



UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Land, Environment Agriculture and Forestry

Second Cycle Degree (MSc)
in Forest Science

**Host tree, bark beetle and blue stain fungi interactions under drought
conditions**

Supervisor

Prof. Andrea Battisti. Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padua.

Co-supervisor

Dipl.-Ing. Dr. Sigrid Netherer. Institute of Forest Entomology, Forest Pathology and Forest Protection, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna.

Submitted by
Giuseppe Morgante
Student n. 1205796

ACADEMIC YEAR 2020-2021

*A mia Madre e a mio Padre,
instancabile e umile forza motrice
di questo mio percorso.*

Contents

1. Introduction.....	7
1.1 <i>The Eurasian spruce bark beetle, I. typographus</i>	7
1.1.1 <i>Spruce bark beetle biology and phenology</i>	8
1.1.2 <i>Important factors triggering spruce bark beetle outbreaks</i>	9
1.1.3 <i>Impact of precipitation deficits and increased temperature conditions on bark beetle outbreaks</i>	10
1.2 <i>The host tree Norway spruce</i>	10
1.2.1 <i>Constitutive and induced defences of Norway spruce</i>	11
1.3 <i>Ophiostomatoid fungi associated with the Eurasian spruce bark beetle</i>	13
1.4 <i>The Rosalia Roof Projects I and II</i>	14
1.5 <i>Olfactory test through the “Petri-dish arena choice experiment”</i>	16
1.6 <i>Research questions</i>	17
1.6.1 <i>Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi at study trees of RRP II</i>	17
1.6.2 <i>Petri dish arena choice experiments in laboratory</i>	18
2. Materials and methods.....	18
2.1 <i>Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi</i>	18
2.1.1 <i>Isolation of fungi and inoculation experiments</i>	18
2.1.2 <i>Control and analysis of hypersensitive wound reaction zones</i>	19
2.1.3 <i>Statistical analysis of WRZs measures</i>	21
2.2 <i>Petri dish arena choice experiments</i>	21
2.2.1 <i>Experimental set-up</i>	22
2.2.2 <i>Monitoring procedure</i>	23
2.2.3 <i>Data analysis</i>	24
2.3 <i>Relationships between results of the different experiments</i>	24
3. Results.....	25
3.1 <i>Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi inoculation experiments</i>	25
3.1.1 <i>Mean Maximum length of WRZs</i>	26
3.1.2 <i>Mean Maximum Width of WRZs</i>	26
3.1.3 <i>Mean Outer Area of WRZs</i>	27
3.1.4 <i>Mean inner area of WRZs</i>	28
3.2 <i>Petri dish arena choice experiments</i>	28
3.2.1 <i>“Zero test” results</i>	29
3.2.2 <i>Choice experiments using RRP II study tree and extra control tree samples (“T versus C” experiments)</i>	29

3.2.3 Choice experiments comparing particular RRP II study trees (“T versus T” experiments).....	31
3.3 Relationships between WRZ size parameters, choices for tree samples found in arena experiments and number of attacks investigated in the course of attack box experiments in the field.....	33
4. Discussion.....	35
4.1 Hypersensitive wound reaction zones (WRZs) in response to fungal inoculations.....	35
4.2 Petri dish arena choice experiments.....	37
4.3 Relationships between tree responses to fungal inoculations and results of Petri dish arena and attack box experiments.....	39
4.4 Conclusions and outlook.....	40
5. References.....	41

Abstract

Bark beetles are known to be highly destructive pests of coniferous stands, their success in colonizing new hosts is normally favoured by their naturally associated symbionts and climatic conditions. The Eurasian spruce bark beetle *Ips typographus* and several species of ophiostomatoid fungi is an already known and studied association that naturally occurs in Norway spruce stands: in their mutualistic symbiosis, they both act in order to overcome host defences, guaranteeing the availability of feeding and breeding material. Conifers are known to have different defensive systems approaches able to repel and to kill their external invaders but, when physiological stresses due to climatic conditions act together with these invaders, plants may not be strong enough to overcome all of them in once. Nevertheless, trees are also known to get benefits from moderate external attacks and stress, enhancing the synthesis and storage of defensive compounds. This thesis work, based on results obtained in the Rosalia Roof Project I and II, aimed to study the capability of Norway spruce trees to face biotic attacks through the inoculation of the ophiostomatoid fungal species *Endoconidiophora polonica* and *Grosmannia penicillata*. The preferences of *I. typographus* in choosing bark cores sampled from these trees in “Petri-dish arena” choice experiments were tested. Results showed how limited water supply, due to the establishment of roofs under canopy, lowered defences of study trees, making them easily colonisable hosts for fungal invaders. At the same time, there was no clear preference of spruce bark beetles for roofed or control trees.

Keywords

Norway spruce, drought stress, bark beetles, *Ips typographus*, ophiostomatoid fungi, *Endoconidiophora polonica*, *Grosmannia penicillata*, inoculation, wound reaction zone, arena choice experiment.

Riassunto

I coleotteri scolitidi sono conosciuti per essere parassiti altamente distruttivi nei popolamenti di conifere, il loro successo nel colonizzare nuovi ospiti è normalmente favorito dai loro simbioti naturali e dalle condizioni climatiche. Il bostrico euroasiatico *Ips typographus* e numerose specie di funghi ofiostomatoidi rappresentano una ormai conosciuta e studiata associazione che si verifica naturalmente nei popolamenti di abete rosso: nella loro simbiosi mutualistica, entrambi agiscono nel sormontare le difese dell'ospite per garantirsi la provvigione di materiale utile al loro nutrimento e accoppiamento. È risaputo che le conifere posseggono diversi sistemi di difesa capaci di repellere e uccidere invasori esterni ma, quando stress fisiologici dovuti alle condizioni climatiche agiscono insieme a tali invasori, le piante potrebbero non essere sufficientemente forti da affrontarli tutti insieme allo stesso tempo. Ciononostante, è anche risaputo che le piante traggono benefici da attacchi esterni contenuti, favorendo la sintesi e lo stoccaggio di composti chimici difensivi. Questo lavoro di tesi, basato sui risultati ottenuti nei Rosalia Roof Project I e II, propone lo studio delle capacità dell'abete rosso di affrontare attacchi di entità biotiche attraverso l'inoculazione dei funghi ofiostomatoidi *Endoconidiophora polonica* e *Grosmannia penicillata*. Sono state inoltre verificate le tendenze dell'*I. typographus* nello scegliere campioni di corteccia raccolti da tali alberi nel "Petri-dish arena choice experiment". I risultati hanno dimostrato come la limitata provvigione di acqua, dovuta alla costruzione di coperture sotto chioma, ha ridotto le difese degli alberi oggetto di studio, rendendoli degli ospiti facilmente colonizzabili da parte dei funghi invasori. Allo stesso tempo, non si sono evidenziate particolari preferenze da parte del bostrico nei confronti degli alberi con copertura o degli alberi di controllo.

Parole-chiave

Abete rosso, stress idrico, coleotteri scolitidi, *Ips typographus*, funghi ofiostomatoidi, *Endoconidiophora polonica*, *Grosmannia penicillata*, inoculazione, zona di reazione della ferita, arena choice experiment.

1. Introduction

This master thesis focuses on relationships between Norway spruce, the Eurasian spruce bark beetle *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae) and its associated ophiostomatoid fungi, *Endoconidiophora polonica* and *Grosmannia penicillata*. The research approach of this study was to induce drought stress in study trees by installing roofs under canopy, as already done in a more comprehensive drought manipulation study: the Rosalia Roof Project I and II, on which this thesis is based.

At trees of the drought manipulation site, ophiostomatoid fungi were inoculated to trigger and to test the trees' defensive reaction. A main part of this thesis was to investigate size parameters of the hypersensitive wound reaction zones established by the study trees in response to fungal growth and whether bark beetle attack and preference of beetles for specific trees were related to the extent of these lesions. The second part of this work comprised olfactory tests by means of Petri-dish arena experiments with *I. typographus* to test for the attractiveness of bark cores sampled from the study trees for male beetles.

1.1 The Eurasian spruce bark beetle, *I. typographus*

Bark beetles play a fundamental role in forest ecosystems. Bark beetle attack of trees promotes the opening of forest gaps, degradation of dead plant material, creation of litter and thus, significantly affects nutrient cycling, and soil structure (Krokene, 2015). As mass outbreaks of some bark beetle species can lead to extensive forest mortality, such aggressive species as the Eurasian spruce bark beetle (*Ips typographus*) are also defined as "landscape engineers" (Raffa et al., 2015; Müller et al., 2008). Despite their importance from a biodiversity and ecological point of view, bark beetles compete with human beings for valued plants and related timber products; due to this competition for resources, they are regarded as pests (Raffa et al., 2015).

The Eurasian spruce bark beetle is one of the most destructive and dangerous pests of Norway spruce (*Picea abies*). Several outbreaks have occurred during the last decades in Northern and Central Europe. For example, from 2012 to 2018 in Poland, Slovakia, Bavaria, Austria and Czech Republic, 87 million of m³ have been infested by *I. typographus*, as reported by Lopatka (2019). In North America, *I. typographus* is considered as quarantine pest (Holderness, 1996). Outbreaks of this bark beetle are triggered by storms and drought events which can result in uncountable economic damages and high mortality rate at landscape level, especially when warm springs and summers enhance brood development (Baier et al., 2007; Seidl et al., 2007). Under favorable

temperature conditions, the number of individuals increase exponentially, triggering density-related competition that force this bark beetle to attack standing, living trees. Such kind of outbreaks can last for years (three to six) (Wermelinger, 2004) and endure as long as feeding and breeding material is available.

1.1.1 Spruce bark beetle biology and phenology

In spring, pioneer male spruce bark beetles leave their overwintering sites and start colonizing Norway spruce trees. At low population densities, beetles preferentially attack fallen trees, logs and slashes, accelerating the decomposition of wood. The male beetles create the nuptial chamber underneath the bark and release conspecific aggregation pheromones that attract further males and females (Wermelinger, 2004). The females build galleries parallel to the phloem fibers. Galleries are constituted by a main gallery along which eggs (up to 50) are laid along both sides. Usually, one male breeds with one to four females. During outbreak periods, the size and the length of maternal galleries are strongly affected by density within the host tree (Wermelinger, 2004). Larvae feed on secondary phloem in larval galleries, perpendicular to maternal galleries, becoming wider as larvae grow and ending up as pupal chambers. Emergence takes place through small round exit holes. Beetles usually overwinter in the adult stage, close to the tree where they had developed, in the forest litter or under the bark of the host tree (Holderness, 1996).

The life cycle of the spruce bark beetle is strictly influenced by temperature. At lower altitudes and latitudes, *I. typographus* populations can develop two or even three generations (bivoltine or trivoltine development) from about April to October. Voltinism depends on the length of the season and the accumulated temperature sums available for brood development. As Wermelinger and Seifert (1998, 1999) found, the optimal temperature range for brood development of *I. typographus* lies between a lower developmental threshold of 8.3°C and 30°C. In order to complete development from egg to adult stage, 573 degree-days above 8.3°C are needed. In particular, the spruce bark beetle needs an accumulated temperature sum of 334 degree-days to reach the pupal stage and 239 degrees-days for maturation feeding above stage-specific developmental thresholds. At higher latitudes, due to cooler temperature conditions, effective temperature sums are reached later and adults need more time to emerge (Wermelinger & Seifert 1998, 1999).

Temperature also influences beetle behavior once *I. typographus* reached the adult stage. Flight

activity and thus, beetle dispersal depends on a minimum air temperature of 16.5°C, with an optimal range between 22°C and 26°C (Wermelinger, 2004), and with the highest activity over noontime and the early afternoon. The spruce bark beetle commonly attacks new trees within a radius of up to 500 m (Wermelinger, 2004), but a certain proportion of the population, especially at high population numbers, can colonize stands which are kilometers away (Kautz et al., 2011).

1.1.2 Important factors triggering spruce bark beetle outbreaks

Natural disturbances such as windthrow and insect outbreaks directly influence forest composition, structure, and functions within an ecosystem. They are highly linked with climate and therefore, strongly affected by climate changes. Recently, forest disturbance regimes have intensified across Europe, bringing along a strong damage increment due to windthrow and bark beetle outbreaks (Seidl et al., 2011). Different hypotheses attribute this intensification to climate change as the main driving force, but also changes in structure and composition of forests due to management aims make forests highly susceptible to disturbance events (Seidl et al., 2011).

Bark beetle population dynamics are particularly driven by temperature conditions, and they are triggered by a range of other climatic (solar irradiation, precipitation, temperature, hydrology) and stand-related parameters (soil features, slope exposure, stand age and density, proportion of species, predisposition to disturbance damages) (Netherer and Nopp-Mayr, 2005). Windstorm is one of the main disturbance events concerning Norway spruce stands and promoting outbreaks of *I. typographus* through the increase of breeding and feeding material availability (Christiansen et al., 1987). Because of the swing action they went through, root systems of trees that resisted windstorms are often harmed. Moreover, the sudden decrease in forest stand density and the creation of gaps expose surviving trees to higher wind turbulence. Thus, those trees who survived a storm event are strongly prone to being harmed in future (Christiansen et al., 1987), increasing the frequency of infestations due to storm damages (Netherer and Nopp-Mayr, 2005). The reproduction of root rot fungi can also be facilitated by windthrow, which lowers the photosynthetic capabilities of the trees and enhances the predisposition of Norway spruce stand to *I. typographus* attacks. The risk of root rot events rises with the age of the forest stand (Christiansen et al., 1987).

Stand density is another factor influencing the risk of storm damage and the susceptibility of trees to spruce bark beetle attack. Due to the competition for nutrients, light, aerial space and crown growth, high density conditions are known to gradually reduce in time the number of trees. While

high stand density induces physiological stress in the plants, increased temperatures in open and sparse stands improve the conditions for bark beetle development (Netherer & Nopp-Mayr, 2005; Netherer et al., 2019). Bark beetle infestations are also linked with stand age. As Netherer & Nopp-Mayr (2005) considered in a predisposition assessment system for *I. typographus*, the older the stand is, the more it is prone to infestations by the spruce bark beetle. Infestations, in fact, were concentrated mostly in Norway spruce stands older than 60 years in a study area located in the High Tatra Mountains. A 100-year-old stand is most vulnerable to bark beetle attack (Netherer & Nopp-Mayr, 2005).

1.1.3 Impact of precipitation deficits and increased temperature conditions on bark beetle outbreaks

The increase in drought events and higher temperatures related to climate change are among the significant factors that directly harm tree growth and defences against biotic invaders such as bark beetles, and which can therefore increase the susceptibility of trees to *I. typographus* attacks. (Netherer et al., 2019). Rising temperatures decrease the development time of *I. typographus*, facilitate its multi-voltinism behaviour, and substantially improve infestation pressure, while drought reduces tree vigour. Similarly, in North America *Dendroctonus ponderosae* infestations have occurred, affecting almost 5 million ha of forests and annually participating to 1.5 billion dollars loss (Hicke et al., 2006).

Local outbreaks of *I. typographus* are usually triggered by common drivers including the deficit of precipitation during summer periods, warm temperatures and, usually, a discrete availability of storm-felled trees from the previous year as a fundamental source of breeding material (Marini et al., 2017). Nevertheless, Faccoli (2009) showed that even with a decrease in precipitation in the south-eastern Alps by about 200 mm (-22%) during March-July from 1922 to 2007 and an increase in mean temperatures by about 2 °C (+13%) during March-July from 1962-2007, damage generated by *I. typographus* was inversely correlated with March-July precipitation in the previous year (Faccoli, 2009). This to show that drought is not necessarily the main driver of outbreaks. They are usually triggered by a range of factors, especially when climate conditions coincide with an increase of stand-related susceptibility (Seidl et al., 2011).

1.2 The host tree Norway spruce

Norway spruce (*Picea abies* (L.) Karst.) (Pinales: Pinaceae) can grow up to 50 m in height and to a width of 100-150 cm at breast height. The tree species has a straight stem, and its crown is of a distinct green-conic shape. Its bark is thin and reddish. Low branches are mostly horizontal while

the upper ones go upward. Needles persist for several years (8-10), they are 15-25 mm long and of dark green color (Gellini and Grossoni, 1996). Cones are cylindrical, straight and, once mature, they are brown and 10-15 cm long. Its root system is very shallow and its thick roots do not descend more than 50 cm underground, thus root development occurs horizontally more than vertically (Gellini and Grossoni, 1996). This feature is the main cause why Norway spruce can be uprooted by wind easily and why, during periods of drought, water supply might be depleted fast due to the drying out of upper soil layers. The natural range of Norway spruce extends from Scandinavia to Siberia in longitude and from 45° to 70° in latitude (Gellini and Grossoni, 1996).

Norway spruce plantations are common in large parts of Europe. Due to its fast growth and high wood quality, this tree species was particularly used for afforestation especially in the decades right after WWII, in particular in Germany and Austria (Aarrestad et al., 2014).

During periods of low precipitation and reduced soil water availability, Norway spruce closes its stomata to prevent further water loss from transpiration (Willmer and Fricker, 1996). In such way, the tree is able to keep xylem water potentials above a critical level. At the same time, however, the availability of carbohydrates for tree defence mechanisms is reduced due to the limited photosynthetic activity (Huang et al., 2020). For example, tree defence mechanisms include the production and storing of resin that can physically drown or delay attacking bark beetles and the accumulation of monoterpenoid compounds that can kill or repel bark beetles and their fungal and bacterial symbionts at high concentrations (Huang et al., 2020). A hypothesis suggests that those stands which are growing on fertile sites with permanent water supply, are more prone to be attacked by *I. typographus* when facing a sudden period of drought (Netherer et al., 2019). Even if many studies have been carried out to support the relationship between precipitation deficits and *I. typographus* outbreaks, the precise mechanisms and causes that make Norway spruce physiologically and biochemically susceptible to bark beetle attacks are still not so well known. More research is needed to understand how drought affects the trees' defence system to keep away insect herbivores and fungal pathogens.

1.2.1 Constitutive and induced defences of Norway spruce

Norway spruce can rely on two types of defences, constitutive defences (mechanical and chemical) and inducible defences (structural and chemical). Constitutive mechanical defences are provided by structural elements of the tree that mechanically provide to its tissue strength or thickness, enhancing the resistance to penetration and degradation through lignin and suberin

impregnation (Krokene, 2015). Mechanical defences are generalized barriers toward a wide range of threats that, over the lifetime of a tree, may lead to perforation of the bark. Constitutive mechanical defences may also feature "spines," as the pointed cauline leaves in monkey puzzle trees, which can be useful against large animals (Franceschi et al., 2005).

Enzymes, various tree metabolites and proteins that have a toxic or inhibitory effect are included in the constitutive chemical defences. These compounds are dispersed across the bark tissues, including the periderms, cortex and secondary phloem, through the resin ducts during the attack (Franceschi et al., 2005).

Through the novel synthesis of chemicals and biochemical agents that are organism-specific, inducible defences increase the overall defence capability of the tree. Mediated defence mechanisms are thought to limit the degree of damage and seal all damaged tissues in response to biological attack or injury. Through this strategy, by synthesizing more and more unique chemicals and biochemicals, plants can acquire some kind of "experience" leading them to a higher degree of tolerance to the most common damage agents of their native region. This process of continuous adaptation of conifer defence to attacks from outside species is occurring since millions of years (Franceschi et al., 2005).

Inducible structural defences are aimed to contain and isolate the invader, repairing the damaged tissues and to limit future invasions. These mechanisms are hypersensitive and highly localized reactions of the tree, directly at the place where the infection or attack is occurring. The production of reactive oxygen species that are intended to kill or to contain the invader, causes the death of all the surrounding cells, which create a barrier of "scorched earth" by the formation of callus tissues and consequent lignification. In that way, the tree is locally protected against future attacks (Franceschi et al., 2005).

Inducible chemical defences are highly different, they include general and specific compounds inhibiting pest species. During the attack, general compounds are released as the tree stimulates its recognition mechanisms to recognise the invader and activate organism-specific defences in order to slow down the invasion (Franceschi et al., 2005).

In a healthy tree, the stages for a successful defence reaction are (a) constitutive defences repelling the invader; (b) if the attack continues and the bark is penetrated, inducible defences start, trying to isolate and to kill the invader. Normally, these two phases act together; (c) the

damaged area is recovered and, (d) to prevent future infections, the wound is sealed (Franceschi et al., 2005).

During their growth, trees must regulate the use of nutrients required for their physiological needs and the development of pest-specific defences. The reason why inducible chemical defences are not created permanently inside a tree is that it is not possible to use nutrients needed to create inducible defences and to promote growth and reproduction at the same time, thus the allocation of resources must be cost-effective (Krokene, 2015). Thus, the ability of trees to face bark beetle attacks and their associated fungi is related to the amount of carbohydrates available for the synthesis of defensive compounds and localized wound reactions. Their production, and thus the total defensive capacity of the tree, is influenced by biotic and abiotic factors that, sometimes, can also act together, increasing the level of stress and susceptibility and leading the plant to death (Huang et al., 2020).

1.3 Ophiostomatoid fungi associated with the Eurasian spruce bark beetle

Fungi have been the subject of various studies among the different species of micro-organisms (nematodes, fungi, yeasts, bacteria) identified as associated with *I. typographus*: *E. polonica*, *G. penicillata*, *Ophiostoma bicolor*, *Grosmannia europhioides* and *Ophiostoma ainoae*. (Kirisits 2007). The majority of bark beetle related fungi are ascomycetes, mainly belonging to the genera *Ophiostoma*, *Ceratocystis* and *Ceratocystiopsis* (Lieutier et al., 2009).

Ophiostomatoid fungi are also called “blue-stain”, “black-stain” or “sap-stain” fungi, because of the bluish-grey colour they give to colonized conifers’ sapwood through their melanised hyphae (Lieutier et al., 2009). Both bark beetles and their related fungi have evolved together in time as symbionts in a mutualistic way, in which two different species get benefits from each other (Kirisits, 2007). Many researchers considered the role of fungi in tree killing and in the depletion of the host's defensive mechanisms as the key mode of action from which bark beetles benefit from the interaction with fungi, the primary evidence in favour of this theory is the high degree of virulence of certain fungal associates to their host trees (Kirisits, 2007). The fading of the leaves, the assessment of sapwood occlusion or sapwood invasion by the fungus are some parameters considered to indicate tree mortality due to fungal mass inoculations (Lieutier et al., 2009).

Plants, when attacked by microorganisms, react with a hypersensitive wound reaction. A necrotic area is created around the point of infection, in order to deprive the invader of the living tissues where it grows. Coniferous trees normally impregnate the necrotic area with resinous and

phenolic compounds, impeding larval development and fungal proliferation. This because resin is particularly toxic to bark beetle eggs and larvae and constrain fungal growth (Christiansen et al., 1987).

A crucial aspect related with resin use is its dosage. Its high concentration around the hypersensitive wound reaction zone in the shortest possible time prevents the breakout of the fungi from this area. In this case, trees may create different additional wound reaction zones outside the first one, in order to better isolate the microorganism (Christiansen et al., 1987). Nevertheless, more attacks, especially in a very short time span, can deplete trees' defences. This has been experimentally proved in *Pinus* and *Picea*: when the number of attacks pass a certain threshold, the amount of resin in every reaction zone decreases, together with carbohydrate reserves and thus, the synthesis of terpenes and phenolic compounds. Some ophiostomatoid fungal species are even able to degrade phenolics and use derivatives as carbon source (Wadke et al., 2016). This decrease in availability and concentration of defence compounds enhance the fungi's spread in the sapwood (Christiansen et al., 1987).

The low moisture content and discoloration caused by the blue-staining fungus within a necrotic zone, make the infected sapwood colour opaque. The inner zone is dark-coloured, due to fungal hyphae penetration; in the outer zone, the resinous reaction, which blocks the fungal growth, creates a lighter colour. A new periderm is formed along the outer edge of the reaction zone and the necrotic body impregnated with resin is eventually removed with the bark (Christiansen et al., 1987).

Experimental inoculation of ophiostomatoid fungi can be used to induce tree resistance and to evaluate tree defence capability by the size of hypersensitive wound reaction zones. Inoculation experiments were, for instance, done by Krokene et al. (1999) and by Netherer et al. (2016) in the Rosalia Roof experiment. Low density and high-density inoculations of *E. polonica* were performed to test the defence mechanism and capability of Norway spruce's phloem and xylem by Krokene et al. (1999), who conducted a dose-response experiment using *E. polonica* in three different dosages to study the different levels of infection and wound reactions.

1.4 The Rosalia Roof Projects I and II

To investigate the dynamics, the effects and the direct influence of drought on Norway spruce physiology and susceptibility to attack by *I. typographus*, the Rosalia Roof Project was launched in 2011. The study plots were established in a mature Norway spruce stand in the Rosalia Mountains,

which are located south of Vienna (Netherer et al., 2015). The first part of the study was performed in the years 2012-2015, the follow-up study started in 2018. In the course of Rosalia Roof Project I (RRP I), variations in the physiological state of the study trees, for instance indicated by twig water potential measured before sunrise (pre-dawn twig water potential) and in defence parameters such as resin flow were investigated. A main question was whether attack activity of *I. typographus* was subjected to seasonal changes and whether the number of successful and defended attacks were related to the drought treatment of the study trees (Netherer et al., 2015).

In both studies, drought was induced by constructing roofs below canopy to keep away precipitation from the study trees. While the experimental set-up for RRP I comprised full cover, semi-cover (50% closure of roofs) and control treatments without roofs (Netherer et al., 2015), only two treatments (roofs, control) were established in the course of Rosalia Roof Project II (RRP II). For RRP I, three Norway spruce trees in the centre of each plot were selected as sample trees (6 roofed, 6 partially roofed and 6 control trees in total), in the follow-up study, each roof embraced one study tree (10 roofed and 10 control trees in total).

Monitoring of bark beetle attack was done by means of newly designed attack boxes, which were made for the purpose of controlled experimental testing of *I. typographus* attacks (Picture 1) (Netherer et al., 2015). Each time the attack experiment was performed, these attack boxes were attached to the trunks of the experimental trees and left there for about 24 hours. Beetles from the institute rearing could move freely within the system and decide whether to attack the trees or not. After removing the box, the exposed bark area was thoroughly examined for fresh boring holes, and the number of attacks prevented by resin flow (defended attacks) and of successful attacks was determined (Netherer et al., 2015).



Picture 1: Roofed tree with attack box attached to the stem of a study tree during the experiment in RRP II

RRP I showed that both resin flow and water content of trees were strongly dependent on soil water supply. As observed over two seasons of drought experiments, the fluctuations in pre-dawn water potentials coincided with lower resistance of trees toward attacks (Netherer et al., 2015). However, despite this coincidence, after having passed a certain stress threshold, the host acceptance by *I. typographus* became lower despite the tree's higher vulnerability level. In the follow-up research project RRP II, the main questions focus on the importance of volatile cues for spruce bark beetle attack and tree defence mechanisms playing a role in attack success. The roofs were established in December 2018, so the drought treatments lasted for more than 20 months when this thesis was performed. In August 2020, inoculation of *E. polonica* and *G. penicillata* as well as of agar controls were performed at the study trees.

1.5 Olfactory test through the "Petri-dish arena choice experiment"

Petri-dish arena choice experiments were thought for studying the reaction of *I. typographus* towards volatile organic compounds emitted by the bark cores sampled from the RRP II study trees. The idea behind this experiment was that these samples might be more or less attractive to male spruce bark beetles based on the different physiological status of the trees at the study site.

Kandasamy et al. (2019) found during such experiments that beetles are attracted by synthetic compounds coming from fungal volatiles. It means that volatile organic compounds emitted by fungi can act as recognition signals able to create the microbial communities in which bark beetles naturally occur (Kandasamy et al. 2019).

In the Petri dish arena choice experiments performed in this thesis, it is assumed that beetles are attracted by the volatile blend of the samples. Although there is no evidence yet that *I. typographus* orients itself on particular volatile blends, some studies such as those of Kalinová et al. (2014) and Zhang and Schlyter (2004) showed that when searching for suitable hosts in flight, especially in mixed forests, bark beetles are subjected to a wide range of different olfactory stimuli coming from suitable and unsuitable hosts. The rejection and the avoidance of non-host trees is based on non-host volatiles recognized by the beetles' olfactory sensory neurons. Different physiological state of trees might modify the blend and concentrations of volatile compounds emitted from the bark, such as monoterpenes (Zhang and Schlyter, 2004). Samples used in the arena choice experiments were taken from the study trees presumably facing different physiological states due to drought and control treatments. Different blends of volatile compounds they emit may create the basis for different choices adopted by the bark beetles when deciding between two samples in a Petri dish arena.

1.6 Research questions

1.6.1 Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi at study trees of RRP II

- Are there differences in the size of hypersensitive wound reaction zones developed in response to the inoculation of the two ophiostomatoid fungal species (*E. polonica*, EP and *G. penicillata*, GP) and of agar controls at the study trees of RRP II?
- Does the size of hypersensitive wound reaction zones developed in response to the inoculation of the two ophiostomatoid fungal species EP and GP, and of agar controls depend on treatment the trees (control trees, roofed trees)?
- Is the size of hypersensitive wound reaction zones developed in response to the inoculation of the two ophiostomatoid fungal species EP and GP, and of agar controls related to attack rates by *I. typographus* in the field (defended and successful attacks as well as attack attempts found in the course of the attack box experiments) and to the choices of beetles made in the Petri dish arena experiments?

1.6.2 Petri dish arena choice experiments in laboratory

- Do beetles prefer any bark sample over an empty space (“zero test”)?
- Is there a preference of spruce bark beetles put in the centre of a petri dish arena for bark samples taken from roofed target trees (4 study trees) and control trees (8 study trees) compared to bark cores of a well water supplied extra control tree, which was not used for any experiment in the field?
- Is there a preference of spruce bark beetles for specific trees in the arena choice experiments?
- Do the choices of beetles for trees as found in the petri dish arena experiments correspond to attack behaviour at particular trees in the field?

2. Materials and methods

2.1 Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi

2.1.1 Isolation of fungi and inoculation experiments

The inoculation of the ophiostomatoid fungal species *Grosmannia penicillata* (GP) and *Endoconidiophora polonica* (EP) as well as of pure agar controls (K) was performed in the course of the Rosalia Roof Project II (RRP II) field experimental work on 06.08.2020, before this thesis was started. Previously, the fungi were isolated from desiccated sapwood of an attacked Norway spruce trap tree nearby the study site on 09.07.2020. The fungi were isolated and identified by Thomas Kirisits. The strains were grown for 13 days on ash leaf malt extract agar (AMEA, Kirisits et al. 2013, but without streptomycin sulphate) in plastic Petri dishes (diameter 5.2 cm). One isolate of *E. polonica* (accession no. LF/Va/3/3 of the fungal culture collection of IFFF-BOKU) and one isolate of *G. penicillata* (LF/Ia/9/1) were used in the inoculation experiment. Sterile AMEA served as control treatment.

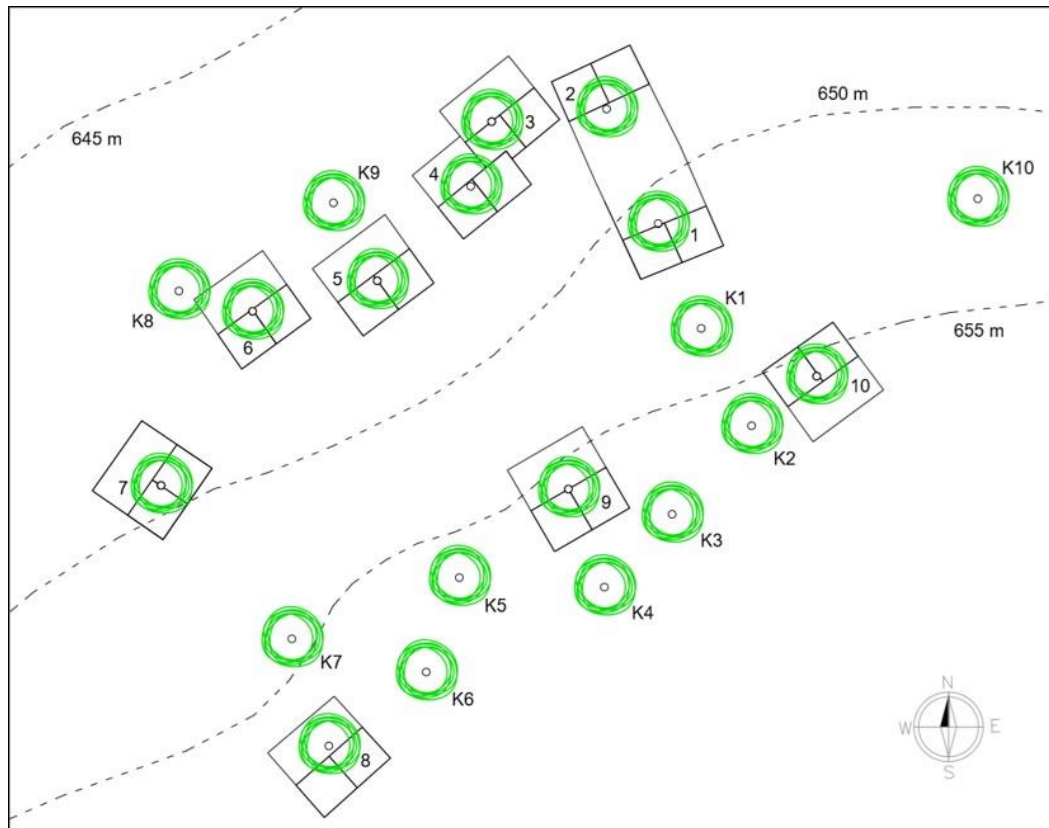


Figure 1: Rosalia Roof Project II (RRP II) study site showing the position and treatment of trees: Roofed trees 1-10 and control trees K1-K10 (figure designed by A. Bachlehner).

In the field experiment, pieces punched out with a cork borer (diameter 3 mm) from fungal cultures or sterile AMEA were used as inoculum. Inoculations were performed at all study trees involving roofed trees (trees number 1-10) and control trees (trees number K1-K10) (Figure 1). At each study tree, 6 samples of GP, 6 samples of EP and 2 agar controls were inoculated in holes of 5 mm diameter. Inoculations of GP and EP were done alternately along the circumference of the trees. Each time six fungal samples and a control were inoculated along a lower ring placed at 160 cm (trees 1-10) or 210 cm (trees K1-K10) stem height and along an upper ring placed at 210 cm (1-10) and 260 cm (K1-K10), respectively.

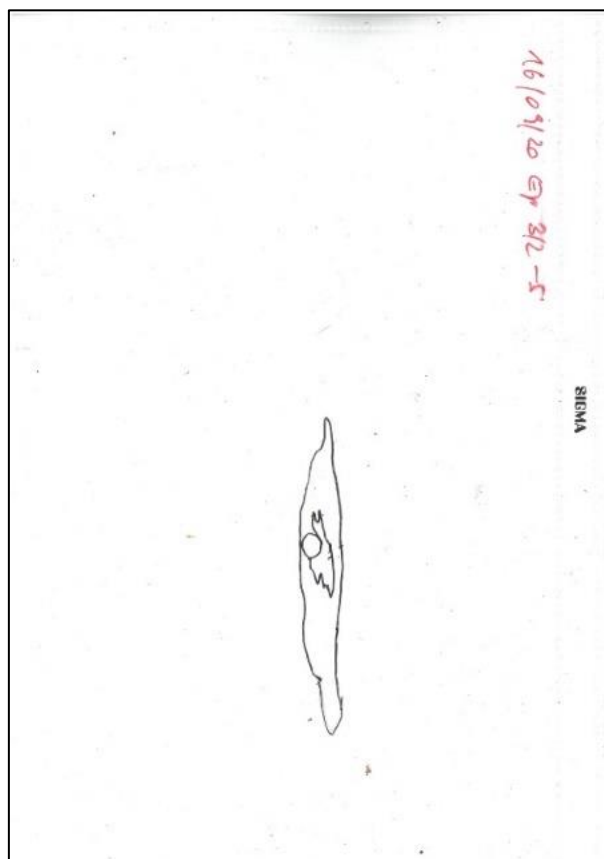
2.1.2 Control and analysis of hypersensitive wound reaction zones

Before this thesis started, the size of hypersensitive wound reaction zones (WRZs) was controlled twice, on 18.08.2020 and on 02.09.2020, each time for 1 GP and 1 EP inoculation by the RRP II study team. These samples were not analysed in the course of this thesis. On 16.09.2020, after a period of 6 weeks from the inoculation experiment, final control of the remaining 4 GP, 4 EP and 2 K inoculations (equally divided between lower and upper ring) took place. For this purpose, the bark was scratched off using a chisel to fully expose the hypersensitive wound reaction zones

(WRZs) (Picture 2). To get the measures of each WRZ, a transparent plastic sheet was placed on the scratched area and the features of the WRZ (inoculation hole, inner and outer demarcation lines) were sketched on the foil using a permanent ink marker (Picture 2).



Picture 2: WRZ (Wound Reaction Zone) beneath the bark after it has been scratched off (left)
Sketching procedure of a WRZ by the use of a permanent ink pen (right)



Picture 3: WRZ (Wound Reaction Zone) sketch after being scanned in a multimedia file.

The sketches were scanned to obtain multimedia files (Picture 3). These pictures were used for analysing the following parameters describing WRZ size: maximum length (ML; mm), maximum width (MW; mm), outer area (i.e. total area according to the outer demarcation line, OA; mm²) and inner area (i.e. the area darkened by growth of fungal hyphae and accumulation of defence compounds limited by an inner demarcation line, only visible at EP samples, IA; mm²). The “Dataf Measure” (Microsoft) software was used for this analysis, data were saved in an Excel sheet.

2.1.3 Statistical analysis of WRZs measures

Analysis of variance (ANOVA) was used to test for statistical differences between the arithmetic mean values of maximum length, maximum width and outer area of WRZs caused by EP, GP and K, for the collectives of control and roofed trees separately. Moreover, statistical differences between control and roofed trees was analysed for EP and GP separately by analysis of variance. Kolmogorov-Smirnov test was used to check for normal distribution of data and Levene statistics to test for homogeneity of variances, required for parametric tests within ANOVA. In case requirements for parametric tests were not fulfilled, non-parametric Tamhane test was applied. P-values <0.05 were regarded as significant, p<0.01 as highly significant.

2.2 Petri dish arena choice experiments

Petri dish arena choice experiments took place at the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF) at BOKU University from October to December 2020. The bark samples (1 cm \varnothing) used for these experiments originated from the RRP II study site, sampled from the study trees of roof and control treatments and from the extra control tree A3, where no field studies except for taking bark samples had been performed. Control tree A3 was part of a tree group located in the triangular area between trees 1, K1 and K10 (Figure 1). The bark samples were collected in September 2020, shock frozen with liquid nitrogen in the field and preserved for the first weeks at -80° in a fridge of the IFFF laboratory. When the petri dish arena experiments started, the samples were transferred to a -20°C fridge.

The test samples (T) were taken from the roofed study trees with numbers 2, 3, 4, and 5 and from the control trees K1, K3, K4, K5, K7, K8, K9, K10. As illustrated by figure 2, these trees showed different numbers of attempted attacks (trials), successful and defended attacks in the course of the “attack box” experiments (methods described in Netherer et al., 2015) conducted at the RRP II study site in 2020.

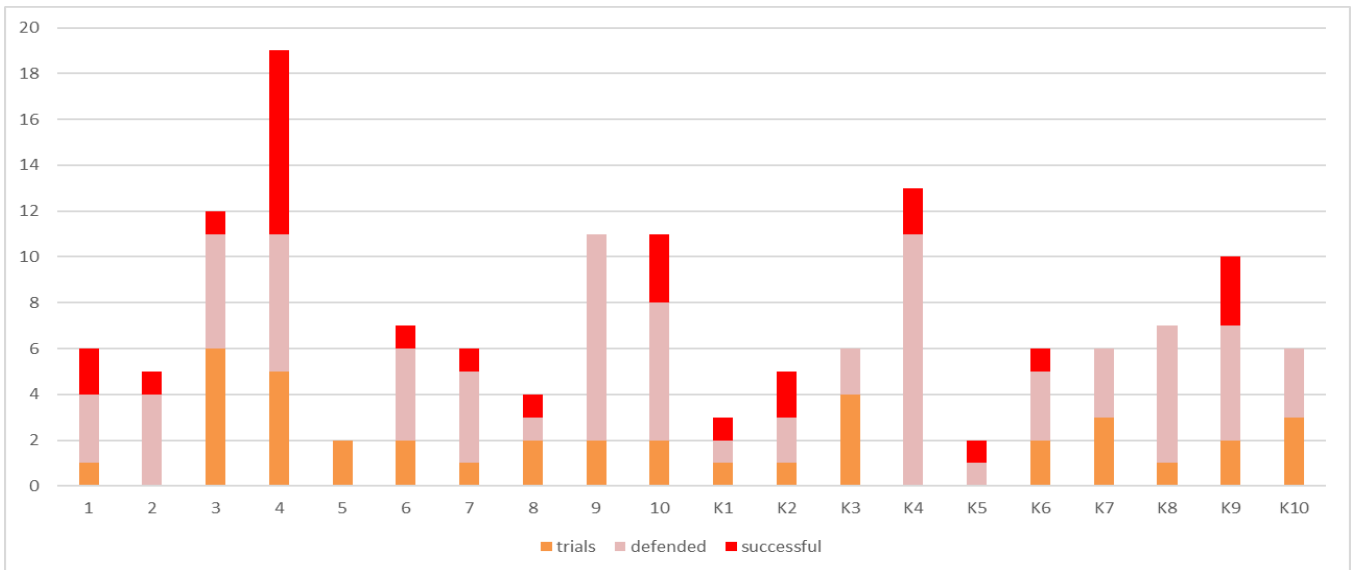
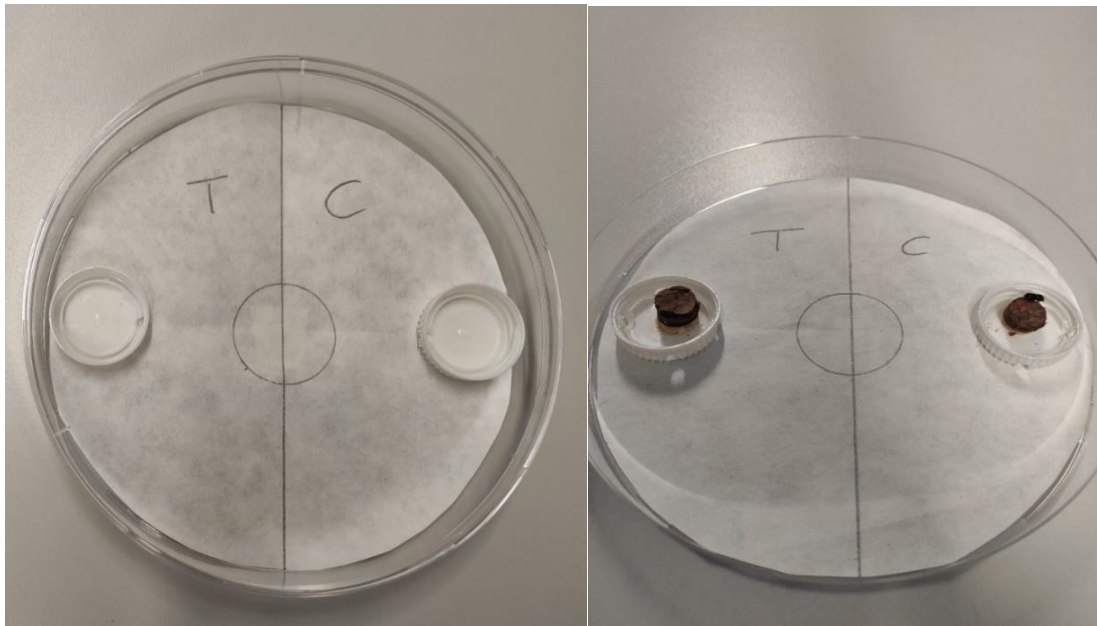


Figure 2: Number of attacks observed at roofed (1-10) and control (K1-K10) trees in the course of attack box experiments performed at RRP II in 2020: trials (beetles just attempted but finally did not attack); defended attacks (beetles were hindered by resin flow from boring); successful attacks.

2.2.1 Experimental set-up

The Petri-dish arenas designed for the experiments were equipped with a white coloured floor of filter paper, to provide homogeneity, reduce distractions and a rough ground for beetles where they could walk on. The space of the Petri dishes was divided into half (T section with study tree samples and C section with control samples originating from extra tree A3 (Picture 4).

In each section of the arena, a white bottle cap was attached, with four entrance holes drilled in. The bark samples were placed inside the caps to test for the preference of spruce bark beetles when deciding between a T and a C sample.



Picture 4: Petri dish arena set-up: empty petri dish (left) and set-up with samples and bark beetle (right)

Ips typographus individuals that had just emerged from breeding galleries were collected from the IFFF institute rearing. The idea was to use only males that had just finished with maturation feeding and were collected right before the experiments in order to avoid their behaviour and final choice to be influenced by stress. The beetles were sexed under a reflected-light microscope based on physical characteristics of males and females. To be certain about their sex, some of the test beetles were dissected after the experiments.

2.2.2 Monitoring procedure

Before the start of each experiment, bark samples (one T sample and one C sample) freshly taken out from the freezer were placed in the Petri-dish arena at room temperature for 10 minutes in order to unfreeze. Afterwards, a mature male of *I. typographus* was placed in the centre of the Petri dish, the dish was closed with a lid, and the timer was started.

The experiment was considered to be terminated when:

- The beetle made a choice by remaining at the cap or on the sample (T or C) for at least 2 minutes
- The beetle made no choice (None) but the maximum duration of the experiment (60 minutes) was reached.
- The beetle was inactive for more than 10 minutes. In this case, the beetle was replaced by a new one, and the experiment started all over again.

For each experiment new bark samples and beetles were used. For the “T versus C” experiments, a minimum number of 10 replicates per T tree were run, but in some cases (as for tree number 2 or K1), there was the need for more replicates. Experiments were repeated in case of inactive beetles, and another set of 10 replicates was done in order to confirm or clarify the results gained in the course of the first round of replicates with specific trees for *I. typographus*.

Results obtained with specific trees (2, 3, K8, K10) showed clear preferences for T or C samples. More Petri-dish arena choice experiments were conducted using the most (3, K8) and the least chosen trees (2, K10) among them. In this series of “T versus T” experiments, both samples originated from the study trees.

In order to test the capability of beetles to perceive olfactory stimuli and to precisely recognize where the source was placed in the Petri dish arena, “zero tests” were conducted. “Zero tests” were run by placing a sample from the A3 tree (C) and leaving the second cap empty (E).

2.2.3 Data analysis

Data analysis of Petri dish arena choice experiment was carried out by summing up the number of choices and calculating proportions per single tree (number and percentage of C, T and None choices, number of experiments stopped due to inactive beetles).

2.3 Relationships between results of the different experiments

To find relationships between particular WRZ size parameters, number of attacks found in the course of the attack box experiments in the field, and the choices made by *I. typographus* for T samples in the petri dish arena experiments, data were compared in scatter plots. Regression lines were drawn in order to check for linear relationships between the datasets.

3. Results

3.1 Hypersensitive wound reaction zones in response to inoculation of ophiostomatoid fungi inoculation experiments

A total number of 200 WRZs were analysed: 80 WRZs established by the RRP II study trees in response to inoculations of EP (40 in roofed trees, 40 in control trees), 80 WRZs developed in response to inoculations of GP (40 in roofed trees, 40 in control trees), and 40 WRZs as tree reaction to pure agar inoculations (K) (20 in roofed trees, 20 in control trees).

Table 1 provides an overview on all WRZ measure results grouped with regard to the size parameter, the inoculated fungal species, and the two different treatments of host trees at the RRP II study site.

Table 1: Summary of WRZ size parameters (mean maximum length, MML, mm; mean maximum width, MMW, mm; mean outer area, MOA, mm; mean inner area, MIA, mm) and standard errors (SE) for the different fungal species (*E. polonica*, EP; *G. penicillata*, GP) and pure agar control (K) and according to treatments (roofed and control trees). MIA were not available (na) for GP and K inoculations.

	Roofed trees							
	MML	± SE	MMW	± SE	MOA	± SE	MIA	± SE
EP	71.51	5.81	13.65	0.54	776.78	92.43	159.98	11.45
GP	217.31	17.87	18.92	0.66	2839.41	268.60	na	na
K	30.55	4.82	9.94	1.11	272.49	54.32	na	na
	Control trees							
	MML	± SE	MMW	± SE	MOA	± SE	MIA	± SE
EP	66.75	4.64	14.71	0.43	698.52	55.76	157.19	13.27
GP	157.86	9.35	18.97	0.69	2063.01	150.06	na	na
K	38.39	3.69	13.46	0.75	382.13	56.68	na	na

The WRZs established by the study trees in response to pure agar controls (K) were much smaller than those established in response to fungal inoculations within both treatments (roofed and control trees). Overall, WRZ size parameters were largest for GP compared to EP and K. The clearest differences in mean WRZ size between roofed and control trees were found for GP with regard to MML and OA. WRZ size parameters are compared in detail in the following sub-sections.

3.1.1 Mean Maximum length of WRZs

Figure 3 shows mean maximum lengths of WRZs established at roofed trees and control trees in response to fungal and agar inoculations. The differences in mean maximum length of WRZs between the two fungal species and the agar control are highly significant within both roofed ($F=54.79$; $p<0.01$) and control ($F=70.09$; $p<0.01$) treatments. While roofed and control trees showed similar WRZ lengths after inoculations of EP and K, a statistically highly significant treatment-related difference was found for GP ($F=8.69$; $p<0.01$). The WRZs in response to GP inoculations were on average 59.45 mm longer at roofed than at control trees. No significant differences were found between roofed trees and control trees related to EP and K inoculations.

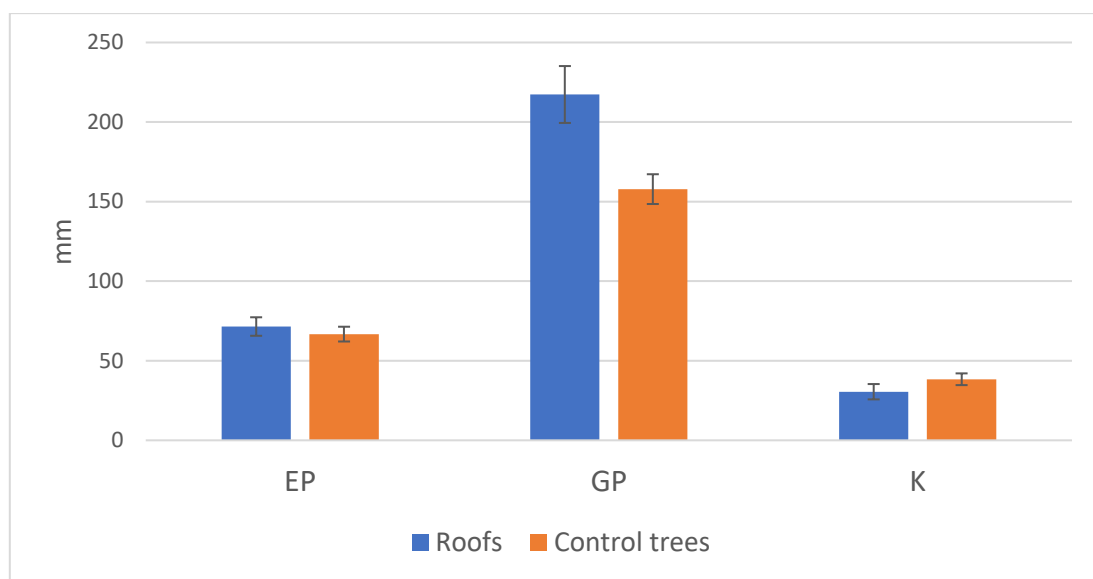


Figure 3: Mean maximum length (mm) \pm standard error of hypersensitive wound reaction zones in response to inoculation of *E. polonica* (EP) and *G. penicillata* (GP) and pure agar controls (K) in trees of the drought stress treatment (roofed trees) and control trees.

3.1.2 Mean Maximum Width of WRZs

Concerning mean maximum width of WRZs established in the study trees in response to fungal inoculations, differences were highly significant between inoculation variants in both treatments (roofs: $F=36.46$; $p<0.01$; controls: $F=21.13$; $p<0.01$) (Figure 4). However, MMW of EP did not differ from K at control trees ($p=0.451$). While there were no treatment-related differences in mean WRZ found for fungal inoculations, MMW related to K differed significantly ($F=6.98$; $p<0.05$) by 3.52 mm between roofed and control trees.

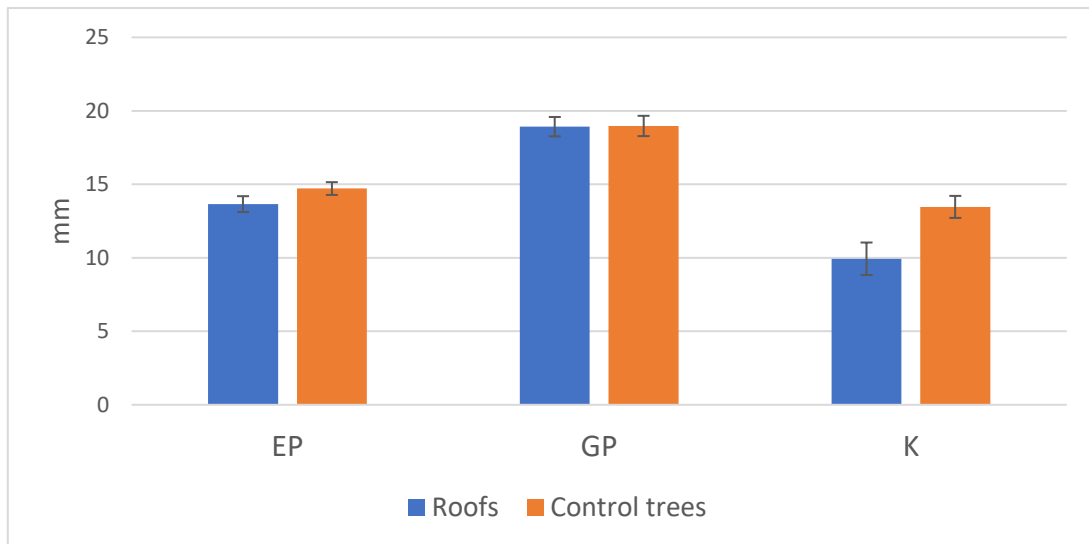


Figure 4: Mean maximum width (mm) of hypersensitive wound reaction zones in response to inoculation of *E. polonica* (EP) and *G. penicillata* (GP) and pure agar controls (K) in trees of the drought stress treatment (roofed trees) and control trees.

3.1.3 Mean Outer Area of WRZs

Figure 5 shows the differences related to mean outer area of WRZs established in response to EP, GP and K inoculations, which were highly significantly different at roofed ($F=46.90$; $p<0.01$) and control trees ($F=62.62$; $p<0.01$). GP inoculations caused significantly largest WRZ areas at roofed trees with a difference in mean value of 776.4 mm^2 compared to control trees ($F=6.37$; $p<0.05$). No clear differences were found for total WRZ size in response to EP and K inoculations according to treatments.

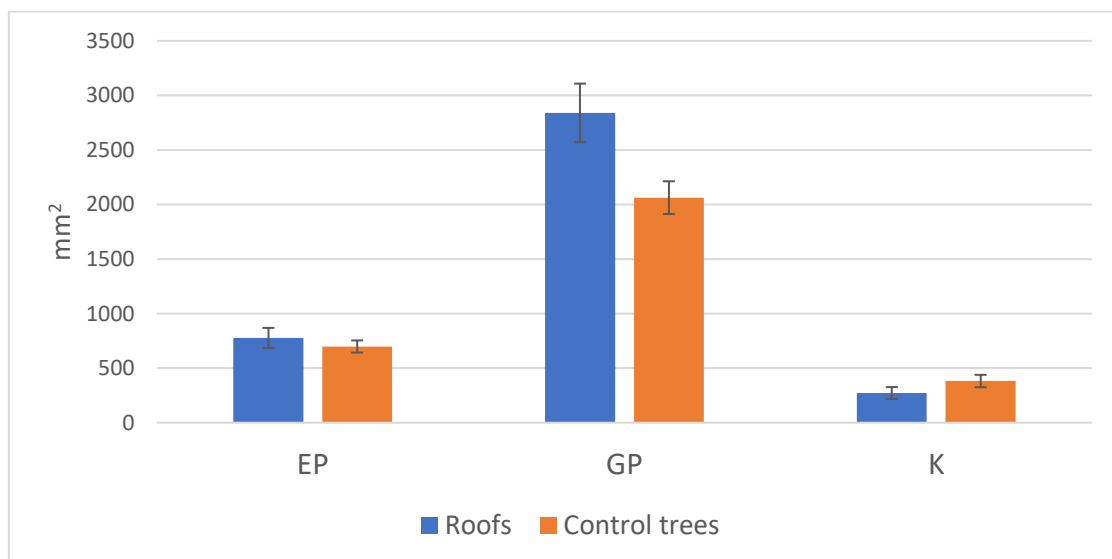


Figure 5: Mean outer area (mm^2) of hypersensitive wound reaction zones in response to inoculation of *E. polonica* (EP) and *G. penicillata* (GP) and pure agar controls (K) in trees of the drought stress treatment (roofed trees) and control trees

3.1.4 Mean inner area of WRZs

The inner, darker area of WRZs was exclusively measurable for EP and not visible in WRZs established after GP and K inoculations. No significant differences were found for this parameter between roofed and control trees, as showed in figure 6.

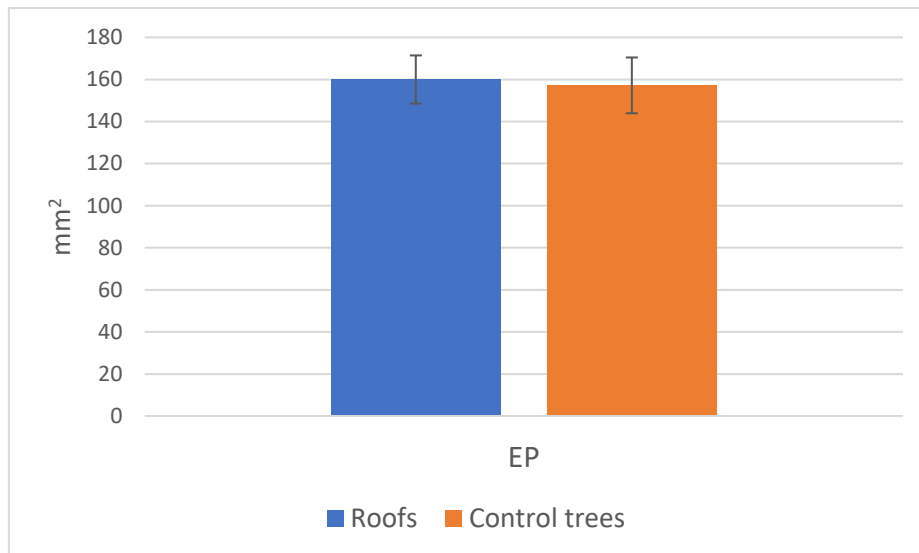


Figure 6: Mean inner area (mm²) of hypersensitive wound reaction zones in response to inoculation of *E. polonica* (EP) in trees of the drought stress treatment (roofed trees) and control trees

3.2 Petri dish arena choice experiments

A total number of 237 arena choice experiments were conducted between October 6th and December 3rd, 2020. As illustrated by table 2, the total number of experiments results from 10 “zero test” runs, 207 “T versus C” tests, and 30 “T versus T” tests. The arena choice experiments were aimed to be performed with solely male *I. typographus*. Yet, in a few cases (6), dissection of gonads after the experiment revealed that the beetles were female.

Table 2: Summary of petri dish arena choice experiments: number of replicates, trees tested, number of males and females according to the different types of tests (“zero test”, “T versus C” and “T versus T” tests).

Choice experiments	no. replicates	trees tested	no. Males	no. Females
zero tests	10	A3	10	0
T versus C	197	2,3,4,5,K1,K3,K4,K5,K7,K8,K9,K10; A3	191	6
T versus T	30	2vs3; 3vsK10; 2vsK8	30	0
In total	237		231	6

3.2.1 “Zero test” results

The “zero tests” were conducted by placing an A3 bark sample in one of the two caps (C) and leaving the other cap empty (E). As shown in figure 7, beetles exclusively preferred the bark sample.

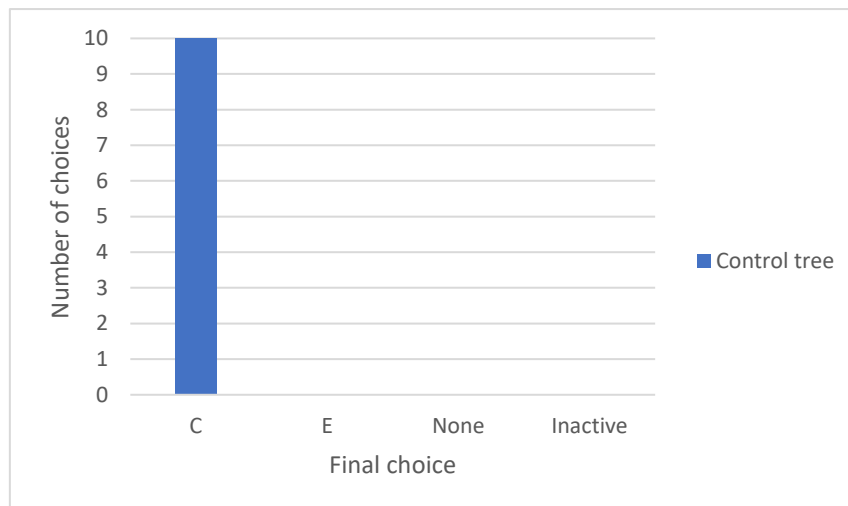


Figure 7: Number of samples (C) chosen by male *I. typographus* when offered a bark core together with an empty cap (E). Number of tests performed =10, experiments performed with 10 males.

3.2.2 Choice experiments using RRP II study tree and extra control tree samples (“T versus C” experiments)

The three different choices possible for spruce bark beetles in the petri dish arena choice experiments were: choice for the test tree (T) sample, the control tree (C) sample, and no choice (None). The choice experiments which were stopped due to inactive beetles are also shown in the figures below, but they were not considered relevant for comparison of tree samples regarding beetle preferences. As extra experiments were performed to replace those with inactive beetles, the total number of replicates for T samples was sometimes more than 10. For the trees number 2, 4, 5, K1, K3, K4, K9, the number of replicates was 20 or more (in case of repeating those experiments stopped due to inactive beetles).

All choices recorded per tree (T) sample are shown in figure 8. Bark samples from study trees number 2, K5, K7 or K10 were rarely chosen by the beetles (high proportions of C choice). Vice versa, in the case of trees number 3 and K8, a clear preference of the beetles towards T samples was examined. A clear preference for the samples of a particular tree was considered in case of at least 7 choices out of 10 for the same tree. No choices (None) were most frequent at tree number

2 samples. Experiments stopped due to inactive beetles were overall rare and only happened with samples of the trees number 2, 3, K3 and K4.

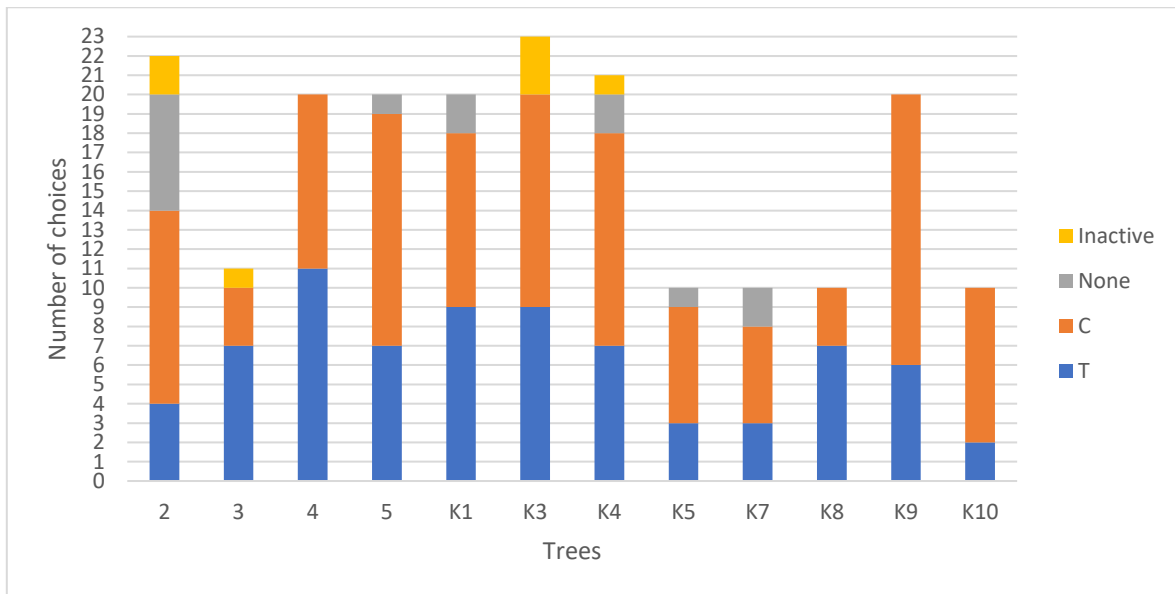


Figure 8: Total number of replicates per tree and related final choices from the “T versus C” experiments

Figure 9 shows in detail the choices of beetles in all “T versus C” tests. A clear choice for T was made with samples from trees number 3 (70%) and K8 (70%) and a trend towards T was investigated for tree number 4 (55%). A clear choice against T was made with samples from trees number 5 and K4 (35%), K5, K7 and K9 (30%), 2 and K10 (20%). A trend for a choice against T was found for the trees K1 and K3 (45%).

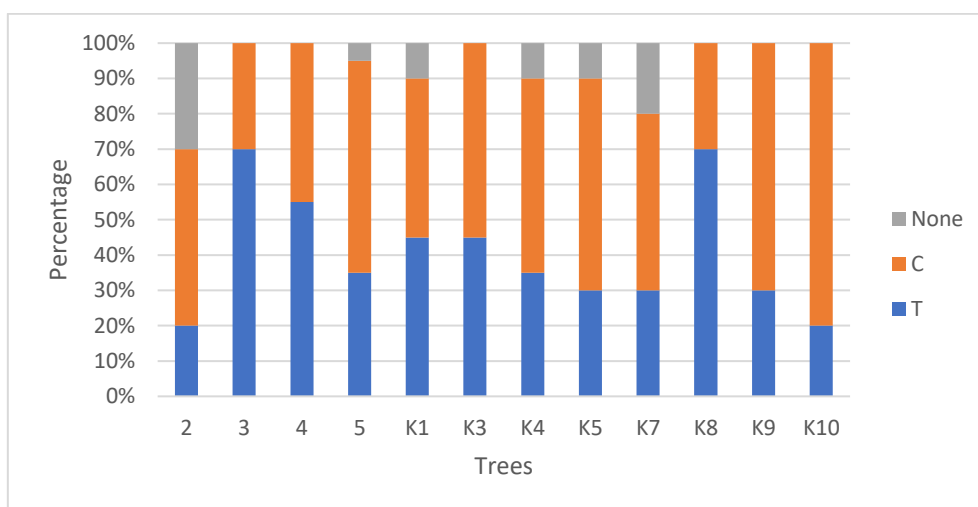


Figure 9: Proportions of choices for T and C and no choice of beetles found in the “T versus C” experiments

It is important to remark that even if Petri-dish arena experiments did not show clear preferences of beetles in relation to roofed or control trees, the C sample used always originated from the same C tree (A3); thus, results must be seen in relation to the obviously high attractiveness of C samples.

3.2.3 Choice experiments comparing particular RRP II study trees (“T versus T” experiments)

Further investigations were conducted by use of samples from particular study trees, comparing the previously most chosen (3, K8) with the least chosen trees (2, K10). The “2 versus K8” experiments (Figure 10) did not repeat the preferences of beetles as found previously.

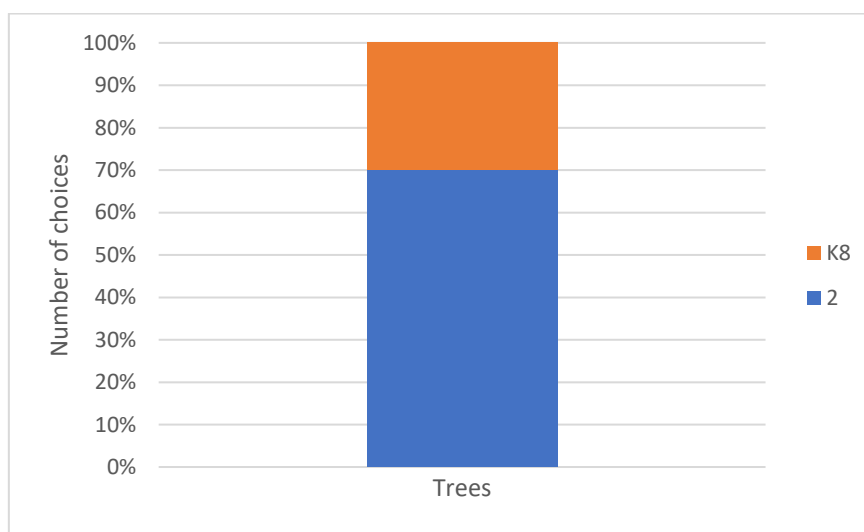


Figure 10: Results of the “2 vs K8” experiments, in which the most chosen was tree number 2

While during the “T versus C” arena choice experiments, no T choice was made by beetles for samples from tree number 2 in the previous 10 experiments), they were chosen 7 times when offered together with samples from K8. On the contrary, samples of the K8 tree, which were chosen 7 out of 10 times during in “T versus C” tests, were less attractive for the beetles in the “2 versus K8” experiments.

The “3 versus K10” experiments (Figure 11) mostly confirmed what had been found during the “T versus C” arena choice experiments: samples from tree number 3, which were the previously most chosen ones (7 out of 10 choices) were found again to be more attractive for the beetles (6 out of 10 choices) when compared with bark cores of tree number K10.

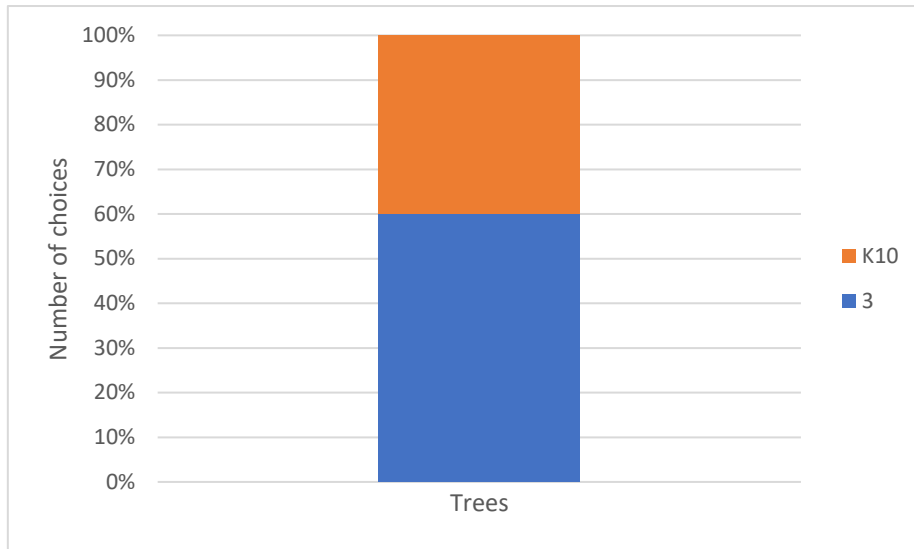


Figure 11: Results of the “3 vs K10” experiments, in which there is no a clear choice confirming the results of the “T vs C” experiments

Finally, further 10 tests with the two most chosen trees from the above described experiments were run (“2 versus 3”). A clear preference was found for the samples taken from tree number 3, which was chosen 8 times out of 10 replicates (Figure 12).

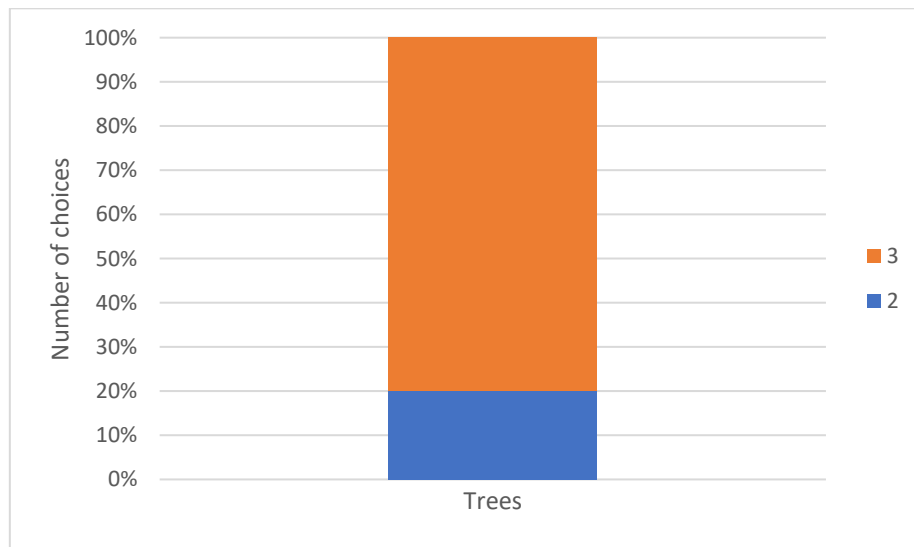


Figure 12: Results of the “2 vs 3” experiments, in which the most chosen has been tree n. 3

3.3 Relationships between WRZ size parameters, choices for tree samples found in arena experiments and number of attacks investigated in the course of attack box experiments in the field

Mean WRZ lengths measured per study tree in response to GP inoculation and the total number of successful and defended attacks found at each tree in the course of attack box experiments at RRP II study site were compared (Figure 13). Although for many trees no clear relationship is visible between these two parameters (left part of point cloud in the figure), there is a tendency for an increasing number of attacks with increasing lesion lengths for about 50% of the study trees (right part of point cloud). Most trees in this group belong to the collective of roofed trees (1, 3, 4, 5, 6, 8, 10). It is important to note that the attack box experiments were repeatedly performed from May to September 2020, while fungal inoculations at the study trees were done in early August. Attack box experiments were conducted at stem sections above the inoculations.

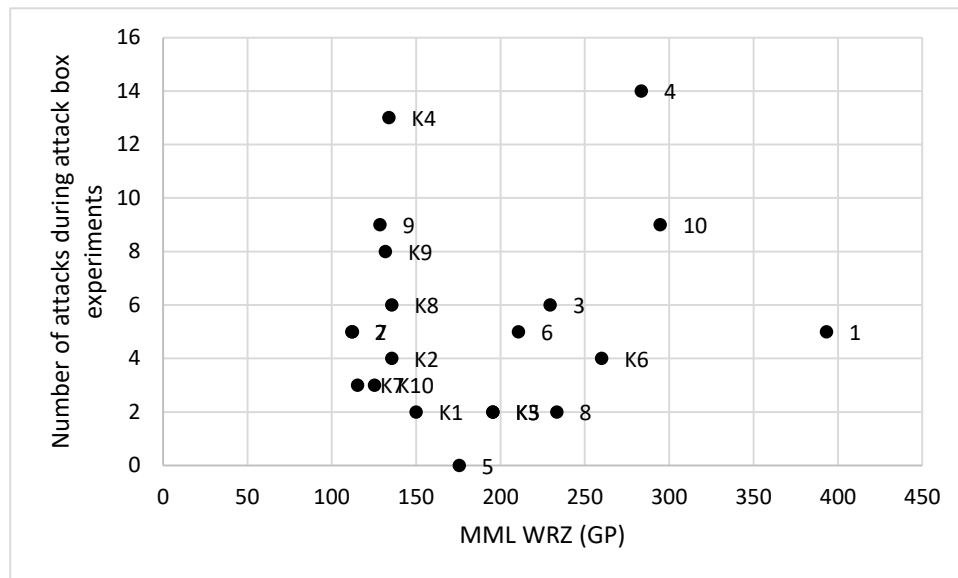


Figure 13: Relationship between number of defended and successful attacks during attack box experiments and mean maximum lengths of hypersensitive wound reaction zones in response to GP inoculations (MML WRZ GP).

The mean outer areas of WRZs measured at the study trees in response to GP inoculation and the proportion of T choices found in the petri dish arena experiments for samples of each study tree were compared (Figure 14). In the “T versus C” experiments, beetles showed a tendency for preferring those trees from an extra control tree (A3) that established larger hypersensitive wound reaction zones.

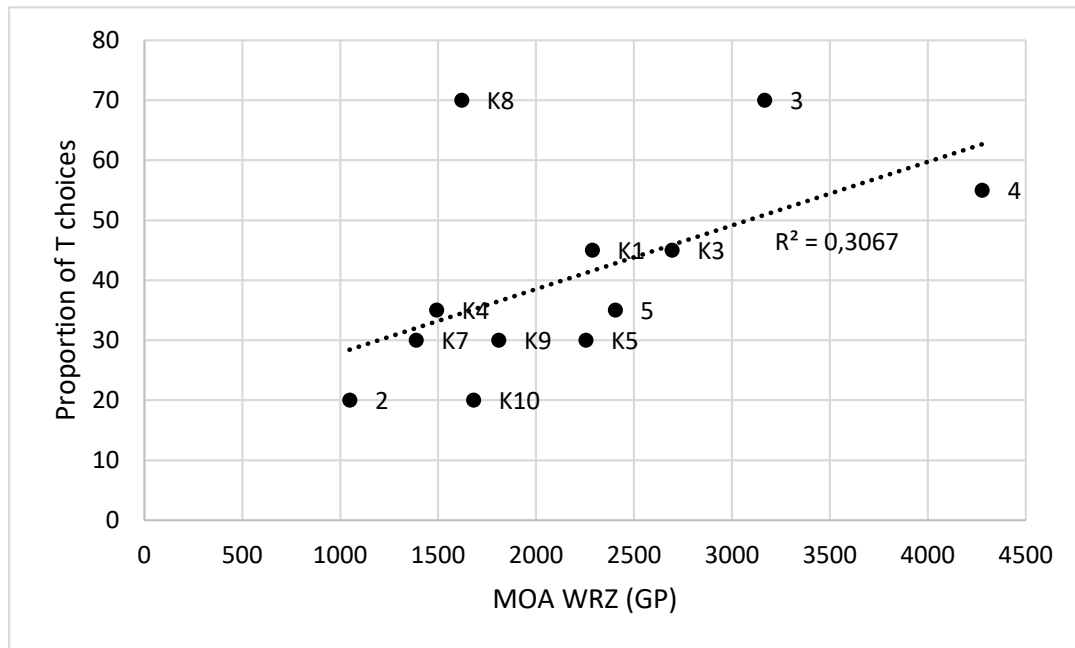


Figure 14: Relationship between the proportion of T choices in petri dish “T versus C” experiments and total (outer) areas of hypersensitive wound reaction zones in response to GP inoculations (MOA WRZ GP).

The relationship between successful, defended and attempted attacks of beetles found at the study trees in the course of attack box experiments and the proportion of choices made for each tree in the laboratory arena experiments are illustrated in figure 15. There is a slight trend for a positive linear relationship between field attacks and choices in the laboratory, but overall, the relationship is not very clear, probably due to the low number of investigated trees.

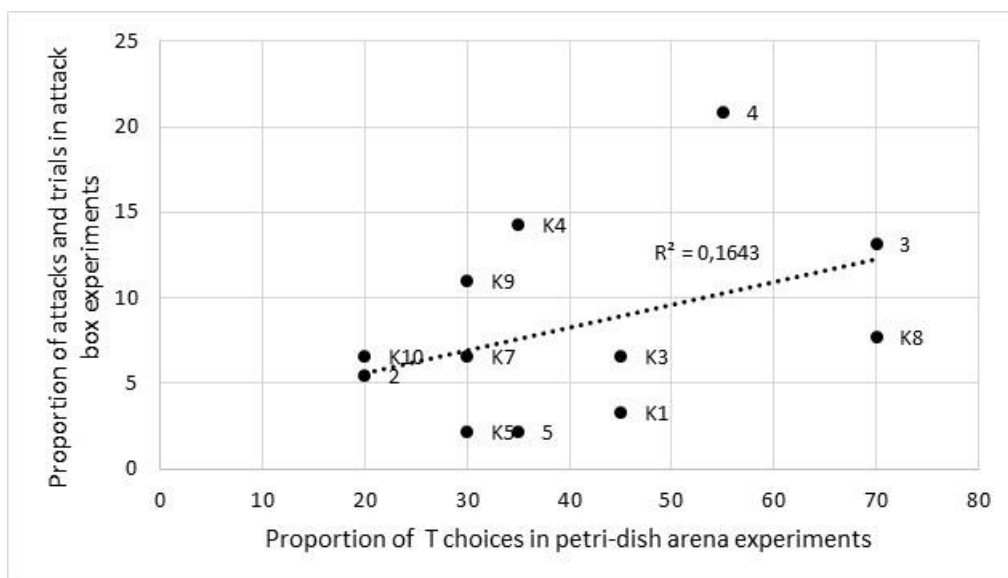


Figure 15: Relation between proportion of attacks and trials attacks in attack box experiment and proportion of T choices in Petri dish arena experiment

4. Discussion

The results obtained in this thesis work showed, for the artificially drought stressed (roofed) study trees of the Rosalia Roof Project (RRP), longer and larger hypersensitive wound reaction zones (WRZs) in response to fungal inoculations and thus, higher susceptibility to the ophiostomatoid fungus *G. penicillata* (GP). All measured WRZ size parameters were significantly smaller for *E. polonica* (EP), which turned out to be less virulent compared to *G. penicillata* in this experiment.

Petri-dish arena choice experiments showed (through the “zero test”) how beetles are able to recognize a precise source of volatile compounds. The “T vs C” experiments showed particular preferences towards specific trees (i.e. trees number 3, K8) while other trees resulted not particularly interesting for them (trees number 2 and K10). Differences in treatments of the trees (roofs, control) did not create a clear difference in terms of choices. Among the control treatment, just tree number K8 got more than 50% of choices, for the roof treatment this was true for trees number 3 and 4.

The extent of WRZs and the Petri dish arena choice experiment results showed a trend towards a positive relationship: large wound reaction zones in response to GP inoculation coincided with higher number of choices of bark core samples from respective trees, pointing to a preference of beetles for trees with reduced defense capability.

4.1 Hypersensitive wound reaction zones (WRZs) in response to fungal inoculations

Clear differences in size parameters of WRZs were found between the different fungal species and agar control inoculations. In particular, the largest and longest WRZs were those developed in response to GP inoculations, similar to the results gained by Schmieder (2017) from his inoculation experiments conducted in a Lower Austrian Norway spruce stand. Schmieder (2017) inoculated three different ophiostomatoid fungal species (*E. polonica*, *G. penicillata*, and *Leptographium* spp.), whereby GP was the one which established the longest WRZs.

In general, the spread of fungi within host tree bark and wood is easier along its fibres than across them perpendicularly (Repe et al., 2015). This explains why, among all WRZ size parameters considered, average lesion lengths were much higher than lesion widths. Moreover, longer WRZs demonstrate that trees are more vulnerable to pathogen colonization or that the pathogen is particularly virulent, while shorter WRZs show that trees can faster resist to pathogen attacks or that the pathogen is less virulent (Christiansen et al., 1987).

The difference in fungus aggressiveness might be addressed to the time at which GP and EP sampling occurred: *E. polonica* can better develop where micro-climatic conditions are cool, moist and in oxygen-limited conditions, while *G. penicillata* is better adapted to drier conditions. As Solheim (1991) found, resin quality and oxygen-deficient conditions are among the discriminants that can enhance or slow down the capability of fungi to reproduce and to overcome trees' defences. Sampling of fungi strains used in this thesis work occurred on 09.07.2020 in a predominantly dry period, which could have favoured the growth and development of *G. penicillata* more than *E. polonica*.

According to expectations, the lengths and total (outer) areas of WRZs established around pure agar inoculations were significantly smaller than those in response to GP inoculations, this because agar controls were nothing else than small wounds without fungus. Interestingly, EP and K inoculations did not differ particularly significant in WRZs size, which again points to the fact that the use EP strain was not very virulent.

Treatment of the study trees at the RRP II site (roofs, controls) significantly affected tree reaction on fungal inoculations, but only for the most virulent fungus GP and regarding maximum length and outer area. In previous inoculation experiments conducted by Netherer et al. (2016) at the RRP I study site, the largest WRZs were as well established by roofed trees, in response to *E. polonica* growth. These trees had been fully covered for two years and were therefore facing physiological stress. The fungal infections in this earlier study developed bigger necrotic areas compared to the agar control inoculations (Netherer et al., 2016). This is partly in accordance with what was found for the WZR studied in this master thesis work, where not all WRZ size parameters related to EP differed significantly from the control. Comparing tree responses to EP inoculations between the former and present study, average WRZ area turned out to be bigger (1069 mm²) in the RRP I study than in this thesis work (776 mm²). Such variations can be due to many variables, such as virulence of the used fungal strains, weather parameters or tree resistance involving the trees' defences induction following attack attempts in the past.

Apart from physiological status, defence reactions of trees depend on genotype. Axelsson et al. (2020) found in their inoculation experiments with *Heterobasidion parviporum* and *E. polonica* that wound reaction zones' length varied in a consistent way between the different Norway spruce genotypes, showing how much the inner features of the single trees can influence success or failure of pathogen colonization. Different genotypes mean also different level of adaptation to

the microclimatic conditions of the site. We must keep in mind that the Norway spruce genotypes used for extensive plantation after WWII, are now facing severe climatic changes and, probably, not all of them are able to adapt to the ongoing scenario to the same extent. Their defensive failure can also be seen as a natural selection process of those genotypes not able to face changes related to new climatic conditions and biotic threats.

4.2 Petri dish arena choice experiments

Ips typographus is capable and quick in recognizing its host tree Norway spruce as clearly shown in the “zero test” experiments, where all beetles preferred the tree sample from the empty space. As hypothesized for the Petri-dish arena choice experiments, beetles track the source of volatile organic compounds such as monoterpenes emitted from bark cores of its host tree and are also able to avoid non-host plants. Andersson et al. (2009) in their study concerning the responses of *I. typographus* towards ecologically relevant odours coming from host and non-host plants, found that among the strongly responding neurons, 106 are olfactory receptor neurons addressed to pheromone and host-volatile recognition; in particular, 37 (40%) of them are involved in the recognition of host volatiles and 24 (25%) are involved in non-host volatiles recognition, demonstrating the large portion of the olfactory system devoted to the avoidance of non-host trees (Andersson et al., 2009).

Petri-dish arena choice experiments were previously conducted by Kandasamy et al. (2019) where immature adult specimens of *I. typographus* could choose between substrates colonized by different associated fungi in which to create feeding galleries and substrate with absence of fungi. Results clearly showed that the fungi-colonized substrates were preferred over the uncolonised substrates. These choices are in accordance with what was found in this master thesis work, namely that bark beetles are able to detect and recognize suitable host tissue for colonization.

In both experiments, only males were chosen in order to guarantee the naturalness of their instinctive behaviour. Spruce bark beetle males are the “pioneers” who are the first to search for suitable hosts. Once they have landed, they establish nuptial chambers and emit aggregation pheromones

to attract conspecific males and females (Wermelinger, 2004). During this Petri-dish arena choice experiments, some females were accidentally used (6), detected afterwards by dissection of gonads. Yet, their behaviour and final choices of samples seemed to be similar to the males' choices, despite their different biological roles in a natural forest environment.

A different experimental set-up for testing behavioural responses of beetles are Y-tube olfactometers. They consist of a release chamber, a Y-tube, a beetle trap and tubes containing the source of the stimulus inside a filter paper. This set-up was used by Blazyte-Cereskiene et al. (2016) to test behavioural response of *I. typographus* to volatile compounds emitted by Norway spruce. In particular, they focused on trans-4-thujanol, a bioactive compound contained in the bark, the concentration of which varies with tree age (the younger the tree, the higher levels of trans-4-thujanol in the bark). According to the authors, this compound plays a fundamental role in bark beetles' tree choices and thus, trees' attractiveness. The samples used in the experiments all originated from mature, i.e. more attractive trees. However, information on concentrations and blends of terpenoid substances in the bark of the studied trees was not available in the present study, as analyses are still ongoing.

It must be taken into consideration that laboratory experiments such as Y-tube olfactometer or the arena choice tests are far from natural conditions in the forest. First, the beetles used in this experiment originated from the university rearing and not from a natural population in the forest, and had thus, been grown in an artificially controlled room environment. This means that the broods developed under artificial light and artificial climatic conditions, so that they missed the real experience of being surrounded by different odours typical for forest environments and presumably playing a role in the development of their natural behaviour in choosing the target plants.

During Petri-dish arena choice experiments, instead of recognizing and approaching a tree through its bark, beetles interacted with a defrosted cork sample that somehow could have confused them despite the by-instinct-already-known volatile compounds. The source of odours they found was something new and unexpected. Moreover, performing experiments with living organisms involves random elements such as natural instincts and personal behaviour of the beetles as well as effects of external factors (light, temperature, season) on the beetles' final choice. The final choice of beetles may be influenced by the sudden change of environment from the rearing room to the laboratory and finally in the Petri-dish arena. Although homogeneous light conditions were ensured, a few beetles seemed to be disturbed, especially over noon, trying to hide in a darker place.

Petri dish arena choice experiments did not show a clear trend according to different treatments (roofed and control trees) when compared with an extra, well water supplied control tree. The

choices of beetles seem to show a quite random distribution among trees and were also quite balanced regarding the total amount of preferences for T or C. With 75 T and 101 C choices, the extra control tree was even more attractive for the beetles than bark cores originating from the study trees. As already mentioned, trees release volatiles that can be specifically recognized by bark beetles; some of them result attractive, some others act as repellent. Usually, trees that faced attacks in the past without being colonized, as consequence, tend to synthesize specific compounds as 1,8-cineole, which acts as aggregation pheromone inhibitor, repelling bark beetles' attacks (Blazyte-Cereskiene et al., 2016). This could explain why, despite the hydrological deficit, tested trees were not the most chosen: the box attacks experiment in the past, likely enhanced their resistance toward future attacks.

4.3 Relationships between tree responses to fungal inoculations and results of Petri dish arena and attack box experiments

Comparing the results of the different experiments performed (fungal inoculations, and petri dish arena choice experiments) and those of the attack box experiments in the field, some interesting relationships came to light. The roofed trees number 3 and 4 stood out in all experiments conducted: they showed higher-than-average WRZ establishment, they both were preferred (> 50% of preferences) in the petri dish arena choice experiments, and the sum of attempted, defended and successful attacks found in the course of the box attack experiments was much higher than for the other trees on average (in particular, tree number 4 registered the highest number; compare figures 14 and 15).

Trees like number 2, K7, K10, on the contrary, lie on the opposite end of the scale: they all established lower-than-average WRZ areas, they were chosen by bark beetles by less than 35% in the petri dish arena and, in the box attack experiments, these trees were rarely attacked by *I. typographus*. A particular case of resistance was observed with tree number K4, which defended by resin flow 11 of 13 attacks in the field, showed comparably smallest WRZs (i.e. was able to stop the inoculated fungi fast), and was rarely chosen (35% of choices) in the petri dish arena choice experiments.

These exemplary results are representative for the observed trends of beetle preferences for samples originating from trees which established larger lesions in response to GP inoculation and which were already attacked to a larger extent in the field. The study trees subjected to drought

treatments (except for tree number 2), were clearly more susceptible to fungal attacks while the control trees with sufficient water supply could better defend biotic invasion.

The observed trends may be as well attributed to the time scale in which the different experiments were conducted: the study trees had the time to adapt to the subjected treatments in the field, involving reduced water supply due to roof establishment, the local inoculation of ophiostomatoid fungi, and bark beetle infestation in the course of the attack box experiments. The bark cores used in the Petri-dish arena choice experiments were sampled after the field experiments had been terminated and thus, although these experiments were performed locally on the stem, trees already had the time to synthesize and to store defensive compounds as possible systemic reaction of previous invasion attempts due to fungal inoculations and bark beetle attacks.

4.4 Conclusions and outlook

Results showed how the lack of adequate water supply can lead trees to an increasing level of difficulty in stopping harmful external attacks. On the other hand, the outcomes of this study seem to emphasise the hypothesis that moderate levels of stress can trigger tree resistance, for instance by the synthesis and the storage of defensive compounds “predisposing” trees for incoming further attacks. More research needs to be addressed to this question, using a bigger sample of trees on which fungal inoculations, attack box experiments and Petri-dish choice arena tests can systematically be performed over a longer time span. Such comprehensive study approach will help to better appreciate possible changes in trees’ responses and physiology during different years and, eventually, to realize at which point external attacks finally overcome trees’ defences.

5. References

- Aarrestad, P.A., Myking, T., Stabbetorp, O.E., Tollefsrud, M.M. (2014). Foreign Norway spruce (*Picea abies*) provenances in Norway and effects on biodiversity. Norwegian Institute for Nature Research. *NINA Report 1075*, 39 pp.
- Andersson, M. N., Larsson, M. C., & Schlyter, F. (2009). Specificity and redundancy in the olfactory system of the bark beetle *Ips typographus*: single-cell responses to ecologically relevant odors. *Journal of Insect Physiology*, *55*, 556-567.
- Axelsson, K., Zendegi-Shiraz, A., Swedjemark, G., Borg-Karlson, A. K., Zhao, T. (2020). Chemical defence responses of Norway spruce to two fungal pathogens. *Forest Pathology*, *50*, e12640.
- Baier, P., Pennerstorfer, J., Schopf, A. (2007). PHENIPS—a comprehensive phenology model of *Ips typographus* (L.) (Col., Scolytinae) as a tool for hazard rating of bark beetle infestation. *Forest Ecology and Management*, *249*, 171-186.
- Blažytė-Čereškienė, L., Apšegaitė, V., Radžiūtė, S., Mozūraitis, R., Būda, V., & Pečiulytė, D. (2016). Electrophysiological and behavioural responses of *Ips typographus* (L.) to trans-4-thujanol—a host tree volatile compound. *Annals of Forest Science*, *73*, 247-256.
- Christiansen, E., Waring, R. H., Berryman, A. A. (1987). Resistance of conifers to bark beetle attack: searching for general relationships. *Forest Ecology and Management*, *22*, 89-106.
- Faccoli, M. (2009). Effect of weather on *Ips typographus* (Coleoptera Curculionidae) phenology, voltinism, and associated spruce mortality in the southeastern Alps. *Environmental Entomology*, *38*, 307-316.
- Franceschi, V. R., Krokene, P., Christiansen, E., Krekling, T. (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist*, *167*, 353-376.
- Gellini, R., Grossoni, P. (1996). Botanica forestale. 1. Gimnosperme. CEDAM-Casa Ed. Milani (71-82) 267 pp.
- Hicke, J. A., Logan, J. A., Powell, J., Ojima, D. S. (2006). Changing temperatures influence suitability for modeled mountain pine beetle (*Dendroctonus ponderosae*) outbreaks in the western United States. *Journal of Geophysical Research: Biogeosciences*, *111*(G2).

Huang, J., Kautz, M., Trowbridge, A. M., Hammerbacher, A., Raffa, K. F., Adams, H. D., Goodsman, D. W., Xu, C., Meddens, A. J., Kandasamy, D., Gershenson, J., Seidl, R., Hartmann, H. (2020). Tree defence and bark beetles in a drying world: carbon partitioning, functioning and modelling. *New Phytologist*, 225, 26-36.

Holderness, M. (1996). Quarantine pests for Europe. Edited by McNamara, D., Smith, I., Scott, P., CAB International, 2nd edition. 1440 pp.

Kalinová, B., Brizova, R., Knizek, M., Turcani, M., Hoskovec, M. (2014). Volatiles from spruce trap-trees detected by *Ips typographus* bark beetles: chemical and electrophysiological analyses. *Arthropod Plant Interactions* 8, 305-316.

Kandasamy, D., Gershenson, J., Andersson, M. N., Hammerbacher, A. (2019). Volatile organic compounds influence the interaction of the Eurasian spruce bark beetle (*Ips typographus*) with its fungal symbionts. *The ISME Journal*, 13, 1788-1800.

Kautz, M., Dworschak, K., Gruppe, A., Schopf, R., 2011. Quantifying spatio-temporal dispersion of bark beetle infestations in epidemic and non-epidemic conditions. *Forest Ecology and Management* 262, 598-608.

Kirisits T. (2007). Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: Lieutier F., Day K.R., Battisti A., Grégoire J.C., Evans H.F. (eds) Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis. Springer, Dordrecht.

Kirisits, T., Dämpfle, L., Kräutler, K. (2013) *Hymenoscyphus albidus* is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media, *Forest Pathology* 43, 386-389.

Krokene, P. (2015). Conifer defense and resistance to bark beetles. In: Bark beetles. Biology and ecology of native and invasive species. Vega, F. E., Hofstetter, R. W. (eds.). Academic Press, Elsevier.

Krokene, P., Christiansen, E., Solheim, H., Franceschi, V. R., Berryman, A. A. (1999). Induced resistance to pathogenic fungi in Norway spruce. *Plant Physiology*, 121, 565-570.

Lieutier, F., Yart, A., Salle, A. (2009). Stimulation of tree defenses by ophiostomatoid fungi can explain attack success of bark beetles on conifers. *Annals of Forest Science*, 66, 801-801.

Lopatka, J. (2019, April 26) Climate change to blame as bark beetles ravage central Europe's forests. *Reuters*, retrieved from <https://www.reuters.com/article/us-centraleurope-environment-barkbeetle/climate-change-to-blame-as-bark-beetles-ravage-central-europes-forests-idUSKCN1S21LA>

Marini, L., Økland, B., Jönsson, A. M., Bentz, B., Carroll, A., Forster, B., Gregoire, J., Hurling, R., Nageleisen, M. L., Netherer, S., Weed, A., Schroeder, M., Ravn, H. P. (2017). Climate drivers of bark beetle outbreak dynamics in Norway spruce forests. *Ecography*, 40, 1426-1435.

Müller, J., Bußler, H., Goßner, M., Rettelbach, T., & Duelli, P. (2008). The European spruce bark beetle *Ips typographus* in a national park: from pest to keystone species. *Biodiversity and Conservation*, 17, 2979-3001.

Netherer, S., Nopp-Mayr, U. (2005). Predisposition assessment systems (PAS) as supportive tools in forest management—rating of site and stand-related hazards of bark beetle infestation in the High Tatra Mountains as an example for system application and verification. *Forest Ecology and Management*, 207, 99-107.

Netherer, S., Ehn, M., Blackwell, E., Kirisits, T. (2016). Defence reactions of mature Norway spruce (*Picea abies*) before and after inoculation of the blue-stain fungus *Endoconidiophora polonica* in a drought stress experiment. *Central European Forestry Journal*, 62, 169-177.

Netherer, S., Matthews, B., Katzensteiner, K., Blackwell, E., Henschke, P., Hietz, P., Pennerstorfer, J., Rosner, S., Kikuta, S., Schume, H., Schopf, A. (2015). Do water-limiting conditions predispose Norway spruce to bark beetle attack? *New Phytologist*, 205, 1128-1141.

Netherer, S., Panassiti, B., Pennerstorfer, J., Matthews, B. (2019). Acute drought is an important driver of bark beetle infestation in Austrian Norway spruce stands. *Frontiers in Forests and Global Change*, 2, 39.

Raffa, K. F., Gregoire, J. C., Lindgren, B. S. (2015). Natural history and ecology of bark beetles. In: *Bark beetles. Biology and ecology of native and invasive species*. Academic Press. Elsevier.

- Repe, A., Bojović, S., Jurc, M. (2015). Pathogenicity of ophiostomatoid fungi on *Picea abies* in Slovenia. *Forest Pathology*, 45, 290-297.
- Seidl, R., Baier, P., Rammer, W., Schopf, A., Lexer, M. J. (2007). Modelling tree mortality by bark beetle infestation in Norway spruce forests. *Ecological Modelling*, 206, 383-399.
- Seidl, R., Schelhaas, M. J., Lexer, M. J. (2011). Unraveling the drivers of intensifying forest disturbance regimes in Europe. *Global Change Biology*, 17, 2842-2852.
- Schmieder, B. (2017). Konstitutive Abwehr und induzierte Wundreaktion von Fichten nach Blattwespenbefall im Revier Heubach (Waldviertel). Master thesis, University of Natural Resources and Life Sciences, Vienna. 93 pp.
- Solheim H. (1991): Oxygen deficiency and spruce resin inhibition of growth of blue stain fungi associated with *Ips typographus*. *Mycological Research* 95, 1387-1392
- Wadke, N., Kandasamy, D., Vogel, H. Lah, V., Wingfield B. D., Paetz, C., Wright, L. P., Gershenzon, J., Hammerbacher, A. (2016). The bark-beetle-associated fungus, *Endoconidiophora polonica*, utilizes the phenolic defense compounds of its host as a carbon source. *Plant Physiology* 171, 914-931.
- Wermelinger, B. (2004). Ecology and management of the spruce bark beetle *Ips typographus*—a review of recent research. *Forest ecology and management*, 202, 67-82.
- Wermelinger, B., Seifert, M. (1998). Analysis of the temperature dependent development of the spruce bark beetle *Ips typographus* (L.) (Col., Scolytidae). *Journal of Applied Entomology* 122, 185-191.
- Wermelinger, B., Seifert, M. (1999). Temperature-dependent reproduction of the spruce bark beetle *Ips typographus*, and analysis of the potential population growth. *Ecological Entomology* 24, 103-110.
- Willmer, C., Fricker, M. (1996). *Stomata* (Vol. 2). Springer Science & Business Media. 375 pp.