Using sniffer dogs for non-invasive detection of *Heterobasidion* root rot from scent stimuli derived from Norway spruce trees

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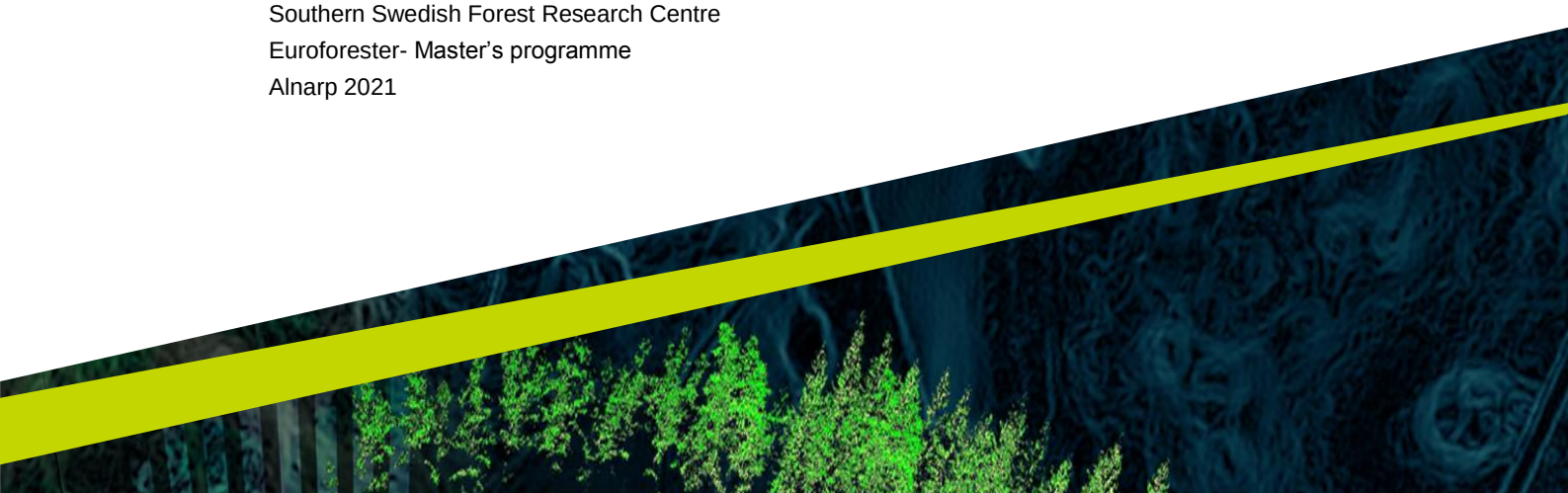
Master's thesis • 30 credits
Swedish University of Agricultural Sciences, SLU
Southern Swedish Forest Research Centre
Euroforester- Master's programme
Alnarp 2021



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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Master's thesis in Forest Management
Course code:	EX0630
Programme:	Euroforester
Course coordinating dept:	Southern Swedish Forest Research Centre
Place of publication:	Alnarp
Year of publication:	2021
Keywords:	**Detection dogs, sniffer dogs, Norway spruce, *Heterobasidion* spp., decay, root rot;**

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Abstract

The fungi of *Heterobasidion* spp. are known to cause root and butt rot disease, and are responsible for major economic losses to forestry sector in Sweden. The fungal infection in Norway spruce (*Piecea abies*(L.)Karst.) is often developing over many years without visible symptoms. Existing detection methods are invasive, costly or not reliable. There is a need for a developing and implementing better methods for detecting *Heterobasidion* spp. infection at an early stage, preferably when the pathogen is still present in the root systems.

The objective of this study was to test sniffing dogs` ability to detect the presence of the scent of an early *Heterobasidion* spp. infection in the spruce.

A field trial was prepared with scent samples in liquid and solid form, extracted from infected spruce trees, and randomly located within experimental blocks with control substrates from healthy tree, as well as blank treatment. Seven teams of dogs and their handlers investigated blocks with five treatments each. The water extracts were applied on the ground surface, solid wood bits were buried under the ground. Test was carried on in three tours to investigate potential changes in dogs` alerts over time.

The infected material was found by the dogs more often than expected by chance. Dogs correctly identified 70% of all infected samples. Combined results for infected and control treatments show 76% of true alerts. Detectability of water and solid samples changed over time. All the extracts from infected tree were detected by dogs in the first round while just 52% of infected solid material was detected. Blank samples were correctly identified in 94% of searches.

Dogs and their owners who were taking part in the field trail were not professionals. Possible development of a synthetic substance mimicking the scent of infection would enable more efficient sniffer training of dogs. This study clearly states, that there is a chance for implementing the use of detection dogs as a non-invasive root rot detection method, nevertheless details regarding training aids, costs or certification need to be refined.

Keywords: Detection dogs, sniffer dogs, Norway spruce, Heterobasidion spp., decay, root rot;

Preface

To all the working dogs.

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Abbreviations

C	Blank, one of the control treatments
FN	False Negative
FP	False Positive
H	Solid sample of wood from a healthy tree
I	Solid sample of wood from an infected tree
spp.	Species pluralis, multiple species
TN	True negative alert
TP	True positive alert
VOCs	Volatile organic compounds
W-	Water extract from a healthy tree
W+	Water extract from an infected tree

1. Introduction

Norway spruce, *Picea abies* (L.) H. Karst, is the most frequent tree species occurring in Swedish forests. It constitutes 40,9% of all species of Swedish productive forests with a growing stock estimated to 1275 mln m³sk (skogsdata 2020 SLU). Root rot, in Sweden caused mostly by the fungal pathogen *Heterobasidion spp.*, leads to reduction in diameter and volume growth (Bendz-Hellgren, and Stenlid, 1997) and wood decay. Annual economic loss in European Union is estimated to reach €500 million (Woodward et al. 1998) with Sweden alone losing around 500mln SEK on timber depravation and predicted growth decrease (Bendz-Hellgren, and Stenlid, 1995). Spruce stands affected by root rot are more prone to windthrow and snow damage, demand extra control measures, entail additional costs related to disease diagnostics, and cause difficulties in silvicultural planning.

Wood decay caused by *Heterobasidion* spp. fungi involves irreversible changes in wood structure resulting in deterioration of timber quality and lower timber volume. Logs with decay can be rejected by timber industry, and rotted or partially rotted timber of Norway spruce is not useful for the pulp industry either (Rönnberg, 2011). This leads to the situation where timber is sold as fire wood with much lower financial residual value than previously assessed by the forest owner.

1.1. Pathogen's characteristics

The *Heterobasidion annosum* s.l. species complex is spread throughout the whole northern hemisphere (Korhonen, Stenlid, 1998) with three species present in Europe. Infections in Norway spruce in Sweden are mostly caused by the species *Heterobasidion parviporum* Niemelä & Korhonen. The less specialised *Heterobasidion annosum* sensu stricto (s.s) (Fr.) Bref., attacks both conifers and broadleaves trees, can also affect spruce stands (Korhonen, 1987) but is more commonly found on Scots pine (*Pinus sylvestris* L.) in Scandinavia.

The primary spread of the pathogen occurs via airborne basidiospores landing on the fresh wounds' surfaces or the freshly cut stumps when temperature exceeds 5˚C (Yde-Andersen, 1962). The secondary infection is vectored by mycelium via root contact between infected and healthy trees (Pukkala et al. 2005). Infection starts in roots with mycelium spreading into root interior, causing the death of the

tree only in the rare cases when mycelium reaches cambium (Schmidt, 2006). Mycelium reaches higher elevations of the stem with growth of 25-40cm/year (Stenlid and Redfern 1998), often without displaying any visible symptoms over the years.

1.2. Wood degradation

Heterobasiodion spp. fungi inhabit the inner parts of trees, in the case of Norway spruce it attacks root interior under its parasitic phase and then heartwood as a saprotroph (Smith, 2006). By secreting various enzymes into the tree interior, the pathogen degrades the structure of cellulose, hemicellulose, starch, pectin but also lignin, freeing energy sources and space for further expansion. The ability to produce enzymes that degrade lignin, which is in most cases highly resistant to attacks by biological factors, classifies *Heterobasidion* spp. as 'white rot fungi'(Asiegbu, et al. 2004, Lundell et al. 2014). The fungi in the genus *Heterobasidion* are known to induce successive white rot, where lignin and hemicellulose is degraded faster than white/bright-coloured cellulose (Asiegbu et al. 1998, Daniel et al. 1998, Schmidt, 2006). Apart from enzymes fungi of *Heterobasidion* spp. produce toxins such as fomannoxin, and fomannosin (Basset et al. 1967, Axelsson et al. 2020) and the host organism secrets substances to defend itself from the pathogens activity. Decomposition of lignocellulose components of plant cell wall realises dissolved sugar and aromatic compounds Volatile organic compounds (VOCs) may be released from wood or originate from the fungi under the process of wood decay (Mali, et al. 2019).

With the progressing wood decay, the physical properties of the wood are undergoing changes. The volume, wood density and water content, as well as the wood colour and odour change (Panshin, de Zeeuw, 1970). Theoretically any of these properties can serve as an assessment criterion. in the wood decay detection.

1.3. Methods of detection

Optimal method for root rot detection ought to be simple, time efficient, accurate and safe to perform, as well as sensitive enough to avoid false positives readings (Fox, 1993), reproducible (Schulze and Bahnweg, 1997), non-destructive and performed by means of portable equipment (Greig and Pratt, 1998). In case of Norway spruce and root rot caused by *Heterobasidion* spp. detection method should be precise enough to detect infection in the early phase, when the pathogen is still located in the root system, before it ascends to the higher parts of the stem. Mobility of the detecting instrument or method is essential when the detection process is transferred into practical forest inventories. Accuracy of the method

influences drastically economic outcomes from the forest land, with false negative indications being possibly the most severe in consequences as infected trees remain in the stand. Multiple methods and tools for root rot detection have been tested and implemented over the years (Pellerin and McDonald, 1993, Greig, 1998, Larsson et al. 2004) but forest sector is still demanding better, less-invasive solutions as presently widely used methods are often destructive. Non-destructive alternatives present on the market involve often complex equipment, making measurements time-consuming and costly.

1.3.1. Destructive methods

Field detection of decay in trees is mostly performed by drilling cores of wood tissue using diverse drilling tools, and analysing sample for signs of discolouration or presence of decay in laboratory conditions (Greig and Pratt, 1998). Boring cause technical defects and can predispose a healthy tree to rot causing fungi (Greig and Pratt, 1998, Rönnberg, 2011). Extracting bore core samples at the breast height- the most convenient level from the worker's perspective, brings high risk of false negative results. According to Rönnberg (2011) about 50% cases of the rot present at the stump height is missed if samples are collected at the breast level, while the number given by Stenlid and Wästerlund (1986) was 40-70%. Another way to verify the severity of pathogen attack in the stand is to count trees with visible rot on the stumps after thinning operations. This practice is not implemented in Sweden and therefore no substantial data has been gathered.

1.3.2. Non-destructive methods

The evident sign of *Heterobasidion* spp. infection in the stand is the presence of the fungus' fruiting bodies localised most often at the lower parts of stumps and dead trees. They are often covered by debris and therefore not easy to notice. Fructifications seldom occur on the living trees and in such case they indicate advanced wood degradation (Greig and Pratt, 1998). Other symptoms of the pathogen activity on living spruce trees, although infrequent, can be resin exudation from the root collar, tree bole tapering and crown discoloration (Greig and Pratt, 1998, Kallio and Tamminen, 1974).

1.3.3. Visual assessment

The validity of the identification method based on the presence of external symptoms of root and butt root on standing trees in Norway spruce stands in Southern Sweden was evaluated in an earlier study and compared with the results of random selection (Vollbrecht and Agestam, 1995). The study demonstrated a

correlation between incidence of butt root and accelerated resin exudation, more frequent tapering of the lower part of the tree, decreasing crown density and its discolouration. However, the assessment made by professional foresters proved to be just slightly more accurate than random selection of affected trees.

1.3.4. Electric methods

With changed water content and released metal ions also the electric properties of the wood change which can be used for identifying decay in standing trees. Electrical resistivity of decayed wood tissue is lower than that of a healthy tree (Shortle and Smith, 1987). Measuring electrical properties of the wood can be therefore utilized in the vitality assessment. Numerous tools based on the measurements of electrical resistance as example: shigometer (Ostrofsky and Shortle, 1993, Humplik et al. 2016), resistograph or even device combining electrical impedance tomography with sonic tomography called PiCUS Treetronic system (Rust et al. 2008) capable of precisely localising decay in a tree trunk (Göcke, 2011) have been developed. However, the use of these devices is either time consuming, costly, causing damage to the structure of the tree (Shigometer, resistograph) or too complex to be implemented as a routine procedure. Four-point resistivity (RISE- Relative Impedance In Situ Examination) method, where one pair of electrodes release alternating current of low frequency passing it through the material while two other electrodes measure differences in the voltage (Popovic and Popovic, 2000) was implemented in the device named ROTFINDER® patented in Sweden (Bengtsson, 1997, Larsson et al. 2004). The device was relatively easy to use, showing the result of measurements on the integrated screen, enabling the user to classify the tree as decayed or healthy *in situ*, without the need for making holes in the tree, as in case of Shigometer. The method however did not reveal the location of the decay in the tree trunk and was depended on the season of the year. Currently, Rotfinder® is not anymore present on the market (situation as at March 2021).

1.3.5. Sniffing dogs as an non-invasive detection method

Dogs are known for their extraordinary olfactory sense. Complex structure of dog's nasal cavities, with a large surface of the sensory epithelium (Sjaastad et al. 2010) enables these animals to recognize an extremely wide range of scents, even when the volatile substances are of very small concentrations. Gadbois and Reeve (2014) define sniffing as an "exploratory behaviour that has many important roles in olfaction: it actively participates in the input of the olfactory stimulus, it can be modulated to account for different odorant concentrations, and it can modulate the pattern of neural activity".

Humans benefit from dogs' superior olfactory sense from the beginning of the species domestication, relaying on their skills during hunting. Nowadays dogs are trained for very specific purposes e.g. for detecting mines (Fjellanger et al. 2002), fire accelerants (Gialamas, 1996) and drugs, searching for disappeared people or even detecting the occurrence of cancer (Elliker et al. 2014), and even viruses (Angle et al. 2016) in humans` and animals` bodies. Dogs smell sense is used for conservation purposes (Beebe et al. 2016) for instance in surveying rare mammals in the wild, which proved to be more efficient than the use of cameras and hair snare methods (Long, 2007) or any other known methods of wildlife surveying (Dematteo, 2009). Dogs are increasingly trained to locate pathogens infesting living plants (Gottwald, 2019) invasive insects (Hoyer-Tomiczek, 2016) or mould in constructions (Kauhanen, 2002). Dogs' ability to cover large areas in rugged terrain during a single search and their high sensitivity in finding a target scent make this method potentially beneficial for the forest sector. A recent Swedish study demonstrated that dogs trained with synthetic pheromones mimicking these of bark beetle (*Ips typographus)* were able to locate trees in the forest that were infested by insects (Johansson, et al. 2019). An earlier attempt of testing detection dogs' ability to find the trees infected by root rot caused by *Heterobasidion* spp. was promising: all the five dogs participating in the test were able to discriminate infected wood from healthy samples with success rates ranging between 70% and 100% (Swedjemark and Morrison, 1987). Unfortunately there is no documentation that would suggest that the Swedish root rot project was carried out to its final phase. Therefore, the need for more studies examining possibilities of using searching dogs in the early detection of the root rot is strong.

2. Aims and objectives of the study

Nowadays detection of *Heterobasidion* spp. presence in the conifer stands is possible almost exclusively after tree felling, which is a costly and problematic method for the forest owners. A non-invasive method of finding trees infected with the pathogen would significantly decrease economic loss, while making the management of conifer forest easier and less hazardous. Assuming that dogs are performing well as a detection 'device' we would be able to test standing trees faster (when the fungus is still in the root system only) and in case of confirmed infection we would gain the time to prepare the necessary measures to protect remaining healthy trees such as stump treatment after cutting (Thor, 2005) or to decide on a new management strategy e.g. shortening the rotation period and rebuilding the future stand with admixtures of resistant species (Korhonen et al. 1998).

The following hypotheses for the study were formulated:

1. Dogs are able to detect scent of infected wood.
2. Dogs are able to detect scent of extracts of infected wood.
3. Dogs alert with different frequency to different treatments.
4. Number of alerts made by dogs as response to wood scent may change with time
5. Number of responses to extracts may change with time.

These hypotheses were tested in a field study that allowed to determine if dogs are able to detect stimuli for early root rot infection and to evaluate their performance under different conditions. To remove false alerts, we assessed sniffing dogs' ability to detect infested material under controlled conditions, whereas the location of infected material was known to the organizers of the case study but not to dog handlers or dogs themselves.

Data on sniffing acuity could contribute to further studies on the volatile substances responsible for the specific scent of infected wood, which could enable the synthetic test stimuli standard in training dogs to detect root rot on a bigger scale.

3. Material and Methods

3.1. Experimental design and study site

The overall experimental design was a randomised block design. A special layout was used to fulfil the requirements of recording the behavioural alerts from dog-handler teams to unknown stimuli buried underground. The layout was constructed as grind of blocks and colour markers of stimuli dug into soil plots within blocks.

The field experiment designed to test the hypothesis was arranged on pasture land near the Asa Experimental Forest and Research station, 37 km north of Växjö, in October 2020. After preparations (on the 9th and 10th of October), the experiment was conducted during one day, on the 10th of October. The experiment took place during one day, on the 10th of October. A field trial for dogs was organized in seven parallel rows, each row consisting of three square-shaped blocks that were marked with ribbons at the perimeter of the square. Each side of the square was 2m long. The distance between two blocks within each row was around 2 m, and the distance between two rows was 3m (Fig.1.). Each of the 21 blocks contained five plots, designed to host the scent samples under the field trail. Each of the 21 blocks contained five plots (holes), designed to host the scent samples under the field trial. The holes were dug in the ground using spade. The dimension of the sample plot was 10cm in depth (in the range of 8-12cm) with 20x20cm long sides.

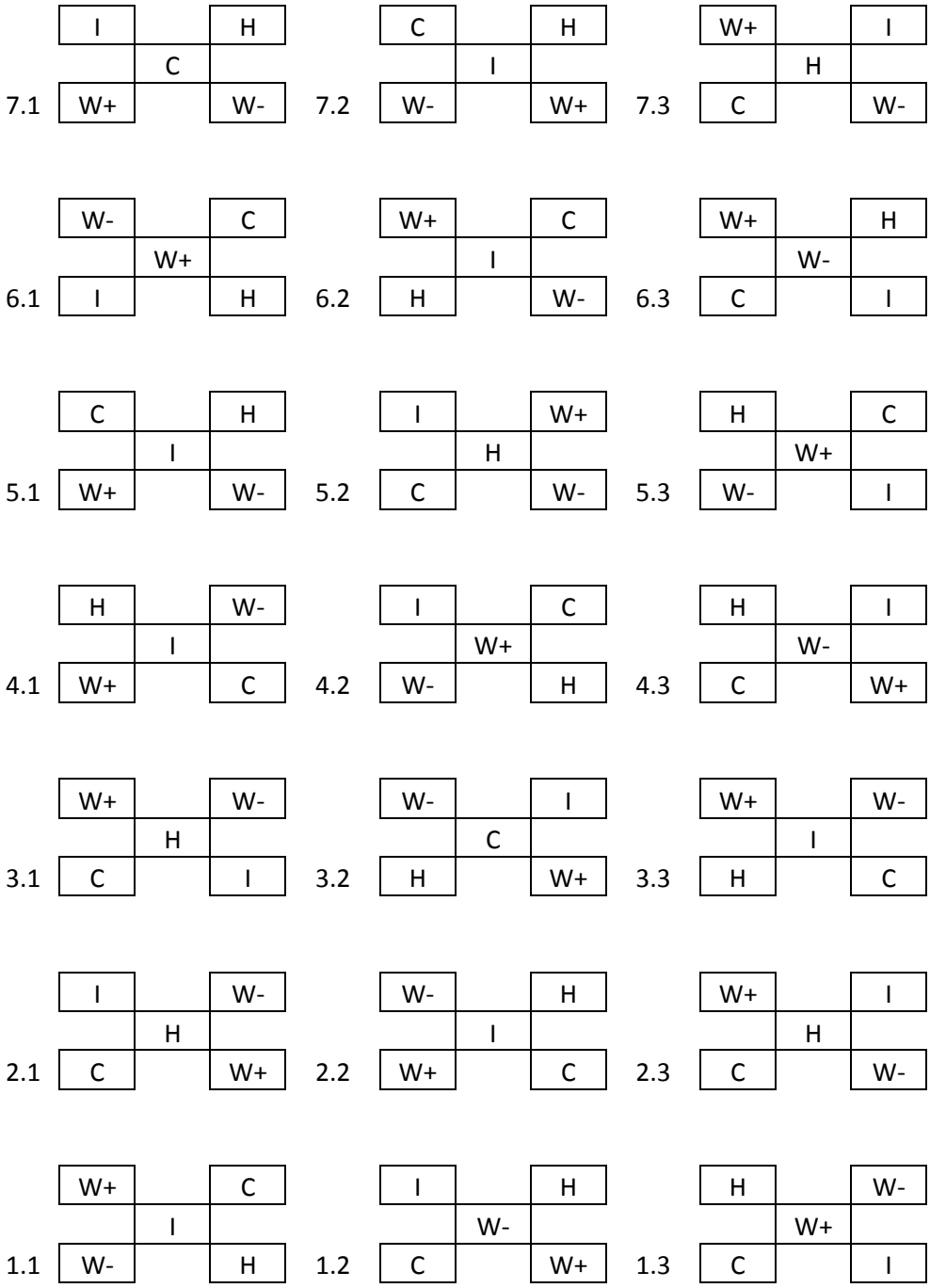

Figure 1: Experimental trial design W+ -water extract from infected material, W- - water extract from a healthy material, I- sample of infected wood, H- sample of healthy wood, C- control, empty spot. Rows are marked with the numbers from 1 to 7, each block in the row is marked with the decimal number.

3.2. Treatments

The following specimens were used to test dogs' ability to detect presence of *Heterobasidion* spp. infection: a block of spruce wood from a healthy tree (referred as H), a wood block sample from a tree previously tested positive for presence of and with visual signs of *Heterobasidion* spp. infection in the wood (referred as I). The tree was previously tested by taking a bore core that was incubated in room temperature for seven days and checked for presence of conidiophores of *Heterobasidion* spp. Water extract from the tree with confirmed infection (referred as W+), water extract from the healthy spruce tissue, referred bellow as W- and blank control (an empty spot). The schematic design of the block (Fig.2.) and a view from the field (Fig.3.) are presented below.

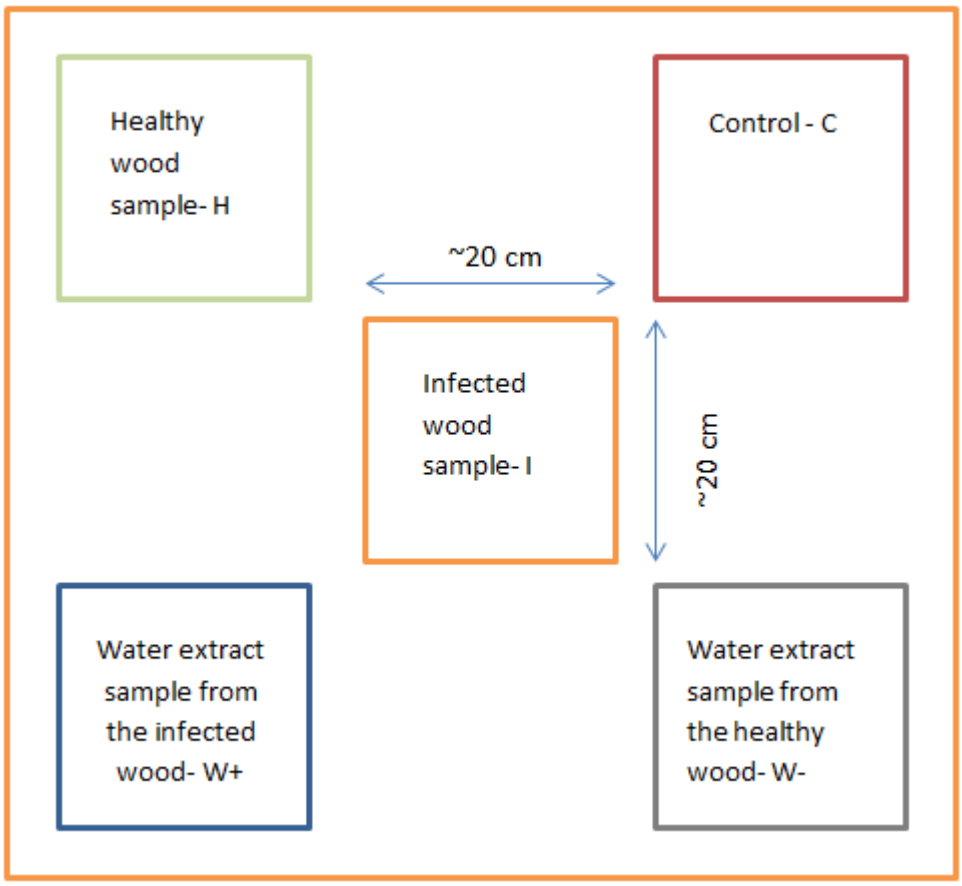

Figure 2: The layout of the experimental block with the stimuli and control (C) located in five spots, each in a form of a hole dug in the ground, not perfectly square. The sequence of positions of the stimuli was randomized within each block.

Figure 3. The experimental block with five spots dedicated for five different treatments. Asa, October 2020. Photo: N.Wysocka.

Figure 4. Water extracts prepared from infected (a) and healthy (b) spruce tree. Photo: A.Johansson.

Wood samples were randomly placed on the bottom of the holes and covered with the remaining soil layer. Once the samples were placed all the remaining free spots were covered with soil.

Water extract were randomly applied directly on the ground surface, within the 20x20cm sample plot, approximately 2-3 minutes before the dog's first test. Control spots (holes without wood or extracts) were also covered with the soil. The location of each sample within each row and block was noted by one designated person and marked on the graphic template (see fig.1.). Each of spots dedicated to single scent sample was additionally marked with sticks of a random colour.

3.2.1. Samples preparation

Samples used in the experiment came from Norway spruce trees in a stand neighbouring Asa Experimental Forest Research station. Prior to the study trees were investigated for the presence of the *Heterobasidion* spp. infection. To assess the occurrence of the pathogen, bore cores were extracted from the spruce trunks with the use of an increment borer. All samples were immediately put into plastic bags and incubated at room temperature for seven days. The presence of *Heterobasidion* spp. was judged by the occurrence of conidiophores using a stereomicroscope at 20 times magnification. After microscopic examination of the bored samples both infected and healthy trees were marked, each type with different paint colour, the holes in the tree trunks were closed to avoid further infections. On the day preceding the field trial one healthy tree and one infected tree were fallen with the use of the chain saw. Approximately 20cm long billets were cut from each tree and stored separately in plastic bags to avoid cross-contamination of the healthy tree samples and then transported to the study site. Before the wood samples were assigned to random spots within each block, tree billets were split into smaller pieces around 10x10x10cm, using an axe. Small fragments of billets taken from H and I trees were put into glass jars (Goss, 2019) filled with water and secured by lid (Fig. 4). The jars were then stored for 24hours to enable the sorption of the scent of the wood into the water (Simon et al. 2020). The water extract, without any visible residues of the wood, was used in the trial.

3.2.2. Identification of *Heterobasidion's* strain

To identify the species of *Heterobasidion* billets from an infected Norway spruce tree cut in Asa one day prior to the field trail were collected after the trial was finished to proceed with further analysis of the material in laboratory conditions.

A 4-5 cm disc from infected tree billet was incubated at room temperature (~ 20 °C) in the dark for ten days. The emerged *Heterobasidion* spp. conidia were picked with a sterile needle and transferred onto a Petri dish containing Hagem agar. Mating tests were conducted to assign isolated strain to *H. annosum* and *H. parviporum*. The tests were based on the isolated strain's ability to heterokaryotize homokaryotic tester (known) strains of *H. annosum* and *H. parviporum* (Korhonen, 1978).

3.2.3. Dogs

The field trial tested detection abilities of seven dogs, owned by six different private persons. Dogs were of diverse age and breed (Tab 1.) and none of the dog handlers is working professionally in the canine sports.

Table 1: Information about dogs tested for early Heterobasidion spp. infection detection
Age as for October, 2020. The number used besides the dog's name can be used in place of the name further in this study.

Dog	Age (years old)	Breed	Additional info
1. Ninja	8	Spanish Water Dog	
2. Smulan	9	Labrador retriever	
3. Azlan	5,5	German Shepherd	
4. Java	3,5	Spanish Water Dog	Shares the owner with Joy
5. Jeff	12	Border collie	
6. Pepper	2,5	Cocker spaniel	
7. Joy	10,5	Spanish Water Dog	Shares the owner with Java

3.3. Training procedure

The dogs were first trained with the use of 1cm long drill cores from infected tries, in a small outdoor search area of 50x50cm in the absence of trees and forest vegetation. When dogs were able to detect these pieces, drill cores from healthy wood were added. Later, dogs' owners were given two pieces of spruce tree trunks, one piece came from an infected tree and the other from a healthy tree. Participants of the programme were advised to store the pieces of wood in the fridge (4˚C) in dark black bags, closed but with the possibility of air circulation into the sample material. To avoid contamination of the samples, wood pieces had to be stored separately and any processing of the sample material had to be carried out with a disinfected drill. Participants were advised to take the wood chips samples with the help of 15-20mm thick spiral borer. In case of infected wood piece, samples had to be bored deep enough to reach the decay zone- close to the

tree pith. The bored chips were taken out slowly, and the wood stem pieces were put back into the fridge in the dark bags. Extracted bore sample chips were then used as a training material outdoors.

The training on the water extracts took place outdoors, in an experimental setting similar to this described in the study. About 3 ml of water extract from an infected tree was applied as a single sample, and then water solution from a healthy tree was introduced when dogs proved to be able to detect samples from infected tree successfully (Johansson, 2021, personal communication).

Presence of *Heterobasidion* spp. in the decayed trees and wood trunk pieces was detected as described in section 3.2.2.

3.4. Test

The experiment was designed to test dogs' ability to detect volatile substances associated with *Heterobasidion* spp. infection in conifer wood.

Dog handlers and dogs were attending Nose Work course (the sport activity where the dog lead by its handler has to find variety of hidden scents) at Sniffer Dogs Sweden, prior to the date (Sniffer Dogs Sweden, n.d.). Each dog had to sniff each of the five samples within the block, with dog handler unaware of positive samples location or quantity. During one round, each dog handler and the dog had to check three blocks, fifteen samples in total. The round was repeated three times. Dogs were not visiting same rows.

Dog handler was informing a referee each time his or her animal indicated detection of infected sample Referee recorded whether the desired scent was present on the indicated plot or the dog's choice was classified as the false positive. If the indication was a success dog received award from the handler- a praise or the possibility to play with its favourite toy.

3.5. Statistical analysis

The results of the study were statistically analysed with the use of the Minitab® software. Microsoft Excel was used for data organisation and visualisation.

The analysis of variance (ANOVA) in general linear model (GLM) was used to compare and to find differences in number of alerts made by dogs during each round, to compare all the participating dogs with each other in term of number of signalised markings as well as to check the differences in number of alerts for particular dog over the particular round.

Tukey's poshoc test for pairwise comparison was added to assess differences in the number of the alarms between the dogs and indicate division into groups among the seven dogs. Test was conducted for total number of alerts (sum of all

the alarms made over the three rounds), as well as for each round separately. The mean number of the alerts was used in comparison and the significance level was 5%.

The mean of true alerts (true positives and true negatives) was calculated for every treatment and every round, and then presented in the graphic form with the use of the colour scale and mapped on the scheme of the experimental trial (Fig.7.).

Sign Test was used to compare pairs of treatments: W+ and W-, I and H, W+ and I in terms of number of the alerts made for both compared treatments in each of investigated blocks. In particular the sign test was used to compare situations when dogs alerted for just one treatment from the pair.the pair. Situations when the dog alerts for none/both of treatments (scenarios ´b´) were removed from this statistical analysis. In case of pair of treatments where both true positive and false positive alerts were possible for the dog to make (W+ W- and I H) the question we wanted to answer was whether the dog alerting just once is making the correct indication (indicating infected sample). The null hypothesis stated that if the dog alerts just once the probability of correct indication is 50%. The alternative hypothesis stated that when alert was made just once for analysed pair of treatments, the probability of alerting on infected material was higher than 50%. Possible outcomes for the alerts within pairs of treatments W+ vs W- and I vs H:

a) dog alerted once, but for the wrong alternative (control)
b) dog alerted for both or for one of treatments, what corresponds to one right and one false alert;
c) dog alerted once and it was for the infected treatment (true positive).

For the pair of treatments W+ vs I three scenarios of single alerts outcomes are possible:

a) dog made false alert for W+ and true alert for I,
b) dog made false alerts for both analysed treatments within the block,
c) dog made false alert for I and true alert for W+.

In the comparison of single alerts for pair of treatments W+ and I the null hypothesis states, that when dog alerts just once, the probability of the alert being given for sample W+ is 50%. If sign test rejects null hypothesis it means that one of analysed treatments either W+ or I, was easier for dogs to identify.

Blank control (C) was excluded from the calculation as this treatment was characterised by high percentage of true alerts, with 59 correct alerts out from 63 possibilities, which could disturb the results if paired with other treatments. Significance level for the test was 5%.

Additionally, Friedman's Test was used to determine whether the median treatment effect differs in a randomized block design. This test is a non-parametric alternative to One-Way ANOVA with blocks.

4. Results

Dogs indicated 70% (88) of the 126 infected samples and left 79% (150 out of 189) of the control samples without alarm. In total, 76% of alerts made by dogs were true positive or true negative alerts. The highest detection rate (94%, i.e. 59 out of 63 samples) was registered for blank controls (Fig.5).

Mating tests assigned isolated material to *Heterobasidion parviporum*, the species the dogs were trained on.

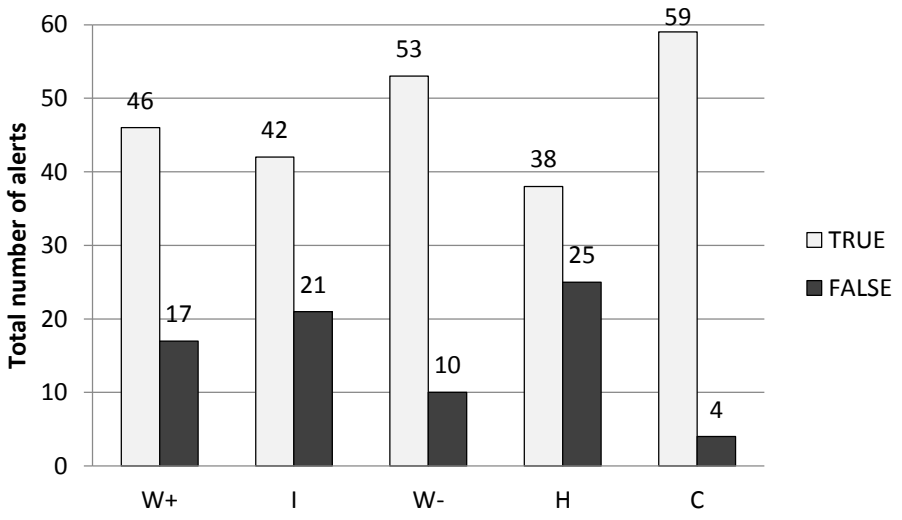

Figure 5: The total number of true and false alerts for each of tested treatments: W+ water infected, I- wood infected, W- water control, H-wood control, C- blank.

4.1. Dogs' ability to detect *Heterobasidion spp.* infection

4.1.1. Wood material

Dogs detected 67% of all the samples of infected wood material (Fig. 5).

Healthy wood sample (H) was the treatment with the highest rate of false positive markings. False positive alerts constituted 40% of all the alarms possible for this treatment (Fig. 5).

4.1.2. Water extracts

During the whole trial dogs were able to detect on 73% of all the prepared samples of water extracts from infected tree, alerting on 30% of control treatment samples (Fig. 5).

4.2. Responses to different treatments

Dogs alerted with different frequency to different treatments (Fig.6). Two dogs, Ninja and Pepper were able to detect all the infected solid wood samples (I). Additionally Pepper detected all the infected material from water solutions (W+) ignoring all the blocks with control water solutions samples (W-).

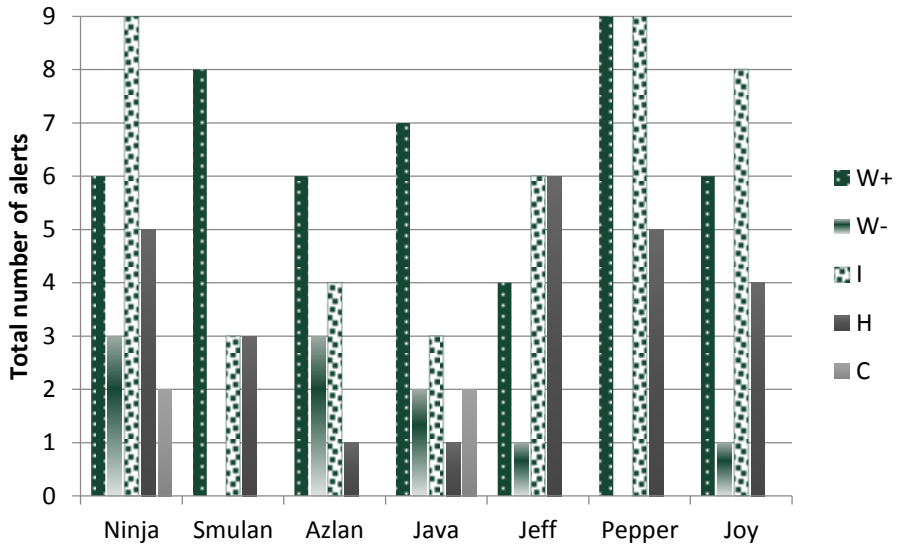

Figure 6: Differences in number of alerts made as a response to treatments for all the participating dogs over three rounds of experiment. Maximum number of alerts for one treatment made by one dog was nine. W+ extract infected tree, W- extract from healthy tree, I- wood from infected tree, H- wood from healthy tree, C- blank.

In a successful search the dog alerts when it finds a material from the found of infected material (true positive alert) and ignores samples collected from healthy trees as well as other controls.

The spatial summary of the success rate (Fig.7.) presents localisation of samples over the field trial as well as the success in their identification after all three rounds. The scheme shows that dogs were most successful in distinguishing control samples (Fig.7). The treatment most problematic for the dogs to identify was healthy wood, with only 38 right answers out of 63 search opportunities. The spot with healthy wood (H) sample in the row number two, plot one (2.1) was

identified as infected wood by three different dogs resulting in 0 successful markings. Three false identifications were made by three different dogs during the investigation of the block 5.2 in case of sample of the infected wood (I). In all the other cases samples were identified correctly by at least one dog over the duration of the trial (Fig.7).

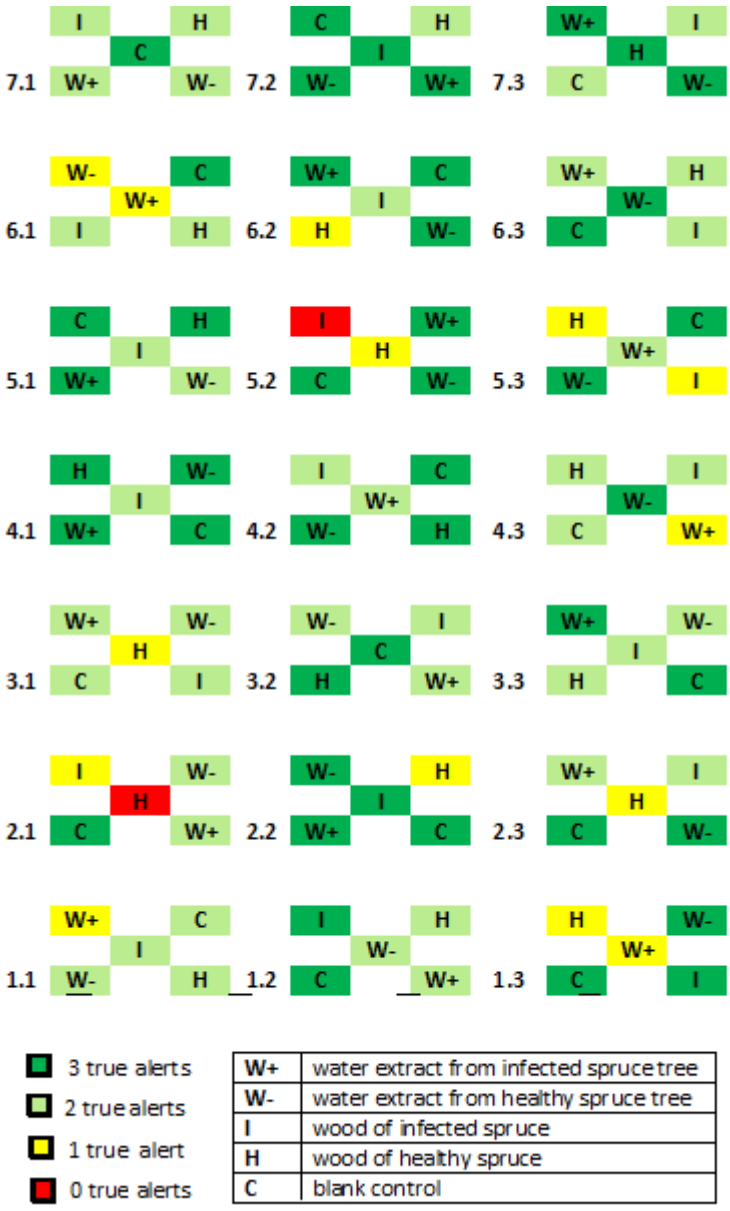

Figure 7: The spatial distribution of dogs' true alerts. The summary comprises the total sum of alerts made over three rounds. Locations of the samples identified three times (every alert made by different dog) are marked with deep green colour, samples identified correctly two times are marked with light green colour, one successful identification was given the yellow colour and situation where none of the dogs succeed with identification of the particular sample is marked in red.

Means of the results of the dogs for the particular treatments were compared using Sign test.

Table 2: Comparison of dogs' alerts for different treatments within one block, situations when dog alerted just for one of the analysed treatments, compared pairwise. Pairs of treatments where both true negative and true positive alerts were possible/one treatment was a control: water extract from infected tree and water extract from healthy tree (W+W-), infected wood- healthy wood (I H). For these pairs following scenarios were possible: a) dog alerted once but for the wrong treatment (control), b) dog alerted for both or for one of treatments, what corresponds to one true and one false alert, c) dog alerted once, and for the infected treatments (true positive alert). H_0: When dog alerted just for one of the analysed treatment, probability of true positive alert was 50%. W_1: When dog alerted just for one of the analysed treatment probability of true positive alert was higher than 50%. Pair of treatments where just true positive alerts were possible: water extract form infected wood and infected wood (W+ I). For this pair following scenarios were possible: a) dog made false alert for W+ and true alert for I, b) dog made false alerts for both analysed treatments, c) dog made true alert for W+ and false alert for I; Control treatment (C) was excluded from the analysis. Sign test, significance level= 5%.

Round	N	a)	b)	c)	p- value
w+ w-					
1	21	0	3	18	<0,001
2	21	2	6	13	0,007
3	21	4	6	11	0,118
I H					
1	21	2	10	9	0,065
2	21	1	15	5	0,219
3	21	4	7	10	0,180
W+ I					
1	21	0	11	10	0,002
2	21	6	10	5	1,000
3	21	7	12	2	0,180

Comparison between results for water extracts from infected and healthy spruce shows significant differences for the first and the second round, but not for the third round (Tab.2).

Dogs did not show significant preference towards one of the treatments from the pair consisted of healthy (H) and infected (I) wood during any of the rounds.

For the comparison of alerts for extract from infected wood (W+) and infected wood (I) only observations from the first round point out significant difference between the outcomes of the alerts (Tab.2), showing that it was easier to identify extracts from infected material (W+) compared to infected solid wood (I).

4.3. The effect of time on the frequency of alerts

4.3.1. Wood

Number of alerts made by dogs as response to the scent from wood samples changed over time. Detectability of infected wood (I) increased by 27% in the second round, reaching an increase of 33% in number of identified samples during the third round when compared to the first round (Fig. 8).

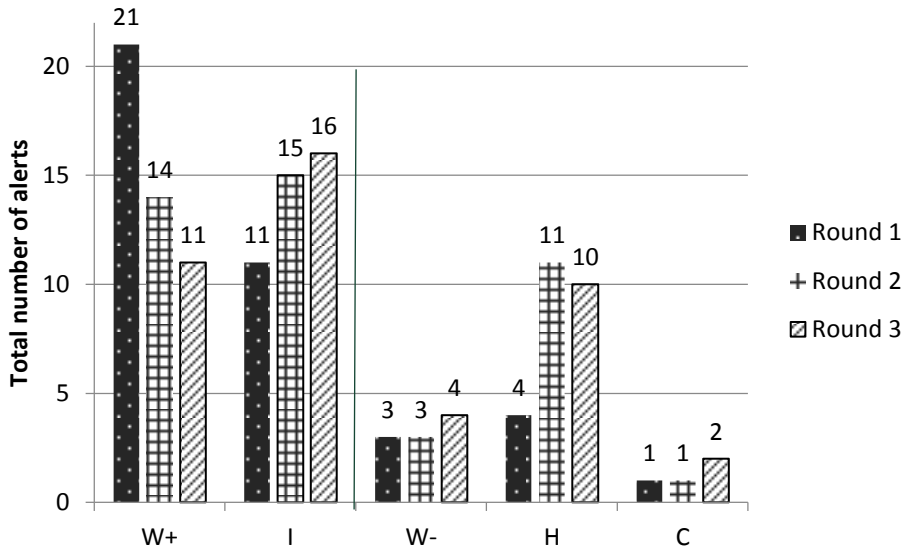

Figure 8: Number of alerts in particular rounds. Treatments to the right from the vertical line are all control treatments, and every alert made for any of the control treatment is classified as false positive one.

Same trend was observed for healthy wood (H) where number of alerts during the second round increased by 64%, decreasing slightly in the third round to the 60% of the initial number of alerts (Fig.8.). Every alert made as a response for healthy wood (H) was by default a false positive (FP) indication.

Results of Friedman Test did not prove that number of alerts for healthy wood treatment (Tab. 3) differed significantly with time (Tab. 3). Noteworthy, both p-value and χ^2 value came up very close to the significance level what can indicate higher variety in number of alerts (Tab. 3).

Table 3: Changes in the number of alerts made by dogs with time. Null hypothesis states that number of alerts for each treatment was equal in each round. Alternative hypothesis states, that the particular treatments` outcomes were not the same for each round. P-values <0,05 and chi-square values greater than 5,99 allow us to reject the null hypothesis.

Friedman's Test result (adjusted for ties)		
Treatment	Chi- square	p-value
Water extract from infected wood (W+)	12,15	0,002
Water extract from healthy tree (W-)	0,22	0,895
Infected wood (I)	2,63	0,269
Healthy wood (H)	5,73	0,057
Control (C)	0,5	0,779

4.3.2. Water extracts

Dogs alerted to all the 21 samples of water extract from infected tree under the first round (detectability=100%). Detectability of W+ samples decreased by 33% under the second round of the trial, reaching just 52% of the initial true alerts number during the third round (Fig.8). Dogs alerted for extracts from healthy wood (W-) three times during the first round and the second round and four times during the third round (Fig. 8).

The influence of the time passed from the application of water extracts on the ground on the number of false alerts made for water extracts is presented in the Tab.4. False alerts constituted 7% of alerts for liquid samples under the first round, 24% of alerts were false ones under second round, reaching 33% of alerts under the third round (Tab. 4). Worth noting factor was an occurrence of heavy rainfall after the first round.

*Table 4: Changes in water extracts **(W+, W-)** detectability in relation to the time from water extract application. Column **W+** presents the number of water extract samples from infected wood, identified by particular dog.*

Dog	Time from extract application (min)	T alerts (TP+TN)	F alerts (FP+FN)	W+
	Round I			
4	3	6	0	3
7	4	6	0	3
5	5	6	0	3
6	6	6	0	3
3	8	4	2	3
1	10	5	1	3
2	10	6	0	3
	Round II			
6	90	6	0	3
7	114	5	1	2
5	117	3	3	0
4	157	5	1	3
3	179	4	2	2
1	189	3	3	1
2	190	6	0	3
	Round III			
7	155	3	3	1
6	162	6	0	3
4	179	3	3	1
5	201	3	3	1
3	214	4	2	1
2	236	5	1	2
1	266	4	2	2

4.4. Differences between the dogs' performance

Comparison of the number of alerts made by dogs over three rounds (Tab.5) indicates statistically important differences between animals. Difference in total number of alerts between three rounds was not significant (Tab. 5). There was no significant correlation between particular dog's performance and the round (Tab.5).

Table 5: ANOVA summary table for a two-way analysis of variance, that was performed on the data for seven dogs, three rounds, and interactions between these variables.

Compared factor	DF	SS	MS	F-Value	P-value
Round	2	0,413	0,206	0,57	0,572
Dog	6	12,984	2,164	5,93	0,000
Round * dog	12	2,254	0,188	0,51	0,893
Error	42	15,333	0,365		
Total	62	30,984			

Significant differences in number of alerts signalized over all the rounds by dogs were also confirmed with Tukey's test (Tab. 6). Three groups could be distinguished among the dogs with the mean number of alerts made within each block as a criterion (Tab. 6).

Table 6: Tukey's Pairwise Comparison of means. Mean indicates the mean number of the alerts made by each dog in all of the investigated blocks (N=9) over the three rounds. Means that do not share a letter are significantly different.

Dog	*N*	*Mean*	*Success (%)*	*True alerts*	*False alerts*	*Grouping*
Ninja (1)	*9*	*2.78*	*71*	*32*	*13*	*A*
Pepper (6)	*9*	*2.56*	*89*	*40*	*5*	*A B*
Joy (7)	*9*	*2.11*	*80*	*36*	*9*	*A B C*
Jeff (5)	*9*	*1.89*	*67*	*30*	*15*	*B C*
Java (4)	*9*	*1.67*	*71*	*32*	*13*	*C*
Smulan (2)	*9*	*1.56*	*78*	*35*	*10*	*C*
Azlan (3)	*9*	*1.56*	*73*	*33*	*12*	*C*

The analysis of the number of alerts signalised by dogs was also conducted with distinction between three rounds of experiment (Tab. 7) Number of alerts signalised by dogs under the investigation of a single block was summarised and then the mean value of the number of alerts was calculated (Tab.7). Both true and false alerts were taken into account. When comparison between means for dogs were conducted significant difference in dogs' reactivity to volatile samples (number of alerts) was found just for the third round (Tab. 7), although there were only two individuals whose results differed from each other: dog nr 1 made in average three alerts within each block and it differs significantly from search results of the dog nr 3., which signalized a found in average 1,3 times under the

block investigation during the third round. The ideal result for the dog would be alerting two times within each block search, as two samples with infected material were placed there by default. Notably, the mean of alerts equal 2.00 does not always indicate the high success rate, as these alerts could as well be false (Tab.7.).

*Table 7: Comparison of means for number of alerts signalised by each dog during single experimental block search. **N**-number of the blocks searched. According to Tukey's Pairwise Comparison means that do not share the letter are significantly different.*

Round	Dog	N	Mean	Success (%)	True alerts	False alerts	Grouping (Tukey's test)	Difference between dogs (ANOVA, p-value)
I	1	3	2.67	87	13	2	A	
	6	3	2.33	93	14	1	A	
	7	3	2.00	100	15	0	A	0,102
	5	3	1.67	80	12	3	A	
	3	3	1.67	67	10	5	A	
	2	3	1.67	80	12	3	A	
	4	3	1.33	73	11	4	A	
II	6	3	3.00	80	12	3	A	
	1	3	2.67	60	9	6	A	
	7	3	2.00	87	13	2	A	
	5	3	2.00	60	9	6	A	0,195
	4	3	2.00	73	11	4	A	
	3	3	1.67	67	10	5	A	
	2	3	1.33	87	13	2	A	
III	1	3	3.00	67	10	5	A	
	7	3	2.33	53	8	7	A B	
	6	3	2.33	93	14	1	A B	
	5	3	2.00	60	9	6	A B	0,016
	4	3	1.67	67	10	5	A B	
	2	3	1.67	67	10	5	A B	
	3	3	1.33	87	13	2	B	

5. Discussion

From this study it is evident that, given the right conditions, dogs are able to perform very well detecting infected samples more often than expected by chance. The results from the study confirmed findings from previous attempt of testing sniffing dogs' ability to find *Heterobasidion* infection (Swedjemark and Morrison, 1989). With the development of the certificated training aids production the dogs' results can be expected to improve (Schlyter, personal communication).

The dogs` ability to correctly alert on infected solid wood samples may be due to an overwhelming smell from the water extract from infected wood. The organizers as well as dogs' trainer hypothesised, that dogs would be able to detect extracts from infected tree to a greater extent than infected wood samples in the first round. The results from the study confirmed this assumption. The superiority of water extracts over wooden material in terms of odour availability (Lazarowski et al. 2020) could explain a much lower detection rate for solid wood samples (I) in the first round with a total of 11 alerts (52%). To test dogs' ability to detect buried scent true material in form of wood billets were used. According to Simon et al. (2020) training aids in form of true materials tends to 'change odor profiles dramatically with time, environment and storage conditions'. Possibly, the time from placing wood samples in the holes to the experimental trial was too long for the odour to remain unchanged, or quite the contrary, too short as:'for a dog to detect buried odor, free molecules must diffuse through soil to the surface' (Lazarowski et al. 2020). Additionally, same author noticed dogs' difficulties with finding buried material, and received numerous reports from handlers about dogs alerting on water sources or plants located near the buried sample ignoring the target scent. This phenomenon could be explained by the movement of free odorants, free molecules can be transported by ground water and even absorbed by vegetation (Lazarowski et al. 2020). Possibly, after the rain with higher moisture of the soil, transport of the odor molecules was to the ground surface was intensified, what could explain higher number of alerts for I treatment after the rainfall (Fig. 8).

The worst results among all the control treatments, as well as for all the tested treatments, were noted for healthy wood (H). While false positive alerts for control water treatment (W-) can be explained by natural process of evaporation

to the air (Goss, 2019), and then even dilution after the rainfall, reasons for low detectability of solid wood treatment (H) must be of other origin. The reason could potentially depend on more practical issues, like the depth of the holes the samples were placed in. Given that the wood samples were buried too deep and the time between preparations and the field trial being too short the part of the scent information could be unreachable for animals. Assumption about problematic local conditions within sample plot could be advocated by findings from the study site, regarding two samples of solid wood material, I and H, located in the blocks 5.2 and 2.1 respectively, where 5 dogs during 6 independent search occasions made false indications (Fig.7). There is a possibility that the soil covering these samples was too compact, or that free odour molecules were transferred to moister location, as the movement of scent molecules depends on the soil structure and moisture (Lazarowski et al. 2020) The local conditions around sample plots was however not specifically tested in this experiment.

Complexity of the factors influencing odour buried underground can also become a problem in a forest where there will be a mishmash of healthy and infected roots to various extents in the ground.

Another explanation for the high false positives rate for wood stimuli treatments could be the limited time for training. While the animals had already practiced with the water extracts, they had not been previously trained on the wooden material buried under the ground. Training on wood had instead been restricted to wood chips from drilling into pieces of wood.

The high (100%) detectability of extracts from infected tree (W+) in the first round could be explained by the dogs' familiarity with the substrate; dogs were trained on water extracts just prior to the field examination. Time from the water extracts application on the ground to the start of the first round of the test did not exceed 10 minutes. The target scent could possibly have been very intense and attractive for the dogs as 'moisture in the water enhances diffusion and increases odor availability' (Lazarowski et al. 2020). In a practical setting this may not be the case. It is expected that the scent from intact roots with infection inside will be less intense and severely complicate the detection for the dogs.

Importance of the time factor for the dogs' response to treatments

The general trend observed in the study was the decreasing number of alerts on water extract (W+) with time, while the number of indications for solid wood material (both infected and control) was increasing after the first round. The heavy rain which occurred just after the first round most likely accelerated the natural process of dilution of the scent from the surface of the ground. The number of false alerts to water extracts treatments increased with time, which can have direct connection with the time that has passed by from the extracts application to the block search (Tab.4).

The correct indications for the infected wood (I) increased with time. Possibly, with the weaker scent from the water extracts dogs became more focused on finding another source of similar scent in order to gain praise (Johansson A., personal communication). The time factor is of big importance, as dogs could have possibly lost their ability to focus in the second and third round. Not being able to find water extract from infected tree as easily as in the first round, the animals could have got bored.

Time of the search

The search of the single block took between 1 to 2 minutes usually. According to the Sniffer dogs' trainer Annette Johansson, the time of the search is an individual characteristic of each dog and should not be included in any statistical analysis, i.e. a dog is not automatically bad because it spends more time on search than another faster dog. During the field trial dog handlers' were allowed to repeat the search within the same block if the handler noticed animal's uncertainty or realised that the team had not checked the whole search area. Additionally, the time each dog spent on playing or receiving verbal praise for a true indication varied greatly. Using search time as a success factor is hence tricky but is in practise of importance when searching bigger areas. In the study on sniffing police dogs line-up training Jezierski et al. (2008) stated that 'the trials resulting in correct indications were shorter in time' as the dogs possibly lose the ability to concentrate.

Distractions

The experimental trial was not placed in a forest stand for practical reasons, but was designed to be challenging for the dogs and mimicking diversified environment. Solid wood samples had been buried to test whether the dogs were

able to detect the scents located underground without a visual hint. The task constituted an introduction to further search for root rot in more realistic setting. The dogs proved to be able to locate buried samples ignoring sources of other intriguing scents such as few rodent holes in some blocks or the nearby sheep herd. The dogs were not influenced by choices made by other dogs visiting investigated blocks before (Tab. 5). Presence of other dogs, as well as many people and even a flock of sheep was not stopping dogs from searching the row.

Recommendations for further development of the method

The challenge and an ultimate goal of further studies is to profile volatile organic compounds (VOCs) of a *Heterobasidion* spp fungus to extract specific chemical components responsible for the 'scent of infection'. Specific scent could possibly originate from the host tree and its defence mechanisms against the fungus.

Previously, an attempt of training dogs on the fommanoxin was made (by people involved into " Hundnäsa mot skogskador" project) as this fungal metabolite was associated with *Heterobasidion* infection (Hansson et al. 2012). Dogs trained on fomannoxin from *Heterobasidion annosum in vitro* culture (Heslin et al. 1983) performed well under the training phase but were not able to detect infested trees in the field. The fommanoxin was chemically detected by solvent extraction only in trace amounts in some of the infected samples, thus biotesting of the whole extracts was chosen for this experiment (Schlyter, 2021, personal communication, 23 June).With known chemistry of host-pathogen interaction, the development of a synthetic substrate mimicking the scent occurring in the early phase of *Heterobasidion* infection would be accelerated. A synthetic substrate available on the market would make dog training more standardised (Schlyter, 2020 personal communication).

Apart from the costs of the training and training materials, other costs, such as veterinary care, food, insurance, dog's handler's salary etc. must be taken into account when decisions about using dogs as a detection method for root rot are being made. Through cross-training (Williams and Johnston, 2002), i.e., by connecting the training for bark-beetle detection with root rot training costs could be reduced. Dogs trained on synthetic semiochemicals successfully detect bark-beetle infested trees in the forests (Johansson et al. 2019, Vošvrdová et al. 2020, unpublished). The method is already successfully implemented into the practice and establishment of seven small companies indicate a success of the concept as a commercially established technique, potentiating forest protection by search-and-pick (Schlyter 2021, personal communication, 23 June).

Not every dog able to determine infected material from controls is a candidate for being a detection dog (Gadbois and Reeve, 2014). The ability to detect infected material (sensitivity) while ignoring non-target odours (specificity) and

other distractions of any form has to be mastered. In case of root rot detection dogs time of the search dedicated to a single tree must be short and communication between dog and the handler clear. The dog nr 5- Pepper, achieved 100% sensitivity rate, detecting all the infected samples over its search rounds, which could suggest the selection of this dog for the purpose of the further, professional training. To validate the results, repetition of the experiment is needed, preferably with the same dogs and handlers involved.

Dog handlers participating in the test suggested changing in the signal for location of scent samples within the block. Instead of single vertical sticks, the sample location should be indicated by visible borders. Sometimes it was unclear which side of the sample was indicated by the stick. This kind of uncertainty, under the stressful conditions of the search, could lead to the situation where the handler sent confusing signal to its dog as where to search. In a real forest setting this would of course also be the case and it is clearly going to be challenging for the dogs, creating additional difficulty in evaluating their performance when roots are underground and normally without open sides letting the scent out.

6. Conclusions and practical implications

The results of this study prove dogs` ability to detect *Heterobasidion* spp. infection from both wood and water solution samples using olfactory sense. Dogs alerted on infected material more often than expected by chance, and frequency of alerts differed depending on treatment and time elapsed.

There is a need for further repetition of the field study to test dogs' detection abilities on material originating from different spruce trees, of varied stages of infection. The common suggestion received from dogs' owners regarding the field study design is to indicate the location of the treatment with visible borders instead of single stick designated to each spot, as was the case in this study in Asa. Dog handlers were unsure where to lead their dogs and dogs were not sure as where to sniff.

Reduction of the costs associated with the method could be achieved with commercialisation of a synthetic volatile substance responsible for characteristic scent of wood infected by a *Heterobasidion* spp. fungi as well as cross-training of pre-selected dogs.

Despite its limitations this pioneering study suggests, that dogs could be a useful tool when detecting root rot in field conditions.

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Acknowledgements

I would like to thank Jonas Rönnberg for being my supervisor, trusting me with the choice of demanding topic of the thesis, and then not giving up on me when things did not turn out as planned.

Fredrik Schlyter, the head supervisor of the research programme 'Rapid olfactory detection of insect and fungal damage in forests', thank you for the long hours you spent on my manuscript, professional advice, factual comments and attempts to stop my graphomania.

Jan-Eric Englund, thank you for supervision of the statistics part of the thesis and for your patience in explaining the rules of this discipline.

Annette Johansson from SnifferDogs Sweden- without you and your love to dogs and forests this project would not have been possible. Thank you for sharing your innovative ideas and experience. I would like to extend my sincere thanks to people without whom the trial could not take place, dog handlers and their lovely dogs. Thank you Håkan and Ninja, Christian and Smulan, Jonas and Azlan, Mattias with Java and Joy, Jenny and Jeff, Magnus and Pepper, it was a pleasure to witness the great cooperation between you and your four-legged friends.

I would like to recognize the assistance and technical help we received from employees of Asa Experimental Forest and Research Station with preparation of the field trial, my special thanks go to Martin Ahlström, the chef of the facility.

Thank you Khaled Youssef for your assistance in Asa and work you have done to recognise the *Heterobasidion* species in laboratory in Alnarp. Ida Nordström thank you for documenting the field trial in spite of bad weather.

I would like to thank Marie Bergqvist from SLU's library in Umeå for helping me with organization of my presentation.

Giulia Attocchi, without your organizational skills and great help I would never be ready. Thank you very much!

Johanna Witzell, your invaluable support is the reason why this thesis came to its final stage. I am very thankful for your guidance and advices.

Financial support was provided by Formas and the Rattsjö foundation.

Appendix 1

Table 8: Descriptive statistics for total number of alerts made by dogs out of 9 searched blocks.

Summary of Data						
	Treatments					
	W+	W-	I	H	C	Total
N	7	7	7	7	7	35
Mean	6.57	1.43	6.00	3.57	0.57	3.63
Std.Dev.	1.62	1.27	2.71	1.99	0.98	2.96

Results details				
Source	SS	df	MS	
Between treatments	199.3143	4	49.8286	F = 15.84254
Within treatments	98.8571	30	3.2952	
Error	75.4857	24	3.1452	

The F-ratio value is 15.84. The p-value is < 0.001. The result is significant at p <0 .05.

Table 9:Frequency of reactions to different treatments for all participating dogs. Numbers present the total sum of alerts made by dogs over all the tree rounds of an experimental trial.

	Ninja (1)	Smulan (2)	Azlan (3)	Java (4)	Jeff (5)	Pepper (6)	Joy (7)	total
W+	6	8	6	7	4	9	6	46
W-	3	0	3	2	1	0	1	10
I	9	3	4	3	6	9	8	42
H	5	3	1	1	6	5	4	25
C	2	0	0	2	0	0	0	4
Total	25	14	14	15	17	23	19	127
st. dev.	2,74	3,27	2,39	2,35	2,79	4,51	3,35	
variance	6	8,56	4,56	4,4	6,24	16,24	8,96	

Table 10: Total amount of alerts: true positives (TP), false negatives (FN), true negatives (FN) and false positives (FP) under the experimental rounds with differentiable sample materials.

Material	TP	FN	TN	FP
Round 1	**32**	**10**	**55**	**8**
Water extract	21	0	18	3
Wood sample	11	10	17	4
Control	-	-	20	1
Round2	**29**	**13**	**48**	**15**
Water extract	14	7	18	3
Wood sample	15	6	10	11
Control	-	-	20	1
Round 3	**27**	**15**	**47**	**16**
Water extract	11	10	17	4
Wood sample	16	5	11	10
Control	-	-	19	2
Total number of reactions= 315	**88**	**38**	**150**	**39**

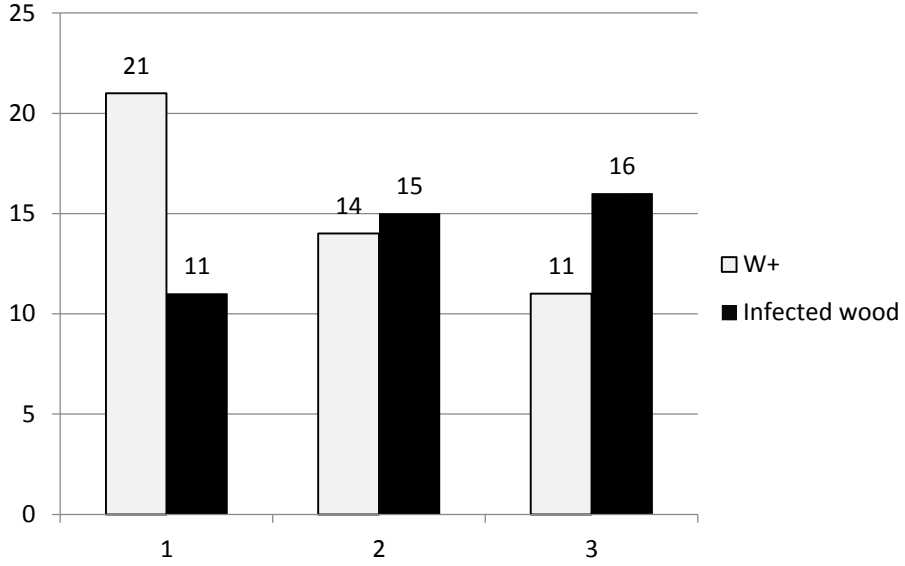

Figure 9: Comparison between true positive indications made on the water extract (W+) and infected wood material (I). Figure shows the sum of true positive results indicated by all the dogs over each round.

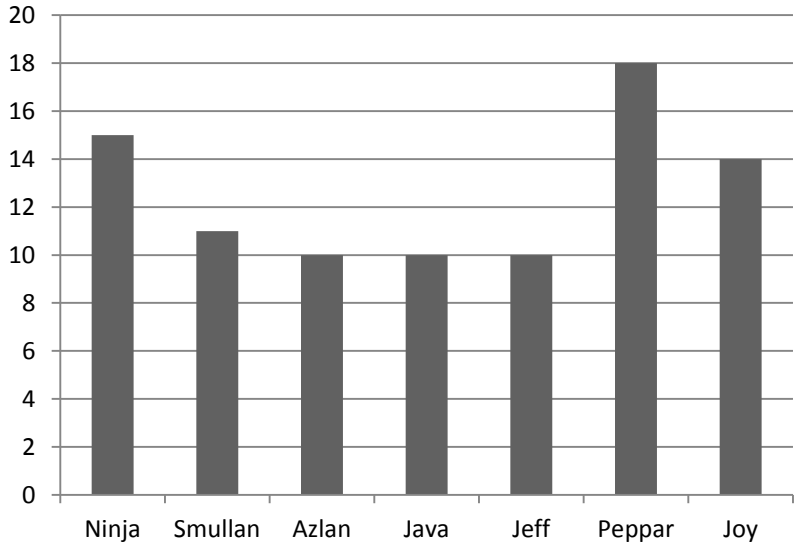

Figure 10: The number of true positive indications made by dogs over three repetitions.

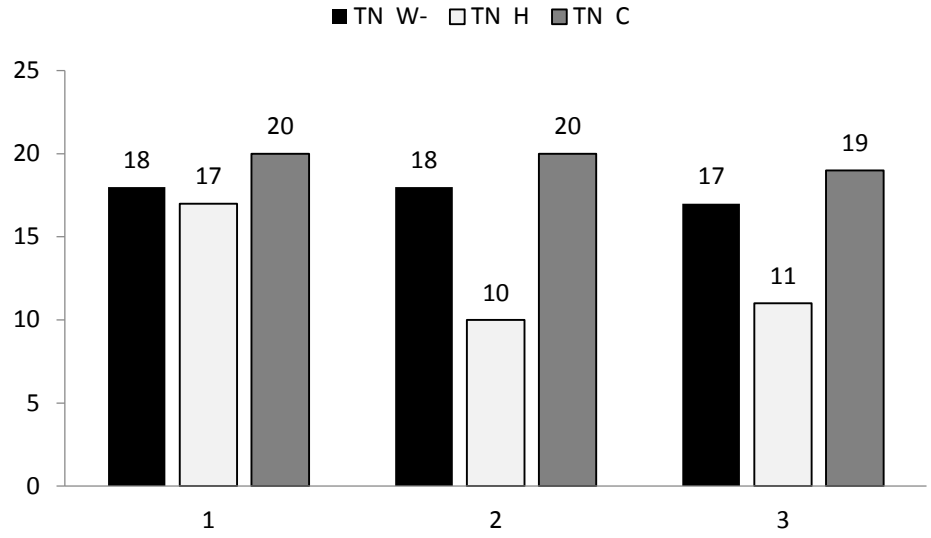

Figure 11: True negative reactions for control of three types: water extract (W-), solid wood control (H) and blank (C) over three rounds.