Norwegian University of Life Sciences
Faculty of Environmental Science and Technology
Department of Ecology and Natural Resource Management

Master thesis 2016
30 credit

The influence of terpenes on growth of blue-stain fungi in vitro

Example of terpene treatment for the blue stain fungus Ophiostoma piceae (Münch) Syd., In Sydow & Sydow, Annls mycol. 17: 43. 1919.
Photo taken by Caritha Lorenza Furnes 09. 12.2016

Caritha Lorenza Furnes
Master program forestry and resource management

Foreword

I thank the Norwegian Institute of Bioeconomy Research (NIBIO) for providing financially support for material needed and space for conducting the experiment in their laboratory. This work was possible to do thanks to the patience and encouragement from my supervisor Paal Krokene at NIBIO and some of the employees at the Norwegian University of Life Sciences (NMBU). I thank co supervisor Halvor Solheim for providing help with the taxonomic status for the fungal cultures used. I thank Gro Wollebæk senior engineer at NIBIO for teaching me to handle the laboratory equipment correctly and for providing help with other practical matters at the NIBO building. I thank E.W.Bergvik for lending me a computer screen and C. Finstad for ordering HDMI cable. I thank; K.L.H. Hansen and C. Finstad for critical reviews on part of the manuscript.

Abstract

Changing climate and increasing global temperatures could lead to a cascade effects on the forest environment. For instance, stretching the range for where insect could find suitable habitat and the use of monoculture in modern forestry might lead to less robust systems to tolerate these changes (Manion., 1981; Perry et al., 1994). Aggressive beetle attacks and associated virulent fungi like the symbiont blue stain fungus that could cause staining on the timber, is source of great economic challenges for the forest industry. Symbiont fungi could be transported on to hosts by aggressive bark beetles through "epizoic (hair and mycangia that are; cavities, tubes, pouches, hollow glands) and endozoic (sticky mucilage, outer shell from dried spores)" transportation (Beaver, 1989). Virulent phytopathogens for example the blue stain fungus *Endoconidiophora polonica* and *Ophiostoma* are suspected to "be involved in overwhelming host defences" that could lead to tree mortality (Krokene & Solheim, 1998; Franceschi., 2005; Six, 2012; Hammerbacher et al., 2013; Wang et al., 2014). The trees have two main defence systems that is; The primary defence system that is active on a general basis, and the secondary defence system which is activated after a trigger is set of. The triggering of the second defence causes the tree to upregulate genetic characteristics that makes it produce chemical compounds, that consumes energy like the nonprotein chemicals; terpenes and phenolics (Vega & Hofstetter 2015; Franceschi., 2005). In a study by Zahao et al., (2011) their result from inoculating virulent fungi associated with bark beetles indicated that terpene induction may aid in tree resistance to bark beetles after 35 days. According to a study from Hammerbacher et al., (2013) "little is known about how effective Norwegian spruce defence compounds are against *E.polonica*, which could be crucial in understanding the success of bark beetle attacks".

In my in vitro experiment the growth inhibiting effects from five terpene treatments were studied on four species of blue stain fungus; *Ophiostoma- piceae*, *Grosmannia- penicillata*, *Ophiostoma- bicolor* and *Endoconidiophora-polonica*. The aim of the experiment was to analyse growth, examine if monoterpenes affected growth differently and to see if different compositions of terpenes could have an impact on growth that differed from each other. Terpene treatment used were; (+)-α-pinene, (-)-β-pinene, (-) limonene, (+) limonene, monoterpene mix of four distinct monoterpenes (MT mix), and a control that was left untreated (C). The actual growth measurements were taken from two perpendicular lines drawn under the bottom of the glass petri dishes for all the replicates made for the treatments. Statistical analyses that were done was residual plot, two-way ANOVA and Tukey HSD. Graphs showing growth per day based on the included data material were made to visualise growth between treatments within an isolate and to present the result in an honest way when dealing with continuous data (Weissgerber., 2015).

The result indicate that the monoterpenes included in the experiment, inhibit growth of blue stain fungus and that their effect on the fungus differ from each other. Inhibition of growth of the fungus is also seen when using terpenes of varying compositions. Differences in growth between fungus were not possible from the result to indicate any unequal growth from, due to variations in growth pace between some of the fungal isolates within one fungi. The main conclusion from the experiment was that growth pace is probably a characteristic that is related to terpene tolerance for blue stain fungi. The findings are consistent with result from other studies, like those mentioned in (Krokene et al., 1996).

Sammendrag

Endret klima med globalt økende temperaturer kan føre til en kaskade effekt for skog økosystemer. Slik som, øke område for hvor insekter kan finne egnede habitat, og bruk av monokultur i moderne skogbruk vil kunne føre til mindre robuste systemer for å tåle disse endringene (Manion., 1981; Perry et al., 1994). Aggressive bille angrep og deres assosierte patogene sopper som symbiont blå ved soppen som kan gi misfarge på tømmer, er en kilde til store økonomiske utfordringer for skog industrien. Symbionte soppen kan bli transportert av bark biller ved "epizoic (hår og mycangia som er; hulrom, rør, lomme, hule kjertler) og endozoic (klebelig slim, ytre skjell tørre sporer) transport (Beaver, 1989). Patogene sopper som *Endoconidiophora polonica* og *Ophiostoma* er mistenkt for å " være involvert i å overvelde vertens forsvar" noe som kan føre til død hos trær (Krokene & Solheim, 1997; Franceschi., 2005; Six, 2012; Hammerbacher et al., 2013; Wang et al., 2014). Trærne har to hoved systemer som er; Det primære forsvaret som er aktivert på en generell basis og det sekundære som forårsaker trær til å oppregulere genetiske egenskaper som får det til å produsere komponenter, som koster energi, slik som ikke protein kjemikalier; terpener og fenoler (Vega & Hofstetter 2015; Franceschi., 2005). I en studie utført av Zahao et al., (2011) indikerte deres resultater ved forsøk med inokulering av patogen sopp assosiert med bark biller at start på terpene produkasjon kanskje kunne hjelpe for trærs motstandsdyktighet mot bark biller etter 35dager. I en studie fra Hammerbacher et al., (2013) " er det lite kunnskap om hvor effektiv norsk gran Piceae abies forsvarkomponenter er mot *E.polonica*, noe som kan være av stor betydning for å forstå bark biller angrep suksess.

I mitt mitt in vitro eksperiment ble effekten av vekstinhibering fra fem ulike terpene behandlinger studert for de fire blådvedsoppene; *Ophiostoma- piceae*, *Grosmannia-penicillata*, *Ophiostoma- bicolor* and *Endoconidiophora-polonica.* Formålet med eksperimentet var å analysere vekst, undersøke om monoterpeners påvirkning på vekst var ulik og se om ulike sammensetning av terpener kunne ha en påvirkning på vekst som var ulik fra hverandre. Terpene behandlinger som ble benyttet var; (+)-α-pinene, (-)-β-pinene, (-) limonene, (+) limonene, monoterpene mix som besto av fire ulike monoterpener (MTmix) og en kontroll som var ubehandlet. Faktisk vekst målinger ble gjennomført ved hjelp av to akser tegnet under glass petriskålen for alle gjentak som ble laget for behandlingene. Statistiske analyser som ble brukt var residualt plot, to veis ANOVA og Tukey HSD: Grafer som viser vekst per dag som var basert på inkludert data materielle ble laget for å visualisere vekst mellom behandlinger innenfor et isolat og for å fremstille resultatene på en ærlig måte når kontinuerlig data var data materiale (Weissgerber., 2015).

Resultatene indikerte at de monoterpener som ble inkludert i eksperimentet, var vekst inhiberende eksperimenter for blå ved sopper og at deres effekt var signifikant ulik fra hverandre. Inhibering av vekst ble også observert når terpenene besto av ulike komponenter. Forskjeller i vekst mellom sopper var ikke mulig utfra resultatene å indikere noe ulike vekst fra, på grunn av variasjonene i vekst hastighet for noen av sopp isolatene for en sopp art. Hoved konklusjonen fra eksperimentet var at vekst hastighet var muligens en egenskap som var relatert til terpene toleranse hos blåvedsopp. Disse resultatene bidrar med forståelse som er i samsvar med forståelse som er fremmet i andre studier slik som i (Krokene et al., 1996).

Introduction

In a times with global trade, increased pressure on primary resources and climate change, it is of great importance to understand what contributing factors could lead to massive mortality in forests. It is known from previous studies (DiGustini 2011; that bark beetle and fungi have a symbiotic relationship that enhance the evolutionary adoption for establishment and proliferation in trees. The bark beetles could have different symbionts like fungi, mites and bacteria that could serve a role as source for nutrition in the galleries where eggs and larvae develop (Vega & Hofstetter 2015). Trees contain primary and secondary defence systems where the primary is active on general everyday basis but the secondary that requires energy to activate needs a trigger for example hundreds of small holes drilled trough the bark by bark beetles (Vega & Hofstetter 2015).

Inoculation experiments like Krokene and Solheim (1998); Krokene and Solheim (1997); Krokene and Solheim (1996) conducted has established a solid understanding of fungi as a contributing factor in tree killing. Other studies indicate that the fungi might manage to overcome the tree defences like (Six, 2012; Hammerbacker et al., 2013; Wang et al., 2014) According to a study from Hammerbacker et al., 2013 “little is known about how effective Norwegian spruce defence compounds are against *E.polonica*, which could be critical in understanding the success of bark beetle attack”.

In my in vitro experiment, the inhibiting of fungal mycelium growth was studied by using five distinct terpene treatments that were compared with a control treatment for all four fungus. The actual growth was measured from two perpendicular lines drawn under the bottom of the glass petri dishes for all the replicates made for the treatments. The aim of my study was to analyse the hypothesises presented in table 1.

Table 1. An overview of the hypothesis (H) that is the focus in my study. Trees resistance from pathogens like blue stain fungus is determined by certain terpene concentrations and I will therefore examine following hypothetical claims as described in H1, H2, H3 in the table.

	An overview of the hypothesis
H1	I will assume that the most virulent blue stain fungus known to grow in trees like *P.abies* will have less growth in my experiment.
H2	Monoterpenes affect growth by blue stain fungus in a different manner
H3	Terpenes with different compositions can affect blue stain fungus growth differently

2. Material and Methods

In this part, the process of how the growth inhibiting experiment was preformed is explained by splitting it up in sections with levels of detailed description. It starts with a general description for the in vitro experiment set up and why it was necessary to test it with a pilot study. Then follows the technical proceedings for the preparation and how the growth inhibiting experiment was carried out. In the end a description of data collection and its processing is given before remarks on which statistical models and analysis were to be conducted.

2.1 General description of the growth inhibiting experiment

Four species of blue stain fungi were used for the growth inhibiting experiment. The four fungi species had two isolates each, that were sampled from different places in Norway and more information about the isolates is given in Appendix 1. The isolates had numbers for the storing place at NIBIO and were given simple letter A or B to ease work usage in the growth inhibiting experiment. In a relatively recent study by De Beer & Wingfield (2013), a huge updated revaluation for the taxonomic status of each species of fungi proposed to be classified as ophiostomatoid fungi have been completed. Table 2 present the species of fungi used in the growth experiment, its two isolate and name for isolate together with known synonym is presented.

An overview of the growth inhibiting experiment set up is presented in a simplified list in table 3. There were six treatments in the experiment, and these were four unique types of monoterpene (MT), one treatment with a mix of four monoterpenes (MT mix) and a control with no content of MT or MT mix added in the growth environment for the blue stain fungi used. 200 µl liquid terpene were added for all eight replicates during a terpene treatment.

Table 2. The species of blue stain fungi used in the growth inhibiting experiments. The taxonomy follows the book "A nomenclator for ophiostomatoid genera and species in the Ophiostomatales and Microascales" by De Beer & Wingfield (2013) and the online database Mycobank (www.mycobank.org/quicksearch.aspx 22.11.2016). * Mycobank species numbers.

Species	Isolation numbers and letter		Synonym names
Ophiostoma piceae (Münch) Syd. Sydow & Sydow, Annals mycol. 17: 43. 1919[MB#433468*]	1998-50/3	A	*Ceratostomella piceae,* Münch, Naturw. Land. Forstw. 5: 547. 1907 [MB#191195] *Ceratocystis piceae* (Münch) Bakshi, Trans. Br. Mycol. Soc. 33: 113. 1950 [MB#294228] *Pesotum piceae* Crane & Schoknecht, Am. J. Bot. 60: 348. 1973
	1980-92/34	B	*Graphium piceae* (Crane & Schoknecht) M.J. Wingf. & W.B. Kendr., Mycol. Res. 95: 1331. 1991
Grosmannia penicillata (Grosmann) Goid. Boll. Staz. Patol. Veg. Roma 15: 156. 1935 [MB#433468 *]	1980-91/54	A	*Ceratostomella penicillata* Grosmann, Hedwigia 72: 190. 1932 *Scopularia penicillata* (Grosmann) Goid., Boll. Staz. Patol. Veg. Roma 15: 156. 1935 *Ophiostoma penicillatum* (Grosmann) Siemaszko, Planta Pol. 7: 24. 1939
	1960-21	B	*Ceratocystis penicillata* (Grosmann) C. Moreau, Rev. Mycol. (Paris), Suppl. Colon. 17: 22. 1952 *Verticicladiella penicillata* (Grosmann) W.B. Kendr., Can. J. Bot. 40: 776. 1962
Ophiostoma bicolor R.W. Davidson & D.E. Wells, In Davidson, Mycologia 47: 63. 1955 [MB#294194*]	1980-48/36	A	*Ceratocystis bicolor* (R.W. Davidson & Wells) R.W. Davidson, Mycologia 50: 665. 1958
	2004-38/1	B	
Endoconidiophora polonica (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf Studies in Mycology 79:211(2014) [MB#810316*]	1994-169/113	A	*Ceratocystis polonica* (Siemaszko) C. Moreau, Revue de Mycologie 17 (Suppl. Col. No. 1): 22 (1952) [MB#294232]
	1993-208/115	B	*Ophiostoma polonicum* Siemaszko, Planta Polonica 7 (3): 33 (1939) [MB#274888]

Table 3. Experimental setup for the growth inhibiting experiment for all blue stain fungus tested. Monoterpene treatment (MT) and monoterpene treatment with four types of monoterpenes blended together (MT mix), are shown in the table with shortened name as MT and MT mix respectively.

Experiment set up for laboratory work.	
Number of species:	4
Number of isolates:	2
Compounds used:	4 MT, 1MT mix
Concentration in treatment:	200 µl
Number of types of control:	1
Number of replicates:	8
Total number of replicates:	384

A small pilot study was conducted with an *E. polonica* isolate with registration number 1980-53/7A. The purpose of the pilot study was to test different ways of making sure that the filter paper needed for encompassing the terpene treatment got attached firmly enough on the glass petri dish lid with a bit of help from some agar gel. Sufficient attachment of the filter paper meant that it endured the manual handling under measurement for a period of several days without falling on the actively growing fungi mycelium at the bottom of the petri dish. *E. polonica* isolate 1980-53/7A was not used later in the growth inhibiting experiment since the pure culture for it died only five days after inoculation was completed on 30.01.14.

2.2 Making a pure start culture

A minimum of five petri dishes with pure start culture were prepared for all isolates to ensure that the mycelium growth rate within the isolates could be standardized and that, possible contamination could be detected. Only one isolate (1994-169/113) had to be prepared from an isolate stored in the ultra-deep freezer -152 °C to make a pure start culture. After pure start culture inoculations were done, the cultures were placed in a dark chamber with a temperature of 25 °C.

2.3 Description of performing the growth inhibiting experiment

Performing the growth inhibiting experiment required several preparation stages such as; sterilization of equipment, making growth medium, preparing glass petri dishes and needed liquid terpenes in an organized manner. From section 2.3.5 the description fit the process of conducting the experiment on two isolates simultaneously.

2.3.1 Sterilization of equipment`s

Some equipment's were autoclaved at 120°C to reduce risk for contamination on the fungal mycelium during the growth inhibiting experiment period and these are listed in table 4 The Kipp automat, mentioned in table 4 consist of a "head" with a hollow "neck" connected to a flask. The "head" is made to measure 25 ml of liquid growth medium out quickly with a hand twist with the "neck" connected inside the flask opening where the liquid growth medium is contained. The stack with 120 pieces of 75mm filter paper were placed in two separate glass petri dishes before the autoclave process so they could be stored inside the glass petri dishes when not in use.

Table 4 Equipment that were autoclaved at 120°C, to reduce contamination risk on the fungal mycelium during growth phase in the growth inhibiting experiment.

Item	Number
Kipp automat	1
Glass petri dishes	104 Pyrex 95mm 84 blue green 100mm
75 mm filter paper (qualitative filter paper 413, medium filtration rate and particle retention 5-13 µm from Vwr European Cat.no 516-081)	2 glass petri dishes 100mm diameter and 3cm high.
Pipette heads size 200 µl and 1 µl	A box full of the pipette heads.
Eppendorf tubes	5

2.3.2 Preparation of maltose extract agar

The growth medium made for the fungi was the maltose extract agar (MEA) which consisted of 1,25 % (w/v) malt extract agar from BD (Becton, Dickson and Company, Sparks, MD 21152 USA) and 2% (w/v) agar from Apotekproduksjon AS Oslo. Prepared growth medium was stored in a heat chamber at 70°C until it was poured into the petri dishes, to prevent the liquid MEA from solidifying.

2.3.3 Method for preparing all glass petri dishes

Preparation of all glass petri dishes with MEA growth medium and filter papers were done inside a sterile bench where sterile technique was applied for all equipment use. The sterilization technique when handling metal equipment was to apply ethanol on them and then let the ethanol burn of after holding the equipment over a small gas flame for a few seconds. Needed equipment's were placed inside the sterile bench and these were; 1 tweezer, 1 bottle of ethanol and all autoclaved equipment needed at a time as described in section 2.3.1. Only one Erlenmeyer flask with liquid MEA was taken out at a time from the heating chamber and poured into the Kipp automat inside the sterile bench. The Kipp automat was used for measuring 25 ml MEA to be poured inside each glass petri dish and the last drops of MEA was saved to be used inside the glass petri dish lid. When all glass petri dishes were prepared with filter paper and MEA growth medium they were stored inside the sterile bench with the ventilation switched on while the agar solidified.

2.3.4 Preparing micropipette with calibration

The 200 µl pipette was calibrated before any terpene treatments were performed on two isolates at a time. The calibration was done with tare weight for the measuring of 200 µl of water.

2.3.5 Preparing monoterpenes

The MT mix was prepared by pipetting 1 ml of each of the four monoterpenes into a 5ml Eppendorf tube. For each individual MT, 4 ml was pipetted into a 5 ml Eppendorf tube. All Eppendorf tubes were branded with name of terpene inside, and placed in a small metal grid before and after use inside the sterile bench. The pipette procedure was done quickly to reduce breathing exposure to the harmful chemicals. All five bottles containing liquid monoterpenes used in the growth inhibiting experiment was to be stored in a refrigerator before and after use. In table 5 an overview of terpenes used in the growth inhibiting experiment is shown.

Table 5 Name, production number and assay concentration value for the monoterpenes used in the growth inhibiting experiment. All monoterpenes were ordered from manufactory Sigma Aldrich. Common synonyms for the terpene chemicals can be seen in Appendix 2

Name of monoterpene	Production number	Concentration for assay
(R)-(+)-Limonene	183164 SIGMA.	97%
(s)-(-)-Limonene	218367 ALDRICH	96%
(+)-(3)-Carene	21986 SIGMA-ALDRICH.	98,5%
(+)-α- Pinene	P45680 ALDRICH.	98%
(-)-β- Pinene	112089 ALDRICH.	99%

2.3.6 The process of conducting the treatment on the blue stain fungus

When inoculating fungal mycelium and applying terpene treatments, only 8-12 glass petri dishes were handled at a time inside the sterile bench. The detailed description of conducting treatment on blue stain fungus is presented in part a-d.

a. Arrangement of glass and plastic petri dishes

The groups of glass petri dishes inside the sterile bench were taken out of the autoclave bags then marked with date, isolate number and type of treatment before they were placed in a row with the lid off. Isolates were marked with different filter pen colours. Minimum five plastic petri dishes containing pure start culture actively growing mycelium from for one isolate were then opened inside the sterile bench by peeling off the plastic foil on the side, which were used for protection against contamination under the storage period. Only one plastic petri dish with pure start culture was placed on the same row as the other glass petri dishes with the lid off, the other plastic petri dishes with pure start culture were stacked on top of each other and placed in a corner inside the sterile bench.

b. Inoculation

Sterilization technique as described in section 2.3.3 was applied on the agar plug stamp, two long metal needles and one tweezer. The agar plug stamp was used to make ca 12 agar-plugs from the actively growing mycelium in the start culture petri dish. A single agar plug was then transferred and placed on the MEA growth medium with the actively growing mycelium facing down at the centre of the glass petri dish with the use of the two long metal

needles. Method for inoculation described in the following text had to be done very quickly to reduce risk for contamination through the air content.

c. Treatments

200 µl of terpene was pipetted with micropipette on to filter paper for replicates within a treatment. Pipetting was done by pressing terpene drops upon the filter paper edge to spread the terpene liquid content even on it. After pipetting terpenes on the first replicate to be made from the row of opened glass petri dishes, the lid was closed. Glass petri dishes had to be closed quickly to minimize highly volatile monoterpenes loss, reduce contamination risk and to prevent filter paper to dry out from the ventilation which would make attachment inside the glass petri dish lid difficult. After pipetting one terpene treatment on the filter paper inside all replicates, the closed glass petri dishes had to be sealed with 7-8 cm bit of Parafilm 2 in x 250 ft PM -992 www.parafilm.com before volatile-tight DuraSeal™ film 1 inch size x 150 ft, Z379026-1EA PCode 10016448043 SIGMA –ALDRICH Inc. P.O 14508, St.Louis, MO 63178 USA 314-771-5750 was strapped on. The control replicates were made by following description in a-b and the two types of sealing with Parafilm and then DuraSeal™ film was applied for these replicates as well.

d. Storage

Replicates in one treatment within one isolate were put inside a plastic bag and sealed before being placed inside a dark incubation chamber which had a constant temperature of 25°C, which were repeated for all replicates in the six treatments within the two isolates.

2.3.7 Differences between the isolates

Under the procedure of performing the GIE there was differences that were made between the isolates of the blue stain fungi used and these are shown in table 5. In the text for figure 4-11 the date for the pure culture inoculation done in advance to be used in the GIE, the date of inoculation for starting the GIE and the date for when the measurement started for every isolate is given.

Table 5. Differences between isolates of blue stain fungi used in the growth inhibition experiment is shown for; type of glass petri dishes used, and challenges that influenced the data collection and content in the monoterpene mix (MT mix). For some isolates measurements were done repeatedly in one specific sequence regarding what the treatment measurements started on and continued with until all six treatments were measured where the number of replicates within a treatment also were measured in one specific sequence. Limited amount of (+)-3-carene monoterpene made it necessary to replace it with the (-) limonene after the growth inhibiting experiment was done for *O.piceae* isolate A and B and G.*penicillata* isolate A. The concentration ratio was close to 1:1:1:1 between the four monoterpenes used in the MT mix. The fungus shown in the table where; Ophiostoma- piceae (O.pic), Grosmannia- penicillata (G.pen), Ophiostoma- bicolor (O.bic) and Endoconidiophora-polonica (E.pol).

Fungi	**Isolate**	**Glass petri dish type**	**Challenges**	**MT mix content**
O.pic	A: 1998-50/3 B: 1980-92/34	Pyrex Glass petri dishes 95mm size ca 104 pieces.	1. Specific sequence for measurement not done. 2. Lots of problems with the use of the old Kipp automat reduced the number of replicates for controls for isolate A and B.	(+)-3-carene (+)-α-pinene (-)-β- pinene (+)-limonene
G.pen	A: 1980-91/54	Blue green in color and 100mm in diameter ca 84 pieces.	1. Specific sequence for measurement not done. 2. Four days after the inoculation, the mycelium grew quickly towards the edge of the pate.	(+)-3-carene (+)-α-pinene (-)-β- pinene (+)-limonene
	B: 1960-21	Blue green in color and 100mm in diameter ca 84 pieces.	1. Specific sequence for measurements not done.	(-)- limonene (+)-α-pinene (-)-β- pinene (+)-limonene
O.bic	A: 1980-48/36 B: 2004-38/1	Pyrex Glass petri dishes 95mm size ca 104 pieces.	1. Specific sequence for measurements done.	(-)- limonene (+)-α-pinene (-)-β- pinene (+)-limonene
E.pol	A: 1994-169/113 B: 1993-208/115	Pyrex Glass petri dishes 95mm size ca 104 pieces.	1. Specific sequence for measurements done.	(-)- limonene (+)-α-pinene (-)-β- pinene (+)-limonene

2.4 Data collection and processing

2.4.1 Data collection

Two perpendicular lines were drawn under the bottom of all the glass petri dishes at the centre of the plate where the inoculation of the agar plug was, before the first growth measurement took place. Fungal growth along the four axes were marked using waterproof felt pens of different colours, with alternate colours used on different days (black, green, red and blue). Criteria for which measurements were to be included to be collected as data, is as follows:

1) It was always the longest hyphae that could be measured along the axes from the two perpendicular lines that was marked as growth.
2) Exception for criteria 1 was when the monoterpene treatment inhibited the growth of mycelium to the extent where only growth zones were possible to be measured along the axes of the two perpendicular lines.
3) If some of the replicates in the growth inhibiting experiment were contaminated, data from the uncontaminated area was to be collected until the contaminated fungi came as close as one cm to the uncontaminated hyphae was measured along the axis. Then the measurement stopped. Measurements collected from a replicate that was contaminated were always compared with the growth of the other replicates. If the growth differed too much, the contaminated replicate was discarded.
4) All data collected had to be written by hand in a scheme like the one shown in Appendix 3. It was strictly forbidden to bring possibly contaminated items inside the laboratory facility, and this included a laptops or mobile phones.

2.4.2 Data processing

Average growth for each time measurement was calculated from the four axes in each replicate within one treatment and for all six treatments in the two isolates. The exact time lapse between two measurements, for example 06.02.14 and 05.02.14 were calculated by subtracting the difference in of minutes and then recalculated to express time as days called "time (days)". Then, the growth in mm per day called "growth (mm)/time (days)" was calculated by dividing the average growth with "time (days). Data that was missing or had too high growth value due to MT or MT mix leakage was not included in the calculation of the average growth from the four axes, and was adjusted for by calculating the average manually.

Objective critera used for discarding measurements from replicates were:

1 Contamination.

2 Useless measurement of data, due to markings that were sometimes rubbed off when the glass petri dishes had to be stacked upon each other in a plastic bag for storage.

3 Leakage from the terpene treated fungus.

Leakage in the growth inhibiting experiment was defined as:

Terpene treated fungi had a higher growth for the same isolate at the same measurement time than the average growth for the control. All measurement from the replicate ware checked with calculation for possibly leakage.

2.4.3 The regression analysis

Description of how values from the regression analysis were obtained is presented in the next paragraph as an example that describes the data process in general for only one replicate in a treatment for one isolate.

Example

The “accumulated growth (mm)/time (days)” and “accumulated time (days)” were calculated by addition of the values from “growth (mm)/time (days)” and “time (days)” from the first time measurement until the last time measurement. Series of time measurements were restricted by growth pace in the control. Then a graph was made to carry out a regression analysis by plotting the “accumulated growth (mm)/time (days)” to “accumulated time (days)”. In figure 1 an example of a graph made to perform regression analysis is shown. The regression equation with its slope value and R^2 value that was obtained by formatting a trend line in the graph using Microsoft Excel.

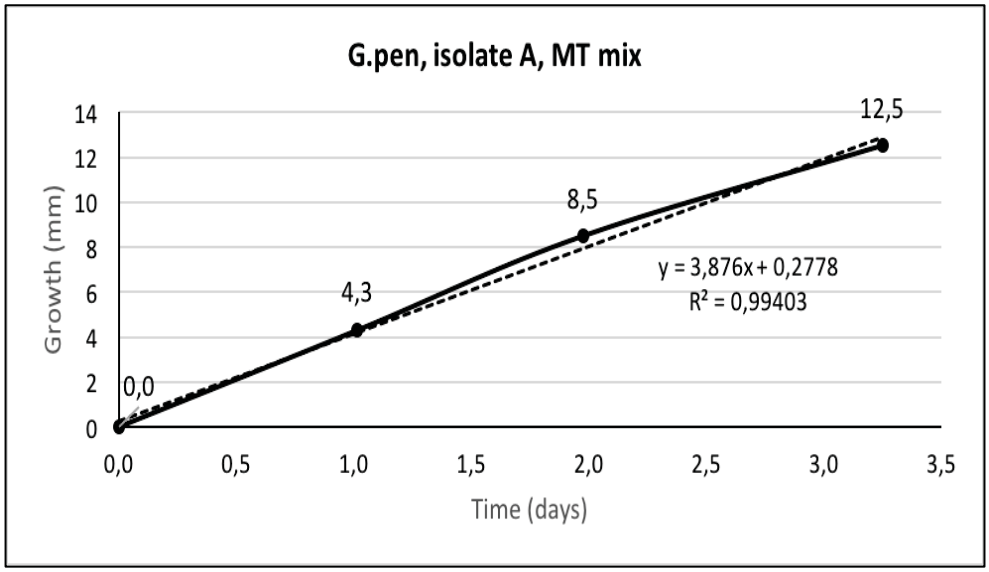

Figure 1. In this figure, the growth curve shown is based on the calculated average accumulated growth (mm) / time (days) in relation to the accumulated time between every time measurement from the collected data within monoterpene mix (MT mix) treatment in the blue stain fungi *G.penicillata* isolate A = 1980-91/54. *Grosmannia penicillata* is shortened to G.pen in the graph. The growth is expressed on the y-axis as; Growth (mm). The time between every measurement is shown on the x-axis as; Time (days). The result from making a regression analysis by formatting a trend line shown as dotted black line on the graph is given with the regression equation y = 3,876x + 0,2778 and the R^2 value 0,99403. R^2 values close to 1 indicate a near perfect fit.

The process described in the example above, were repeated for all replicates in all the six treatment for the eight isolates where the growth slope value was collected to be used in statistical analysis after the data was properly organized in a new Excel sheet. In Appendix 6 an overview of the regression equation, R^2 value and standard error value from the average "accumulated growth mm/time (day)" to "accumulated growth (mm)/time (day)" based on the number of replicates within each six treatment for the eight isolates is shown.

2.4.4. Growth curves

The growth curves displayed in figure 4-11 were made using the program MATLAB.

2.4.5. Precentage

The percentages were calculated on the assumption that the average growth slope value from the regression equation based on the replicates from the control in each specific isolate could describe the potential 100 % growth. The average growth slope value based on replicates within terpene treatments were then divided by the average growth slope value from the replicates in the control to compared the different percentages for all the five terpene treatments.

2.5 Statistical methods

The whole growth inhibiting experiment can be explained as a block experimental design which is described in general terms in (Mendenhall & Sincich 2012;Montgomery 2013). Tests for model assumptions were done before the chosen model was applied in further statistical analysis. The complete model for the growth inhibiting experiment could be described as shown in model 1 under. The program R version 3.3.2 and JMP pro 13 were used for the statistical analysis.

Model 1:

$$y_{ijkl} = \mu + \tau_i + \beta_j + \gamma_k + \tau\beta_{ij} + \tau\gamma_{ik} + \beta\gamma_{jk} + \tau\beta\gamma_{ijk} + \varepsilon_{ijkl} \quad \begin{cases} i = 1,2,3,4,5,6 \\ j = 1,2,3,4 \\ k = 1,2 \\ l = 1,2,3,4,5,6, \end{cases}$$

y_{ijkl}	=	Growth rate explained from the slope in the regression lines
μ	=	Overall sample mean
τ_i	=	Treatment where i, is number of treatments and 1= (+)- α-Pinene, 2= (-)-β-Pinene, 3= (-)- Limonene, 4= (+)-Limonene, 5= MT mix (monoterpene mix), 6= Control
β_j	=	Fungi species where j is number of fungi and 1 = *O.piceae*, 2= *G.penicillata*, 3= *O.bicolor*, 4= *E.polonica*
γ_k	=	Isolate where k is number of isolates and 1= A, 2= B
$\tau\beta_{ij}$	=	Treatment x Fungi species
$\tau\gamma_{ik}$	=	Treatment x Isolate
$\beta\gamma_{jk}$	=	Fungi species x Isolate
$\tau\beta\gamma_{ijk}$	=	Treatment x Fungi species x Isolate
ε_{ijkl}	~	Random error term; NID = normally and independently distributed with mean 0, and variance σ^2.

The complete model was reduced to contain only the relevant factors to examine hypothesis H1 and H3 with two-way analysis of variance (ANOVA).

Model 2 for H1:

$$y_{ijkl} = \mu + \tau_i + \cancel{\beta_j} + \gamma_k + \cancel{\tau\beta_{ij}} + \tau\gamma_{ik} + \cancel{\beta\gamma_{jk}} + \cancel{\tau\beta\gamma_{ijk}} + \varepsilon_{ijkl} \quad \begin{cases} i = 1,2,3,4 \\ k = 1,2 \end{cases}$$

Model 3 for H3:

$$y_{ijkl} = \mu + \tau_i + \cancel{\beta_j} + \gamma_k + \cancel{\tau\beta_{ij}} + \tau\gamma_{ik} + \cancel{\beta\gamma_{jk}} + \cancel{\tau\beta\gamma_{ijk}} + \varepsilon_{ijkl} \quad \begin{cases} i = 1,2,3,4,5,6 \\ k = 1,2 \end{cases}$$

When model 2 and 3 showed significant low p value in the two-way ANOVA, then a follow up post hoc analysis like Tukey Multiple Comparisons of Means with honest significant difference (Tukey HSD) was to be included, so the effect from the factors could be examined more specifically (Mendenhall & Sincich 2012; Tukey 1949).

3. Result

3.1 Plot of mean

Two different mean plots were made to look for possible unequal growth before any statistical testing. The two mean plots were calculated with r commander based on the dataset with linear regression value obtained from all replicates within monoterpene treated isolate A and B for all blue stain fungi used in the growth inhibiting experiment.

The mean plot comparing the response to the monoterpene treatments for all the fungi that were tested can be seen in Appendix 4 The plot indicated that (-) limonene might be the most growth inhibiting monoterpene treatment for the two blue stain fungi *E.polonica* and *G.penicillata.* The plot also indicated that *G.penicillata* might had the highest potential for average growth under influence from all the monoterpene treatments in the growth inhibiting experiment.

The second plot of mean that compares the average growth response from the two isolates A and B for each fungi tested in the growth inhibiting experiment can be seen in Appendix 5 The plot revealed that the different average growth between the two isolate A and B for all four fungi tested differed, which were especially visible for *O.bicolor* and *G.penicillata.*

3.2 Model adequacy test

Figure 2 A-D sums up the results from the summary of fit using Model 2. Model adequacy was checked efficiently with residual plots before two-way ANOVA was conducted as recommended in Montgomery (2013)., p 80. Highest RMSE = 0,5536 was found for *E.polonica* implying more random errors made when measuring growth in this fungi than for the other three fungus.

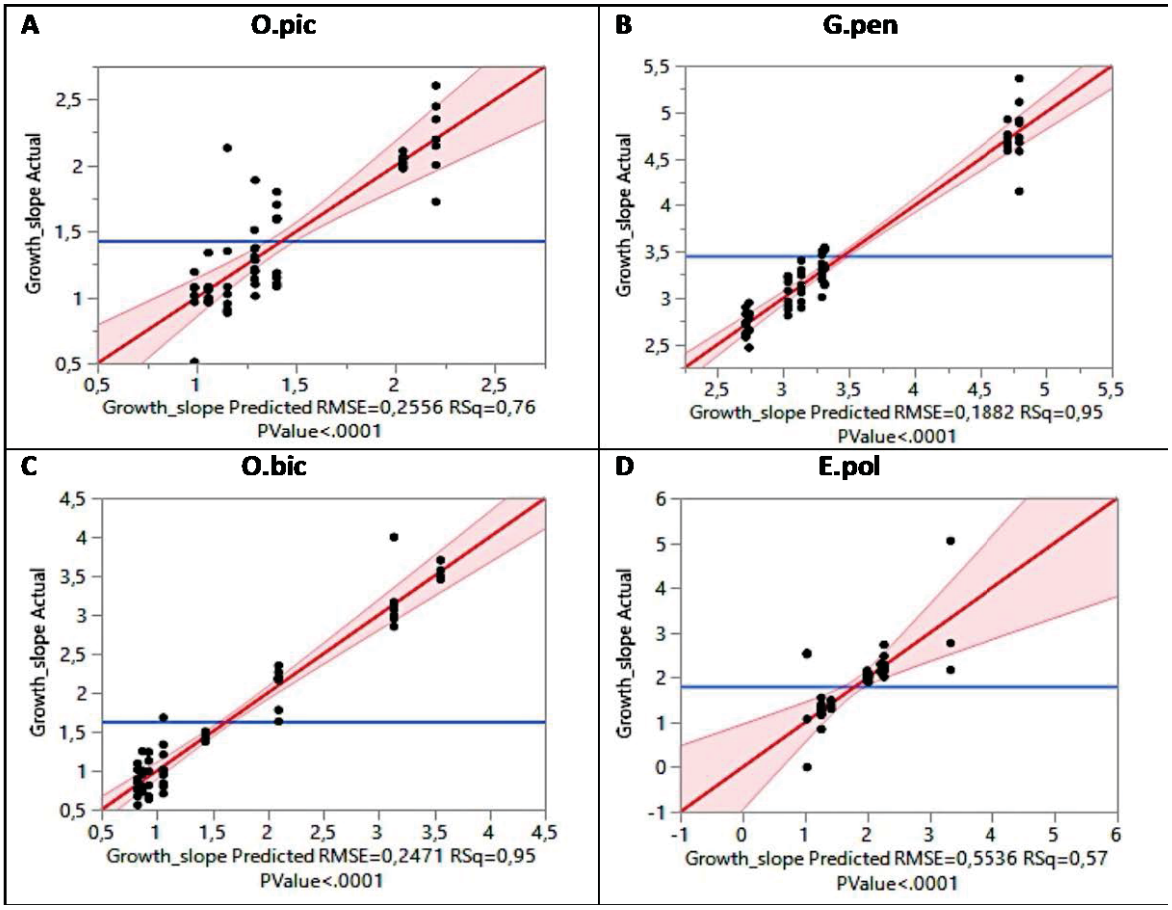

Figure 2. Actual by predicted plot made with JMP pro 13. The graphics display the response variable growth_slope on the y-axis as a function of the design factors described in detail for Model 2 in section 2.5. Dataset was based on the calculated growth slope value obtained by regression analysis done for all replicates within the four monoterpene treatments for all four blue stain fungi. The actual by predicted plot indicate lower fit for Model 2 in both *E.polonica* with R^2 = 0,57 and *O.piceae* with R^2 = 0,76 than Model 2 does for *G.penicillata* and *O.bicolor* which had a similar R^2 value = 0,95. R^2 closer to 1 indicates a better fit of the model than R^2 closer to 0. RMSE = 0,5536 for *E.polonica* indicating more random errors imposed on the observed data for this fungi compared to the other fungus.. The four fungal species shown in separate panales were; Ophiostoma- piceae (O.pic), Grosmannia-penicillata (G.pen), Ophiostoma- bicolor (O.bic) and Endoconidiophora-polonica (E.pol).

3.4 Result from the two-way ANOVA test

Table 6 sums up the result from the two-way ANOVA test in Model 2 using JUMP pro 13. The two-way ANOVA test in Model 2 revealed almost p < 0, 05 for all source variables, apart from the source variable “Treatment x Isolate” in *O.piceae* and “Isolate” in *E.polonica*. Low p values in the two-way ANOVA indicated that there was need to use post hoc analysis to understand where possible differences in the growth response variables were to be found within the source variables.

Table 6. **Overview two-way ANOVA test result.** For most source variables in Model 2, the p values were < 0,05, apart from the source variable "Treatment * Isolate" for O.pic and "Isolate" for E.pol. The four fungi in this table were; Ophiostoma- piceae (O.pic), Grosmannia- penicillata (G.pen), Ophiostoma- bicolor (O.bic) and Endoconidiophora-

Fungi	Source variable	DF	Sum of Squares	F Ratio	Prob > F
O.pic	Isolate	1	0,36	5.52	0.0226
	Treatment	3	9,64	49.16	<.0001
	Treatment*Isolate	3	0,52	2.67	0.0573
G.pen	Isolate	1	1,32	37.36	<.0001
	Treatment	3	36,58	344.34	<.0001
	Treatment*Isolate	3	0,51	4.79	0.0048
O.bic	Isolate	1	24,34	398.73	<.0001
	Treatment	3	8,56	46.74	<.0001
	Treatment*Isolate	3	16,05	87.65	<.0001
E.pol	Isolate	1	1,06	3.46	0.0698
	Treatment	3	4,38	4.76	0.0059
	Treatment*Isolate	3	15,22	16.55	<.0001

polonica (E.pol). The p values < 0,05 are highlighted in red.

3.5 Result from Tukey HSD test

Figure... sums up the result from the Tukey HSD test. The Tukey HSD shown in figure 3 indicated that some MT treatments affected mycelium growth significantly different when compared with an isolate, between isolates A and B within a fungus and between the four fungi. It is interesting that response to MT treatments for isolate A and B in *E.polonica* and *O.bicolor* were not as similar as for isolate A and B in *O.piceae* and *G.penicillata*.

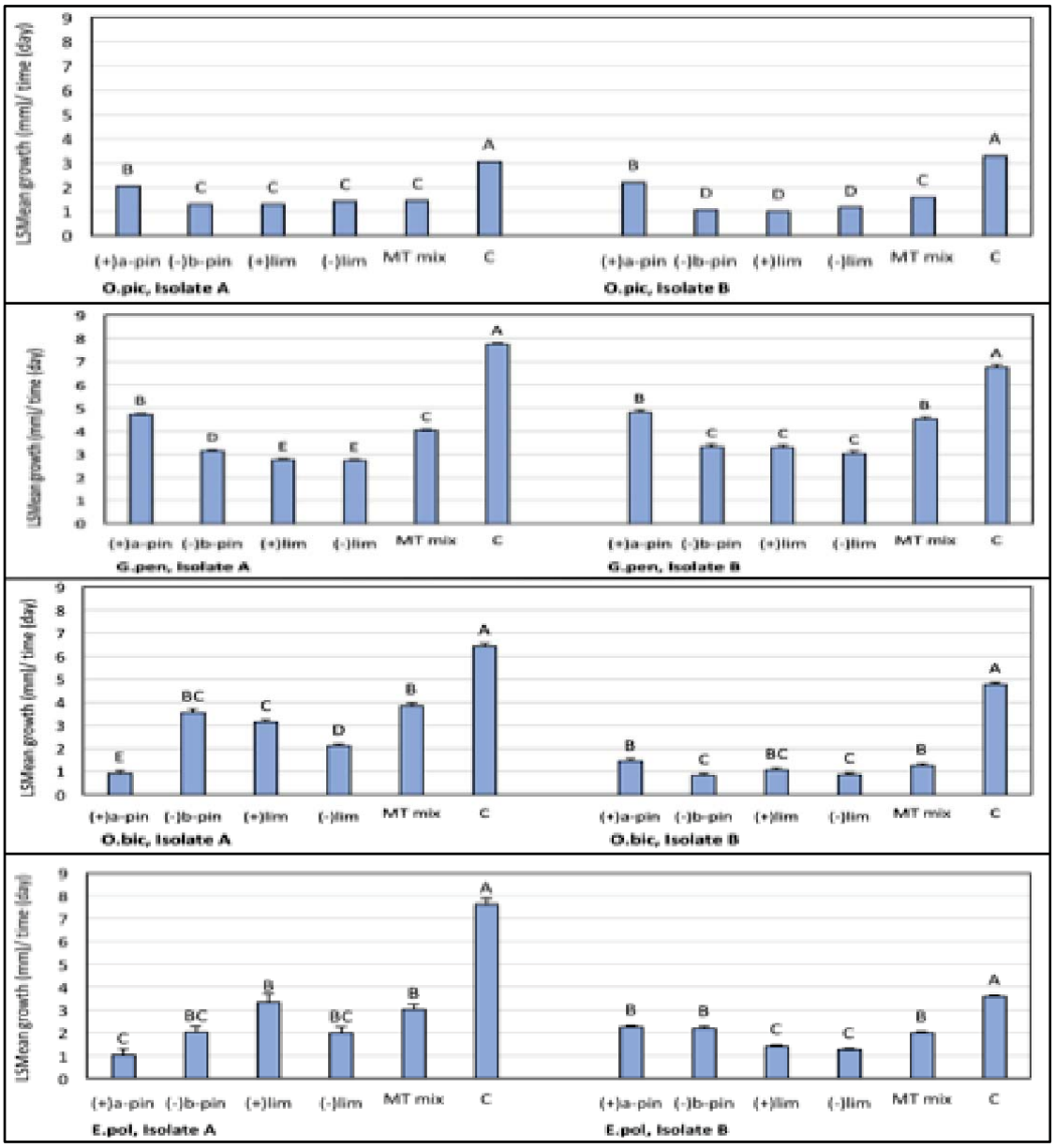

Figure 3. Tukey HSD test result for the source variable treatment in Model 2 for all isolates with an α =0,05. Different capital letters over the bars signalize significant differences in growth response to the terpene treatments used in the growth inhibiting experiment. The standard error is shown as error bars over the bars. Growth is expressed on the y-axis as; LS mean growth (mm)/ time (day). On the x-axis the six treatments in the growth inhibiting experiment for two isolates; (+)-α-pinene ((+) a-pin), (-)-β-pinene ((-) b-pin), (-) limonene ((-) lim), (+) limonene ((+) lim), monoterpene mix (MT mix), control (C). The fungal species shown in separate panels were; *Ophiostoma- piceae* (O.pic) isolate A = 1998-50/3 and isolate B =1980-92/34, *Grosmannia- penicillata* (G.pen) isolate A =1980-91/54 and isolate B =1960-21, *Ophiostoma- bicolor* (O.bic) isolate A = 1980-48/36 and isolate B =2004-38/1 and *Endoconidiophora-polonica* (E.pol) isolate A = 1994-169/113 and isolate B = 1993-208/115

3.6 Result from percetnage calculation

Table 7 shows all percentages differences of growth rate between terpene treatment and control within all isolates in the five terpene treatments. High value of percentage indicates a strong growth inhibiting effect from terpene treatments on the fungi. Isolate A and B in each fungus had not the same growth rate when treated with terpenes as can be seen from the percentage values in table 7. The variation in growth rate within isolate A and B also reflected variation in growth rate for the fungus under terpene treatments.

Table 7. Percentage of unequal growth rate between terpene treatment and control within all isolates in the growth inhibiting experiment and total average from each terpene treatment. The percentage value was found by using the growth slope value from linear regression in all replicates within every treatment and using its average value to calculate the relation between terpene treated mycelium and control, expressed as % in the table. High % value indicates strong growth inhibiting effect. The fungal species were; *Ophiostoma-piceae* (O.pic) isolate A = 1998-50/3 and isolate B =1980-92/34, *Grosmannia- penicillata* (G.pen) isolate A =1980-91/54 and isolate B =1960-21, *Ophiostoma- bicolor* (O.bic) isolate A = 1980-48/36 and isolate B =2004-38/1 and *Endoconidiophora-polonica* (E.pol) isolate A = 1994-169/113 and isolate B = 1993-208/115. The six treatments were;(+)-α-pinene ((+) a-pin), (-)-β-pinene ((-) b-pin), (-) limonene ((-) lim), (+) limonene ((+) lim), monoterpene mix (MT mix), control (C). Detail of MT mix content for isolates can be seen in section 2.3.7

Fungi	Isolate	MT mix	(+) a-pin	(-) lim	(-) b-pin	(+) lim
O.pic	A	53 %	33 %	54 %	58 %	58 %
O.pic	B	52 %	33 %	65 %	68 %	70 %
G.pen	A	48 %	40 %	63 %	57 %	66 %
G.pen	B	23 %	24 %	53 %	53 %	48 %
O.bic	A	40 %	86 %	64 %	46 %	52 %
O.bic	B	75 %	63 %	78 %	80 %	69 %
E.pol	A	61 %	86 %	75 %	74 %	64 %
E.pol	B	50 %	31 %	62 %	9 %	58 %

3.7 Fungal growth

Figures bellow shows how the fungal growth (mm)/time (days) was for all included replicates. The growth curve for isolate A in *O.bicolor* and *E.polonica* can seem odd, like time measurement suddenly stopped and is missing when compared to isolate B. The growth curve for isolate A in *O.bicolor* and *E.polonica* grew fast, so for the first time measurement the growth was several mm but when growth (mm)/time (days) were

calculated this effect is masked since the data from first time measurement becomes a cero start reference for growth between time measurements.

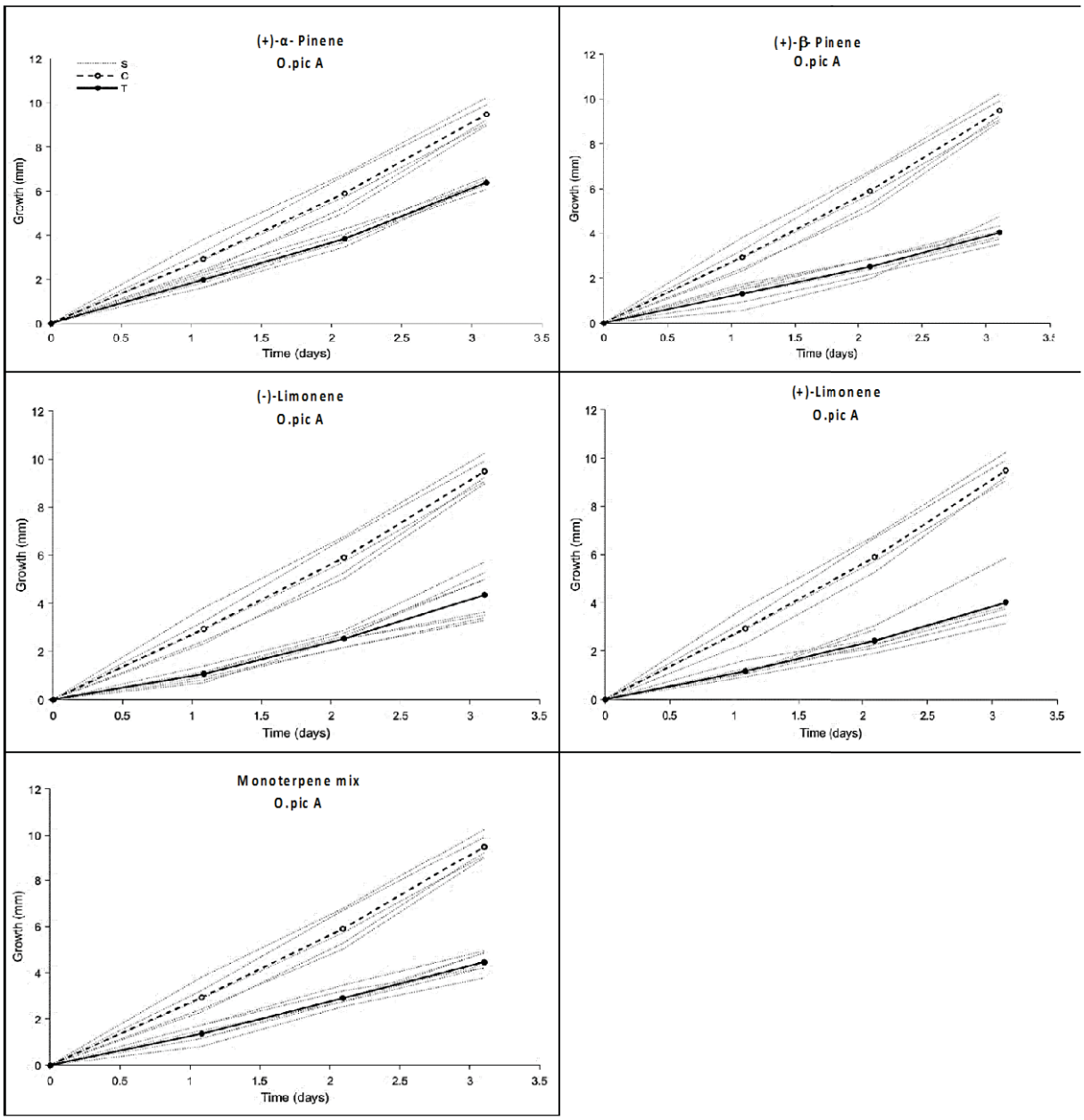

Figure 4. Growth inhibiting experiment with five selected terpene treatments for one strain of *Ophiostoma piceae* (O.pic A =1998-50/3). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (+)-3-Carene). Control replicates were left untreated. Pure culture was made 17.01.2014, inoculation from pure culture for growth inhibiting experiment was done 01.02.14 and the first radial growth measurement happened at 05.02.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure lines ⋯, -o- , —● shown together with initials **S**, **C** and **T** in the figure legend respectively, stand for the observed growth given by the collected radial growth measurements were; **S**= single replicate growth, C= average growth from the control replicates, **T**= average growth from the terpene treated replicates.

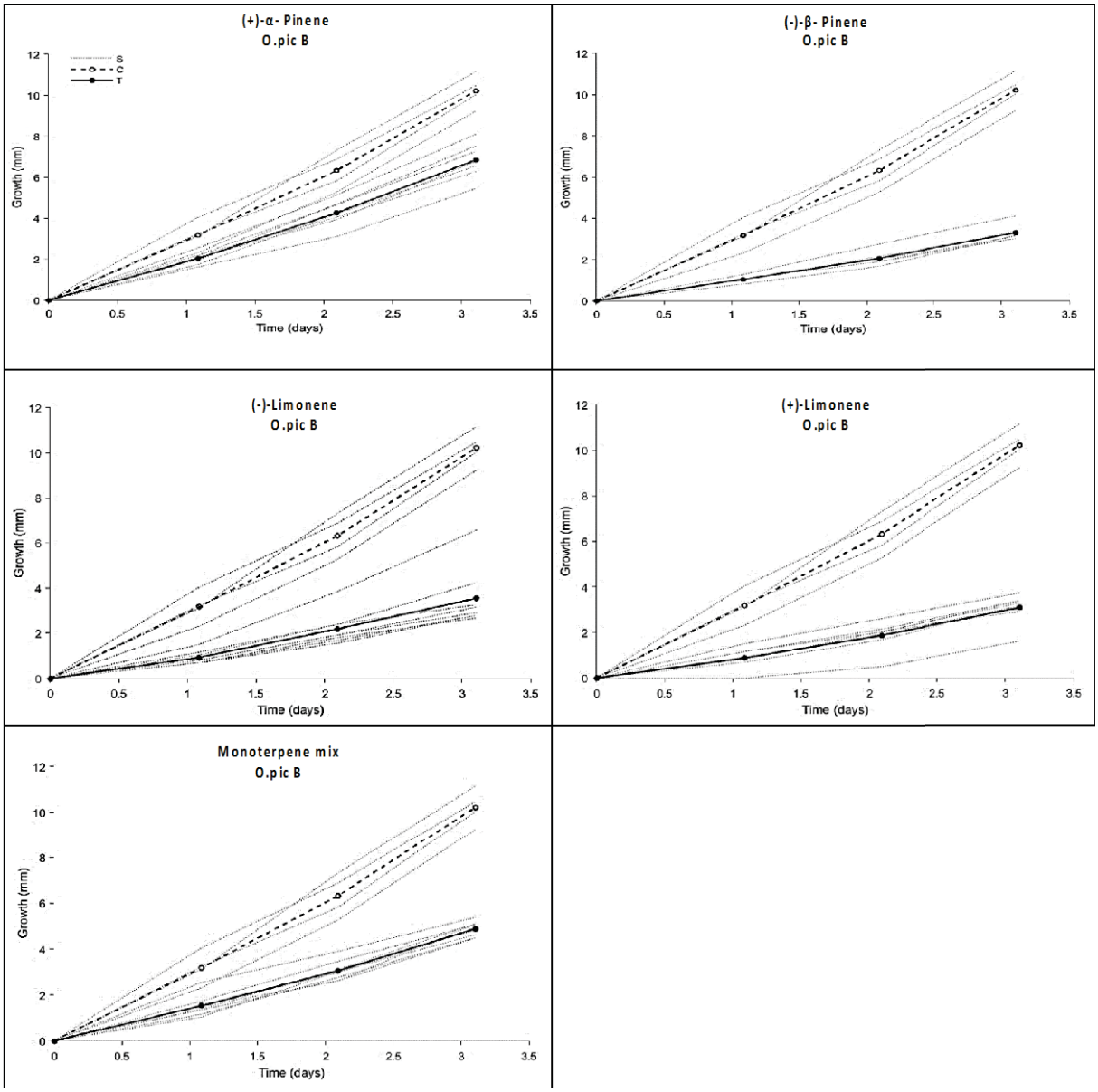

Figure 5.Growth inhibiting experiment with five selected terpene treatments for one strain of *Ophiostoma piceae* (O.pic B = 1980-92/34). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (+)-3-Carene). Control replicates were left untreated. Pure culture was made 17.01.2014, inoculation from pure culture for growth inhibition experiment was done 01.02.14 and the first radial growth measurement happened at 05.02.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines , , shown together with initials **S**, **C** and **T** in the figure legend respectively, stand for the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

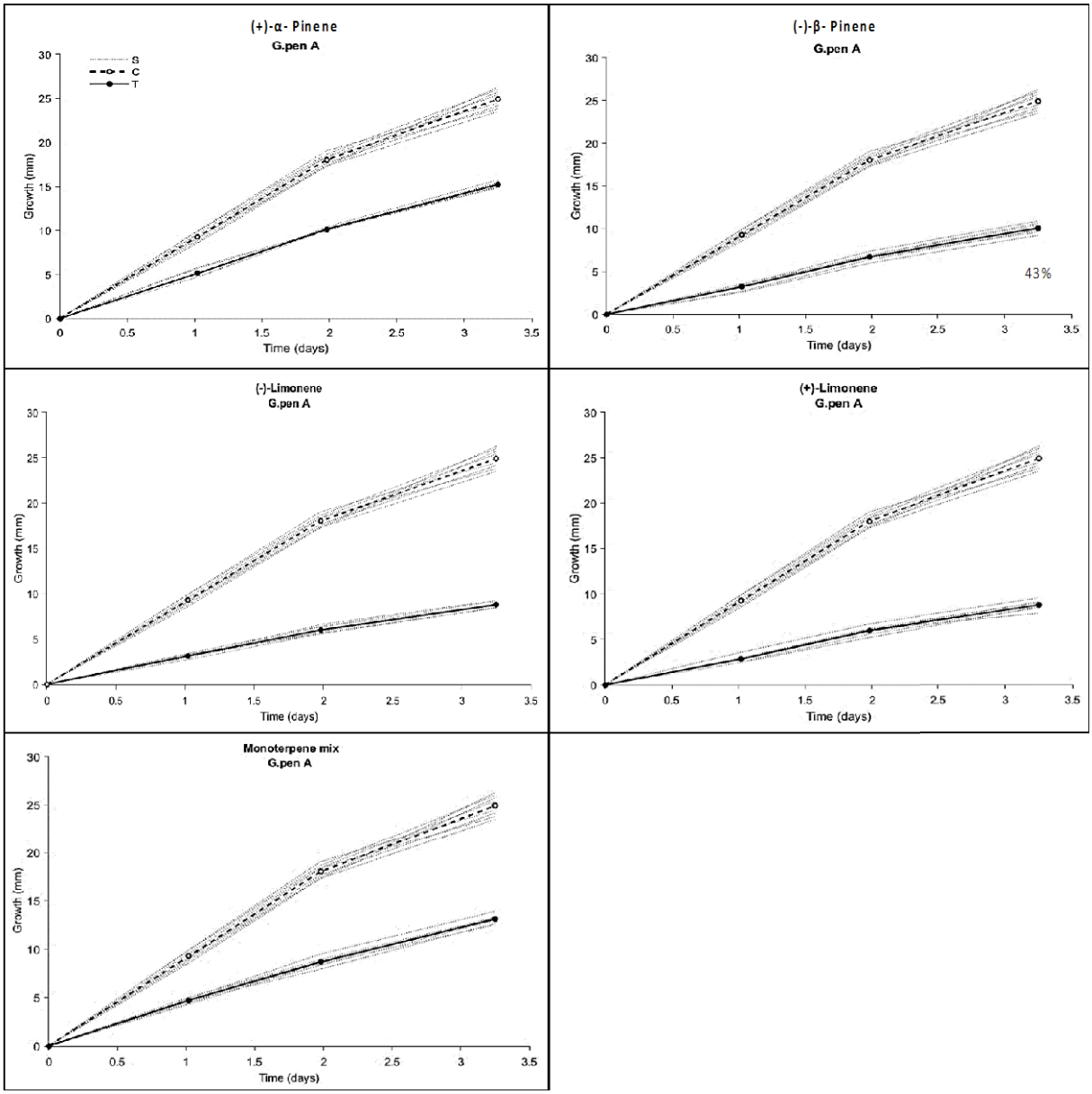

Figure 6. Growth inhibiting experiment with five selected terpene treatments for one strain of *Grosmannia penicillata* (G.pen A = 1980-91/54). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (+)-3-Carene). Control replicates were left untreated. Pure culture was made 17.01.2014, inoculation from pure culture for growth inhibition experiment was done 01.02.14 and the first radial growth measurement happened at 05.02.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ,-o-,— shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

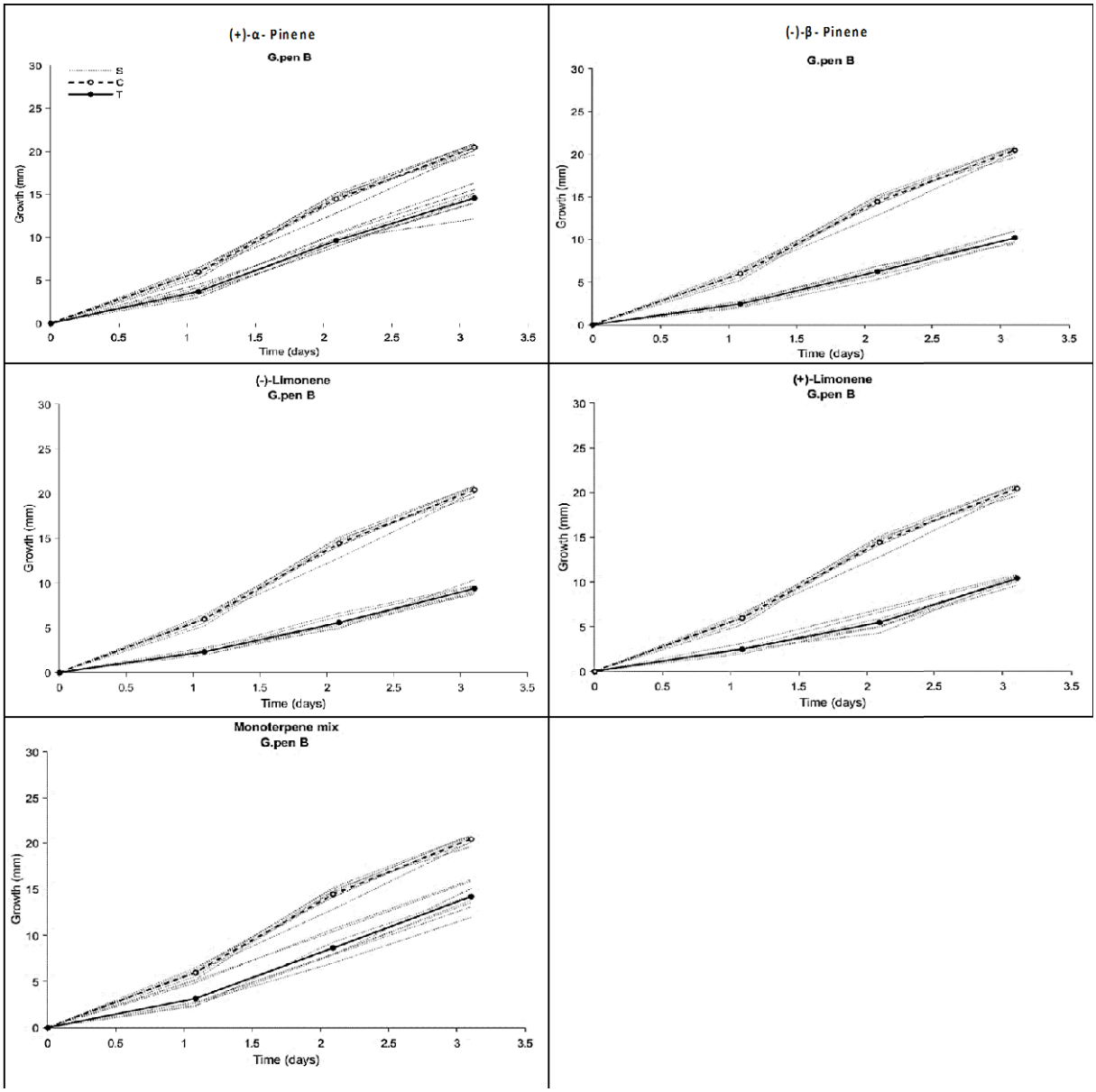

Figure 7. Growth inhibiting experiment with five selected terpene treatments for one strain of *Grosmannia penicillata* (G.pen B =1960-21). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (-)-Limonene). Control replicates were left untreated. Pure culture was made 01.05.2014, inoculation from pure culture for growth inhibition experiment was done 17.05.14 and the first radial growth measurement happened at 20.05.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ⋯, -o-, —● shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

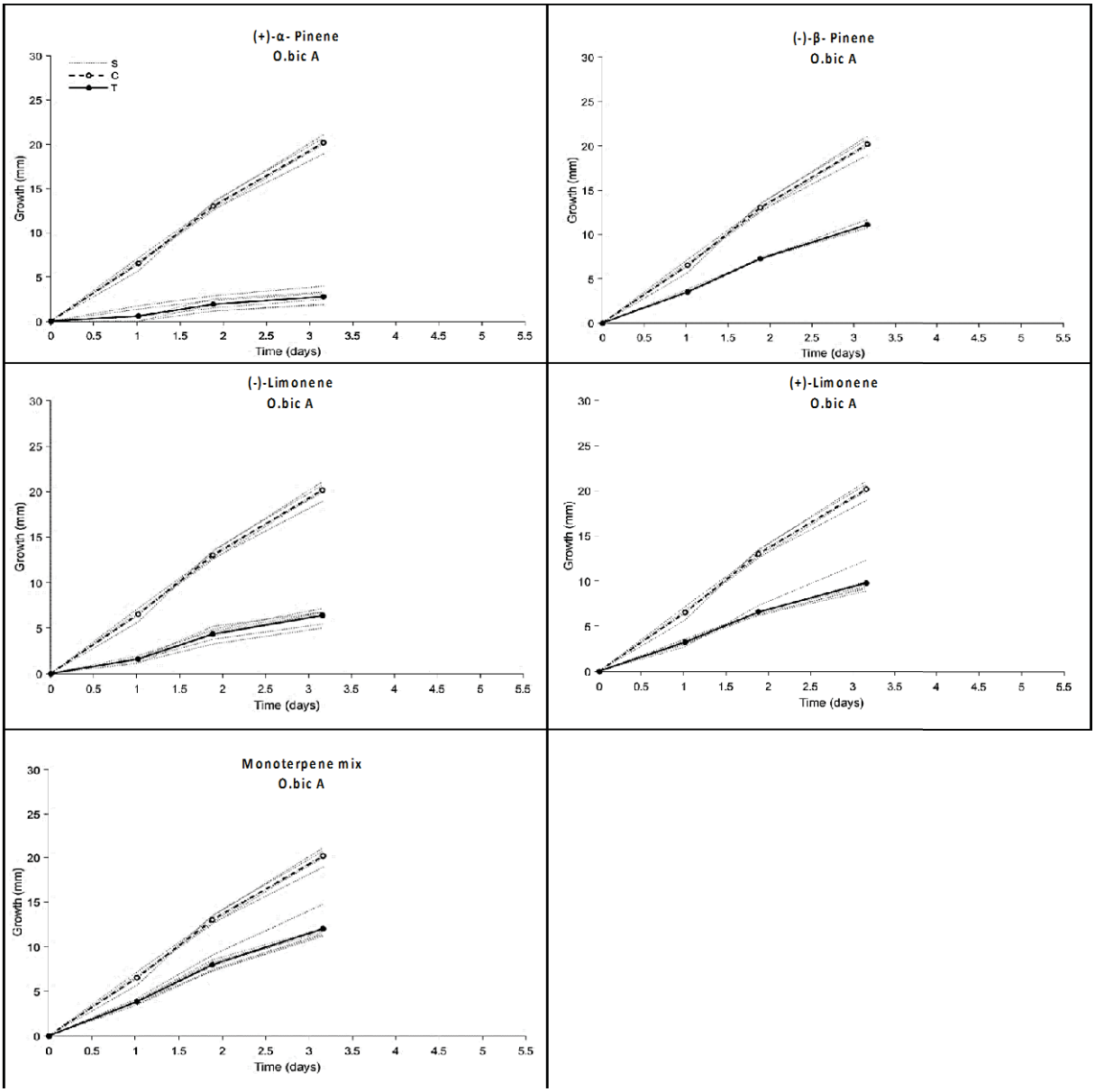

Figure 8. **Growth inhibiting experiment with five selected terpene treatments for one strain of *Ophiostoma bicolor* (O.bic A = 1980-48/36).** Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (+)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (-)-Limonene). Control replicates were left untreated. Pure culture was made 01.05.2014, inoculation from pure culture for growth inhibition experiment was done 17.05.14 and the first radial growth measurement happened at 20.05.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ⋯, -o- , ⟶ shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

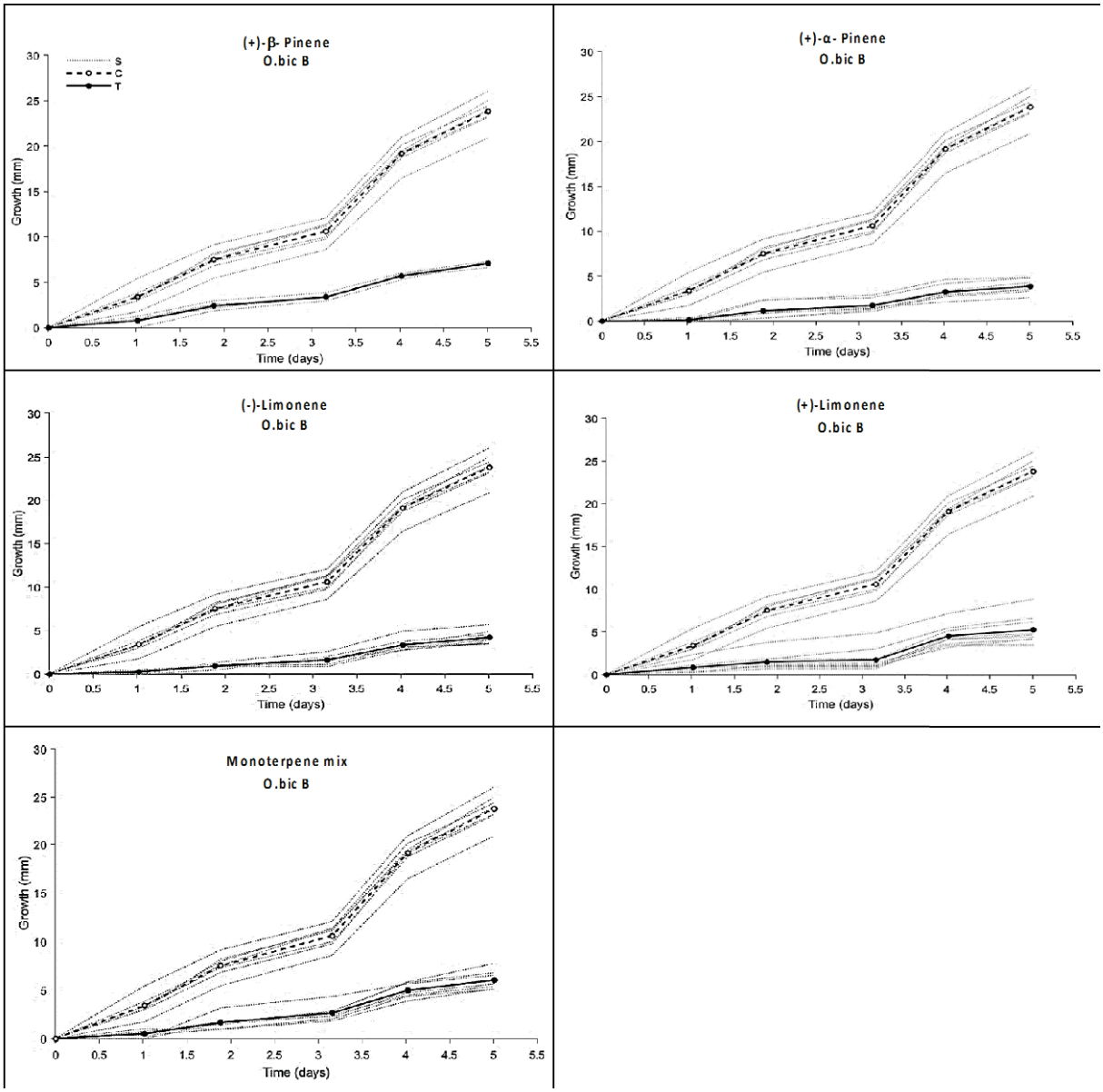

Figure 9. Growth inhibiting experiment with five selected terpene treatments for one strain of *Ophiostoma bicolor* (O.bi B = 2004-38/1). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (-)-Limonene). Control replicates were left untreated. Pure culture was made 01.05.2014, inoculation from pure culture for growth inhibition experiment was done 17.05.14 and the first radial growth measurement happened at 20.05.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ⋯, -o-, ⟶ shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

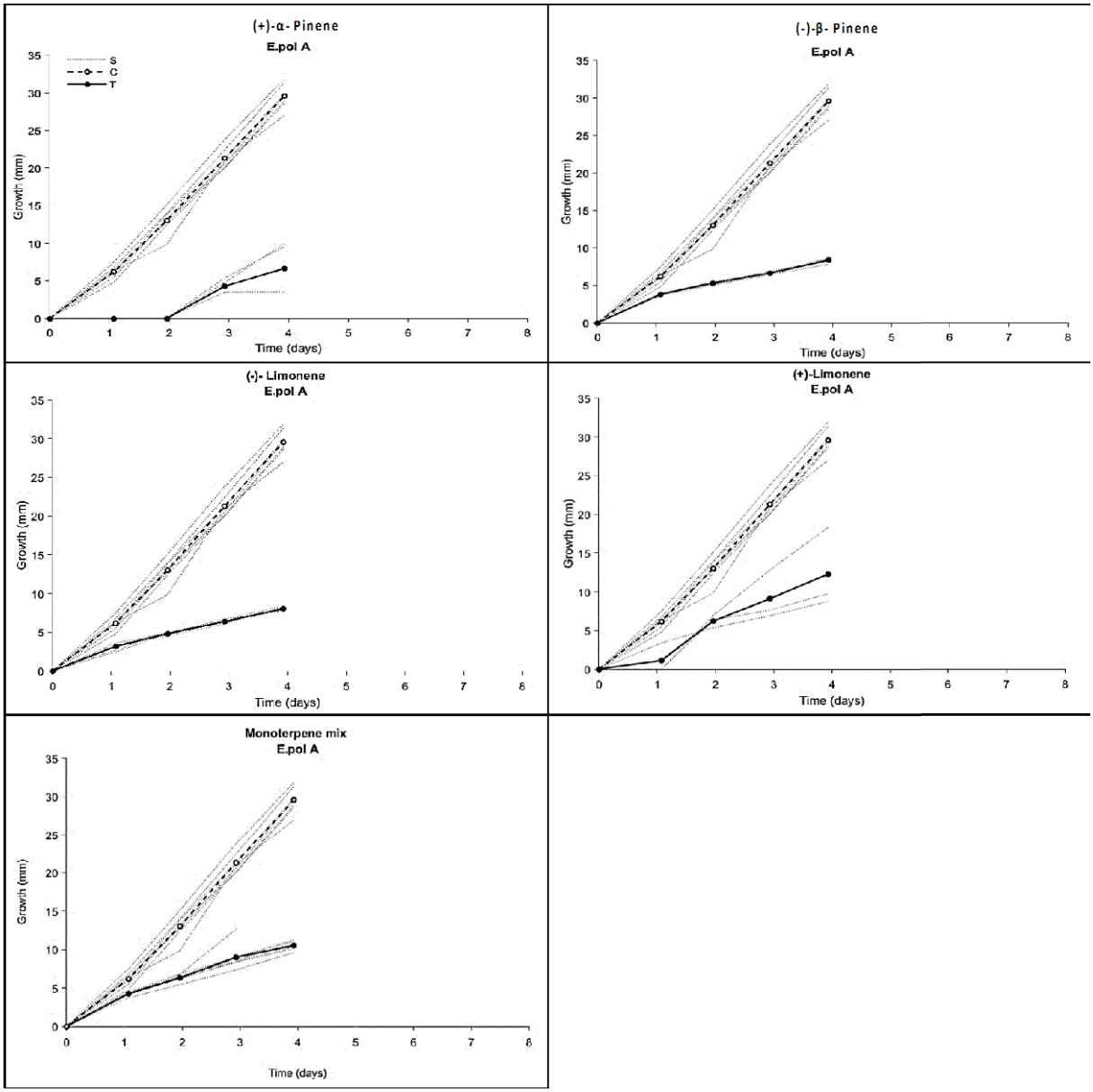

Figure 10. Growth inhibiting experiment with five selected terpene treatments for one strain of *Endoconidiophora polonica* (E.pol *A* = 1994-169/113). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (-)-Limonene). Control replicates were left untreated. Pure culture was made 20.07.2014, inoculation from pure culture for growth inhibition experiment was done 09.08.14 and the first radial growth measurement happened at 11.08.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ····· , -o- - , —●— shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements were; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

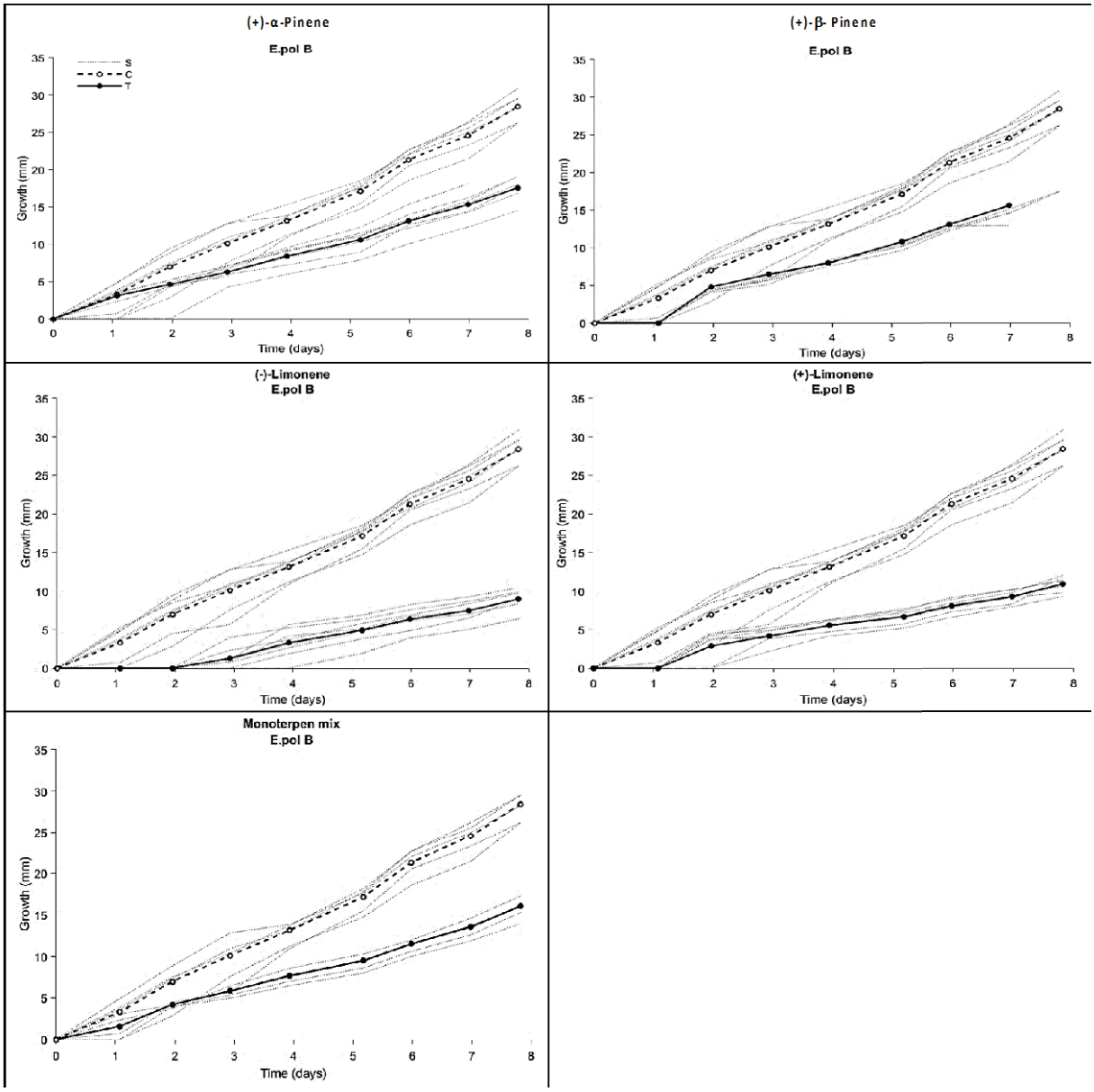

Figure 11. Growth inhibiting experiment with five selected terpene treatments for one strain of *Endoconidiophora polonica* (E.pol B = 1993-208/115). Replicates with terpene treatment in mycelium growth environment consisted of 200 µl neat individual monoterpenes like (+)-α-pinene or (-)-β-pinene or (+) limonene or (-) limonene or monoterpene mix (ca 1:1:1:1 concentration of (+)-α-Pinene, (-)-β-Pinene, (+) Limonene and (-)-Limonene). Control replicates were left untreated. Pure culture was made 20.07.2014, inoculation from pure culture for growth inhibition experiment was done 09.08.14 and the first radial growth measurement happened at 11.08.14. When mycelium in control treatment reached the edge of the plate, radial growth measurements stopped. The figure the lines ⋯, -o-, —● shown together with initials **S**, **C** and **T** in the figure legend respectively, represents the observed growth given by the collected radial growth measurements where; **S**= singe replicate growth, **C**= average growth from the control replicates, **T**= average growth from the treated replicates.

4. Discussion

All blue stain fungi had less growth under the influence of terpene treatments which is seen with the capital letter A over the bar for the control treatment in figure ..... from the Tukey HSD test. Isolate A grew almost twice as fast as isolate B for *E.polonica* and *O.bicolor* and from figure ... it is possible to see that the isolate A for both fungi were a bit less affected by the terpene treatments, than isolates that had more similar growth pace which was the case for isolate A and B in *O.piceae* and *G.penicillata*. The result discussed above might indicate that growth capacity is a characteristic for terpene tolerance which also is mentioned in Krokene et al., 1996 as follows " Pythopathogenic blue stain fungi have several adaptions that increase their ability to grow in fresh host tissues, among them higher growth rates and the ability to grow at lower oxygen pressures than most other blue stain fungi".

From the Tukey HSD test shown in figure 3, MT treatments had different effect on the isolates tested and between the fungi. It was expected to find clear inhibition from the MT treatment (-) limonene as a study from Novak et al.,2013 indicated that (-) limonene inhibited fungal growth where they suggested "that it is an important induceddefense metabolite involved in Norway spruce resistance to primary fungal invaders such as *E. polonica*". In the Tukey HSD analysis for this growth inhibiting experiment isolate B in *E.polonica* had the highest growth inhibiting effect while (+)-α-pinene was the most inhibiting terpene treatment for isolate A.

For the MT mix that consisted of different MT compounds the Tukey HSD clearly indicated an effect that differed from the control but between other MT treatments only in *G.penicillata* isolate A there was significant differences from the other MT treatments.

The weaknesses for conducting this growth inhibiting experiment was the lack of air tight containers since the volatile terpenes leaked out from the sealing and because the sealing kind of melted away because of the interaction between the plastic and volatile terpene molecules. This caused problems because leakages could be found perhaps long time after it appeared due to small size on the hole from the sealing plastic around the glass petri dishes. Leakages could perhaps upregulate growth for the blue stain fungi when normal O_2 levels increased in the growth environment.

Having only two samples of isolates for each fungus was too small sample size to begin to evaluate what kind of fungi had the highest of lowest growth rate. An approach to the evaluate growth between the fungi was done by calculating percentages. But these did not show any clear differences too since the isolates had high variation in growth rate.

In conclusion, MT treatments affect growth differently and MT mix did not have much significantly effect on fungal growth when compared to the MT treatments.

Litteratur:

(DiGustini, 2011)

(Hammerbacker et al., 2013)

(Krokene and Solheim, 1998)

(Krokene and Solheim, 1997)

(Krokene and Solheim, 1996)

(Novak et al.,2013)

(Vega & Hofstetter 2015).

(Six, 2012)

(Wang et al., 2014)

Litterature, abstract included:

(Beaver, 1989). R. A. Beaver. “Insect –Fungus Relationship in the Bark and Ambrosia Beetles” in book: Insects – fungus interactions, Chapter 5, Editors; Wilding, Collings, Hammond, Webber, pp. 121-143

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Program used:

R version 3.3.2 (2016-10-31) -- "Sincere Pumpkin Patch" Copyright (C) 2016 The R Foundation for Statistical Computing. Platform: x86_64-w64-mingw32/x64 (64-bit)

JMP Statistical Discovery. ™ From SAS. JMP ® Pro 13.0.0 Organization: NMBU-JMP PRO WIN 64. Site ID 70102514. Expires 14.12.2017

MATLAB ® R2015a (8.3.0.197613) 64-bit (win 64) February 12, 2015. License Number: 969765

Appendix 1

Information about the isolates

Isolat navn:	2004-38/1
Sopp art	*Ophiostoma bicolor* R.W. Davidson & D.E. Wells, In Davidson, Mycologia 47: 63. 1955
Sted	Norge: Akershus: Ås: Nær Årungen
Substrat:	*Orthotomicus laricis.*
Samlet av:	Halvor Solheim, Darko Dubak 15.06.2004
Isolert av:	Jolanda Roux 15.06.2004
Konserveringsmedium	MA (10.01.2007)
Konserveringsmetode	Ultra- dyp- fryser (-152°), fryser- ID: 4/29/E5 (16.01.2007)
Bestemt av:	Halvor Solheim

Isolat navn:	1980-48/36
Sopp art	*Ophiostoma bicolor* R.W. Davidson & D.E. Wells, In Davidson, Mycologia 47: 63. 1955
Sted	Norge: Akershus: Ås : Sløstad
Substrat:	Picea abies (Gran). Stamme, stående- Ved. *P.abies*, drept av *Ips typographus*, blåvedfarget ved.
Samlet av:	
Isoleringstype:	W
Konserveringsmedium	MA (14.12.2006)
Konserveringsmetode	Ultra dyp fryser (-152°), fryser- ID: 4/29/E5 (16.01.2007)
Bestemt av:	Halvor Solheim

Isolat navn:	1980-91/54
Sopp art	*Grosmannia penicillata* (Grosmann) Goid., Boll. Staz. Patol. Veg. Roma 15: 156. 1935
Sted	Norge: Akershus: Ås: Slørstad
Substrat:	*Picea abies* (Gran). Stamme, stående- Ved. Blå ved i billedrept tre
Samlet av:	Halvor Solheim
Isoleringstype:	W
Konserveringsmedium	PCA medium + *P.abies* (14.03.2007)
Konserveringsmetode	Ultra dyp fryster (-152°), fryser- ID: 2/15/H7 (25.09.2000)
Bestemt av:	Halvor Solheim (*Ophiostoma penicillatum*)

Isolat navn:	1960-21
Sopp art	*Grosmannia penicillata* (Grosmann) Goid., Boll. Staz. Patol. Veg. Roma 15: 156. 1935
Sted	Sverige: Jämtlands län: Strömsund: Strömsund:
Substrat:	*Picea abies* (Gran). Isolat fra ganger av *Ips typographus*.
Samlet av:	Aino Käärik
Isoleringstype:	W
Konserveringsmedium	MA(14.03.2007)
Konserveringsmetode	Ultra dyp fryser (-152°), fryser- ID: 2/15/H7 (25.09.2000)

Isolat navn:	1980-92/34
Sopp art	*Ophiostoma piceae* (Münch) Syd., In Sydow & Sydow, Annls mycol. 17: 43. 1919
Sted	Norge: Akershus: Ås: Sløstad
Substrat:	*Picea abies* (Gran).
Samlet av:	Halvor Solheim
Isoleringstype:	W
Konserveringsmedium	MA
Konserveringsmetode	Ultra dyp fryser (-152°), fryser- ID: 4/29/E5 (16.01.2007)
Bestemt av:	Halvor Solheim (20.03.1981)

Isolat navn:	1998-50/3
Sopp art	*Ophiostoma piceae* (Münch) Syd., In Sydow & Sydow, Annls mycol. 17: 43. 1919
Sted	Norge: Oppland:Gjøvik:Vardal
Substrat:	*Picea abies* (Gran). Såringsforsøk. Merket V1/3/18
Samlet av:	
Isoleringstype:	W
Konserveringsmedium	MA (21.03.2007)
Konserveringsmetode	Ultra dyp fryser (-152°), fryser- ID: 4/30/G6 (19.04.2007)
Bestemt av:	Halvor Solheim

Isolat navn:	**1993-208/115**
Sopp art	***Endoconidiophora polonica*** **(Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf**
Sted	**Norway: Akershus: Ås**
Substrat:	***Picea abies*** **(Gran). Stamme, stående- ved.**
Samlet av:	**Paal Krokene 24.08.1993**
Isoleringstype:	**W**
Konserveringsmedium	**MA (14.12.2006)**
Konserveringsmetode	**Ultra dyp fryser (-152°), fryser- ID: 2/14/F4, ID2: 4/28/A7**
Bestemt av:	**Paal Krokene, Halvor Solheim (*Ophiostoma polonicum*)**

Isolat navn:	**1980-53/7A**
Sopp art	***Endoconidiophora polonica*** **(Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf**
Sted	**Norge: Akershus: Ås: Slørstad**
Substrat:	***Picea abies*** **(Gran). Levende tre angrepet av *Ips typographus***
Samlet av:	**C. Libach 17.06.1980**
Isoleringstype:	**W**
Konserveringsmedium	**MA (08.09.2006)**
Konserveringsmetode	**Ultra dyp fryser (-152°), fryser- ID: 2/14/G1 (23.08.2000)**
Bestemt av:	**Halvor Solheim**

* MA; Malt extract agar
* PCA; Potatoe carrot extract agar.
* 10 % of the liquid in the sample stored in the deep freezer (-152°), contained glycerol said senior engineer Gro Wollbæk at NIBO.

Appendix 2

Common synonyms for the terpene chemicals

Name for terpen used	Synonym for terpen used:
(*R*)-(+)-Limonene	(+)-*p*-Mentha-1,8-diene (+)-Carvene, (*R*)-4-Isopropenyl-1-methyl-1-cyclohexene
(*S*)-(−)-Limonene	(−)-*p*-Mentha-1,8-diene (−)-Carvene (*S*)-4-Isopropenyl-1-methyl cyclohexene
(+)-3-Carene	1*S*)-3,7,7-Trimethylbicyclo[4.1.0]hept-3-en
(+) -α-Pinene	(1*R*,5*R*)-2-Pinene, (1*R*,5*R*)-2,6,6 Trimethylbicyclo[3.1.1]hept-2-ene
(−)-β-Pinene	(-)-beta-Pinene (1*S*)-(−)-β-Pinene (1*S*,5*S*)-2(10)-Pinene (1*S*,5*S*)-6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane

Appendix 3

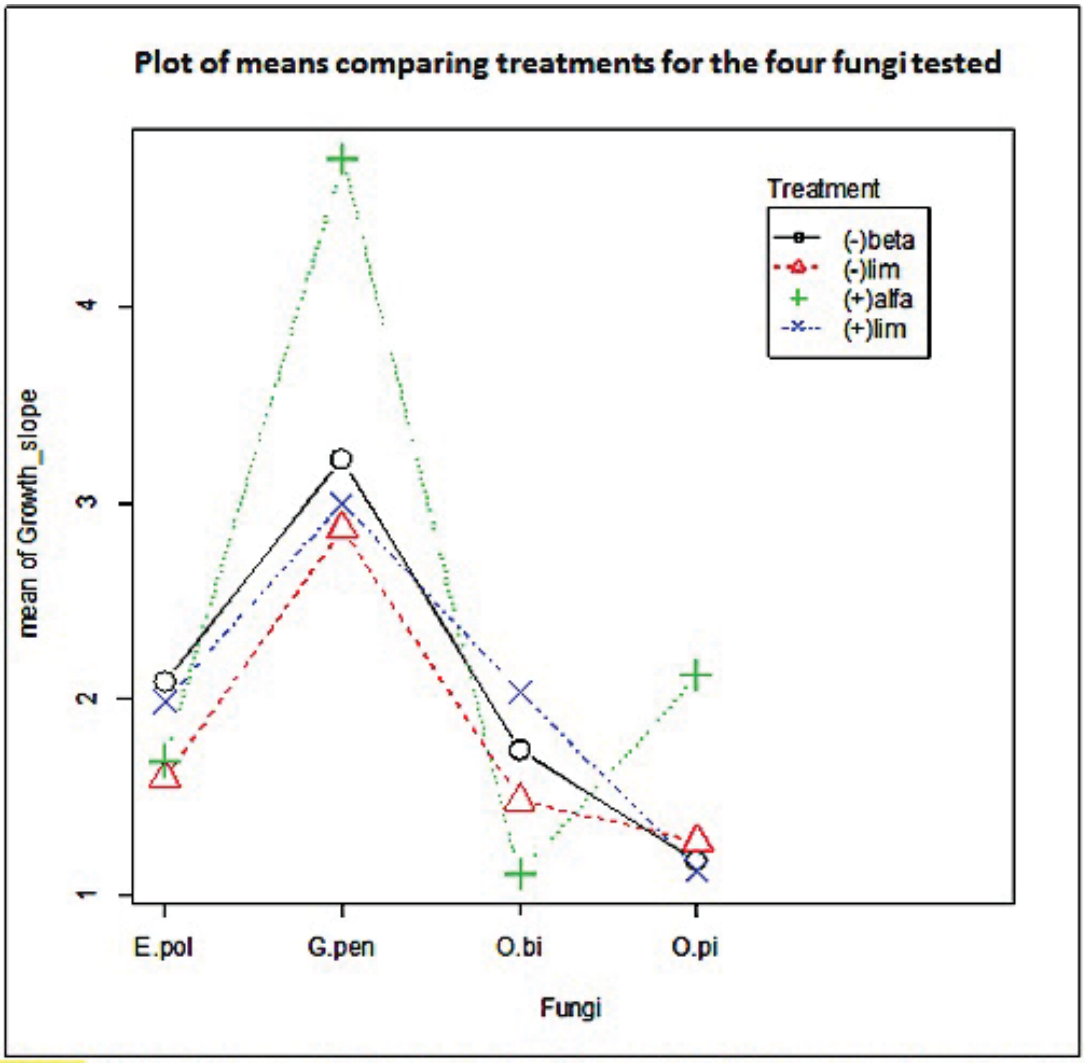
Type of experiment Date started:	Pilot growth inhibiting experiment Onsdag 08.01.2014
Date finished:	Tirsdag 21.01.2014
Species of fungi tested:	*E.polonica*

Treatment	4 MT mix. (α-pinene, -α pinene, -β pinene og + limonene)				4 MT mix. (α-pinene, -α pinene, -β pinene og + limonene)			
Sample nr	1				2			
Axis →								
Date and time ↓	1	2	3	4	1	2	3	4
13.01.14 16:00								
15.01.14 11:15								
17.01.14 16:20								
20.01.14 18:30								
21.01.14 16:20								

Scheme for collecting data from samples (replicates with treatments) in the growth inhibition laboratory experiment

Appendix 4

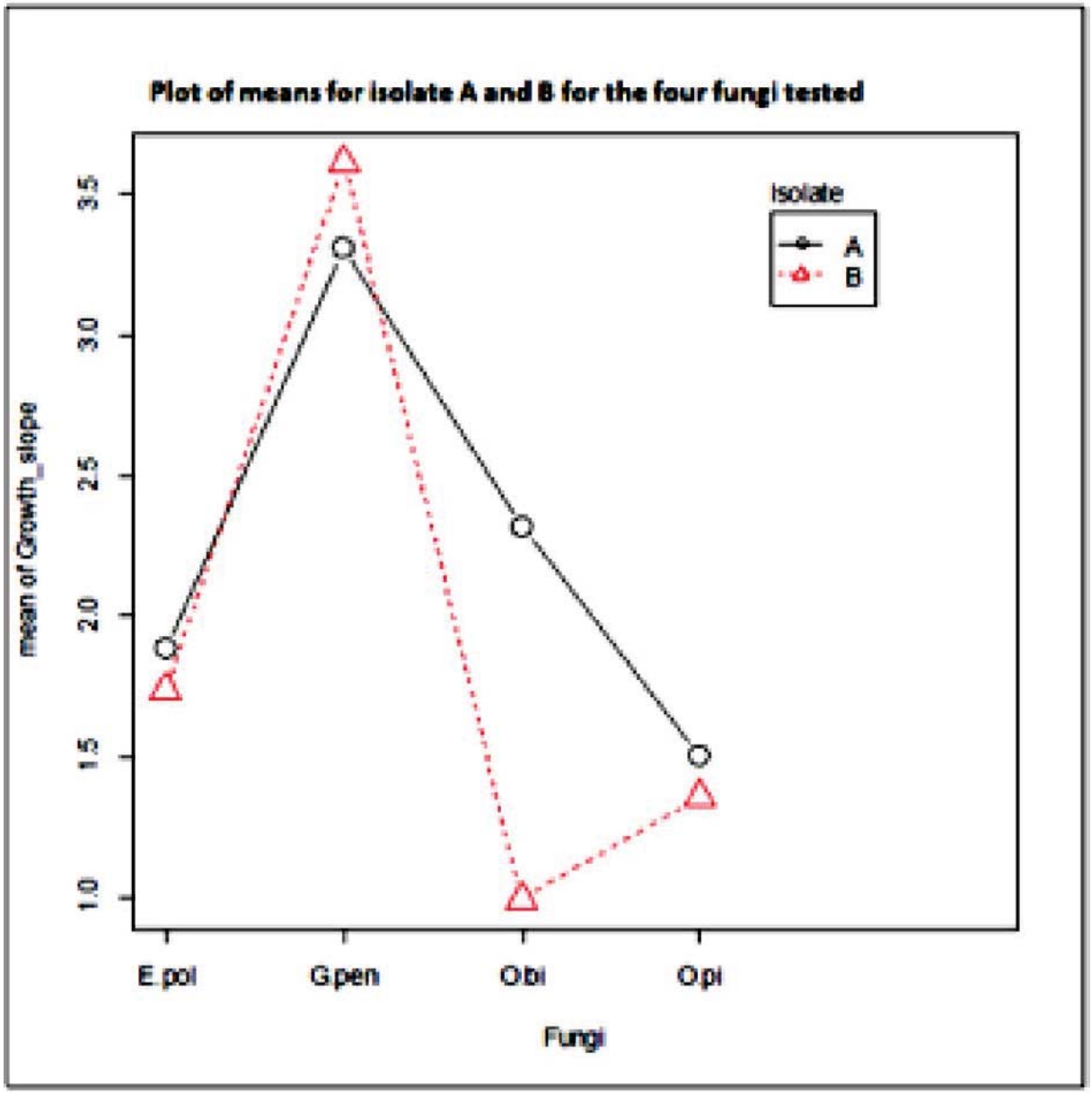
Plot of means comparing treatments for the four fungi tested



Plot of means made to check after observable effects from treatments for each blue stain fungi species tested. The plot of means was made from the data available to test statistical model 2 as shown in section 2.5. On the x-axis the shortened name for the blue stain fungi used is; E.pol, G.pen , O.bi, O.pi which stands for *E.polonica*, *G.penicillata*, *O.bicolor*, *O.penicillata* respectively. In the y-axis, the average mean growth slope from all replicates within every monoterpene treatment for each type blue stain fungi could be viewed in (mm)/ time (day). The legend box named "Treatment" shows the shortened name for the monoterpene treatment used in the growth experiment where; (+)-α-pinene ((+) a-pin), (-)-β-pinene ((-) b-pin), (-) limonene ((-) lim), (+) limonene ((+) lim), monoterpene mix (MT mix), control (C). The plot indicates that the terpene treatment (-) limonene, might be one of the most inhibiting monoterpene treatment in the growth inhibition experiment for the two blue stain fungi *E.polonica* and *G.penicillata*.

Appendix 5

Plot of means for isolate A and B for the four fungi tested



Plot of means for checking how different the growth for the two isolates for one species of blue stain fungus tested was. The plot of means was made from the data available to test statistical model 2 as shown in section 2.5. On the x-axis the shortened name for the blue stain fungi used is; E.pol, G.pen, O.bi and O.pi which stands for *E.polonica*, *G.penicillata*, *O.bicolor*, *O.penicillata* respectively. On the y-axis the mean growth slope from all replicates within one isolate either A or B for each type of blue stain fungi tested could be viewed in cm. From the plot, there is indication that isolate E.pol and O.pi have the most similar growth between the two isolate A and B. In G.pen some difference between the two isolate A and B could was indicated, but for O.bi the plot indicated huge differences in the average growth rate between the two isolate A and B.

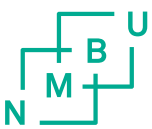
Appendix 6.

Average regression analysis values for all treatments in the growth inhibiting experiment.

Table continue next page

Fungi	Isolate	Letter	Treatment	Equation	R^2	Gr_slope	Av SD error
O.pi	503	A	K	y = 3,046x - 0,1951	0,996	3,046	0,426
O.pi	9234	B	K	y = 3,2783x - 0,2075	0,996	3,278	0,561
G.pen	9154	A	K	y = 7,8672x + 0,8273	0,989	7,867	0,655
G.pen	6021	B	K	y = 6,7552x - 0,3953	0,994	6,755	0,721
O.bi	4536	A	K	y = 6,4409x + 0,1502	0,997	6,441	0,601
O.bi	381	B	K	y = 3,3185x +1,7661	0,910	3,319	0,550
C.pol	169	A	K	y = 7,6332x - 1,0976	0,994	7,633	1,217
C.pol	208	B	K	y = 3,6271x - 0,0844	0,997	3,626	0,918
O.pi	503	A	MT mix	y = 1,4459x - 0,09	0,998	1,446	0,289
O.pi	9234	B	MT mix	y = 1,5671x - 0,0936	0,997	1,567	0,351
G.pen	9154	A	MT mix	y = 4,0074x + 0,3044	0,997	4,007	0,303
G.pen	6021	B	MT mix	y = 4,6445x - 0,8029	0,981	4,645	0,855
O.bi	4536	A	MT mix	y = 3,8598x + 0,0988	0,994	3,860	0,469
O.bi	381	B	MT mix	y = 0,7925x + 0,108	0,898	0,793	0,454
C.pol	169	A	MT mix	y = 2,9253x + 0,4822	0,992	2,925	1,020
C.pol	208	B	MT mix	y = 2,3394x - 0,8312	0,989	2,339	1,092
O.pi	503	A	(-) Alfa	y = 2,1652x - 0,1869	0,993	2,165	0,441
O.pi	9234	B	(-) Alfa	y = 2,202x - 0,1735	0,995	2,202	0,335
G.pen	9154	A	(-) Alfa	y = 4,7092x + 0,2629	0,996	4,709	0,409
G.pen	6021	B	(-) Alfa	y = 4,7996x - 0,5696	0,990	4,800	0,722
O.bi	4536	A	(-) Alfa	y = 0,925x - 0,0859	0,969	0,925	0,420
O.bi	381	B	(-) Alfa	y = 1,1069x + 0,2799	0,955	1,107	0,372
C.pol	169	A	(-) Alfa	y = 1,0294x - 0,7898	0,797	1,029	1,176
C.pol	208	B	(-) Alfa	y = 2,2892x - 0,4511	0,994	2,289	0,818
O.pi	503	A	(-) Beta	y = 1,2527x + 0,0177	1,000	1,253	0,182
O.pi	9234	B	(-) Beta	y = 1,0577x - 0,0602	0,997	1,058	0,177
G.pen	9154	A	(-) Beta	y = 3,1349x + 0,1067	0,107	3,135	0,282
G.pen	6021	B	(-) Beta	y = 3,312x - 0,5041	0,985	3,312	0,477
O.bi	4536	A	(-) Beta	y = 3,6435x + 0,17603	0,995	3,644	0,286
O.bi	381	B	(-) Beta	y = 0,5555x - 0,0007	0,923	0,556	0,383
C.pol	169	A	(-) Beta	y = 2,019x + 0,8268	0,956	2,019	0,175
C.pol	208	B	(-) Beta	y = 2,5385x - 1,3813	0,981	2,539	0,709
O.pi	503	A	(+) Lim	y = 1,3855x - 0,1897	0,981	1,386	0,518
O.pi	9234	B	(+) Lim	y = 1,0254x - 0,0597	0,996	1,025	0,234
G.pen	9154	A	(+) Lim	y = 2,7374x + 0,1236	0,994	2,737	0,349
G.pen	6021	B	(+) Lim	y =3,2913x - 0,5915	0,969	3,291	0,667
O.bi	4536	A	(+) Lim	y = 3,1379x + 0,1313	0,993	3,138	0,764
O.bi	381	B	(+) Lim	y = 0,8126x + 0,0637	0,924	0,813	0,492
C.pol	169	A	(+) Lim	y = 3,3282x - 0,8482	0,967	3,328	2,318
C.pol	208	B	(+) Lim	y = 1,4151x - 0,3705	0,982	1,415	0,640

O.pi	503	A	(-) Lim	y = 1,4024x - 0,2219	0,982	1,402	0,402
O.pi	9234	B	(-) Lim	y = 1,1531x - 0,1441	0,990	1,153	0,462
G.pen	9154	A	(-) Lim	y = 2,7126x + 0,2383	0,993	2,713	0,258
G.pen	6021	B	(-) Lim	y = 3,0333x - 0,4548	0,985	3,033	0,547
O.bi	4536	A	(-) Lim	y = 2,0963x - 0,1042	0,981	2,096	0,424
O.bi	381	B	(-) Lim	y = 0,6713x - 0,0676	0,938	0,671	0,431
C.pol	169	A	(-) Lim	y = 1,9904x + 0,5372	0,980	1,990	0,384
C.pol	208	B	(-) Lim	y = 1,2585x - 1,4279	0,953	1,259	0,520



Norges miljø- og biovitenskapelig universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway