviewed by Morath et al. 2012).
- **Inducible civilian defenses:** they allow the plant to compensate for the reduction in photosynthetic capacity. It includes up-regulation of photosynthetic rates in unaffected organs, alteration of growth patterns and shift in resource allocation.

IR can be active through the whole plant resulting in **systemic induced resistance (SIR)**, which is activated via one or more signaling molecules such as salicylic acid (SA), jasmonic acid (JA) or ethylene (ET). IR may be attributable to a priming effect that enable a previously attacked plant to respond to later attack, so plants that survive an initial attack by an herbivore or pathogen are often more resistant to second attacker (see also Stout et al. 2006). However, the signaling molecules implicated may differ between fungal lifestyles and insect feeding guild (see section 1.2.3 for further information).

Plant defense production, however, is not free for the plant (Heil 2002). Resource allocation to plant defenses after a first attack is assumed to reduce availability of these resources for plant growth and reproduction (Koricheva 2002, Strauss et al. 2002) or even for a second attack (Thaler et al. 1999, Heil 2002). There also some trade-offs between different types of chemical defenses, between chemical and mechanical defenses, and between constitutive and induced defenses, so one may ask the question ‘are plants jacks-of-all-trades?’ (Koricheva et al. 2004). In a meta-analysis of 31 studies which included plant defensive traits against herbivores, Koricheva et al. (2004) found that plants appear to be able to produce several types of defense without considerable trade-offs, which were only significant for constitutive

and induced defenses. However, little is known about trade-offs between defenses against pathogens *vs.* herbivores (but see Heil 2002). Answering this question may improve our understanding and predictions about the outcome of tripartite interactions between plants, herbivores and fungal pathogens.

1.2 Direct and plant-mediated interactions between fungi and insects

1.2.1 Effects of fungi on insect herbivores

Fungal plant pathogens and endophytes may have direct effects on insect herbivores. For example, some insects cultivate and/or feed on fungi, which mycelium or spores, which constitute a supplementary source of nutrients for the insect (*e.g.*, ambrosia beetles, Batra 1966). Plant infection by a fungus may indirectly affect insect herbivores through changes induced in the host plant by the infection process. Fungal infection may change plant nutritive quality for the insect, thus increasing (*e.g.* sugar content higher on infected plants, Cardoza et al. 2003) or decreasing (*e.g.* reducing nitrogen content on infected plants, Hatcher et al. 1994) nutritive value for the insect. Also, fungal infection may activate plant direct defenses thus increasing (*e.g.*, Moran 1998) or decreasing (*e.g.*, Cardoza et al. 2003a) levels of secondary compounds such as phenols.

These effects of fungal plant pathogens on herbivore insects will be further evaluated and discussed in the first chapter of this thesis.

1.2.2 Effects of insects on plant fungi

Direct effects of insects on fungi include those produced by the insect itself (Hatcher et al. 1995). Some insects are known for being vectors of plant pathogens (*e.g.*, ambrosia bark beetles, reviewed in Ploetz et al. 2013) and some facilitate fungus penetration in plant tissues through injuries or damage they cause to plants (Paine et al. 1997). Insects may also be an additional source of nutrients for some fungus that feed on insects attacking plants (*i.e.*, entomopathogenic fungus). In addition, some insects, like aphids, can produce honeydew which is used by some pathogens for growing (Diener et al. 1987) and even stimulate their germination.

Insect feeding may also induce changes in the host plant which may indirectly impact fungi (reviewed by Hatcher 1995, and Stout et al. 2006). For example, Simon and Hilker (2003), showed a lower infection by *Melampsora alli-fragilis* on undamaged willow leaves as compared to leaves damaged on the same plant by the leaf beetle *Plagioderia versicolora*.

Both direct and plant-mediated interactions may result in induced susceptibility (*e.g.*, Leath and Byers 1977, Simon and Hilker 2003), induced resistance (*e.g.*, Karban et al. 1987,

Hatcher et al. 1994) or neutral (*e.g.*, Ajlan and Potter 1991, Rostas and Hilker 2002) effects on pathogen infection.

1.2.3 Effect of dual attack of the same host plant by fungal pathogens and herbivore insects

The outcome of tripartite interactions is mediated by a crosstalk between defense reaction pathways (Al-Naemi and Hatcher 2013, Mouttet et al. 2013a, Lazebnik et al. 2014). As discussed in section 1.1.3, hormonal pathways implicated in induced resistance to pests and pathogens depend on fungus and insect lifestyles. Biotrophic pathogens and sap-feeding insects often activate and respond to SA pathway while necrotrophs and wounding insects mainly activate and respond to JA pathway. Moreover, a crosstalk between these two hormonal pathways (box 3) have been found in several systems,

with SA being under-regulated by JA pathway activation and *vice versa* (Thaler et al. 2012). Consequently, we may expect that plant attack by a sap-feeding insect may up-regulate SA levels thereby under-regulating JA which would benefit necrotrophs and be detrimental to biotrophs (Figure 5 A). In the contrary, plant attack by a wounding insect may up-regulate JA levels and under-regulate SA, thus benefiting biotrophs and being detrimental to necrotrophs (Figure 5 A). Similarly, plant infection by a biotroph may up-regulate SA levels thereby under-regulating JA and thus being beneficial to sap-feeders but detrimental to wounding insects (Figure 5 B). Finally, infection by a necrotroph may up-regulate JA levels, under-regulating SA levels, and thus being beneficial to sap feeders and detrimental to chewers (Figure 5 B).

Box 3. Glossary

Hormone cross talk:

interactions of hormone signal transduction pathways, via shared signaling components or downstream of signal transduction at the gene expression level (Pieterse et al. 2012).

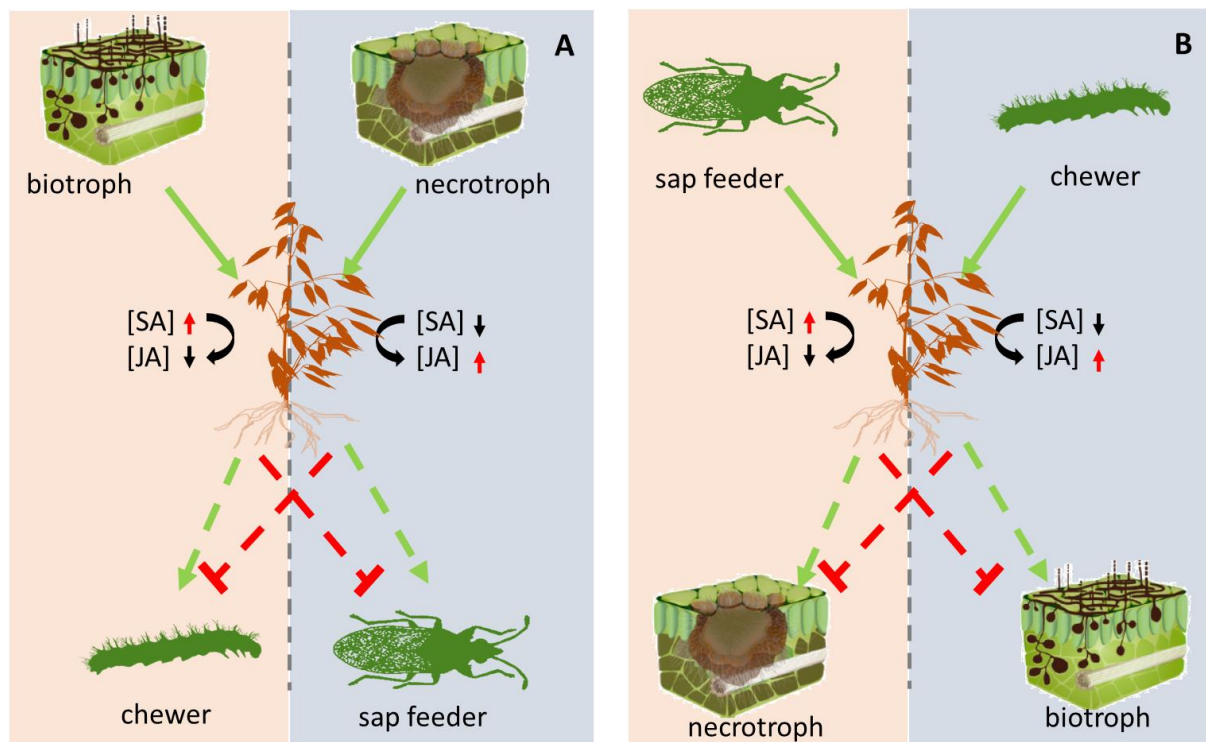


Figure 4. Predictions for insect and fungus interactions outcomes based on the crosstalk between SA and JA dependent defense reaction pathways and different fungus lifestyle and insect feeding guild, when plant are first attacked by a pathogen (A) or an insect (B). Lines represent a plant-mediated effect on the following attackers (green for positive and red for negative). Pictograms for biotrophic and necrotrophic fungus are from Garcia-Guzman and Heil (2014).

Fungus-insect interactions can result in **synergistic effects** on host plants when their combined impact is more severe than the sum of impacts of either attacker alone (e.g. Turner et al. 2010 Biol. Control).

At the opposite, dual attack may have **antagonistic** effects when combined effect of both attackers is less severe than the sum of both. That may happen when one attacker has a negative impact on the other one (e.g. one attacker may decrease host plant quality for the second one by increasing defensive compounds level or decreasing nutrient content). For example, Hatcher et al. 1994, found that sequential feeding with the beetle *Gastrophysa viridula* followed by the rust *Uromyces rumicis* on *Rumex crispus* resulted in damage 40% weaker than predicted by the sum of both.

Finally, their combined impacts can also be **independent and additive** when the impact of both pathogens and insects is the sum of both (Hauser et al. 2013). Independent effects may occur when the insect and the fungus do not interact at all, when they have a reciprocal effect on each other (e.g. if the first attacker have a positive effect on the second one, which in return have a similar negative effect on the first one) or when plants compensate for their damage (Hauser et al. 2013; Schuldt et al. 2017).

Independent and additive effects on plants have been shown to be the general pattern in a meta-analysis of 35 published articles reporting effects of fungus and insect on plant characteristics (Hauser et al. 2013). However combined impacts of fungi and insects were

synergistic for the size and number of plant parts (*i.e.*, number of shoots, tubers...), additive for plant population growth and reproduction and antagonistic on whole plant biomass.

The outcome of these interactions may also vary with **time** (Tack et al. 2012; Hatcher et al. 1994; Mouttet et al. 2011; 2013). Not all aggressors may attack the host plant at the same time but they can still impact each other. For example, Lapalainen, Helander & Palokangas (1995) showed that infection by the rust *Melampsoridium botulinum* on birch trees (*Betula pubescens*) impacted the larval performances of the moth *Epirrita autumnata* even one year after the infection. How different aggressors impact each other may also vary during the interaction period. For example, the leaf miner *Tuta absoluta* increased the fungal lesion of the powdery mildew (*Oidium neolycopersici*) on tomato plants (*Solanum lycopersicum*) after four days of herbivory but not the first three days.

Similarly, the **spatial scale** of interactions may importantly affect the outcome of tripartite interactions. This scale can range from different parts of the same organ (*e.g.* infected and healthy parts the same leaf, Simon & Hilker 2003) to the whole plant (Kruess 2002; Apyranto and Potter 1990), for instance when root pathogens have effects on leaf herbivores, or conversely. When insect and fungi attack the same plant organ, interactions are more likely to be direct and plant mediated, while when they attack different organs or tissues interactions are mostly plant-mediated. For example, Hatcher et al. 1994 found that foliar grazing by the beetle *Gastrophysa viridula* on leaves of *Rumex* spp infected by the fungus *Uromyces rumicis* reduced pustules densities of about 80%. In addition, beetle feeding also increased plant resistance to fungus on undamaged parts of challenged leaves, with these effects being lower on undamaged leaves. Moreover, plant defenses are not always induced in the same ways. While some species of herbivores and pathogens may induce plant defenses locally, other species may induce systemic defenses in the whole plant. The spatial location of both aggressors may have important consequences on tri-partite interactions, especially when interactions between herbivores and pathogens occur within different compartments, *i.e.* aboveground-belowground interactions (see also Appendix I for further information about cross-compartment interactions).

Thus, tripartite interactions may be very diverse and complex. In addition, plants do not only interact with insects and pathogens but also with other plants from the same or different species. Plant diversity may independently affect plant defenses, plant infection by fungi and attack by herbivores and thus their interactions.

1.3 Plant diversity effects on associated organisms

Plant-plant interactions, such as facilitation or competition, may impact host plant growth and chemistry, thus affecting associated organisms. These plant interactions may vary with genetic (see also supplementary material Appendix II) and species diversity of plant species present in a same area. Hereafter, we will focus on the effect of plant species diversity on associated herbivore insects and fungi.

1.3.1 Plant diversity effects on insect herbivore

Pest regulation is one of the ecosystem services provided by plant diversity. An increasing plant diversity generally correlates with a decrease in herbivory (Jactel & Brockerhoff 2007; Castagneyrol et al. 2013), which is known as **associational resistance** (AR, Barbosa et al. 2009). However, the opposite pattern, that is, an increase in likelihood of focal plants of being attacked by herbivores when surrounded by heterospecific neighbours, known as **associational susceptibility** (AS), have also been reported in the literature (Schuldt et al. 2010,2015; White & Whitham 2000). Understanding the mechanisms behind AR and AS is crucial to better predict how plant diversity will impact tri-partite interactions.

Several mechanisms and hypothesis have been proposed to explain **associational effects** (Figure 5). The first ones are based on how plant diversity may change host plant location by the insects. The “**resource concentration hypothesis**” (Root 1973) predicts that herbivores are more able to locate and remain on hosts that are growing in high density or in nearly pure stands than in more diverse stands where their host plants are more diluted (Hambäck et al. 2000, 2014). Heterospecific neighbors may disrupt both visual (Dulaurent et al. 2012, Damien et al. 2016) and chemical (Zhang and Schlyter 2004, Jactel et al. 2011) cues used by herbivore insects to locate and colonize a host tree, thus reducing their **apparency**. Conversely, an ‘**herbivore spill-over**’ (and then AS) may happen when the focal plant is more consumed when growing near more apparent or more palatable plant neighbors (White and Whitham 2000, Hahn and Orrock 2016).

Plant diversity may also influence the top-down control of insect herbivores by their natural enemies. The ‘**enemies hypothesis**’ (Elton 1958, Root 1973) posits that plant communities with higher species richness provide more resources and habitats and thus can shelter more diverse predator or parasitoid communities (Wilby and Thomas 2002, Schuldt et al. 2011, Castagneyrol and Jactel 2012), which could in turn provide a better control of herbivore populations (Riihimäki et al. 2005, Leles et al. 2017).

Finally, plant neighbors may change host plant resource quality for insects. A more diversified diet may increase herbivore performance by a complementary acquisition of deficient nutrients or a reduction in toxins ingestion (*i.e.*, “**mixing diet**”; Bernays et al. 1994) thus increasing herbivory levels. In addition, facilitation or competition and changes in micro-environmental conditions by plant neighbors may change the expression of plant traits such as the leaf area, toughness or thickness (Castagneyrol et al. 2017), plant chemistry (Kos et al. 2015) and defensive compounds (Mraja et al. 2011, Moreira et al. 2014), thus impacting associated herbivore insects.

Two recent meta-analysis (Jactel and Brockerhoff 2007b, Castagneyrol et al. 2014b) have suggested that AR is the most common pattern, however, there are several factors that may explain the variability in outcomes of insect responses to plant diversity. Diversity effects may depend on **herbivore specialization** (Jactel & Brockerhoff 2007; Castagneyrol et al. 2014). While mixing tree species generally significantly decreases herbivory by mono and oligophagous insects, the effect on generalist is more variable (Castagneyrol et al. 2014). The **phylogenetic distance** between plant neighbors and the focal plant have also important consequences in terms of herbivory, with diversity effects being more effective when plants are phylogenetically more distant (*i.e.*, by mixing broadleaves and conifers; Castagneyrol et al. 2014).

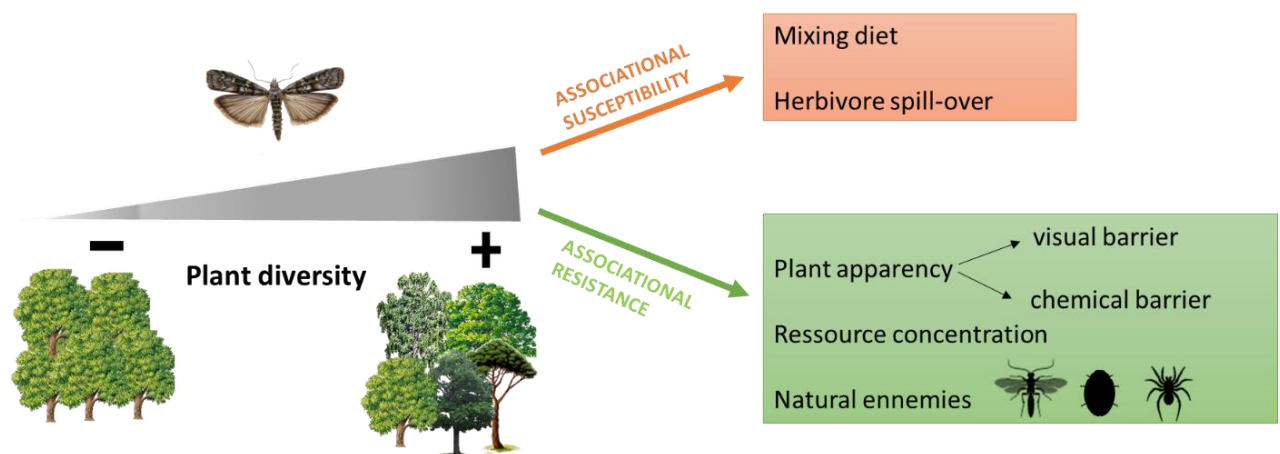


Figure 5. Theoretical mechanisms of plant diversity effects on herbivore insects.

1.3.2 Plant diversity effects on fungal pathogens

A reduced pathogen damage in mixed forest compared with monospecific forest stands have been reported in several cases (reviewed by Jactel et al. 2017). The disease-diversity hypothesis (Elton 1958) posits that communities with high genetic or species diversity in a community confers disease resistance (Mitchell et al. 2002, Hantsch et al. 2013b). Even if mechanisms of AR and AS have been defined for herbivores (Barbosa et al. 2009), we may

expect that mechanisms and hypotheses of associational effects also apply to pathogens (Jactel et al. 2017).

A reduction of pathogen disease rates on mixed forest maybe explained by a **host dilution** (*i.e.*, resource concentration hypothesis, Root 1973) by mixture of non-hosts and host species, or by mixtures of trees species of different susceptibilities (Peri et al. 1990, Gerlach et al. 1997). Indeed non-host or less susceptible species may decrease pathogen infection risk in the stand by reducing pathogen load and transmission (Hantsch et al. 2013b, Parker et al. 2015). Fungi dissemination to new hosts (horizontal transmission) is mediated by spore dispersal in air, water or soil, by mycelial spread, root contacts or by vectors as insects. In the last case, associational effects on insect may also mediate associational effects on vectored pathogens having the same host range.

Horizontal transmission may depend on the host density. For example, Mitchell et al. (2002) showed that foliar pathogen load in plant communities was correlated to host plant density. For fungal pathogens, this host density is reduced in mixed stands. However, Hantsch et al. (2014a) found a positive effect of tree diversity reducing specialized fungal pathogens of *Tilia cordata* and *Quercus petraea* which was independent on host proportion in the stand.

A disease reduction is not always the general pattern (Hantsch et al. 2014c). Hantsch et al. (2014b) did not find any significant effect of tree species functional diversity neither on fungal species richness nor pathogen load of tree leaves on a diversity experiment. In some cases, fungi need two different host species to complete their life cycle (*i.e.* heteroxenic pathogens), and thus an increase in biodiversity may facilitate their development. For example, the pine twisting rust, *Melampsora pinitorqua*, could be benefited in pine and aspen mixtures, as aspen trees can acts as alternate host species for pathogen development (Mattila 2002). In addition, most pathogens infect more than one species, which may result in a **pathogen spill-over** in some mixtures, especially when host plants are closely related (Gilbert and Webb 2007, Parker et al. 2015).

Indeed, disease pressures at local scales are strongly correlated with phylogenetic distance between host plants in the stand and relative abundance of host plants (Parker et al. 2015; Figure 6), so we can expect that mixing plants more phylogenetically distant may provide some associational resistance to pathogens.



Figure 6. Theoretical mechanisms for plant diversity effect on pathogenic fungi.

Plant diversity may also change the local environment for fungal dispersion and infection. These changes in micro-environment may also change plant traits, and thus affect plant quality for the fungus. Moreover, not all fungi exploit their host plant in the same way (see section 1.1.2) so they may be differently affected by changes in host plant quality. Fungi lifestyle may also play an important role in associational resistance to pathogens, however, this hypothesis have not been formally tested.

As for insects, there is also a top-down control of fungal pathogens by other fungi (endophytes, or hyperparasites), by some bacteria and virus. Plant mixtures can accommodate antagonistic microorganism of fungal pathogens, so the ‘enemies hypothesis’ (Elton 1958, Root 1973) may also be applied to fungal pathogens (Jactel et al. 2017). For example, mixing Douglas-fir (*Pseudotsuga menziensis*) trees with paper birch (*Betula papyrifera*) can provide the first one with some protection against the root pathogen *Armillaria ostoyae*. This is due to the fact that paper birch, which is less susceptible to this pathogen, also provides more favorable environment for fluorescent pseudomonads which have a negative impact on *A. ostoyae*, by reducing its radial growth (DeLong et al. 2002). However, little is known about plant diversity effect on their microbiome (but see Nguyen et al. 2017 and chapter 4 of this thesis).

To conclude, as for insect herbivores, the plant diversity effects on associated pathogenic fungi are probably dependent on pathogen dispersion mode, host specificity, the relative proportion and density of host plants, and neighbors identity.

1.3.3 The case of invasive insect and fungus in forests

Biological invasions are big threats for forest health. For example, forest in virtually all regions of the world are being affected by invasions of non-native insects (reviewed by Brockerhoff and Liebhold 2017) and most of the fungal pathogens are alien in regions where

they were recorded (Pimentel et al. 2001). Pathogenic fungi provide several examples of devastating biological invasions and of management strategies attempting to mitigate them (reviewed by Desprez-Loustau et al. 2007).

Plant diversity has also been shown to drive ecosystem resistance to invasion (the diversity-invasibility relationship, Elton 1958). Biotic resistance has long attracted interest in research and management, but exotic pest and pathogens remains understudied (reviewed by Nunez-Mir et al. 2017a). A reduced invader abundance, survival, fertility and diversity with increasing plant diversity was found as general pattern in a meta-analysis (Balvanera et al. 2006), but most of these studies concerned invasive plant species. For instance, forest susceptibility to a non-native beetle was independent on forest community composition and structure (Smith et al. 2015).

In several cases (discussed in sections 1.3.1 and 1.3.2), diversity may provide AR to pest and fungal pathogens. Mechanisms of biotic resistance to non-native pest may be also similar to described for AR to native species (Jactel et al. 2006, 2017, Guyot et al. 2015).

For example, one of the proposed reasons for the success of exotic species relates to the ‘**enemy release hypothesis**’. This hypothesis have been initially formulated in the case of invasive plants (Shea and Chesson 2002, Keane and Crawley 2002) and posits that “non-native plants escape damage from herbivores and thereby achieve a greater fitness in their novel range” (Keane and Crawley 2002). If we take this hypothesis in the context of invasive insect pest or fungal pathogens (Nunez-Mir et al. 2017a, Brockerhoff and Liebhold 2017, Jactel et al. 2017) we should generalize that invasive species may achieve a greater fitness in their novel range because of the absence of co-evolved natural enemies. By contrast, the “**exotic prey naïveté hypothesis**” (Sih et al. 2010) posits that exotic, naïve species may suffer from higher predation pressures than native prey species because of their naïveté towards native enemies (Li et al. 2011). In any cases, in an AR context, the ‘**enemies hypothesis**’ (Elton 1958, Root 1973) predicts that communities with greater plant diversity may shelter more diverse and abundant predator and parasitoids, thus increasing the probability of natural enemies being able to shift onto exotic

Box 4. Glossary

Alien (non-native, exotic, non-indigenous, foreign): a species, subspecies or lower taxon occurring outside of its natural range and dispersal potential (from IUCN, see <http://www.issg.org>).

Invasive species (invader): an alien species that becomes established in natural or semi-natural ecosystems or habitat, is an agent of change and threatens native biological diversity (from IUCN; see <http://www.issg.org>).

Invasiveness: the ability of an organism to arrive, spread beyond its introduction site and become established in new locations where it might provide a deleterious or harmful effect on the resident organisms and ecosystem (Desprez-Loustau et al. 2007).

Invasibility: the vulnerability or susceptibility of a community or ecosystem to invasions, resulting from its intrinsic properties.

Biotic resistance: ability of communities to resist to exotic invasions (Nunez-Mir et al. 2017).

prey and reduce their damage. However, this hypothesis needs further research. For instance, Jactel et al. (2006) showed that an exotic insect, *Matsucoccus pini*, was more likely to be preyed by native bugs (*Elatophilus nigricornis*) in mixed stands of maritime pine and Corsican black pine because the latter is the host species of *Matsucoccus pini*, a native scale species serving as main prey for *E. nigricornis*.

At this point, we may state that there are still several questions that remain to be better addressed:

- *which is the general outcome of plant-fungus-insect interactions?*
- *which factors explain general patterns in tripartite interactions?*
- *what are the effects of plant diversity on plant-insect and plant-fungi interactions?*
- *and in the particular case of non-native bio-aggressors?*
- *which are the likely mechanisms explaining this effects?*

An interesting model of study for such tripartite interactions in the case of non-native bio-aggressors may be the fungal pathogen *Cryphonectria parasitica*, the gall wasp *Dryocosmus kuriphilus* and their host plant, the sweet Chestnut (*Castanea sativa*), which are now co-occurring in Southern Europe. We already have some evidence of direct interactions between these two aggressors, as *C. parasitica* seems to benefit of *D. kuriphilus* emergence holes in galls to penetrate in chestnut tissues (Meyer et al. 2015). However, plant-mediated interactions are also likely to occur. In addition, *D. kuriphilus* damage have been shown to decrease with tree species diversity in the stand (Guyot et al. 2015). However, likely mechanisms, in particular spill-overs of native enemies are still unknown.

In next section 1.4, I present details about this model system and questions about tri-partite interactions and biodiversity effects will be further evaluated on Chapters I, II, III and IV.

1.4 Model system

1.4.1 The European chestnut, *Castanea sativa*

The European chestnut or sweet chestnut (*Castanea sativa* Mill.) is a deciduous tree species from the *Fagaceae* family.

Origin and distribution area:

Its diffusion and active management makes it difficult to trace its original range (see also Conedera et al. 2004).

Its current distribution area ranges from Southern Europe (Iberian Peninsula, Italy, Balkans, Mediterranean islands) and North Africa (Morocco), to North-Western Europe (England, Belgium, Netherlands) and eastward to Western Asia (North East Turkey, Armenia, Georgia, Azerbaijan, Syria) (Conedera et al. 2016).

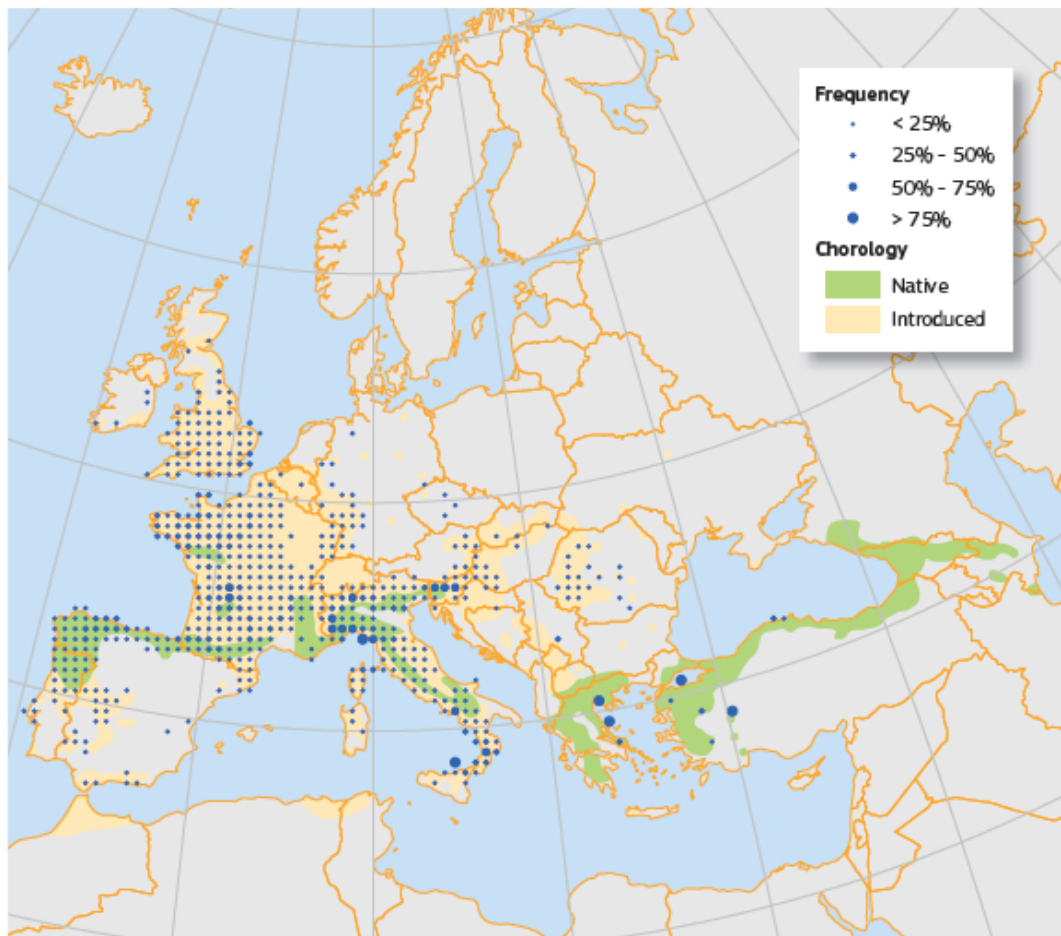


Figure 7. Map of plot distribution and simplified chorology for *Castanea sativa* in Europe. From Conedera et al. (2016). In: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), *European Atlas of Forest Tree Species*. Publ. Off. EU, Luxembourg, pp. e0125e0+.

Habitat and Ecology:

C. sativa is a warm-temperate tree species that likes mean annual temperatures that range from 8°C to 15°C and minimum rainfall of 600-800mm (Conedera et al. 2016). It is very sensitive to high temperatures combined with the lack of precipitations (Conedera et al. 2004). Trees grow better in poor, well-drained soils, ranging from very acidic to neutral.

Uses:

European chestnut forests have been managed since the medieval times for the production of nuts and timber but also secondary goods and products such as fuel wood, food for cattle and honey. It has also an important ecological and structural role in coppice forests, and is of high cultural relevance in Mediterranean regions.

Diseases and threats:

Several insects and fungi are known to attack chestnut trees in Europe, particularly those damaging nuts like the weevil *Curculio elephas*, the nut moth (*Carpocapse*) or the nut rot diseases caused by *Gnomonipsis spp.* or *Phomopsis spp.*

Two main diseases caused by exotic species have resulted in severe chestnut decline in the XXth century, the ink disease, caused by the oomycetes *Phytophthora cinnamomi* and *P. cambivora*, and the chestnut blight, caused by the fungus *Cryphonectria parasitica* (see section 1.4.2).

Recently, a non-native insect pest, the chestnut gall wasp, *Dryocosmus kuriphilus* has been accidentally introduced in Europe and he is currently spreading across the continent (see 1.5.3).

1.4.2 Chestnut blight, *Cryphonectria parasitica*

The chestnut pathogen, *Cryphonectria parasitica* (Murr.) (Figure 8) is an invasive fungus from the family *Cryphonectriaceae* (Order Diaporthales) which is responsible of blight disease (see the reviews of Robin and Heiniger 2001, and Rigling and Prospero 2017 for detailed information and references).

Invasion history:

C. parasitica is native to Eastern Asia. It was first reported in the American chestnut (*Castanea dentata*) in the United States in 1904 where it developed a large outbreak that lead to the quasi-extinction of the host species. In Europe, multiple introductions have been identified (Dutech et al. 2012). *C. parasitica* was first reported in 1938 in Italy



Figure 8. Chestnut bark infected by *C. parasitica*

near Genoa, where it was probably introduced from North America. Additional introductions occurred directly from Asia, certainly in western Spain or France (Dutech et al. 2012). Then, the disease rapidly spread throughout the chestnut distribution area in Europe (Figure 9). In contrast to the US, the epidemics did not result in chestnut extinction, especially in the first colonized areas where the disease incidence is high but severity low. This disease severity regulation may be explained by the appearance of *C. parasitica* hypovirulence, which allows trees to recover (cf. Disease management).

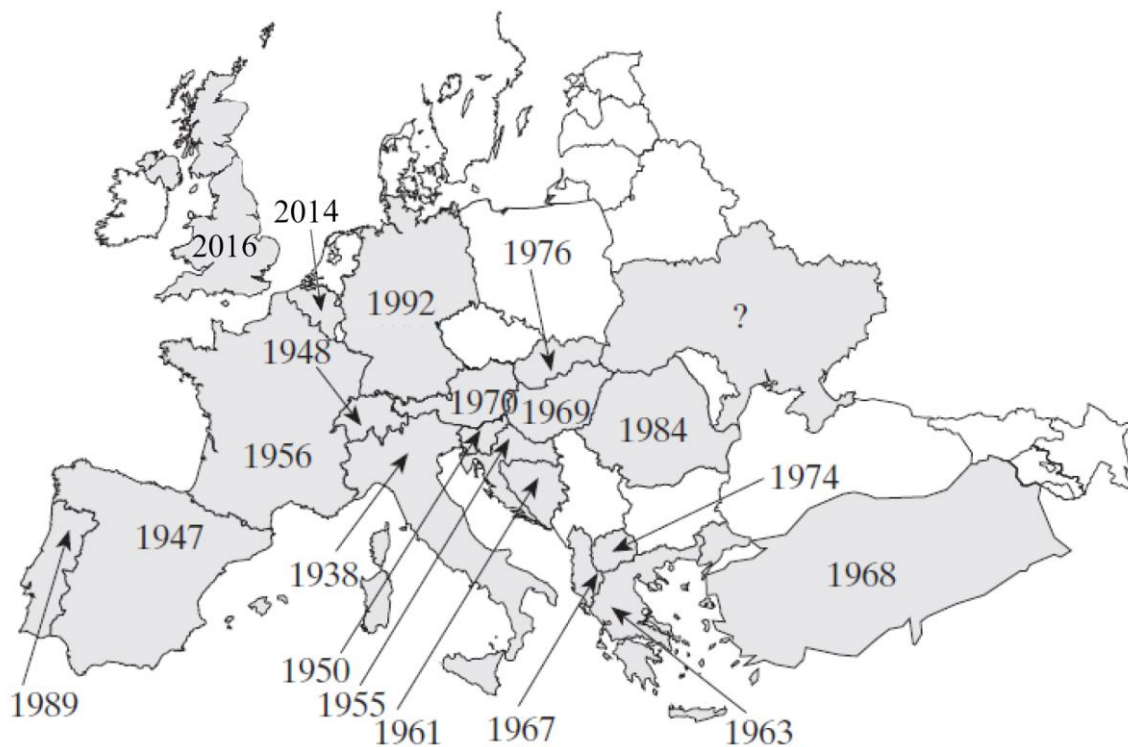


Figure 9. Map of *C. parasitica* invasion in Europe and year of its first observation. Modified from Robin and Heininger (2001).

Life cycle (Figure 10):

Cyphonectria parasitica is a necrotrophic pathogen and, as such, needs wounds or death plant tissues to penetrate in the host plant. On susceptible trees, *C. parasitica* produces mycelial fans, which penetrate intercellularly into the bark and cambium to form a canker. Once established, it produces fructification bodies resulting from sexual or asexual reproduction (perithecia and pycnidia, respectively). Asexual (conidia) and sexual (ascospores) spores can be transported over short and long distances through rain splashes, birds, insects, mites and wind borne dust and infect new trees. On susceptible trees, *C. parasitica* produces mycelial fans, which penetrate intracellularly into the bark and cambium to form a canker. *C. parasitica* can infect stem, branches, and also twigs, thereby altering the functioning of these tissues (sap flow) in susceptible chestnut species such as *Castanea sativa*. Generally, following infection, tree leaves become yellow or brown and remain hanging on the dead branches which is called flag symptoms. In addition, trees produce numerous epicormics

shoots below the cankers. The severity of symptoms in *C. sativa* varies with the age of infected stem or branch and with the virulence of the fungal strain involved.

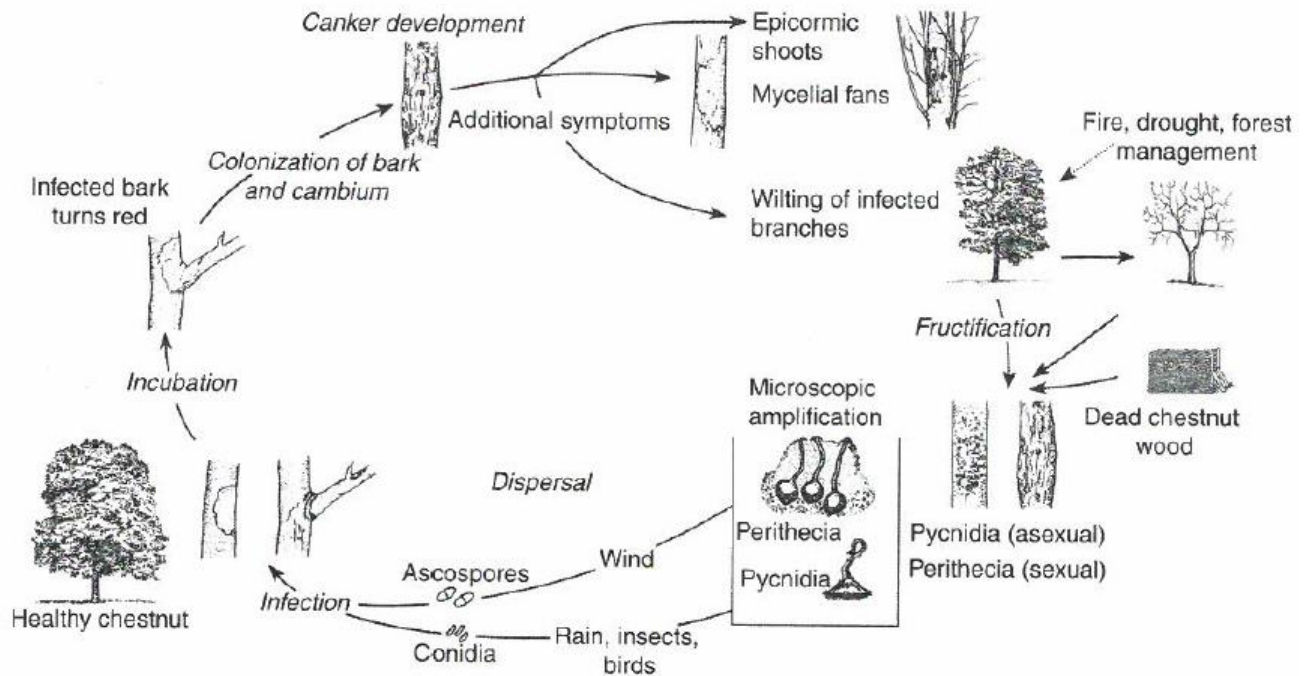


Figure 10. *Cryphonectria parasitica* life cycle. From Prospero & Rigling 2013 (book chapter 5)

Disease management:

In Europe, the EPPO (European and Mediterranean Plant Protection Organization) still recommends the regulation of *C. parasitica* as an A2 quarantine organism. However, once *C. parasitica* is established into an area, control measures can be undertaken to protect trees to infection (wound protection), to decrease the inoculum pressure (by cutting and burning the infected branches). These measures may be effective in orchards isolated from chestnut forests, with a low disease incidence. Fungicide applications are too difficult and expensive to represent a practicable option and are not registered for chestnut blight.

There are several breeding programs for developing chestnut blight resistant varieties based in crosses between susceptible American or European chestnuts with Asian chestnuts (in America there is also transgenic chestnuts resistant to blight). In Europe, these crosses have resulted in hybrid trees with different susceptibility levels, some of them being used as some interspecific as fruit varieties in orchards.

In Europe, the most effective management method is the biological control with hypovirus-infected fungal strains. The hypovirus CHV-1 is the best studied and widespread in Europe and also in Asia Minor. Blight biocontrol treatment consists in artificially injecting infected tree with fungus strains carrying the hypovirus. Then the mycovirus is horizontally

transmitted to the virus free fungus (causing the canker) via hyphal anastomosis. When the hypovirus is present, trees are able to create new bark layers under the canker, the outer bark cracks, and the canker takes a swollen appearance (*i.e.* healed cankers).

1.4.3 Asian chestnut gall wasp, *Dryocosmus kuriphilus*

The Asian Chestnut Gall Wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae) is a micro-hymenoptera (2-3 mm) belonging to the Cynipidae family (Figure 11). ACGW is classified as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO 2005).

Colonization history:

This pest, native to China, was first introduced to Japan in 1958 (Murakami et al. 1980), then to Korea in 1958 (Cho & Lee, 1963) and in USA in 1974 (Payne et al. 1976). It was firstly reported in Europe (Piedmont, Italy) in 2002 (Brussino et al. 2002) but its introduction can be traced back to 2-3 years, with the importation of contaminated nursery material from China (Quacchia et al., 2008). To date ACGW have colonized almost all the European chestnut geographical range (Aebi et al. 2007; Battisti et al. 2014). Dispersal is due to human movement of infected plants and scions (Rieske 2007; Graziosi & Santi 2008) but also to active adult flight (about 24-25 km per year, Rieske 2007).



Figure 11. *Dryocosmus kuriphilus* adult. ©INRA

Life cycle (Figure 12):

ACGW is a univoltine and thelytokous species (*i.e.*, its populations are composed only of females which reproduce asexually once a year). It is a gall maker, specialized on chestnuts with a life cycle synchronized with its host phenology (Bernardo et al. 2013). At the time of chestnut bud-burst (around mid-April), ACGW larvae induce the formation of galls within which they develop and pupate. Between mid-June and mid-August adults hatch from the galls and lay eggs in new preformed chestnut buds. Adult longevity can be up to 10 days. Each female has a mean egg load of 268 eggs (Graziosi and Rieske 2014). Eggs hatch in summer and first instar larvae overwinter within dormant buds until the following season (Bernardo et al. 2013).

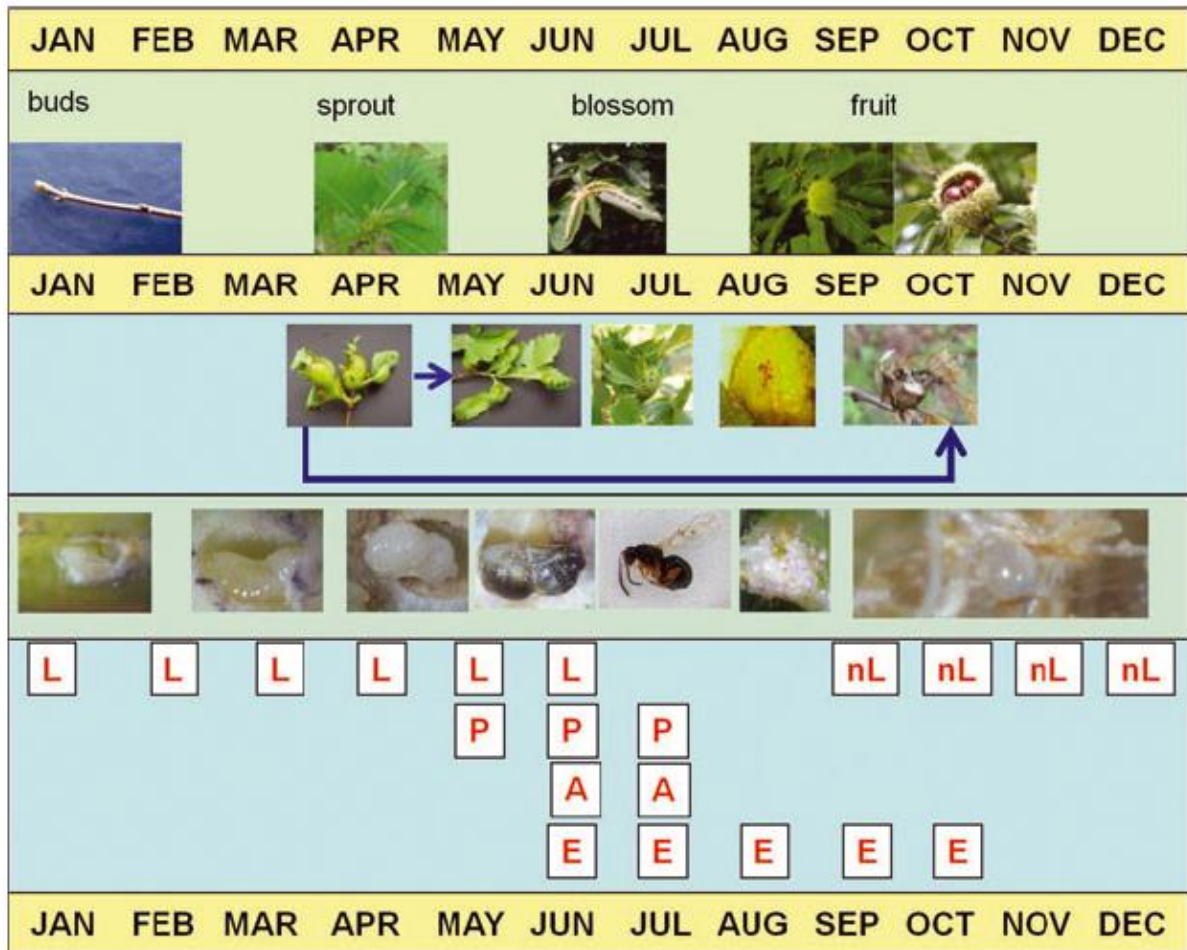


Figure 12. *Dryocosmus kuriphilus* life cycle linked to *Castanea sativa* phenology in Italy E, eggs; L, larvae; P, pupae; A, adults; nL, young larvae (first instar). From Bernardo et al. (2013).

Damages:

ACGW is considered the most important pest of *Castanea* species worldwide (Payne 1983; Moriya 1990). Galls can develop on chestnut leaves, stipulas, dormant buds and shoots, with different damage levels (for more details on damage classification and estimation see also Maltoni et al. 2012). Gall formation prevent normal development of plant tissues and reduces leaf photosynthetic area. In galled tissues, there is a reduction of about 30% of CO₂ assimilation and stomatal conductance (Ugolini et al. 2014). This can result in a reduced vigor of infected shoots. Severe and repeated infestations on young trees can even lead to tree death (Moriya et al. 2003). Damaged trees are also less vigorous and thus more susceptible to other pathologies as chestnut blight (Ugolini et al.2014).

ACGW attacks have profound consequences on wood and nut production, with losses in nut yield that can be up to 80% (Battisti et al. 2013).

Control strategies:

Immature stages of the ACGW are protected by the gall which makes it difficult the utilization of chemicals (Murakami, 1981). Mechanical removal of galls and some pruning methods may however help to control their populations in chestnut orchards (Maltoni et al. 2012).

Variability to ACGW susceptibility has been observed in *C. sativa* and among the interspecific hybrids. First breeding programs for chestnut resistance took place in Japan, where ACGW was first established. This had an initial success but resistance was overcome by some ecotypes of the insect (Sartor et al. 2015).

To date, the most successful tool for controlling this pest seems to be the parasitoid *Torymus sinensis* (Hymenoptera, Torymidae). This parasitoid is native to China, like its host, and has a well synchronized lifecycle with ACGW. It was first released as biocontrol agent in Japan where it reduced local infestation levels (Aebi et al 2007), and more recently in Europe, in 2005 (see also Gibbs et al. 2011, Aebi et al. 2007; Borowiec et al 2014).

Other hymenopteran insects, naturally present in Europe as parasitoids of oak gall wasps (Cynipids), have been also reported on ACGW galls (Panzavolta et al. 2013; Palmeri et al. 2014; Francati et al. 2015; Quacchia 2013). These parasitoids have a great potential for ACGW biological control but a better understanding in the biology and ecology of these parasitoids is needed (see Chapter III).

In addition, some endophytic fungus are known to produce necrosis in ACGW galls such as *Gnomonopsis castanea*, and some of them are promising biocontrol agents as some *Fusarium spp.* (Tosi et al. 2015).

1.5 Research questions and manuscript structure

Along the following chapters of this thesis I aimed at improving our knowledge on tripartite interactions, tree diversity effects and associated mechanisms by using the chestnut - *C. parasitica* - ACGW model.

For this aim, I structured the content of this manuscript in four chapters:

Chapter I: provides a theoretical framework of tri-partite interactions. More specifically, I performed a meta-analysis on fungal infection effects on insect preference and performance when they both co-occur within the same host plant.

Chapter II: I experimentally tested tripartite interactions between chestnut trees, *C. parasitica* and ACGW. Specifically, we tested the effect of fungal infection in ACGW infestation rates and adult fitness, reciprocal effect of ACGW presence on later *C. parasitica* infection and their single and combined impacts on tree growth. We also performed chemical analysis in order to study whether fungal or insects infections change host plant quality or quantity.

Chapter III: I addressed the question of plant diversity and AR to ACGW in presence or absence of blight symptoms on chestnut tree trunks in an observational study in natural mixed forests. We put special attention in ACGW natural enemies in order to provide some experimental support to the ‘enemies hypothesis’ in the framework of biological invasions (see section 1.3.4).

Chapter IV: I provided a preliminary study of the ‘enemies hypothesis’ by studying the fungal endophytic communities in galls caused by ACGW: I compared these communities to those found in surrounding leaf tissues and studied the effect of stand species diversity on their composition.

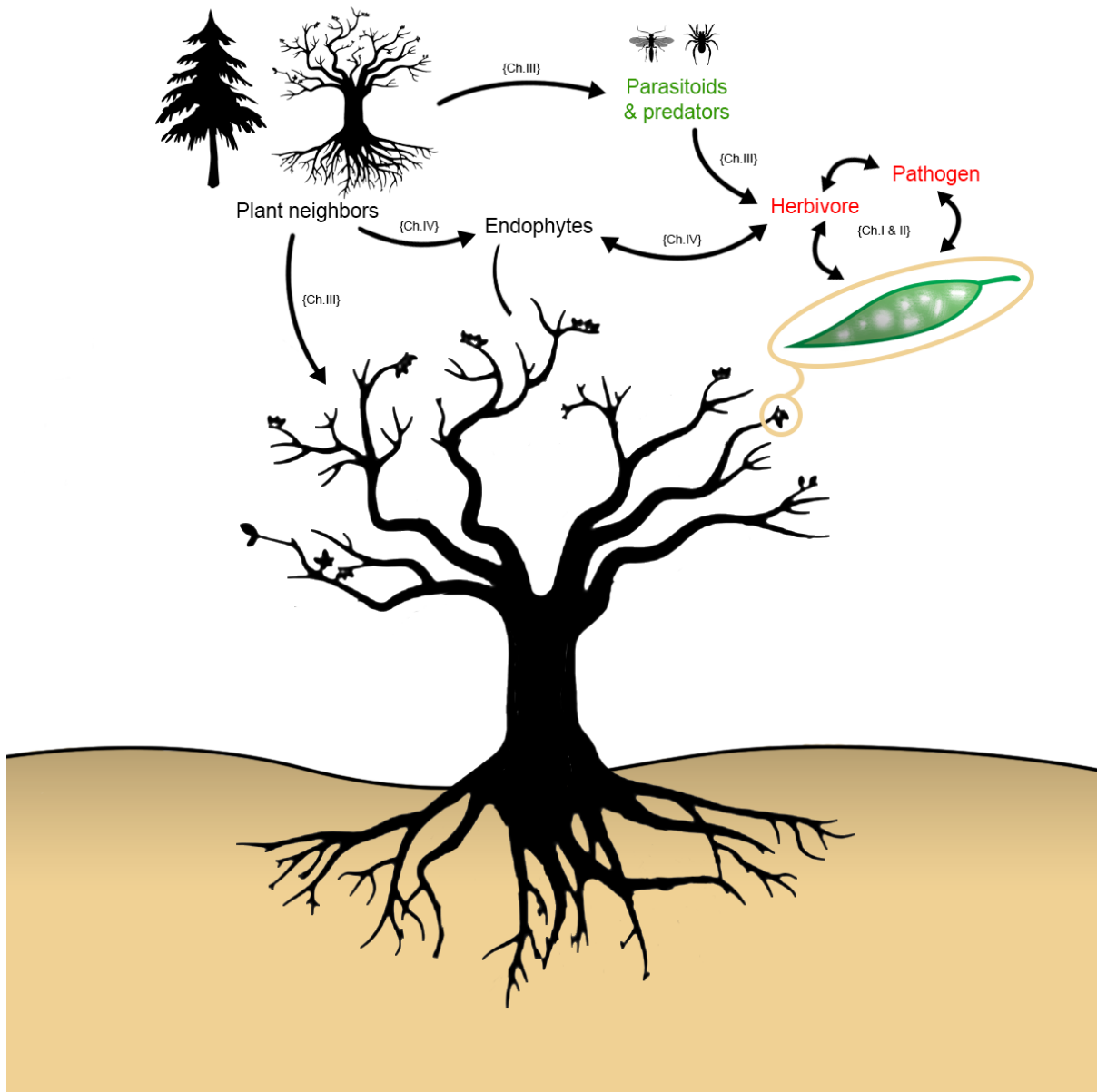


Figure 13. Plant interactions addressed in this thesis and their distribution in the different chapters.

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2. CHAPTER I

Fungi reduce preference and performance of insect herbivores on challenged plants

Ecology. In press

Fungi reduce preference and performance of insect herbivores on challenged plants

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ABSTRACT

Although insect herbivores and fungal pathogens frequently share the same individual host plant, we lack general insights in how fungal infection affects insect preference and performance. We addressed this question in a meta-analysis of 1,113 case studies gathered from 101 primary papers that compared preference or performance of insect herbivores on control *vs.* fungus challenged plants.

Generally, insects preferred, and performed better on, not challenged plants, regardless of experimental conditions. Insect response to fungus infection significantly differed according to fungus lifestyle, insect feeding guild and the spatial scale of the interaction (local/distant). Insect performance was reduced on plants challenged by biotrophic pathogens or endophytes but not by necrotrophic pathogens. For both chewing and piercing-sucking insects, performance was reduced on challenged plants when interactions occurred locally but not distantly. In plants challenged by biotrophic pathogens, both preference and performance of herbivores were negatively impacted, whereas infection by necrotrophic pathogens reduced herbivore preference more than performance and endophyte infection reduced only herbivore performance.

Our study demonstrates that fungi are may be important but hitherto overlooked drivers of plant-herbivore interactions, suggesting both direct and plant-mediated effects of fungi on insect's behavior and development.

Key-words: biotrophic pathogens, endophytes, meta-analysis, necrotrophic pathogens, plant defense, plant-mediated indirect interactions, tripartite interactions

2.1. Introduction

Plant-associated fungi and herbivorous insects often co-occur on the same host plant. While most of the attention so far focused on their independent and additive effects on plant fitness (Hauser et al. 2013), direct and plant mediated effects of fungi on herbivorous insects are less well understood. Plant-associated fungi may modify plant functional traits and quality, and thus indirectly affect herbivorous insects (Friesen et al. 2011). Fungal infection can either increase (e.g., by releasing soluble sugars, Cardoza et al. 2003a) or decrease (e.g., through reduced nitrogen content, Hatcher et al. 1994a) the quantity and within-plant distribution of nutrients, thus affecting the performance of insects (Tinney et al. 1998, Cardoza et al. 2003a). Fungal infection can thus be detrimental (Hatcher et al. 1994a, Kruess 2002), beneficial (Friedli and Bacher 2001, Cardoza et al. 2002, 2003a) or neutral (Kok et al. 1996, Saikkonen et al. 2001) to herbivores. However, we still lack a quantitative estimate of the overall effect of fungal infection on insect preference and performance on challenged plants. Moreover, how these tripartite interactions depend on the feeding guild of the insect, lifestyle of the fungus and the spatial scale of the interaction remains to be comprehensively explored (Hatcher 1995, Stout et al. 2006, Tack and Dicke 2013).

The way by which fungi obtain resources from their living host plants may have profound consequences for insect herbivores. Among the five main functional groups of plant-associated fungi (mycorrhizae, epiphytes, endophytes, biotrophic pathogens and necrotrophic pathogens, Porrás-Alfaro and Bayman 2011), mycorrhizae is the group that received the greatest attention with respect to its effect on herbivores. Koricheva et al. (2009) showed that the impact of mycorrhizal-infected plants on insect performance depends on herbivore feeding specialization. Mycorrhizal infection generally increase the performance of mono- and oligophagous chewers and decrease the performance of polyphagous chewers. For sucking insects, mycorrhizal infection positively affects phloem feeders but has a negative impact on the performance of mesophyll feeders. Although they also received substantial interest over the past decades, the direct and indirect effects of plant infection by pathogenic fungi and endophytes on insect herbivores are far less well understood (Hatcher 1995, Stout et al. 2006, Raman et al. 2012, Tack and Dicke 2013). Our study focuses on biotrophic and necrotrophic pathogens and endophytes. Biotrophic pathogens develop and extract their nutrients from living plant tissues (Delaye et al. 2013, García-Guzmán and Heil 2014) while necrotrophic pathogens secrete enzymes and toxins that degrade and kill the host cells and then live and feed on the dead plant tissue (Spoel et al. 2007, Delaye et al. 2013, García-Guzmán and Heil 2014). Both biotrophic and necrotrophic pathogens may produce molecules that are deterrent or toxic to herbivores, but are also frequently consumed by the herbivore while feeding on the host plant. Endophytes correspond to another lifestyle and are defined as ‘microorganisms that live at least during a part of their life cycle inside living plant tissue without causing visible disease symptoms’ (Partida-Martínez and Heil 2011b). Although their status along the continuum between parasitism and mutualism is evolutionarily and

ecologically unstable (Arnold 2007, Delaye et al. 2013), some endophytes are known to strongly influence the outcome of plant-herbivore interactions (Clay 1996, Kuldau and Bacon 2008, Saikkonen et al. 2010). Although endophyte-free plants do not exist (Arnold 2017, Partida-Martínez and Heil 2011, Peñuelas and Terradas 2014), the presence of particular endophyte species can have important consequences on plant functioning. Given the differences in the way that biotrophic pathogens, necrotrophic pathogens and endophytic fungi exploit plants, we predict that the direction and magnitude of fungal infection effects on insect herbivores depends on the fungus lifestyle.

Plants evolved common molecular mechanisms against aggression from both herbivores and fungal pathogens. Yet, not all mechanisms are elicited by, nor are effective against all aggressors. For instance, while the salicylic acid pathway is usually induced by and efficient against biotrophic pathogens and sucking herbivores, necrotrophic pathogens and chewing herbivores principally activate and respond to the jasmonic acid pathway (Spoel et al. 2007, Ali and Agrawal 2012, Thaler et al. 2012a, Al-Naemi and Hatcher 2013). Given these specificities, we predict that the response of herbivores to plant infection by fungi will depend interactively on fungus lifestyle and herbivore feeding guild, with stronger negative effects of necrotrophic fungi on chewing than on sap feeding insects, but conversely more negative effects of biotrophic fungi on sap feeding insects. Mouttet et al. (2013) found partial support for this hypothesis. They showed a reciprocal negative effect of the sap-feeding whitefly *Bemisia tabaci* and the biotrophic pathogen *Oidium neolycopersici* in tomato plants, which is consistent with JA-SA crosstalk, but an asymmetrical positive effect of the leaf miner *Tuta absoluta* on tomato powdery mildew, which is inconsistent with JA-SA crosstalk.

Herbivores have often been reported to discriminate between “not challenged” (non-infected by a specific endophyte or pathogen) and “challenged” (by a specific endophyte or pathogen) plants, and thus exhibit preference. A likely reason is that fungal infection modifies the visual (Rizvi et al. 2015a) or chemical cues (Rostás et al. 2015) that herbivores use to locate and select their host. However, there is no consensus about the consequences of such changes on herbivore behaviour: fungus challenged plants may be more (Johnson et al. 2003, Cardoza et al. 2003b, Jallow et al. 2008), less (Kruess 2002, Laine 2004, Menjivar et al. 2012) or as attractive as not challenged plants (Jallow et al. 2004, Spafford Jacob et al. 2007). Although it is generally assumed that insect preference matches insect performance (Gripenberg et al. 2010), fungal infection may break down the preference-performance relationship by modifying only herbivore preference or performance, or, alternatively, affect preference and performance in opposite directions (for example, the fungus may increase insect preference but decrease insect performance). For instance, Kruess (2002) showed an increase in the preference and performance of the leaf beetle *Cassida rubiginosa* on creeping thistle *Cirsium arvense* challenged by the necrotrophic fungus *Phoma destructiva*. By contrast, Jallow et al. (2004) found reduced performance of the polyphagous moth *Helicoverpa armigera* on endophyte challenged tomato plants but no significant differences in foliage consumed on inoculated vs. control plants in choice tests. Given these discrepancies among studies, a general overview of patterns and mechanisms is needed.

At a within-plant scale, herbivores can discriminate between not challenged and challenged organs from the same host plant, and even between not challenged and challenged tissue of the same organ (e.g. the same leaf, Simon and Hilker 2003a). But not all herbivores may be able to discriminate and avoid challenged organs or tissues. Notably, when the fungus and insect share the same plant organ, the effect of the fungus on the insect herbivore may be direct (through production of supplementary nutrients or toxins), indirect (i.e., fungal-induced changes in the host plant) or both. However, when a herbivorous insect feeds on not challenged organs of a fungal challenged plant, the effect of the fungus on insect performance is mainly indirect and mediated by changes in the host plant. As the impact of fungal infection on plant quality (direct and indirect) and fungal biomass may decrease with increasing distance from the site of infection, the effect of fungal infection on herbivore performance may then depend on the spatial scale of fungus-insect interactions (Simon and Hilker 2003a, Tack and Dicke 2013, Mouttet et al. 2013a). We thus hypothesize that the effect of fungal infection on herbivorous insects is stronger for local (i.e., when feeding on the same plant organ) than for distant (i.e., when feeding on the same plant) interactions.

Studies have tested how herbivore preference and/or performance are affected by fungal infection under both laboratory / greenhouse conditions (Friedli and Bacher 2001, Cardoza et al. 2003a) and in field experiments (Hatcher et al. 1994b, Kluth et al. 2001, Tack et al. 2012a). Yet, field conditions are often more variable and the impact of fungal infection on the insect herbivore may therefore be obscured by confounding factors such as climatic conditions (e.g., water availability, Bultman and Bell 2003, Miranda et al. 2011) or local species pools of insects and fungi. We therefore hypothesize that the impact of fungal infection on insect herbivores is easier to detect, and has a stronger effect, under more controlled experimental conditions, i.e., in laboratory or greenhouse experiments.

The main objectives of the present study were thus (1) to provide a quantitative estimate of the overall effect of fungal infection on the preference and performance of insect herbivores and (2) to explore the sources of variation in the magnitude of the fungus effect, by testing how insect feeding guild, fungus lifestyle, spatial scale of the interaction and experimental conditions impact on fungus-herbivore interactions. Detailed hypotheses and predictions are listed in Table 1.

Table 1: Effects of plant fungal infection on insect herbivores. For each hypothesis tested in the meta-analysis, the dataset used, main results and key references are given.

k = number of case-studies (in parentheses, the number of corresponding articles).

	Hypotheses	Dataset used	Result	Key references
Overall response of insect to plant infection by fungi	H1: Fungal infection has a negative impact on insect herbivores associated with the same host plant	Full data set <i>k</i> = 1113 (101)	Insect preference and performance are negatively affected by fungal infection	(Hatcher 1995, Rostas et al. 2003, Tack and Dicke 2013)
Experimental conditions	H2: The impact of fungal infection on insect herbivores is easier to detect and quantify under laboratory and greenhouse conditions than in the field		There are no statistical differences between experimental conditions (Table 2)	(Kluth et al. 2001, Stout et al. 2006, Tack et al. 2012a, Keathley and Potter 2012)
Fungus lifestyle	H3: The magnitude of herbivore response to fungal infection depends on the fungus lifestyle	Laboratory and greenhouse studies on insect performance <i>k</i> = 678 (67)	Biotrophic pathogens and endophytes negatively impacted insect performance, but there was no effect of necrotrophic pathogens (Fig. 1a)	(Clay 1996, Al-Naemi and Hatcher 2013, García-Guzmán and Heil 2014)
Cross-talk hypothesis	H4: Herbivore response to fungal infection is stronger for chewing herbivores when the plant is challenged by a necrotrophic fungus and for sucking herbivores when the plant is challenged by a biotrophic fungus		No significant interaction between insect feeding guild and fungus lifestyle on herbivore performance (Table 2)	(Ali and Agrawal 2012, Thaler et al. 2012a, Al-Naemi and Hatcher 2013, Mouttet et al. 2013a, Lazebnik et al. 2014)
Spatial scale of the fungus-herbivore interaction	H5: The effect of fungal infection is larger at the local scale		The effect of fungal infection is, for chewing insects, larger at the local scale (Fig. 1b)	(Hatcher et al. 1994b, Rostas and Hilker 2002a, Mouttet et al. 2011, 2013a)
Differences between insect preference and performance	H6: The impact of fungal infection differs between preference and performance	Laboratory and greenhouse studies for chewing insects at the local scale <i>k</i> = 415 (45)	Insect preference and performance differ between plants challenged by different fungus lifestyles (Fig. 2)	(Gripenberg et al. 2010, Crawford et al. 2010, Tack and Dicke 2013)

2.2. Materials and methods

Data collection

We searched the published literature reporting fungal effects on herbivorous insects sharing the same host plant. A first set of studies was initially identified from Tack and Dicke's review (2013) on plant-pathogen-herbivore interactions, which was used to define keywords to be searched in the Web of Science (ISI) electronic bibliographic database. We applied combinations of relevant terms such as: “(*Plant* or *tree*) and (*insect*) and (*preference* or *performance* or *choice*) and (*fung** or *oomyc**) not *bacteri** not *virus* not **mycorrh**”. We retained only articles, book chapters, reviews, theses, dissertations and abstracts published in English. To further limit the search to relevant papers, we filtered outputs to retain only those matching with the following research areas: plant sciences, environmental sciences, ecology, pathology, agriculture, zoology, forestry, chemistry, physiology, behavioral sciences, microbiology, entomology, biochemistry, molecular biology, parasitology and mycology.

The search was limited to the period 1950 – 2015. Our initial search yielded 1092 papers (the number of papers retained at each stage is reported in the PRISMA flow diagram, Fig. S1, Appendix S1 in Supporting Information). To complete our dataset, we surveyed the cited references in the articles retained and in the main reviews about plant-fungus-arthropod interactions (Rostas et al. 2003, Stout et al. 2006, Tack and Dicke 2013) and additionally screened the articles that cited these three review papers.

To be retained in the meta-analysis, studies had to meet the following criteria: (i) report insect preference for, or performance on, plants infected by the studied fungus (hereafter referred to as challenged plants) vs plants non infected by this specific fungus (hereafter referred to as not challenged plants), (ii) report taxonomic information about plant, insect and fungus, at least at the genus level and (iii) provide a measure of the mean and variability (i.e., variance, standard error or standard deviation) and the sample size in either the text, figures, tables or appendices. When needed, data were extracted from figures following digitalization using the open office extension *Oodigitizer* version 1.2.1 and *ImageJ*. We finally retained 1,113 study cases from 101 primary papers (see also Appendix S1). List of corresponding references are available in Supporting Information, Appendix S2.

Moderators

For each study case, we extracted the following moderators (explanatory variables): plant, insect and fungus species identity (at least at the genus level); fungus lifestyle (*biotrophic pathogen*, *necrotrophic pathogen*, or *endophyte*); insect feeding guild (*chewing*, *piercing-sucking*, *phloem feeding* and *sucking*, *cell-content sucking*, *sap-feeding*, *stem-boring*, *root-boring*, *pollen-feeding*, *bud-feeding* and *seed-feeding*); experimental conditions (field and greenhouse or laboratory study); spatial scale of the interaction (*local*, when insects targeted organs challenged by the fungus, *distant* when insects targeted organs not challenged by the fungus, and missing data, *NA*, when organ infection was not explicitly indicated in primary

papers); type of insect response (*abundance, acceptance, attraction, body size, resource consumption, density, development rate, development time, digestibility, egg hatching, emergence, fecundity, generation length, growth, longevity, mortality, oviposition, oviposition deterrence, population growth, population size, pupation, reproduction, survival, weight*). We eventually grouped insect responses into two categories: *preference* and *performance*. In some cases, the distinction between preference and performance was not straightforward. We decided to code corresponding cases as missing data (*NA*) to avoid spurious classification.

In addition to moderators, each study case was attributed a single identifier (*Case ID*) and assigned to one original paper (*Study ID*) and one study system (*System ID*). A *Study ID* corresponded to a single published paper retained in our analysis. A *System ID* was the combination of plant, fungus and insect species. Within a paper, each combination of plant, fungus and insect species was thus assigned to a specific study system (*System ID*). Within each *Study ID*, we considered as a *Case ID* any response variable measured for each pair of challenged and control plants. In most studies, more than one insect response variable was measured for the same system. Although variables from the same study were not strictly independent (*e.g.*, insect weight and survival), we used all variables to avoid possible bias due to *a priori* exclusion of some variables or losing valuable information. Non-independence among case studies was accounted for in the analyses using two independent and complementary approaches (see below, Statistical Analyses).

Statistical analyses

For each study case, we calculated an effect size using the Hedges' *d* metric and its variance (Hedges 1981) as estimated with the '*metafor*' package 1.9-8 version in R 3.2.3 (Viechtbauer 2010, R Core Team 2015). See Supporting Information (Appendix S3) for details of effect size calculation. First, we estimated the grand mean effect size using the complete data set. Second, we selected subsets of data for which there were enough observations for each level of moderators to enable testing their effects (Table 1). For instance, we excluded case studies on root-feeding ($k = 78$), seed feeding ($k = 2$), stem-boring ($k = 32$), pollen-feeding ($k = 5$) and bud-feeding insects ($k = 4$), and thereby only retained case studies on defoliators (chewing and mining insects) and piercing-sucking insects (including phloem feeders and suckers, cell-content suckers and sap-feeders).

To avoid confounding factors, moderators were tested using a hierarchical approach (Castagneyrol and Jactel 2012, Ferreira et al. 2015). Because results from field and laboratory/greenhouse studies may yield different results, we first tested the effect of experimental setting on effect sizes. Further analyses were restricted to laboratory/greenhouse studies for which it was possible to address the hierarchical effect of additional moderators (Table S1).

In laboratory/greenhouse experiments, case studies were not evenly distributed among moderators (Table S1). For instance, there were no case studies addressing the effect of plant infection by biotrophic pathogens on the preference of piercing-sucking insects. To avoid confounding the effects of fungus lifestyle and the type of insect response, we therefore used two independent models. We first tested how the effect size of fungal infection on insect

performance was affected by fungus lifestyle (necrotrophic vs. biotrophic pathogen vs. endophytic fungi), insect feeding guild (chewers vs. piercing-sucking) and spatial scale of interaction (local vs. distant). Next, we compared the impact of fungal infection on insect preference vs. performance retaining only case studies where fungi and chewing insects interacted at the local scale, while accounting for fungus lifestyle (Table S1).

For both models, all two- and three-ways interactions were included in the full model. We applied model simplification by sequentially removing non-significant interactions, starting with highest order interactions. For model comparison, parameters were estimated using Maximum Likelihood. Parameters of the final model were estimated using Restricted Maximum Likelihood (REML).

Most primary studies provided more than one single study case. Multiple outcomes from the same study are correlated, which is likely to increase the variance of model parameter estimates (Koricheva et al. 2013). We accounted for non-independence among effect sizes by conducting multi-level error meta-analyses, using two moderators as random factors. In particular, different measurements of insects (e.g., survival, body mass, number of eggs) were frequently taken in the same study for the same combination of plant, fungus and insect species. Because measurements taken from the same model species were likely correlated, we used *System ID* (i.e., the combination of plant, fungus and insect species corresponding to each effect size) as a random factor. We used *Case ID* nested within *Study ID* as an additional random factor to account for correlation among multiple case studies within the same primary study.

To ensure that our results were robust and unbiased by non-independence among effect sizes, we additionally conducted a sensitivity analysis. We randomly selected one study case per primary study, system and moderator level and re-ran models (those selected by simplification procedures). This procedure was repeated 1000 times. We compared parameter estimates from the complete dataset to the distribution of 1000 estimates obtained from random subsets of case studies.

We finally used four different approaches to verify that our results were not affected by publication bias (Koricheva et al. 2013): (1) inspection of funnel plots, (2) cumulative meta-analysis, (3) calculation of fail-safe number and (4) exploration of the relationship between effect-sizes and journal impact factor (Murtaugh 2002).

All analyses were conducted in *R* (R Core Team 2015). Model parameters were estimated using the *rma.mv* function from the *metafor* package (Viechtbauer 2010). Post-hoc comparisons were done using the *linearHypothesis* function from the *car* package (Fox and Weisberg 2011).

2.3. Results

We identified a total of 1,113 case studies (*k*) obtained from 101 original (primary) papers that quantified the effects of plant infection by fungi on insect preference and/or performance.

This included 63 different plant species (84% being herbaceous), 65 fungal species and 99 insect species for a total of 205 different plant-fungus-insect combinations (*i.e.*, 205 *study systems*).

The grand mean effect size [\pm 95% CI] calculated with the full data set ($k = 1113$) was significantly negative and equaled $-0.42 \pm$ CI $[-0.64; -0.20]$, indicating that, generally, insects avoid and perform worse on challenged plants than on control, not challenged plants (H1 in Table 1).

Studies performed in the field or under laboratory experimental conditions provided qualitatively similar results ($k= 137$, mean= $-0.36 \pm [-0.69; -0.02]$ and $k= 976$, mean= $-0.44 \pm [-0.68; -0.21]$, respectively; H2 in Table 2), but effect sizes were notably of higher magnitude and less variable in laboratory studies than in field studies.

Studies on chewing and piercing-sucking insects in laboratory studies represented 75% of the case studies ($k= 839$). For these insects, the grand mean effect sizes were consistently negative and significantly different from zero, even when only including case studies using laboratory and greenhouse conditions ($-0.38 \pm [-0.63; -0.12]$). In the analysis on overall effect size, there was a large amount of residual heterogeneity ($Q_E= 8137.26$, $P < 0.0001$) that could be further accounted for by moderators. From this point on, all results will refer to studies conducted on leaf chewing and piercing-sucking insects under laboratory conditions because data on other insect types and under field conditions were too few to allow for robust tests of moderators (see Methods).

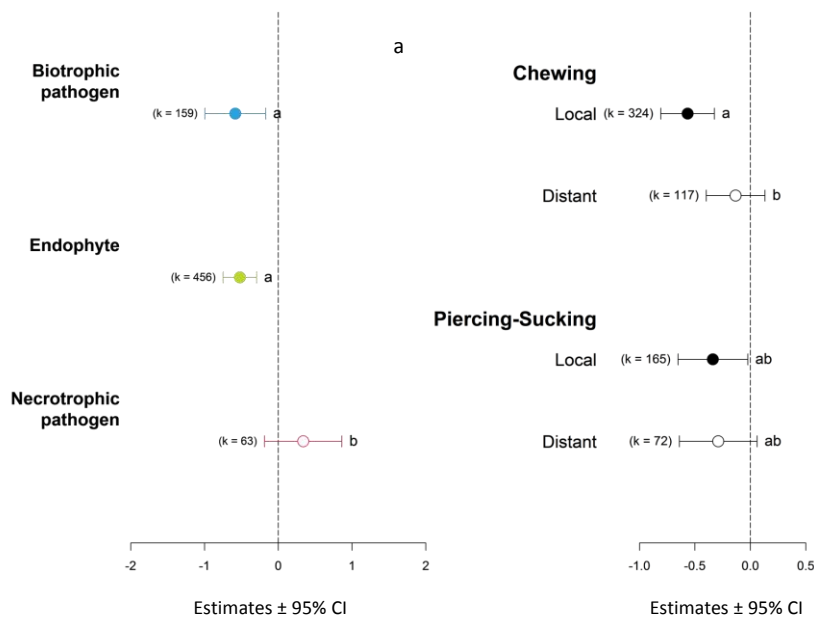
Effect of fungal infection on insect performance

The impact of fungal infection on insect performance was dependent on the lifestyle of the fungus (H3, Table 2): insect performance was significantly reduced on plants challenged by biotrophic pathogens and endophytes, whereas insect performance was unaffected by infection with necrotrophic pathogens (Fig. 1a). Contrary to our prediction, we detected no interaction between fungal lifestyle and insect feeding guild (H4, Table 2). However, we detected a two-way interaction between insect feeding guild and the spatial scale of the interaction between insects and fungi (H5, Table 2). In particular, fungal infection strongly reduced the performance of chewing insects at a local scale (but not at a distant scale), whereas the piercing sucking insects responded in similarly to both local and distant interactions (Fig. 1b). Finally, we did not detect a three-way interaction between fungal lifestyle, insect feeding guild and spatial scale on the response of insects to plant infection ($QM=3.34$, $k=678$, $P=0.188$).

Table 2: Summary of model values for the different moderators tested. Given are the moderator, hypothesis tested, number of case studies (k), model heterogeneity (Q_M) and associated P -value.

Moderators	Hypothesis tested	Q_M	k	P-value
<i>Experimental conditions</i> (Field vs. greenhouse/laboratory)	H2	0.34	1113	0.560
<i>Fungus lifestyle</i> (endophytes vs. necrotrophic pathogens vs. biotrophic pathogens)	H3	7.04	678	0.030
<i>Fungus lifestyle</i> × <i>Insect feeding guild</i> (endophytes vs. necrotrophic pathogens vs. biotrophic pathogens) × (chewing vs. piercing-sucking herbivores)	H4	1.36	678	0.507
<i>Insect feeding guild</i> × <i>Spatial scale</i> (chewing vs. piercing-sucking herbivores) × (local vs. distant)	H5	9.96	678	0.002
<i>Fungus lifestyle</i> × <i>Response Type</i> (endophytes vs. necrotrophic pathogens vs. biotrophic pathogens) × (preference vs. performance)	H6	34.43	415	<0.0001

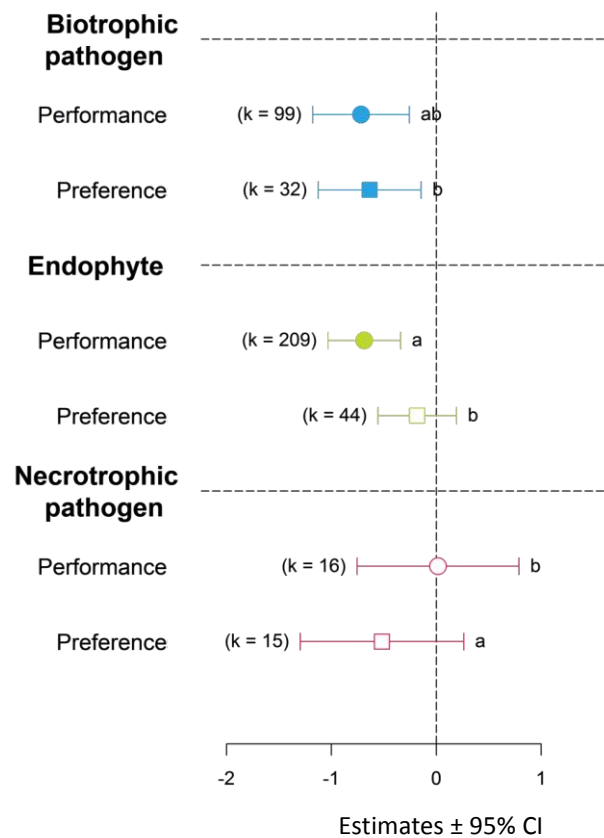
Figure 1: Response of insect performance to plant infection by fungal pathogens as a function of (a) fungus lifestyle and (b) insect feeding guild and spatial scale of interaction. Circles and error bars represent model parameter estimates and corresponding 95% CI. k is the number of case studies. The vertical dashed line centered on zero represents the null hypothesis (i.e., no difference between insect response to not challenged vs. challenged plants). Filled and empty circles represent significant and non-significant effect sizes, respectively. Different letters indicate significant differences between moderator levels.



Effect of fungal infection on preference vs. performance in chewing insects

Generally, both preference and performance of chewing insects were reduced in challenged plants as compared to not challenged plants (Fig. 2). However, the magnitude of the insect response depended on fungus lifestyle (H6, significant *Fungus lifestyle* × *Response type* interaction, see Table 2; Fig. 2). Plant infection by biotrophic fungi reduced both insect preference and performance to a similar degree, whereas plant infection by endophytes had a stronger negative effect on insect performance than on insect preference (Fig. 2). Plant infection by necrotrophic fungi did not significantly affect either preference or performance of chewing insects (Fig. 2).

Figure 2: Effects of fungal infection on preference and performance of chewing insects on fungus-challenged plants. Dots and error bars represent model parameter estimates and corresponding 95% CI. k is the number of case studies. The vertical dashed line centered on zero represents the null hypothesis (i.e., no difference between insect response to not challenged vs. challenged plants). Filled and empty dots represent significant and non-significant effect sizes, respectively. Different letters indicate significant differences between moderator levels.



Publication bias and sensitivity analyses

Visual assessment of funnel plots confirmed a symmetrical distribution of effect sizes (Fig. S2, supporting information), which makes publication bias unlikely. The Rosenberg's fail safe number was 292 725, which was much greater than the critical conservative value of $5 \times k + 10 = 5 575$. This result does not prove the lack of publication bias but indicates that, if present, publication bias can safely be ignored (Rosenberg 2005). There was no temporal tendency in combined effect sizes; sequentially aggregating case studies across years only contributed to increase in the accuracy around the grand mean in the cumulative meta-analysis (Fig.S3). Finally, the Pearson's coefficient of correlation between effect sizes and impact factors of journals from which they were retrieved was weakly positive ($r= 0.061$, $P= 0.043$). Altogether, these analyses indicate that our findings were robust to selective reporting and dissemination bias.

Model parameters estimated with the original dataset (i.e., with multiple outcomes taken from the same primary study) were within the range of the 95% distribution of the 1000 parameters estimated after random drawing of only one case per combination of study, system and

moderator level (Fig. S4). Our initial predictions were therefore robust and unlikely biased by multiple measurements on the same individuals.

2.4. Discussion

Our meta-analysis, based on several hundreds of case-studies, unequivocally demonstrates that plant infection by pathogenic and endophytic fungi, on average, reduces preference and performance of insect herbivores. Even though some primary studies reported a positive effect of fungal infection on insect preference and performance (e.g., by reducing phenolic content and increasing soluble sugar content, Cardoza et al. 2003a), the overall effect size is pervasively negative and consistent across a large set of plants, herbivores, fungi and methodological approaches. And, although most of the studies used do not necessarily reflect the whole complexity of these interactions (*i.e.*, with plants being infected by several fungi and insects at the same time), they do imply overall strong effects of the fungi on insects. Importantly, and as discussed in detail below, we detected several sources of variation in the magnitude of plant-fungus-insect interactions providing new insights on underlying mechanisms (*i.e.*, spatial scale of interactions, insect feeding guild and fungus lifestyle).

Insect performance on fungus-challenged plants are dependent of fungus lifestyle

Biotrophic pathogens and endophytes reduced herbivore performance more than necrotrophic pathogens did. The way endophytes exploit their resources is more similar to biotrophic pathogens as they both develop in living plant tissues (Partida-Martínez and Heil 2011b, García-Guzmán and Heil 2014). Such a similarity may therefore be the likely explanation of their similar negative effect on insect performance. On the contrary, necrotrophic pathogens produce cell-wall degrading enzymes which may contribute to the release of plant carbohydrates. A rapid increase of soluble sugars and others plant nutrients can have a positive effect on insect performance which could explain the tendency of necrotrophic fungi to increase insect performance (Cardoza et al. 2003a, Johnson et al. 2003). For example, Johnson et al. (2003) showed a positive effect of the necrotrophic pathogen *Marssonina betulae* on performance of the aphid *Euceraphis betulae* when co-occurring in silver birch trees, which was correlated with a higher concentration of free-amino acids following the degradation of leaf mesophyll cells by fungus enzymes.

A strong negative impact of endophytes on insect performance was expected, given that some of them are considered potential biocontrol agents (Gurulingappa et al. 2010, Akello and Sikora 2012, Castillo Lopez et al. 2014, Lopez and Sword 2015). Endophytes are a very diverse group (Rodríguez et al. 2009) which are present in virtually all plants (Partida-Martínez and Heil 2011). Here we show that the proven presence in plants of some particular endophyte species may have a negative impact on insect performance. Additionally, some variability in endophyte effect may be explained by their division in two major groups: clavicipitaceous and non-clavicipitaceous endophytes (Partida-Martínez and Heil 2011b), the

former being known to have a negative impact on insect herbivores in some grass systems (Clay 1996, Kuldau and Bacon 2008). However, we found no evidence for a stronger negative effect of clavicipitaceous endophytes than non-clavicipitaceous endophytes on herbivores, which makes unlikely that our result is blurred by a lack of taxonomic resolution in this particular group (for more information, see Appendix S4). Little is known about the effect of the whole community of endophytes on insect performance in challenged plants (Peñuelas and Terradas 2014) and we cannot exclude that some plants defined as not challenged and used as control in primary studies were actually colonized by one or several endophytic fungi. In addition, many fungi can act as endophytes and pathogens depending on the host plant, environment, and biotic interactions (Arnold 2007). We classified them following information provided in the primary studies we used. However, we acknowledge that taking environmental context into account in further studies will deeply improve our understanding of how fungal pathogens influence herbivorous insects on shared hosts. Moreover, plants are infected by a wide community of microorganisms that may play an important role in their extended phenotype (Partida-Martínez and Heil 2011b). However, little is known about the effect of the whole microbiome on insect preferences and performances. This question will require further attention, which will surely benefit from the rapid development of new generation sequencing methods (Lindahl et al. 2013).

Fungus-insect interactions are scale dependent and insect guild-specific

The magnitude of negative effects of plant pathogens on insect herbivores varies with insect feeding guild and spatial scale of fungus-insect interactions. Performance of both chewing and piercing-sucking herbivores were more reduced when they fed on fungus challenged organs (*i.e.*, local interaction) than when they fed on not challenged organs of challenged plants (*i.e.*, distant interaction). However, this difference was significant only for chewing insects. When interacting locally, chewing herbivores may consume both the plant and the fungal material (Moran 1998, Rostas et al. 2003, Mondy and Corio-Costet 2004). Yet, fungi may produce mycotoxins that are toxic to insects and thus directly contribute to a reduction of insect performance (Dowd 1989, Bultman and Bell 2003). This may be mainly harmful to chewing herbivores that indiscriminately consume plant and fungus tissue, but less to piercing-sucking herbivores that only consume sap.

When insects and pathogens feed on different plant organs, the insect-fungus interaction is usually presumed to be plant mediated. Plant-mediated indirect interactions may then result from the fungus reducing plant growth (*e.g.*, Al-Naemi and Hatcher 2013) or nutritional quality to herbivores (Tinney et al. 1998). Fungal infection may also trigger systemic defense responses against both fungi and herbivores (Simon and Hilker 2003a, Stout et al. 2006). Although only few studies clearly distinguished between direct and plant mediated effects of fungi on insect herbivores (*e.g.* by infecting a part of the leaf and subsequently allowing the insect to only feed on the uninfected part of the same leaf, Simon and Hilker 2003a), the additive contribution of direct and plant mediated effects may explain the stronger negative impact of fungal infection observed on chewing and piercing-sucking herbivores in local interactions.

Guild specific response to fungus infection does not depend on fungus lifestyle

How plants respond to multiple aggressors has been widely debated (reviewed by Thaler et al. 2012a) and our results contribute to this debate. Current thinking often states that while the pathway involving salicylic acid (SA) is usually induced by and effective against biotrophic pathogens and sucking herbivores (Ali and Agrawal 2012, Thaler et al. 2012a, Al-Naemi and Hatcher 2013), necrotrophic pathogens and chewing herbivores principally activate and negatively respond to the jasmonic acid (JA) pathway (Ali and Agrawal 2012, Thaler et al. 2012a). Empirical evidence shows reciprocal antagonism between the SA and JA signaling pathways (reviewed by Thaler et al. 2012a). If such cross-talk between these two defense-related hormonal pathways (Stout et al. 2006) is a general pattern, then piercing-sucking herbivores should perform worse on plants challenged by biotrophic pathogens than by necrotrophic pathogens, whereas chewing herbivores should have lower performance on plants challenged by necrotrophic fungi. This hypothesis received some experimental support (Al-Naemi and Hatcher 2013, Mouttet et al. 2013a). For instance, Al-Naemi and Hatcher (2013) showed inhibitory effect of the necrotrophic pathogen *Botrytis cinerea* on individual aphid *Aphis fabae* performances while the biotrophic rust *Uromyces viciae-fabae* infection enhanced aphid performance. In addition, when both fungi were applied simultaneously to the same organ they generally cancelled out each other's effect, resulting in comparable performance of aphids on dually challenged plants and challenged, control plants. Our meta-analysis did not find support for the trade-off between JA and SA. In our study, differences in performance of chewing and piercing-sucking insects on not challenged vs. challenged plants were comparable in both direction and magnitude, irrespective of fungus lifestyle. The lack of interaction between insect feeding guild and fungus lifestyle on insect performance to pathogen infection suggests that JA or SA pleiotropic effects may not be universal (Thaler et al. 2012a), that they can act together, or that mechanisms other than changes in plant defenses explain the observed differences in plant-mediated effect of fungal infections.

Fungal infection differentially affects herbivore preference and performance

There is a general agreement that insect preference should match their performance (Gripenberg et al. 2010). Consistently, we show that plant infection by fungal pathogens reduces (or has no effect on) both the preference and performance of chewing herbivores. However, the difference between herbivore preference for and performance on not challenged vs. challenged plants did vary with the lifestyle of fungi. Such a difference may result from different fungus lifestyles differentially affecting plant traits involved in plant selection (preference) or plant quality for herbivores (performance).

While insect performance is mainly affected by a change in nutritional quality and defense of host plants (Hatcher 1995, Tack and Dicke 2013), preference is frequently affected by host selection cues, like attractive colors or odors (Schoonhoven et al. 2005, Tasin et al. 2012, Rizvi et al. 2015a). In plants challenged by biotrophic fungi, the negative impact of the fungus was as strong for insect preference as for performance. However, the effect of endophyte infection was stronger for insect performance than insect preference. Plant infection by endophytes is, in contrast to biotrophic pathogens, basically symptomless (Partida-Martínez and Heil 2011b) and thus the endophyte is unlikely to modify host plant visual cues for insect location. We therefore suggest that reduced insect preference may be mainly driven by

endophyte mediated changes in host chemical cues (*e.g.*, volatile emissions, Rostás et al. 2015).

Finally, we did not detect any significant effect of plant infection by necrotrophic pathogens on insect preference or performance, although there was a notable tendency for preference to be more reduced than performance. Necrotrophic fungi can change visual and chemical cues used by herbivores to locate and select their host plant (Rizvi et al. 2015a) which can explain the stronger negative effect of necrotrophic pathogens on insect preference than on insect performance. Moreover, there is a greater variability of insect response to necrotrophic plant infection. This is due to the fact that several papers reported a positive effect of necrotrophic fungus infection on associated insects, which was explained by the release of soluble sugars (Cardoza et al. 2003a), amino acids (Johnson et al. 2003) or volatile compounds that could enhance insect oviposition and feeding behaviors (Cardoza et al. 2002). Alternatively, variability in the magnitude of necrotrophic pathogen effects on herbivores could result from differences in the severity of induced symptoms, which may vary with the quantity and quality of infective fungal propagules. The time lag between infection by a pathogen and the expression of symptoms by the plant may also depend on the specific plant - fungus interaction. In general, larger and older necrotic lesions may produce more modified plant tissues and necrotic tissue, resulting in a stronger negative impact on herbivores. However, this possibility remains poorly addressed in the literature (Jaber and Vidal 2009, Mouttet et al. 2011, Akello and Sikora 2012).

Conclusion and future research directions

Meta-analyses enable testing hypotheses that cannot be addressed in a single primary study. Here, we could unequivocally show that plant infection by fungal pathogens is generally detrimental to insect herbivores, reducing both their preference and performance. Importantly, we unravel some biological mechanisms behind the variability among published studies. In particular, we show that the magnitude of insect negative response to plant infection by fungi varies with insect feeding guild, fungus life history traits and the spatial scale of insect-fungus interactions. However, some aspects remain unclear. We identified particular gaps in knowledge that would require more experimental studies in order to better explain and predict the outcomes of such complex, tripartite interactions. An important future avenue will be to compare the relative importance of fungi on tripartite interactions, as compared to other abiotic and biotic drivers of plant-herbivore interactions and herbivore demographics.

What are the molecular mechanisms at work? Only few studies (Cardoza et al. 2002, 2003a) reported solid evidence for fungus-induced change in metabolites content of plant organs. In particular, the pivotal and pleiotropic role that phytohormones play in these interactions remains unclear as most studies did not measure their levels (but see Cardoza et al. 2003a). In this respect, we believe that developments in transcriptomic analyses will pave the way for a better understanding of plant physiological responses to single and multiple biotic stresses (Lazebnik et al. 2014).

Does timing and disease progression matter? Insect response to fungal infection was shown to vary with the time elapsed since the first fungal infection (Mouttet et al. 2013a) and the

phase of fungal disease. Yet, there are only few studies to date that explicitly took time, disease phase or infection severity into account (Jaber and Vidal 2009, Mouttet et al. 2011, Akello and Sikora 2012). More studies controlling the timing of interactions between herbivores and fungi are required to better understand how insect response to fungal infection varies along a time gradient.

How are plant-fungus-insect interactions modified by other biotic factors? Most of our current knowledge is based on highly controlled laboratory or greenhouse studies (ca 87% of our dataset). Such studies are definitely useful to isolate the effects of different treatments, but they fail to address the real complexity of interactions at play. For instance, natural enemies of herbivores may respond directly or indirectly to plant fungal infection. For example, Tack et al. (2012) showed higher parasitism rates in the leaf miner *Tischeria ekebladella* on mildew-infected oak leaves. Fungal infection can change attraction of insect predators and parasitoids by modifying volatile emissions (Cardoza et al. 2003b, Hare 2011) or prey resource quality (Omacini et al. 2001). Fungi can also modify host plant protection provided to insect herbivores against natural enemies by altering refuge structures such as fruits and galls (Biere et al. 2002a). This needs to be further evaluated (but see Bultman et al. 2003, 2012, Härrri et al. 2009, Miranda et al. 2011, Bixby-Brosi and Potter 2012, Tack and Dicke 2013).

How do plant-fungus-insect interactions scale up to the community level? Herbivores sharing the same host plant interact with each other, either directly or indirectly (e.g., via resource depletion) (Crawford et al. 2007, Kaplan and Denno 2007, Wielgoss et al. 2012). Plant interactions with fungi may thus indirectly affect the whole insect community structure (Tack et al. 2012a), triggering changes in herbivory through competitive or facilitative processes.

Are tripartite interactions symmetrical? So far, most studies addressed the additive and interactive effects of herbivores and pathogens on plants (Hauser et al. 2013). The present meta-analysis expands our understanding to the effects of fungi on herbivores within the same plant (Koricheva et al. 2009). Yet, very little is known about the reciprocal effects, i.e., the plant-mediated effects of insect herbivores on fungus infection (Eyles et al. 2007, Rayamaghi et al. 2006, Rostas and Hilker, Simon and Hilker 2003, Tack and Dicke 2013) or on plant susceptibility to other pathogens such as bacteria and viruses.

In natural and agricultural ecosystems, plants have to deal with a large variability of antagonistic organisms, including pathogens and insects. A better understanding of plant-fungus-insect tripartite interactions is therefore crucial to improve management and control strategies of pests and diseases in these ecosystems. While our quantitative synthesis provides new insights into plant-fungus-insect interactions, a complete understanding of tripartite interactions will require expanding the results of our meta-analysis (i) with current knowledge on additive and interactive effects of herbivores and pathogens on plants (Hauser et al. 2013) and (ii) with a review of the reciprocal effect of insect herbivores on fungi (Eyles et al. 2007, Rayamaghi et al. 2006, Rostas and Hilker, Simon and Hilker 2003, Tack and Dicke 2013) and other pathogens like bacteria and viruses.

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2.6. Appendix S1 – Supplementary figures and tables

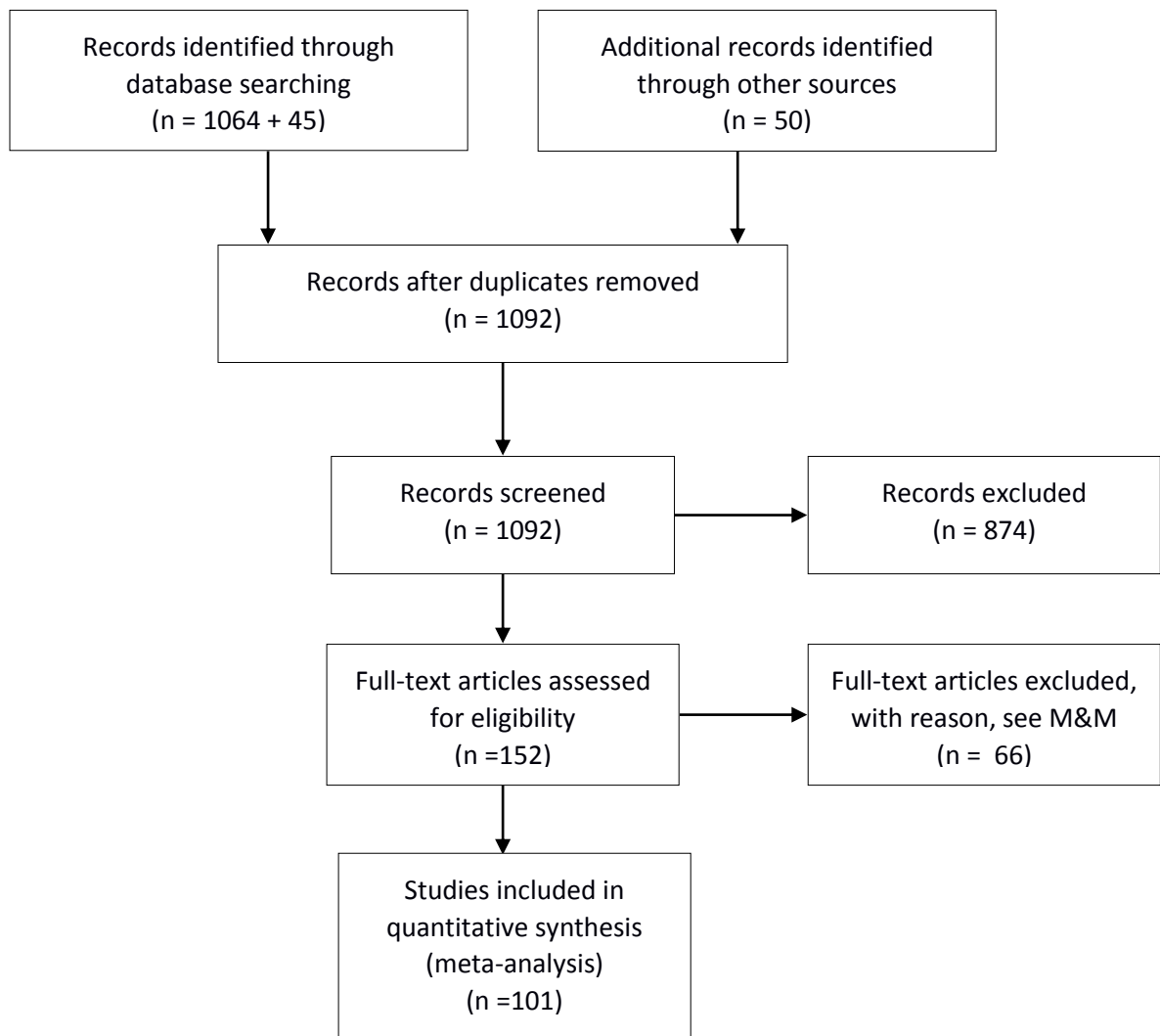


Figure S1: PRISMA flow diagram for our data-set

Table S1: Number of case studies retained in the meta-analysis of moderators. Numbers within brackets correspond to the number of primary studies. Mismatches in case study number on columns correspond to not retained moderators in insect feeding guilds and missing data.

Experimental conditions	Fungus functional group	Insect feeding-guild	Spatial scale	Insect Performance	Insect Preference
Laboratory and greenhouse n = 982	Biotrophic pathogen n = 241	Chewer n = 186	Local	99 (13)	32 (6)
			Distant	26 (5)	6 (1)
		Piercing-Sucking n = 51	Local	29 (3)	0
			Distant	7 (2)	0
	Necrotrophic pathogen n = 93	Chewer n = 56	Local	16 (4)	15 (5)
			Distant	19 (6)	6 (2)
		Piercing-Sucking n = 36	Local	22 (3)	2 (2)
			Distant	6(2)	2 (2)
	Endophyte n = 648	Chewer n = 390	Local	226 (23)	47 (10)
			Distant	75 (4)	38 (2)
		Piercing-Sucking n = 251	Local	121 (17)	4 (4)
			Distant	60 (3)	10 (1)

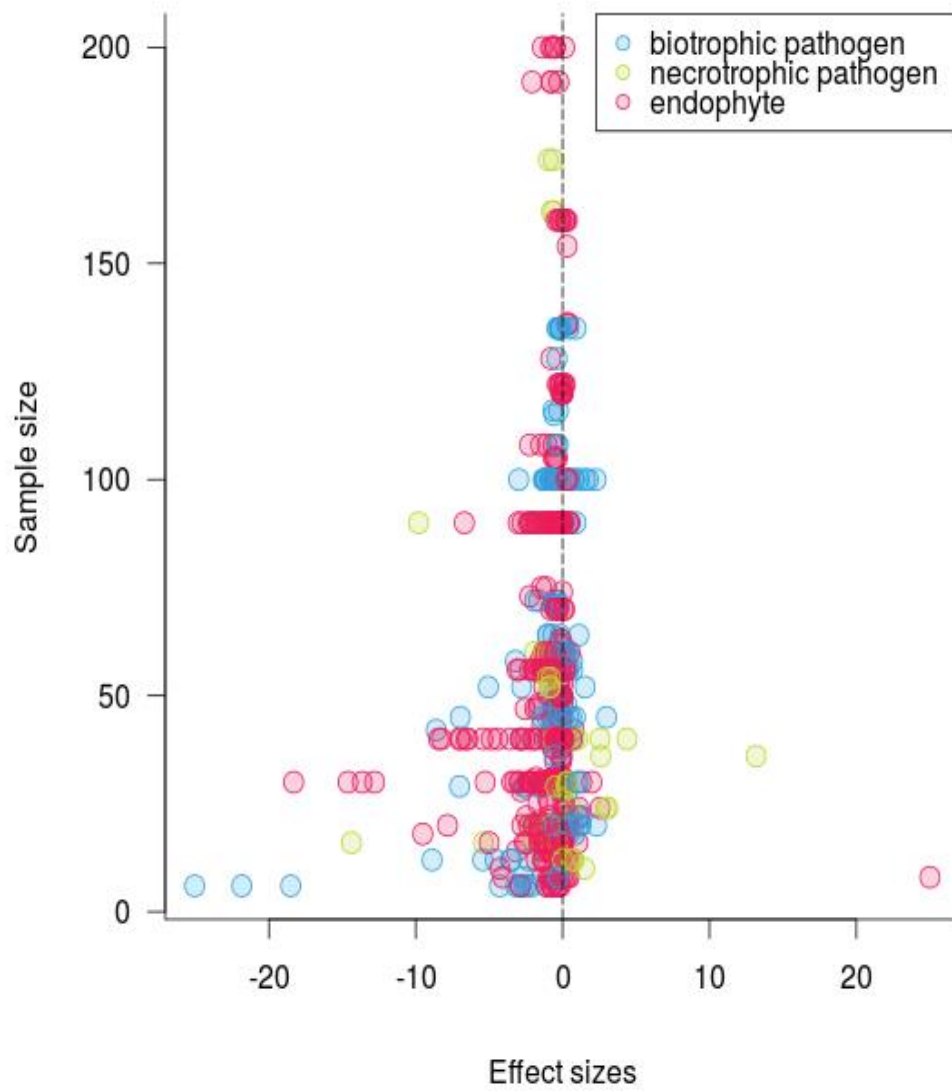


Figure S2: Funnel plot showing the relationship between individual effect size and sample size for each fungus functional group

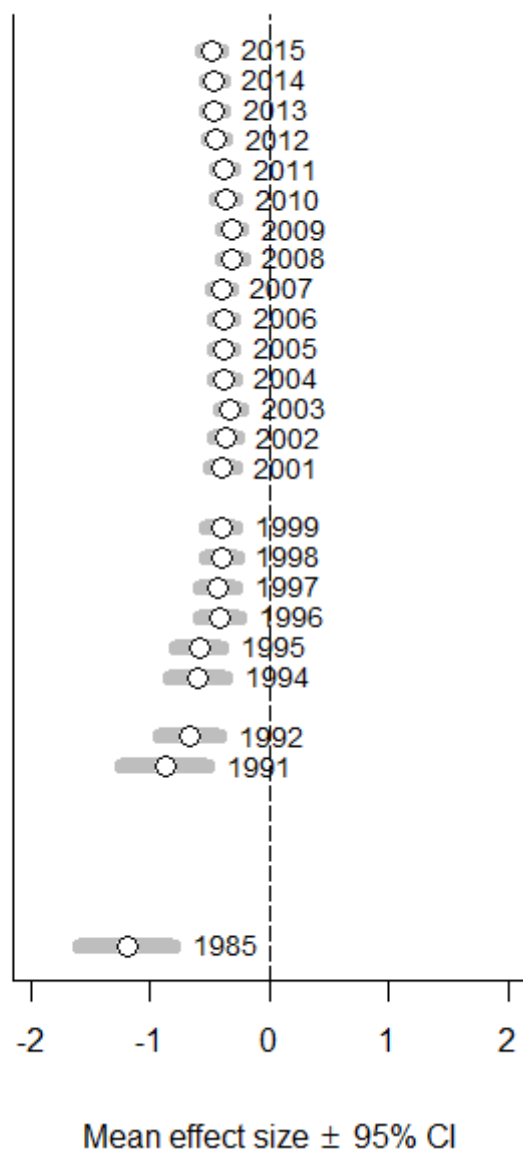


Figure S3: Temporal trend in combined effect sizes through cumulative meta-analysis.

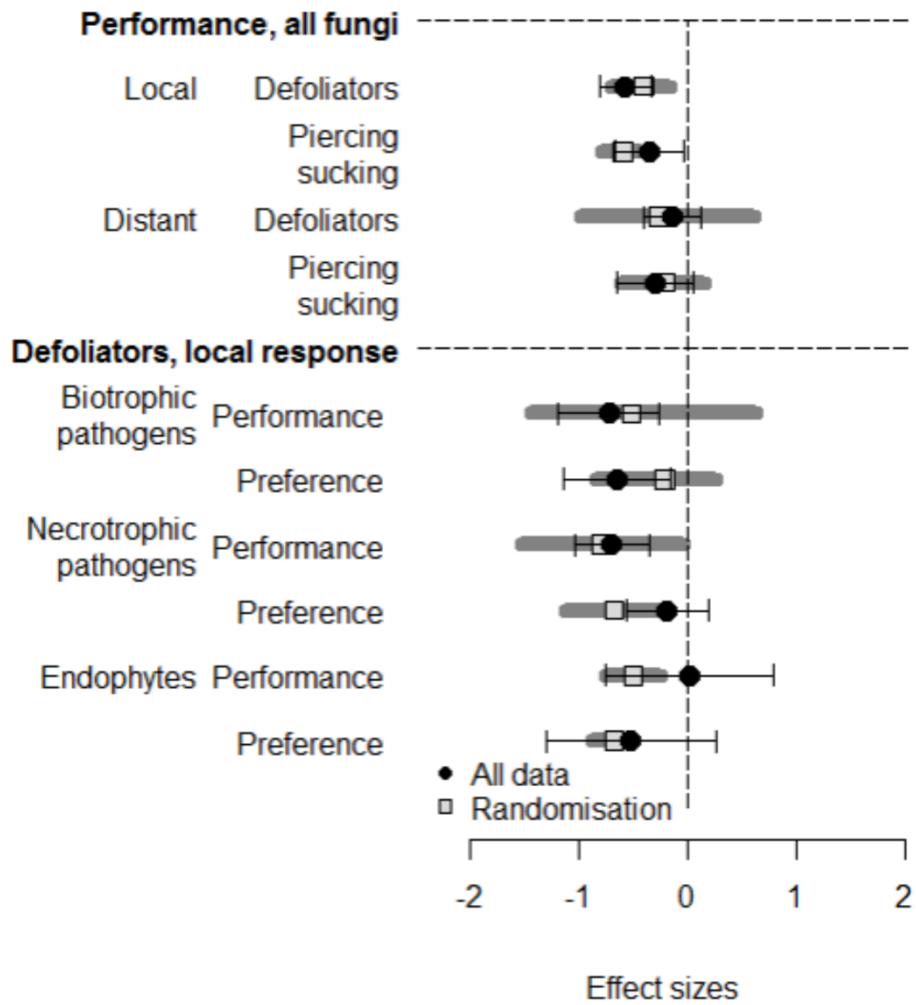


Figure S4: Sensitivity analysis. Dots and thin error bars represent model parameters and corresponding 95% CI estimated with the original dataset. Squares represent the mean of 1000 combined effect sizes calculated after random selection of one case study per combination of primary study, system and moderator level. Thick error bars represent the 95% distribution of combined effect sizes estimated after random drawing.

2.7. Appendix S2 – List of studies included in the quantitative synthesis

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2.8. Appendix S3 – Effect size calculation

For each case study, we calculated effect size using the Hedges' d metric and its variance (Hedges 1981):

$$d = J \frac{\bar{x} - \bar{x}_{\text{control}}}{\sqrt{\sigma}}$$

where $x_{\text{treatment}}$ refers to mean insect response on fungus challenged plants and x_{control} to mean insect response on not challenged, control plants, with

$$J = 1 - \frac{3}{4(n_{\text{treatment}} - n_{\text{control}} - 2) - 1}$$

where $n_{\text{treatment}}$ and n_{control} are the sample sizes for fungus challenged and not challenged plants and with

$$\sigma_{\text{pooled}} = \frac{(n_{\text{treatment}} - 1)\sigma_{\text{treatment}}^2 + (n_{\text{control}} - 1)\sigma_{\text{control}}^2}{n + n - 2}$$

where σ refers to the variance of insect response.

Hedges' d was preferred to other metrics of effect size such as the log-response ratio because it is corrected for bias due to small sample size and enables having control or experimental means equal to zero (Koricheva et al. 2013). For several insect response variables reported in primary studies (*i.e.*, development time, mortality, oviposition deterrence), positive values indicated deterrence or lower performance on fungus-challenged than on control plants. For these studies, d_i was multiplied by -1 to make interpretations consistent across studies. Negative values therefore indicate that herbivores avoided or performed worse on fungus-challenged plants as compared to control plants. Positive values indicate higher preference for or better performance on challenged plants.

In some papers, several experimental conditions were compared to the same control (e.g., plants challenged by different fungal species or strains compared to the same not challenged control plant). Non-independent effect sizes may underestimate sampling variance, which was therefore corrected to account for multiple comparison to the same control using the following equation:

$$v = \frac{1}{n_{\text{treatment}}} + \frac{1}{n} + \frac{d^2}{2N}$$

where d is the Hedges' effect size, and N the total sample size of the corresponding study.

Effect sizes and their corresponding variances were calculated in R using the '*metafor*' package (Viechtbauer 2010; R Core Team 2012).

2.9. Appendix S4 – Endophyte groups

About 53% of case studies with endophytes corresponded to Clavicipitaceous fungal endophytes, which are known to be usually detrimental to insect herbivores. They belong to the Clavicipitaceae family and are vertically transmitted. They infect the whole plant and are well known for their harmfulness to herbivores through the induction of chemical defenses in the challenged plants (Clay 1996, Rudgers and Clay 2008, Partida-Martínez and Heil 2011). As such, they are used as biocontrol agents. The other endophytes or non-clavicipitaceous (or class 3, following Rodriguez et al. 2009) are horizontally transmitted fungi (Partida-Martínez and Heil 2011). Despite possible differences in the way clavicipitaceous and non-clavicipitaceous endophytes exploit their host plant, they did not differ in their effect on herbivores (Figure S5). Hence, considering four different fungus lifestyles (*i.e.*, biotrophic pathogens, necrotrophic pathogens, clavicipitaceous endophytes and non-clavicipitaceous endophytes) instead of three (Figure 1, main document) did not change the outcomes of our meta-analysis.

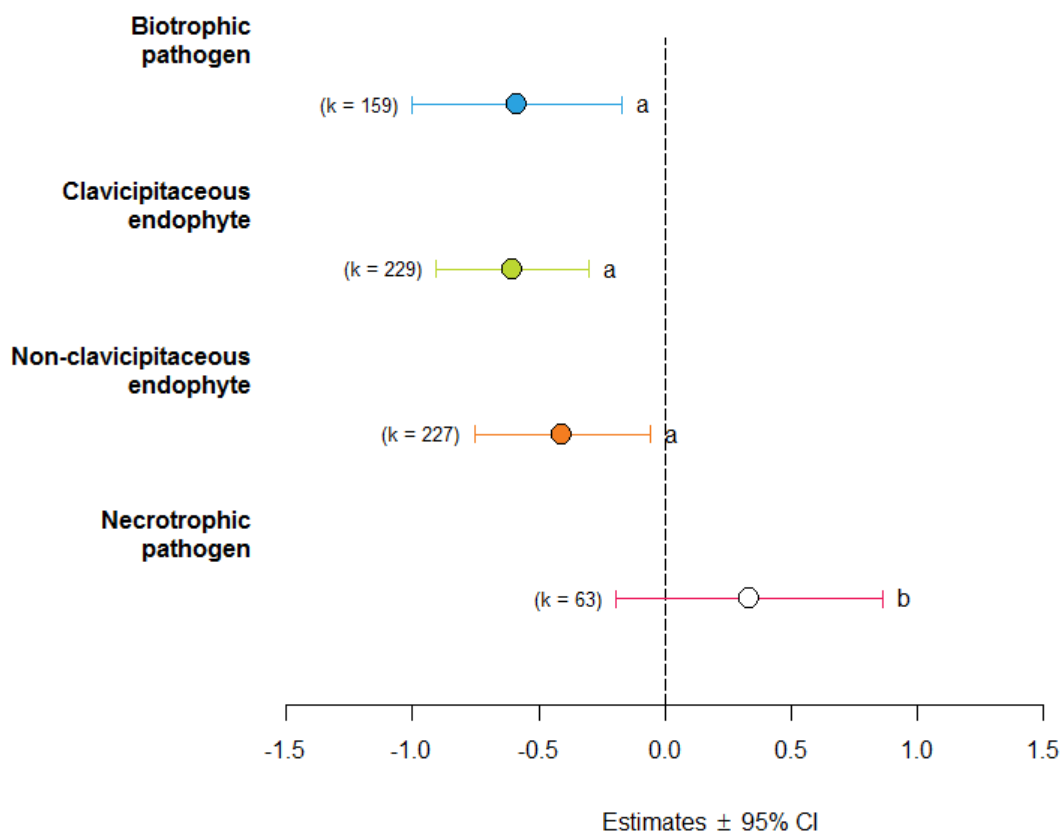


Figure S1: Response of insect performance to plant infection by fungal pathogens as a function of fungus lifestyle. Circles and error bars represent model parameter estimates and corresponding 95% CI. k is the number of case studies. The vertical dashed line centered on zero represents the null hypothesis (*i.e.*, no difference between insect response to not challenged vs. challenged plants). Filled and empty circles represent significant and non-significant effect sizes, respectively. Different letters indicate significant differences between moderator levels.

3. CHAPTER II

Tripartite interactions between an invasive gall wasp, an invasive pathogenic fungus and their new host plant

Agricultural and Forest Entomology. In prep.

Tripartite interactions between an invasive gall wasp, an invasive pathogenic fungus and their new host plant

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ABSTRACT

Insects and fungal pathogens often co-occur within the same host plant. We examined tripartite interactions between a necrotrophic fungus (*Cryphonectria parasitica*) infected or not by Cryphonectria Hypo Virus, a gall-maker insect (*Dryocosmus kuriphilus*), and their common host plant, the European chestnut (*Castanea sativa*). Specifically, we experimentally tested (i) whether the infection of chestnut saplings by *C. parasitica* affects natural infestation rate by *D. kuriphilus* through changes in host plant quality (i) whether prior *D. kuriphilus* infestation affects *C. parasitica* performance; and (iii) the consequences of their single and dual attacks on chestnut trees.

Plant infection by *C. parasitica* did not impact chestnut quality (hormones, nitrogen content and phenolics) for *D. kuriphilus* or its infestation rate. *Dryocosmus kuriphilus* gall modified plant leaf chemistry (phenolic composition and nitrogen content) independently on *C. parasitica* infection status but it did not affect *C. parasitica* disease progression, whatever the virus infection. Finally, their single and dual attacks did not affect chestnut growth during the first year of infection.

Thus, our results demonstrate neutral outcomes of interactions for an invasive fungal pathogen, an invasive insect and their host plant for all of the players after one year of interaction. Long-term experiments are needed to better understand how tripartite interactions between exotic aggressors in a new host plant evolve with time and severity of their infections.

Key words: plant-fungus-insect tripartite interactions, plant defenses, *Castanea sativa*, *Dryocosmus kuriphilus*, *Cryphonectria parasitica*, gall-maker, necrotrophic fungus

3.1. Introduction

Plants are often simultaneously or sequentially attacked by pathogens and herbivores (de Nooij et al. 1992) and therefore need to cope with both aggressors. Plants have evolved a variety of mechanisms to defend themselves, such as morphological structures or chemical weapons like secondary metabolites (Hatcher et al. 1995).

Chemical defensive responses of plants to both insect and fungus are mediated by two main biochemical pathways, the salicylic acid (SA) pathway and the jasmonic acid (JA) pathway. Yet, not all mechanisms are elicited by and effective against all aggressors. For instance, while the SA pathway is usually activated against biotrophic pathogens and sucking herbivores, necrotrophic pathogens and chewing herbivores principally activate and respond to the JA pathway (Ali & Agrawal 2012; Thaler, Humphrey & Whiteman 2012; Al-Naemi & Hatcher 2013). As a consequence, pathogens and herbivores inducing defenses through the same pathways are expected to antagonize each other. For instance, such a theory predicts that necrotrophic pathogens and leaf-chewing herbivores attacking the same plant would antagonize each other. However, this simple assumption is only partly supported by the literature (Lazebnik et al. 2014) and a recent meta-analysis revealed that the strength and direction of plant-fungus-herbivore interactions greatly varies with the lifestyle of fungi and herbivores with the type of interaction, being local or distant (Fernandez-Conradi et al. 2017), and besides these general patterns, very little is known about the tripartite interactions involving fungal pathogens, plants and gall-making insects.

Galls are tumor-like plant tissues which provides gall-inducing herbivores with nutrients and shelter from natural enemies (Stone and Schönrogge 2003). Gall-making herbivores usually trigger profound changes in hormone expression profiles, generally increasing the expression of cytokines and auxins, and decreasing production of abscisic acid, JA and SA (Giron et al. 2016 and references therein). The cross-talk hypothesis (Thaler et al. 2012) therefore predicts a reciprocal effect of fungus pathogens on gall-making herbivores, and *vice versa*, the direction of which depending on the fungus lifestyle and hormonal induction by the gall-making herbivore (Lazenbick et al. 2014). In addition, galls are metabolic sinks, with higher concentrations of nutrients (Giron et al. 2016). As a consequence, indirect plant mediated interactions between gall-making insects and plant pathogens may not only be mediated by the interaction between the JA and SA pathways, but also by metabolic changes affecting the whole plant physiology. Reciprocally, plant modification by a gall-maker may impact fungi infecting the same host plant. Insects have been shown to have positive, negative or neutral effects on fungal infection (reviewed by Hatcher et al. 1995a; Stout et al. 2006). These effects may be direct, for example by vectoring fungal spores (Ullman et al. 1997) or facilitating pathogen penetration through wounds (Raffa and Smalley 1995), or mediated by changes in their host plant (Hatcher et al 1995a; Stout et al 2006). Although possible since gall-makers

are known to divert plant metabolism to their own profit, to the best of our knowledge, the plant-mediated impact of a gall-maker on a fungal pathogen have not been addressed yet.

As a consequence of these complex interactions, dual attacks by insect and pathogens may result in synergistic, additive or antagonistic effects on plant performance. Synergistic effects occur when the combined impact of fungus and insect is more severe than the sum of impacts of each attacker alone (*e.g.*, Turner et al. 2010). Antagonistic effects occur when dual impacts are less severe than single, individual attacks (*e.g.*, Hatcher et al. 1994). Fungus and insect combined impact may also be independent and additive, *i.e.* their combined impact is the sum of both (*e.g.*, Hauser et al. 2013, Schuldt et al. 2017). Despite large variability in plant response to dual attacks by herbivores and pathogens depending on experimental setups, plant size or growth rate, additive effects of dual attacks have been shown to be the most common outcome of tripartite interactions between plants, pathogens and herbivores in a recent meta-analysis of 35 published papers (Hauser et al. 2013).

In this study, we focused on the tripartite interactions involving a fungal pathogen (*Cryphonectria parasitica* Murrill.), a gall wasp (the Asian Chestnut Gall Wasp [ACGW], *Dryocosmus kuriphilus* Yasumatsu) and their common host plant, the European chestnut, *Castanea sativa* Mill. We addressed the reciprocal effect of the pathogen on the herbivore, and *vice versa* and the consequences of single vs. dual attacks on leaf traits and performance of the host plant. Being a necrotrophic pathogen, *C. parasitica* is expected to increase JA levels (Lazembick et al 2014; Spoel et al 2007, Thaler et al. 2012), which may antagonize the SA pathway effective against gall-making herbivores and therefore result in positive effects on the ACGW (Cooper & Rieske 2011). At the opposite *C. parasitica*, may also have other indirect effects on plant physiology, independent of plant defenses, in particular through inducing chestnut xylem dysfunction (McNamus & Evers 1990), which is expected to reduce ACGW performance. Reciprocally, ACGW, as a gall maker, is supposed to induce defenses through the SA pathway (Giron et al. 2016), and thus, according to the cross-talk hypothesis, decrease JA levels (Thaler et al. 2012). Being a necrotrophic pathogen sensitive to defenses induced by the JA pathway, host infection by the ACGW is expected to benefit *C. parasitica* infestation not only directly, by facilitating its penetration through wounds (Meyer et al. 2015), but also indirectly, by decreasing chestnut defenses. The dual attack of the pest and the pathogen may severely impact their host plant. If, as hypothesized, based only on plant defenses, each aggressor positively impacts the second one, we may expect that the dual effect on chestnut plants is synergistic, with ACGW and *C. parasitica* having more negative consequences on host plant when acting together than predicted by the sum of impacts of each aggressor alone. In addition, *C. parasitica* and ACGW being both non-native species, ‘invasional meltdown’ processes (*i.e.* mutualistic interactions between the two invaders, Simberloff and Van Holle, 1999) are likely to occur. Based in this hypothesis, trees infected by *C. parasitica* should suffer higher ACGW attacks, and similarly, plants attacked by ACGW should be more susceptible to *C. parasitica* infection.

We addressed the consequences of tripartite interactions between the ACGW, *C. parasitica* and their host on each partner in two manipulative experiments with chestnut potted saplings. In the first experiment, we artificially infected half of chestnut saplings with *C. parasitica* and allowed ACGW to naturally infect fungus-infected and control trees. We noted ACGW infestation rates and adult fitness. We additionally collected bud, leaves and gall samples in both types of trees and quantified hormonal expression (JA and SA) and secondary metabolite contents. In the second, reciprocal experiment, we infected chestnut saplings with ACGW and later with two strains of *C. parasitica*, carrying or not the CHV-1 virus, and compared canker development and healing on ACGW infected vs. non infected chestnuts. We eventually measured chestnut diameter at the beginning and the end of the current year growing season to test the effect of single vs. dual attacks on chestnut growth. By simultaneously addressing the reciprocal effect of both attackers on each other and measuring both changes in host plant chemistry and performance, we provide new insights to the growing field of tripartite interactions between plants, pathogens and herbivores.

3.2. Materials and Methods

Model organisms

The Asian chestnut gall wasp (ACGW), *D. kuriphilus*, is an invasive exotic insect, considered the most important pest of *Castanea* spp. worldwide. ACGW is native to China and it has been introduced in Italy in the early 2000's from where it colonized almost all the European chestnut geographical range (Aebi et al. 2007, Quacchia et al. 2008). ACGW galls can develop in chestnut leaves, stipulas, dormant buds and shoots, with different damage levels (Maltoni et al. 2012). ACGW is a univoltine and thelytokous species (*i.e.*, its populations are composed only of females which reproduce asexually once a year). At the time of chestnut bud-burst (around mid-April), ACGW larvae induce the formation of galls within which they develop and pupate. Between mid-June and mid-August adults emerge from the galls and lay eggs in new chestnut buds. Eggs hatch in summer and first instar larvae overwinter within dormant buds until the following season (Bernardo et al. 2013). When ACGW adults emerge, the emergence holes in ACGW galls can serve as entry points for *Cryphonectria parasitica*, the causal agent of chestnut blight (Meyer et al. 2015).

Cryphonectria parasitica induces necrotic lesions (*i.e.*, cankers) in bark and cambium of susceptible trees (Prospero and Rigling 2012). It is native to Eastern Asia (Rigling and Prospero 2017) and it was first reported on American chestnut (*Castanea dentata*) in the United States in 1904 where it almost eradicated this species (Griffin 2000). In Europe, it was first reported in 1938 in Northern Italy, where it was probably introduced from North America (Dutech et al. 2012). In Europe, *C. parasitica* is infected by a mycovirus, *Cryphonectria hypovirus 1* (CHV-1), which reduces *C. parasitica* virulence. Virus-infected strains cause limited bark necrosis and superficial cankers which can be healed by *C. sativa* trees and which do not induce the dieback of infected trees (Heininger and Rigling 1994).

Chestnut blight disease regulation by CHV-1 was observed in Europe. Moreover virus-infected strains are used as biocontrol agents in orchards as therapeutic treatments of cankers induced by *C. parasitica* (Rigling and Prospero 2017), the aim of this biocontrol method being to transmit the virus to uninfected fungal strains

Plant material

A total of 500 two years-old chestnut plants (bought in a commercial nursery) were planted in 5.5 liters plastic pots (20 cm diameter) containing 1:2 mixture soil of Klasmann substrate number four (blond peat, black peat and clay mixture) and substrate number five (peat and perlite mixture), respectively.

C. parasitica inoculation

Plants were inoculated by placing a 8mm plug of *C. parasitica* culture in Potato Dextrose Agar (PDA) into a hole of the same diameter, made with a sterile corn borer (Guérin and Robin 2003). For the experiment 1 (see below), in May 2016, we inoculated chestnut stems with a virus-infected strain (STC33.5A, from Dordogne, France) because we needed to keep chestnut plants alive during at least 18 months. Plants were inoculated at 10-15cm above the ground level and, in Mai 2017 we made a second inoculation five to ten cm higher than the first one, in the opposite stem side. Inoculation wounds were covered after each infection with a plastic film to prevent desiccation and removed after one month. For the experiment 2, we made only one inoculation in June 2017 at 10-15 cm above the ground level by either the CHV-1 infected *C. parasitica* strain or the virus free strain XAN7A (from Lot-et-Garonne, France). Control plants were equally wounded and wounds were filled with a plug of sterile PDA.

Experimental design and sampling

Effects of C. parasitica infection on ACGW (experiment 1)

In spring 2016, we transferred 354 potted saplings to a chestnut orchard at the INRA experimental station (44.790667°N and -0.578474°W) where ACGW populations established in 2005. Half of the plants were inoculated by *C. parasitica* strain STC33.5A. Pots were placed 80 cm from each other in a regular pattern, alternating control and *C. parasitica* infected saplings. For this experiment, we used *C. parasitica* carrying CHV-1 virus to ensure the survival of saplings main stems during a year. Pots were organized in four rows separated by 80 cm. In summer 2016, ACGW adults were allowed to freely infect all buds of potted trees.

In spring 2017, we estimated ACGW infestation rates on all 354 saplings as the proportion of current year shoots (*i.e.*, those preformed in buds during the 2016 growing season) with galls. We randomly selected 40 saplings (20 *C. parasitica* infected and 20 non-infected, control chestnuts), among those with higher infestation rate by ACGW, for chemical analyses of galls and ACGW performance estimation.



Figure 1. Experimental site at INRA of Bordeaux.

ACGW performance

To do that, in early summer 2017, we installed 60 × 120 cm fine meshed bags around three branches of the 40 saplings used for chemical analyses in order to collect emerging ACGW adults. However, we did not succeed to collect enough ACGW adults because at this period we had a heat wave and a problem with automatic irrigation so most of the chestnut branches and galls dried before adult came out. We suppose that the 50 adults we collected should have come out before drought damage was too severe. In order to estimate insect fitness, we measured ACGW metasomal width which is supposed to be highly correlated with number of eggs ($r^2 = 0.63$ according to Graziosi et al. 2014).

*Effect of ACGW attack on *C. parasitica* (experiment 2)*

Potted chestnuts not used in the first experiment were kept in a tunnel during the 2016 growing season. Among them, we selected 124 saplings that were kept within insect proof cages to prevent ACGW colonization. In spring 2016, we put five additional chestnut potted trees (not further used for this experiment) already infected by ACGW and 200 cut ACGW galls in a half of the cages to provide sources of ACGW inoculum. The other 62 saplings were kept under insect proof cages without any ACGW.

In late spring 2017, all trees were moved from the cages and half of the saplings were infected with a single *C. parasitica* strain, either infected or not by CHV-1 virus. In late spring 2017, all trees were removed from the cages.

In total, we tested six different modalities: control saplings with no ACGW nor *C. parasitica* infection (thereafter named as ‘control’); saplings with ACGW but no *C. parasitica* infection (‘ACGW’); saplings with no ACGW and with virus free *C. parasitica* strain (‘*C. parasitica*’); saplings with no ACGW and with a virus infected *C. parasitica* strain (‘*C. parasitica* + CHV-1’); saplings with ACGW and with virus free *C. parasitica* strain (‘ACGW + *C. parasitica*’); and saplings with ACGW and a virus infected *C. parasitica* strain (‘ACGW + *C. parasitica* + CHV-1’). *C. parasitica* lesions (*i.e.*, cankers) length and width were then measured 11, 26, 40 and 52 days following infection with a digital caliper. At the last day, bark was scrapped out to measure the necrotic lesion at the cambium level. Canker healing, *i.e.* restrictions of fungal mycelium development by healing swelling which recover the necrotic cambial area, was also noted during the last canker length assessment.

To measure separated and combined impact of ACGW and the two different *C. parasitica* strains on chestnut growth we recorded stem diameter at ground level in April (before fungal inoculation) and at the end of October 2017, *i.e.* at the beginning of the chestnut growing season and at the end of the experiment. Two perpendicular stem measures were made with a digital caliper, then averaged. Chestnut basal area (S) was calculated by approximating stem surface to a circle.

$$S = \pi r^2$$

With r being the mean of the two diameter measures divided by two.

Chestnut growth was estimated as the difference between chestnut basal area in October and April.

Chemical analyses

In autumn 2016, we collected buds on 64 saplings (32 *C. parasitica* infected and 32 control chestnuts) for chemical analyses. In each of the 64 sapling we collected 25 random buds for phenols and carbon-to-nitrogen content analysis and 25 buds for the analysis of SA and JA hormone concentrations. Buds collected for hormone quantification were immediately frozen in liquid nitrogen to prevent any alteration of hormonal profile.

In spring 2017, for each of the 40 selected saplings, we collected four galled and three ungalled leaves, (directly frozen with liquid Nitrogen). The three ungalled leaves sampled per tree were thereafter pooled. For galled leaves, we separated galls and surrounding leaf tissue. The three galls were pooled for each tree, and so were the three surrounding leaves, such that we analyzed the chemical content of three samples per tree (*i.e.*, on one sample per type of tissue: gall, surrounding leaf and ungalled leaf). The fourth gall was used for water content estimation.

Water content in galls was calculated as the difference between gall fresh weight and dry weight, after 24h in a freeze dryer (Alpha 1-2 LD plus; SciQuip ©).

Phenolics were extracted from plant tissues with 1mL of Ethanol: water: formic acid (70:29.5: 0.5 v/v) solution in an ultrasonic bath for 15 min. For chestnut buds, 10mg of dry material was used. For ACGW galls, surrounding leaves and ungalled leaves, fresh material was finely ground in liquid N and 100mg of fresh weight were used. After that, samples were centrifuged at 18 000×g during five minutes and 350µl of each solution was placed in a HPLC vial. We used ultrahigh-pressure liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) to detect phenolic compounds and other secondary metabolites using a protocol adapted from Moreira et al. (2017). The separation was carried out on a 50 × 2.1 mm Acquity UPLC BEH C18 column (Waters, Milford, CT, USA) thermostated at 25°C. Solvents were water + 0.05% vol. formic acid (A), and acetonitrile + 0.05% vol. formic acid (B). The gradient program was performed at a flow rate of 0.4 mL/min under the following conditions: 5-30% B for 6 min, 30-100% B for 2 min, holding at 100% B for 2 min followed by re-equilibration at 5% B for 2 min with an injection volume of 2 µl. The QTOF-MS was operated in MSE negative mode over an m/z range of 85-1200 Da with the following parameters: capillary voltage at -2.5 kV, cone voltage -25 V, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow 800 L/hr. The instrument was internally calibrated by infusing a solution of leucine-enkephaline at 400 ng/mL at a flow rate of 15 µL/min through the Lock Spray™ probe. Peak picking was performed in Markerlynx XS (Waters) as in Gaillard et al (New Phytologist, in press). The obtained list of features, characterized by their retention time and mass-to-charge ratio, was normalized to unit norm (i.e. to the total integrated area per sample) and imported into R software where data were mean-centred and Pareto/UV scaled before applying principal component analysis (PCA). Significant features highlighted by PCA are currently under investigation for tentative identification on the basis of their molecular formula and MS/MS fragments, and comparison with existing databases.

Hormones (JA and SA) were extracted and quantified from 20 mg of fresh buds, finely ground in liquid N by following Glauser et al. (2014). Briefly, samples were extracted with 990µl of a solution of EtOAc: formic acid (99.5:0.5 v/v) and 10µl of internal standard solution containing isotopically labeled hormones at concentration of 100ng/mL. Then five to ten glass beads were added to each sample and extracted in a mixer mill at a frequency of 30 Hz for three minutes. Samples were centrifuged, supernatant recuperated and pellet re-extracted with 0.5 mL of the extraction solution. The extraction solution was therefore evaporated in a centrifugal evaporator and re-suspended in 100µL of MeOH 70 %. After centrifugation (1.5 min at 14 000×g), samples were transferred to a conical glass insert and placed in a HPLC vial. Samples were analyzed with an optimized ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The separation was carried in ACQUITY BEH C18 column (Waters, Milford, CT, USA) at 35°C using the following solvents: (A) water: formic acid (95.95: 0.05 v/v) and (B) acetonitrile: formic acid (95.95: 0.05 v/v). The injection volume was 2µl.

Carbon-to-nitrogen ratio (C: N). Carbon and nitrogen tissue content was measured on 2-3mg of dry finely grounded tissues by using an elemental analyzer (NV-2500 from CE Instrument).

Statistical analysis

Effects of C. parasitica on ACGW and chestnut bud and galls chemical content

We first tested the effect of fungal treatment (*C. parasitica* + CHV-1 vs. control) on ACGW infestation rate by using a GLM with a quasibinomial error family where the response variable was the combination of number of shoots with at least one gall vs. ungalled shoots. Bud chemistry in late 2016 (water content, hormone levels, N content or C:N ratio) between *C. parasitica*+CHV-1 and control treatments was compared with *t*-tests. We then used mixed effect models (LMM) with tissue (gall vs. galled leaf vs. ungalled leaf) × fungus treatment (*C. parasitica*+CHV-1 vs. control) as fixed effects and tree identity as a random effect to compare leaf chemistry in spring 2018 (water content, hormone levels, N content or C:N ratio) between control and fungus infected plants. Non-significant interactions were removed prior to estimating main effects with REML. Finally, we used non-metric multidimensional scaling (NMDS) and PERMANOVA procedure to compare phenolic composition among tree tissues (*i.e.*, galls, galled leaves or ungalled leaves) and fungal treatments. Tree identity was included as strata factor in order to account for the correlation between different types of tissues from a same tree.

Effect of ACGW on C. parasitica

We used non-parametric Kruskal-Wallis' tests to compare differences in healing rates among ACGW and *C. parasitica* treatments. Post-hoc comparisons among fungus × ACGW treatments were done with Wilcoxon's tests with Bonferroni's adjustment of P-values.

In order to assess individual and combined impact of *C. parasitica* strain on plant growth, we performed a linear model with the basal area increment as a response variable and the initial stem basal area, *C. parasitica* strain, ACGW presence and the last two variables interactions as fixed effects.

All analyses were performed in R (version 3.4.1). Multivariate analyses were done with package *vegan*. (Oksanen et al. 2017). LMM were run with package *lme4* (Bates et al. 2015). Test results were considered significant if $P < 0.05$.

3.3. Results

Experiment 1: effects of *C. parasitica* on ACGW

Infestation rates by the ACGW were independent of fungal treatment (Figure 1A; $\chi^2= 0.01$; $df= 1$; $P= 0.967$). *Cryphonectria parasitica* infection did not impact insect fitness (Figure 1B; $t= 0.99$; $df= 13.74$; $P= 0.340$).

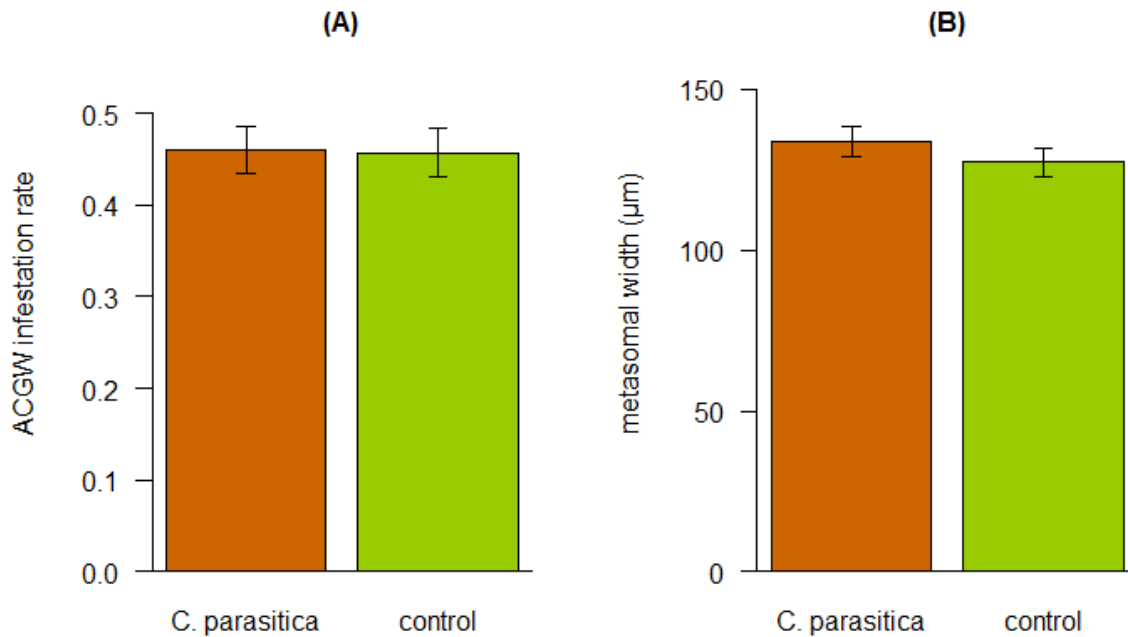


Figure 2. ACGW infestation rates (A) and fitness (B) in fungal infected plants (*C. parasitica*) or uninfected plants (control). Error bars represent the standard error.

The chemical content of buds collected at the end of the 2016 growing season did not differ between control and *C. parasitica* infected chestnut trees (Table 1; JA: $t= 0.28$, $df= 54.81$, $P= 0.779$; SA: $t=-0.38$, $df= 58.31$, $P= 0.702$; N: $t= 1.00$, $df= 51.82$, $P= 0.322$; C:N ratio: $t= 0.77$, $df= 56.90$, $P= 0.447$; phenolic composition: PERMANOVA: $F= 0.60$; $df= 1$; $P= 0.621$).

In spring 2017, the C:N ratio varied between leaf tissues ($\chi^2= 11.42$; $df= 2$; $P= 0.003$), being lower in infected leaves than in galls and healthy leaves. The phenolic content also varied between tissues (PERMANOVA: $F= 14.21$, $df= 2$, $P= 0.001$), with a phenolic profile differing between healthy and galled leaves, whereas the phenolic content was similar between galls and surrounding galled leaves (Figure 3).

Table 1. Mean (\pm SD) amounts of metabolites for different chestnut tissues in *C. parasitica*^{CHV-1} infected and control plants.

Plant tissue	Treatment	SA (ng/g	JA (ng/g	N (%)	C : N
		fresh weight)	fresh weight)		
Bud (autumn 2016)	control	4 029 \pm 1 544	49 \pm 47	0.98 \pm 0.11	48.42 \pm 5.27
	<i>C. parasitica</i> ^{CHV-1}	4 200 \pm 1 960	47 \pm 31	0.94 \pm 0.15	47.36 \pm 5.31
Gall (spring 2017)	control	-	-	1.86 \pm 0.32	22.93 \pm 3.74
	<i>C. parasitica</i> ^{CHV-1}	-	-	2.01 \pm 0.42	21.77 \pm 5.00
galled leaf (spring 2017)	control	-	-	2.14 \pm 0.38	20.87 \pm 2.82
	<i>C. parasitica</i> ^{CHV-1}	-	-	2.11 \pm 0.37	19.84 \pm 2.32
healthy leaf (spring 2017)	control	-	-	2.00 \pm 0.38	22.67 \pm 3.78
	<i>C. parasitica</i> ^{CHV-1}	-	-	2.04 \pm 0.36	22.78 \pm 3.97

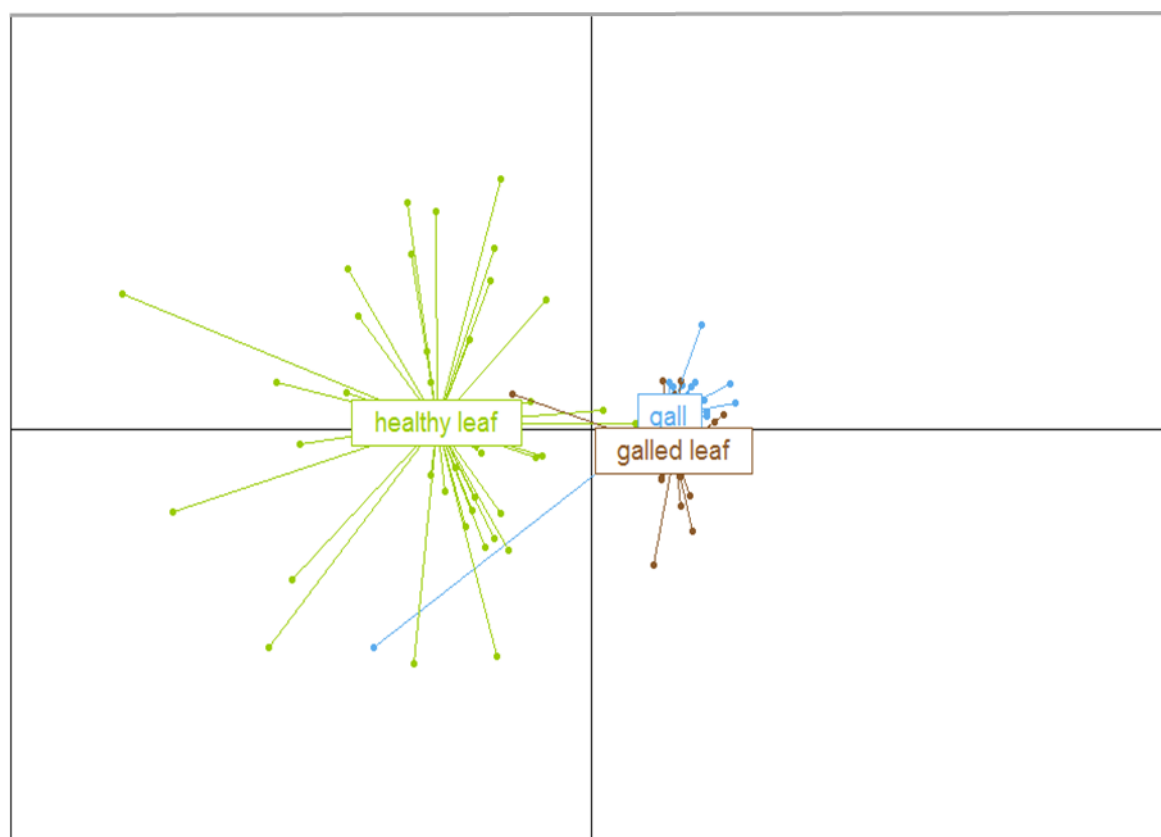


Figure 3. NMDS representing phenolic content of different tissues of chestnut trees infected by ACGW. Stress value associated with this representation was 0.166.

Experiment 2: effect of ACGW on *C. parasitica*

Canker length varied between *C. parasitica* strains ($F= 34.47$; $df= 1$; $P< 0.001$), being on average 37 % higher in saplings infected by virus infected strain (*C. parasitica*+CHV-1) than in saplings infected by the virus free strain (*C. parasitica*). Canker length tended to be lower in ACGW infested plants, but this difference was not significant ($F= 2.45$; $df= 1$; $P= 0.121$). There was no significant interaction between *C. parasitica* strain and ACGW infestation ($F= 1.83$; $df= 1$; $P= 0.180$).

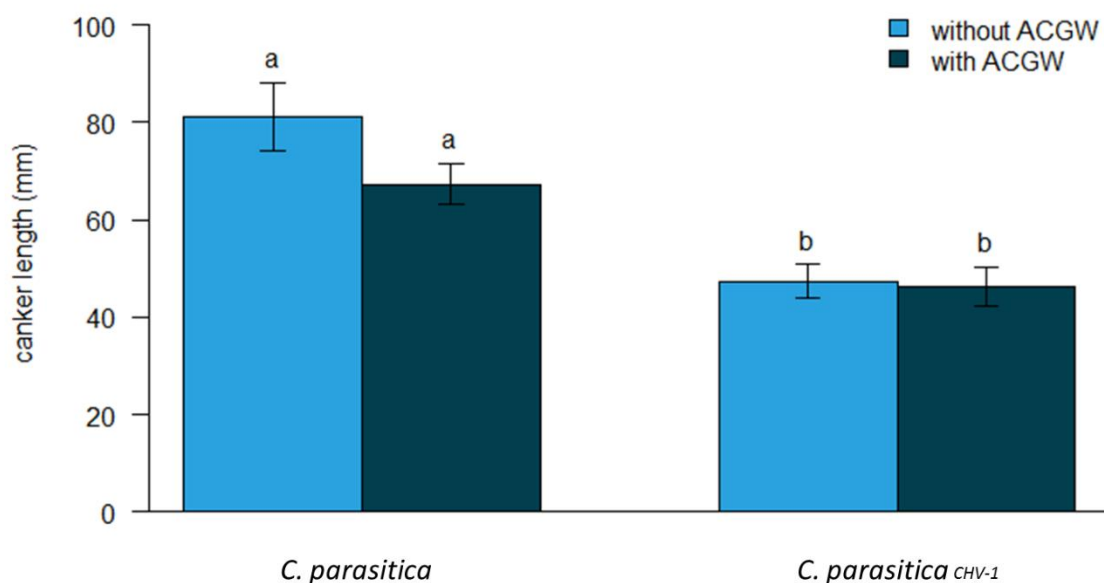


Figure 4. Canker length for CHV-1 virus free (*C. parasitica*) and virus infested (*C. parasitica* CHV-1) fungal strains in chestnut plants with or without ACGW galls. Error bars represent the standard error.

Canker healing (active vs. healed canker) varied between *C. parasitica* strains ($\chi^2= 42.49$; $df= 1$; $P< 0.001$) but not between control and ACGW-infested plants ($\chi^2= 0.02$; $df= 1$; $P= 0.898$).

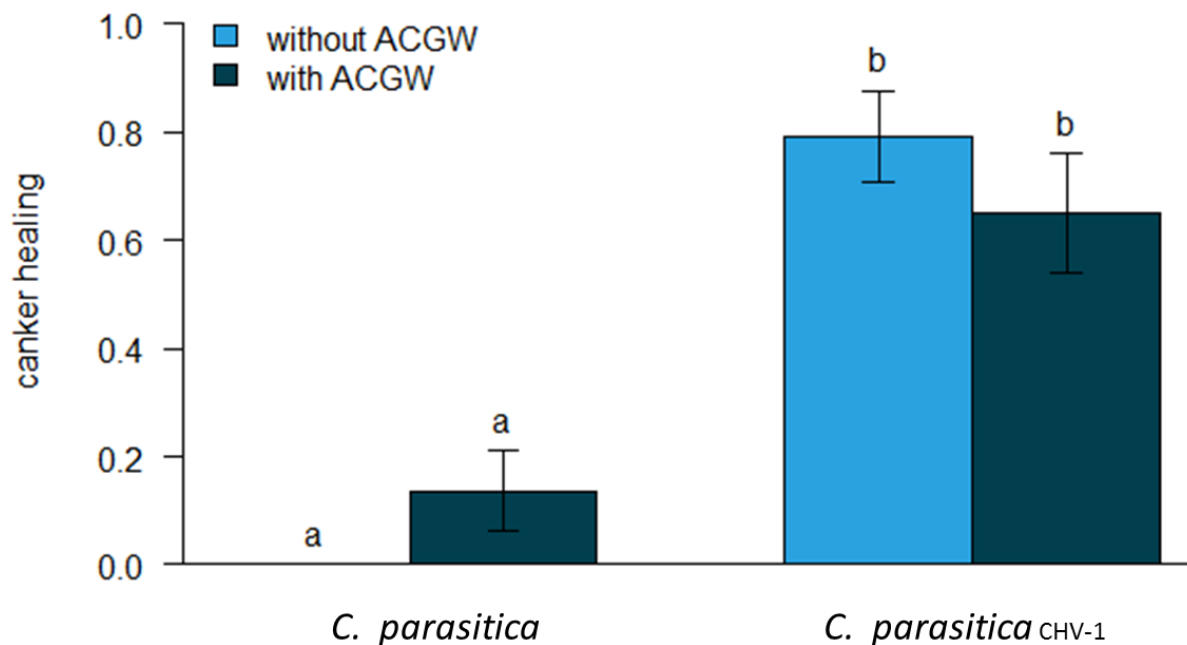


Figure 5. Proportion of plants with a healed canker for the different *C. parasitica* virus free strain (*C. parasitica*) and virus infected (*C. parasitica*^{CHV-1}) on plants infected or not by ACGW.

Individual and combined effects of ACGW and blight on chestnut tree growth

There was no effect of *C. parasitica* strain (Figure 6; $F= 0.16$; $df= 2$; $P= 0.853$), ACGW infestation (Figure 6; $F= 0.002$; $df= 1$; $P= 0.097$) nor their interaction ($F= 0.16$; $df= 2$; $P= 0.849$) on chestnut growth.

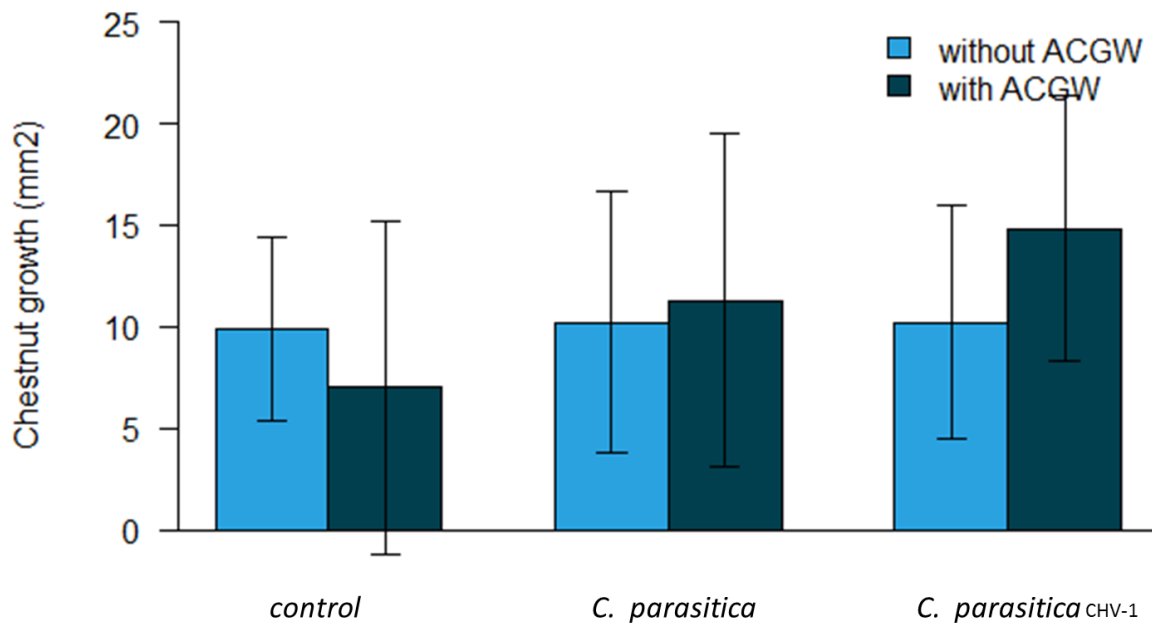


Figure 6. Chestnut growth for plants without *C. parasitica* infection (control), infected with a *C. parasitica* strain without CHV-1 virus (*C. parasitica*) and with a *C. parasitica* strain with CHV-1 virus (*C. parasitica*^{CHV-1}), for plants with or without ACGW galls. Error bars represent the standard error.

3.4. Discussion

We did not find any significant effect of *C. parasitica* on ACGW preference for chestnut plants. The nutritive quality or defensive compounds in chestnut buds were not affected either. Likewise, we found no symmetrical effect of ACGW on *C. parasitica* and no evidence that chestnut growth was affected by any of these two antagonists at the end of this two years experiment.

Fungal pathogens generally have negative effects on the preference and performance of insect herbivores attacking fungus-infected plants (Fernandez-Conradi et al. 2017, Hatcher et al. 1994, Kruess 2002, Simon and Hilker 2003, Rizvi et al. 2015). Yet, a recent meta-analysis showed that tripartite interactions between fungal pathogens, plants and herbivores are contingent on fungus lifestyle (Fernandez-Conradi et al. 2017). In particular, necrotrophic pathogens were shown to have highly variable and, on average, no significant effects on leaf-chewing and sap-feeding herbivores. Although this meta-analysis did not address the particular case of gall-making herbivores, our result is in line with this meta-analysis. However, *Cryphonectria parasitica* infection has been shown to induce stomatal closure in susceptible *Castanea dentata* trees, possibly as a direct result of xylem dysfunction and a deficient water transport (McManus and Evers 1990). Virus infected *C. parasitica* strains, as used in our experiment, are known to have less pronounced effects on water relations than CHV-1 virus free cankers (McManus and Evers 1990). It may explain why we could not

detect any effect of *C. parasitica* infection on water content in galls or adult performance. We cannot discard the possibility that an infection with a virus free *C. parasitica* fungal strain would have a significant effect on water content and on ACGW performances and/or on other, unmeasured variables such as amino acid or soluble sugar content

Chestnut defensive responses to *C. parasitica* infection (virus infected or virus free strains) can be activated systemically, and may prime chestnut defenses (Schafleitner and Wilhelm 1997). However, these responses did not seem to affect ACGW. In addition, we did not find any significant increase in JA or SA levels in buds of *C. parasitica* infected plants compared to controls. In our inoculation assays, plant-mediated interactions between ACGW and *C. parasitica* were distant (*i.e.*, ACGW was present in buds or galls, while cankers developed on stem). We cannot exclude that more local interactions between both aggressors (*i.e.*, cankers developing on branches with ACGW galls) would have impacted more ACGW. Indeed, significant negative effects of pathogen infection on insects have been found for local interactions but not for distant interactions in a recent meta-analysis (Fernandez-Conradi et al. 2017). Such more local interactions may occur in the canopy of adult chestnut trees, when *C. parasitica* infection in branches are located near shoots having buds susceptible to be chosen by ACGW as oviposition sites.

Detecting effects of necrotrophic pathogens, such as *C. parasitica*, on herbivorous insects, can be time dependent. For example, Cardoza et al. (2003), found a positive effect of plant infection by the necrotrophic fungus, *Sclerotium rolfsii*, on the development of *Spodoptera exigua* caterpillars compared to uninfected plants. This effect was correlated with an increase in soluble sugars compounds and a decrease in phenolic contents, leading to an initial increase of the herbivore performance on fungus-infected plants. Yet, this necrotrophic pathogen ultimately kills its host and thus the positive effect found in the first stages of infection eventually reverses with disease progression. By using virus infected *C. parasitica* strains, we reduced the virulence of the pathogen to avoid chestnut mortality before ACGW infestation. It is thus possible that ACGW is more adversely affected in natural conditions, when chestnut trees are infected by more virulent, virus free *C. parasitica* strains.

ACGW produced changes in the chemical composition of infected leaves and galls independently of *C. parasitica* infection. The ‘nutrition hypothesis’ (Price et al. 1987, Bronner 1992) states that gall induction leads to concentration of nutrients in the gall, which becomes a better food source than ungalled plant tissue (Allison and Schultz 2005, Nabity et al. 2013, Giron et al. 2016). However, we found that nitrogen content in galls were lower than in surrounding leaf tissue and similar to that in ungalled leaf. This is consistent with the result of a study performed on 20 gall makers on 11 different plant species (Hartley 1998). This may be due to the fact that increase nitrogen levels in gall not always results in better performance of their inhabitants (Gange and Nice 1997). Polyphenolics also differed between galled and un-galled leaves and were comparable between galls and galled leaves. However, the effect of secondary compounds on performance of gall-maker herbivores is still unclear, with some studies showing that high levels of phenolics in the host plant may be an effective defense against galling insects (*e.g.*, Westphal et al. 1981) while other states that gall-makers get some benefit from actively sequestering phenolic and tannins in gall tissues (*e.g.*, Hartley 1998). In

fact, the “enemy hypothesis” (Stone et al. 2002) proposes that higher levels of tannins and phenols in galls may protect the gall-maker from mortality due to pathogenic fungi or parasitoids attack (Taper and Case 1987, Price and Pschorn-Walcher 1988).

Despite tendencies toward lower development of *C. parasitica* on ACGW infected plants, prior infection of seedlings by ACGW did not have a significant effect on *C. parasitica* canker development or healing for none of the two fungal strains used. We provided evidence that ACGW affected plant chemistry by modifying nitrogen content and phenolic composition of galls and surrounding leaves compared to ungalled leaves. Pathogens and herbivores attacking above-ground plant parts have well documented effects on plant traits, both above- and below-ground (Castagneyrol et al. 2017). It is therefore likely that these changes extend beyond leaves and that massive chestnut infection by ACGW also has systemic effects on the chemistry of other tissues. For instance, ACGW infestation in *C. crenata* plants has been shown to affect performance of *Myzocallis kuricola* aphid by affecting host plant quality (Triyogo and Yasuda 2013). Aphid fecundity and body weight were lower when feeding on leaves of a galled shoot than on an ungalled shoots. It is possible that ACGW effect on *C. parasitica* would be more visible when occurring in the same phytomere (e.g. same branch) or following a more severe or prolonged ACGW infestation than in our experiment. In any case, ACGW infestation did not compromise biocontrol activity of *C. parasitica* by CHV-1 virus, as we could observe canker healing even in plants infected by ACGW.

ACGW and *C. parasitica* alone or together had no significant impact on chestnut growth. This is surprising as ACGW is known to reduce photosynthesis, shoot vigor and bud development (Ugolini et al. 2014) reducing nut yield and even killing the host plant when infections are repeated and severe (Battisti et al. 2014). Here, infestation by ACGW were moderate (around 45% of infested shoots) and only recent (first time for the saplings) so maybe not strong enough to adversely affect chestnut growth on the short term, especially as saplings benefitted from a good water and nutrient supply in their pots. All artificial inoculations of *C. parasitica* produced cankers, with several of them completely girdling chestnut stems. However the infection period was probably not long enough to allow detecting any effect on sapling growth. More severe and prolonged ACGW and *C. parasitica* infections are thus needed to better evaluate their single and combined effects on host tree physiology.

In conclusion, we showed that *C. parasitica* infection did not produce any change in plant bud or leaf chemistry and ACGW induced changes in leaf and gall chemistry independently of *C. parasitica* infection status. Interestingly, the outcome of ACGW, *C. parasitica* and chestnut interactions was neutral for each of the tree players. However, long-term experiments are needed to better understand how interactions between exotic aggressors in a new host plant evolve with time and severity of their infections.

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4. CHAPTER III

Plant neighbour identity and invasive pathogen infection affect associational resistance to an invasive gall wasp

Biological Invasions. Under review

Plant neighbour identity and invasive pathogen infection affect associational resistance to an invasive gall wasp

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ABSTRACT

Theory predicts that mixed forests are more resistant to native pests than pure forests (*i.e.* associational resistance) because of reduced host accessibility and increased top-down control by natural enemies. Yet, whether the same mechanisms also apply to invasive pests remains to be verified.

We tested the hypothesis of associational resistance against the invasive Asian chestnut gall wasp (ACGW, *Dryocosmus kuriphilus*) by comparing ACGW infestation rates on chestnuts (*Castanea sativa*) in stands varying in species composition (chestnut alone or associated with oaks, pines or ashes). We investigated the effects of reduced chestnut density and frequency in mixed stands, as well as the effect of biotic interactions between ACGW, its parasitoids and the chestnut blight disease (caused by *Cryphonectria parasitica*).

ACGW infestation rates were significantly lower in chestnut-oak and chestnut-ash mixtures than in pure chestnut stands and chestnut-pine mixtures. Infestation rate decreased with decreasing chestnut relative proportion. The composition of native parasitoid communities emerged from galls significantly differed between pure and mixed chestnut stands, but not the species richness or abundance of parasitoids. The abundance of the introduced parasitoid *Torymus sinensis* was not correlated with ACGW infestation rates and was independent of stand composition. Blight symptoms modified ACGW infestation rates with taller trees being preferred when they were asymptomatic but avoided when they presented blight disease damage.

Our results suggest that conservation biological control based on tree species mixtures could contribute to reducing the damage of invasive forest pests.

Keywords: biodiversity, associational resistance, invasive pest, *Dryocosmus kuriphilus*, *Cryphonectria parasitica*, natural enemies

4.1. Introduction

Tree diversity provides support to multiple functions and services in forest ecosystems (Balvanera et al. 2006, Gamfeldt et al. 2013). Yet, biodiversity is increasingly threatened by invasive species, which have already caused alarming decline and local extinctions of native species (Clavero and Garcíaberthou 2005, Galil 2007). A common view in invasion ecology is that communities with higher species richness are more resistant to the establishment and spread of invasive species (the diversity-invasibility relationship, Elton 1958). This idea received support (Case 1990, Erneberg 1999, Kennedy et al. 2002) mainly from plant ecologists (Levine et al. 2004), while much less is known about ecosystem resistance to invasive pest insects (Wilsey and Polley 2002). A few recent studies suggest that tree diversity in native communities may reduce the establishment and spread of invasive pests (Rigot et al. 2014, Guyot et al. 2015), but identifying the underlying mechanisms remains a major challenge (Nunez-Mir et al. 2017b).

Associational resistance (*AR*) is a likely mechanism explaining the greater resistance of species-rich plant communities to invasion by herbivorous insects. *AR* describes the lower risk of a given plant being infested when surrounded by heterospecific neighbours (Jactel and Brockerhoff 2007a, Barbosa et al. 2009). Although the opposite pattern – associational susceptibility (*AS*) – can also be observed (White and Whitham 2000, Schuldt et al. 2015), *AR* seems a more common phenomenon in forest ecosystems (Jactel and Brockerhoff 2007a, Castagneyrol et al. 2014a, but see Kambach et al. 2016). *AR* has been described mainly for native pest species but it can be hypothesized that similar mechanisms are involved for invasive pests (Rigot et al. 2014, Guyot et al. 2015).

AR primarily occurs when the presence of heterospecific neighbours reduces the probability of a plant being colonized by herbivores, because these neighbours can reduce host plant concentration (*i.e.*, species-specific density), frequency (*i.e.*, relative proportion) and apparency (*i.e.*, relative size). These mechanisms, however, are more likely to explain *AR* to herbivore specialists, with heterospecific neighbours being unsuitable hosts (Castagneyrol et al. 2014). The “resource concentration hypothesis” (Tahvanainen and Root 1972, Hambäck et al. 2014) posits that herbivores are more able to locate and remain on hosts that are growing in high density or in nearly pure stands than in more diverse stands where their host plants are more diluted. How easily a host plant is found by herbivores, *i.e.* its apparency (Strauss et al. 2015), has been described as a key mechanism driving *AR* (Castagneyrol et al. 2013). Both visual (Dulaurent et al. 2012, Damien et al. 2016) and chemical (Zhang and Schlyter 2004, Jactel et al. 2011) cues used by insect herbivores to locate and colonize a host tree can be disrupted by the presence of heterospecific neighbours.

Host apparency may further be altered by biotic and abiotic factors interacting with plant diversity. For instance, fungal infections may modify plant quality (e.g., Hatcher et al. 1994a, Cardoza et al. 2003a), defensive traits (e.g., Cardoza et al. 2003a), or even the emission of plant volatile compounds (e.g., Cardoza et al. 2002), that can be used by herbivores' natural enemies (e.g., Tack et al. 2012b), resulting in the potential reduction of herbivorous insect performance and preference for a determined host plant (Tack and Dicke 2013). Infection of a host tree by fungal pathogens is thus an important but often neglected factor that can drive associational resistance.

Associational effects may also result from top-down biotic interactions involving herbivores' enemies. The “enemies hypothesis” (Elton 1958, Root 1973) posits that plant communities with higher species richness provide more resources and habitats and thus can shelter more diverse predator or parasitoid communities (Wilby and Thomas 2002, Schuldt et al. 2011, Castagneyrol and Jactel 2012), which could in turn provide a better control of herbivore populations (Riihimäki et al. 2005, Leles et al. 2017b). A spill-over of natural enemies from associated to target trees is expected if associated and target trees share common or alternative prey or hosts (Cappuccino et al. 1998). However, a greater abundance or diversity of herbivores' enemies does not necessarily result in higher predation rates as there is a wide range of enemy-enemy interactions that can be positive, negative or neutral (reviewed by Letourneau et al. 2009). Moreover, one of the proposed reasons for the success of alien herbivores (Shea and Chesson 2002) relates to the “enemy release hypothesis” (Keane and Crawley 2002) which states that their co-evolved natural enemies are virtually absent in newly colonized areas (Meijer et al. 2016). By contrast, the “exotic prey naïveté hypothesis” (Sih et al. 2010) posits that exotic, naïve species may suffer from higher predation pressures than native prey species because of its naïveté towards native enemies (Li et al. 2011). However, a few studies have taken into account pressure by native natural enemies on exotic species in invaded areas (but see Cox and Lima 2006, Li et al. 2011, Cabrera-Guzmán et al. 2012, Wilcox and Fletcher 2016).

Since 2002, European chestnut (*Castanea sativa*, Mill.) forests and orchards have been severely affected by the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). ACGW is considered the most important pest of *Castanea* species worldwide. It can cause chestnut productivity losses up to 80% (Battisti et al. 2014). Severe and repeated infestations on young trees can even lead to tree death (Moriya et al. 2003). Chestnut tree mortality may also occur when ACGW infestations are combined with strong chestnut blight infections (*Cryphonectria parasitica*, Murrill). ACGW is a micro-Hymenoptera (2-3 mm) native to China, which was introduced to Italy in early the 2000's from where it colonized almost all the European chestnut geographical range (Aebi et al. 2007, Quacchia et al. 2008). All members of the Cynipidae family are obligatory parasites of plants, either by inducing gall formation or by developing as inquilines within galls induced by other gall wasps (Stone et al. 2002).

Guyot et al. (2015) showed that crown defoliation caused by ACGW was significantly reduced in mixed chestnut stands compared to pure stands. However, little is known about underlying mechanisms of this AR and particularly the role of other interacting organisms. For example, the chestnut blight, is now widely spread in all chestnut forests in Europe (Rigling and Prospero 2017). This invasive fungus causes extensive necrosis in the cortical tissue (cankers) which can girdle trunks and branches and result in the death of the distal part. Recently, Meyer et al. (2015) showed that attacks by ACGW could increase the incidence of chestnut blight, because the latter can colonize abandoned galls and then establish in shoots usually not affected by the fungus. A reciprocal effect of chestnut blight on ACGW searching behavior may also occur but has not been documented yet. Interactions between two invaders in a new area may result in an ‘invasional meltdown’ process, *i.e.* invader-invader mutualism (Simberloff and Von Holle, 1999). In particular a non-native pathogen (*e.g.*, chestnut blight) may facilitate the establishment of a subsequent invader (*e.g.*, ACGW), leading to exacerbated impact on native ecosystems (Simberloff and Von Holle, 1999; Simberloff 2006). However the associational effects on co-occurring invasive pests remain largely unknown.

The aim of this study was to test the effect of non-host trees on the non-native ACGW infestation in chestnuts previously infected by a non-native pathogen and to investigate mechanisms responsible for the AR effects reported by Guyot et al. (2015) in natural stands. Although located in the same area, we designed a complementary study to specifically address mechanisms responsible for AR. In particular, we predicted that: (i) heterospecific neighbours trigger AR to ACGW; (ii) AR results from the dilution, *i.e.* reduced density and relative abundance, of chestnuts in mixed stands, (iii) AR is stronger in the presence of tree species also infected by Cynipidae galls that are likely to share parasitoids with ACGW, in particular *Quercus* species, and (iv) infection by chestnut blight may modify ACGW infestation through changes in chestnut apparency. To test these hypotheses, we assessed ACGW infestation in chestnut trees naturally growing in pure *versus* two-species mixtures of different compositions. In the mixed stands, chestnut trees were associated with one of the three following native tree species: the turkey oak (*Quercus cerris* L., Fagaceae), a broadleaved species colonized by a high diversity of oak cynipid gall wasps, with associated parasitoids that have been already observed in ACGW galls (Aebi et al. 2007) and for which ACGW could present a “naïve prey”; the flowering ash (*Fraxinus ornus* L., Oleaceae), another broadleaved tree which is not known to be attacked by cynipid gall wasps, and the maritime pine (*Pinus pinaster* Aiton, Pinaceae), a conifer for which no cynipid gall maker is known and which is phylogenetically much more distant from chestnut than ash is. In each forest plot, we measured the density and relative abundance of chestnut trees. On each sampled chestnut tree we estimated ACGW infestation, chestnut blight severity, tree height and collected galls of ACGW to characterize parasitoids community.

4.2. Materials and Methods

Study species

The current distribution of European chestnut ranges from Southern Europe (Iberian Peninsula, Italy, Balkans, Mediterranean islands) and North Africa (Morocco), to North-Western Europe (England, Belgium, Netherlands) and eastward to Western Asia (North East Turkey, Armenia, Georgia, Azerbaijan, Syria) (Conedera et al. 2016).

ACGW is a thelytokous and univoltine species (*i.e.*, its populations are composed only by females which reproduce asexually once a year). At the time of chestnut bud-burst (around mid-April), ACGW larvae induce the formation of galls on different vegetative organs (Maltoni et al. 2012) within which they feed, develop and pupate. Between mid-June and mid-August adults hatch from the galls and lay eggs in new developing chestnut buds. Adult longevity can be up to 10 days. Following eggs hatching in summer, first instar larvae overwinter within dormant buds until the following spring (Bernardo et al. 2013).

ACGW populations are mainly regulated by hymenopteran parasitoids (Cooper and Rieske 2007). In Europe, ACGW galls are parasitized by native parasitoids of oak gall wasps (Panzavolta et al. 2013, Palmeri et al. 2014, Francati et al. 2015), and by a parasitoid wasp from its natural range, *Torymus sinensis* (Hymenoptera, Torymidae), which has been introduced in Europe as a biocontrol agent (see also Aebi et al. 2007, Borowiec et al. 2014, Matošević et al. 2014). Native to China, this parasitoid is univoltine (with about 3% of their population following a 12 months diapause period, Quacchia et al. 2014, Ferracini et al. 2015), with adults emerging in early spring and females laying eggs in newly-formed ACGW galls. The ectoparasitoid larva feeds on the host larva and adults emerge in the following spring (Quacchia et al. 2014).

Cryphonectria parasitica is an invasive plant pathogen native to Eastern Asia (Rigling and Prospero 2017). Its native range partly overlaps with that of ACGW in China (Zhang et al. 2009). This necrotrophic pathogenic fungus infects in the cortical tissues, causing extensive necrosis and cankers which can girdle trunks and branches. This results in the blight and then the death of the distal part (Rigling and Prospero 2017). Blight was first reported in American chestnut (*Castanea dentata*) in the United States in 1904 and in European chestnuts in Italy in 1938 (Rigling and Prospero 2017). It is a necrotrophic pathogen, which colonizes bark and cambium of chestnut trees through wounds, thereby destroying the function of these tissues in susceptible chestnut species such as *Castanea sativa* (Rigling and Prospero 2017).

Study area

The study was performed in natural forest stands in Southern Tuscany (Italy). These forests are mainly composed of chestnut (*Castanea sativa*), European hornbeam (*Ostrya carpinifolia*), oaks (*Quercus ilex*, *Q. petraea*, *Q. suber*, *Q. pubescens* and *Q. cerris*), ash

(*Fraxinus ornus*) and pines (*Pinus pinaster*). In these stands, the chestnut was part of the original mixed broadleaved forests (native), then transformed into mostly pure stands for the production of wood and fruits. ACGW was officially reported in the study area for the first time in 2009 and *T. sinensis* was first released in 2010 (Maltoni, *personal communication*).

In February 2015, we selected 32 forest plots (30 × 30 m) representing 10 chestnut monocultures and 22 two-species mixtures (Fig. S1). Mixtures associated chestnut with *Q. cerris* (n = 10), *F. ornus* (n = 6) or *P. pinaster* (n = 6). Mean plot elevation was 438 m above sea level. All plots were in similar conditions of soil and type of parent rock, e.g. siliceous crystalline quartzites and anagenites of "Verrucano" formation that are widespread in the study area (Lazzarotto 1993, Carmignani and Lazzarotto 2004). Soils deriving from this formation belong to the broad category of Cambisols (V.V.A.A. 2008), and are generally nutrient-poor and subject to acidification and moderate summer drought. Comparable site conditions among plots was also recently verified for the purpose of the FundivEurope project which involved the study of 36 forest plots close to those used in the present study (Baeten et al. 2013). We initiated plot selection with plots previously used by Guyot et al. (2015). However, given specific hypotheses regarding mechanisms responsible for AR to the ACGW, only one of these plots met our criteria such that the present study is fully independent of Guyot et al. (2015).

In each plot, we sampled three focal chestnut trees, with either conspecific neighbours only (in monocultures) or both conspecific and heterospecific (in mixtures) neighbours in their immediate vicinity (*i.e.*, tree canopies overlap; total: n = 96 sampled chestnut trees). Spatial coordinates of each focal chestnut tree and corners of plots were recorded using a Trimble® Geo 7X. We measured the diameter at breast height of all trees present in the plots. We also measured the total height of each focal chestnut tree, which was comparable for each type of plot composition (Table 1; see Table S1 for more details).

Table 1. Mean (\pm SD) plot characteristics for each type of composition. CS: *Castanea sativa* (monocultures); CS-PP: *C. sativa* + *P. pinaster*; CS-FO: *C. sativa* + *Fraxinus ornus*; CS-QC: *C. sativa* + *Quercus cerris*.

Plot composition	Chestnut basal area (cm ² /m ² plot)	Total trees basal area (cm ² /m ² plot)	Chestnut relative proportion (in basal area)	Focal chestnut height (m)	<i>C. parasitica</i> symptomatic trees
CS (n=10)	44.59 \pm 44.58	45.66 \pm 45.18	0.97 \pm 0.05	11.92 \pm 1.83	60% (n= 30)
CS-FO (n=6)	13.50 \pm 11.52	24.29 \pm 28.32	0.62 \pm 0.23	10.36 \pm 3.85	56% (n= 18)
CS-PP (n=6)	43.80 \pm 32.86	70.92 \pm 43.17	0.60 \pm 0.20	10.89 \pm 4.33	72% (n=18)
CS-QC (n=10)	23.35 \pm 30.13	46.24 \pm 29.31	0.38 \pm 0.26	10.13 \pm 3.03	50% (n= 30)

Assessment of blight symptoms

Severity of chestnut blight was assessed by recording cankers on stems and main branches. *Cryphonectria parasitica* was isolated in all accessible cankers, confirming they were caused by this pathogenic fungus.

Assessment of ACGW infestation

In May 2015, we collected 15 branches per focal chestnut tree, randomly selected around the tree crown. For each collected branch, we recorded the number of galls per shoot in all new shoots. New shoots, *i.e.* those produced during spring 2015, corresponded to buds of the previous year, *i.e.* those susceptible to be attacked by ACGW the previous year. As such, gall infestation observed in 2015 corresponded to eggs laid in summer 2014. Infestation rate by ACGW was estimated as the number of infested shoots (*i.e.*, presenting at least one gall).

Parasitoid collection

In winter 2015, we randomly collected 25 galls on eight branches per focal chestnut tree (total: $n = 200$ galls per tree). In laboratory "winter galls" were stored in cardboard boxes (one box per tree) with extractable tubes exposed to light. Boxes were maintained in a climatic chamber at 25°C, with 12h day-light. Boxes were surveyed every two to three days and emerged parasitoids were collected, stored at -20°C, and then transferred in 96% ethanol for further identification. In May 2015, we also collected 100 green galls per tree. These "spring galls" were surveyed for parasitoid emergence as described above for the winter galls.

Parasitoids emerging from winter and spring galls were identified at least to the genus level under a stereomicroscope (Leica M205C). Winter galls were expected to host overwintering parasitoids, and in particular *T. sinensis*. Spring galls were sampled to collect native parasitoids of oak gall wasps that were expected to parasitize ACGW larvae. They belong to six main families of chalcid wasps (Eulophidae, Eupelmidae, Eurytomidae, Ormyridae, Pteromalidae, Torymidae), and have been identified on oak cynipids (Askew and Thuroczy 1998, Askew et al. 2013) or on ACGW (Aebi et al. 2006, 2007, Panzavolta et al. 2013, Al Khatib et al. 2014, 2015, 2016, Palmeri et al. 2014). It is important to note that for some wasps, molecular characterization (Cytochrome Oxydase I) revealed the presence of probable cryptic species (Borowiec, unpubl. data), especially for *Ormyrus nitidulus* and *O. pomaceus* (Kartinen et al. 2010, Lotfalizadeh et al. 2012, Gómez et al. 2017). For this reason, specimens of *Ormyrus* were not identified at species level.

Statistical analysis

General approach. Data analysis was performed with generalized linear mixed models (GLMMs) following the procedure recommended by Zuur et al. (2009). Plot identity was included as a random factor (1|Plot.ID in R syntax) to account for the correlated data structure

arising from the three replicate focal trees sampled per plot (Schielzeth and Nakagawa 2013). Significance of parameters was assessed using χ^2 tests by comparing models with and without the term to be tested. We applied model simplification by starting with the highest order interaction and sequentially removing non-significant predictors. After the most parsimonious model had been reached, parameter estimates corresponding to fixed effects were estimated by restricted maximum likelihood (REML). Contrast analyses were used to compare levels of significant factors. To estimate model fit, R^2 were calculated following Nakagawa and Schielzeth (2013). For each model, we calculated the marginal R^2 (R^2_m , corresponding to the proportion of variance explained by fixed effects) and the conditional R^2 (R^2_c , corresponding to variance explained by fixed *plus* random effects). All analyses were conducted in 3.2.3 version of R (R Core Team 2015), using the *glmer* function from the *lme4* package (Bates et al. 2015).

First, we tested the effect of chestnut forest composition as a categorical fixed-effect factor with four levels (pure chestnut stand, CS; mixture of chestnut and ash, CS-FO; mixture chestnut and pine, CS-PP; mixture chestnut and oak, CS-QC) on ACGW infestation. We analyzed ACGW infestation rate as the proportion of shoots with at least one gall by using a bivariate response variable consisting in number of shoots with at least one gall *vs.* number of shoots with no gall. We used a GLMM with binomial error distribution and logit function such that fitted values were bounded between 0 and 1.

Second, to assess mechanisms that might explain the effect of chestnut forest composition, we ran another model with the general structure described above but with different predictors. This model included three types of predictors referring to (1) tree species composition, (2) bottom-up and (3) top-down associational resistance effects. The composition effect (1) was described by two continuous variables: chestnut density (*i.e.*, chestnut basal area) and chestnut frequency, as its relative proportion to other species in the plot (measured as the ratio between chestnut basal area and basal area of all tree species present in the plot). Bottom-up processes (2) were accounted for, at the tree level, by including as fixed effects the presence/absence of blight symptoms, chestnut height and their interaction. Top-down processes (3) were accounted for, at the tree level, by including the number of parasitoids that emerged from winter and spring galls as separate fixed effects. This approach resulted in one model that was simplified as explained above. Despite the weak correlation between chestnut density and proportion (Pearson's $r= 0.22$), both were introduced in the same model (Dormann et al. 2013). A marginal test of predictor significance was used by testing whether some variance remained explained by each predictor once other sources of variation in the response variable were accounted for. *Torymus sinensis* was the most common parasitoid emerging from winter galls (94% of individuals). So, in order to avoid correlation between *T. sinensis* and total winter parasitoid abundance, the effect of *T. sinensis* as biocontrol agent on ACGW was specifically tested in a separated model with ACGW infestation rates as binomial response variable, as explained above.

Third, we analyzed the effect of plot composition on parasitoid abundance (number of parasitoids from winter galls per tree in one model, number of *T. sinensis* specifically in a second one, and number of parasitoids from spring galls in a third one) with a *Poisson* error distribution.

Finally, a non-metric multidimensional scaling (NMDS) was performed on parasitoid data in order to test whether and how the composition of spring gall parasitoid communities varied according to the tree species composition of plots. This was not investigated with the communities of parasitoids emerging from winter galls because they were dominated by *T. sinensis*. The significance of tree composition in the plot on parasitoid community composition was tested with a PERMANOVA (Anderson 2005) using the ‘*adonis*’ function from package ‘*vegan*’ (Oksanen et al. 2017). We performed 1000 permutations using Bray-Curtis’ dissimilarity measure. Parasitoid diversity was estimated by calculating Shannon’s index with the ‘*diversity*’ function. Significance of composition effect on Shannon indices was tested by using GLMMs as described above, using *Gaussian* error distribution, after linearization of the index to obtain the ‘effective species number’ (Jost 2006).

4.3. Results

All the 96 sampled chestnut trees were infested by ACGW. The proportion of shoots infested by ACGW (*i.e.*, infestation rate) was on average 78% and varied between 30% and 100% per tree. Infestation rates significantly differed among plots of different tree species composition ($\chi^2= 15.66$, $df= 3$, $P= 0.001$, $R^2_{m=}$ 0.15, $R^2_c = 0.34$) being significantly lower in ash- and oak-chestnut mixtures as compared to chestnut monocultures (Fig. 1A). In pine-chestnut mixtures, infection rates were intermediate between chestnut monocultures and chestnut-ash mixtures (Fig. 1A).

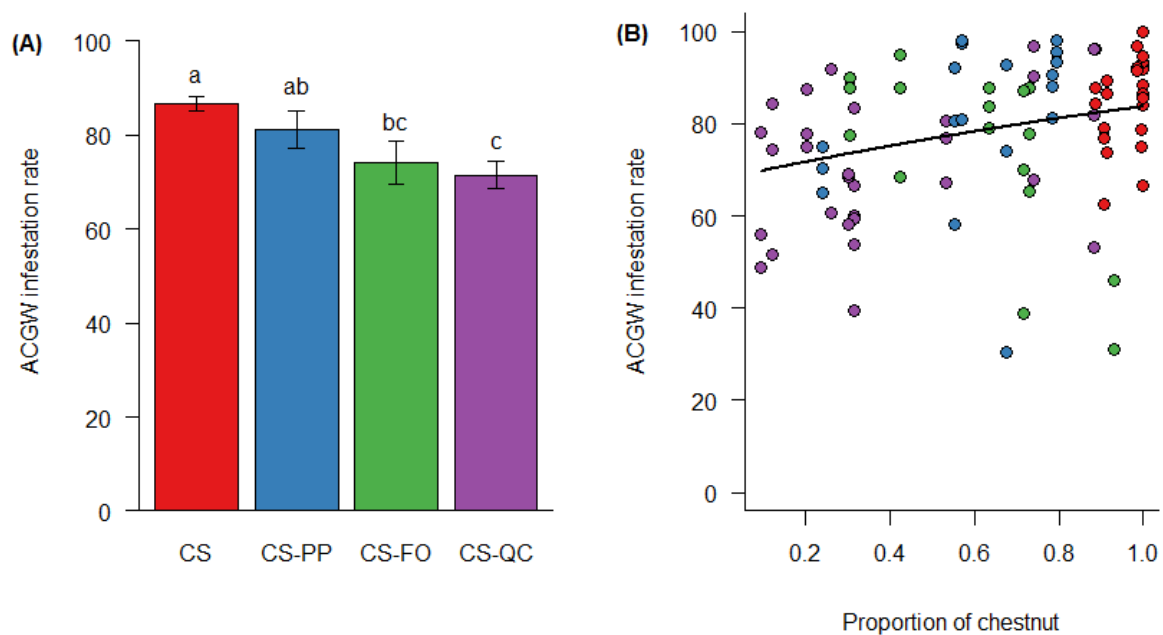


Figure 1. Mean ACGW infestation rate per chestnut tree as a function of (A) tree species composition and (B) chestnut frequency (i.e., relative proportion of chestnut basal area to basal area of all tree species in the plot). In (A), different letters indicate significant differences between treatments (at $P < 0.05$). In (B) the line represents model predictions after back-transformation of the logit link applied to binomial GLMMs. CS: *Castanea sativa* (monocultures); CS-PP: *C. sativa* + *Pinus pinaster*; CS-FO: *C. sativa* + *Fraxinus ornus*; CS-QC: *C. sativa* + *Quercus cerris*. Colors in B correspond to tree species compositions in A.

The infestation rate by ACGW significantly increased with the relative proportion of chestnut trees in plots (Table 2, Fig. 1B), but was independent of chestnut density (Table 2).

Table 2. Summary of generalized linear mixed effect model evaluating the effects of stand structure and composition, natural enemies pressure and two-way interactions, on ACGW infestation rate. Explanatory variables in bold characters had a significant effect for χ^2 values (at $P < 0.05$).

Fixed effects	χ^2	df	P	Estimate	SE
Intercept				1.31	0.14
chestnut density	2.24	1	0.137	0.20	0.13
chestnut relative proportion	4.05	1	0.046	0.27	0.13
chestnut blight symptoms (symptomatic)	5.82	1	0.015	-0.21	0.13
height	0.03	1	0.918	-0.20	0.09
parasitoids abundance in spring galls	0.09	1	0.838	0.01	0.06
parasitoids abundance in winter galls	0.14	1	0.651	0.03	0.07
chestnut density × relative proportion	0.65	1	0.419	-0.16	0.20
chestnut blight symptoms × height	16.49	1	<0.001	-0.47	0.12

Note: Model parameter estimates and standard errors for the intercept correspond to the reference level for chestnut blight: asymptomatic trees. Marginal R^2_m represents the variance explained by fixed factors, while conditional R^2_c is interpreted as variance explained by both fixed and random factors. For the final model (retained after model selection), they equaled 0.14 and 0.36, respectively.

The infestation rate by ACGW varied with chestnut blight symptoms in interaction with tree height (Table 2). In asymptomatic trees, ACGW infestation rate increased with chestnut tree height (Fig.2; Table 2) while ACGW infestation rates decreased with chestnut height in symptomatic trees (Fig.2; Table 2).

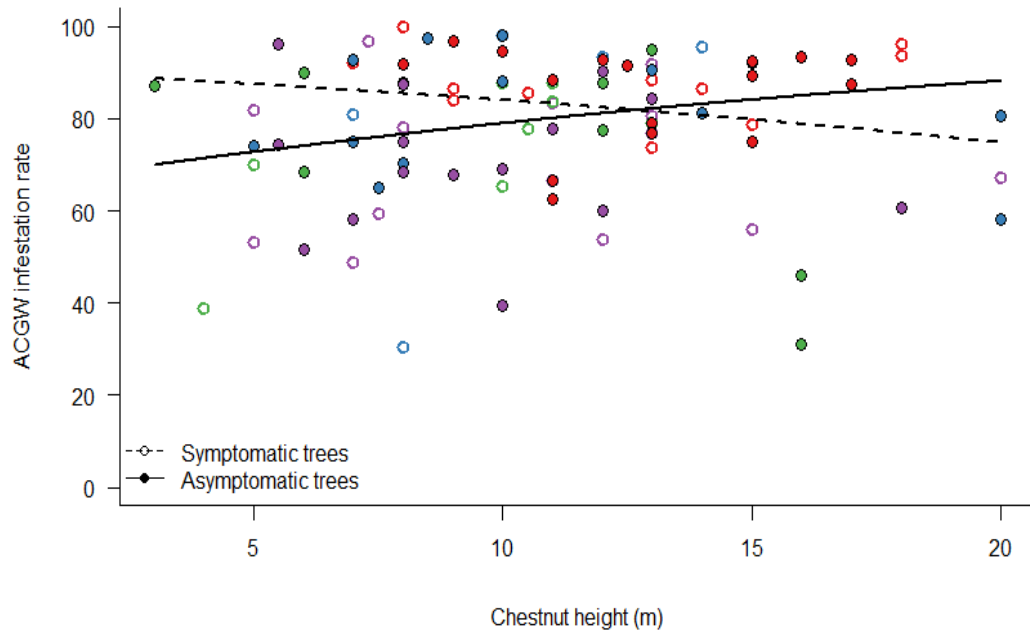


Figure 2. ACGW infestation rate per chestnut tree as a function of chestnut height, in symptomatic vs. asymptomatic chestnut blight trees. Lines represent model predictions after back-transformation of the logit link applied to binomial GLMMs. Different point colors represent different plot composition: chestnut monocultures (red) and pine (blue), ash (green) and oak (purple) two species chestnut mixtures.

A total of 1 619 parasitoids were collected from winter galls, with a mean number of 16 (varying from 0 to 300) parasitoids emerging from 200 winter galls per tree. Nine trees did not provide any parasitoid. In winter galls, *T. sinensis* represented 94% of total parasitoid abundance.

From spring galls, a total of 814 parasitoids emerged, with a mean number of 8 parasitoids per 100 galls (varying from 0 to 35). Ten trees had no parasitoids in their spring galls. The most abundant species were *Torymus auratus* and *Torymus flavipes* that represented respectively 34% and 26% of the total number of parasitoids collected (see also Table S2 for additional information about parasitoids species found). The effect of tree species composition of plots was significant but explained only 16% of variance in the composition of parasitoid communities emerging from spring galls (PERMANOVA $pseudo-F= 1.76$, $P= 0.021$, Fig. 3).

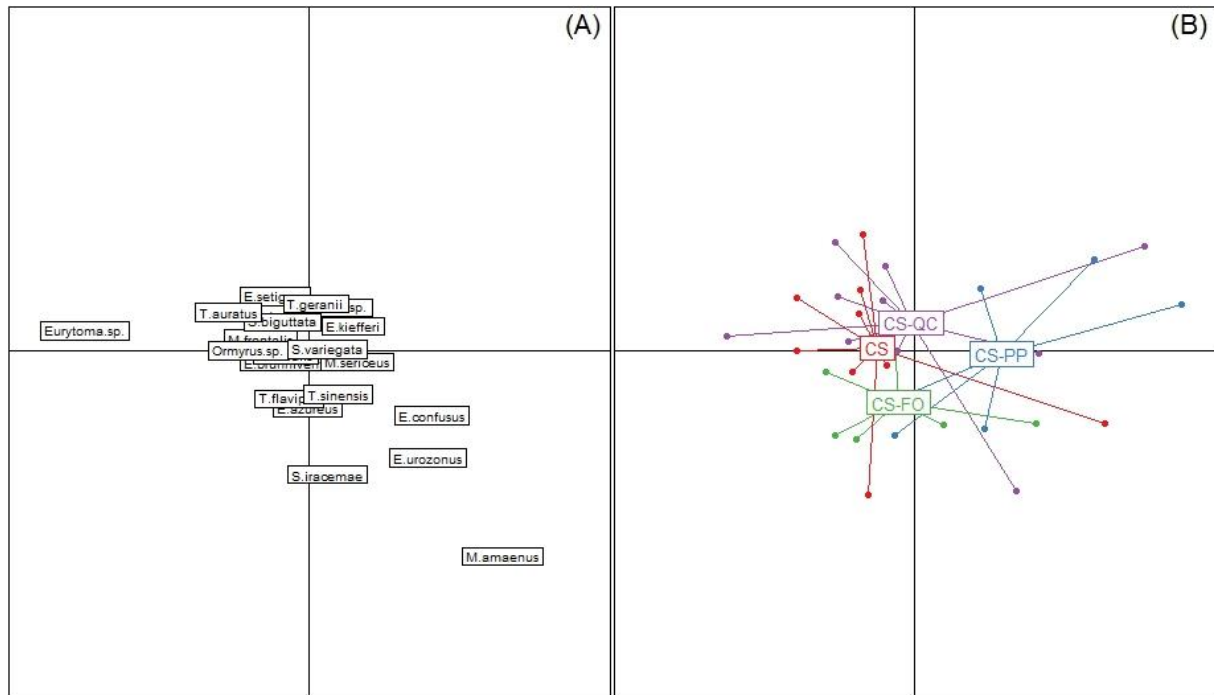


Figure 3. NMDS of parasitoid communities from spring ACGW galls. In (A) species identity distribution and in (B) sites relative distribution. CS: *Castanea sativa* (monocultures); CS-PP: *C. sativa* + *Pinus pinaster*; CS-FO: *C. sativa* + *Fraxinus ornus*; CS-QC: *C. sativa* + *Quercus cerris*. The stress value associated with this representation was 0.167.

The infestation rate by ACGW was independent of total parasitoid abundance in winter or spring galls (Table 2). ACGW infestation rate was also independent of *T. sinensis* abundance ($\chi^2= 1.78$, $df= 1$, $P= 0.18$). Total parasitoid abundance was independent of plot composition for winter ($\chi^2= 4.22$, $df= 3$, $P= 0.238$, $R^2_m= 0.08$, $R^2_c = 0.58$) and spring galls ($\chi^2= 2.77$, $df= 3$, $P= 0.428$, $R^2_m= 0.03$, $R^2_c = 0.27$). The species richness and diversity of parasitoids were also independent of plot composition ($\chi^2= 0.877$, $df= 3$, $P= 0.831$ and $\chi^2= 1.08$, $df = 3$, $P= 0.781$ respectively; Table 3). The abundance of *T. sinensis* found in winter galls was also independent of plot composition ($\chi^2= 6.31$, $df= 3$, $P= 0.098$).

Table 3. Mean parasitoid abundance ($\pm SE$), species richness and Shannon diversity per plot for each plot composition: chestnuts monocultures (CS) and ash (CS-FO), pine (CS-PP) and oak (CS-QC) mixtures.

	CS (n=10)	CS-PP (n=6)	CS-FO (n=6)	CS-QC (n=10)
Abundance	27.10 \pm 1.30	15.00 \pm 4.99	23.50 \pm 4.65	27.10 \pm 0.68
Species richness	5.70 \pm 3.16	5.33 \pm 0.99	5.00 \pm 0.97	6.00 \pm 0.68
Shannon diversity	1.23 \pm 0.04	1.44 \pm 0.20	1.16 \pm 0.18	1.29 \pm 0.13

4.4. Discussion

ACGW infestation rates depend on tree neighbour identity

We not only confirmed previous observations reporting AR to the ACGW (Guyot et al. 2015), we provide evidence that this effect is dependent on the identity of the tree species associated with chestnut trees. AR occurred only in the two-species mixtures where chestnut trees were associated to another broadleaved species, with an infestation rates on average 13.9% lower than in monocultures (Figure 1A). Such reduction in ACGW infestation may be important for chestnut survival, even if mean infestation rates in mixtures with oak and ashes were still high (71.5% and 74.1% respectively; Figure 1A). Infestation rates in chestnut-pine mixtures, however, were comparable to that in monocultures (Figure 1A). This last finding conflicts with the prediction that AR should be stronger in mixtures of more distantly related species such as mixtures of conifers and broadleaved species (Castagneyrol et al. 2014a). However, ACGW is a highly specialized pest with only one host species in the study area (Bernardo et al. 2013). In this context, Castagneyrol et al. (2014a) showed that host frequency should be the main driver of AR.

Host dilution in mixed stands resulted in lower ACGW infestation rates

Chestnut density *per se* had no significant effect on ACGW infestation rates (Table 2), which goes against the resource concentration hypothesis (Root 1973, Hambäck et al. 2014). However, we demonstrated that chestnut infestation by ACGW was reduced when chestnut was less frequent, *i.e.* more diluted among non-host neighbours (Figure 1B; Table 2). An increasing dilution between non-host trees is an important driver of AR (Hambäck and Beckerman 2003, Jactel et al. 2006, Damien et al. 2016). This dilution effect on ACGW infestation rates could be explained by heterospecific neighbours acting as physical (Castagneyrol et al. 2014b, Damien et al. 2016) or chemical barriers (Tahvanainen and Root 1972, Randlkofer et al. 2010, Jactel et al. 2011) to chestnut localisation by ACGW (*see also* Germinara et al. 2011).

Blight symptoms in interaction with chestnut apparency modifies ACGW infestations

In our study, we show that ACGW infestation rates were higher in taller trees than on smaller ones for trees without blight symptoms (Figure 2). This is consistent with previous observations by Maltoni et al. (2012), who found that vigorous chestnut trees are more attacked than dominated or weak chestnuts. These results suggest that taller, more apparent and more vigorous trees are preferred by female ACGW for laying eggs. However, because we counted galls and not oviposition events, we cannot exclude that differences in observed infestation rates were driven by differential mortality after oviposition. It remains possible

that ACGW females did not choose among stands and trees, but larvae developed more successfully in more vigorous trees.

However, the host apparency effect was not observed on trees severely infected by chestnut blight. In these trees, ACGW infestation rates were higher in smaller trees than in taller ones (Figure 2). The presence of *C. parasitica* in cankers of American chestnut (*C. dentata*) results in stomatal closure, possibly as a direct result of xylem dysfunction (McNamus and Evers 1990), thus, in trees with blight symptoms water transport might be more impaired than in asymptomatic trees. This might be more severe in large than in small trees. As a consequence, foliar tissues might be of lower quality as breeding substrate for ACGW in large and blight symptomatic trees. Fungal infection of a plant can also modify host visual and chemical cues used for the insect to locate its host (Cardoza et al. 2002, 2003a, Rizvi et al. 2015b), thus interfering with tree apparency. Knowing how chestnut blight modifies chestnut traits used by ACGW to locate and exploit host trees (e.g., Germinara et al. 2011) would provide useful information about their combined impact.

The co-occurrence of blight and ACGW on smaller chestnut trees could be a case of invasional meltdown, with a more severe, combined adverse effect on survival and growth of young chestnut trees, thus jeopardizing natural regeneration. However, our data does not allow us to properly test such invasional meltdown as we could not reported independent impact of each exotic aggressors (all trees were infected by ACGW and likely by *C. parasitica*).

Abundances of ACGW parasitoids were independent of forest composition

Invasion success of exotic pests are usually attributed to the absence of co-evolved natural enemies in the new colonized area ('enemy release hypothesis', e.g., Keane and Crawley 2002). At the time of its establishment in Italy, ACGW found an 'enemy free space', allowing the pest to rapidly build up its populations. However few years later a spill-over of native parasitoids was already observed from oak gall onto ACGW galls (Aebi et al. 2006, 2007; Panzavolta et al. 2013), suggesting that ACGW became a 'naïve prey' (Sih et al. 2010) for local, native enemies.

Due to this spill-over process, we expected a better top-down control of ACGW by natural enemies in chestnut-oak mixed stands ('enemies hypothesis', Elton 1958; Root 1973). However, we did not detect any correlation between parasitoid abundance and ACGW infestation rate (Table 2), and the parasitoid abundance was independent of stand composition. We found native parasitoids in all stands (Figure 3), even though no cynipid gall wasps are known on *Pinus* or *Fraxinus* species (Csoka et al. 2005). The more likely explanation is the presence of oak species (*Q. petraea*, *Q. pubescens*) in the vicinity of our plots, which was not controlled in our study, and may have represented nearby sources of native cynipid parasitoids.

Moreover, the parasitism rate by native species was on average of *ca.* 8 individuals per 100 galls, which was relatively low compared with the mean parasitism rate of oak cynipids as reported in the literature (e.g., Fernandes and Price 1992). This suggests that oak trees may have also acted as a sink for native parasitoids, *i.e.* oak galls being more suitable hosts. Some parasitoid species could have indeed avoided ACGW galls and concentrated on native hosts as infecting a new introduced host may incur a fitness cost for native parasitoids (Jones et al. 2008; Knoll et al. 2017).

We did not find either any effect of *T. sinensis* abundance on ACGW infestation rate. However, the introduction of this exotic parasitoid, originating from the native area of ACGW in Asia, in Tuscany was relatively recent (2010), which is consistent with the low abundance of *T. sinensis* observed on ACGW galls (on average 15 parasitoids for 200 galls). Yet, it took eight years following the first releases of *T. sinensis* to achieve an effective reduction of 75% of ACGW infestations in the Piedmont Region, Italy (Quacchia et al. 2014b). We may expect that the combined effects of native and exotic parasitoids will contribute to better reduce ACGW populations in the future. However, whether native parasitoids only (those produced by oak galls) would have been effective enough to control ACGW outbreaks in mixed forests remains an open question.

Last, it must be also acknowledged that parasitoids represent a fraction of ACGW' enemies and that variability in ACGW infestation rates could be explained by the effects of endophytic fungi (ex. *Gnomoniopsis castanea*, Vannini et al. 2017) or of competing generalist herbivores (Cooper and Rieske 2010, Tosi et al. 2015).

4.5. Conclusion

Our study provides new empirical data supporting the hypothesis that mixed forests are more resistant to biological invasions by exotic pests than tree monocultures. Our results suggest that the strength of associational resistance to invasive insects depends on both the quality (*i.e.* the identity of associated tree species) and the quantity of non-host trees in mixed stands. In addition, our observations suggest that complex biotic interactions might mediate these mechanisms. For example, plant infection by a pathogenic fungi may modify *AR* to pest.

Classical biological control, *i.e.* the introduction of natural enemies native to the area of origin of the pest for its permanent establishment in a new area (Eilenberg et al. 2001), is the most common strategy used to regulate ACGW populations worldwide. However, although *T. sinensis* was present in winter galls from almost all trees that we sampled, the parasitism rates are still low and we thus observed a high level of ACGW damage, ranging from 30% to 100% infested shoots. Our findings suggest that forest management practices, based on tree species mixtures, would provide a complementary protection against ACGW, along the lines of the conservation biological control strategy (Barbosa 1998). As additional advantage mixed-

species forests represent a preventive method which might help to reduce the risk posed by other natural disturbances, including further biological invasions (Jactel et al. 2017).

4.6. References

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4.7. Supplementary Material

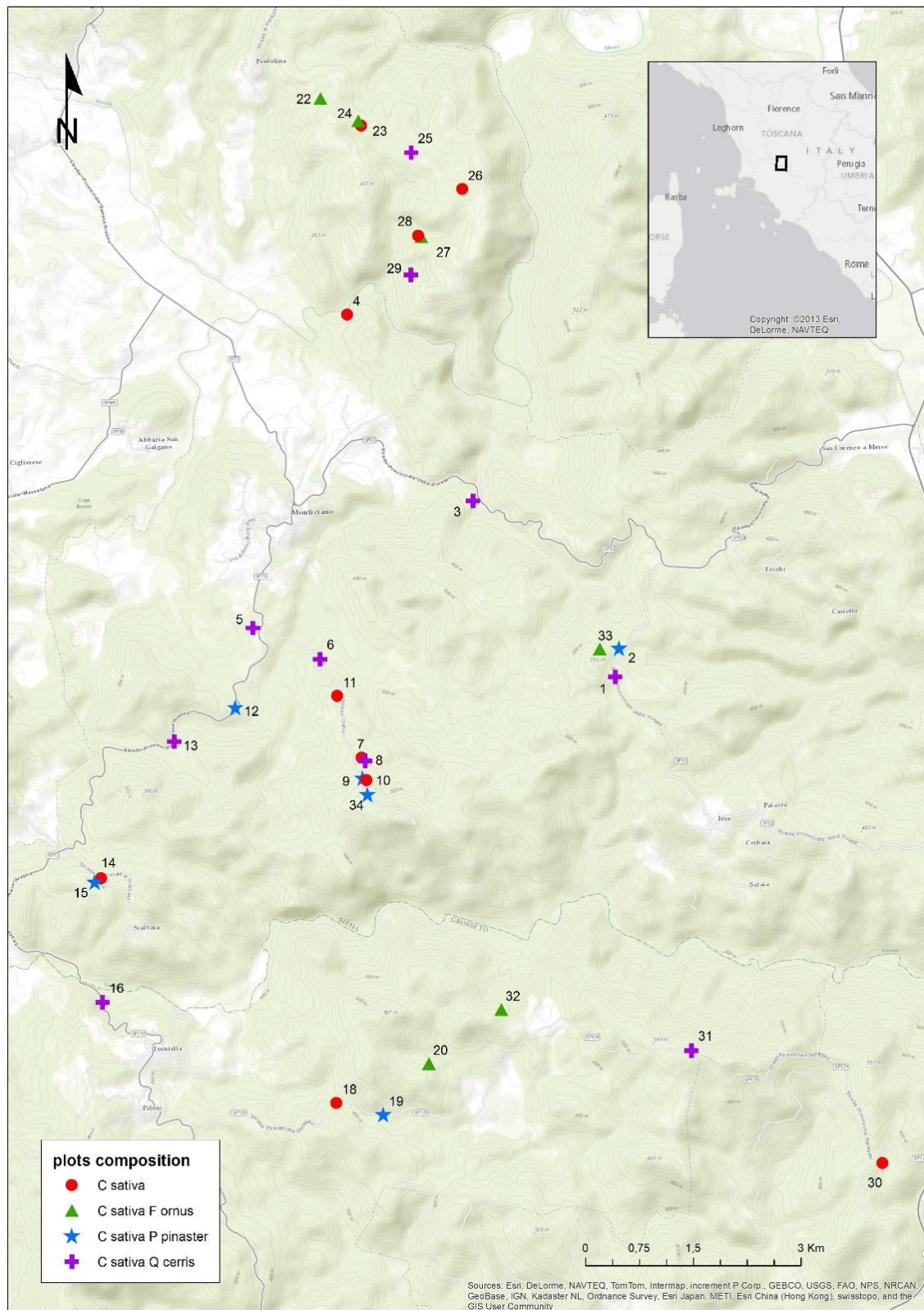


Figure S1. Plot distribution in study area. Pure chestnut plots (circles) and ash (triangles), pine (stars) and oak (cross) mixtures are represented.

Table S1. Plot descriptors: plot identity (see Figure S1), tree composition, spatial coordinates in UTM 32N (EPSG 32432), chestnut basal area, relative proportion of chestnut trees to other tree species and mean height of focal chestnut trees.

Plot	Composition	X- Coordinate	Y- Coordinate	Chestnut basal (cm ² plot)	Total basal area (cm ² /m ² plot)	trees area (cm ² /m ² proportion	Chestnut relative proportion	Mean focal chestnut height (m)
4	<i>C. sativa</i>	678062	4781538	30	34	0,89	13	
7	<i>C. sativa</i>	678252	4775360	27	30	0,91	12	
10	<i>C. sativa</i>	678343	4775051	54	55	0,99	15	
11	<i>C. sativa</i>	677926	4776227	163	163	1,00	12	
14	<i>C. sativa</i>	674635	4773679	20	20	1,00	12	
18	<i>C. sativa</i>	677911	4770552	22	22	1,00	9	
23	<i>C. sativa</i>	678253	4784167	25	27	0,91	12	
26	<i>C. sativa</i>	679665	4783278	36	36	1,00	12	
30	<i>C. sativa</i>	685513	4769708	23	24	0,99	10	
2	<i>C. sativa</i> + <i>P. pinaster</i>	681831	4776887	12	51	0,24	8	
9	<i>C. sativa</i> + <i>P. pinaster</i>	678281	4775066	67	121	0,55	18	
12	<i>C. sativa</i> + <i>P. pinaster</i>	676498	4776062	89	113	0,79	12	
15	<i>C. sativa</i> + <i>P. pinaster</i>	674537	4773634	42	74	0,57	9	
19	<i>C. sativa</i> + <i>P. pinaster</i>	678556	4770382	2	3	0,68	7	
34	<i>C. sativa</i> + <i>P. pinaster</i>	678333	4774843	51	65	0,78	12	
20	<i>C. sativa</i> + <i>F. ornus</i>	679199	4771103	6	9	0,73	10	
22	<i>C. sativa</i> + <i>F. ornus</i>	677685	4784541	34	81	0,42	10	
24	<i>C. sativa</i> + <i>F. ornus</i>	678214	4784223	16	22	0,72	4	
27	<i>C. sativa</i> + <i>F. ornus</i>	679096	4782613	2	6	0,31	10	

32	<i>C. sativa</i> + <i>F.</i> 680201 <i>ornus</i>	4771846	7	11	0,63	12
33	<i>C. sativa</i> + <i>F.</i> 681571 <i>ornus</i>	4776866	16	17	0,93	16
1	<i>C. sativa</i> + <i>Q.</i> 681789 <i>cerris</i>	4776478	21	65	0,32	11
3	<i>C. sativa</i> + <i>Q.</i> 679816 <i>cerris</i>	4778934	21	39	0,53	15
5	<i>C. sativa</i> + <i>Q.</i> 676750 <i>cerris</i>	4777166	8	39	0,20	9
6	<i>C. sativa</i> + <i>Q.</i> 677681 <i>cerris</i>	4776732	54	73	0,74	9
8	<i>C. sativa</i> + <i>Q.</i> 678307 <i>cerris</i>	4775312	12	39	0,32	10
13	<i>C. sativa</i> + <i>Q.</i> 675648 <i>cerris</i>	4775580	9	36	0,26	15
16	<i>C. sativa</i> + <i>Q.</i> 674645 <i>cerris</i>	4771950	98	111	0,88	5
25	<i>C. sativa</i> + <i>Q.</i> 678948 <i>cerris</i>	4783781	1	7	0,12	8
29	<i>C. sativa</i> + <i>Q.</i> 678948 <i>cerris</i>	4782082	8	27	0,30	8
31	<i>C. sativa</i> + <i>Q.</i> 682849 <i>cerris</i>	4771275	2	27	0,09	10

Table S2. Information about parasitoids founds in ACGW galls collected in winter and spring.

Family	Species	Total in 'spring galls'	Total in 'winter galls'	Primary (Order and levels)	Hosts Parasitoid Family and Family levels)	Hosts (Order and Family levels)
Eulophidae	Aulogymnus arsame (Walker, 1838)	0	1	Hymenoptera (Cynipidae)	-	
	Aulogymnus obscuripes (Mayr, 1877)	0	3	Hymenoptera (Cynipidae)	-	
	Minotetrastichus frontalis (Nees, 1834)	1	2	Coleoptera (Curculionidae), Hymenoptera (Cimbicidae, Cynipidae, Tenthredinidae), Lepidoptera (Coleophoridae, Eriocraniidae, Gracillariidae, Heliozelidae, Lyonetiidae, Nepticulidae, Notodontidae, Tischeriidae)	Hymenoptera (Braconidae, Eulophidae)	
Eupelmidae	Eupelmus annulatus (Nees, 1834)	0	2	Coleoptera (Buprestidae, Coccinellidae, Curculionidae), Hymenoptera (Cynipidae, Diprionidae), Lepidoptera (Gelechiidae, Lymantriidae, Momphidae, Psychidae, Tortricidae)	Hymenoptera (Braconidae, Cynipidae, Ichneumonidae)	
	Eupelmus azureus (Ratzeburg, 1844)	3	4	Hymenoptera (Cynipidae)	-	
	Eupelmus confusus (Al Khatib 2015)	2	1	Dipera (Cecidomyiidae, Tephritidae),	-	

				Hymenoptera (Cynipidae, Eurytomidae), Lepidoptera (Gelechiidae, Pyrilidae)	
	Eupelmus kiefferi De Stefani, 1898	34	29	Coleoptera (Bruchidae, Coccinellidae, Curculionidae), Diptera (Cecidomyiidae, Tephritidae), Hemiptera (Aphididae, Coccidae), Hymenoptera (Cynipidae, Tenthredinidae), Lepidoptera (Gelechiidae, Gracillariidae, Tortricidae)	Hymenoptera (Braconidae, Ichneumonidae)
	Eupelmus urozonus Dalman, 1820	3	5	Coleoptera (Bruchidae, Curculionidae, Scolytidae), Diptera (Agromyzidae, Cecidomyiidae, Tephritidae), Neuroptera (Chrysopidae), Hymenoptera (Cynipidae, Tenthredinidae), Lepidoptera (Gelechiidae, Oecophoridae, Tortricidae)	Hymenoptera (Braconidae, Ichneumonidae)
Eurytomidae	Eurytoma brunniventris Ratzeburg, 1852	49	0	Hymenoptera (Cynipidae)	Hymenoptera (Cynipidae, Eulophidae, Eurytomidae, Pteromalidae, Torymidae)
	Eurytoma setigera Mayr, 1878	1	21	Hymenoptera (Cynipidae, Torymidae)	Hymenoptera (Torymidae)

	Sycophila biguttata (Swederus, 1795)	43	0	Hymenoptera (Cynipidae, Torymidae)	Hymenoptera (Eulophidae)
	Sycophila iracemae Nieves Aldrey, 1984	2	0	Hymenoptera (Cynipidae)	-
	Sycophils variegata (Curtis, 1831)	5	0	Hymenoptera (Cynipidae)	Hymenoptera (Cynipidae)
Ormyridae	Ormyrus sp. (pomaceus group)*	69	10	Hymenoptera (Cynipidae)	-
Pteromalidae	Mesopolobus amaenus (Walker, 1834)	3	0	Diptera (Cecidomyiidae), Hymenoptera (Cynipidae), Lepidoptera (Lymantriidae)	Hymenoptera (Cynipidae)
	Mesopolobus sericeus (Forster, 1770)	55	0	Hymenoptera (Cynipidae)	Hymenoptera (Cynipidae, Eurytomidae)
	Mesopolobus tibialis (Westwood, 1833)	11	0	Diptera (Cecidomyiidae), Hymenoptera (Cynipidae), Lepidoptera (Tortricidae)	Hymenoptera (Cynipidae, Eulophidae, Pteromalidae)
Torymidae	Megastigmus dorsalis (Fabricius, 1798)	6	0	Hymenoptera (Cynipidae)	Hymenoptera (Cynipidae, Torymidae)
	Microdontomerus annulatus Spinola, 1808	0	2	Diptera (Cecidomyiidae, Tephritidae), Hymenoptera (Cynipidae), Lepidoptera (Tortricidae)	-
	Torymus auratus (Müller, 1764)	270	1	Hymenoptera (Cynipidae)	Hymenoptera (Cynipidae, Eurytomidae)
	Torymus flavipes (Walker, 1833)	216	0	Diptera (Cecidomyiidae, Tephritidae), Hymenoptera	Hymenoptera (Cynipidae, Eulophidae, Eurytomidae, Pteromalidae, Torymidae)

			(Cynipidae)	
Torymus	geranii 4	0	Diptera	-
(Walker, 1833)			(Cecidomyiidae),	
			Hymenoptera	
			(Cynipidae)	
Torymus	sinensis 10	1518	Hymenoptera	-
Kamijo, 1982			(Cynipidae)	

* Probable presence of a cryptic species complex (Kaartinen et al. 2010; Lotfalizadeh et al. 2012; Gomez et al. 2017)

5. CHAPITRE IV

Fungal endophyte communities differ
between chestnut galls and leaves

Fungal Ecology, in prep

Fungal endophyte communities differ between chestnut galls and leaves

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Statement of authorship:

CR, TF and PFC conceived and designed the experiments. PFC performed the experiment. TF performed bioinformatics analysis. TF and PFC performed statistical analysis. PFC wrote the first draft of the manuscript and all authors contributed substantially to reviewing and editing.

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ABSTRACT

Fungal endophytes are potential bio-control agents for pest insects. Despite empirical evidence, little is known about their diversity and functions. We explored fungal endophyte diversity within galls induced by an invasive insect, *Dryocosmus kuriphilus*, and in surrounding chestnut leaf tissues, sampled in mature forest plots where chestnuts were growing alone or mixed with pines, oaks or ashes. We hypothesized that endophyte communities in galls differ among plot composition and that galls tissues shelter richer and more diverse endophyte communities than leaf tissues.

We selected 28 forest stands consisting in eight chestnut monocultures and 20 two-species mixtures associating chestnut with pine, ash or oak. In each site, we sampled galls and leaf tissues (3 samples per tree and 3 trees per site of each type). Fungal endophytes were characterized by Illumina sequencing of the Internal Transcribed Spacer 1 (ITS1) region.

We found a total of 1,378 different OTUs. The most common OTU corresponded to *Gnomoniopsis castanea* which has been previously described to be responsible for *D. kuriphilus* gall necrosis. The richness, diversity and composition of endophyte communities differed between galls and surrounding leaf tissues but were independent of forest stand composition. Endophytes richness and diversity in gall tissues were reduced compared to surrounding leaf tissues. Most of differences between the composition of endophyte communities between leaves and galls were due to OTU turnover.

These results suggest that the physiology of insect-induced galls acts as an ecological filter for endophytic micro-organisms, regardless of forest species composition. A better understanding of their functioning is important to improve biocontrol agents for galling insects.

Key words: endophytes, galls, diversity, plant-microbe-insect interactions, next generation sequencing methods

5.1. Introduction

Plants are colonized by a wide variety of fungi among which pathogens can cause major damage to their host plant. Fungus pathogens differ by important life-history traits such as dispersal mechanisms, reproduction and modes of parasitism (García-Guzmán and Heil 2014). Three major lifestyles of plant-associated fungal pathogens are commonly described: (i) biotrophic pathogens, which develop and extract their nutrients from living plant tissues (Delaye et al. 2013; García-Guzmán and Heil 2014); (ii) necrotrophic pathogens, which secrete enzymes and toxins that degrade and kill the host cells and then live and feed on the dead plant tissue (Spoel et al. 2007; Delaye et al. 2013; García-Guzmán and Heil 2014) and (iii) endophytes, which are the focus of this paper. Fungal endophytes are microorganisms that live at least during a part of their life cycle inside living plant tissue without causing visible disease symptoms (Partida-Martínez and Heil 2011). All major lineages of vascular plants worldwide are colonized by endophytes (Arnold 2007; Hardoim et al. 2015). In some cases, their presence may increase plant fitness through improved plant resistance to abiotic (e.g. drought or salinity tolerance, Redman et al. 2002; Sherameti et al. 2008) and biotic stressors (e.g., resistance to insects and fungal pathogens, Clay 1996; Ownley et al. 2010; Combès et al. 2012). For these reasons they have been usually recognized as ‘beneficial’ microbes (e.g. Pineda et al. 2013). However, plant-endophytes interactions may also result in pathogenicity depending on biotic and abiotic factors, such as plant and microbe genotypes or environmental conditions like drought (Hardoim et al. 2015). Because of the complexity of the endophyte-pathogen-saprophyte continuum in fungi (Arnold 2007; Delaye et al. 2013), understanding how endophytes and their host plant interact requires further investigations. In particular, it is critical to determine how endophytes interact with plant antagonists and which factors structure endophyte communities and their feedbacks on other bio-aggressors.

Endophytic fungi have been shown as one of the key drivers of plant-herbivore interactions, with several studies reporting negative impacts of endophytic fungi on plant associated herbivorous insects (Fernandez-Conradi et al. 2017; Clay 1996; Clay and Schardl 2002; Kuldau and Bacon 2008; Rudgers and Clay 2008; Saikkonen et al. 2010; but see Faeth and Saari 2012). Plant associated fungi may induce changes in plant chemical composition, thus indirectly affecting insects by changing the quality of feeding resources (Hatcher 1995; Tack and Dicke 2013; Raman and Suryanarayanan 2017). In addition, some fungal endophytes may produce chemical compounds, such as alkaloids, which are generally toxic for insects (Clay 1996; Clay and Schardl 2002). For these reasons, several endophyte species or strains have been used for pest biocontrol (Gurulingappa et al. 2010; Akello and Sikora 2012; Castillo Lopez et al. 2014; Lopez and Sword 2015). However, the reciprocal effect of insects on the structure of endophyte communities associated to the same host plant is still little understood. As fungi, insects may also induce changes in host plant quality and defensive compounds (reviewed by Hatcher 1995; Stout et al. 2006; Appendix I, Supplementary material) which may impact plant associated fungal endophytes.

Galls are good examples of plant manipulation by insects which may impact plant-associated fungi. Gall-makers are known to manipulate and reprogram host plant development, inducing spectacular morphological and physiological changes in host plant tissues (Giron et al. 2016). Galls are particular modified, tumor-like growth of plant tissues which provide their inhabitants with nutriment and shelter from natural enemies (Stone and Schönrogge 2003). Galls may act as plant metabolic sinks (Hata and Futai 1995; Allison and Schultz 2005; Giron et al. 2016), thus affecting host plant quality for fungi. In addition, insect larvae inside galls can represent supplementary source of nutriment for endophytic fungi, since several endophytes have been reported as entomopathogen (Wilson 1995). The variability of endophyte communities between plant organs can be, in some cases, greater than between plants from different geographical locations (Mishra et al. 2012). Endophyte infections can be strictly localized in one type of plant tissue while some species are systemic and found in all host tissues (Kumar and Hyde 2004). Because galls are formed by special plant modified tissues, one can expect their endophyte community to strongly differ from that of other plant organs (Lawson et al. 2014; Washburn and Van Bael 2017).

To date, most of studies on plant-insect-endophyte interactions have been carried out to understand the effect of endophytes on plant-associated insects (Clay 1996; Clay and Scharndl 2002; Saikkonen et al. 2010; Faeth and Saari 2012) or the combined effect of a plant endophyte and an insect on their host plant (*e.g.*, Kruess 2002). Studies on the impact of insect herbivores on fungal endophytes are comparatively scarce (but see Lawson et al. 2014; Washburn and Van Bael 2017). Understanding how endophytes communities vary between galls and adjacent leaf tissues may help better understand tripartite interactions between galling insects, endophytic fungi and their host plant. In addition, studies of plant-endophyte interactions are commonly based on controlled conditions manipulating one endophyte species under given biotic and abiotic conditions. By contrast, although plants are naturally infected by a wide community of endophytes composed of very different fungal species or strains which may interact with each other and with other organisms, only very few studies have addressed tripartite interactions in the field.

Tripartite interactions between plants, endophytic fungi and insects may depend on other biotic conditions such as plant biodiversity. For instance, it is increasingly recognized that plant diversity plays an important role on plant-insect interactions (Koricheva et al. 2000; Jactel and Brockerhoff 2007; Barbosa et al. 2009; Castagnyrol et al. 2014; Moreira et al. 2016). However, the effect of plant diversity on endophytic fungal communities is still poorly understood. For instance, Müller and Hallaksela (1998) found that fungal endophyte diversity in Norway spruce needles varies with tree diversity in the stand, whereby fungal endophyte diversity was higher in pure spruce stands compared to spruce mixtures with birch or Scots pine. By contrast, Nguyen et al. (2016) found that a significant effect of tree diversity on the structure of foliar fungal communities of Norway spruce trees, composed of fungal pathogens and endophytes, was not a general phenomenon in European forests. They suggested that tree species identity and tree species composition could blur the sole effect of tree diversity, but this question remains to be explored.

In this study, we analyzed fungal endophyte communities in galls induced by an invasive insect, the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), and in surrounding leaf tissues of chestnuts sampled in forest stands with different tree species composition. ACGW is a micro-Hymenoptera native to China and considered the most important pest of *Castanea* species worldwide (Moriya et al. 2003). Classical biological control, *i.e.* the introduction of natural enemies native to the area of origin of the pest for their permanent establishment in a new area (Eilenberg et al. 2001), is the most common strategy used to manage ACGW populations worldwide. Classical biological control is currently performed with the exotic parasitoid *Torymus sinensis* Kamijo (Gibbs et al. 2011). Concomitantly, there is an increasing research on the potential use of entomopathogenic fungi like *Fusarium proliferatum* as biocontrol agents for the ACGW (Tosi et al. 2015). Recently, necrosis found in ACGW galls in Italy were attributed to the endophytic fungus *Gnomoniopsis castanea*, which is seen as a putative biocontrol agent (Vannini et al. 2017). Another complementary method for controlling ACGW is the conservation biological control strategy (Barbosa 1998) which relies on the reinforcement of biological control by native natural enemies through habitat improvement. ACGW infestations have been shown to be lower in mixed chestnut forests (Guyot et al 2015; Chapter III), with empirical evidence suggesting a putative role of parasitoids (Leles et al. 2017; Chapter III). However, whether and how endophytes could be the hidden link between tree diversity and chestnut resistance to ACGW remains to be evaluated (see Chapter III).

The aim of this study was thus to compare the diversity and composition of fungal endophytes communities in ACGW galls and surrounding leaf tissues in chestnut monocultures vs. mixtures. We sampled galled chestnut leaves in natural mature forest plots where chestnuts were growing alone or mixed with pines, oaks or ashes. We extracted DNA and used Illumina sequencing to estimate endophytes diversity and richness. We hypothesized that (i) galls tissues shelter a richer and more diverse endophyte community than surrounding leaf tissues because of higher nutrient content and (ii) endophytes communities richness and composition in ACGW galls and surrounding tissues depend on forest stand composition. By addressing the above, this study builds towards a better understanding of plant-endophyte-insect interactions and aim to help developing more efficient biocontrol strategies against the ACGW.

5.2. Materials and Methods

Sampling design

The study was performed in over-rotation coppice stands in Southern Tuscany (Italy) as described in Chapter III. These forests are mainly composed of European chestnut (*Castanea sativa*), European hornbeam (*Ostrya carpinifolia*), oaks (*Quercus ilex*, *Q. petraea*, *Q. suber*,

Q. pubescens and *Q. cerris*), ash (*Fraxinus ornus*) and maritime pine (*Pinus pinaster*). We selected 28 chestnut plots consisting in eight chestnut monocultures and 20 two-species mixtures. Mixtures associated *C. sativa* with *Q. cerris* (n = 8), *F. ornus* (n = 8) or *P. pinaster* (n = 4). In each plot, we randomly sampled three leaves bearing ACGW galls on three chestnut trees per site, in June 2015. Trees were the same as those sampled by Fernandez-Conradi et al. (Chapter III). Leaf and gall samples were dried in plastic bags with silica gel and kept frozen at -80°C until DNA extraction.

DNA extraction and sequencing

We prevented sample contamination by exposing to 30 min of UV light in a laminar flow hood all the tools and materials required for sample processing and DNA extraction. Galls and surrounding leaf tissues of each sampled leaf were separated using a sterile razor blade. Four discs (each 8.0 mm in diameter) were cut randomly from each leaf and gall, with a hole-punch sterilized by flaming with 95% ethanol. Discs were sterilized according to Bálint et al. (2015): they were first immersed in 4% sodium hydrochloride solution for 1 min and then washed twice in a sterile solution containing 0.1% of Tween ® 20 (Sigma-aldrich company) to break surface tension. Discs from the same tree were pooled in two separate autoclaved sample tubes further named 'leaf' and 'gall'. Two tubes were left empty for negative controls.

Total DNA for each sample (including the empty tubes for negative controls) was extracted with the DNeasy 96 Plant Kit (QIAGEN). DNA samples from the same tree were pooled before DNA amplification. Fungal Internal Transcribed Spacer 1 (ITS1) barcode was amplified with the ITS1 (forward) and ITS4 (reverse) primer pair (Gardes and Bruns 1993). Paired-end sequencing (300 bp) was performed in a single run of an Illumina MiSeq sequencer, on the basis of V3 chemistry. Polymerase Chain Reaction (PCR) amplification, barcodes and MiSeq adapters addition, library sequencing and data preprocessing were carried out by the LGC Genomics sequencing service (Berlin, Germany).

Bioinformatic analysis

Sequences were first demultiplexed and filtered. All sequences with tag mismatches, missing tags, one-sided tags or conflicting tag pairs were discarded. Tags and Illumina TruSeq adapters were then clipped from all sequences, and sequences with final length shorter than 100 bases were discarded. All sequences with more than three mismatches with the ITS1 and ITS4 primers were discarded. Primers were then clipped and sequence fragments were placed in forward-reverse primer orientation. Forward and reverse reads were then combined with the BBmerge software. Read pair sequences that could not be combined were discarded.

The remaining high quality sequences were processed following the pipeline developed by Bálint et al. (2014). The ITS1 sequence was first extracted from each read with the FungalITSextractor (Nilsson et al., 2010). All the sequences were then concatenated in a single fasta file, after adding the sample code in the label of each sequence. The sequences were dereplicated, sorted and singletons were discarded with VSEARCH (<https://github.com/torognes/vsearch>). The sequences were then clustered into molecular

operational taxonomic units (OTUs) with the UPARSE algorithm implemented in USEARCH v8 (Edgar 2013), with a minimum identity threshold of 97%. Additional chimera detection was performed against the UNITE database (Kõljalg et al. 2013), with the UCHIME algorithm implemented in USEARCH v8 (Edgar et al. 2011). The OTU table, giving the number of sequences in each OTU for each sample, was created with USEARCH v8.

OTUs were taxonomically assigned with the online BLAST web interface (Madden 2013) against the GenBank database, by excluding environmental and metagenome sequences. Only the assignment with the lowest *e*-value was retained. The full taxonomic lineage of each assignment was retrieved from the GI number information provided by NCBI. All the OTUs assigned to plants or other organisms, and all unassigned OTUs were removed, to ensure that only fungal OTUs were retained.

Statistical analyses

All statistical analyses were performed in the 3.4.1 version in *R*. One hundred random rarefied OTU matrices were computed, using the smallest number of sequences per sample as rarefaction threshold. OTU richness, Shannon diversity index and Bray-Curtis dissimilarity index were calculated for each rarefied matrix, and averaged across the 100 matrices (Jakuschkin et al. 2016; Fort et al. 2016).

Linear mixed effect models were used to assess the effect of tissue (leaf vs. gall) and plot composition on OTU richness and diversity, including the sampled trees and plot as random factors to account for the non-independence of the three samples per plot (Schielzeth and Nakagawa 2013). We applied model simplification by removing non-significant interactions prior estimating significant of principal effects. We used the *lmer* function from the *lmer4* package (Bates et al. 2015) after linearization of the index (richness and Shannon diversity) to obtain the effective species number (Jost 2006). Package *car* (Fox and Weisberg 2011) was used to assess the significance of fixed effects.

PCoA and PERMANOVA were used to assess the effect of stand composition on endophytic community composition for both chestnut leaves and galls. Bray-Curtis dissimilarities in endophytic community composition were tested with Principal Coordinates Analysis (PCoA). Permutational Analysis of Variance (PERMANOVA) was used to investigate differences in OTU composition between chestnut galls and surrounding leaf tissues. The sampling plot was included as strata factor in the PERMANOVA.

An indicator species analysis was performed in order to identify OTUs that were characteristic of each chestnut tissue (Cáceres and Legendre 2009). Beta-diversity partitioning was used to separate the turnover and nestedness-resultant components of endophytic community composition between chestnut galls and surrounding foliar tissues (Baselga 2010).

5.3. Results

Taxonomic description of fungal communities

We found a total of 6,401,652 high-quality sequences, which clustered into 1,378 OTUs. Overall, 97 OTUs were not taxonomically assigned to fungi BLAST, corresponding to 55,306 sequences (0.86% of the raw OTU table). Among them, a single OTU was assigned to the Eumetazoa kingdom, another one to the Chlorophyta division and five to the Tracheophyta division, from *Quercus*, *Castanopsis* and *Fraxinus* genera. These OTUs were discarded. Negative control samples contained 22 fungal OTUs, corresponding to 8,745 sequences (0.14% of the total fungal sequences). At this time, there is no consensus on how to deal with sequences in negative controls (Nguyen 2015). We thus decided to keep those OTUs in the dataset. Five samples containing very few sequences (less than 500 sequences) were removed. The final OTU table used for the analyses contained 181 samples (97 foliar and 84 gall communities), and 6,336,807 sequences representing 1,274 fungal OTUs. The mean number of sequences per sample was 35,010 ranging from 717 to 188,765. This OTU table was used for taxonomical description.

The 1,274 fungal OTUs could be assigned to 670 different fungal species in NCBI database. The fungal communities were largely dominated by ascomycetes (Figure 1, Table 1): sequences assigned to the Ascomycota division accounted for 93.0%. Basidiomycota division only represented 5.5% and other divisions and unassigned sequences at the division level represented together 1.5% of the sequences.

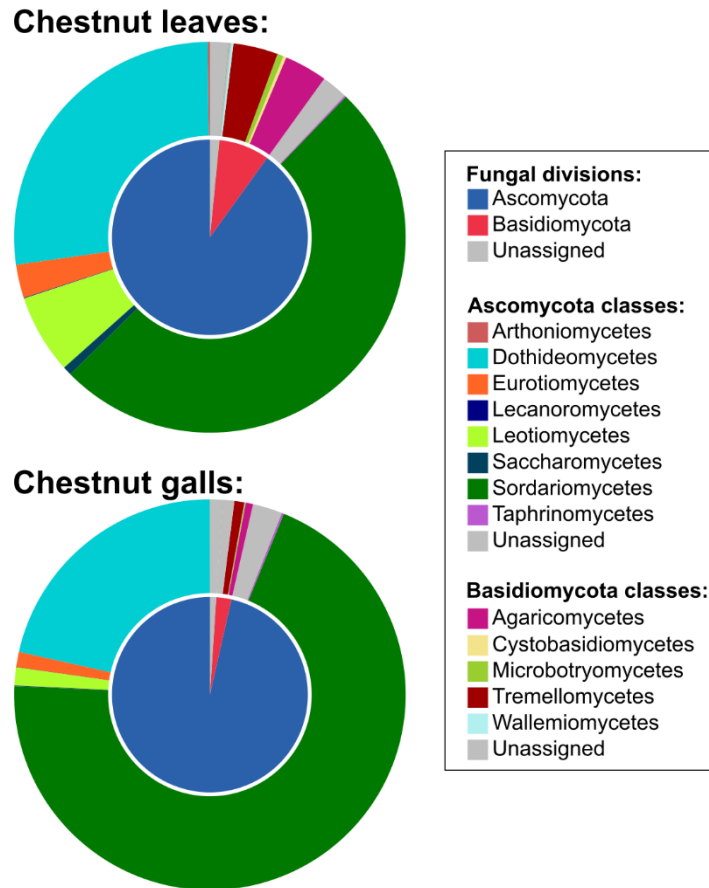


Figure 1. Taxonomic composition of fungal endophytic communities in (A) chestnut leaves and (B) ACGW galls. The inner disc shows the proportion of sequences assigned to each taxonomic division, and the outer disc the proportion of sequences assigned to each class of the Ascomycota and Basidiomycota divisions.

We found 763 different fungal OTUs in ACGW galls. Among them, OTU 1 represented 46.91% of reads. This OTU was assigned to *Gnomoniopsis castanea*. OTUs 955, 1 051, 1 058, 1 112, 1 165 and 1 168 were also assigned to this species. The most frequent OTUs were also detected in leaves.

Table 1. Taxonomic assignment of the 12 most abundant OTUs in ACGW galls (representing more than 1% of our sequences) by the online BLAST analysis against the GenBank database.

OTU number	Class	Putative taxon	Total abundance	Relative abundance
1	<i>Sordariomycetes</i>	<i>Gnomoniopsis castanea</i>	1, 845,964	55.89
2	<i>Sordariomycetes</i>	<i>Trichothecium roseum</i>	187,145	5.67
3	<i>Dothideomycetes</i>	<i>Alternaria</i> sp.	170,375	5.16
4	<i>Dothideomycetes</i>	<i>Diplodia seriata</i>	111,741	3.38
7	<i>Dothideomycetes</i>	<i>Stemphylium vesicarium</i>	86,460	2.62
14	Unknown	<i>Ascomycota</i> sp. D7	73,807	2.23
10	<i>Dothideomycetes</i>	<i>Botryosphaeria dothidea</i>	69,926	2.12

OTU number	Class	Putative taxon	Total abundance	Relative abundance
6	<i>Sordariomycetes</i>	<i>Sordario sp.</i>	66,545	2.01
13	<i>Dothideomycetes</i>	<i>Pyrenochaeta cava</i>	53,327	1.61
15	<i>Sordariomycetes</i>	<i>Trichothecium roseum</i>	50,226	1.52
22	<i>Sordariomycetes</i>	<i>Diaporthe sp. G360</i>	44,255	1.34
5	<i>Letiomycetes</i>	<i>Botryotinia pelargonii</i>	43,446	1.32

Differences between fungal endophyte communities in chestnut galls vs. surrounding leaf tissues

The richness and Shannon diversity of fungal endophytes OTUs differed significantly between ACGW galls and surrounding leaf tissues ($\chi^2= 12.778$; $df= 1$; $P < 0.001$ and $\chi^2= 78.038$; $df=1$; $P < 0.001$ respectively), both being higher in chestnut leaf tissues than in galls (Figure 2).

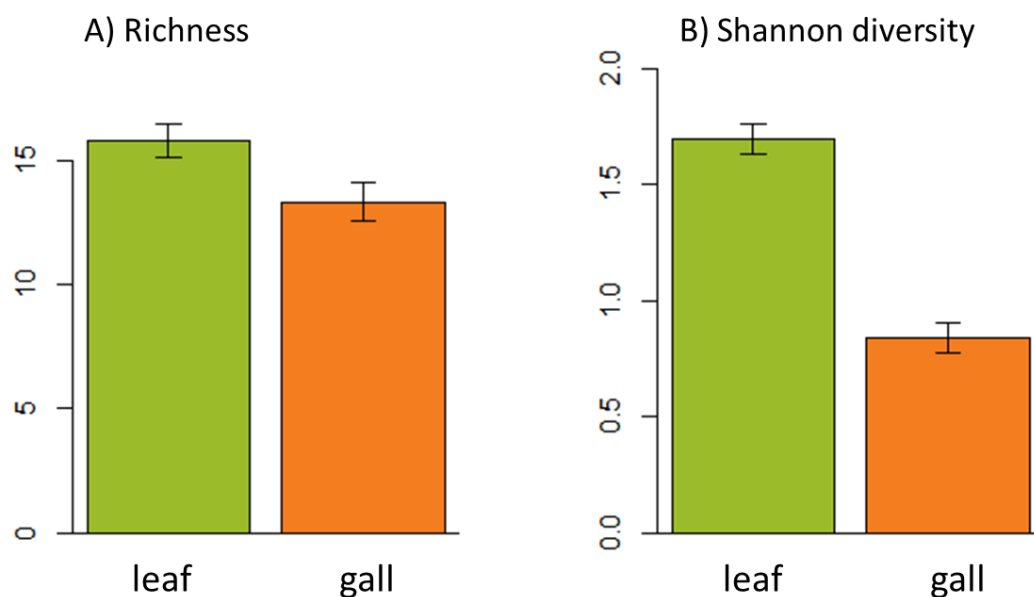


Figure 2. OTU richness (A) and Shannon diversity (B) of fungal endophytic communities in chestnut galls and surrounding leaves tissues. Error bars represent the standard error of the mean ($n= 84$ leaves and 84 galls).

The first two PCoA axes explained 28.2 and 6.7% of variance in dissimilarities between foliar and gall fungal communities respectively (Figure 3). The type of chestnut tissue (*i.e.* leaf vs. gall) had a significant effect on fungal endophytic community composition (PERMANOVA: $F= 15.31$; $R^2= 0.09$; $P= 0.001$).

The indicator species analysis revealed that 25 OTUs were significantly associated with chestnut foliar tissues, and 15 OTUs with the galls (Table 2).

PCoA: galls and leaves

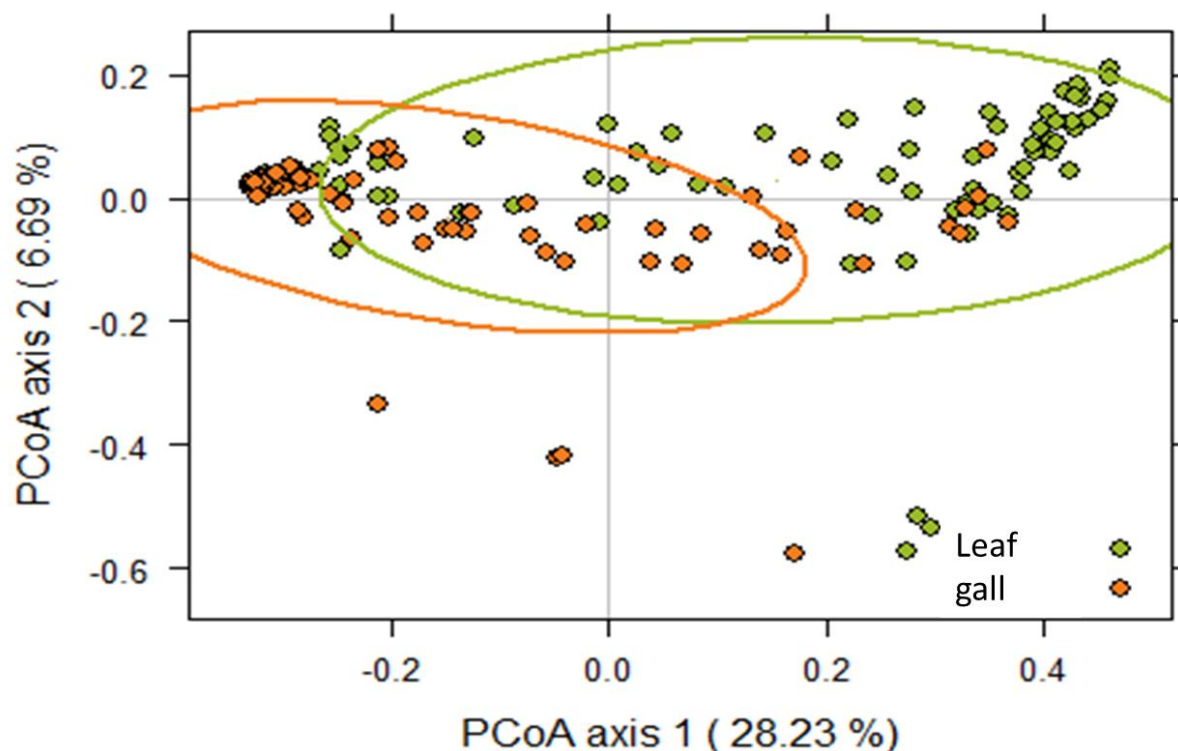


Figure 2. PCoA representing OTUs distribution in chestnut galls (in orange) and surrounding leaf tissues (in green). Dissimilarities between samples were computed with the Bray-Curtis index, averaged over 1000 random permutations.

Table 2. Taxonomic assignment by the online BLAST analysis against the GenBank database of fungal OTUs with a significant indicator value (Indval) for a type of chestnut tissue.

Tissue type	Indval	P-val	OTU number	Assigned species
Leaf	0.846	0.005	47	<i>Erysiphe necator</i>
	0.820	0.005	28	<i>Cortinarius infractus</i>
	0.467	0.005	1170	<i>Epicoccum nigrum</i>
	0.429	0.005	17	<i>Cylindrium sp. 1 ICMP 18787</i>
	0.272	0.005	125	<i>Filobasidium magnum</i>
	0.446	0.010	68	<i>Cortinarius sp.</i>
	0.365	0.010	639	<i>Alternaria sp.</i>
	0.353	0.010	630	<i>Cladosporium subinflatum</i>
	0.314	0.010	263	<i>Penicillium expansum</i>
	0.294	0.010	116	<i>Aspergillus flavus</i>
	0.307	0.015	801	<i>Cortinarius sp. LM03</i>
	0.282	0.020	939	<i>Ramularia endophylla</i>
	0.268	0.020	752	<i>Cladosporium subinflatum</i>
	0.455	0.025	11	<i>Trichoderma atroviride</i>
	0.329	0.025	630	<i>Cladosporium subinflatum</i>
0.329	0.025	119	<i>Wallemia canadensis</i>	

Tissue type	Indval	P-val	OTU number	Assigned species	
	0.248	0.025	85	<i>Holtermanniella wattica</i>	
	0.248	0.025	216	Unidentified	
	0.305	0.030	939	<i>Ramularia endophylla</i>	
	0.272	0.030	193	<i>Vishniacozyma tephrensis</i>	
	0.489	0.035	639	<i>Alternaria sp.</i>	
	0.222	0.035	144	<i>Nectria sp.</i>	
	0.248	0.040	498	<i>Clypeophysalospora latitans</i>	
	0.248	0.050	305	<i>Hygrophorus arbustivus</i>	
	Gall	0.677	0.005	27	Unidentified
		0.656	0.005	13	<i>Pyrenochaeta cava</i>
		0.571	0.005	49	<i>Diplodina castaneae</i>
		0.492	0.010	704	<i>Diplodina castaneae</i>
		0.362	0.010	10	<i>Botryosphaeria dothidea</i>
		0.327	0.010	1165	<i>Gnomoniopsis castanea</i>
		0.422	0.015	774	<i>Pyrenochaeta sp G41</i>
		0.550	0.020	14	Unidentified
0.321		0.020	94	<i>Colletotrichum fioriniae</i>	
0.267		0.020	36	<i>Clonostachys sp.</i>	
0.319	0.025	739	<i>Aureobasidium sp.</i>		
0.267	0.025	789	<i>Neophaeomoniella niveniae</i>		

The β -diversity partitioning revealed that dissimilarity between the fungal communities of leaves and galls was mostly explained by a turnover (87.1% of the total dissimilarity) with a low nestedness (12.9%).

Effect of stand composition on leaf and galls communities

Fungal endophytes OTUs richness and Shannon diversity in ACGW galls did not depend on stand composition ($F= 0.42$; $df= 3$; $P= 0.738$ and $F= 0.24$; $df= 3$; $P= 0.867$ respectively). Likewise, OTUs richness and Shannon diversity in chestnut leaves were independent of plot tree species composition ($F= 1.90$; $df= 3$; $P= 0.135$ and $F= 0.99$; $df= 3$; $P= 0.403$ respectively).

Tree species composition was not a significant driver of the structure of endophytic fungal OTUs communities neither in galls (Figure 4a; $F= 1.21$; $R^2= 0.04$; $P= 1.000$) nor in leaves (Figure 4b; $F= 1.38$; $R^2=0.04$; $P= 1.111$).

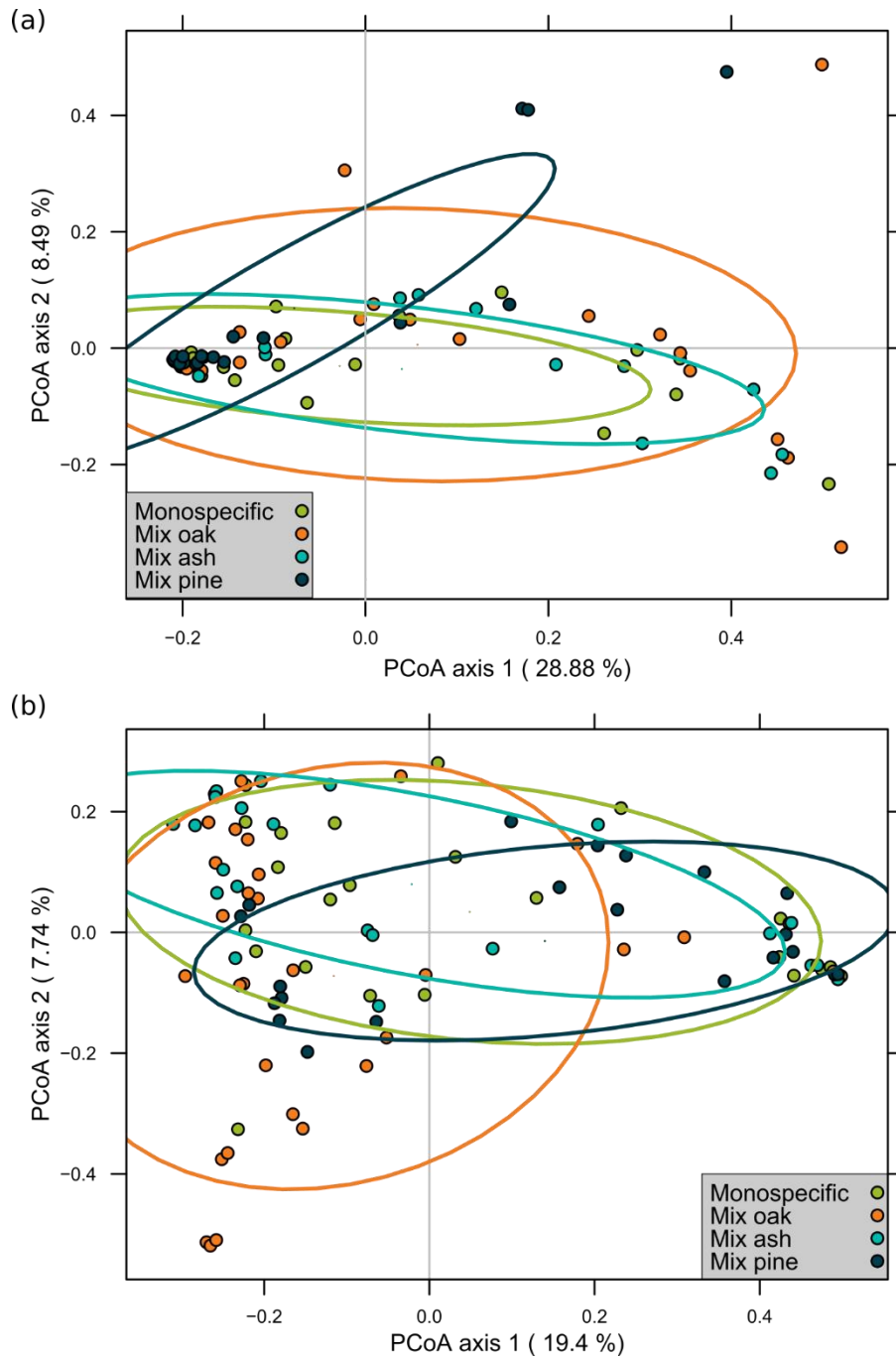


Figure 4. PCoA representing dissimilarities in the composition of fungal endophytic communities in (A) chestnut galls and (B) surrounding leaf tissues. Different colors and ellipses represent the plot composition in tree species. Dissimilarities between samples were computed with the Bray-Curtis index, averaged over 1000 random permutations.

5.4. Discussion

Our study is one of the rare to describe endophytic fungal communities associated with insect-induced galls. We found a large amount of fungal endophytes sequences in ACGW galls belonging to 763 different fungal OTUs. The number of fungal OTUs found is much higher than that found by Lawson et al. (2014) and Washburn and Van Bael (2017) in other insect galls (62 endophytes species and 34 OTUs respectively). This is probably due to the use of a more powerful identification method, as we applied new generation sequencing techniques instead of more classical fungus culture.

Endophytic fungal communities differed between galls and surrounding tissues

Endophyte fungi OTU richness and diversity differed significantly between chestnut galls and surrounding leaf tissues and contrary to our expectation, were lower in galls. Endophytic community composition were also different between the two tissues. This is consistent with the patterns reported by Lawson et al. (2014) in aphid-induced galls in poplars and Washburn and Van Bael (2017) in midges induced galls in bald cypress trees.

Differences in the composition of endophytic communities between galls and surrounding leaves may have two sources. First, these differences between leaves and galls may be primarily due to the insect bringing some fungal species during oviposition that may not have been associated with the plant in the absence of galls (Wilson 1995; Washburn and Van Bael 2017). This explanation would be consistent with the observation that in several cases, insects can act as vectors of plant pathogenic fungi (Kluth et al. 2002). However, the hypothesis of galling insects as a source of plant endophytic fungi received only indirect evidence. For instance, Washburn and Van Bael (2017) found that diversity and community composition of fungi differed in galls from which midges emerged or not. In our study, we found that endophyte community composition in galls differed from surrounding leaf tissues also providing indirect evidence for this hypothesis (Lawson et al. 2014).

Second, galling insect may act as an ecological filter selecting particular endophytic species from a pool of species initially present in plant buds or galls. This may explain why the diversity and richness of fungal endophyte communities in galls is lower than in surrounding leaves. Galling insects are able to divert plant nutrients to the galls, which thus act as nutrient sinks (Allison and Schultz 2005; Giron et al. 2016). Some authors proposed that the species richness of endophytic fungi would increase in galls as a result of increased nutrient availability. It is possible that, at the contrary, such rich environments would select particularly competitive species excluding less competitive species, thus resulting in less species rich communities in galls as compared to surrounding tissues. Alternatively, galls are also known to express high levels of secondary compounds (Hartley 1998), which may contribute to filter (here, exclude) some fungal species. This hypothesis is however controversial. The “nutrition hypothesis” (Price et al. 1987; Bronner 1992) states that gall tissues have higher nutrient contents and lower amount of secondary compounds compared to ungalled plant tissue (*e.g.*, Nyman and Julkunen-Tiitto 2000; Allison and Schultz 2005) while

the “enemy hypothesis” (Stone et al. 2002) proposes that higher levels of tannins and phenols in galls can protect the gall-maker from mortality due to pathogenic fungi or parasitoids attack (Taper and Case 1987; Price and Pschorn-Walcher 1988). In chapter II, we confirmed that N content was lower in galls and that polyphenolics differed between galls and surrounding leaves. Differences in endophytic communities between galls and surrounding leaves are consistent with the idea that galls may act as an ecological filter on endophytic communities, yet, expected that the endophytic community associated to galls to be a nested subset of the endophytic community associated with leaves. This was not the case as most of differences in endophytic communities between the two tissues were mainly due to species turnover. This suggests that galls and leaves provided different habitat conditions to the local pool of endophytes, which could be either physical (barriers to spore contact) or chemical (barriers to germination or infection).

Gall and leaf endophytic communities were independent of stand composition

Forest composition did not influence the structure of endophyte communities in ACGW galls or chestnut leaves. Fungal endophytes of trees are usually horizontally transmitted (Partida-Martínez and Heil 2011). As such, if endophytes are generalists, one can expect a contagion from other neighboring species present in mixed plots, thus resulting in higher endophyte richness in mixed-species forests. At the contrary, if endophytes are mainly host specialists, non-host tree species may act as barrier to spore transmission, thus reducing endophytes richness on a given target tree species as a result of host dilution in mixed forests. We found no overall effect of stand specific composition on endophytic communities, which could result from endophytic communities being a mix of generalist and specialist endophytes (Saikkonen 2007), as recently suggested by Nguyen et al. (2017) who focused on foliar fungi.

ACGW biocontrol by endophytes

Using the same forest stands we showed that ACGW infestation rates generally decreased in tree mixtures compared to monocultures (this thesis, Chapter III, see also Guyot et al. 2015). We found that in this case associational resistance depended on tree neighbor identity, being stronger in oak- and ash- chestnut mixtures, particularly as non-host density increased in mixed stands. The fact the composition of endophytic communities was independent of stand composition suggests that associational resistance was not mediated by changes in endophytic diversity.

Although we found no clear evidence for a conservation biological control of ACGW mediated by endophytic fungi, the latter may be still relevant for classical biological control approaches.

Interestingly, the most common OTU present in our chestnut gall samples was *Gnomoniopsis castanea* (Gnomoniaceae, Diaporthales, synonym *Gnomoniopsis smthogilyvi*) which represented about 56% of OTU sequences. This fungus, causing nut rot in *C. sativa*, has been described based on morphology and analysis of ribosomal DNA of the ITS region and EF-1a

locus (Visentin et al. 2012; Shuttleworth et al. 2016, in Italy and in Australia respectively). *Gnomoniopsis castanea* is now considered a latent pathogen, which can infect flowers, leaves and chestnut branches (Shuttleworth and Guest 2017, Vannini et al. 2017) and can cause necrosis in branches, leaves and fruits (Pasche et al. 2016). It is also associated to ACGW gall necrosis (Magro et al. 2010; Vannini et al. 2017; Seddaiu et al. 2017). According to Vannini et al. (2017) gall necrosis could have a strong impact on vitality of adult ACGW inside galls. They proposed that *G. castanea* could be one of the most efficient sources of natural biological control of ACGW in Europe. However, Lione et al. (2016) reported a large number of ACGW emergence from galls colonized by *G. castanea*, suggesting that despite frequent gall infection by *G. castanea*, this fungus may fail to provide an efficient biocontrol of the ACGW. We found *G. castanea* in all living galls (and also in winter, where galls were abandoned and dried, see also Appendix B in Supplementary Material) even if they were not necrotic. This result suggests that *G. castanea* can opportunistically shift from endophytic to saprophytic life style (Wilson 1995) thus explaining the conflicting results on its effectiveness as a biocontrol agent. In any case, the fact that *G. castanea* can also cause direct damage to *Castanea* species (nut and kernel rot) makes it an unsuitable biocontrol agent of the gall wasp in orchards.

Another potential biocontrol agent of ACGW is the endophyte *Fusarium spp.* (Addario et al. 2011). For example, *F. proliferatum* was shown to be associated with high ACGW mortality rates in the laboratory and to be not pathogenic to chestnut trees (Tosi et al. 2015). The genus *Fusarium* only represent about 1.5% of OTU sequences in our samples. These sequences matched with *Fusarium lateritium* (also found in ACGW galls by Seddaiu et al. 2017), *F. ciliatum*, *F. oxysporum* and other unidentified *Fusarium spp.* However, none of our sequences matched with *F. proliferatum*. This might be due to a low frequency of this fungus on ACGW galls or to methodological problems in identifying and differentiating species of this genera. Despite its potential to act as a biocontrol agent as suggested by laboratory studies, our results suggests that it is unlikely to currently contribute to control ACGW populations in the study area.

5.5. Conclusion

Our study contributes to the understanding of how galling insects can affect endophytic fungal communities in their common host plant tissues. Endophytic communities in insect galls are very different in terms of richness and composition from that of surrounding leaf tissues. This suggests that physiological changes in plant tissues triggered by the gall formation are important ecological filters acting upon endophyte assemblages. How these changes can modify the physical and chemical requirements of endophyte infection remains an important challenge to verify this hypothesis. The fact that gall-induced filtering processes were independent of tree species diversity and composition of the forests is probably due to the large variability in endophytes species traits, particularly their host specificity. More

research on endophytes functional diversity is clearly needed to better disentangle the mechanisms underlying plant-insect-fungi interactions in forests.

5.6. References

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5.7. Supplementary material

5.7.1. APPENDIX A: Endophytic fungi OTUs differed between chestnut leaves and tree neighbor species in our plots

In our study, we also collected three leaves from each three chestnut neighbors per plot. These leaves were processed as chestnut leaf and gall samples, with the difference than we pooled them after DNA extraction and the DNA amplification was performed on the pooled samples.

We found fungal OTUs shared and specific to chestnut leaves and galls and their neighbors leaves (Figure S1).



Figure S1. Venn diagram giving the number of OTUs shared between chestnut leaves ($n= 84$), galls ($n= 84$) and leaves from chestnut neighboring tree species: *Fraxinus ornus* ($n= 8$), *Pinus pinaster* ($n= 4$) and *Quercus cerris* ($n=8$)

Even if our sampling was not equilibrated between chestnut and other tree species, which may explain the greater variability in fungal OTUs in chestnut, endophytic communities strongly differed between the different tree species (Figure S1; PERMANOVA: $F= 4.266$; $df= 3$; $P= 0.001$).

Feuilles - PCoA - Bray - Effet hôte

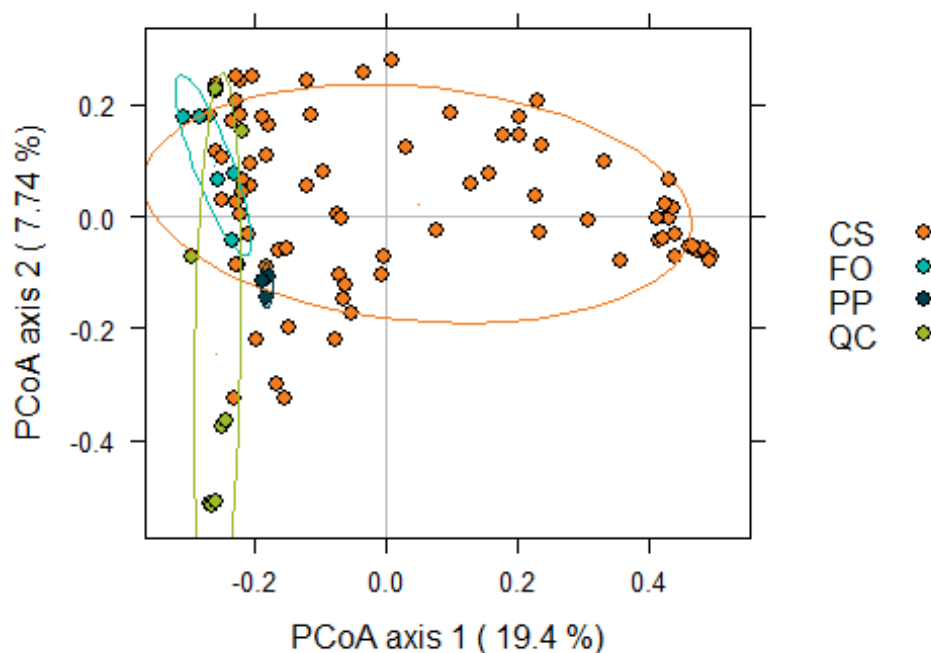


Figure S2: PCoA representing OTUs distribution between *Castanea sativa* (CS, $n= 84$), *Fraxinus ornus* (FO, $n= 8$), *Pinus pinaster* (PP, $n= 4$) and *Quercus cerris* (QC, $n=8$) leaves. Dissimilarities between samples were computed with the Bray-Curtis index, averaged over 1000 random permutations.

5.7.2. APPENDIX B: List of fungal endophytes found in ACGW winter galls

- *Methods*

In winter 2015, we also sampled three ACGW dry galls per tree ($n= 288$ galls; 96 trees). For each gall, we cut eight small pieces of 2-3 mm and we disinfected them as described in 'materials and methods' section for green galls. After surface sterilization, gall pieces were placed in petri dishes containing potato dextrose agar (PDA at 39g/liter, Difco Laboratoires, Detroit). Petri dishes were then sealed with Parafilm and incubated for a week at 20°C under complete dark or light conditions (8h light - 16h dark conditions). Each different fungus was then replicated in a new Petri dish in order to have a single fungal type per dish. We allowed the fungus to growth in the same conditions described above and we thereafter grouped them in different morphotypes based on their size and pigmentation. Three samples of each defined morphotype, when available, were then replicated again in new dishes, this time containing filters over the PDA. Fungal mycelium was then scraped with a sterile razor blade and transferred to Eppendorf tubes. After lyophilization of fungal mycelium, DNA was extracted using Invisorb® DNA extraction kit following manufacturer protocol. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using the forward primer ITS1 and the reverse primer ITS4. For DNA amplification, we used 2 μ l of the DNA extraction product,

mixed with 18.3µl of master mix containing 2µl of MgCL₂, 2µl of BSA (bovine serum albumin) at 10ng/µl, 0.8 µl of dNTP at 5mM, 0.15µl of Taq polymerase, 2 µl of buffer 10x, 11.55µl of deionized water and 0.3µl of each ITS1 forward and ITS4 reverse primers. DNA amplification reactions were performed for 3 minutes at 94°C initial denaturation, 35 cycles of 30 seconds at 94°C denaturation, 40 seconds at 55°C annealing and 7 minutes at 72°C extension. DNA products were sent for sequencing to an external service.

For species identification, sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database ([http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)).

- **Results:**

He found 57 different fungal morphotypes which were assigned to 13 different fungal groups (Table S2).

Table S2. Taxonomic assignments of endophytic fungi found in ACGW dry winter galls and the number of times we cultured them from different galls.

Taxonomic assignments	Times observed in DRy galls
TRICHODERMA SP.	17
BOTRYOSPHAERIA DOTHIDEA	15
GNOMONIOPSIS SMITHOGILVYI	15
FUSARIUM SP.	14
PENICILLIUM SP.	14
BOTRYOSPHAERIA SP.	8
PARACONIOTHYRIUM SP.	7
ALTERNARIA SP.	3
CRYPHONECTRIA PARASITICA	2
BISCOGNIAUXIA SP.	1
COLLETOTRICHUM ACUTATUM	1
EPICOCCUM NIGRUM	1
GLOMERELLA CINGULATA	1

6. DISCUSSION

6.1. Tripartite interactions: How do fungal pathogens affect co-occurring insects on host plants and vice versa?

Infection by a fungus has important consequences on herbivorous insects feeding on the same host plant. By using a meta-analysis, we found that, in general, insects preferred and performed better on unchallenged plants (Chapter I). However, the detrimental effect of fungi was only significant in the case of endophytic and biotrophic fungal pathogens, whereas a greater variability was observed with necrotrophic pathogens and remains to be explained.

In our literature review, we found 17 papers that studied the effect of plant infection by a necrotrophic fungus (12 different species) on herbivorous insects. Among them, eight papers reported negative (*e.g.*, Simon and Hilker 2003, Rayamajhi et al. 2006, Mouttet et al. 2011, Al-Naemi and Hatcher 2013), five positive (Siemens and Mitchell-Olds 1996, Cardoza et al. 2002, 2003a, 2003b, Johnson et al. 2003), two neutral (Ajlan and Potter 1991, Rostás et al. 2006) and two reported both positive and negative effects on preference or performance related variables (Rizvi et al. 2015, Abreha et al. 2015). Based on this pattern, we can suppose that the lack of significant effect of necrotrophic fungi on insect is due to a great variability between model systems and/or an important influence of experimental conditions such as the timing of interactions and disease severity (see next section). The outcomes of our experimental study on tripartite interactions involving the chestnut blight confirm the non-significant general effect of insect response to plant infection by a necrotrophic fungus after one whole year of interactions (Chapter II).

We still lack quantitative studies and a theoretical framework for a better understanding of reciprocal plant-mediated effects of insect attacks on plant infection and colonization processes by fungi. For instance, Stout et al. (2006) reported 12 cases of negative, 7 positive and 17 neutral effects of herbivore arthropods on plant-associated fungal pathogens. But this is vote counting and cannot be used to infer on overall effects. In our model system, we found neutral plant-mediated effects of previous plant infection by ACGW on *C. parasitica* canker development or healing (Chapter II) but again, this is only one additional case that would need to be included in a future meta-analysis, when more data will be available, in order to get better insight onto quantitative effects of insects on fungi sharing the same plant.

In my opinion, the different outcomes found in literature on tri-partite interactions may be explained, at least in part, by the variability in the temporal and spatial scales studied. In addition, most of published experiments (including ours) have focused on interactions between one fungal pathogen, one insect on a given plant. Yet, all plants are virtually infected by many fungi and insects, not talking about all microbes, which adds a degree of complexity in multitrophic interactions studies.

6.1.1. Temporal variability of interactions

The outcome of tripartite interactions is strongly dependent on the time spent since the first infection, together with disease severity. For example, the beetle *Plagioderia versicolora* avoided healthy halves of willow leaves infected by the rust *Melapsora alli-fragilis* only 16 days after fungal infection, but before that, it showed no preference for fungus infected or control leaves (Simon and Hilker 2003).

Several studies also suggest that the effect of initial fungus infection on subsequent herbivores feeding on the same plant may depend on the severity of fungus infection. For example, Cardoza et al. (2003a) found an increase in soluble sugar content in peanut plants following plant infection by the pathogen *Sclerotium rolsfii*, which positively affects beet armyworm (*Spodoptera exigua*). However, as acknowledged by the authors, levels of fungal inoculum were low in their experiment and only caused sub-lethal infections in plants. If conditions are favorable, this fungus can kill the host plant and, thus, will probably have negatively impact plant-associated insects. Similarly, the negative effects of *Vicia faba* plants infection by the necrotrophic fungus *Botrytis cinerea* on *Aphis fabae* performance were linearly related to fungal lesion density (Al-Naemi and Hatcher 2013). Likewise, Rizvi et al. (2015) found that negative effects of *Vitis vinifera* foliar infection by *Botrytis cinerea* on *Ephiphyas postvittana* moth oviposition depended on intensity of infection, whereby moth laid fewer eggs on moderately and intensely infected leaves than on low, mildly or uninfected control leaves.

The conditions of disease severity and progression in nature need to be further evaluated in order to perform experimental set-ups that can mimic realistic processes. However, this is not always an easy task. For example, in our experimental study, *C. parasitica* was infected by the CHV-1 virus which decreases fungal virulence. We may assume that by using CHV-1 virus we have underestimated the effects that *C. parasitica* could have on ACGW in natural conditions. However had we used virus free *C. parasitica* strains, saplings would have probably died (or at least main stem) before ACGW could emerge from galls. But what are the most frequent interactions in natural systems? The reality is more complex than our experimental set up. In chestnut orchards and forests, *C. parasitica* is more frequently observed on adult trees, on trunk and branches. Multiple infections can occur within a single tree and even mixtures of virus free and virus infected strains can be found (C. Robin, *perso comm*).

In my opinion, one reason of the greater variability found in insect responses to necrotrophic pathogen infections is that the influence of driving factors (*i.e.*, timing of infection, disease severity and progression) in fungus-plant-insect interactions may be stronger in this group. Necrotrophic fungi first destroy cells in which they fed by producing phytotoxins and cell degrading enzymes, which may release plant nutrients (Pieterse et al. 2012) that can benefit to herbivores in the first stage of infection (Johnson et al. 2003). But effects of necrotrophic fungi may turn to be detrimental as disease severity increases and plant eventually dies.

Similarly, plant-mediated effects of insect on fungal infection may probably depend on the severity of insect attacks. However, how insect damage severity and attack accumulation affect plant sensibility to subsequent fungal infection have not been texted yet.

6.1.2. Spatial scale of interactions

The strength of effect of fungal infection on insects may also vary with the spatial location of fungus and insect interactions within their host plant (Chapter I). We found negative effects of fungal infection when insect and fungus co-occurred within the same plant organ (local interactions) but not when they interacted indirectly by attacking different plant parts (distant or systemic interactions). However, there was a large variability in the spatiality of fungus-insect interactions in our review that ranged from infected and uninfected parts of a same leaf (Simon and Hilker 2005) to different plant compartments (McNee et al. 2003), even if, in most of the cases, they referred to different plant leaves (Rostas and Hilker 2002, 2003, Mouttet et al. 2013) or different aboveground plant organs such as leaves and stem (Cardoza et al. 2003a, 2003b). The impact of a fungus on its host plant can be highly localized to the infected tissue or organ, which is more or less distant from the infection. For example, Simon and Hilker (2005) showed that negative effects of plant infection by the rust fungus *Melampsora allii-fragilis* on the beetle *Plagioderia versicolora* extended one leaf position up and two down from the infection site but not to more distant leaves.

In our model system *C. parasitica* did not affect chestnut buds, galls or leaves' chemistry for the measured metabolites (nitrogen content, hormones or phenolic). The induced changes in chestnut quality by *C. parasitica* infesting stem are thus probably local, which could explain why it did not affected ACGW that develop on distant buds. Similarly, the lack of significant effect of ACGW on *C. parasitica* canker development or healing may be explained by the fact that ACGW developed in chestnut buds and galls while *C. parasitica* infection are located in chestnut stems. Although we did not quantify the systemic changes in plant chemistry, we may assume that ACGW infection did not prime tree defenses to *C. parasitica*, as canker development and healing were independent of ACGW infection. Induced plant resistance to ACGW was probably activated only locally.

Contrary to this example of limited spatial influence of initial fungus infection on subsequent attack by herbivores, several examples of belowground-aboveground interactions between pathogens and herbivores suggest that fungus infection may have large systemic effects on herbivores, whatever the organ they feed on and their distance to infection (see Appendix I).

6.1.3. Mechanisms of tri-partite interactions

6.1.3.1. Plant defenses and hormone-induced defensive pathways

Why should fungi and herbivore interact both locally and distantly when attacking the same host plant? Several studies discussed the implication of systemic induced defenses mediated by hormonal pathways on the outcome of tripartite interactions (Rostas and Hilker 2003, Simon and Hilker 2005, Al-Naemi and Hatcher 2013, Mouttet et al. 2013). In fact, most of these studies indirectly tested the presence of systemic induced defenses by testing whether there were or not significant effects of fungal infection on insect located in distant parts of the shared host plant (*e.g.*, Mouttet et al. 2013). Only one paper used in our meta-analysis did measure hormone levels, showing no impact of the fungi on SA levels (Cardoza et al. 2003a).

Several papers predicted or discussed the outcome of tripartite interactions based on a theoretical framework for defenses induction by different hormonal pathways, in function of fungi lifestyles and insects feeding guilds (Stout et al. 2006, Al-Naemi and Hatcher 2013, Mouttet et al. 2013). They suggested that the effect of necrotrophic fungi should be more important on chewing insects, while the effect of a biotrophic fungi would be more important for piercing-sucking insects (Stout et al. 2006, Thaler et al. 2012). Our meta-analysis made it possible to test this theoretical framework of hormonal cross-talk, using a large dataset. However, we did not find any significant interactive effect of the fungi lifestyle and the feeding guild of the insect on insect performance or preference (Chapter I). In addition, when we experimentally measured hormones levels in buds of chestnut plants infected or not by *C. parasitica*, we could not find any significant difference (Chapter II).

As discussed in Chapter I, the pleiotropic effects of hormone-dependent induced defenses may not be universal. Alternatively, differences in insect responses to plant infection by a fungi may depend on other mechanisms, together or not with the changes in plant defenses. Another possibility is that the common view of necrotrophic pathogens and chewing insects inducing JA pathway and biotrophic pathogens and piercing-sucking inducing SA pathway is not always true. For example, aphids have been shown to induce both JA and SA pathways (see also Lazebnik et al. 2014 and references therein). In addition, plant defenses to insects and pathogens may be induced by other plant hormones mediated pathways, such as ethylene or abscisic acid (Thaler and Bostock 2004, Erb et al. 2011a, Pineda et al. 2013, Lazebnik et al. 2014), although the latter have received relatively less attention in the tri-partite interactions literature.

In my opinion, it remains difficult to predict the outcome of tripartite interactions based only on two hormones-induced pathways and their crosstalk. Plant defenses against insect and pathogens are very complex, and they likely vary with the timing of induction and experimental conditions (Pineda et al. 2013). Moreover, many studies focusing on the hormonal pathway did not manipulate both pathogens and herbivores but, instead, applied synthetic hormones on plants. This is an important issue because the timing and intensity of

artificial defense inductions may be very different from what actually occurs in nature. Real infection or real herbivory may simultaneously trigger different pathways with different intensities, which is not well reflected by this type of experimental approach. Such studies are very valuable to understand the mechanisms underlying tripartite interactions, but our current understanding is still in its infancy and probably lacks important ecological insights. There are also several defense-independent mechanisms that may link pathogens with herbivores and these mechanisms are worth exploring.

6.1.3.2. Defense-independent mechanisms

In several cases, positive or negative effects of one aggressor on a second one have been shown to be mediated by an increase in plant nutritive quality. For example, Johnson et al. (2003) reported an increase in amino acid content of birch leaves infected by the necrotrophic fungus *Marssonina betulae* which is the most likely reason for a positive effect of fungal infection on *Eucera aphidis* aphids.

As discussed above, literature reviews on tri-partite interactions have mainly focused on change in plant defenses induced by a first attacker (Hatcher et al. 2004, Pieterse and Dicke 2007, Lazebnik et al. 2014, Biere and Goverse 2016). However, the importance of fungus-induced change of plant resource allocation, and thus its capacity to overcompensate a first attack, has been neglected so far (see also Appendix I in supplementary material).

In my opinion, these changes in plant resources and nutritive quality are as important as, or even more important than, changes in defenses induction. These changes may even mediate cross-compartment interactions. For example, McNee et al. (2003) reported a negative effect of pine infection by the root pathogenic fungi *Heterobasidium annosum* on *Ips paraconfusus* insect, which was correlated to the transport of toxic compounds produced by the fungus through plant xylem.

6.1.4. How could we predict the outcome of plant- fungi-insect tripartite interactions? Knowledge gaps and research perspectives

We identified general patterns and several sources of variability in insect response to plant infection by fungi (chapter I). Based on some factors like fungi lifestyle, insect feeding guild and spatial location of interactions we could make predictions about the effect of a fungi on a plant associated herbivore. However, in order to be able to predict the outcome of tri-partite interactions we also need to expand our knowledge on the reciprocal effects of insects on plant-associated fungi, as well as on the additive and interactive effects of herbivores and pathogens on plants (Hauser et al. 2013).

Accurate reviews and quantitative syntheses of studies accounting for the effects of herbivore insects on plant-associated fungi are needed to identify major patterns for these interactions.

For instance, the last review on this topic was performed in 2006, and already reported 36 studies differing in the outcome of fungi response to insect (Stout et al. 2006). Last years, tripartite interactions have been addressed by several papers (Stout et al. 2006, Tack and Dicke 2013, Shikano et al. 2017, Franco et al. 2017) which could be used to conduct an up-to-date review of insect effects on fungi ability to colonize and damage plants.

Hauser et al. (2013), performed a bibliographic synthesis of studies reporting data about combined effects of insect and pathogens on plant performance. They identified several sources of variability in the outcome of fungi and insect interactions on plants such as measured plant traits and experimental conditions. However, this meta-analysis was based on 35 published papers only and outcomes of more recent experimental research on this topic (e.g., Drakulic et al. 2015, Willsey et al. 2017; Chapter II) need to be included to better understand the variability in plant response to joint pest and pathogen attacks.

In addition, we still lack more experimental studies, especially under natural or semi-natural conditions. For example, they could test the effect of the sequence of arrival of plant attackers. Changes in plant quality and defenses not only depend on the combination of attackers but also on their sequence of attacks (Blossey and Hunt-Joshi 2003). This have received some attention in the case of insect-insect interactions (e.g., Erb et al. 2011b), but not in the case of insect-fungi interactions.

We are also missing studies focusing on belowground plant compartments. In fact, most of experimental studies have been performed on aboveground plant organs (see Chapter I), probably because of the experimental complexity of working on roots (but see Willsey et al. 2017 and references in SM, Appendix I).

6.2. Multipartite interactions: plant-fungus-herbivore interactions from a community wide perspective

6.2.1. From tripartite to multipartite interactions

6.2.1.1. Effects of single infections of fungi or insects on co-occurring multiple insects or fungi, respectively

The fact that fungi may modify insect preference and performance may result in changes in interactions within the community of other plant-associated insects. For example, Tack et al. (2012) found that plant infection by powdery mildew (*Erisiphe alphitoides*) resulted in changes in insect communities of oak trees (*Quercus robur*) at a local scale.

Similarly, since insect attack may have important consequences in fungi performance, it can in turn impact fungus-fungus interactions (reviewed in Kemen 2014), making plants more or less susceptible to multiple fungal infections.

6.2.1.2. *Effects of single infections of fungi or insects on their natural enemies*

The presence of fungi can modify insect interactions with other organisms such as insect predators or parasitoids (Biere et al. 2002, Miranda et al. 2011, Tack et al. 2012). For example, the specialist leaf herbivore *Pieris brassicae* suffered lower parasitism rates by the wasp *Cortesia gromerata* in mildew infected *Brassica rapa* leaves, which suppress herbivore-induced plant volatiles (Desurmont et al. 2016).

Similarly, insects may influence fungi interactions with other organisms such as their hyper parasites, however, to my knowledge, this have not been experimentally tested yet. For instance, we showed that the effect of CHV-1 virus on *C. parasitica* was the same on plants attacked by ACGW and plants without ACGW (Chapter II), however there is a higher prevalence of *C. parasitica* without CHV-1 virus in the colonized galls (Meyer et al. 2015), probably because they are mainly colonized by asexual ascospores, which do not carry the virus.

6.2.1.3. *Effects of multiple infections of fungi or insects on co-occurring single insect or fungus, respectively*

Plants are usually attacked by more than one fungus species at once. Each fungus may have different impacts which may result in additive, antagonistic or synergistic effects on insects. For example, plant infection by the rust fungus *Uromyces vicia-fabae* resulted in enhanced performance of *Aphid fabae* on *Vicia faba* plants, whereas infection by *Botritis cinerea* negatively impacted aphid performance. Plant infection by both pathogens resulted in similar aphid performance on infected than on control plants, but with a reduced aphid fecundity, indicating lower plant carrying capacity when infected by both pathogens at the same time.

Symmetrically, plants can be infected by different, interacting insects. Fungus performance may then depend on the outcome of these insect-insect interactions, *i.e.* their individual and combined effects. For example, the leaf-miner *Tuta absoluta* positively affects powdery mildew in oak plants while the whitefly *Bemisia tabaci* has negative impacts on lesion size (Mouttet et al. 2013). Both insects often co-occur within the same plants, and they have been shown to impact each other's performance (Mouttet et al. 2013), thus powdery mildew infection may not only depends on the impact of each insect but also their interactions and combined effects.

6.2.1.4. *Plant-mediated effects of insect attacks on plant microbiota*

Insect infection may also affect the whole fungal endophytic community in plant tissues (Lawson et al. 2014; Chapter IV). In chapter IV, we showed that endophytic communities of galls differed in composition, richness and diversity from those of surrounding leaf tissues. Local changes in chestnut tissues (*i.e.* nitrogen content and polyphenolics composition) may have produced these differences (Chapter II). However, how changes in plant chemistry impact endophytic fungi still remains little explored. For instance, Unterseher et al.(2016)

found that diversity and composition of foliar mycobiome in beech (*Fagus sylvatica*) depended on its biochemistry (chlorophylls and flavonoids content). In chapter II, we found a reduction in nitrogen content and a change in polyphenolics composition in galls tissues compared to surrounding leaves. A diminution in fungal richness and diversity in galls (Chapter IV) could be related to this decrease in N content, making the galls of poor resource quality for fungi. It could also be linked to a change in phenolic composition, which may act as a filter for some endophytic species. In fact, the “enemy hypothesis” (Stone et al. 2002) proposes that higher levels of tannins and phenols in galls can protect the gall-maker from mortality due to pathogenic fungi or parasitoids attack (Taper and Case 1987, Price and Pschorn-Walcher 1988). Although we have not quantified yet these polyphenols levels, ACGW could have modified plant polyphenolics (at least their composition) in order to protect themselves against endophytic fungi.

6.2.1.5. *Effects of non-fungal pathogens on insects*

Inducing plant systemic defenses by non-fungal pathogens like bacteria, nematodes and virus has been shown to impact herbivore insects, even when they are attacking different plant compartments (Lee et al. 2012, De Roissart et al. 2013, Kammerhofer et al. 2015, see also Appendix I in Supplementary Material). But there still several unresolved questions. Which are the main patterns of bacteria-plant-herbivore interactions? And nematode-plant-herbivore? Are results for plant-pathogenic fungi-insect interactions transposable to other pathogens?

6.2.1.6. *Effects of beneficial microbes on insect and pathogens*

Plant-mutualistic organism such as mycorrhizal fungi and plant-growth-promoting rhizobacteria can help improve plant tolerance to herbivorous insects and pathogens, however these effects seem to be highly context-dependent (reviewed by Pineda et al. 2013). Plant microbes may range along a continuum from mutualism to parasitism (Hoeksema et al. 2010). Similarly, their plant-mediated effects on insect and pathogens can range from negative, as they can suppress pathogens locally and induce systemic resistance against several diseases and herbivorous insects, to positive, via the improved quality and quantity of their host plant (see Pineda et al. 2013 and references therein). Because microbes can affect both insect and pathogens, they may also influence their interactions and their combined effects on the host plant. Most of studies performed on this topic focused only on one microbe-plant-insect or microbe-plant-pathogen interactions, while little is known about microbe-plants-pathogens-fungi quadri-partite interactions.

6.2.1.7. *Impacts on plant community diversity and structure*

Outcome of interactions between fungi and insects may have profound consequences on plant community diversity and structure. Different plant genotypes or species may respond differently to several parasites. That may affect plant relative competitive or facilitative abilities, thus resulting in changes of relative abundances, proportion, dynamics and survival. For example, Hatcher et al. (1994) found that combined negative effects of plant infection by the rust fungus *Uromyces rumicis* and the beetle *Gastrophysa viridula* was more important for *Rumex crispus* than *Rumex obtusifoliosus* plants.

6.2.2. *The mediating effect of plant community diversity and structure*

Plant-plant interactions may also influence plant relationship with the community of detrimental and beneficial organisms living on them. These plant-plant interactions may vary with their specific or genetic diversity and can provide ecosystems with increased resistance to abiotic and biotic disturbances such as windstorms, fires, pathogens and pests (reviewed in Jactel et al. 2017).

Plant diversity have been shown to affect plant-insect and plant-fungus interactions (Mitchell et al. 2002, Jactel and Brockerhoff 2007, Barbosa et al. 2009, Castagneyrol et al. 2014, Hantsch et al. 2014, Jactel et al. 2017). As such, we can expect that plant diversity also has an effect on plant-insect-fungi tripartite interactions, directly or indirectly by modifying the biotic and abiotic environment in which they co-occur. To my knowledge, only one paper addressed the question of tripartite interactions in a plant biodiversity experiment (Schuldt et al. 2017). They reported a positive relationship between several foliar herbivores damage and foliar pathogens. Tree species richness leaded to *AS* to insects but *AR* to pathogens with an overall reduced combined impact of both damaging agents on tree growth (Schuldt et al. 2017). In this paper, authors suggested that additive effects results from the capacity of plants to (over-)compensate for damage from several attackers, but they did not test how herbivores and pathogens impacted each other.

Plant genetic diversity may also influence tripartite interactions. Several ecosystems functions and services of plant ecosystems are driven by both species and genetic diversity effects (Cardinale et al. 2011, Cook-Patton et al. 2011, Brockerhoff et al. 2017; see also appendix II in SM), and some plant-fungus-insect interactions have been shown to vary with plant genotype (Saikkonen et al. 2001, Meister et al. 2006). Understanding how plant genetic and specific diversity modifies insect and fungus interactions may improve our understanding of ecosystem functioning in ‘real field conditions’ where plants are usually attacked by several, distinct organisms.

6.2.3. The particular case of non-native pests and pathogens

A common view in invasion ecology is that communities with higher species richness are more resistant to the establishment and spread of invasive species (the diversity-invasibility relationship, Elton 1958). However, literature have mainly focused on invasive plants while biotic resistance to invasive fungus and insect received less attention (reviewed in Nunez-Mir et al. 2017, Jactel et al. 2017).

6.2.3.1. Mechanisms of plant diversity effects on ACGW

One of the proposed reasons for invasive species achieving greater fitness in their novel range is the absence of co-evolved natural enemies (the ‘enemy release hypothesis, Shea and Chesson 2002, Keane and Crawley 2002).

In the case of ACGW, a spill-over of local, native insect parasitoids have been already reported (Aebi et al. 2006, 2007) and there are also several native endophytes that could cause ACGW mortality. In this case, we can expect that associational resistance to exotic pests is mediated by generalist natural enemies (‘enemies hypothesis’, Elton 1958, Root 1973), in a similar way as for native pests. However, based on our results we cannot completely confirm that the observed tree diversity effects on ACGW infestations (Guyot et al. 2016; chapter III) was due to reinforced top-down control by natural enemies. The abundance and diversity of native parasitoids were statistically independent of plot composition (see discussion Chapter III), and we did not find any correlation between their abundance (or abundance of the introduced parasitoid, *Torymus sinensis*) and ACGW infestation rates. Although the composition of communities of ACGW native parasitoids did vary with plot composition, we did not find any parasitoid species significantly associated to one type of plot composition. Similarly, the community composition, richness and diversity of endophytic fungus found in ACGW galls did not depend on plot composition neither (Chapter IV). However, we cannot exclude that tree diversity or chestnut forest composition might have enhanced the control of ACGW by one the many parasitoid or endophyte species, species that remain to be identified.

6.2.3.2. Plant diversity effects on chestnut blight

In natural chestnut forest stands (Chapter III), we also recorded the presence of blight symptoms in chestnut stem and main branches. Based on our *C. parasitica* notations, we did not find any significant effect of plot composition on the abundance of blight symptoms. However, we were not able to properly test the effect of tree diversity on this pathogen, and draw any conclusion on AS or AR, because *C. parasitica* was probably present in all trees, even those recorded as asymptomatic. Indeed, *C. parasitica* can affect all branches of chestnut trees but we only noted the larger ones, and even infect chestnut tissues as endophyte (Bissegger and Sieber 1994). In addition, *C. parasitica* and CHV-1 are well established in the

sampled region, which makes it difficult to assess precisely *C. parasitica* (virus free or virus infected) incidence on mature and high trees (Turchetti et al. 2008).

C. parasitica is widely established across the distribution area of *Castanea spp.* worldwide. Plant diversity effects on this pathogen may thus be similar to native fungal pathogens. However, it is difficult to predict how plant diversity could drive AR or AS to this pathogen. As discussed in the *Introduction*, non-host neighbors may reduce host concentration and act as barriers to spore dispersion reducing fungal spread to new host plants. Moreover, top-down control by the CHV-1 virus may depend on its dispersal and the diversity of *C. parasitica* vegetative compatibility groups. Virus dispersal is associated to fungal sexual conidia dispersion and contact between *C. parasitica* hyphae from a same vegetative compatibility group carrying or not CHV-1. Thus, the same mechanisms that may be involved in reduction of *C. parasitica* spread may also reduce CHV-1 virus spread in mixed forests, increasing the severity of disease symptoms.

6.2.3.3. Interactions between exotic species in the invaded area

Why interaction between exotic species should be different from interactions between native species?

The ‘invasional meltdown’ theory proposes that two invasive species in a new area enhance the impact and/or probability of establishment and spread of the other, having together more negative impacts on the native ecosystem (Simberloff and Holle 1999, Simberloff 2006). Thus, the presence of *C. parasitica* in chestnut forest could have facilitated the establishment of ACGW on their invaded area, and reciprocally, ACGW could have benefited *C. parasitica* spread in its already invaded area, and even compromise *C. parasitica* biocontrol by CHV-1 virus.

In natural conditions, we observed that ACGW infestation rates were higher in small trees when they presented *C. parasitica* symptoms but not on asymptomatic trees (Chapter III). We discussed the possibility that the interaction between both exotic aggressors resulted in an ‘invasional meltdown’ (Simberloff and Holle 1999, Simberloff 2006), at the expense of small trees, compromising chestnut survival at young stage. Unfortunately, our data on ACGW and blight infections in natural forest did not allow us to confirm or refute this ‘invasional meltdown’ theory, as we were not able to measure the impact of separated *vs.* combined aggressors on chestnut trees because all trees were infected by ACGW (and probably by *C. parasitica* too).

However, in experimental conditions (Chapter II), we found that combined or separate effects of ACGW and *C. parasitica* on chestnut sapling growth were negligible. The period of infection by *C. parasitica* was relatively short (from June to October), and chestnut growth was too variable among treatments. In this experiment, we did not find any effect of *C. parasitica* on infection by ACGW or vice versa. Moreover, we showed that canker development depended on the presence of CHV-1 being more important when the virus was absent. This was expected but, interestingly, our result showed that the infestation by ACGW

is unlikely to compromise the natural regulation of chestnut blight by CHV1 and its use for biological control. However, it is possible that ACGW improve *C. parasitica* long distance wind dissemination as abandoned galls provide new infection sites in tree canopy (Meyer et al. 2015).

6.3. Some avenues for future research

For a better understanding of the functioning of ecosystems and of the complexity of species interactions at play, we need more studies taking into account the plant microbiote, the diversity of plant attackers and the biotic and abiotic conditions of these interactions. Studies involving multi-trophic interactions are also important to better understand mechanisms used by plants to defend themselves against multiple aggressors, develop new strategies of pest and disease control and create new genetically improved plant varieties. With the development of new generation sequencing methods it is possible to extend investigations from few isolated species to complex plant microbiomes (Hardoim et al. 2015), and to improve our understanding of interactions between plant microbiome (Vacher et al. 2016).

In my opinion, an interesting research topic to further test several of the questions proposed in this thesis would be to explore the effects of plant diversity on biotic interactions between ambrosia beetles, their fungal symbionts, their host trees and the associated microbiote. Ambrosia beetles and their associated fungi have been widely studied since years as they can cause several economically important diseases of trees (reviewed in Ploetz et al. 2013). Ambrosia beetles are a very diverse group which include ca. 3 400 different species. They are known to be fungal farmers with some species being only saprophytes of their associated fungi (*e.g.*, Batra 1966). Ambrosia species have evolved particular organs, called mycangia, dedicated to the transport of fungal spores. Most of them do not cause important damage to forest as they preferentially feed on dead or stressed plants, however, several species may feed on xylem of living trees and transfer the symbiotic microbiota to infected trees.

Several recent tree disease emergences have been associated to fungi transported by ambrosia beetles with an increasing number of exotic species arriving in new areas and infecting naïve trees (*e.g.* Fusarium dieback of avocado trees in California, Eskalen et al. 2013). However, to the best of my knowledge, there is not any study about tree biotic resistance (the effect of tree species diversity on associational resistance) to invasive ambrosia beetle species and their associated microbiota. In addition, ambrosia beetles, their associated fungi and their host plants provide good examples of tri-partite interactions with both direct (*e.g.* by insect facilitating fungal penetration in host tissues or by fungi supplying additional nutrients to the insect) and plant mediated reciprocal effects (*e.g.*, by changes in plant defenses caused by some pathogenic fungi or by insect attack). Moreover, thanks to new generation sequencing methods, we could study the whole community of ambrosia beetles symbiotic fungi, how they

affect plant microbiota (bacterial and fungal), and whether there is any effect of tree diversity on ambrosia beetle attacks, emergences of fungal diseases or propagation.

For that, observational studies in experimental or natural mixed forests would be needed. Insect abundance and disease symptoms should be noted, and plants sampled, for analyses of insect and plant microbiota in the laboratory. It would also be useful to note how plant neighbors modifies plant defenses and other traits, and how this affect disease development and insect-fungi interactions. Experiments in greenhouses should also be performed in order to study fine interactions and better understand in which conditions ambrosia beetles endophytic symbionts cause disease symptoms and which plant endophytes may antagonize them.

6.4. References

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GENERAL SUMMARY IN FRENCH

RESUME GENERAL EN FRANCAIS

Les plantes sont au centre d'une grande diversité d'interactions biotiques entre organismes plus ou moins proches qui les exploitent en tant que ressources. Mieux comprendre ces interactions est essentiel pour améliorer la gestion des écosystèmes terrestres et leurs services associés. Alors que les interactions plantes-insectes sont étudiées depuis plusieurs décennies dans plusieurs domaines de la biologie, notre compréhension des mécanismes qui contrôlent les dégâts causés aux plantes par les insectes herbivores dans les environnements naturels reste limitée. De plus, les insectes coexistent avec des différents organismes associés aux plantes, tel que les champignons phyto-pathogènes et endophytes, pouvant établir des différentes interactions plantes-microbes-herbivores, affectant aussi les dégâts causés aux plantes. L'objectif de cette thèse a été de comprendre comment les infections fongiques de la plante et la diversité des arbres en forêt modifient les interactions arbres-insectes.

Le châtaignier (*Castanea sativa*) et ses deux principaux agresseurs exotiques, le cynips (*Dryocosmus kuriphylus*) et le chancre du châtaignier (*Cryphonectria parasitica*) ont servi de modèle à l'étude des interactions plantes-pathogènes-herbivores. Le cynips du châtaignier est un micro-hyménoptère originaire de Chine produisant des galles au niveau des feuilles, des bourgeons et des branches de châtaignier et occasionnant des baisses de vigueur des arbres, des pertes dans la production de bois et châtaignes, allant jusqu'à la mortalité des arbres quand les infections sont sévères et répétées. Le chancre du châtaignier est une maladie occasionnée par le champignon phytopathogène *C. parasitica*, originaire d'Asie. Ce champignon produit des nécroses dans l'écorce et cambium du châtaignier en réduisant les flux de sève chez les variétés d'arbres sensibles. Ces bio-agresseurs sont tous deux actuellement présents dans le sud-est de l'Europe et causent d'importants dégâts dans les châtaigneraies. On soupçonne l'existence d'interactions directes entre ces deux agresseurs. En effet, *C. parasitica* semble bénéficier de la présence des trous d'émergence des cynips des galles pour pénétrer dans les tissus des châtaigniers. La présence d'interactions indirectes entre *D. kuriphylus* et *C. parasitica*, du fait de leurs effets mutuels sur leur hôte commun, demande à être testée. Par ailleurs, il a été récemment observé que la défoliation due au cynips diminue avec une augmentation de la diversité d'essences forestières dans les parcelles de châtaigniers. Cependant, les mécanismes impliqués dans cette diminution, tels qu'un « spill-over » d'ennemis naturels du cynips provenant d'autres espèces natives de *Cynipidae*, n'ont pas été élucidés.

Dans le **chapitre I**, j'ai posé le cadre théorique sur les effets indirects des infections fongiques sur les insectes herbivores associés aux mêmes plantes hôtes. En effet, l'infection d'une plante par un champignon peut modifier la teneur de la plante en certains nutriments, comme l'azote ou le phosphore, ou des composés défensifs, tels que les phénols ou les tanins, en impactant les performances des insectes (*i.e.* poids, survie, temps de développement...). Cet effet peut être positif, si par exemple l'infection fongique augmente la qualité nutritive de la plante pour l'insecte ou diminue la production de composés de défenses ; ou négatif, si elle diminue la valeur nutritive de la plante ou augmente ses défenses. De la même manière, une

infection fongique peut modifier la production par la plantes de signaux visuels ou chimiques utilisés par les insectes pour reconnaître leur plante hôte et ainsi modifier leurs préférences pour un hôte donné (*i.e.* consommation différentielle, oviposition...).

Dans la littérature, plusieurs études rapportent des effets positifs, négatifs ou neutres de l'infection d'une plante par un champignon sur les préférences ou performances des insectes. Pour obtenir les tendances générales et mieux comprendre les sources de variations entre ces différents résultats, nous avons tout d'abord effectué une méta-analyse sur les articles publiés dans la littérature à ce sujet. Nous avons trouvé 101 articles rapportant 1113 cas d'études dans lesquels les auteurs comparent une variable de préférence ou de performance d'un insecte sur une plante avec ou sans infection par un champignon. Nous avons restreint cette analyse aux champignons pathogènes biotrophes, qui se nourrissent des tissus vivants de la plante, aux pathogènes nécrotrophes, qui produisent toxines ou enzymes dégradant les parois cellulaires pour se nourrir ensuite des tissus morts de la plante, et aux champignons endophytes, qui pendant au moins une partie de leur cycle de vie se trouvent à l'intérieur des cellules végétales sans entraîner de symptômes de maladie.

En moyenne, l'effet de l'infection préalable des plantes par les champignons sur les préférences et performances des insectes s'avère négatif : les insectes préfèrent des plantes qui ne sont pas infectées par un champignon pathogène et y ont de meilleures performances. Cependant, la magnitude de l'effet délétère du pathogène sur l'herbivore varie selon le mode de vie du champignon, la guildes trophique de l'insecte et la spatialité des interactions (interactions locales *vs* distantes).

Les performances des insectes sont moins bonnes dans des plantes infectées par des champignons pathogènes biotrophes et des endophytes mais pas par des pathogènes nécrotrophes. De plus, les préférences et les performances des insectes herbivores sont impactées différemment en fonction du mode de vie du champignon. Alors que dans des plantes infectées par des pathogènes biotrophes, les préférences et les performances des insectes herbivores sont impactées négativement, l'infection par des pathogènes nécrotrophes diminue plus la préférence des herbivores que la performance. Finalement, l'infection des plantes par des champignons endophytes réduit seulement la performance des herbivores mais elle est sans effet sur leur préférence.

La magnitude de ces interactions dépend aussi de la guildes de l'insecte et la localisation des interactions. La performance des insectes défoliateurs et piqueurs-suceurs est réduite dans des plantes infectées quand les interactions se produisent de manière locale (l'insecte et le champignon étant présents dans le même tissu de la plante) mais pas de manière distante (ils ne sont pas dans un même tissu).

Cette étude démontre que les champignons pathogènes des plantes jouent un rôle important de médiateur des interactions plantes herbivores. Un rôle jusqu'ici négligé.

Dans le **chapitre II** de ce manuscrit, nous avons analysé de façon expérimentale les interactions tripartites entre le châtaignier européen (*Castanea sativa*), le cynips (*Dryocosmus kuriphilus*), et *Cryphonectria parasitica*. Plus concrètement, nous avons testé (i) comment

l'infection des plants de châtaignier par *C. parasitica* impacte le taux d'infection des plants par *D. kuriphilus* à travers des changements dans la qualité de l'hôte ; (ii) comment l'infection préalable par *D. kuriphilus* affecte la performance de *C. parasitica* ; et (iii) les conséquences de leurs attaques séparées ou conjointes sur les plants de châtaigniers.

Pour cela, nous avons réalisé des tests en conditions semi-contrôlées avec des châtaigniers élevés en pots. Dans une première partie, nous avons infecté artificiellement des châtaigniers avec *C. parasitica* et soumis ces plants à des populations de *D. kuriphilus* pour faciliter leur infection par le cynips. Simultanément, nous avons inoculé *C. parasitica* sur des châtaigniers infectés ou non, l'année précédente par le cynips. Nous avons utilisé deux souches de *C. parasitica*, une seule étant infectée par l'hypovirus CHV-1 (*Cryphonectia hypovirus 1*). Ce virus diminue l'agressivité de *C. parasitica* et permet ainsi aux châtaigniers de résister à l'infection et guérir. L'utilisation de souches virosées comme agents de lutte biologique est actuellement la seule méthode efficace pour réguler la maladie en vergers.

L'infection des plantes par *C. parasitica* a été sans effet sur la qualité du châtaignier en tant que ressource pour *D. kuriphilus* (hormones, teneur en azote et phénols). Les galles de *D. kuriphilus* modifient la chimie de la feuille (phénols et teneur en azote) indépendamment du statut d'infection par *C. parasitica*, sans que cela n'affecte la progression de la maladie, et ce quel que ce soit l'état d'infection par le virus CHV-1. Finalement, l'impact de *D. kuriphilus* et *C. parasitica*, de manière séparée ou conjointe n'affecte pas la croissance des châtaigniers pendant la première année d'infection.

Par conséquent, nos résultats montrent des interactions neutres entre ce champignon pathogène, cet insecte, et leur plante hôte après un an d'interaction. Ces résultats sont en accord avec les résultats de la méta-analyse concernant les champignons nécrotrophes et les interactions distantes. Cependant, des expériences à long terme sont nécessaires pour mieux comprendre comment les interactions tripartites entre des agresseurs exotiques et leur nouvel hôte évoluent avec le temps et la sévérité des interactions.

Dans le **chapitre III**, j'ai étudié les variations dans les taux d'infection par le cynips en fonction de la composition spécifique en essences forestières des forêts de châtaignier atteintes de chancre. L'hypothèse de la résistance par association suggère que les forêts mélangées seraient plus résistantes aux ravageurs que les forêts mono-spécifiques du fait d'une diminution de l'accessibilité à l'hôte et d'une augmentation du contrôle des populations d'herbivores par les ennemis naturels. Cependant, peu d'études ont porté sur la résistance à des ravageurs exotiques tels que le cynips.

Nous avons testé cette hypothèse de résistance par association en comparant les taux d'infection du cynips du châtaignier sur des forêts naturelles en Toscane (Italie). Nous avons choisi des parcelles où le châtaignier se trouvait comme essence majoritaire (plus de 90% de surface terrière) ou en association avec le pin (*Pinus pinea*), le chêne (*Quercus cerris*) ou le

frêne (*Fraxinus ornus*). Le chêne et le frêne sont des essences connues pour être parasitées par des cynips eux-mêmes potentiellement attaqués par des parasitoïdes susceptibles de parasiter également les larves de *D. kuriphilus*. Nous avons évalué la densité et la fréquence des châtaigniers dans les parcelles mélangées, ainsi que l'effet des interactions biotiques entre le cynips, ses parasitoïdes et la maladie du chancre, causée par *C. parasitica*.

Les taux d'infection du cynips sont significativement plus faibles dans les mélanges châtaignier-chêne et châtaignier-frêne que dans les monocultures ou les mélanges châtaignier-pin. Les taux d'infection augmentent avec la proportion relative de châtaignier par rapport aux autres essences. La composition des communautés de parasitoïdes natifs associés aux galles du cynips diffère significativement entre les parcelles mono-spécifiques et les parcelles associant le châtaignier à une autre essence, mais pas leur richesse spécifique ou leur abondance.

L'abondance du parasitoïde introduit pour le bio-contrôle du cynips, *Torymus sinensis*, n'est pas corrélée avec les taux d'infection de *D. kuriphilus* et est indépendante de la composition des parcelles. Les infections de tronc et branches basses par *C. parasitica* modifient les taux d'infection du cynips en fonction de la taille des arbres, les arbres plus grands étant plus infectés quand ils sont asymptomatiques.

Ces résultats suggèrent qu'un contrôle biologique par conservation de la diversité basée sur des mélanges d'espèces peut contribuer à réduire les dégâts dus aux ravageurs forestiers exotiques.

Dans le **chapitre IV**, les effets de la diversité des essences forestières sur les communautés de champignons endophytes des galles du cynips ont été étudiés. Certains de ces champignons peuvent avoir un effet antagoniste sur le cynips et expliquer une partie des effets de la composition en essences sur les taux d'infections observés dans le chapitre précédent.

De façon générale, les champignons endophytes sont des agents de bio-contrôle potentiels pour les insectes ravageurs. Cependant, leur diversité et leur fonctionnement restent encore méconnus. Par exemple, les interactions avec leurs hôtes peuvent varier entre mutualisme, parasitisme et saprophytisme, en fonction des conditions biotiques et abiotiques de leurs interactions.

Nous avons exploré la diversité des champignons endophytes présents dans les galles induites par *D. kuriphilus* et les tissus foliaires adjacents, échantillonnés dans des forêts de châtaigniers purs ou en mélange avec du chêne, frêne ou pin. Nous avons testé l'hypothèse selon laquelle (i) les communautés des champignons endophytes des galles diffèrent en fonction de la composition des parcelles et que (ii) les tissus des galles contiennent des communautés d'endophytes plus riches et diverses que les feuilles adjacentes.

Nous avons sélectionné 28 parcelles forestières composées par 8 monocultures et 20 mélanges à deux espèces associant le châtaignier avec du pin, du frêne ou du chêne. Cette étude s'est basée sur les mêmes arbres que ceux étudiés dans le chapitre III. Sur chaque site, nous avons

prélevé des échantillons des tissus foliaires et de galles et caractérisé les champignons endophytes en utilisant comme gène barcode l'Espaceur Transcrit Interne 1 (ITS1), et une méthode de séquençage nouvelle génération (NGS, séquençage Illumina).

Nous avons identifié 1 378 OTUs (*Operational Taxonomic Units*). L'OTU retrouvée le plus souvent correspond à *Gnomoniopsis castanea*, qui a été précédemment décrit comme l'un des agents responsables de la nécrose des galles du cynips. La richesse, la diversité et la composition des communautés d'endophytes diffèrent entre les galles du cynips et les tissus foliaires adjacents, mais est indépendante de la composition en essences forestières des parcelles. La richesse et diversité d'endophytes présentes dans les galles sont réduites en comparaison avec les tissus foliaires adjacents. Les différences de composition entre communautés d'endophytes des galles et feuilles sont principalement dues à de OTUs spécifiques de chacun des tissus.

Ces résultats suggèrent que la physiologie des galles induites par des insectes agit comme des filtres écologiques pour des microorganismes endophytes indépendamment de la composition spécifique des essences forestières des parcelles. Une meilleure compréhension du fonctionnement de ces communautés d'endophytes est importante pour améliorer les agents de lutte par biocontrôle des insectes galligènes.

Pour conclure, nous avons ainsi apporté de nouvelles preuves que la diversité des plantes et les champignons pathogènes sont des facteurs clés déterminant les interactions plantes-insectes. Etudier comment les plantes interagissent avec leurs insectes et champignons associés, et les mécanismes sous-jacents à l'effet de la diversité des plantes sur ces interactions, doit permettre de mieux comprendre les relations entre diversité et fonctionnement des écosystèmes et de proposer des applications pour la gestion des bio-agresseurs forestiers natifs et exotiques.

7. SUPPLEMENTARY MATERIAL

BELOWGROUND-ABOVEGROUND INTERACTIONS BETWEEN PATHOGENS AND HERBIVORES

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ABSTRACT

Plants are attacked by pathogens and herbivores with a wide range of lifestyles, both belowground and aboveground. These pathogens and herbivores often co-occur on the same host plant, even though one of them may be in the roots and the other in the shoots. It has long been known that pathogens and herbivores can affect each other when sharing the same part of the plant, but more recently it has been shown that these interactions can span the belowground-aboveground divide. Root pathogens, for instance, can affect foliar herbivores, and, *vice versa*, foliar herbivores can affect root pathogens. Likewise, root herbivores can affect foliar pathogens and, *vice versa*, foliar pathogens can affect root herbivores. Such cross-compartment interactions are indirect (i.e., plant-mediated) and may involve induction and priming of common plant defenses, or altered plant quality. This chapter will review the literature and present a framework for this novel type of aboveground-belowground interactions between pathogens and herbivores.

7.1.1. Introduction

In this Chapter, the main focus will be on belowground-aboveground (BG-AG) interactions between plant-associated pathogens and herbivores. Most of herbivores studied in this context are arthropods. Hence, unless stated otherwise, the term *herbivore* will be used to refer to plant-feeding arthropods. It is now largely accepted that plant pathogens can interact strongly with herbivores when co-occurring on aboveground plant parts. Pathogens generally

reduce the preference and performance of herbivores (Fernandez-Conradi et al. in review), which can have cascading effects on the structure of insect communities found on terrestrial plants (Tack et al. 2012; Tack and Dicke 2013). The reciprocal effect of herbivores on pathogens has also been addressed, but there is no consensus yet on how pathogens respond to herbivore attack on the shared plant: studies have reported either positive, neutral or negative effects of herbivores on pathogens (Hatcher 1995).

Cross-compartment interactions between herbivores and pathogens differ from interactions involving BG beneficial microbes and AG herbivores as they involve two plant antagonists that compete for a shared, limited and defended resource. Likewise, cross-compartment pathogen-herbivore interactions differ from pathogen-herbivore interactions within the same compartment: while within-compartment pathogen-herbivore interactions may be both direct (e.g., herbivores acquiring supplementary nutrients from pathogens or being exposed to their toxins) and indirect (e.g., plant-mediated interactions), cross-compartment interactions among pathogens and herbivores are inevitably indirect. Indirect interactions may involve changes in primary and secondary metabolites within the shared host plant. It is also important to stress that a cross-compartment interaction between a pathogen and an herbivore is just one type of indirect interaction between two organisms using the same resource. In real life such species interactions are embedded within highly diverse plant-based food webs: for example, BG pathogens and herbivores could interact with a range of organisms aboveground, like herbivores, pathogens, endophytes, as well as their natural enemies; likewise, AG pathogens and herbivores could interact with the entire belowground community, and not only with organisms tightly associated with their host's roots.

Here, we explore whether pathogen-herbivore interactions may also play an important role when the organisms are separated by the soil surface. These interactions have received little attention as compared to interactions between BG beneficial micro-organisms and AG herbivores, a discrepancy that may be explained by the focus on BG beneficial organisms in studies for biocontrol development. However, as the outcome of plant attack by multiple attackers is not necessarily additive, recent studies in both community ecology and agroecology have increasingly focused on the outcome of tripartite interactions between plants, pathogens and herbivores, and its consequences for community dynamics and plant yield. In this chapter we will explore the scant available literature on belowground-aboveground interactions between herbivores and pathogens, and outline promising areas for future research. Throughout, given the scarcity of published studies on BG-AG interactions between herbivores and pathogens, we draw partly on findings, ideas and insights from three related research areas that are accompanied by a wealth of published articles: i) the study of interactions between BG and AG herbivores, ii) the study of interactions among pathogens and herbivores that both attack AG parts of the plant (Fernandez-Conradi et al. under review; Tack and Dicke 2013) and iii) interactions between BG mutualistic microbes and AG herbivores.

In this chapter, we aim to: (1) provide a comprehensive overview of the literature on BG-AG interactions among herbivores and pathogens, by tabulating the available studies and discussing the patterns, (2) explore the sources of variation in the strength, direction and symmetry of these interactions, including the role of the abiotic environment and the life-

history of both pathogens and herbivores, and therefore assess whether cross-compartment interactions among herbivores and pathogens are predictable, and (3) address the consequences of cross-compartment interactions on the ecology and evolution of plant-based communities.

7.1.2. Consequences of plant-pathogen-herbivore interactions for three players: a review of patterns

To review patterns of cross-compartment interactions involving BG pathogens and AG herbivores or BG herbivores and AG pathogens (Fig. 1), we first screened reference lists of the recent reviews discussing plant-microbe-herbivore interactions (Wondafrash et al. 2013; Tack and Dicke 2013; Hauser et al. 2013; Biere and Goverse 2016). We further searched additional references in the web of science database (January 23, 2017) using the following combination of keywords: ‘aboveground’ AND ‘belowground’ AND ‘plant’ AND ‘pathogen’ AND ‘herbivor*’ AND ‘insect’.

Plant parasitic nematodes are by far the most studied BG antagonists of plants in the context of BG-AG pathogen-herbivore interactions (reviewed by Wondafrash et al. 2013). However, classifying nematodes as herbivores or pathogens is debatable. Plant parasitic nematodes include ectoparasites, which live outside the plant and puncture cell walls feeding on cell material using their stylet, as well as migratory endoparasites, which penetrate the root and continuously move through the root cells while feeding through the puncturing of cell walls. These plant parasitic nematodes can cause cell death, similar to what is seen in some leafhoppers (e.g., Hunter and Backus 1989). In contrast, sedentary endoparasites penetrate roots and induce permanent giant feeding cells within the plants, the most typical being the root-knot and cyst nematodes. By inducing permanent feeding cells that are not killed, such nematodes are more similar to gall-forming pathogens and galling herbivores, such as the ovary smut fungus *Ustilago maydis* on maize or oak gall wasps. As interactions involving nematodes have been thoroughly reviewed, and their placement within the current framework is unclear, we did not include them in Table 1. However, we will refer to the key patterns involving nematodes within the text, and refer readers interested in a comprehensive overview of relevant nematode studies to Table 1 in Wondafrash *et al.* (2013). We did not include viruses, as they cannot be unambiguously defined as belowground or aboveground pathogens.

Pathogens were the most studied BG antagonists (Table 1) and belonged to different taxonomic groups, including bacteria (Yang et al. 2011; Song et al. 2015), necrotrophic fungi (Leath and Byers 1977; Godfrey and Yeargan 1987; McNee et al. 2003) and oomycetes (Landgraf et al. 2012; Milanović et al. 2015). Most of the studied AG herbivores were suckers (mainly aphids) or leaf-chewers (Lepidoptera, Coleoptera) (Table 1). (Table 1, see also Biere and Goverse 2016).

7.1.2.1. *The impact of single and dual attack on plant performance*

Both pathogens and herbivores are, on their own, harmful to plants. Harmful effects of dual attack by BG herbivores and AG pathogens (or, inversely, BG pathogens and AG herbivores) attacking distant compartments have been reported for plant growth (Alexander et al. 1981), biomass production (De Roissart et al. 2013; Saravesi et al. 2015), survival (Leath and Byers 1977), reproductive output (Barber et al. 2015) and crop yield (Godfrey and Yeargan 1987). Yet, this general tendency hides an important variability in plant-pathogen-herbivore interactions, with examples of antagonistic (i.e., the plant being less damaged than expected based on single attacks, Godfrey and Yeargan 1987; Yang et al. 2011), synergistic (i.e., the plant being more damaged than expected based on single attacks, Leath and Byers 1977) and additive effects of dual attack on plant performance (reviewed in Hauser et al. 2013).

Current knowledge on plant-pathogen-herbivore interactions in general suggests that most of dual attacks result in additive effects on plant performances, with surprisingly little evidence for synergistic effects of pathogens and herbivores on plant performance (Hauser et al. 2013). To predict the particular effect of dual attack by BG and AG attackers on plant performance, which currently seems like a distant future, we probably need an accurate knowledge of the reciprocal impact of BG and AG attackers on each other's performance. If one attacker has a positive effect on the second one, dual attack is likely to have a stronger negative impact on plant fitness than single attacks. If there is a negative effect of one attacker on the other, dual attack is likely to be less harmful than single attack. Finally, if the attackers do not affect each other's fitness, dual attack may simply have an additive impact on their host plant.

7.1.2.2. *Interactions between BG pathogens and AG herbivores*

7.1.2.2.1 *Effect of BG pathogens on AG herbivores*

The effects of BG pathogens on AG herbivores were shown to be negative (McNee et al. 2003; Hong et al. 2011; McCarville et al. 2012; Kammerhofer et al. 2015), positive (De Roissart et al. 2013; Milanović et al. 2015; Kammerhofer et al. 2015) or neutral (Godfrey and Yeargan 1989) (Table 1). Wondafrash et al. (2013) extensively reviewed cross-compartment interactions between BG nematodes and AG herbivores and showed that the outcome of their distant interactions was contingent upon the feeding strategies of both nematodes (migratory *vs.* sedentary) and herbivores (leaf chewers *vs.* phloem feeders), with migratory nematodes principally reducing performance of phloem feeders such as aphids (Bezemer et al. 2005; Wurst and van der Putten 2007), while the impact of root infection by sedentary nematodes on AG herbivores is more variable (Wondafrash et al. 2013). Most of available literature addresses the effect of BG nematodes on AG herbivores. The few studies focusing on fungus pathogens are highlighted in Table 1. While evidence is lacking, current knowledge on plant-pathogen-herbivore interactions within the same compartment (Lazebnik et al. 2014) suggests that the direction and strength of the effect of BG pathogens on AG herbivores may depend on the pathogen lifestyle (necrotrophic *vs.* biotrophic). Likewise, it may depend on herbivore

feeding guild, with different responses by chewers and sap-sucking insects. These differences and corresponding predictions will be detailed in section 3.1.1 (Figure 2G and 2H).

[7.1.2.2 Effect of AG herbivores on BG pathogens](#)

AG herbivores may both positively and negatively affect BG pathogens. Several studies show that AG herbivores facilitate root colonization by BG pathogens and are associated with greater pathogen severity (Leath and Byers 1977; Alexander et al. 1981; Burrill et al. 1999; Saravesi et al. 2015; Kammerhofer et al. 2015). For instance, in a study by Leath and Byers (1977), it was found that root rot caused by the necrotrophic fungus *Fusarium roseum* was more severe when the plant was simultaneously attacked by aboveground aphids. Similar positive effects of AG herbivores on BG pathogens have been found for the southern pine beetle *Dendroctonus frontalis*, which increased colonization levels of the BG necrotrophic fungus *Heterobasidion annosum* on *Pinus taeda* roots (Alexander et al. 1981). On the other hand, AG herbivores may trigger systemic defenses effective in roots that may act against BG pathogens (Yang et al. 2011; Landgraf et al. 2012), thus reducing their incidence and severity (Song et al. 2015). The same variability in the response to AG herbivores is also reported for nematodes, where AG herbivores have been found to lower the number of plant nematodes (Kutyniok and Müller 2013) or, on the contrary, to make roots more attractive to nematodes (Kammerhofer et al. 2015).

[7.1.2.3 Interactions between AG pathogens and BG herbivores](#)

Very little is known on the interaction between AG pathogens and BG herbivores. In fact, we could not find a single example of the impact of AG pathogens on BG herbivores. Hence, all examples of interactions between AG pathogens and BG herbivores presented in Table 1 refer to the effect of BG herbivores on AG pathogens. For instance, root damage by BG larvae of the chrysomelid *Diabrotica virgifera* was shown to induce defenses in maize leaves against the necrotrophic pathogen *Setosphaeria turcica* (Erb et al. 2009). Likewise, root herbivory by the specialist herbivore *Acalymma vitatum* was shown to increase cucumber leaf resistance to downy mildew *Pseudoperonospora cubensis* (Barber et al. 2015). This effect was stronger with higher herbivore abundance. Although these examples are consistent with previous studies reporting negative effects of root herbivory on AG herbivory (Erb et al. 2008), it is clearly premature to draw any generalizations at this stage.

[7.1.2.4 Symmetry of cross-compartment pathogen-herbivore interactions](#)

An important question is whether species interactions between BG and AG antagonists are symmetric. Symmetry could take two forms: i) species A negatively affects species B and species B also negatively affect species A; ii) species A positively affects species B and species B positively affect species A. Unfortunately, few studies simultaneously addressed the effect of a BG attacker on an AG attacker and the reciprocal effect of an AG attacker on a BG attacker (see Table 1: Godfrey and Yeargan 1989; McCarville et al. 2012; Lee et al. 2012; Kammerhofer et al. 2015). As one example, Leath and Byers (1977) reported an

increased severity of the BG fungal pathogen *Fusarium roseum* when the host plant was simultaneously colonized by aphids, whereas aphid population size decreased on *Fusarium*-infected plants. McCarville et al. (2012) found that dual attack by aphids and the fungus *Cadophora gregata* increased plant infestation by root nematodes, whereas co-infection by nematodes and the fungal pathogen reduced aphid population growth.

7.1.2.5. General patterns

Our understanding of BG-AG plant-pathogen-herbivore interactions is still in its infancy, and current evidence reveals a large diversity of interaction outcomes, with both positive, neutral and negative effects reported on each of the three players. Given the varying responses, identifying general patterns and the factors that modify the direction and strength of the effect will require a large(r) number of studies. Beyond patterns, we may also change our focus to the mechanisms at play and develop a predictive framework. This will be the focus of the next section.

7.1.3. Mechanisms shaping BG-AG interactions between pathogens and herbivores

Few studies in Table 1 explore the mechanisms underlying BG-AG interactions between herbivores and pathogens. However, given that a plant's response to herbivores, pathogens and other organisms involves common signaling pathways and secondary compounds, we may assume that i) interactions between pathogens (Blodgett et al. 2007), ii) interactions between herbivores (Erb et al. 2008; Johnson et al. 2012), iii) interactions between herbivores and mutualists (Koricheva et al. 2009) and iv) within-compartment interactions between pathogens and herbivores (Fernandez-Conradi et al. in review) can help to predict the outcome of cross-compartment interactions between pathogens and herbivores (Van der Putten et al. 2001; van Dam and Heil 2011; Biere and Goverse 2016). Notably, while changes in defense-related hormonal pathways received a massive interest, other mechanisms like changes in plant quality, the possible interplay between biotic attackers and abiotic stressors, as well as the ecological and evolutionary consequences of dual infection, are relatively poorly addressed.

In this section, we recapitulate the recognized and putative mechanisms linking pathogens and herbivores across BG and AG compartments. However, as this topic has been extensively reviewed, we aim to be brief, and we refer readers interested in the fine hormonal and physiological mechanisms to the recent and extensive reviews on this topic (e.g., Wondafrash et al. 2013; Lazebnik et al. 2014; Biere and Goverse 2016). Importantly, while this section focuses on mechanisms related to primary and secondary chemistry, interactions may equally likely be mediated by changes in the quality and quantity of the shared resource (the host plant) or, as discussed in section 4, by interactions mediated by other members of the plant-associated food web.

7.1.3.1. *Plant-mediated AG-BG interactions*

7.1.3.1.1. *Effects mediated by shared defenses and cross-compartment signaling*

Transportation, induction and priming. There are three non-exclusive mechanisms by which herbivory or pathogen infection in one compartment can make the other compartment increase its defense or readiness for attack: transportation of defensive secondary compounds, defense induction and defense priming. Induction is the increase in concentration of secondary metabolites involved in defenses immediately following attack. Defense priming is the pre-activation of mechanisms that make plants able to better or more rapidly mount defense responses against attackers (Prime-A-Plant Group et al. 2006; Martinez-Medina et al. 2016). While translocation and induction directly result in an increase of basal defense levels, priming does not and may go unnoticed if only defensive compounds are targeted. If cross-compartment interactions rely – based on their spatial separation – more on defense priming than within-compartment interactions, BG-AG cross-compartment interactions may have been underestimated because of methodological issues (i.e. a focus on increased levels of compounds).

Several defense compounds such as nicotine (an alkaloid) are exclusively produced in the roots but are effective against foliar herbivores, and can migrate through long-distance transportation to AG parts (Dawson 1941; Kaplan et al. 2008; Bezemer et al. 2013). In tobacco plants, Kaplan *et al.* (2008) showed that the concentration of alkaloids decreased in shoots after plants were attacked BG by the root-knot nematode *Meloidogyne incognita*, whereas concentrations of chemical compounds synthesized in the shoots increased. From the literature addressing cross-compartment interactions between BG and AG herbivores, it is clear that root herbivory is commonly followed by an increase in basal levels of defenses in shoots, even in the absence of AG damage (reviewed by Erb et al. 2008), which can result from translocation, induction or both.

The opposite, increase of basal defenses in roots following attacks in shoots, is also possible but more variable in terms of direction and intensity (Erb et al. 2008). AG herbivores and pathogens can induce the production and storage of defensive compounds in roots (which is common for alkaloids such as nicotine, Kaplan et al. 2008), or activate defense-related pathways resulting in the priming or induction of defenses in BG organs (Yang et al. 2011; Landgraf et al. 2012). For example, AG herbivory by the whitefly *Bemisia tabaci* activates the SA-dependent signaling in AG and BG organs, eliciting induced resistance of pepper plants to the soil-borne pathogen *Ralstonia solanacearum* (Yang et al. 2011).

Hormone signaling and shoot-root integration. Plant BG and AG parts are tightly interconnected by the plant vascular system, allowing long-distance communication between roots and shoots. Although plants respond locally to herbivore attack or pathogen infection, plant-level resistance to both pathogens and herbivores requires a complex integration at the plant scale, including root-to-shoot-to-root or shoot-to-root-to-shoot communication loops (reviewed by Biere and Goverse 2016). Such compartments' share of defenses involve uni- or bidirectional exchanges of molecules (for example, RNA, peptides, phytohormones or alkaloids) through xylem and phloem vessels (Lucas et al. 2013).

The induction of systemic resistance to herbivores and pathogens is mainly based on chemical defense pathways involving three key hormones acting as major players: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). A certain specificity in their induction by and effectiveness against different groups of herbivores and pathogens has long been assumed. For example, the SA pathway is usually induced by and efficient against biotrophic pathogens and sucking herbivores, whereas the JA pathway is principally activated by, and effective against, necrotrophic pathogens and leaf-chewers (Spoel et al. 2007; Ali and Agrawal 2012; Thaler et al. 2012; Lazebnik et al. 2014). In addition, there is a reciprocal cross-talk consisting of an antagonism between SA and JA signaling pathways in several systems (Thaler et al. 2012).

When such cross-talk exists, the impact of dual attack may result in either negative or positive interactions between herbivores and pathogens, where the direction of the interaction is predicted to depend on the specific combination of herbivore feeding guild and pathogen lifestyle (Fig. 2). For instance, it has been postulated that plant attack by a BG or AG chewing herbivore may activate the JA-pathway, thereby suppressing SA production, which may be detrimental to necrotrophic pathogens and beneficial to biotrophs in the other compartment (Fig. 2, panels A and E). On the contrary, plant attack by sucking herbivores may increase SA levels, and decrease levels of JA, which would benefit necrotrophs and be detrimental to biotrophs (Fig. 2, panels B and F). Similarly, plant infection by an AG or BG necrotrophic pathogen may increase JA levels and reduce SA levels, which may benefit piercing-sucking herbivores but be detrimental to chewing herbivores (Fig. 2, panels C and G). Finally, infection by an AG or BG biotrophic pathogen may upregulate the SA-pathway and downregulate the JA-pathway, which would be beneficial to chewers and detrimental to piercing-sucking herbivores (Fig. 2, panels D and H).

7.1.3.1.2. Effects mediated by altered plant nutritional quality and the abiotic environment

Changes in plant nutritional quality and defenses can hardly be teased apart (Van der Putten et al. 2001), both concurring to shape defense syndromes (Agrawal and Fishbein 2006). Indeed, nutrient uptake by the roots does not only affect plant quality, but frequently affects both direct and indirect defenses (*i.e.*, involving a third trophic level). As one example, the density of trichomes, which act as physical barriers against herbivores, as well as volatile compounds, which may be used for parasitoid recruitment, increase with nitrogen uptake (Bernays 1994; Van der Putten et al. 2001). As a consequence, changes in nutrient uptake resulting from root herbivory, infection by pathogens, or changes in abiotic conditions due to N fertilization, may have important consequences in terms of both host plant quality and subsequent defense production.

Effects of BG damage and abiotic factors on AG tissues and AG organisms (Figure 3A)

BG attackers can have multiple effects on AG plant quality. These include both changes in primary and secondary metabolites (Hatcher 1995; Van der Putten et al. 2001; Cipollini et al. 2002) and alteration of plant growth pattern and architecture (Bernays 1994, Van der Putten et al. 2001). Yet, these mechanisms can be triggered both by BG attackers, abiotic stresses, or

a combination of both. It is therefore critical to acknowledge that spatial and temporal variation in the abiotic environment can impact the outcome of cross-compartment plant-pathogen-herbivore interactions.

BG attackers often cause nutrient or water stress (Figure 3A). As such, they can mimic the well known effects of both abiotic stresses on AG plant parts. For instance, water stress and root infection by *Phytophthora cinnamomi* have similar effects on stomatal conductance and the concentration of abscisic acid, a hormone involved in plant response to drought, in the xylem of chestnut (Maurel et al. 2004). Similarly, Erb et al. (2011) showed that the root herbivore *Diabrotica virgifera* induced changes in the quality of AG tissues that were mediated by the production and translocation of abscisic acid.

Stress-like effects of BG attackers on AG plant parts may cascade on AG herbivores and pathogens. The plant stress hypothesis (White 1974, 2009) predicts an increase in herbivore performances on drought-stressed plants (Gange and Brown 1989)). However, whether these stress-induced changes are beneficial or detrimental to AG attackers may depend on their feeding habits (Huberty and Denno 2004), and in particular on whether they target foliage or wood, and healthy or declining trees (Jactel et al. 2012). BG herbivores and pathogens may, similar to water stress (White 1974, 2009), have contrasting effects on AG herbivores depending on the type of tissues they feed on: for example, AG herbivores feeding on young and actively growing leaves (i.e., flush-feeders, *sensu* White 2009) may be more hampered by BG attackers than AG herbivores that feed on older, senescent organs (i.e., senescence-feeders). Indeed, the latter herbivores may even benefit from regulatory mechanisms resulting in the release of soluble sugars and free amino-acids in cells (Gutbrodt et al. 2011, Ximénez-Embún et al. 2016).

Abiotic stresses and BG herbivores and pathogens can interactively shape plant-pathogen and plant-herbivore interactions in AG plant parts. For instance, the strength of BG-AG interactions between the nematode *Heterodera schachtii* and aphids was found to be dependent on N-fertilization (Kutyniok and Müller 2013; Kutyniok et al. 2014): in low N-soil, nematodes had no effect on *Brevicoryne brassicae* aphids, whereas aphids increased nematode abundance in roots; in contrast, under high N, aphids reduced nematode abundance and cyst formation (Kutyniok and Müller 2013). On the contrary, the presence of nematodes decreased the abundance of the shoot-infesting aphid *Myzus persicae* only when N supply was low (Kutyniok et al. 2014). A direct consequence of such an interaction between the effects of BG attackers and abiotic factors on AG attackers is that the direction, strength and underlying mechanisms of cross-compartment pathogen-herbivore interactions are expected to vary along abiotic gradients.

Altogether, the presence of spatial and temporal variation in the abiotic environment weakens our ability to infer general patterns on plant-pathogen-herbivore interactions. Yet, this role of the environment in mediating species interactions may have massive implications in agricultural systems where such biotic and abiotic stresses may or may not be controlled (through irrigation, fertilization and pesticides). It therefore appears urgent to better address how abiotic factors can mediate BG-AG plant-pathogen-herbivore interactions.

Effects of AG damage on the quality of BG tissues and BG organisms (Fig. 3B).

AG herbivores and pathogens can strongly affect carbon dynamics and alter carbon allocation to AG and BG compartments (Orians et al. 2011). Foliar herbivory commonly increases resource allocation to roots, thereby reducing its availability to AG plant attackers, which is referred to as *induced resource sequestration* (Orians et al. 2011). Although this strategy may be seen as a way to secure resources and make them unreachable to AG herbivores and pathogens, induced resource sequestration may also have indirect effects on BG pathogens and BG herbivores. These indirect effects can range from positive, when roots act as a sink for photoassimilates, to negative, when these resources are invested and stored in roots as defensive compounds (see section 4.1.1. and Biere and Goverse 2016). For instance, AG herbivory was found to increase levels of defensive secondary metabolites in roots, which can reduce plant quality to root herbivores and nematodes (Van Dam et al. 2005).

AG-BG interactions involving changes in plant nutritional quality are, generally, asymmetrical. BG herbivores and pathogens consume or destroy root tissues which directly reduces the plant's ability to take up water and nutrients. The effects of root consumption propagates through the plant to AG parts, resulting in changes in the nutritional quality of AG plant tissues (e.g., changes in water content or concentration of free amino acids and soluble sugars). While AG herbivores and AG pathogens have also been shown to affect root quality, their systemic effect is generally weaker (Kaplan et al. 2008). Bezemer and van Dam (2005) proposed that such an asymmetry may further result from roots being exposed to herbivores early in the season before leaves are available to herbivores, making the plant ready to face AG herbivores and pathogens before they attack.

7.1.3.2. Intensity and timing of damage

7.1.3.2.1. The intensity of herbivory and pathogen infection

The consequences of BG or AG damage on plant quality, and hence on AG or BG attackers, depend on the amount of damage. However, very few studies manipulated, or even clearly reported, the amount of herbivory or the intensity of the infection (Marçais and Bréda 2006). This seems surprising, as herbivory can range from a few percent to full defoliation, and infections can range from a few lesions, which may increase plant quality due to the mobilization of nutrients, to entirely necrotic foliage or rotting roots (Agrios 2005). In the extreme case, the plants may die, which will dramatically affect the performance or the survival of other organisms feeding on the same plant, with a shift from biotrophic toward necrotrophic (i.e., hemi-biotrophs) and then saprotrophic species. For example, while Cardoza et al. (2003) found a positive effect of the necrotrophic fungus *Sclerotium rolfsii* on the development of *Spodoptera exigua* caterpillars when developing on fungus-infected peanut plants, this pathogen will ultimately kill its host, and the positive effect of infection may then reverse with increased inoculation density and disease progression. As one example, the effect of birch defoliation by geometrid moths on the birch fungal root community differed with the intensity and frequency of the attacks (Saravesi et al. 2015).

[7.1.3.2.2. Does it matter who comes first?](#)

The strength and direction of BG-AG interactions between herbivores and pathogens may also be affected by the relative timing of attacks: herbivores and pathogens may attack the plant at the same time, or one of the species may arrive before the other (Moultet et al. 2013). Indeed, in the most extreme case one of the attackers may already be gone from the plant before the other attacker arrives. This naturally excludes any reciprocal effect, and leaves us to probe the impact of the first on the second attacker. While this sounds trivial, we stress that this may be rather common in nature, where herbivores may move around and pathogens often have a restricted growing season. Indeed, early-season herbivores are known to have a pronounced impact on herbivore preference, performance, and community structure later in the season, where "later" can be hours, days, weeks, months or even years (Van Zandt and Agrawal 2004; Stam et al. 2014). Importantly, the plant responses linking the first attacker to the second attacker may take place at different time scales: while induced defenses may take minutes to hours, changes in plant quality and quantity may take longer. Thus, even when the attackers are separated in time, it may be important to take into account the amount of time that has passed between the attack by the first and second attacker.

However, the majority of studies on species interactions focuses on cases where the timing of the two attackers at least partly overlaps. Here, the meta-analysis by Johnson et al. (2012) reported that AG herbivores had strong negative effects on BG herbivores when they attacked first in laboratory studies. In contrast, primary attacks by BG herbivores had only moderately positive and non-significant effects on AG herbivores.

It is critical to acknowledge that the effects of BG attackers on AG attackers, and *vice versa*, may vary non-linearly with both the intensity of damage and with time. While the hormonal signaling may be relatively fast (section 3.1.1.), the impact of damage on the quality of root and aerial tissues, or changes mediated by the composition of the other plant-associated biota, may take longer to establish and may last long after the initial damage was caused (section 3.1.2.). It is therefore not only the identity of the first attacker and the attacked compartment that matters for the second player, but also the type of changes it induced in the host plant by the time it arrives. (e.g. Li et al. 2016)

[7.1.3.2.3. Annual vs. perennial plants: does it matter if interactions are reset every year?](#)

During their lifetime, perennial plants are exposed to a greater abundance and diversity of pathogens and herbivores than annual plants. Moreover, they experience profound ontogenetic changes in constitutive and induced defenses against different attackers (Boege and Marquis 2005; Barton and Koricheva 2010). They may also be more difficult to study, or at least, there might be a bias toward more observational field studies for perennial plants such as trees (Marçais and Bréda 2006; Saravesi et al. 2015), and short-term, highly controlled studies for annual plants, including crops (Table 1).

The timing and diversity of attackers may differ strongly between annual and perennial plants, and results from short-term highly controlled studies may therefore lack relevance for perennial plants. After emergence, the first attacker of annual plants may have a

large impact on how the plant will respond to future attacks in the same and opposite compartment (see section 3.2.2.). In contrast, the response of perennial plants to the first attack of the season may be weakened by a legacy of attacks by pathogens and herbivores in the previous year. Likewise, as large perennials like trees are attacked by a diverse community of herbivores and pathogens, the attack by a single herbivore or pathogen may leave a very weak imprint. In such cases, it seems hard to extrapolate the outcome and effect sizes of short-term and highly controlled laboratory experiments on annual or crop plants to the diversity and complexity of interactions occurring on long-living plants. Notably, there may also be intergenerational legacy effects in annual plants: induced changes in defenses in year $t-1$ may affect the composition of soil microbial communities, which indirectly affects the next generation of the plant growing within the same soil (Kostenko et al. 2012).

Despite the scarcity of studies documenting cross-compartment interactions among trees, pathogens and herbivores, forest ecologists have long recognized the importance of dual attacks for tree health. They defined primary pests as those pathogens and herbivores being able to successfully develop and reproduce on healthy trees (Wainhouse 2005). In contrast, secondary pests can only exploit trees that are first weakened by attack from primary pests or by an abiotic stress. For instance, severe defoliation by the Gypsy moth *Lymantria dispar* was shown to alter root chemistry and facilitate root colonization by *Armillaria* spp., a taxon that includes several secondary fungal pathogens and causes root rot (Burrill et al. 1999; Young and Giese 2003; Marçais and Bréda 2006).

7.1.4. Upscaling plant-pathogen-herbivore interactions: from individuals to communities and ecosystems

In the previous section we saw that spatial and temporal variation in the **abiotic** environment affects cross-compartment pathogen-herbivore interactions. This section will focus on how the **biotic** environment affects cross-compartment pathogen-herbivore interactions, and, *vice versa*, how cross-compartment pathogen-herbivore interactions affect the biotic environment. At the same time, we raise questions about the importance of BG-AG interactions among herbivores and pathogens within a community context.

7.1.4.1. How do BG-AG interactions among herbivores and pathogens compare to other types of interactions?

As evidenced by Table 1, the majority of controlled greenhouse and field studies have demonstrated that herbivores and pathogens can strongly interact with each other, despite the spatial (and in some instances temporal) separation between the herbivore and the pathogen. However, a demonstration in the lab does not automatically translate into relevance in a natural setting. As may be evident, the data available to date does not allow to unambiguously answer the question raised in the section header. Nonetheless, we here make

a first attempt to explore the relevance of these BG-AG interactions between herbivores and pathogens in understanding the dynamics of communities in the natural environment.

Comparing within- and between-compartment interactions between pathogens and herbivores (Fig 4A). The BG and AG plant parts are frequently attacked by a diverse set of pathogens and herbivores, and reviews have highlighted that pathogen-pathogen interactions, herbivore-herbivore interactions, and interactions between herbivores and pathogens within the same compartment can have a major impact on plant-associated community structure (Kaplan and Denno 2007; Tack and Dicke 2013). But if plants are already attacked by a diverse set of herbivores and pathogens within the same compartment, how important and how different are cross-compartment interactions between herbivores and pathogens?

To answer this question, we can compare the relevance (effect size) of BG-AG interactions between pathogens and herbivores with the relevance (effect size) of interactions between pathogens and herbivores within the same compartment. Ideally, we would carry out a meta-analysis and compare studies within and between compartments: for instance, we can investigate whether the effect of BG pathogen infection similarly affects BG and AG herbivores. However, while there is a considerable number of studies on pathogen-herbivore interactions that can be compared through meta-analyses (Fernandez-Conradi et al. *under review*), most focus on within compartment interactions. Among the very few studies dedicated to cross-compartment interactions, results are conflicting. For instance, the root necrotrophic pathogen *Heterobasidion annosum* produces phloem metabolites that negatively impact the bark beetle *Ips paraconfusus* (McNee et al. 2003). Interestingly, the effect size of this cross-compartment interaction is -1.22 (SD: ± 0.31), which is slightly stronger than the overall effect size for within-compartment interactions (mean \pm 95%-CI: -0.42 [-0.64,-0.20]). In contrast, Milanovic et al. (2015) found that the performance of Gypsy moth larvae (*Lymantria dispar*) was higher when fed leaves from *Phytophthora*-infected trees than when fed leaves from healthy red oaks. Given the diversity of mechanisms shaping BG-AG interactions among pathogens and herbivores, and their dependency on abiotic factors, it is obvious that these two studies need to be backed up by further research.

Comparing within- and between-kingdom interactions. Studies of interkingdom interactions between herbivores and pathogens are relatively few, as a common approach in entomology and pathology has been to isolate the effect of the focal organism group (insects or pathogens) by the use of insecticides, fungicides or enclosures (Tack and Dicke 2013). This may be due to the fact that competition for resources has been assumed to increase with species similarity, which precluded much enthusiasm for studies of interactions among species with widely different lifestyles.

While relatively few studies exist on interactions between plant pathogens (Marçais et al. 2011; Kemen 2014), there is a wealth of literature on the interactions between insect herbivores (Denno et al. 1995; Kaplan and Denno 2007). Interestingly, the review by Kaplan & Denno (2007) has demonstrated that interactions among herbivores are highly variable, are similar in magnitude within and among feeding guilds (e.g. sap-sucking herbivores and chewers), and can range from negative to positive. Importantly, the effect sizes reported for these herbivore-herbivore interactions (e.g. Figure 3 in Kaplan and Denno 2007) are within

the same range as those reported by Fernandez-Conradi et al. (under review) for the effect of pathogens on herbivores. Barber et al. (2015) showed that root herbivory of cucumber plants did not affect leaf herbivory, whereas it did reduce infection by downy mildew, illustrating that between-kingdom interactions can be stronger than within-kingdom interactions. Hence, we feel confident to postulate that – from the perspective of either plant, pathogen or herbivore – it does not matter whether the partners involved are pathogens or herbivores. What does matter is the identity, or possibly the lifestyle, of the attackers involved and the changes that the attackers induce in the plant, which, amongst others, can include priming and induction of defenses (see section 3).

7.1.4.2. *How do BG-AG interactions among herbivores and pathogens affect other members of the community, and vice versa?*

Plants are associated with a diverse plant-based community of organisms belonging to different trophic levels (Fig. 4B). Moreover, plants are not growing alone, but are embedded within plant communities. This community context may strongly mediate the interactions between pathogens and herbivores. At the same time, interactions between pathogens and herbivores will shape the surrounding community. Clearly, we need a community perspective. So how does the community context affect BG-AG interactions among pathogens and herbivores? And, *vice versa*, how do BG-AG interactions among pathogens and herbivores affect the surrounding community?

Plant community structure. The plant community surrounding the focal plant may affect the outcome of single and dual attack by herbivores and pathogens (Fig. 4B). For instance, Damicone et al. (1987) reported a significant interaction between fungicide, insecticide and herbicide treatments, such that yield and survival of *Asparagus officinale* was strongly (and non-additively) reduced by dual-attack of the AG herbivore and BG pathogen in the absence of competitors, whereas dual attack resulted in additive effects on asparagus yield in the presence of competitors (Damicone et al. 1987). This study then suggests that the consequences of single and dual attack by pathogens and herbivores can be modified by the presence of competitors of the host plant. Moreover, the surrounding plant community can affect the likelihood and severity of BG-AG interactions among pathogens and herbivores: the risk of attack by both herbivores and pathogens on a given plant can be lower (i.e., associational resistance) or higher (i.e., associational susceptibility) in the presence of heterospecific neighbors (Underwood 2010; Hantsch et al. 2014; Nguyen et al. 2016). One fascinating direction would also be to focus on plant competitors that are attacked by the same pathogen and herbivore species.

The outcome of single and dual attacks by herbivores and pathogens may also affect the competitive ability of plants relative to conspecifics or heterospecifics, and thereby affect the structure of the plant community (Fig. 4B). In one example, Godfrey and Yeargan (1987) showed how interactions of early season pests and pathogens changed the density of the surrounding plant community (‘weed density’) within alfalfa fields. Hopefully, future studies will target natural systems to explore whether BG-AG interactions among pathogens and herbivores result in changes in natural plant communities.

Herbivore community structure. When herbivore species respond differently to attack by a pathogen in the other compartment this will result in a change in herbivore community structure. This may be either due to differences in herbivore preference or performance. As an example from an aboveground study, Tack et al. (2012) showed that the community structure of leaf miners and gallers associated with the oak *Quercus robur* changed with the intensity of infection by the powdery mildew *Erysiphe alphitoides* (a foliar biotrophic pathogen).

Given the highly variable response of herbivores to the presence of a pathogen within the same compartment (Tack and Dicke 2013) we can *a priori* expect that pathogen infection will differentially affect some members of the herbivore community in the opposite compartment, and infection will thereby result in changes in the herbivore community structure.

Lifestyle of the pathogen and herbivore may underlie some of the differences among herbivores in their response to infection (figure 2). In addition, part of the variability in the cross-talk between AG and BG plant parts could be explained by the degree of herbivore specialization (Kaplan et al. 2008; Ali and Agrawal 2012). As generalist and specialist herbivores differ in their effect on, and response to, qualitative and quantitative defenses (Ali and Agrawal 2012), the nature of changes in foliar quality induced by BG specialists and generalists may profoundly influence the nature of the response of AG specialists and generalists, and *vice versa*.

No studies have yet addressed how herbivore community structure would affect BG-AG interactions between pathogens and herbivores.

Pathogen community structure. No studies in Table 1 have measured the response of multiple pathogen species to herbivore attack. However, as explained in detail in section 3, we may expect differences in response of pathogens to herbivore attack to be affected by the lifestyle of the pathogen (e.g. necrotrophic *versus* biotrophic pathogens). Similar to the herbivores, we therefore expect pathogens to respond differently to attack by herbivores within the other compartment.

No studies have yet addressed how pathogen community structure would affect BG-AG interactions between pathogens and herbivores.

Microbial community structure. Soil biota may mediate the interactions between BG and AG attackers. For instance, root herbivores can affect root colonization by mycorrhizal fungi (reviewed by Johnson and Rasmann 2015), with consequences for plant nutrition and defense (Gange 2000). Such changes in the BG community of plant-associated beneficial organism may provide an indirect link between BG and AG attackers. However, the direction and strength of the effect of BG herbivores and pathogens on mycorrhizal fungi was reported to range from negative (Bennett et al. 2013), to neutral (Gange 2001) or even positive (Currie et al. 2006). Hence, predicting the strength and direction of mycorrhiza-mediated effects of BG herbivores and pathogens on AG attackers may be difficult (Chapter V).

Likewise, AG herbivores and pathogens may change the quality and defense of BG plant tissue, with consequences for the soil biota (Gehring and Bennett 2009; Heath and Lau 2011). For example, defoliation of mountain birches by geometrid moths caused subsequent

changes in taxonomic and functional composition of root fungal communities (Saravesi et al. 2015), and resource sequestration in roots following herbivore damage in AG organs was shown to influence root exudation by the grass *Poa pratensis* (Hamilton et al. 2008), which in turn may impact associated soil microorganisms (Kostenko et al. 2012). In an interesting study, Barber et al. (2015) assessed the impact of root herbivory on both root colonization by arbuscular mycorrhizal fungi and leaf infection by downy mildew; while both response variables were affected by root herbivory, it seems unlikely that arbuscular mycorrhizal fungi mediated the response of the pathogen to the root herbivore: root colonization was lowest at intermediate herbivory, whereas foliar infection was highest in the absence of herbivory.

We predict that the aboveground microbial community, including bacterial and fungal endophytes and epiphytes, may act as the aboveground equivalent of the soil biota, and play an equally important role in mediating interactions between the BG and AG compartments (Jaber and Vidal 2010; Menjivar et al. 2012; Vacher et al. 2016). Overall, the role of microbes in mediating the response of the plant to BG and AG attack would be a promising avenue for future research.

Higher trophic levels. BG-AG interactions among pathogens and herbivores may also affect higher trophic levels (Bezemer et al. 2005). The attack of roots by pathogens and herbivores may induce volatile organic compounds (VOCs) that attract the natural enemies of herbivores, like parasitoids or entomopathogenic nematodes (Rasmann et al. 2005). Root exudates may play a similar signaling role within the belowground compartment. Notably, the impact of AG pathogens and herbivores on belowground natural enemies may involve both BG parasitoids and parasitic nematodes, as the latter play a particularly important role in the soil community (Strong et al. 1999). However, most of the existing studies taking into account natural enemies focused on within-compartment interactions between pathogens and herbivores (e.g., Cardoza et al. 2003; Tack et al. 2012) or cross-compartment interactions between herbivores (Soler et al. 2005, 2007).

We hope that future studies will address the impact of cross-compartment interactions between pathogens and herbivores on both natural enemy attack and the multitrophic community structure. Likewise, future studies may investigate whether induced changes in plant quality, VOCs and root exudates also affect the performance of natural enemies of pathogens (e.g. fungal hyperparasites and snails). To our knowledge, no study has been dedicated to this topic.

7.1.4.3. *How do BG-AG interactions among herbivores and pathogens affect ecosystem dynamics?*

Given the strong impact of BG-AG interactions among herbivores and pathogens on plant performance and community composition, it seems likely that such interactions will also affect ecosystem processes like carbon dynamics, water dynamics, and decomposition in the litter layer. Alternatively, even a strong pathogen-herbivore interaction may leave only a weak imprint at the ecosystem-level. We eagerly await studies that explore this.

7.1.5. Further avenues for future research

As stated above, the available literature specifically addressing cross-compartment interactions between pathogens and herbivores is very small (Table 1). Hence, despite the speculations and predictions provided in this chapter, we think that no general patterns can be safely inferred, and we refrain from a final synthesis. In the previous section, we already discussed the need to assess the relative importance of plant-pathogen-herbivore interactions within a community and ecosystem context. Here, we hope to stimulate future research by outlining additional open questions with tentative predictions.

7.1.5.1. *Are the outcomes of short-term laboratory experiments and observational studies in natural and agricultural systems comparable?*

Prediction: The evidence seems inconsistent. Focusing on herbivores, Johnson et al. (2012) reported stronger cross-compartment interactions in short-term experiments than in observational studies. In contrast, Fernandez-Conradi et al. (*under review*) reported similar effect sizes for the impact of plant pathogens on herbivores when studies were conducted under highly controlled experiments or in the field. Overall, we expect that short-term experiments will be reflected to some degree in the field, although the effect sizes may generally be lower: short-term experiments do not take into account all variation or complexity (e.g. neighboring plants, abiotic and biotic variation in the environment), and thereby are sometimes informative, and sometimes not.

7.1.5.2. *Can we predict the outcome of BG-AG interactions between pathogens and herbivores?*

Prediction: Yes, but only to some degree. Interactions may vary predictably as based on the pathogen and herbivore lifestyle (Figure 2; Thaler et al. 2012, Biere and Goverse 2016) and specialization (Ali and Agrawal 2012; Thaler et al. 2012; Biere and Goverse 2016). Superimposed on this are the idiosyncrasies of the study system and variation in the outcome due to the abiotic and biotic environment (sections 3 and 4).

Suggestion for future studies: To improve our understanding of the generality and mechanisms at play, we recommend studies to consider multiple herbivores or pathogens within the same study system (e.g., Kaplan et al. 2008, McCarville et al. 2012, Barber et al. 2015). To facilitate meta-analyses, we ask authors to systematically report detailed information on the biology of the studied attackers (notably their degree of specialization and the plant organs they damage), and include the sample size, the mean and the variability for each experimental result, even for differences that are not statistically significant among treatments.

7.1.5.3. *What about other animals, like viruses and mammalian herbivores?*

Prediction: Other organisms, like viruses and mammalian herbivores, are of major importance. In particular, there is an extensive literature on vector-transmitted viruses, which we ignored in this book chapter for two reasons. First, the distinction between BG and AG viruses is frequently unclear (admittedly, bacterial and fungal pathogens can also become systemic). Second, many viruses are transmitted by vectors, and the interaction between viruses and herbivores becomes very complex. Hence, we think that viruses are best treated separately. We did not find any studies on BG-AG interactions among pathogens and mammalian herbivores. But as both BG mammalian herbivores (like meadow voles eating roots) and AG mammalian herbivores (like grazers) play an important role in plant performance, we do think that BG-AG interactions between pathogens and mammalian herbivores are worth exploring. The strong impact of grass endophytes on grazers provides one example of the potential role of microbes on grazers; conversely, mammals may facilitate the entrance of pathogens into their plant host.

7.1.5.4. *Are BG-AG interactions between pathogens and herbivores symmetric?*

Prediction: We predict the absence of a general pattern of symmetry in BG-AG interactions among pathogens and herbivores. Symmetry in the direction of the effect may depend on the lifestyle of the pathogen and herbivore (Figure 2). Symmetry in the strength of the effect (e.g. effect size) has not been studied for pathogen-herbivore interactions, but was notably absent for herbivore-herbivore interactions (Kaplan and Denno 2007). Because BG-AG interactions between herbivores and pathogens partially involve the same signaling pathways and may have comparable effects on the shared host plant, we expect symmetry in the strength of the effect to be absent for pathogen-herbivore interactions too. However, we note that the different metrics of herbivore and pathogen performance makes a quantitative comparison more difficult.

Most studies investigate unidirectional effects. While this is logical for studies where the first attacker is gone before the arrival of the second attacker, it seems more surprising for cases where attack by the herbivore and pathogen (partly) overlap.

7.1.5.5. *Are there AG-BG-AG or BG-AG-BG feedbacks?*

Prediction: Feedbacks are – within the context of BG-AG interactions among pathogens and herbivores – *terra incognita*. It would be fascinating to explore whether, for example, an early-season root herbivore can affect a foliar pathogen later in the season, which in turn affects BG herbivory.

7.1.5.6. What is the role of the abiotic and biotic environment in mediating BG-AG interactions among pathogens and herbivores? Can we predict the impact of climate change?

Prediction: There are several studies showing that not only the strength, but also the sign, of species interactions can change with the abiotic or biotic environment (Chamberlain et al. 2014). This may be due to the fact that the hormonal signaling pathways involved in responses to herbivores and pathogens such as SA, JA or even ethylene are often also involved in and show cross-talk with hormones involved in responses to abiotic stresses such as ABA (Pieterse et al. 2012). As such, we predict that BG-AG interactions among pathogens and herbivores are variable in space and time. However, we feel it is too early to postulate in what context, and what way, the environment matters. It would be interesting to explore the relative importance of the abiotic environment and the biotic environment (and their interactions) on cross-compartment plant-pathogen-herbivore interactions. While it is to be expected that BG-AG plant-pathogen-herbivore interactions will be modified by climate change, we have no explicit predictions for what may happen.

7.1.5.7. What are the evolutionary consequences of BG-AG interactions among herbivores and pathogens?

The outcome of BG-AG interactions among pathogens and herbivores may be affected by genetic variation within both the plant, pathogen and herbivore (Biere and Tack 2013). However, few studies on pathogen-herbivore interactions have used multiple genotypes. McCarville et al. (2012) used six cultivars of soybean *Glycine max* that varied in their resistance to the soybean cyst nematode *Heterodera glycines*, and showed that the interaction between the AG herbivore *Aphis glycines* and the BG pathogen *Cadophora gregata* varied between resistant vs. sensitive cultivars.

The non-additivity of single and dual attack on plant performance may affect selection on plant resistance (Biere and Tack 2013). As a hypothetical example, the negative impact of a common BG plant pathogen on plant performance may turn neutral, or even positive, in the presence of an AG herbivore. If so, the plant would not undergo selection for increased resistance to the pathogen in the presence of the AG herbivore. Moreover, negative effects of herbivore and pathogen attack on plant performance may be offset by beneficial indirect effects on other community members. However, in a study on the effects of root herbivory on the associated community of cucumber, Barber et al. (Barber et al. 2015) showed that direct negative interactions on plant fitness were more important than indirect interactions with other community members: direct damage inflicted by a root herbivore was not compensated by indirect effects on mycorrhizal colonization, pollination or foliar infection rates. The impact of single and dual attack by pathogens and herbivores on the evolution of plant resistance and tolerance would be an interesting research direction.

Likewise, BG-AG interactions among herbivores and pathogens may affect selection on both the pathogen and the herbivore (Biere and Tack 2013). As an empirical example, the selection pressure exerted by the presence of root-feeding nematodes on the common bean

Phaseolus vulgaris resulted in spider mites (*Tetranychus urticae*) adapted to perform better on nematode-infected than nematode-free plants within the time span of ten mite generations (Bonte et al. 2010). As another example of soil-mediated selection, the perennial herb *Plantago lanceolata* showed higher resistance against its specialist powdery mildew *Podosphaera plantaginis* when growing in association with its local soil biota (Mursinoff and Tack 2017).

7.1.6. References

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Table 1: Overview of BG-AG plant-pathogen-herbivore interactions. The table includes key features of each primary study, taxonomic and functional identity of BG and AG attackers, and consequences of BG-AG interactions for the plant, the pathogen and the herbivore. 'Compartment attacked first' indicates the order of the attack: the first attack was belowground ($BG \rightarrow AG$), the first attack was aboveground ($AG \rightarrow BG$), or below- and aboveground attack took place at the same time ($BG \leftrightarrow AG$). References are organized to mirror numbers in Figure 1. NA indicates that the information could not be extracted from the study.

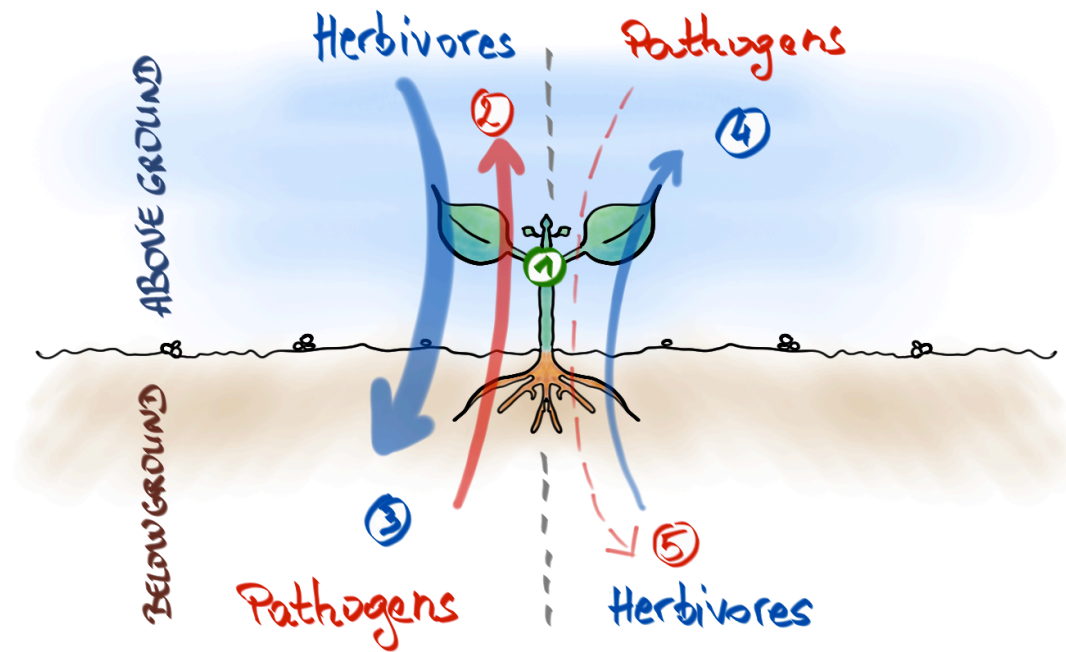


Figure 1: Cross-compartment interactions between pathogens and herbivores. Blue arrows represent the effect of herbivores on pathogens. The reciprocal effect of pathogens on herbivores is shown by red arrows. (1) consequences of dual-attack on the host plant; (2) effects of BG pathogens on AG herbivores; (3) effects of AG herbivores on BG pathogens; (4) effects of BG herbivores on AG pathogens; (5) effects of AG pathogens on BG herbivores. Arrow width is proportional to the number of studies specifically addressing corresponding interactions.

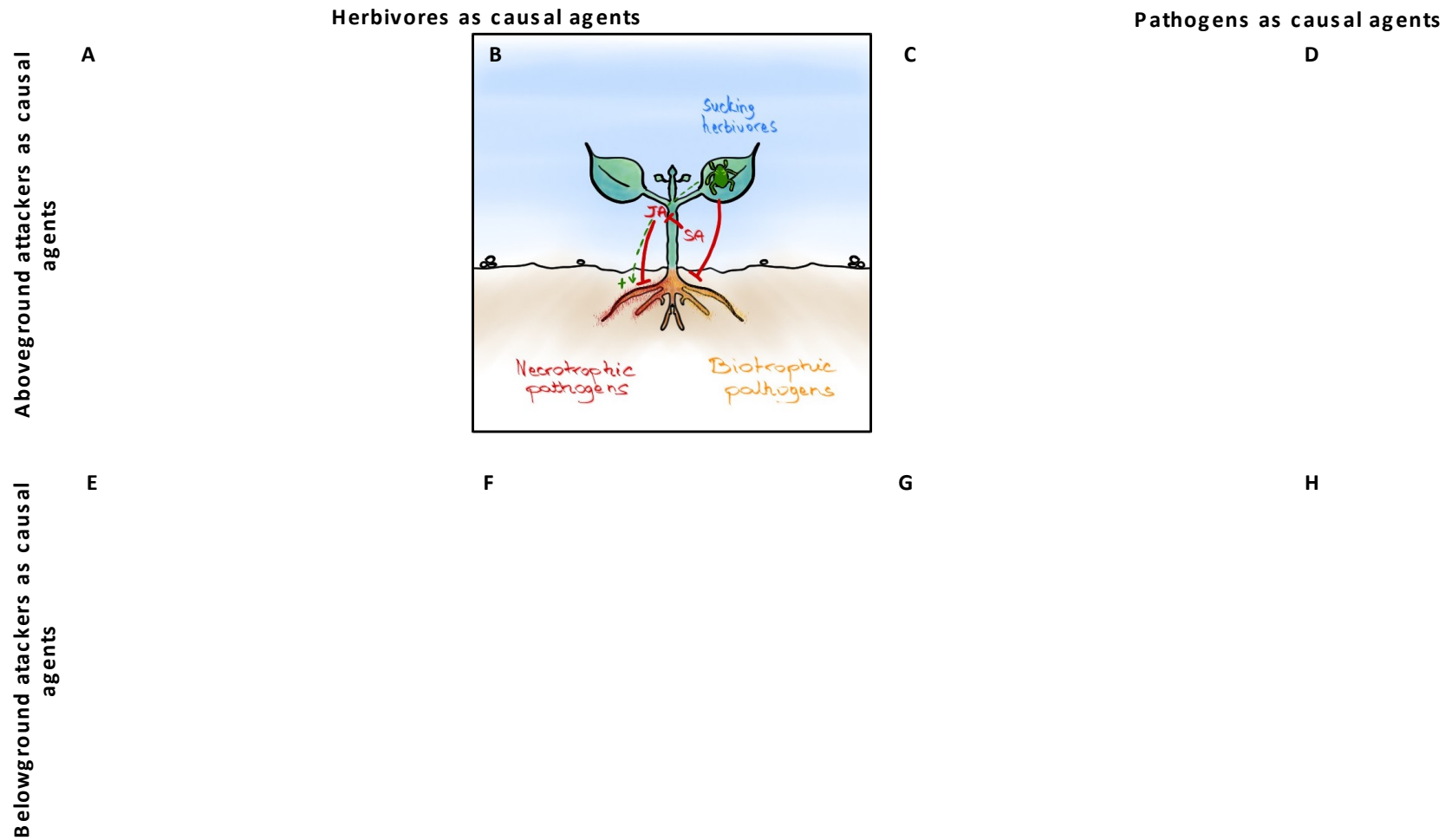


Figure 2: Summary of hormonal pathways and cross-talk antagonisms involving BG and AG herbivores and pathogens. Arrows originating from one compartment indicate the causal effect of the corresponding attacker on the second attacker in the other compartment. Red and green arrows are for *predicted* negative and positive effects, respectively. Panels C, D and F are faded to indicate scenarios that are likely but for which no specific case study was retrieved.

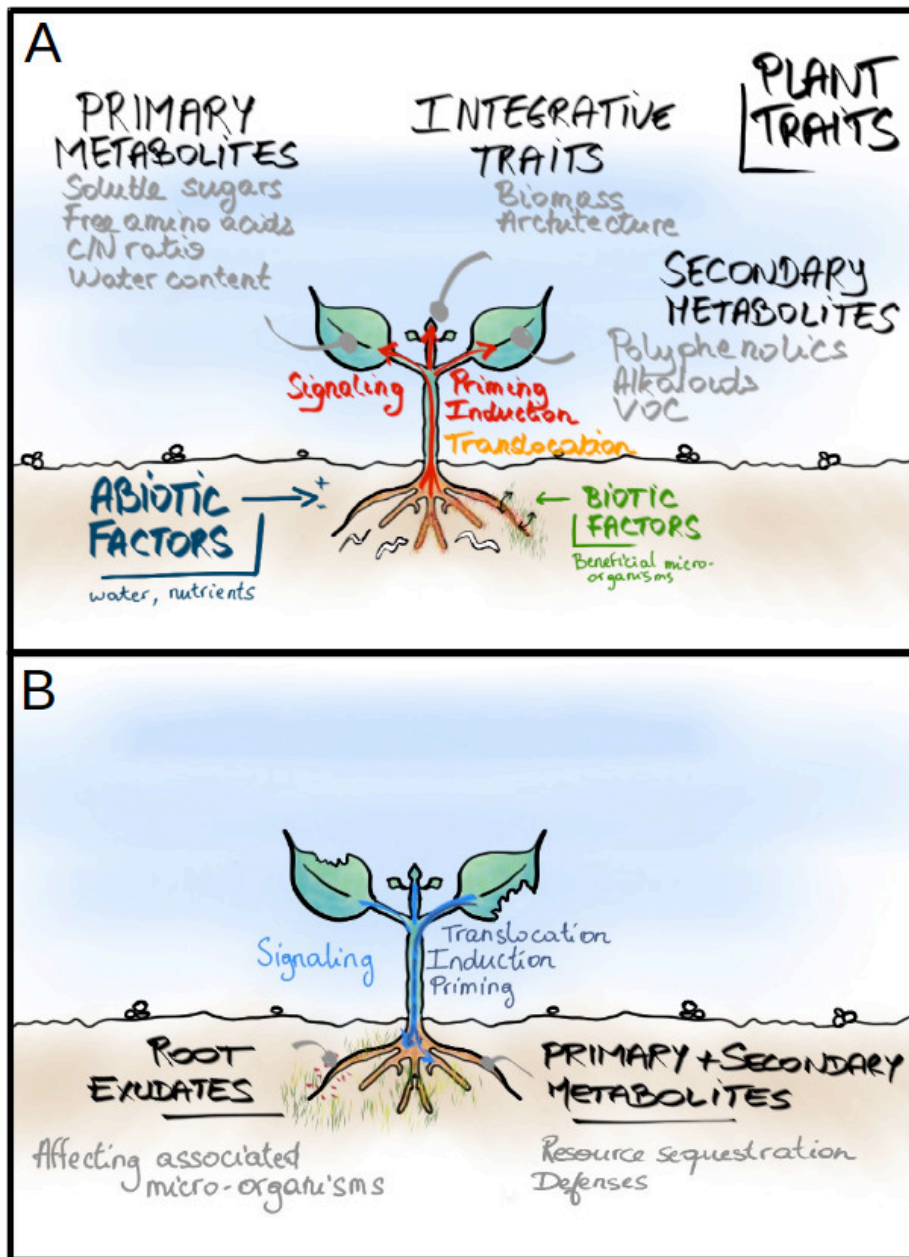


Figure 3: Summary of mechanisms involved in cross-compartment interactions between herbivores and pathogens. (A) AG response to BG damage and (B) BG response to AG damage.

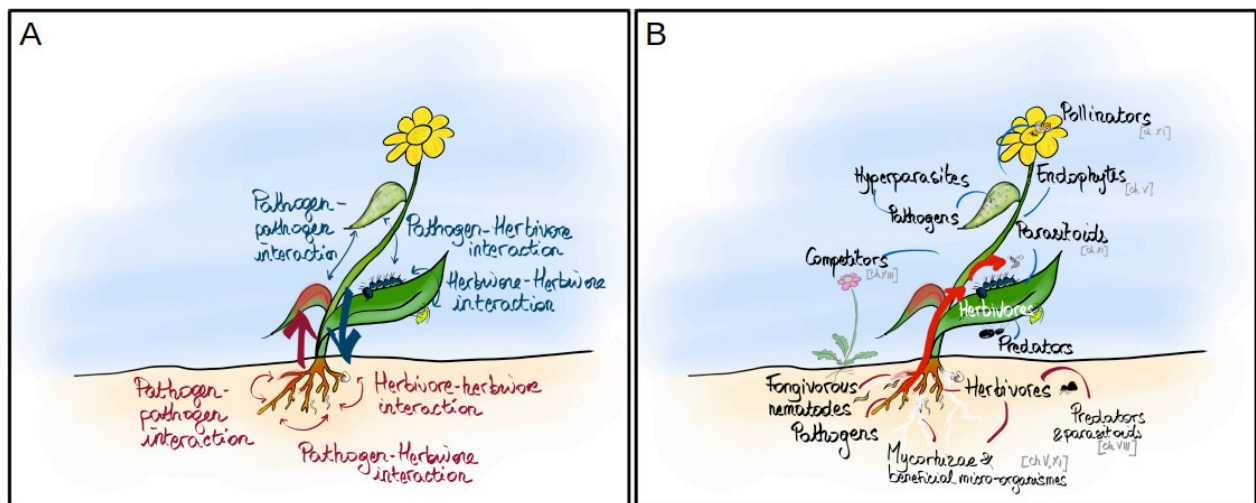


Figure 4. Upscaling plant-pathogen-herbivore interactions to the community level. In panel A are shown both the interactions among pathogens and herbivores within the same compartment (thin arrows) as well as the interactions among compartments (thick arrows). A major challenge will be to assess the relative importance of within- versus between-compartment interactions, and within- versus between-kingdom interactions. In other words: which types of interactions are most important within a community context? Panel B illustrates the complex web of multitrophic interactions within which belowground-aboveground interactions are embedded. The red arrows illustrate one possible interaction cascade, where a belowground pathogen affects the preference and density of an aboveground herbivore, which in turn affects the rate of attack by the parasitoid. The response of the parasitoid may be density-mediated (i.e., in response to changes in the density of the herbivore) or trait-mediated (for example, due to changes in the volatile composition of the plant or changes in the behavior of the herbivore). In panel B, the roman numerals (in grey font) refer to other chapters within this book.

The effect of tree genetic diversity on insect herbivory varies with insect abundance

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ABSTRACT

Variance in edibility among plant genotypes is expected to be a key driver of plant genetic diversity (PGD) effects on abundance of insect herbivores and resulting herbivory. Yet, herbivore foraging behavior and leaf consumption may be also context dependent, and in particular influenced by herbivore density, which remains unexplored. We used a combination of field and laboratory experiments with saplings from four half-sib families (henceforth, families) of pedunculate oak (*Quercus robur*) to test how PGD and herbivore density interactively affect herbivory. Insect herbivory was assessed in a common garden experiment with plots containing all possible combinations of individuals from one to four oak families. Herbivore density was manipulated by spraying insecticide in a factorial design. Complementary feeding trials with gypsy moth larvae (*Lymantria dispar*) were used to further explore the mechanisms underlying observed patterns in the field. Herbivory decreased with increasing PGD under normal herbivore density, but not under reduced herbivore abundance. The most damaged oak family in the field was also the most consumed in non-choice tests and was consistently preferred over other families in choice tests. Trials showed that the presence of less edible families in the diet reduced overall consumption by gypsy moth larvae. Under field conditions, the most edible family consistently benefited most from being associated to less edible, neighboring genotypes. Our results demonstrate that small-scale PGD can provide associational resistance to insect herbivory, probably through change in herbivore foraging activity. Importantly, they also reveal that the magnitude of genetic diversity effect depends on herbivore density.

Keywords: Associational resistance; Mixed diet; Oak; Regeneration; Variance in edibility

7.2.1. Introduction

Plant diversity is a key driver of terrestrial ecosystem functioning (Cardinale et al. 2011). Intraspecific genetic diversity is an essential component of biodiversity and its significance for ecosystem functioning seems to be of similar magnitude than species diversity in many aspects (Cook-Patton et al. 2011). For instance, greater plant genetic diversity (PGD) has been shown to enhance biomass production (Stachowicz et al. 2013), community stability (Booth and Grime 2003) and resistance to stress or perturbation (Jung et al. 2014). Assemblages of different plant genotypes also shelter richer insect communities (Crutsinger et al. 2008). So far, herbivore richness and abundance were mainly considered as a response variable and was shown to increase with PGD (Crutsinger et al. 2008, Cook-Patton et al. 2011, Crawford and Rudgers 2013, Pohjanmies et al. 2015). However it remains uncertain to what extent PGD also influences the activity of insect herbivores and resulting plant consumption (Castagneyrol et al. 2012, McArt and Thaler 2013, Barton et al. 2014).

Plant associational effects against herbivores occur when damage on a given plant is a function of the identity and abundance of its heterospecific neighbors (Underwood et al. 2014). It is now clear that intra-specific variability in plant traits is large enough to explain why PGD could result in such associational effects (Hughes et al. 2008, Hughes 2014, Barbour et al. 2015). However, evidence about their direction and magnitude is still conflicting since genetically based associational effects ranges from associational resistance, when plants from a given genotype suffer less damage when surrounded by conspecifics of different genotypes (McArt and Thaler 2013, Barton et al. 2015), to neutral (Moreira et al. 2014) or even the opposite, i.e. associational susceptibility, when a plant experience more damage when surrounded by distinct genotypes of the same species (Castagneyrol et al. 2012, Moreira and Mooney 2013).

Discrepancies among studies may result from insect guild-specific responses to PGD (Abdala-Roberts et al. 2015, Barton et al. 2015), spatial factors like host patch connectivity (Pohjanmies et al. 2015) or uncontrolled environmental factors (Tack et al. 2010, 2012) among which local herbivore density has been so far overlooked. Yet, it can critically change the direction and strength of associational effects. For instance, insect foraging behavior and relative preference for a given host plant may be influenced by the intensity of inter- and intraspecific competition between herbivores (Underwood 2010, Utsumi et al. 2011, Karban et al. 2013, Parent et al. 2014, Carrasco et al. 2015). The amount of competitors on a given plant, together with induced plant defenses are likely to change rank order preference in foraging insects (Utsumi et al. 2013, Carrasco et al. 2015) and thus PGD associational effects on herbivory.

In addition to herbivore density, PGD based associational effects may also depend on the genetic identity and relative abundance of conspecific neighbors. Assuming differences in palatability among plant genotypes, the '*variance in edibility hypothesis*' (Liebold 1989)

posits that a genotypically more diverse plant population is more likely to experience reduced herbivory on some genotypes and exacerbated herbivory on others as some genotypes could be preferred by herbivores over the others (Hambäck et al. 2014). Less defended plants could benefit from the vicinity of more resistant neighbors that deter or repel herbivores, i.e. associational resistance (McArt and Thaler 2013). In contrast, less preferred genotypes could be more attacked when growing near more edible and hence more attractive neighboring genotypes, as a result of herbivores spill-over (White and Whitham 2000, Castagneyrol et al. 2012). Moreover, the strength of associational effects likely depends on the relative abundance of genotypes varying in palatability: associational resistance is expected to be stronger for more palatable plants increasingly diluted among less palatable neighbors (Hahn and Orrock 2016).

Some studies have shown that herbivores can adjust the amount of consumed leaf biomass according to plant quality (Mody et al. 2007, Kotowska et al. 2010, McArt and Thaler 2013). The ‘*dietary mixing hypothesis*’ (Bernays et al. 1994) states that herbivores achieve better performance when feeding on a mix of plant resources due to complementary acquisition of deficient nutrients or reduced ingestion of toxins. The consequences of this process for plants is less well known. Having access to a mixed diet could result in lower overall herbivory in more diverse plant assemblages as in monocultures herbivores would compensate suboptimal nutrition by consuming more plant tissues that they would need in a mix of plant resources (McArt and Thaler 2013). Alternatively, a mixed diet may also result in higher plant consumption because of a reduction of toxins and better insect performances, although there is little evidence supporting this ‘*toxins dilution hypothesis*’ (Marsh et al. 2006, Mason et al. 2014).

All these hypotheses suggest that the diversity of traits involved in plant defence could exert idiosyncratic effects on insect herbivore activity. However, the interpretation of herbivory pattern will also depend on whether the individual plant or the plant population is considered. Some individual plants may be more severely attacked in mixtures while overall herbivory at the population level is lower, if the rest of the plant population is less damaged (than in monocultures). Upscaling effects of PGD on herbivory from the individual plant genotype to the population level thus remains an important challenge in community genetics (Utsumi et al. 2011, Barton et al. 2014).

In the present study, we performed a manipulative field experiment with different mixtures of one to four half-sib families of pedunculate oak (*Quercus robur*, Fagaceae) planted in a common garden in order to test the density-dependency effect of PGD on insect herbivory. Manipulation of herbivore abundance in the field was complemented with feeding bioassays and choice tests in the lab, in order to further explore mechanisms responsible for observed patterns in the field. In particular, we tested the following hypotheses: (i) insect herbivory, herbivore preference and performance vary among oak families, (ii) insect herbivory decreases with increasing PGD, and (iii) the magnitude of PGD effects on herbivory changes with herbivore abundance. Our study is therefore one of the few that explicitly tested mechanisms responsible for associational effects resulting from plant genetic diversity in the field.

7.2.2. Materials and methods

7.2.2.1. Experimental site and field experiment

The study was carried out in 2013 in a previously established experimental common garden located 40 km southwest of Bordeaux (44°440 N, 00°460 W). The experimental design has been described in detail by Castagneyrol et al. (2012). Briefly, oak saplings were grown from acorns collected in 2007 on four pedunculate oak trees within a 10 km radius around the experimental site. Saplings had been kept in the greenhouse in 2008 and treated with insecticide to prevent herbivore damage until being planted into the field in March 2009. The field site was a clearing surrounded by pine (*Pinus pinaster*) and broadleaved (*Quercus robur*, *Q. rubra* and *Betula pendula*) forest stands. It was fenced to prevent grazing by mammalian herbivores. The four source trees will hereafter be referred to as ‘mother trees’, and saplings from the same mother tree (being either full-sibs or, more likely, half-sibs) as ‘family’. Saplings from a same family were genetically and phenotypically more similar than those from different trees (see Castagneyrol et al. 2012). We therefore used the number of oak families per plot as a proxy of genetic diversity.

The common garden consisted of six different blocks established in a factorial design (see Figure S1 in Castagneyrol et al. 2012). Each block contained 15 plots with 12 saplings each (*i.e.*, four rows of three saplings planted 0.2 m apart from each other), corresponding to one of the 15 possible family combinations of one to four families: four family monocultures, six mixtures of two families, four mixtures of three families and one mixture of all four families. Saplings from different families were planted at equal distance in a regular alternate pattern so that saplings from the same family were never adjacent to each other in mixed plots. Plots were separated by a distance of 3 m and were randomly distributed within blocks. Blocks were located 4 m apart from each other.

In 2013, we manipulated herbivore density by applying three treatments to the experiment. Blocks 1 and 2 were kept as control. All plots in blocks 3 and 4 were sprayed with pyrethroid insecticides (alternating Decis Protech[®], 15 g of deltamethrin per liter diluted at 3 mL.L⁻¹, and Fastac[®], 50 g of alphasulphatrin per liter, diluted at 25 mL.L⁻¹) every fortnight from March to September in order to reduce insect herbivore density. These insecticides have a large action spectrum ensuring an efficient reduction of abundance of herbivores belonging to different taxonomic groups. In blocks 5 and 6, each sapling received three fifth instar larvae of gypsy moth (*Lymantria dispar*) in late May 2013 that fed in the plot for *ca.* 10 days until pupation. The gypsy moth is a generalist herbivore naturally present in our field area, which usually feeds on oaks. Larvae were obtained from eggs collected in the wild. From egg hatching to installation in the field, larvae were fed a wheat germ-based artificial diet in the laboratory (Bioserv product # F9630B).

Additional treatments were applied at the block level for technical reasons. Given the short distance between plots, insecticide was spread on all plots of two adjacent blocks to reduce the risk of spray drift that might affect neighboring control and herbivore enriched plots. We set up plastic barriers, 30 cm high and sprayed with glue, around blocks with gypsy moth

larvae to prevent their spill over onto adjacent blocks. Herbivore enriched (blocks 5-6) and control blocks (1-2) were separated by blocks sprayed with insecticide to further reduce the risk of spill over. This result in a split-plot experiment (Altman & Krzywinski 2015), where additional factors (insecticide and herbivore addition) were applied at the whole-block level, while genetic diversity was manipulated at the sub-block (plot) level (see below, *Data analyses*).

7.2.2.2. Insect herbivory assessment

A total of 20 leaves were collected on each sapling in August 2013. Five leaves were picked up at the tip and five at the base of two randomly chosen branches from the top and two from the lower part of the sapling, respectively. Leaves were placed into a paper bag and dried for 48h at 55°C for further examination in the lab. Preliminary tests confirmed that this treatment does not affect the assessment of herbivore damage. Herbivory was visually assessed as the percentage of leaf area removed by chewing and skeletonizing herbivores (% LAR), the most abundant insect herbivores. We used six defoliation classes (0, 1-5, 6-15, 16-25, 26-50, 51-75 and >76%). The midpoint of each class was used to calculate the mean defoliation per tree. Given most of damage at the leaf level are usually smaller than 25%, having more classes for small damage provides a better estimate of mean herbivory at the individual level (Johnson et al. 2016).

A total of 40 individual saplings of the initial experimental design were dead in previous years and 43 of the 1040 remaining saplings had less than 20 leaves. The last ones were not collected to avoid complete defoliation, and herbivory was only assessed on 997 saplings. The missing data was randomly distributed among blocks and plots and all families were represented by at least one individual in all plots.

7.2.2.3. Herbivore preferences and performance

A feeding trial and choice test was carried out between 12 and 17 May 2014 with second instar larvae of gypsy moth reared on a wheat germ-based artificial diet (Bioserv product # F9630B). Tests were performed in a climatic chamber with L16:D8 photoperiod at 23°C. A complete description of the method is provided as supplementary material (Appendix S1). An overview is given here.

We designed five experimental feeding treatments, each one replicated ten times. Replicates consisted in three larvae feeding on four oak leaves in a transparent plastic box. The four leaves came either from the same family (four single diet treatments) or from each of the four families (one mixed diet treatment). The mixed diet treatment was included to test the dietary mixing hypothesis and also to evaluate gypsy moth preferences among families (*i.e.*, as choice test).

Every morning, 50 intact mature leaves were randomly collected from saplings in monoculture plots of each oak family. Plots were selected within a single block to avoid

possible block effects on leaf quality. Leaves were scanned every day before and after consumption by larvae. Total remaining leaf area per family in single and mixed diet treatments was measured using the software ImageJ. After consumption (24h), leaves were dried at 55°C for 48h and weighted. We estimated the leaf area-biomass ratio and used it to estimate biomass consumption from leaf area consumption (see Appendix S1 for details). Larvae were kept in starvation for 24h before the experiment and weighted at the start and at the end of the feeding trial to calculate mean larval weight gain in each replicate. The Relative Growth Rate of larvae was calculated as: $RGR = (\text{final weight} - \text{initial weight}) / \text{initial weight}$.

To test for gypsy moth larvae preferences for a given oak family, we used the method developed by Larrinaga (2010) for simultaneous, multiple-choice food trials. This approach summarizes the relative consumption of a food item, given the total amount of available food (Eq. 1) and overcomes the lack of independence of data derived from repeatedly measuring the preference for several food types by the same individuals. We calculated a preference index (p_i) as:

$$p_i = (C_i / A_i) / T \quad (\text{Eq. 1})$$

where C_i and A_i are the total amount of consumed and available food for oak family i , respectively, and T is:

$$T = \frac{\sum_{i=1}^N C_i}{\sum_{i=1}^N A_i} \quad (\text{Eq. 2})$$

with N being the total number of families.

Values of $p_i > 1$ and $p_i < 1$ indicate relative preference and avoidance for the corresponding family, given the choice offered.

7.2.2.4. *Data analyses*

Herbivory in the field

Preliminary analyses of defoliation data showed that the insecticide treatment did not kill all insect herbivores. However, it consistently reduced herbivory by 55% as compared to control (mean % leaf area consumed \pm SE: 4.6 ± 0.2 % and 10.2 ± 0.3 %, respectively). By contrast, there was no difference in mean herbivory between the control and the herbivore addition treatment (10.2 ± 0.3 % vs. 8.1 ± 0.2 %). These were hence pooled and the following analyses only distinguished plots with no insecticide treatment (hereafter +H, as more herbivory) vs. plots with insecticide (hereafter –H as less herbivory).

There might be no effect of PGD on herbivory at the plot level if herbivores have opposite preferences for different families. In order to unravel likely hidden effects, we used two complementary analyses to assess insect herbivory at the level of individual plants and of

experimental plots. Individual-level analysis allowed testing family specific resistance to herbivores and interactions between family identity and PGD. Aggregating data at the plot level made it possible to test possible non-additive effects of PGD on herbivory (Barton et al. 2014).

Insecticide treatment being applied at the block level (i.e., *whole block*), our design corresponds to a split-plot experiment which requires adapting the calculation of degrees of freedom and mean sum of squares of residuals (Altman and Krzywinski 2015). This was achieved using linear mixed effect models (LMM), with Block and Block \times Insecticide as random factors (1|Block: Insecticide in R syntax). At the individual sapling level, plot identity was included as an additional random factor, nested within block, to account for the fact that individual trees from the same family were pseudo-replicates within plots (Schielzeth and Nakagawa 2013). Mother tree identity (MT), insecticide treatment (+H vs. -H), PGD of the plot and their interactions were declared as fixed effects. The full model was simplified by sequentially removing non-significant interaction terms, starting with the highest order interaction, to finally retain the least parameterized models including only simple terms and significant interaction terms. Significance of parameters was assessed using χ^2 tests by comparing models with and without the term to be tested. Parameters corresponding to fixed effects were estimated by maximum likelihood. A log+1 transformation was applied to herbivory data to meet assumptions of homogeneity in variance and normality in residuals.

Analyses at the plot level were carried out using the method developed by Loreau and Hector (2001) and adapted by Unsicker et al (2008) to partition the net effect of PGD on herbivory into a complementarity effect (CE) and a selection effect (SE). Net, complementarity and selection effects were used to upscale observations from the individual plant to the plot level while accounting for family-specific differences. The net effect compares observed vs. expected damage in a given mixture, where expected damage is the mean of damage observed in component monocultures weighted by the proportion of families in the mixture. The full description of these indices is provided as supplementary material (Appendix S2). We used analyses of variance (ANOVA) to test for differences in herbivory for each effect (net, complementarity and selection effects), and applied two sided *t*-tests to determine if these effects were significantly different from zero.

Herbivore performance and preferences in feeding trials

Performance of gypsy moth larvae in single vs. mixed diet treatments was compared using ANCOVA (Raubenheimer 1995) with diet type as factor, initial larval weight as continuous covariate, and final weight as dependant variable. Biomass consumption per diet type was assessed using linear mixed effect models (LMM) with the replicate (i.e., rearing box identity) as random factor to account for the repeated measurements of the same set of three larvae. Feeding preferences were tested using LMM with oak family as fixed effect factor, replicate as random factor and preference index (p_i) as dependent variable.

All analyses were conducted in 3.0.2 version of R (R core Team 2013), using the *lmer* function from the *lme4* package (Bates et al. 2013). Contrast analyses were used to compare factor levels. To estimate model adjustments, R^2 were calculated following Nakagawa and

Schielzeth (2013). For each model, we calculated the marginal R^2 (R^2_m) corresponding to the proportion of variance explained by fixed effects and the conditional R^2 (R^2_c) corresponding to variance explained by fixed *plus* random effects.

7.2.3. Results

7.2.3.1. Data analyses

Herbivory at individual sapling level

In no insecticide plots (+H), herbivory was on average $9.1 \pm 0.2\%$ (mean \pm SE) of leaf area removed and ranged between 1.3 and 37.8%. Application of insecticide resulted in 50% reduction of herbivory ($4.6 \pm 0.2\%$).

Herbivory differed among oak families with the family MT2 being on average 1.27 times more damaged than the other three families (among which no difference was observed; Fig. 1A). Family-specific differences in herbivory were independent of the number of families in the plot (PGD), as indicated by the non-significant interaction with the mother tree variable (MT \times PGD, Table 1) and of insecticide treatment (no significant MT \times IT interaction).

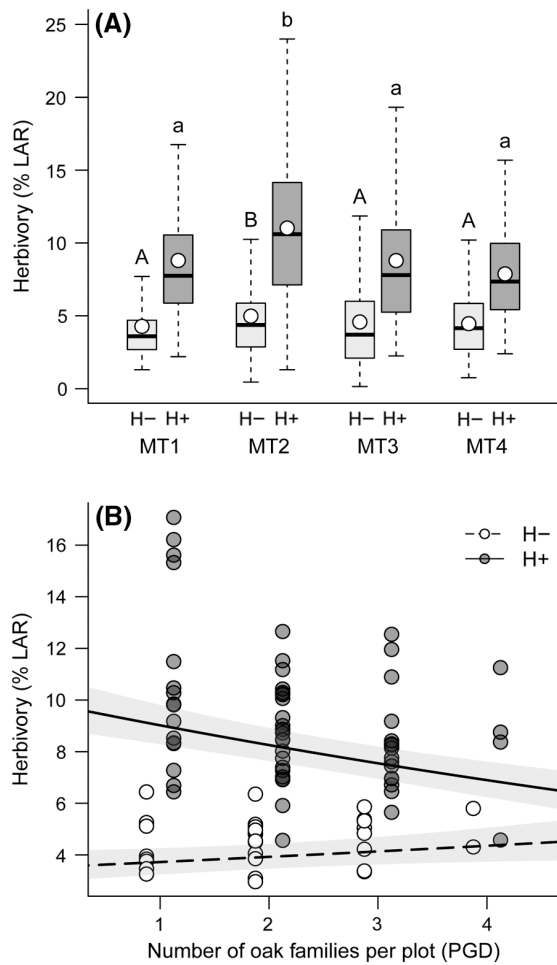


Figure 1: Effects of mother tree identity (A), number of oak families per plot (B) and insecticide treatment on insect herbivory at individual oak sapling level. (A) Boxes represent first and third quartiles. The horizontal line represents the median while dots correspond to the mean, across all plots. Different letters above boxes indicate significant differences in herbivory among families. (B) Dots represent mean herbivory in plots with different herbivore abundance. Regression lines and corresponding SE (indicated as shaded area) are predictions from mixed effect models averaged across the four families.

Table 1: Summary of linear mixed effect models evaluating the effects of insecticide treatment (IT, i.e. +H vs. -H), mother tree identity (MT, i.e. MT1, MT2, MT3 and MT4), plant genetic diversity (PGD) and their interactions on insect herbivory on oak saplings. Explanatory variables in bold character correspond to those retained in the final model after model simplification. Significance thresholds for χ^2 values: (.) $P < 0.1$, (*) $P < 0.01$, (**) $P < 0.001$, (***) $P < 0.0001$.

Model parameter estimates and standard error for the intercept correspond to the reference level for IT (-H) and MT (MT4).

Marginal R^2_m represents the variance explained by fixed factors while conditional R^2_c is interpreted as variance explained by both fixed and random factors. For the final model retained after model selection, they equalled 0.32 and 0.41 respectively.

Explanatory variables	χ^2	Parameter	Estimate	SE	df
		Intercept (MT4, -H)	1.541	0.145	69,8
IT	41,16 (***)	IT: H+	0.753	0.178	69,7
		MT: MT3	-0.056	0.175	991
MT	35,01 (***)	MT: MT2	-0.057	0.178	991,1
		MT: MT1	0.003	0.177	991
PGD	6,67 (*)	PGD	0.022	0.053	991
		IT: H+ \times MT: MT3	-0.039	0.214	991
IT \times MT	5,64 (ns)	IT: H+ \times MT: MT2	0.504	0.217	991,1
		IT: H+ \times MT: MT1	0.012	0.217	991,1
IT \times PGD	12,93 (***)	IT: H+ \times PGD	-0.108	0.066	991
		MT: MT3 \times PGD	0.019	0.075	991
MT \times PGD	4,67 (ns)	MT: MT2 \times PGD	0.063	0.076	991
		MT: MT1 \times PGD	-0.008	0.076	991
		IT : H+ \times MT: MT3 \times PGD	0.061	0.092	991
IT \times MT \times PGD	5,72 (ns)	IT : H+ \times MT: MT2 \times PGD	-0.143	0.093	991,1
		IT : H+ \times MT: MT1 \times PGD	0.032	0.094	991,1

The effect of PGD on herbivory was dependent on insecticide treatment (significant IT \times PGD, Table 1). In plots with no insecticide treatment (+H), herbivore damage decreased with the number of families (Fig. 1B). On the contrary, there was no effect of PGD on herbivory in blocks where the insecticide treatment had reduced herbivore density (-H).

Herbivory at plot level

Insecticide treatment affected the net and complementarity effects of PGD on insect herbivory but not the selection effect (Table 2).

Table 2: Effects of insecticide treatment on the net (NE), complementarity (CE) and selection effects (SE) of Plant Genetic Diversity (PGD) on insect herbivory on oak saplings. The table reports *F* values from ANOVAs and estimated means and 95% CI from *t*-tests ($\mu = 0$). Bold characters indicate significant differences between factor levels. Significance thresholds: (.) $P < 0.1$, (*) $P < 0.01$, (**) $P < 0.001$, (***) $P < 0.0001$.

		NE	CE	SE
ANOVAs	Insecticide treatment	F_{1,60} = 34.81 (***)	F_{1,60} = 29.58 (***)	F _{1,60} = 0.26 (<i>ns</i>)
	Block	F_{4,60} = 7.36 (***)	F_{4,60} = 7.68 (***)	F _{4,60} = 0.9 (<i>ns</i>)
<i>t</i> -tests	Insecticide (-H)	4.8 [-1.14, 10.73]	8.45 [2.17, 14.74]	-3.66 [-5.01, -2.3]
	No insecticide (+H)	-26.86 [-36.63, -17.08]	-20.66 [-30.78, -10.53]	-6.2 [-8.62, -3.78]

In blocks with high herbivore density (+H), mean defoliation was significantly lower in mixed plots than expected from the corresponding monocultures, *i.e.*, associational resistance. Three out of four families showed a reduction of herbivory in mixtures compared to their respective monocultures (Fig.2A). The negative net effect arose from both a negative complementarity and a negative selection effect (Table 2). The negative selection effect was mainly driven by MT2. It was the most susceptible family in monocultures (Fig. 1A) and the family for which the deviation from the 1:1 line (equal mean herbivory in monocultures and in mixtures) was the greatest, showing a large associational resistance effect (Fig. 2A).

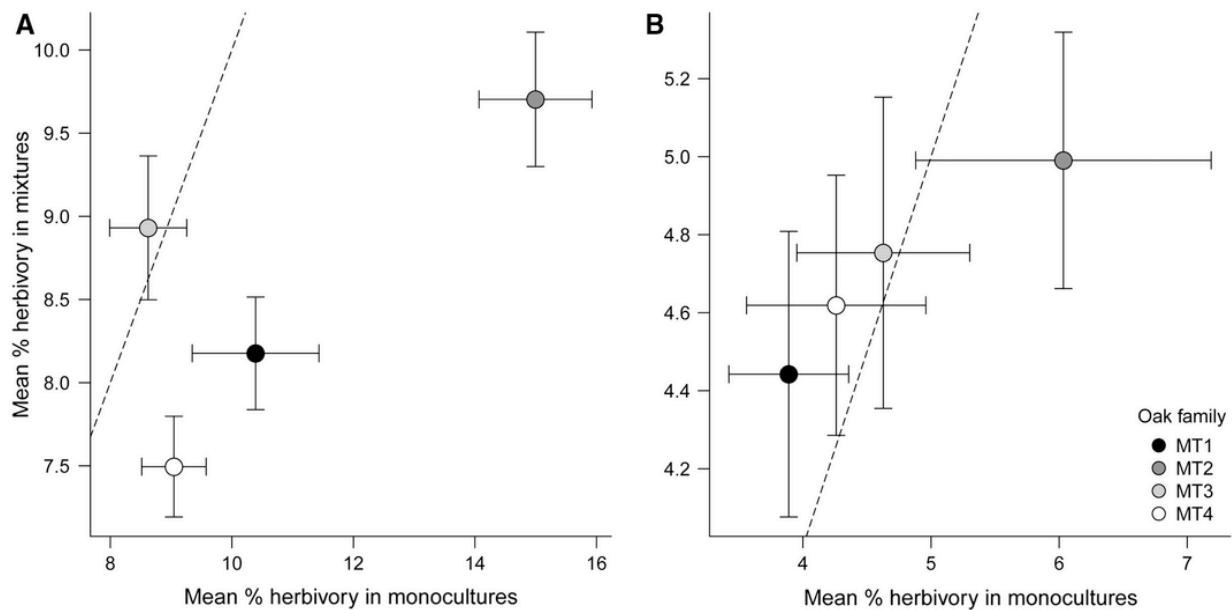


Figure 2: Mean insect herbivory on oak saplings of each family growing in monoculture or in mixtures in no insecticide plots (A) and insecticide plots (B). Dotted lines correspond to the $y = x$ line (not shown in diagonal to improve the distinction of families). Error bars indicate standard errors.

In blocks with lower herbivory density (–H), complementarity and selection effects had opposite signs, resulting in a non-significant net effect of family mixtures (Table 2). The significant, positive complementarity effect indicates that on average all families suffered higher herbivory than expected from component monocultures (indicating the existence of associational susceptibility). The negative selection effect indicates that the most resistant oak family in monoculture experienced disproportionately less damage in mixtures. Yet, family specific differences in herbivory between monocultures and mixtures were less pronounced than in plots with no insecticide treatment (Fig. 2B). Only MT2, the most susceptible family overall, showed a reduction of herbivory in mixed plots compared to monocultures.

7.2.3.2. Herbivore preferences and performance in feeding trials

Gypsy moth larvae clearly distinguished among the four oak families when offered in mixed diet ($F_{3,214} = 29.8$, $P < 0.0001$). The relative consumption of MT2 and MT3 leaves was higher than their proportion in the offered diet, indicating preferential feeding on these families (Fig. 3A). Conversely, larvae avoided feeding on leaves of MT1 while they were indifferent to leaves of MT4 (Fig. 3A). This preference pattern mirrored the difference in herbivory observed on oak families in the field experiment.

The type of diet (four single plus one mixed diets) had a clear effect on leaf consumption ($F_{4,283} = 12.5$, $P < 0.0001$). MT1 was the least consumed family in single diet treatments

(Fig. 3B). Interestingly, the mixed diet treatment was the one with lowest overall leaf consumption (Fig. 3B).

The relative growth rate of gypsy moth larvae differed among the five diet types ($F_{4,44} = 4.5$, $P = 0.004$, Fig. 3B). Larvae grew best on MT2 leaves and least on MT1 leaves, which was in accordance with preferences observed in the feeding choice experiment. Larvae consuming a mixture of leaves from the four families showed an intermediate growth rate (Fig. 3B).

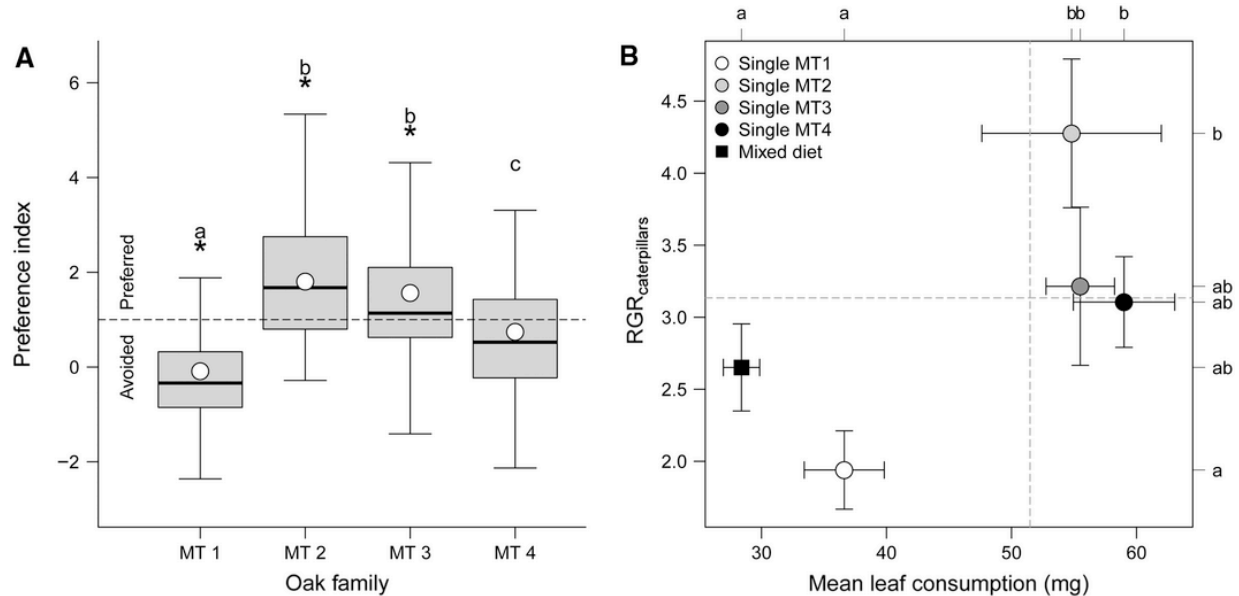


Figure 3: Preference and performance of gypsy moth larvae in single vs. mixed diet treatments. (A) The preference index indicates the relative consumption of a specific oak family by gypsy moth larvae in the mixed diet treatment. Boxplots represent median, 25th and 75th percentiles, respectively. The dashed horizontal line at $y = 1$ corresponds to the null hypothesis of neither preference nor avoidance. Asterisks indicate significant differences from $\mu = 1$ according to t -tests ($P < 0.05$). Different letters indicate differences between families in the mixed diet treatment. (B) Effects of diet type on leaf consumption and larval growth. Circles represent consumption and RGR in single diet treatments, with corresponding SE. Mean consumption and RGR in single diet treatments are shown by vertical and horizontal dashed lines, respectively. The effect of diet type was tested separately for consumption and growth using ANCOVAs. Letters on the top and right edges of the panel refer to contrast comparisons between treatments for leaf consumption (top) and RGR (right). Different letters indicate significant differences between treatments (at $P < 0.05$).

7.2.4. Discussion

We found experimental evidence that the amount of damage caused by insect herbivores on a given plant varies with the number of neighboring conspecific genotypes. However, the magnitude and direction of this relationship depend on both herbivore density (i.e., through insecticide treatment) and identity of plant genotypes.

Insect herbivory varies with oak genetic identity and diversity in interaction with herbivore density

In accordance with our first hypothesis, herbivory, herbivore preference and performance varied between oak families. In particular, oaks grown from mother tree MT2 experienced

more damage than the three other families in the field. Its leaves were also more consumed in non-choice feeding trials and were preferred over leaves of other families in choice trials. These results confirm the genetically-based variability in plant susceptibility to insect herbivores that has been observed in many other systems (Barbour et al. 2009, Barton et al. 2014) and suggest that despite the low number of families in the experiment, intra-specific variability in oak traits could be large enough to allow associational effects. Gypsy moth larvae fed on MT2 leaves also had greater growth rate as compared to larvae fed other leaves, which confirms that differences in plant resistance are consistent with differences in herbivore performances. Although patterns in the field were consistent with results from feeding trials, not all herbivores respond in the same way to plant genotype identity and the response of a single species (here *L. dispar*) may not be representative of the response of the whole herbivore community.

In addition to this identity effect we detected a diversity effect. Increasing the number of oak families per plot caused an overall decrease in herbivory, both at the individual and plot levels. This was however only observed when herbivore density was medium (i.e., in no insecticide plots, +H), whereas the effect of PGD was null in case of low herbivore density (i.e., in insecticide plots, -H). The observed decrease of insect herbivory with increasing plant genetic diversity is consistent with previous studies on willow (Peacock et al. 2001), evening primrose (Parker et al. 2010, McArt and Thaler 2013) and different crops (Tooker and Frank 2012). These results contrast with other studies reporting opposite or neutral effects of PGD on insect herbivory (Tack and Roslin 2011, Castagneyrol et al. 2012, Barton et al. 2014, Maldonado-López et al. 2015). None of these studies (to the best of our knowledge) has however assessed PGD effects under contrasted herbivore densities. Yet, it is increasingly acknowledged that herbivore population dynamics may depend on both herbivore density and variance in plant quality (Underwood 2004, 2010, Parent et al. 2014).

Mechanisms underlying associational resistance

Lower herbivory in mixed plots could arise from three non-exclusive mechanisms acting at different spatial scales: i) a relocation of herbivores within plots, sparing the three most resistant families at the expense of the most susceptible one, ii) an overall reduced consumption due to more effective exploitation of mixed diets by herbivores and/or iii) an active avoidance of plots containing less edible individuals. All three mechanisms rely on the same two premises: oaks from different families should differ in edibility and herbivores should be able to choose among them. Our field and lab experiments indicate that both premises are met in our study system, although the three ecological mechanisms received varying empirical support.

We cannot formally exclude the alternative hypotheses that variability in herbivory resulted from neighbor-mediated changes in plant traits such as anti-herbivore defenses (Moreira et al. 2014) or differential pressure of natural enemies upon herbivores (Moreira and Mooney 2013, Abdala-Roberts and Mooney 2014). However, we do not have data to test these assumptions.

Choice of individual plants within plots

A relocation of herbivores within plots was not supported by our individual level analysis, as we observed no family (MT) \times PGD interaction. This lack of interaction suggests that, on average, all oaks benefited from growing among neighbors from other families, regardless of their family identity. This interpretation is further supported by the observed negative complementarity effect, which indicates that, on average, all saplings experienced less damage in family mixtures than in component monocultures. Such an associational resistance has been observed in several other studies (Unsicker et al. 2008, Barbosa et al. 2009, McArt and Thaler 2013).

Avoidance of less suitable plots

We found some support for an active avoidance of entire plots containing less edible individuals. Theory predicts that associational resistance would be stronger for most sensible plants, and weaker for more resistant ones (Hambäck et al. 2014, Hahn and Orrock 2016). Family MT2 was consistently preferred in feeding trials and the most damaged in monocultures in the field. At the same time, the negative selection effect observed at the plot scale suggests that more susceptible families (i.e., especially MT2) benefited most from growing together with more resistant neighbors. Our result is therefore in line with the ‘*variance in edibility hypothesis*’ (Liebold 1989): more resistant plants can contribute to reduce herbivore recruitment in mixed plots more than expected from their sole abundance by ‘protecting’ more edible neighboring plants (Jiang et al. 2008).

Dietary mixing and reduced herbivore consumption

We found clearer evidence that the observed relationship between PGD and herbivory could have been driven by an overall reduced consumption of mixed diets by herbivores, as predicted by the dietary mixing hypothesis (Bernays et al. 1994, McArt and Thaler 2013). Our feeding trials revealed that leaf consumption was on average lower in the mixed diet treatment than in any single diet treatments (although not significantly different from consumption of the less edible family, MT1). Despite this reduced consumption, the growth of gypsy moth larvae was not lower in the mixed diet treatment than in single diet treatments.

Effects of host genetic diversity on herbivory are weak and herbivore density-dependent

The effect of PGD on insect herbivory was significant, but weak. It was only observed in "no insecticide" plots, where defoliation decreased from 10% in monocultures to 8% in four-family mixtures. Although low, such defoliation levels are quite common and consistent with background herbivory observed in trees at a global scale (Kozlov et al. 2015). Yet, even low levels of herbivory may have substantial negative effect on plant growth, especially in long-living trees (Zvereva et al. 2012).

So far, herbivore density has been studied as a response variable to local conditions and it was shown to be better explained by local environmental drivers than by host genetic diversity (Tack et al. 2010, Pohjanmies et al. 2015). Yet, the response of herbivore abundance and damage to plant diversity are poorly related (Rhainds and English-Loeb 2003, Barbosa et al. 2009, Utsumi et al. 2011, Karban et al. 2013, Parent et al. 2014, Carrasco et al. 2015). The

distinction between both aspects of herbivore response to PGD is not trivial because herbivore recruitment and actual plant consumption likely respond to different drivers (e.g., relative frequency of more or less palatable plants, plant nutritional quality, top-down control of natural enemies, Moreira et al. 2016). By addressing herbivory while controlling for herbivore density, our results provide new evidence that PGD effects on herbivory are density dependent. We cannot completely exclude that observed pattern resulted from the specificity of the insecticide action on particular herbivore species and further research will be needed to fully disentangle the effects of herbivore density from the composition of herbivore community. However, assuming that more herbivores exert a stronger pressure upon host plants, the density-dependent effect is consistent with other studies highlighting that effects of PGD on ecosystem functioning vary along ecological gradients and are often stronger in harsher environments where plants have to face stronger biotic (e.g., herbivory) or abiotic pressures (e.g., drought) pressures (Hughes and Stachowicz 2009, Kanaga et al. 2009, Parker et al. 2010, but see Drummond and Vellend 2012).

The overlooked density-dependency of plant-herbivore interactions may explain why previous studies addressing effects of plant genetic diversity on insect herbivory provided conflicting results. It is a promising direction for unravelling causes of ‘context-dependency’ in diversity-resistance relationships (Moreira et al. 2016). However, a deeper understanding of mechanisms at play will require a better experimental control of herbivore density. In particular, larger gradient of herbivore abundance should be used in order to compare the effects of PGD under background herbivory vs. outbreak conditions.

7.2.5. Acknowledgements

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