Eesti Maaülikooli doktoritööd

Doctoral Theses of the
Estonian University of Life Sciences

Eesti Maaülikool
Estonian University of Life Sciences

INTERACTION BETWEEN LARGE PINE WEEVIL (*HYLOBIUS ABIETIS* L.), PATHOGENIC AND SAPROTROPHIC FUNGI AND VIRUSES

HARILIKU MÄNNIKÄRSAKA (*HYLOBIUS ABIETIS* L.) SEOS PATOGEENSETE JA SAPROTROOFSETE SEENTE NING VIIRUSTEGA

TIIA DRENKHAN

A Thesis
for applying for the degree of Doctor of Philosophy in Forestry

Väitekiri
filosoofiadoktori kraadi taotlemiseks metsanduse erialal

Tartu 2017

Institute of Forestry and Rural Engineering
Estonian University of Life Sciences

According to verdict No 6-14/2-2 of 17 April 2017, the Defence Board of PhD theses in Forestry of the Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor of Philosophy in Forestry.

Opponent: **Research Professor Isabella Børja, PhD**
Biotechnology and Plant Health
Norwegian Institute of Bioeconomy Research

Supervisors: **Assoc. Prof. Kaljo Voolma, PhD** (Entomology)
Institute of Forestry and Rural Engineering
Estonian University of Life Sciences

Assoc. Prof. Ivar Sibul, Doctor of Science (Forestry)
Institute of Forestry and Rural Engineering
Estonian University of Life Sciences

Assoc. Prof. Risto Kasanen, Doctor of Science
(Forestry)
Department of Forest Sciences
University of Helsinki

Defence of the thesis:
Estonian University of Life Sciences, room 2A1, Kreutzwaldi 5, Tartu, on 19 June, 2017, at 10:00.

The English language was edited by Dr. Ingrid H. Williams and Estonian by Mrs. Urve Ansip.

Publication of this dissertation is supported by the Estonian University of Life Sciences

© Tiia Drenkhan, 2017
ISSN 2382-7076
ISBN 978-9949-569-81-6 (trükis)
ISBN 978-9949-569-82-3 (pdf)

CONTENTS

LIST OF ORIGINAL PUBLICATIONS 7
ABBREVIATIONS. 8
1. INTRODUCTION 9
2. REVIEW OF THE LITERATURE 12
2.1 *Hylobius* spp. 12
2.2 The biology of large pine weevil. 13
2.3 Feeding behaviour of *Hylobius abietis*. 15
2.4 Control of large pine weevil. 16
2.5 Species complex *Heterobasidion annosum sensu lato* 17
2.6 Infection cycle of *H. annosum s.l.*. 18
2.7 Viruses in *H. annosum s.l.* 19
2.8 *Diplodia sapinea* (Fr.) Fuckel 20
2.9 *Phlebiopsis gigantea* (Fr.) Jülich. 21
2.10 Mycoviruses. 23
2.11 Partitiviridae 25
3. AIMS OF THE STUDY. 26
4. MATERIALS AND METHODS 27
4.1 Sample collection 27
4.2 Fungal isolates 27
4.3 Laboratory feeding experiment 28
4.4 Genotype determination (I, II) 30
4.5 Counting of oidial spores (II) 31
4.6 Isolation of hyphal tips and single oidial spores (II) 31
4.7 Growth rate measurements and statistical testing (II). 32
4.8 Virus detection by RT-PCR. 32
4.9 DNA extraction and fungal species identification (III). 34
5. RESULTS 35
5.1 The laboratory feeding experiment (I–III) 35
5.2 Occurrence of viruses from insect faeces (I–II). 36
5.3 CF11 chromatography (I) 37
5.4 Growth rate measurements and oidial counting (II). 37

5.5 Field collected insects (III) 38
6. DISCUSSION 39
6.1 Viruses in pathogenic and saprotrophic fungi 39
6.2 Viruses and fungi transmitted by insects 40
6.3 Insects as vectors of fungi. 42
7. CONCLUSIONS 44
REFERENCES 45
SUMMARY IN ESTONIAN 66
ACKNOWLEDGEMENTS 78
ORIGINAL PUBLICATIONS 81
CURRICULUM VITAE. 113
ELULOOKIRJELDUS 117
LIST OF PUBLICATIONS 121

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which are referred to in text by their Roman numerals.

I **Drenkhan, T.**, Sibul, I., Kasanen, R., Vainio, E.J. 2013. Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. Forest Pathology, 43: 317–323.

II **Drenkhan, T**., Kasanen, R., Vainio, E.J. 2016. *Phlebiopsis gigantea* and associated viruses survive passing through the digestive tract of *Hylobius abietis*. Biocontrol Science and Technology, 26: 320–330.

III **Drenkhan, T.**, Voolma, K., Adamson, K., Sibul, I., Drenkhan, R. 2017. The large pine weevil *Hylobius abietis* (L.) as a potential vector of the pathogenic fungus *Diplodia sapinea* (Fr.) Fuckel. Agricultural and Forest Entomology, 19: 4–9.

The contributions of the authors to the papers were as follows:

	I	II	III
Original idea	RK, EV, **TD**, IS	EV, RK, **TD**	RD, **TD**
Study design	**TD**, EV	EV, **TD**	**TD**, RD
Data collection	**TD**	**TD**	**TD**, RD
Data analysis	EV	EV, **TD**	**TD**
Manuscript preparation	EV, **TD**, RK, IS	EV, **TD**, RK	**TD**, RD, IS, KA, KV

TD – Tiia Drenkhan, EV – Eeva J. Vainio, RK – Risto Kasanen, IS – Ivar Sibul, KV – Kaljo Voolma, KA – Kalev Adamson, RD – Rein Drenkhan

ABBREVIATIONS

CF11	cellulose affinity chromatography
CP	coat protein
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
HetPV2-pa1	*Heterobasidion* partitivirus 2
HetPV4-pa1	*Heterobasidion* partitivirus 4
HetRV6-pa17	*Heterobasidion* RNA virus 6
mt SSU rDNA	mitochondrial small subunit ribosomal DNA
ORF	one long open reading frame
PCR	polymerase chain reaction
PgLV-1	*Phlebiopsis gigantea* large virus-1
RAPD	random amplification of polymorphic DNA
RDRP	RNA dependent RNA polymerase
RNA	ribonucleic acid
RT	reverse transcription
RT-PCR	reverse transcription - polymerase chain reaction
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
SSPP	species-specific polymerase chain reaction (PCR) priming

1. INTRODUCTION

The interaction between insects and fungi to break down woody substrate is crucial in nutrient cycling in forest ecosystems (Mahendrappa *et al.*, 1986). It also has been recognized as one of the most serious risks threatening the health of forests. A striking example of realization of such risk is the unexcpected outbreak of mountain bark beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae) and associated fungus, causing massive forest decline in Northern America (Dhar *et al.*, 2016).

The kingdom Fungi is one of the most diverse groups of organisms on earth; the fungi are integral ecosystem agents that govern soil carbon cycling, plant nutrition, and pathology (Tedersoo *et al.*, 2014). In Estonia, 7,000–8,000 species of fungi are known. When species identified in environmental samples using molecular methods are also included, the number exceeds 100,000 species (Käärt, 2016).

Almost all types of microorganisms (fungi, bacteria, viruses, nematodes, protozoa, phytoplasmas) can be transmitted by insects (Agrios, 2008). The important association between fungi and insects (bark beetles) is especially well known (Nuorteva, Laine, 1972; Viiri, Lieutier, 2004; Harrington, 2005; Linnakoski *et al.*, 2008, 2012). Many of these fungi are pathogens causing diseases on leaves, stems, twigs, branches and roots (Phillips, Burdekin, 1992). Fungi show adaptations for insect dispersal, as they produce asexual or sexual spores in sticky drops at the tips of fungal fruiting structures. Some fungi (e.g. *Phlebiopsis gigantea* (Fr.) Jülich) have an evolutionary conidial state which is probably an adaptation for insect dissemination that supplements wind dissemination of basidiospores (Harrington, 2005).

Insects as vectors can introduce pathogens such as fungi, bacteria or viruses into a plant and cause an infection (D'Arcy, Nault, 1982; Purcell, Almeida, 2005; Hogenhout *et al.*, 2008). Generally, insects transmit pathogens in on of three main ways: (i) by feeding or walking through a plant area infected with bacteria or fungal spores thus carrying the infection to the same or another plant; (ii) by feeding on infected plant tissue and carrying the pathogen on their mouthparts to other plants; (iii) by sucking up the pathogen (viruses, phytoplasmas, protozoa, nematodes, some bacteria) with the plant sap on which they feed (Agrios, 2008; Büttner *et al.*, 2013).

Many insects are not only agents of dissemination but also very effective agents of inoculation (Leach, 1935). Insect transmission occurs while wounding plants during oviposition or most frequently, when plants are wounded during feeding (Agrios, 2008). Pathogens and viruses can be transmitted to insects horizontally by contact, ingestion, and by vectors, and vertically from parents to their offspring (Wegensteiner, 2004).

Viruses are widespread (Ahn, Lee, 2001; Bao, Roossinck, 2013; Ghabrial, 2013) small, infectious pathogens attacking animals, plants, bacteria, archaea, fungi (Agrios, 2005; Cho *et al.*, 2013) and viruses. The viruses have been detected in different environments, such as woody and herbaceous plants, soil, surface waters, glacier ice, seawater and clouds (Büttner *et al.*, 2013). Mycoviruses (fungal viruses) are a specific group of viruses which naturally infect and replicate in fungi (Özkan, Coutts, 2015). Mycoviruses are transmitted by cytoplasmic mixing following hyphal fusion (anastomosis) or by the formation of asexual spores (Nuss, 2005). Estimates of mycovirus incidence suggest that 30–80% of fungal species may be infected (Ghabrial, Suzuki, 2009).

The fungal viruses (mycoviruses) have known vectors carried by insects and mites (Özkan, Coutts, 2015; Bouneb *et al.*, 2016; Liu *et al.*, 2016) while plant viruses have many insect vectors: from the order Homoptera, aphids (family Aphididae), whiteflies (family Aleyrodidae), leafhoppers (family Cicadellidae), planthoppers (family Fulgoroidea), mealybugs (family Pseudococcidae), thrips (order Thysanoptera), leaf beetles (family Chrysomelidae), weevils (family Curculionidae), and other beetles (order Coleoptera) (Agrios, 2005; Purcell, Almeida, 2005; Büttner *et al.*, 2013); other vectors are nematodes (phylum Nematoda), fungi and mites (family Eriophyidae) (Hohn, 2007; Büttner *et al.*, 2013).

Insects moving on the forest floor are contaminated internally and externally with viruses, fungi, bacteria, nematodes. Insects may also carry pathogens externally on their legs, mouthparts, bodies or internally through their digestive tract. Fungal spores are located on the dorsal and lateral sides of the pronotum, especially anteriorly. Sticky masses of spores are situated in cuticular depressions associated with the pronotal setae (Viiri, 2004). Weevils (Coleoptera: Curculionidae) can carry the spores of fungi to their host trees and vector blue-stain fungi and resin top disease (Lévieux *et al.*, 1994; Pappinen, Weissenberg, 1994). The large pine weevil (*Hylobius abietis* L., Coleoptera: Curculionidae) is able to carry viable hyphae or conidia

of *Heterobasidion* spp. (Fr.) Bref. (Nuorteva, Laine, 1968, 1972; Kadlec *et al*., 1992) and species of *Leptographium* (Piou, 1993; Lévieux *et al*., 1994; Jankowiak, Bilański, 2013a, 2013b). The resin top disease *Endocronartium pini* is known to be often associated with the pine-top weevil, *Pissodes piniphilus* (Pappinen, Weissenberg, 1994). The extensively analysed Dutch elm disease (DED) is a well-known example of a disease, caused by elm bark beetles (*Scolytus* spp.) (Coleoptera: Curculionidae, Scolytinae) and associated fungal pathogens *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier, 2001; Jacobi *et al*., 2013; Santini, Faccoli, 2015; Menkis *et al*., 2016).

This thesis is a synthesis of three papers. All papers (**I**, **II**, **III**) show evidence of the ability of the large pine weevil (*H. abietis*) to carry the pathogenic (**I**, **III**) or saprotrophic (**II**) fungi and viruses related to fungi.

2. REVIEW OF THE LITERATURE

2.1 *Hylobius* spp.

Pine weevils of the genus *Hylobius* (Coleoptera: Curculionidae) are widespread, abundant and economically harmful insect pests in reforestation areas in boreal coniferous forests across the northern Palearctic region (Leather *et al.*, 1999; Långström, Day, 2004). In Estonia, four species of *Hylobius* have been found. The large pine weevil, *H. abietis* (Linnaeus 1758) prefers dry areas where pine and spruce are clear cut. The lesser pine weevil *H. pinastri* (Gyllenhal 1813) lives in moist spruce stands whereas the large spruce weevil, *H. piceus* (De Geer 1775) inhabits old pine trees and is quite rare in Estonia (Maavara *et al.*, 1961; Sibul, 2000). A new species for Estonia is a root-mining weevil, *H. transversovittatus* (Goeze 1777) which inhabits marshy banks of water bodies, natural wet meadows and river flood-lands (Bukejs, Balalaikins, 2011). In Estonia, the large pine weevil is the most dispersed, abundant and destructive pest of conifer plantations (Voolma, 1994, 2001; Sibul, 2000).

The large pine weevil is dark brown as a young adult, but later the colour turns blackish. A hard chitinous cover protects the body. The scales on the body and on the elytra are covered in light yellow spots and patches. There are patches of yellow scales on the wing cases and on the thorax. The thorax is slightly wider than long, strongly convex and constricted at the front. The head is extended to form a strong snout with mandibles at the tip. The antennae are elbowed and attached to the snout near the end (Maavara *et al.*, 1961). The adult weevil is 10 to 14 mm long (Eidmann, 1974) and lives 1–4 years (Thomas, 2008; Thorpe, Day, 2008). The average lifetime of the pine weevil is 2–3 years (Maavara *et al.*, 1961; Bejer-Petersen *et al.*, 1962).

In Estonia, during the period from May to September, baited pitfall traps from a freshly clear cut area yielded 10,900 *H. abietis* specimens per hectare (Sibul, 2000; Sibul, Voolma, 2004). In Sweden and in the UK, pine weevil density on a freshly clear-cut area is estimated at 14,000 and 15,440 weevils per hectare, respectively (Nordlander *et al.*, 2003b). Data from Ireland have shown more than 100,000 adult weevils per hectare (Dillon *et al.*, 2006). Due to the high population densities, the economical losses caused by weevil feeding and subsequent death of planted seedlings

are high. The cost of the resulting *H. abietis* damage across Europe in 2004 has been estimated to be approximately 140 million euros per year (Långström, Day, 2004).

2.2 The biology of large pine weevil

Adult insects migrate over long distances to find stumps and dying roots of conifers suitable for breeding (Viiri, 2004). In their migratory phase adult *H. abietis* can fly up to 10 km in search of suitable breeding material (Solbreck, 1980) or can walk on the ground (Eidmann, 1997). Flying weevils are attracted to freshly cut areas by the volatiles emanating from fresh conifer stumps (Örlander *et al.*, 1997). Attractive volatiles include ethanol (Nordenhem, Eidmann, 1991) and monoterpenes: α-pinene, β-pinene and 3-carene (Zagatti *et al.*, 1997; Schlyter, 2004; Dillon, Griffin, 2008). Adult weevils have a period of maturation feeding before ovipositing in the spring (Leather *et al.*, 1999), usually achieved in the pine stumps (Daniel, 1935). The damage caused by *H. abietis* is extensive on clear cut areas of former conifer stands which have been reforested by planting pine or spruce (Örlander, Nilsson, 1999; Day *et al.*, 2004). The weevils are found to be abundant up to 5-year-old clear-cut areas (Örlander *et al.*, 1997; Sibul, 2000).

After mating, females oviposit in fresh conifer stumps (Figure 1); eggs are laid in small notches on the bark of roots excavated by them in late spring (Nordenhem, Nordlander, 1994). Oviposition mostly occurs in the top 100 mm of stumps, rarely deeper than 400 mm (Leather *et al.*, 1999). The oviposition phase lasts for 10–49 days (Thorpe, Day, 2008). Both spruce and pine stumps are suitable for laying eggs, although in spruce stumps development of the larva is slower (Wainhouse *et al.*, 2001; Inward *et al.*, 2012). During this period, an individual female is able to produce 0.1–3.7 eggs per day (Thorpe, Day, 2008), approximately 60–100 eggs during the season (Maavara *et al.*, 1961; Bylund *et al.*, 2004). The oviposition period generally lasts 5 months (from May to September) (Nordenhem, 1989). The preferred temperature for oviposition is +22 °C (Christiansen, Bakke, 1968).

The large pine weevil passes through four larval moults before pupation (Leather *et al.*, 1999). The larvae develop on the roots of fresh stumps (Day *et al.*, 2004). Larval development depends on temperature and climate,

varying from one year in Western Europe to four years in the Northern Europe (Bejer-Petersen *et al.*, 1962; Leather *et al.*, 1999) and lasts 14–15 months in Estonia (Daniel, 1935; Karu, 1939; Voolma, 1986).

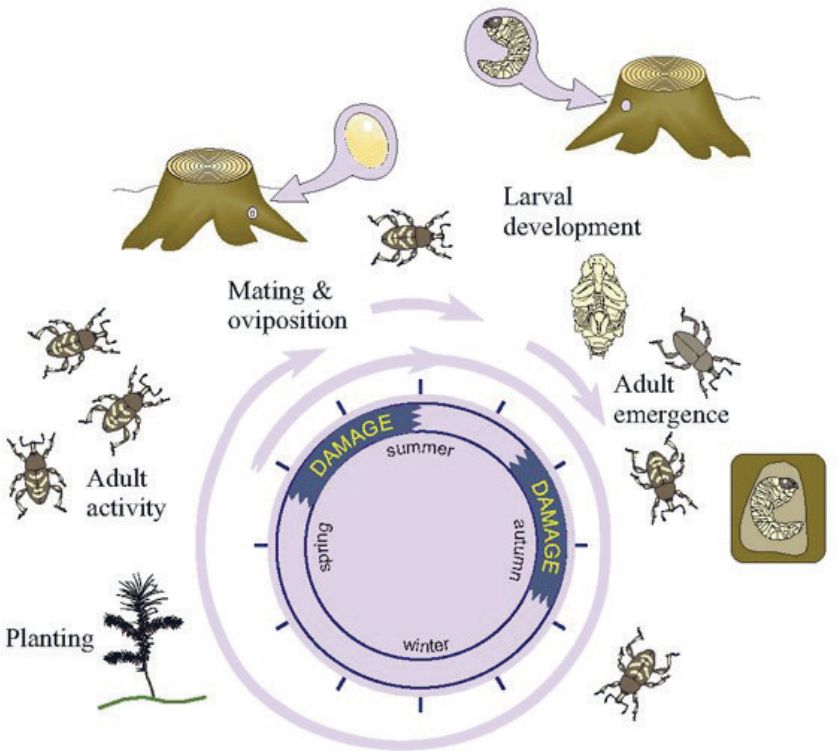

Figure 1. The weevil lifecycle (Forestry Commission, 2017)

Freshly hatched larvae migrate from oviposition sites in soil to suitable feeding sites in bark (Nordenhem, Nordlander, 1994). The larvae feed under the bark of roots of recently dead or dying conifer trees and in fresh stumps (Day *et al.*, 2004). The seedlings are damaged by the adult weevils, while larvae are harmless (Karu, 1939). Adult weevils and new generation of weevils remain on clearcut areas until spring of the second year after oviposition and then appear on the clearcut areas (Örlander *et al.*, 1997). The roots of trees are suitable for oviposition from the time of felling until the following summer (Sibul, 2005).

Air temperature, humidity and light strongly affect pine weevil activity

(Christiansen, Bakke, 1968, 1971; Sibul *et al.*, 1999). Activity increases with temperature (Örlander *et al.*, 1997) up to +25 °C (Christiansen, Bakke, 1968). The pine weevil population depends on the amount of breeding material (Eidmann, 1974); critical is also the nutritional status of the ovipositing females (Örlander *et al.*, 2001). They hibernate below ground in the soil and litter. The weevils emerge from hibernation in spring when temperatures reach +8–9 °C (Nordenhem, 1989; Leather *et al.*, 1999).

2.3 Feeding behaviour of *Hylobius abietis*

Adult *H. abietis* weevils have a broad host range, but Scots pine (*Pinus sylvestris*) is the preferred food source (Dillon, Griffin, 2008). Weevils may also feed on other conifer seedlings (*Picea abies*, *P. sitchensis*, *Pseudotsuga menziesii*) (Leather *et al.*, 1994; Örlander, Nilsson, 1999; Wallertz *et al.*, 2014) and on broadleaved trees (*Acer pseudoplatanus*, *Alnus* spp., *Betula pendula*, *Fraxinus excelsior*, *Sorbus aucuparia*, *Salix* spp.) (Manlove *et al.*, 1997; Månsson, Schlyter, 2004). The weevils feed on the bark of (2)3–6(7) year old conifer seedlings (Daniel, 1935; Nordlander *et al.*, 2003b; Toivonen, Viiri, 2006). The seedlings under shelterwood are generally less damaged by pine weevils because shelterwood provides food resources other than seedlings on clearcuts (Nordlander *et al.*, 2003a, 2003b). The weevils feed on conifer tree bark of branches and roots and ground vegetation (e.g. bilberry, *Vaccinum myrtillus*) (Örlander *et al.*, 2001; Wallertz *et al.*, 2006). Bark of living as well as of recently dead trees is utilized (Nordlander *et al.*, 2005) and feeding can occur also on fallen tops and branches of conifers lying on the ground (Munro, 1928). The *H. abietis* feed on the bark of twigs in the crowns of mature coniferous trees and migrate to new breeding sites in spring and early summer to seek females reaching sexual maturity (Örlander *et al.*, 2000, 2001). The presence of humus and vegetation provides cover for pine weevils and contributes to feeding (Björklund *et al.*, 2003) so that seedlings in mineral soil are less damaged (Långström, Day, 2004; Petersson, 2004). Some data have shown that scarification reduces weevil attacks (von Sydow, 1997).

Weevils make large patches of feeding scars with their chewing mouthparts (Viiri, 2004), feeding on approximately 0.2 cm^2 bark per weevil per day (Örlander *et al.*, 2000). The rate of feeding has been estimated as 62 mm^2 per day for females and 40 mm^2 per day for males on *P. sylvestris*

twigs (Bylund *et al.*, 2004). However, the feeding activity depends on the species of conifer, the depth of bark and temperature (Day *et al.*, 2004). The weevils move and feed more at twilight. Males and females differ in their feeding activity (Merivee *et al.*, 1998; Sibul *et al.*, 1998) and during the day the weevils are not active (Merivee *et al.*, 1998; Sibul *et al.*, 1998; Fedderwitz *et al.*, 2012). Feeding extends over the summer until adult weevils are ready to hibernate (Bylund *et al.*, 2004).

2.4 Control of large pine weevil

Various methods have been attempted to control *H. abietis* including microbial pathogens, fungi, entomopathogenic nematodes, parasitoids, predators or predatory beetles and vertebrates and to reduce pest populations (Leather *et al.*, 1999; Dillon, Griffin, 2008; Hoy, 2008; Nordlander *et al.*, 2009; Popowska-Nowak *et al.*, 2016). Prevalent natural enemies of *H. abietis* are braconid wasps (*Bracon hylobii*, *B. brachyceros*, *Perilitus areolaris*, *P. rutilus*), ichneumonid wasps (*Dolichomitus tuberculatus*), nematodes (*Steinernema* spp., *Dirhabdilaimus leukartis*) and fungi (*Beauvaria bassiana, Sporotrichum globuliferum*) (Brixey, 1997; Faccoli, Henry, 2003; Williams *et al.*, 2013). They affect *H. abietis* in different lifestages: larva, pupa and adult. The level of natural parasitism is usually too low to reduce the number of weevil adults emerging from stumps to below economically damaging levels (Dillon, Griffin, 2008). Earlier, synthetic pyrethroids (Voolma, 2003), neonicotinoids (Olenici *et al.*, 2014), ditches and bark of conifers (Daniel, 1935) have been used to decrease the amount of large pine weevil. The use of pesticides in critical areas for environmental and health reasons should be minimised or banned (European Commission, 2017). Many alternative repellents are implemented to protect seedlings against weevils. The environmentally more friendly defence mechanism are physical protection by plastic collar (Lindström *et al.*, 1986; Eidmann *et al.*, 1996), latex or wax like paste (Zumr, Stary, 1995; Sibul, 2013; Sibul, Ploomi, 2016) or biopesticide protecting the conifer seedlings against gnawing of the large pine weevil (Luik *et al.*, 1995; Sibul *et al.*, 2009; Gupta, Dikshit, 2010). Conifer seedlings covered with a flexible sand coating (Conniflex) is used (Nordlander *et al.*, 2009). Botanical insecticides (environmentally friendly treatment) have a short-term influence and plants need to be treated repeatedly (Luik *et al.*, 1995, 2000; Sibul *et al.*, 2009; Khater, 2012). This makes the treatment of seedlings more expensive. Therefore, forest management combined with novel seedlings protective methods are used.

2.5 Species complex *Heterobasidion annosum sensu lato*

The species complex *Heterobasidion annosum* (Fr.) Bref. *sensu lato* (Basidiomycota, Bondarzewiaceae) includes some of the most destructive forest pathogens of the northern hemisphere, where they cause root rot and stem decay in conifers (Woodward *et al*., 1998). It is of major concern in several areas of the world, especially where intensive forest management is practised (Gonthier, Thor, 2013). The fungus *H. annosum s.l.* can be divided into five species – three in Eurasia and two in North America (Korhonen, 1978; Capretti *et al*., 1990). The Eurasian group was named as *H. annosum sensu stricto* (Fr.) Bref. infecting many conifer species including pines (*Pinus* spp.), especially Scots pine (*P. sylvestris*), juniper (*Juniperus communis*) (Korhonen, 1978) and also several broadleaved tree species (*Betula*, *Fraxinus*, *Corylus*, *Alnus*, *Acer*, *Salix*, *Ligustrum*, *Quercus*, *Prunus*) (Sedlák, Tomšovský, 2014). *H. parviporum* Niemelä & Korhonen infects mainly Norway spruce (*P. abies*) and Sitka spruce (*P. sitchensis*). *H. abietinum* Niemelä & Korhonen is associated mostly with Silver fir (*Abies alba*) and other species in the genus *Abies* in central and southern Europe (Niemelä, Korhonen, 1998).

North American groups are described as *H. irregulare* (Underw.) Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel. (Otrosina, Garbelotto, 2010). In eastern and southern Asia and Oceania: *H. araucariae* Buchanan and species forming the *H. insulare sensu lato* (Murr.) Ryv. species complex (*H. insulare*, *H. ecrustosum*, *H. australe*, *H. orientale*, *H. linzhiense*, *H. amyloideum* and *H. tibeticum*) are found (Vainio, Hantula, 2015a). The *H. insulare s.l.* (Murr.) Ryv. and *H. araucariae* Buchanan are able to grow at a fast rate in sapwood of spruce and pine seedlings and stem pieces (Johansson *et al*., 2004). The species are saprophytes in their normal environment (Niemelä, Korhonen, 1998), but may turn pathogenic to our main conifers, similar or worse than *H. annosum* (Johansson *et al*., 2004). Financial losses caused by this species complex have been estimated to be approximately 800 million euros in Europe (Woodward *et al*., 1998).

The disease caused by *Heterobasidion* spp. has been studied in many parts of Europe, particularly in Finland, Sweden, Italy, Britain, Lithuania, Poland, Switzerland, Spain, Bulgaria, Belarus, Slovenia and Estonia (Korhonen *et al*., 1992; Munda, 1994; Korhonen, Stenlid, 1998; La Porta *et al*., 1998; Gonthier *et al*., 2001; Bendel *et al*., 2006; Mesanza, Iturritxa, 2012).

2.6 Infection cycle of *H. annosum s.l.*

H. annosum s.l. is able to infect healthy conifers, while spore mediated infections occur frequently on fresh stump surfaces (Stenlid, Redfern, 1998). The fungus can infect uninjured trees by vegetative growth of mycelia through root contacts or grafts (Stenlid, 1985; Stenlid, Rayner, 1989; Gonthier, Thor, 2013). The germination of a single basidiospore results in the formation of a homokaryotic, haploid, primary mycelium while a compatible mating between two homokaryotic mycelia produces a heterokaryotic, diploid, secondary mycelium (Vainio, Hantula, 2015a). The spread of mycelia or conidiospores may have been facilitated by insect vectors (Stenlid, 1985). The majority of airborne infections are caused by sexual spores rather than by conidia (Redfern, Stenlid, 1998). Conidia are important in short distance transmission in substrates or when they are vectored by root-feeding insects (Kadlec *et al.*, 1992; Gonthier, Thor, 2013). The conidia are introduced into damaged wood where the beetles have fed, and then initiate colonization (Stenlid, Rayner, 1989). The growth in stump roots of Norway spruce infected by *H. parviporum* increases the rate of spread of the fungus in the root system of felled trees (Pettersson *et al.*, 2003). However, it appears that the risk of spore infection transfer to the next tree generation varies (Piri, Korhonen, 2007; Rönnberg *et al.*, 2007). Primary infection can be reduced by stump treatment (see 2.9) and secondary infection by reducing the number of contacts between roots of host trees, e.g. by mixing host trees with non host trees (Hodges, 1969).

Young and adult trees are susceptible to a white root rot (Gonthier, Thor, 2013); two-thirds of a root system may be killed by *H. annosum s.l.* before symptoms appear in the crown (Greig, 1998). The *P. sylvestris* is more resistant while pine rays are more loaded with polymeric phenolic compounds and resins forming a barrier against fungal hyphae (Johansson *et al.*, 2004) while Norway spruce is more susceptible. Werner and Łakomy (2002) has reported that an inoculation experiment of Scots pine (*P. sylvestris*) and Norway spruce (*P. abies*) seedlings with *H. annosum* pine (P), spruce (S) and fir (F) isolates, indicated that all the strains were more virulent on spruce than on pine seedlings.

2.7 Viruses in *H. annosum s.l.*

Several virus species have been described from *H. annosum s.l.* species, belonging to the family Partitiviridae (Ihrmark, 2001; Ihrmark *et al.*, 2002; Vainio *et al.*, 2010, 2011b) throughout the world (Europe, Asia and North America) and in both pathogenic and saprotrophic *Heterobasidion* species (all species of *H. annosum s.l.* as well as two species of the *H. insulare* species complex) (Vainio *et al.*, 2011a). To date, fourteen different species of partitiviruses have been described in *Heterobasidion* spp. (Vainio, Hantula, 2015a) and all can be assigned to the genus *Alphapartitivirus* or *Betapartitivirus* (Nibert *et al.*, 2014).

It is known that approximately 15% of European and western Asian *H. annosum s.l.* isolates are infected by dsRNA viruses (Ihrmark, 2001). Partitiviruses infecting *Heterobasidion* spp. have bisegmented dsRNA genomes encoding a coat protein (CP) and an RNA-dependent RNA polumerase (RdRp), packed in separate virions (Vainio, Hantula, 2015a). The most common mycovirus species in *Heterobasidion* spp. is a taxonomically unassigned virus species *Heterobasidion* RNA virus 6 (HetRV6), found from four species of *Heterobasidion* (Vainio *et al.*, 2012). HetRV6 seems to be a single virus species with relatively low overall sequence polymorphism (Vainio, Hantula, 2015a) and distantly related to the Curvularia thermal tolerance virus (CThTV) (Márquez *et al.*, 2007). Nevertheless, paritivituses are rare and constitute less than 30% of dsRNA infections hosted by *H. annosum s.l.* and occurred in only about 5% of the isolates in culture collections (Vainio *et al.*, 2011a). Most viruses appear to have no effect on the pathogenicity of their host fungi, but the dsRNA in fruitbodies of *H. parviporum* resulted in a reduced germination frequency of basidiospores (Ihrmark *et al.*, 2004; Vainio *et al.*, 2011b) and 10–84% of the germinated basidiospores contained dsRNA viruses (Ihrmark *et al.*, 2004). Viruses of *H. annosum s.l.* are capable of dispersal via basidiospores and conidia as well as vegetatively by hyphal contact (Ihrmark *et al.*, 2002, 2004). Basidiospores and vegetative growth are important for spread of the dsRNA in *H. annosum s.l.* (Ihrmark, 2001) because virus transmission between somatically incompatible hosts in natural conditions is possible (Vainio *et al.*, 2013). Virus transmission may occur via mating between two homokaryotic mycelia or a homokaryon and a heterokaryon or cellular contact between two somatically incompatible heterokaryons (Ihrmark *et al.*, 2002; Vainio *et al.*, 2010).

Usually, the partitiviruses disperse slowly by hyphal contact between *Heterobasidion* mycelia, while some strains are able to rapidly infect new clones via airborne spores and stumps have a higher viral incidence than standing trees (Vainio *et al.*, 2015b). The *H. annosum s.l.* complex has several possibilities for horizontal virus transfer while *Heterobasidion* species share common host tree species. The transmission of viruses is rather usual in the laboratory which suggests they could be transmitted between *Heterobasidion* species also in nature (Vainio *et al.*, 2010). The significance of the asexual conidial spores of *Heterobasidion* is unknown but they are probably able to travel short distances by lateral transfer of transient anastomosis even between somatically incompatible isolates of *Heterobasidion* (Ihrmark *et al.*, 2002; Vainio *et al.*, 2010).

2.8 *Diplodia sapinea* (Fr.) Fuckel

Diplodia sapinea (Fr.) Fuckel (syn. *D. pinea* (Desm.) Kickx., *Sphaeropsis sapinea* /Fr.: Fr./ Dyko & Sutton) is one of the most common and widely distributed pathogens of conifers worldwide (Whitehill *et al.*, 2007) causing *Diplodia* tip blight (Phillips *et al.*, 2013).

The pathogen is known to occur on pines and other conifers across the world (Stanosz *et al.*, 1999; Flowers *et al.*, 2001; Aday *et al.*, 2012; Oliva *et al.*, 2013; Adamson *et al.*, 2015) as an endophyte (Stanosz *et al.*, 1997, 1999; Luchi *et al.*, 2014), or asymptomatic in green needles (Diminic, Jurc, 1999). The pathogen was recorded on cone scales of Austrian pine (*P. nigra*) in Estonia in 2007 (Hanso, Drenkhan, 2009) and in 2012 from native Scots pine (*P. sylvestris*) (Adamason *et al.*, 2015). *D. sapinea* is able to damage the stems, buds, immature cones and bracts of mature cones (Flowers *et al.*, 2001; Burgess *et al.*, 2004; Stanosz *et al.*, 2005; Decourcelle *et al.*, 2014). The pathogen is strongly associated with cones and cone infection is a specific adaptation of the fungus (Munck *et al.*, 2009; Munck, Stanosz, 2010). *D. sapinea* is an important sapstain fungus that injures logs and decreases wood quality (Thwaites *et al.*, 2004).

D. sapinea has different strategies for dispersal: over short distances with conidia by rainfall and long range dispersal with colonized tissues of plants or trees, wood, water flow, animals, birds, arthropods, insects and plant material (Parry, 1990; Tainter, Baker, 1996; Flowers *et al.*, 2001; Shigesada, Kawasaki, 2001; Bihon *et al.*, 2011).

The pathogen is also known to be carried by bark beetles (Coleoptera: Scolytinae) (Whitehill *et al.*, 2007), the invasive seed insect *Leptoglossus occidentalis* (Heteroptera: Coreidae) (Luchi *et al.*, 2012), root feeding bark beetles *Hylastes* spp. (Coleoptera: Scolytidae) (Jankowiak, Bilański, 2013b) and by *H. abietis* (**III**).

D. sapinea is known to contain members of the virus families Narnaviridae, Totiviridae, Partitiviridae (Preisig *et al.*, 1998; Steenkamp *et al.*, 1998; Adams *et al.*, 2002). Two viruses with dsRNA genomes, *Sphaeropsis sapinea* RNA virus 1 and 2 (SsRV1 and SsRV2) are found in *D. sapinea* (Preisig *et al.*, 1998; De Wet *et al.*, 2008).

2.9 *Phlebiopsis gigantea* (Fr.) Jülich

The basidiomycete *Phlebiopsis gigantea* (Fr.) Jül. (syn. *Phlebia gigantea* (Fr.) Donk, *Phanerochaete gigantea* (Fr.) S.S. Rattan) is a common saprophytic basidiomycete fungus in coniferous wood successfully colonizing stumps and fallen trunks and causing white rot of logs and stumps (Meredith, 1959; Kallio, 1965). *P. gigantea* is a typical wood decay fungus and its basidiospores are adapted for wind dispersal (Hsiau, Harrington, 2003). The fungus is easily recognised in pure culture by the presence of oidial chains, encrusted upright aerial hyphae, numerous encrusted cystidia and multiple clamp connections (Stalpers, 1978; Eriksson *et al.*, 1981).

Since 1950s, conifer tree stump treatment against *Heterobasidion* spp. infection has been carried out using *P. gigantea* (Risbeth, 1952, 1963; Greig, 1976; Korhonen *et al.*, 1994) to prevent penetration of *H. annosum* through the cut surface (Roy *et al.*, 1997). A strain of *P. gigantea*, isolated from spruce wood in Finland, has been formulated into a powder or liquid registered as a biological control agent (Korhonen *et al.*, 1994) Rotstop® (Verdera Oy) and applied to conifer stumps after logging in England (Risbeth, 1975; Greig, 1976), Finland (Korhonen, 1993), Sweden (Korhonen *et al.*, 1994), Poland (Rykowski, Sierota, 1983), Estonia (Drenkhan *et al.*, 2008), Latvia (Kenigsvalde *et al.*, 2015). The fungus *P. gigantea* is an effective and a highly competitive primary colonizer (Roy *et al.*, 1997), but the use of a commercial biocontrol agent (Rotstop) has been effective in preventing *Heterobasidion* spp. infections in Scots pine stumps (Korhonen *et al.*, 1994; Drenkhan *et al.*, 2008), whereas in Norway spruce stumps the effectiveness has been lower (Korhonen *et*

al., 1994; Nicolotti *et al.*, 1999; Berglund, Rönnberg, 2004). *P. gigantea* is less effective in stands where *Heterobasidion* spp. is already present in stumps (Pettersson *et al.*, 2003), but in stands that are more or less free of infection, stump treatment may be a way of reducing the build-up of infection in subsequent rotations (Rönnberg *et al.*, 2007).

P. gigantea is a true biological species with one intersterility group (Vainio *et al.*, 1998). The fungus has a clear genetic differentiation between the European and North American populations (Vainio, Hantula, 2000) but has a low geographic differentiation around the Baltic Sea (Samils *et al.*, 2009). Based on RAPDs analysis the *P. gigantea* population was composed of a large number of genetically distinct individuals (Roy *et al.*, 1997). The strain of *P. gigantea* used in preparation of the biocontrol agent Rotstop is not likely to cause any immediate threat to the genetic diversity of *P. gigantea* (Vainio *et al.*, 2001, 2005). However, *P. gigantea* migration between the continents for biocontrol purposes should be avoided (Vainio, Hantula, 2000).

Mycovirus coinfections have been found in *P. gigantea* (Kozlakidis *et al.*, 2009). The *P. gigantea* strain TW-2 was isolated from Scots pine, initially used as a biopesticide in the UK. It was reported to have two large double-stranded RNAs (dsRNAs) 10–12 kbp in size, nominated as *P. gigantea* large virus-1 (PgLV-1 dsRNA1) and *P. gigantea* large virus-2 (PgLV-2 dsRNA2) and they are members of a new, yet unassigned virus family (Kozlakidis *et al.*, 2009; Lim *et al.*, 2011). However, a new genus named *Phlegivirus* containing PgLV1 and other viruses (TtV1, RfV1 and LeV) is proposed (Petrzik *et al.*, 2016). The growth rates of uninfected *P. gigantea* isolates and virus-infected types seem to be similar (Lim *et al.*, 2011).

In addition to the sexual state, *P. gigantea* can also form a dense layer of asexual spores (conidia or arthrospores), and these spores may be suitable for grazing by beetles and for dispersal by insects (Hsiau, Harrington, 2003). However, little is known of the relation between *P. gigantea* and insects. *P. gigantea* oidia have been found in arthropod galleries (Hunt, Cobb, 1982) and the fungus is carried by *H. abietis* (Coleoptera: Curculionidae) (**II**). The colonization of *P. gigantea* fungus inhibits the attractiveness of pine branches to large pine weevil because *P. gigantea* changes the composition of the volatiles emanating from pine (Skrzecz, Moore, 1997; Šmits, Gaitnieks, 2013).

2.10 Mycoviruses

Mycoviruses (fungal viruses) are believed to be of ancient origin and widespread in all major taxa of fungi (Ghabrial, 1998; Ghabrial *et al.*, 2015), including representatives of Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (Vainio *et al.*, 2010, 2011b). The mycoviruses that infect plant-pathogenic fungi, yeasts, and mushrooms are composed of single-stranded (ss) RNAs (Cho *et al.*, 2013), double-stranded (ds) RNAs (Ghabrial, 1998), positive- or negative-strand ssRNAs (Ghabrial *et al.*, 2015) and ssDNAs (Yu *et al.*, 2013; Ghabrial *et al.*, 2015; Krupovic *et al.*, 2016). Fungal viruses are isometric particles 25–50 nm in diameter (Ghabrial, 1998). The dsRNAs are formed during virus replication, and consist of full length genomes. Large molecules of dsRNA do not normally occur in fungal cells and therefore, their presence is a sign of a viral infection (Márquez *et al.*, 2007; Büttner *et al.*, 2013).

The life cycle of mycoviruses consists of the following steps: (1) propagation within a host cell; (2) cell-to-cell spread in the direction of hyphal extension after cell division; (3) lateral spread between fused hyphal branches within an interconnected mycelial network; and (4) horizontal transmission via hyphal fusion (anastomosis) between fungal strains belonging to the same vegetative compatibility group or mycelial compatibility group; vertical transmission occurs via sporulation (Yaegashi *et al.*, 2013). The extracellular phase in the mycovirus life cycle has been discovered (Yu *et al.*, 2013; Liu *et al.*, 2016). The heterokaryon formation in basidiomycetes gives mycoviruses the opportunity to transfer to vegetative mycelia that other fungal groups lack (Ihrmark *et al.*, 2002); vegetative spread by cell-to-cell contacts is also common (Ihrmark *et al.*, 2002, 2004).

Fungal viruses are divided according to genomes made of linear dsRNA into seven families (Chryso-, Endorna-, Megabirna-, Quadri-, Partiti- and Totiviridae); linear positive-sense ssRNA (+RNA) into five families (Alphaflexi-, Barna-, Gammaflexi-, Hypo-, and Narnaviridae); linear negative-sense ssRNA (-RNA) (family Mycomononegaviridae); circular ssDNA (Genomoviridae) (International Committee on Taxonomy of Viruses, ICTV).

Most mycoviruses are cryptic (latent) and seem to cause no phenotypic effects on their host fungi (McCabe *et al.*, 1999; Vainio *et al.*, 2012) or they have symptomless infections and/or reduce the virulence in their

fungal hosts (Ghabrial, Suzuki, 2009). The potential mutualistic roles of mycoviruses in nature are poorly studied (Márquez *et al.*, 2007). Hypothetically, mycoviruses are a potential transportable factor that can provide their partners with more flexibility for rapid adaptation and a favourable trait during temporal or spatial environmental changes (Bao, Roossinck, 2013).

Fungal viruses have been used to control disease caused by pathogenic fungi (Preisig *et al.*, 1998) and one possibility is to use mycoviruses as biocontrol agents (Nuss, 2005; Dawe, Nuss, 2013; Vainio, Hantula, 2015a, 2015b). The viruses are used at the molecular level and it is possible to use them for control of *H. annosum s.l.* (Ihrmark, 2001; Nuss, 2005; Vainio, Hantula, 2015a), but an obstacle is that mycoviruses limit transfer from one fungal strain to another (Vainio *et al.*, 2010). Viruses which are virulent to their hosts are used as virocontrol agents (biological control using viruses) (Kondo *et al.*, 2013). Liu *et al.* (2016) has found that the exploration of mycoviruses to control fungal diseases may play an important role in the transmission and distribution of newly emerging viruses. Mycovirus virulence of their phytopathogenic fungal hosts is of considerable interest for the development of novel biocontrol strategies (Ghabrial *et al.*, 2015). The first described mycovirus caused dieback disease in cultivated mushrooms (*Agaricus bisporus* (Lange) Sing.) and was termed "La France disease" or "X-disease" or "brown disease" (Hollings, 1962; Eicker *et al.*, 1990).

Some mycoviruses have the ability to attenuate the disease caused by fungal plant pathogens in a phenomenon referred to as hypovirulence (Huang, Ghabrial, 1996; Cho *et al.*, 2013) but only a limited number of mycoviruses cause hypovirulence (Cho *et al.*, 2013). Hypovirulence-associated mycoviruses have double-stranded (ds) or single-stranded (ss) RNA genomes (Nuss, 2005). The best studied example of hypovirulence caused by mycoviruses used for biological control is the Chestnut Blight *Cryphonectria parasitica hypovirus 1* (CHV1) (Murrill) Barr, belonging to the family Hypoviridae (contains a single genus, *Hypovirus*) (Anagnostakis, 1982; Heiniger, Rigling, 1994; Ihrmark *et al.*, 2002; Deng *et al.*, 2003; Nibert *et al.*, 2013), and some other examples like partitivirus *Rosellinia necatrix partitivirus* 1-W8 (RnPV1-W8) (Kanematsu *et al.*, 2010), *Sclerotinia sclerotiorum partitivirus* 1 (SsPV1/WF-1), conferred hypovirulence on their natural plant-pathogenic fungal host, *Sclerotinia sclerotiorum* strain WF-1 (Xiao *et al.*, 2014). Most hypoviruses reduce fungal virulence and/

or other phenotypes, including pigmentation, sporulation, and laccase activity (Yaegashi *et al.*, 2012).

2.11 Partitiviridae

The investigated viruses of *Heterobasidion* spp. belong to the family Partitiviridae. In addition to partitiviruses, the studied viruses included HetRV6 and the 'phlegivirus' of *Phlebiopsis*. Members of the family Partitiviridae are typically isometric and have bisegmented dsRNA genomes, with two segments of similar size (linear dsRNA molecules, each 1.4–2.3 kbp) and with a total genome length of 4–6 kbp, where one segment codes for an RNA-dependent RNA polymerase (RdRp) and the other segment codes for a coat protein (Ihrmark, 2001), packed in separate virions (Vainio, Hantula, 2015a). The virus possesses two dsRNA genome segments, dsRNA1 (encoding RNA dependant RNA polymerase (RdRp) and ds RNA2 (encoding the capsid/coat protein (CP), each 1300–2500 bp in length and containing one long open reading frame (ORF) on one of the RNA strands. Viruses in the family Partitiviridae infect plants, fungi and protozoa (Nibert *et al.*, 2014). Also, the uncharacterized A78 partitivirus is pathogenic to the human pathogenic fungus *Aspergillus fumigatus* Fresenius 1863 (Özkan, Coutts, 2015). Partitiviruses have natural transmission between host cells only through intracellular means during cell division (mitosis, meiosis) or cell–cell fusion (hyphal anastomosis). This allows transfer of multiple virus particles to each new cell (Nibert *et al.*, 2013).

3. AIMS OF THE STUDY

The aim of this study was to investigate the ability of the large pine weevil, *H. abietis* to carry saprotrophic and pathogenic fungi and viruses. Experiments examined the ability of fungi and virus to survive in the digestive tract of the weevil. Large pine weevils were collected from natural conditions and their ability to carry the fungus *D. sapinea* was analysed.

Hypotheses:

1. Pathogenic and saprotrophic fungi are able to survive and preserve viability after passing through the digestive tract of the large pine weevil (**I**, **II**, **III**);
2. *Heterobasidion* spp. associated viruses preserve the stability after passing through their large pine weevil digestive tract (**I**);
3. *P. gigantea* associated viruses retain their stability after passing through the digestive tract of the large pine weevil (**II**);
4. The viability of the *P. gigantea* strain 93073 infected with virus PgLV-1 is lower than that of the *P. gigantea* strain without viruses (**II**);
5. *D. sapinea* can be dispersed in natural conditions by the large pine weevil (**III**).

The specific aims of this study were:

1. To determine the ability of the pathogenic and saprotrophic fungi to pass through the digestive tract of the large pine weevil (**I**, **II**, **III**);
2. To determine the ability of the large pine weevil to carry *Heterobasidion* spp. and associated viruses through the digestive tract (**I**);
3. To investigate the large pine weevil's transmission ability of *P. gigantea* and associated viruses (**II**);
4. To examine the stability of the virus during insect digestion (**II**);
5. To investigate virus infected and virus free strains of *P. gigantea* (**II**);
6. To study the large pine weevil's ability to carry *D. sapinea* in natural conditions (**III**).

4. MATERIALS AND METHODS

4.1 Sample collection

The large pine weevils (*H. abietis*) were collected from a *Vaccinum-Myrtillus* site type in a Scots pine (*P. sylvestris*) clear cut area in the forest district Järvselja (East Estonia 58°27′ N, 27°33′ E) (**I**, **II**, **III**), Kaitsemõisa in Tartu county (East Estonia, 58°34′ N, 26°49′ E) and Ränna in Võru county (South Estonia, 57°45′ N, 26°29′ E) (**III**), by using ground pitfall traps ((20)25 × 40 × 50 cm). The weevils removed from the traps were transferred to the laboratory and stored in a fridge (+4 °C) (**I**, **II**, **III**) or at –20 °C until DNA extraction (**III**). Insects were weighed before and after experimentation and their gender determined (**I**, **II**). In paper **III** the gender of weevils for the laboratory feeding experiment was determined. The light regime during experiment was L18:D6. Together, 154 large pine weevils were used and analysed in laboratory experiments: 60 in experiment **I**, 40 in study **II** and 54 in experiment **III**.

Scots pine and Norway spruce branches were collected from healthy pine and spruce stands in Tartu county (South Estonia, 58°18′ N, 26°39′ E). Mostly Scots pine was used (**I**, **II III**), whereas in experiment **II**, Norway spruce branches were infected with virus infected *P. gigantea* and Rotstop® strain. The branches (diameter 3–5 mm; length 6–7 cm) were cut with secateurs from pine or spruce trees, cleaned of needles, and transported to the laboratory in a plastic bag and stored in the fridge (+4 °C) (**I**, **II**, **III**). The conifer branches were autoclaved for 30 min at +106 °C (**II**, **III**) and +120 °C (**I**).

4.2 Fungal isolates

4.2.1 *Heterobasidion parviporum* (I)

Three *H. parviporum* strains were used, hosted by diverse viruses. 60 replicates of *H. parviporum*, 20 plates for each virus type were used. The *H. parviporum* strain RT3.49C hosted the partitivirus HetPV4-pa1, strain 7R18 the partitivirus HetPV2-pa1 (Vainio, Hantula, 2015a) and strain 136212 the partitivirus HetRV6-pa17 (Vainio *et al.*, 2012).

The isolate HetPV2-pa1 (*H. parviporum* strain 7R18) was isolated from spruce dominated Norway spruce forest in southern Finland (Ruotsinkylä, Vantaa) heavily infected with *Heterobasidion* spp. (Piri, Korhonen, 2008; Vainio *et al.*, 2011b). The isolate HetPV4-pa1 (*H. parviporum* strain RT3.49C) was isolated from Finland and HetRV6-pa17 (*H. parviporum* strain 136212) from Estonia.

4.2.2 *Phlebiopsis gigantea* (II)

The *P. gigantea* strain (93073) infected with the virus strain PgLV-1 (Lim *et al.*, 2011) and the Rotstop® strain (Verdera Oy) without any viruses, were isolated by K. Korhonen from spruce wood in Finland (Korhonen *et al.*, 1994). In experiments, 40 replicates of *P. gigantea*, 20 plates for each strain were used.

4.2.3 *Diplodia sapinea* (III)

The *D. sapiena* strain used in experiments was collected from Vormsi Island (western Estonia, 58°59′ N, 23°14′ E) from a Scots pine tree. The fruitbodies of *D. sapinea* were surface sterilizied in ethanol (96%) and inoculated onto filtered Scots pine needle agar media. The plates were incubated for ca 2–3 weeks at +20 °C. The mycelia of *D. sapinea* from culture edges were removed and stored at –20 °C until used for DNA isolation. 20 replicates of *D. sapinea* in the laboratory experiment were conducted. After that, 34 insects were analysed in the laboratory.

4.3 Laboratory feeding experiment

The plates were inoculated with *H. parviporum* **I**, *P. gigantea* **II** or *D. sapinea* **III** to the 2% malt extract agar (40 g malt extract, OXOID Ltd., England; 28 g agar technical, Becton, Dickinson & Co., 2 L water). After 2 weeks incubation at room temperature, the branches were totally covered by fungal mycelia (**I**, **II**, **III**).

The conifer branches were wrapped with foil perforated with small holes (0.3 × 0.3 cm) to allow feeding by the weevils and to prevent insect contamination from conidia or hyphae on their legs or exoskeleton. To keep the Petri dishes clean, the paper inside the dishes was changed.

Before the experiment, the insects were thoroughly purified both internally and externally. Insect excrements and impurities attached to their legs were removed by allowing them to adhere to moist filter papers at the bottom of 90 mm diameter glass Petri dishes (Figure 2). After 24 h, the papers were changed and more water was added. In order to empty their digestive tracts, the insects were kept without food for 48 h prior to the experiments to reduce the load of pre-existing fungal spores in the faeces, although fresh water was supplied (**I**, **II**, **III**).

Figure 2. *H. abietis* individual on a plate before experiment

One insect was placed on each experimental plate (90 mm). The pine weevils were allowed to feed on conifer branches colonized by fungal hyphae for 48 h. After feeding, the insects were removed and their faeces collected from the Petri dishes containing the feeding branch (**I**) or the insects were placed on new Petri dishes for 24 h and their faeces were collected from the Petri dishes without a feeding branch (**II**, **III**). From each insect 5 (**I**, **II**) or 4 (**III**) faeces were selected randomly. The faeces were handled with tweezers, surface sterilized for a few second in alcohol (96%), followed by rinsing in sterile water. The faeces from each weevil were disseminated onto new agar plates (MEA) in the laminar cabinet (Kojair Tech, Finland) in sterile conditions. The antibiotic thiabendazole

(20 g malt extract, 15 g agar, 1 L water, autoclave sterilization for 20 min by 120 °C, 230 mg thiabendazole, 1 mL lactic acid, small amount (4 mL) of sterile water for making the thiabendazole soluble) (ICN Biomedicals Inc., USA) was added to the plates. They were then incubated for two weeks at room temperature. Thereafter, the faeces were examined under a dissecting microscope and emerging hyphae (Figure 3) of *H. parviporum* (**I**), *P. gigantea* (**II**), *D. sapinea* (**III**) were harvested and inoculated onto new Petri dishes with malt extract agar (2% MEA).

Figure 3. The emerging fungus growing from *H. abietis* faeces

The harvested isolates of fungi from one plate (per 5 or 4 faeces) were inoculated onto the same Petri dishes because all the faeces on single plates originated from the same insect.

4.4 Genotype determination (I, II)

The somatic incompatibility (Stenlid, 1985) of the emerged fungal strains was tested to compare isolates cultured from the insect faeces. The isolates were paired with the corresponding original strains of *H. parviporum* (7R18, RT3.49C, 136212) (**I**) and *P. gigantea* (93073, Rotstop) (**II**). The cultures were inoculated on 2% MEA. The original strains and faecal isolates were disseminated at a distance of 70 mm from each other. The plates were incubated at room temperature until mycelial contact between

colonies had occurred (**II**) and the plates were checked after 14 and 28 days (**I**). Somatic compatibility was assumed when fusion of the hyphae without a detectable incompatibility zone was evident. Somatic compatibility indicates that the emergent strains were identical in genotype with the original strains used for the feeding experiments. When the paired strains were somatically incompatible a clear demarcation zone between the colonies formed (**II**). The genetic equality or inequality of the faecal *H. parviporum* isolates with the original strains was determined based on somatic incompatibility (**I**).

4.5 Counting of oidial spores (II)

The oidial counting was done according to Sun *et al.* (2009). The 0.1 g mycelia of *P. gigantea* was added to 500 µL water in an Eppendorf tube, followed by shaking of the suspension for 1 min. Then, 5 µL of the suspension was applied to the microscope glass slide, and the oidia were counted using 200 times magnification.

4.6 Isolation of hyphal tips and single oidial spores (II)

To investigate the stability of PgLV-1 in *P. gigantea* 93073, we tested whether the virus could be excluded from the host using hyphal tip isolation, oidial isolation or temperature treatment. Individual hyphal tips were picked under the microscope from *P. gigantea* 93073 cultivated on 2% MEA plates for 1–2 days at +20–25 °C using a modified Pasteur pipette as described in Vainio *et al.* (2011b).

Additionally, single oidial spores of *P. gigantea* 93073 were isolated. Fungal isolates were cultivated onto 2% MEA plates for ca. 3 weeks at +20–25 °C. Thereafter, spores were collected by washing with 10 mL of deionised sterile water and by moving a glass triangle on the colony surface for a short time. A few droplets (50–100 µL) of the suspension were then spread with a sterile glass triangle onto a Petri dish containing water agar. After 1–2 days of incubation, germinating single spores were transferred using a modified Pasteur pipette onto new MEA plates. To analyse the temperature treatment the plates were inoculated (2% MEA) and incubated at +31 °C for 10 days.

4.7 Growth rate measurements and statistical testing (II)

The growth rate of virus infected *P. gigantea* 93073 hosting virus PgLV-1 was compared with that of virus free single oidial isolation (strains OI023, OI028, OI029) and by hyphal tip isolate (strain HT05). Similarly, Rot-stop strain was included. For this purpose, a piece of mycelium (5 mm in diameter) was inoculated in the middle of a MEA plate (2%) and allowed to grow for 3 days. Six replicate cultures were analysed for each fungal isolate. The area occupied by fungal mycelia was measured (cm^2) using a planimeter (Tamaya Planix 10S Marble, Topgeo Oy, Finland). The effect of PgLV-1 on the growth rate of *P. gigantea* 93073 was analysed using the Student's t-test (Student, 1908).

4.8 Virus detection by RT-PCR

4.8.1 H. *parviporum* (I)

Total nucleic acid samples were used to conduct reverse transcription (RT) using the protocol of Vainio *et al*. (1998) with some modifications. Briefly, the fungal cells were first disrupted using at FastPrep FP120 homogenizer and 1- to 2-mm quartz sand grains and then purified by phenol–chloroform extractions. Nucleic acids were then precipitated using polyethylene glycol. In order to prevent RNA degradation, the cell lysate was not incubated at +65 °C after cell lysis. The RT reactions were set up using 5–12 µL of the nucleic acid sample (denatured in a boiling water bath for 5 min and chilled at –80 °C), 0.2 µg of random hexamer primer (Fermentas GmbH, Germany), 200 U of RevertAid MuLV reverse transcriptase (Fermentas GmbH, Germany) and dNTPs and buffer as recommended by the manufacturer.

4.8.2 PCR with virus specific primers (I)

The presence of viruses after the fungal isolates had passed through the digestive tract was screened by RT-PCR with specific primers. Three different primer pairs were used to investigate the presence of viruses in the *H. parviporum* isolates. The virus strain HetRV4-pa1 was screened using the specific primer pair RTmidF and RTmidR, while HetRV2 was screened using the primers 7RPForB and 7RPRevB. The HetRV6 was screened using the consensus primer pair HV6F1A and HV6Re2 as described in

Vainio *et al*. (2012). The PCRs were conducted in volumes of 50 µL, including 1–2 µl of the cDNA product, 25 pmol of each primer, 1 U of Dynazyme TM 451 II DNA polymerase (Finnzymes, Finland) and 10 nmol of dNTPs. The amplification conditions for 7RPForB/7RPRevB were as follows: 10 min at +95 °C, followed by 35 cycles of 30 s at +95 °C, 45 s at +53 °C, 2 min at +72 °C; and a final extension of 7 min at +72 °C. For RTmidF/RTmidR, a touch-down cycling protocol was used: 10 min at +95 °C and 13 cycles of 30 s at +95 °C, 45 s at 65 –1 °C per cycle, 2 min at +72 °C; and 20 times of 30 s at +95 °C, 45 s at +52 °C, 2 min at +72 °C, with a final extension of 7 min at +72 °C. The original host isolates (RT3.49C, 7R18 and 136212) were used as positive controls, and each PCR batch included a negative control without template. At least two independent nucleic acid preparations were reverse-transcribed and subjected to RT-PCR from each isolate.

4.8.3 CF11 chromatography (I)

The presence of viral genomic dsRNA in the *H. parviporum* isolates from insect faeces was confirmed using CF11 cellulose affinity chromatography (Morris, Dodds, 1979). Briefly, about 2–3 g of mycelia were grown on modified orange serum agar plates supplemented with cellophane membranes, harvested and homogenized in a lysis buffer. The samples were then extracted by phenol and chloroform and precipitated using CF11 cellulose powder in 15% ethanol. The presence or absence of dsRNA was analysed by agarose gel electrophoresis.

4.8.4 *P. gigantea* (II)

The presence of virus PgLV-1 of *P. gigantea* strain 93073 was analysed in the hyphal tips, thermal treated mycelia and oidial isolates. Reverse transcription (RT) was conducted using total nucleic acid samples prepared using the protocol of Vainio *et al.* (2013), including phenol–chloroform extractions and nucleic acid precipitation using polyethylene glycol. The RT reactions were conducted using random hexamer priming and RevertAid MuLV reverse transcriptase (Fermentas GmbH) as described earlier (Vainio *et al*., 2011b).

The primer pair (5'-GGTTACGAAGGTGCGTCTGC-3') and (5'-GGATAGGTGCCGCGTACAGC-3') was used (Lim *et al*., 2011). The PCRs were conducted in volumes of 50 µl, including 1–2 µL of the comple-

mentary DNA (cDNA) produced in the RT reaction, 25 pmol of each primer, 1.25 U of DreamTaq DNA polymerase (Thermo Scientific) and 10 nmol of dNTPs. The amplification conditions were as follows: 5 min at +95 °C, followed by 35 cycles of 30 s at +95 °C, 45 s at +61 °C, 1 min at +72 °C; and a final extension of 7 min at +72 °C. Positive (*P. gigantea* strain 93073) and negative (PCR samples without template cDNA) controls were used. Nucleic acid extraction and RT-PCR were repeated to confirm the absence of virus PgLV-1.

4.9 DNA extraction and fungal species identification (III)

DNA extraction was prepared in two ways: (1) fungal DNA isolation from field-collected and frozen insects thereby crushing entire weevils without surface sterilization. Cell disruption was performed in a Retsch MM400 homogenizer (Retsch GmbH, Germany) using a mix of quartz sand and metal beads (2.5 mm). DNA was extracted using the E.Z.N.A Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, Georgia, USA); (2) fungal mycelia growing from the weevil faeces were analysed to determine the occurrence of *D. sapinea*. DNA was extracted from cultures using the method described by Drenkhan *et al.* (2014). The identity of fungi in either case was confirmed by species-specific polymerase chain reaction (PCR) priming (SSPP). SSPP was carried out in 20-µL reactions volumes as described by Smith and Stanosz (2006) with minor modifications (see **III**). Cycling conditions were reported by Smith and Stanosz (2006) with one modification: the length of the denaturation step was 10 min.

For taxonomical assignment, the SSU region of samples was sequenced at the Estonian Biocentre in Tartu using the DpF (Smith, Stanosz, 2006). The sequences were edited using BioEdit, version 7.0.9.0 (Hall, 1999). The database at GenBank was used to determine the identity of SSU sequences for fungal taxon confirmation.

5. RESULTS

5.1 The laboratory feeding experiment (I–III)

5.1.1 Insect weight gain during experiments (I–III)

The inoculated twigs were successfully colonized and visible mycelial coverage was observed after two weeks. *H. abietis* insects feed effectively on conifer branches and gained weight from 8 mg (**III**) to 10–13 mg (**I**) or ca. 22% (**II**) during the experiments. The plates of pure culture were checked before inoculation and no contaminations were observed.

5.1.2 *H. parviporum* (I)

Determination of the survival of *H. parviporum* strains 7R18, RT3.49C, 136212 after passing through the digestive tract of the weevils showed that *Heterobasidion* spp. grew on 50% of plates. The number of fungal colonies passing through the insects were 5, 9, 16 faecal isolates from the fungal hosts RT3.49C, 136212, 7R18, respectively. Survival rates of *H. parviporum* inside *H. abietis* was 25–80% (Table 1).

The somatic incompatibility test proved that all the isolates originated from insect faeces were identical with the corresponding original strains (7R18, RT3.49C, 136212). The insect derived cultures represented the introduced strains and were not originally present inside the insects.

5.1.3 *P. gigantea* (II)

The Rotstop strain of *P. gigantea* survived the insect feeding with 100% viability. The *P. gigantea* strain 93073 infected with the virus PgLV-1 survived with a rate of 35%. Four of the survived strains originated from Norway spruce (*P. abies*) and three from Scots pine (*P. sylvestris*) branches. The somatic incompatibility test proved that emerging strains were compatible with the original isolate (both cases: Rotstop and 93073).

5.1.4 *D. sapinea* (III)

After incubation of faeces, mycelium of *D. sapinea* was identified from 14/20 plates. The survival rate of fungus after passing through the diges-

tive tract of the pine weevil was 70%. The male and female *H. abietis* insects were equally included and no difference was observed between sexes. Whilst females are heavier than males, the damage caused did not differ significantly between the sexes.

D. sapinea presence in the faeces was identified by species-specific PCR priming (SSPP). The primers targeted the mitochondrial small subunit ribosomal DNA (mt SSU rDNA) of fungus and were used to amplify a 700 bp fragment.

Table 1. Number of analysed fungal and mycovirus strains and their survival rates

	H. parviporum			*P. gigantea*		*D. sapinea*
	RT3.49C	136212	7R18	Rotstop	PgLV-1	
The number of fungal strains passing through the insect digestive tract (from 20)	5	9	16	20	7	14
The fungal strains survival rates (%)		25–80%		100%	35%	70%
The number of viruses from fungal strains after insect passage	0	6	10	*	7	*
Viruses survival rate (%)	0	67%	63%	*	100%	*

* - viruses are not determined

5.2 Occurrence of viruses from insect faeces (I–II)

The presence of viruses is likely to be detected with molecular analysis while the tested viruses do not confer a phenotypic effect on their fungal host. *Heterobasidion* isolates were screened twice by RT-PCR using two independent cDNA samples (**I**). The virus persistence varied between 0–67% depending on the fungal strain investigated (Table 1). The virus specific primer pairs amplified PCR products of the same lengths from the original strains. PCR products with lengths 610 or 714 bp were obtained from 10 and 6 faecal isolates from strains 7R18 and 136212 (**I**), accor-

dingly. Strain RT3.49C did not have any amplification products.

All seven *P. gigantea* 93073 (PgLV-1) isolates detected from *H. abietis* faeces using the virus-specific primer pair successfully amplified a PCR product of the expected length (ca 450 bp). The virus persistence was 100% during passage through the alimentary tract.

The 13 hyphal tip isolates were examined using RT-PCR and 12 contained PgLV-1 infection (**II**). The virus (PgLV-1) was identified from 29 oidial isolates (frequency 90%), while 3 were virus-free. Temperature treatment did not significantly decrease the infection level with viruses (**II**). Fungal growth was reduced at +31 °C.

5.3 CF11 chromatography (I)

High virus concentration was detected in strain 7R18 among 7 of the 16 isolates analysed (44%). The same isolates were virus positive by RT-PCR. Three isolates of the same strain were positive by RT-PCR, but the virus was not detectable as dsRNA.

Low virus concentration was detected in strain 136212, the virus dsRNA was detected from 2 faecal isolates (22%). Both isolates of strain 136212 were positive by RT-PCR. All the samples of strain RT3.49C were virus negative analysed by CF11 chromatography and RT-PCR.

5.4 Growth rate measurements and oidial counting (II)

Comparison of the growth of the virus infected strain (PgLV-1) over 3 days with virus free isolates (HT05, OI023, OI028, OI029) revealed that the presence of virus did not significantly affect the growth rate of the host strain. The difference in area colonised by the original virus-infected *P. gigantea* strain was statistically not significant when compared with isogenic virus-free strains ($0.09<p<0.77$ using the Student's t-test).

The PgLV-1 strain had a higher concentration of spores (32.36 million spores per plate) than the Rotstop strain (24.27 million spores per plate). Nevertheless, the Rotstop strain had a faster growth rate than the virus infected PgLV-1.

5.5 Field collected insects (III)

The 34 adult pine weevils were analysed for the presence of *D. sapinea*. PCR amplification showed the presence of *D. sapinea* in 8 *H. abietis* insects (24%). The insects were analysed by SSPP. Five of the weevils carrying the DNA of *D. sapinea* originated from Kaitsemõisa (31%) and 3 from Ränna (17%).

Sequencing of the nuclear mt SSU rDNA region was used to confirm the identity of the fungus in the insect samples. All eight amplicons shared 100% similarity with sequences of *D. sapinea* in GenBank. The *D. sapinea* sequences from insect samples were deposited in GenBank under accession numbers KU954092, KU954093, KU954094, KU954095, KU954096, KU954097, KU954098 and KU954099.

6. DISCUSSION

6.1 Viruses in pathogenic and saprotrophic fungi

The studies demonstrated the ability of the large pine weevil to carry the propagules of fungi, as well as viruses inside the fungal host. The investigated fungi and the majority of viruses were able to pass through the alimentary tract of the large pine weevil and survive digestion by the large pine weevil. The presence of fungi and associated viruses were examined using various molecular methods.

The virus species HetPV2-pa1 (7R18) and HetRV6-pa17 (136212) are taxonomically different. HetRV6-pa17 is the most common virus species found from *H. annosum s.l.* (Vainio *et al.*, 2012), whilst the partitivirus species HetPV2-pa1 is rare (Vainio *et al.*, 2011b). The viruses HetPV2-pa1 and HetRV6-pa17 were present in *Heterobasidion* isolates from excrements of *H. abietis* examined using RT-PCR. The presence of viruses was 63% and 67%, respectively. However, only 22–44% of the isolates contained sufficient dsRNA to be detected by CF11 chromatography. The RT-PCR virus-specific primers identified only the specific viruses and the amount of viral template used can be low. However, CF11 chromatography requires a higher quantity of dsRNA in the host isolate than the RT-PCR. Therefore, the determination of viruses using CF11 chromatography is probably lower. The virus HetPV4-pa1 (in host *H. parviporum* RT3.49C) was not found in insect faeces.

Previously it was known that the fungal viruses are transmitted intracellularly, and therefore they should have no contact with the alimentary tract during insect feeding. However, the results have shown that virus passage through the insects might result in virus loss (**I**). This loss has been previously observed when viruses are transmitted vertically into sexual or asexual spores of *Heterobasidion* spp. species (Ihrmark *et al.*, 2002, 2004). It might also result from uneven distribution of viruses in the fungal mycelium and no viruses might be carried over if only minute amounts of hyphae survive inside the digestive tract of the insect. *Heterobasidion* spp. viruses are typically absent from part of the hyphal tips even in virus infected fungal strains (Ihrmark *et al.*, 2002). The cultural conditions can possibly affect the virus content of many fungal species, and elevated temperature or cyclohexamide treatment can diminish dsRNA replication

(Ahn, Lee, 2001; Vainio *et al.*, 2010).

Both strains (virus-free and with virus PgLV-1) of *P. gigantea* survived passage through the alimentary tract of *H. abietis*. Comparing the viability of virus-infected and virus-free strains after insect passage, 7 viable isolates of *P. gigantea* from the virus infected strain 93073 were obtained, while 13 isolates were declining. Moreover, all the virus free isolates (Rotstop, N=20) survived. The results have shown the lower viability of the virus infected strain. It is known that viruses might diminish the *P. gigantea* strain ability to function as a biocontrol agent (Lim *et al.*, 2011). The laboratory experiment has indicated that the virus free strain, biocontrol strain of Rotstop has a higher growth rate than the virus infected strain. Despite this, on media, the growth rate of oidial or hyphal tip isolates of both strains of *P. gigantea* seemed to be similar.

Most viruses infect the host fungus without alternation of fungal phenotype, others have clear effects on the host, including changes in pigmentation, mycelial growth, sporulation, and virulence (Ghabrial, Suzuki, 2009). Hyder *et al.* (2013) concluded that virus infection generally had a negative effect on the competitive ability of the host; the viruses seem to be either solely harmful or beneficial to their host. Jurvansuu *et al.* (2014) found that *H. annosum s.l.* partitivirus infections are generally symptomless, but under some conditions, i.e. new host, interfere with fungal growth. The characteristics of *D. sapinea* double-stranded RNA (dsRNA) presence are reduced virulence, reduced growth rate, lack of pigmentation, altered colony morphology, and suppressed conidiation (Steenkmap *et al.*, 1998). The fungal virus slowing down the mycelial growth of the pathogen might have a significant economic value in practical forestry (Vainio *et al.*, 2010).

6.2 Viruses and fungi transmitted by insects

Insects as virus transmission vectors are very common in nature, as insects transmit many plant and animal viruses (Gray, Banerjee, 1999). Generally, the viruses move through the insect vector, from the gut lumen into the haemolymph or other tissues into the salivary glands and during insect feeding back to the host (Hogenhout *et al.*, 2008). Plant and animal viruses in different host species have a horizontal transmission and are transmitted between species in extracellular routes (airborne, waterborne, vector-borne transmission and physical contact) (Yaegashi *et al.*, 2013).

It was presumed that the entire life cycle of mycoviruses occurs within the cytoplasm of host fungi and any vector organism was known (Kanematsu *et al*., 2010). Lately, extracellular transmission was discovered and described the insect *Lycoriella ingenua*, whose larvae fed on virus infected fungus and found that viruliferous adults could transmit SsHADV-1 – DNA virus transovarially suppressing the production of repellent volatile substances to attract adults to lay eggs on its colony (Liu *et al*., 2016). Similarly, the mycovirus *Cryphonectria hypovirus* (CHV1) was detected in excrements of *Thyreophagus corticalis* (Acari, Sarcoptiformes, Acaridae) mite that feed on virus infected fungus and thus the mite has a potential role as vector (Bouneb *et al*., 2016). Özkan and Coutts (2015) described the ability of the greater wax moth *Galleria mellonella* L. larvae to retain the pathogenicity of the fungus *Aspergillus fumigatus*. Viruses (partitiviruses, totiviruses, mitoviruses) have been found previously from insect-vectored ophiomastoid fungi, including the Dutch elm disease fungi *O. ulmi* and *O. novo-ulmi* (Buck *et al*., 2002). Our results shows the transmission of mycoviruses (**I**, **II**) via *H. abietis* digestive tract in laboratory conditions. The vectors for mycoviruses are likely to exist in nature (Bouneb *et al*., 2016; Petrzik *et al*., 2016) as many fungi could release volatiles to attract insects (Hung *et al*., 2015).

H. abietis insects collected from nature carried in their digestive tracts *D. sapinea* (**III**). *D. sapinea* as an opportunistic pathogen damages different parts of trees and causes severe damage to pines and especially after stress events, e.g. water deficiency, insect damage etc. (Sinclair, Lyon, 2005). While the habitats of large pine weevils and *D. sapinea* overlap, the mortality rate of seedlings in afforestation sites is the combined effect of weevil feeding and fungus infection (Lévieux *et al*., 1994). The seedlings are stressed after insect damage becoming more susceptible to infection by pathogens. Although, the weevil can girdle the seedling at its base and kill it, a vigorous seedling may survive insect damage as long it is not girdled. In such a case the seedling may be stressed and may decline due to other factors, e.g. stress caused by drought. Drought periods can promote spread of *D. sapinea* (Blaschke, Cech, 2007) and droughts have probably promoted establishment of this fungus in Estonia (Drenkhan, Hanso, 2009).

6.3 Insects as vectors of fungi

Large pine weevils are contaminated with various fungal spores internally (in the digestive tract) and externally (Viiri, 2004; Jankowiak, Bilański, 2013a) as the spores of fungi are naturally present everywhere. Several species of fungus have been shown to be carried by the large pine weevil (Viiri, 2004): *H. annosum s.l.* (Nuorteva, Laine, 1968, 1972; Kadlec *et al.*, 1992; Leather *et al.*, 1999; Drenkhan *et al.*, 2013) (**I**), *Leptographium* (Piou, 1993; Viiri, 2004; Jankowiak, Bilański, 2013a, 2013b), *P. gigantea* (**II**) and *D. sapinea* (**III**). The fungal spores or mycelia can be attached to the body surface or to the legs of the weevils. The fungi probably have a chance of infecting the pine seedlings while feeding (Leather *et al.*, 1999). The inocula can originate from roots of mature trees or from tree harvest debris. The large pine weevil's ability to carry fungi via faeces to seedlings in nature is not known (Lévieux *et al.*, 1994). Damage to seedlings must be considered in conjunction with weevil population density and environmental factors (Örlander *et al.*, 1997). Important factors include whether the planting area was previously forested, the time since harvesting and whether the seedlings were naturally regenerated or planted (Nordlander *et al.*, 2003b). Naturally regenerated seedlings display a higher resistance to weevil damage than planted ones (Långström, Day, 2004).

Many other bark beetles and weevils (family Curculionidae) are known as vectors for many fungal species, which transmit via insect feeding and faeces. It has been concluded that there is an association between primary scolytid species and bark beetles (*Pityogenes chalcographus*, *I. typographus*) with *P. gigantea* (Persson *et al.*, 2011; Strid *et al.*, 2014). The common pine shoot beetle (*Tomicus piniperda* L.) carries the fungi *Leptographium* spp., *Ophiostoma* spp. and *D. sapinea* (Hausner *et al.*, 2005). Ascospores of *O. ips* (Rumbold) Nannf. in the gut and faeces of adult *Ips* species (Harrington, 2005) or on the body of insect *Ips sexdentatus* (Bueno *et al.*, 2010) are known. *Hylastes ater* was found to vector sapstain fungi (*Leptographium* spp. and *Ophiostoma* spp.) to seedlings during feeding attempts (Reay *et al.*, 2001). The blue-stain fungus *Ceratocystis polonica* (Siemaszko) C. Moreau has been carried by beetles and their excised parts and detected also in the digestive tract of *I. typographus* L. (Furniss *et al.*, 1990; Viiri, Lieutier, 2004) and to be pathogenic to Norway spruce (Krokene, Solheim, 1998).

Insects are important vectors of fungi. The large pine weevil is a common insect, an important vector of fungi causing economically notable costs.

Countries using clear felling and planting as their main forest management strategy are reporting the worst pine weevil problems and likely have more diseases carried by the large pine weevil as well. Silvicultural systems based on clear-cutting seem to provide suitable conditions for large pine weevils (Daniel, 1935; Karu, 1939; Örlander *et al.*, 1997). It is clear that fungi benefit from their relationship with insects, although the benefits to the insect are not always apparent (Harrington, 2005).

7. CONCLUSIONS

The main hypothesis was proved: the saprotrophic and pathogenic fungi persisted after passing through the alimentary tract of the large pine weevil (**I**, **II**, **III**).

The second hypothesis was partly proved: the *Heterobasidion* spp. and two taxonomically different mycoviruses survived passage through the digestive tract of the large pine weevil inside their fungal hosts (**I**). However, one virus (HetPV4-pa1) did not survive. The goal of *Heterobasidion* spp. virus research is to find new virus-based methods to control root and butt rot problems in boreal forests (Hyder *et al.*, 2013). As a practical conclusion, the stability of the viruses inside the insects suggest that if similar viruses are to be used as biological control of *Heterobasidion* species, it is likely that insect feeding does not considerably decrease the virus effect.

The third and fourth hypotheses were proved: *P. gigantea* and virus PgLV-1 resisted passage through the alimentary tract of the large pine weevil inside the fungal host and retained stability after passing through the large pine weevil. The *P. gigantea* strain 93073 infected with virus PgLV-1 showed a lower viability and growth rate than the virus-free biocontrol strain. The virus did not drastically alter the growth rate of *P. gigantea* strain 93073 and virus infected plates have a higher concentration of spores. Nevertheless, a small proportion of hyphal tips and oidial spores of virus-infected mycelia seemed to be devoid of PgLV-1. The stability of viral infections is important in case the presence of viruses threatens the viability and/or efficacy of *P. gigantea* biocontrol strains.

The results support the possibility of insect-mediated transfer for *P. gigantea*. Based on this study, insect feeding does not present a comparably limiting 'bottle neck' treatment resulting in frequent virus loss, and virus infections seem to be stable in *P. gigantea*. The results indicate that large pine weevil is a potential vector of *D. sapinea* in managed pine forest. The insect could contribute to the long-range dispersal of the pathogen. The significance of large pine weevil in forestry may increase as we have more information on the ability of the insect to transmit the pathogenic fungi. The occurrence, distribution and effect of viruses in *D. sapinea* populations still requires further investigations.

REFERENCES

Adams, G.C., Wu, N.-T., Eisenberg, B.E. 2002. Virulence and double-stranded RNA in *Sphaeropsis sapinea*. Forest Pathology, 32: 309–329.

Adamson, K., Klavina, D., Drenkhan, R., Gaitinieks, T., Hanso, M. 2015. *Diplodia sapinea* is colonizing the native Scots pine (*Pinus sylvestris*) in the northern Baltics. European Journal of Plant Pathology, 143: 343–350.

Aday, A.G., Lehtijärvi, A., Doğmuş-Lehtijärvi, H.T. 2012. Frequency of virus in some *Diplodia pinea* and *Gremmeniella abietina* isolates originated from Turkey. Journal of Agricultural Extension and Rural Development, 4: 181–183.

Anagnostakis, S.L. 1982. Biological control of chestnut blight. Science, 215: 466–471.

Agrios, G.N. 2005. Plant diseases caused by viruses. In: Agrios, G.N. (ed.). Plant Pathology, fifth edition. Academic Press Inc., London, 724–820.

Agrios, G.N. 2008. Transmission of plant diseases by insects. In: Capinera, J.L. (ed.). Encyclopedia of Entomology, second edition. University of Florida, Springer, 3853–3885.

Ahn, I.-P., Lee, Y.-H. 2001. A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. Molecular Plant-Microbe Interactions, 14: 496–507.

Bao, X., Roossinck, M.J. 2013. Multiplexed interactions: Viruses of endophytic fungi. In: Ghabrial, S.A. (ed.). Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 37–54.

Bendel, M., Kienast, F., Bugmann, H., Rigling, D. 2006. Incidence and distribution of *Heterobasidion* and *Armillaria* and their influence on canopy gap formation in unmanaged mountain pine forests in the Swiss Alps. European Journal of Plant Pathology, 116: 85–93.

Berglund, M., Rönnberg, J. 2004. Effectiveness of treatment of Norway spruce stumps with *Phlebiopsis gigantea* at different rates of coverage for the control of *Heterobasidion*. Forest Pathology, 34: 233–243.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M.J., Wingfield, B.D. 2011. Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. Forest Pathology, 41: 370–375.

Bejer-Petersen, B., Juutinen, P., Kangas, E., Bakke, A., Butovitsch, V., Eidmann, H., Heqvist, K. J., Lekander, B. 1962. Studies on *Hylobius abietis* L. I. Development and life cycle in the Nordic countries. Acta

Entomologica Fennica, 17: 1–107.

Björklund, N., Nordlander, G., Bylund, H. 2003. Host-plant acceptance on mineral soil and humus by the pine weevil *Hylobius abietis* (L.). Agricultural and Forest Entomology, 5: 61–65.

Blaschke, M., Cech, T. 2007. Absterbende Weisskiefern – eine langfristige Folge des Trockenjahres 2003? Forstschutz Aktuell, 40: 32–34. [In German with English abstract]

Bouneb, M., Turchetti, T., Nannelli, R., Roversi, P.F., Paoli, F., Danti, R., Simoni, S. 2016. Occurrence and transmission of mycovirus *Cryphonectri*a hypovirus 1 from dejecta of *Thyreophagus corticalis* (Acari, Acaridae). Fungal Biology, 120: 351–351.

Brasier, C.M. 2001. Rapid evolution of introduced plant pathogens via interspecific hybridization. BioScience, 51: 123–133.

Brixey, J. 1997. The potential for biological control to reduce *Hylobius abietis* damage. Forestry Commission Research Information Note, 273, 7 p.

Buck, K.W., Brasier, C.M., Paoletti, M., Crawford, L. 2002. Virus transmission between two species of the Dutch elm disease fungus, *Ophiostoma ulmi* and *O. novo-ulmi*: d-factors as selective agents for gene introgression. In: Halls, R. (ed.). Genes and the Environment. Oxford, Blackwell Science Ltd, UK, 26–45.

Bueno, A., Diez, J.J., Fernández, M.M. 2010. Ophiostomatoid fungi transported by *Ips sexdentatus* (Coleoptera; Scolytidae) in *Pinus pinaster* in NW Spain. Silva Fennica, 44: 387–397.

Bukejs, A., Balalaikins, M. 2011. New records of beetles (Insecta: Coleoptera) in Estonia. Acta Zoologica Lituanica, 21: 235–237.

Burgess, T.I., Wingfield, M.J., Wingfield, B.D. 2004. Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. Australasian Plant Pathology, 33: 513–519.

Büttner, C., Bargen, von S., Bandte, M., Mühlbach, H.-P. 2013. Forest diseases caused by viruses. In: Gonthier, P., Nicolotti, G. (eds.). Infectious Forest Diseases, 50–75.

Bylund, H., Nordlander, G., Nordenhem, H. 2004. Feeding and oviposition rates in the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). Bulletin of Entomological Research, 94: 307–317.

Capretti, P., Korhonen, K., Mugnai, L., Romagnoli, C. 1990. An intersterility group of *Heterobasidion annosum* specialized to *Abies alba*. European Journal of Forest Pathology, 20: 231–240.

Cho, W.K., Lee, K.-M., Yu, J., Son, M., Kim, K.-H. 2013. Insight into mycoviruses infecting *Fusarium* species. In: Ghabrial, S.A. (ed.). Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 273–285.

Christiansen, E., Bakke, A. 1968. Temperature preference in adults of *Hylobius abietis* L. (Coleoptera: Curculionidae) during feeding and oviposition. Journal of Applied Entomology, 62: 83–89.

Christiansen, E., Bakke, A. 1971. Feeding activity of the pine weevil, *Hylobius abietis* L. (Col., Curculionidae), during a hot period. Norsk Entomologisk Tidsskrift, 18: 109–111.

Daniel, O. 1935. Metsakaitse. Tartu, 210 p. [In Estonian]

D'Arcy, C.J., Nault, L.R. 1982. Insect transmission of plant viruses and mycoplasmalike and rickettsialike organisms. Plant Disease, 66: 99–104.

Day, K.R., Nordlander, G., Kenis, M., Halldorson, G. 2004. General biology and life cycles of bark weevils. 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. Kluwer Academic Publishers, Dordrecht, Boston, London, 331–349.

Dawe, A.L., Nuss, D.L. 2013. Hypovirus molecular biology: From Koch's postulates to host self-recognition genes that restrict virus transmission. In: Ghabrial, S.A. (ed.). Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 109–140.

Decourcelle, T., Piou, D., Desprez-Loustau, M.-L. 2014. Detection of *Diplodia sapinea* in Corsican pine seeds. Plant Pathology, 64: 442–449.

Deng, F., Xu, R., Boland, G.J. 2003. Hypovirulence-associated double-stranded RNA from *Sclerotinia homoeocarpa* is conspecific with *Ophiostoma novo-ulmi* Mitovirus 3a-Ld. Phytopathology, 93: 1407–1414.

De Wet, J., Preisig, O., Wingfiled, B.D., Wingfield, M.J. 2008. Patterns of multiple virus infections in the conifer pathogenic fungi, *Diplodia pinea* and *Diplodia scrobiculata*. Journal of Phytopathology, 156: 725–731.

Dhar, A., Parrott, L., Hawkins, C.D.B. 2016. Aftermath of mountain pine beetle outbreak in British Columbia: Stand dynamics, management response and ecosystem resilience. Review. Forests, 7: 1–19.

Dillon, A.B., Ward, D., Downes, M.J., Griffin, C.T. 2006. Suppression of the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) in pine stumps by entomopathogenic nematodes with different foraging strategies. Biological Control, 38: 217–226.

Dillon, A., Griffin, C. 2008. Controlling the large pine weevil, *Hylobius abietis*, using natural enemies. Silviculture/Management No. 15, 8 pp.

Diminic, D., Jurc, M. 1999. Some aspects of *Sphaeropsis sapinea* presence on Austrian pine in Croatia and Slovenia. In: Vilhar, B., Grill, D., Guttenberger, H. (eds.). 2nd Slovenian symposium on plant physiology with international participation, Gozd Martuljek, Slovenia, September 30 – October 2, 1998. Phyton (Horn), 39: 231–234.

Drenkhan, R., Hanso, M. 2009. Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. Forestry Studies, 51: 49–64.

Drenkhan, R., Adamson, K., Jürimaa, K., Hanso, M. 2014. *Dothistroma septosporum* on firs (*Abies* spp.) in the northern Baltics. Forest Pathology, 44: 250–254.

Drenkhan, T., Hanso, S., Hanso, M. 2008. Effect of the stump treatment with *Phlebiopsis gigantea* against root rot in Estonia. Baltic Forestry, 14: 16–26.

Drenkhan, T., Sibul, I., Kasanen, R., Vainio, E.J. 2013. Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. Forest Pathology, 43: 317–323.

Eicker, A., Botha, W.J., Coetzee, J. 1990. The occurrence of a virus-associated disease in the cultivated mushroom *Agaricus Bisporus* in South Africa. Phytophylactica, 22: 311–315.

Eidmann, H.H. 1974. *Hylobius* Schönh. In: Schwenke, W. (ed.). Die Forstschädlinge Europas, vol. 2. Verlag Paul Parey, Hamburg, Berlin, 275–293. [In German]

Eidmann, H.H., Nordenhem, H., Weslien, J. 1996. Physical protection of conifer seedlings against pine weevil feeding. Scandinavian Journal of Forest Research, 11: 68–75.

Eidmann, H.H. 1997. Assessment of pine weevil numbers on clear-cut and forest sites with shelter boards and pitfall traps. Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz, 70: 68–72.

Eriksson, J., Hjortstam, K., Ryvarden, L. 1981. The Corticiaceae of North Europe, Vol. 6. *Phlebia-Sarcodontia*. Fungiflora, Oslo, Norway, 1048–1276.

European Commission. 2017. Sustainable use of pesticides. http://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides_en [Accessed: 27.01.2017]

Faccoli, M., Henry, C.J. 2003. Host location by chemical stimuli in *Bracon hylobii* (Ratzeburg) (Hymenoptera: Braconidae), a larval parasitoid of *Hylobius abietis* (L.) (Coleoptera: Curculionidae). Annales de la Societe

Entomologique de France, 39: 247–256.

Fedderwitz, F., Björklund, N., Ninkovic, V., Nordlander, G. 2012. Pine weevil (*Hylobius abietis*) feeding pattern on conifer seedlings. In: Spink, A., Grieco, F., Krips, O., Loijens, L., Noldus, L., Zimmerman, P. (eds.). Proceedings of Measuring Behaviour 2012. 8th International Conference on Methods and Techniques in Behavioral Research. Utrecht, The Netherlands, August 28–31, 2012, 333–337.

Flowers, J., Nuckles, E., Hartman, J., Vaillancourt, L. 2001. Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. Plant Disease, 85: 1107–1112.

Forestry Commission. 2017. Weevil biology. http://www.forestry.gov.uk/forestry/infd-5tuf7e [Accessed: 20.01.2017].

Furniss, M.M., Solheim, H., Christiansen, E. 1990. Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce. Annals of the Entomological Society of America, 83: 712–716.

GenBank. 2017. http://www.ncbi.nlm.nih.gov/genbank [Accessed: 20.01.2017].

Ghabrial, S.A. 1998. Origin, adaptation and evolutionary pathways of fungal viruses. Virus Genes, 16: 119–131.

Ghabrial, S.A., Suzuki, N. 2009. Viruses of plant pathogenic fungi. Annual Review of Phytopathology, 47: 353–384.

Ghabrial, S.A. (ed.) 2013. Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 386 p.

Ghabrial, S.A., Castón, J.R., Jiang, D., Nibert, M.L., Suzuki, N. 2015. 50-plus years of fungal viruses. Virology, 479–480: 356–368.

Gonthier, P., Garbelotto, M., Varese, G.C., Nicolotti, G. 2001. Relative abundance and potential dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests. Canadian Journal of Botany, 79: 1057–1065.

Gonthier, P., Thor, M. 2013. Annosus root and butt rots. In: Gonthier, P., Nicolotti, G. (eds.). Infectious Forest Diseases. CAB International, London, UK, 128–158.

Gray, S.M., Banerjee, N. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbiology and Molecular Biology Reviews, 63: 128–148.

Greig, B.J.W. 1976. Biological control of *Fomes annosus* by *Peniophora gigantea*. European Journal of Forest Pathology, 6: 65–71.

Greig, B.J.W. 1998. Field recognition and diagnosis of *Heterobasidion annosum*. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (eds.). *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, UK, 35–41.

Gupta, S., Dikshit, A.K. 2010. Biopesticides: An eco-friendly approach for pest control. Journal of Biopesticides, 3: 186–188.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95–98.

Hanso, M., Drenkhan, R. 2009. *Diplodia pinea* is a new pathogen on Austrian pine (*Pinus nigra*) in Estonia. Plant Pathology, 58: 797.

Harrington, T.C. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Vega, F.E., Blackwell, M. (eds.). Ecological and Evolutionary Advances in Insect-Fungal Associations. UK, Oxford University Press, 257–291.

Hausner, G., Iranpour, M., Kim, J.J., Breuil, C., Davis, C.N., Gibb, E.A., Reid, J., Loewen, P.C., Hopkin, A.A. 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium terebrantis*, *Leptographium truncatum*, and *Leptographium wingfieldii*. Canadian Journal of Botany, 83: 1222–1237.

Heiniger, U., Rigling, D. 1994. Biological control of chestnut blight in Europe. Annual Review of Phytopathology, 32: 581–599.

Hodges, C.S. 1969. Modes of infection and spread of *Fomes annosus*. Annual Review of Phytopathology, 7: 247–266.

Hogenhout, S.A., Ammar, El-D., Whitfield, A.E., Redinbaugh, M.G. 2008. Insect vector interactions with persistently transmitted viruses. Annual Review of Phytopathology, 46: 327–359.

Hohn, T. 2007. Plant virus transmission from the insect point of view. Proceedings of the National Academy of Sciences of the United States of America, 104: 17905–17906.

Hollings, M. 1962. Viruses associated with a dieback disease of cultivated mushroom. Nature, 196: 962–965.

Hoy, M.A. 2008. Classical biological control. In: Capinera, J.L. (ed.). Encyclopedia of Entomology, second edition. University of Florida, Springer, 906–923.

Hsiau, P.T.W., Harrington, T.C. 2003. Phylogenetics and adaptations of Basidiomycetous fungi fed upon by bark beetles (Coleoptera: Scolyti-

dae). Symbiosis, 34: 111–131.

Huang, S., Ghabrial, S.A. 1996. Organization and expression of the double-stranded RNA genome of *Helminthosporium victoriae* 190S virus, a totivirus infecting a plant pathogenic filamentous fungus. Proceedings of the National Academy of Sciences of the United States of America, 93: 12541–12546.

Hung, R., Lee, S., Bennett, J.W. 2015. Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology, 99: 3395–3405.

Hunt, R.S., Cobb, F.W. Jr. 1982. Potential arthropod vectors and competing fungi of *Fomes annosus* in pine stumps. Canadian Journal of Plant Pathology, 4: 247–253.

Hyder, R., Pennanen, T., Hamberg, L., Vainio, E.J., Piri, T., Hantula, J. 2013. Two viruses of *Heterobasidion* confer beneficial, cryptic or detrimental effects to their hosts in different situations. Fungal Ecology, 6: 387–396.

International Committee on Taxonomy of Viruses. 2015. http://www.ictvonline.org/ [Accessed: 20.01.2017].

Ihrmark, K. 2001. Double-stranded RNA elements in the root rot fungus *Heterobasidion annosum*. Dissertation, Swedish University of Agricultural Sciences, 36 pp.

Ihrmark, K., Johannesson, H., Stenström, E., Stenlid, J. 2002. Transmission of double-stranded RNA in *Heterobasidion annosum*. Fungal Genetics and Biology, 36: 147–154.

Ihrmark, K., Stenström, E., Stenlid, J. 2004. Double-stranded RNA transmission through basidiospores of *Heterobasidion annosum*. Mycological Research, 108: 149–153.

Inward, D.J.G., Wainhouse, D., Peace, A. 2012. The effect of temperature on the development and life cycle regulation of the pine weevil *Hylobius abietis* and the potential impacts of climate change. Agricultural and Forest Entomology, 14: 348–357.

Jacobi, W.R., Koski, R.D., Negron, J.F. 2013. Dutch elm disease pathogen transmission by the banded elm bark beetle *Scolytus schevyrewi*. Forest Pathology, 43: 232–237.

Jankowiak, R., Bilański, P. 2013a. Diversity of ophiostomatoid fungi associated with the large pine weevil, *Hylobius abietis*, and infested Scots pine seedlings in Poland. Annals of Forest Science, 70: 391–402.

Jankowiak, R., Bilański, P. 2013b. Ophiostomatoid fungi associated with

root-feeding bark beetles on Scots pine in Poland. Forest Pathology, 43: 422–428.

Johansson, S.M., Lundgren, L.N., Asiegbu, F.O. 2004. Initial reactions in sapwood of Norway spruce and Scots pine after wounding and infection by *Heterobasidion parviporum* and *H. annosum*. Forest Pathology, 34: 197–210.

Jurvansuu, J., Kashif, M., Vaario, L., Vainio, E., Hantula, J. 2014. Partitiviruses of a fungal forest pathogen have species-specific quantities of genome segments and transcripts. Virology, 462–463: 25–33.

Kadlec, Z., Starý, P., Zumr, V. 1992. Field evidence for the large pine weevil, *Hylobius abietis* as a vector of *Heterobasidion annosum*. European Journal of Forest Pathology, 22: 316–318.

Kallio, T. 1965. Studies on the biology of distribution and possibilities to control *Fomes annosus* in southern Finland. Acta Forestalia Fennica, 78: 1–18. [In Finnish with English summary]

Kanematsu, S., Sasaki, A., Onoue, M., Oikawa, Y., Ito, T. 2010. Extending the fungal host range of a *Partitivirus* and a *Mycoreovirus* from *Rosellinia necatrix* by inoculation of protoplasts with virus particles. Phytopathology, 100: 922–930.

Karu, A. 1939. Männikärsakad ja juureüraskid – tähtsaimad okaspuuuuenduste kahjurid. Eesti Mets, 10: 357–363. [In Estonian]

Kenigsvalde, K., Brauners, I., Korhonen, K., Zaluma, A., Mihailova, A., Gaitnieks, T. 2015. Evaluation of the biological control agent Rotstop in controlling the infection of spruce and pine stumps by *Heterobasidion* in Latvia. Scandinavian Journal of Forest Research, 31: 254–261.

Khater, H.F. 2012. Prospects of botanical biopesticides in insect pest management. Pharmacologia, 3: 641–656.

Kondo, H., Kanematsu, S., Suzuki, N. 2013. Viruses of the white root rot fungus, *Rosellinia necatrix*. In: Ghabrial, S.A. (ed.). Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 177–214.

Korhonen, K. 1978. Intersterility groups of *Heterobasidion annosum*. Communicationes Instituti Forestalis Fenniae, 94: 1–25.

Korhonen, K. 1993. Juurikäävän torjunta. In: Kurkela, T., Lipponen, K. (eds.). Metsänsuojelututkimuksen tuloksia. Metsäntutkimuspäivä Vantaalla 9.12.1992. Metsäntutkimuslaitoksen tiedonantoja, Research Papers 460. The Finnish Forest Research Institute, 53–60. [In Finnish]

Korhonen, K., Bobko, I., Hanso, S., Piri, T., Vasiliauskas, R. 1992. Intersterility groups of *Heterobasidion annosum* in some spruce and

pine stands in Byelorussia, Lithuania and Estonia. European Journal of Forest Pathology, 22: 384–391.

Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., Seiskari, P., Niemi, M. 1994. Control of *Heterobasidion annosum* by stump treatment with 'Rotstop', a new commercial formulation of *Phlebiopsis gigantea*. In: Johansson, M., Stenlid, J. (eds.). Proceedings of the 8th International Conference on root and butt rots, Wik, Sweden & Haikko, Finland, 9–16 August 1993. Swedish University of Agricultural Sciences, 675–685.

Korhonen, K., Stenlid, J. 1998. Biology of *Heterobasidion annosum*. In: Woodward, S., Stenlid, J., Karjalainen, R., Huttermann, A. (eds.). *Heterobasidion annosum*: Biology, Ecology, Impact, and Control. CAB International, Wallingford, UK, 43–70.

Kozlakidis, Z., Hacker, C.V., Bradley, D., Jamal, A., Phoon, X., Webber, J., Brasier, C.M., Buck, K.W., Coutts, R.H.A. 2009. Molecular characterisation of two novel double-stranded RNA elements from *Phlebiopsis gigantea*. Virus Genes, 39: 132–136.

Krokene, P., Solheim, H. 1998. Pathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles. Phytopathology, 88: 39–44.

Krupovic, M., Ghabrial, S.A., Jiang, D., Varsani, A. 2016. *Genomoviridae*: a new family of widespread single-stranded DNA viruses. Archives of Virology, 161: 2633–2643.

Käärt, U. 2016. Seeneriigi varjatud elurikkus. Ajaleht Sirp, 14.10.2016. [In Estonian]

La Porta, N., Apostolov, K., Korhonen, K. 1998. Intersterility groups of *Heterobasidion annosum* and their host specificity in Bulgaria. European Journal of Forest Pathology, 28: 1–9.

Långström, B., Day, K.R. 2004. Damage, control and management of weevil pests, especially *Hylobius abietis*. 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. Kluwer Academic Publishers, Dordrecht, Boston, London, 415–444.

Leach, J.G. 1935. Insects in relation to plant diseases. Botanical Review, 1: 448–466.

Leather, S.R., Ahmed, S.I., Hogan, L. 1994. Adult feeding preferences of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). European Journal of Entomology, 91: 385–389.

Leather, S.R., Day, K.R., Salisbury, A.N. 1999. The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? Bulletin of Entomological Research, 89: 3–16.

Lévieux, J., Piou, D., Cassier, P., André, M., Guillaumin, D. 1994. Association of phytopathogenic fungi for the Scots pine (*Pinus sylvestris* L.) with the European pine weevil *Hylobius abietis* (L.) (Col. Curculionidae). The Canadian Entomologist, 126: 929–936.

Lim, J.M., Jamal, A., Phoon, X., Korhonen, K., Coutts, R.H.A. 2011. Incidence of *Phlebiopsis gigantea* large virus-1 in a collection of *Phlebiopsis gigantea* isolates. Archives of Virology, 156: 2091–2094.

Lindström, A., Hellqvist, C., Gyldberg, B., Långström, B., Mattsson, A. 1986. Field performance of a protective collar against damage by *Hylobius abietis*. Scandinavian Journal of Forest Research, 1: 3–15.

Linnakoski, R., De Beer, Z.W., Rousi, M., Niemelä, P., Pappinen, A., Wingfield, M.J. 2008. Fungi, including *Ophiostoma karelicum* sp. nov., associated with *Scolytus ratzeburgi* infesting birch in Finland and Russia. Mycological Research, 112: 1475–1488.

Linnakoski, R., De Beer, Z.W., Duong, T.A., Niemelä, P., Pappinen, A., Wingfield, M.J. 2012. *Grosmannia* and *Leptographium* spp. associated with conifer-infesting bark beetles in Finland and Russia, including *Leptographium taigense* sp. nov. Antonie van Leeuwenhoek, 102: 375–399.

Liu, S., Xie, J., Cheng, J., Li, B., Chen, T., Fu, Y., Li, G., Wang, M., Jin, H., Wan, H., Jiang, D. 2016. Fungal DNA virus infects a mycophagous insect and utilizes it as a transmission vector. Proceedings of the National Academy of Sciences of the United States of America, 113: 12803–1208.

Luchi, N., Mancini, V., Feducci, M., Santini, A., Capretti, P. 2012. *Leptoglossus occidentalis* and *Diplodia pinea*: a new insect-fungus association in Mediterranean forests. Forest Pathology, 42: 246–251.

Luchi, N., Oliveira Longa, C.M., Danti, R., Capretti, P., Maresi, G. 2014. *Diplodia sapinea*: the main fungal species involved in the colonization of pine shoots in Italy. Forest Pathology, 5: 372–381.

Luik, A., Voolma, K., Őunap, H. 1995. Taimsete vesitõmmiste mõjust hariliku männikärsaka (*Hylobius abietis* L.) küpsussööma-käitumisele. (The influence of some plant water extracts on the maturation feeding behaviour of *Hylobius abietis* L.). EPMÜ teadustööde kogumik, 181: 85–98. [In Estonian with English summary].

Luik, A., Sibul, I., Voolma, K. 2000. Influence of some plant extracts and neem preparations on the maturation feeding of the large pine weevil,

Hylobius abietis L. Baltic Forestry, 6: 53–58.

Maavara, V., Merihein, A., Parmas, H., Parmasto, E. 1961. Metsakaitse. Tallinn, Eesti Riiklik Kirjastus, 734 pp. [In Estonian]

Mahendrappa, M.K., Foster, N.W., Weetman, G.F., Krause, H.H. 1986. Nutrient cycling and availability in forest soils. Canadian Journal of Soil Science, 66: 547–572.

Manlove, J.D., Styles, J., Leather, S.R. 1997. Feeding of the adults of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). European Journal of Entomology, 94: 153–156.

Månsson, P.E., Schlyter, F. 2004. *Hylobius* pine weevils adult host selection and antifeedants: feeding behaviour on host and non-host woody Scandinavian plants. Agricultural and Forest Entomology, 6: 165–171.

Márquez, L.M., Redman, R.S., Rodriguez, R.J., Roossinck, M.J. 2007. A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance. Science, 315: 513–515.

McCabe, P.M., Pfeiffer, P., van Alfen, N.K. 1999. The influence of dsRNA viruses on the biology of plant pathogenic fungi. Trends in Microbiology, 7: 377–381.

Menkis, A., Östbrant, I.-L., Davydenko, K., Bakys, R., Balalaikins, M., Vasaitis, R. 2016. *Scolytus multistriatus* associated with Dutch elm disease on the island of Gotland: phenology and communities of vectored fungi. Mycological Progress, 15: 1–8.

Meredith, D.S. 1959. The infection of pine stumps by *Fomes annosus* and other fungi. Annals of Botany, 23: 455–476.

Merivee, E., Sibul, I., Luik, A. 1998. Die1 dynamics of feeding and locomotor activities in the large pine weevil, *Hylobius abietis*. Baltic Forestry, 4: 59–62.

Mesanza, N., Iturritxa, E. 2012: Root and butt rot caused by *Heterobasidion annosum* in Atlantic coniferous ecosystems of Spain. Forest Pathology, 42: 514–520.

Morris, T.J., Dodds, J.A. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology, 69: 854–858.

Munck, I.A., Smith, D.R., Sickley, T., Stanosz, G.R. 2009. Site-related influences on cone-borne inoculum and asymptomatic persistence of *Diplodia* shoot blight fungi on or in mature red pines. Forest Ecology and Management, 257: 812–819.

Munck, I.A., Stanosz, G.R. 2010. Longevity of inoculum production by *Diplodia pinea* on red pine cones. Forest Pathology, 40: 58–63.

Munda, A. 1994. Preliminary report on the distribution of *Heterobasidion annosum* intersterility groups in Slovenia. In: Johansson, M., Stenlid, J. (eds.). Proceedings of the 8th International Conference on root and butt rots, Wik, Sweden & Haikko, Finland, 9–16 August 1993. Swedish University of Agricultural Sciences, 272–275.

Munro, J.W. 1928. The biology and control of *Hylobius abietis* L. Part I. Forestry, 2: 31–39. [Abstract]

Nibert, M.L., Tang, J., Xie, J., Collier, A.M., Ghabrial, S.A., Baker, T.S., Tao, A.M. 2013. 3D Structures of fungal partitiviruses. In: Ghabrial, S.A. (ed.). Advances in Virus Research 86: Mycoviruses. Academic Press, Elsevier, 59–81.

Nibert, M.L., Ghabrial, S.A., Maiss, E., Lesker, T., Vainio, E.J., Jiang, D., Suzuki, N. 2014. Taxonomic reorganization if family *Partitiviridae* and other recent progress in partitivirus research. Review. Virus Research, 188: 128–141.

Nicolotti, G., Gonthier, P., Varese, G.C. 1999. Effectiveness of some biocontrol and chemical treatments against *Heterobasidion annosum* on Norway spruce stumps. European Journal of Forest Pathology, 29: 339–346.

Niemelä, T., Korhonen, K. 1998. Taxonomy of the genus Heterobasidion. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (eds.). *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, UK, 27–41.

Nordenhem, H. 1989. Age, sexual development, and seasonal occurrence of the pine weevil *Hylobius abietis* (L.). Journal of Applied Entomology, 108: 260–270.

Nordenhem, H., Eidmann, H.H. 1991. Response of the pine weevil *Hylobius abietis* L. (Col., Curculionidae) to host volatiles in different phases of its adult life cycle. Journal of Applied Entomology, 112: 353–358.

Nordenhem, H., Nordlander, G. 1994. Olfactory oriented migration through soil by root-living *Hylobius abietis* (L.) larvae (Col., Curculionidae). Journal of Applied Entomology, 117: 457–462.

Nordlander, G., Örlander, G., Langvall, O. 2003a. Feeding by the pine weevil *Hylobius abietis* in the relation to sun exposure and distance to forest edges. Agricultural and Forest Entomology, 5: 191–198.

Nordlander, G., Bylund, H., Örlander, G., Wallertz, K. 2003b. Pine weevil

population density and damage to coniferous seedlings in a regeneration areas with and without shelterwood. Scandinavian Journal of Forest Research, 18: 438–448.

Nordlander, G., Bylund, H., Björklund, N. 2005. Soil type and microtopography influencing feeding above and below ground by the pine weevil *Hylobius abietis*. Agricultural and Forest Entomology, 7: 107–113.

Nordlander, G., Nordenhem, H., Hellqvist, C. 2009. A flexible sand coating (Conniflex) for the protection of conifer seedlings against damage by the pine weevil *Hylobius abietis*. Agricultural and Forest Entomology, 11: 91–100.

Nuorteva, M., Laine, L. 1968. Über die Möglichkeiten der Insekten als Überträger des Wurzelschwamms (*Fomes annosus* (Fr.) Cooke). Annales Entomologici Fennici, 34: 113–135. [In German]

Nuorteva, M., Laine, L. 1972. Lebensfähige Diasporen des Wurzelschwamms (*Fomes annosus* (Fr.) Cooke) in den Exkrementen von *Hylobius abietis* L. (Col., Curculionidae). Annales Entomologici Fennici, 38: 119–121. [In German]

Nuss, D.L. 2005. Hypovirulence: Mycoviruses at the fungal–plant interface. Nature Reviews. Microbiology, 3: 632–642.

Olenici, N., Olenici, V., Manea, A.I., Tomescu, R. 2014. Efficacy of conifer seedling against pine weevil damage using neonicotinoids and metaflumizone insecticides. Bulletin of the Transilvania University of Braşov. Series II: Forestry, 7: 29–36.

Oliva, J., Boberg, J., Stenlid, J. 2013. First report of *Sphaeropsis sapinea* on Scots pine (*Pinus sylvestris*) and Austrian pine (*P. nigra*) in Sweden. New Disease Reports, 27: 23.

Örlander, G., Nilsson, U., Nordlander, G. 1997. Pine weevil abundance on clear-cuttings of different ages: a 6-year study using pitfall traps. Scandinavian Journal of Forest Research, 12: 225–240.

Örlander, G., Nilsson, U. 1999. Effect of reforestation methods on pine weevil (*Hylobius abietis*) damage and seedling survival. Scandinavian Journal of Forest Research, 14: 341–354.

Örlander, G., Nordlander, G., Wallertz, K., Nordenhem, H. 2000. Feeding in the crowns of Scots pine trees by the pine weevil *Hylobius abietis*. Scandinavian Journal of Forest Research, 15: 194–201.

Örlander, G., Nordlander, G., Wallertz, K. 2001. Extra food supply decreases damage by the pine weevil *Hylobius abietis*. Scandinavian Journal of Forest Research, 16: 450–454.

Özkan, S., Coutts, R.H.A. 2015. *Aspergillus fumigatus* mycovirus causes mild hypervirulent effect on pathogenicity when tested on *Galleria mellonella*. Fungal Genetics and Biology, 76: 20–26.

Otrosina, W.J., Garbelotto, M. 2010. *Heterobasidion occidentale* sp. nov. and *Heterobasidion irregular* nom. nov.: a disposition of North American *Heterobasidion* biological species. Fungal Biology, 114: 16–25.

Pappinen, A., Weissenberg, K. 1994. The ability of the pine-top weevil to carry spores and infect Scots pine with *Endocronartium pini*. European Journal of Forest Pathology, 24: 258–263.

Parry, D.W. 1990. Plant Pathology in Agriculture. Cambridge University Press, UK, 385 p.

Persson, Y., Ihrmark, K., Stenlid, J. 2011. Do bark beetles facilitate the establishment of rot fungi in Norway spruce? Fungal Ecology, 4: 262–269.

Petersson, M. 2004. Regeneration methods to reduce pine weevil damage to conifer seedlings. Doctoral thesis, Swedish University of Agricultural Sciences, 34 p.

Petrzik, K., Sarkisova, T., Starý, J., Koloniuk, I., Hrabáková, L., Kubešová, O. 2016. Molecular characterization of a new monopartite dsRNA mycovirus from mycorrhizal *Thelephora terrestris* (Ehrh.) and its detection in soil oribatid mites (Acari: Oribatida). Virology, 489: 12–19.

Pettersson, M., Rönnberg, J., Vollbrecht, G., Gemmel, P. 2003. Effect of thinning and *Phlebiopsis gigantea* stump treatment on the growth of *Heterobasidion parviporum* inoculated in *Picea abies*. Scandinavian Journal of Forest Research, 18: 362–367.

Phillips, D.H., Burdekin, D.A. 1992. Diseases of forest and ornamental trees, second edition, The MacMillan Press Ltd., London and Basingstoke, 581 p.

Phillips, A.J.L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J., Groenewald, J., Crous, P. 2013. The *Botryosphaeriaceae*: genera and species known from culture. Studies in Mycology, 76: 51–167.

Piou, D. 1993. Rôle d'*Hylobius abietis* (L.) (Col, Curculionidae) dans le transport de *Leptographium procerum* (Kendr) Wingfet son inoculation au pin sylvestre. Annales des Sciences Forestière, 50: 297–308. [In French with English summary]

Piri, T., Korhonen, K. 2007. Spatial distribution and persistence of *Heterobasidion parviporum* genets on a Norway spruce site. Forest Pathology, 37: 1–8.

Piri, T., Korhonen, K. 2008. The effect of winter thinning on the spread of

Heterobasidion parviporum in Norway spruce stands. Canadian Journal of Forest Research, 38: 2589–2595.

Popowska-Nowak, E., Skrzecz, I., Tumialis, D., Pezowicz, E., Samborska, I., Góral, K. 2016. Entomopathogenic fungi in the soils of forest plantations: towards the control of large pine weevil, *Hylobius abietis*. Baltic Forestry, 22: 8–15.

Preisig, O., Wingfield, B.D., Wingfield, M.J. 1998. Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. Virology, 252: 399–406.

Purcell, A.H., Almeida, R.P.P. 2005. Insects as vectors of disease agents. In: Goodman, R.M. (ed.). Encyclopedia of Plant and Crop Science, Taylor & Francis Inc., 1–5.

Reay, S.D., Thwaites, J.M., Farrell, R.L., Walsh, P.J. 2001. The role of the bark beetle, *Hylastes ater* (Coleoptera: Scolytidae), as a sapstain fungi vector to *Pinus radiata* seedlings: A crisis for the New Zealand forestry industry? Integrated Pest Management Reviews, 6: 283–291.

Redfern, D.B., Stenlid, J. 1998. Spore dispersal and infection. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (eds.). *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, UK, 105–124.

Risbeth, J. 1952. Control of *Fomes annosus* Fr. Forestry, 25: 41–50.

Risbeth, J. 1963. Stump protection against *Fomes annosus*. III. Inoculation with *Peniophora gigantea*. Annals of Applied Biology, 52: 63–77.

Rishbeth, J. 1975. Stump inoculation: A biological control of *Fomes annosus*. In: Bruehl, G.W. (ed.). Biology and control of soil borne plant pathogens. International symposium, American Phytopathological Society, St Paul, MN, 158–162.

Roy, G., Cormier, M., Hamelin, R.C., Dessureault, M. 1997. Comparison of RAPD technique and somatic incompatibility tests for the identification of *Phlebiopsis gigantea* strains. Canadian Journal of Botany, 75: 2097–2104.

Rönnberg, J., Berglund, M., Johansson, U. 2007. Incidence of butt rot at final felling and a first thinning of the subsequent rotation of Norway spruce stands in South-Western Sweden. Silva Fennica, 41: 639–647.

Rykowski, K., Sierota, Z. 1983. A biologically active preparation for protecting stands against root rot. Polish Technical Review, 5: 15.

Santini, A., Faccoli, M. 2015. Dutch elm disease and elm bark beetles: a century of association. iForest, 8: 126–134.

Samils, N., Vasaitis, R., Stenlid, J. 2009. Impact of the biological control agent *Phlebiopsis gigantea* on its resident genetic structure in the Baltic Sea. Biocontrol Science and Technology, 19: 263–276.

Schlyter, F. 2004. Semiochemicals in the life of bark feeding weevils. 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. Kluwer Academic Publishers, Dordrecht, Boston, London, 351–364.

Sedlák, P., Tomšovský, M. 2014. Species distribution, host affinity and genetic variability of *Heterobasidion annosum sensu lato* in the Czech Republic. Forest Pathology, 44: 310–319.

Shigesada, N., Kawasaki, K. 2001. Invasion and the range expansion of species: effects of long-distance dispersal. In: Bullock, J.M., Kenward, R.E., Hails, R.S. (eds.). Dispersal Ecology. The 42nd Symposium of the British Ecological Society at the University of Reading, 2–5 April 2001, Blackwell Science Ltd, UK, 459 p.

Sibul, I. 2000. Abundance and sex ratio of pine weevils, *Hylobius abietis* and *H. pinastri* (Coleoptera: Curculionidae) in pine clear-cuttings of different ages. In: Metspalu, L., Mitt, S. (eds.). Proceedings of the International Conference. Tartu, Estonia, September 28–29, 2000. Transactions of the Estonian Agricultural University, 209: 186–189.

Sibul, I. 2005. The effects of some botanical insecticides and environmental factors to the behaviour and physiology of the large pine weevil, *Hylobius abietis* (L.) (Coleoptera, Curculionidae). Doctoral Dissertation, Estonian Agricultural University, 173 p.

Sibul, I. 2013. Männikärsakatõrje uued ohud ja võimalused. Maamessi Aastaraamat ja -kataloog. AS Postimees, 47–49. [In Estonian]

Sibul, I., Merivee, E., Luik, A. 1998. Hariliku männikärsaka (*Hylobius abietis* L.) ööpäevasest liikumis- ja toitumisaktiivsusest. Forestry Studies, 29: 136–145. [In Estonian with English summary]

Sibul, I., Merivee, E., Luik, A. 1999. On diurnal locomotor activity in the large pine weevil, *Hylobius abietis* L. (Coleoptera, Curculionidae). In: Elberg, K., Martin, M., Pekkarinen, A. (eds.). Proceeding of XXIV Nordic Congress of Entomology, August 8–11, 1997. Tartu, Eesti Loodusfoto, 163–166.

Sibul, I., Voolma, K. 2004. The abundance, seasonal dynamics and sex ratio of the large pine weevil, *Hylobius abietis* (L.), in reforestation areas: assessment by ground pitfalls and baited pitfall traps. In: Kangur, A. (ed.). Proceedings of the International Conference, Tartu, Estonia, May 27–29, 2004. Transactions of the Faculty of Forestry, Estonian

Agricultural University, 37: 56–62.

Sibul, I., Ploomi, A., Voolma, K. 2009. Influence of neem oil on the large pine weevil, *Hylobius abietis* L. (Coleoptera: Curculionidae). Baltic Forestry, 15: 255–261.

Sibul, I., Ploomi, A. 2016. Kaitsevaha mõju hariliku männikärsaka (*Hylobius abietis*) (Coleoptera: Curculionidae) toitumiskäitumisele laboritingimustes. In: Metspalu, L., Jõgar, K., Veromann, E., Mänd, M. (eds.). Eesti taimekaitse 95. Tartu, Eesti Maaülikool. Eesti Loodusfoto, 19–24. [In Estonian]

Sinclair, W.A., Lyon, H.H. 2005. Diseases of trees and shrubs, 2nd edition. Ithaca, Cornell University Press, United States, 660 p.

Skrzecz, I., Moore, R. 1997. The attractiveness of pine branches infected with selected wood-colonising fungi to the large pine weevil (*Hylobius abietis*). In: Gregoire, J.C., Liebhold, A.M., Stephen, F.M., Day, K.R., Salom, S.M. (eds.). Proceedings: Integrating cultural tactics into the management of bark beetle and reforestation pests. USDA Forest Service General Technical Report NE-236, 146–152.

Smith, D.R., Stanosz, G.R. 2006. A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. Plant Disease, 90: 307–313.

Solbreck, C. 1980. Dispersal distances of migrating pine weevils, *Hylobius abietis*, Coleoptera: Curculionidae. Entomologia Experimentalis et Applicata, 28: 123–131.

Stalpers, J.A. 1978. Identification of wood-inhabiting Aphyllophorales in pure culture. Studies in Mycology, 16, 248 p.

Stanosz, G.R., Smith, D.R., Guthmiller, M.A., Stanosz, J.C. 1997. Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. Mycologia, 89: 525–530.

Stanosz, G.R., Swart, W., Smith, D. 1999. RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. Mycological Research, 103: 1193–1202.

Stanosz, G.R., Smith, D.R., Albers, J.S. 2005. Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from Seven Great Lakes region nurseries. Forest Pathology, 35: 233–244.

Steenkamp, E.T., Wingfield, B.D., Swart, W.J., Wingfield, M.J. 1998. Double-stranded RNA and associated virulence in South African isolates of *Sphaeropsis sapinea*. Canadian Journal of Botany, 76: 1412–1417.

Stenlid, J. 1985. Population structure of *Heterobasidion annosum* as deter-

mined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. Canadian Journal of Botany, 63: 2268–2273.

Stenlid, J., Rayner, A.D.M. 1989. Environmental and endogenous controls of developmental pathways: variation and its significance in the forest pathogen, *Heterobasidion annosum*. Tansley Review No. 19. New Phytologist, 113: 245–258.

Stenlid, J., Redfern, D.B. 1998. Spread within the tree and stand. In: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (eds.). *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, UK, 125–141.

Strid, Y., Schroeder, M., Lindahl, B., Ihrmark, K., Stenlid, J. 2014. Bark beetles have a decisive impact on fungal communities in Norway spruce stem sections. Fungal Ecology, 7: 47–58.

Student, 1908. The probable error of a mean. Biometrika, 6: 1–25.

Sun, H., Korhonen, K., Hantula, J., Asiegbu, F.O., Kasanen, R. 2009. Use of a breeding approach for improving biocontrol efficacy of *Phlebiopsis gigantea* strains against *Heterobasidion* infection of Norway spruce stumps. FEMS Microbiology Ecology, 69: 266–273.

Šmits, A., Gaitnieks, T. 2013. Effect of conifer stump treatment with *Phlebiopsis gigantea* (Fr.) Jülich spores on development success of *Hylobius abietis* L. Mežzinātne, 27: 103–112. [In Latvian with English abstract]

von Sydow, F. 1997. Abundance of pine weevils (*Hylobius abietis*) and damage to conifer seedlings in relation to silvicultural practices. Scandinavian Journal of Forest Research, 12: 157–167.

Tainter, F.H., Baker, F.A. 1996. Foliage pathology. In: Tainter, F.H., Baker, F.A. (eds.). Principles of Forest Pathology. John Wiley and Sons, Hoboken, New Jersey, 444–497.

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., *et al*. 2014. Global diversity and geography of soil fungi. Science, 346: 1–10.

Thomas, M.C. 2008. Beetles (Coleoptera). In: Capinera, J.L. (ed.). Encyclopedia of Entomology, second edition. University of Florida, Springer, 437–447.

Thorpe, K., Day, K. 2008. Reproductive maturation in the large pine weevil *Hylobius abietis*: the relative importance of larval and adult diet. Agricultural and Forest Entomology, 10: 53–59.

Thwaites, J.M., Farrell, R.L., Hata, K., Carter, P., Lausberg, M. 2004. Sapstain fungi on *Pinus radiata* logs – from New Zealand forest to export in Japan. Journal of Wood Science, 50: 459–465.

Toivonen, R., Viiri, H. 2006. Adult large pine weevils *Hylobius abietis* feed on silver birch *Betula pendula* even in the presence of conifer seedlings. Agricultural and Forest Entomology, 8: 121–128.

Tuomivirta, T., Hantula, J. 2003. Two unrelated double-stranded RNA molecule patterns in *Gremmeniella abietina* type. A code for putative viruses of the families Totiviridae and Partitiviridae. Archives of Virology, 148: 2293–2305.

Vainio, E.J., Korhonen, K., Hantula, J. 1998. Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycological Research, 102: 187–192.

Vainio, E.J., Hantula, J. 2000. Genetic differentiation between European and North American populations of *Phlebiopsis gigantea*. Mycologia, 92: 436–446.

Vainio, E.J., Lipponen, K., Hantula, J. 2001. Persistence of a biocontrol strain of *Phlebiopsis gigantea* in conifer stumps and its effects on within-species genetic diversity. Forest Pathology, 31: 285–295.

Vainio, E.J., Hallaksela, A.-M., Lipponen, K., Hantula, J. 2005. Direct analysis of ribosomal DNA in denaturing gradients: application on the effects of *Phlebiopsis gigantea* treatment on fungal communities of conifer stumps. Mycological Research, 109: 103–114.

Vainio, E.J., Korhonen, K., Tuomivirta, T.T., Hantula, J. 2010. A novel putative partitivirus of the saprotrophic fungus *Heterobasidion ecrustosum* infects pathogenic species of the *Heterobasidion annosum* complex. Fungal Biology, 114: 955–965.

Vainio, E.J., Hakanpää, J., Dai, Y.-C., Hansen, E., Korhonen, K., Hantula, J. 2011a. Species of *Heterobasidion* host a diverse pool of partitiviruses with global distribution and interspecies transmission. Fungal Biology, 115: 1234–1243.

Vainio, E.J., Keriö, S., Hantula, J. 2011b. Description of a new putative virus infecting the conifer pathogenic fungus *Heterobasidion parviporum* with resemblance to *Heterobasidion annosum* P-type partitivirus. Archives of Virology, 156: 79–86.

Vainio, E.J., Hyder, R., Aday, G., Hansen, E., Piri, T., Doğmuş-Lehtijärvi, T., Lehtijärvi, A., Korhonen, K., Hantula, J. 2012. Population structure of a novel putative mycovirus infecting the conifer root-rot fungus *Heterobasidion annosum sensu lato*. Virology, 422: 366–376.

Vainio, E.J., Piri, T., Hantula, J. 2013. Virus community dynamics in the conifer pathogenic fungus *Heterobasidion parviporum* following an artificial introduction of a Partitivirus. Microbial Ecology, 65: 28–38.

Vaino, E.J., Hantula, J. 2015a. Taxonomy, biogeography and importance of *Heterobasidion* viruses. Virus Research, 219: 2–10.

Vainio, E.J., Müller, M.M., Korhonen, K., Piri, T., Hantula, J. 2015b. Viruses accumulate in aging infection centers of a fungal forest pathogen. The ISME Journal, 9: 497–507.

Viiri, H. 2004. Fungi associated with *Hylobius abietis* and other weevils. In: Lieutier, F., Day, K.R., Battisti, A., Grégoire, J.-C., H. F., Evans, H.F. (eds.). Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer Academic Publishers, Dordrecht, Boston, London, 381–393.

Viiri, H., Lieutier, F. 2004. *Ophiostomatoid* fungi associated with the spruce bark beetle, *Ips typographus*, in three areas in France. Annals of Forest Science, 61: 215–219.

Voolma, K. 1986. Raiestike puhastamisviisi mõjust männikärsakate arvukusele. – Metsamajandus 1984. Tallinn, 134–142. [In Estonian]

Voolma, K. 1994. Atraktantpüüniste kasutamine männikärsakate ja juureüraskite arvukuse hindamiseks. (Use of baited ground traps for monitoring pine weevil, *Hylobius abietis*, and root-colonizing bark beetles). Metsanduslikud Uurimused (Forestry Studies), 26: 96–109. [In Estonian with English summary]

Voolma, K. 2001. Harilik männikärsakas (*Hylobius abietis* L.) Räpina metskonna raiestikel: uurimus atraktantpüünistega. (The large pine weevil, *Hylobius abietis* L., in the felling areas of the Räpina forest district: a case study with baited ground traps). Metsanduslikud Uurimused (Forestry Studies), 35: 172–178. [In Estonian with English summary]

Voolma, K. 2003. Okaspuukultuure ähvardab männikärsakas. Eesti Mets, 1: 46–48. [In Estonian]

Wainhouse, D., Ashburner, R., Boswell, R. 2001. Reproductive development and maternal effects in the pine weevil *Hylobius abietis*. Ecological Entomology, 26: 655–661.

Wallertz, K., Nordlander, G., Örlander, G. 2006. Feeding on roots in the humus layer by adult pine weevil, *Hylobius abietis*. Agricultural and Forest Entomology, 8: 273–279.

Wallertz, K., Nordenhem, H., Nordlander, G. 2014. Damage by the pine weevil *Hylobius abietis* to seedlings of two native and five introduced tree species in Sweden. Silva Fennica, 48: 1–14.

Wegensteiner, R. 2004. Pathogens in bark beetles. In: Lieutier, F., Day, K.R., Battisti, A., Gregoire, J.-C., Evans, H.F. (eds.). Bark and wood

boring insects in living trees in Europe, a synthesis. Kluwer Academic Publishers, Dordrecht, 291–313.

Werner, A., Łakomy, P. 2002. Intraspecific variation in *Heterobasidion annosum* for mortality rate on *Pinus sylvestris* and *Picea abies* seedlings grown in pure culture. Mycologia, 94: 856–861.

Whitehill, J.G.A., Lehman, J.S., Bonello, P. 2007. *Ips pini* (Curculionidae: Scolytinae) is a vector of the fungal pathogen, *Sphaeropsis sapinea* (Coelomycetes), to Austrian pines, *Pinus nigra* (Pinaceae). Environmental Entomology, 36: 114–120.

Williams, C.D., Dillon, A.B., Harvey, C.D., Hennessy, R., McNamara, L., Griffin, C.T. 2013. Control of a major pest of forestry, *Hylobius abietis*, with entomopathogenic nematodes and fungi using eradicant and prophylactic strategies. Forest Ecology and Management, 305: 212–222.

Woodward, S., Stenlid, J., Karjalainen, R., Hütterman, A. (eds.). 1998. *Heterobasidion annosum*: Biology, Ecology, Impact and Control. CAB International, Wallingford, UK, 589 p.

Xiao, X., Cheng, J., Tang, J., Fu, Y., Jiang, D., Baker, T.S., Ghabrial, S.A., Xie, J. 2014. A novel partitivirus that confers hypovirulence on plant pathogenic fungi. Journal of Virology, 88: 10120–10133.

Yaegashi, H., Kanematsu, S., Ito, T. 2012. Molecular characterization of a new hypovirus infecting a phytopathogenic fungus, *Valsa ceratosperma*. Virus Research, 165: 143–150.

Yaegashi, H., Nakamura, H., Sawahata, T., Sasaki, A., Iwanami, Y., Ito, T., Kanematsu, S. 2013. Appearance of mycovirus-like double-stranded RNAs in the white root rot fungus, *Rosellinia necatrix*, in an apple orchard. FEMS Microbiological Ecology, 83: 49–62.

Yu, X., Li, B., Fu, Y., Xie, J., Cheng, J., Ghabrial, S.A., Li, G., Yi, X., Jiang, D. 2013. Extracellular transmission of a DNA mycovirus and its use as a natural fungicide. Proceedings of the National Academy of Sciences, 110: 1452–1457.

Zagatti, P., Lemperiere, G., Malosse, C. 1997. Monoterpenes emitted by the large pine weevil, *Hylobius abietis* (L.) feeding on Scots pine, *Pinus sylvestris* L. Physiological Entomology, 22: 394–400.

Zumr, V., Stary, P. 1995. LATEX paint as an antifeedant against *Hylobius abietis* (L.) (Col., Curculionidae) on conifer seedlings. Anzeiger für Schädlingskunde. Pflanzenschutz, Umweltschutz, 68: 42–43.

SUMMARY IN ESTONIAN

HARILIKU MÄNNIKÄRSAKA (*HYLOBIUS ABIETIS* L.) SEOS PATOGEENSETE JA SAPROTROOFSETE SEENTE NING VIIRUSTEGA

Sissejuhatus

Putukate ja patogeensete seente koosesinemine ohustab metsade tervist, samas võib see vastastikmõju metsakaitse seisukohalt kujuneda ka positiivseks või neutraalseks. Teadaolevalt nakatab ja surmab putukaid või mõjutab otseselt nende elutegevust umbes 1000 liiki erinevate perekondade seeni. Seevastu on ka arvukalt nii patogeenseid kui ka saprotroofseid seeneliike, mis läbivad putuka seedetrakti peremeesorganismi kahjustamata. Seega on putukad olulised patogeensete ja saprotroofsete seeneeoste edasikandjad. Kärsaklaste sugukonda (Coleoptera: Curculionidae) männikärsaka perekonda (*Hylobius*) kuuluvad liigid on võimelised levitama seeneeoseid, olles seega metsakahjustusi tekitavate seente vektoriks. Seosed putukate ja seente vahel on üldiselt tuntud. Näiteks võivad maltsaüraskid (*Scolytus* spp.) ja jalaka perekonna (*Ulmus* spp.) liikidel esinevad üraskiliigid (*Hylurgopinus rufipes*) kanda haigestunud puudelt jalakasurma tekitavaid seeni (*Ophiostoma ulmi*, *O. novo-ulmi*) tervetele puudele. Samuti on varasematest uuringutest teada, et hariliku männikärsaka (*H. abietis* L.) valmikud võivad edasi kanda juurepessu (*Heterobasidion* spp.) nakkust.

Tugeva kitiinse kehakatte ja tumepruuni põhivärvusega, umbes 1 cm pikkune mardikas – harilik männikärsakas on levinud üle kogu Euraasia, tekitades majandatavate metsade 1–5 aasta vanustes hariliku männi (*Pinus sylvestris* L.) ja hariliku kuuse (*Picea abies* (L.) Karst.) istutuskultuurides suurt majanduslikku kahju. Lisaks võib putukas suure asustustiheduse korral tarvitada toiduks teisigi okaspuid (*Juniperus communis* (L.), *Larix* spp., *Pseudotsuga* spp.) ning toidupuudusel toituda isegi lehtpuudel, närides arukase (*Betula pendula* (Roth)), hariliku saare (*Fraxinus excelsior* (L.)), lepa (*Alnus* spp.), hariliku pihlaka (*Sorbus aucuparia* (L.)) ja paju (*Salix* spp.) koort. Hariliku männikärsaka valmikud toituvad raiesmikel peamiselt okaspuutaimede tüvekoorest, põhjustades seetõttu tihti taimede ulatuslikku suremust. Mardikad võivad maapinnal liikudes nakatuda seeneeostega ning kanda haigusetekitajaid edasi jalgadega, kehapinnal ja seedetraktis. Seeneeoste siirutamine võib toimuda nii ühe langi piires kui

ka lankide vahel, sest männikärsaka valmikud migreeruvad ühelt langilt teisele, lennates soodsates oludes tuule abil isegi kümnete ja sadade kilomeetrite kaugusele. Pärast maist-juunist augustini toimuvat intensiivset küpsussööma toidutaimedel munevad emasmardikad raiesmikul olevatele värsketele, peamiselt okaspuukändude pindmistele, kuid mulla ja metsakõduga kaetud juurtele, mullaga seotud lamapuidule ja jämedamate okste alaküljele. Pärast vastsete koorumist kaevandavad need pruuni peakapsliga jalutud valkjad tõugud käike ning toituvad kännujuurte ja raiejäätmete koore all, tungides seejuures ka puitu, kus vastsed järgmise aasta suvel nukkuvad. Noormardikad väljuvad puidus olevast nukuhällist alates juulikuust ja asuvad toitumispaika otsima suve lõpul või sügisel, mõned ka järgmise aasta kevadel. Valmikud talvituvad raiesmikul mullas ja metsakõdus. Hariliku männikärsaka arvukus ja levik on otseselt seotud okaspuu-uuendusraietega, mistõttu on putukas Eestis ja kogu Euroopas muutunud ohtlikuks metsakahjuriks. Mardika tekitatava majandusliku kahju suurus Eestis on hinnanguliselt pool kuni miljon eurot aastas, Euroopas on tema põhjustatud kahju hinnatud ligikaudu 140 miljonile eurole aastas.

Juuremädanikke põhjustav patogeen juurepess (*Heterobasidion annosum sensu lato*) on kõige mõjukam majanduslikku kahju põhjustav seeneliik põhjapoolkera okaspuumetsades. Eestis on levinud männi-juurepess (*H. annosum sensu stricto* (Fr.) Bref.), mis nakatab mitmeid okaspuuliike, eriti harilikku mändi ja kadakat, samuti mitmeid lehtpuuliike (peamiselt arukaske). Kuuse-juurepessu (*H. parviporum* Niemelä & Korhonen) nakkus levib peamiselt harilikul ja sitka kuusel (*P. sitchensis* (Bong.) Carr.). Euroopas on levinud ka kolmas juurepessu liik – nulu-juurepess (*H. abietinum* Niemelä & Korhonen), mis kahjustab peamiselt euroopa nulgu (*Abies alba* Mill.) ja teisi sama perekonna liike. Juurepessu liikidest tingitud majandusliku kahju suurust hinnatakse Euroopas ligikaudu 800 miljoni euroni aastas. Juurepessu esmane nakkus tungib puitu eostega, mis nakatavad värskeid okaspuukände, millelt omakorda levib nakkus edasi puujuurtesse. Sekundaarse nakatumise käigus kandub patogeen juurekontaktide kaudu puult puule. Juurepessu eoste või mütseeli levimist võib soodustada putukvektor. Putukad siirutavad seeneeoseid puudele või rohttaimedele ning selle tulemusena toimubki nakatumine. Iseäranis on patogeeni suhtes vastuvõtlikud kevadel raiesmikele istutatud okaspuutaimed, mis võivad nakkuse tagajärjel hukkuda.

Teadaolevalt on ligikaudu 15% Euroopa ja Aasia juurepessu tüvedest vii-

rustega nakatunud. Juurepessu eosed kannavad viirusnakkust, mis levib seeneeostega ning vegetatiivselt seeneniidistiku kaudu. Viirustel ei ole peremeesorganismile (seenele) patogeenset toimet, kuid viiruse olemasolu juurepessu viljakehas võib pärssida patogeeni eoste idanemist.

Seeneliikidest on viirustega nakatunud 30–80%, kuid seeneviiruste (mükoviiruste) vektorite kohta on andmeid vähe. Mükoviiruste vektorid on teadaolevalt mõned putukad ja lestad. Näiteks *Lycoriella ingenua* (Diptera: Sciaridae), kes levitab *Sclerotinia sclerotiorum* (Lib.) de Bary DNA viirust (SsHADV-1). Taimeviiruseid kannavad edasi mitmed putukvektorid: valdavalt ülemsugukonnast Fulgoroidea ja sugukondadest Aphididae, Aleyrodidae, Cicadellidae, Chrysomelidae, Curculionidae, Pseudococcidae ning seltsidest Coleoptera, Homoptera, Thysanoptera. Taimeviiruseid siirutavad ka nematoodid, seened ja lestad (näiteks Eriophyidae).

Mükoviirused on levinud kõikides peamistes seene perekondades. Viirused koosnevad peamiselt kas üksikahelalisest või kaksikahelalisest RNA-st, harvem DNA-st. Mükoviirused on isomeetrilised osakesed läbimõõduga 25–50 nm. Viirusele iseloomulikke suuremõõtmelisi virione seenerakkudes tavapäraselt ei esine. Kui seenerakk on nakatunud mükoviirustega, on osakesed nähtavad.

Viiruste olemasolu seenerakkudes tehakse kindlaks molekulaarsete meetoditega, tuvastades RNA või DNA järjestusi. Viirused paljunevad elusates rakkudes kiiresti ja saavad küpseks nakatatud peremeesraku RNAd ning valgusünteesi ära kasutades. Mükoviiruste rühma kuuluvatel viirustel on täheldatud võimet pärssida seenhaiguste (patogeensete seente) arengut ja kasvu ning vähendada haiguste virulentsust peremeesorganismile. Mükoviirustega on tehtud katseid patogeensete seente tekitatud haiguste kontrollimiseks. Teada on, et mükoviirused on enamasti pigem kasulikud, soodustades peremeestaime kiiremat kohanemist keskkonnas toimuvate muutustega. Mõned mükoviirused vähendavad aga patogeenide põhjustatud haigussümptomeid. Seda nähtust nimetatakse hüpovirulentsuseks.

Diplodia sapinea (Fr.) Fuckel (sün. *D. pinea* (Desm.) Kickx.) on kogu maailmas okaspuudel (peamiselt männi erinevatel liikidel) levinud patogeen, mis põhjustab võrsete närbumist, kahjustab tüve, pungasid, käbisid ja tekitab puidusinetust. *D. sapinea* levib eostega lühikesi vahemaid sademete abil, kaugemale levimine toimub nakatunud puitmaterjali, taimede, vee, loomade, lindude ja putukatega. Sealhulgas on patogeeni levitajateks

erinevad juureüraskite (Coleoptera: Scolytinae) liigid, samuti kärsaklaste sugukonda kuuluv harilik männikärsakas.

Hiidkoorik (*Phlebiopsis gigantea* (Fr.) Jül. (sün. *Phlebia gigantea* (Fr.) Donk, *Phanerochaete gigantea* (Fr.) S.S. Rattan) on tavaline saprotroofne kandseen okaspuumetsades, mis asustab kände ja maapinnal olevaid oksi, põhjustades puidu valgemädanikku. Hiidkoorik on tõhus kändude esmane koloniseerija ja juurepessu antagonist. Hiidkoorik toodab rohkelt eoseid (oiide), mistõttu hiidkooriku baasil hakati valmistama preparaati Rotstop® (Verdera OY, Soome) mida kasutatakse juurepessu biotõrjel okaspuumetsades. Peamiselt kasutatakse Rotstopi harilikul männil ja harilikul kuusel, kusjuures harilikul männil on preparaadi toime osutunud tõhusamaks. Kui juurepessu nakkus puistus puudub või esineb seda vähesel määral, siis on biopreparaadi toime tõhusam. Preparaadi toimet võib pärssida eelnev tugev juurepessu nakkus. Hiidkoorikul esinevad viirused (PgLV-1, PgLV-2) võivad mõjutada hiidkooriku kasvu ja biotõrje omadusi. Viiruste pärssivat mõju seente kasvule on täheldatud ka juurepessu (*Heterobasidion* spp.) ja *D. sapinea* puhul.

Mitmed uurijad on leidnud, et nendel lageraie aladel, kus kändusid on pärast raiet Rotstopiga töödeldud, on kahanenud männikärsakate arvukus ning vähenenud kahjustatud taimede hulk. Arvatavasti on põhjus selles, et hiidkoorikuga nakatunud kännud ei ole männikärsakale haudme rajamiseks sobilikud.

Doktoritöös uuriti hariliku männikärsaka (*H. abietis*) võimet levitada saprotroofseid ja patogeenseid seeni ning nendega seotud viiruseid. Laboratoorsete katsete käigus analüüsiti seente ja viiruste elujõulisust pärast hariliku männikärsaka seedetrakti läbimist. Lisaks selgitati hariliku männikärsaka valmikute võimet levitada patogeenset seent *D. sapinea*.

Hüpoteesid

1. Patogeensed ja saprotroofsed seened on võimelised hariliku männikärsaka seedetrakti läbimisel säilitama elujõulisuse (**I**, **II**, **III**);
2. Juurepessuga seotud viirused säilitavad pärast hariliku männikärsaka seedetrakti läbimist stabiilsuse (**I**);
3. Viirustega nakatunud hiidkooriku tüve elujõulisus on nõrgem võrreldes viirusevaba hiidkooriku tüvega (**II**);
4. Viirus PgLV-1 säilitab putuka seedetrakti läbimisel nakatamisvõime (**II**);

5. Harilik männikärsakas kannab looduslikes tingimustes edasi *D. sapinea* nakkust (**III**).

Eesmärgid

1. Uurida patogeensete ja saprotroofsete seente võimet läbida elujõuliselt hariliku männikärsaka seedetrakti (**I**, **II**, **III**);
2. Selgitada hariliku männikärsaka potentsiaali kanda seedetraktis elujõulisena edasi juurepessu eoseid ja viiruseid (**I**);
3. Teha kindlaks hariliku männikärsaka tähtsus vektorina hiidkooriku ja seenega seotud viiruste edasikandmisel (**II**);
4. Teha kindlaks hiidkooriku viiruste püsivus pärast männikärsaka seedetrakti läbimist (**II**);
5. Selgitada välja viirusega nakatunud ja viirusevaba hiidkooriku tüve nakatamisvõime pärast katseputuka seedetrakti läbimist (**II**);
6. Uurida hariliku männikärsaka võimet edasi kanda *D. sapinea* nakkust looduslikes tingimustes (**III**).

Materjal ja metoodika

Putukad, seene isolaadid ja viirused (**I–III**)

Hariliku männikärsaka valmikud koguti hariliku männi lageraie aladelt Järvselja Õppe- ja Katsemetskonnast (58°27′ N, 27°33′ E) (**I**, **II**), Tartu maakonnast Kaitsemõisast (58°34′ N, 26°49′ E) ning Võru maakonnast Rännalt (57°45′ N, 26°29′ E) (**III**). Putukate kogumiseks kasutati pinnasesse kaevatud püünisauke mõõtmetega 25 × 40 × 50 cm. Püünistest leitud hariliku männikärsaka valmikud viidi laborisse ja hoiustati katsete alguseni külmkapis (+4 °C) (**I**, **II**, **III**) või surmati ja säilitati temperatuuril –20 °C kuni DNA eraldamiseni (**III**). Kõik katseputukad kaaluti enne ja pärast katset ning määrati nende sugu (**I**, **II**).

Laborikatsetes kasutati hariliku männi (*P. sylvestris*) (**I**, **II**, **III**) ja hariliku kuuse (*P. abies*) (**II**) võrseid. Võrsed (läbimõõt 3–5 mm, pikkus 6–7 cm) varuti 14- ja 17-aasta vanustest männi- ja kuusepuistutest (58°18′ N, 26°39′ E), puhastati okastest, viidi laborisse ning hoiti katseni külmkapis (+4 °C) (**I**, **II**, **III**). Vahetult enne katse läbiviimist autoklaaviti (steriliseeriti) võrseid 30 minutit +106 °C (**II**, **III**) või +120 °C juures (**I**).

Laborikatsetes kasutati kolme kuuse-juurepessu (*H. parviporum*) tüve, mis oli eelnevalt nakatunud erinevate viirustega: tüvi RT3.49C viirusega HetPV4-pa1, 7R18 viirusega HetPV2-pa1 ja 136 212 viirusega HetRV6-pa17. Iga kasutatava tüve kohta tehti 20 külvi, kokku 60 külvi (**I**).

Hiidkooriku (*P. gigantea*) tüvi (93073) oli nakatunud viirusega PgLV-1. Võrdluseks kasutati biotõrje preparaadi Rotstopi aluseks olevat hiidkooriku tüve, millest viiruseid ei ole määratud. Mõlema tüve kohta tehti 20 korduskülvi, kokku 40 külvi (**II**).

D. sapinea nakatunud hariliku männi käbid koguti Vormsi saarelt (58°59′ N, 23°14′ E). Seen isoleeriti viljakehast ja määrati liik. Laborikatseks tehti kokku 20 seenekülvi. Lisaks analüüsiti 34 kogutud hariliku männikärsaka valmikut (**III**).

Laboratoorsed katsed (**I–III**)

Kuuse-juurepessu (**I**), hiidkooriku (**II**) ja *D. sapinea* (**III**) külvid tehti 2% virdeagari söötmele (40 g linnaseekstrakti, 28 g tehnilist agarit ja 2 L vett). Petri tassid suleti Parafilm kilega ja inkubeeriti kaks nädalat toatemperatuuril.

Enne katset puhastati putukad sisemiselt ja välimiselt. Selleks asetati Petri tassi põhja niiske paber, mille külge jäi putukate jalgade ja kehakatete küljes olev mustus. Putukate kehakatetel ja jalgadel oleva mustuse eemaldamiseks vahetati tassi põhjas olevat paberit ning putukate dehüdratiseerumise vältimiseks lisati sellele vajadusel destilleeritud vett. Pärast püünisaukudest kogumist hoiustati putukaid laboratoorsete töödeni külmkapis (+4 °C). Enne katse algust tõsteti valmikud 12 tunniks toatemperatuurile. Pärast kogumist ja enne katse algust valmikuid ei toidetud vältimaks seedetraktis olnud seeneeoste esinemist putuka ekskrementideskatse jooksul.

Katse alguses asetati igasse Petri tassi üks putukas ja üks hariliku männi (**I–III**) või hariliku kuuse (**II**) võrse. Võrsed kaeti fooliumiga, mille sisse tehti kaks auku (0,3 × 0,3 cm). Aukude kaudu oli putukatel võimalik võrsete koorest toituda, kuid fooliumiga katmine aitas vältida võrsete nakatamist putukate jalgadel või seedetraktis olevate seeneeoste või -hüüfidega. Valmikud toitusid võrsetel 48 tunni jooksul. Seejärel eemaldati putukas tassist ja samalt tassilt koguti putuka ekskremendid (**I**) või pandi

putukad puhtasse Petri tassi ning eemaldati sealt 24 tunni möödudes, misjärel koguti tassi põhjal olevad ekskremendid (**II**, **III**). Igast tassist võeti pintsettide abil juhuslikkuse meetodil viis (**I**, **II**) või neli (**III**) ekskrementi. Ekskremente steriliseeriti mõne sekundi jooksul piirituses (96%) ja seejärel destilleeritud vees. Ekskremendid külvati 2% virdeagarile, millele oli lisatud antibiootikumi thiabendazole (20 g linnaseekstrakti, 15 g tehnilist agarit, 1 L vett autoklaaviti 20 min +120 °C juures, lisati 230 mg thiabendazole, 1 mL piimhapet ja 4 mL steriliseeritud vett). Ekskrementidega Petri tasse inkubeeriti toatemperatuuril kaks nädalat, Seejärel vaadeldi ekskremente mikroskoobi all. Seente (kuuse-juurepess, hiidkoorik ja *D. sapinea*) tüved inokuleeriti ekskrementidelt (**I**, **II**, **III**) uuele virdeagarile. Ühest tassist (putukast) pärinevatest ekskrementidest välja kasvanud seeneisolaadid külvati kokku ühte tassi.

Seente genotüübi uurimine (**I**, **II**)

Võrreldi seenetüvede omavahelist vastasmõju, milleks tehti somaatilise sobimatuse (inkompetibiilsuse ehk kokkusobimatuse) testid. Sama liigi kaks seenetüve (algne ja putuka ekskremendist väljakasvanud) külvati virdeagarile teineteisest ligikaudu 70 mm kaugusele. Kultuure hoiustati toatemperatuuril ning 14 ja 28 päeva hiljem kontrolliti tulemusi. Kui kahe seenetüve vahel tõkketsooni ei teki, siis on tegemist sama seenetüvega ehk seenetüved on geneetiliselt identsed. Kui tegemist on erinevate seenetüvedega, siis tekib seenetüvede vahele selge eristav tsoon (s.o somaatiline inkompetibiilsus) ehk seenetüved on geneetiliselt erinevad (**I**, **II**).

Hiidkooriku hüüfitippude ja oiidide eraldamine, kasvukiiruse mõõtmine ning statistiline analüüs (**II**)

Hiidkooriku tüve nr 93073 viiruse PgLV1 stabiilsuse uurimiseks eemaldati viirus peremeestaimest, isoleerides hüüfitipud ja oiidid. Uuriti, kas temperatuuri erinevus võib mõjutada viiruse stabiilsust. Selleks võrreldi hiidkooriku tüve 93073 (nakatunud viirusega PgLV-1) kasvukiirust viirusevabade oiidide (tüved OI023, OI028, OI029) ja hüüfitippude (tüvi HT05) isoleerimise teel. Arvutati hiidkooriku oiidide hulk mõlema hiidkooriku tüve puhul. Samuti jälgiti Rotstopi preparaadi aluseks oleva hiidkooriku tüve kasvukiirust ja oiidide idanemise tõhusust. Seenemütseeli hõivatud ala mõõdeti planimeetri (cm^2) abil. Viiruse PgLV-1 mõju hiidkooriku tüve 93073 kasvule analüüsiti Student t-testi abil.

Viiruste määramine spetsiifiliste praimeritega (**I**, **II**) ja CF11 kromatograafia abil (**I**)

Pöördtranskriptsiooni (RT) käigus toimub geneetilise informatsiooni ülekanne RNA-lt DNA-le RNA-sõltuva DNA polümeraasi abil. Pöördtranskriptsiooni (RT) läbiviimiseks järgiti tootja soovituste kohaselt protokolli (Vainio *et al*., 1998). Viiruste olemasolu tehti kindlaks pöördtranskriptsiooni polümeraasahelreaktsiooni (RT-PCR) abil, kasutades liigispetsiifilisi praimereid. Kuuse-juurepessu viiruste määramiseks kasutati kolme erinevat praimerite paari (RTmidF ja RTmidR; 7RPForB ja 7RPRevB; HV6F1A ja HV6Re2) (**I**). PCR-i teostamisel järgiti olemasolevat protokolli. Algtüvesid (RT3.49C, 7R18 ja 136212) kasutati positiivse kontrollina ja lisati negatiivne kontroll (**I**).

Hiidkooriku viiruste määramiseks tehti Vainio jt (2013) protokollile vastav protseduur (RT PCR). Viiruse kindlakstegemiseks kasutati praimerite paari (5'-GGTTACGAAGGTGCGTCTGC-3') ja (5'-GGATAGGTGCCGCGTACAGC-3'). PCR-i läbiviimisel lähtuti samuti protokollist. PCR-il lisati proovidesse positiivne (hiidkooriku tüvi 93073) ja negatiivne kontroll (**II**). Mõlemal juhul analüüsiti proove kahes korduses (**I**, **II**).

Viiruseosakeste (dsRNA) olemasolu kuuse-juurepessu tüvedes määrati CF11 kromatograafia abil, järgides vastavat protokolli (Tuomivirta, Hantula, 2003; Vainio *et al*., 2010). Viiruseosakeste olemasolu või puudumine visualiseeriti agaroos-geelelektroforeesi abil (**I**).

D. sapinea eraldamine kultuurist ja putukatest (**III**)

Hariliku männikärsaka valmikutest eraldati *D. sapinea* seejuures putukaid eelnevalt steriliseerimata. Putukad purustati Retsch MM400 homogenisaatori abil (Retsch GmbH, Saksamaa), kasutades liiva- ja metallkuule (2,5 mm). DNA eraldamiseks kasutati E.Z.N.A. DNA eraldamise komplekti (Omega Bio-Tek Inc., Norcross, Georgia, USA).

Putuka ekskrementidest välja kasvanud *D. sapinea* puhaskultuuridest patogeeni määramiseks kasutati Drenkhan jt (2014) metoodikat ning liigispetsiifilisi praimereid BotR (5′-GCTTACACTTTCATTTATAGACC-3′) ja DpF (5′-CTTATATATCAAACTATGCTTTGTA-3′) (Smith, Stanosz, 2006). Proove analüüsiti kahes korduses, lisades negatiivse ja positiivse kontrolli. PCR-i produkt visualiseeriti 1% agaroosgeelis, kasutades

Quantum ST4 süsteemi. Liigid määrati sekveneerimise (DNA-järjestuse määramine) abil Eesti Biokeskuses, kasutades praimerit DpF. Saadud tulemusi analüüsiti programmiga BioEdit 7.0.9.0 (Hall, 1999). Seenetaksoni vastavuse kinnitus saadi geenipanga andmebaasist.

Tulemused

Kõik katses kasutatud putukad toitusid männi- ja kuusevõrsetel ning saavutasid katse jooksul kaalutõusu 8 mg (III) kuni 10–13 mg (I) või 22% (II). Kuuse-juurepessu tüved (RT3.49C, 136212, 7R18) säilitasid pärast katseputuka seedetrakti läbimist elujõulisuse. Isoleeritud seenetüvede hulk oli vastavalt 5, 9, 16 isolaati ning seene elujõulisus vahemikus 25–80%. Viirusevaba hiidkooriku tüve 20 kultuurist säilitasid pärast putuka seedetrakti läbimist elujõulisuse kõik katses kasutatud kultuurid, seene elujõulisus oli 100%. Hiidkooriku tüve nr 93073 puhul õnnestus seen eraldada seitsmest kultuurist (külve tehti 20 tassi) ja seene elujõulisuseks määrati 35% (II). Juurepessu ja hiidkooriku puhul osutusid ekskrementidest isoleeritud tüved sarnaseks algtüvedega (seenetüved olid geneetiliselt identsed) (I, II). D. sapinea elujõulisus pärast putuka seedetrakti läbimist oli 70%. Erinevused putukate sugude vahel puudusid (III).

Viiruste esinemine (**I–II**) hüüfitippudes ja oiidides (**II**)

Viiruste esinemine kuuse-juurepessu tüvedes oli vahemikus 0–67%. Tüvedes 7R18 ja 136212 määrati viiruste olemasolu vastavalt kümne ja kuue putuka ekskrementidest isoleeritud seenekultuuridest. Tüvest RT3.49C viiruseid ei määratud. Määramiseks kasutati PCR-i produkti pikkusega 610 või 714 aluspaari (bp) (**I**). CF11 kromatograafia abil määrati 22–44% isolaatidest dsRNA esinemine. See meetod aitab tuvastada viiruste kontsentratsiooni seene tüvedes. Suurim oli dsRNA esinemine tüves 7R18 (44%), väikseim 136212 tüves (22%) ja tüves RT3.49C puudus.

Viirustel puudub otsene kontakt putuka seedetraktiga, kuigi tulemused näitavad, et putuka seedetrakti läbimine võib tekitada viiruse kadu (**I**). Käesolevas töös on viiruste olemasolu määratud laboritingimustes, kuid viirused võivad juurepessu liikide vahel levida ka looduses (Vainio *et al.*, 2010). Viirus võib peremeestaime suhtes olla negatiivse toimega, neutraalne või kasulik. Viirustel, mis vähendavad patogeense seene kasvu, võib praktilises metsamajanduses olla suur majanduslik mõju (Vainio *et*

al., 2010; Hyder *et al.*, 2013).

Hiidkooriku tüvest nr 93073 määrati seitsmest elujõulisuse säilitanud kultuurist viiruse olemasolu, kasutades selleks viirus-spetsiifilisi praimereid (PCR-i produkt 450 aluspaari (bp)). Antud katse tulemusel oli hiidkooriku viiruse (PgLV-1) nakatamisvõime 100% (**II**). Teadaolevalt võib viiruste olemasolu mõjutada hiidkooriku arengut ja seega ka biotõrje omadusi (Lim *et al.*, 2011). Ka käesoleva uuringu tulemustest selgus, et viirusega nakatunud hiidkooriku tüvi (93073) on ellujäämisel väiksema osakaaluga, sest 20 kultuurist säilitasid nakatamisvõime vaid 7 kultuuri.

Hiidkooriku tüve 93073 hüüfitippude ja oiidide isoleerimisel määrati nendes viiruse (PgLV-1) esinemine. Võrreldes hiidkooriku 93073 tüve oiidide kasvukiirust viirusevabade oiidide (HT05, OI023, OI028, OI029) kasvukiirusega, selgus, et viiruse olemasolu hüüfitippude ja oiidide kasvukiirust ei mõjutanud. Samuti ei esinenud olulist erinevust kahe erineva hiidkooriku tüve oiidide ja hüüfitippude analüüsimisel ($0,09<p<0,77$) (**II**). Hiidkooriku tüve 93073 oiidide kontsentratsioon oli suurem (32,36 miljon eost) kui Rotstopi preparaadi aluseks oleva hiidkooriku tüve oiidide kontsentratsioon (24,27 miljon eost). Sellest hoolimata oli Rotstopi preparaadi aluseks oleval hiidkooriku tüve elujõulisus suurem (100%) kui viirusega nakatunud hiidkooriku tüvel.

D. sapinea määramine hariliku männikärsaka valmikutest ning ekskrementidest (**III**)

Analüüsitud 34 hariliku männikärsaka valmikust määrati *D. sapinea* esinemine kaheksal putukal, s.o 24%. *D. sapinea* esinemine oli 100% vastavuses geenipanga andmetega. Harilikelt männikärsakatelt määratud *D. sapinea* järjestused numereeriti andmebaasis järgmiselt: KU954092, KU954093, KU954094, KU954095, KU954096, KU954097, KU954098 ja KU954099.

Arutelu

Viiruse tüvi HetRV6-pa17 (136212) on kõige sagedamini esinev juurepessu viirus, harvem esinevad HetPV2-pa1 (7R18) ja HetPV4-pa1 (RT3.49C). Kui tüvedest HetPV2-pa1 ja HetRV6-pa17 määrati viiruste esinemine, siis juurepessu viiruse HetPV4-pa1 tüve hariliku männikärsaka ekskrementi-

dest ei leitud. Seetõttu võib hariliku männikärsaka valmikute seedetrakti läbimine põhjustada juurepessu viiruste kadu.

Hiidkooriku viirusega nakatunud tüve (PgLV-1) elujõulisus oli 35%, mis on palju väiksem võrreldes viirusevaba tüve (Rotstopi aluseks olev hiidkooriku tüvi) elujõulisusega (100%). Viirused võivad hiidkooriku elujõulisust vähendada, mis on oluline juhul, kui viirusega nakatunud tüve kasutatakse juurepessu biotõrjel. Viiruse olemasolu seenes võib tähendada biotõrje väiksemat tõhusust.

Putukad on levinud seente ja nendega seotud viiruste edasikandjad. Kärsaklaste (Curculionidae) sugukonda kuuluv harilik männikärsakas (*H. abietis*) siirutab seeneeoseid kehakatetega või kehast väljutatud ekskrementidega. Hariliku männikärsaka valmikud on võimelised elujõulisena levitama mitmeid erinevaid seeni, sealhulgas juurepessu (*Heterobasidion* spp.) (**I**), hiidkoorikut (**II**) ja *D. sapinea*'t (**III**). Toitudes okaspuu taimede koorest, tekitab harilik männikärsakas taimedele haavandeid levitades samal ajal haavanditesse seeneeoseid. Okaspuutaimed on valmikute sööma tõttu patogeenidele vastuvõtlikud ning okaspuu kultuuride hukkumine on tingitud nii valmikute toitumisest puukoorel kui ka levitatavast seennakkusest. Taimede hukkumine istutuskultuurides on kompleksne nähtus, mis on mõjutatud nii hariliku männikärsaka tekitatud kahjustustest, seennakkusest, taimede vastuvõtlikkusest kui ka keskkonnateguritest.

Järeldused

Peamine püstitatud hüpotees leidis katseliselt kinnitust: patogeensed ja saprotroofsed seened ning nendega seotud taksonoomiliselt erinevad viirused on võimelised läbima hariliku männikärsaka valmikute seedetrakti ning säilitama sealjuures elujõulisuse. Teine töös esitatud hüpotees kinnitust ei leidnud: kõik uuritud juurepessu viirused ei säilitanud hariliku männikärsaka seedetrakti läbimisel stabiilsust.

Juurepessuga seotud viiruste uurimise eesmärk on leida uusi võimalusi juuremädaniku leviku piiramiseks. Viiruste olemasolu ja stabiilsus võimaldaks neid tulevikus kasutada juurepessu vastase bioloogilise tõrjevahendina. Töö tulemustest selgus, et uuritud viiruste puhul võib hariliku männikärsaka valmikute seedetrakti läbimine vähendada viiruste nakatamisvõimet.

Kolmas ja neljas esitatud hüpotees leidsid kinnitust: juurepessu antagonisti, hiidkooriku viirusega nakatunud tüve nr 93073 puhul oli seene elujõulisus väiksem kui Rotstopi preparaadi aluseks oleval hiidkooriku tüvel. Seega, kui hiidkoorik on viirusega nakatunud, võib tema elujõulisus olla pärsitud. Samas võib viirus putuka seedetrakti läbida kadudeta ja tõhusalt säilitades seejuures stabiilsuse. Hiidkooriku oiidide hulk oli vastavuses varasemate uuringute tulemustega (Sun *et al.*, 2009). Viiruse püsivus on tähtis hiidkooriku kasutamisel biotõrjes, sest viiruse võime vähendada seene stabiilsust võib mõjutada biopreparaadi tõhusust.

Töö olulise tulemusena leidis katseliselt kinnitust, et hariliku männikärsaka valmikud levitavad universaalse patogeeni *D. sapinea* nakkust. Kui patogeen läbib eoste abil lühikesi vahemaid, siis putuka abil on seenpatogeenil võimalik läbida pikemaid distantse. Hariliku männikärsaka osa majandatavates metsades võib tulevikus veelgi suureneda, sest seenhaiguste hulk looduses kasvab ning metsakahjurina tuntud mardikas on võimeline levitama seennakkust.

ACKNOWLEDGEMENTS

This doctoral thesis is dedicated to my miraculous son Eerik. He has given me the motivation and energy to complete the thesis.

This thesis owes its existence to the help, support and inspiration of many people. Firstly, a special thanks goes to my supervisor Dr. Risto Kasanen and colleague Dr. Eeva J. Vainio. They gave me inspiration and supported me throughout research. I am very grateful to both of you!

I am thankful to my supervisors Dr. Ivar Sibul and Dr. Kaljo Voolma, who helped me to discover the wonderful world of insects and made comments during the preparation of the thesis.

For helpful discussion and valuable comments of this thesis, I thank Dr. Aare Abroi. The work of language editors Dr. Ingrid H. Williams, Dr. Floortje Vodde and Mrs. Urve Ansip is highly appreciated.

In particular, I would like to thank the entire forest pathology group in our institute. My special thanks go to Rein. We have had a lot of discussion when I was writing my articles or applications. He always supported me very enthusiastically. Thank you for that! I would like to thank Katrin and Kalev. We worked together in laboratory, during fieldwork and we discussed different topics. They helped me a lot to complete many of my activities.

Furthermore, I would like to express my deepest appreciation and gratitude to Dr. Märt Hanso. He is the „grand man" in forest pathology, he inspired me to work more deeply with forest diseases. I like very much his devotion and passion for his profession. I am thankful to Silja Hanso. She gave me valuable suggestions in laboratory work in the beginning of my academic activity and explained all the details very patiently.

I am deeply grateful to Piret. She has always been helpful, supportive, understanding and the best colleague one could wish for.

I am grateful to all colleagues from our institute, especially from the departments of Forest Biology and Silviculture. I have been working with you for many years and this time has been educative and interesting.

I would like to thank the students whom I have supervised. It has been a great opportunity to work with you.

Minu siirad tänud perekonnale nende toetuse, kannatlikkuse ja mõistmise eest töö koostamiseks kulunud aja jooksul.

The studies in this thesis were financially supported by the Environment Investment Centre under the projects 8-2/T15020MIMK, 8-2/T12155MIMB, 8-2/T11009MIMB, 8-2/T10030MIMI and 8-2/T7026MIMI; by the Forest Management Centre project 8-2/T12115MIMK, by the Institutional Research Funding IUT21-04 and by Norwegian Financial Mechanism 2009–2014 under the project EMP162.

Dissertation is supported by the Doctoral School of Earth Sciences and Ecology created under the auspices of the European Social Fund.

I

ORIGINAL PUBLICATIONS

Drenkhan, T., Sibul, I., Kasanen, R., Vainio, E.J. 2013. Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. Forest Pathology, 43: 317–323.

Forest Pathology

For. Path. 43 (2013) 317–323 doi: 10.1111/efp.12033
© 2013 Blackwell Verlag GmbH

Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*

By T. Drenkhan[1,4], I. Sibul[1], R. Kasanen[2,3], and E. J. Vainio[3]

[1]Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, F. R. Kreutzwaldi 5, Tartu, 51014, Estonia; [2]Department of Forest Sciences, University of Helsinki, Helsinki, Finland; [3]Finnish Forest Research Institute, Vantaa Research Unit, Vantaa, Finland; [4]E-mail: tiia.drenkhan@emu.ee (for correspondence)

Summary

Heterobasidion annosum (Fr.) Bref. *sensu lato* is an important fungal parasite of coniferous trees throughout the temperate regions of the world. Approximately 15% of *Heterobasidion* isolates are infected by dsRNA viruses, which are considered as obligately intracellular and transmit vertically into both basidiospores and asexual conidia. Insects such as *H. abietis* and its larvae feeding on wood colonized by *Heterobasidion* fungi may carry *Heterobasidion* conidia and hyphae. In this study, we used *H. abietis* as a model species to reveal whether *Heterobasidion* viruses resist in their fungal host passing through the digestive tract of insects that may potentially serve as disseminators of fungal propagules. *Pinus sylvestris* branches were inoculated by three different strains of *Heterobasidion parviporum*, hosting taxonomically diverse virus species: *Heterobasidion* RNA virus 2, HetRV4 and HetRV6. Then, the inoculated branches were fed to *H. abietis* insects, and beetle excrements were investigated for *Heterobasidion* infections. All the inoculated fungal strains survived passage through the alimentary tract of the insects (survival rate 25–80%). The passage rates of the viruses inside their hosts varied considerably, ranging from 0 to 67%. Two different mycoviruses, HetRV2 and HetRV6, survived the intestinal passage of their fungal host, while the virus species HetRV4 was detected among none of the five *H. parviporum* isolates retrieved from insect faeces. The relative stability of fungi harbouring viruses suggests that if viruses are to be used for biological control against *Heterobasidion* species, it is likely that insect feeding does not considerably decrease the virus effect, but might instead enhance short-range dispersal of the viral biocontrol agent.

1 Introduction

The basidiomycetous species cluster *Heterobasidion annosum s.l.* is economically the most important disease agent of coniferous forests in northern temperate regions (Korhonen and Stenlid 1998) and has been a major concern in Nordic forestry for over 100 years (Bendz-Hellgren et al. 1998). Two *Heterobasidion* species occur in Nordic countries: the polyphagous *H. annosum s.str.* prefers pines (*Pinus* spp.), while *H. parviporum* infects mainly spruce trees (*Picea* spp.). Both young conifer seedlings and mature trees are affected by a characteristic root and butt rot (Hodges 1969; Korhonen 1978). *H. annosum s.l.* enters a healthy stand by airborne basidiospores, which germinate on newly exposed wood such as fresh stump surfaces, colonize the stump and spread via root contacts into neighbouring healthy trees (Korhonen and Stenlid 1998). The infected stumps are substrates for fruiting bodies and sporulation, which increases the risk of stump infections in subsequent thinnings (Swedjemark and Stenlid 1993; Redfern et al. 2001). After final cutting, the fungus is able to live in spruce stumps for several decades, infecting trees of the next generation through root contacts (Piri 1996). The disease is responsible for annual losses of approximately 800 million Euros in Europe (Woodward et al. 1998).

The interaction between insects and fungi poses major threats to conifer forests (Schowalter and Filip 1993), and many devastating forest pathogens such as *Ophiostoma ulmi and novo-ulmi* (Buck et al. 2002) are carried to new hosts by insect vectors. Also, for *Heterobasidion* species, the possibility of insect- or arthropod-mediated dispersal has been debated over decades. Already in 1950s, Bakshi (1950) observed conidiophores of *Heterobasidion annosum* (at the time named as *Oedocephalum lineatum*) in breeding tunnels of the wood-boring beetle *Trypodendron lineatum* and showed that *O. lineatum* is the conidial stage of *Fomes annosus* syn. *H. annosum* (Bakshi 1952). Moreover, Nuorteva and Laine (1972) demonstrated that either viable *Heterobasidion* hyphae or conidia were carried over into excrements of the large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) when the insects were fed with pine phloem tissue covered by hyphae. In turn, Kadlec et al. (1992) were able to detect *H. annosum s.l.* from the excrements of *H. abietis* insects isolated in the field. Although the actual insect-transmitted new infections on healthy trees are questionable, it is likely that insects such as the large pine weevil (*H. abietis*) and its larvae feeding on trees colonized by *H. annosum* carry conidia and hyphae, which would facilitate the colonization process in dead or infected wood. *H. abietis* is also known as one of the most common and important pests of reforested sites in Northern and Eastern Europe (Leather et al. 1999; Långström and Day 2004). It is the most abundant and widespread of the three species of the genus *Hylobius* that occur in Estonia (Voolma 1994, 2001; Sibul 2000). In Northern European forests, managed on a cycle of clearfelling and replanting, adult weevils feed on bark on the main stem of transplants, which leads to decreased growth and increased mortality. Female adult weevils lay their eggs on nearby tree stumps (Nordlander et al. 1997). Larvae develop in stumps, living and feeding just under the bark. They pass through four larval moults over a period of 12–36 months prior to pupation and emergence of adults (Leather et al. 1999). In 2004, it was estimated that if pesticides were not used, the cost of the resulting *H. abietis* damage across Europe would be approximately 140 million Euros per year (Långström and Day 2004).

Received: 26.6.2012; accepted: 4.2.2013; editor: O. Holdenrieder

Fungal viruses (mycoviruses) may confer negative effects on the fitness of their fungal hosts. The phenomenon of virus-mediated hypovirulence (reduced pathogenicity) in plant pathogenic fungi has received growing interest during the last few years and occurs in several basidiomycetous and ascomycetous fungal species (Ghabrial and Suzuki 2009). The best known example is the hypovirulence-causing virus CHV1, which has been successfully utilized as a biocontrol agent in attempts of rescuing the European chestnuts from the invasive pathogen *Cryphonectria parasitica* (MacDonald and Fulbright 1991). It is known that approximately 15% of *Heterobasidion* isolates are infected by dsRNA viruses. The newly described virus species *Heterobasidion* RNA virus 6 constitutes about 70% of all dsRNA infections in *H. annosum s.l.* (Vainio et al. 2012a), and members of *Partitiviridae* are also relatively common and taxonomically highly diverse (Ihrmark 2001; Vainio et al. 2010, 2011a,b). While most of these viruses are considered more or less cryptic, both negative and slightly positive interactions with the fungal hosts have been described (Ihrmark et al. 2004; Vainio et al. 2010, 2012b). The viruses are obligately intracellular and transmit laterally by anastomosis contacts and vertically into sexual and asexual spores, but virus transmission frequency into basidiospores and conidia is highly variable among *Heterobasidion* individuals (Ihrmark et al. 2002, 2004). In case hypovirulence-causing viruses are to be utilized in biological control against *Heterobasidion* species, it is crucial to study virus stability and transmission rate in the nature in more detail. As *Heterobasidion* strains are able to survive in woody tissues for decades, it is highly probable that their hyphae encounter xylem-feeding insects. We used *H. abietis* in this study as a model species to reveal whether *Heterobasidion* viruses resist passing through the digestive tract of insects that may potentially serve as disseminators of fungal propagules.

2 Material and methods

2.1 Experimental set-up

2.1.1 Inoculation of Pinus sylvestris branches by Heterobasidion parviporum

Three *H. parviporum* strains hosting taxonomically diverse viruses were used to infect pine branches to be fed to *H. abietis* insects. Two of the fungal strains were of Finnish origin: *H. parviporum* RT3.49C hosted the partitivirus strain HetRV4-pa1 (Vainio et al. 2011b), while *H. parviporum* 7R18 contained the partitivirus strain HetRV2-pa1 (Vainio et al. 2011a). The third strain, *H. parviporum* 136212, originated from Estonia and harboured the virus strain HetRV6-pa17 (Vainio et al. 2012a). HetRV6 is a novel virus species that has not been assigned to any known virus family. These virus-containing *H. parviporum* strains were inoculated on malt extract agar plates (40 g malt extract, OXOID Ltd., England: LP0039; 28 g agar technical, Becton, Dickinson & Co., BBL™, 2 l water), each fungal strain as 20 replicates. The plates were incubated for two weeks at room temperature and then used for infecting branches of *Pinus sylvestris*.

Fresh branches of *P. sylvestris* were collected from a 14-year-old Scots pine forest from South Estonia (Tartu county, Ülenurme, 58°18′N, 26°39′E). The branches (diameter 3–5 mm and length 6–7 cm) were cut with nippers from healthy pine trees, cleaned from needles, transported to the laboratory in a plastic bag and stored in the fridge. The branches were then autoclaved for 30 min at 120°C and placed to the agar plates containing *H. parviporum* mycelia (one branch per plate). The dishes were closed with Parafilm (Pechiney Plastic Packaging Company). After two weeks, the pine branches were totally covered by *Heterobasidion* mycelia (Fig. 1a). All the tools and instruments used in the experiment were autoclaved and sterilized before each experiment and sprayed with alcohol (96%) and left to dry.

2.1.2 Cleansing the insects from putative contaminants

Freshly collected *H. abietis* insects were used in the laboratory experiments. The pine weevils were collected from a fresh clear-cut area in the forest district of Järvselja, East Estonia (58°27′N, 27°33′E), by using ground pitfalls (25 × 40 × 50 cm). The trapped, living weevils were removed from the traps and transferred to the laboratory and stored

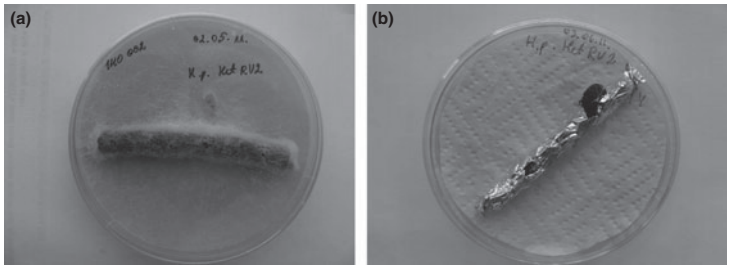

Fig. 1. The experimental setup for feeding *Heterobasidion abietis* adults with pine branches colonized by *H. parviporum*. (a) Inoculation of pine branch pieces with *H. parviporum* mycelia; (b) *H. abietis* individual feeding on a colonized pine branch covered with foil and containing two feeding holes (photos Tiia Drenkhan).

in the fridge (+4°C). Before the experiment, the insects were thoroughly purified both internally and externally. Insect excrements and impurities attached to the insect legs were removed by allowing them to adhere to moist filter papers at the bottom of 90-mm-diameter glass Petri dishes (Figure S1). After twenty-four hours, the papers were exchanged and more water was added. In order to empty their digestive tracts, the insects were also kept without food for 48 h prior to the experiments, while fresh water was supplemented. The insects were then weighted (mean body mass = 0.115 g, SD = 0.03, N = 60) and their gender was determined (Table 1).

2.1.3 Feeding the insects with pine branches covered by H. parviporum

New filter paper was placed inside sterile Petri dishes. The pine branches were wrapped with foil using dispensable gloves. The tin-foil paper was added in order to prevent the insects from obtaining conidia or hyphae in their legs or exoskeleton. Two holes (0.3 × 0.3 cm) were made in the tin-foil paper (Fig. 1b) to allow feeding. One pine branch and one insect were placed on each Petri dish.

The pine weevils were allowed to feed on the *Heterobasidion*-infected pine branches for 48 h. After feeding, the insects were removed. The excrements were collected from the Petri dishes containing the feeding branches. From each insect, 5 excrements were selected at random (Figure S2). Surface sterilization of the excrements was made in alcohol (96%) and in sterile water. Excrements were handled with tweezers and immersed for a few seconds in alcohol and in sterile water after that. The excrements were placed onto new agar plates in the laminar flow cabinet (Kojair Tech) in sterile conditions. The plates were supplemented with the antibiotic thiabendazole (20 g malt extract, 15 g agar, 1 l water, autoclave sterilization for 20 min by 120°C, add 230 mg thiabendazole, 1 ml lactic acid and a small amount (4 ml) of sterile water for making the thiabendazole soluble) (ICN Biomedicals). The agar plates were incubated two weeks at room temperature. The excrements were investigated under microscope and all emerging *Heterobasidon* isolates were harvested and inoculated on new Petri dishes with malt extract agar (MEA). Each excrement was not analysed individually. All the harvested isolates from one plate (per 5 excrements) of *Heterobasidion parviporum* were disseminated to the same Petri dishes as all the excrements on single plates originated from the same insect. The cultures emerging from the five excrements of each insect were allowed to fuse on the MEA plates and stored as a pooled stock culture. Altogether, twenty replicate plates were therefore analysed for each virus–host combination (Table 1).

2.2 Fungal genotype determination

The identity of the emerged fungal strains was confirmed based on somatic compatibility as described by Stenlid (1985). Briefly, the *Heterobasidion* isolates cultured from the insect faeces were paired with the corresponding original strains (7R18, RT3.49C and 136212), and the appearance of a demarcation zone was checked after fourteen days and after about four weeks. Fusion of the hyphae without a detectable incompatibility zone was deemed as an indication of somatic compatibility.

2.3 Detection of viruses by molecular methods

2.3.1 Isolation of total nucleic acids

Reverse transcription (RT) was conducted using total nucleic acid samples prepared using the protocol of Vainio et al. (1998) with some modifications. Briefly, the fungal cells were first disrupted using the FastPrep FP120 homogenizer and 1- to 2-mm quartz sand grains and then purified by phenol–chloroform extractions. Nucleic acids were then precipitated using polyethylene glycol. In order to prevent RNA degradation, the cell lysate was not incubated at 65°C after cell lysis.

Table 1. Survival of the *Heterobasidion parviporum* isolates and persistence of viruses after passage through the digestive tract of insects.

Fungal host	Virus	Average weight of insects before experiment (g)	Average weight of insects after experiment (g)	Gender of insect	*H. parviporum* survival[1]	Somatic compatibility	Virus persistence by RT-PCR	Virus detected as dsRNA by CF11 chromatography
7R18	HetRV2	0.112 ± 0.03	0.125 ± 0.04	Male - 10 Female - 10	80% (16/20)	100%	63% (10/16)	44% (7/16)
RT3.49C	HetRV4	0.117 ± 0.03	0.127 ± 0.03	Male - 11 Female - 9	25% (5/20)	100%	0% (0/5)	0% (0/5)
136212	HetRV6	0.133 ± 0.02	0.143 ± 0.03	Male - 7 Female - 13	45% (9/20)	100%	67% (6/9)	22% (2/9)

[1]Twenty replicate plates were investigated for each virus-infected host isolate.

2.3.2 Reverse transcription

The RT reactions were set up using 5–12 μl of the nucleic acid sample (denatured in a boiling water bath for 5 min and chilled at −80°C), 0.2 μg of random hexamer primer (Fermentas GmbH), 200 U of RevertAid MuLV reverse transcriptase (Fermentas GmbH) and dNTPs and buffer as recommended by the manufacturer.

2.3.3 PCR with virus-specific primers

Three different primer pairs were used to investigate the presence of viruses in the *H. parviporum* isolates. The virus strain HetRV4-pa1 was screened using the specific primer pair RTmidF and RTmidR, while HetRV2 was screened using the primers 7RPForB and 7RPRevB (Table 2). The third virus species HetRV6 was screened using the consensus primer pair HV6F1A and HV6Re2 as described earlier (Vainio et al. 2012a). The PCRs were conducted in volumes of 50 μl, including 1–2 μl of the cDNA product, 25 pmol of each primer, 1 U of Dynazyme TM 451 II DNA polymerase (Finnzymes, Finland) and 10 nmol of dNTPs. The amplification conditions for 7RPForB/7RPRevB were as follows: 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at 53°C, 2 min at 72°C; and a final extension of 7 min at 72°C. For RTmidF/RTmidR, a touch-down cycling protocol was used: 10 min at 95°C and 13 cycles of 30 s at 95°C, 45 s at 65 −1°C per cycle, 2 min at 72°C; and 20 times of 30 s at 95°C, 45 s at 52°C, 2 min at 72°C, with a final extension of 7 min at 72°C. The original host isolates (RT3.49C, 7R18 and 136212) were used as positive controls, and each PCR batch included a negative control without template. At least two independent nucleic acid preparations were reverse-transcribed and subjected to RT-PCR from each isolate.

2.3.4 CF11 chromatography

The occurrence and quantity of dsRNA in the *Heterobasidion* isolates was investigated using CF11 cellulose affinity chromatography as described earlier (Tuomivirta and Hantula 2003; Vainio et al. 2010). Briefly, about 2–3 g of mycelia was grown on modified orange serum agar plates supplemented with cellophane membranes, harvested and homogenized in a lysis buffer. The samples were then extracted by phenol and chloroform and precipitated using CF11 cellulose powder in 15% ethanol. The presence or absence of dsRNA was analysed by agarose gel electrophoresis.

3 Results

3.1 The feeding experiment

We successfully infected *P. sylvestris* branches by three different strains of *Heterobasidion parviporum*, and all the inoculated branches were covered by mycelia after two weeks (Fig. 1a). The insects feed on the colonized branches effectively, resulting in weight gain of approximately 7.5–12% (10–13 mg) during the experiment (Table 1). Before plating, the insect excrements had been rinsed with alcohol and sterile water and incubated for two weeks in sterile conditions, and no contaminations (e.g. *Penicillium* spp., bacteria) were observed during the experiments.

3.2 Survival of *Heterobasidion* strains inside *H. abietis*

We tested three different *H. parviporum* strains for their survival while passing through *H. abietis* intestinal tract. After the passage, *Heterobasidion* growth was observed on 30 plates of the original 60 plates (Table 1). Although all the fungal strains survived passage through insects, their passage rates were different: 5, 9 or 16 faecal isolates were obtained from the fungal hosts RT3.49C, 136212 or 7R18, respectively. The passage rates were therefore 25%, 45% or 80% (Table 1).

3.3 Genotype determination

The genetic equality or inequality of the faecal *H. parviporum* isolates with the original strains was determined based on somatic incompatibility. All the isolates derived from faeces were somatically compatible with the corresponding original strains (RT3.49C, 7R18 or 136212), which confirmed that the insect-derived cultures represented the introduced strains and were not originally present inside the insects.

Table 2. Primers used for the screening of viruses from the *H. parviporum* cultures.

Virus	Primer name	Primer sequence (5′→ 3′)	Product length
HetRV4	RTmidF	TTC AAA AAG GCC AAG TTT TC	335 bp
	RTmidR	ATT GTG CAG TTC TGC GTT TA	
HetRV2	7RPForB	CCC TTC CAA CGC CAT AAA	610 bp
	7RPRevB	CGT CAG GAG TGA CGA AAA CC	
HetRV6	HV6F1A	TTG AAT CAC CTG GAC CGT TT	714 bp
	HV6Re2	CAT CAA CCC ATT ATC CAG GT	

3.4 Occurrence of viruses in the *Heterobasidion* cultures derived from insect excrements

The tested viruses do not confer noticeable phenotypic effects on their fungal hosts, and their presence cannot be detected without molecular analysis. Therefore, the presence of viruses after the passage was screened by RT-PCR with specific primers. All the 30 recovered *Heterobasidion* isolates were screened twice by RT-PCR using two independent cDNA samples. The three virus-specific primer pairs successfully amplified PCR products of the expected lengths from the original strains 7R18, RT3.49C and 136212 (data not shown). PCR products of the same lengths (610 or 714 bp) were also obtained from altogether 10 and 6 faecal isolates representing strains 7R18 and 136212, respectively (Fig. 2a, Table 1). No amplification products, however, were obtained from isolates representing strain RT3.49C. Therefore, the virus persistence varied between 0–67% depending on the fungal strain investigated.

In order to detect viral genomic dsRNA in the isolates from excrements, the *Heterobasidion* isolates were analysed by CF11 chromatography (Fig. 2b). In the case of HetRV2, we detected high virus concentrations among seven of the sixteen isolates analysed (44%). These isolates were also virus positive by RT-PCR. In addition, three isolates were conditionally HetRV2 positive by RT-PCR, although not detectable as dsRNA. As for HetRV6, the virus is present in low concentration in its original host (136212), and after passage, we detected dsRNA only among two faecal isolates (22%). Both isolates were virus positive by RT-PCR. Moreover, four isolates were found repeatedly HetRV6 positive by RT-PCR. In the case of HetRV4, all isolates were virus negative by both CF11 chromatography and RT-PCR (both replicate cDNA samples).

4 Discussion

In this study, we used two different molecular methods to investigate whether *Heterobasidion* viruses are able to resist passing through the alimentary tract of *H. abietis* inside their fungal hosts. Based on both methods, the virus species HetRV2 and HetRV6 were present in part of the *Heterobasidion* isolates recovered from the excrements of *H. abietis.* Based on RT-PCR, 63 and 67% of the faecal isolates were infected by HetRV2 and HetRV6 after the experiment, respectively. However, only 22–44% of the isolates revealed to contain enough dsRNA to be detected by CF11 chromatography. In the case of *H. parviporum* HetRV4, we found no viruses in the five host isolates discovered in the insect faeces. Methodologically, RT-PCR with virus-specific primers specifically recognizes only the virus species being investigated and allows the detection of even minute amounts of viral templates. In turn, CF11 chromatography reveals the quantity of dsRNA present in the host isolate, but sometimes fails to detect virus infections with low concentrations that are consistently detected by RT-PCR with specific primers (e.g. Vainio et al. 2012b).

The results of this study suggest that passage through insects might result in some degree of virus loss. A similar phenomenon has been previously observed when viruses are transmitted vertically into sexual or asexual spores of *Heterobasidion* species (Ihrmark et al. 2002, 2004). Part of the virus loss might also result from uneven distribution of viruses in the fungal mycelium. *Heterobasidion* mycoviruses are typically absent from part of the hyphal tips even in virus-infected fungal strains (Ihrmark et al. 2002), and therefore, no viruses might be carried over if only minute amounts of hyphae survive inside the intestinal tract of an insect. Cultural conditions have also been shown to affect the virus content of many fungal species, and elevated temperature or cycloheximide treatment can even be used to remove viruses from their fungal hosts (Fulbright 1984; Elias and Cotty 1996; Zhou and Boland 1997; Ahn and Lee 2001; Vainio et al. 2010). Therefore, it would not be unexpected that passage through insects might influence the virus content of the fungal host.

In general, the passage rate of the *Heterobasidion* host strains varied considerably (25–80%). Nuorteva and Laine (1972) observed that *Heterobasidion* passed through ~70% of the *H. abietis* insects that feeded on *Pinus* phloem supplemented with pure culture of *Heterobasidion*. In the present study, the *P. sylvestris* branches were inoculated with *H. parviporum*, which is a spruce pathogen in the nature, although *H. abietis* feeds on both spruce and pine wood and therefore is

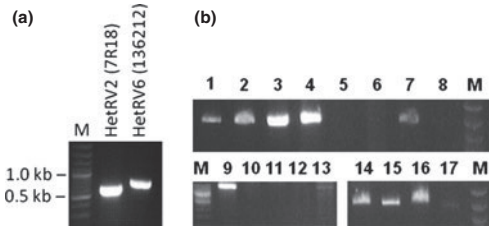

Fig. 2. (a) Agarose gel electrophoresis of RT-PCR amplification products obtained from two fungal isolates after passage through the insects. The products were obtained using the primer pairs 7RPForB/7RpRevB and HV6F1A/HV6Re2, respectively. (b) CF11 chromatography profiles obtained from *Heterobasidion* isolates before and after passage through the insects: (1) 7R18 (original host of HetRV2); (2) RV2-20; (3) RV2-18, (4) RV2-11; (5) RV2-5; (6) RV2-1; (7) RV2-10; (8) RV6-11; (9) RT3.49C (host of HetRV4); (10) RV4-2; (11) RV4-3; (12) RV2-14; (13) RV6-16; (14) RV2-17; (15) RV2-15; (16) RV2-8; (17) 136212 (host of HetRV6). The sample names indicate the virus strain investigated and the replicate plate analyzed (RV2-20 is HetRV2 replicate 20). M indicates the molecular marker (the GeneRuler 100 bp DNA Ladder Plus, Fermentas GmbH, Germany). The sizes of the dsRNAs are as follows: HetRV2, 2238/2290 bp (capsid protein/polymerase); HetRV4, ~2000 bp; HetRV6,~2050 bp.

suspected to encounter both *H. parviporum* and *H. annosum s.str.* In experimental conditions, *H. parviporum* has also been shown to infect Scots pine sapwood, roots and seedlings (Johansson et al. 2004).

The two virus species that survived the passage through insect intestines, HetRV2 and HetRV6, are taxonomically very different. However, similar viruses have been found from *H. annosum s.str.*: the nucleotide sequence of the polymerase gene of HetRV6-pa17 is 93% similar to the virus strain HetRV6-an4 from a Finnish *H. annosum s.str.* isolate (Vainio et al. 2012a), while HetRV2-pa1 resembles the HaV-P from *H. annosum s.str.* by about 70% (Ihrmark 2001). Overall, HetRV6 is the most common virus species found from *Heterobasidion annosum s.l.* thus far (Vainio et al. 2012a), while the partitivirus species HetRV2 seems to be relatively rare (Vainio et al. 2011a). Viruses of *Heterobasidion* species infect basidiospores and may potentially travel hundreds of kilometres (Kallio 1970). In contrast, the biological significance of the asexual conidial spores of *Heterobasidion* is still largely unknown and they are considered to travel only short distances. Short-distance lateral transfer of mycoviruses takes place by transient anastomosis contacts that form readily even between somatically incompatible isolates of *Heterobasidion* (Ihrmark et al. 2002; Vainio et al. 2010).

On the other hand, very little is known about the putative role of insects, micro-arthropods or nematodes feeding on fungal tissue in the context of mycovirus dispersal. Partitiviruses, totiviruses and mitoviruses have been described from insect-vectored ophiostomatoid fungi, including the Dutch elm disease fungi *Ophiostoma ulmi*, *O. novo-ulmi* and *O. himal-ulmi* (Buck et al. 2002; Doherty et al. 2007). Moreover, ascospores of *O. ips* are present in the gut and faeces of adult *Ips* species (Harrington 2005), and the blue-stain fungus *Ceratocystis polonica* has been detected in the digestive tract of *I. typographus* (Furniss et al. 1990). However, to our knowledge, it has not been previously studied whether mycoviruses persist inside their fungal host during passage through the alimentary tract of bark beetles. This study shows that two taxonomically different mycoviruses were able to survive passing through the digestive tract of bark beetles inside their fungal hosts. As a practical conclusion, the stability of the viruses inside the insects suggests that if similar viruses are to be used as biological control of *Heterobasidion* species, it is likely that insect feeding does not considerably decrease the virus effect, although there seems to be differences between virus strains.

Acknowledgements

The research was funded by Estonian Environmental Investment Centre, the Finnish Forest Research Institute and the Academy of Finland (decision number 251193). We would like to thank Dr Rein Drenkhan for comments and Mrs. Anne Tartu for improving the English. We thank Ms. Marja-Leena Santanen, Ms. Sonja Sarsila and Mr. Mirsad Krasniqi for their skilful technical assistance in the laboratory.

References

Ahn, I.-P.; Lee, Y.-H., 2001: A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. Mol. Plant Microbe Interact. **14**, 496–507.

Bakshi, B. K., 1950: Fungi associated with ambrosia beetles in Great Britain. Trans. British Mycol. Soc. **33**, 111–120.

Bakshi, B. K., 1952: *Oedocephalum lineatum* is a conidial stage of *Fomes annosus*. Trans. British Mycol. Soc. **35**, 192–195.

Bendz-Hellgren, M.; Lipponen, K.; Solheim, H.; Thomsen, I. M., 1998: The Nordic Countries. In: Heterobasidion annosum. Biology, Ecology, Impact and Control (Chapter 17). Ed. by: Woodward, S.; Stenlid, J.; Karjalainen, R.; Hüttermann, A. Wallingford, UK: CAB International, pp. 333–345.

Buck, K. W.; Brasier, C. M.; Paoletti, M.; Crawford, L., 2002: Virus transmission between two species of the Dutch elm disease fungus, *Ophiostoma ulmi* and *O. novo-ulmi*: d-factors as selective agents for gene introgression. In: Genes and the Environment. Ed. by Halls, R. Oxford, UK: Blackwell Science Ltd, pp. 26–45.

Doherty, M.; Sanganee, K.; Kozlakidis, Z.; Coutts, R. H. A.; Brasier, C. M.; Buck, K. W., 2007: Molecular characterization of a Totivirus and a Partitivirus from the genus *Ophiostoma*. J. Phytopathol. **155**, 188–192.

Elias, K. S.; Cotty, P. J., 1996: Incidence and stability of infection by double-stranded RNA genetic elements in Aspergillus section flavi and effects on aflatoxigenicity. Can. J. Bot. **74**, 716–725.

Fulbright, D. W., 1984: Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. Phytopathol. **74**, 722–724.

Furniss, M. M.; Solheim, H.; Christiansen, E., 1990: Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce. Ann. Entomol. Soc. Am. **83**, 712–716.

Ghabrial, S. A.; Suzuki, N., 2009: Viruses of plant pathogenic fungi. Annu. Rev. Phytopathol. **47**, 353–384.

Harrington, T. C., 2005: Ecology and evolution of mycophagous bark beetles and their fungal partners. In: Ecological and Evolutionary Advances in Insect-Fungal Associations. Ed. by Vega, F. E.; Blackwell, M. UK: Oxford University Press, pp. 257–291.

Hodges, C. S., 1969: Modes of infection and spread of *Fomes annosus*. Ann. Rev. Phytopathol. **7**, 247–266.

Ihrmark, K., 2001: Double-stranded RNA elements in the root rot fungus *Heterobasidion annosum*. PhD dissertation, Uppsala, Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences. ISSN 1401–6230, ISBN 91-576-6094-8, pp. 36.

Ihrmark, K.; Johannesson, H.; Stenström, E.; Stenlid, J., 2002: Transmission of double-stranded RNA in *Heterobasidion annosum*. Fungal Gene. Biol. **36**, 147–154.

Ihrmark, K.; Stenström, E.; Stenlid, J., 2004: Double-stranded RNA transmission through basidiospores of *Heterobasidion annosum*. Mycol. Res. **108**, 149–153.

Johansson, S. M.; Lundgren, L. N.; Asiegbu, F. O., 2004: Initial reactions in sapwood of Norway spruce and Scots pine after wounding and infection by *Heterobasidion parviporum* and *H. annosum*. Forest Pathol. **34**, 197–210.

Kadlec, Z.; Starý, P.; Zumr, V., 1992: Field evidence for the large pine weevil, *Hylobius abietis* as a vector of *Heterobasidion annosum*. Euro. J. Forest Pathol. **22**, 316–318.

Kallio, T., 1970: Aerial distribution of the root-rot fungus *Fomes annosus* (fr.) Cooke in Finland. Acta For. Fenn. **107**, 1–55.

Korhonen, K., 1978: Intersterility groups of *Heterobasidion annosum*. Commun. Inst. For. Fenn. **8**, 236–239.

Korhonen, K.; Stenlid, J., 1998: Biology of *Heterobasidion annosum*. In: Heterobasidion annosum. Biology, Ecology, Impact and Control (Chapter 4). Ed. By Woodward, S.; Stenlid, J.; Karjalainen, R.; Hüttermann, A. Wallingford, CAB International, pp. 43–70.

Långström, B.; Day, K. R., 2004: Damage, control and management of weevil pests, especially *Hylobius abietis*. In: Bark and Wood Boring Insects in Living Trees in Europe: A Synthesis. Ed. by: Lieutier, F.; Day, K. R.; Battisti, A.; Grégoire, J.-C.; Evans, H. F. The Netherlands, Kluwer Academic Publishers, pp. 415–444.

Leather, S. R.; Day, K. R.; Salisbury, A. N., 1999: The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? Bull. Entomol. Res. **89**, 3–16.

MacDonald, W.; Fulbright, D., 1991: Biological control of chestnut blight: use and limitations of transmissible hypovirulence. Plant Dis. **75**, 656–661.

Nordlander, G.; Nordenhem, H.; Bylund, H., 1997: Oviposition patterns of the pine weevil *Hylobius abietis*. Entomol. Exp. Appl. **85**, 1–9.

Nuorteva, M.; Laine, L., 1972: Lebensfähige Diasporen des Wurzelschwamms (*Fomes annosus* (Fr.) Cooke) in den Exkrementen von *Hylobius abietis* L. (Col., Curculionidae). Ann. Entomol. Fennici **38**, 119–121.

Piri, T., 1996: The spreading of the S type of *Heterobasidion annosum* from Norway spruce stumps to the subsequent tree stand. Eur. J. For. Path. **26**, 193–204.

Redfern, D. B.; Pratt, J. E.; Gregory, S. C.; MacAskill, G. A., 2001: Natural infection of Sitka spruce thinning stumps in Britain by spores of *Heterobasidion annosum* and long-term survival of the fungus. Forestry **74**, 53–71.

Schowalter, T. D.; Filip, G. M. (eds), 1993: Beetle-Pathogen Interactions in Conifer Forests. Academic Press, Harcourt Brace & Company, London, San Diego, New York, Boston, Sydney, Tokyo, Toronto.

Sibul, I., 2000: Abundance and sex ratio of pine weevils, *Hylobius abietis* and *H. pinastri* (Coleoptera: Curculionidae) in pine clear-cuttings of different ages.. Proceedings of the International Conference. Tartu, Estonia, Sept. 28–29, 2000. Trans. Estonian Agricul. Univ. **209**, 186–189.

Stenlid, J., 1985: Population structure of Heterobasidion annosum as determined by somatic incompatibility, sexual incompatibility, and isoenzyme patterns. Can. J. Bot. **63**, 2268–2273.

Swedjemark, G.; Stenlid, J., 1993: Population dynamics of the root rot fungus *Heterobasidion annosum* following thinning of *Picea abies*. Oikos **66**, 247–254.

Tuomivirta, T.; Hantula, J., 2003: Two unrelated double-stranded RNA molecule patterns in *Gremmeniella abietina* type A code for putative viruses of the families *Totiviridae* and *Partitiviridae*. Arch. Virol. **148**, 2293–2305.

Vainio, E.; Korhonen, K.; Hantula, J., 1998: Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. Mycol. Res. **102**, 187–192.

Vainio, E. J.; Korhonen, K.; Tuomivirta, T. T.; Hantula, J., 2010: A novel putative partitivirus of the saprotrophic fungus *Heterobasidion ecrustosum* infects pathogenic species of the *Heterobasidion annosum* complex. Fungal Biol. **114**, 955–965.

Vainio, E. J.; Keriö, S.; Hantula, J., 2011a: Description of a new putative virus infecting the conifer pathogenic fungus *Heterobasidion parviporum* with resemblance to *Heterobasidion annsoum* P-type partitivirus. Arch. Virol. **156**, 79–86.

Vainio, E. J.; Hakanpää, J.; Dai, Y.-C.; Hansen, E.; Hantula, J., 2011b: Species of *Heterobasidion* host a diverse pool of partitiviruses with global distribution and interspecies transmission. Fungal Biol. **115**, 1234–1243.

Vainio, E. J.; Hyder, R.; Aday, G.; Hansen, E.; Piri, T.; Doğmuş-Lehtijärvi, T.; Lehtijärvi, A.; Korhonen, K.; Hantula, J., 2012a: Population structure of a novel putative mycovirus infecting the conifer root-rot fungus *Heterobasidon annosum* sensu lato. Virology **422**, 366–376.

Vainio, E. J.; Piri, T.; Hantula, J., 2012b: Virus community dynamics in the conifer pathogenic fungus *Heterobasidion parviporum* following an artificial introduction of a partitivirus. Microbial Ecol. **65**, 28–38. In press doi: 10.1007/s00248-012-0118-7.

Voolma, K., 1994: Atraktantpüüniste kasutamine männikärsakate ja juureüraskite arvukuse hindamiseks. (Use of baited ground traps for monitoring pine weevil, *Hylobius abietis*, and root-colonizing bark beetles). Metsanduslikud Uurimused (Forestry Studies) **26**, 96–109 (In Estonian with English summary).

Voolma, K., 2001: Harilik männikärsakas (*Hylobius abietis* L.) Räpina metskonna raiestikel: uurimus atraktantpüünistega. (The large pine weevil, *Hylobius abietis* L., in the felling areas of the Räpina forest district: a case study with baited ground traps). Metsanduslikud Uurimused (Forestry Studies) **35**, 172–178 (In Estonian with English summary).

Woodward, S.; Stenlid, J.; Karjalainen, R.; Hütterman, A. (eds) 1998: *Heterobasidion annosum*: Biology, Ecology, Impact and Control. Wallingford, UK: CAB International.

Zhou, T.; Boland, G. J., 1997: Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. Phytopathol. **87**, 147–153.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Insect excrements and impurities were removed using moist filter papers on the bottom of Petri dishes (photo Tiia Drenkhan).

Figure S2. From each insect, 5 excrements were selected at randomly and disseminated onto agar plates (photo Tiia Drenkhan).

II

Drenkhan, T., Kasanen, R., Vainio, E.J. 2015.
Phlebiopsis gigantea and associated viruses survive passing
through the digestive tract of *Hylobius abietis*.
Biocontrol Science and Technology, 26: 320–330.

BIOCONTROL SCIENCE AND TECHNOLOGY, 2016
VOL. 26, NO. 3, 320–330
http://dx.doi.org/10.1080/09583157.2015.1111998

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Phlebiopsis gigantea and associated viruses survive passing through the digestive tract of *Hylobius abietis*

Tiia Drenkhan[a], Risto Kasanen[b] and Eeva J. Vainio[c]

[a]Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, Tartu, Estonia; [b]Department of Forest Sciences, University of Helsinki, Helsinki, Finland; [c]Natural Resources Institute Finland (Luke), Bio-based Business and Industry, Bioprocessing, Jokiniemenkuja 1, Vantaa, Finland

ABSTRACT

Phlebiopsis gigantea (Fr.) Jül. is one of the most common fungal species in coniferous forests and commonly used as a biological control agent to prevent aerial infections of conifers by *Heterobasidion* fungi. We used feeding experiments to examine whether *Hylobius abietis* L. (Coleoptera: Curculionidae) could serve as a vector for *P. gigantea* and associated viruses by disseminating the fungus in its faecal pellets. Two different *P. gigantea* strains were used in the experiments: the virus-free Rotsop biocontrol strain and *P. gigantea* 93073 infected with the virus strain PgLV-1. The Rotstop strain showed 100% viability during insect feeding, while the viability of the virus-infected strain 93073 was only 35%. Virus persistence was 100% during the passage of the host fungus through the alimentary tract. Based on growth experiments using virus-infected and virus-free strains obtained by hyphal tip or oidial isolation, the presence of PgLV-1 did not significantly affect the growth rate of the host fungus.

ARTICLE HISTORY
Received 7 August 2015
Revised 19 October 2015
Accepted 20 October 2015

KEYWORDS
Heterobasidion; biocontrol; insect vector; mycovirus

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

1. Introduction

Phlebiopsis gigantea (Fr.) Jül. (syn. Phlebia gigantea (Fr.) Donk, *Phanerochaete gigantea* (Fr.) S.S. Rattan) is one of the most common fungal species in coniferous wood remains in the Boreal region, especially in managed forests (Kallio, 1965; Meredith, 1959). This saprotrophic white-rot fungus is a primary wood coloniser in Europe, North America, South Africa, Australia and New Zealand (Grillo, Hantula, & Korhonen, 2005). The European *P. gigantea* population is variable and freely interbreeding (Grillo et al., 2005; Korhonen, Kannelsuo, Vainio, & Hantula, 1997; Korhonen & Kauppila, 1988; Vainio & Hantula, 2000; Vainio, Korhonen, & Hantula, 1998), and *P. gigantea* is considered to be a true biological species with only one intersterility group (Vainio et al., 1998). The fungus is easily recognised in pure culture by the presence of oidial chains, encrusted upright aerial hyphae, numerous encrusted cystidia and multiple clamp connections (Eriksson, Hjortstam, & Ryvarden, 1981; Stalpers, 1978).

P. gigantea is commonly used as a biological control agent to prevent aerial infections of *Heterobasidion* spp. Bref. which cause serious root and butt rot disease in conifers in

CONTACT Tiia Drenkhan tiia.drenkhan@emu.ee
© 2016 Taylor & Francis

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

temperate and boreal forests throughout the northern hemisphere (Holdenrieder & Greig, 1998; Korhonen et al., 1994; Rishbeth, 1963). Freshly cut pine and spruce stumps are commonly treated with biocontrol preparations containing oidial spores of *P. gigantea* in several countries, such as England (Greig, 1976; Rishbeth, 1975), Finland (Korhonen, 1993), Sweden (Korhonen et al., 1994), Poland (Rykowski & Sierota, 1983) and Estonia (Drenkhan, Hanso, & Hanso, 2008). In Finland, a single heterokaryotic *P. gigantea* strain called 'Rotstop' (Verdera Oy, Lallemand Inc.) has been used for stump treatments since 1991 (Korhonen et al., 1994; Sun, Korhonen, Hantula, Asiegbu, & Kasanen, 2009).

P. gigantea oidia are believed to spread only short distances, and their biological function is still unclear. In North America, *P. gigantea* oidia have been found in large quantities in arthropod galleries (Hunt & Cobb, 1982), which suggests the possibility of insect-mediated transfer. The large pine weevil (*Hylobius abietis* L., Coleoptera: Curculionidae) occurs commonly throughout northern and eastern Europe and Asia. This bark-feeding insect causes substantial economic losses to forestry in 1–5-year-old pine and spruce plantations (Langström & Day, 2004; Leather, Day, & Salisbury, 1999; Toivonen & Viiri, 2006). Adult weevils damage or kill conifer seedlings by feeding on the bark and phloem of stems (Örlander, Nordlander, Wallertz, & Nordenhem, 2000; Wainhouse & Brough, 2007). The damage caused by *H. abietis* is most extensive on clear-cut areas of former conifer stands which have been reforested by planting pine or spruce (Day, Nordlander, Kenis, & Halldórsson, 2004; Örlander & Nilsson, 1999; Sibul, Ploomi, & Voolma, 2009). The adults lay eggs in the cortex of roots and larvae of the insect feed on dying roots of damaged conifers or conifer stumps, where typically a number of decay fungi co-occur. As the habitats of the fungus and insect overlap, it can be hypothesised that *H. abietis* could serve as a vector for *P. gigantea*.

Fungal viruses (mycoviruses) are ubiquitous among fungi and may mediate diverse phenotypic changes in their hosts (Ghabrial & Suzuki, 2009; Hyder et al., 2013; Márquez, Redman, Rodriguez, & Roossinck, 2007). Hypoviruses infecting the chestnut blight fungus (*Cryphonectria parasitica* (Murrill) M. E. Barr) are used as biocontrol agents against their plant pathogenic host fungus in Europe (MacDonald & Fulbright, 1991). *P. gigantea* hosts two yet unassigned large (10–12 kbp) dsRNA viruses: *P. gigantea* large virus-1 (PgLV-1) and PgLV-2 (Kozlakidis et al., 2009). These virus strains somewhat resemble members of the virus family *Totiviridae* but most likely are members of a new, yet unassigned virus family (Kozlakidis et al., 2009; Lim, Jamal, Phoon, Korhonen, & Coutts, 2011). The *P. gigantea* strain 'TW2' hosting both PgLV-1 and PgLV-2 is reported to have altered morphology (irregular colony margin, slowed growth and diminished production of asexual spores). The TW2 strain has been used as a biocontrol fungus against *Heterobasidion* spp., but its performance has been reported to have diminished during repeated use (Lim et al., 2011). Whether the growth debilitation of the host fungus is caused by the viral infections is yet unknown.

In this study, our aim was to investigate the role of *H. abietis* as a vector for *P. gigantea* and associated viruses. Insect feeding experiments were conducted to assess whether the large pine weevil is able to disseminate *P. gigantea* via passage through its digestive tract. The stability of PgLV-1 infections was examined during insect digestion and among *P. gigantea* oidial spores and hyphal tips. Putative phenotypic effects caused by the virus were tested using virus-infected and virus-free strains obtained by isolation of hyphal tips or oidial spores.

2. Materials and methods

2.1. Feeding experiment with P. gigantea and H. abietis

Two strains of *P. gigantea* were used to infect pine and spruce branches to be fed to *H. abietis* insects. Strain 93073 and the Rotstop strain (Verdera Oy) were both originally isolated by K. Korhonen from spruce wood in Finland (Korhonen et al., 1994). *P. gigantea* 93073 is infected with the virus strain PgLV-1 (Lim et al., 2011), whereas Rotstop does not harbour any known viruses. The *P. gigantea* strains were inoculated on 2% malt extract agar (MEA) plates, each fungal strain as 20 replicates. The plates were incubated at room temperature for 2 weeks and used for infecting conifer branches. Specifically, 10 *Pinus sylvestris* branches and 10 *Picea abies* branches were infected with *P. gigantea* 93073 and an equal amount of branches was infected with the Rotstop strain (the total number of branches used was therefore 40).

Fresh branches of *P. sylvestris* and *P. abies* were collected from a 17-year-old forest from South Estonia (Tartu County, Ülenurme, 58°18N, 26°39E). The branches (diameter 3–5 mm and length 6–7 cm) were cut with nippers from healthy trees, cleaned from needles, transported to the laboratory in a plastic bag and stored in the fridge. The branches were then autoclaved for 30 min at 106°C and placed on agar plates containing *P. gigantea* mycelia (one branch per plate).

The pine weevils were collected from a fresh clear-cut area in the forest district of Järvselja, East Estonia (58°27N, 27°33E), by using ground pitfalls ($25 \times 40 \times 50$ cm). The insects were weighed before the experiment (mean body mass = 0.117 g, SD = 0.03, N = 40) and their gender was determined. In total, 26 female and 14 male insects were included in the experiment. Before the experiment, the insects were washed externally as described in Drenkhan, Sibul, Kasanen, and Vainio (2013). In order to empty their digestive tracts, the insects were starved for 48 h prior to the experiments, while water was supplemented.

One insect was placed on each experimental plate containing a conifer branch infected with *P. gigantea*, and the pine weevils were allowed to feed on the branches for 48 h. The branches were wrapped with tin foil supplemented with small holes to prevent contamination of insect legs or exosceleton (Drenkhan et al., 2013). After feeding, the insects were removed and placed onto new agar plates to collect the excrement. The mean body mass of the insects was 0.139 g (SD = 0.03, N = 40) after the experiment. From each insect, five excrements were selected at random. Surface sterilisation of the excrements was conducted using 96% alcohol, followed by rinsing in sterile water. The excrements were placed onto new agar plates (MEA plates supplemented with thiabendazole, 0.023%, w/v) in the laminar flow cabinet (Kojair Tech). All emerging isolates of *P. gigantea* were harvested and inoculated on new agar plates (2% MEA).

2.2. Genotype determination for P. gigantea

Dual cultures were used to identify the *P. gigantea* strains emerging from the insect faeces and to exclude the possibility of contamination with natural *P. gigantea* isolates. The original strains and corresponding faecal isolates were inoculated at a distance of 70 mm from each other on 2% MEA plates, the plates were sealed with parafilm and incubated at room temperature until there was mycelial contact between the colonies. Complete mycelial fusion was interpreted as a sign of somatic compatibility, indicating that the emergent

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

strains were identical in genotype with the original strains (93073 or Rotstop) used for the feeding experiments. As a control, isolates of 93073 and Rotstop were paired and found to be somatically incompatible as indicated by the formation of a clearing zone between the colonies.

2.3. Counting of oidial spores

Oidial counting was conducted by adding 0.1 g of *P. gigantea* mycelia and 500 µl water to the Eppendorf tube, followed by roiling of the suspension for 1 min. Then, 5 µl of the suspension was applied to the microscope glass slide, and the oidia were counted using 200 times magnification. Counting of oidial spores was based on the method of Sun et al. (2009).

2.4. Isolation of hyphal tips and single oidial spores and temperature treatment

To investigate the stability of PgLV-1 in *P. gigantea* 93073, we tested whether the virus can be excluded from the host using hyphal tip isolation, oidial isolation or temperature treatment. Individual hyphal tips were picked under microscope from *P. gigantea* 93073 cultivated on 2% MEA plates for 1–2 days at 20–25°C using a modified Pasteur pipette as described previously (Vainio, Keriö, & Hantula, 2011).

Single oidial spores of *P. gigantea* 93073 were isolated. Briefly, fungal isolates were cultivated on 2% MEA plates for ca. 3 weeks at 20–25°C. Spores were then collected by washing with 10 ml of deionised sterile water and by moving a glass triangle on the colony surface for a short time. A few droplets (50–100 µl) of the suspension were then spread with a sterile glass triangle onto a Petri dish containing water agar. After 1–2 days of incubation, germinating single spores were transferred using a modified Pasteur pipette onto new MEA plates.

Temperature treatment was conducted by inoculating *P. gigantea* 93073 onto 2% MEA plates, followed by incubation in 31°C for 10 days. At this temperature, the growth of the host fungus slows down considerably.

2.5. Virus detection by RT-PCR

The presence of PgLV-1 in the hyphal tips, thermal-treated mycelia and oidial isolates as well as *P. gigantea* 93073 strains emerged after insect feeding were examined by RT-PCR. Reverse transcription was conducted using total nucleic acid samples prepared using the protocol of Vainio, Piri, and Hantula (2013), including phenol–chloroform extractions and nucleic acid precipitation using polyethylene glycol. The RT reactions were conducted using random hexamer priming and RevertAid MuLV reverse transcriptase (Fermentas GmbH) as described earlier (Vainio et al., 2011).

The primer pair used for detecting the presence of PgLV-1 in the *P. gigantea* isolates has been described by Lim et al. (2011). The PCRs were conducted in volumes of 50 µl, including 1–2 µl of the complementary DNA (cDNA) produced in the RT reaction, 25 pmol of each primer, 1.25 U of DreamTaq DNA polymerase (Thermo Scientific) and 10 nmol of dNTPs. The amplification conditions were as follows: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at 61°C, 1 min at 72°C; and a final extension of 7 min at 72°C.

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

P. gigantea 93073 was used as a positive control, and PCR samples without template cDNA were used as negative controls. The absence of PgLV-1 was confirmed by repeating the nucleic acid extraction and RT-PCR.

2.6. Growth rate measurements and statistical testing

The growth rate of *P. gigantea* 93073 hosting PgLV-1 was compared to four virus-free oidial or hyphal tip isolates. The Rotstop strain was also included in the experiment. A mycelial plug of 5 mm in diameter was inoculated on the centre of each 2% MEA plate and allowed to grow for 3 days. After this, the area occupied by fungal mycelia was measured (in cm^2) using a planimeter (Tamaya Planix 10S Marble, Topgeo Oy). Six replicate cultures were analysed for each fungal isolate. The effect of PgLV-1 on the growth rate of *P. gigantea* 93073 was investigated with Student's *t*-test (Student, 1908) using Microsoft Excel 2010 (two-sample unequal variance, two-tailed distribution).

3. Results

3.1. Survival of P. gigantea *and associated viruses in the alimentary tract of* H. abietis

Insects feeding on conifer branches infected with *P. gigantea* gained ca. 22% of weight during the experiment. *P. gigantea* growth was observed on all the plates containing faeces of *H. abietis* fed with the Rotstop strain ($n = 20$). However, only 7 of the 20 plates containing faeces of *H. abietis* fed with the PgLV-1-infected *P. gigantea* 93073 had viable mycelia (35% viability). These viable cultures originated from insects fed on both Norway spruce (four plates) and from Scots pine (three plates). All the emerging strains were somatically compatible with the original isolate fed to the insects (93073 or Rotstop).

The virus-specific primer pair successfully amplified a PCR product of the expected length (ca. 450 bp) from all the seven *P. gigantea* 93073 isolates recovered from *H. abietis* faeces. Therefore, virus persistence was 100% during passage through the alimentary tract.

3.2. Presence of PgLV-1 in hyphal tips, oidial spores and temperature-treated culture

We examined a total of 13 hyphal tip isolates, 12 of which contained PgLV-1 infection as revealed using RT-PCR. Moreover, we observed a frequency of 90% for the PgLV-1 among a total of 29 oidial isolates, and only 3 oidial isolates were found to be virus-free based on RT-PCR (Figure 1). Temperature treatment at 31°C for 10 days did not cure the virus from *P. gigantea* 93073 although the growth of the fungus was nearly halted.

3.3. Growth rate measurements and oidial counting

The effects of PgLV-1 were assessed using the virus-infected *P. gigantea* 93073 and the four virus-free isolates generated by hyphal tip isolation (strain HT05) and single oidial

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

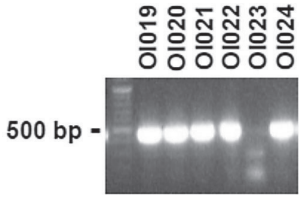

Figure 1. (Colour online) Examples of RT-PCR amplification products obtained from six oidial isolates of *P. gigantea* 93073 as detected by agarose gel electrophoresis. The GeneRuler 100 bp DNA Ladder Plus (Fermentas) was used as a size standard.

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

isolation (strains OI023, OI028 and OI029). Based on 3 days growth on 2% MEA plates, the presence of PgLV-1 did not significantly affect the growth rate of the host strain: comparison of the area colonised by the original virus-infected *P. gigantea* strain to that of isogenic virus-free strains yielded *p*-values of $0.09 < P < 0.77$ using the Student's *t*-test (Figure 2).

The growth of the Rotstop strain was considerably faster than that of *P. gigantea* 93073 (Figure 2), whereas the oidial counts for both fungal strains were normal (see Sun et al., 2009), and somewhat higher for *P. gigantea* 93073 (32.36 million spores/plate) than for Rotstop (24.27 million spores/plate).

4. Discussion

This is the first study showing that mycelium and oidia of *P. gigantea* can survive during the passage through the alimentary tract of *H. abietis*. The results support the possibility of beetle-mediated transfer for *P. gigantea*. Some recent publications have also suggested associations between primary scolytid species or bark beetles (*Pityogenes chalcographus* and *Ips typographus*) and *P. gigantea* (Persson, Ihrmark, & Stenlid, 2011; Strid, Schroeder, Lindahl, Ihrmark, & Stenlid, 2014). Similar experiments have been conducted earlier for

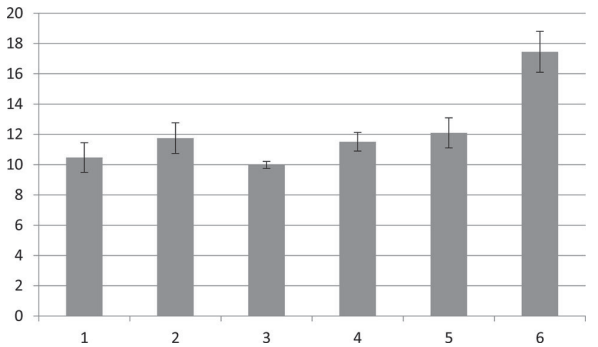

Figure 2. (Colour online) Growth (cm^2/3 days) of virus-free and virus-infected isolates of *P. gigantea*. The error bars show standard deviation. 1, OI023 (virus-free 93073); 2, OI028 (virus-free 93073); 3, OI029 (virus-free 93073); 4, HT05 (virus-free 93073); 5, virus-infected 93073; 6, Rotstop (virus-free). Virus-free strains were generated by isolation of hyphal tips and single oidial spores of *P. gigantea* 93073.

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

Heterobasidion spp. (Drenkhan et al., 2013; Nuorteva & Laine, 1972) and revealed that strains of *Heterobasidion* are also able to survive digestion by *H. abietis*. It can be speculated that the possibility of insect-mediated transfer could contribute significantly to local spread of the *Heterobasidion* pathogen as well as the biocontrol fungus used against it (*P. gigantea*), but the ecological significance of these observations needs to be studied further. In general, bark beetles serve as vectors for numerous fungal species, but relatively few species are transmitted *via* insect feeding. Best known examples are ascomycetes transported by bark beetles of family Scolytidae. Ascospores of *Ophiostoma ips* (Rumbold) Nannf. have been detected in the gut and faeces of adult *Ips* species (Harrington, 2005), and the blue-stain fungus *Ceratocystis polonica* (Siemaszko) C. Moreau has been found in the digestive tract of *I. typographus* L. (Furniss, Solheim, & Christiansen, 1990). In addition to our earlier report (Drenkhan et al., 2013), this study demonstrated that basidiomycetes with diverse ecological strategies are transported by a common and economically important insect pest of the family Curculionidae.

Here, we showed that the virus PgLV-1 infecting *P. gigantea* is able to resist passage through the alimentary tract of the large pine weevil (*H. abietis*) inside the fungal host. We have earlier shown that viruses infecting the conifer pathogen *Heterobasidion parviporum* Niemelä & Korhonen similarly resist passage through *H. abietis*, but there was variability in the transmission rate between the *Heterobasidion* viruses, and the passage resulted in some degree of virus loss (Drenkhan et al., 2013). In principle, mycoviruses are transmitted intracellularly, and they should have no contact with the alimentary tract during insect feeding. However, we can hypothesise that virus loss might result from unfavourable conditions during the passage. Mycovirus titers can often be lowered using chemicals or culturing conditions that hinder dsRNA replication (e.g. cycloheximide, elevated temperature) (Ahn & Lee, 2001; Elias & Cotty, 1996; Fulbright, 1984; Vainio, Korhonen, Tuomivirta, & Hantula, 2010; Zhou & Boland, 1997). In addition, mycoviruses are frequently absent from sexual or asexual spores or actively growing hyphal tips. Virus loss might therefore result from uneven distribution of viruses in the fungal mycelium, especially if only minute amounts of hyphae or spores survive inside the digestive tract of an insect. Based on this study, insect feeding does not present a comparably limiting 'bottle neck' treatment resulting in frequent virus loss, and virus infections seem to be highly stable in *P. gigantea*. Nevertheless, a small proportion of hyphal tips and oidial spores of virus-infected mycelia seemed to be devoid of PgLV-1. The stability of viral infections is important in case the presence of viruses threatens the viability and/or efficacy of *P. gigantea* biocontrol strains.

It has been suggested that viruses might be a reason why some *P. gigantea* biocontrol strains seem to deteriorate in time (Lim et al., 2011). In this study, we compared the viability of a virus-infected *P. gigantea* strain to the virus-free Rotstop strain after passing through the digestive tract of *H. abietis*. After insect passage, we obtained 7 viable isolates of *P. gigantea* 93073, while 13 isolates did not survive through the alimentary tract. In the case of the Rotstop strain, all experiments ($n = 20$) yielded viable cultures after insect passage. Therefore, the viability of the virus-infected *P. gigantea* isolate seemed to be lower compared to the Rotstop strain. It should be noted that this difference might be due to the presence of the virus or it might result from differences between the nuclear genotypes of the fungal strains. The Rotstop biocontrol strain also showed considerably

higher growth rate than any isolates derived from *P. gigantea* 93073, which reflects the high fitness of the biocontrol strain. Nevertheless, the growth rate of virus-free oidial or hyphal tip isolates of *P. gigantea* 93073 seemed to be approximately similar to the original virus-infected isolate on laboratory culturing media. The presence of viruses in airborne basidiospores may enable efficient long-range dispersal of mycoviruses (Vainio, Müller, Korhonen, Piri, & Hantula, 2015), but it is yet unknown whether viruses are present in *P. gigantea* basidiospores.

In conclusion, we found that: (i) *P. gigantea* was able to survive during the passage through the alimentary tract of *H. abietis*, (ii) PgLV-1 infecting *P. gigantea* was able to resist passing through the alimentary tract of *H. abietis* inside the fungal host, (iii) *P. gigantea* 93073 infected with PgLV-1 showed a lower viability and growth rate than the virus-free Rotstop biocontrol strain, but the virus did not drastically alter the growth rate of *P. gigantea* 93073 when isogenic virus-free and virus-infected strains were investigated.

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

Acknowledgements

We thank Mr Ivar Sibul for providing the *H. abietis* insects and Kalev Adamson, Marja-Leena Santanen, Sonja Sarsila, Ingrid Tobreluts and Joonas Karppinen for assistance in the laboratory.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was financially supported by the Estonian Environmental Investment Centre [project no. 3-2_8/18-7/2012], Natural Resources Institute Finland (Luke) and the Academy of Finland [decision number 251193].

References

Ahn, I. P., & Lee, Y. H. (2001). A viral double-stranded RNA up regulates the fungal virulence of *Nectria radicicola*. *MPMI*, *14*, 496–507. Retrieved from http://dx.doi.org/10.1094/MPMI.2001.14.4.496

Day, K. R., Nordlander, G., Kenis, M., & Halldórsson, G. (2004). General biology and life cycles of bark weevils. In F. Lieutier, K. R. Day, A. Battisti, J.-C. Grégoire, H. F. Evans (Eds.), *Bark and wood boring insects in living trees in Europe, a synthesis* (pp. 331–349). Dordrecht: Kluwer Academic.

Drenkhan, T., Hanso, S., & Hanso, M. (2008). Effect of the stump treatment with *Phlebiopsis gigantea* against root rot in Estonia. *Baltic Forestry*, *14*, 16–26.

Drenkhan, T., Sibul, I., Kasanen, R., & Vainio, E. J. (2013). Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. *Forest Pathology*, *43*, 317–323. doi:10.1111/efp.12033

Elias, K. S., & Cotty, P. J. (1996). Incidence and stability of infection by double-stranded RNA genetic elements in *Aspergillus* section flavi and effects on aflatoxigenicity. *Canadian Journal of Botany*, *74*, 716–725. doi:10.1139/b96-090

Eriksson, J., Hjortstam, K., & Ryvarden, L. (1981). *The Corticiaceae of North Europe, Vol. 6: Phlebia-Sarcodontia* (pp. 1048–1276). Oslo: Fungiflora.

Fulbright, D. W. (1984). Effect of eliminating dsRNA in hypovirulent *Endothia parasitica*. *Phytopathology*, *74*, 722–724. Retrieved from http://www.apsnet.org/publications/phytopathology/backissues/Documents/1984Articles/Phyto74n06_722.pdf

Furniss, M. M., Solheim, H., & Christiansen, E. (1990). Transmission of blue-stain fungi by *Ips typographus* (Coleoptera: Scolytidae) in Norway spruce. *Annals of the Entomological Society of America*, *83*, 712–716. Retrieved from http://dx.doi.org/10.1093/aesa/83.4.712

Ghabrial, S. A., & Suzuki, N. (2009). Viruses of plant pathogenic fungi. *Annual Review of Phytopathlogy*, *47*, 353–384. doi:10.1146/annurev-phyto-080508-081932

Greig, B. J. W. (1976). Biological control of *Fomes annosus by Peniophora gigantea*. *European Journal of Forest Pathology*, *6*, 65–71. doi:10.1111/j.1439-0329.1976.tb00508.x

Grillo, R., Hantula, J., & Korhonen, K. (2005). Interfertility between North American and European strains of *Phlebiopsis gigantea*. *Forest Pathology*, *35*, 173–182. doi:10.1111/j.1439-0329.2005.00399.x

Harrington, T. C. (2005). Ecology and evolution of mycophagous bark beetles and their fungal partners. In F. E. Vega, M. Blackwell (Eds.), *Insect-fungal associations: Ecology and evolution* (pp. 257–291). New York: Oxford University Press.

Holdenrieder, O., & Greig, B. J. W. (1998). Biological methods of control. In S. Woodward, J. Stenlid, R. Karjalainen, A. Hüttermann (Eds.), *Heterobasidion annosum: Biology, ecology, impact and control* (pp. 235–258). Wallingford, UK: CAB International,Wallingford.

Hunt, R. S., & Cobb, F. W. Jr. (1982). Potential arthropod vectors and competing fungi of *Fomes annosus* in pine stumps. *Canadian Journal of Plant Pathology*, *4*, 247–253. doi:10.1080/07060668209501290

Hyder, R., Pennanen, T., Hamberg, L., Vainio, E. J., Piri, T., & Hantula, J. (2013). Two viruses of *Heterobasidion* confer beneficial, cryptic or detrimental effects to their hosts in different situations. *Fungal Ecology*, *6*, 387–396. doi:10.1016/j.funeco.2013.05.005

Kallio, T. (1965). Studies on the biology of distribution and possibilities to control *Fomes annosus* in southern Finland. *Acta Forestalia Fennica*, *78*, 1–18. (in Finnish, English summary)

Korhonen, K. (1993). Juurikäävän torjunta. In T. Kurkela, K. Lipponen (Eds.), *Metsänsuojelututkimuksen tuloksia. Metsäntutkimuspäivä Vantaalla 9.12.1992. Metsäntutkimuslaitoksen tiedonantoja* (pp. 53–60) (Research Papers 460). The Finnish Forest Research Institute (In Finnish).

Korhonen, K., Kannelsuo, S., Vainio, E., & Hantula, J. (1997). Population structure of *Phlebiopsis gigantea* in Europe. Root and Butt Rots of Forest Trees. In C. Delatour, J. J. Guillaumin, B. Lung-Escarmant, & B. Marcais (Eds.), *Proceedings of the 9th international conference on root and butt rots* (p. 438). Carcans-Maubuisson, France, September 1–7, 1997. Paris: INRA Editions, Les Colloques.

Korhonen, K., & Kauppila, P. (1988). The sexuality of *Phlebiopsis gigantea*. *Karstenia*, *27*, 23–30. ISSN 0453–3402

Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., … Niemi, M. (1994). Control of *Heterobasidion annosum* by stump treatment with 'Rotstop', a new commercial formulation of *Phlebiopsis gigantea*. In M. Johansson, J. Stenlid (Eds.), *Proceedings of the 8th international conference on root and butt rots* (pp. 675–682). Finland: Wik, Sweden and Haikko.

Kozlakidis, Z., Hacker, C. V., Bradley, D., Jamal, A., Phoon, X., Webber, J., … Coutts, R. H. A. (2009). Molecular characterisation of two novel double-stranded dsRNA elements from *Phlebiopsis gigantea*. *Virus Genes*, *39*, 132–136. doi:10.1007/s11262-009-0364-z

Langström, B., & Day, K. R. (2004). Damage, control and management of weevil pests, especially *Hylobius abietis*. In F. Lieutier, K. R. Day, A. Battisti, J.-C. Gregoire, & H. F. Evans (Eds.), *Bark and wood boring insects in living trees in Europe: A synthesis* (pp. 415–444). Dordrecht: Kluwer Academic.

Leather, S. R., Day, K. R., & Salisbury, A. N. (1999). The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bulletin of Entomological Research*, *89*, 3–16. doi:10.1017/S0007485399000024

Lim, J. M., Jamal, A., Phoon, X., Korhonen, K., & Coutts, R. H. A. (2011). Incidence of *Phlebiopsis gigantea* large virus-1 in a collection of *Phlebiopsis gigantea* isolates. *Archives of Virology*, *156*, 2091–2094. doi:10.1007/s00705-011-1086-9

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

MacDonald, W. L., & Fulbright, D. W. (1991). Biological control of chestnut blight: Use and limitations of transmissible hypovirulence. *Plant Disease*, *75*, 653–661. Retrieved from http://www.apsnet.org/publications/PlantDisease/BackIssues/Documents/1991Articles/PlantDisease75n07_656.pdf

Márquez, L. M., Redman, R. S., Rodriguez, R. J., & Roossinck, M. J. (2007). A virus in a fungus in a plant: Three-way symbiosis required for thermal tolerance. *Science*, *315*, 513–515. doi:10.1126/science.1136237

Meredith, D. S. (1959). The infection of pine stumps by *Fomes annosus* and other fungi. *Annals of Botany*, *23*, 455–476. doi:10.1093/aob/23.3.455

Nuorteva, M., & Laine, L. (1972). Lebensfähige Diasporen des Wurzelschwamms (*Fomes annosus* (Fr.) Cooke) in den Exkrementen von *Hylobius abietis* L. (Col., Curculionidae). *Annales Entomologici Fennici*, *38*, 119–121.

Örlander, G., & Nilsson, U. (1999). Effect of reforestation methods on pine weevil (*Hylobius abietis*) damage and seedling survival. *Scandinavian Journal of Forest Research*, *14*, 341–354. doi:10.1080/02827589950152665

Örlander, G., Nordlander, G., Wallertz, K., & Nordenhem, H. (2000). Feeding in the crowns of scots pine trees by the pine weevil *Hylobius abietis*. *Scandinavian Journal of Forest Research*, *15*, 194–201. doi:10.1080/028275800750015000

Persson, Y., Ihrmark, K., & Stenlid, J. (2011). Do bark beetles facilitate the establishment of rot fungi in Norway spruce? *Fungal Ecology*, *4*, 262–269. doi:10.1016/j.funeco.2011.01.005

Rishbeth, J. (1963). Stump protection against Fomes annosus. III. Inoculation with Peniophora gigantea. *Annals of Applied Biology*, *52*, 63–77. doi:10.1111/j.1744-7348.1963.tb03728.x

Rishbeth, J. (1975). Stump inoculation: A biological control of *Fomes annosus*. In G. W. Bruehl (Ed.), *Biology and control of soil borne plant pathogens international symposium. American Phytopathological Society* (pp. 158–162). St Paul, MN: American Phytopathological Society.

Rykowski, K., & Sierota, Z. (1983). A biologically active preparation for protecting stands against root rot. *Polish Technical Review*, *5*, 15.

Sibul, I., Ploomi, A., & Voolma, K. (2009). Influence of neem oil on the large pine weevil, *Hylobius* abietis L. (Coleoptera, Curculionidae). *Baltic Forestry*, *15*, 255–261.

Stalpers, J. A. (1978). Identification of wood-inhabiting fungi in pure culture. *Studies in Mycology*, 248 pp. Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

Strid, Y., Schroeder, M., Lindahl, B., Ihrmark, K., & Stenlid, J. (2014). Bark beetles have a decisive impact on fungal communities in Norway spruce stem sections. *Fungal Ecology*, *7*, 47–58. doi:10.1016/j.funeco.2013.09.003

Student (1908). The probable error of a mean. *Biometrika*, *6*, 1–25. doi:10.1093/biomet/6.1.1

Sun, H., Korhonen, K., Hantula, J., Asiegbu, F. O., & Kasanen, R. (2009). Use of a breeding approach for improving biocontrol efficacy of *Phlebiopsis gigantea* strains against *Heterobasidion* infection of Norway spruce stumps. *FEMS Microbiology Ecology*, *69*, 266–273. doi:10.1111/j.1574-6941.2009.00711.x

Toivonen, R., & Viiri, H. (2006). Adult large pine weevils *Hylobius abietis* feed on silver birch *Betula pendula* even in the presence of conifer seedlings. *Agricultural and Forest Entomology*, *8*, 121–128. doi:10.1111/j.1461–9563.2006.00290.x

Vainio, E. J., & Hantula, J. (2000). Genetic differentiation between European and North American populations of *Phlebiopsis gigantea*. *Mycologia*, *92*, 436–446. doi:10.2307/3761502

Vainio, E. J., Keriö, S., & Hantula, J. (2011). Description of a new putative virus infecting the conifer pathogenic fungus *Heterobasidion parviporum* with resemblance to *Heterobasidion annsoum* P-type partitivirus. *Archives of Virology*, *156*, 79–86. doi:10.1007/s00705-010-0823-9

Vainio, E. J., Korhonen, K., & Hantula, J. (1998). Genetic variation in *Phlebiopsis gigantea* as detected with random amplified microsatellite (RAMS) markers. *Mycological Research*, *102*, 187–192. doi:10.1017/S0953756297004577

Vainio, E. J., Korhonen, K., Tuomivirta, T. T., & Hantula, J. (2010). A novel putative partitivirus of the saprotrophic fungus *Heterobasidion ecrustosum* infects pathogenic species of the *Heterobasidion annosum* complex. *Fungal Biology*, *114*, 955–965. doi:10.1016/j.funbio.2010.09.006

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

Vainio, E. J., Müller, M. M., Korhonen, K., Piri, T., & Hantula, J. (2015). Viruses accumulate in aging infection centers of a fungal forest pathogen. *The ISME Journal*, *9*, 497–507. doi:10.1038/ismej.2014.145

Vainio, E. J., Piri, T., & Hantula, J. (2013). Virus community dynamics in the conifer pathogenic fungus *Heterobasidion parviporum* following an artificial introduction of a partitivirus. *Microbial Ecology*, *65*, 28–38. doi:10.1007/s00248-012-0118-7

Wainhouse, D., & Brough, S. (2007, May 2015). Resistance of young conifers to feeding damage by pine weevil. *Information Note*, Forestry Commission. Retrieved from http://www.forestry.gov.uk/pdf/fcin087.pdf/$FILE/fcin087.pdf.

Zhou, T., & Boland, G. J. (1997). Hypovirulence and double-stranded RNA in *Sclerotinia homoeocarpa*. *Phytopathology*, *87*, 147–153. Retrieved from http://dx.doi.org/10.1094/PHYTO.1997.87.2.147

Downloaded by [Estonian University of Life Sciences] at 05:32 10 June 2016

III

Drenkhan, T., Voolma, K., Adamson, K., Sibul, I., Drenkhan, R. 2017. The large pine weevil *Hylobius abietis* (L.) as a potential vector of the pathogenic fungus *Diplodia sapinea* (Fr.) Fuckel. Agricultural and Forest Entomology, 19: 4–9.

Agricultural and Forest Entomology

Agricultural and Forest Entomology (2017), **19**, 4–9 DOI: 10.1111/afe.12173

The large pine weevil *Hylobius abietis* (L.) as a potential vector of the pathogenic fungus *Diplodia sapinea* (Fr.) Fuckel

Tiia Drenkhan, Kaljo Voolma, Kalev Adamson, Ivar Sibul and Rein Drenkhan

Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, F.R. Kreutzwaldi 5, 51014 Tartu, Estonia

Abstract

1 *Diplodia sapinea*, an important pathogen of various conifer species, was recently recorded in the northern Baltic region. The pathogen can disperse over short distances via rain or wind, whereas long range dispersal occurs via movement of contaminated plant material and seeds by humans, as well as by insects.

2 *Hylobius abietis* is one of the most important forest pests over large areas of Europe. Adult weevils feed on the bark of seedlings of young conifers, causing injury and often death.

3 Weevils were collected from fresh clear-cut areas and near the symptomatic conifer stands to analyze the presence of *D. sapinea*. Entire weevils collected from natural environments were crushed without surface washing.

4 The identity of *D. sapinea* and the fungal DNA extraction from the insects was confirmed by species-specific polymerase chain reaction priming. Eight *H. abietis* individuals were determined to be infected with *D. sapinea.* In a laboratory experiment, pine branches were infected with *D. sapinea* and were fed to adult *H. abietis*.

5 The results show that the pathogen survived in the digestive tract of *H. abietis* in the laboratory experiment, and also that the isolation of fungus from the faeces was successful. In addition, the results demonstrate that the large pine weevil may be a potential vector of pathogenic fungus *D. sapinea*.

Keywords Insect vector, invasive pathogen, *Pinus* spp., species-specific PCR (SSPP), *Sphaeropsis sapinea*.

Introduction

Diplodia sapinea (Fr.) Fuckel (syn. *D. pinea* (Desm.) Kickx. *Sphaeropsis sapinea* / Fr.: Fr./ Dyko & Sutton) (Ascomycota: Botryosphaeriaceae) is the widely distributed pathogen of conifers causing *Diplodia* tip blight (syn. *Sphaeropsis* tip blight) (Phillips *et al.*, 2013). This pathogen is known to occur on pines and other conifers across the world (Stanosz *et al.*, 1999; Flowers *et al.*, 2001). In Estonia, *D. sapinea* was first found on Austrian pine (*Pinus nigra* J. F. Arnold) in 2007 (Hanso & Drenkhan, 2009), and later on native Scots pine (*Pinus sylvestris* L.) in 2012 (Adamson *et al.*, 2015). *Diplodia sapinea* is also known to have an endophytic life strategy, which is a part of the pathogen life-cycle (Stanosz *et al.*, 1997, 1999; Luchi *et al.*, 2014), occurring in asymptomatic green needles (Diminic & Jurc, 1999), stems, buds, immature cones and bracts of mature cones (Flowers *et al.*, 2001; Burgess *et al.*, 2004; Stanosz *et al.*, 2005; Decourcelle *et al.*, 2014). Over short distances *D. sapinea* spreads through conidia mostly by rainfall (Tainter & Baker, 1996; Shigesada & Kawasaki, 2001). Wind plays a very limited role dispersing conidia (Swart & Wingfield, 1991). Long-range dispersal can occur from all colonized tissues of plants or trees (e.g. needles, cones, branches, seeds and the pith of cones), as well as by wood (Flowers *et al.*, 2001; Bihon *et al.*, 2011) or water flow, animals, birds, arthropods, insects (Shigesada & Kawasaki, 2001) and international trade in plant material (e.g. seeds, horticultural stock and fruit) (Parry, 1990).

The large pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) is a common and important pest of conifer reforestation, occurring across the northern Palaearctic region (Leather *et al.*, 1999; Långström & Day, 2004), including Estonia (Sibul, 2000; Sibul & Voolma, 2004). *Hylobius abietis* develops in the stumps and roots of dying and dead conifer trees, when females are laying eggs in the bark of fresh conifer stumps (Leather *et al.*, 1999). The adult weevils hibernate below ground in the soil and litter and emerge from hibernation in spring when temperatures reach 8–9 °C (Nordenhem, 1989; Leather *et al.*,

Correspondence: Tiia Drenkhan. Tel.: +372 731 3795; e-mail: tiia.drenkhan@emu.ee

© 2016 The Royal Entomological Society

1999). Before and after oviposition in spring, *H. abietis* adults feed on seedlings, on the bark of young transplants or branches of conifers, on the canopy of mature trees (Örlander *et al.*, 2000) and on the bark of tree roots (Nordlander *et al.*, 2003; Tan *et al.*, 2011).

Reforestation areas are attacked during spring migration, when adult *H. abietis* insects may disperse up to 10 km to find stumps and dying roots of conifers suitable for breeding (Solbreck, 1980). Individual weevils may also remain at a particular clear cut site for a number of months, although movement patterns and residence times have not been well documented (Leather *et al.*, 1995). In 2004, the cost of the resulting *H. abietis* damage across Europe was estimated to be approximately 140 million Euros per year (Långström & Day, 2004).

The association between *D. sapinea* and insect damage is observed in many countries and is generally related to bark beetles (Coleoptera: Scolytinae) (Swart & Wingfield, 1991; Lombardero *et al.*, 2006; Reay *et al.*, 2006; Whitehill *et al.*, 2007). Less is known about the vector relationships between *D. sapinea* and *Hylobius* species, particularly the large pine weevil.

The present study aimed to: (i) investigate the ability of *H. abietis* to carry *D. sapinea* under natural conditions and (ii) determine whether the fungus survives through the digestive tract of the weevils.

Materials and methods

Isolates of D. sapinea

The *D. sapinea* strain used in the experiment (GenBank accession number KJ143982) was collected from a Scots pine tree on Vormsi Island, Estonia (58°59′N, 23°14′E). After surface sterilizing of cone scales in 96% ethanol and squeezing the fruiting body onto pine needle agar media, samples were incubated for 1 week at room temperature. The pine needle agar consisted of 1 L of filtered Scots pine needle extract (100 g fresh weight/L tap water boiled for 20 min) and 14 g of OXOID Malt Extract, LP0039 (Oxoid Ltd, U.K.) autoclaved at 106 °C for 30 min. Some mycelia were transferred to fresh pine needle agar plates and, after several transfers to fresh agar plates and cultivation, all isolates were transferred onto agar plates covered with a sterile cellophane membrane (British Cellophane Ltd, U.K.). After an incubation time of approximately 2 to 3 weeks at 20 °C, hyphal mass from culture edges was removed and stored at −20 °C until used for DNA isolation (Drenkhan *et al.*, 2013a).

Hylobius abietis insects

To determine the ability of the pine weevils to carry *D. sapinea* under natural conditions, insects were collected in May 2015 from two different location: Kaitsemõisa in Tartu county (plot 1) (eastern Estonia, 58°34′N, 26°49′E) and Ränna in Võru county (plot 2) (southern Estonia, 57°45′N, 26°29′E); the distance between the locations is 117 km. Plot 1 was located on an old clear-cut area and plot 2 was on former agricultural land, close to pine trees (up to 65 years old) infected by *D. sapinea*. The weevils were collected from six ground pitfall trap-holes ($20 \times 40 \times 45\ cm^3$) within 15 m of each other; both plots contained an equal number of traps. In both locations, the insects were collected over an interval of 5 days and a total of 34 weevil adults were collected. The living weevils were removed from the traps, transferred to the laboratory and stored at −20 °C until DNA extraction.

For the laboratory feeding experiment, *H. abietis* adults were collected from Järvselja Forest District (southeast Estonia, 58°27′N, 27°33′E) in May 2013, the first year after clear cutting (0.9 ha). To collect moving weevils, five ground pitfall trap-holes ($25 \times 40 \times 50\ cm^3$) with a spacing of 15 m were located in a *Vaccinium-Myrtillus* site type in a Scots pine clear-cut area. The living weevils were removed from the traps, transferred to the laboratory and stored at +4 °C until required.

Inoculation of P. sylvestris *branches with* D. sapinea *for the feeding experiment*

Fresh branches of *P. sylvestris* were collected from a 15-year-old Scots pine forest in southern Estonia (Tartu County, Ülenurme, 58°18′N, 26°39′E). The branches (diameter 3–5 mm; length 6–7 cm) were cut with secateurs from healthy pine trees, cleaned of needles, and transported to the laboratory in a plastic bag and stored at +4 °C. The branches were autoclaved for 30 min at 106 °C and placed onto the malt extract agar (MEA) overgrown by *D. sapinea* mycelium (one branch per Petri dish). The dishes were closed with Parafilm (Pechiney Plastic Packaging Company, Chicago, Illinois). After 2 weeks, the pine branches were totally covered by *D. sapinea* mycelium. All the tools and instruments used in the experiment were autoclaved and sterilized before each experiment and then sprayed with alcohol (96%) and left to dry (Drenkhan *et al.*, 2013b).

Insect feeding experiment and isolation of D. sapinea *from insect excrement*

Before the experiment, the insects were thoroughly purified both internally and externally. Insect excrement and impurities attached to the insect legs were removed by allowing them to adhere to moist filter papers at the bottom of 90-mm diameter Petri dishes. After 24 h, the papers were exchanged and more water was added. To empty their digestive tracts, the insects were starved for 48 h before the experiments to reduce the load of pre-existing fungal spores in the faeces, at the same time as being supplemented with fresh water (Drenkhan *et al.*, 2013b, 2015).

New filter paper was placed inside sterile Petri dishes. The pine branches were wrapped in aluminium foil using disposable gloves. Foil was added to prevent the insects from obtaining conidia or hyphae in their legs or exoskeleton. Two holes ($0.3 \times 0.3\ cm^2$) were made in the foil to allow feeding (Fig. 1). One pine branch and one insect were placed on each Petri dish (90 mm). Seven male and seven female *H. abietis* were used in the feeding experiments.

The pine weevils were allowed to feed on the *Diplodia*-infected pine branches for 48 h. After feeding, the insects were placed on new Petri dishes for 24 h. The excrement was collected from the Petri dishes without the feeding branches.

Four discrete faeces were collected at random from each insect; excrement was handled with tweezers and surface sterilized for a few seconds in alcohol (96%) and rinsed in sterile water. All

Figure 1 *Hylobius abietis* individual feeding on a colonized pine branch covered with foil and containing two feeding holes (photo Tiia Drenkhan). [Colour figure can be viewed at wileyonlinelibrary.com].

four faeces samples from each weevil were placed onto new agar plates in the laminar flow cabinet under sterile conditions. The fresh MEA plates were supplemented with the antibiotic thiabendazole (20 g of malt extract, 15 g of agar, 1 L of water, autoclave sterilization for 20 min by 120 °C, with the addition of 230 mg of thiabendazole, 1 mL of lactic acid and a small amount (4 mL) of sterile water to make the thiabendazole soluble) (ICN Biomedicals Inc., Irvine , California). After incubation for 2 weeks at room temperature, the excrement and fungal cultures were examined under a dissecting microscope and *Diplodia* isolates were harvested and inoculated on new Petri dishes with MEA. All isolates of *Diplodia* from each weevil were inoculated onto the same Petri dishes as all of the excrement on single plates originating from the same insect. Altogether, 20 replicate plates were included in the experiment initially, although no *D. pinea* was present in 6 of 20 cultures after 2 weeks; these Petri dishes were excluded from further work. The experiment was continued with 14 cultures.

DNA extraction and fungal species identification

For the isolation of fungal DNA from field-collected and frozen insects, entire weevils were crushed without surface sterilization. Cell disruption was performed in a Retsch MM400 homogenizer (Retsch GmbH, Germany) using a mix of quartz sand and metal beads (2.5 mm). DNA was extracted using the E.Z.N.A Fungal DNA Mini Kit (Omega Bio-Tek Inc., Norcross, Georgia).

Fungal mycelia growing from the weevil faeces were analyzed to determine the occurrence of *D. sapinea*. DNA was extracted from cultures using the method described by Drenkhan *et al.* (2014). The identity of fungi in either case was confirmed by species-specific polymerase chain reaction (PCR) priming (SSPP). SSPP was carried out in 20-μL reactions volumes as described by Smith and Stanosz (2006) with minor modifications: 5 μL of 5 × HOT FIREPol Blend Master MixRTL with 7.5 mM $MgCl_2$ (Solis BioDyne, Estonia), 0.5 μL of both primers: (20 μM) BotR (5′-GCTTACACTTTCATTTATAGACC-3′) and DpF (5′-CTTATATATCAAACTATGCTTTGTA - 3′), 1 μL of template DNA and 13 μL of PCR grade water. Cycling conditions were as reported by Smith and Stanosz (2006) with one modification: the length of the denaturation step was 10 min. The PCR product was visualized on 1% agarose (SeaKemLE Agarose; Lonza Group Ltd, Portsmouth, New Hampshire) gels under ultraviolet light. The resulting PCR products were visualized using the Quantum ST4 system (VilberLourmat SAS, France). All amplifications were performed at least twice; negative and positive controls were included.

For taxonomical assignment, the SSU region of samples was sequenced in the Estonian Biocentre in Tartu using the DpF (Smith & Stanosz, 2006). The sequences were edited using BIOEDIT, version 7.0.9.0 (Hall, 1999). The database at GenBank (http://www.ncbi.nlm.nih.gov/genbank) was used to determine the identity of SSU sequences for fungal taxon confirmation.

Results

Laboratory experiment

Scots pine branches infected by *D. sapinea* were fed to 20 individuals of *H. abietis*. Mycelium of *D. sapinea* grew out in MEA plates from the faeces of 14 weevils (70%) after passing through the digestive tract of the insects. There was no difference in the abilities of male (seven insects) and female (seven insects) insects to carry the fungus.

Before the experiment, the weevils were weighed and the mean body mass was 0.101 ± 0.027 g ($n = 20$). During the experiment (48 h), the insects gained approximately 8 mg of weight, increasing to 0.109 ± 0.024 g ($n = 20$); the weight of the insects was significantly higher ($P < 0.05$) after feeding. The identity of the fungus in the excrement was confirmed by SSPP (Fig. 2). The primers targeted the mitochondrial small subunit ribosomal DNA (mt SSU rDNA) of *D. sapinea* and were used to amplify a 700-bp fragment.

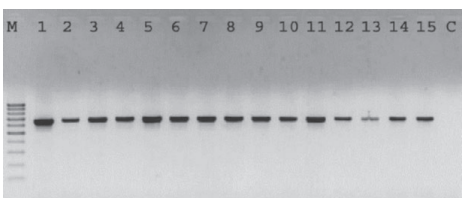

Figure 2 Species-specific polymerase chain reaction priming amplification products of *Diplodia sapinea* after passage through the insects as detected by agarose gel electrophoresis. M, molecular marker (100 bp Range DNA Ladder, Naxo Ltd, Estonia); 1–14, *D. sapinea* isolated from insect excrement; 15, Reference of *D. sapinea*; C, Control.

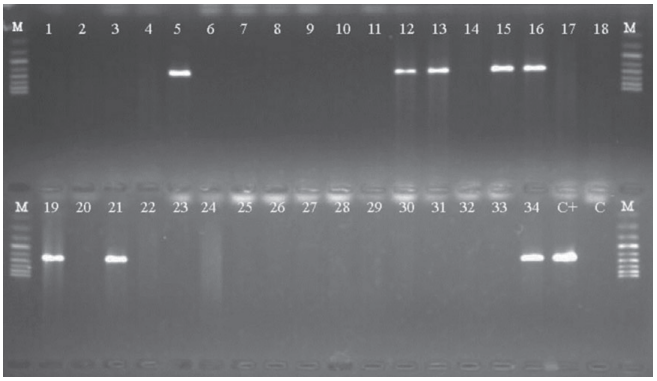

Figure 3 Species-specific polymerase chain reaction priming amplification products of *Diplodia sapinea* occur on adult weevils collected in natural conditions as detected by agarose gel electrophoresis. M, molecular marker (100 bp Range DNA Ladder, Naxo Ltd, Estonia); 5, 12, 13, 15, 16, 19, 21 and 34, *D. sapinea* isolated from weevils; C+, Reference of *D. sapinea*; C, Control.

Field-collected insects

The presence of *D. sapinea* was examined from 34 adult individuals of *H. abietis* collected from two forest sites located in eastern and southern Estonia. Primarily, whole crushed insects were analyzed by SSPP. According to PCR amplification, eight samples of the weevils collected (i.e. 24%) carried the DNA of *D. sapinea* (Fig. 3). In Kaitsemõisa (plot 1), the presence of *D. sapinea* was identified in five weevils (31%), whereas, in Ränna (plot 2), three weevils (17%) were infected by *D. sapinea.* Sequencing of the nuclear mt SSU rDNA region was used to confirm the identity of the fungus in the insect samples. All eight amplicons shared 100% similarity with sequences of *D. sapinea* in GenBank. All of the *D. sapinea* sequences from insect samples were deposited in GenBank under accession numbers KU954092, KU954093, KU954094, KU954095, KU954096, KU954097, KU954098 and KU954099.

Discussion

In the present study, we demonstrate that the large pine weevil may carry propagules of the forest pathogenic fungus *D. sapinea*. We detected *D. sapinea* DNA in insects collected from two forest sampling sites (plot 1 and plot 2), indicating the presence of the fungus either in the exoskeleton or digestive tract of the insect. To investigate whether the fungus remains viable when passing through the alimentary tract of the weevil, we conducted a laboratory feeding experiment, which demonstrated living mycelia of *D. sapinea* in the excrement of *H. abietis*. The laboratory experiment has shown that *D. sapinea* survive in the digestive tract of *H. abietis* and the successful isolation of fungus from the faeces indicates a potential means for spread of pathogen.

According to previous studies (Viiri, 2004; Jankowiak & Bilański, 2013a), most weevils analyzed were contaminated with different fungal spores both internally (within the digestive tract) and externally. Although the spores of *D. sapinea* are naturally present, it is possible that the fungus is picked up by emerging adults of *H. abietis*, which then inoculate pine seedlings during feeding (Leather *et al.*, 1999). *Hylobius abietis* wounds conifer seedlings and breeds in the roots of stumps of recently felled mature conifer trees or dying and dead conifer trees, laying its eggs on the bark of roots or in the soil near the roots (Nordlander *et al.*, 1997; Bylund *et al.*, 2004). The infection of *D. sapinea* to *H. abietis* may originate from roots of mature trees infected by *D. sapinea* or from the harvest debris. Germinable conidia of *Diplodia* species were obtained from debris collected 6 months to 5 years after harvest (Oblinger *et al.*, 2011). The *D. sapinea* is isolated from logs, timber, stumps, veneer and wood chips (Thwaites *et al.*, 2005). The presence of *D. sapinea* in these insects is probably the result of conidia or mycelia on the body surface or attached to legs of the beetles.

The possible role of the *H. abietis* faeces themselves directly transferring phytopathogenic fungi to seedlings in nature is not known (Lévieux *et al.*, 1994) and this aspect needs further investigation. It can be hypothesized that insects drop faeces on the stumps and roots of dying and dead conifer trees when females are laying eggs in bark of conifers. Root bark containing egg cavities with faeces is avoided as food by other individuals of this species (Borg-Karlson *et al.*, 2006).

However, it is clear that the habitats of the *D. sapinea* and *H. abietis* overlap, and so the mortality rate of seedlings that occurs in afforestation sites could be the combined effect of weevils feeding and fungus infection (Lévieux *et al.*, 1994). Weevils feeding on the stem bark of young conifer seedlings often cause growth loss and high mortality (Örlander & Nilsson, 1999; Day *et al.*, 2004; Wallertz *et al.*, 2014). Seedlings stressed after insect damage become more susceptible and can easily be infected by pathogens. *Diplodia sapinea* is able to damage trees of different ages, including seedlings (Stanosz *et al.*, 2005), and infection of *D. sapinea* affects trees that are wounded (Swart & Wingfield, 1991). The weevil feeding activity is increasing at the same time as the length of the overwintering period is reduced as a result of climate warming (Tan *et al.*, 2010, 2011; Wainhouse *et al.*, 2014).

It has been reported previously that several species of fungi have been found in association with *H. abietis* (Viiri, 2004). The large pine weevil may act as a vector of pathogenic fungi such as *Heterobasidion annosum* (Fries) Brefeld (Nuorteva & Laine, 1968, 1972; Kadlec *et al.*, 1992; Leather *et al.*, 1999; Drenkhan *et al.*, 2013b) and several species of *Leptographium* (Piou, 1993; Viiri, 2004; Jankowiak & Bilański, 2013a, 2013b). Little work has been reported on the long-range dispersal of *D. sapinea*,

however, and the possibility that it is entomochorous (i.e. spread by insects) has received little attention to date (Luchi *et al.*, 2012). Spread of the fungus may be aided further by adults being able to recognize trees that have been treated with insecticides and moving onto other trees after initial feeding, thus increasing encounter rates (Rose *et al.*, 2005). Many insects facilitate the dissemination and infection of pathogenic fungi (Lévieux *et al.*, 1994; Whitehill *et al.*, 2007; Jankowiak & Bilański, 2013a, 2013b), particularly via wounds on host plants (Palmer, 1991; Feci *et al.*, 2003). Some insects have previously been associated with the transfer of *D. sapinea*, such as bark beetles (*Ips* spp.) (Whitehill *et al.*, 2007); an invasive seed insect in Europe, *Leptoglossus occidentalis* may also be a vector for this pathogen (Luchi *et al.*, 2012), as well as root-feeding bark beetles *Hylastes* spp. (Jankowiak & Bilański, 2013b). Whether *D. sapinea* would survive passage through the digestive tract was previously unknown, and no information was available with respect to whether *D. sapinea* was coexisting with the large pine weevil under natural conditions.

In conclusion, the results of the present study show that *H. abietis* is a potential vector of *D. sapinea* in managed pine forests. In this case, the insect could contribute to the long-range dispersal of the pathogen. Furthermore, the significance of *H. abietis* in production forestry may increase as we gain more information on the ability of the insect to transmit the pathogenic fungus.

Acknowledgements

We thank Mr Terry Bush (Madison, Wisconsin) for revision of the English, as well as Dr Eeva J. Vainio (LUKE, Finland), Dr Risto Kasanen (University of Helsinki, Finland) and Dr Märt Hanso (Estonian University of Life Sciences, Estonia) for reading and correcting the manuscript submitted for publication. The study was supported by the Estonian Environmental Investments Centre project no. 3-2_8/18-7/2012, the State Forest Management Centre project no. 8-2/T12115 (2012–2015) and the Institutional Research Funding IUT21-04.

References

Adamson, K., Klavina, D., Drenkhan, R., Gaitinieks, T. & Hanso, M. (2015) *Diplodia sapinea* is colonizing the native Scots pine (*Pinus sylvestris*) in the northern Baltics. *European Journal of Plant Pathology*, **143**, 343–350.

Bihon, W., Slippers, B., Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2011) Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa. *Forest Pathology*, **41**, 370–375.

Borg-Karlson, A.-K., Nordlander, G., Mudalige, A., Nordenhem, H. & Unelius, C.R. (2006) Antifeedants in the feces of the pine weevil *Hylobius abietis*: identification and biological activity. *Journal of Chemical Ecology*, **32**, 943–957.

Burgess, T.I., Wingfield, M.J. & Wingfield, B.D. (2004) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australasian Plant Pathology*, **33**, 513–519.

Bylund, H., Nordlander, G. & Nordenhem, H. (2004) Feeding and oviposition rates in the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *Bulletin of Entomological Research*, **94**, 307–317.

Day, K.R., Nordlander, G., Kenis, M. & Halldórsson, G. (2004) General biology and life cycles of bark weevils. *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis* (ed. by F. Lieutier, K. R. Day, A. Battisti, J.-C. Grégoire and H. F. Evans), pp. 331–349. Kluwer Academic Publishers, The Netherlands.

Decourcelle, T., Piou, D. & Desprez-Loustau, M.-L. (2014) Detection of *Diplodia sapinea* in Corsican pine seeds. *Plant Pathology*, **64**, 442–449.

Diminic, D. & Jurc, M. (1999) Some aspects of *Sphaeropsis sapinea* presence on Austrian pine in Croatia and Slovenia Phyton (Austria)Special issue: *Plant Physiology*, **39**, 231–234.

Drenkhan, R., Hantula, J., Vuorinen, M., Jankovský, L. & Müller, M.M. (2013a) Genetic diversity of *Dothistroma septosporum* in Estonia, Finland and Czech Republic. *European Journal of Plant Pathology*, **136**, 71–85.

Drenkhan, T., Sibul, I., Kasanen, R. & Vainio, E.J. (2013b) Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. *Forest Pathology*, **43**, 317–323.

Drenkhan, R., Adamson, K., Jürimaa, K. & Hanso, M. (2014) *Dothistroma septosporum* on firs (*Abies* spp.) in the northern Baltics. *Forest Pathology*, **44**, 250–254.

Drenkhan, T., Kasanen, R. & Vainio, E.J. (2015) *Phlebiopsis gigantea* and associated viruses survive passing through the digestive tract of *Hylobius abietis*. *Biocontrol Science and Technology*, **26**, 320–330.

Feci, E., Smith, D. & Stanosz, G.R. (2003) Association of *Sphaeropsis sapinea* with insect damaged red pine shoots and cones. *Forest Pathology*, **33**, 7–13.

Flowers, J., Nuckles, E., Hartman, J. & Vaillancourt, L. (2001) Latent infection of Austrian and Scots pine tissues by *Sphaeropsis sapinea*. *Plant Disease*, **85**, 1107–1112.

Hall, T. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hanso, M. & Drenkhan, R. (2009) *Diplodia pinea* is a new pathogen on Austrian pine (*Pinus nigra*) in Estonia. *Plant Pathology*, **58**, 797.

Jankowiak, R. & Bilański, P. (2013a) Diversity of ophiostomatoid fungi associated with the large pine weevil, *Hylobius abietis*, and infested Scots pine seedlings in Poland. *Annals of Forest Science*, **70**, 391–402.

Jankowiak, R. & Bilański, P. (2013b) Ophiostomatoid fungi associated with root-feeding bark beetles on Scots pine in Poland. *Forest Pathology*, **43**, 422–428.

Kadlec, Z., Stary, P. & Zumr, V. (1992) Field evidence for the large pine weevil, *Hylobius abietis* as a vector of *Heterobasidion annosum*. *European Journal of Forest Pathology*, **22**, 316–318.

Långström, B. & Day, K.R. (2004) Damage, control and management of weevil pests, especially *Hylobius abietis*. *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis* (ed. by F. Lieutier, K. R. Day, A. Battisti, J.-C. Grégoire and H. F. Evans), pp. 415–444. Kluwer Academic Publishers, The Netherlands.

Leather, S.R., Small, A.A. & Bøgh, S. (1995) Seasonal variation in local abundance of adults of the large pine weevil, *Hylobius abietis* (Col., Curculionidae). *Journal of Applied Entomology*, **119**, 511–513.

Leather, S.R., Day, K.R. & Salisbury, A.N. (1999) The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bulletin of Entomological Research*, **89**, 3–16.

Lévieux, J., Piou, D., Cassier, P., André, M. & Guillaumin, D. (1994) Association of phytopathogenic fungi for the Scots pine (*Pinus sylvestris* L.) with the European pine weevil *Hylobius abietis* (L.) (Col. Curculionidae). *The Canadian Entomologist*, **126**, 929–936.

Lombardero, M.J., Ayres, M.P. & Ayres, B.D. (2006) Effects of fire and mechanical wounding on *Pinus resinosa* resin defenses, beetle attacks, and pathogens. *Forest Ecology and Management*, **225**, 349–358.

Luchi, N., Mancini, V., Feducci, M., Santini, A. & Capretti, P. (2012) *Leptoglossus occidentalis* and *Diplodia pinea*: a new insect-fungus association in Mediterranean forests. *Forest Pathology*, **42**, 246–251.

Luchi, N., Oliveira Longa, C.M., Danti, R., Capretti, P. & Maresi, G. (2014) *Diplodia sapinea*: the main fungal species involved in the colonization of pine shoots in Italy. *Forest Pathology*, **5**, 372–381.

Nordenhem, H. (1989) Age, sexual development, and seasonal occurrence of the pine weevil *Hylobius abietis* (L.) *Journal of Applied Entomology*, **108**, 260–270.

Nordlander, G., Nordenhem, H. & Bylund, H. (1997) Oviposition patterns of the pine weevil *Hylobius abietis*. *Entomologia Experimentalis et Applicata*, **851**, 1–9.

Nordlander, G., Örlander, G. & Langvall, O. (2003) Feeding by the pine weevil *Hylobius abietis* in relation to sun exposure and distance to forest edges. *Agricultural and Forest Entomology*, **5**, 191–198.

Nuorteva, M. & Laine, L. (1968) Über die möglichkeiten der insekten als überträger des wurzelschwamms (*Fomes annosus* (Fr.) Cooke). *Annales Entomologici Fennici*, **34**, 113–135.

Nuorteva, M. & Laine, L. (1972) Lebensfähige diasporen des wurzelschwamms (*Fomes annosus* (Fr.) Cooke) in den exkrementen von *Hylobius abietis* L. (Col., Curculionidae). *Annales Entomologici Fennici*, **38**, 119–121.

Oblinger, B.W., Smith, D.R. & Stanosz, G.R. (2011) Red pine harvest debris as a potential source of inoculum of *Diplodia* shoot blight pathogens. *Forest Ecology and Management*, **262**, 663–670.

Örlander, G. & Nilsson, U. (1999) Effect of reforestation methods on pine weevil (*Hylobius abietis*) damage and seedling survival. *Scandinavian Journal of Forest Research*, **14**, 341–354.

Örlander, G., Nordlander, G., Wallertz, K. & Nordenhem, H. (2000) Feeding in the crowns of Scots pine trees by the pine weevil *Hylobius abietis*. *Scandinavian Journal of Forest Research*, **15**, 194–201.

Palmer, M.A. (1991) Isolate types of *Sphaeropsis sapinea* associated with main stem cankers and top-kill of *Pinus resinosa* in Minnesota and Wisconsin. *Plant Disease*, **75**, 507–510.

Parry, D.W. (1990) *Plant Pathology in Agriculture*. Cambridge University Press, U.K.

Phillips, A.J.L., Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J., Groenewald, J. & Crous, P. (2013) The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, **76**, 51–167.

Piou, D. (1993) Rôle d'*Hylobius abietis* (L.) (Col, Curculionidae) dans le transport de *Leptographium procerum* (Kendr) Wingfet son inoculation au pin sylvestre. *Annales des Sciences Forestière*, **50**, 297–308 (in French with English summary).

Reay, S.D., Thwaites, J.M., Farrell, R.L. & Glare, T.R. (2006) The lack of persistence of *Ophiostomataceae* fungi in *Pinus radiata* 3 years after damage by the bark beetle *Hylastes ater*, and the subsequent colonisation by *Sphaeropsis sapinea*. *Forest Ecology and Management*, **233**, 149–152.

Rose, D., Leather, S.R. & Matthews, G.A. (2005) Recognition and avoidance of insecticide-treated Scots pine (*Pinus sylvestris*) by *Hylobius abietis* (Coleoptera: Curculionidae): implications for pest management strategies. *Agricultural and Forest Entomology*, **7**, 187–191.

Shigesada, N. & Kawasaki, K. (2001) Invasion and the range expansion of species: effects of long-distance dispersal. *Dispersal Ecology* (ed. by J. M. Bullock, R. E. Kenward and R. S. Hails), *The 42nd Symposium of the British Ecological Society at the University of Reading*, *2–5 April 2001*, p. 459 p. Blackwell Science Ltd, U.K.

Sibul, I. (2000) Abundance and sex ratio of pine weevils, *Hylobius abietis* and *H. pinastri* (Coleoptera: Curculionidae) in pine clear-cuttings of different ages. *Transactions of the Estonian Agricultural University*, **209**, 186–189.

Sibul, I. & Voolma, K. (2004) The abundance, seasonal dynamics and sex ratio of the large pine weevil, *Hylobius abietis* (L.), in reforestation areas: assessment by ground pitfalls and baited pitfall traps. *Transactions of the Faculty of Forestry, Estonian Agricultural University*, **37**, 56–62.

Smith, D.R. & Stanosz, G.R. (2006) A species-specific PCR assay for detection of *Diplodia pinea* and *D. scrobiculata* in dead red and jack pines with collar rot symptoms. *Plant Disease*, **90**, 307–313.

Solbreck, C. (1980) Dispersal distances of migrating pine weevils, *Hylobius abietis*, Coleoptera: Curculionidae. *Entomologia Experimentalis et Applicata*, **28**, 123–131.

Stanosz, G.R., Smith, D.R., Guthmiller, M.A. & Stanosz, J.C. (1997) Persistence of *Sphaeropsis sapinea* on or in asymptomatic shoots of red and jack pines. *Mycologia*, **89**, 525–530.

Stanosz, G.R., Swart, W. & Smith, D. (1999) RAPD marker and isozyme characterization of *Sphaeropsis sapinea* from diverse coniferous hosts and locations. *Mycological Research*, **103**, 1193–1202.

Stanosz, G.R., Smith, D.R. & Albers, J.S. (2005) Surveys for asymptomatic persistence of *Sphaeropsis sapinea* on or in stems of red pine seedlings from Seven Great Lakes region nurseries. *Forest Pathology*, **35**, 233–244.

Swart, W.J. & Wingfield, M.J. (1991) Biology and control of *Sphaeropsis sapinea* on *Pinus* species in South Africa. *Plant Disease*, **75**, 761–766.

Tainter, F.H. & Baker, F.A. (1996) Foliage pathology. *Principles of Forest Pathology* (ed. by F. H. Tainter and F. A. Baker), pp. 444–497. John Wiley and Sons, Hoboken, New Jersey.

Tan, J.Y., Wainhouse, D., Morgan, G. & Day, K.R. (2010) Flight ability and reproductive development in newly-emerged pine weevil *Hylobius abietis* and the potential effects of climate change. *Agricultural and Forest Entomology*, **12**, 427–434.

Tan, J.Y., Wainhouse, D., Morgan, G. & Day, K.R. (2011) Interaction between flight, reproductive development and oviposition in the pine weevil *Hylobius abietis*. *Agricultural and Forest Entomology*, **13**, 149–156.

Thwaites, J.M., Farrell, R.L., Duncan, S.M. *et al.* (2005) Survey of potential sapstain fungi on Pinus radiata in New Zealand. *New Zealand Journal of Botany*, **43**, 653–663.

Viiri, H. (2004) Fungi associated with *Hylobius abietis* and other weevils. *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis* (ed. by F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire and H. F. Evans), pp. 381–393. Kluwer Academic Publishers, The Netherlands.

Wainhouse, D., Inward, D.J.G. & Morgan, G. (2014) Modelling geographical variation in voltinism of *Hylobius abietis* under climate change and implications for management. *Agricultural and Forest Entomology*, **16**, 136–146.

Wallertz, K., Nordenhem, H. & Nordlander, G. (2014) Damage by the pine weevil *Hylobius abietis* to seedlings of two native and five introduced tree species in Sweden. *Silva Fennica*, **48**, 1–14.

Whitehill, J.G.A., Lehman, J.S. & Bonello, P. (2007) *Ips pini* (Curculionidae: Scolytinae) is a vector of the fungal pathogen, *Sphaeropsis sapinea* (Coelomycetes), to Austrian pines, *Pinus nigra* (Pinaceae). *Environmental Entomology*, **36**, 114–120.

Accepted 22 June 2016
First published online 18 July 2016

CURRICULUM VITAE

First name:	Tiia
Surname:	Drenkhan
Date of birth:	28.03.1982
Address:	Institute of Forestry and Rural Engineering, Estonian University of Life Sciences, Kreutzwaldi 5, 51014 Tartu
E-mail:	tiia.drenkhan@emu.ee

Education:

2006–2017	PhD studies in Forestry, Institute of Forestry and Rural Engineering, Estonian University of Life Sciences
2004–2006	Master studies in Forest Management, Institute of Forestry and Rural Engineering, Estonian University of Life Sciences
2000–2004	Bachelor studies in Forest Management, Faculty of Forestry, Estonian Agricultural University
1989–2000	Antsla Gymnasium
1988–1989	Kaika Primary School

Academic degree:

2006	Master's Degree, Thesis: „The use of bioregulator ROTSTOP® in the control of *Heterobasidion* root rot", Estonian University of Life Sciences

Professional employment:

Since 2006	Estonian University of Life Sciences, Institute of Forestry and Rural Engineering, Department of Forest Biology, specialist
2005–2006	Estonian University of Life Sciences, Institute of Forestry and Rural Engineering, Department of Forest Biology, laboratory assistant
2004–2005	Estonian Agricultural University, Faculty of Forestry, laboratory assistant

Research interests:

Forest diseases, forest protection; especially root rots; control of root rots; invasive pathogens; the interactions between fungi and pests; viruses in fungi

Foreign languages: English, German, Russian

Training and special courses:

2013	NOVA PhD course „Invasive and Emerging Plant Diseases", Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden
2011	NOVA PhD course "Plant Disease; from survival to epidemics, and management strategies", Norwegian University of Life Sciences, Ås, Norway
2011	DNA based research to determine the viruses of *Heterobasidion* spp., METLA, Finland
2011	Course „PlutoF", University of Tartu, Estonia
2011	Course „Statistical models in Natural Science", University of Tartu, Estonia
2008	Course „Polymerase Chain reaction", Estonian University of Life Sciences, Estonia
2006	NOVA PhD course „Forest Pathology", University of Helsinki, Hyytiälä, Finland

Awards:

2005	Baltic Agro Scholarship for research "The use of bioregulator ROTSTOP® in the control of *Heterobasidion* root rot"

Projects:

2016–2019	8T160148MIMK The impact of different cutting time and practices to pathogens distribution and biodiversity in spruce stands on fertile site types, Forest Management Centre, principal investigator
2014–2019	IUT21-4 The carbon dynamics in Estonian forests affected by sustainable management, Estonian Ministry of Education and Research, investigator
2017–2018	8T160020MIMB Analysis of significant root rots in conifer stands, Environmental Investment Centre, responsible investigator

2015–2016	8-2/T15020MIMK Analyses of riskiness of new invasive pathogen *Diplodia sapinea* in Estonia, Environmental Investment Centre, investigator
2014–2016	EMP 162 DNA-based early detection and diagnostics of alien invasive forest pathogens and tracing of their introduction pathways into northern Europe, Norwegian Financial Mechanism EMP 162, investigator
2013–2015	8-2/T13088MIMK Preventive and restorative measures to reduce damage on forests – *Phythopthora* diseases in focus, Nordic Forest Research (SNS), principal investigator
2012–2015	8-2/T12115MIMK Forest protection problems associated with reforestation in Estonian forestry and their prevention with environmentally sustainable methods, Forest Management Centre, principal investigator
2012–2014	8-2/T12155MIMB Significant root rot pathogen and damages in Estonia, Environmental Investment Centre, responsible investigator
2011–2012	8-2/T11009MIMB Analysis of fungal root diseases (incl. invasive diseases) on forest and trees in a park in context of climate change, Environmental Investment Centre, principal investigator
2010–2011	8-2/T10030MIMI The development and impact of new invasive (incl. quarantine) forest diseases to our forests on the wave of climate change, Environmental Investment Centre, investigator
2006–2009	ETF6781 Biological control of pine weevils (*Hylobius* spp.) in forestry, Estonian Science Foundation, investigator
2005–2008	ETF6087 Animal-caused disturbances and their consequences in forest ecosystems, Estonian Science Foundation, investigator
2007–2008	8-2/T7026MIMI The connection between *Heterobasidion* spp., *Phlebiopsis gigantea* and *Hylobius abietis* in forest, Environmental Investment Centre, responsible investigator
2006–2007	Project No. 13 „The use of bioregulator ROTSTOP® in forestry“, Environmental Investment Centre,

	principal investigator
2005–2006	Project No. 66 „The use of bioregulator ROTSTOP® in forestry“, Environmental Investment Centre, principal investigator

The science organizational activity:

2015–2019	COST Action FP1406 Pine pitch canker – strategies for management of *Gibberella circinata* in greenhouses and forests (PINESTRENGTH)
2014–2018	COST Action FP1401 A global Network of nurseries as early warning system against alien tree pests (Global Warning)
2012–2016	COST FP1103 *Fraxinus* dieback in Europe: elaborating guidelines and strategies for sustainable management (FRAXBACK)

MSc dissertations supervised:

2016	Elisabeth Rähn, „Fungal pathogens distribution and dynamics in managed conifer stands after final felling“ (co-supervisors: Ivar Sibul, Rein Drenkhan)
2015	Signe Sabas, „*Phytophthora* spp. detection on trees and water bodiesi n Estonia“ (co-supervisor: Rein Drenkhan)
2014	Katri Ahtijäinen, „Research of root rot in Scots pine stands“ (co-supervisor: Rein Drenkhan)

ELULOOKIRJELDUS

Eesnimi:	Tiia
Perekonnanimi:	Drenkhan
Sünniaeg:	28.03.1982
Aadress:	Metsandus- ja maaehitusinstituut, Eesti Maaülikool, Kreutzwaldi 5, 51014 Tartu
E-post:	tiia.drenkhan@emu.ee

Hariduskäik:

2006–2017	Eesti Maaülikool, metsandus- ja maaehitusinstituut, metsandus, doktoriõpe
2004–2006	Eesti Maaülikool, metsandus- ja maaehitusinstituut, metsamajandus, magistriõpe
2000–2004	Eesti Põllumajandusülikool, metsandusteaduskond, metsamajandus, bakalaureuseõpe
1989–2000	Antsla Keskkool
1988–1989	Kaika Algkool

Teaduskraad:

2006	Teadusmagister, väitekiri: „Bioregulaator Rotstop'i kasutamine juurepessu profülaktikas", Eesti Maaülikool

Teenistuskäik:

Alates 2006	Eesti Maaülikool, metsandus- ja maaehitusinstituut, metsabioloogia osakond, spetsialist
2005–2006	Eesti Maaülikool, metsandus- ja maaehitusinstituut, metsabioloogia osakond, vanemlaborant
2004–2005	Eesti Põllumajandusülikool, metsandusteaduskond, vanemlaborant

Teadustöö põhisuunad:

Metsahaigused ja -kaitse (juuremädanikud, juurepessu biotõrje, invasiivsed patogeenid, patogeensete seente ja putuka vastastikune mõju, viirused seentel)

Võõrkeelte oskus: inglise, saksa, vene

Täiendkoolitused:

2013	NOVA doktorikursus „Invasive and Emerging Plant Diseases", Rootsi Põllumajandusülikool (SLU), Uppsala, Rootsi
2011	NOVA doktorikursus "Plant Disease; from survival to epidemics, and management strategies", Norra Maaülikool, Ås, Norra
2011	DNA alaste uuringute teostamine juurepessu viiruste määramisel, METLA, Soome
2011	Kursus „PlutoF kasutamine", Tartu Ülikool, Eesti
2011	Kursus „Statistilised mudelid loodusteadustes", Tartu Ülikool, Eesti
2008	Kursus „Polymerase Chain reaction", Eesti Maaülikool, Tartu
2006	NOVA „Välikursus metsapatoloogias", Helsingi Ülikool, Hyytiälä, Soome

Tunnustused:

2005	Baltic Agro stipendium uurimistöö eest "Bioregulaatorite kasutamine metsanduses preparaadi Rotstop näitel"

Projektid:

2016–2019	8T160148MIMK Kuusikute raieaja ja raieviiside mõju patogeenide levikule ja arvukusele ning puistu elurikkusele viljakates kasvukohatüüpides, Riigimetsa Majandamise Keskus, põhitäitja
2014–2019	IUT21-4 Eesti metsade süsiniku dünaamika ja jätkusuutlik majandmine, Haridus- ja Teadusministeerium, täitja
2017–2018	8T160020MIMB Olulisemate juuremädanike kahjustuste uuring okaspuu puistutes, SA Keskkonnainvesteeringute Keskus, vastutav täitja
2015–2016	8-2/T15020MIMK Uue invasiivse patogeeni *Diplodia sapinea* ohtlikkuse analüüs Eestis, SA Keskkonnainvesteeringute Keskus, täitja
2014–2016	EMP 162 DNA-based early detection and diagnostics of alien invasive forest pathogens and tracing of their introduction pathways into northern Europe, Norra ja EMP finantsmehhanismide teaduskoostöö toetus, täitja

2013–2015	8-2/T13088MIMK Preventive and restorative measures to reduce damage on forests – *Phythopthora* diseases in focus, Nordic Forest Research (SNS), põhitäitja
2012–2015	8-2/T12115MIMK Metsakultiveerimisega seotud metsakaitseprobleemid Eesti metsanduses ning nende vältimine keskkonnasäästlike tõrjevõtetega, Riigimetsa Majandamise Keskus, põhitäitja
2012–2014	8-2/T12155MIMB Olulisemate juuremädanike tekitajate leviku ja kahjustuste uuring Eestis, SA Keskkonnainvesteeringute Keskus, vastutav täitja
2011–2012	8-2/T11009MIMB Juuremädanike-seenpatogeenide ning invasiivsete seenhaiguste kahjustuste analüüs metsa- ja pargipuudel kliimamuutuste kontekstis, SA Keskkonnainvesteeringute Keskus, põhitäitja
2010–2011	8-2/T10030MIMI Uute invasiivsete (s.h. karantiinsete) metsahaiguste areng ja mõju meie metsaloodusele kliimamuutuste taustal, SA Keskkonnainvesteeringute Keskus, täitja
2006–2009	ETF6781 Männikärsakate (*Hylobius* spp.) biotõrje metsamajanduses, Sihtasutus Eesti Teadusfond, täitja
2005–2008	ETF6087 Loomsed häiringud ja nende tagajärjed metsaökosüsteemides, Sihtasutus Eesti Teadusfond, täitja
2007–2008	8-2/T7026MIMI Juurepessu, hiidkooriku ja männikärsaka vahelised seosed metsas, SA Keskkonnainvesteeringute Keskus, vastutav täitja
2006–2007	Metsanduse programmi jätkuprojekt nr. 13 Bioregulaatorite kasutamine metsanduses preparaadi ROTSTOP® näitel, SA Keskkonnainvesteeringute Keskus, põhitäitja
2005–2006	Metsanduse programmi projekt nr. 66 Bioregulaatorite kasutamine metsanduses preparaadi ROTSTOP® näitel, SA Keskkonnainvesteeringute Keskus, põhitäitja

Teadusorganisatsiooniline tegevus:

2015–2019	COST Action FP1406 Pine pitch canker – strategies for management of *Gibberella circinata* in greenhouses and forests (PINESTRENGTH)

2014–2018	COST Action FP1401 A global Network of nurseries as early warning system against alien tree pests (Global Warning)
2012–2016	COST FP1103 *Fraxinus* dieback in Europe: elaborating guidelines and strategies for sustainable management (FRAXBACK)

Juhendatud magistritööd:

2016	Elisabeth Rähn, „Okaspuuenamusega majandusmetsade seenpatogeenide levik ja dünaamika lõppraiete järgses metsamullas“ (kaasjuhendajad: Ivar Sibul, Rein Drenkhan)
2015	Signe Sabas, „*Phytophthora* spp. leidudest puudel ja veekogudes Eestis“ (kaasjuhendaja: Rein Drenkhan)
2014	Katri Ahtijäinen, „Juuremädanike uuringud hariliku männi puistutes“ (kaasjuhendaja: Rein Drenkhan)

LIST OF PUBLICATIONS

Publications indexed in the Thomson Reuters Web of Science database

Drenkhan, T., Voolma, K., Adamson, K., Sibul, I., Drenkhan, R. 2017. The large pine weevil *Hylobius abietis* (L.) as a potential vector of the pathogenic fungus *Diplodia sapinea* (Fr.) Fuckel. Agricultural and Forest Entomology, 19: 4–9.

Drenkhan, T., Kasanen, R.,Vainio, E.J. 2016. Phlebiopsis gigantea and associated viruses survive passing through the digestive tract of *Hylobius abietis*. Biocontrol Science and Technology, 26: 320–330.

Drenkhan, R., Solheim, H., Bogacheva, A., Riit, T., Adamson, K., **Drenkhan, T.**, Maaten, T., Hietala, A.M. 2016. *Hymenoscyphus fraxineus* is a leaf pathogen of local *Fraxinus* species in the Russian Far East. Plant Pathology, doi: 10.1111 /ppa.12588.

Drenkhan, T., Sibul, I., Kasanen, R., Vainio, E. 2013. Viruses of *Heterobasidion parviporum* persist within their fungal host during passage through the alimentary tract of *Hylobius abietis*. Forest Pathology, 43: 317–323.

Kõljalg, U., Nilsson, H., Abarenkov, K., Tedersoo, L., Taylor, A., Bahram, M., Bates, S., Bruns, T., Bengtsson-Palme, J., O`Callaghan, T., Douglas, B., **Drenkhan, T.**, Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G., Hartmann, M., Kirk, P., Kohout, P., Larsson, E., Lindahl, B., Luecking, R., Martín, M., Matheny, B., Nguyen, N., Niskanen, T., Oja, J., Peay, K., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schlüsser, A., Scott, J., Senés, C., Smith, M., Suija, A., Taylor, D.L., Telleria, M., Weiss, M., Larsson, K.-H. 2013. Towards a unified paradigm for sequence-based identification of Fungi. Molecular Ecology, 22: 5271–5277.

Drenkhan, T., Hanso, S., Hanso, M. 2008. Stump treatment against *Heterobasidion* root rot with *Phlebiopsis gigantea* in Estonia. Baltic Forestry, 14: 16–25.

Chapter in a peer-reviewed book

Drenkhan, R., Agan, A., Palm, K., Rosenvald, R., Jürisoo, L., Maaten, T., Padari, A., **Drenkhan, T.** 2017. Overview of ash and ash dieback in Estonia. In: Vasaitis, R., Enderle, R. (Ed.). Dieback of European

Ash (*Fraxinus* spp.) – Consequences and Guidelines for Sustainable Management. Sweden, Uppsala, 115–124.

Published meeting abstracts

Drenkhan, T., Adamson, K., Maaten, T., Drenkhan, R. 2015. *Hymenoschypus fraxineus* on intorduced ash species in Estonia: research in perspective of ongoing COST Action FP1103 FRAXBACK. 5th International workshop on the genetics of tree-parasite interactions, 23–28 August 2015, Orleans, France, 9.

Popular-scientific publications

Drenkhan, T., Rähn, E., Jürimaa, K., Adamson, K., Padari, A., Drenkhan, R., Pilt, E. 2014. Juuremädanike levik on arvatust ulatuslikum. Eesti Mets, 4: 45–49.

Drenkhan, T. 2013. Seentevaheline konkurents päästab inimese kaasabil kuuski ja mände. Päärt, V., Rajalo, S. (Toim.) Maateaduste ja ökoloogia doktorikool (Teadusportaal Novaator), Tartu Ülikool, 78–80.

Drenkhan, T., Drenkhan, R., Hanso, M. 2012. Saaresurma tekitaja on hoopis teine, invasiivne kottseen. Eesti Loodus, 10: 46–48.

Drenkhan, T. 2011. Pritsiga juurepessu vastu. Sinu Mets, 2/3: 8–9.

Drenkhan, T. 2011. Bioregulaator Rotstop juurepessu vastu. Eesti Mets, 2: 32–34.

Drenkhan, T. 2008. Kolm aastat katsetatud bioregulaator. Eesti Mets, 1: 36–38.

Hanso, M., **Drenkhan, T.** 2006. Bioregulaator ROTSTOP on tõhus juurepessu tõrjuja, Eesti Mets, 1: 50–53.

Hanso, M., **Drenkhan, T.** 2005. Seenega seene vastu. Eesti Loodus, 1: 16–19.

Hanso, M., **Drenkhan, T.** 2004. Kas leitud pisiseen on haigusetekitaja või mitte? Eesti Mets, 1: 40–41.

PRESENTATIONS

Presentations at international conferences and meetings

Drenkhan, T., Sibul, I., Kasanen, R., Vainio J.E. 2012. Viruses of the conifer pathogenic fungus *Heterobasidion parviporum* resist passing through the alimentary tract of the large pine weevil, *Hylobius abietis* L.

IUFRO 7.03.10 „Methodology of forest insect and disease survey“ IUFRO WP 7.03.06 „Integrated management of forest defoliating insects“ Working Party Meeting, 10–14 September 2012, Palanga, Lithuania, 28.

Sibul, I., **Drenkhan, T**. 2012. The large pine weevil (*Hylobius abietis*) as a vector of saprophytic fungi. Pathology: XXIV International Congress of Entomology, August 19–25, 2012, Daegu, Korea, 196.

Drenkhan, T. 2011. Pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae) as a potential fungi spores spreader in the forest. SNS meeting 06.09.2011, Uppsala, Sweden.

Drenkhan, T., Hanso, S., Hanso, M. 2007. Stump treatment against *Heterobasidion* root rot with *Phlebiopsis gigantea*. Two years of experiment in Estonia. SNS Forest Pathology meeting, 26.–29.8.2007, Hyytiälä, Finland.

Presentations at local meetings

Drenkhan, T., Pilt, E., Adamson, K., Jürimaa, K., Rähn, E., Drenkhan, R. 2014. Juuremädanike levikust viljakates kasvukohatüüpides. RMK ja maateaduste ökoloogia doktorikooli teadusseminar, Tartu, 14.02.2014.

Drenkhan, T., Drenkhan, R. 2013. Saaresurmast Eestis ja mujal Euroopas. ELUS-i metsandussektsiooni koosolek, Tartu, 13.02.2013

Drenkhan, T. 2013. Saaresurmast (*Chalara fraxinea* T. Kowalski) Eestis ja mujal Euroopas. Loodusteaduste doktorantide konverents, Voore, 18.–19.05.2013.

Drenkhan, T., Sibul, I. 2012. Harilik männikärsakas, *Hylobius abietis* (L.) (Coleoptera: Curculionidae) – kas võimalik seeneeoste levitaja metsas? RMK ja maateaduste ökoloogia doktorikooli teadusseminar, Tartu, 02.02.2012.

Drenkhan, T. 2011. Seenhaigused, juurepess ja külmaseen, looalade metsades ja nende kahjustuste ennetamine ja tõrjevõimalused. Metsakaitse õppepäev, Rapla, 24.05.2011.

Drenkhan, T. 2007. Juurepessu (*Heterobasidion* spp.) ja hiidkooriku (*Phlebiopsis gigantea*) mütseeli elujõulisus hariliku männikärsaka (*Hylobius abietis*) seedetraktis. Doktorantide ettekannete päev, Tartu, 10.05.2007.

Drenkhan, T., Hanso, M. 2006. Seenega seene vastu – ROTSTOPi kasutamise esimesi kogemusi Eestis. Mükoloogia ühingu aastakonverents, Tartu, 09.12.2006.