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Inoculation success of *Inonotus obliquus* (Ach. ex Pers.) Pilát in birch (*Betula pendula* Roth, *B. pubescens* Ehrh.) and alder (*Alnus incana* (L.) Moench, *A. glutinosa* (L.) Gaertn.) in Estonia

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Abstract

Key message This is the first study to investigate the success of inoculation of *Alnus incana* and *A. glutinosa* with *Inonotus obliquus* (Ach. ex Pers.) Pilát, providing new insights about the potential cultivation of this valuable fungus under field conditions. Results show that conks developed slowly under natural conditions. The development of the fungus observed in *A. glutinosa* suggests that this species may be as suitable a host for *I. obliquus* than birches. Furthermore, visible stem symptoms proved unreliable for detecting infection, highlighting the importance of molecular diagnostics for accurate assessment.

Context *Inonotus obliquus* (Ach. ex Pers.) Pilát (commonly known as Chaga) is a medicinal fungus traditionally harvested from birch trees. Artificial cultivation is increasingly considered due to a rising commercial demand. However, our knowledge remains limited regarding infection dynamics and conk development on different host species.

Aims We assessed the success of *I. obliquus* inoculation in *Betula pendula*, *B. pubescens*, *Alnus incana*, and *A. glutinosa* stands 2 years after inoculation in order to: (i) identify the most susceptible host tree species and the most effective *I. obliquus* strains for cultivation; (ii) assess the frequency of conk formation under hemiboreal conditions; investigate whether (iii) visible symptoms on stems resulted from *I. obliquus* infection and (iv) mechanical wounding influenced fungal establishment.

Methods The experiment was conducted at two locations in Estonia, encompassing a total of six forest sites, with inoculation success assessed using species-specific qPCR. Sterile conk development was additionally monitored visually 4 years post-inoculation.

Results Inoculation success differed between the two locations, with an infection rate of 18% observed at Järvselja and 3% at Võrumaa. Three fungal strains consistently achieved higher infection rates among the six tested ones. Conks developed only on *A. glutinosa* trees and with specific strains. Only the identity of the fungal strains resulted in a significant effect on infection ess. Visible stem symptoms, such as bleeding and bulging, were unreliable indicators of the infection rate.

Conclusion *I. obliquus* conks developed more rapidly on *A. glutinosa* compared to *B. pendula*, *B. pubescens*, and *A. incana*. Moreover, fungal strains play a crucial role in determining cultivation success.

Keywords Chaga, *Betula* spp., *Alnus* spp., Medicinal fungi, Artificial inoculation, Non-timber forest products

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1 Introduction

Medicinal fungi have been a cornerstone of European ethnomedicine for centuries, valued for their therapeutic properties and deeply rooted in traditional healing practices. Over the years, these fungi have attracted growing scientific interest due to their pharmacological potential. Despite considerable research efforts, however, the transformation of these natural compounds into clinically approved medicines continues to pose complex scientific and regulatory challenges (Gründemann et al. 2020). Globally, medicinal fungi are widely recognized and utilized for their immune-modulating, antiviral, and other bioactive effects, highlighting their continued relevance in both traditional and modern healthcare (Zhang et al. 2022).

Inonotus obliquus (Ach. ex Pers.) Pilát, commonly known as Chaga, has been used for millennia by indigenous populations across northern and eastern Europe and Asia (Saar 1991; Perevedentseva 2013; Sak et al. 2014; Szychowski et al. 2020). Historically, *I. obliquus* has served as a remedy for various ailments, most notably for pain relief in cancer treatment (Phillips and Burdekin 1992) and for addressing gastrointestinal disorders (Szychowski et al. 2020). Its therapeutic efficacy is attributed to diverse bioactive compounds, including triterpenoids such as inotodiol, betulin, and betulinic acid, as well as polysaccharides, polyphenols, and melanin (Peng and Shahidi 2020; Thomas et al. 2020). Recent studies have further advanced this understanding; for instance, Raal et al. (2024) demonstrated significant cytotoxic effects of *I. obliquus* extracts, particularly against human hepatocellular carcinoma and thyroid anaplastic carcinoma cells, with the most pronounced activity observed when the fungus was collected from *Betula pendula* and *B. pubescens*. This growing evidence has contributed to a marked increase in scientific publications focused on *I. obliquus* over the past decade (Lu et al. 2021).

I. obliquus is a basidiomycete fungus belonging to the family *Hymenochaetaceae*, order *Hymenochaetales*, and class *Agaricomycetes*, predominantly found in the Northern Hemisphere, where it primarily infects broadleaved tree species (Kalamees 2000; Lee et al. 2008; Kirk et al. 2008). While birches (*Betula* spp.) are its principal hosts, the fungus also occurs on other deciduous trees such as alders (*Alnus* spp.) (Ryvarden and Gilbertson 1993; Niemelä 2008; Drenkhan et al. 2022). The life cycle of the fungus includes the production of basidiospores, which are released during summer and early autumn—typically at night—under Estonian climatic conditions (Adamson et al. 2024). These spores originate from basidiocarps that form beneath the bark of standing dead trees, and the process eventually leads to bark detachment and the exposure of decayed wood (Kalamees 2000; Niemelä 2008; Lõhmus and Lõhmus 2024).

The infection process caused by *I. obliquus* results in white rot, which weakens tree structure and increases susceptibility to windthrow and premature mortality (Salo et al. 2007; Raal et al. 2018; Drenkhan et al. 2020). On living trees, the fungus produces distinctive sterile mycelial masses known as black conks (Katayevskaya 1928; Salo et al. 2007). These begin to form approximately 3–5 years after natural infection and typically 4–5 years following artificial inoculation (Pilz 2004; Miina et al. 2021). A single host tree may bear one to six conks, with numbers exceeding six often indicating extensive internal decay (Sinclair and Lyon 2005). Due to its relatively slow growth and patchy natural occurrence, locating *I. obliquus* in nature can be difficult (Zheng et al. 2010). In areas where it does occur, harvesting from natural populations is frequently already underway (Pietka and Grzywacz 2006; Thomas et al. 2020).

In response to increasing demand and conservation concerns, the intentional cultivation of *I. obliquus* through artificial inoculation of living trees has gained traction as a viable alternative. In Northern Europe, forest owners are encouraged to inoculate birch stands using wooden dowels colonized with *I. obliquus* mycelium (Vanhanen et al. 2014). In Finland, this method is particularly recommended for low-yielding *B. pubescens* stands and peatland areas designated for set-aside use (Vanhanen et al. 2014; Miina et al. 2021). Monitoring efforts in Estonia have revealed that *I. obliquus* conks form with comparable frequency in both birch and alder stands (Adamson et al. 2024), and chemical analyses indicate that conks harvested from alders contain bioactive compounds similar to those found in birch-derived *I. obliquus* (Drenkhan et al. 2022). These findings suggest that the inoculation of low-value *A. incana* stands could be part of similarly profitable practices.

Several experimental studies have investigated the artificial cultivation of *I. obliquus* (Blanchette 1982; Piętka and Grzywacz 2006); Park et al. 2010; Ka et al. 2017; Silvan and Sarjala 2017; Miina et al. 2021). These studies collectively indicate that inoculation outcomes are influenced by a range of ecological and management factors. Miina et al. (2021) identified key predictors of success, including southerly geographic location, mineral soil type, smaller mean tree diameter, greater stand basal area, and early-season inoculation timing. Furthermore, both Silvan and Sarjala (2017) and Miina et al. (2021) emphasized the critical role of fungal strain selection in

determining both infection rates and conk productivity. While Adamson et al. (2023) have characterized fast- and slow-growing strains under laboratory conditions, their behavior under natural outdoor conditions remains poorly understood. In addition, it remains unknown how quickly sterile conks develop on different tree species, particularly *Alnus* spp., after artificial inoculation.

Despite increasing interest in cultivating *I. obliquus*, our knowledge remains limited regarding its infection dynamics and conk development on different host species under hemiboreal Northern European conditions. To address these knowledge gaps, the main aims of this study were to (a) evaluate the success of *I. obliquus* inoculation in *B. pendula*, *B. pubescens*, *A. incana*, and *A. glutinosa* stands 2 years after inoculation; (b) identify the most susceptible host tree species and the most effective *I. obliquus* strains for cultivation; (c) assess the frequency of sterile conk formation under hemiboreal conditions; and (d) explore whether visible stem symptoms after inoculation reflect fungal infection, as well as whether mechanical wounding influences fungal establishment.

2 Materials and methods

2.1 Study area

In this study, six forest sites were selected at two different locations in Estonia (Fig. 1, Table 1). Three sites (JS243, JS109, JS331) are located in southeast Estonia, at Järvselja (Kütt et al. 2025a), and three sites (AS085, SR356, AS020)

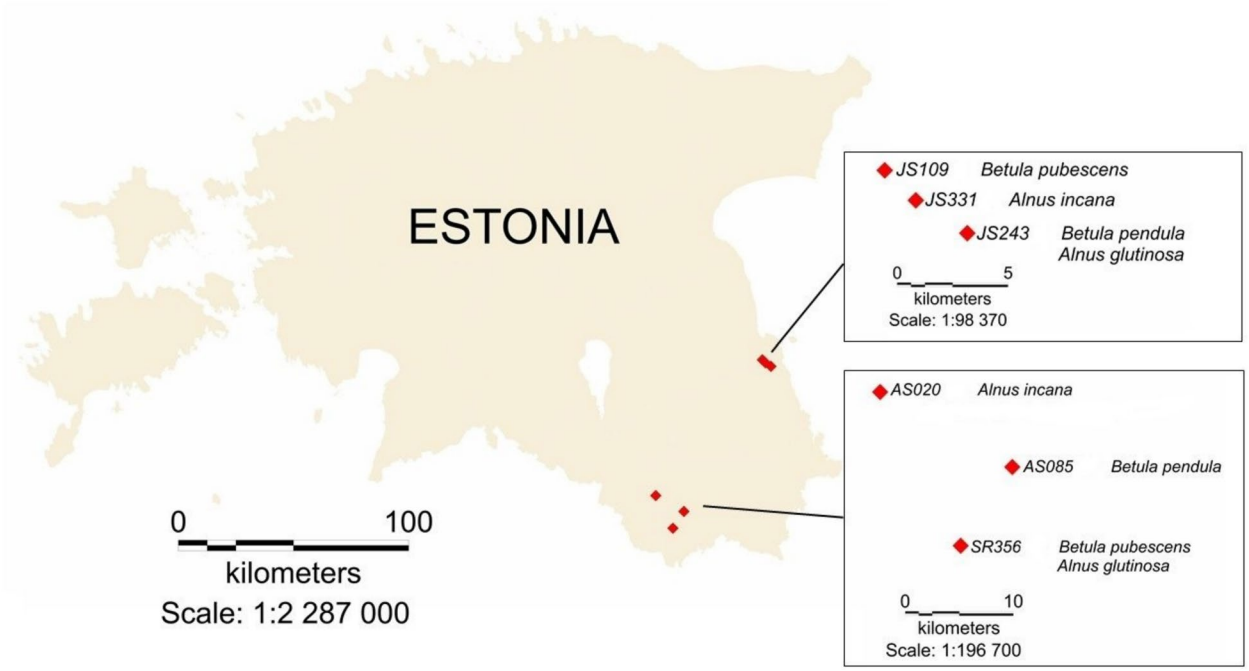


Fig. 1 Location of six sites where birch and alder trees were inoculated with *I. obliquus* in Estonia, using MapInfo Pro v 17.0

Table 1 Sites used for *I. obliquus* inoculation experiment including forest site code, geographical coordinates, forest site type, main tree species, DBH (diameter at breast height, measured 1.3 m above ground), tree age, and H (average stand height on the first layer). Forest site types follow the classification by Lõhmus (1984)

	Geographical coordinates						
Forest site code	***N***	**E**	**Forest site type**	**Main tree species**	**DBH (cm)**	**Age (years)**	**H (m)**
JS243	58.26983	27.32471	*Aegopodium*	*B. pendula*	26	66	25.9
JS109	58.28778	27.25962	*Alnus-Betula* fen	*B. pubescens*	14	59	14.0
JS331	58.28919	27.25922	*Aegopodium*	*A. incana*	14	37	16.1
AS085	57.71637	26.60527	*Oxalis*	*B. pendula*	25	53	24.8
SR356	57.65325	26.58648	*Carex-Filipendula*	*A. glutinosa*	25	60	21.8
AS020	57.78778	26.44537	*Aegopodium*	*A. incana*	18	37	19.5

in southern Estonia, at Võrumaa (Kütt et al. 2025b). At both locations, sites dominated by four tree species (*B. pendula, B. pubescens, A. incana, A. glutinosa*) were selected on four different forest site types (Lõhmus 1984) with an average stand age of 37–66 years and an average stand height from 14.0 to 25.9 m (Table 1). Basic climatic data (mean annual air temperature and annual precipitation) for 2020–2024 were obtained from the Estonian Weather Service (2025) Annual Maps for the nearest meteorological stations in Tõravere and Võru (Table 2).

2.2 Isolation and identification of the fungal strains used in the experiment

Nine *I. obliquus* strains were collected during the years 2019–2021 across the forests in Estonia (Table 3) and transported to the laboratory. Conks were stored at + 4 °C until isolation. To obtain pure cultures, a small piece of mycelia from each conk was placed onto a 2% MEA (Malt Extract Agar, Biolife, Italy) plate, under the laminar flow. Isolates were purified by subculturing until monocultures were obtained and were grown at room temperature (22 °C). Approximately 0.04 g of mycelium was transferred into 2.0 ml microcentrifuge tubes that were stored at −20 °C until DNA extraction.

The mycelia from pure culture were homogenized using ca 5 metal beads (∅ 2.5 mm) in a Retsch MM400 homogenizer (Retsch GmbH, Haan, Germany). DNA was extracted from mycelia using the GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics UAB, Lithuania) following the manufacturer's protocol. The identity of each fungal strain was confirmed by sequencing of the Internal Transcribed Spacer (ITS) region. ITS-PCR was performed using the fungal-specific primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), carried out as described by Drenkhan et al. (2016). Electrophoresis of PCR products was conducted at 75 V for 55 min using 1% agarose (SeaKem® LE Agarose; Lonza, Rockland, ME, USA) gels. PCR products were visualized under UV light using Quantum ST4 Express v16.04 (Vilber Lourmat SAS, Marne-la-Vallée, France). PCR products were sequenced at the Estonian Biocentre in Tartu, using the primers ITS5 and ITS4 (White et al. 1990). The sequences were edited using the BioEdit version 7.2.5 (Hall 1999). BLAST searches for the fungal

Table 2 Mean annual air temperature (°C) and annual precipitation (mm) recorded at the nearest meteorological stations during the years of inoculation experiments (2020–2024). Tõravere station represents the closest station to the Järvselja sites, while Võru station is the closest to the Võrumaa sites

Year	Mean air temperature in Tõravere, °C	Mean air temperature in Võru, °C	Annual precipitation in Tõravere, mm	Annual precipitation in Võru, mm
2020	8.1	8.3	697	597
2021	6.3	6.4	682	606
2022	6.9	7.0	551	561
2023	7.1	7.3	722	675
2024	8.1	8.3	587	608

Table 3 *Inonotus obliquus* strains used in this study, including strain number, sampling date, host species, collector, fungal collection code (TFC—Tartu Fungal Collection in Estonian University of Life Sciences, Estonia), and GenBank accession number for ITS sequences (NCBI)

Strain no.	Sampling date	Host species	Collector	Fungal collection code	Accession no. in GenBank
PAT22865	13.01.2019	*A. incana*	Kalev Adamson	TFC101265	OP942247
PAT21061	18.11.2019	*A. incana*	Kalev Adamson	TFC101264	OP942246
PAT24478	18.02.2020	*A. incana*	unknown	TFC101325	PV740223
PAT24474	22.03.2020	*A. incana*	Eilike Sõber	TFC101269	OP942251
PAT29045	21.12.2019	*B. pendula*	Maidu Silm	TFC101258	OP019325
PAT24473	22.03.2020	*B. pendula*	Eilike Sõber	-	PV740222
PATKA880	09.11.2021	*B. pendula*	Kalev Adamson	TFC101274	OP942256
PATKA896	11.11.2021	*B. pendula*	Kalev Adamson	TFC101277	OP942259
PATKA900	11.11.2021	*B. pubescens*	Kalev Adamson	TFC101278	OP942260

taxa confirmation were performed at GenBank (NCBI). All the ITS sequences of *I. obliquus* from Estonia were deposited in GenBank (Table 3).

2.3 Inoculation of trees

For inoculation, *Betula* spp. dowels with a size of 10×40 mm were prepared according to Adamson et al. (2023). First, grain mycelium was prepared. For that, wheat grains were first boiled at low heat for 30 min and then placed into 815 ml mushroom-growing jars and sterilized at 121 °C for 60 min. Cooled grains were inoculated with *I. obliquus* mycelium and incubated at room temperature for 30 days. Dowels were then autoclaved for 60 min at 121 °C. After cooling, dowels were inoculated with 40 g of *I. obliquus* grain mycelium (per 100 dowels) and incubated at room temperature until dowels were covered with mycelium.

Birch and alder trees were inoculated with *I. obliquus* dowels by drilling five 12-mm-diameter holes into each stem. Three holes were drilled on one side of each trunk at approximately 40, 110, and 180 cm and two on the opposite side at approximately 75, 135 cm above ground level. In the Järvselja sites, wax (Lac-Balsam®, Compo, Germany) was applied to the inoculation holes, whereas no wax was used in the Võrumaa sites (Table 4). Between use at every tree, the drill was cleaned and sterilized with 96% alcohol and flaming.

During early July 2020 at Järvselja, a total of 240 trees were inoculated with *I. obliquus* across three sites (JS243, JS109, JS331), including 60 trees each of *B. pendula*, *B. pubescens*, *A. incana*, and *A. glutinosa*. Four fungal strains were selected: two faster-growing (PAT24473, PAT22865) and two slower-growing ones (PAT24474, PAT24478). These strains were selected based on their growth rates at 9 °C and 18 °C (unpublished data, only PAT24474 having been previously published in Adamson et al. 2023). These four genetically different *I. obliquus* strains originated from *B. pendula* and *A. incana* (Table 3).

During early June 2022 at Võrumaa, a total of 360 trees were inoculated with *I. obliquus* across three sites (AS085, SR356, AS020), including 90 trees each of *B. pendula*, *B. pubescens*, *A. incana*, and *A. glutinosa*. Six fungal strains were selected based on their growth rates at different temperatures: 9 °C, 18 °C, and 22 °C (Adamson et al. 2023), including four faster-growing strains (PAT21061, PAT29045, PATKA880, PATKA900) and two slower-growing ones (PATKA896, PAT24474). These six *I. obliquus* strains originated from *B. pendula*, *B. pubescens*, and *A. incana* (Table 3).

Each fungal strain was used to inoculate 15 trees of each tree species. At every site, 15 control trees per species were inoculated with five sterilized dowels, and an additional 15 trees were mechanically wounded with an axe to simulate natural injuries that may facilitate fungal entry. Fungal establishment was assessed to compare inoculated, wounded, and control trees.

2.4 Sample collection, visual assessment of trees and *I. obliquus* specific qPCR assay

To monitor the infection rate and assess the success of artificial inoculation with *I. obliquus,* sawdust samples were collected prior to inoculation and 2 years after inoculation. Pre-inoculation sampling (840 samples) aimed to confirm whether trees were uninfected or naturally infected by *I. obliquus*. These samples were collected from 12 mm diameter drill holes prepared for dowel placement, a total of 360 samples at the Järvselja sites in July 2020, and 480 samples at the Võrumaa sites in June 2022. Post-inoculation samples were taken 2 years

Table 4 Inoculation of birches and alders at six experimental sites in Estonia, including forest site code, inoculated tree species, number of inoculated, control, and mechanically wounded trees, fungal strains, wax treatment, date of inoculation, and date of sample collection before and after inoculation

Forest site code	Inoculated tree species	No. of inoculated/control/mech. wounded trees	Fungal strain	Wax treatment	Date of inoculation and sample collection (before inoculation)	Date of sample collection (after inoculation)
JS243	*B. pendula*	60/15/15	PAT24473, PAT22865, PAT24474, PAT24478	Yes	03.07.2020	28.06.2022
	A. glutinosa	60/15/15		Yes	03.07.2020	28.06.2022
JS109	*B. pubescens*	60/15/15		Yes	06.07.2020	28.06.2022
JS331	*A. incana*	60/15/15		Yes	02.07.2020	28.06.2022
AS085	*B. pendula*	90/15/15	PAT21061, PAT29045, PATKA880, PATKA900, PATKA896, PAT24474	No	10.06.2022	Destroyed by the storm
SR356	*B. pubescens*	90/15/15		No	09.06.2022	07.06.2024
	A. glutinosa	90/15/15		No	09.06.2022	07.06.2024
AS020	*A. incana*	90/15/15		No	09.06.2022	06.06.2024

after inoculation approximately 5 cm above the inoculation dowel holes on one side of the tree stem to minimise trees injury. At Järvselja, 359 post-inoculation samples were collected in June 2022; one *B. pubescens* tree had been damaged by a storm and was excluded from sampling. A storm on August 31, 2023, destroyed the *B. pendula* experimental site AS085 in Võrumaa. Consequently, 2 years after inoculation, only the sites of *B. pubescens*, *A. incana*, and *A. glutinosa* in Võrumaa were available for analysis. In June 2024, a total of 359 sawdust samples were collected from these sites. One *A. incana* tree had been felled, and only its stump could be identified. For each tree, sawdust samples were stored at − 20 °C until laboratory analysis. To avoid cross-contamination, the drill was sterilized with 96% ethanol and flaming between use at every tree. DNA was extracted from sawdust samples following the same protocol as described in Section 2.2. The presence of *I. obliquus* was verified with a species-specific qPCR assay according to Adamson et al. (2024).

In addition, each sampled tree was individually marked and assessed in the field. DBH was measured, and decay was initially evaluated visually based on the appearance and color of the drilled sawdust. Injuries and any fungal fruiting bodies observed on the stem surface were recorded. Two years after inoculation, the health status of all inoculated *Betula* and *Alnus* trees at both locations was assessed (alive or dead) as well as visible symptoms. Stem bleeding was indicated by dark-colored fluid oozing from the bark, while trees showing signs of outward bulging and/or cracked bark were classified as bulging. The formation of conks was assessed and measured to estimate their volume based on height, width, and depth. In October 2024, 4 years after inoculation, trees at the Järvselja sites were revisited and inspected for external signs of *I. obliquus* conk development.

2.5 Statistical analyses

Post hoc comparisons were performed using Tukey's HSD test with a significance threshold of $p < 0.05$, conducted separately for the two locations using Statistica v 13 software. The tree species and fungal strain interaction was analyzed for Järvselja sites only. Data visualization, including charts and graphical summaries, was conducted using Microsoft Excel.

To assess the factors influencing the success of *I. obliquus* inoculation, a generalized linear model (GLM) with a binomial error distribution and logit link function was employed. Logistic regression was selected due to the binary nature of the response variable (infection detected or not detected via qPCR). The model included the following explanatory variables:

- Presence or absence of decay (categorical),
- Tree diameter at breast height (DBH, continuous),
- Fungal strain (categorical),
- Host tree species (categorical).

Model fitting and significance testing were conducted in R using the `glm()` function from the base stats package. To assess whether visible stem symptoms after inoculation could serve as reliable indicators of infection, an additional binomial logistic regression was conducted using a combined symptom variable, defined as the presence of either bulging/cracking or stem bleeding (binary response: 0 = absent, 1 = present; predictor: qPCR infection status). Statistical models were constructed using data from the Järvselja sites only, as the number of positive infection cases in Võrumaa was too low for meaningful model fitting.

3 Results

3.1 Inoculation success at the two locations

qPCR analysis did not reveal any pre-existing infection of *I. obliquus* in any of the trees prior to experimental inoculations. Two years after inoculation, at Järvselja, the presence of *I. obliquus* was confirmed by qPCR analysis in 44 out of 239 inoculated trees across all tree species and sites JS243, JS109, and JS331, corresponding to an overall inoculation success rate of 18%. At Võrumaa, 7 out of 269 inoculated trees in *B. pubescens*, *A. incana*, and *A. glutinosa* tested positive for *I. obliquus*, with an inoculation success rate of 3%.

3.2 Results at Võrumaa

At the Võrumaa sites, inoculation success was uniformly low, with the most effective strain—PAT21061—resulting in 3 (7%) infected trees (Fig. 2). Strains PAT29045, PATKA896, and PATKA900 yielded 1–2 (2–4%) infected trees each, while no infection (0%) was detected for strains PAT24474 and PATKA880. Mechanically wounded trees and control treatment remained uninfected by *I. obliquus*.

At the Võrumaa sites SR356 and AS020, the number of infected trees was low across all species (Fig. 3).

3.3 Results at Järvselja

3.3.1 Inoculation success of I. obliquus strains

At the Järvselja sites, inoculation success varied substantially among the tested strains (Fig. 4). The highest infection rate was observed with strain PAT24473, which successfully colonized 16 (27%) trees out of 60. This was followed by PAT22865 with 13 (22%) and PAT24478 with 12 (20%) infected trees, and both of these strains showed consistent infection rates across replicate inoculations. In contrast, PAT24474 exhibited markedly lower

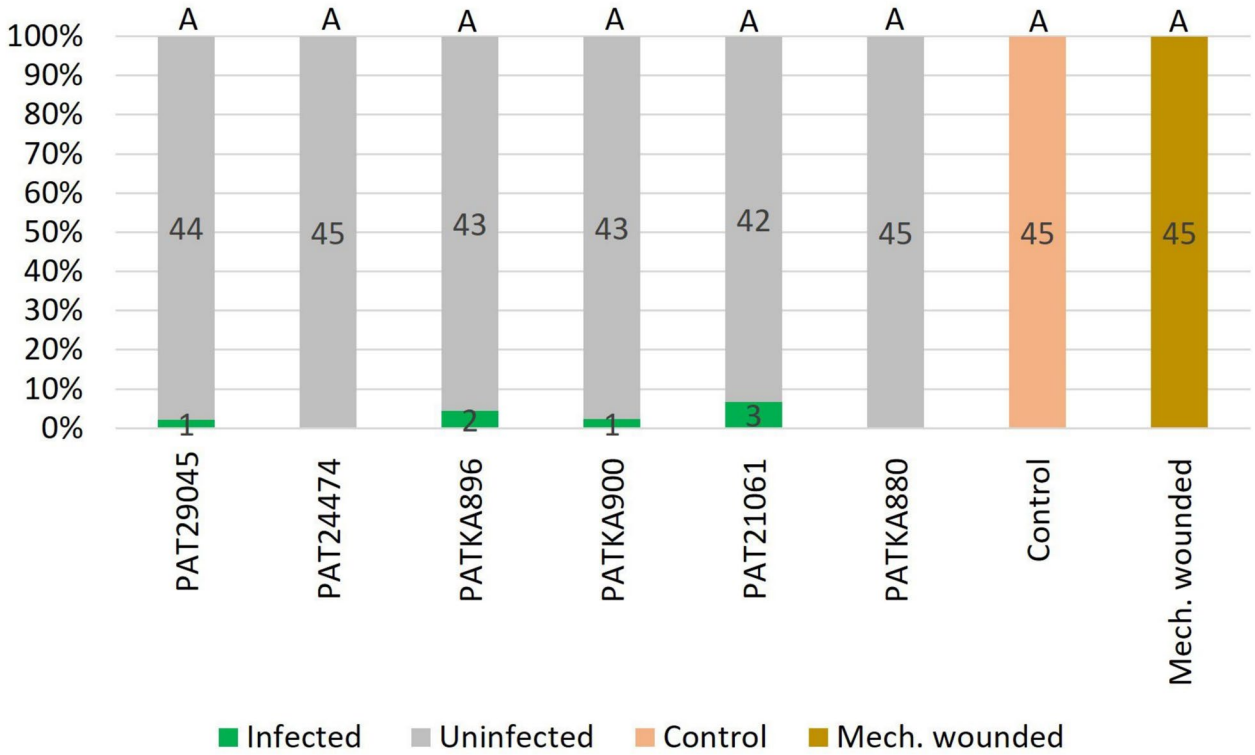


Fig. 2 Success rate of inoculation with different fungal strains, assessed by qPCR analysis of sawdust samples collected 2 years post-inoculation at the Võrumaa sites. The same capital letter at the top of the bar indicates the lack of statistical difference ($p > 0.05$). Numbers in the bars indicate infected/uninfected trees and control (uninoculated) and mechanically wounded trees

effectiveness, with only 3 (5%) trees testing positive for *I. obliquus.*

Statistical analysis indicated that strains PAT24473, PAT22865, and PAT24478 resulted in similar rates of inoculation success ($p > 0.05$). Infection rates for PAT24474 were similar to those observed in the uninoculated or mechanically wounded control groups ($p > 0.05$), suggesting limited inoculation potential or poor compatibility of this strain under field conditions.

3.3.2 Host tree susceptibility to I. obliquus infection

At Järvselja (Fig. 5), 10 (17%) out of 60 *B. pendula* trees and 7 (12%) out of 59 *B. pubescens* trees became infected. In addition, 12 (20%) out of 60 *A. incana* trees and 15 (25%) out of 60 *A. glutinosa* trees tested positive for *I. obliquus*. Infection rate was similar among the four host species ($p > 0.05$).

3.3.3 Host-strain interactions and predictors of I. obliquus inoculation success

In *B. pendula*, two fungal strain groups (A, B) with statistically significant differences between them ($p < 0.05$) were identified (Fig. 6). The first group included strains PAT24473, PAT22865, and PAT24478, while the second group comprised strains PAT24473, PAT22865, PAT24474, along with the control and mechanically wounded trees. In *B. pubescens*, inoculation success remained low, and the strains showed similar inoculation success ($p > 0.05$), indicating a low-level infection rate within this species. In *A. incana*, infection frequencies were slightly higher compared to *B. pubescens* with however similar inoculation success among strains ($p > 0.05$). *A. glutinosa* exhibited generally higher infection rates. Two groups were identified ($p < 0.05$): the first included PAT24473, PAT22865, PAT24478, and PAT24474, and the second PAT24473, PAT24478, PAT24474, as well as control and mechanically wounded trees (Fig. 6).

Throughout all host species, both control and mechanically wounded trees remained consistently uninfected with *I. obliquus*, thereby confirming the effectiveness of sterile procedures and the absence of incidental or spontaneous infections during the experiment.

To evaluate the combined influences on inoculation success, a logistic regression model was fitted using the binary infection outcome (infected vs. not infected) as the response variable (Table 5). The explanatory variables included the presence of pre-existing wood decay in the tree stem, tree diameter at breast height (DBH), fungal strain identity, and

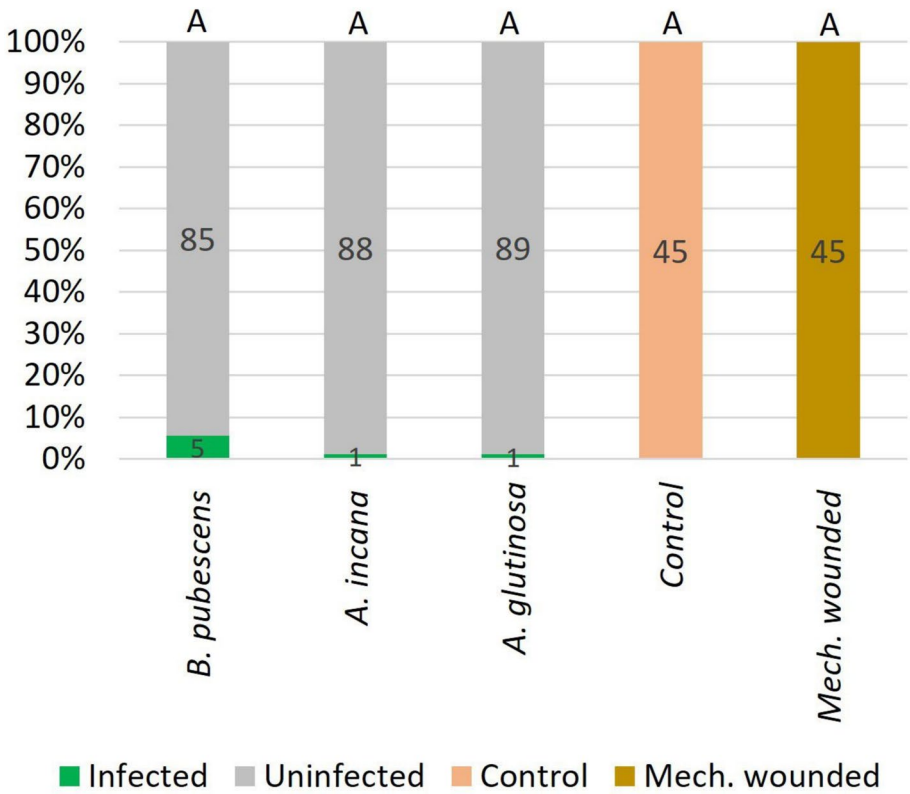


Fig. 3 Inoculation success in different tree species, assessed by qPCR analysis of sawdust samples collected 2 years post-inoculation in Võrumaa sites SR356 and AS020. The same capital letter (A) at the top of the bar indicates the lack of statistical difference ($p > 0.05$). Numbers in the bars indicate infected/uninfected trees and control (uninoculated) and mechanically wounded trees

host tree species. The model's residual deviance (227.53 on 354 degrees of freedom) and AIC (237.53) indicate an acceptable fit, with relatively low unexplained variance.

Among the tested predictors, only fungal strain identity had a statistically significant effect on infection probability (Estimate = 0.000195, $p = 0.014$). This result indicates that variation among *I. obliquus* strains plays a key role in determining inoculation success, even when accounting for differences in host species and tree condition. In contrast, pre-existing wood decay ($p = 0.453$), tree diameter ($p = 0.697$), and host species identity ($p = 0.184$) were not significant predictors, suggesting that under the conditions of this study, host-related factors had limited influence on inoculation success.

To further assess the potential of observable traits, we examined whether the presence of visual stem symptoms after inoculation could reliably indicate infection status. No statistically significant association was found between visual symptom presence and infection status determined by qPCR ($p = 0.557$). The low predictive value of these external symptoms suggests that they are unreliable indicators of *I. obliquus* colonization in the tree stem. These findings underscore the importance of molecular diagnostic tools, such as qPCR, for accurate and early detection of fungal infection in inoculated trees.

3.4 Formation of *I. obliquus* conks on *A. glutinosa*

The first conks of *I. obliquus* were observed on five *A. glutinosa* trees 4 years after artificial inoculation at Järvselja JS243 (Fig. 7), resulting in a conk formation rate of 8.3% (5 out of 60 inoculated trees). One of the inoculated trees showed three conks, another developed two, and three trees each exhibited a single conk (Table 6). The smallest conk volume was 4.5 cm^3, while the largest reached 48 cm^3. Three of these five trees had been inoculated with strain PAT22865, while the remaining two trees were inoculated with strains PAT24478 and PAT24474, respectively. Additionally, no signs of bark cracking or stem bleeding were observed in any of the conk-bearing trees, and no internal decay was detected prior to inoculation.

4 Discussion

Our study compared the inoculation success of *I. obliquus* in alder and birch trees, and to our knowledge, it was the first attempt to cultivate the fungus in alder trees. The overall inoculation success of *I. obliquus* 2 years after

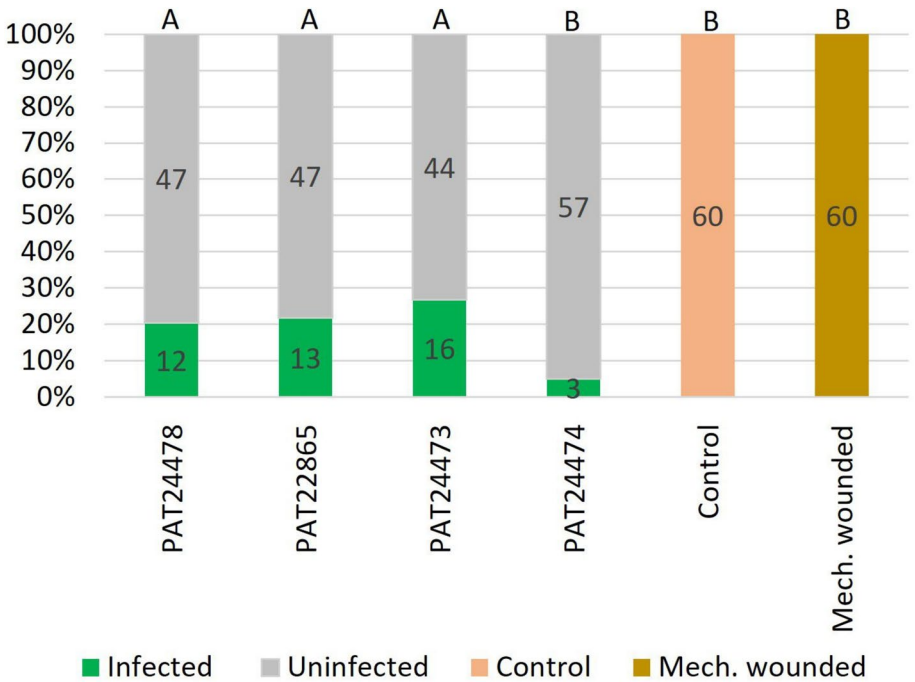


Fig. 4 Success rate of inoculation with different fungal strains, assessed by qPCR analysis of sawdust samples collected 2 years post-inoculation in Järvselja sites. The same capital letter (A–B) at the top of the bar indicates the lack of statistical difference ($p > 0.05$). Numbers in the bars indicate infected/uninfected trees and control (uninoculated) and mechanically wounded trees

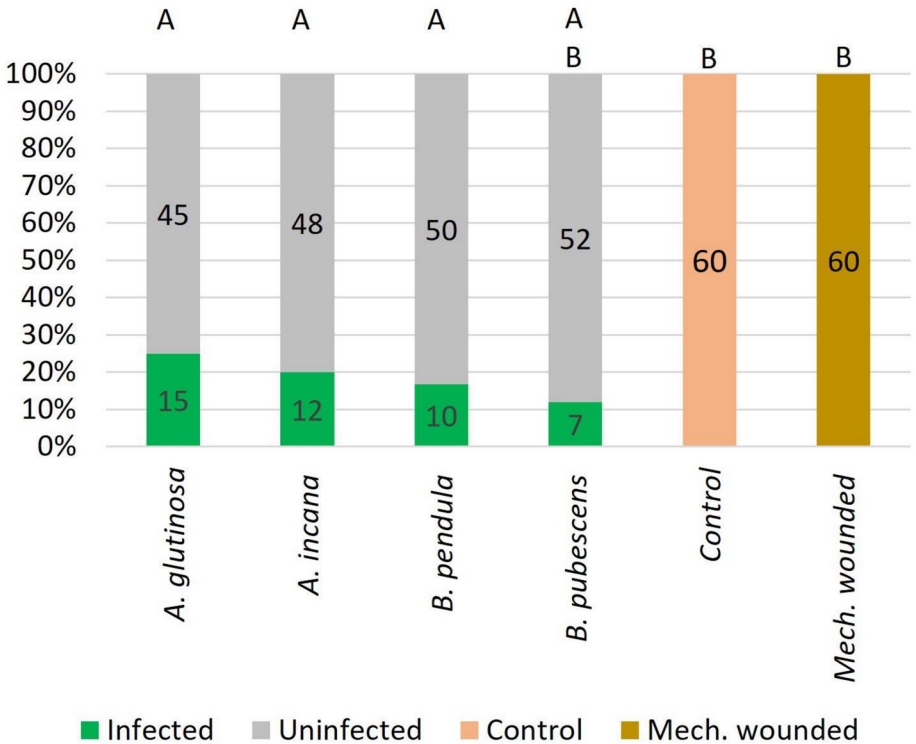


Fig. 5 Inoculation success in different tree species, assessed by qPCR analysis of sawdust samples collected 2 years post-inoculation in Järvselja sites JS243, JS109, and JS331. The same capital letter (A–B) at the top of the bar indicates the lack of statistical difference ($p > 0.05$). Numbers in the bars indicate infected/uninfected trees and control (uninoculated) and mechanically wounded trees

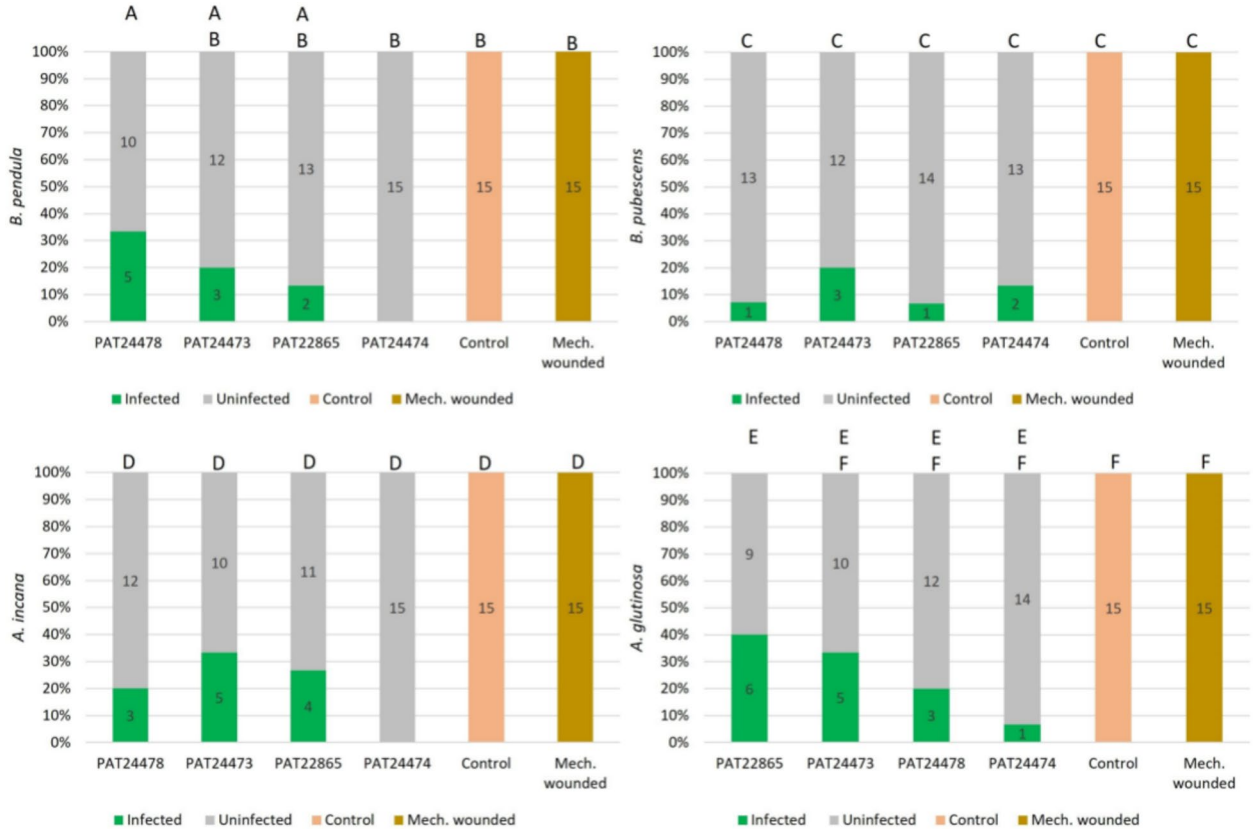


Fig. 6 Inoculation success at Järvselja sites JS243, JS109, and JS331 based on fungal strains, assessed by qPCR analysis of sawdust samples collected 2 years post-inoculation. The same capital letter (A–F) at the top of the bar indicates statistical similarity ($p > 0.05$). Numbers in the bars indicate infected/uninfected trees and control (uninoculated) and mechanically wounded trees

Table 5 Results of the logistic regression model assessing the effects of wood decay, DBH, fungal strain, and host tree species on the inoculation success of *I. obliquus*. Statistically significant predictors ($p < 0.05$) are marked in bold. The analysis included data from 359 trees across Järvselja forest sites, for which sawdust samples were collected 2 years after inoculation

Predictor	Estimate	Std. error	*P*-value
Intercept	**−6.940**	**2.030**	**<0.001**
Decayed wood	−0.400	0.530	0.453
DBH	0.020	0.040	0.697
Fungal strain	**0.00019**	**0.00008**	**0.014**
Tree species	0.210	0.160	0.184

artificial inoculation under natural forest conditions in hemiboreal Estonia was 18% at the Järvselja sites and only 3% at the Võrumaa sites. The infection rate remained low overall, and these results highlight the need for further research to optimize inoculation methods, timing, and strain selection before any practical application or large-scale use can be considered.

These results indicate a lower inoculation success rate than reported in previous studies. For example, Miina et al. (2021) reported an average inoculation success of 79% in *Betula* spp. across 24 stands, with individual stand-level success rates ranging from 53 to 100%. Our estimates may have been affected by sampling limitations: only one side of each inoculated tree stem was sampled near a single inoculation point, meaning some infections may have gone undetected. The absence of detectable *I. obliquus* DNA in these samples does not necessarily rule out infection elsewhere in the tree. Therefore, the actual inoculation rate may be higher, and future studies should consider more comprehensive sampling across all inoculated trees.

In addition to evaluating infection outcomes, we assessed the expression of external symptoms on inoculated trees. Visual indicators such as stem bleeding, bark bulging, or cracking were occasionally observed. However, these symptoms showed no correlation with molecular (qPCR) detection of the presence of the fungus. In several cases, trees that lacked any visible symptoms tested positive for *I. obliquus* DNA, while some trees



Fig. 7 Sterile conks of *I. obliquus* on four different *A. glutinosa* trees at the Järvselja site JS243. Photo: Karin Kütt, 31 October 2024

that exhibited apparent external changes tested negative. This indicates that such symptoms, while possibly reflecting general physiological responses to wounding or fungal presence, are not reliable indicators of successful inoculation. These findings reinforce concerns about relying solely on visual cues for infection assessment. For instance, Miina et al. (2021) reported a 79% inoculation success rate in birch based on external symptom observation. However, our results suggest that such rates may be overestimated when based exclusively on visual signs, especially given the lack of consistent correlation with molecular confirmation. It is possible that some of the trees classified as infected in Miina et al. (2021) were not actually colonized by *I. obliquus*, but instead showed physiological responses induced by wounding. This underscores the need for caution when interpreting external symptoms as direct evidence of infection. Therefore, we recommend that molecular diagnostics such as qPCR be routinely integrated into monitoring protocols to improve the accuracy of infection rate measurement. While visual assessments may remain useful as preliminary tools in field settings, they cannot be used as the sole method to estimate inoculation success.

Table 6 Developed *I. obliquus* conks on *A. glutinosa* trees at the Järvselja site JS243, assessed visually 4 years post-inoculation

Strain no	DBH (cm)	1 st conk Dimensions (cm)/(cm^3)	2nd conk	3rd conk
PAT24474	14	5×3×3/45		
PAT24478	24	4×3×2/24	3×3×1/9	3×3×1/9
PAT22865	19	3×3×1.5/13.5		
PAT22865	19	3×1.5×1/4.5		
PAT22865	19	5×4×2/40	4×4×3/48	

Beyond host characteristics, we also evaluated whether mechanical wounding alone could lead to natural infection. No *I. obliquus*-specific symptoms were observed on trees subjected solely to mechanical wounding, suggesting that injury alone was not sufficient for fungus inoculation under the given conditions. In our study, wounds were made on 9–10 June 2022 in Võrumaa and on 2, 3, and 6 July 2020 in Järvselja, whereas basidiospores were detected only on a few occasions later in the season—at the end of July (2019) and early September (2018) (Adamson et al. 2024). Since *I. obliquus* typically infects through frost cracks formed in winter or other injuries, and spores appear in late summer or autumn, infections may also occur via older wounds if viable spores arrive later.

Although all tested tree species exhibited some susceptibility to *I. obliquus* infection, we were unable to detect any species-related difference in inoculation success. Instead, colonization outcomes were more strongly influenced by the fungal strain. The three strains PAT24473, PAT24478, and PAT22865 resulted in similar inoculation success. In contrast, strain PATKA880 failed to cause any infection. The complete lack of colonization may be due to poor fungal viability, lack of host compatibility, or site-related inhibitory factors. Similarly, other strains such as PAT24474, PAT29045, PATKA896, and PATKA900 exhibited low inoculation success. Among these, only PAT24474 induced conk formation, and even then, only on an *A. glutinosa*. This suggests that these strains have limited practical value for cultivation purposes. Strain PAT21061 exhibited a moderate level of inoculation success, although no conks were observed from this strain either.

However, conk development was observed only on *A. glutinosa* at the Järvselja site, with the first visible conks

appearing 4 years after inoculation. Conk sizes ranged from 4.5 to 48 cm^3. In comparison, Silvan and Sarjala (2017) reported conks on *B. pubescens* within 3 years, with sizes up to 10×20 cm. Miina et al. (2021) found that 6% of *B. pendula* and *B. pubescens* developed conks within 4–5 years, averaging 54 ± 64 cm^3. Ka et al. (2017) observed 9 cm conks after 9 years on *B. platyphylla* var. *japonica*. While our findings suggest slower development on *A. glutinosa* compared to birch species, conclusions remain tentative due to data from only one site. Conks in our study formed on five trees: three inoculated with strain PAT22865, and one each with PAT24478 and PAT24474. Notably, PAT22865 and PAT24478 were also among the strains with the highest infection rates based on species-specific qPCR detection. This overlap suggests that strains with greater inoculation success may also have a higher potential to induce sterile conks under suitable conditions. These findings offer a preliminary indication that selecting such strains—particularly PAT22865 and PAT24478—could enhance practical outcomes in cultivation, although further multisite studies are needed to confirm this pattern.

Our results highlight the complex interplay between fungal strain, site conditions, and host characteristics in determining the inoculation success of *I. obliquus* under natural forest conditions. While infection rates were generally low, certain strains and forest types demonstrated higher success, emphasizing the need for tailored approaches in future artificial inoculation efforts.

5 Conclusions

This study provided novel insights into the artificial inoculation dynamics of *I. obliquus* in four broadleaved tree species under natural forest conditions in Estonia. Among the tested fungal strains, three -PAT24473, PAT24478, and PAT22865- achieved higher infection rates. Only fungal strains had a significant effect on infection probability, and not host tree species; tree diameter at breast height and pre-existing wood decay in tree stem had no effect on infection probability. Visible symptoms were unreliable indicators of fungal colonization in trees, highlighting the necessity of molecular diagnostics for inoculation success. The development of sterile conks exclusively on *A. glutinosa* 4 years after inoculation indicates that this species and the *Alnus* genus is also a promising host for *I. obliquus* production compared to birches. However, the extended period to conk emergence highlights the need for better planning in *I. obliquus* cultivation strategies. These findings contribute to a more refined understanding of host-pathogen interactions and lay essential groundwork for future optimization of *I. obliquus* cultivation practices in diverse forest environments.

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Declaration of generative AI and AI-assisted technologies in the writing process
During the preparation of this work, the authors used ChatGPT (OpenAI) to improve the clarity and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Authors' contributions
Conceptualization and methodology: R.D., K.A.; sampling: K.K., K.A., K.J., A.S., R.D.; formal and statistical analysis: K.K.; writing—original draft: K.K., K.A., R.D.; writing—review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

Data availability
The data supporting this study have been uploaded to the PlutoF platform and are publicly available under the following DOIs:
Järvselja forest sites: https://doi.org/10.15156/BIO/3301250.
Võrumaa forest sites: https://doi.org/10.15156/BIO/3301251.

Declarations

Competing interests
The authors declare no competing interests.

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