In vitro optimisation of Swedish chaga (*Inonotus obliquus*)

The path to chaga cultivation in Sweden

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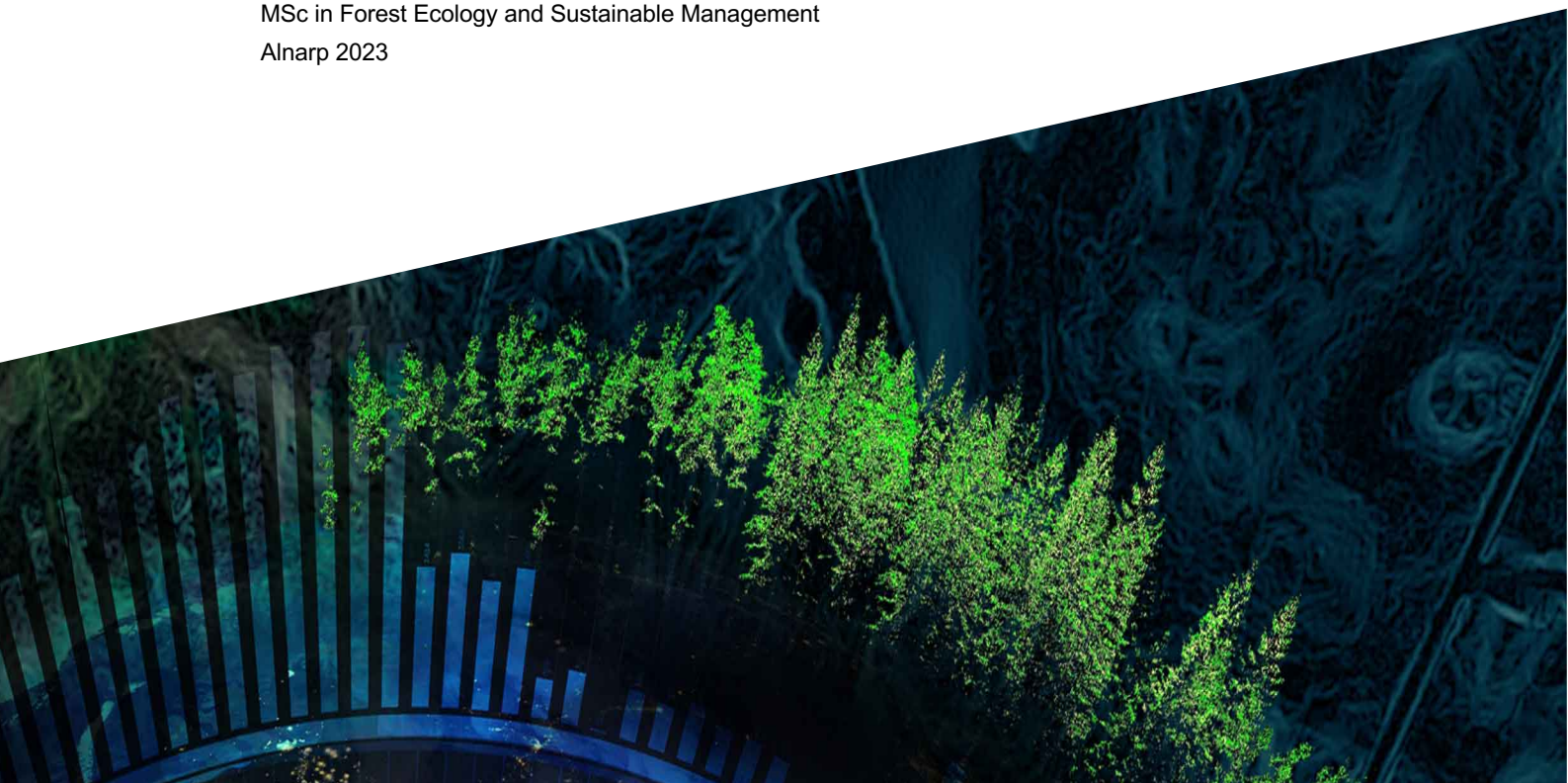
Master's Thesis • 30 credits
Swedish University of Agricultural Sciences, SLU
Southern Swedish Forest Research Centre
MSc in Forest Ecology and Sustainable Management
Alnarp 2023



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Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Master's thesis in Forest Science
Course code:	EX0984
Programme/education:	MSc in Forest Ecology and Sustainable Management
Course coordinating dept:	Southern Swedish Forest Research Centre
Place of publication:	Alnarp
Year of publication:	2023

Keywords:	chaga, *Inototus obliquu*s, *in vitro* optimisation, sustainable chaga production

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Abstract

Chaga has become more popular in recent years as a beneficial natural supplement and wellness tea. Understanding chaga source limitations and its harvest dynamics as well as it's potential revenue streams would be important for future chaga growers in Sweden. Under natural conditions, the fungus *Inonotus obliquus* takes many years to establish and develop sterile conks that are then able to be harvested. Cultivating the fungus in forests for profitability therefore would be best optimized by choosing isolates that can establish more quickly. In this study, the growth of *I. obliquus* was analysed from 10 samples collected across a climatic gradient in Sweden, on three different nutrient media and at three different temperatures, in order to select superior isolates that can be used for cultivation and under optimal conditions for mass inoculum production. The results showed significantly higher mycelium growth on yeast agar (YA) and potato dextrose agar (PDA), compared to birch agar (BA). No significant difference was found in growth rates between samples originating from the North (64° North) and the South (56° North) of Sweden. Highest growth rate occurred at 16°C, followed by 20°C and finally 26°C and this was consistent across all three media tested. In addition to the in vitro growth trials, the degree of pigmentation was assessed in each culture, as pigmentation is commonly an indicator of the presence of melanins which are known bioactive secondary metabolites. Results showed that the fungus growth on PDA and YA exhibited more pigmentation than BA. Interestingly the isolate showing highest growth (S4) showed least pigmentation, and conversely the isolate showing least growth (S3) produced the highest number of highly pigmented petri plates. This may indicate a potential trade-off between primary and secondary metabolite production and ought to be investigated in more detail in future growth optimisation experiments. From the results we recommend future *in vitro* cultivation of chaga *Inonotus obliquus* in YA at least as low as 16°C. Further *in vitro* optimisation experiments could investigate lower temperatures to find the optimal growing temperature.

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Abbreviations

BA	Birch agar
DNA	Deoxyribonucleic acid
HTS	High-throughput sequencing
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
YA	Yeast extract agar

1. Introduction

1.1 Morphology and Pathophysiology

Chaga, *Inonotus obliquus,* is described as a phytoparasitic, white-rot fungi that infects living trees of the *Betulaceae* and *Populaceae* genus and can cause or at least contribute to host tree mortality (Lee *et al,* 2008, Piętka, J., & Grzywacz, A, 2013).

Until the tree dies, successfully colonised fungal mycelium aggregates and erupts into its anamorphic (asexual) state; a slerotium, or sterile conk which is a composite material containing both woody material and fungal mycelium (Fig.1) (Miina *et al* 2021, Lee *et al,* 2008). It is these structures that are harvested and processed into traditional medicinal preparations which has grown in popularity recently (Szychowski, 2021, Tridge, 2021).

It has a strong traditional use amongst the indigenous Ugric Khanty peoples with various medicinal applications (Saar, 1991), as well as by the Saami in Fennoscandia (Svanberg, 2018). Chaga is currently experiencing a revival of this traditional use in complementary and herbal medicine, leading to increased consumption and demand (Pilz, 2004, Tridge, 2021, Globenewswire, 2023)).

Figure 1. Chaga sclerotium on a birch tree showing the outer melanised, coal-like layer. A crack exposes the orangey cork layer within. (Photo: Čekanavičius, 2009)

Figure 2. A chaga piece showing the protective outer black melanised coal-like structure (left), with the inner orange cork layer shown (right), containing the mycelium of Inonotus obliquus. (Photos courtesy of Linda Lindström, 2021)

The mycelium which is present in the corky lower layer (Fig. 2, right) establishes itself in its host, digesting lignin as well as perhaps preferentially hemicellulose in the heartwood (Beltrame *et al,* 2022), while mostly avoiding the sapwood (Fig. 3) (Lee *et al,* 2008, Blanchette, 1982).

Infection on host trees usually occurs through a wound in the bark, which is also the site of conk formation (Blanchette, 1982). Once the fungus infects the host, it stimulates a defense response, with both biochemical and anatomical responses to the injury, including compartmentalization. The fungus can sometimes evade this host response, which results in a sclerotium being formed 3-5 years post-infection (Lee *et al,* 2008, Blanchette, 1982, Miina *et al*, 2021).

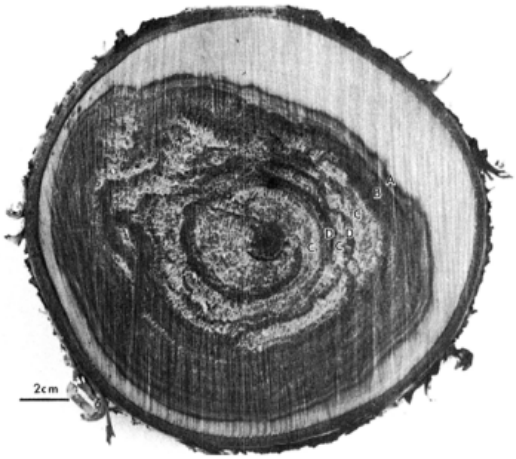

Figure 3. A cross-section of a stem disc showing discoloration from colonisation of mainly heartwood by I. obliquus. Sapwood is left mostly intact, except in the region around the conk and infection site (see line adjoining cross section). Image from Blanchette, 1982.

These sterile sclerotia are perennial meaning that their growth is additive by up to 14 mm annually and it does not produce sexual reproductive structures such as basidiospores until the tree, or at least a part of the tree, dies (Lee, 2008). After that, the lifestyle of this parasitic fungus can switch to its sexual state and resupinate basidiospores are formed, and sporulation can commence (Fig. 4). Its spores are at an upwardly oblique angle (hence the name *I. obliquus*), of around 20-30°, with spore dispersal occurring via wind and insects (Lee *et al,* 2008).

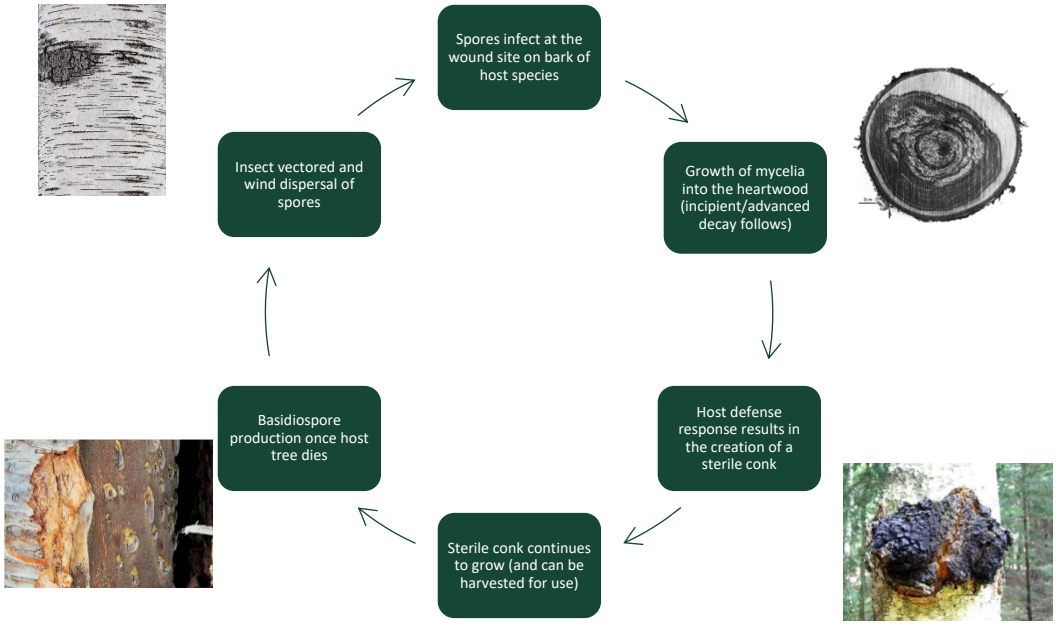

Figure 4. Lifecylce of I. obliquus (Lee et al, 2008, Blanchette, 1982), Images from Čekanavičius, 2009, Blanchette, 1982 and Niemala et al, 1995)

1.2 Distribution of *I. obliquus*

Chaga has a circumpolar distribution that encompasses the boreal region Scandinavia and Russia, temperate regions of North America and Eastern Europe (Fig. 5). Several different tree species can serve as a host for the fungus, among the most common belonging to the genus *Betula*, *Acer*, and *Quercus* (Global Biodiversity Information Facility, 2022, Thomas et al, 2021, Lee *et al*, 2008). The vast majority, around 90%, of host tree species falls within the *Betula* genus (Mycology Collections Data Portal, 2022, Thomas et al, 2021).

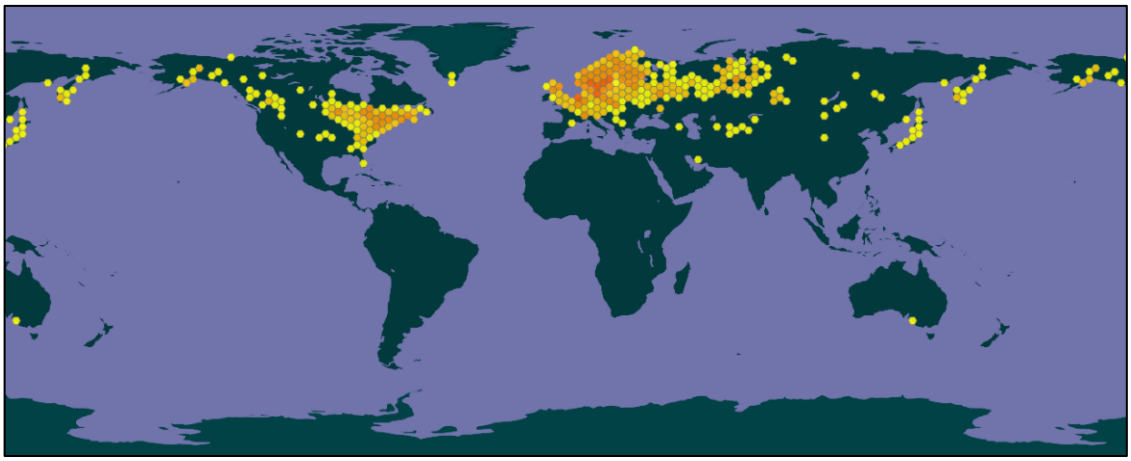

Figure 5: The circumporal distribution in mainly boreal and temperate forest ecosystems of Chaga from 7,516 geotagged records from the Global Biodiversity Information Facility

1.3 Medicinal Constituents

Chaga is popular for having bioactive medicinal compounds (Stamets, 2005, Géry *et al,* 2018), which are soluble in water (Wold *et al,* 2020), and therefore liable to effective extraction as a 'tea' or hot water-based extraction. The most widely used traditional method of consumption is producing a chaga tea or coffee, as well as alcoholic tinctures which would also capture the lipophilic fractions (Géry *et al,* 2018, Pilz 2004, Lu *et al,* 2021, Szychowski *et al,* 2021).

Among the known compounds are polysaccharides (primarily beta glucans), phenolic compounds, triterpenoids and melanins (Kim *et al,* 2015), where the medicinal effects of these compounds are numerous (Kim *et al,* 2008; Peng and Shahidi, 2020; Sun *et al* 2014).

The high value of chaga in connection to its health benefits has sparked the interest in the market for chaga production, which could be seen potentially as another revenue stream for forest owners in Sweden, an opportunity to diversify income and contribute to increasing ecological value to managed forests. However, there are some concerns that the supply from wild harvested sources may not be able to adequately meet the growing demands for it. Efforts to optimize cultivation of chaga have not, to the author's knowledge, been made in Sweden, but have in other countries such as Finland and Estonia, (Miina *et al,* 2021, ChagaHealth, 2022, MarketWatch, 2022, Thomas *et al,* 2020, IUCN Global Fungi Red List Initiative, 2020).

1.3.1 Melanins

There are many anti-oxidant melanin compounds, including allomelanins responsible for the dark pigmentation of the chaga outer crust (Mattoon, 2021). Functionally it has been described as 'fungal armour' since it is able to protect the fungal mycelium from a variety of externally damaging agents. This is known to promote survival and enhance virulence for wood-decay fungi responsible for wood spalting such as *Kretzchmaria deusta* and *Trametes versicolor*. The latter *T. versicolor* is also considered a medicinal fungus (Morris *et al,* 2021, Gómez & Nosanchuk, 2003, Benson *et al*, 2019).

This pigmentation can be visualized when the fungus grows in solid media *in vitro*. Melanin is a relevant and important medicinal constituent of chaga with potent immunomodulatory properties (Wold *et al,* 2020), that can be screened for using *in vitro* laboratory tests.

1.4 Nutraceutical Current Market

It is the medicinal properties of chaga that give it its market value justified by the growing level of medical research and literature establishing its health benefits (Kim *et al,* 2008, Lu *et al,* 2021). Chaga is predominantly harvested from the wild (Vanhanen *et al,* 2014, Pilz, 2004)) and there are limitations to wild sourcing due to the slow growing nature of chaga and access rights, impacting the supply chain and, therefore, responsible for it's high market value. The cultivation of other medicial species such as shiitake (*Lentinula edodes*), matsutake (*Tricholoma matsutake*), reishi (*Ganoderma lucidum*), lion's mane (*Hericium erinaceus*) and turkey tail (*Trametes versicolor*) demonstrate the market adapting to limitation of wild-sourced medicinal mushrooms (Venturella *et al,* 2021).

Currently the market exports of medicinal and gourmet mushrooms (excluding *Agaricus spp*.) are dominated by China with values worth US$ 260M exported in 2021. Europe is the primary importer. In 2021, Germany, France, Italy and the UK imported around US$ 350M (Tridge, 2021). This shows that the market demand for medicinal and edible fungi in Europe is high and could potentially benefit from more locally sourced Chaga from Northern Europe rather than via imports. For chaga specifically, the current market value globally is estimated at US$ 28.2 billion and is expected to grow to US$ 87 billion by 2034 (Globenewswire, 2023).

Identifying fast growing strains of *I. obliquus* that could be used for cultivation would allow some stability and predictability to the supply chain and provide some assurance to forest owners interested to diversify their forestry operations and income, especially on sites that produce low quality timber. Chaga production by artificial inoculation allows some control over the supply chain. It will also allow forest owners the knowledge of when they will be able to harvest and get a return

on their investment, rather than if they were to just rely on wild-harvested Chaga alone. Increasing the supply can help meet the increasing demands and affect market economies (ChagaHealth, 2020, Brydon Williams *et al,* 2021).

Cultivating chaga rather than wild harvesting does also come with concerns. For example, growers would be introducing a pathogenic fungus to a forest that might presumably be healthy otherwise. This could cause an issue if inadvertent infection is undesired, for example if a particular use of the birch stem is required. Another concern is in reducing the genetic diversity of chaga morphotypes if only one or two strains are used for inoculating trees. A third is the understudied ecological, logistic and economic implications for a changing of land/forestry use and how chaga inoculation could interfere with current forestry uses, or whether it could be added to existing regimes. All of these concerns require further analysis and longer term follow up studies (Miina *et al,* 2021, Moyers *et al*, 2017, Pilz, 2004).

1.5 Previous cultivation attempts

Cultivation attempts have been made, however not always successfully. Unsuccessful attempts have been made, as a conservation measure, whereby artificial inoculation of trees was conducted to prevent the establishment of other pathogenic organisms (Piętka and Grzywacz, 2006). The inoculation was attempted on dead trees however and the trees were colonized by other more common saprotrophic organisms such a *Fomes fomentarius* and *Trametes versicolor*, which gave evidence that successful inoculation of *I. obliquus* is a function of obligate relationship with a living host (Piętka and Grzywacz, 2006, Lee *et al*, 2008).

Figure 6: Chaga sclerotium erupting from a Betula platyphylla var.japonica at Gyeonggido, South Korea after inoculation. (Park et al , 2010)

However Park *et al,* (2010) successfully inoculated *I. obliquus* on *B. platyphylla* var. *japonica* in South Korea (Fig. 6). Successful inoculation was also reported in *B. pendula* in Finland where conk formation was 4% (30 in 679 trees) but with a higher infection rate (62%), characterised by stem bleeding (Miina *et al,* 2021). This demonstrates multiple bottlenecks for the cultivation process which require

more in-depth study, including mechanisms by which *I. obliquus* mycelium successfully overcome host tree defences (Blanchette, 1982).

1.5.1 Cultivation of chaga in vitro

Understanding optimal conditions for growth of chaga *in vitro* is the first stage of chaga cultivation. Some developments in this area have already been achieved, demonstrating the optimal conditions required for *in vitro* cultivation of, for example, South Korean strains.

Other medicinal wood-decaying fungi that have both a boreal and temperate distribution include *Pleurotus ostreatus*, *Trametes versicolor and Ganoderma lucidum* (Global Biodiversity Information Facility, 2022, Benson *et al,* 2019, Venturella *et al*, 2021). Hoa and Wang, (2015) found that the optimal growing conditions for *Pleurotus ostreatus* is at 28°C on various media when tested in a temperature trial ranging from 16°C to 36°C. Mycelial growth was higher for *P. ostreatus* grown on PDA (potato dextrose agar) and YDA (yeast dextrose agar) than other media, including MEA -malt extract agar. This was likely due to there being more nutr[i]ients available for fungal growth (Hoa and Wang, 2015). *Trametes versicolor* has been shown to have optimal growth on MEA at 30°C when tested in a temperature trial ranging from 15°C- 40°C (Sagar *et al,* 2020). Reishi, (*Ganoderma lucidum),* has an optimum growth *in vitro* between 28-32°C, and peaking at 30°C, on PDB (potato dextrose broth) when tested across a temperature range of 9°C-32°C (Subedi *et al,* 2021).

The literature on the *in vitro* optimization of Chaga is rather sparse, especially in the English language while research is available in the Chinese and Russian languages. Chang et al. (2001) showed that optimal growth of *I. obliquus in* vitro was 30°C, however other authors in South Korea have found the optimal range to be between 25-29°C (Shin, 2001), and therefore conducted their growth experiments at 26°C (Cho and Shin, 2005).

Chang et al. (2001) showed that mycelium growth and density of *I. obliquus* was highest on birch dextrose agar (BDA) compared to potato dextrose agar (PDA), yeast agar (YA), and malt extract agar (MEA). Better growth on BDA may be attributable to the fact that BDA may contain compounds which the fungi are adapted to and have co-evolved with. It has been shown that fungal steroids in *I. obliquus* are stimulated to be synthesised by aqueous extracts of birch bark which would contain polysaccharide, polyphenol and triterpene fractions (Wang *et al*, 2014).

Other studies from South Korea found that PDA was the most favourable for growth, followed by MEA and Czapek-Dox media (Cho and Shin, 2005) and this was confirmed by Russian studies in which PDA was again shown as the best media compared with dextrose-peptone agar and oatmeal broth agar, for maximum daily

growth rate. In the same trial, an onset of pigmentation occurred after 8 days (Sysoeva *et al,* 2020).

The objective of this research is to identify the relationship, if any, between the latitudinal origin of Chaga conks and their growth rate and pigmentation *in vitro*. In this study we compared the growth rate of multiple *I.obliquus* isolates collected from the north and south of Sweden to see if there is an obseravble difference in growth rate due to the climatic range the chaga is from.

2. Research aims and hypothesis

Research Questions:

1. Is growth rate of *I. obliquus* mycelium affected by the temperature, agar type or latitudinal origin of samples?
2. Is pigmentation of *I. obliquus* mycelia affected by the temperature, agar type or latitudinal origin of samples?
3. Is there a relationship between growth rate and pigmentation?

Hypotheses:

In relation to research objectives it is hypothesised that, when tested under different temperatures, southern isolates of *I. obliquus,* compared to northern isolates will have a higher;

1. Growth rate (H1)
2. Biomass (H2)

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In relation to research objectives it is hypothesised that, when tested under different media, isolates of *I. obliquus* grown on BA and YA, compared to PDA, will have a higher;

3. Growth rate (H4)
4. Biomass (H5)
5. Degree of pigmentation (H6)

In relation to research objectives it is hypothesised that, when tested under different temperatures, isolates of *I. obliquus* grown 26°C compared to 16°C, 20°C, will have a higher;

6. Growth rate (H7)
7. Biomass (H8)

3. Materials and Methods

Research Steps:

In order to assess these research questions, the following steps were taken:

1. Collect sterile conks at different latitudes across Sweden (North, South);
2. Obtain individual isolates using culturing techniques;
3. To identify which isolates are growing best at particular temperatures and therefore could be 'prescribed' for a particular latitude in Sweden;
4. To test growth of *I. obliquus* on different types of media and temperature for optimisation *in vitro.*;
5. To assess pigmentation by melanin of selected individuals across all treatments; media, temperature and latitudinal origin.

3.1 Materials

3.1.1 Field work and sampling

Samples of *I. obliquus* were collected from symptomatic trees in the northern and southern Sweden (Fig. 7) by cutting off the growing conks from trees of *Betula spp.* (approximately 40-80-years-old host tree). The location of trees growing in different forest types is shown in Table 1. Five samples were collected from the north and five from the south for a total of 10 conk samples. All samples were visually insecpted to verify the presenece of two distinct layers, an inner mycelial and outer, melanised crust layer before being sent to the Forest Pathology laboratory in Alnarp for further analyses.

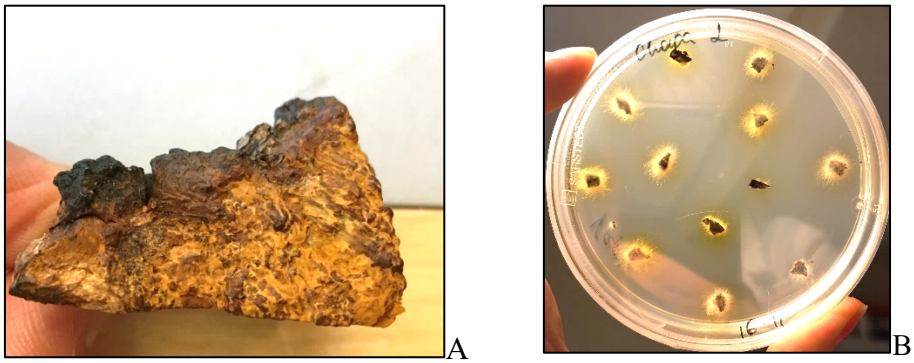
Figure 7. Google Map of Inonotus obliquus sampling points throughout Sweden. The five samples from the North (63.8 °N) are shown in blue and the five from the South (55.6 ° N), shown in red.

Table 1. Samples harvesting locations and presence of host trees. Approximate age of trees is given as is the forest type hosting them.

Sample	***Location***	***Forest Type***	***Host Tree***	***Tree Age***
N1	Brannland, Umea	Individual old birch	*Betula pubescens*	80+ years
N2	Gammlia, Umea	Pine-Birch mixed forest	*Betula pubescens*	40-80 years
N3	Langvattnet, Storuman	Spruce-Birch mixed forest	*Betula pubescens*	50+ years
N4	Storuman	Spruce- Birch mixed forest	*Betula pubescens*	100+ years
N5	Grotjaur, Storuman	Pine dominated plantation	*Betula pubescens*	60 years
S1	Hackeberga	Beech- Oak dominated forest	*Betula pendula*	*Not assessed*
S2	Hackeberga	Beech- Oak dominated forest	*Betula pendula*	*Not assessed*
S3	Hackeberga	Beech- Oak dominated forest	*Betula pendula*	*Not assessed*
S4	Hackeberga	Beech- Oak dominated forest	*Betula pendula*	*Not assessed*
S5	Hackeberga	Beech- Oak dominated forest	*Betula pendula*	*Not assessed*

3.1.2 Isolation of *I. obliquus*

All sampled conks of *I. obliquus* were sterilized with 97% ethanol using a spray bottle, and dried on sterile paper in a laminar flow hood before the isolation process to avoid contamination between samples and exposing inner layers of conks. The inner mycelium was cut aseptically into 3x3 mm pieces, and plated onto 9 cm Petri dishes (5-7 plates per conk) containing PDA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) under sterile conditions in the laminar flow hood and then incubated at room temperature, in dark conditions for 3 weeks (Fig. 8).



A B

Figure 8. Isolation process of I. obliquus: A - two distinct layers - a melanised outer layer, observable from the outside of the tree, and an inner cork layer, containing culturable mycelium within a matrix of woody material before plating; B – chaga hyphae developing after one week of incubation on PDA media

Petri dishes were examined regularly under an OLYMPUS BX45 microscope (magnification 15-20x) to observe hyphae development (Fig. 8B). When growth was observed, small agar plugs of mycelium at the colony margin were then transferred onto new PDA media to obtain pure cultures where they were maintained. Isolates were grouped according to sampled locations, and then 5 representative samples of each conk were selected to identify species through DNA sequencing.

3.1.3 Molecular identification of *I. obliquus* isolates

DNA extraction. Fungal mycelia of 20 isolates (2 isolates selected per conk) was obtained from liquid media cultures and were separated from their agar plugs with sterile tweezers, as pr the biomass protocol. The fungal mass of each was placed in 2 mL centrifuge tubes, frozen and homogenized to a fine powder using a Rescht MM400 ball mill (Retsch, Haan, Germany). Mycelial DNA was extracted using the EZNA Plant DNA Kit (Omega Bio-tek) according to the manufacturer's

instructions. The concentration of the DNA extracts was measured using NanoDrop® ND1000 (Wilmington, USA).

Amplification of the rDNA ITS region and Sanger sequencing. The region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) of the ribosomal DNA was amplified by PCR using the primer ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990, Gardes and Bruns 1993).

PCR was performed in 25 µL reaction volume containing 12.5 µL of DreamTaqPCR Master Mix (2X) (Thermo Fisher Scientific), 1 µL of each primer ITS1 and ITS4 and 8.5 µL of sterile distilled water and 2µL of 2 ng/µL template DNA (25 µL total). The PCR amplification conditions were: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 1 min at 95 °C, 45 s at 55 °C, and 1.5 min at 72 °C, with a final 5 min at 72 °C.

The presence of fungal DNA in samples was confirmed with gel electrophoresis in which DNA fragments of different lengths migrate under the influence of an electric field. Subsequently, they can be properly distinguished in comparison with the known length of DNA fragments from the DNA marker (Lee *et al*. 2012). The resulting PCR products were sent to Macrogen (Amsterdam, Netherlands) for purification and sequencing in both directions by automated Sanger sequencing using the same primers.

Sequence analysis and identification. Molecular Evolutionary Genetic Analysis (MEGA - X) was used to trim the low quality ends and to align forward and reverse DNA sequences into consensus sequences. The Basic Local Alignment Search Tool (BLAST) was used for comparing obtained sequences to available data at the National Center for Biotechnology Information (NCBI) database (Geer *et al,* 2010).

3.2 *In vitro* experiments

Three week old cultures of the 50 isolates (5 individuals per conk) from the north and south of Sweden were used for the temperature-controlled experiment on solid media (section 3.2.1) as well as biomass experiment in liquid media (section 3.2.2).

3.2.1 Solid culture experiment

Three types of media for agar culture, PDA (potato-dextrose agar), BA (birch agar) and YA (yeast agar), were tested for the 50 Swedish *I. obliquus* isolates (5 per each of the 10 individual conks) at three different temperature conditions (+16ºC, +21-22ºC, +26-28ºC). These three different temperatures are representative of the north-south average summer temperatures in Sweden; 16°C (Kiruna), Ambient 20-21°C (Umea) and 24°C (Stockholm/Malmo) (Climate-Data.org, 2022).

For each medium and temperature, the growth of *I. obliquus* was observed and measured using a graduated ruler, measuring in four directions, and an average calculated. This was performed every 7 days for 21 days (450 plates were measured in total).

The schematic design of solid culture experiment is shown below in Fig. 9.

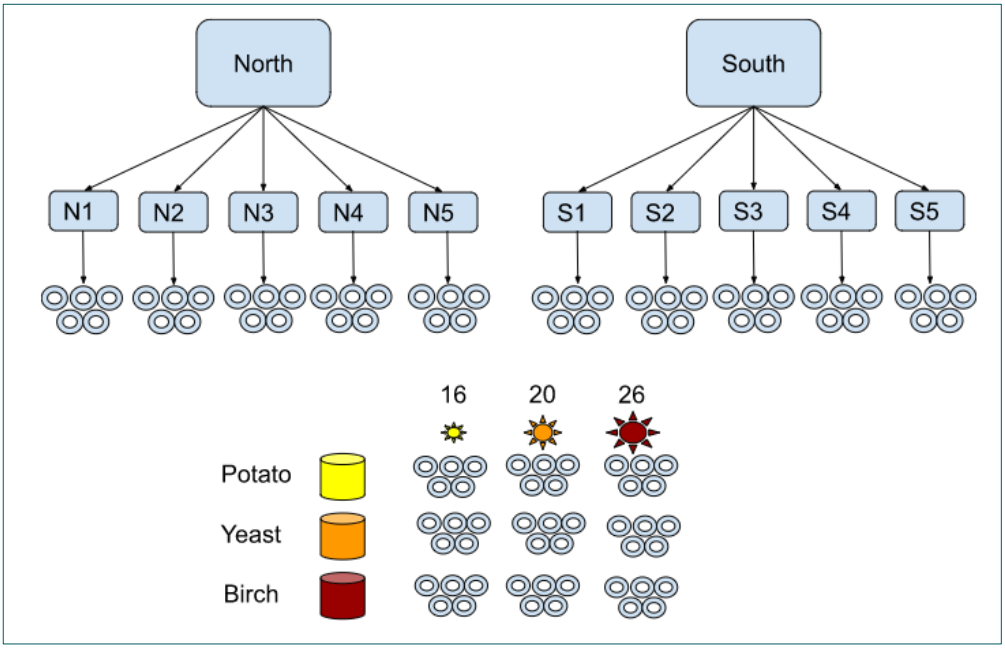


Figure 9. Schematic showing the solid culture experimental design. Note that the media and temperature treatments occur with every isolate. There are 5 replicates per isolate, therefore 25 plates for each isolate from the north and the south, giving 50 in each treatment group, and 450 plates overall.

PDA was prepared in the same way as for fungal isolation (see subchapter 3.1.2). The YA consisted 30 g of dextrose (Sigma-Aldrich), 5 g of yeast extract (Sigma-Aldrich), 1 g KH_2PO_4, 0.5 g $MgSO_4$ (Sigma-Aldrich) and 15 g of agar powder. The BA was made by combining 25 g of birch bark strips and 25 g of birch heartwood (2 cm lengths), obtained from *B. pendula*, 20 g of agar, 20 g of D-glucose and 1 L of distilled water (Sysoeva *et al,* 2020). All media were autoclaved at 121°C for 20 mins and poured at approximately 20 mL/plate.

Plates in the 16°C treatment were incubated in a digital incubator INCU-Line® (VWR International Ltd, England). Plates in the temperature treatment +21-22ºC were kept on the bench in the laboratory room and were subject to the temperature daily variations. Plates in the 26°C treatment were placed in the SLU greenhouse in plastic boxes lined with Vaseline to prevent mite infestation (Song *et al,* 2018, Chang, 2001).

Some plates became contaminated with other fungal or bacterial organisms and were discarded, however all isolates have at least 3 replicates (n=3) with the vast majority having 5 replicates (n=5) (See appendix, Table 6).

In solid media experiments, 5 mm inoculum plugs excised by a sterilised cork borer were taken from the edge of 3-week-old colonies grown on PDA plates and placed in the centre of the plates. This was done with the three types of media and incubated at selected temperatures over one month (28 days) to give growth plates (Fig. 10). The plates were visually analysed and the growth rate was measured radially in four directions every week and then an average growth for that whole period was calculated.

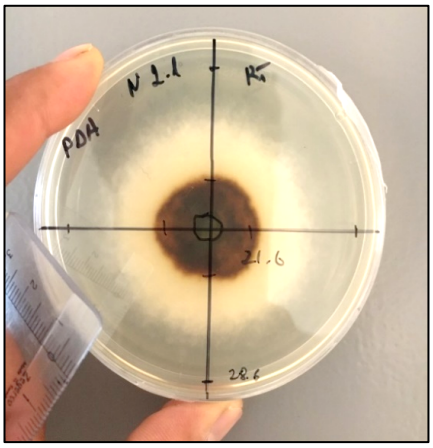


Figure 10. An example of a chaga isolate growth in a plate experiment. The markings denote: the agar type PDA - potato dextrose agar; RT – room temperature 20°C, sample number N 2.1 – North, sample 2, replicate 1. An average of measurements in four directions was taken.

The degree of mycelium pigmentation was determined visually as either low, medium or high pigmentation based on the approximate proportion of the plate that was pigmented (Low: ≤ 25%, Medium: 25 – 75%, High: ≥ 75%) (Fig. 11).

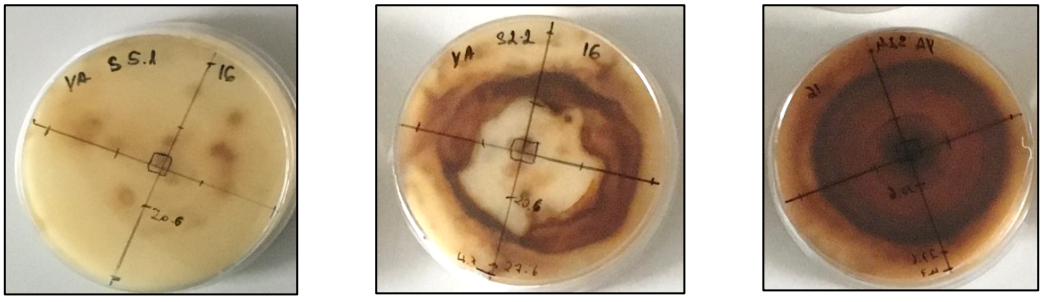


Figure 11. Low (left), medium (centre) and high (right) classifications of mycelium pigmentation

3.2.2 Liquid Culture Experiment

The same three types of media (PDA, YA and BA) were used for the liquid culture experiment to determine the mycelial biomass growth. All media were prepared in the same way as for solid culture experiment but without adding agar. For each liquid medium, 30 mL aliquots were delivered into 50 mL falcon tubes.

The schematic design of liquid culture experiment is shown below (Fig. 12).

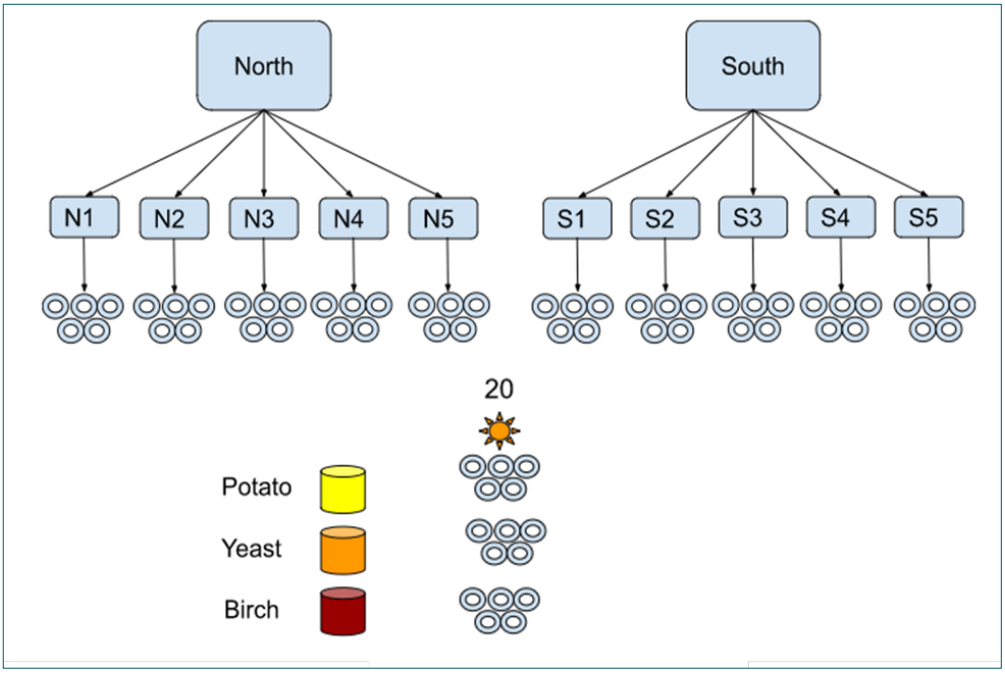


Figure 12. Schematic showing the liquid culture experimental design. Note that there is no temperature treatment for the liquid culture, all samples were at room temperature.

In the liquid media experiment, *circa* 1 cm^2 inoculum plugs were prepared using a sterilize cork borer taken from the edges of 3-week-old colonies, grown on PDA plates. These were put into the falcon tubes and incubated on the orbital shaker (IKA® KS 130 Basic, IKA®-Werke GmbH & Co. KG, Germany) at room temperature for a month. After one month of incubation, residues of agar were removed and the mycelia were separated from the agar plug with sterile tweezers in the laminar flow hood. Mycelia were transferred to preweighed 2 mL Eppendorf tubes and lyophilized for 24 hours (Scanvac CoolSafe 55-9 PRO 4lt), and measured on an analytical balance (Analitik OHAUS PA 224C) to obtain the dry weights.

3.3 Statistical Analysis

All statistical tests were conducted in R (R Core Team 2018). Statistical tests were considered significant at an $\alpha \leq 0.05$ level.

Data on the average final colony diameter (mm) and dry biomass (mg) after 3 weeks, was confirmed for their normal distribution using density plotting. The effects of media, temperature and origin of cultures on the average final colony diameter (mm) after 3 weeks were assessed with linear regression using lm function. A three way analysis was performed to this end. The interaction of media and origin and temperature and origin were included in the model to test if the effect of media and temperature on the response variable was consistent across origin.

Linear regression was also used to assess the differences in dry biomass between media and origin, including their interactions.

Ordinal logistic regression using polr function from MASS package was used to analyse the effects of media, temperature and origin of isolates on the pigmentation of the isolates.

A three way ordinal logistic regression using MASS package was performed but not included due to rank deficiency which would result in misleading results.

When models were significant, Tukey's honestly significance difference test (HSD) was used to determine which significantly different groups was done using function lsmeans from lsmeans package. The same package was used to calculate model predictions. Graphic visualisations were produced with ggplot function from ggplot2 package.

4. Results

4.1 Identification *I. obliquus* isolates

It total, 269 plates were plated on media for fungal isolation from 10 sampled *I. obliquus* sclerotium conks, from southern and northern parts of Sweden, which yielded 288 isolates.

The sequenced ITS regions of the 20 selected isolates had 98-100% match to *I. obliquus* sequences in the NCBI database (Table 2). Only one isolate (S2.2) was sequenced with 98% similarity to GenBank closest assession number, the rest were 99-100% matches.

Table 2. Identification of obtained chaga isolates from sampled sclerotium conks

No.	Isolate number	Identified taxon	Closest GenBank Accession No	Similarity %
1	N1.2	*I. obliquus*	OK586158.1	99
2	N2.3	*I. obliquus*	KY949235.1	100
3	N3.5	*I. obliquus*	OK586158.1	99
4	N4.1	*I. obliquus*	OK586158.1	100
5	N5.4	*I. obliquus*	GU903006.1	100
6	N1.2	*I. obliquus*	DQ103883.1	99
7	N2.4	*I. obliquus*	OK586158.1	99
8	N3.4	*I. obliquus*	KY949235.1	99
9	N4.1	*I. obliquus*	OK586158.1	100
10	N4.2	*I. obliquus*	OK586158.1	99
11	S1.2	*I. obliquus*	KY949235.1	99
12	S2.2	*I. obliquus*	OK586158.1	98
13	S3.2	*I. obliquus*	KY949235.1	99
14	S3.4	*I. obliquus*	KY949235.1	99
15	S5.4	*I. obliquus*	OK586158.1	99
16	S1.1	*I. obliquus*	KY949235.1	100
17	S1.2	*I. obliquus*	KY949235.1	100
18	S1.3	*I. obliquus*	KY949235.1	100
19	S3.2	*I. obliquus*	KY949235.1	99
20	S5.2	*I. obliquus*	OK586158.1	100

4.2 Solid Media Growth of *I. obliquus*

Mycelial growth measurements are the average colony diameter at 3 weeks. Size on the Y axis refers to final average radius of colony after 3 weeks in mm.

A three way model was employed to look at the effecst of orign, media and temperature on radial growth, as shown below (Fig. 13). No significant interaction occurred between media and temperature (p=0.90), nor between media and origin (p=0.15), nor between origin, media and temperature (p=0.88). (See appendix Table 3).

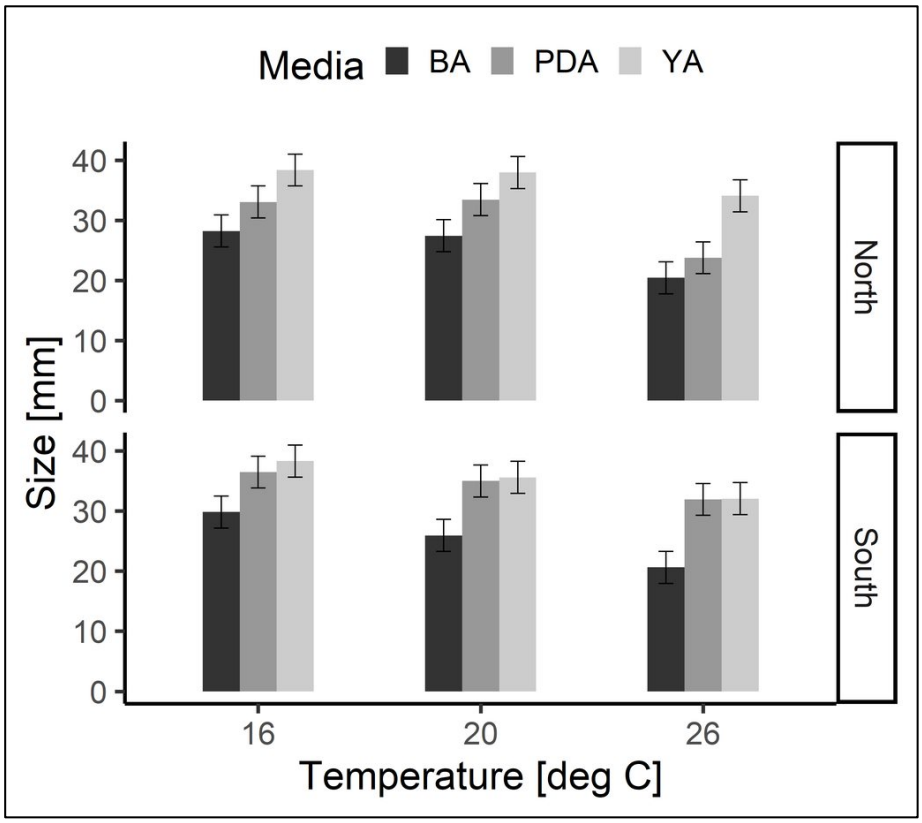
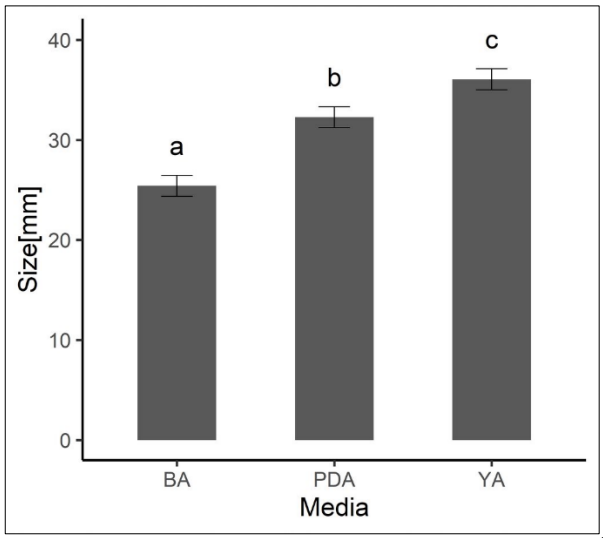
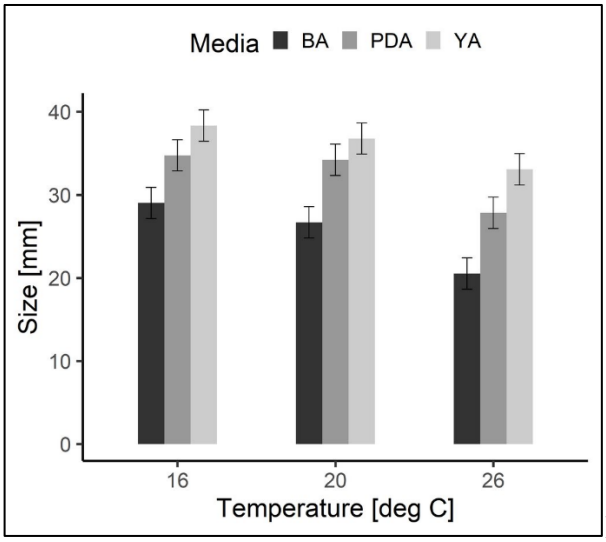


Figure 13. Final average plate growth (mm) showing isolates from both North and South, in every media; YA, PDA and BA, across all temperature treatments. Size on the Y axis refers to final average radius of colony after 4 weeks in mm.

Media type

Statistically significant differences were found between all of the media types, with YA having the fastest growth, followed by PDA and then BA, which showed the least average radial growth after three weeks ($p<0.0001$, $df=2$) (Fig.14A).


40
30
20
10
0
Size[mm]
a
b
c
BA
PDA
YA
Media
A


Media
BA
PDA
YA
40
30
20
10
0
Size [mm]
16
20
26
Temperature [deg C]
B

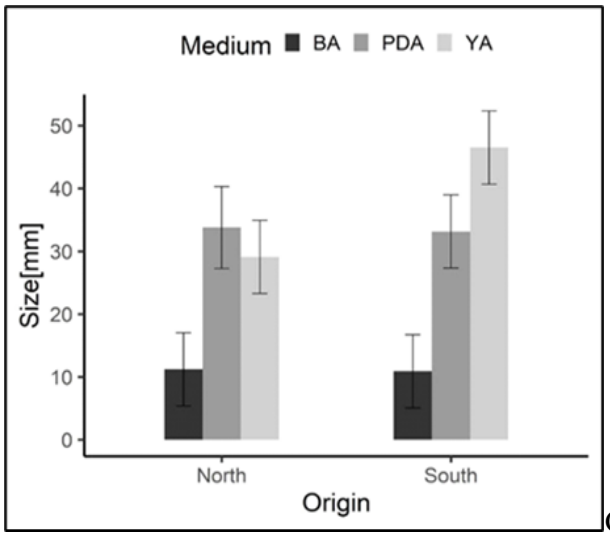
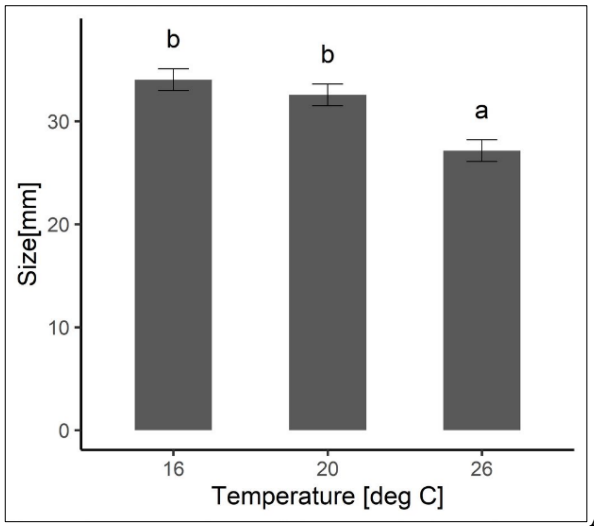

Medium
BA
PDA
YA
50
40
30
20
10
0
Size[mm]
North
South
Origin
C

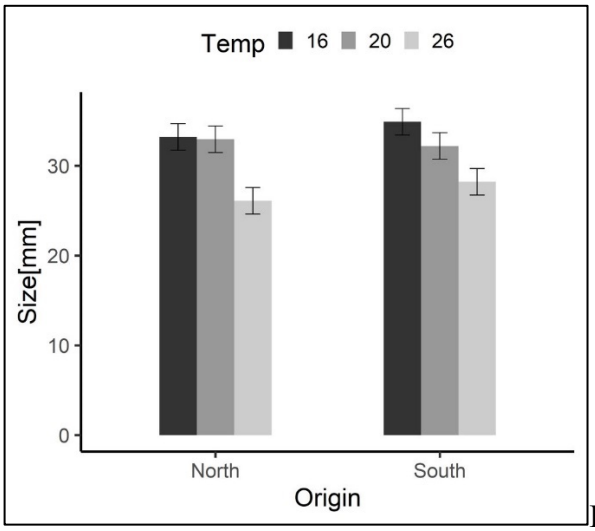
Figure 14. A: Bar graphs showing the average radial growth of I. obliquus after 21 days, in plates of different solid media, birch agar (BA), potato dextrose agar (PDA) and yeast agar (YA) . B: showing the effect of media by temperature treatments for radial growth. C showing the differences in media growth amongst isolates from the north and south. Of note is the higher radial growth seen in YA in southern isolates. Statistical lettering determined by Tukey's HSD post hoc test. (p<0.001). Size on the Y axis refers to final average radius of colony after 4 weeks in mm.

Temperature

A statistically significant difference, ($p<0.0001$, $df= 2$), between temperatures was found, whereby an inverse relationship between temperature and growth rate exists over the temperature range tested. As we decrease the temperature, the greater the growth, in general. 16°C was not statistically different to the plates at 20°C but they were both faster growing than samples at 26°C (Fig. 15A). No interaction was found between origin and temperature ($p=0.60$)



A



B

Figure 15. Bar graphs showing the average radial growth of I. obliquus after 4 weeks at the three tested temperatures (16, 20 and 26°C). B shows the effect of temperature within the origin treatment. Statistical lettering, determined by Tukey's HSD post hoc test. Size on the Y axis refers to final average radius of colony after 3 weeks in mm.

Origin

No statistical difference was found between growth of isolates as a function of their origin, *i.e.* whether samples were from the north or south (p=0.42, df=1) (Fig. 16), since they grew at similar rates of radial growth after 3 weeks, and therefore we cannot make any inference of climatic gradient influencing the growth of chaga in solid culture experiments.

No interaction was found between origin and media (p=0.15), nor origin and temperature (p=0.58).

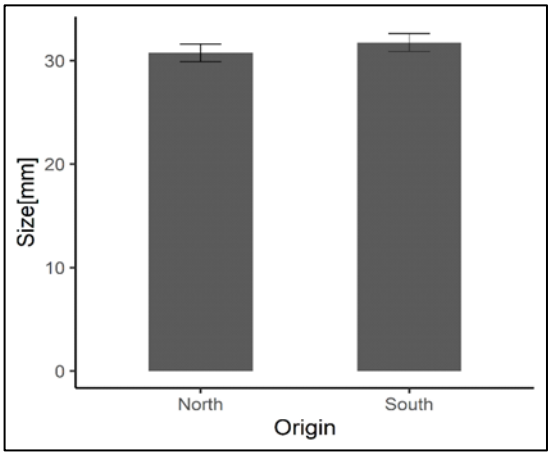


Figure 16. Mycelial growth of northern (64° N) and southern (56° N) isolates with no significant difference found between samples grown at these two different latitudes (p>0.404). No significant interactions occur between other variables tested, (See appendixm table 2).

Growth curves showing the radial growth of mycelium over the three week growth period is shown below (Fig. 17) and shows the growth dybnamics of the mycelium in the different media and at different temperatures. It can be noted that the highest growth can be seen at lower temperatures and the lowest growth can be seen in all BA media treatments.

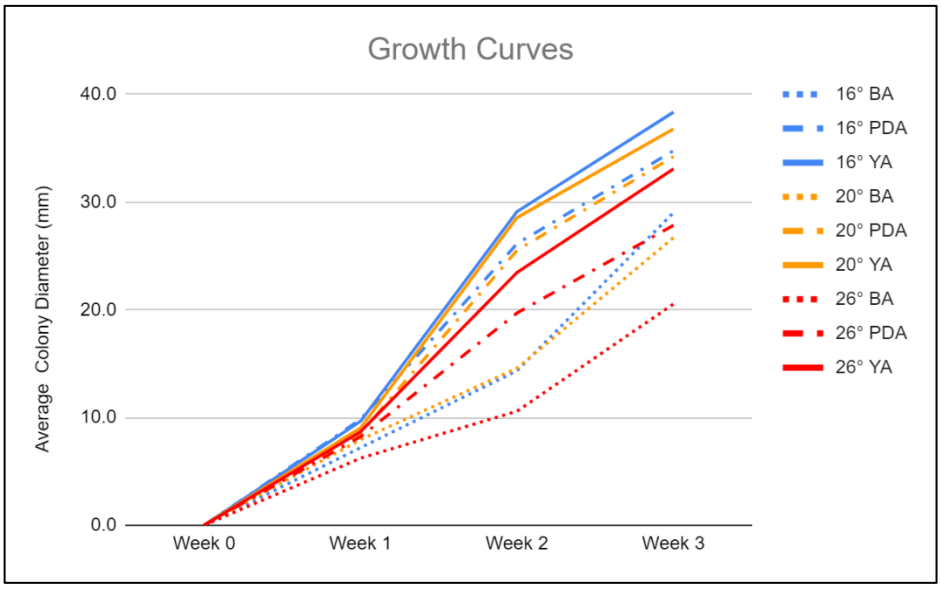


Figure 17. Growth curves showing growth of mycelium (mm) on solid media over 3 weeks. All temperatures and all media are shown. Measurement are averages in 4 directions of each replicate. Data points are averages of all isolates values. Data is not split by origin due to the lack of significance of origin on growth rate.

A comparison of how isolates performed in solid agar is shown below (Fig. 18), whereby the highest radial growth after three weeks (mm) is seen in South 4, South 5 and then North 1 isolate.

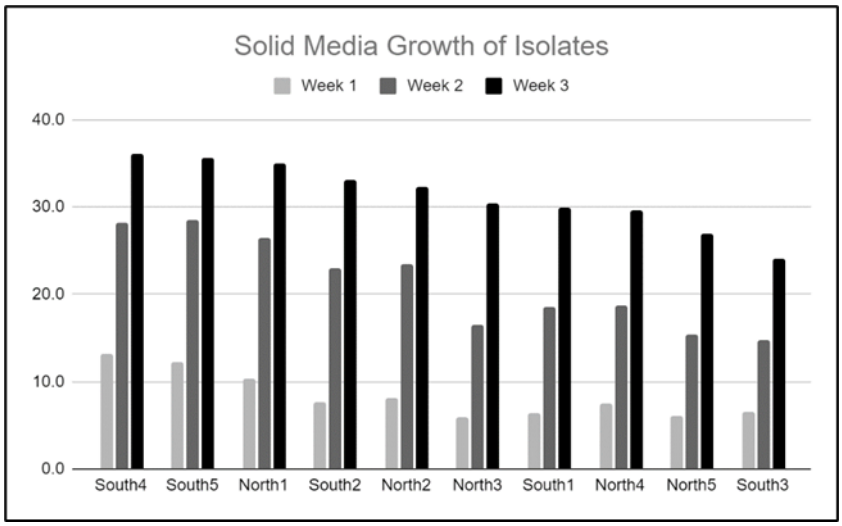


Figure 18. Solid media growth by isolates where each data point is an average of replicates. Y axis is average radial growth in mm at 3 weeks.

Birch Agar

Considering that birch agar media can be the closest conditions that resemble the live *Betula* host, then it is of interest to note which isolates grow best in the media; South 4, followed by South 5 and North 1 were the three isolates that showed the highest end-point growth on solid birch agar (Fig. 19).

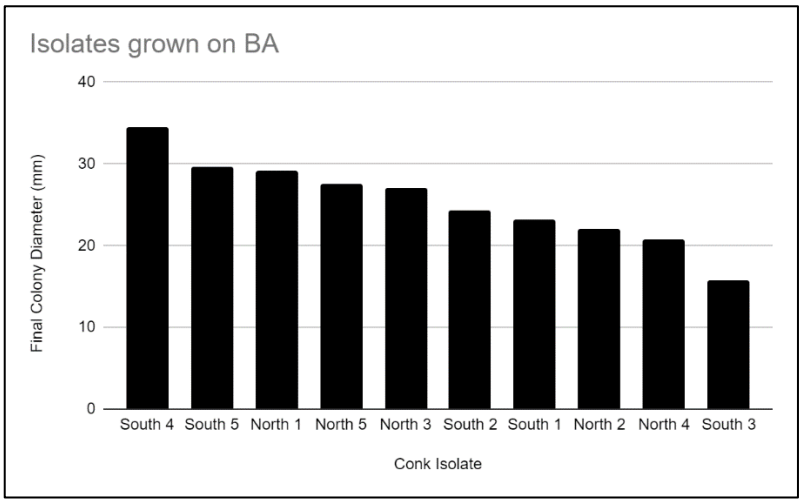


Figure 19. Growth on solid BA for all conks. Ranking is by final average radial growth (mm) at week 3.Isolate replicates have been averaged and used as data points here.

4.3 Pigmentation assessment of *I.* obliquus on solid media

The media that promoted the highest proportion of *high* pigmentation plates was; YA (58%)> PDA (43%) > BA (9%) ($p<0.0001$) (Fig. 20). There was however an interaction between the media and origin of the isolate ($p<0.0001$). (See appendix table 5).

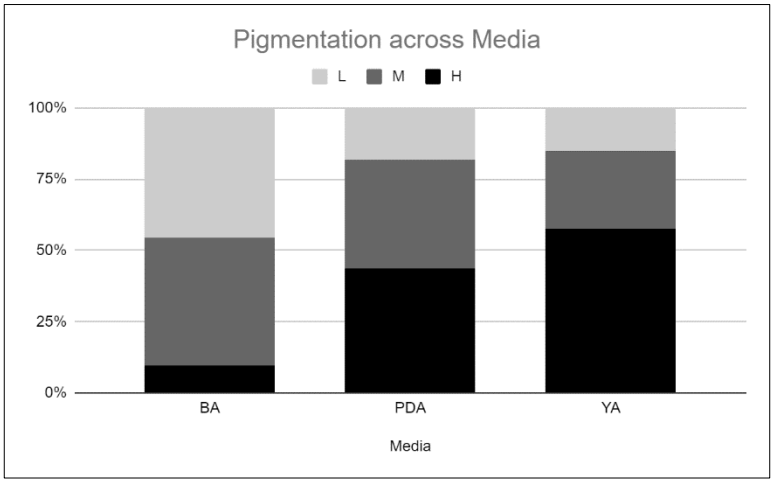


Figure 20. Proportion of Low (L), Medium (M) and High (H) pigmentation of plates observed across the three tested media ($p<0.0001$). Interaction of media with origin was significant, although not hypothesised ($p<0.0001$).

Temperature

There was not any statistical difference between the temperature treatment and pigmentation of plates ($p=0.317$) (Fig. 21).

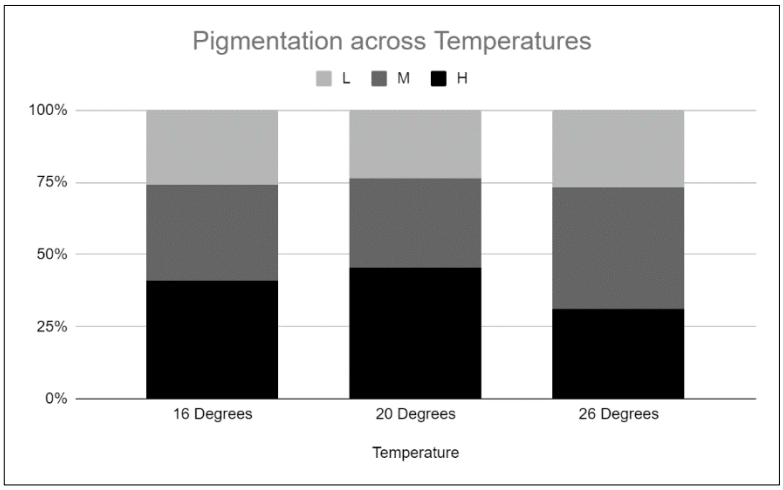


Figure 21. Proportion of Low (L), Medium (M) and High (H) pigmentation of plates observed across the three tested temperatures

Origin.

There was not a statistically significant difference between the pigmentation of those plates growing samples from the North and those from the South ($p=0.059$), although ,as mentioned earlier, an interaction between media and origin exists ($p<0.0001$) (Fig. 22).

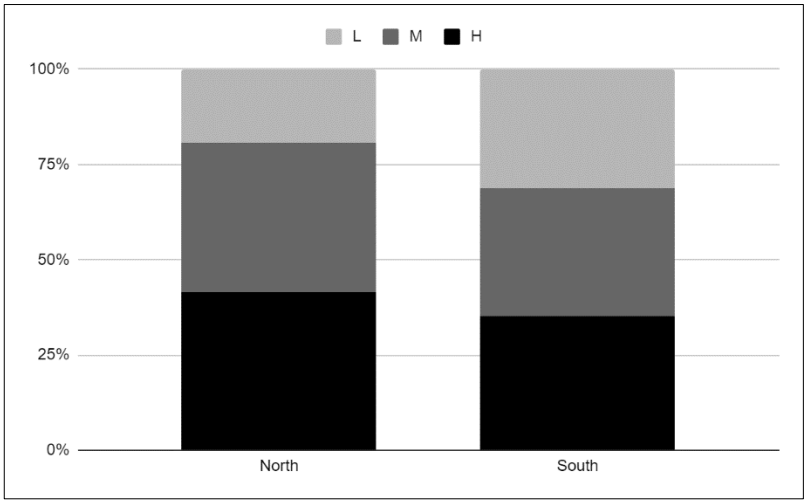


Figure 22. Proportion of Low (L), Medium (M) and High (H) pigmentation of plates observed for Northern and Southern isolates

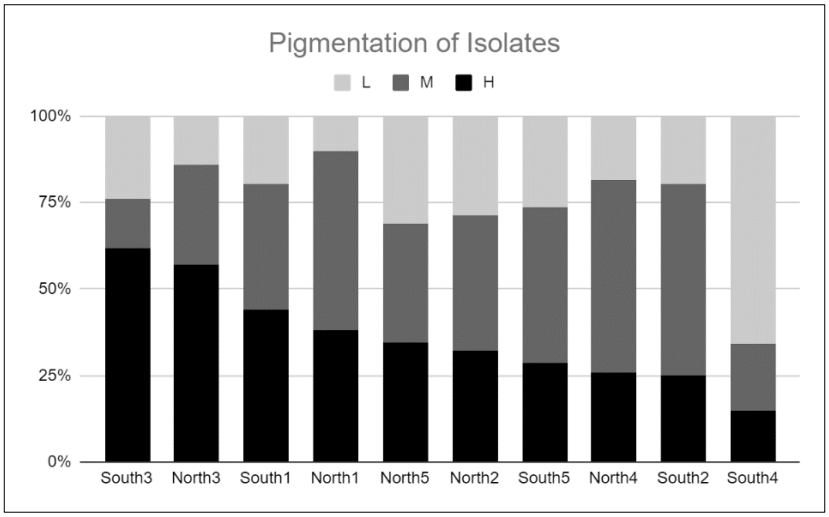


Figure 23. Proportion of plates graded Low (L), Medium (M) and High (H) for each isolate, ordered by the proportion of High (H) graded plates.

The S3 isolate had the most High (H) pigmented plates (62%) whereas the S4 isolate had the lowest number of H plates (15%) (Fig.23).

A Spearman's rank correlation coefficient was calculated for the growth rate on solid media and the pigmentation (High ranking plates) to give a value of -0.59. This value is considered non-significant negative correlation, considering the degrees of freedom (df=8).

4.4 Biomass assessment of *I. obliquus* in liquid media

Media

In biomass measurements, the highest growth after four weeks in liquid media was observed in YA > PDA > BA. There was a significant difference in growth between YA and PDA as compared to BA ($p<0.001$) (Fig. 24).

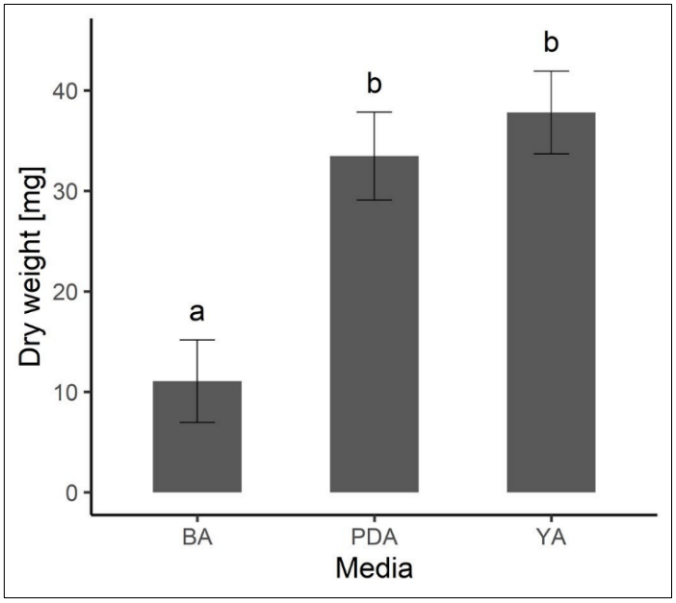
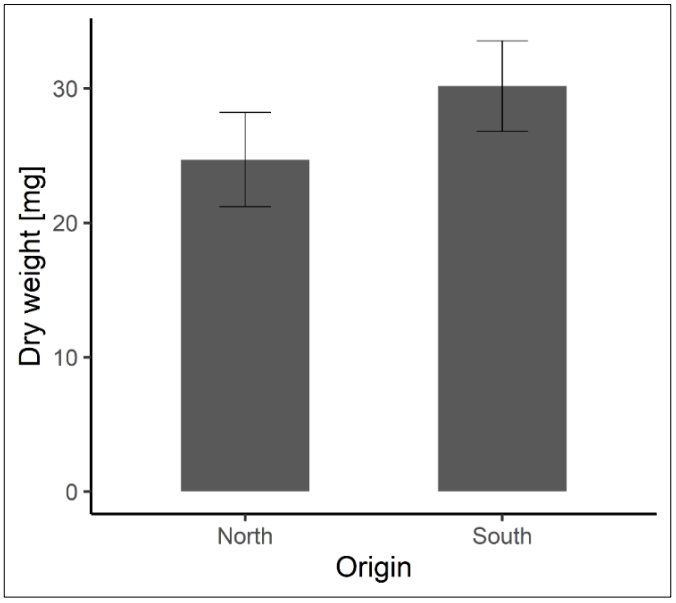


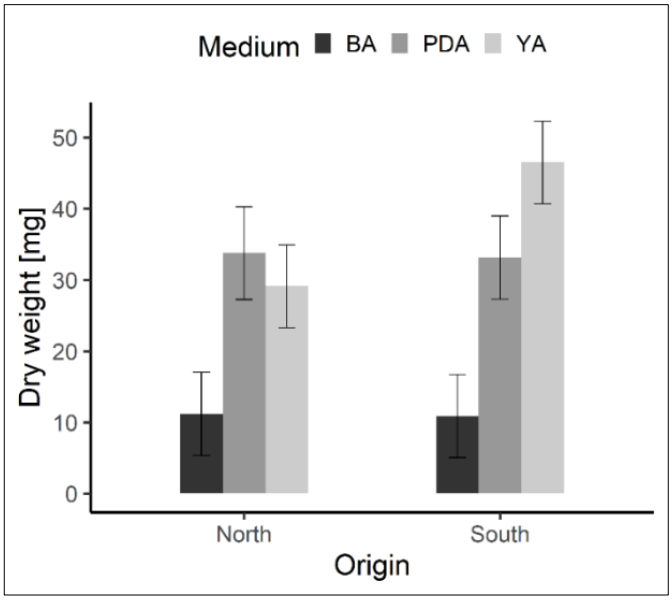
Figure 24. Graph showing Biomass growth was highest in; YA = PDA > BA with a statistical significance showing higher growth of YA and PDA over BA ($p<0.0001$).

Origin

Although growth in the Southern samples was observed to be higher, this was mostly due to YA growths (Fig. 25A) and not consistent over the various treatments (Fig.25B), hence a non-statistical difference overall was observed ($p=0.25$).



A



B

Figure 25. A: bar graphs showing the main effect of isolate origin on biomass. There was not a statistically significant difference observed between them (p=0.25) B: Graph showing biomass growth by North and South, and the media treatment associated with them. The highest growth values were observed in South YA treatments

Although samples from the south have been collected from a geospatially smaller area than those from the north, the variation in growth as measured by biomass, between samples in the south is much greater than those from the north, as shown below (Fig. 26).

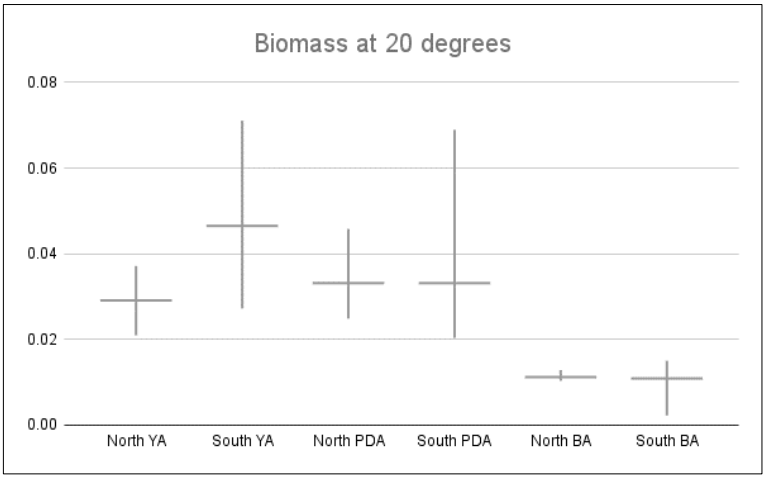


Figure 26. Biomass growth measured by dry weight (mg). Averages and ranges shown within replicates for isolates. Y axis is dry weight (mg).

BA media encouraged the least growth, whereas the PDA and YA were the better media for *I. obliquus* growth *in vitro* ($p<0.001$, $df=2$) with the southern samples having a much larger variation in growth rate.

5. Discussion

Solid media

There was no significant difference in growth rate dependent upon the latitudinal origin of the samples in this study, and therefore no climatic insights can be drawn on the growth of chaga *in vitro*. Other meteorological and climatic variables can come into play when attempting to establish such a hypothesis, for example light, concentrations of CO_2, number of frost days etc. that could affect the growth rate of the chaga fungus in the field, dependently or independently of its host, and these could be modelled and tested in future research. Larger scale studies could be conducted to confirm this trend and it would be interesting to look more at local climate adaptations in this species.

The highest growth on solid media was observed on YA samples, and it is this media type that can be suggested for use in further *in vitro* cultivation of chaga on solid media. There are many variables that need to be considered when translating these results to *in vivo* growth of *I. obliquus* in it's *Betula* host, and it does not necessarily follow that strains that grow fastest on YA *in vitro* will grow the fastest in the host tree in the field. It may be more valuable to know which isolate grew fastest on BA, even if that was the poorest performing substrate, since it is the media that is potentially closest to the field conditions. The conk S4, followed by S5, was the conk that showed the highest average radial growth at week 3 on solid BA (See Graph. 24). It could be argued that it is these conk strains that are taken forward into the field, even though growth on BA was the lowest of other tested media *in vitro.*

At every temperature, there was no statistically significant difference between YA and PDA. This high growth in the YA was observed in other papers such as Chang *et al,* 2001 and is likely due to the presence of certain compounds in the YA, such as B vitamins,that encouraged metabolic processes and growth, as has been reported in the literature (Cho and Shin, 2005). There was no significant difference due to latitudinal origin of the samples.

At each temperature treatment, isolates growing on BA had the least radial growth. BA was observed and reported in previous *in vitro* culturing of *I. obliquus*, to result in the highest growth (Chang *et al,* 2001). Our contradictory result and is likely due to the differences in preparing BA;, our method does not exclude tannins.

The preparation of BA in future experiments should take into consideration the potential for fungal inhibition by tannins.

Tannins are present at different concentrations in different species but in birch bark they are found at approximately 1.6% weight of crude bark (Holonec, 2012), and can exert a concentration-dependent fungal inhibitory effect (Zhu *et al,* 2019). Aqueous extracts of birch bark reported in Wang *et al* (2014) have been used. As a water extract, which would be assumed also to contain tannins and other polyphenolic compounds, it still demonstrated increased *I. obliquus* mycelial growth and steroid production (Wang *et al,* 2014).

In every media, there was an inverse relationship between temperature and growth rate, which was counter to our temperature hypothesis (H1). The north isolates at 16°C, were a little slower growing than the same samples at 20°C, but faster growing than those at 26°C and show perhaps a lag effect, where these samples are perhaps growing at an optimal rate after the 3 weeks which our experiment was limited to. Isolates grown on BA and YA from the north at 16°C seem to 'catch up' to their southern counterparts and did actually end at a higher overall growth than the other higher temperatures in the same media (Graph.). This supports the hypothesis that *I. obliquus* is adapted to grow at cooler temperatures. The optimal temperature for growth *in vitro* could not however be elucidated since we did not test growth at temperatures lower than 16°C.

The samples at 26°C were much slower growing and therefore would not be encouraged at *in vitro* optimisation experiments in the future. There was no difference in growth at 16°C and 20°C and therefore we can recommend future growth experiments at this temperature, however, this is rather different to the reported optimal temperature by Chang, (2001), who reported an optimal growth around 30°C. This may be due to different isolates having different growth dynamics and optimal growth temperatures due to local adaptations. It also is a lower optimal temperature than with the other parasitic medicinal species that can grow in this climatic range such as *Trametes versicolor, Pleurotus ostreatus* and *Lentinula edodes*. These species all grew better at higher temperature ranges even though they are also adapted to grow at cooler Boreal and temperate climates (Benson *et al,* 2019, Hoa and Wang, 2015, Subedi *et al,* 2021). This could be due to better local adaptation by chaga in these regions or an inaccuracy in results analysis due to inadequate temperature control.

Unfortunately, due to equipment constraints, the plates may not have been been maintained at the desired temperature, and no temperature log was kept to ensure that they were. Plates at 16°C were kept in an incubator without adequate cooling function, although in theory, it was kept at 16°C (notes on digital display), in all likelihood, they could have been at a higher temperature. Plates for the 26°C treatment arm were kept in the greenhouse, which reached higher temperatures than 26°C, and would have undoubtedly affected the plates. They were however kept in

opaque, sealed boxes, so the effects of humidity and light in the greenhouse would have been minimal. All in all, this makes the results less reliable and warrants a repeating of the experiment.

The samples reach the edge of the Petri dish and therefore the final week 3 values used are unreliable in representing a growth rate. 40 data points were like this and were used anyway. It would have been an option to use values from previous weeks as a cut off, but growth dynamics seen in growth curves showed that the growth rate accelerated in many plates after week 2 which would then also not be captured if we used an earlier week cut-off point. This posed a limitation in the interpretation of the results. In future, larger plates can be used, which reduced the chances of the edge being reached by week 3.

Pigmentation

There was an interaction between the media and origin of the isolates ($p<0.0001$), perhaps due to limiting nutrient content amongst locally adapted strains (See appendix table 5).

From the author's understanding of fungal pigmentation and literature on *I. obliquus* pigmentation, it has been assumed that pigmentation in our samples is from presence of melanins (Mattoon, 2021).

From visual pigmentation analysis there is a qualitative aspect where melanin, a phenolic compound, has marked anti-oxidant properties, demonstrating clinical activity in cancer cell lines and in metabolic conditions such as diabetes mellitus (Lu *et al,* 2021, Lee *et al,* 2014).

Interestingly the sample S4 (south #4) recorded the highest growth over both solid and liquid growth experiments, while also having the lowest amount of pigmentation. Conversely, sample S3 (south #3) performed poorly on growth experiments, however was consistently highly pigmented. This indicates some trade off with regards to growth and pigmentation, whereby energy used for primary metabolite production in growth of mycelium is potentially prioritised over secondary metabolite production. However, the Sperarmans ranked correlation coefficient value of -0.59 shows a close, but non-significant negative correlation (-0.7 would have been considered significant). This value warrants a larger scale comparison from more conks, their *in vitro* growth rates, and pigmentation, (or other secondary metabolite production).

It is known that fungal melanins have many actions, and it is also a virulence factor for fungal species (Casadevall *et al,* 2000, Mattoon *et al*, 2021). This is likely true for chaga, although to the author's knowledge, has never been studied directly. An implication in decreasing melanin production by selecting for faster growing strains could result in producing lower melanin producing strains, and therefore less virulent strains. This could may well mean that in the field, the faster growing strains would be less effective at overcoming host immune defences, negatively

affecting infection rates and conk formation. This needs to be confirmed by future, rigorously designed studies.

Chemical analysis of melanin present in our fungal isolates was not undertaken but assumed to be responsible for pigmentation of our samples (Casadevall *et al,* 2000, Mattoon *et al*, 2021).

To assess pigmentation in future experiments, colorimetry instruments and spectrophotometers could be used to assess the percent coverage of mycelium and determine the intensity of pigmentation. The analysis in this experiment was performed by eye, but the method accuracy could be markedly improved by incorporating such instrumentation.

Biomass production in liquid media

YA and PDA demonstrated the most growth within the 4 week liquid culture experiment, and is likely due to them both being able to supply nutrients to the mycelium without any obvious detrimental metabolite being present, e.g. tannins. Although we did not chemically analyse the media for polyphenolic compounds, it could be the case that BA contained high enough concentrations of tannins to inhibit growth sufficiently so as to create a statistically significant difference between the plates in this treatment group and the PDA and YA treatment arms. Compounds in birch extract have been shown to stimulate the growth of Chaga mycelium (Wang *et al*, 2014), but this effect was not seen in our experiment, presumably due to tannin-mediated inhibition. This can occur via cell wall mediated disruption (Zhu *et al*, 2019), and in future experiments, it could be an important consideration to prepare tannin-free extracts for birch media, so as to observe the effects of the triterpene compounds as growth stimulators on chaga.

A larger variation between Southern samples than Northern samples has been observed in the biomass experiments and this can usually be explained by genetic variation within a sample set. However it is interesting since the Southern samples were from a much smaller collection zone (see Fig. 7), and so it could be assumed a narrower genetic pool. Further studies on morphotypes of these samples are required to fully delineate and explain the variation observed in growth rate between these samples. It may be that regardless of geographical placement, more genetically diverse individuals are able to be found in the southern Swedish climatic zone.

6. Conclusion

It can be concluded from the results here that YA at 16°C or 20°C are the conditions that produces most growth *in vitro* for *I. obliquus* and this can be recommended for future *in vitro* cultivation experiments.

Growth experiments at temperatures over a lower range would help to establish the optimal growth of Swedish strains of chaga. Limitations of our results due to our lack of temperature control needs to be taken into consideration and warrants repeating experiments under more rigidly controlled conditions.

It can be concluded that conks South 4, South 5 and North 1 respectively exhibited the highest growth on solid media, including for BA, and could be suggested as strains to be taken forward for further study for optimized growth trials.

We've observed, at least for two isolates, low pigmentation in faster growing strains, and vice versa, and this indicates a trade-off between nutrient use supporting increased primary metabolite production and growth rate with that of secondary metabolite production, for example medicinal compounds. Larger studies could perhaps show a significant effect, but it would be prudent in any case to have a parallel process of strain selection on the basis of both growth and medicinal compound biosynthesis, which is also recommend by other researchers (Pilz, 2004). For melanin production, the conk South 3 could be recommended for future trials.

The results of this study are revealing the new knowledge about *in vitro* cultivation of chaga in Sweden. They will demonstrate the efficacy of various conditions to identify the best cultivation protocol for Swedish isolates.

7. Future Experiments and Recommendations for Chaga Optimisation and Inoculation

Chemical analysis

Chaga is currently traded by currency/weight/crude mass. This is important sinceit is possible that other phenotypes could be lost in a growth rate selection process, for example the biosynthesis or accumulation of medicinal compounds mentioned above. It has been suggested that the efforts of strain isolation of *I. obliquus* should be focused on preservation or improvement of medicinal compound concentrations (Pilz *et al,* 2004, Roger and Wasser, 2012), since this makes the market for such strains resilient in the face of changing criteria for selection and consumption of chaga at the personal level and also at the market level. This will be in the sense that a particular extract will be standardised or guaranteed to contain a certain percentage of a particular bioactive compound, or even a profile of them. It can be that there is a range of compounds with known medicinal efficacy, but one of them is used as an indicator or proxy for the others, based on common biosynthetic pathways or other variables. For example, the triterpenoid fraction includes betulinic acid, trametenolic acid and inotodiol which all have established demonstrable medicinal efficacy (Kim *et al*, 2020) but could be assessed by just inotodiol, for example.

Inotodiol and trametenolic acid have demonstrated cytotoxic effects on multiple breast cancer cell lines, via the induction of autophagic pathways, as has an aqueous chaga mushroom extract (CME). Moreover, these have not interfered with cytotoxic chemotherapeutics, which increasingly supports the use of *Inonotus obliquus* in breast cancer alongside standard chemotherapeutic regimes (Lee *et al,* 2021). In the case of inotodiol, it is reported that products are already being standardised to concentrations of this well researched compound (Rogers and Wasser, 2012, Pilz, 2004), and so any research effort to find a cultivatable strain would do well to couple it to a quantitative analysis of these key bioactive compounds.

It has been shown that chaga from different regions contains different concentrations of compounds which affect their activity; samples from Normandy,

France contained concentrations of betulinic acid and inotodiol resulting in a cytotoxicity of normal transformed BEAS-2B cell, but far less of an effect on lung cancer cells (A549) (Géry *et al,* 2018). This has a multitude of implications since the origin of the chaga seems to determine the chemical composition and therefore medical application. Whether this is due to genetic variation of morphotypes, external factors or epigenetic, is to be determined by future studies, but is pertinent to consider here since there could very well be qualitative differences between strains from different regions within Sweden.

Many nutraceuticals currently on the market are standardized to a specific compound; for example turmeric (*Curcuma longa*) extracts are standardised to curcumin concentration, and Frankincense (*Boswellia spp.*) are standardised to Boswellic acids. The same is true for chaga (*Inonotus obliquus*) with medicinal constituents such as inotodiol and mycopolysaccharide concentrations, and this is set to likely become increasingly important, especially if cultivated strains are being used, or if mycelium is being used instead of the actual sclerotium.

We attempt to address it at the qualitative level concerning melanins and pigmentation of our plates and have pointed to a potential trade-off whereby growth rate may compromise melanin production, however, more in depth chemical analysis must necessarily accompany such studies.

Biodiversity

A direct issue regards the use of a particular *I. obliquus* strain and inserting into into a genetically diverse context and considering the genetic implications of domestication and an apparent and potential homogenization of *I. obliquus.* This can be considered in similar veins to other domesticated species in agriculture and is well reviewed (Moyers *et al,* 2017).

In relation to prevailing/existing forests, is there a net increase in biological and ecological value by introducing *I. obliquus* to these forests? Should forest owners be given different advice on taking up such practices depending on the forest types and current management systems that are in place? Should solely birch stands be inoculated in the same way as birch-pine stands or other mixed stands? It becomes crucial if this is to be a large scale, commonplace practice, that the ecological implications are well understood.

The presence of live, standing birch itself alone likely contributes to the increased biological and ecological value, following the reasoning and success of Bednarz *et al,* 2013.

Dead wood and the necrotrophic cycle

Increased amounts of dead wood should be taken into consideration considering the biodiversity question. The cubic metres of dead wood per inoculated area should be compared to that of non-inoculated areas, and this increase (if any) taken as an

added benefit for the ecosystem services made available. These include increased soil regeneration, increased biodiversity, increased water retention via increased humus (soil quality), increased microbiological load of the soil (soil quality), diversifying structures available for wildlife (biodiversity) and the increase of necrotrophic species, e.g. saproxylic fungi and beetles (Bauhaus, 2018, Stokland, 2012). Inoculation of live trees is not currently an established method in fungiculture, since many culinary species in fungiculture are mycorrhizal and saprophytic, and therefore do not require inoculation of live trees (Miina *et al,* 2021). Live inoculation of trees has been performed for ecological purposes, and to improve biodiversity in forests (Bednarz *et al,* 2013) and strengthens the ecological case for inoculation with *I. obliquus*.

Fire

Encouraging the retention of deciduous species, especially particularly fire-resistant species such as *Betula spp.*, would offer an otherwise mainly Spruce or Pine plantation the advantage of additional fire protection, since they have some natural resistance to wildfires (Ascoli and Bovio, 2010). Birch, due to its particularly high water content has also been used in urban areas as a fire break, for example in Umea, Sweden, now called the City of Birches, after major fires destroyed much of the wooden infrastructure of the city, Birches now line the streets as fire guardians (Hoel, 2016).

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8. Appendix

8.1 Statistical analysis data

8.1.1 Solid culture experiment

Table 3. Statistical tables from three way linear model regression of solid plate growth experiments.

FACTOR	SUM.SQ	DF	F.VALUE	PR..F.
ORIGIN	23,13779012	1	0,651661287	0,422178156
MEDIA	1749,011599	2	24,62990509	**7,0821E-09**
TEMP	789,5244876	2	11,11822998	**6,19565E-05**
ORIGIN:MEDIA	137,7847469	2	1,940310311	0,151097889
ORIGIN:TEMP	36,02308025	2	0,507283684	0,604264787
MEDIA:TEMP	37,29441975	4	0,262593461	0,901009507
ORIGIN:MEDIA:TEMP	41,96349383	4	0,295468844	0,880000158
RESIDUALS	2556,421444	72		

8.1.2 Biomass in liquid media

Table 4.. Statistical tables from ANOVA of liquid media experiments.

FACTOR	SUM.SQ	DF	F.VALUE	PR..F.
MEDIUM	4044,359279	2	11,91916125	**0,000280514**
ORIGIN	236,1152804	1	1,391714191	0,250174602
MEDIUM:ORIGIN	521,7614696	2	1,5376871	0,236166772
RESIDUALS	3902,13126	23		

8.1.3 Pigmentation

Table 5. Statistical tables from chi-squared regression analysis of solid media experiments.

FACTOR	LR.CHISQ	DF	PR..CHISQ.
MEDIA	30,80692683	2	**2,04344E-07**
ORIGIN	3,565822303	1	0,058980521
TEMP	2,300465182	2	0,316563131
MEDIA:ORIGIN	21,46246778	2	**2,18517E-05**
ORIGIN:TEMP	2,4831005	2	0,28893594

8.2 Plate data

8.2.1 Contaminated Plates

Table 6. Plates that were discarded due to contamination and therefore fewer replicates for that individual isolate could be used

Temp	*Origin*	*Number*	*Media*
20 Degrees	North	N9.1	BA
26 Degrees	North	N9.1	PDA
26 Degrees	North	N9.1	BA
16 Degrees	South	S1.5	YA
20 Degrees	South	S1.5	BA
26 Degrees	South	S2.2	PDA
26 Degrees	South	S2.3	YA
20 Degrees	South	S2.4	YA
20 Degrees	South	S2.5	BA
26 Degrees	South	S2.5	BA
20 Degrees	South	S4.4	PDA
20 Degrees	South	S4.5	PDA

8.2.2 Overgrown Plates

Table 7. Plates that had reached the edge, or very close to the edge of the plate (≥35mm) by week 2

Temp	*Origin*	*Replicate*	*Media*
16 Degrees	North	1.2	PDA
16 Degrees	North	2.3	PDA
16 Degrees	North	2.5	PDA
16 Degrees	North	2.4	PDA

16 Degrees	North	2.1	YA
16 Degrees	South	4.4	PDA
16 Degrees	South	4.5	PDA
16 Degrees	South	5.1	PDA
16 Degrees	South	5.2	PDA
16 Degrees	South	5.3	PDA
16 Degrees	South	5.4	PDA
16 Degrees	South	5.5	PDA
16 Degrees	South	2.1	YA
16 Degrees	South	4.1	YA
16 Degrees	South	4.2	YA
16 Degrees	South	4.3	YA
16 Degrees	South	4.4	YA
16 Degrees	South	4.5	YA
16 Degrees	South	5.1	YA
16 Degrees	South	5.2	YA
16 Degrees	South	5.3	YA
16 Degrees	South	5.4	YA
16 Degrees	South	5.5	YA
20 Degrees	North	1.1	PDA
20 Degrees	North	1.2	PDA
20 Degrees	North	2.4	PDA
20 Degrees	North	2.5	PDA
20 Degrees	North	1.1	YA
20 Degrees	North	1.2	YA
20 Degrees	North	1.3	YA
20 Degrees	North	1.4	YA
20 Degrees	North	1.5	YA
20 Degrees	North	2.1	YA
20 Degrees	North	2.2	YA
20 Degrees	North	2.4	YA
20 Degrees	South	2.2	YA
20 Degrees	South	4.1	YA
20 Degrees	South	5.3	YA
26 Degrees	North	1.5	YA
26 Degrees	North	2.1	YA

Popular science summary

Chaga has become more popular in recent years as a beneficial natural supplement and wellness tea. Understanding chaga source limitations and its harvest dynamics as well as potential revenue streams from chaga would be important for chaga growers in Sweden. Under natural conditions, the fungus *Inonotus obliquus* takes years to establish and develop these growths that are then harvested. Cultivating the fungus in forests for profitability therefore would be best optimized by choosing isolates that can establish quickly. In this study, the growth of *I. obliquus* was analysed from 10 samples collected across a climatic gradient in Sweden, on three different nutrient media and at three different temperatures, in order to select superior isolates that can be used for cultivation and under optimal conditions for mass inoculum production. The results showed significantly higher mycelium growth on yeast agar (YA) and potato dextrose agar (PDA), compared to birch agar (BA). No significant difference was found in growth rates between samples originating from the North (64° North) and the South (56° North) of Sweden. Highest growth occurred at 16°C, followed by 20°C and finally 26°C and this was consistent across all three media tested. In addition, to the *in vitro* growth trials, the degree of pigmentation was assessed in each culture, as pigmentation is commonly an indicator of the presence of melanin which has established therapeutic effects. Results showed that the fungus growth on PDA and YA exhibited more pigmentation than BA.

Interestingly the isolate showing highest growth (S4) showed least pigmentation, and conversely the isolate showing least growth (S3) produced the highest number of highly pigmented petri plates. This may suggest a potential trade-off between growth rate and production of medicinal compounds.

From the results we recommend future *in vitro* cultivation of chaga *Inonotus obliquus* in YA at least as low as 16°C. Further *in vitro* optimisation experiments could investigate lower temperatures to find the optimal growing temperatures.

Keywords**:** chaga, *Inonotus obliquus*, *in vitro* optimisation, sustainable production of chaga, NTFPs

Acknowledgements

I would like to thank my main supervisor Iryna Matsiakh for her enthusiasm with which she has helped me invaluably throughout this project. Her knowledge and skill in the forest pathology laboratory has been inspirational.

I would like to thank Morgan Karlsson, for so diligently collecting our Chaga samples from the North of Sweden. Thank you friend.

I would like to thank Iva Franic for her assistance with statistical analysis and for being available on hand in the lab. Mostly I am grateful for her friendship.

Gratitude to Alex Huttunen for his help with data manipulation and organsiation throughout the project.

I would like to thank Michelle Cleary for her thorough and dedicated proofreading and for making the time and giving her energy to help me improve this manuscript, despite her many other commitments.

Thanks also to Gudmund Vollbrecht for his insights and helpful comments.

I would like to thank Patrick Sherwood for his razor sharp insights and throroughness in proposing edits to better this manuscript.

Thank you to all in the Forest Pathology lab who were always willing to offer advice and a helping hand when needed; Patrick Sherwood, Diana Marciulyniene, Dusan Sadikovic, Milos, Khaled Youssef and Wiktoria Breza.

Thanks to Marlis, Alex, Hannah, Rike, Will, Odd, Kaspar and Anna for their friendship, support and hospitality during these times, and for welcoming me so warmly into your space for these many months.

Gratitude to Sir Thomas and Katarina for your generosity in inviting me to stay at Turmalinen. Without your good hearts, much would not have been possible these past years and I will always remember this. Next stop,the Nobel prize. .

Thanks to Giulia Attochi for background organisation of this thesis, my defence and final editing of the manuscript.

Lastly, I would like to dedicate this thesis to my son Rafi, to whom I wish to impart what knowledge I have gained during this Master's course. I would like to thank his mother, Adrienn Gönczi, for taking such good care of him in my absence. Although it has been challenging in innumerable ways, the sacrifice to be away from him has been the most difficult. I hope for him and his generation it will be worth it, one day.

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