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Local neighbourhood tree species composition effects in young forest stands

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Local neighbourhood tree species composition effects in young forest stands

Impact of stress, species identity and multitrophic interactions

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by

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Dutch translation of the title: *Effecten van naburige boomsoortensamenstelling in jonge bosopstanden: impact van stress, soortidentiteit en multitrofe interacties*

Cover images:

Front, top: Side view of a phytometer plot in the FORBIO site of Zedelgem;

Front, bottom: Markings of larval ladybird mycophagy on a leaf of *Quercus robur* severely infected by oak powery mildew.

Back, top: On the left, mating *Psyllobora vigintiduopunctata* on an infected oak leaf. On the right, hatching ladybird eggs under microscope (picture Martijn Buyse).

Back, bottom: Side view of a mixed clone plot in the Short Rotation Coppice plantation in Zedelgem, containing the Swedish willow clones Klara and Gudrun.

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One does not simply finish a PhD. It is four years of fieldwork under the blistering rage of the sun. It is four years of fieldwork growing accustomed to the pleas of mosquitoes and horseflies, unless fate finds you an unexpected, radar-stealthy torrent of rain. Four years of embracing the punctuality of the Belgian rail services. Four years of staring at computer screens, making sense out of excel quirks, R errors and organically grown Matlab script cities, while wishing pain and misfortune to many a software developer. Four years of trying to keep tabs on an inevitable caffeine addiction, from uplifting milestone to manic panic as the deadlines byte past. Four years of scrounging the scientific literature for thousands of interesting and (less) interesting papers from Abrahamson to Zvereva, et al. Four years of seemingly never-ending chaos and drama and tremendous amounts of fun. Four years of meeting talking working and, above all, having fun with awesome people from all over the biosphere (or should I already say anthroposphere?). Four years of unforgettable stories that will last a lifetime. Four years of stress and panic and suffering, yet still all so amusing in retrospect. One does not simply finish a PhD. One lives through it, a quest for knowledge and experience, while aided by wizards and rangers and rogues from all sorts of backgrounds. So many people who played a role.

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Samenvatting

In de laatste twee decennia werd in verscheidene ecosystemen een positieve relatie tussen biodiversiteit en het functioneren van ecosystemen geobserveerd. Aangezien verlies van biodiversiteit nu optreedt op globale schaal, zou dit kunnen impliceren dat dergelijke ecosysteefuncties als productie van biomassa, nutriëntenretentie en fixeren van koolstof hierdoor negatief beïnvloed kunnen worden. Deze ecosysteefuncties zijn vaak belangrijk voor de mens, aangezien ze gelieerd zijn aan ecosysteemdiensten waar mensen afhankelijk van zijn. In het geval van soortenarme, beheerde productiesystemen zoals in landbouw of plantagebosbouw, kan het bijmengen van verschillende soorten resulteren in grotere productiviteit of sterkere resiliëntie jegens extreme weerfenomenen of uitbraken van plagen en ziektes. Bossen zijn opmerkelijke ecosystemen in deze context, vanwege hun lange levensduur en complexiteit. Dit maakt hen moeilijker te bestuderen, maar ook potentieel gevoeliger voor enige interactie tussen verhoogde stress en verminderde diversiteit: in hun lange levens is het risico op het voorkomen van ernstige stressfenomenen groter en de capaciteit zich hieraan aan te passen kleiner. Positieve effecten van het mengen van boomsoorten zijn geobserveerd in verscheidene bosccosystemen en sterkere diversiteitseffecten werden bevonden onder strengere abiotische condities. Tegelijkertijd benadrukte de lagere densiteit van individu's en diversiteit van boomsoorten in gematigde bossen het belang van de identiteit van welke soorten aanwezig waren.

De interactie tussen compositie-effecten en stress is in het bijzonder belangrijk met oog op andere globale veranderingen die de biosfeer aantasten tegelijkertijd met biodiversiteitsverlies, zoals koolstofverrijking van de atmosfeer en de oceanen. Van deze veranderingen wordt verwacht dat ze het risico op extreme abiotische gebeurtenissen zoals droogtes verhogen alsook het optreden van biotische uitbraken, vaak door uitheemse soorten. Positieve interacties tussen verschillende soorten worden geacht frequenter of belangrijker te worden onder dergelijke stressvollere condities, een idee dat vaak de Stress Gradiënt Hypothese wordt genoemd (SGH) en deels door onderzoek ondersteund wordt. Dit suggereert dat de positieve relatie tussen biodiversiteit en het functioneren van ecosystemen nog belangrijker zou kunnen worden in de toekomst, wanneer het optreden en de intensiteit van stress in ecosystemen verwacht worden te zullen toenemen.

Op verschillende manieren heeft men reeds de studie aangepakt van de impact van verschillende boomsoortensamenstellingen en stress op het functioneren van bosccosystemen. Anders dan bij metingen in bestaande bossen hebben nieuw aangelegde experimentele plantages het voordeel dat ze vaag of niet gekende variabelen zoals vroeger landgebruik of –beheer vermijden en toelaten specifieke, interessante soortensamenstellingen na te gaan en te vergelijken. Ze zullen echter

enkel representatief zijn voor bestanden van gelijkaardige leeftijd, waardoor het decennia zal duren om zo informatie te verzamelen over de impact van verschillende samenstellingen op volwassen bos. Toch blijven ze representatief op korte termijn voor jongere bestanden en het is bekend dat vooral bij jongere bomen plagen en ziektes aanzienlijke schade kunnen veroorzaken door groeivermindering en sterfte. Dit maakt hen tot ideale systemen om na te gaan hoe het aanpassen van boomsoortensamenstelling biotische schade zou kunnen mitigeren.

Verskillende hypothesen trachten te verklaren hoe soortensamenstelling de impact van plagen en ziektes zou kunnen beïnvloeden. Verschillen in vatbaarheid voor sommige plagen tussen verschillende boomsoorten kan hun vermogen de gevoelige soorten te beschadigen verminderen, een mechanisme dat bekend staat als associatieve weerstand onder de Grondstof Concentratie Hypothese. Ook kan een hogere boomsoortenrijkdom de activiteit van predators en parasitoïden beïnvloeden, door het aanbieden van alternatieve voedselbronnen en beschutting volgens de Natuurlijke Vijanden Hypothese. Jonge bomen zullen eveneens waarschijnlijker positieve effecten van beschaduwning door sneller groeiende buren ondervinden, een vaak bestudeerd geval van facilitatie in SGH onderzoek. De mechanismen die achter deze interacties tussen boomsoortencompositie, abiotische stress en multitrofe interacties met plagen en ziektes liggen blijven echter weinig bekend.

In deze thesis is het voornaamste onderzoeksdoel de effecten op groei van verschillende boomsoortensamenstellingen in jonge bosbestanden na te gaan, alsook of deze samenstellingseffecten gemoduleerd worden door stress zoals voorspeld door de Stress Gradiënt Hypothese. Ik wilde ook meer duidelijkheid scheppen over de mechanismen die achter deze effecten schuilen, specifiek de impact van plagen en/of ziektes en de rol van hun natuurlijke vijanden, maar ook het belang van beschaduwning, verschillen in biomassa allocatie en andere bestandskarakteristieken. Om deze doelen te halen, volgde ik drie jonge bosbestanden in België op: een Korte Omloophout plantage bestaande uit drie verschillende wilgenklonen en twee sites van het FORBIO project. Beide FORBIO sites bevatten vijf verschillende boomsoorten in plots bestaande uit één tot en met vier verschillende soorten. Om de Stress Gradiënt Hypothese te evalueren werden zaailingen van eik (*Quercus robur* en *petraea*) in potten geplant binnen de FORBIO sites en blootgesteld aan een gradiënt van verminderde regenval gedurende drie groeiseizoenen. Ten slotte werd een labo-experiment opgezet met eikenzaailingen, eikenmeeldauw (PM, veroorzaakt door *Erysiphe* sp.) en een obligaat mycofaag lieveheersbeestje (*Psyllobora vigintiduopunctata*) om meer inzicht te verkrijgen in deze weinig bekende multitrofe interactie en hoe ze beïnvloed werd door watercondities van de plant.

In de Korte Omloophoutplantage was de biomassaproductiviteit algemeen hoger in genetisch meer diverse plots, maar dit diversiteitseffect was statistisch niet significant.

Dit was deels te wijten aan een hoge, onverklaarde variabiliteit van de productiviteit, die niet duidelijk verbonden was aan klonale samenstelling, bodemkarakteristieken of bladoppervlaktetemperaturen. Desondanks waren voor die gemengde plots die wel meer opbrengst vertoonden, effecten van complementariteit algemeen sterker dan deze van selectie vanwege de aanwezigheid van een productievere kloon. Bestandskarakteristieken zoals de verdeling van biomassa tussen de stoven werden ook beïnvloed door de klonale samenstelling, met een minder gelijke verdeling in monoculturen van een minder productieve kloon. Ook al zou men dergelijk verschillen verwachten op basis van de verschillen in vitaliteit, kunnen deze nog steeds gevolgen hebben voor hoe deze systemen verder zullen functioneren, aangezien ze meer open plekken veroorzaken en mogelijk de kwaliteit van de te oogsten biomassa doen dalen.

In de FORBIO sites was er maar weinig effect van de regenvalverminderende gradiënt op relatieve groei of infecties door meeldauw, de meest voorkomende en schadelijke ziekte in beide sites. Dit was waarschijnlijk omdat de regenvalverminderende behandeling te weinig effect had op bodemvocht of omdat de behandeling gecompenseerd werd door water dat vanuit de bodem onderin de potten stroomde. Noch was er een effect van soortenrijkdom op relatieve groei. Voor de zaailingen die het minst regenwater kregen vond ik dat mycofagie door lieveheersbeestjes positief beïnvloed werd door boomsoortenrijkdom in de nabije omgeving, in Zedelgem in September 2014 tijdens een heel droge periode. Onder labo condities was mycofagie door lieveheersbeestjes op een gelijkaardige manier heel gevoelig aan veranderingen in watervoorziening voor de plant. Op een alsnog onbekende manier werd de kwetsbaarheid van de schimmel voor mycofagie door bewatering beïnvloed. Meeldauwinfecties werden op zich niet door bewatering beïnvloed. De hoge waarden voor mycofagie in mijn experimenten suggereren een potentieel voor biologische controle door deze lieveheersbeestjes, die vrij eenvoudig te kweken zijn. Er is nog echter onderzoek nodig naar daadwerkelijke impact van deze mycofagie op vitaliteit en groei van de plant.

Meeldauwinfecties waren erger in 2013 en 2015 in monoculturen in de abiotisch minder optimale site van Gedinne, ook al was de impact van meeldauw op groei hier eveneens minder. Boomsoortensamenstelling had behalve dit weinig impact op groei of meeldauw in deze site. In de rijkere site van Zedelgem had een Janzen-Connell effect van de aanwezigheid van eik zeer negatieve effecten op groei, gelieerd met ergere meeldauwinfecties. Gelijkaardige identiteitseffecten van de aanwezigheid van beuk en linde zorgden voor een hogere relatieve groei, waarschijnlijk vanwege minder competitie voor licht bij deze soorten op deze leeftijd. Een sterkere beschaduwning had aanzienlijk negatieve gevolgen voor groei in deze site. Contrasterend had meer beschaduwning initieel positieve effecten op groei in Gedinne. Beide sites verschilden in focale boomsoort (*Q. robur* in Zedelgem vs *Q. petraea* in Gedinne), maar in Gedinne

lagen de relatieve groeicijfers ook lager terwijl allocatie van biomassa naar de wortels en mortaliteit van de nabije FORBIO bomen veel hoger was. Dit suggereert dat verschillen in abiotische condities soortensamenstellingseffecten zouden kunnen modiëren. In dit geval zouden ze hun belang verminderen, hoewel de verschillen tussen beide sites te veelvoudig zijn om hier een sterk besluit uit te trekken. Het belang van identiteitseffecten onder optimalere condities, vooral het Janzen-Connell effect, zou kunnen suggereren dat het individueel mengen van verschillende boomsoorten in nieuwe plantages voordelig is. Desondanks moet men zien te vermijden dat competitievere soorten of klonen anderen doen verdwijnen en de negatieve effecten van competitie voor licht te minimaliseren.

Summary

In the last two decades, a positive relationship between biodiversity and the functioning of ecosystems has been observed in various ecosystems. Considering biodiversity is being lost now at a global scale, this could imply that such ecosystem functions as production of biomass, nutrient retention and carbon fixation may be negatively impacted as a result. These ecosystem functions are often important to mankind, as they are linked to ecosystem services humans rely on. In the case of species-poor managed production systems such as in agriculture or plantation forestry, admixing of different species may result in greater productivity or greater resilience against extreme weather events or outbreaks of pests or diseases. Forests are notable ecosystems in this context, due to their longevity and complexity. This makes them more difficult to study, but also potentially more sensitive to any interaction between greater stress and reduced diversity: in their long lives, the risk of great spells of stress occurring is higher and the capacity for adaptation to them lower. Positive effects of mixing tree species have been observed in multiple forest ecosystems and stronger diversity effects were noted under harsher abiotic conditions. At the same time, the lower density of individuals and diversity of tree species in temperate forests emphasized the importance of the identity of which species were present.

The interaction between composition effects and stress is particularly important in the face of other global changes affecting the biosphere concurrent with biodiversity loss, such as carbon enrichment of atmosphere and ocean. These changes are expected to increase the risk of extreme abiotic events such as droughts and the occurrence of biotic outbreaks, often by nonnative species. Positive interactions between different species are expected to become more frequent or important under such more stressful conditions, a notion often called the Stress Gradient Hypothesis (SGH) and partially supported by past scientific research. This suggests that the positive relationship between biodiversity and ecosystem functioning may become even more important in the future, when occurrence and intensity of stress in ecosystems are expected to increase.

Different approaches have been taken to study the impact of different tree species compositions and stress on forest ecosystem functioning. Unlike measurements in extant forests, newly established experimental plantations have the advantages of avoiding poorly known confounding variables such as past land use or management and allowing the assessment and comparison of specific species compositions of interest. However, they will only be representative for stands of similar age, so that it would take decades to collect information this way on the impact of different compositions in mature forests. Still, they are representative for young stands and it is known that in particular in younger trees, pests and pathogens can cause substantial

damage through reduced growth and mortality. This renders them ideal systems to assess how modifying tree species composition might mitigate biotic damage.

Different hypotheses attempt to explain how species composition might influence the impact of pests or pathogens. Differences in susceptibility to certain pests by different tree species may reduce their ability to damage susceptible ones, a mechanism known as associational resistance under the Resource Concentration Hypothesis. Increased richness of tree species may also impact the activity of predators and parasitoids, offering alternative food sources and shelter, under the Natural Enemies Hypothesis. Young trees are also more likely to enjoy positive effects from shading by faster growing neighbours, an often-studied instance of facilitation in SGH research. However, the mechanisms underlying these interactions between tree species composition, abiotic stress and multitrophic interactions with pests and pathogens are still poorly known.

In this thesis, the main research objective was to assess the effects on growth of different tree species compositions in young forest plantations and whether these composition effects were modulated by environmental stress as predicted by the Stress Gradient Hypothesis. I also wished to shed light on the mechanisms driving these composition effects, specifically the impact of pests and/or pathogens and the role of their natural enemies, but also the role of shading, biomass allocation and other stand dynamics. To address these objectives, I monitored three young forest stands in Belgium: a Short Rotation Coppice (SRC) plantation with three different willow clones and two sites from the FORBIO project, both containing five different tree species in plots consisting of one up to four different species. To evaluate the Stress Gradient Hypothesis, saplings of oak (*Quercus robur* and *petraea*) were planted in pots within the FORBIO sites and subjected to a gradient of reduced rainfall during three growing seasons. Finally, a laboratory experiment with oak saplings, oak powdery mildew (PM, caused by *Erysiphe* sp.) and obligate mycophagous ladybirds (*Psyllobora vigintiduopunctata*) was set up to gain more insight into this poorly known multitrophic interaction and how it was influenced by plant water conditions.

In the SRC plantation, biomass productivity was generally higher in more genetically diverse plots, but this diversity effect was not statistically significant. This was partially caused by high unexplained variability in productivity, not clearly linked to clonal composition, soil characteristics or leaf surface temperatures. Nevertheless, for these plots where overyielding compared to monocultures occurred, complementarity effects were generally stronger than selection effects of the presence of highly productive clones. Also, stand characteristics such as the distribution of biomass between stools were found to be affected by clonal composition, being more uneven in monocultures of a less productive clone. While such a difference could be expected based on differences in vitality, it might still have repercussions for the functioning of these systems, causing more gaps and possibly harvestable biomass of lower quality.

In the FORBIO sites, there was little effect of the reduced rainfall gradient on relative growth rates or PM infections, the most abundant and impactful pathogen in both sites. This was probably because the rainfall interception treatment had insufficient effect on soil moisture conditions or was compensated by water flowing from the soil below into the pots. Neither was there an effect of species richness on relative growth rates. For saplings receiving the least rainfall, ladybird mycophagy was found to be positively influenced by neighbourhood tree species richness when the weather was dry in Zedelgem in September 2014. Under laboratory conditions, ladybird mycophagy was found to be similarly sensitive to changes in water conditions, somehow increasing the vulnerability of the fungus to grazing by the ladybird. PM levels did not differ between different watering rates. High rates of mycophagy in my experiments suggest a potential for biological control of these ladybirds, which can be bred relatively easily, although the impact on plant vitality and growth remains to be investigated.

PM infection rates were higher in 2013 and 2015 in monocultures in the abiotically less favorable site of Gedinne, although the impact of PM on growth was lower here as well. Otherwise, tree species composition had little effect on growth or PM levels in this site. In the more favorable site of Zedelgem, a Janzen-Connell effect of oak presence associated with greater PM infectivity had strong negative effects on growth. Similar identity effects of beech and lime were associated with greater relative growth rates, probably due to reduced competition for light at this age. Greater levels of shading had substantial negative impacts on growth rates in this site. In contrast, greater shading had initially positive effects on growth in Gedinne. Both sites differed in focal tree species (*Q. robur* in Zedelgem vs *Q. petraea* in Gedinne), but in Gedinne growth rates were also lower, while biomass allocation to roots and mortality of the neighbouring trees was higher. This suggests differences in abiotic conditions may modify composition effects, in this case decreasing their impact, although the differences between both sites were too diverse to establish any strong claim. The importance of identity effects under more optimal conditions, in particular the Janzen-Connell effect, might suggest individual mixing of different tree species in new plantations to be beneficial. Still, care should be taken to avoid displacement by more competitive species or clones and to minimize the negative effects of competition for light.

List of abbreviations

BAI _r :	Relative Basal Area Increment
BEF:	Biodiversity and Ecosystem Functioning
EV:	Evenness of dry biomass per stool
FORBIO:	FORest BIOdiversity and Ecosystem Functioning
HI _r :	Relative Height Increment
LOX:	Lipoxygenase
MEA:	Millennium Ecosystem Assessment
MP:	Mycophagy
N:	Number of shoots
PCA:	Principal Components Analysis
PM:	Powdery Mildew
R:	Belowground dry woody biomass
S:	Number of stools
SGH:	Stress Gradient Hypothesis
SH:	Degree of shading
SLA:	Specific Leaf Area
SMC:	Soil Moisture Content
SP:	Species Presence
SR:	Species Richness
SRC:	Short Rotation Coppice
TBM:	Total dry woody biomass
TLA:	Total Leaf Area
UAV:	Unmanned Aerial Vehicle
VOC:	Volatile Organic Compound
W:	Aboveground dry woody biomass

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Chapter 1: General introduction

1. Biodiversity and Ecosystem Functioning

1.1. Background

During the last two decades, a considerable body of research brought about evidence of a positive relationship between biodiversity and ecosystem functioning (BEF) (Cardinale et al., 2012; Tilman et al., 2014). This may have important repercussions for the global trend of biodiversity loss that is currently underway (Barnosky et al., 2011; Ceballos et al., 2015), for it means that local disappearance of species may have a negative impact on ecosystem functions and the ecosystem services to man related to those functions (Millennium Ecosystem Assessment, 2005). Such negative impacts do not seem to be insignificant when compared to other global change drivers, such as climate change or acidification (Hooper et al., 2012; Tilman et al., 2012). Multiple possible mechanisms have been proposed and studied as explanations for the BEF relationship (Srivastava and Vellend, 2005). Different species may have different affinities for niches present in a system, meaning that their coexistence leads to a more efficient exploitation of the available resources – a mechanism often called niche complementarity (Liang et al., 2015). Morin et al. (2011) provide an example of how such a mechanism could operate in forest ecosystems, based on differences in shade tolerance and growth rate. Some species may also have a positive impact on the functioning of others (facilitation), for instance by ameliorating stressful growing conditions or diminishing the pressure of herbivores and pathogens (Callaway, 2007).

On the other hand, the inclusion of more species leads to a higher probability that a species with a very strong effect on ecosystem functioning is among them – a phenomenon that is often called the selection effect (Hector et al., 2002). This was initially considered an artifact of how BEF experiments were designed and not representative of actual ecosystems, which do not consist of random species assemblages (Wardle et al., 2000). Later authors attempted to address this issue by quantifying the selection effect separately (Loreau and Hector, 2001) and still found persistent, positive BEF relationships due to other mechanisms (Cardinale et al., 2007; Fargione et al., 2007; van Ruijven and Berendse, 2009). Additionally, greater diversity leads to a higher probability that in the long term a species with a very strong effect on functioning under certain environmental conditions will be present. This can be linked to an increased stability of these ecosystem functions, due to insurance and possibly compensatory feedback effects by resistant species (Isbell et al., 2009; Srivastava and Vellend, 2005). Still, other contentions continue to be raised concerning the representativity of the methods employed to study BEF relationships (Eisenhauer et al., 2016; Wardle, 2016). Typically, experiments induce random removal of species (e.g. Flombaum and Sala, 2008) or design random species assemblages (e.g. Marquard et

al., 2009), which may not be representative for real biodiversity loss scenario's (De Laender et al., 2016). In addition, the assumption behind these kind of experiments and studies is that local diversity is globally on the decline, whereas there is evidence that this might generally not be the case at all (Sax and Gaines, 2003; Vellend et al., 2016, 2013).

However, the importance of this research field stretches further than merely an attempt to understand the potential consequences of global biodiversity loss (Vellend et al., 2013; Verheyen et al., 2015). Humans have been engineering the ecosystems they live in for their own good at least since the dawn of sedentary agriculture (Ellis, 2015). Gradually and in particular during the industrial revolutions of the last two centuries, this practice has become more widespread, more intensive and in general more important, both to the humans dependent on it and in terms of how this practice influences the processes of the entire biosphere (Ellis et al., 2010; Kareiva et al., 2007; Vitousek et al., 1997). This trend is expected to continue, as demand for ecosystem services is expected to grow while the supply is considered surprisingly constant (Foley et al., 2011; Rockström et al., 2009; Running, 2012). If there is some sort of positive relationship between biodiversity and the functioning of ecosystems, introducing or maintaining diversity in these human ecosystems may be interesting or even crucial for optimal delivery of their services to man (Bommarco et al., 2013; Paquette and Messier, 2010).

On the other hand, positive effects of greater species diversity may not outweigh ecosystem services delivered by monocultures of dominant species, such as typical cash crops. Historically, production systems in the 20th century have seen a strong trend towards specialization (i.e. the Green Revolution) and this trend is considered to be still ongoing (Davies, 2003; Sanchez et al., 2009). This has allowed agriculture to strongly improve its yields per unit area, at the expense of increased management intensity and services other than productivity the systems provided (e.g. supporting biodiversity and pollination, Davari et al., 2010). For forests, a similar though less spectacular picture can be found in the form of conversion to or establishment of plantation forestry of high yielding or high value species (Fox et al., 2004; Paquette and Messier, 2010; Sheil et al., 2009). As theoretical support for this approach, Grime's 'Humped-back' model of the relationship between productivity and realized species diversity (Fraser et al., 2015; Grime, 1973) suggests that, at least for the ecosystem service of biomass production, the most optimal systems will be rather species-poor. The rarity of transgressive overyielding, where a mixture's productivity is greater than that of any monoculture of its constituent species, also seems to support specialized species-poor production systems. While the Humped-back model has recently been disputed (Adler et al., 2011; Laanisto and Hutchings, 2015), its predictions may not hold in the long term either, when environmental and ecological conditions are subject to

change. This is of particular importance for long-lived systems such as forests, where adaptation in the short term is impossible.

1.2. BEF in forests

Mixing tree species to improve productivity and other functions was considered by early forestry scientists, but was mostly dismissed as maximal yields were found for monocultures of highly productive species (Pretzsch, 2005). In the wake of the pioneering BEF experiments in grasslands, interest into mixing tree species in plantations resurfaced, partially driven too by concern for the impact of climate change and invasive diseases (Carnol et al., 2014; Thompson et al., 2009). There was also increased attention for increasing the multifunctionality of tree plantations beyond production of woody biomass, which is expected to be associated with greater positive impact of mixing species (Gamfeldt et al., 2013; Paquette and Messier, 2010; van der Plas et al., 2016a). Some early positive effects of increased tree species on productivity and stability of productivity were found in forest stands (Caspersen and Pacala, 2001; Forrester et al., 2006; Potvin and Gotelli, 2008).

However, the slow development of these systems and their enormous size render research time-consuming, expensive and subject to more potentially confounding variables (Leuschner et al., 2009). In addition, forests differ in multiple ways from systems that are faster to develop and easier to study, such as grasslands (Scherer-Lorenzen et al., 2005a). The most important way in which trees differ from herbaceous plants is their life cycle, as it operates on the scale of decades and often much longer, rather than within the boundaries of one or a few years (Scherer-Lorenzen et al., 2007). This increases the risk of an extreme event of abiotic or biotic pressure taking place in an individual tree's lifetime. The impact of such events is also more severe for the services delivered by these stands, as these services are often tied to functions that depend on cumulative processes such as woody biomass production and carbon fixation. Hence, events causing dieback, mortality or growth reduction can be expected to have longer lasting impact on functioning, as compensatory dynamics or adaptation through regrowth or recruitment are much slower than in grasslands (Forrester and Buhus, 2016). Other than a higher risk and frequency of major disturbances within a tree's life cycle, forests will also be subjected more to the effects of gradual directional changes, such as rising temperatures and changing weather conditions due to Climate Change (Lindner et al., 2008). This greater stability of functioning in the face of stress or disturbance in more diverse forests may outweigh the greater maximal yields of highly productive monocultures (Knoke et al., 2005).

Mature tree ecosystems are dominated by a few individuals with large biomass (Scherer-Lorenzen et al., 2005a). In this light, they are more likely to be subject to the mass ratio hypothesis (Grime, 1998), which asserts that identity effects of dominant

species will have the bulk of the impact on ecosystem functioning rather than the diversity within the entire community (Diaz et al., 2007). Diversity among tree species in the forests of temperate Europe in particular is known to be quite low (FOREST EUROPE, 2015; Leuschner et al., 2009), more so when these trees need to be adapted to a certain set of growing conditions. Other eco-regions at the same latitude such as eastern North America have more species, possibly because they faced less evolutionary bottle-necks in the recent past (Little Jr, 1983) or because of differences in climate not related to latitude (Adams and Woodward, 1989). Lower latitudes generally have much greater numbers of species, as described by the Latitudinal Diversity Gradient (Hillebrand, 2004). But it is in comparison with herbaceous systems, which can also support a number of individuals some orders of magnitude larger, where this difference in species pool is most striking (Little Jr, 1983). It can hence be expected that there will be a lower frequency of interspecific interactions between dominant individuals in these forest ecosystems. This facilitates the study of mechanisms that give rise to these interactions, already reinforced by their greater identifiability (Scherer-Lorenzen et al., 2007), but also emphasizes the role of which species are present (i.e. identity effects) perhaps more so than how many.

Large (meta-)analyses in forests have shown overall positive effects of mixing tree species on productivity (Liang et al., 2016; Paquette and Messier, 2011; Vilà et al., 2013; Zhang et al., 2012) and other ecosystem functions or services (Felton et al., 2016; Gamfeldt et al., 2013; van der Plas et al., 2016b). However, Nadrowski et al. (2010) reviewed 13 studies that considered identity as well as diversity effects in forests and found that identity effects were stronger in general. Other authors have also reported stronger effects of identity than richness on the functioning of forest ecosystems (Firn et al., 2007; Ratcliffe et al., 2015; Setiawan et al., 2016; Sobek et al., 2009; Tobner et al., 2016). Most studies on the influence of tree species diversity reported humped or stabilizing relationships with biomass production, suggesting considerable functional redundancy in forest ecosystems (Forrester and Bauhus, 2016; Vilà et al., 2013; Zhang et al., 2012). Nevertheless, these findings of positive effects of mixing tree species on various ecosystem functions and their stability in particular, are still considered to support the implementation of greater tree diversity into managed systems, even in those plantations where productivity remains the most important service (Liang et al., 2016; Piotta, 2008).

In some cases, more diversity may turn out to have negative effects on ecosystem functioning and associated services. For instance, complementarity in resource use such as different rooting depths may lead to more efficient use of soil resources, which may exacerbate conditions of scarcity (Grossiord et al., 2014). Generalist herbivores may benefit from increased fitness if the diversity of their food is higher (Castagneyrol et al., 2012; Schuldt et al., 2015; Vehviläinen et al., 2007). The potential link between host

diversity and pathogen transmission - described by some authors as negative (Ostfeld and Keesing, 2012) – has raised much debate, with recent reviews (Civitello et al., 2015a, 2015b; Salkeld et al., 2013; Wood and Lafferty, 2013) suggesting the relationship to be much more complex. Pathogens requiring multiple hosts for their life cycle may benefit from increased diversity and more diverse systems may set the stage for streamlined bugs posing serious ecological challenges when they can invade other areas (Cheatham et al., 2009). Most of these downsides have in common that they are a result of trophic interactions. This sort of diversity disservice is unavoidable, as the MEA concepts have been defined with respect to a human standard (Millennium Ecosystem Assessment, 2005). If humans are to benefit from maintaining diversity, organisms living off of similar niches as humans will likely benefit as well. Maintaining genetic or even species diversity among the crops in production systems could benefit man, but if so this will also benefit organisms that thrive within the same niche.

2. Stress Gradient Hypothesis

2.1. Background

One hypothesis that has been evaluated in literature almost at the same time as the BEF-relationship is the Stress Gradient Hypothesis (SGH), which proposes that positive interactions between individual organisms (facilitation) become more frequent as the degree of stress or disturbance *sensu* Grime (1977) in the system increases (Bertness and Callaway, 1994). Under mild conditions, plants growing with rival plants nearby suffer from negative, competitive interactions. Under unfavorable conditions such as increased water stress or consumer pressure, the negative competitive effects of these neighbours, if any, are outweighed by the positive impacts they can have, such as shelter to diminish evapo-transpiration or associational unpalatability to diminish herbivore pressure. Most of SGH research has focused on abiotic stress, in particular water stress. While multiple authors found supporting evidence in a variety of plant systems (Callaway et al., 2002; Eränen and Kozlov, 2008; Forey et al., 2016; He et al., 2013; Michalet et al., 2014), conflicting results have also been published, often suggesting a breakdown of facilitation in very extreme conditions (Koyama and Tsuyuzaki, 2013; Maestre et al., 2005; Soliveres et al., 2014). This lead to calls for a more refined approach to predicting species interactions along environmental gradients, taking into account nonlinearity of the shifts, the role of species functional traits and the nature of the stress gradient (Butterfield et al., 2015; Maestre et al., 2009; Soliveres et al., 2014). Some authors described how inclusion of biotic disturbances could modify the theoretical predictions of the hypothesis (Bulleri et al., 2011; Smit et al., 2009).

Initially, Bertness and Callaway (1994) referred to the frequency of interactions between individuals within a community or an ecosystem. Yet most later studies looked at either the intensity or the importance of these interactions (López et al., 2016; Maestre et al.,

2009; Soliveres et al., 2014), two separate concepts which find their origin in a paper by Welden and Slauson (1986). Intensity refers to the relative or absolute effect an interaction (be it competition or facilitation) has on a property of the focal individual, whereas importance scales this effect to an optimal value of that property and hence puts it in context next to other drivers (Brooker et al., 2005). Both intensity and importance have typically been quantified by studying pairwise interactions between benefactors and beneficiaries of facilitation. It is not always clear how such pairwise interactions may scale up towards the community or ecosystem level. Assessments at the community level also show only partial support for the SGH (López et al., 2016; Maestre et al., 2010; Soliveres and Maestre, 2014).

2.2. BEF and SGH?

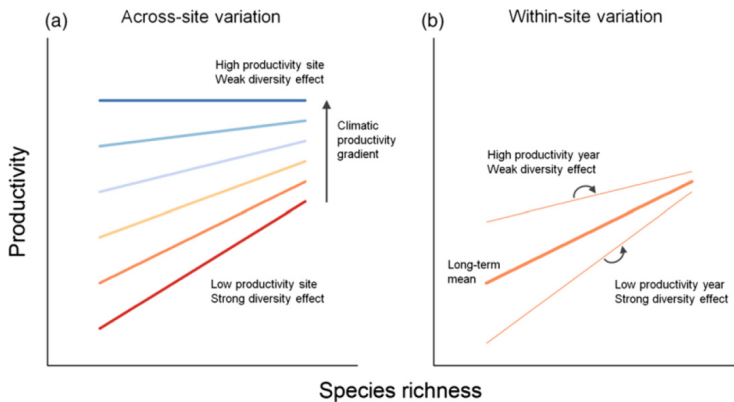


Fig. 1: Interaction between species richness and changes of productivity in space (a) and time (b) due to the occurrence of stress, as predicted from positive BEF relationships and the SGH. Figure from Jucker et al. (2015).

One thing both research into BEF and SGH have in common is facilitation, which is predicted according to the SGH to be more common, more intense and/or more important under stressful conditions. In BEF research, it is a mechanism that can explain the presence of diversity effects (Srivastava and Vellend, 2005), i.e. the added effect of a benefactor species improving a property of a beneficiary species so that its expected value is higher than found when the benefactor species is replaced with a neutral one (Callaway, 2007). Therefore, if the implications of both hypotheses hold in a given system and at least a part of the diversity effect can be attributed to facilitation, the SGH would suggest increased stress or disturbance to strengthen the impact of the diversity effect on ecosystem functioning (Fig. 1). Steudel et al. (2012) found evidence of this sort of interplay between stress and diversity for algae in microcosm experiments along two different stress gradients. The analysis by Paquette and Messier (2011) of

Canadian forest inventory data also suggested this sort of interaction along a latitudinal gradient. Other researchers have found similar results for forest ecosystems along climatic and productivity gradients (Jucker et al., 2015; Toïgo et al., 2015).

In the context of anthropogenic global change, this interplay between stress and diversity effects might become more emphasized. If, for instance, extreme weather events such as heat waves or drought spells grow more severe and/or frequent as a consequence of Climate Change (Dai et al., 2004; Meehl and Tebaldi, 2004), an increased presence of facilitating interactions between different species under such circumstances may play a more important role in maintaining the functioning of ecosystems (Pretzsch et al., 2012). Moreover, this sort of higher level interaction is not as likely to be noted in classic BEF experimental setups, where environmental fluctuations are supposed to be minimal as to not confound the experimental results (Steudel et al., 2012). In this sense, facilitation can also be considered a mechanism that explains ecosystem stability.

Changes in consumer pressure can similarly interact with diversity effects: invasive herbivores and pathogens may reinforce the importance of positive interactions hampering their invasion success (Guyot et al., 2015; Kennedy et al., 2002), like associational unpalatability (reduced consumer pressure due to the presence of unpalatable species) or more efficient exploitation of all available niches (Davis et al., 2000; Stachowicz and Byrnes, 2006). Native herbivores or pathogens too may be more active or abundant as a consequence of Climate Change (Lindner et al., 2008; Lu et al., 2015; Netherer and Schopf, 2010).

3. Research approaches

Confirmation of the validity and extent of BEF and SGH relationships can be found in multiple ways, each with their own strengths and weaknesses. Nadrowski et al. (2010) called these properties representativity (for real ecosystems), comprehensiveness (i.e. including all variables of interest) and orthogonality (i.e. controlling for confounding variables). Results can be obtained most quickly by applying ecosystem models to predict biotic interactions and their consequences for ecosystem properties (e.g. Morin et al., 2011; Yachi and Loreau, 2007). This approach may reveal the theoretical potential for positive species interactions across long time scales and under different environmental conditions, but may not be wholly representative of ecological reality (Morin et al., 2014). To this end, models may be supported by analyzing existing datasets such as national forest inventories, which tend to be quite representative of their subject ecosystems. Yet, such datasets may not include all variables of interest and their underlying methodology may not allow the elimination of certain confounding variables (Forrester and Pretzsch, 2015). To this end, new data can be collected from existing ecosystems with specifically positive species interactions in mind. To diminish

the impact of confounding variables even further, new experiments can be established where specific treatments can be evaluated (Scherer-Lorenzen et al., 2005b). Such experiments may also allow the assessment of expected environmental conditions from global change scenario's or ecological conditions that are otherwise rare or suspected to be strongly confounded, such as certain species combinations (Verheyen et al., 2013). However, due to their specified design, experiments may not be very representative of ecological reality. They are also the most difficult to account for temporal effects, as they will be studied in real time, which is particularly problematic for long-lived systems such as forests.

To compensate for the various downsides of each technique, it might be best to adopt them all and integrate their different results (Scherer-Lorenzen, 2014). A European project looking into effects of tree species diversity on ecosystem functioning (FunDivEurope) did exactly that (Baeten et al., 2013), combining data from forest inventories, from an exploratory platform in multiple European forests and from multiple recent plantations with orthogonal species composition gradients (Verheyen et al., 2015). An alternative approach is to interpret results from these different approaches within their scope and limitations. The temporal constraints on newly planted forest experiments are much less severe if interactions between species at the current successional stage are of interest. Young stands differ in lots of ways from mature forests, where dominance of individuals is much higher and hence the occurrence of pairwise interactions between trees much more limited. Young trees may also differ in their susceptibility to stress, herbivory and pathogens, with less reserves to survive through extreme events or strengthen their defenses (Barton and Hanley, 2013).

4. Mechanisms

Another advantage of monitoring carefully designed experiments is that it becomes easier to determine more specifically the mechanisms that are driving composition effects. This is in particular interest in forest ecosystems, where the dominant trees tend to have lower density and species diversity than other plant systems (Scherer-Lorenzen et al., 2007). In addition to increasing the importance of identity effects of certain species, this also makes it easier to investigate and even quantify species-specific pairwise interactions (Pretzsch et al., 2015). Crown interactions may already occur before crown closure as a result of different juvenile growth rates of different species, which may have negative effects of light interception but also positive effects of shelter (Castagneyrol et al., 2013; Valladares et al., 2016). Indirect effects such as mitigating herbivory and disease may additionally play a more important role, in particular because such pressures are often worse in younger trees (Barton and Hanley, 2013; Sobek et al., 2009).

4.1. Herbivores and pathogens

Two hypotheses attempt to explain why a higher diversity may reduce the impact or efficacy of herbivores and pathogens: the Resource Concentration Hypothesis and the Natural Enemies Hypothesis (Castagneyrol et al., 2013; Jactel et al., 2005; Sobek et al., 2009). Under the former, the reduced density in mixtures of hosts for specialist herbivores and pathogens increases travel time, risk and energy cost required for feeding or colonization. It also reduces the chance and frequency of attack by physically or chemically hindering herbivores from finding their preferential species and causing phenological mismatch with at least a part of the host population (Jactel et al., 2005; Peacock et al., 1999). According to the Natural Enemies Hypothesis, more diverse communities support more predators or more effective hunting behavior, either through providing shelter and necessary food supplements or allowing for (temporal) diet mixing (Jactel et al., 2005). It should be noted that the mechanism of resource concentration may also impair the behavior of predators, in particular if they have a fairly specialist diet themselves.

Pathogens typically have no natural enemies – although powdery mildew is an interesting exception (Sutherland and Parrella, 2009). They can be expected to be affected to some extent by resource concentration, but their colonization behavior is often passive, ruling out many of the mechanisms behind this hypothesis. Peacock et al. (2001) compared the phenology and severity of damage by leaf beetles (*P. vulgatissima*) and by leaf rust (*Melampsora* sp.) along gradients of willow (*Salix* sp.) clonal mixing in a Short Rotation Coppice plantation. They found less damage as genotype diversity increased, but also different colonization patterns. Leaf beetles showed more directional colonization to host plants, whereas rust spread radially from infected trees, causing high correlation between infection rates within one tree. This suggests that a physical barrier effect of resistant individuals can be an important driver of diminished infection rates of pathogens with similar dispersal mechanisms, such as powdery mildew. This way, the presence of non-host species amidst a passively spreading infectious species could reduce infection rates because a larger fraction of spores will be captured by insusceptible individuals (Hantsch et al., 2013).

4.2. Impact of stress

Louthan et al. (2013) list three hypotheses governing the effects of herbivores across abiotic stress gradients. The Compensatory Continuum Model emphasizes the ability of plants to compensate for biomass losses to herbivory. As stress will impair this ability, herbivory will be worse on plants subjected to more stress. In contrast, the Herbivore Pressure Hypothesis proposes that herbivory will have a greater impact in unstressed systems, because the abundance and diversity of herbivores will be greater there. Similarly, the Differential Growth Rate Hypothesis also predicts a greater impact with

lower stress, but as a result of the higher potential growth rate of plants under these conditions. The latter two are obviously related, but they emphasize different sides of the system as causing the discrepancy along gradients of stress.

The interaction between environmental (abiotic) stress and biotic stress in respect to plant interactions has been discussed by Smit et al. (2009). They referred to earlier studies incorporating biotic stress and thus finding a humped relationship between biotic stress and positive interactions, the optimum being intermediate due to a 'collapse' at the extreme end of the gradient. Using theoretical examples based on the life strategy distinction proposed by Maestre et al. (2009), they show that the interplay between biotic and abiotic stress can strongly alter an expected relationship with only one of these drivers. Based on this, they propose two hypotheses. The first is similar to the latter two in Louthan et al. (2013) in that biotic stress is expected to be most severe at lower abiotic stress levels, due to the greater abundance of food at low stress levels and a correlation between stress tolerance and unpalatability. From this they propose that the unimportance of biotic stress at high abiotic stress levels diminishes the impact of associational resistance mechanisms under these circumstances and that thus the importance of associational resistance increases with decreasing abiotic stress.

The second hypothesis they propose is related to the Exploitation Ecosystem Hypothesis (Oksanen and Oksanen, 2000), which states that herbivore control is most important at intermediate abiotic stress levels. At high levels of abiotic stress, these stressors will be the major drivers of many ecosystem processes. At low levels, herbivore populations will be sufficiently large to support top-down control by predators. Combining both hypotheses leads to a modification of the Stress Gradient Hypothesis to a humped relationship, with facilitation being most important at intermediate levels of abiotic stress corresponding to the most impactful levels of biotic stress.

This model assumes that the abiotic stress is of equal importance for every species and individual in the system. Patch dynamics, age differences and species traits may undermine this assumption. In the extreme, where a focal individual is stressed amidst relatively unstressed circumstances, the direct relationship between level of stress and herbivore population is irrelevant. Under such circumstances, a mechanism more akin to the Compensatory Continuum Model could be expected, with herbivory being worse in the presence of abiotic stress *ceteris paribus* and any mechanism of associational resistance subsequently more important. However, if phenotypic variation as a result of abiotic stress correlates with resistance to herbivory, the model as detailed by Smit et al. (2009) could still hold.

5. Research Questions

The main research objective of this thesis is to assess the effects of different local neighbourhood compositions on growth in young temperate forest systems and whether

these composition effects are influenced by stress as derived from the Stress Gradient Hypothesis. Furthermore, I wish to shed light on the mechanisms underlying these composition effects by looking at different tree and stand characteristics, including symptoms of damage by herbivores and pathogens on leaves and the degree of shading. To assess these composition effects, I monitor two young forest systems (Fig. 2): a multiclonal willow (*Salix* sp.) Short Rotation Coppice plantation (**Chapter 2**) and a multitrophic system of young oak (*Quercus*), oak powdery mildew and mycophagous ladybirds subjected to a local neighbourhood composition gradient and a stress gradient of reduced rainfall under field conditions (**Chapter 3-6**).

In **Chapter 2**, I test whether clonal diversity has a positive effect on productivity and other stand characteristics in a willow Short Rotation Coppice plantation in the North-West of Belgium, consisting of monocultures and mixtures of three different Swedish willow clones. Measurements were performed in 2011 and 2013, i.e. two and four years after planting. I differentiated between effects of complementarity and selection of highly productive clones. Additionally, I gathered data of leaf temperatures using an Unmanned Aerial Vehicle (or 'drone') to determine a suspected abiotic gradient in soil conditions and evaluate its impact on productivity and clonal interactions.

In **Chapter 3**, I describe an experiment performed in two young forest plantations in the North-West and South of Belgium. Both forest plantations are a part of the FORBIO project, itself a part of the TreeDivNet network, and contained monocultures and mixtures of up to four different species out of a pool of five site-adapted and silviculturally relevant tree species. Within these experiments, I planted young oak (*Quercus robur* and *petraea*) saplings in pots and subjected them to a three-level stress gradient of reduced rainfall throughout 3 growing seasons (2013-2015). Linking relative annual diameter and height growth rates to neighbourhood tree species composition and to oak powdery mildew (*Erysiphe* sp., PM) infection assessments as well as the degree of shading, I investigate the role of species composition, how these biotic interactions may arise and how they interact with the reduced rainfall gradient. In particular, I separated composition effects of richness from effects of identity of the tree species in the local neighbourhood of the focal saplings.

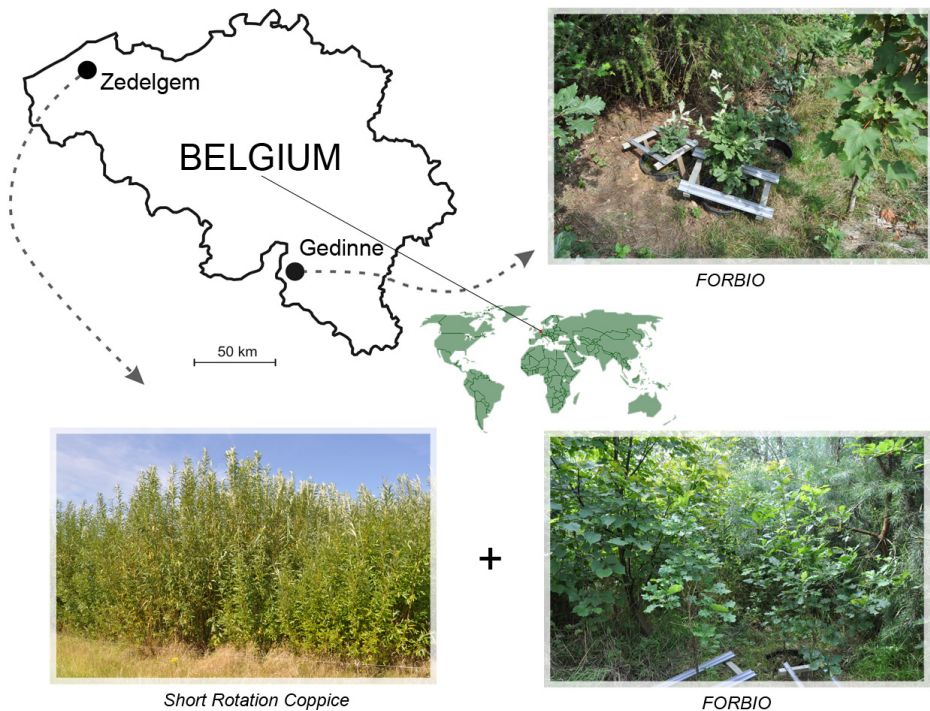


Fig. 1: Location of the different field sites in Belgium I made use of in this thesis. Two of the FORBIO sites were used, in Zedelgem and in Gedinne. Also in Zedelgem, we monitored a Short Rotation Coppice experimental site with mixtures of *Salix* clones.

In **Chapter 4**, I add another trophic level to the experiment of chapter 3 by monitoring the amount of mycophagy on oak PM by a native obligate mycophagous ladybird species (*Psyllobora vigintiduopunctata*) in one of the two sites in 2014. I attempt to link mycophagy to tree species composition as well as the reduced rainfall gradient and their interaction. I also describe a lab experiment where I monitor ladybird mycophagy in function of different watering treatments in a better controlled environment. Ladybirds used for this lab experiment had to be kept in captivity and bred, a program which I describe in detail in **Chapter 5**. In this chapter, I also describe various biological and ecological characteristics I observed of this poorly known ladybird species.

Finally, in **Chapter 6** I measure different wood and leaf characteristics of the saplings from Chapter 3 and link them to species composition, the reduced rainfall gradient, oak PM and shading, in an attempt to elucidate underlying mechanisms of species richness and identity effects.

Chapter 2: Productivity, stand dynamics and the selection effect in a mixed willow clone Short Rotation Coppice plantation.

Dillen, M., Vanhellemont, M., Verdonckt, P., Maes, W.H., Steppe, K., Verheyen, K., 2016. Productivity, stand dynamics and the selection effect in a mixed willow clone short rotation coppice plantation. Biomass and Bioenergy 87, 46–54.

Abstract

Short Rotation Coppice (SRC) is a promising method of biomass production for energy purposes, but there have been concerns about the low diversity of these stands, including the risk for pest outbreaks. Mixing different clones has been advised as a way to tackle this problem and improve yields through positive diversity effects. Recent research into the relationship between biodiversity and ecosystem functioning supports these recommendations, but also raises worries about mechanisms that may confound results, such as a selection effect due to dominant clones outcompeting weaker ones. However, the few available studies on diversity effects in SRC plantations did not allow the disentangling of the mechanisms at play.

We used data from an experimental SRC site, which incorporated three Swedish *Salix* clones in a row-based mixing design. Productivity was expected to be greater in mixtures and we attempted to elucidate whether this was due to a complementarity or a selection effect. We found that complementarity effects were generally larger than selection effects, yet the total diversity effect on yield was not significant. Leaf surface temperature measurements indicated that drought stress was unlikely to be the factor underlying this distorted diversity productivity relationship. We also found that a less-productive clone (Gudrun) had a different stem biomass distribution when in monoculture, which may have repercussions for the quality of the harvested product and points at a so far less recognized potential benefit of mixing.

1. Introduction

The use of woody biomass for energy production has been a mainstay throughout the history of human civilization and only in recent, industrial times its importance has plummeted in favor of fossil fuels (Tillman, 1978; Wrigley, 2010). Questions concerning the continuity of the supply of these fossil carbon stocks (Hubbert, 1949; Jakobsson et al., 2012) and the impact on the environment of their liberation has led to renewed interest in alternative sources of energy (Bruckner et al., 2014), including woody biomass (Mantau et al., 2010). The recent interest in developed countries for dedicated production systems of woody biomass for energy can be linked to these concerns (Wickham et al., 2010).

Short Rotation Coppice (SRC) is a silvicultural system developed for efficient production of woody biomass. Fast-growing woody species are planted in high density, harvested in short cycles of 2-5 years and replaced when productivity starts to decrease. SRC incorporates some of the agricultural developments of the 20th century, such as science-based breeding of varieties, mechanized field management and the (limited) use of agro-chemicals (Wickham et al., 2010). Management of SRC sites is extensive when compared to other cultivation systems for energy production, which often operate in cycles of one year or less. Fertilization is less important as relatively few nutrients are removed with the biomass, which is harvested after leaves are shed (Baum et al., 2009). The only important management intervention during growth is weeding in the first year after planting, sometimes using herbicides (Broeckx et al., 2012; Caslin et al., 2010). In addition to the relatively low intensity of management, SRC systems possess greater structural diversity than annual crops, resulting in a generally greater associated (functional) biodiversity (Dimitriou et al., 2011; Verheyen et al., 2014; Volk et al., 2004). SRC can also provide other ecosystem services next to biomass production, such as phytoremediation (Volk et al., 2004).

Despite the successful trials of SRC that have been undertaken across the globe (Abrahamson et al., 2012; Dimitriou et al., 2011; Lindegaard et al., 2011), some issues still threaten its ecological and economical sustainability. One of these is the low diversity of these cultures, in particular because often genetically identical high-yielding clones are used (Wickham et al., 2010). A lack of diversity results in increased pest pressure in agricultural systems (Matson, 1997; Zhu et al., 2000), which typically operate at a temporal scale of less than one year. SRC systems have longer harvest cycles and individual plants are intended to last up to seven such cycles (Caslin et al., 2010), which increases the risk and associated costs of devastating disturbances such as pests or droughts. It is also not as easy or desirable – ecologically or financially – to treat woody (energy) crops with pesticides.

Recent research suggests that introducing mixtures of genetically distinct clones in SRC could be an effective measure to address these risks. The past two decades, evidence has been found in multiple ecosystems of a positive relationship between (genetic) diversity and ecosystem functions such as productivity (Cardinale et al., 2012; Roger et al., 2012; Scherer-Lorenzen et al., 2007). This field of research is now known as *Biodiversity and Ecosystem Functioning* (BEF). Several mechanisms were proposed to explain these results, including facilitation, complementarity and the selection effect. Facilitation is the interaction between species where at least one species benefits and neither of them is harmed by the presence of the other (Bruno et al., 2003). Complementarity allows more diverse systems to make more efficient use of resources, as long as the niches of some of their species differ sufficiently (Srivastava and Vellend, 2005). Finally, the selection effect may occur because the probability of dominant species being present increases as the number of species present increases (Hector et al., 2007; Huston, 1997; Thompson et al., 2005; Wardle et al., 2000).

Only few studies have been undertaken on clonal mixtures in SRC plantations, mainly in the UK (Peacock et al., 2001) and in Northern Ireland (McCracken et al., 2011; McCracken and Dawson, 1998). These studies found that clonal mixtures of willow (*Salix* sp.) tended to be more productive (McCracken et al., 2011) and less susceptible to pests (Peacock et al., 2001) than monoculture stands of the clones the mixtures were composed of. These findings might be explained by the mechanisms described in BEF literature (Srivastava and Vellend, 2005). Facilitation could mitigate the impact of pests through *associational resistance*: differences in susceptibility to pests among clones reduce the overall biotic pressure in the plantation, for instance because it takes longer for pests to spread (Jactel et al., 2005). Complementarity, e.g. through differences in shade tolerance, could increase the system's efficiency of resource use (Morin et al., 2011). In the study by McCracken. *et al.* (McCracken et al., 2011), nested mixtures of five, ten, fifteen and twenty clones were compared with the monocultures of their constituent clones. Relatively poor yields of three out of the five clones that were present in all these mixtures could explain the positive diversity effect they found when comparing the five-way mixture with the three others as a selection effect. Comparisons with monocultures cannot be considered accurate, if some of the clones are virtually eliminated as the mixed treatments age and the high-yielding (dominant) clones quickly fill the gaps left this way – as was observed in the study.

In our study, we aimed at evaluating whether multiclonal SRC plantations had higher productivity, and whether this higher productivity was due to complementarity effects, rather than or in addition to selection effects. We set up an experimental SRC plantation in which the contributions of the different willow clones to the overall yield could be separated. Furthermore, we determined possible other effects of mixing clones by comparing the stand dynamics of mixtures and monocultures. Conventional sampling

methods to assess productivity and soil characteristics were complemented with thermal imagery obtained with an Unmanned Aerial Vehicle (UAV) at the end of a long dry period. The thermal maps were used as indirect estimator of transpiration levels and drought stress severity (Maes and Steppe, 2012) of the different plots.

2. Materials and Methods

2.1 Study site

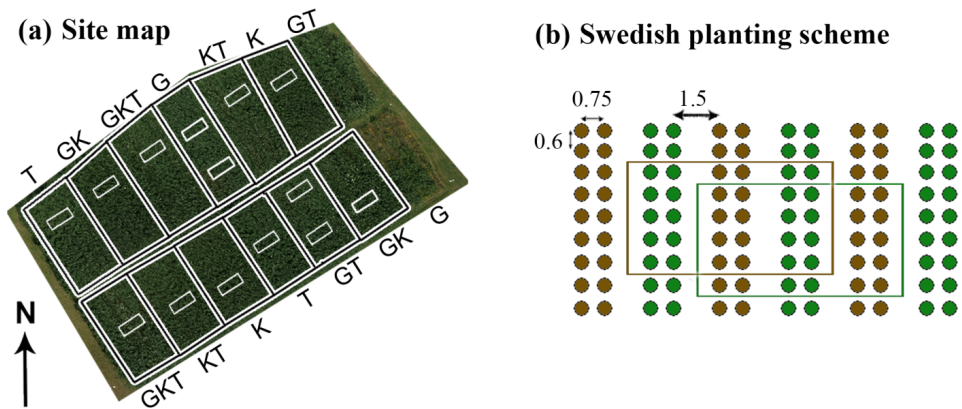


Fig.1: (a) Map of the experimental site. Plots and subplots are indicated. G, K and T represent the clones Gudrun, Klara and Tordis. The two plots in the most eastern part of the site (G and GT) were not sampled. To compensate for this, their replicates were sampled twice. (b) An example of the Swedish planting scheme used in the experiment for a two-clone mixture. The two rectangles show the subplots as they would be demarcated in the two different plots of the same clonal composition.

In March 2010, an experimental Short Rotation Coppice (SRC) plantation was established in Zedelgem, Belgium (51°15' N, 3°13' E). The study site has a temperate maritime climate with a mean annual rainfall of 855 mm and air temperature of 10.5°C (1981-2010, KMI Belgium). Four small former agricultural fields with a sandy soil were combined into an area of 2.28 ha. Before planting, the soil of the site was surveyed. The pH (KCl, 4.8) was below the optimal zone for willow, while the K and Mg content were at the lower end. Therefore, the site was treated with dolomitic lime (3 t ha⁻¹) and fertilized with manure (cattle, 23 t ha⁻¹). In the first year after planting, manual weed management was performed three times, based on previous research and management guidelines (Broeckx et al., 2012; Caslin et al., 2010). The site was not cut back after the first growing season, because of the high cost of harvest in the region the site was located.

The site was planted with Swedish willow clone cuttings and saplings of indigenous species. We will focus on the willow clones only, which comprised half of the site. Willow clones have been used in SRC plantations for some time now and breeding

stations in Sweden and in the UK have produced varieties with SRC as a specific focus (Wickham et al., 2010). Swedish clones gave better results than locally-adapted varieties at former SRC experiments in Belgium (Enerpedia.be, in Dutch). The three clones planted at our study site were Tordis, Klara and Gudrun. Gudrun is a clonal variety of *Salix dasyclados*, whereas Tordis is a hybrid of *Salix viminalis* and *Salix schwerinii* clones. Klara is a hybrid with a complex lineage, including clones of *S. viminalis*, *S. dasyclados* and *S. schwerinii*. Gudrun is advertised for its low susceptibility to frost, leaf rust (*Melampsora* sp.) and leaf beetles (*Phratora* sp.), Tordis for its tolerance to dry soils and Klara for its frost tolerance and high yield. More information on these clones (including their full pedigrees if known) can be found in the Willow Varietal Identification Guide from Teagasc, Ireland and AFBI, Northern Ireland (Caslin et al., 2012).

The three clones were planted in a full factorial mixing design, i.e. all seven possible combinations of the three clones were present. All the diversity treatments were replicated, which resulted in fourteen different plots distributed over two blocks (Fig. 1a). The spatial distribution of the plots was decided randomly, although each block contained seven different clone combinations. The mean plot size was 33 m x 12 m. Room was left for one access path between the two blocks and machine-turning strips around them. The Swedish planting scheme was used. This scheme consists of double rows of trees, roughly parallel to the north-south axis at our site, with planting distances of 1.5 m between the double rows and 0.75 m between the two rows of a double row. Within each row, the planting distance between the cuttings was about 60 cm (Fig. 1b). This pattern resulted in a planting density of almost 15.000 cuttings per ha and a total of 6 double rows per plot. Mixing was (double) row-based, so that the double rows in a plot contained only one clone.

2.2 Data collection

In late August – September 2011 and in December 2013, subplots of 6.75 m x 3 m were set up in twelve of the fourteen plots (Fig. 1). Every subplot contained three double rows and an estimated total of 30 planted cuttings. Because the planting distance of 60 cm inside a row varied at times, deviations from this number did not necessarily reflect mortality. A subplot always had a double row adjacent to it that was still a part of the plot it was in. Subplots were also at a distance of at least the tree height from the access paths and turning strips (Fig. 1a). In mixtures of two clones, each clone was present in one of the three rows in one subplot and in two rows in its replicate (Fig. 1b). As some trees were harvested for biomass determination, the subplots of 2013 were not at the same location as those of 2011.

The two most eastern plots (Fig. 1a) were not measured as the overall survival and vitality of the clones was low. In addition, one of the plots – a Gudrun monoculture – had

to be replanted in 2011 and the other – a mixture of Gudrun and Tordis – was too small to enable the use of buffer rows. For these two treatments, we installed two subplots in their replicates (Fig. 1a).

In every subplot, the diameter (average of two perpendicular measurements at 20 cm above ground level) of each stem taller than 170 cm or with a diameter larger than 20 mm was measured. We indicated which stems belonged to the same stool. In 2011, the height of each stem was measured using a 7 m measuring pole and the dominant height per double row was calculated as the average height of the 5 highest stems. We could not do this in 2013 as the trees had grown too high. Instead, we used a Vertex hypsometer (Vertex III and Transponder T3, Häglof) to measure the height of one canopy-forming stem per double row.

In 2011 and 2013, one stem in every double row of every subplot was harvested for a total of 42 stems each year. To ensure a minimal degree of diameter variation for the harvested stems of each clone, we randomly allocated one of three diameter classes to each double row in advance. The 42 harvested stems were weighed *in situ* (accuracy of 0.05 kg). To determine dry weight, a subset of 9 stems – one for each clone and diameter class – was dried at 70°C until constant weight.

In 2011, a mixed sample of the top 20 cm of the soil was taken in every subplot, composed of 10 subsamples taken randomly throughout the subplot. The soil samples were dried at 40°C until constant weight, sieved at mesh size 2 mm and then analysed for pH-H₂O, pH-KCl, total P, Olsen P, N percentage by weight and C percentage by weight. pH was assessed using a glass electrode (Orion, model 920A) after extraction of 14 ml soil in a 70 ml H₂O or 1 M KCl solution. Total P content was measured according to the colorimetric malachite green procedure (Lajtha et al., 1999) after wet, acidic digestion of a 0.2 g sample with an equimolar mixture of HClO₄, HNO₃ and H₂SO₄ in Teflon reservoirs for 4 hours at 150°C. Bio-available, inorganic phosphate (Olsen P) was assessed using the same procedure, after shaking a 2 g sample with 0.5 M NaHCO₃ at a pH of 8.5 for half an hour. C and N fractions were determined making use of elementary analysis (Variomax CNS, Germany). Also in 2011, the terrain height in the four corners of every plot was measured using a precision GPS (resolution up to 2 cm). Establishing the elevation within the plots was generally not possible as the closed canopy blocked the GPS signal.

On August 2nd and 23rd of 2013, flights were performed with a UAV, the AT8 octocopter of AerialTronics (Scheveningen, The Netherlands), equipped with a thermal and a visual camera. A pre-programmed waypoint flight was used to fly each block in two parallel scan lines at about 50 m altitude. The visual camera (Canon S110, Canon, Japan) was programmed with CHDK to log continuously every 0.5 seconds. The thermal camera, a Flir SC305 (FLIR Systems, Inc., Wilsonville, OR, USA) has a resolution of 320x240

pixels, a thermal accuracy of $\pm 2^{\circ}\text{C}$ and a thermal sensitivity of 0.05°C ; equipped with a 10 mm lens, it has a field of view (FOV) of $45^{\circ} \times 34^{\circ}$. The camera is controlled through in-house developed Python-based software from an on-board linux computer (Olimex), logging continuously every 2 to 2.5 seconds.

For further analysis, visual images only from the August 2nd flight were used, as a hardware problem during the second flight rendered its visual images unusable. Thermal images were used from the August 23rd flight, however, as there was significantly less wind that day and hence these images were much sharper than those of August 2.

The GPX-logfile from the UAV was used to estimate the GPS position and altitude of the UAV of each visual image. AgiSoft PhotoScan Professional (AgiSoft LLC, St Petersburg, Russia) was used to mosaick the visual images and a georeferenced orthophoto was extracted. The thermal images were converted to canopy temperature images as described by Maes *et al.* (2014). Due to the relatively low resolution, low overlap and limited contrast between the images, the thermal images could not be mosaicked properly with AgiSoft. Thermal images were therefore overlaid on the visual map in ArcGIS (ESRI, Redlands, Ca, USA) using the Georeference Tool and later mosaicked to form a thermal map. The thermal map covered the entire experiment except for a small gap in a Gudrun-Klara mixture (Fig. 2).

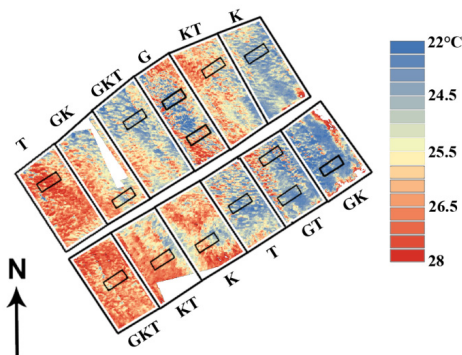


Fig.2: Leaf surface temperature map of the experimental site. G, K and T stand for the clones Gudrun, Klara and Tordis. Using ArcGIS, the data was classified into 16 classes with an equal amount of pixels in each class ('quantile'), hence the nonlinearity of the temperature scale.

2.3 Data analysis

To estimate standing biomass and productivity, we established allometric relationships between stem diameter (D, mm) and dry biomass (DM, kg) for every clone and

separately for the two measuring years by fitting power relationships with the loglinear regression formula $DM = aD^b$ (Arevalo et al., 2007). We then estimated the dry biomass for every stem and summed these values into standing biomass per stool, per double row and per subplot. The latter two values were transformed into productivity in dry tonnes per hectare per year ($t DM ha^{-1} yr^{-1}$), a metric regularly used in SRC literature (Abrahamson et al., 2012; Lindegaard et al., 2011; Vande Walle et al., 2007).

To explain potential variations in productivity caused by abiotic factors, we performed a Principal Component Analysis (PCA) with the soil variables and the GPS-determined terrain height. We also included a spatial variable that indicated the relative position of the plots from west to east, as there was a visible loss of vitality along this gradient, particularly in 2011. We then used linear regression to look for relationships between subplot productivity ($t DM ha^{-1} yr^{-1}$) and the first two principal components. We also incorporated the planted fraction of Gudrun as a second variable, because initial surveys led us to expect that this clone was considerably less productive than the other two.

To derive corresponding data from the thermal image, subplots of equal size and orientation as described in the previous section were drawn using ArcGIS (Fig. 2), with each subplot corresponding to ca. 718 pixels. These digital subplots were placed as close as possible to the location of the subplots during field measurements in 2013, except for the Gudrun-Klara mixture in the first block due to a thermal data gap (cf. 2.2). Average values and standard deviations of leaf surface temperature were determined for each subplot. Differentiation between double rows was not possible because these elements could not be consistently identified. The average values were related to the field measurements of productivity as well as to soil and spatial variables using linear regression, again including the Gudrun fraction as an additional variable.

Diversity effects were assessed for multiple characteristics other than productivity. This analysis was performed at the double row level rather than the subplot level, because clonal identity effects were expected. The number of stools (S) in a double row was used as a proxy for survival, although the deviations in planting distance may confound this relationship. As the plantation was still in its first rotation and the stools were not cut back after the first year to induce multiple stem formation (as in (McCracken et al., 2011)), we could assume that different stools originated from different cuttings. An indicator for apical dominance was estimated by calculating the ratio between the total number of stems (N) and S . Averages of S , N and N/S were calculated at the double row level. The evenness (EV) of stool biomass for each double row was calculated as:

$$EV = - \sum_{i=0}^S \frac{p_i \ln(p_i)}{\ln(S)}$$

with p_i the fraction of the total biomass for the i^{th} stool and S the number of stools in the double row. Lower values of EV indicate an imbalance of stool biomass within the subplot and suggest that some stools are not doing well and/or other stools are taking advantage of the gaps.

The significance of the diversity effect was tested using two-way ANOVA with clone as the second factor. We incorporated the interaction term, as diversity effects can be clone-specific. Tukey's Honest Significant Difference test (HSD) was used if significant factors had more than two levels or interactions were significant. Because the mixtures with three clones would distort the balance of the dataset and thus undermine the applicability of Tukey's HSD, they were combined with the two-clone mixtures into one single level. Analyses were performed for productivity (Y), dominant height (H), number of stools (S), number of stems (N), N/S and the evenness of biomass (EV).

To differentiate the complementarity (CMP) and selection (SEL) effects, we calculated the diversity effect on productivity in the mixed subplots using the method of Loreau & Hector (Loreau and Hector, 2001). These effects can be calculated for each mixed subplot using the following equations:

$$\Delta RY_i = RY_{Oi} - RY_{Ei} = \frac{Y_{Oi}}{M_i} - \frac{Y_{Ei}}{M_i} = \frac{Y_{Oi}}{M_i} - P_{Ai}$$

$$CMP = N \overline{\Delta RY M}$$

$$SEL = N cov(\Delta RY, M)$$

RY_{Oi} is the relative observed productivity for clone i in a mixed subplot, calculated by dividing the observed productivity of this clone in this subplot (Y_{Oi}) by M_i , the average observed productivity of clone i in monoculture subplots (both in $t \text{ DM ha}^{-1} \text{ yr}^{-1}$). RY_{Ei} is the relative expected productivity of clone i in mixture based on monoculture performance, or simply the proportion of the area planted with this clone (P_{Ai}). CMP and SEL are calculated by taking the average and the covariance of the ΔRY_i and the M_i values for all N clones present in the mixture.

All statistical analyses were performed using the statistical package R 3.1.0 (R Core Team, 2014).

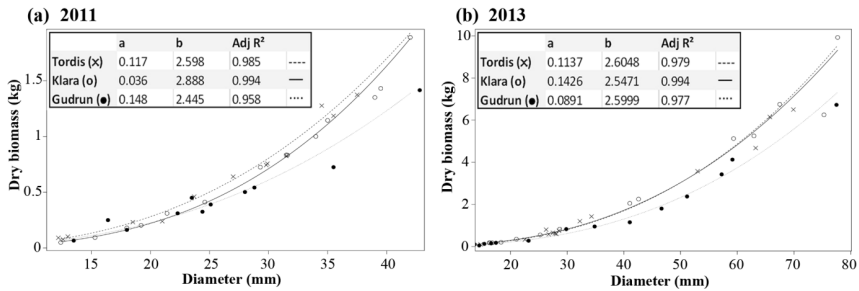


Fig. 3: The allometric relationships between stem diameter and dry biomass of the three clones in 2011 (a) and 2013 (b). The parameter estimates and adjusted R² values can be found in the embedded tables.

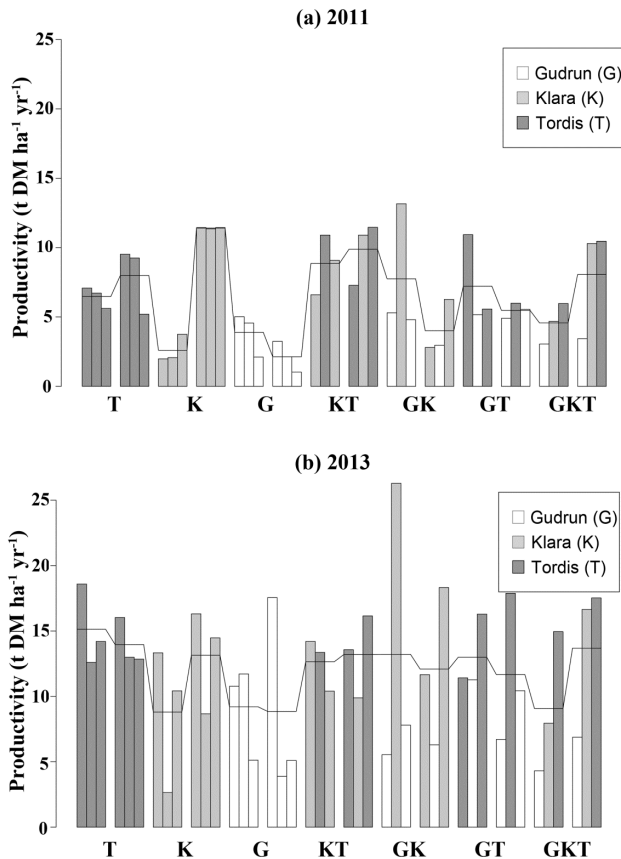


Fig. 4: Productivities in 2011 (a) and 2013 (b). The bars denote the three sampled double rows in the subplots, whereas the curve indicates the average productivity of the three double rows in each subplot.

3. Results

3.1 Productivity

The allometric relationships between diameter and dry biomass held up very well in both years ($\text{adj } R^2 > 0.985$, Fig. 3). Gudrun showed a consistently lower biomass for equal diameters than the other two clones. The relationship for Tordis was similar in both years. The other two clones showed divergent patterns between years, overestimating Klara biomass and underestimating Gudrun biomass if the relationships from 2011 would be extrapolated to 2013 (data not shown).

Gudrun had significantly lower productivity (Fig. 4; Table 1) and was lower in height (Table 1) than the other two clones. One subplot with Klara and one of the three-clone mixtures, both in the first block, were notably underproductive in both years, while the Klara row in the first Gudrun-Klara mixture was a strong and consistent over-productive outlier. The replicate of the Gudrun-Klara mixture with two Klara rows was poorly productive in 2011, but not in 2013. The productivity differences between the subplots were relatively larger in 2011 than in 2013, as can be seen in the slopes of the subplot average curve in Fig. 4.

3.2 Soil variables and leaf temperature

The first two axes of the PCA of the soil variables explained together 66% of the total variance. The first axis (40%) was strongly correlated with total P, Olsen P, terrain height, C% and N%. The second axis (26%) was correlated with pH (KCl: -0.87, H₂O: -0.76) and the spatial variable (0.64). The first axis showed no relationship with productivity. The second axis scores did show a pattern where lower pH and more eastern subplots were less productive, but they were not significantly related to subplot productivity in 2011 ($p=0.25$) or 2013 ($p=0.07$) when the fraction of planted Gudrun was included in the model.

Leaf temperatures decreased from west to east in the site (Fig. 2). This spatial relationship was significant ($p=0.01$, Fig. 5a) and not due to an effect of the planted Gudrun fraction ($p=0.16$). In 2013, productivity tended to increase with leaf temperature (Fig. 5b) rather than decrease, though this relationship was only marginally significant ($p=0.08$). Again, no effect of Gudrun ($p=0.17$) was observed. The Gudrun-Klara mixture in the most eastern part of the second block had the lowest leaf temperatures. The Gudrun monocultures had the highest variance in leaf surface temperatures.

Table 1: Averages of the descriptive statistics per double row for 2011 and 2013. **H** is dominant height in m, **Y** is productivity in t DM ha⁻¹ yr⁻¹, **S** is the total number of stools, **N** the total number of stems and **EV** the evenness of biomass among stools. The second column indicates the diversity level, i.e. the number of clones present in the subplot where the double row is located. Two-way ANOVA tests were performed to find significant differences between clones, diversity levels and their interaction for all variables. Significant differences with Tukey's HSD ($p < 0.05$) after two-way ANOVA are indicated.

2011		H	Y	S	N	N/S	EV
Tordis	1	5.96 ^a	7.23 ^a	8.67	14	1.62	0.93
	2+3	5.90 ^a	8.57 ^a	8.88	18.25	2.09	0.94
Gudrun	1	3.25 ^b	3.02 ^b	7.33	17.5	2.41	0.92
	2+3	4.16 ^b	4.39 ^b	8	15.63	1.94	0.95
Klara	1	5.41 ^a	7.01 ^a	7.67	12.33	1.6	0.95
	2+3	5.80 ^a	7.97 ^a	8.38	13.88	1.66	0.93
all	1	4.87	5.75	7.89	14.61	1.87	0.93
	2+3	5.29	6.98	8.42	15.92	1.9	0.94
2013		H	Y	S	N	N/S	EV
Tordis	1	9.13 ^a	14.54 ^a	8.83	14 ^{ab}	1.59 ^a	0.95 ^a
	2+3	8.65 ^a	15.14 ^a	8.38	15.25 ^{ab}	1.84 ^{ab}	0.93 ^a
Gudrun	1	7.25 ^b	9.02 ^b	7.67	26.17 ^c	3.38 ^c	0.76 ^b
	2+3	7.23 ^b	7.40 ^b	7.34	18.75 ^{ab}	2.53 ^b	0.92 ^a
Klara	1	9.03 ^a	10.98 ^a	7.67	11.5 ^a	1.54 ^a	0.87 ^a
	2+3	9.04 ^a	14.42 ^a	8.38	13.25 ^{ab}	1.58 ^a	0.91 ^a
all	1	8.47	11.51	8.06	17.22	2.17	0.86
	2+3	8.3	12.32	8.04	15.75	1.98	0.92

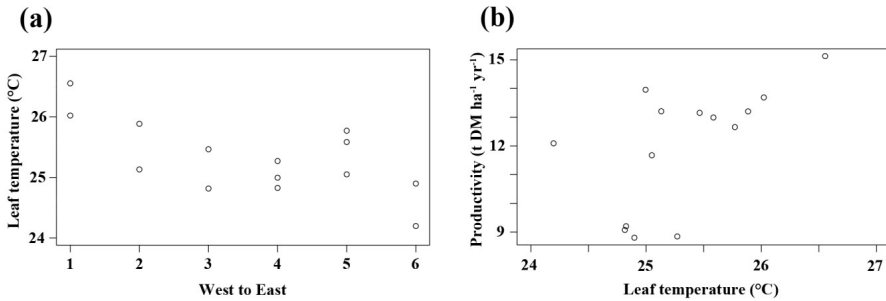


Fig. 5: (a) Subplot average leaf surface temperatures from west to east. An index of 1 refers to the most western plots; of 6 to the most eastern plots that were measured (cf. also Fig. 1a). (b) Subplot average leaf surface temperatures plotted against 2013 productivities.

3.3 Diversity effects

Although the difference was never significant, the productivity of any clone was generally higher in mixture than in monoculture – except for Gudrun in 2013 (Table 1). Gudrun had a greater number of stems per stool (N/S) in both years, which was also reflected in its total stem count (N) in 2013 but not in 2011. In 2013, the monocultures of Gudrun showed a significantly higher stem count and lower evenness of biomass (EV) than all the other treatments. To better understand this result, we compiled the stem biomass distribution of Gudrun for this year (Fig. 6). There were much more small stems in the monocultures (stem biomass less than 0.5 kg) and this effect was compensated in terms of standing biomass by more intermediate stems in the mixtures (biomass between 1 and 2.5-3 kg). The largest stems (>5 kg) all occurred in the monocultures again. Gudrun showed a relatively similar pattern (data not shown) in 2011, except that the largest stems were then in the mixtures.

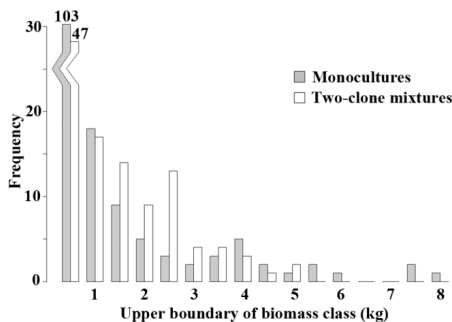


Fig. 6: The distribution of stem biomass for Gudrun in 2013. The biomass classes on the x axis are indicated by their upper boundary. The interval size is 500 g.

The diversity effect as calculated with the method of Loreau & Hector (Loreau and Hector, 2001) was generally positive, with the notable exception of one three-clone mixture (1GKT) in both years and one Gudrun-Klara mixture (2GK) in 2011 (Table 2). As one monoculture of Klara (1K) had an unusually low productivity (cf. 3.1), the analysis of diversity effects was repeated omitting the data from this subplot. The average diversity effect diminishes for both years this way, even becoming negative in 2011 (Table A1). The selection effect tended to be negative in 2011 and positive in 2013. The complementarity effect was generally larger in 2011 than in 2013 and always larger than the selection effect, as can be seen in the ratio of their absolute values (Table 2). When the Klara monoculture was omitted from the dataset (Table A1), the complementarity effect was still larger than the selection effect for most subplots. However, the median of the ratio's decreased, suggesting an increase in the relative contribution of the selection effect.

Table 2: Diversity effects according to the method of Loreau & Hector (2001). ΔRY_i is the difference in relative observed and expected productivity for clone i . The first clone in each two-clone mixture is the one with two out of three double rows. K is Klara, T is Tordis and G is Gudrun. CMP is the complementarity and SEL the selection diversity effect; both are in $t DM ha^{-1} yr^{-1}$. The ratio between the absolute values of CMP and SEL is listed to indicate their relative contribution. The sum of both CMP and SEL is also listed to indicate the total diversity effect.

		2011					2013				
		ΔRY_i	CMP	SEL	Ratio	Total	ΔRY_i	CMP	SEL	Ratio	Total
1KT	K	0.08					0.08				
	T	0.17	1.77	0.01	174	1.78	-0.03	0.68	-0.19	3.56	0.49
2KT	T	0.20					0.01				
	K	0.19	2.72	0.00	2038	2.72	-0.03	-0.24	0.09	2.78	-0.15
1GK	G	0.45					-0.17				
	K	0.29	3.72	-0.31	11.9	3.41	0.46	2.91	0.62	4.66	3.53
2GK	K	-0.24					0.24				
	G	-0.01	-1.21	-0.46	2.64	-1.67	-0.10	1.43	0.34	4.25	1.76
1GT	T	0.09					-0.03				
	G	0.24	1.70	-0.30	5.61	1.39	0.08	0.60	-0.32	1.91	0.29
2GT	G	0.49					-0.03				
	T	-0.06	2.21	-1.15	1.92	1.06	0.08	0.51	0.30	1.67	0.81
1GKT	G	0.00					-0.17				
	K	-0.11					-0.09				
2GKT	T	-0.06	-0.95	-0.23	4.03	-1.18	0.01	-2.95	0.51	5.78	-2.44
	G	0.05					-0.08				
	K	0.16					0.17				
	T	0.15	2.02	0.29	6.94	2.31	0.07	1.86	0.31	3.82	2.17
Median					6.28					3.90	
Average			1.50	-0.27		1.23		0.60	0.21		0.81

4. Discussion

4.1 Productivity variability

Gudrun has been reported to have a slow initial growth, which eventually catches up with those of other clones (Caslin et al., 2012; Lindegaard et al., 2011). We observed that Gudrun's productivity shortfall was lower in 2013, which seems to corroborate this. Tordis was less variable in its productivity than the other two clones (Fig. 4). This could be due to its genetic characteristics, as it is one of the clones promoted for drier areas by its producer. If drought conditions occur unevenly throughout the plantation, less drought-tolerant clones such as Klara and Gudrun would show greater differences in their productivity. The extreme outlier of Klara in one double row (Fig. 4) suggests that Klara has potential for very high productivity in optimal circumstances. Klara also grew taller than Tordis in the subplots where it did well.

An explanation for the low productivities in some plots could not immediately be found in soil and terrain data. The pH-KCl was 5.7 at the lowest which is still within the optimal range for willow (Tahvanainen and Rytönen, 1999). A spatial link might explain the poor productivity of the first Klara monoculture, in particular in the face of the neighbouring plots to the east that were not sampled in part due to their poor vitality (Fig. 1) and the decreasing leaf temperature trend from west to east (Fig. 5a). However, a spatial link does not explain the consistent productivity shortfall of the three-clone mixture in block 1. The absence of any major pest (leaf beetle, *Phratora* spp.) or pathogen (rust, *Melampsora* spp.) of willow in the plantation (pers. obs.) suggests that an abiotic cause is the most likely driver of these low productivities.

Drought could be a potential explanation: whereas Tordis (promoted as a drought-tolerant clone) was consistent in its productivity, Gudrun (discouraged on dry soils) showed low values. However, leaf temperatures were not negatively related to productivity, which would be expected if drought stress was the cause of lower productivities. This may be due to a lack of drought stress at the time of measuring leaf surface temperatures and does not rule out the occurrence of drought stress during drier parts of the growing season. However, August 2013 was an unusually dry month in the region where the site is located and the weather was dry in the days leading up to the UAV flight (KMI, Belgium). It is possible that greater productivity is related to greater rates of transpiration, which will deplete soil water more quickly under dry conditions and therefore result in faster stomatal closure, increasing leaf surface temperatures. However, even if this is the case, it would still imply that drought stress is not the limiting factor of productivity over the course of the plantation's four growing seasons.

Productivities for Klara and Gudrun fell within the expected range as based on previous trials if the poorly yielding plots were ignored. The Flemish research organization Inagro

sets its expected productivity for SRC in Flanders at 12 t DM ha⁻¹ yr⁻¹ after three years, based on their own trials (cf. Enerpedia.be, in Dutch), which is close to the average this site attained (Table 1). Findings from Sweden, Ireland and the UK are also within this range (Caslin et al., 2010; Lindegaard et al., 2011). Yet, the age of a plantation is crucial in comparing its yield with other sites; in our experiment, growth in 2010-2011 strongly differed from growth in 2012-2013. The average annual productivity roughly doubled from the first two to the last two years, implying that three times as much biomass was produced in the last two years. Hence, it might be expected that yearly average yields would be lower for a three year period.

4.2 Clone-specific analysis

Gudrun's deviant characteristics compared to the other clones were most pronounced in 2013, with the exception of productivity (Table 1). The total stem count was not different between clones in 2011, which can possibly be explained by the criteria used for including a stem in our measurements. Very small stems were not measured in both years, but as time progresses more stems will attain the minimum diameter and height.

A lower evenness of biomass was found in the monocultures of Gudrun in 2013 (Table 1) as well as a greater amount of both small and large stems (Fig. 6). The variance in leaf surface temperature was also highest in the Gudrun monocultures (data not shown). A possible explanation for this phenomenon could be that in our plantation, Gudrun generally suffered from greater mortality or more instances of poor vitality regardless of any non-additive diversity effect in the form of facilitation or complementarity (Srivastava and Vellend, 2005). This would distort the evenness of biomass locally, as still fit stools compensate for their less fortunate neighbours and hence unevenness increases in two ways: fit stools grow larger, less fit stools stay smaller. This mechanism can be expected to be more common in monocultures even if survival/vitality was not noticeably different between monocultures and mixtures, as Gudrun double rows will be surrounded by similarly deficient rows in monocultures but not in mixtures. Willows grow fast enough for such compensation between rows to be already apparent after four years of growth (Heinsoo et al., 2009), though possibly not after two years (cf. the ΔRY_i values as discussed in section 4.3) explaining why this phenomenon could not be found in 2011.

This mechanism of unevenness through generally poor vitality also explains why the most massive Gudrun stems were found in monocultures in 2013, though this is not necessarily true for the overall greater stem number. It is possible that this latter effect is related to the poor growth of Gudrun, resulting in less inhibition of lateral growth through shading and less apical dominance in monocultures. It is unclear what implications such a diversity effect on stand dynamics could have for SRC systems. Stem diameters play a role in the choice of harvesters and harvest optimization techniques, as well as the

quality of the final product. Smaller stems may be more optimal for modified forage harvesters, as they are less likely to jam the header (Spinelli, 2007). Larger stems generally have a lower bark fraction and hence a lower ash content, which improves fuel quality and decreases nutrient losses (Tharakan et al., 2003).

4.3 Complementarity and selection effect

The selection effects were generally smaller than the complementarity effects (cf. the ratios in Table 1). This relationship persisted when the data of the Klara monoculture were omitted (Table S1). Klara edged out Gudrun in their more productive mixtures in 2013 (cf. the ΔRY_i values in Table 1), which means that Klara's generally greater productivity increases its specific productivity in mixture at the expense of Gudrun's. However, this selection effect does not seem to completely or even significantly explain the differences between observed and expected productivity (Table 1). Hence, we might conclude that the diversity effect cannot be explained by the presence of a more productive clone.

Some selection effects were negative, primarily in 2011. A negative selection effect means that a clone that is less-productive in monoculture yields relatively more in a mixture than the more productive other clones present in that mixture. Hence, in such a mixture, the clones do not behave as would be expected from their dominance hierarchies based on their monoculture performance. Competition between double rows (and thus clones) was probably less strong or even absent in 2011, when the plantation was just at the end of its second growing season. The mixture that was most extreme in terms of productivity (1GK) reflects this in its ΔRY_i values (Table 1), which were positive for both clones in 2011 but had diverged substantially in 2013.

5. Conclusion

The objective of this research was to identify diversity effects on productivity in a SRC stand and to assess the role of a selection effect. We found indications of a diversity effect on productivity that was not caused by a selection effect, but this effect was not significant due to unexplained variance in productivity. When taking a closer look at the stand characteristics of the studied systems, diversity effects were found for a less productive clone in 2013. Such effects might be expected even in the absence of a non-additive diversity effect on productivity and can still have real impacts on system qualities, such as harvest efficiency or quality of the harvested product. Future research should attempt to keep within-treatment variation as low as possible, necessitating a larger research site or data from multiple sites. Longer time series including harvest years are also recommended, as biotic pressure is expected to be highest just after this traumatic event. Finally, clonal diversity gradients should be widened to more different clones with larger differences in their lineage, as advised by the Willow Best Practice Guide (Caslin et al., 2010).

Appendix

Table A1: Diversity effects according to the method of Loreau and Hector (2001). One poorly yielding Klara monoculture was not used in this analysis. ΔRY_i is the difference in relative observed and expected productivity for clone i . The first clone in each two-clone mixture is the one with two out of three double rows. K is Klara, T is Tordis and G is Gudrun. CMP is the complementarity and SEL the selection diversity effect; both are in $t DM ha^{-1} yr^{-1}$. The ratio between the absolute values of CMP and SEL is listed to indicate their relative contribution. The sum of both CMP and SEL is also listed to indicate the total diversity effect.

		2011					2013				
	ΔRY_i	CMP	SEL	Ratio	Total	ΔRY_i	CMP	SEL	Ratio	Total	
1KT	K	-0.21				-0.04					
	T	0.17	-0.37	-0.79	0.47	-1.16	-0.03	-0.97	0.01	86.9	-0.96
2KT	T	0.20				0.01					
	K	-0.01	1.70	-0.44	3.83	1.26	-0.08	-0.94	0.07	13.9	-0.88
1GK	G	0.45				-0.17					
	K	0.05	3.61	-1.67	2.16	1.94	0.33	1.76	1.05	1.68	2.81
2GK	K	-0.40				0.09					
	G	-0.01	-2.94	-1.66	1.77	-4.61	-0.10	-0.09	0.40	0.21	0.32
1GT	T	0.09				-0.03					
	G	0.24	1.70	-0.30	5.61	1.39	0.08	0.60	-0.32	1.91	0.29
2GT	G	0.49				-0.03					
	T	-0.06	2.21	-1.15	1.92	1.06	0.08	0.51	0.30	1.67	0.81
1GKT	G	0.00				-0.17					
	K	-0.20				-0.13					
	T	-0.06	-1.81	-0.84	2.15	-2.65	0.01	-3.63	0.46	7.87	-3.17
2GKT	G	0.05				-0.08					
	K	-0.03				0.09					
	T	0.15	1.17	-0.33	3.56	0.84	0.07	0.96	0.49	1.94	1.45
Median				2.16					1.93		
Average		0.66	-0.90		-0.24		-0.23	0.31		0.08	

Chapter 3: Identity rather than richness drives local neighbourhood species composition effects on oak sapling growth in a young forest

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Abstract

In light of global biodiversity loss, the influence of species composition on ecosystem functioning has attracted increasing attention. However, questions remain whether neighbourhood species richness or identity is more important and what mechanisms drive these composition effects. We investigated the role of local species richness and identity on oak sapling growth in young forest stands, and attempted to link growth with two commonly expected mechanisms: oak powdery mildew (PM), a host-specific leaf pathogen, and degree of shading. Furthermore, we studied how such effects might interact with reduced rainfall.

We established a reduced rainfall gradient on oak (*Quercus*) saplings planted within two sites of a young tree diversity experiment (FORBIO). We found no effect of species richness on growth, whereas presence of particular species had a significant impact. In one site, we found an identity effect on growth through negative effects of oak (*Q. robur*) presence, which was linked to increased PM infection severity. Such link was less clear in the other site, where the impact of PM and oak (*Q. petraea*) presence was lower and there was no relation between the two. Other identity effects were related to levels of shading, which were themselves influenced by fast early growth rates of the neighbouring trees. We found no consistent effects of reduced rainfall on growth. Host-mediated effects through oak PM and functional size-mediated effects through shading were the most important composition effects in our young forest experiment, with no support for species richness effects through local environmental conditions or natural enemies.

1. Introduction

In the last two decades, more and more research was undertaken into effects of species diversity and species composition on ecosystem functioning (Cardinale et al., 2012; Hooper et al., 2005; Tilman et al., 2001, 2014). After initial experiments with synthetic species assemblages in grasslands (Hector et al., 1999; Weigelt et al., 2010), the scope was broadened to include many other types of ecosystems, including forests (Scherer-Lorenzen et al. 2007; Zhang et al. 2012; Vilà et al. 2013). The original impetus to investigate these effects of biodiversity was the strong ongoing decline of biodiversity on a global scale, linked to the widespread environmental changes of the Anthropocene (Hooper et al., 2012). However, some researchers have cast doubt on the link between biodiversity loss at a local scale, as studied in the mentioned experiments, and the observed biodiversity loss at a regional or global scale (Sax and Gaines, 2003; Vellend et al., 2013). Nevertheless, in systems such as grasslands or forests, species composition can often be influenced or even controlled by the manager. Hence studies covering the impact of local scale biodiversity on ecosystem functioning will have implications for the management of these ecosystems (Tilman et al. 2006; Pretzsch and Schütze 2009; Gamfeldt et al. 2013).

Studies into composition effects in forests can take multiple approaches (Nadrowski et al., 2010). This includes looking at existing inventory data (Gamfeldt et al., 2013; Paquette and Messier, 2011; Vilà et al., 2007), gathering data from different compositions in extant forest (Baeten et al., 2013; Firn et al., 2007) and establishing new plantations designed with compositional comparisons in mind (Verheyen et al., 2015). New plantations have the advantage of a well-controlled composition gradient, with fewer (unknown) confounding variables such as differences in site history (Leuschner et al., 2009; Scherer-Lorenzen et al., 2005b). However, the slow growth of trees makes the representativeness to mature forest of such experiments poor in the short term, as tree interaction mechanisms, such as root competition, will have much less of an impact initially than they are expected to have in mature forest (Domisch et al., 2015; Meinen et al., 2009a).

While some mechanisms such as root competition do not play a big role yet, it is known that young trees are much more susceptible to certain pests and diseases because they have less reserves to fall back on in severe years or to support defense mechanisms (Barton and Hanley 2013). For instance, the oak powdery mildew fungus (PM, predominantly *Erysiphe alphitoides*) is not considered a severe problem for mature trees, but can cause substantial mortality and growth reduction in young saplings (Marçais and Desprez-Loustau, 2012). Hence, composition-based mechanisms related to mitigating the impact of pests or pathogens are expected to play a more important role in younger stands.

Examples of these mechanisms are the dilution of resources favored or required by these pests as the density of nonsusceptible species increases and an enriched top-down control by natural enemies, who may benefit from greater structural diversity or diet mixing in more species diverse neighbourhoods (Jactel and Brockerhoff, 2007; Sobek et al., 2009). In the case of oak PM, which is a specialist biotrophic leaf parasite of various *Quercus* species, an increased density of nonsusceptible trees may decrease the spore load for the susceptible ones. This may be particularly so for oak PM, as the most virulent species of this fungus, *E. alphitoides*, tends to hibernate in sexual form outside the host, which necessitates the fungus to infect its hosts anew with ascospores every year (Feau et al., 2012). The fungus also spreads on a leaf-by-leaf basis during a growing season by continuously producing asexual spores as fast as possible from newly infected leaves (Marçais et al., 2009). Hence, reducing the availability of suitable host trees at the local scale would reduce the initial spore load and therefore reduce the probability of infection as well as the proportion of initially infected leaves, whereas oak leaves are only susceptible to PM in the first two weeks of their life (Edwards and Ayres, 1982). Changes in neighbourhood composition may also influence natural predators of PM, such as mycophagous ladybugs by offering them alternate food sources such as pollen or powdery mildew fungi of other plant species that may be available earlier in the growing season (Sutherland and Parrella, 2009).

Another composition mechanism is the Janzen-Connell effect (Bagchi et al., 2010), where proximity to the mother tree increases the pressure of pests and pathogens on offspring. The presence or density of conspecifics in the local neighbourhood can be expected to have a strong impact, by facilitating host-specific pest dispersal as well as increasing patch attractivity (Jactel et al., 2005). Similar effects of species identity could also be associated with other, allospecific tree species with certain high impact functional traits such as nitrogen fixation (Forrester et al., 2006). Such impacts of species identity in the local neighbourhood have been found in multiple studies (Firn et al., 2007; Hantsch et al., 2013; Nadrowski et al., 2010; Setiawan et al., 2014), related to traits such as susceptibility to specialist pathogens (Hantsch et al., 2013) or tree size and its relationship to microclimate and apparency for insects (Castagneyrol et al., 2013; Hantsch et al., 2014). Tree size is obviously related to shading, which may have negative consequences for growth, but it also influences microclimate, possibly reducing the likelihood of spore-dispersing pathogens to find a host (Bock et al., 2010).

More recently, researchers have been looking into how positive effects of neighbourhood composition are influenced by other ecosystem characteristics, such as the resource stress the species present are suffering from (Jucker et al., 2015; Paquette and Messier, 2011; Steudel et al., 2012; Toïgo et al., 2015). They considered that such diversity effects might be stronger or more important under more stressful circumstances, for instance because this opens up more opportunities for (and therefore

more expected instances of) facilitation between species (Bertness and Callaway, 1994). Evidence supporting this so-called Stress Gradient Hypothesis (SGH) was found in multiple forest studies, finding stronger composition effects under less favorable site conditions (Eränen and Kozlov, 2008; Jucker et al., 2015; Paquette and Messier, 2011; Toïgo et al., 2015). If such a relationship were to be quite generic, it could have serious implications for conservation ecology and production ecosystems as these are associated with a trend of biodiversity loss or a low imposed species diversity respectively. Both are also expected to be subjected to increasing stress and disturbance events through the various mechanisms of Global Change, including climate change, invasive (pathogenic) species and changes in land use (Rockström et al., 2009). The realized diversity of both natural and synthetic ecosystems could therefore prove to be more important than currently perceived.

Here, we attempted to elucidate the relationship between local neighbourhood composition, growth and reduced rainfall in two young forest stands, which were each incorporated with a balanced tree species diversity gradient (Verheyen et al., 2013). Potted oak saplings were planted within the plantation and subjected to a reduced rainfall gradient. We hypothesized that local neighbourhood tree species richness would have a positive effect on tree growth or that the presence of certain tree species in the local neighbourhood influenced growth as an identity effect, in particular if this species was the same as the focal species (oak), due to the Janzen-Connell effect. To assess the mechanisms by which either such relationship might come to be, we compared both indices of neighbourhood composition to the severity of powdery mildew and to the degree of shading the focal tree was subjected to, as we expected these to be the most likely to operate on our potted oak saplings. We expected a negative effect of reduced rainfall on growth and stronger positive effects of neighbourhood composition along the reduced rainfall gradient.

2. Materials and Methods

2.1. Sites

Our experiment was set up within two sites of the FORBIO project, Zedelgem and Gedinne. FORBIO (FORest BIODiversity and Ecosystem Functioning) is a network of three relatively large (ca. 9 ha) experimental forest sites in Belgium, 'designed specifically to test the effects of tree species diversity on forest ecosystem functioning' (Verheyen et al., 2013). In each site, young saplings of five different tree species were planted in compositions that ranged from monocultures to up to four different species mixed together. Due to differences in juvenile growth, the trees were always planted in groups of 3 by 3, sometimes 4 by 3. This would make it possible to sustain mixtures for a longer time, by reducing strong competitive interspecific interactions. All monocultures and four-species mixtures were represented, as well as 5 randomly selected

compositions for the other two species richness levels (Table A.1). These 20 compositions were replicated once for a total of 40 plots. Planting distance was 1.5 m x 1.5 m and typical plot size was 42 m x 42 m. 13 plots in Gedinne were only 42 m x 37.5 m (Fig. 1). Each site also had a few extra plots containing different provenances of *Quercus* (Zedelgem) or *Fagus* (Gedinne). These were not included in this experiment, which was limited to plots 1 - 41 in each site, omitting plot 38.

The site of Zedelgem was planted in late 2009 and early 2010 on former agricultural field with a sandy to loamy sandy soil. The five species used here are pedunculate oak (*Quercus robur*), European beech (*Fagus sylvatica*), Scots pine (*Pinus sylvestris*), small-leaved lime (*Tilia cordata*) and silver birch (*Betula pendula*). The site of Gedinne was divided into two parts, about 4 km from each other and all composition levels were present in each site. The part called Gribelle has a stony soil and is more exposed than Gouverneurs, which has a shallow soil. Both were planted on Norway spruce (*Picea abies*) clearcuts in early spring 2010. The species used here are European beech, sessile oak (*Quercus petraea*), sycamore maple (*Acer pseudoplatanus*), hybrid larch (*Larix x eurolepis*) and Douglas fir (*Pseudotsuga menziesii*). Monthly rainfall data for 2013-2014, based on weather station proxies (KMI, Belgium), can be found for the oak growing season months in Table A.2. The summer of 2013 was rather dry, in particular in August, whereas the summer of 2014 was much wetter than normal. 2015 fell somewhere in between, with July being a bit drier than average but not as severe as August 2013. More info on the FORBIO sites can be found in Verheyen et al. (2013).

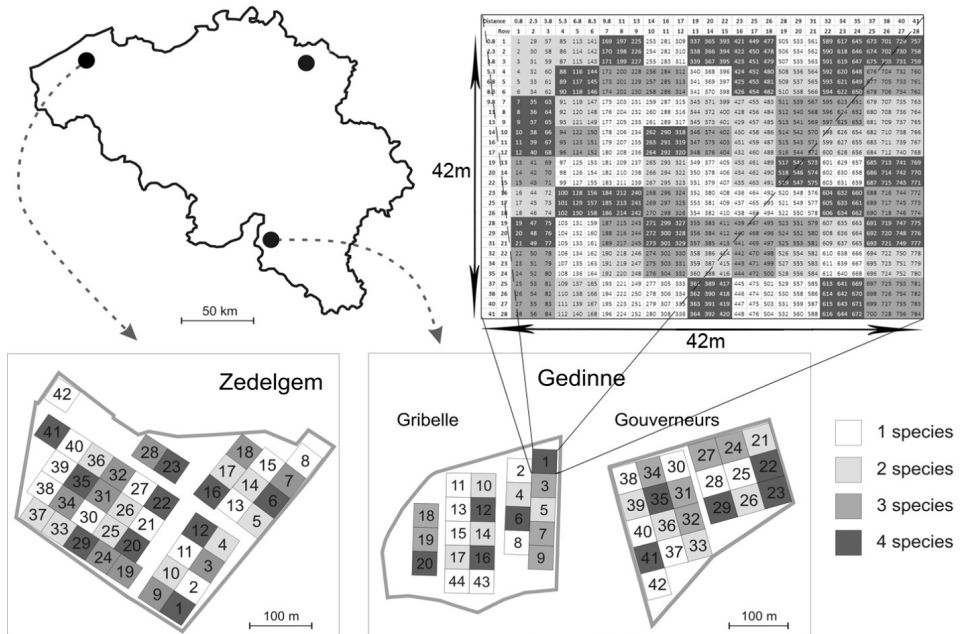


Fig. 1: Experimental design of the FORBIO sites of Zedelgem and Gedinne, with their location inside Belgium depicted in the top left corner. Each site consists of at least 40 plots with composition of 1 up to 4 different tree species. Our experiment was performed only in plots 1 - 41, not including plot 38. Each plot was set up similar to the grid structure depicted in the top right corner, where the different colors represent different tree species groups. Plots with two species were planted with the 3x3 groups alternating regularly like a checkerboard. Plots with three or four species had the species groups alternating randomly as in the example grid. Our phytometer plots were planted at points where the four adjacent FORBIO trees corresponded to the plot's species composition. This was not always possible at the center, in particular because four of the center spots had been allocated there already as permanent sampling plots for long-term monitoring and were therefore not usable for our experiment.

2.2. Experimental setup

In late April and early May 2013, one-year old saplings of oak were planted in pots between the trees of the FORBIO site. The same oak species was used as present in the site, but not the same provenance as some plots in Zedelgem contained multiple *Q. robur* provenances. Three such 'phytometers' were planted in 20 liter black pots and placed together between four of the older, larger trees (1-3 m higher) in each of the 40 plots (Fig. 1). These black pots had drainage holes in the bottom and were placed in the ground to reduce solar heat absorption and ensure that the soil level was more or less equal in and outside of the pots. The four neighbouring FORBIO trees were always representative of the whole plot's composition. We set up our phytometers as far from the plot edge as possible (i.e. close to the center), but in practice this was not always

the case for mixtures of 3 or 4 species (cfr. Fig. A.1 and A.2). In those plots, the crossings were the surrounding composition matched were limited and often already claimed as permanent sampling plots for long term study of the sites. Soil from the sites themselves was used, collected from an unused patch in Zedelgem and from two separate patches for the two blocks in Gedinne for logistic reasons. No potting soil was used as a difference in nutrient status might influence the attractivity of the phytometers to herbivore pests (White, 1984).

Late May (Zedelgem) and late June (Gedinne) in the same year, a reduced rainfall treatment was installed on these phytometers. Small PVC gutters with a diameter of 3.2 cm were placed above the pot surface but below the tree canopy in two of the three phytometers of every plot. One and two pairs of gutters were used as can be seen in Fig. 2. The theoretical interception rates based on pot surface covered are 18 and 35%, mimicking average summer precipitation reduction expectations for the region according to climate model predictions (Willems et al., 2009). The interception treatments were defined as the control (0% interception), the medium treatment (18%) and the severe treatment (35%). In 5 of the 40 plots, collectors were installed at the gutter ends (Fig. 2) to obtain estimates of the rainfall removal.



Fig. 2: Example of a plot with three potted Quercus phytometers. One and two gutters on each side of the sapling correspond to the medium (-18%) and severe (-35%) rainfall reduction treatment respectively. Surrounding the three pots are four larger FORBIO trees, always in mixtures at the crossing of four different three by three planting blocks.

The pots were kept free of major understorey influence by frequent weeding and the collectors were measured and emptied regularly. A full precipitation assessment of the growing season was not possible, as the four-gutter collectors could overflow after one major rain event (max. 33 mm per collector). The rainfall reduction treatment was removed at the end of each growing season (late October, early November) and reinstated in early May, because winter precipitation reductions per climate change are unexpected for this region (Willems et al., 2009).

In July and August of 2015, volumetric soil moisture content was measured in all pots using a ML3 ThetaProbe (Delta-T Devices). For each pot, two measurements were made at the pot surface (0-6 cm) and two at a depth of 14-20 cm, using a small PVC tube to gather soil from this depth and subsequently inserting the ThetaProbe's sensor pins into the entubed soil to perform the measurement. This depth measurement was not possible in the Gribelle part of the Gedinne site due to the high number of rocks in the soil. A third measurement at the surface was made instead. The standard conversion parameters for a mineral soil type were used for both sites, as the carbon content fell within the suggested interval.

2.3. Monitoring protocol

To assess annual growth rates, stem diameters were measured using digital calipers (0.01 mm accuracy, rounded to 0.1 mm) at the start of the experiment and between growing seasons. Each time, two perpendicular measurements were made at the level of the pot surface and averaged. The level of the pot surface was chosen as a fixed point for repeated measurements. The height (cm) was assessed as the length of the main (or longest) shoot between the growing seasons, but not at the start of the experiment.

A monitoring for leaf damage symptoms was undertaken in July, August, October 2013; July, September 2014 and August 2015 in Zedelgem as well as in July and August 2013, 2014 and August 2015 in Gedinne. First order shoots, i.e. shoots on the main stem or, in unclear cases, all main stems, were scored for different leaf damage symptoms using four % classes: 0-5%, 5-30%, 30-60% and 60-100% of leaf area affected. Different symptoms were scored separately. To correct for increasingly differing sizes of the first order shoots, from October 2013 onwards relative leaf area weights were estimated for each shoot. To determine these weights, we assumed an average-sized single oak leaf from the lower canopy to have a leaf area of 1 and subsequently approximated the total leaf area of each first order shoot in multiplications of such an oak leaf unit (e.g. a single shoot might have a total leaf area of approximately 12 average-sized leaves). We used a typical lower canopy leaf as our single unit, because leaves growing alone from the main stem were mostly only found there. These

weights were later used to calculate a weighted average symptom score at the whole tree level.

Other than oak powdery mildew (PM), the following leaf damage symptoms were also assessed: chewing, skeletonization, mining and brown discoloration. Leaf galls were counted at the shoot level; no gall species differentiation was made, but a grand majority were *Cynips divisa*. This differentiation was based on results from previous surveys on the FORBIO trees (Setiawan et al., 2014) and a pilot survey on the phytometers. During this pilot survey, the presence of these symptoms on the older FORBIO trees was also ascertained. Symptoms were only assessed during dry weather conditions, as PM hyphae become less visible when a leaf is wet. Monitoring of all symptoms was continued for all measuring moments, but PM was the most important symptom by far. Leaf chewing overall only minimally reduced leaf area and was impossible to quantify accurately when it was significant, as this typically surmounted to whole leaf or shoot loss. Leaf mining was rare, while skeletonization was limited mostly to the end of the growing season only, when its impact on growth was expected to be minimal. Brown discoloration was more abundant, but could be linked to various different, not very easily differentiable causes, including late spring frost, drought stress, PM-induced mortality and early onset of autumn. Galls were only found in less than 20% of our trees. Hence, we opted to only take the PM data into account for further analysis. Data of all symptoms can be found in the supplemented data files for this paper.

In July and August of 2015, the degree of shading for each phytometer was estimated using a spherical densiometer (Baudry et al., 2014). A % value for East, South and West was determined separately above each sapling. Collinearity between these values was high, so an average was calculated for each phytometer and used in the further analysis.

2.4. Calculations

Class scores of leaf damage symptoms were scaled up to phytometer level by averaging the class mids (0, 17.5, 45 and 80 %), using the relative shoot size scores as weights if available. For the lowest 0-5% class, 0 was used instead of the class mid as this class was supposed to correct for the difficulty in determining very small or beginning infestations. Hence, using the class mid would distort the meaning of the 0 value in this case. The galls count data were not averaged but summed.

The diameters were transformed into (circular) basal area (mm^2) and its relative increment (BAI_t) calculated for the 2013, 2014 and 2015 growing seasons as well as across all three seasons. Relative height increment (HI_t) was determined similarly, though only from winter 2013-2014 onwards:

$$BAI_r = \frac{BA_2 - BA_1}{BA_1}; HI_r = \frac{H_2 - H_1}{H_1}$$

One phytometer with the severe reduced rainfall treatment in a beech monoculture was dead in Gedinne by the 2014 growing seasons (cause unknown). Six phytometers were dead in Zedelgem by 2014, mostly because of attack by macro-herbivores (hare, rabbit, possibly wild boar). Three of these were in the same plot, a 4-species mixture without birch. The other three were in an oak monoculture (medium rainfall reduction treatment), an oak-beech mixture (medium) and a 4-species mixture without lime (severe). All of these were omitted from all analyses. Three incorrect measurements (two diameter, one height) in Zedelgem were also omitted from the growth models.

2.5. Statistical analysis

All statistical analyses were performed using R (R Core Team, 2015). Mixed models were run using the package *lme4* (Bates et al., 2015), with *lmerTest* extension (Kuznetsova et al., 2015) to determine Satterthwaite approximated p-values.

The soil moisture content (SMC) data were compared to the reduced rainfall treatment using a linear mixed model with intercept. In Zedelgem, weather conditions preceding the measurements in block 2 were much drier than those in block 1. In Gedinne, the stone density of the soil in block 1 made measurements at a depth of 14-20 cm impossible. The model was therefore run separately for the two sites and for the two replication blocks within each site. For post hoc analysis, the model was always run a second time with a different reduced rainfall treatment as reference value. Alpha was corrected using the Šidák correction. Conditional and marginal pseudo-R² values were determined using the approach of Nakagawa & Schielzeth (2013), implemented in the R package *MuMIn* (Barton, 2016).

$$SMC (\%) = RR + D + SH + RR * D + RR * SH + D * SH$$

RR: *The three level reduced rainfall treatment.*

D: *Factor for the depth the measurement was made at (0-6 cm and 14-20 cm).*

SH: *Degree of shading in %; centered around the mean.*

The analysis of the growth, powdery mildew and shading data was performed for each site separately, as the conditions of soil, climate and tree species composition were all different. Composition was determined for the local neighbourhood, which was defined as the 4 FORBIO trees surrounding the phytometer plots. Due to the 3x3 group-planted FORBIO design, this also corresponds to the tree composition of the 36 FORBIO trees closest to the phytometer plot. All models were linear mixed models with plot as random factor and including an intercept.

First, the relationship between the annual or total relative increments and neighbourhood composition was tested. The model used also incorporated the reduced rainfall treatment and its interaction with composition, to test for the Stress Gradient Hypothesis. The indices of composition, richness and identity, could not be tested together in the same model, as richness would always be a linear combination of all identity effects.

$$BAI_r \text{ or } HI_r = SR + RR + SR * RR + B$$

$$BAI_r \text{ or } HI_r = \sum_{i=1}^5 SP_i + RR + \sum_{i=1}^5 SP_i * RR + B$$

SR: Tree Species Richness in the local neighbourhood, gradient from 1 to 4.

B: The two replicates ('blocks') of all treatments.

SP_i: The presence/absence in the local neighbourhood of the five species present in the site.

Second, similar models tested the relationship between neighbourhood composition and the two potential driving mechanisms: PM infection severity and the level of shading (SH) in 2015. PM levels of the different measuring moments were included separately, as PM infections can occur throughout the growing season while the Lammas shoots develop.

$$PM \text{ or } SH = SR + RR + SR * RR + B$$

$$PM \text{ or } SH = \sum_{i=1}^5 SP_i + RR + \sum_{i=1}^5 SP_i * RR + B$$

PM: PM infection severity (% of total leaf area affected).

SH: Degree of shading in 2015 (%).

Finally, we linked the driving mechanisms directly to relative increment. The mechanism variables were individually centered around their mean and rescaled from percentages to fractions, both to facilitate interpretation of their effect sizes.

$$BAI_r \text{ or } HI_r = SH + \sum_{i=1}^n PM_i + B$$

SH: Degree of shading, as a fraction between 0 and 1. Centered around the mean.

PM_i: PM infection severity, as a fraction between 0 and 1. Centered around the mean.

If residual heterogeneity was present, square root transformations were applied, adding a constant equal to the lowest value of the response variable if it was negative.

Multicollinearity was evaluated with Variance Inflation Factors, using a modified function of the package *rms* (<https://github.com/aufrank/R-hacks/blob/master/mer-utils.R>).

3. Results

3.1. Treatment validation

A combined dataset of all ratios of reduced rainfall treatment collectors compared to the in-plot control collector yielded 67 values for the pots with one pair of gutters and 61 for those with two pairs. Average interception rates were estimated in confidence intervals of $12.95 \pm 0.9\%$ and $26.13 \pm 1.6\%$, respectively, which correspond relatively well with the theoretical interception rates of 18% and 35%. These values were all determined for Zedelgem, as there was not enough good data for a similar assessment in Gedinne due to frequent collector failure. However, as the same design was used in both sites, we assume the Zedelgem values to be fairly representative.

The results of the soil moisture content models can be found in Table A.3. A barchart differentiating the most important predictors can be found in Fig. 3. The difference in soil moisture content was 2 to 3%; always significant between the control and the most severe reduced rainfall treatment, but the medium treatment did not always differ from the severe or control treatment. Measurements at 14-20 cm depth in Zedelgem showed larger differences than those at the surface, although this interaction was not significant. The level of shading had little impact on soil moisture content (Table A.3).

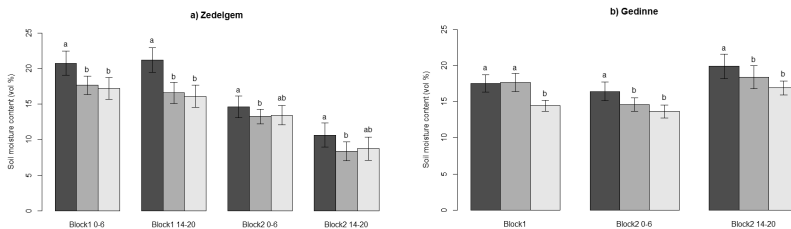


Fig. 3: Soil moisture contents and their confidence intervals separated between block and measurement depth for the sites of a) Zedelgem and b) Gedinne. Black bars indicate the control, light grey bars the most severe reduced rainfall treatment. There were no measurements at 14-20 cm in block 1 of Gedinne ('Gribelle') due to the high stone content. Significant differences according to the models is indicated using the digits a and b. Note that the model was not run separately for different depths of measurement and that the interaction between depth and level of rainfall reduction was never significant (Table A3). Hence, the graphical interpretation of the confidence intervals may differ from the mixed model results.

In our composition model results, there were little to no effects of the reduced rainfall treatment in Zedelgem or Gedinne. Our collector data supported that precipitation was

removed as expected and we did see an effect of the treatment on soil moisture content (SMC). However, the effect on SMC was only minor and did not consistently follow our interception gradient. Therefore, we also ran all models without the reduced rainfall treatment or its interactions to facilitate interpretation of our other two hypotheses. The results of these simplified models can be found in Tables 1 to 3. The original results, including the reduced rainfall effects, can be found in Tables A.4 to A.7.

3.2. Model results

Neither BA_r or HI_r were influenced by species richness in either of the two sites (Table 1). In contrast, particularly in Zedelgem, there were strong effects of identity. From 2014 on the presence of oak had in Zedelgem an increasingly negative significant impact on BA_r as well as on HI_r in the 2015 growing season. A less strong, but still overall negative impact of oak was found in Gedinne, but this was only significant in the 2014 growing season (Fig. 4). Other identity effects were less strong or consistent, though the presence of beech and lime did seem to be generally beneficial in Zedelgem, while the presence of larch improved HI_r in Gedinne.

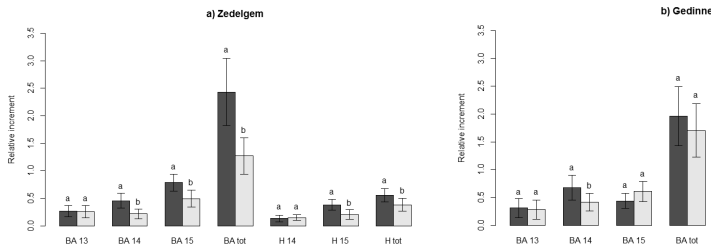


Fig. 4: Relative increment of basal area (BA) and height (H) in a) Zedelgem and b) Gedinne from 2013 to 2015 and across the three years. Bars in dark are phytometers without oak in the local neighbourhood, whereas grey bars represent phytometers with oak. Confidence intervals at 95% are indicated. Significant differences according to the models is indicated using the digits a and b.

Species richness did have a negative impact on PM symptoms in Gedinne in August 2013 and 2015, while the trend was negative at other times as well (Table 2). Graphic exploration and running the model with species richness as a factor variable revealed that this was caused by a strong difference between monocultures and the three other richness levels. Increasing richness also had a strong negative influence on the level of shading in Zedelgem, but did not seem to influence PM levels. Identity effects increasing the infectivity of PM were found for oak and to a lesser extent lime in Zedelgem, and for none of the five species in Gedinne. Mitigating effects were found for pine and to a lesser extent beech in Zedelgem, and weakly for larch and beech in

Gedinne. Strikingly, maple in Gedinne had a mitigating effect in 2013 and 2015, while its effect was (not-significantly) positive in 2014. Unexpectedly, oak in Gedinne had barely any impact on PM levels, with the small effect sizes even being mostly negative. Levels of shading in Zedelgem differed markedly between the two extremes of either birch and pine or beech and lime. Oak fell somewhere in between. In Gedinne, larch caused most shading by far, while maple was associated with the least – most likely related to its high mortality in the site with 20% of the local neighbourhood maples deceased.

Negative effects of PM on BAI_r were found in both sites (Table 3). Correlation within years as measured with VIF's was high in Gedinne, but not in Zedelgem. Because of this, the August data for Gedinne were dropped from the models. Negative effects were strongest in Zedelgem in July 2014, which seemed to dominate all other PM responses despite the poor level of multicollinearity and also had a negative impact on the following year both in terms of BAI_r and HI_r . PM effects were less strong in Gedinne and did not have an effect on growth in 2015. Shading had a negative impact on BAI_r in Zedelgem, contrasting with a positive impact on both BAI_r and HI_r in Gedinne.

Table 1: Table with the species richness and identity model results for basal area and height increment in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. B is the blocking factor. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. Square root (sqrt) transformations to compensate for residual heterogeneity are indicated, with an * indicating that the response variable was first pre-transformed by adding a constant to have a minimum of 0. These minima can be found in the rightmost column.

		Zedelgem											
		Species Richness					Species Identity						Min
tf	SR	B	C	Oak	Beech	Lime	Birch	Pine	B	C			
BAI13	sqrt*	0.03 °	-0.05	0.82***	sqrt*	-0.02	0.09 **	0.10 **	-0.02	-0.01	-0.05 °	0.82***	-0.35
BAI14	sqrt*	-0.01	-0.06	0.88***	sqrt*	-0.12 **	0.00	0.04	-0.02	0.05	-0.05	0.88***	-0.32
BAI15	sqrt*	0.01	-0.02	1.18***	sqrt*	-0.13 *	0.12 *	0.08	-0.03	0.00	-0.02	1.18***	-0.83
BAItot	sqrt*	0.01	-0.19	1.64***	sqrt*	-0.34 **	0.27 *	0.24 *	-0.09	0.00	-0.20 °	1.64***	-0.85
HI14		0.00	-0.07 *	0.18***	sqrt*	0.00	-0.02	0.02	-0.02	0.02	-0.04 *	0.86***	-0.20
HI15		0.01	-0.06	0.29*	sqrt*	-0.11 *	0.05	0.07	0.06	0.01	0.00	0.92***	-0.68
HIItot		0.03	-0.14	0.48***	sqrt*	-0.11 °	0.05	0.12 *	0.04	0.04	-0.03	0.94***	-0.57
		Gedinne											
		Maple					Larch Douglas						
BAI13	sqrt*	-0.01	-0.05	1.09***	sqrt*	-0.02	-0.04	-0.04	0.07	-0.01	-0.05	1.09***	-0.76
BAI14	sqrt*	0.01	0.16 **	0.77***	sqrt*	-0.12 *	0.04	-0.01	0.00	0.12 *	0.16 **	0.77***	-0.62
BAI15	sqrt*	0.01	0.02	1.06***	sqrt*	0.07	-0.07	-0.01	0.03	0.00	0.02	1.06***	-0.74
BAItot	sqrt*	0.00	0.17	1.30***	sqrt*	-0.07	-0.10	-0.08	0.12	0.13	0.17	1.30***	-0.78
HI14	sqrt*	-0.01	0.02	0.68***	sqrt*	-0.05	-0.05	-0.01	0.07	0.03	0.01	0.69***	-0.33
HI15	sqrt*	0.01	0.02	0.73***	sqrt*	0.02	-0.03	-0.03	0.08 *	0.00	0.02	0.73***	-0.43
HIItot	sqrt*	0.00	0.04	0.90***	sqrt*	-0.01	-0.08	-0.05	0.13 *	0.04	0.04	0.90***	-0.55

Table 2: Table with the species richness and identity model results for PM and shading levels in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, **, and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. B is the blocking factor. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. Square root (sqrt) transformations to compensate for residual heterogeneity are indicated.

		Zedelgem											
		Species Richness				Species Identity							
tf	SR	B	C	tf	Oak	Beech	Lime	Birch	Pine	B	C		
Jul 13	sqrt	-0.09	1.09**	1.64**	0.45	-0.49	0.54	-0.29	-0.69*	1.07**	1.66***		
Aug 13	sqrt	0.00	0.44	2.90***	-0.15	-0.35	0.73**	0.24	-0.55*	0.45°	2.91***		
Oct 13	sqrt	0.07	0.06	3.81***	0.64*	-0.32	0.42	-0.10	-0.30	0.05	3.83***		
Jul 14	sqrt	0.01	-0.07	4.36***	1.59***	-0.36	-0.39	-0.18	-0.58	-0.09	4.38***		
Sep 14	sqrt	0.10	1.36**	3.06***	0.65	-0.42	0.46	0.33	-0.59	1.36**	3.09***		
Shade		9.2**	4.71	37.52***	4.33	-6.04	-7.23	31.10***	20.11***	6.01	38.12***		
		Gedinne											
		Species Richness				Species Identity							
tf	SR	B	C	tf	Maple	Larch	Douglas						
Jul 13	sqrt	-2.09	4.35	26.73***	-0.39	-0.36	-0.42	0.11	-0.58	0.50	4.96***		
Aug 13	sqrt	-3.06*	3.56	38.21***	-0.10	-0.33	-0.57*	-0.37	-0.02	0.30	6.07***		
Jul 14	sqrt	-0.04	-1.00*	4.47***	-0.44	-0.29	0.60	-0.46	0.42	-1.00*	4.46***		
Aug 14	sqrt	-0.17	-0.93*	5.56***	0.13	-0.50	0.41	-0.92*	0.03	-0.92*	5.55***		
Aug 15	sqrt	-0.54**	-0.09	4.30***	-0.26	-1.08*	-0.73°	-0.34	-0.29	-0.08	4.29***		
Shade		0.30	0.71	3.92*	0.58	-0.59	-1.85**	2.67***	0.68	0.71	3.92**		

Table 3: Table with the PM and shading level results for basal area and height increment in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. B is the blocking factor. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. Square root (sqrt) transformations to compensate for residual heterogeneity are indicated, with an * indicating that the response variable was first pre-transformed by adding a constant to have a minimum of 0. These minima can be found in the rightmost column. Empty cells indicate that the PM predictor was not used in this model, either because it was a measurement from a later year than the growth took place or because the collinearity with other PM predictors was too high, as was the case for August 13 and 14 in Gedinne (Variance Inflation Factor > 2). No measurements were undertaken in Gedinne in October 2013 or September 2014, or in Zedelgem in August 2014. PM was mostly absent from Zedelgem in August 2015.

		Zedelgem											Min
tf	Shade	B	Jul 13	Aug 13	Oct 13	Jul 14	Sep 14	Aug 15	C				
BAI13	sqrt*	-0.12°	-0.05	-0.03	0.24	-0.2			0.89***				-0.35
BAI14	sqrt*	-0.07	-0.07°	-0.27	0.13	-0.07	-0.39***	0.24°	0.94***				-0.32
BAI15	sqrt*	-0.18*	-0.01	-0.27	0.03	-0.02	-0.58***	0.10	1.20***				-0.83
BAItot	sqrt*	-0.38*	-0.20°	-0.42	0.15	-0.15	-1.13***	0.30	1.68***				-0.85
HI14		-0.02	-0.06°	-0.14	-0.10	-0.02	-0.10	0.02	0.18***				
HI15		0.03	-0.08	0.27	0.02	-0.06	-0.40*	0.03	0.33***				
HIItot		0.00	-0.15	-0.28	-0.09	0.13	-0.72**	0.03	0.55***				
		Gedinne											
BAI13	sqrt*	0.22*	-0.06	-0.13					1.08***				-0.76
BAI14	sqrt*	-0.11	0.14*	-0.20		-0.51*			0.82***				-0.62
BAI15	sqrt*	0.07	0.03	-0.11		0.15		-0.01	1.06***				-0.74
BAItot	sqrt*	0.32°	0.13	-0.46°		-0.56		-0.22	1.35***				-0.78
HI14	sqrt*	-0.07	0.03	0.02		0.13			0.64***				-0.33
HI15	sqrt*	0.16*	0.01	-0.13		-0.16		-0.09	0.78***				-0.43
HIItot	sqrt*	0.14	0.03	-0.06		-0.04		-0.19	0.93***				-0.55

4. Discussion

In our experiment, effects of identity were much more important than those of species richness, which did not seem to have an influence on growth at all in either site. While other studies have found positive effects of increased species richness on basal area (Piotto, 2008) and height increment (Haase et al., 2015) of young trees, these effects were generally small and mostly a difference between monoculture and mixture, not a gradient effect. However, such a binary effect on growth was not seen in our experiment either (data not shown). In our design, increasing species richness did not correspond to increased dilution of hosts for specialist herbivores and pathogens, as the probability of a specific species being present increased along the richness gradient, while species density was constant across all richness levels. Hence, we would not expect a species richness effect through host dilution, whereas such host-density mediated effects have been found elsewhere (Hantsch et al., 2013; Peacock et al., 2001). This suggests that, at least in young forests, the influence of neighbourhood tree species richness lies more with resource dilution for specialist herbivores and pathogens, rather than effects of altered local environment (Hantsch et al., 2014) or by increased support for natural enemies (Jactel et al., 2005).

Support for a negative identity effect of the presence of oak on growth was found in both sites, but the strongest effect by far was in Zedelgem. While mortality was too low to assess a Janzen-Connell effect *sensu stricto*, growth reductions were substantial, with BAI_r from 2013 to 2015 being 54% lower and HI_r 61% from 2014 to 2015, when comparing plots without to plots with oak in Zedelgem (Table 1). This effect only became apparent from 2014 onwards, when a late frost in May killed the majority of first flush leaves in both sites. A second leaf flush was formed earlier than normal in June and was swiftly colonized by oak PM, as evidenced by the high levels seen in July in Zedelgem. This is not surprising, as initial oak PM sexual spore production is known to peak about a month after bud burst (Feau et al., 2012) and oak leaves are most vulnerable to PM infection in the first two weeks of their development (Edwards and Ayres, 1982).

Oak PM was mostly absent from Zedelgem in 2015, suggesting a carry-over effect from 2014's poor growth rates or mechanisms by other pests or pathogens to explain the ongoing negative effect of oak presence. However, we found no evidence of significant damage by other pests and we found strong negative relationships between oak PM levels and growth rates, most strongly the PM levels of July 2014. Other authors have not found such host-mediated impacts on oak PM levels (Hantsch et al., 2014). This might be explained by a different approach to identity effects, as we looked at absence or presence, whereas they looked at host density. The *Quercus* species studied by Hantsch et al. (2014), *Q. petraea*, is also known to be less susceptible to oak PM (Marçais and Desprez-Loustau, 2012) than the one present in Zedelgem, *Q. robur*. If an

identity effect is weaker and predominantly an asymptotic effect of presence, it might not be identified using density metrics.

In Gedinne, we also used *Q. petraea* rather than *Q. robur* and the negative effect of oak presence on growth was less strong there, being only significant in 2014, reverting to positive in 2015 and having no effect on height increment. Similarly, the negative effects of oak PM on growth were also lower and did not cross over into 2015, despite oak PM infections still being significant that year. The most likely explanation is, again, the lower susceptibility of *Q. petraea* to oak PM (Marçais and Desprez-Loustau, 2012). Yet, PM rates in Gedinne were found to be often higher in monocultures than in mixtures, which cannot be explained by a Janzen-Connell effect as monoculture neighbourhoods in our experiment could also consist of a different species and implied the lowest probability of encountering a specific species. A possible explanation could be that the monotonous environment as a result of a low functional diversity generates a stable physical environment that facilitates infections. A previous study found only a weak effect of functional diversity which even promoted herbivory instead (Haase et al., 2015), but this was attributed to associational susceptibility where the herbivores could benefit from a more diverse diet. This would not be the case for oak PM, a specialist parasite. A study covering oak PM on *Q. petraea* found a mitigating effect of tree species richness on PM infections that was not related to host dilution, but they could link it to identity effects of certain non-host tree species (Hantsch et al., 2014). In their experiment, an increasing proportion of coniferous species decreased fungal infestation, while *Fraxinus excelsior*, which suffered severely from ash dieback, had the opposite effect. Diseased trees with poorly developed crowns might have a reduced ability to conceal their neighbours from infectious spores, analogous to an increased apparency for herbivores (Castagneyrol et al., 2013). However, this contrasts with the mitigating effect of maple on PM infection in our experiment, as the species also suffered from poor vitality and even mortality (Van de Peer et al., 2016). Further supporting the theory that environmental conditions, rather than effective dispersal, were limiting to oak PM in this site is the absence of a link between oak presence and oak PM infection levels. A link to natural enemies seems unlikely too, as almost no signs of PM grazing were found and mycophagous ladybirds (Sutherland and Parrella, 2009) were only very rarely spotted within this site.

The presence of beech and lime in Zedelgem had positive effects on growth, though it is not immediately clear why. While lime had a positive effect on BAI_r and HI_r, beech had no influence on HI_r. In contrast with the effect of oak, both species did not have an impact in 2014. Beech and lime were associated with lower levels of shading than the other three species, in particular pine and birch (Table 3). Hence, an effect of diminished light competition might be at work here, in particular as we do not find these identity effects in 2014. That year, growth was suboptimal due to late spring frost and subsequent severe oak PM infections. These drivers would have been limiting rather

than competition for resources. Both a facilitative nursing effect and a mechanism through pathogen mediation seem less supported by our data, as we would expect drought to be more severe under more open canopies and we found contrasting results for the impact of beech and lime presence on oak PM levels (Table 2).

The presence of larch in Gedinne was positively related to height increment in 2015. Larch was the most shade-casting species, but greater levels of shading are not known to influence juvenile height growth in *Quercus* (Jensen et al., 2012; Niinemets, 1998). Nevertheless, greater levels of shading were also associated with greater HI_t in 2015 in Gedinne. Possibly a protective microclimate effect by large, nursing larches is an explanation, though we do not see this relationship to hold for BAI_t.

Data from the soil moisture content measurements and the lack of strong, consistent effects in our models suggests that our reduced rainfall treatment had little effect on the drought stress of our phytometers, even in very dry months such as August 2013. Interception rates as measured by the collectors fit our expectations, with the lower values found explainable by evaporation from the gutters and possibly even the dug-in collectors themselves. The soil moisture content values were generally 2 to 3% lower in the most severe treatment as compared to the control, and this effect generally increased at the depth of 14 to 20 cm as suggested by the effect size of the interaction between measurement depth and interception treatment (Table S2). Hence, there was also less water inside the treated pots than in the controls, but possibly not sufficient to become limiting even during dry spells. In addition, oak is considered to be quite tolerant of drought and not at great risk concerning expected climatic changes in summer precipitation (Campioli et al., 2012). However, such risks are typically evaluated in terms of mortality, not growth. Drought stress has been found to reduce growth for oak, even if compensatory leaf flushes follow the dry spell (Kuster et al., 2011; Scharnweber et al., 2011; Spieß et al., 2012).

5. Conclusion

In this study, we found a negative identity effect on growth of oak presence in the local neighbourhood of a young forest, which can be seen as a Janzen-Connell effect working primarily through the host-specific pathogen powdery mildew. This effect was seen in both our experimental sites but differed considerably in its strength, which might be explained by contrasting abiotic conditions or by the lower susceptibility to PM of *Q. petraea*. Other identity effects could be related to different levels of shading as a result of different juvenile growth rates of the tree species in the local neighbourhood. We found no effect of species richness influencing growth, but we did find higher PM levels in monocultures regardless of whether the neighbour species was the same species as our phytometers. We found no effects of reduced rainfall but this might change if a treatment with a stronger impact on soil moisture conditions is applied. We did find

contrasting effects of shading on growth between the sites of Zedelgem and Gedinne, which might be related to their differences in edaphic and microclimatic conditions. More research is needed to disentangle the influence of environmental conditions such as shading or rainfall on growth and oak PM infections, in particular concerning the impact of tree species that show poor vitality (such as *F. excelsior* due to ash dieback) and may therefore cast less shade or have lower apparency. The implications of our findings may not hold for other species than *Quercus* or for older stands, in particular given the strong impact of the specialist PM fungus. However, even if the contemporary impact of PM infections decreases with tree age, it might still be interesting to study the effect of young sapling infections on vitality and size in older trees. If severe PM infections are expected in a plantation, (temporarily) mixing the oak stand with other tree species at an individual level can be recommended to mitigate the spread of spores and reduce the odds of initial infection, as well as opting for *Q. petraea* over *Q. robur*.

6. Acknowledgments

MD was supported in this research by an FWO and an Ubbo Emmius grant. We would like to thank Luc Willems, Kris Ceunen and Filip Ceunen for their help with setting up the experiment and performing the fieldwork, as well as Evy Ampoorter for help with the statistical analysis.

Appendix

Table A1: Species combinations used for the two- and three-species mixtures in Zedelgem (Z) and Gedinne (G). Species are listed by their abbreviated scientific names: Quercus, Fagus, Tilia, Betula, Pinus, Acer, Larix and Pseudotsuga. Designs were similar in both sites, hence the symmetry of the matrices with only three of the five species replaced by others. x indicates species which were combined, A – E represent the five three-species mixtures.

Species combinations (2)

Z		Be	Qu	Fa	Ti	Pi
Z	G	La	Qu	Fa	Ac	Ps
Be	La		x			x
Qu	Qu	x		x		
Fa	Fa		x		x	
Ti	Ac			x		x
Pi	Ps	x			x	

Species combinations (3)

Z		Be	Qu	Fa	Ti	Pi
Z	G	La	Qu	Fa	Ac	Ps
Be	La		A	DE	AD	E
Qu	Qu	A		B	AC	BC
Fa	Fa	DE	B		D	BE
Ti	Ac	AD	AC	D		C
Pi	Ps	E	BC	BE	C	

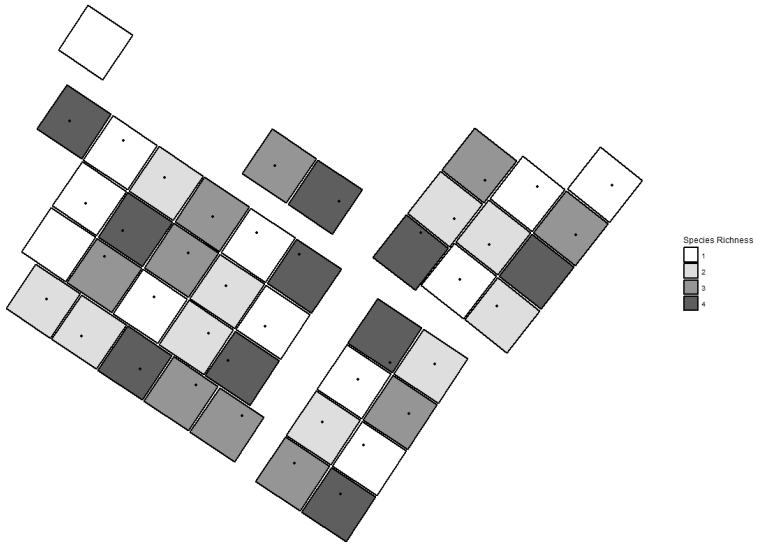


Fig. A1: Location of the phytometer plots in Zedelgem. The plot numbers have been removed to see the locations more clearly, they can be found in Fig. 1 in the manuscript.

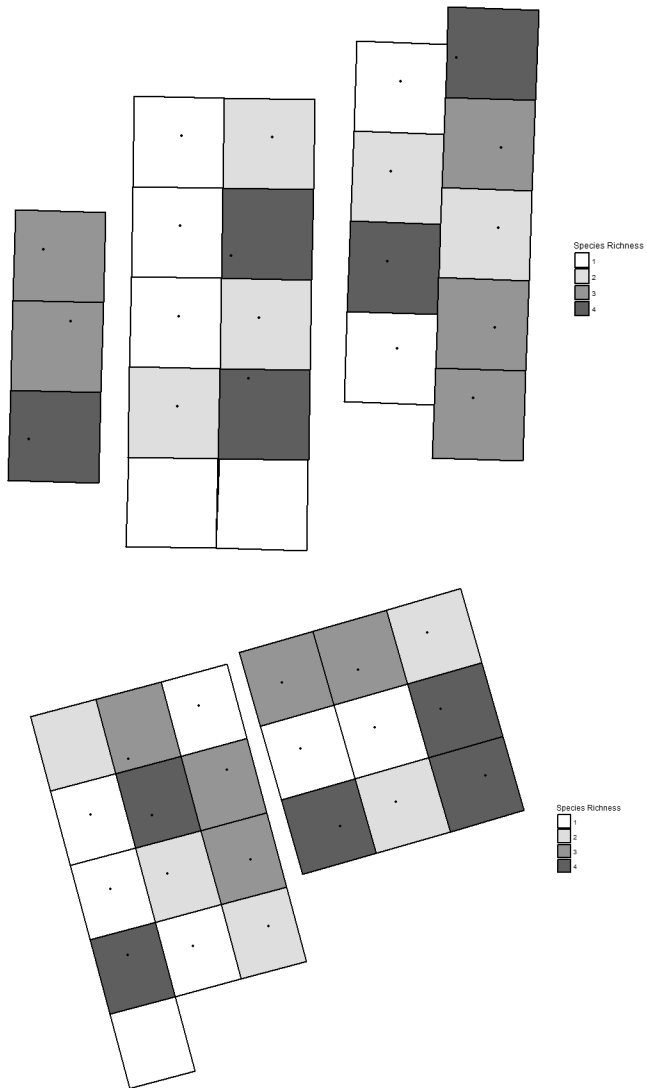


Fig. A2: Location of the phytometer plots in Gribelle (left) and Gouverneurs (right). The plot numbers have been removed to see the locations more clearly, they can be found in Fig. 1 in the manuscript.

Table A2: Monthly rainfall in mm for the growing season months of oak in the experiment, based on data from weather stations nearby (approximated straight distance from the sites indicated).

	Zedelgem				Gedinne			
	Lichtervelde 14 km				Bièvre 5-7 km			
	2013	2014	2015	1981-2010	2013	2014	2015	1981-2010
May	82	84	73.3	62	145	108	56.5	90
June	43	75	34.1	70	104	94	48.4	88
July	75	153	51.4	72	68	133	70.3	94
August	27	112	119.3	76	43	155	130.5	94
September	90	8	61.4	76	134	35.5	117.1	86
October	141	66	35.7	85	139	121	40.7	114

Table A3: Results of the soil moisture content models for Zedelgem and Gedinne. ***, ** and * imply p-values of <0.001, <0.01 and <0.05 respectively. The third column describes the results of retesting the models with a different reduced rainfall treatment as intercept, with the Šidák correction applied to α . 0, 1 and 2 refer to the three reduced rainfall treatments, with 0 the control and 2 the most severe treatment. SH is the shading % and was centered around its mean. Depth (d) was not a factor in Block 1 of Gedinne as all measurements there were taken at the surface.

	ZEDELGEM				GEDINNE			
	Block 1		Block 2		Block 1		Block 2	
R ² (marginal)	0.17		0.23		0.14		0.36	
R ² (conditional)	0.57		0.68		0.60		0.62	
0 (intercept)	20.78	*** a	14.84	*** a	17.55	*** a	16.62	*** a
1	-3.11	*** b	-1.94	* b	0.06	a	-2.08	** b
2	-3.60	*** b	-1.39	ab	-3.09	*** b	-2.92	*** b
d	0.45		-3.80	***			3.48	***
SH	-0.01		-0.05		0.01		0.14	***
1*d	-1.50		-1.01				0.34	
2*d	-1.55		-0.89				-0.21	
1*SH	0.02		-0.02		0.03		-0.10	*** b
2*SH	0.02		0.04	*	-0.02		-0.08	*** b
d*SH	0.01		0.01				0.00	

Table A4: Table with the species richness (SR) and reduced rainfall gradient model results for basal area and height increment in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. The reduced rainfall effects are indicated as 1 (medium) and 2 (severe treatment). B is the block factor. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. Square root transformations to compensate for residual heterogeneity are indicated, with an * indicating that the response variable was first pre-transformed by adding a constant to have a minimum of 0.

		Zedelgem						
	tf	SR	1	2	1*SR	2*SR	B	C
BAI13	sqrt*	0.00	-0.17°	0.03	0.09*	-0.01	-0.06°	0.87***
BAI14	sqrt*	-0.01	0.04	0.04	-0.01	0.01	-0.05	0.86***
BAI15	sqrt*	0.06°	0.23*	0.26**	-0.07°	-0.09**	-0.02	1.02***
BAItot	sqrt*	0.05	0.11	0.37*	0.00	-0.11°	-0.20	1.48***
HI14		0.01	0.08	0.01	-0.02	-0.01	-0.07*	0.15*
HI15		0.02	-0.01	0.06	-0.01	-0.01	-0.06	0.26°
HIItot		0.08	0.25	0.24	-0.08	-0.08	-0.15	0.32*
		Gedinne						
	tf	SR	1	2	1*SR	2*SR	B	C
BAI13	sqrt*	0.01	0.16	0.05	-0.05	-0.00	-0.05	1.02***
BAI14	sqrt*	0.00	-0.02	0.23	0.04	-0.02	0.16*	0.71***
BAI15	sqrt*	0.04	0.11	-0.07	-0.07°	-0.02	0.02	1.05***
BAItot	sqrt*	0.03	0.25	0.23	-0.07	-0.04	0.16	1.15***
HI14	sqrt*	0.01	0.05	-0.05	-0.05°	0.01	0.02	0.68***
HI15	sqrt*	0.04°	0.12	0.13	-0.06°	-0.04	0.02	0.65***
HIItot	sqrt*	0.04	0.12	0.05	-0.09*	-0.03	0.04	0.85***

Table A5: Table with the species identity and reduced rainfall gradient model results for basal area and height increment in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. Species are listed by their abbreviated scientific names: Quercus, Fagus, Tilia, Betula, Pinus, Acer, Larix and Pseudotsuga. The reduced rainfall effects are indicated as 1 (medium) and 2 (severe treatment). B is the block factor. The intercept C was always significant (***), and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. All response variables were square root transformed to compensate for residual heterogeneity, but also first pre-transformed by adding a constant to have a minimum of 0.

Zedelgem																			
	Qu	Fa	Ti	Be	Pi	1	2	Qu*1	Qu*2	Fa*1	Fa*2	Ti*1	Ti*2	Be*1	Be*2	Pi*1	Pi*2	B	C
BAI13	-0.03	0.03	0.14*	-0.03	-0.07	-0.17°	0.03	0.08	-0.04	0.18*	0.01	-0.01	-0.10	0.03	0.02	0.15°	0.03	-0.06°	0.87
BAI14	-0.14*	0.01	0.07	-0.03	0.03	0.03	0.05	0.06	0.00	-0.07	0.03	-0.05	-0.05	-0.03	0.05	0.06	-0.01	-0.06	0.85
BAI15	-0.09	0.12°	0.22**	0.00	0.06	0.23*	0.26**	-0.09	-0.02	0.06	-0.04	-0.22**	-0.21**	-0.01	-0.09	-0.08	-0.11	-0.02	1.02
BAItot	-0.29*	0.16	0.52***	-0.08	-0.01	0.13	0.39*	-0.04	-0.09	0.28°	0.05	-0.36*	-0.45**	-0.01	-0.04	0.12	-0.07	-0.21°	1.46
HI14	0.03	-0.03	0.06°	-0.02	0.01	0.05	0.01	-0.03	-0.04	0.00	0.04	-0.04	-0.06	-0.01	0.03	0.02	0.00	-0.04*	0.83
HI15	-0.13*	0.03	0.11°	0.06	0.05	0.01	0.07	0.00	0.06	0.01	0.04	-0.06	-0.06	0.04	-0.03	-0.04	-0.08°	0.00	0.89
HIItot	-0.09	0.03	0.22**	0.01	0.10	0.15	0.14	-0.04	-0.02	-0.02	0.06	-0.16*	-0.17*	0.05	0.03	-0.06	-0.12	-0.03	0.84
Gedinne																			
	Qu	Fa	Ac	La	Ps	1	2	Qu*1	Qu*2	Fa*1	Fa*2	Ac*1	Ac*2	La*1	La*2	Ps*1	Ps*2	B	C
BAI13	0.04	-0.1	0.04	0.06	0.04	0.16	0.05	-0.07	-0.11	0.17	0.09	-0.17	-0.06	-0.06	0.1	-0.1	-0.03	-0.05	1.01
BAI14	-0.18*	0.12	0	-0.01	0.1	-0.02	0.22	0.15	0.03	-0.05	-0.2	0.15	-0.07	-0.03	0.06	0	0.08	0.16*	0.71
BAI15	0.07	-0.1	0.01	0.16*	0.03	0.11	-0.07	0.05	-0.05	-0.06	0.10	-0.09	0.04	-0.25**	-0.12	-0.02	-0.07	0.02	1.05
BAItot	-0.09	-0.2	0	0.23	0.19	0.25	0.22	0.19	-0.15	0.11	0.03	-0.10	-0.10	-0.39*	0.06	-0.18	-0.01	0.17	1.15
HI14	-0.04	-0.1	0.00	0.10	0.08	0.05	-0.06	0.02	-0.01	0.00	0.05	-0.09	0.01	-0.15*	0.04	-0.05	-0.05	0.02	0.68
HI15	-0.01	0.01	0.01	0.15**	0.05	0.12	0.13	0.04	0.06	-0.09	-0.01	-0.11	-0.02	-0.09	-0.12°	-0.05	-0.11	0.02	0.65
HIItot	-0.04	-0.1	0.00	0.23**	0.11	0.12	0.05	0.04	0.04	-0.06	0.04	-0.16°	0.00	-0.20*	-0.08	-0.08	-0.14	0.04	0.85

Table A6: Table with the species richness (SR) and reduced rainfall gradient model results for PM and shading levels in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. The reduced rainfall effects are indicated as 1 (medium) and 2 (severe treatment). B is the block factor. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. Square root transformations to compensate for residual heterogeneity are indicated.

		Zedelgem						
	tf	SR	1	2	1*SR	2*SR	B	C
Jul 13	sqrt	-0.30	-0.32	-1.20	0.08	0.56°	1.10**	2.12**
Aug 13	sqrt	-0.16	0.16	0.01	0.18	0.31	0.44	2.84
Oct 13	sqrt	0.05	-0.43	0.84	0.23	-0.18	0.07	3.66***
Jul 14	sqrt	-0.07	-1.34	-0.17	0.36	-0.10	-0.06	4.83
Sep 14	sqrt	-0.07	-0.22	-0.35	0.19	0.34	1.37**	3.24***
Shade		7.49*	-14.79*	-6.15	3.73	1.73	4.97	43.97***
		Gedinne						
	tf	SR	1	2	1*SR	2*SR	B	C
Jul 13		-4.16	-10.87	-8.27	4.20	2.06	4.38	32.99***
Aug 13		-4.31°	-6.41	-4.20	2.42	1.35	3.57	41.72***
Jul 14	sqrt	0.06	-0.90	0.23	0.12	-0.40	-1.00*	4.70***
Aug 14	sqrt	-0.02	-0.19	0.23	-0.13	-0.33	-0.93*	5.55***
Aug 15	sqrt	-0.48*	0.94	-1.18	-0.38	0.22	-0.07	4.32***
Shade	sqrt	0.42	0.38	0.68	-0.10	-0.26	0.71	3.58*

Table A7: Table with the species identity and reduced rainfall gradient model results for PM and shading levels in Zedelgem and Gedinne. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. Species are listed by their abbreviated scientific names: Quercus, Fagus, Tilia, Betula, Pinus, Acer, Larix and Pseudotsuga. The reduced rainfall effects are indicated as 1 (medium) and 2 (severe treatment). B is the block factor. The intercept C was always significant (***, unless otherwise indicated) and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. All response variables were square root transformed to compensate for residual heterogeneity, with the exception of shade in Zedelgem.

		Zedelgem																		
		Qu	Fa	Ti	Be	Pi	1	2	Qu*1	Qu*2	Fa*1	Fa*2	Ti*1	Ti*2	Be*1	Be*2	Pi*1	Pi*2	B	C
Jul 13		0.45	-0.28	-0.32	-0.78	-0.48	-0.30	-1.13	-0.21	0.23	-0.51	-0.10	1.34°	1.23°	0.40	1.15°	-0.68	0.10	1.09**	2.10**
Aug 13		-0.24	-0.53	0.24	0.21	-0.56	0.20	0.08	0.7	-0.33	0.31	0.24	0.43	0.95	-0.62	0.62	0.09	-0.14	0.42	2.87
Oct 13		0.52	-0.47	-0.03	-0.2	0.48	-0.32	0.89	0.55	-0.21	0.69	-0.22	0.89°	0.44	0.07	0.17	-1.2*	-1.19*	0.03	3.66
Jul 14		1.22*	0.09	-1.31*	-0.16	-0.16	-1.24	-0.16	0.68	0.29	-0.67	-0.76	1.36°	1.54*	0.50	-0.56	-0.25	-1.01	-0.09	4.85
Sep 14		0.55	-0.72	-0.40	0.20	-0.02	-0.16	-0.3	0.27	0.03	0.19	0.69	0.96	1.62*	0.07	0.29	-0.62	-1.11	1.35**	3.26
Shade		2.52	-2.24	-7.45	25.19***	16.37**	-14.91*	-6.04	1.17	3.00	-5.24	-6.24	1.62	0.17	12.86*	6.28	7.38	5.08	6.48	44.24
		Gedinne																		
		Qu	Fa	Ac	La	Ps	1	2	Qu*1	Qu*2	Fa*1	Fa*2	Ac*1	Ac*2	La*1	La*2	Ps*1	Ps*2	B	C
Jul 13		-0.87	-0.44	-0.88	0.17	-0.68	-0.88	-0.76	0.83	0.59	-0.31	0.55	0.99	0.37	0.17	-0.39	0.31	-0.02	0.49	5.53
Aug 13		0.21	-0.5	-0.99*	-0.62	0.16	-0.30	-0.17	-0.11	-0.84	-0.06	0.61	0.73	0.49	0.59	0.14	-0.5	-0.04	0.29	6.25
Jul 14		0.27	-0.74	0.21	-0.30	0.85	-0.90	0.35	-0.25	-1.94*	0.17	1.27	0.55	0.54	0.04	-0.61	0.08	-1.43°	-1.02*	4.73
Aug 14		0.77	-0.79	0.05	-0.49	0.37	-0.19	0.30	-0.13	-1.83**	0.15	0.79	0.35	0.67	-0.81	-0.53	-0.22	-0.84	-0.94*	5.56
Aug 15		-0.19	-1.18*	-1.01°	0.22	-0.24	0.94	-1.18	-0.69	0.53	-0.3	0.56	0.21	0.67	-1.10°	-0.54	0.00	-0.11	-0.07	4.32
Shade		1.04°	-0.48	-1.67**	2.80***	0.38	0.38	0.68	-0.43	-0.96**	-0.12	-0.19	-0.42	-0.13	0.19	-0.61°	0.27	0.61°	0.71	3.58

Chapter 4: Stronger diversity effects with increased environmental stress: A study of multitrophic interactions between oak, powdery mildew and ladybirds

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Abstract

Recent research has suggested that increasing neighbourhood tree species diversity may mitigate the impact of pests or pathogens, by supporting the activities of their natural enemies and/or reducing the density of available hosts. In this study, we attempted to assess these mechanisms in a multitrophic study system of young oak (*Quercus*), oak powdery mildew (PM, caused by *Erysiphe* spp.) and a mycophagous ladybird (*Psyllobora vigintiduopunctata*). We assessed ladybird mycophagy on oak PM in function of different neighbourhood tree species compositions. We also evaluated whether these species interactions were modulated by environmental conditions as suggested by the Stress Gradient Hypothesis. We adopted a complementary approach of a field experiment, where we monitored oak saplings subjected to a reduced rainfall gradient in a young planted forest consisting of different tree species mixtures, as well as a lab experiment, where we independently evaluated the effect of different watering treatments on PM infections and ladybird mycophagy.

In the field experiment, we found effects of neighbourhood tree species richness on ladybird mycophagy becoming more positive as the target trees received less water. This effect was only found as weather conditions grew drier. In the lab experiment, we found a preference of ladybirds to graze on infected leaves from trees that received less water. We discuss potential mechanisms that might explain this preference, such as emissions of volatile leaf chemicals. Our results are in line with the expectations of the Natural Enemies Hypothesis and support the hypothesis that biodiversity effects become stronger with increased environmental stress.

1. Introduction

In the last two decades, there have been an increasing number of studies concerning the influence of species composition on the functioning of ecosystems (Cardinale et al., 2012; Hooper et al., 2005; Tilman et al., 2014). Positive effects of increasing diversity were found in many different systems, including forests, and for many different ecosystem functions, including biomass production (Liang et al., 2016) and mitigated impacts of pests and pathogens – also known as associational resistance (Jactel et al., 2005; Jactel and Brockerhoff, 2007). Many mechanisms have been proposed to explain these community effects, including host dilution for specialist pests (Castagneyrol et al., 2013), complementarity of resource use (Morin et al., 2011) and direct facilitation such as through fertilizing effects of legumes (Schmidtke et al., 2010). Research has also suggested an important role of multitrophic interactions through natural enemies of pests (Sobek et al., 2009). According to the Natural Enemies Hypothesis, predators may diversify their diet in more diverse stands nutritionally as well as temporally and have access to more diverse habitats for shelters or oviposition (Jactel et al., 2005; Letourneau, 1987). However, questions remain concerning the role of the ecological traits of these natural enemies, as increasing species richness in a stand is often associated with increasing pest richness, which may impair the efficacy of specialist natural enemies such as most hyperparasites (Haase et al., 2015; Scherber et al., 2010).

Oak powdery mildew (PM, caused by *Erysiphe* spp.) is the most important disease of West European oak (*Quercus*) species. As the disease is caused by a biotrophic fungal leaf pathogen, it may reduce net carbon assimilation as well as leaf lifespan and is associated with reduced radial growth or even increased mortality, especially in saplings (Bert et al., 2016; Dillen et al., 2016; Hajji et al., 2009; Marçais and Desprez-Loustau, 2012). First sighted in Europe in 1907 (Mougou et al., 2008), the disease may play a role in the poor shade tolerance and regeneration of young oak saplings under canopy (Rackham, 2003) and is the chief reason for frequent fungicide use on oaks in tree nurseries (Marçais and Desprez-Loustau, 2012; Pap et al., 2012). Recent research into the causal agents of oak PM differentiated at least four different species (*E. alphitoides*, *E. quercicola*, *E. hypophylla* and *Phyllactinia roboris*), of which *E. alphitoides* is the most frequently observed (Feau et al., 2012; Hamelin et al., 2016).

Grazing of powdery mildews by mycophagous ladybirds (Weber and Lundgren, 2009) has been observed across the world (de Almeida and Milléo, 1998; Kumar et al., 2010; Satti, 2015; Sutherland and Parrella, 2009; Younes et al., 2015). While several species of aphidophagous ladybird were found to include powdery mildews as a minor part of their diet (Giorgi et al., 2009), ladybirds of the tribe Halyziini are strongly suspected of being obligate mycophages, possibly even limited to only mildew species (Sutherland and Parrella, 2009). The ladybird *Psyllobora vigintiduopunctata* is a member of this tribe

and occurs relatively abundantly in Western European woodland elements and young forest patches. Given that tree saplings will suffer the strongest impact of mildew infections (Marçais and Desprez-Loustau, 2012), these ladybirds might play an important ecological role in mitigating the impact of PM infections. Furthermore, given that PM is caused by a specialist fungal pathogen and these ladybirds may be specialized feeders on mildew, this system could shed light on the relationship between the composition effect hypotheses of host dilution and natural enemies.

Effects of neighbourhood species composition might be dependent on environmental factors (Haase et al., 2015; Nguyen et al., 2016). Recent research into the context dependency of neighbourhood species richness effects raised some evidence for the Stress Gradient Hypothesis, where positive interactions between species are more frequent in stressful conditions (Jucker et al., 2015; Paquette and Messier, 2011; Toïgo et al., 2015). Therefore, if the Stress Gradient Hypothesis applies, a greater incidence of stressful conditions would increase the impact of neighbourhood composition effects, as more facilitative interactions will be expected. Such an interaction between plant stress and composition may have important ramifications in the light of Anthropogenic Global Change, which is expected to increase the frequency of stressful conditions such as drought or outbreaks of invasive pests (Rockström et al., 2009). Hence, it might become more important to increase diversity in species-poor systems or attempt to halt the global trend of biodiversity loss to preserve ecosystem functioning (Thompson et al., 2009; Tilman et al., 2014), as positive interactions would play a greater role under the changing conditions than expected from current assessments. While previous research found no great impact of dry weather conditions (Marçais and Desprez-Loustau, 2012) or air humidity (Pap et al., 2013) on mildew infection rates, the response of mycophagous ladybirds to changing plant water conditions is unknown. Changing water conditions may affect palatability of the fungus or the ability of the ladybird to find infected leaves by increased emission of signal molecules (Tabata et al., 2011).

In this study, we set out to elucidate the impact of natural enemies on oak PM infections of oak (*Q. robur*) saplings in a young planted forest stand. We looked specifically at the ladybird *P. vigintiduopunctata*, due to its abundance in the study area, in particular on *Quercus*, and because previous studies already indicated that relatives of this species were capable of causing substantial reductions in infected leaf area through mildew grazing (Sutherland and Parrella, 2009). We hypothesized that increasing tree species richness had a mitigating effect on oak PM levels. Attempting to link composition effects to the Natural Enemies Hypothesis, we hypothesized that PM mycophagy and ladybird counts were similarly related to richness effects. To investigate the interplay between composition effects and the Stress Gradient Hypothesis, we established a rainfall reduction gradient for our focal saplings, predicting that effects of species richness on ladybird activity would be more important under more stressful conditions. Given the

little knowledge available on the relationship between plant water status and mycophagy by ladybirds, we also conducted a lab study where the impact of neighbourhood species composition was eliminated. We specifically looked at the impact of different watering rates on PM infection severity and on the attractivity of mycelia for the ladybirds.

2. Materials and methods

2.1. Field experiment

Our experiment was conducted as part of a larger study into tree species composition effects and took place in Zedelgem, one of the three FORBIO sites in Belgium (Verheyen et al., 2013). This site was planted in late 2009 and early 2010 with monocultures and mixtures of saplings of *Quercus robur*, *Fagus sylvatica*, *Pinus sylvestris*, *Tilia cordata* and *Betula pendula*, planted in groups of 3 by 3. In a total of 40 plots, 5 compositions for each species richness level from 1 to 4 were represented, including all five monocultures and 4-species mixtures; each composition was replicated once. In early May 2013, one-year old *Q. robur* saplings were planted in separate pots between 4 of the older FORBIO trees. Saplings were planted in groups of 3, each subjected to a different treatment of reduced rainfall by placing 0, 1 or 2 pairs of gutters above the pot surface but below the sapling canopy (Fig. 1). A more extensive description of the experimental setup can be found in Dillen et al. (2016).



Fig. 1: Example of a phytometer plot with the three rainfall reduction treatments. Three of the four surrounding FORBIO trees are visible.

In 20 of the Zedelgem plots, a monitoring for oak PM, PM mycophagy and mycophagous ladybird occurrence was performed bi-weekly four times in August and the first half of September 2014. All first order shoots were assessed using affected area classes of 0-5%, 6-30%, 31-60% and 61-100%. We also estimated a size weight for each shoot, indicative of relative differences in leaf area between first order shoots using the same approach as in Dillen et al. (2016). The PM scores included the PM grazing marks (Fig. 2), so they could be used as a control variable for initial PM infected area. Mycophagy was assessed as % of total leaf area, not the area infected by PM. A weighted average of the class scores of the shoots was subsequently calculated to get PM infected leaf area percentages at the sapling level, using the shoot size weights and the class mids, except 0% for the smallest class (i.e. 0, 17.5, 45 and 80%). *P. vigintiduopunctata* occurrence was assessed by counting all present individuals that could be found on a sapling (in their 3rd growing season at the time) in 1 minute. We differentiated between larvae, pupae and adults, but did not look for eggs as these are very small and therefore very difficult to find and identify.



Fig.2: Example of ladybird grazing marks on oak PM.

All statistical analyses were performed using R (R Core Team, 2015). For mixed models, we used the R package *lme4* (Bates et al., 2015), with *lmerTest* extension (Kuznetsova et al., 2015) to determine Satterthwaite approximated p-values. Testing the

influence of composition, reduced rainfall (DR) and their interaction on PM infection levels was done using mixed models, with plot as a random factor. Models were run separately for the four monitoring moments, as weather conditions changed between them. Models were also run separately testing the influence of neighbourhood species richness (SR) and species identity (SP), quantified as species presence or absence.

$$PM (\%) = SR + RR + SR * RR$$

$$PM (\%) = \sum_{i=1}^5 SP_i + RR + \sum_{i=1}^5 SP_i * RR$$

Where **PM** is the weighted average of PM infected leaf area % at the sapling level, **SR** is the species richness gradient (1-4), **RR** is the three-level rainfall reduction gradient and **SP** is the absence/presence in the local neighbourhood of the five FORBIO three species. Similar models were run testing the influence of neighbourhood species composition on PM mycophagy, but including a covariate for PM levels as the potential for mycophagy could be expected to be greater if more PM was available. This covariate was always centered around its mean to improve interpretability of the parameter estimates.

The influence of composition and reduced rainfall on ladybird counts was modeled as a Generalized Linear Mixed Model using *lme4*, assuming a Poisson distribution. A negative binomial distribution was assumed if overdispersion was suspected, as indicated by a χ^2 test on the ratio between the residual deviance and degrees of freedom using the function found at <http://glmm.wikidot.com/faq/>. The high frequency of zeroes and stochasticity of the data due to (adult) ladybird mobility lead us to sum the counts across all four measuring moments. Larvae were already very rare at this time of year and were treated as adults when summing the data.

2.2. Lab experiment

In December 2014, 21 oak saplings were planted in pots of 15 L and top diameter of 32 cm, using potting soil with N-P-K content of 14-16-18 and 13% organic matter. The saplings were already in hibernation and had faced subzero temperatures before being removed from the field. All were placed in a climate chamber with 15 h of light and 9 h dark at constant temperature of 21°C. Each pot received weekly watering of 1 L, corresponding to a monthly precipitation level of about 56 mm. Monthly summer precipitation (June – September) averaged 73 mm in Zedelgem in the last 8 years, with 56 mm being the low end of the 95% confidence interval (KMI, Belgium). The lower estimate was chosen as poorer drainage was expected in the lab conditions.

Half January the first leaves started to appear. Three older oak saplings infected with PM had been removed from the field in late summer and were kept in the climate chamber to serve as inoculation for the other saplings. These older saplings still had

fresh leaves and live PM mycelia by the time the last of the 21 saplings started to leaf out. One month after initial leafing out, all trees had fully developed their leaves and all were infected with PM. On from this point, two groups of 7 saplings received only 0.8 L and 0.6 L of water per week respectively, mimicking the reduced rainfall treatment used in the field. Saplings were sorted in the different watering treatment groups (i.e. 1, 0.8 and 0.6 L per week) in a random manner. Soil moisture content by volume was measured from the topsoil two weeks, six weeks and ten weeks after watering treatment began, using a ML3 ThetaProbe (Delta-T Devices) set with the standard conversion parameters for an organic soil. The crown area infected by PM was also evaluated three times: three, six and nine weeks after the watering treatment began. The protocol used was similar to the one used in the field, but because of the smaller crowns and lower amount of trees, classes of 10% were used.



Fig.3: Example of a ladybird choice feeding experiment, picture taken after 24 hours. The ladybird was removed to take this picture, but evidence of mycophagy can be noted in particular on the two leaf segments on the left. The leaf on the bottom also contains multiple particles of feces.

In late April and early May, four experiments on mycophagy by *P. vigintiduopunctata* were performed using leaves from the 21 saplings. Leaves of four saplings of each watering treatment were harvested and cut into segments that could fit in a Petri dish (Fig. 3). Leaves were picked to minimize differences in PM infected area and leaf age (as lammas shoots had already formed in most saplings) between the watering treatments. Three leaf segments of the different watering treatments were placed on agar substrate (Micro Agar M1002) in a Petri dish with a ventilation hole (Fig. 3). An adult *P. vigintiduopunctata* was placed at equal distance from the three segments and monitored for mycophagy with a handheld camera every 24 hours, including an initial

snapshot and lasting up to 96 hours by which time leaves started to show signs of wilting. Ladybirds were removed as pictures were taken and reintroduced afterwards at their initial starting point. In each of the four experiments, four male and four female ladybirds were monitored. The male and female group each received leaf segments originating from the same 12 saplings. The ladybirds used for the experiment were adults who had been captured in the autumn of 2014 and kept fed in a climate chamber (16/8 light and dark treatment and constant temperature of 20°C) with fresh PM-infected leaves of *Q. robur*, *Convolvulus sp.* and *Sonchus oleraceus*. Before the feeding experiment, these ladybirds were subjected to a four month hibernation treatment of 4°C to ensure they would not be in diapause. Mycophagy was quantified using image analysis with APS Assess 2.0 (Lamari 2008). For each image, the PM infected area was digitally selected by modifying saturation. Differences in infected area were defined as mycophagy.

The influence of the water treatment was assessed in *lme4* (Bates et al., 2015) using a repeated measures mixed model, with the four different experiments and the ladybird used as random factors:

$$MP = RR + T + G + PM + RR * T + RR * G + T * G$$

Where MP is the mycophagy in cm², RR is the three-level watering treatment, T is the time of measurement (24-48-72-96h), G is gender of the ladybird and PM is initial PM affected area in cm², centered around its mean. We also tested separately using a General Linear Model whether there was any difference in initial PM affected area between the three watering treatments.

3. Results

3.1. Field experiment

Table 1: Parameter estimates of the mixed models of the PM and mycophagy scores (%) and total number of ladybirds over 4 measuring moments in function of rainfall reduction (RR) and species richness (SR). C is the intercept, PM.c is the PM score covariate centered around its mean when applicable. Statistical significance is indicated using °, *, ** and *** indicating $p < 0.1$, 0.05, 0.01 and 0.001 respectively. Overdispersion was present for the ladybird count model as indicated by the ratio and its χ^2 test.

		RR1	RR2	SR	RR1*SR	RR2*SR	PM.c (%)	C	
PM	Week 1	0.5	-5.86	0.92	-0.24	1.6		29.13**	
	Week 2	-0.48	-9.15	0.67	0.78	2.76		32.39***	
	Week 3	6.47	-0.64	2.79	-2.36	-1.15		17.76***	
	Week 4	0.48	-3.8	-0.2	0.66	1.07		22.03***	
Mycophagy	Week 1	-1.94	-1.77	-0.16	0.49	1.29	0.4***	16.82***	
	Week 2	-4.03	0.73	-0.44	1.73	0.05	0.36***	15.84***	
	Week 3	-5.51*	-4.38°	-1.16°	2.09*	1.55°	0.39***	16.03***	
	Week 4	1.78	-3.63°	-0.5	-0.69	1.37*	0.38***	13.22***	
Ladybirds	Poisson	0.08	0.75**	0.03	0.07	-0.15		1.62***	Ratio = 1.74***
	Negat Bin	0	0.72	0.02	0.1	-0.15		1.68***	

We found no influence of reduced rainfall or of species richness on PM infection rates (Table 1). We did see consistently higher PM rates if *Tilia* was present in the neighbourhood, but only for the phytometers receiving reduced rainfall (Table A1). PM was about 10% lower in the last two weeks than it was in the first two. In the last two weeks, we found an interaction between species richness and reduced rainfall impacting mycophagy (Fig. 4). Mycophagy overall did not change if rainfall was more strongly reduced (Fig. 4), but the impact of neighbourhood species richness increased – resulting in less mycophagy in monocultures but more in mixtures when comparing control phytometers to those receiving less rainfall. As expected, phytometers with greater PM infected area also showed greater mycophagy (Table 1). In the presence of *Fagus*, mycophagy was reduced but only on phytometers under the control treatment (Table A1).

We found no significant influence of reduced rainfall or composition on ladybird occurrence. There were generally more ladybirds (larvae + adults) on the phytometers receiving less rainfall (121, 158 and 176 total over 4 weeks respectively), but this effect was not significant when using a negative binomial distribution instead of Poisson after overdispersion was found (Table 1). Across the four monitoring sessions, we found no pupae and only few larvae of which only 1 in the control trees, compared to more than 10 on the trees receiving less rainfall. An identity model for ladybird count data yielded

no significant effects, but the high complexity of the mixed model also resulted in a lack of convergence.

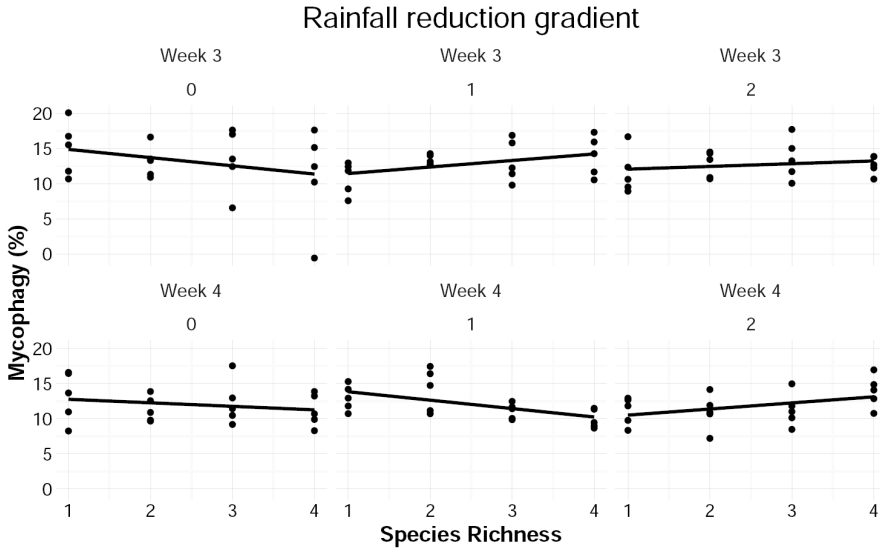


Fig.4: PM mycophagy in function of species richness in weeks 3 and 4 of monitoring, when a significant interaction was found. The mycophagy scores were transformed according to the effect of the PM covariate.

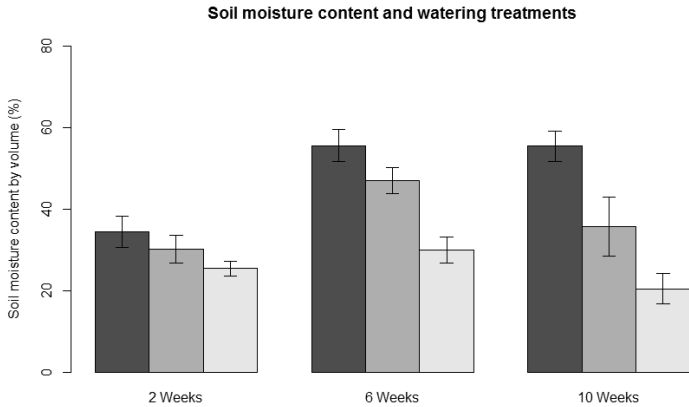


Fig. 5: Soil moisture content by volume of the lab experiment oak trees for the different watering treatments (black = weekly 1L, dark grey = 0.8 L and light grey 0.6 L) with their 95% confidence intervals.

3.2. Lab experiment

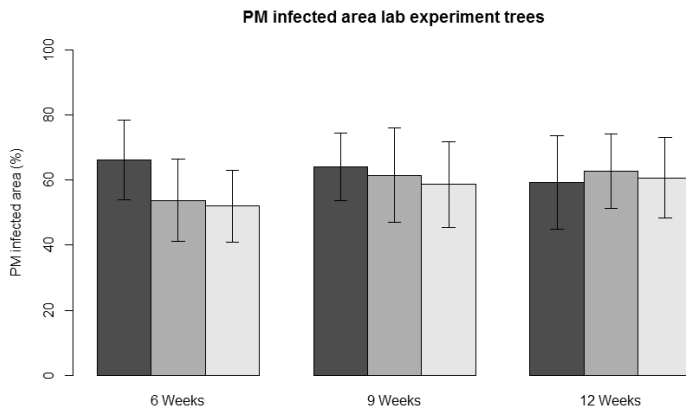


Fig. 6: Average PM infected area of the lab experiment oak trees for the different watering treatments (black = 1 L, dark grey = 0.8 L and light grey 0.6 L per week) with their 95% confidence intervals.

The difference in soil moisture content between the three watering treatments continued to grow as the experiment ran and was significant ($p < 0.01$ in one-way ANOVA) before the choice feeding experiments began (Fig 5.). The moisture content values were all rather high, rarely dropping below 20%. There was a small, insignificant initial difference in PM infected area between the trees receiving less water and the control ($p=0.06$ in one-way ANOVA), but this was not found again during later measurements (Fig 6.).

In the choice feeding experiment, we found that PM infected leaves from trees that received less water were significantly more grazed upon than the control (Fig. 7). The effect followed the gradient we applied, mycophagy increasing by 0.33 and 0.67 cm² for the 0.8 and 0.6 L treatments respectively ($p < 0.01$ in the full model; extensive model results can be found in Table A2). The overall average PM consumption after 96 hours was 1.54 cm² per leaf segment and 4.61 cm² per ladybird. This represents on average 51% of the PM on each leaf segment and 50% of all PM available for each ladybird. Complete removal by grazing of all PM on a single leaf was rare and not related to watering treatment, though it did not occur at all on the control leaves (data not shown). After 24 hours, males removed 1.95 cm² and females 2.31 cm² on average. Thus, females appeared to graze more than males and the difference increased with time (Fig. 7), but this difference was not significant in our model ($p = 0.18$). While initial PM area positively influenced mycophagy ($p=0.001$), it did not differ significantly between the three watering treatments ($p=0.94$, one-way ANOVA). There were no significant interactions between the watering treatment (Table A2), gender and running time of the

experiment, though females did seem to keep up more steady mycophagy rates than males (Fig. 7).

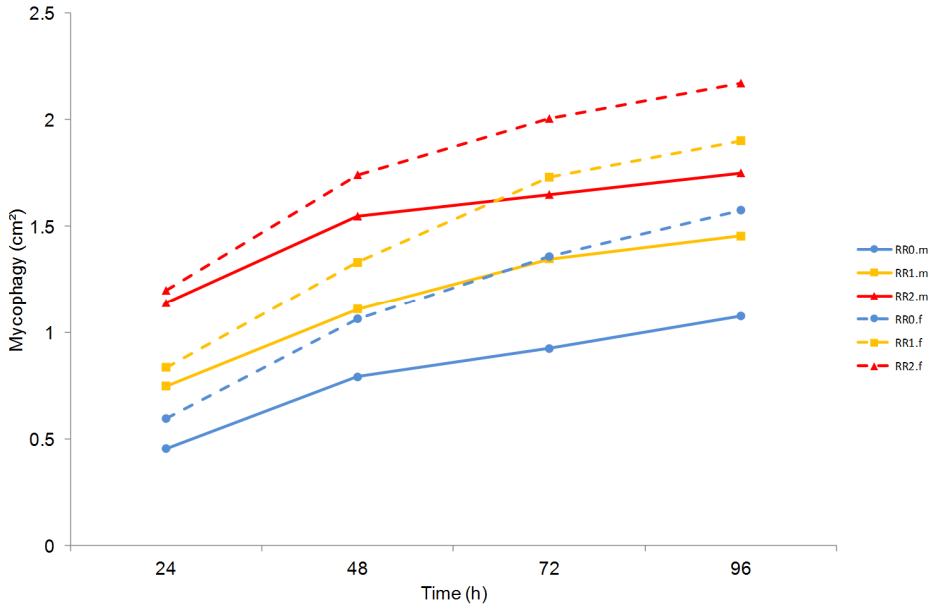


Fig. 7: Mycophagy in function of choice experiment running time, for different watering treatments (RR0, 1 and 2 corresponding to weekly watering of 1, 0.8 and 0.6 L respectively) and ladybird gender (m = male, f = female). Values were determined based on fixed parameter estimates of the full mixed model at average initial PM infected area.

4. Discussion

In our field experiment, we found effects of increasing species richness on mycophagy becoming stronger as more rainfall was removed along a stress gradient – corresponding to expectations of interplay between biodiversity effects and the Stress Gradient Hypothesis. This can be seen as evidence of an associational resistance effect through the actions of natural enemies, modulated by an environmental variable. However, we did not find an effect of species richness on ladybird counts. Hence, this would imply a mechanism by which greater species richness renders PM fungi more vulnerable, but not more attractive, to mycophagous ladybirds and only if the fungus is also (directly or indirectly) affected by lower plant water availability. This effect was apparently too small to have an impact on initial PM levels – as indicated by the lack of an effect of species richness or our reduced rainfall gradient on PM infected leaf area.

The lack of an effect on initial PM levels in our field study might be explained by the higher than average precipitation in August 2014 (112 mm, compared to a 1981-2010

monthly average of 76 mm), which would make mechanisms through plant water status less clear. September 2014 was extremely dry (8 mm compared to a monthly average of 76 mm) and only then did we find an interactive effect of reduced rainfall and neighbourhood species richness. As we scored PM infected area to include freshly grazed mycelia, it is likely that our initial (total) PM scores corresponded to those of the previous wet month. An alternative possibility is that mycelia loss due to mycophagy is swiftly replaced by the fungus. This would be supported by the results of Dillen et al. (2016), where still no interaction between species richness and the reduced rainfall gradient on levels of PM was found for the very dry month of August 2013. If this is the case, the impact of mycophagy on PM may be limited. Previous studies where ladybird PM mycophagy was quantified focused on short-term removal rates, mostly under laboratory conditions (Sutherland and Parrella, 2009; Younes et al., 2015). However, in a study of mycophagous mites on grape PM, Melidossian et al. (2005) did note a significant trophic impact on the disease over a three-year period, but they specified that this effect was strongest if biological control was possible at the early stages of PM infection – whereas our measurements occurred later in the growing season.

In the lab choice feeding experiment, a higher preference of ladybirds for PM on infected leaves originating from trees that received less water was found. Our PM removal rates after 24 hours and the gender difference were consistent with results in another study using the same ladybird species but other PM species and other environmental conditions (Younes et al., 2015). Similar to the field experiment, we found no impact of watering rate on PM infected area, furthermore suggesting that the mechanism of different watering rates does not impact PM infections directly, only their being grazed upon by ladybirds. Possibly, a reduced access to water may impair the fungus's defenses against mycophagy or increase its nutritional quality for the ladybird. While oak PM is known as a xerophilic pathogen (Marçais and Desprez-Loustau, 2012), this is largely because of its tolerance of dry meteorological conditions and vulnerability to the physical effects of precipitation – which were not altered by our stress gradient. Pap et al. (2013) found germination of PM conidia even at very dry levels of air humidity (down to 10%), but they also noted that high levels of humidity (90%) were associated with maximal germ tube length.

Another possibility is that plant water levels influence plant hormonal pathways that attract ladybirds to PM infected trees or reinforce resistance to fungal infection. Tabata et al. (2011) noted strong attraction of *Psyllobora* ladybirds to 1-octen-3-ol, a Volatile Organic Compound (VOC). This alcoholic VOC is well-known as a fungal metabolite of the lipoxygenase (LOX) pathway, producing a moldy odor. However, in a study system of aphids, *Arabidopsis* and water stress, Truong et al. (2014) found greater emissions of this and other alcohols under water stress and in the absence of aphid activity, while Kigathi et al. (2009) noted 1-octen-3-ol as an Herbivore Induced Plant Volatile (HIPV)

produced in certain (non-woody) Fabaceae species, possibly as a defense compound against herbivory. There is no known evidence of 1-octen-3-ol production in *Quercus*, other than as a result of fungal infections such as in cork from *Q. suber* (Rocha et al., 1996). Copolovici et al. (2014) noted that PM-infected *Q. robur* leaves emitted less isoprene and more LOX products, which were chemically similar but not identical to 1-octen-3-ol. In another experiment with *Q. rubra*, higher emissions of LOX products were associated with heat stress (Copolovici et al., 2015). However, they also noted that it is very difficult in practice to distinguish between emissions originating from the plant and from the fungus. Still, even if there is no endogenous 1-octen-3-ol production by the oak, changing water conditions inside the leaf might yet influence the VOC production by the fungus. As the hormonal pathways underlying the plant responses to different stressors interact (Aimar et al., 2011; Niinemets et al., 2013), it would not be surprising to find a (positive or negative) impact of the leaf response to changing conditions of watering on its suitability for PM infection, which in turn could influence the palatability of the fungus for its fungivores. Such interactions between pathogens and water conditions may have implications in the light of future environmental changes associated with increased droughts, such as Climate Change, but more research into the underlying pathways is needed to offer more confident predictions.

5. Conclusion

In this study, we found evidence of mitigating effects of species richness on oak PM, being modulated by environmental factors. These modulated mitigating effects were linked to multitrophic interactions with a mycophagous ladybird, which seemed to prefer feeding on infected leaves from trees with lower water status under laboratory conditions. These findings are in line with expectations of how the Stress Gradient Hypothesis interacts with positive effects of species richness on ecosystem functioning, in this case the mitigation of pathogen infection severity through trophic control. Previous studies have found little response of PM infections to dry or wet meteorological conditions, other than physical inhibition of spore germination by rain drops, and conflicting results for the effects of neighbourhood tree species richness on PM infections, but our results suggest that the same may not hold for ladybird mycophagous activity. Interactions between plant hormonal responses to abiotic and biotic stressors may explain this environmental modulation of trophic control, but more research is needed to elucidate these mechanisms. The substantial mycophagy rates we noted in both our field and lab study suggest that these mycophagous ladybirds may play a considerable role in mitigating the impact of oak powdery mildew. While we did not find a significant relationship between species composition and PM levels, our results suggest that mixing tree species may have a mitigating effect through enhanced ladybird activity under drier climatic conditions. In addition, given our observations in the field and during our lab study, promoting the presence of plant species vulnerable to

other powdery mildew species, such as *Heracleum sphondylium* and *Sonchus oleraceus*, may strengthen ladybird activity by increasing their numbers and allow them to mix their diet.

6. Acknowledgments

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Appendix

Table A1: Parameter estimates for the identity models of the field experiment. MP is mycophagy and PM is powdery mildew crown area %. Qu, Fa, Ti, Be and Pi represent respectively Quercus, Fagus, Tilia, Betula and Pinus presence in the local neighbourhood and C is the intercept. Interactions with the rainfall reduction (RR) treatment are indicated by 1 (medium) and 2 (severe reduction). Statistical significance is indicated using °, *, ** and *** indicating $p < 0.1$, 0.05, 0.01 and 0.001 respectively. All response variables were square root transformed due to residual heterogeneity.

	RR1	RR2	Qu	Fa	Ti	Be	Pi	PM.c	Qu1	Qu2	Fa1	Fa2	Ti1	Ti2	Be1	Be2	Pi1	Pi2	C
Week 1	-0.63	-0.79	0.68	0.79	-0.44	-0.44	-0.05		-0.46	0.13	-0.09	-0.71	1.78°	1.10	0.44	0.46	-0.89	-0.10	5.32***
Week 2	-0.20	-0.88	0.68	0.08	-0.27	-0.26	0.10		-0.42	-0.05	-0.19	-0.76	1.43°	1.11	0.82	1.37	-1.29	-0.64	5.59***
Week 3	0.58	-0.04	0.87	0.41	-0.16	-0.06	0.56		-1.22	-0.93	-0.78	-1.28	1.15	1.18	0.78	0.71	-1.44°	-0.79	4.11***
Week 4	-0.04	-0.47	0.40	0.38	-0.70	-0.11	0.17		-0.28	-0.18	-0.54	-0.55	1.30*	1.43*	0.80	0.42	-1.16°	-0.72	4.58***
Week 1	-0.53	-0.31	0.17	-0.29	0.00	-0.08	-0.03	0.05***	0.24	0.19	0.23	0.42	-0.13	0.37	0.26	-0.15	0.14	0.04	4.12***
Week 2	-0.34	0.19	0.34	-0.58*	0.21	0.14	-0.46°	0.05***	0.04	-0.33	0.43	0.62°	-0.38	-0.23	-0.11	-0.50	0.88*	0.49	3.85***
Week 3	-0.71°	-0.75*	-0.12	-0.67**	-0.18	0.43*	-0.41°	0.06***	0.58°	0.32	0.67*	0.69*	0.10	0.50	-0.32	-0.27	0.45	0.19	3.95***
Week 4	0.23	-0.56°	0.21	-0.52**	-0.03	0.20	-0.31°	0.07***	-0.44°	0.11	0.41°	0.72*	-0.26	-0.10	-0.44°	-0.06	0.27	0.49*	3.65***

Table A2: Results of the full model for the choice feeding experiment.

	Estimate	Std. Error	df	t value	Pr(> t)
(Intercept)	0.34	0.25299	23.5	1.327	0.19729
t48h	0.34	0.24552	334.7	1.366	0.1729
t72h	0.47	0.24552	334.7	1.907	0.05735 .
t96h	0.62	0.24552	334.7	2.53	0.01186 *
RR1	0.29	0.23872	336.3	1.228	0.22039
RR2	0.68	0.23794	335.1	2.877	0.00427 **
gender.female	0.14	0.28931	84.2	0.486	0.62835
PM (centered)	0.12	0.03654	235.2	3.309	0.00108 **
t48h:RR1	0.03	0.30069	334.7	0.089	0.92886
t72h:RR1	0.13	0.30069	334.7	0.438	0.66185
t96h:RR1	0.08	0.30069	334.7	0.281	0.77873
t48h:RR2	0.07	0.30069	334.7	0.243	0.80844
t72h:RR2	0.04	0.30069	334.7	0.138	0.89004
t96h:RR2	-0	0.30069	334.7	-0.035	0.97194
t48h:gender.f	0.13	0.24552	334.7	0.54	0.5893
t72h:gender.f	0.29	0.24552	334.7	1.199	0.23118
t96h:gender.f	0.36	0.24552	334.7	1.463	0.1445
RR1:gender.f	-0.1	0.21541	339.5	-0.246	0.80554
RR2:gender.f	-0.1	0.21386	336.9	-0.372	0.71007

Chapter 5: Biology and breeding of *Psyllobora vigintiduopunctata*, a mycophagous ladybird

Based on Buyse, M., 2015. (In Dutch) Biologische controle van eikenmeeldauw door lieveheersbeestjes en interacties met droogtestress. MSc Thesis. Ghent University.

Abstract

Psyllobora vigintiduopunctata, also known as the 22-spot ladybird, is a small yellow ladybird with 22 black spots native in Western Europe. Unlike most other ladybirds, it feeds mostly and probably exclusively on fungi, in particular powdery mildew fungi. We studied the ladybird's natural behavior, feeding rate and life cycle in the field and under laboratory conditions during a breeding program in 2014-2015.



Fig. 1: *Psyllobora vigintiduopunctata* adults.

1. Introduction

Ladybirds (Coccinellidae) are one of the best known biological control organisms. While mostly known for their feeding on aphids, their diet is often considerably broader, including certain species of other beetles, mites and fungi and even plant pollen (Weber and Lundgren, 2009). Some ladybird species, specifically members of the Halyziini family, have been found to be obligate mycophages, having adapted to a diet of powdery mildew (Erysiphales) fungi (Samways et al., 1997; Sutherland and Parrella, 2009). A few experimental findings suggest that both at the larval stage and as adult these species are dependent on powdery mildew as a sole food source (Davidson, 1921; Giorgi et al., 2009; Sutherland and Parrella, 2009). Recently, feeding on a leaf rust fungus (Uredinales) by *Psyllobora rufosignata* was observed in Brazil (Culik et al., 2011).

Powdery mildew is one of the most important foliar diseases in agriculture, horticulture and forestry all over the world (Glawe, 2008). It is caused by a biotrophic fungus which forms white mycelium mats on the top layer of leaves, with haustoria growing inside the leaf to take up nutrients and carbon assimilates (Divon and Fluhr, 2007). In addition to this loss of nutrients and carbon, the fungal mycelia reduce the light transparency of the leaf, influence water dynamics and reduce leaf lifespan (Hajji et al., 2009; Pap et al., 2014). Powdery mildew can lead to reduced growth and eventual mortality this way (Bert et al., 2016; Marçais and Desprez-Loustau, 2012), which has induced widespread fungicide use on economically important plant species, such as cucurbits, grapes and oak saplings in tree nurseries (Gadoury et al., 2003; McGrath, 2001; Pap et al., 2012).

The most studied obligate mycophagous ladybird species is *Psyllobora vigintimaculata*, native to most of North America (Davidson, 1921; Sutherland et al., 2010; Sutherland and Parrella, 2009; Tabata et al., 2011). Other species have also been subjected to

research, such as *P. vigintiduopunctata* (Samways et al., 1997; Younes et al., 2015), *P. gratiosa* (de Almeida and Milléo, 1998) and *P. bisoetnotata* (Kumar et al., 2010; Satti, 2015; Younes et al., 2015). However, research is often restricted to records of observations and anatomical descriptions. Only a few studies have addressed the ecology of these species, their impact on their fungal prey and indirectly on the infested plant or the possibility of breeding them for biological control purposes. This chapter covers the part of Buyse (2015) that is about the biology and breeding of *P. vigintiduopunctata* (Fig. 1) in the northwest of Belgium. This species was studied in its natural habitat and during a breeding program in a climate chamber.

2. Material and methods

2.1. Field observations

To initiate the breeding program, about 200 adults of *P. vigintiduopunctata* were collected from powdery mildew infected plants in young forest stands, mainly composed of *Q. robur*, located in the northwest of Belgium, during August and September of 2014. During the collections, the habitat and natural behavior of larvae and adults of the ladybird were studied. The stop of egg deposition was assessed, as well as overwintering behavior and habitat.



Fig. 2: Opened breeding box for adult ladybirds.

2.2. Breeding program

The first breeding program started in September 2014 and ended in December 2014. Fresh leaves from plants infected with powdery mildew and the collected ladybird adults were placed together in groups of 30 on sheets of absorbent paper in ventilated plastic

breeding boxes (14.5 x 31.5 x 10 cm, Fig. 2). These boxes were placed in a climate chamber at constant 20°C and with a light regime of 16 hours light and 8 hours dark. Mostly fresh leaf material from infected *Convolvulus arvensis* and *Q. robur* was used as feed, as well as *Sonchus oleraceus*. From October onwards, only *C. arvensis* growing as a weed in a greenhouse was still available. Feeding with pollen (Nutrimite™, Biobest) was attempted, but the pollen remained untouched. Plant material was initially renewed once or twice a week. The absorbent paper was also renewed once a week and feces and other debris removed. A wet wad of cotton wool was placed as a source of water. After noticing that many eggs were deposited on these wads once they were dry, more dry wads of cotton wool were added and more eggs were subsequently deposited on the wads.



Fig. 3: Breeding box for egg hatching and rearing of larval stadia. At the center and on the enlarged picture eggs on a wad of cotton wool can be observed.

Initially, no copulation was observed and 50 ladybirds were stored in a refrigerator at 4°C to break possible diapause. Subsequently, replacement of infected plant material was done more frequently (up to every two days), which resulted swiftly in the occurrence of copulation and oviposition. Some mating couples were separated and microscopically studied, in order to identify differences in gender. They were also weighed individually, using a Sartorius genius ME 235S with an accuracy of 0.01 mg. To avoid adults eating the eggs, each day or each two days the breeding boxes were searched thoroughly and all deposited eggs were removed and placed in smaller breeding boxes (Fig. 3). They were placed on mildew-infected leaves lying on wetted absorbent paper. Each deposited group of eggs was put in a separate breeding box. Every two or three days, the infected plant material in these boxes was renewed and each week the absorbent paper as well. In those smaller breeding boxes, the development of all eggs and their mortality was followed individually every 24 or 48 hours.

Following this program, a second breeding setup was undertaken starting in February 2015 using the 50 ladybirds stored at 4°C since October 2014. These ladybirds were fed solely with infected *Q. robur* leaves, collected from infected saplings growing in a climate chamber. The protocol in this program was identical to the one used in the first, except that larvae were reared on a congealed agar substrate (Micro Agar M1002), to keep infected leaves fresh for a longer time. Nine of these larvae were monitored with greater scrutiny as they developed through the four different stadia.



Fig. 4: Larva of *P. vigintiduopunctata* (4th instar), grazing on oak powdery mildew.

2.3. Feeding quantification

During the breeding program, ladybird mycophagy (Fig. 4) was also studied in greater detail. For each larval stadium and the adult stadium, for both sexes, the amount of powdery mildew that was daily grazed was quantified in terms of reduced infected surface area and weight gain of the ladybirds. Cumulative assessments could not be made as the larvae often molted towards the next stage during the course of the experiment, resulting in occasional mixed compositions of different instars and in reduced mildew consumption during the change. An earlier experiment with *C. arvensis* leaves did not use agar but only moistened paper to keep the leaves fresh. This setup included two ladybirds for every Petri dish and could not run longer than 72 hours. For the experiments with *Q. robur*, infected leaves were put on an agar in extra-deep Petri dishes. This setup allowed leaves to conserve for longer periods. The larva or adult was placed on the agar and for 96 hours, a picture was taken every 24 hours. The leaf and

powdery mildew surface area in each picture were digitally determined using APS Assess 2.0 (Lamari, 2008). The more elaborate setup for the adult feeding is described in greater detail in Chapter 4.

All graphs were made using the *ggplot2* (Wickham, 2009) package in R (R Core Team, 2015).

3. Results

3.1. Field Study

P. vigintiduopunctata larvae and adults were mainly found on plants infected by powdery mildew species, in young forest stands, forest edges and roadsides. In our study area, they could predominantly be found on infected leaves of *Heracleum sphondylium* and young *Q. robur*. To a lesser extent, they were also noted on leaves of *Trifolium*, *Rubus* and small *Acer* species. Often, feeding tracks could be discerned from the infected leaves (Fig. 4). Oviposition occurred at the bottom side of mildew-infected leaves. The last time eggs were noticed in 2014 was at the end of July. Larvae could be found until early September. Late September, adults began to congregate and settle at the bottom sides of leaves of young *Q. robur*, typically under leaves close to the ground partially covered by herbaceous vegetation. From late September until mid December, groups of 10 to 20 adults could often be found this way.

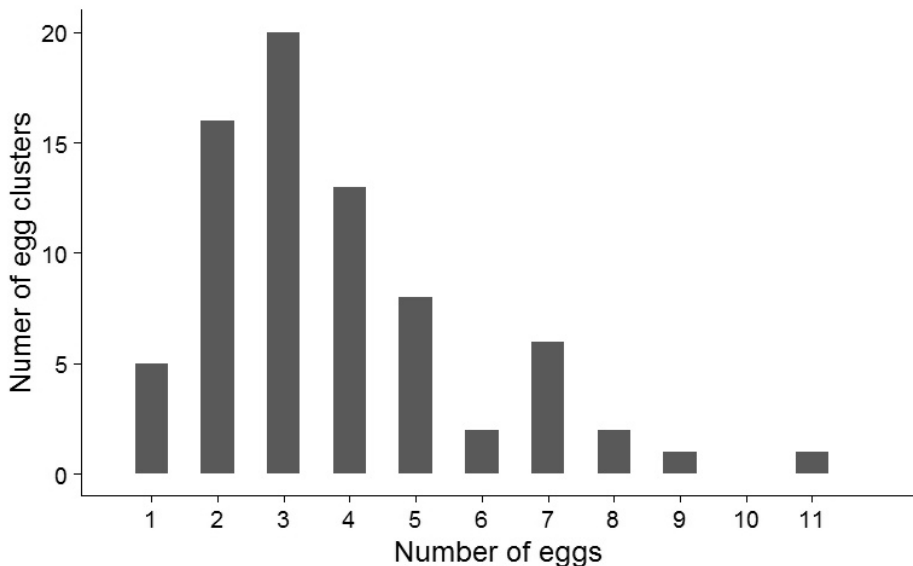


Fig. 5: Distribution of number of eggs among egg clutches.

3.2. Breeding program

During the first breeding program, 279 eggs were deposited in groups of 1 to 11 (Fig. 5). Eggs were mostly deposited on the cotton wads (65% of clutches). 236 of the eggs hatched (85%), but only 62 survived the four larval stages (22%) and reached the pupa stage. 43 emerged as adults (15%). The length of each stage is depicted in Fig. 6. The periodicity of the measurements (typically 24 to 48 hours) and difficulty in identifying individuals caused the variability of time estimates and the differences in sample size, respectively. Only estimates for which both the time of beginning and end were known were included in the figures. The average time between oviposition and emergence from the pupa was 45 days. Hatching typically took about 8 days, while the larval stages lasted 27 days and pupation 10 days. These calculations are based on available data and using the average if the phase change was found to have happened between two points in time. This variation can be seen in Fig. 6.

The ladybirds used in the second breeding program recovered from their hibernation with low mortality after two weeks in a climate chamber supplied with infected oak leaves. Copulation started anew. One clutch of 6 eggs and another of 3 eggs were monitored in greater detail. One larva from the second clutch died after a few days, but all others made it to emergence. Larvae from the first clutch had greater initial weight (0.18 ± 0.03 mg) and developed faster (Fig. 7) than those from clutch 2 (0.11 ± 0.04 mg). There was still considerable variation within both clutches.

When studying copulation, a few possible differences between males and female adults were noted. Males often showed a V-shaped spot pattern on the top of their heads (Fig. 8), which was never seen in females although some males had a less obvious pattern. Female adults tended to be larger and weighed more than males, 6.90 ± 0.60 mg compared to 5.34 ± 0.61 mg for males.

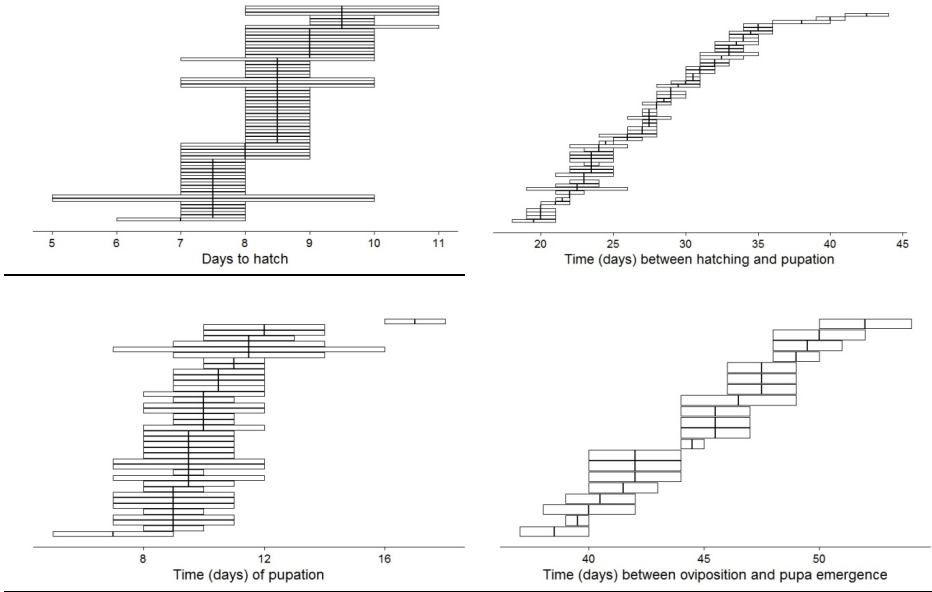


Fig. 6: Development times at different stages of life. Each crossbar depicts the time a single individual spent in that stage. The longer the crossbar, the greater the uncertainty of the exact starting and/or ending date of that phase.

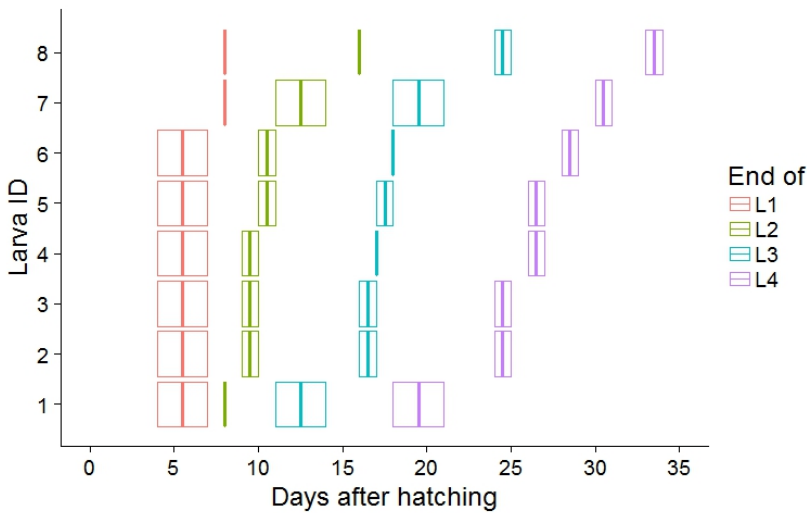


Fig. 7: Development of 8 larvae through the four larval life stages (also known as instars, L1-4). The crossbars depict the estimated ending time of each instar. Larvae 7 and 8 were of the second clutch.

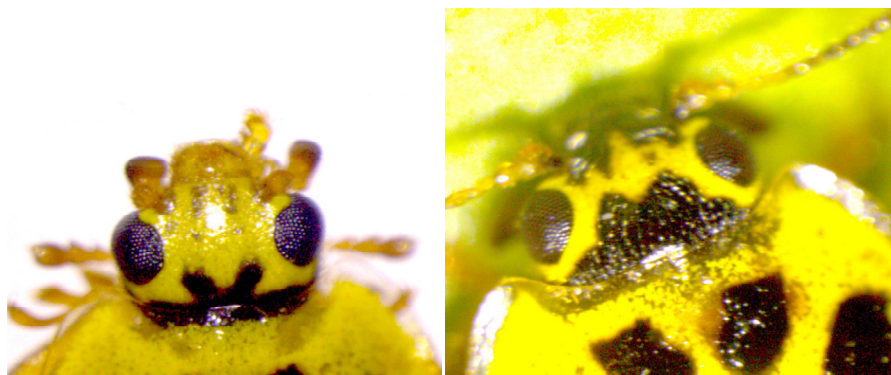


Fig. 8: Head of adult male (left) and female (right) ladybird. Note the different shape of the spot, which is more V-shaped on the male.

3.3. Feeding quantification

When observing mycophagy of powdery mildew on *C. arvensis*, it was noted that chasmothecia were left untouched. Two adult ladybirds grazed on average an area of 0.62 cm² per day. Results for adult ladybird feeding on oak powdery mildew are discussed in detail elsewhere in Chapter 4. On average, a single adult grazed away 4.61 cm² of mildew after 96 hours, representing about 50% of all mildew available during that time. After the first 24 hours, males had already grazed away 1.95 cm² and females 2.31 cm². This gender difference increased with time, while the grazed mildew area decreased.

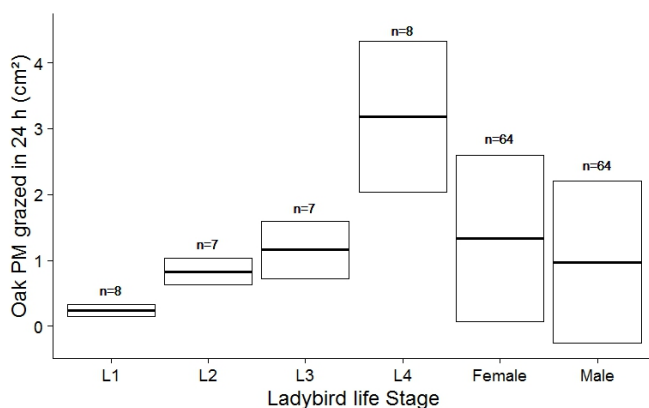


Fig. 9: Mean grazing rates at different stages of life and their standard deviations. Sample sizes are indicated. Female and male refer to the adults. Identification of gender during the four larval phases (L1-4) was impossible.

When comparing grazing by the different larval stages and adults, it was found that larvae, in particular at later stages, consumed more than adults (Fig. 9). Only data of 24 hour periods when all larvae were of the same instar and when initial powdery mildew levels were not close to depletion were included. As a consequence, much more data was available for adults, which did not molt anymore and were always alone.

4. Discussion

We successfully kept *P. vigintiduopunctata* adults alive and procreating under laboratory conditions. Critical were the availability of fresh leaves infected with powdery mildew and the availability of substrates fit for oviposition, such as dry cotton wads. Cannibalism of eggs was frequent at our population densities, necessitating incubation in separation. High mortality at the larval stage in our first breeding program, compared to only one dead larva in the second program is likely explained by the more frequent individual monitoring and feeding during the latter. The presence of the agar substrate allowing the leaves to be preserved for longer times likely played a large role as well, as also evidenced by the much lower feeding rate on infected *C. arvensis* leaves. The high fecundity found during the first breeding program renders it unlikely the quality of powdery mildew plays a major role, at least for the two species we utilized.

Our observations in the lab indicated that this species does not go into diapause, but climatic conditions in the northwest of Belgium probably prevent more than one generation from developing every year. Larvae of this species are solely encountered in Belgium from June to September (Waarnemingen.be, <http://waarnemingen.be>). This should be enough time in theory for two generations in a growing season, considering the average development time of 45 days, so we cannot rule out this happening during warm, mildew-rich summers, especially in the light of the changing climate.

In a study on *P. vigintimaculata*, Sutherland and Parrella (2009) noted average PM feeding of an area of 6.32 cm² during development from egg to pupa. Given our daily rates, which sum to be slightly lower, our figures are likely to be much higher, considering the average 27 days of larval development. We cannot compare the numbers directly, however, as we do not have data for a single individual's feeding throughout all larval stages. Younes et al. (2015) describe the biology and feeding of *P. vigintiduopunctata* in Syria, finding only half of our development times and higher feeding rates. These can be explained by the difference in climate (25°C temperature and 12/12 light regime, rather than our 20°C and 16/8).

In this study, we describe a successful breeding program of *Psyllobora vigintiduopunctata*, a mycophagous ladybird feeding chiefly on powdery mildew fungi. This offers prospects for using this species in biological control of mildew fungi, in particular considering its easy rearing in the presence of fresh mildew-infected leaves and the suitability of a mildew species from a common agricultural weed, *Convolvulus*

arvensis. The negative impact of fungicide use on *Psyllobora* activity (Sutherland et al., 2010) could weigh considerably on cost-benefit analyses, but the species may still prove of great interest for managers of less intensive plant systems and organic farmers.

Chapter 6: How does neighbourhood tree species composition affect growth characteristics of oak saplings?

Dillen, M., Smit, C., Verheyen, K. How does neighbourhood tree species composition affect growth characteristics of oak saplings? Submitted to Forest Ecology and Management

Abstract

Recent research into positive effects of species diversity has renewed interest into mixing tree species in managed forests. Mixing tree species may have positive effects on productivity and other forest ecosystem functions, while also reducing the impact of extreme weather events or disease – both expected to become worse in the context of global change. In particular saplings are very vulnerable to attacks by pathogens and positive effects at this stage may play a substantial role in shaping later forest dynamics, biomass yields and other ecosystem services. While positive effects of mixing tree species have been found, high influence of which species were mixed specifically was noted and it was often unclear just how these interactions work and what their impact is on tree physiology.

In this study, we investigated the impact of local neighbourhood tree species composition on various above- and belowground growth characteristics of four year old oak (*Quercus robur* and *petraea*) saplings in two sites with contrasting abiotic conditions. To evaluate specific mechanisms underlying composition effects, we attempted to link these characteristics to the degree of oak powdery mildew infections and shading cast by local neighbourhood trees, two important factors influencing oak sapling survival and growth. Our results showed no impact of neighbourhood tree species richness, but there were strong effects of species identity on dry biomass production and total leaf area in an abiotically more favorable site. These effects of species presence were related to a strong negative impact of powdery mildew and the degree of shading, both affected differently by different tree species. Effects of composition, mildew and shading were much weaker in the abiotically less favorable site, but we could not disentangle this effect from a difference in oak species. Our results suggest that admixing certain tree species can have considerable positive impact. Mixing of species on individual basis can be generally recommended, particularly if high impact specialist pathogens such as oak powdery mildew are to be expected. However, differences in juvenile growth rates need to be taken into account, as they may lead to strong adverse effects of shading.

1. Introduction

Mixed tree species composition in managed forests has been a contentious topic since the dawn of modern forestry in the 18th century. As modern forestry initially revolved around the sustainable optimization of wood and biomass production, mono-specific stands of highly productive (often coniferous) species quickly became the norm (Pretzsch, 2005; Scherer-Lorenzen, 2014). Recently, worries concerning global loss of biodiversity and research into positive effects of biodiversity on the functioning of ecosystems have reinvigorated this discussion (Cardinale et al., 2012; Hillebrand and Matthiessen, 2009; Hooper et al., 2005; Zhang et al., 2012). Case studies, analyses into forest inventory data and meta-analyses showed positive effects of mixing tree species on the functioning of forest ecosystems (Liang et al., 2016; Paquette and Messier, 2011; van der Plas et al., 2016b; Vilà et al., 2007; Zhang et al., 2012).

Mechanisms proposed to explain these positive effects of mixing tree species include complementarity of resource use in time and/or space (Morin et al., 2011; Pretzsch and Schütze, 2009; Ratcliffe et al., 2015) or through facilitation such as hydraulic or nutrient lift (Pretzsch and Schütze, 2009; Zapater et al., 2011) or nitrogen fixation (Forrester et al., 2006). Mixing tree species has also been associated with decreased pressure of pests and pathogens, mitigating their negative impact and reducing the risk of catastrophic infestation episodes (Hantsch et al., 2013; Jactel et al., 2005; Jactel and Brockerhoff, 2007). Similarly, mixing species may also hedge against other calamities such as severe droughts, wildfires or spring frost through a portfolio effect of variant susceptibilities (Isbell et al., 2009; Van de Peer et al., 2016). Direct facilitation is also possible here, for instance by reducing physical transmission rates (Peacock et al., 2001) or tempering the local microclimate (Hantsch et al., 2014). This has been of particular interest as the various abiotic and biotic processes giving rise to the Anthropocene, such as greenhouse gas emissions and the spread of invasive species, are expected to increase the frequency and severity of such extremes (Niinemets, 2010; Spathelf et al., 2014).

One of the most critical phases of tree development is the sapling stage, when trees are more accessible to herbivores and have less reserves to compensate for growth- and assimilation-impairing processes such as spring frost, drought and diseases (Barton and Hanley, 2013; Sobek et al., 2009). Under these stressful conditions, facilitation between organisms may be promoted as suggested by the Stress Gradient Hypothesis (Bertness and Callaway, 1994) and found in practice in various ecosystems, including forests (He et al., 2013; Paquette and Messier, 2011; Toigo et al., 2015). The potential for facilitation is likely to be greater in more diverse stands, as size asymmetry due to different growth rates allows for sheltering effects (Castagneyrol et al., 2013; Eränen and Kozlov, 2008; Smit et al., 2006) and different susceptibilities (often resistance) to

pests or pathogens reduces their potential populations and likelihood to encounter hosts (Guyot et al., 2015; Sobek et al., 2009).

Such mitigating effects of facilitation in the sapling stage may have repercussions for future tree vitality and growth, as effects of stress can carry over to later years (Bert et al., 2016; Marçais and Desprez-Loustau, 2012). For example, the biotrophic oak powdery mildew fungus (*Erysiphe* sp.) infects the leaves of oaks (*Quercus* sp.) reducing leaf lifespan and photosynthesis (Hajji et al., 2009). Besides a negative impact on growth rates, it is responsible for substantial mortality in young oak saplings, who lack the reserves to compensate for these productivity losses while struggling in competition with other trees and understorey species (Bert et al., 2016; Marçais and Desprez-Loustau, 2012; Pap et al., 2012). As powdery mildew is a host-specific disease which requires asexual spore production during the growing season for maximal infection rates (Feau et al., 2012), reducing the availability of hosts and increasing the frequency of 'dead ends' for the spores may limit the disease's impact in more tree species rich stands. Supporting findings for this mechanism have been found (Dillen et al., 2016; Hantsch et al., 2013), as well as for similar diseases such as leaf rusts (Peacock et al., 2001). Size asymmetry between smaller oak saplings and sheltering pioneer species of similar age might mitigate negative effects of wind or late spring frost (Gemmell et al., 1996; Setiawan et al., 2016) or reduce apparency for herbivores (Castagnyrol et al., 2013), but may also reduce light transmission and therefore negatively impact growth rates (Gemmell et al., 1996).

While trees through their size are more easily individually observable than non-woody plants (Scherer-Lorenzen et al., 2007), their slow growth makes experiments run long and more easily confoundable through changing abiotic conditions and stand dynamics throughout the years (Baeten et al., 2013). Their slow growth also makes harvesting less practical and desirable, which is particularly problematic for root characteristics (Meinen et al., 2009b) and differences in allocation of biomass to different parts of the tree (Domisch et al., 2015). To look at these characteristics in greater detail, we harvested four-year old oak saplings which had grown for three years within two experimental tree diversity plantations of the FORBIO experiment in Belgium (Verheyen et al., 2013). We investigated the influence of neighbourhood tree species composition on multiple characteristics of oak saplings linked to growth. Specifically, we looked at above- and belowground coarse and fine dry biomass (and their ratio), specific leaf area, total leaf area and leaf nutrient contents of carbon (C) and nitrogen (N). We defined tree species composition both as species richness and species identity, i.e. the absence or presence of certain tree species. Additionally, to refine our understanding of which mechanisms were at play, we linked these growth characteristics to two important factors influencing oak sapling survival and growth: powdery mildew infection severity and degree of shading.

2. Materials and methods

2.1. Field sites

Our study took place in two of the three sites of the FORBIO project in Belgium. FORBIO (FORest BIODiversity and Ecosystem Functioning) is a network of three relatively large (ca. 9 ha) experimental forest sites, 'designed specifically to test the effects of tree species diversity on forest ecosystem functioning' (Verheyen et al., 2013). We worked within the sites in Zedelgem and Gedinne, both of which were planted in winter-early spring of 2009-2010. One to three year old saplings of five different tree species were planted with a planting distance of 1.5 m x 1.5 m in 40 plots of typically 42 m x 42 m (Fig. 1). Plots corresponded to compositions of one up to four species and consisted of 81 planted groups of 3 by 3 (4 by 3 at two of the four plot edges) or 784 trees total. Groups were distributed randomly for the three- and four species mixtures, but evenly for the two species mixtures. All five monocultures and four-species mixtures were represented; five randomly selected compositions were chosen for the two- and three species richness level. All 20 compositions were replicated once for the total of 40 plots. A few extra plots were present in both sites for differences in provenance but these were not used in this study. The tree species used differed between sites due to their different edaphic conditions and because species important in local silviculture were preferred, to make the experiment more representative (Verheyen et al., 2013). In Zedelgem, pedunculate oak (*Quercus robur*), European beech (*Fagus sylvatica*), Scots pine (*Pinus sylvestris*), small-leaved lime (*Tilia cordata*) and silver birch (*Betula pendula*) were planted; in Gedinne, European beech, sessile oak (*Quercus petraea*), sycamore maple (*Acer pseudoplatanus*), hybrid larch (*Larix x eurolepis*) and Douglas fir (*Pseudotsuga menziesii*) were planted.

The site of Zedelgem is located on a former agricultural field and has a sandy to loamy sandy soil. It is located not far from the North Sea and has a very mild, temperate climate. The site of Gedinne consists of two separate subsites, called Gribelle and Gouverneurs, separated by about 4 km and each containing all different species compositions. Both subsites were planted on former Norway spruce (*Picea abies*) forest after clearcut. Their soils are more organic, with the soil in Gribelle more exposed and with a higher stone content. Monthly rainfall data during the course of our study can be found in (Dillen et al., 2016). More info on the FORBIO sites can be found in Verheyen et al. (2013).



Row	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42																																																																																																																																																																																																																																																																																																																												
1	129	17	83	113	141	199	197	215	253	281	309	317	365	393	421	449	477	505	533	561	589	617	645	673	701	729	757	785	813	841	869	897	925	953	981	1009	1037	1065	1093	1121	1149	1177	1205	1233	1261	1289	1317	1345	1373	1401	1429	1457	1485	1513	1541	1569	1597	1625	1653	1681	1709	1737	1765	1793	1821	1849	1877	1905	1933	1961	1989	2017	2045	2073	2101	2129	2157	2185	2213	2241	2269	2297	2325	2353	2381	2409	2437	2465	2493	2521	2549	2577	2605	2633	2661	2689	2717	2745	2773	2801	2829	2857	2885	2913	2941	2969	2997	3025	3053	3081	3109	3137	3165	3193	3221	3249	3277	3305	3333	3361	3389	3417	3445	3473	3501	3529	3557	3585	3613	3641	3669	3697	3725	3753	3781	3809	3837	3865	3893	3921	3949	3977	4005	4033	4061	4089	4117	4145	4173	4201	4229	4257	4285	4313	4341	4369	4397	4425	4453	4481	4509	4537	4565	4593	4621	4649	4677	4705	4733	4761	4789	4817	4845	4873	4901	4929	4957	4985	5013	5041	5069	5097	5125	5153	5181	5209	5237	5265	5293	5321	5349	5377	5405	5433	5461	5489	5517	5545	5573	5601	5629	5657	5685	5713	5741	5769	5797	5825	5853	5881	5909	5937	5965	5993	6021	6049	6077	6105	6133	6161	6189	6217	6245	6273	6301	6329	6357	6385	6413	6441	6469	6497	6525	6553	6581	6609	6637	6665	6693	6721	6749	6777	6805	6833	6861	6889	6917	6945	6973	7001	7029	7057	7085	7113	7141	7169	7197	7225	7253	7281	7309	7337	7365	7393	7421	7449	7477	7505	7533	7561	7589	7617	7645	7673	7701	7729	7757	7785	7813	7841	7869	7897	7925	7953	7981	8009	8037	8065	8093	8121	8149	8177	8205	8233	8261	8289	8317	8345	8373	8401	8429	8457	8485	8513	8541	8569	8597	8625	8653	8681	8709	8737	8765	8793	8821	8849	8877	8905	8933	8961	8989	9017	9045	9073	9101	9129	9157	9185	9213	9241	9269	9297	9325	9353	9381	9409	9437	9465	9493	9521	9549	9577	9605	9633	9661	9689	9717	9745	9773	9801	9829	9857	9885	9913	9941	9969	9997	10000

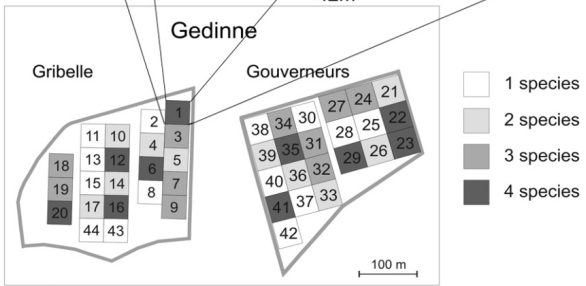
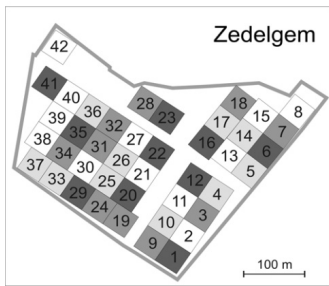


Fig. 1: Lay-out of the experimental setup in the two FORBIO sites. In the example of a four-species plot in the top right, each numbered cell represents a tree and each color represents its species. Three oak saplings were planted between four such trees, in one of the black rectangles where the plot composition matches, as can be seen in the top left. There were only three saplings per plot so only one of those rectangles was used.

2.2. Experimental setup

To study the effects of different compositions on oak saplings specifically, one-year old saplings were planted between the FORBIO trees in April-May 2013. We used the same oak species as was present in the site (i.e. *Q. robur* in Zedelgem, *Q. petraea* in Gedinne). Three such saplings were planted together between four of the FORBIO trees in all 40 plots, for a total of 120 saplings per site (Fig. 1). The saplings were planted in black pots of 20 L to make it possible to subject them to a gradient of rainfall reduction. The pots had holes in the bottom to prevent waterlogging and were dug in so as to minimize solar heating and equalize the soil levels in and outside the pot. Soil from the (sub)sites themselves was used, collected from unplanted patches. A treatment reducing rainfall was added in May-June of 2013 and, after being removed each winter, installed again around the same time in the subsequent two years. Small PVC gutters were placed above the pot surface but below the sapling's crown to intercept precipitation. A control and one to two pairs of gutters were used as can be seen in Fig. 1. In a previous analysis of growth data, this rainfall reduction treatment was found to

have no impact on growth rates or powdery mildew infections and only minor impact on soil moisture conditions (Dillen et al., 2016). Therefore the rainfall reduction gradient was only included in this analysis as a covariate.

2.3. Measurements of powdery mildew and shading

Monitoring for powdery mildew was performed in Zedelgem in July, August and October of 2013 and July and September of 2014. There was virtually no powdery mildew in Zedelgem during the 2015 growing season. In Gedinne, mildew monitoring was performed in July and August of 2013 and 2014 as well as in August 2015. Estimates of leaf area affected by the fungus were made for each first order shoots using four classes: 0-5%, 6-30%, 31-60% and 61-100%. From October 2013 onwards, these first order shoot units were also given relative weights estimated to reflect their size as part of the crown to reflect the rapidly increasing size asymmetry between shoots. Shoot scores were subsequently scaled up to the sapling level by calculating a (weighted) average using the shoot size weights if available and the class mids for each class except that 0 was used for the lowest class. Other symptoms of leaf damage, such as mining and galls, were also evaluated, but found to be far less important than powdery mildew. Shading was measured once using a spherical densiometer (Baudry et al., 2014) in July and August of 2015. A value in % was obtained right above the crown of each sapling for light from the East, South and West, but collinearity was high so that the average of those three values was used in subsequent analysis. More info on this monitoring protocol can be found in (Dillen et al., 2016).

2.4. Measurements of growth characteristics

From September to November 2015, all 240 saplings planted in the experiment were removed from the sites. In Zedelgem, six saplings were dead and had been at least since 2014. Three were in one plot, a four-species mixture without birch. The others were in a monoculture of oak, a mixture of oak and beech and a four-species mixture without lime. These were all omitted from further analysis. One dead phytometer was noted in Gedinne in a monoculture of beech and also omitted.

In September, all leaves were harvested from all saplings. To determine Specific Leaf Area (SLA, leaf area/leaf dry weight), a selection of ten leaves per sapling representative for the whole sample was scanned fresh using a copying machine. The selected leaves were then dried and weighed individually (0.001 g precision). The remaining leaves were also dried at 70°C and weighed together to determine a total crown leaf weight per sapling (excluding the selected leaves). Before weighing, leaf galls were removed from all leaves. The total crown leaf weight samples were then grinded to prepare them for chemical analysis. Total C and N concentrations were then

measured by high-temperature combustion at 1,150°C using an elemental analyzer (Vario MACRO cube CNS, Elementar, Hanau, Germany).

The surface area of the scanned leaves was determined digitally using a batch macro in ImageJ (Rasband, 2016), first thresholding the scans to binary images then using the built-in *Analyze Particles* function with minimal size (1000) and circularity (0.1) restrictions to count the number of pixels per leaf. These were subsequently converted to mm² using the resolution of 300 DPI. The data for leaf surface area and dry weight were then matched in R (R Core Team, 2015) to obtain the SLA's, assuming the same hierarchy for each ten values, using the *dplyr* package (Wickham and Francois, 2015). The Total Leaf Area (TLA) was determined by multiplying the average SLA of the ten selected leaves with the total crown leaf weight.

Between September and November 2015, the woody biomass of all saplings was collected and dried at 70°C until constant weight. Roots were removed from the soil but not washed before drying. After drying, aboveground biomass with diameter < and \geq 3 mm was weighed separately per sapling. The cut-off point between above- and belowground was defined as the soil line marked on the stem. Belowground biomass was cleaned of soil, stones and herbaceous roots manually, before separately weighing it for roots < and \geq 2 mm.

2.5. Statistical analysis

Our statistical analysis was performed in R (R Core Team, 2015). To evaluate the shoot weight approach of the PM monitoring, we compared the determined TLA to the sum of all weights as estimated in August 2015 in both sites. We also made allometric relationships between diameter in September 2015 (as measured by Dillen et al. (2016)) and belowground, aboveground and total dry biomass for both sites. To derive these relationships, we made a linear regression between the natural logarithm of the diameter and the different types of dry biomass. Results for both validations can be found in the Appendix.

For the analysis of the influence of species composition on growth characteristics, we used linear mixed models from the *lme4* package (Bates et al., 2015) with *lmerTest* extension (Kuznetsova et al., 2015) to determine Satterthwaithe approximated p-values. We ran models separately for data from the two sites, due to the difference in oak species, the neighbourhood tree species compositions and abiotic conditions. Composition in the local neighbourhood was defined as the four surrounding FORBIO trees, which also corresponded to the composition of the 36 closest FORBIO trees. For each site, the following three models were determined

$$Y = \sum_{i=1}^n Y_i$$

$$Y = \sum_{i=1}^n Y_i + \sum_{j=1}^m Y_j$$

$$Y = Y_0 + \sum_{i=1}^n Y_i$$

Table 1: List of all growth characteristics (Y) used in the models

Symbol	Description	Unit
Rf	Fine belowground dry biomass (< 2 mm)	mg
Rc	Coarse belowground dry biomass (≥ 2 mm)	mg
Wf	Fine aboveground dry biomass (< 3 mm)	mg
Wc	Coarse aboveground dry biomass (≥ 3 mm)	mg
TBM	Total dry biomass	mg
R/W	Below-/aboveground dry biomass ratio	
SLA	Average Specific Leaf Area	mm ² /mg
TLA	Total Leaf Area	cm ²
leaf N	Leaf N concentration by weight	%
leaf C	Leaf C concentration by weight	%
leaf C/N	Leaf C to N ratio	

SR is the species richness (1-4), **SP** is the absence or presence of each of the five species, **SH** is the degree of shading (between 0 and 1) and **PM_i** constitutes all different powdery mildew scores (between 0 and 1). The list of variables for Y can be found in Table 1. All models included an intercept and plot as a random factor. They also included a fixed factor for the rainfall reduction gradient and for the replication of the different composition treatments, which also corresponded to the two subsites in Gedinne. We applied log transformations if residual heterogeneity was noted. Species richness and species identity were modeled separately as the former is always a linear combination of all the latter. Multicollinearity in the last model was evaluated with Variance Inflation Factors (VIF), using a modified function of the package *rms* (<https://github.com/aufrank/R-hacks/blob/master/mer-utils.R>).

3. Results

General results for all growth characteristics and scores of powdery mildew and shading can be found in Table 2. As expected, TBM was lower in the abiotically less favorable site of Gedinne. Biomass was disproportionally lower aboveground than belowground in this site, as reflected in a R/W ratio that was twice as high as in Zedelgem. Leaf C varied little, so that the leaf C/N ratio was very strongly correlated to leaf N ($r = -0.98$ in Zedelgem and -0.96 in Gedinne). Values for shading were a fair bit lower in Gedinne

(39%) than in Zedelgem (63%), while powdery mildew scores were generally lower in Zedelgem (with the notable exception of July 2014).

Results for the three models per site can be found in Tables 3 and 4. All variables were log transformed. Species richness had virtually no effects, only correlating with R/W ratio and SLA in Zedelgem. Conversely, species identities had strong impact in this site, on biomass variables as well as TLA. The presence of oak, and to a lesser extent birch, had a negative impact on biomass characteristics, whereas the other three species and in particular beech showed positive effects. Negative effects of oak presence were slightly stronger on aboveground than belowground biomass (41 and 37% reductions in coarse BM respectively), but the difference was not significant given the small, insignificant effect on the R/W ratio. The presence of pine much like beech positively influenced aboveground biomass, in particular fine BM and TLA, but also had similar effects as birch on R/W ratio and SLA. In contrast, identity effects in the site of Gedinne were much weaker, finding only effects for R/W ratio, SLA and leaf N characteristics.

Table 2 Mean values and standard deviations for the growth characteristics measured, the different PM scores and the degree of shading. Note that the units used here may differ from those used in the statistical models. Descriptions of the symbols can be found in Table 1.

	Zedelgem		Gedinne	
	Mean	SD	Mean	SD
Rf (g)	9.47	4.83	6.88	3.20
Rc (g)	31.57	23.23	25.76	14.36
Wf (g)	8.43	5.22	4.58	2.28
Wc (g)	30.40	21.29	12.41	9.83
TBM (g)	79.87	51.49	49.63	27.04
R/W	1.14	0.35	2.14	0.75
SLA (mm ² /mg)	17.88	2.94	14.44	2.77
TLA (dm ²)	24.16	15.23	13.47	8.76
leaf N (%)	2.48	0.28	2.06	0.37
leaf C/N	20.15	2.18	25.27	4.84
leaf C (%)	49.39	0.67	50.29	0.96
PM Jul 13 (%)	6.82	8.27	28.03	18.18
PM Aug 13 (%)	11.74	9.52	35.88	16.20
PM Jul 14 (%)	22.61	16.80	12.16	13.08
PM Aug 15 (%)	2.08	6.13	10.87	13.6
Shade (%)	62.84	25.75	39.24	27.47

Collinearity for the PM scores in the third model was large (VIF>2) in both sites and hence variables with the highest VIF were omitted. The impact of the most severe PM infections of July 2014 in Zedelgem is dominant on all biomass traits and TLA (Fig. 2).

Shade also had a notable negative effect, increasing average SLA and leaf N as well, but not affecting TLA or fine aboveground BM. The average infection with PM in July 2014 is associated with 29% less total biomass at the end of 2015 compared to an uninfected sapling. The average degree of shading results in 36% less biomass compared to no shade. In Gedinne, the negative impact of PM on biomass was visible in both 2013 and 2014 (Fig. 2), but not 2015. PM in 2015 did have an effect on the leaf traits of SLA and leaf N. Negative effects of shading on BM were less pronounced in this site, with total BM reductions of 13%, 13% and 17% for shading and PM in July 2013 and July 2014 respectively (Fig. 2).

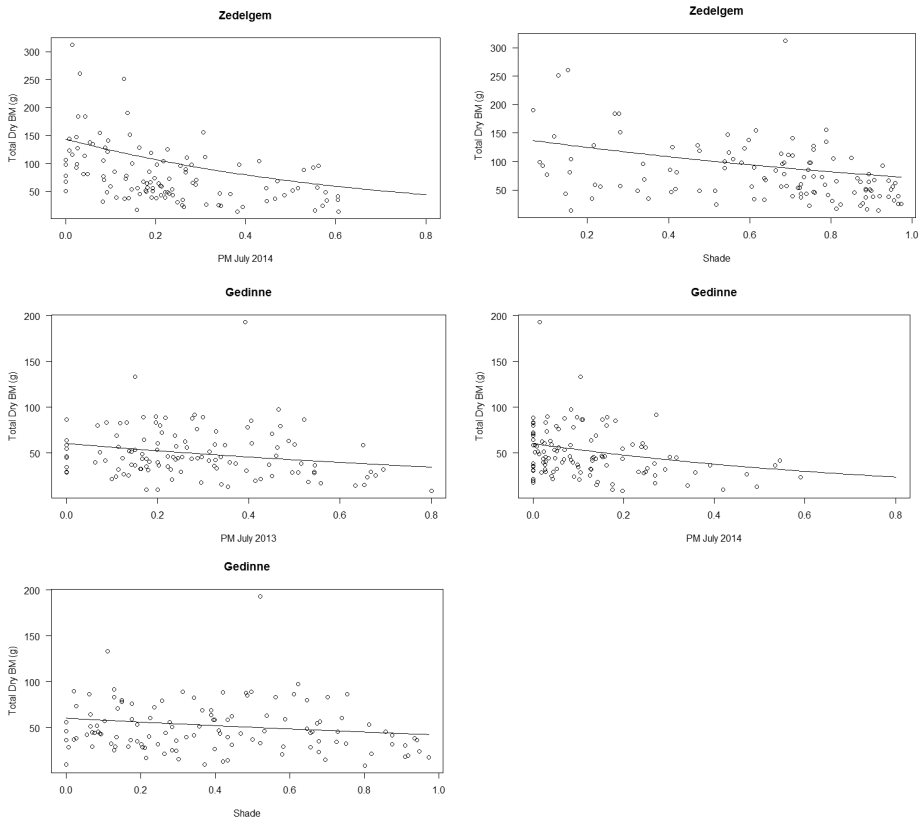


Fig. 2 Scores for total sapling BM in function of PM affected leaf area and degree of shading in both sites. The lines shown are model predictions based on the full model results in table 2, assuming all other parameters to be equal to 0.

Table 3: Results of the three models in Zedelgem. Symbol descriptions can be found in Table 1. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. It was more or less identical in value for the species richness and identity models, as were the estimates of the reduced rainfall treatment (RR). Log transformations were performed on all response variables to compensate for residual heterogeneity. PM scores that are not listed were omitted from analysis due to high VIF's.

	Species Richness			Species Identity					PM and shading					
	C	SR	RR1	RR2	Oak	Beech	Pine	Lime	Birch	C	Jul13	Aug13	Jul14	Shading
Rf (mg)	8.91***	-0.03	0.24**	0.14	-0.37**	0.22	0.13	0.09	-0.20	9.52***	-0.15	-0.36	-1.08***	-0.47*
Rc (mg)	10.15***	-0.06	0.25*	0.14	-0.46**	0.31*	0.01	0.26°	-0.36*	11.24***	0.03	-0.26	-1.69***	-1.14***
Wf (mg)	8.57***	0.03	0.15	0.20°	-0.59**	0.24	0.36*	0.19	-0.01	9.12***	1.39°	0.01	-1.46***	-0.14
Wc (mg)	9.98***	0.01	0.27*	0.16	-0.52**	0.29°	0.20	0.25	-0.14	10.86***	0.39	-0.23	-1.55***	-0.58*
TBM (mg)	11.02***	-0.02	0.24*	0.15	-0.48**	0.28°	0.13	0.22	-0.22	11.88***	0.34	-0.28	-1.47***	-0.70**
RW	0.19°	-0.06*	0.00	-0.02	0.09	0.02	-0.19**	-0.01	-0.21**	0.31**	-0.36	-0.27	0.13	-0.44***
SLA (mm ² /mg)	2.77***	0.04*	-0.03	0.00	-0.04	-0.02	0.15***	-0.05	0.14***	2.66***	-0.09	-0.13	-0.07	0.40***
TLA (cm ²)	4.93***	0.08	0.20°	0.15	-0.62***	0.34*	0.40*	0.21	0.08	5.75***	0.81	-0.16	-1.85***	-0.15
leaf N (%)	0.89***	0.01	0.00	-0.02	0.04	-0.01	0.01	-0.03	0.05*	0.83***	0.23°	-0.18°	0.07	0.12**
leaf C/N	3.02***	-0.01	0.00	0.02	-0.04	0.01	-0.02	0.03	-0.05*	3.08***	-0.23°	0.19°	-0.07	-0.14**
Leaf C (%)	3.90***	0.00	0.00	0.00	0.00	0.00	-0.01*	0.00	0.00	3.91***	0.00	0.00	0.00	-0.01°

Table 4: Results of the three models in Gedinne. Symbol descriptions can be found in Table 1. Significant or semi-significant parameter estimates are shown in bold, with °, *, ** and *** indicating a significance of <0.1, <0.05, <0.01 and <0.001 respectively. The intercept C was virtually always significant and was included in the table to allow interpreting the order of magnitude of the different parameter effect sizes. It was more or less identical in value for the species richness and identity models, as were the estimates of the reduced rainfall treatment (RR). Log transformations were performed on all response variables to compensate for residual heterogeneity. PM scores that are not listed were omitted from analysis due to high VIF's.

	Species richness					Species identity					PM and shading				
	C	SR	RR1	RR2		Oak	Beech	Douglas	Maple	Larch	C	Jul13	Jul14	Aug15	Shading
Rf (mg)	8.67***	0.01	-0.07	0.06	-0.12	0.23*	0.09	-0.08	-0.08	-0.06	9.18***	-0.66**	-0.97**	-0.22	-0.36*
Rc (mg)	9.86***	0.03	-0.22°	-0.03	-0.01	0.18	0.11	-0.01	-0.13	-0.13	10.48***	-0.70*	-1.37***	-0.52	-0.56*
Wf (mg)	8.15***	-0.02	-0.17°	0.00	-0.05	-0.04	0.08	-0.16	0.06	0.06	8.29***	-0.76**	-0.38	0.00	-0.04
Wc (mg)	9.02***	-0.05	-0.20	0.07	-0.06	-0.07	0.11	-0.24	0.03	0.03	9.28***	-0.71*	-1.13*	-0.08	-0.07
TBM (mg)	10.54***	0.00	-0.20°	0.00	-0.05	0.10	0.10	-0.09	-0.06	-0.06	11.01***	-0.70**	-1.17**	-0.33	-0.36°
R/W	0.74***	0.06°	0.00	-0.05	0.02	0.25***	0.00	0.19**	-0.15*	-0.15*	1.13***	0.02	-0.42°	-0.40°	-0.46***
SLA (mm ² /mg)	2.69***	-0.02	0.04	-0.02	-0.03	-0.10*	-0.02	-0.07°	0.10*	0.10*	2.54***	0.02	-0.05	0.33**	0.29***
T/LA (cm ²)	4.53***	0.02	-0.15	-0.06	0.06	0.01	0.13	-0.23	0.11	0.11	4.93***	-0.83**	-1.34**	0.09	0.10
leaf N (%)	0.72***	-0.01	0.00	0.02	0.04	-0.06	0.02	-0.11*	0.08°	0.08°	0.64***	0.06	-0.03	0.29*	0.14°
leaf C/N	3.19***	0.01	0.00	0.00	-0.04	0.07	-0.02	0.11*	-0.08°	-0.08°	3.29***	-0.08	0.03	-0.28*	-0.16*
Leaf C (%)	3.91***	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-0.01	-0.01	3.92***	-0.02°	0.00	0.00	-0.02*

4. Discussion

Neighbourhood species richness had no effect on any of our measurements, as was previously found in the same sites for relative growth data (Dillen et al., 2016) as well as growth of the older FORBIO trees (Setiawan et al., 2016). The identity of the neighbourhood species had quite pronounced effects in the site of Zedelgem, but not in Gedinne. Negative effects of the presence of oak on all aspects of dry biomass and total leaf area in Zedelgem might be explained by the equally strong effects of powdery mildew. The oak-specific pathogenic fungus was found to cause substantial infections at least once in each site during the course of the experiment, most notably in July 2014 in Zedelgem after late spring frost.

In Zedelgem, the positive identity effect of beech and to a slightly lesser extent of small-leaved lime on all aspects of biomass might be partially explained by the low degree of shading cast by these species at this age (Dillen et al., 2016), while shading had a pronounced negative effect on biomass in this site. However, shading had a disproportionate impact on root biomass, which is not seen in the species identity effects of lime and beech, while the most shade-casting species, silver birch, did have stronger negative effects on belowground biomass. The influence of shading on leaf traits, increasing SLA and reducing leaf C/N (Jensen et al., 2012; Niinemets, 1998; van Hees, 1997), could be found for birch, but was also not mirrored for the identity effects of beech and lime. This suggests another mechanism of facilitation besides shading may be at work here, although complementarity for resources at the root level is unlikely as all oak saplings were planted in pots.

The other shade-casting species in Zedelgem, Scots pine, had similar effects as birch on SLA and R/W ratio, but not leaf C/N or BM. While this may partially be due to a diminished association of presence with shading in this site compared to birch – 20 and 31% increase, respectively (Dillen et al., 2016) – the positive effects on fine aboveground BM and TLA are remarkable. Despite the large difference in degree of shading, pine was on average of similar height as the other three species present (ca. 2 m in 2014), whereas birch was almost twice as tall and very close to crown closure (Setiawan et al., 2016, Appendix A). Hence, the shade cast by birches from much higher was inescapable for the rarely taller than 1 m oak saplings, whereas pines cast their shade from much lower and grew more horizontally, keeping the incentive for stem growth and crown development stronger. This does not explain the discrepancy between a greater SLA yet no lower leaf C/N; however, this may be due to the greater sensitivity to changing light conditions of SLA than leaf N in *Q. robur* (Niinemets, 1998).

Species identity effects were much less prominent in the site of Gedinne. The characteristics related to shading (R/W ratio, SLA and leaf C/N) were associated with the most shade-casting species hybrid larch in this site, as well as inversely with

sycamore maple and beech, which suffered from high mortality and slow juvenile growth rate, respectively (Setiawan et al., 2016; Van de Peer et al., 2016). Yet there were no strong associations with BM characteristics or TLA, not even of oak despite the negative effects of PM both in 2013 and 2014 on all aspects of BM and leaf area. A likely explanation is lower susceptibility of *Q. petraea* to PM than *Q. robur* (Dillen et al., 2016; Marçais and Desprez-Loustau, 2012). This is supported by the lower impact of PM on BM in July 2013 in Gedinne than in July 2014 in Zedelgem, despite the average affected area in Gedinne being over 50% greater. We cannot disentangle this hypothesis from the different abiotic conditions between both sites, however, as illustrated by the much less negative impact of shading on BM and total dry BM per sapling being over 60% greater in Zedelgem than in Gedinne. The higher R/W ratio might reflect incentive for stronger root development, such as water or nutrient shortages, or less incentive for shoot development due to the lower degree of shading and its lower impact on biomass.

The occurrence of PM in Gedinne in 2015 reveals a strong impact of the disease on leaf characteristics, increasing SLA and reducing leaf C/N. Previous research found opposite response of leaf C/N, with leaf C increasing and leaf N decreasing with increasingly severe infection, and no response of SLA (Hajji et al., 2009; Tack et al., 2012). However, their results also show that leaf N was elevated in infected leaves when compared to uninfected ones. This difference dwarves the trend among infected leaves, which was strongest for severe infections of more than 25% of leaf area affected, compared to the 11% in our data. Another study did not look at differences in leaf area, but found higher leaf dry BM in infected leaves, which does not support our results for SLA either (Pap et al., 2014).

5. Conclusion

In this study, we looked at the influence of neighbourhood tree species composition, defined as either species richness or absence/presence of certain species, on various growth characteristics of oak saplings. We also linked these characteristics to scores of leaf area infected by powdery mildew and the degree of shading. We found no effects of species richness in neither site, yet strong effects of species presence in the abiotically more favorable site of Zedelgem. These effects could be linked to the strong negative impact on biomass of powdery mildew and shading, which were both associated differently with the five species. Effects of shading differed between above- and belowground biomass and varied depending on which shade-casting species was present. Higher biomass values in the presence of slower growing and therefore – while young – less shade-casting species like European beech and small-leaved lime could not be fully linked to this absence of shading. The abiotically less favorable site of Gedinne showed little effects of species composition on oak saplings and less negative impact of shading or powdery mildew. This can be explained by poorer susceptibility of

Q. petraea to oak PM than *Q. robur* or by a smaller impact of the disease under harsher abiotic conditions.

Our results suggest that, while increased tree species richness showed little impact on dry biomass production or other growth characteristics per se, admixing certain species in the local neighbourhood can have a considerable negative or positive impact. Mixing of species on individual basis can be recommended in particular if high impact specialist pathogens such as oak powdery mildew are to be expected. *Q. petraea* may also be a preferable species to *Q. robur* in high risk circumstances. Even though we found little effect of species richness *sensu stricto*, our study did not look into impacts on other ecosystem functions or on longer time scales. The substantial impact of disease in our experiment strengthens the argument for mixing species as risk mitigation against future calamities, in particular in the light of globalization and its associated greater risk of invasive pests and pathogens.

6. Acknowledgments

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Appendix

Results of the crown size estimate validation can be found in Figure A.1. Adjusted R^2 was 0.78 for Zedelgem and 0.69 for Gedinne, with residual variance increasing with greater crown size. Results for the allometric relationships can be found on Figure A.2. Adjusted R^2 values for Zedelgem were 0.82, 0.83 and 0.72 for TBM, W and R biomass respectively. For Gedinne, they were 0.74, 0.80 and 0.62. Variance was notably higher for Gedinne belowground biomass.

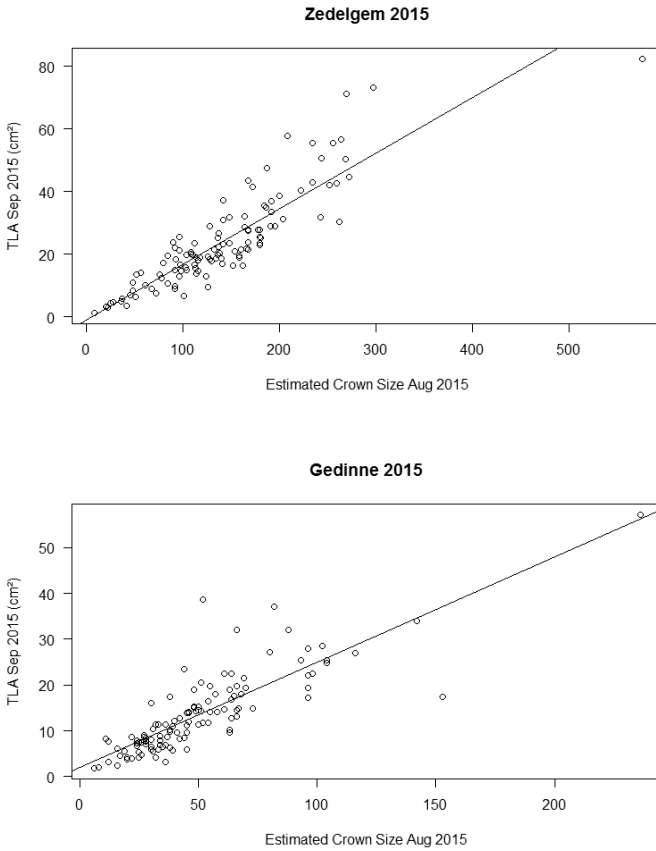


Fig. A.2 Linear regression between Total Leaf Area as determined after harvest in September 2015 and estimated crown size as estimated by the sum of shoot weights used during monitoring for PM. Adjusted R^2 was 0.78 for Zedelgem and 0.69 for Gedinne.

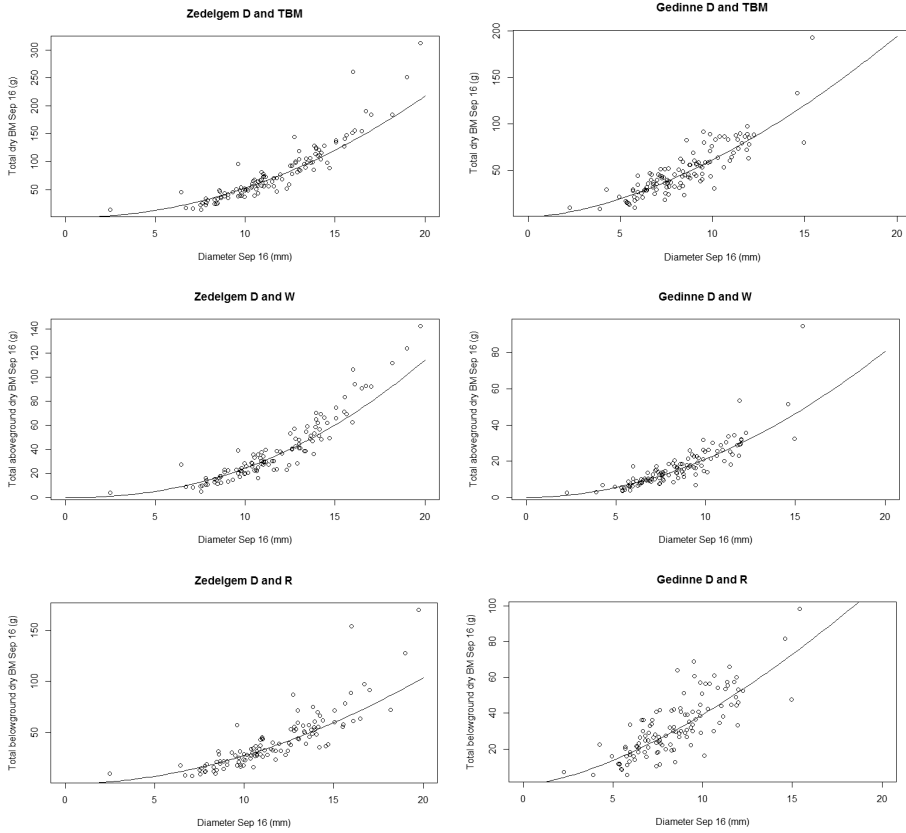


Fig. A.3 Allometric relationships between diameter and dry biomass, respectively total (TBM), aboveground (W) and belowground (R), in 2016. Adjusted R^2 values for Zedelgem were 0.82, 0.83 and 0.72 respectively. For Gedinne, they were 0.74, 0.80 and 0.62 respectively.

Chapter 7: Synthesis and conclusion

Global loss of biodiversity has raised concerns about the potential impact of reduced diversity on the functioning of ecosystems (Ceballos et al., 2015). After roughly two decades of research, evidence has been found in multiple systems, including forests, that a greater species diversity is associated with greater ecosystem functioning (Cardinale et al., 2012; Liang et al., 2016; Tilman et al., 2014). Concurrent with biodiversity loss, other processes such as climate change and biotic homogenization are expected to alter environmental conditions of multiple ecosystems, possibly increasing the amount and frequency of stress organisms within them will experience (Rockström et al., 2009; Turcsan et al., 2016; van der Plas et al., 2016b). Theory suggests positive interactions between organisms to be more frequent and possibly more important under more stressful conditions (Bertness and Callaway, 1994; He et al., 2013; Soliveres et al., 2014), which may result in positive effects of greater diversity on the functioning of ecosystems to be even more prominent under future, more stressful conditions. A few studies have found evidence of such an interplay between diversity, stress and ecosystem functioning (Stuedel et al., 2012), also in forests (Jucker et al., 2015; Paquette and Messier, 2011; Toïgo et al., 2015). But much remains unclear about how universal these relationships really are and the mechanisms driving them.

In this thesis, I shed light on how local neighbourhood composition may influence growth and productivity in young forest systems and how these composition effects may be influenced by an environmental stress gradient. Second, I looked at potential mechanisms driving these composition effects, including multitrophic interactions with natural enemies of prominent tree pests. To address my research questions, I monitored productivity of trees in young forest stands in function of their local neighbourhood tree species composition, contrasting effects of species richness with those of different species identities. To explore interactions between diversity relationships and stress, I subjected saplings to a stress gradient of reduced rainfall. Finally, to shed light on the mechanisms behind composition effects, I looked at symptoms of leaf damage and the trophic interaction between a leaf pathogen and its natural enemy in function of composition, as well as multiple tree and stand characteristics related to biomass and leaf traits. In this final chapter, I will summarize the most important results I found of each chapter and link the findings from different chapters with each other. I will also offer recommendations for future research as well as for forest management.

1. Main Findings

In **Chapter 2**, I assessed effects of clonal diversity in a willow Short Rotation Coppice (SRC) stand. While biomass productivity was generally found to be higher in more genetically diverse plots, this diversity effect was not significant. Variability in biomass

production between plots was very high due to an unidentified confounding variable, which could not clearly be linked to measured soil characteristics or to leaf surface temperatures obtained using an UAV. Nevertheless, complementarity effects were generally stronger than selection effects for those plots where overyielding was found. Differences in stand characteristics such as stem count per stool and evenness of biomass were found between monocultures and mixed plots containing less-productive clones. These effects were most likely related to differences in productivity and vitality between clones, causing more gaps of dead or poor stools and therefore more instances of increased productivity of stools adjacent to these gaps. This resulted in less symmetric stands and could possibly influence the quality of the harvested biomass. Hence, mere differences in productivity may still have repercussions for the functioning of these systems – in particular relatively intensively managed forest systems such as SRC.

In **Chapter 3**, I measured relative growth rates of oak saplings in two young forest plantations consisting of various monocultures and mixtures of five different tree species. The saplings were also subjected to a gradient of reduced rainfall and they were scored for severity of powdery mildew (PM) infection and other leaf pests or pathogens, as well as how much shading they received from neighbouring trees. The reduced rainfall gradient had little to no effect on growth or PM infection, likely because its impact on soil moisture conditions was not strong enough. Species richness had no impact on growth in neither of the two sites, but PM rates were elevated in 2013 and 2015 in monocultures in the site of Gedinne, where growth was slower and PM had less impact on it. Species identity had strong effects in the other site of Zedelgem, most notably a Janzen-Connell effect of the presence of oak associated with PM infections. Presence of beech and lime had positive effects on growth, likely related to comparably reduced levels of shading they cast at this young age. The degree of shading had strong negative effects on growth in Zedelgem, as did PM infections. In contrast, shading had positive effects on growth in Gedinne. The different responses to neighbourhood composition between sites were ascribed to the difference in oak species (*Q. robur* versus *Q. petraea*), but could also be related to differences in abiotic conditions between sites. These results emphasize the importance of identity effects, in particular Janzen-Connell effects if high impact pathogens such as powdery mildew are expected. While the effect of stress on composition effects could not properly be evaluated, indications of more facilitation were found in the less favorable site of Gedinne, including PM mitigation in mixed stands.

In **Chapter 4**, I looked into how the activity of adult mycophagous ladybirds (*Psyllobora vigintiduopunctata*), which are considered natural enemies of PM, is influenced by local neighbourhood tree species composition and by a stress gradient. I combined a field monitoring campaign in the experiment of **Chapter 3** with a lab choice feeding

experiment to gain insight into the direct impact of water stress on PM and ladybird mycophagy. An interaction between the reduced rainfall gradient and tree species richness was found during the drier second half of our monitoring period: When controlling for initial PM levels, the effect of species richness on the rate of mycophagy became (more) positive as the treatment intercepted more rainfall. This interaction was found to be due to a somehow increased vulnerability of PM to mycophagy, rather than increased ladybird numbers. In the lab experiment, PM levels were not influenced by different watering rates, while mycophagy was considerably higher on leaves of saplings which received less water. Both results support positive (intertrophic) species interactions becoming more important along a stress gradient, although the mechanisms behind this particular interaction are not yet clear. Mycophagous ladybirds had significant impact on PM levels and might be an interesting species group for biological control.

Chapter 5 is a brief chapter detailing breeding of the mycophagous ladybirds required for the lab experiment of **Chapter 4**. Breeding was ultimately successful, but some important considerations were noted. Their life cycle was described and quantified, both the durations of each life stage and associated PM mycophagy rates. Larvae of the final instar were found to have greater rates of mycophagy than the adults. We found no evidence of the species going into diapause or preferences for certain species of PM, but other food sources than PM were left untouched.

Finally, in **Chapter 6** I described results from the analysis of different sapling biomass and leaf traits in function of local neighbourhood composition, PM levels and shading, after harvesting the saplings at the end of the experiment of **Chapter 3**. Effects on total biomass were similar to those on relative growth, with neighbourhood species richness having little impact whereas neighbourhood identity had a strong impact in Zedelgem, particularly oak, beech and lime. Positive effects of beech and lime could not be fully related to reduced shading rates, suggesting facilitation or complementarity by another mechanism. The negative effect of shading in the site of Zedelgem was much more apparent on biomass traits, although effects contrasted between identity effects of birch and pine. This was probably a consequence of their different juvenile growth strategies, with pine showing less height growth than birch. A much greater fraction of belowground biomass in Gedinne provided further evidence for the influence of abiotic limitation in this site. The influence of PM on leaf traits only partially concurred with findings from previous studies.

2. Composition effects in forests

In the experiments that were a part of this thesis, I found no significant effects of local neighbourhood tree species richness on productivity and some effects on the severity of PM infections or PM grazing by ladybirds. There was an increased severity of PM in

monocultures in Gedinne in 2013 and 2015 and there were increased levels of mycophagy with greater species richness in Zedelgem in 2014 at the more extreme end of the reduced rainfall gradient. However, these effects did not translate into altered growth rates or realized biomass at the end of the experiment in the autumn of 2015. PM had overall less impact on growth in the site of Gedinne, despite a greater percent of leaf area affected by the fungus. The effect of richness on PM might be related to the extreme microclimatic conditions in monocultures, associated with either very low or very high incidence of shade and exposure to the elements, whereas in mixtures different functional traits result in conditions somewhere in between (Hantsch et al., 2014). Yet, this would seem to suggest some link between these functional traits and PM infection, which we did not see anywhere else in our study and is also reported to be weak at best in literature (Marçais and Desprez-Loustau, 2012; Pap et al., 2013). The lack of a similar pattern for 2014 growth rates and species composition as found for mycophagy suggests the effect of this sort of biological control on the impact of PM on growth to be rather weak. Yet I did not assess mycophagy earlier in the 2014 growing season, when PM infections were worse and the presence of ladybird larvae could have had a greater impact.

The lack of species richness effects on growth in the FORBIO sites might be explained by the lack of dilution along the richness gradient. Oak density was not maximal in monocultures, which included oak monocultures but also monocultures of other species, and the fraction of plots that included oak was actually higher with greater species richness. The strong negative effect of oak presence on growth in one of the two sites suggests the Janzen-Connell effect to play a major role (Scherer-Lorenzen et al., 2005b). As the most important noted pathogen in both sites, PM is the most likely cause, although I cannot rule out other, less apparent pests and pathogens of oak. This negative identity effect implies there may be considerable benefit in mixing species, mitigating the impact of pests and pathogens by host dilution (Hantsch et al., 2013). Hence, rather than suggesting there is no effect of mixing different tree species, the lack of species richness effects on productivity is more indicative of little impact of mechanisms such as natural enemies activity, associational susceptibility or species richness-linked alterations of the local microclimate. Natural enemies in this system were very specialized, limiting the expected impact of species richness by mechanisms such as diet mixing (Jactel et al., 2005).

Productivity was greater in mixtures of different clones in the SRC site, but this effect was not significant. The presence of one or more confounding drivers that could not be identified precludes making definite conclusions concerning the impact of clonal mixing in SRC. Monitoring for longer time periods, including one or more harvests of aboveground biomass, is needed to provide a comprehensive picture. Still, I did not see negative effects of mixing and when overyielding occurred, complementarity was a

greater contributor than the selection effect (per the approach of Loreau and Hector (2001)). I did find positive selection effects after four years of growth, which seems to support another proposed benefit of mixing: compensatory growth by a more fit clone. Mixing clones individually rather than by rows may increase the potency of this dynamic even more and could also be more effective in hampering the spread of pests such as leaf rust or beetles (Peacock et al., 2001). However, this will increase competition between different clones, increasing the odds that more dominant clones will start to edge out less competitive ones, such as Gudrun in this study. This may be problematic, if these less competitive clones are important due to their resistance during expected extreme events, such as drought, storm or severe pest outbreaks, including breakdown of resistance by the currently most productive clone (Lindegaard et al., 2011).

A similar picture can be seen in the FORBIO site of Zedelgem. Individual mixing would maximize the positive impact of certain species interactions, such as we could note for beech and lime, as well as the associational resistance effect against a specialist pathogen such as PM. But these positive interactions are likely related to reduced competition at this life stage, given the strong negative impact of shading, and this can be expected to change in particular in the case of oak-beech mixtures. As individual mixing maximizes interspecific interactions, competition would also be stronger with consequentially faster and more frequent competitive displacement of some species by others. Hence, while we might expect stronger facilitative interactions for individual mixing in these youthful stages, stronger competitive interactions in the longer term could lead to reduced species richness and therefore potentially reduced resilience against adverse abiotic conditions and pest outbreaks. The strong negative effects of shading and the tree species associated with it (mostly birch and pine) constitute another trade-off to argue against individual mixing, as shading was noted to have an even more adverse impact than PM. Still, shading may also have some positive, facilitative effects under different conditions (Valladares et al., 2016), such as associated with larch in the site of Gedinne.

Effects of species composition were less prominent in the FORBIO site of Gedinne. The Janzen-Connell effect of oak here was only significant for growth in 2014 and had no significant impact on any biomass trait. There was also no strong relationship between PM infection rates and presence of oak. Other identity effects were not consistent other than larch being associated with greater relative height increment, probably due to the much greater amount of shade it cast than the other four species. Shading had initially a positive impact on relative growth rates and no negative effect on aboveground biomass at the end of the experiment. PM also had less negative effects on sapling growth than in Zedelgem, despite overall greater mildew infection severity. This might be the consequence of a difference in oak species, as *Q. petraea* is known to be less vulnerable to the disease (Marçais and Desprez-Loustau, 2012). Another explanation is

that other, abiotic factors were more limiting to growth in this site, supported by the higher allocation to root biomass and the less prominent negative impact of shading. There was overall much less shading in this site due to the presence of only one fast-growing species (larch), one very slow-growing (beech) and one suffering from fairly high mortality (maple). Both FORBIO sites also differed in other ways from each other, such as previous land use (agricultural for Zedelgem, *Picea abies* forest for Gedinne) and topography, making any conclusion concerning changing composition effects along gradients of stress even more confounded.

3. Role of the Stress Gradient Hypothesis

Except for mycophagy, I did not find interactions between species richness and stress. In the Short Rotation Coppice plantation, the inability to identify the source of stress made the determination of diversity effects convoluted, never mind its interaction with an unquantified stress gradient. A suggestion was raised later by managers that remnant herbicides in the soil might have played a role, as the site was formerly used for intensive agriculture, but this was never tested. In the FORBIO sites, the limited effect of the reduced rainfall treatment on soil moisture content, even less so during drier conditions, suggests that this treatment did not have sufficient impact to cause water limitations. This despite the fact that rainfall interception rates were more or less as strong as expected. Possibly, capillary rise of water through the holes in the bottom of the pots dampened the effect of reduced rainfall. Roots sometimes grew outside the pots, but this was the case only for the largest saplings (mostly in Zedelgem). These were associated with lower soil moisture content inside the pots, most likely due to their larger canopy and root system allowing greater transpiration. Shade cast by the gutters could have reduced soil evaporation and reduced light availability for the soil within the pots, potentially having an impact on soil microbiology and limiting development of herbaceous vegetation there – although the latter is not a likely factor as the pots were regularly weeded. Soil microbiology was assessed as part of another, ongoing research project, which may shed some light on whether this mechanism played a major role. Either way, this implementation of a stress gradient did not have the desired impact on soil and tree, despite rainfall interception rates conforming to expectations. This emphasizes the importance of validating the effectivity of a stress treatment in this kind of research. Future studies considering a similar approach to simulating water stress might wish to ensure intercepted water is deposited further from the target saplings and that water inflow through the holes in the pots is limited or prevented, for instance by depositing a layer of coarser substrate below the pots.

I did note an interaction between the reduced rainfall gradient and tree species richness on ladybird mycophagy of PM, which was surprising considering the otherwise lack of effects of either gradient. The lab experiment suggest mycophagy of PM to be quite sensitive to water conditions of the host tree, regardless of actual plant water stress as

the soil moisture contents of the saplings used in this experiment did not indicate severe water stress. The absence of a link with ladybird numbers in the field suggests the mechanism to be mostly related to an increased susceptibility of PM to mycophagy when trees have a better water status. The link with species richness is less clear, as none of the other tree species were infected by PM fungi. A few herbaceous species in the site were susceptible to PM, such as *Sonchus*, *Trifolium* and *Rubus* sp., and could therefore have been alternative sources of food, but the extent of their PM infections and link to tree species richness is not known. The fraction of plots containing *Quercus robur* did increase along the species richness gradient, but this did not seem to influence ladybird numbers. Rates of mycophagy were also not elevated with increased density of *Quercus*. Other mechanisms by which increased species richness may enhance the activity of natural enemies, such as better shelter structures and availability of alternative food sources (Jactel et al., 2005), do not seem to be supported by my results either. I found shelter to be mostly associated with *Quercus* as well and during the breeding of the ladybirds, no alternative food sources were accepted, which was also observed by other authors (Sutherland and Parrella, 2009).

4. Insights concerning mechanisms

In line with the Natural Enemies Hypothesis, I found an increase in mycophagy of oak PM in more species-rich plots, but this positive association occurred only in saplings which were subjected to rainfall interception – a treatment that did not seem to influence sapling characteristics otherwise. Species richness did not influence these characteristics otherwise either, suggesting that composition-mediated trophic control might not be as important for sapling growth and survival in this system. Still, we only assessed mycophagy close to the end of the growing season, whereas its impact might be expected to be greater in the period of June-July, when PM impact on growth seems to be higher and ladybird larvae are still around. The grazing rates we observed in the choice feeding experiment suggest substantial mycelia removal is possible, but the exact impact on the fungus and therefore its impact on the tree remains unknown. This association would best be studied in a multiannual experiment quantifying PM infections, PM mycophagy and tree growth or vitality in a controlled environment such as a greenhouse or climate chamber. Field observations will be very strongly confounded, even if just by rain, and pose considerable logistic difficulties as multiple monitoring sessions per growing season will be required. Mycophagy of oak PM may yet play a significant role in reducing the impact of the disease in young oaks, in particular in young plantations which are still sufficiently open to allow the growth of large forbs susceptible to PM diseases.

Effects of host dilution could not be clearly quantified with this experimental setup, but we noted strong effects of host presence in the site where the impact of PM was worst. This suggests that by maximizing planting distances between susceptible species by

planting non-susceptible species between them, PM infections could be mitigated in young stands. Indications of mitigating shelter effects by larger coniferous species such as pine or larch were noted, but not as strong as the Janzen-Connell effect. There were also conflicting results for a species not associated with shelter, beech, still consistently but not significantly reducing PM, as well as the most sheltering species of all in Zedelgem, birch, not having any effect on PM infections. Also, in Gedinne the negative impact of oak presence on growth was less severe and host presence was not significantly related to PM severity. The oak species present in this site, *Q. petraea*, is known to be less susceptible to PM, but this translated in our experiment to a reduced impact on growth rates and biomass, not reduced infection severities. Hence, the reduced sensitivity to host presence is more likely to be related to a different infection pattern in this site, with the impact of other drivers such as local microclimate more important than proximity to host trees. Further supporting this is the increased severity of PM in monocultures in this site, which is more strongly tied to differences in local microclimate than to differences in host presence. Saplings in this site were much more exposed to wind and therefore PM spores, as FORBIO trees were overall substantially smaller than those in Zedelgem, with larch as the sole exception. The understorey was also not as well-developed, with the exception of common bracken (*Pteridium aquilinum*) which was mowed every year. Differences in sporal inoculum from outside the experimental sites is not an explanation, as both were surrounded by mature forest, with more likely a greater density of oak in Zedelgem than in Gedinne.

Levels of shading were associated differently with the presence of different tree species, as expected based on their juvenile growth rates and to a lesser extent mortality. While shading had strong negative effects in Zedelgem and initially mildly positive effects in Gedinne on relative growth rates, this did not translate to presence effects of species which cast most shade, i.e. birch, pine and larch. This may suggest that individual species mixing is not as much of a problem as the negative effects of shading on growth seem to suggest, but, as stated before, competitive interactions are expected to only grow more severe as the stand ages. Mortality of the oak saplings in the FORBIO experiments was low and mostly related to more stochastic types of damage by macroherbivores such as rabbit or hare. However, I would expect mortality to have become higher the years after the experiment was finished, when increased competition would result in self-thinning of the smaller trees. Such a longer run was not possible with my setup, as the larger saplings were already starting to outgrow the pots after three years by the end of 2015. My protocol will also have prevented some mortality, as it restricted root interactions with the FORBIO trees and, more importantly, competition with the herbaceous understorey vegetation, which was frequently weeded. Hence, the negative impacts on growth and biomass I noted in my experiments might very well result in mortality under typical plantation field conditions. On the other hand, the absence of this driver may have also resulted in the impact of shading seeming more

severe than under normal conditions, as positive effects of shading by restricting understorey growth are left unrepresented.

5. General outlook

Multiple recent analyses have noted positive effects on productivity of mixing tree species in forests. Results from this thesis are partially in line with those findings, but emphasize the role of species identities, in particular the Janzen-Connell effect. These findings provide support for individual mixing of different tree species or clones in young stands, maximizing positive interspecific interactions and compensatory growth rates. However, also some of the drawbacks are revealed, such as the risk of competitive displacement reducing eventually realized richness. Aside from a strong Janzen-Connell effect supporting the Resource Concentration Hypothesis, notable biological control of powdery mildew by mycophagous ladybirds was observed, with considerable sensitivity to plant water conditions. However, the significance of this mycophagy for the potentially very negative effects of powdery mildew on plants remains unknown. Insight into the impact of ladybird mycophagy on growth and survival of oak saplings could also shed further light on mechanisms behind tree species composition effects related to oak powdery mildew.

Differences in abiotic conditions seemed to have an impact on interspecific interactions, but difficulty in establishing and identifying stress gradients rendered it impossible to make conclusions concerning the implications of the Stress Gradient Hypothesis for mixing of species. Further research into this topic is needed, in particular for the case of specific abiotic gradients, such as reduced rainfall or warmer temperatures, rather than comparing sites or years differing in multiple ways. Care should be taken to implement well-controlled gradients of stress and their negative impact on the target trees should be regularly evaluated.

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Setiawan, N.N., Vanhellefont, M., Baeten, L., Dillen, M., Verheyen, K., 2014. *The effects of local neighbourhood diversity on pest and disease damage of trees in a young experimental forest*. For. Ecol. Manage. 334, 1–9.

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Dillen, M., Verheyen, K., Smit, C. 2013. *The interplay of biodiversity and environmental stress for ecosystem functioning*. FORBIO meeting, Zedelgem

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Dillen M., Smit C., Buyse M., Höfte, M., De Clercq P., Verheyen K. 2017. *Do biodiversity effects become more important with stress? A study of multitrophic interactions between oak, powdery mildew and mycophagous ladybirds*. NAEM 2017, Lunteren

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Dillen, M., Verheyen, K., Smit, C. 2014. *Drought stress, tree diversity and ecosystem functioning? Leaf damage symptoms of oak saplings in a phytometer experiment*. NAEM 2014, Lunteren

Supervision of MSc students

Buyse, M., 2014-15. *Biological control of oak powdery mildew by ladybirds in interaction with drought stress (In Dutch)*. Ghent University.