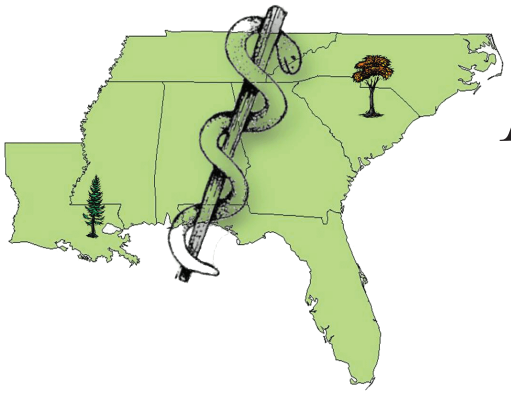

AUBURN UNIVERSITY
FOREST HEALTH COOPERATIVE

RESEARCH REPORT 17-03

EFFECT OF GROWTH RATE ON *AMYLOSTEREUM* SPP. FUNGUS BY TERPENES

by
Andrea Cole Wahl and Lori G. Eckhardt

ABSTRACT
Sirex noctilio is a species of woodwasp native to Europe that has been identified as invasive in Australia, South Africa, South America, New Zealand, and the northeastern United States. This pest has caused significant economic and ecological damage, and in some cases mortality of previously healthy trees. Females attack *Pinus* spp. by drilling into the xylem to oviposit eggs, venom, and a mutualistic fungus, causing trees to begin to die within days of inoculation. Certain chemicals emitted by stressed pines have been observed to serve as chemical attractants to the wasps. As a means of exploring pine resistance to *Sirex* associated fungi, the effect of these mentioned host plant secondary metabolites on the growth of these fungi were tested. Eighteen isolates of *Amylostereum* spp. were grown in saturated atmospheres or in direct contact with pure monoterpenes for 7 days. Fungal growth in the saturated atmosphere was measured on day 7 while the tactile experiment was measured at 3, 5, and 7 days. These experiments showed that certain metabolites such as 4AA, α-Phellandrene, (+) Camphene, and (-) Limonene significantly reduced growth of isolates compared to control treatments. Conversely, α-Pinene and β-Pinene treatments tended to increase growth rates of the fungal isolates. A difference in growth rates between isolates from the northern hemisphere and southern hemisphere also was observed. The treatments (+) α-Pinene and β- Myrcene resulted in the highest percentage of fungal growth for all isolates tested when comparing fungal growth as a percent area relative to the controls.

INTRODUCTION
Different species of *Amylostereum* fungi are associated with multiple *Sirex* (Hymenoptera: Siricidae) species from around the world, including *A. areolatum* (Chaillet) Boidin, *A. chailletii* (Pers.: Fries) Boidin, A. ferreum (Berk. & Curt.) Boidin & Lanquetin, and A. laevigatum (Fries) Boidin (Hurley et al., 2007; Slippers et al., 2003). *Amylostereum chailletti* is generally thought to be the species most often associated with the North American native *S. nigricornis* Fabricius, while *A. areolatum* is found in accordance with *S. noctilio* Fabricius. (Nielson et al., 2009). Worldwide, there are 100 species in the family Siricidae. Twenty three species of woodwasps are currently found in North America, including invasive species (Schiff et al., 2006). *Sirex noctilio* is an aggressively invasive species that has been associated with the symbiotic fungus *Amylostereum*, which along with phytotoxic venom causes wood of affected trees to rot (Bordeaux et al., 2014; Hurley et al., 2007). Adult females oviposit *Amylostereum* into the xylem tissue of the tree where it secretes cellulases, giving the siricid larvae a substrate to feed upon (Ayres et al., 2009; Kukor and Martin, 1983b).

Once a tree has been attacked by a *Sirex* spp. wasp, the tree begins to exhibit defensive behavior.

This behavior includes the production of a series of commonly produced terpenes by southern pines. Stress chemicals such as α-Pinene and β-Pinene are given off when a tree is under stress, and defense chemicals such as α-Phellandrene, and 4-allylanisole (4-AA), emitted by healthy pines, and are found in higher concentrations when trees are not under stress (Eckhardt et al., 2009). These chemicals, oleoresins, are found in different concentrations in each species of the *Pinus* genus. When produced, oleoresins of different chemistries have been shown to be capable of both inhibiting and encouraging fungal colonization, depending on species of fungus and chemical compound (Eckhardt et al., 2009; Himejima et al., 1992; Gershenzon and Dudareva, 2007). Paine et al. (1987) states that Phellandrene and Myrcene both have been shown to significantly impact the biota of microorganisms living within the host tree.

Chemicals such as those mentioned above may be artificially used as lure to bait live siricids to traps, indicating that these chemicals are attracting adult siricids to oviposit in stressed pine stands (Barnes et al., 2014). While previous studies have determined the interaction of terpenes with other plant parasitic fungi, there is still a lack of understanding how defense and stress chemicals found in pine trees affect fungal species associated with the invasive species *S. noctilio*. Since this species is rapidly spreading through pine stands in the southern hemisphere, and has been documented in the northeastern United States, a greater understanding of this fungal genus is warranted (Nielson et al., 2009). The primary objective of this study was to determine how different terpenes commonly produced by southern pine trees affect the growth of *Amylostereum* spp. fungi isolated from several locations worldwide.

MATERIALS AND METHODS

Isolation

Standard protocol has been established for the dissection of siricids to isolate fungal samples. Thomsen and Harding (2011) describe in detail these standard procedures. They were kept in a cooler in glass vials from the time of collection to the time of dissection. Wasps were killed with ethyl acetate, and then the exoskeleton was surface sterilized by brushing with 95% ethanol. Wasps were pinned and placed under a dissection microscope. Curved bladed micro- scissors were sterilized, and then inserted between the last tergal and sternal plate on either side of the ovipositor. The abdomen of the wasps was opened and pinned so that the dissector could remove eggs and body contents lying on top of the mycangia and venom sac. The mycangia were removed, gently brushed with 95% ethanol, and plated on a potato dextrose agar (PDA) plate. The mycangia were gently prodded until the spore mass is exuded on the plate. The spore mass was then transferred to another PDA plate. Thomsen and Harding (2011) suggested culturing two to three plates from each mycangia of each wasp dissected. This methodology was followed to obtain the two isolates of fungus from Alabama. The other isolates were obtained from the culture collection at the University of Pretoria, Forest and Agricultural Biotechnology Institute, South Africa.

Project design

Inoculation methods and chamber set up are in accordance with Eckhardt et al. (2009). Terpenes utilized included commonly produced stress and defense chemicals in pine trees: (+) α-Pinene, (-) α-Pinene, (+/-) α-Pinene, (-) β-Pinene, β-Myrcene, (+) Camphene, (+) Limonene, (-) Limonene, α-Phellandrene, and 4-AA (Sigma-Aldrich, St. Louis, MO, USA). Two studies were conducted to test how the terpenes affected the growth rates of the fungus via both direct (tactile) and indirect

(volatile) contact. For the tactile study, 1 mL of terpene treatment was pipetted onto each PDA (potato-dextrose agar – Difco, Voigt Global Distribution, Lawrence, KS, USA) plate and was allowed to dry before a 4 mm inoculum punched with a cork borer was placed in the center. Isolates used for inoculation can be found in Table 1. The growth of inoculum was traced onto transparencies at three, five, and seven days after inoculation. The growth of the leading edge of each tracing was measured in millimeters with a digital planimeter (Lasico 1281-12; Lasico, Los Angeles, CA, USA). Six replications of each of the terpene treatments plus six replications of control plates with 1 mL of deionized water added were performed.

The second study included setting up vapor chambers to test how volatilized chemicals affected the inoculum without direct contact. Glass petri dishes poured with PDA were inoculated with the same isolates from the tactile study, tops removed, and stacked inside a standard paint can (Fig. 4.3a) (Freund Container #1800T01; Freund Container and Supply, Lisle, IL, USA) under sterile conditions. A smaller petri dish containing 2 mL of the tested terpene was placed at the bottom of the paint can without a lid to allow volatilization. Two different control methods were used, one set of cans with 2mL-deionized water, and one set of cans with no added liquid. Cans were then sealed (Fig. 4.3b). Seven days after inoculation, the plates were removed from the sealed cans where the growth was traced onto transparencies and measured with the digital planimeter (Fig 4.3c). Six replications of plates were carried out, arranged so that three paint can chambers of each treatment were created. The chambers were arranged so that there were two plates of each inoculum randomly placed in each can.

Statistical Analyses

Post- Hoc analysis of growth rate between fungal and chemical treatments was conducted using a Tukey HSD [Honest Significant Difference] test. All statistical analyses were run using the SAS program (SAS 9.4, 2013) and graphs were drawn using STATISTICA (version 13.0, 2015).

RESULTS

The isolates collected from the Northern Hemisphere were slower growing compared to the fungal isolates collected from the Southern Hemisphere in both trials. Alabama isolates of *A. chailletii* (Fig. 4.1, Fig. 4.4) performed similarly to the *A. areolatum* isolates (Figs. 4.2, 4.3, 4.5, 4.6). Chemical treatments 4-AA, (+) Camphene, (-) Limonene, and α- Phellandrene significantly reduced the growth of isolates compared to the control and pinenes. The exception was that β-Myrcene was found to significantly increase growth of fungal isolates for the tactile trial (Figs.4.7, 4.8, 4.9), this trend did not occur in the atmospheric study.

In the atmospheric trial, (+) α- Pinene and β- Myrcene resulted in the highest percentage of fungal growth compared the control for all isolates of *Amylostereum* spp. (Fig. 4.6). The defense chemical terpenes α- Phellandrene and 4-AA resulted in the greatest reduction of growth of *A. areolatum* in this trial (Figs. 4.6, 4.7). The enantiomers of α- Pinene and β- Pinene showed a greater increase in growth in the atmospheric trial compared to the tactile trial.

DISCUSSION

For the majority of the results, the volatile and tactile trial results were concurrent. The exception

to these findings was the way that the fungus responded to β -Myrcene. In this study, growth of all *Amylostereum* isolates were significantly reduced by the terpenes deemed defense chemicals, especially 4-AA and α-Phellandrene. The terpenes deemed stress chemicals, such as both enantiomers of α- Pinene and (-) β- Pinene, tended to increase the growth of the fungal cultures when compared to the control treatment in the volatile trial. Eckhardt et al. (2009) also identified these same chemicals as reducing and stimulating fungal growth, respectively. Conversely, Klepzieg (1994) found that the presence of α- Pinene could be linked to reduced growth rates of fungal isolates. Those findings are more in line with the results of the tactile trial, which yielded a reduced growth of fungus on α- Pinene and (-) β- Pinene. Further studies are warranted in looking at how these chemicals directly affect fungal growth. It is hypothesized that this is partially due to the direct contact with these chemicals, as they were harsh enough to melt the plastic petri dishes. If this experiment were to be performed again, glass petri dishes may be recommended.

This study yielded results that the same terpenes that are known to be emitted when trees are already under stress and are known to draw insects into stressed stands are the same compounds that increase growth rates of the fungal species associated with the genus *Sirex*. The terpenes α-Pinene and β- Pinene are commonly used to trap and bait woodwasps (Barnes et al., 2014), and are the same treatments that allowed for the highest percentage of fungal growth in relation to the control treatments. This is an unfortunate cycle, which leads to attracting more woodwasps to the area to colonize the stressed trees. These trees are then a hospitable environment for the fungal mycelia to colonize. Conversely, defense chemicals found in higher concentrations in healthy trees significantly reduced the growth of the same isolates of *Amylostereum* than the stress terpenes found in higher concentrations in stressed trees.

CONCLUSIONS

It could be hypothesized that certain terpenes, such as 4-AA and α-Phellandrene could be used to control growth rates of *Amylostereum* species. It seems as though these chemicals would be naturally suppressing the growth of this fungus when inoculated into a pine tree, if the tree is healthy and not stressed. It would most likely not be feasible to try to use these chemicals to control growth rates if applied artificially, as these chemicals are very expensive. Identifying chemicals that could control growth of *Amylostereum* spp. is substantial because this pathogen rots the affected trees from within, degrading the tissues of the tree, and can eventually lead to the death of the tree (Coutts, 1969). Currently, the procedure to control the *Sirex / Amylostereum* complex is to control wasp populations with various biological control agents, including inoculating infested stands of pine trees with *Deladenus* spp. nematodes (Ryan et al., 2012a).

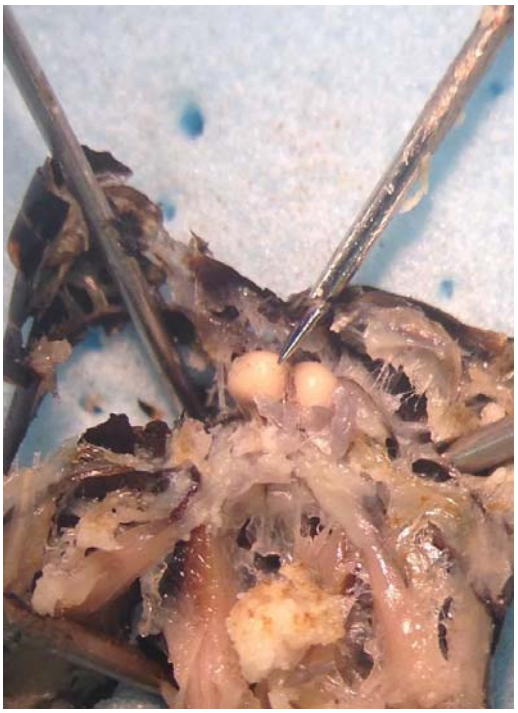

Figure 4.1 Dissected abdomen of a female *S. noctilio* from South Africa that was preserved in 95% ethanol. The pointer draws attention to the two mycangia that house *Amylostereum* spp. spores.

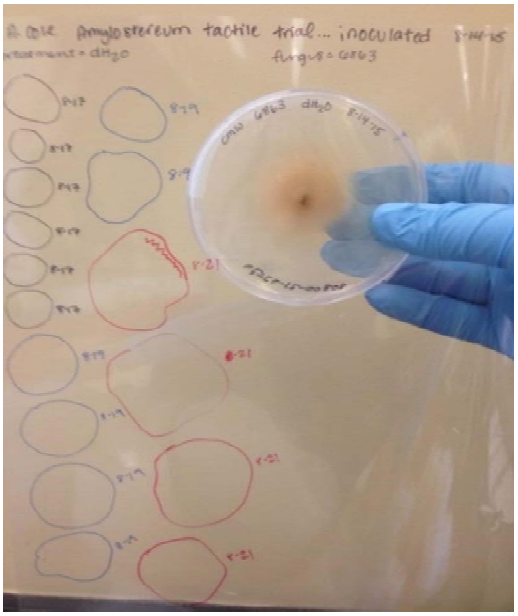

Figure 4.2. Traced hyphae of *Amylostereum areolatum* culture during the tactile trial. The leading edge of the hyphae was traced onto transparency every other day, and was later measured using a digital planimeter

Figure 4.3. Glass plates inoculated with *Amylostereum* spp. were stacked inside paint cans (A) to create atmospheric chambers (B). After the 7 day growth period was completed, plates were removed from paint can chambers (C). The leading edge of hyphae was traced after the seventh day of treatment.

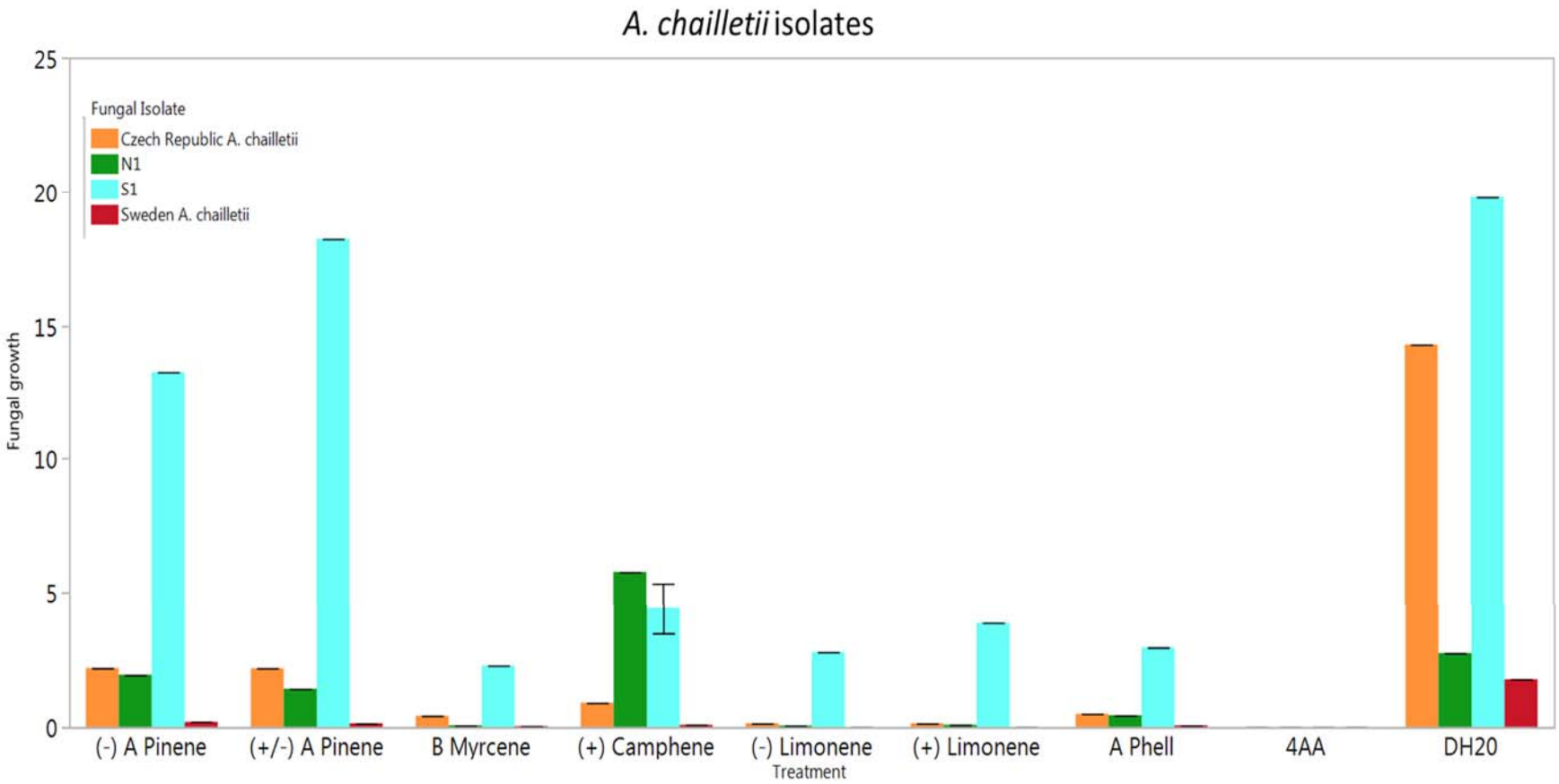

Figure 4.4. Effects of the atmospheric study on unknown isolates from *Sirex nigricornis* trapped in Tuskegee, Alabama. Alabama 1 is isolate S1, and Alabama 2 is isolate N1, both *A. chailletii*.

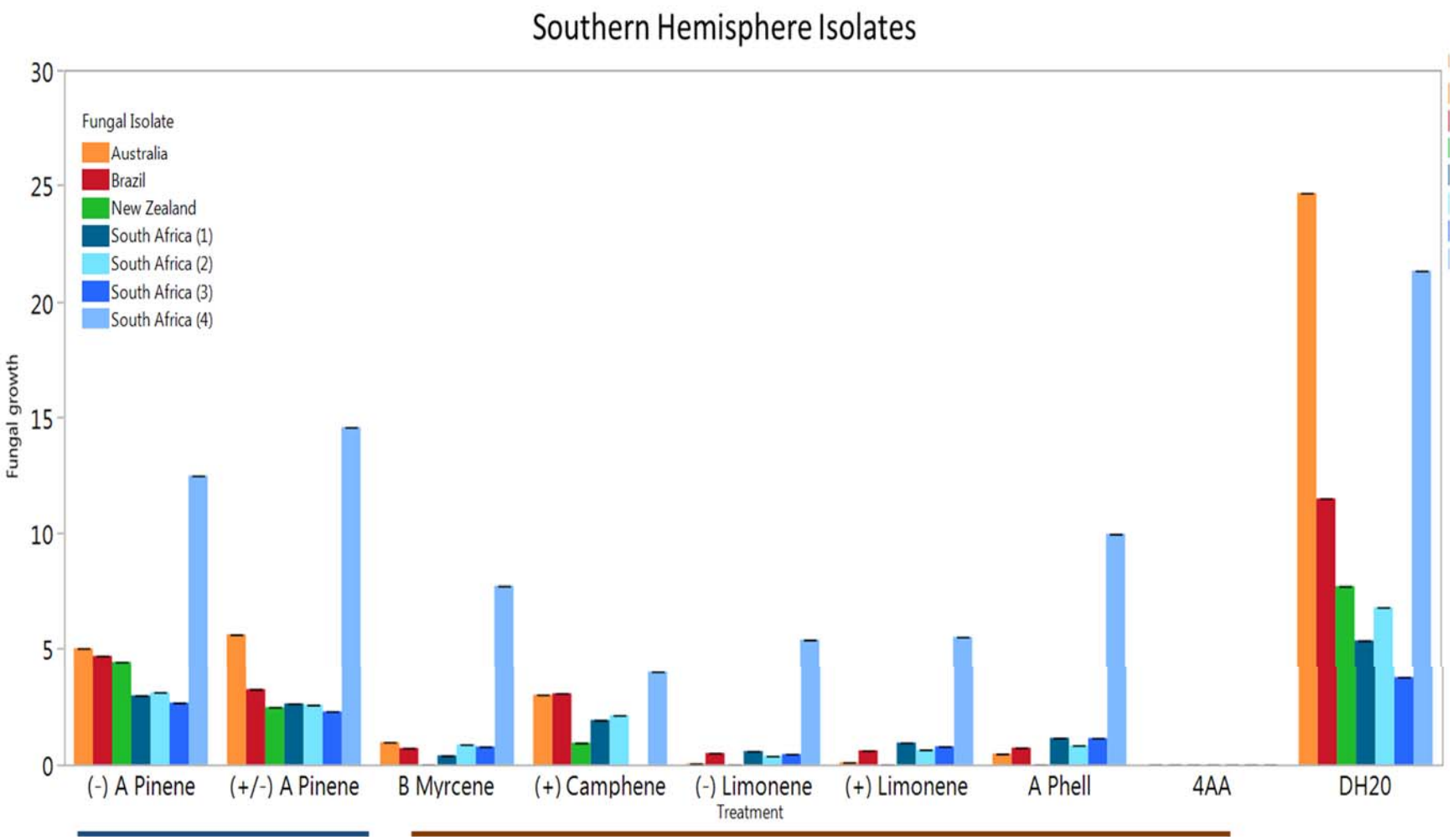

Figure 4.5. Effects of the atmospheric study on isolates of *Amylostereum areolatum* from the Southern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. B-Myrcene and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

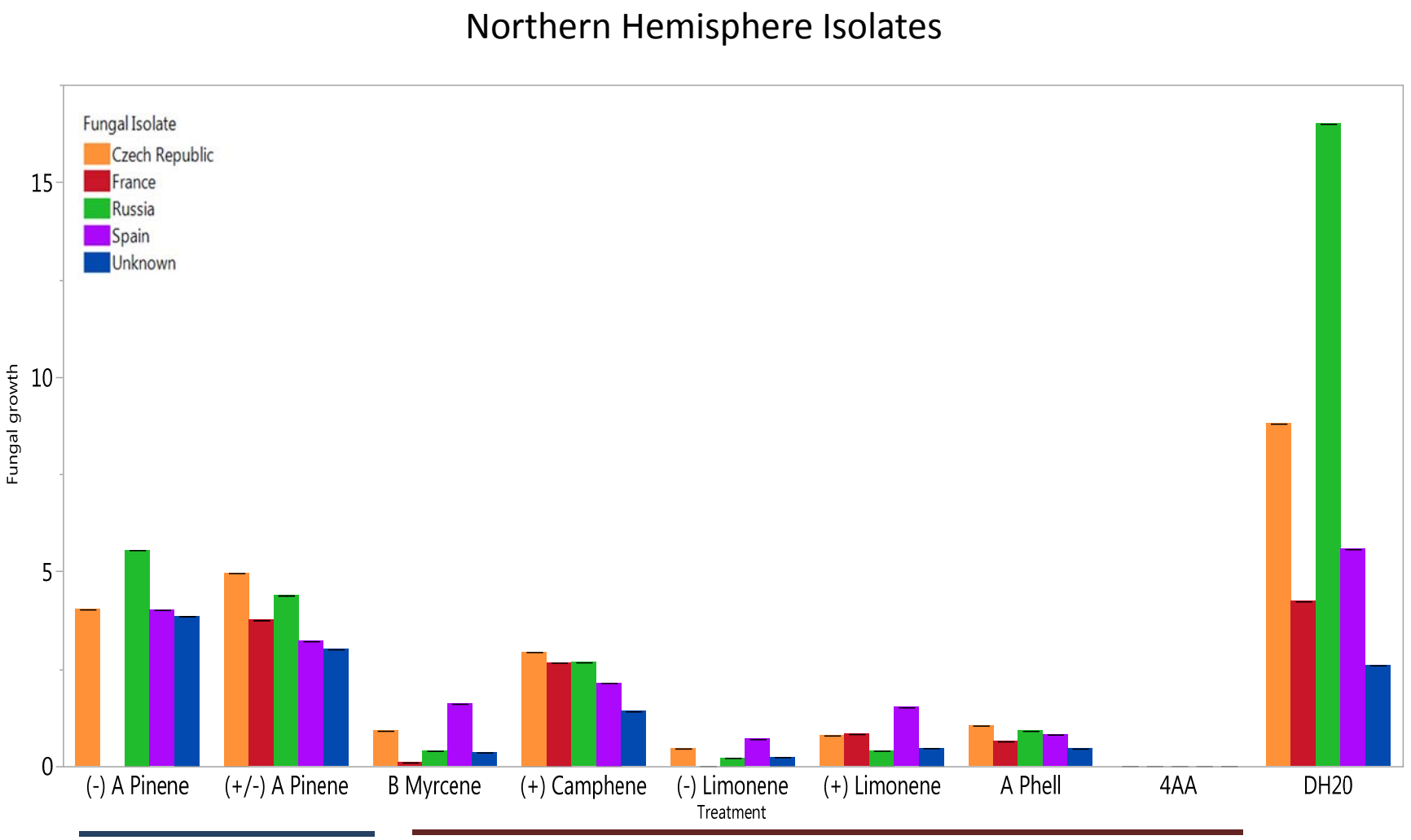

Figure 4.6. Effects of the atmospheric study of *A. areolatum* isolates from the Northern Hemisphere. Isolates grow slower than Southern Hemisphere isolates. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. B-Myrcene and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

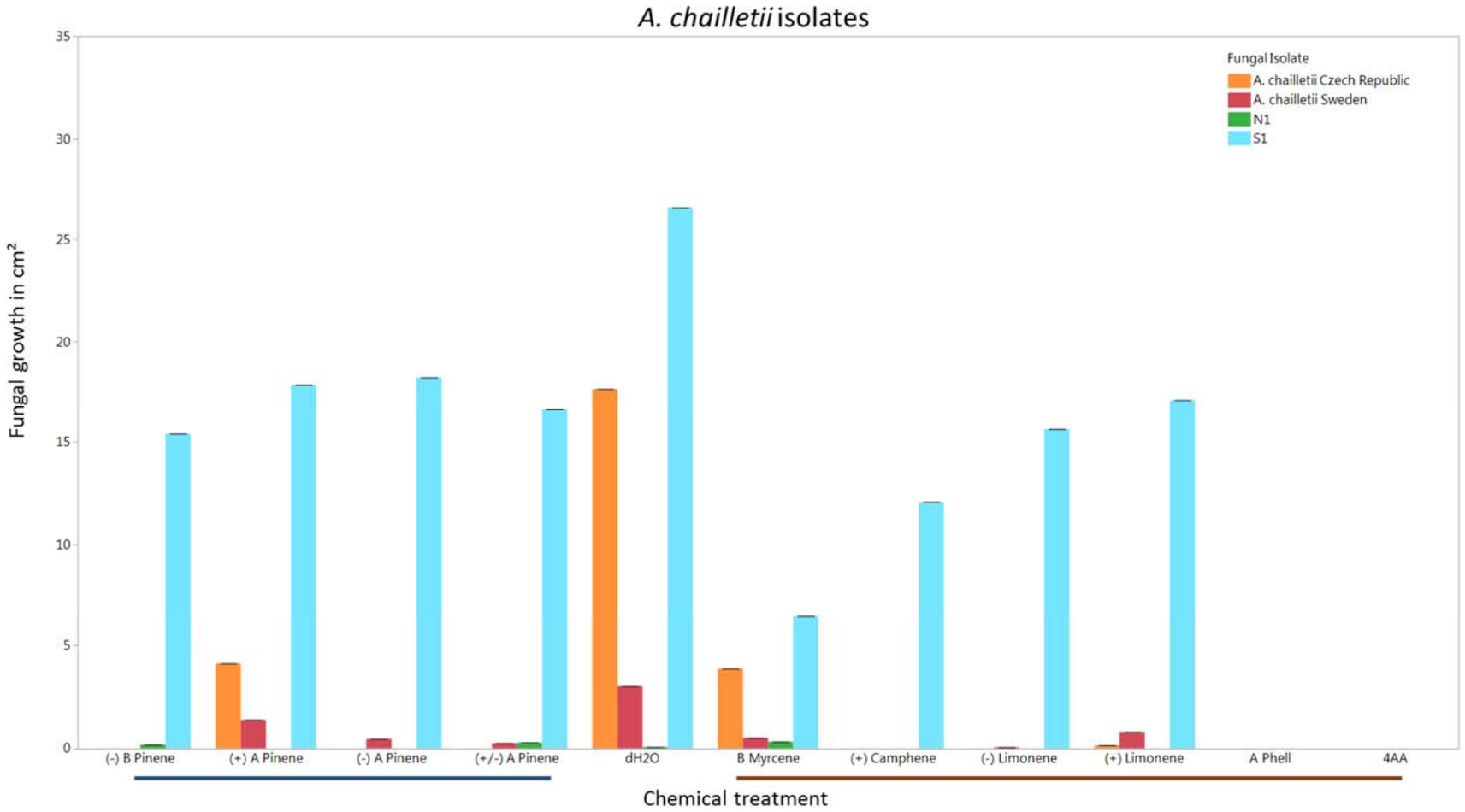

Fig. 4.7 Effects of the tactile study on unknown isolates from *S. nigricornis* trapped in Tuskegee, Alabama. Alabama 1 represents isolate S1, Alabama 2 represents isolate N1. Treatments α-Phellandrene and 4-AA significantly reduced growth in both isolates.

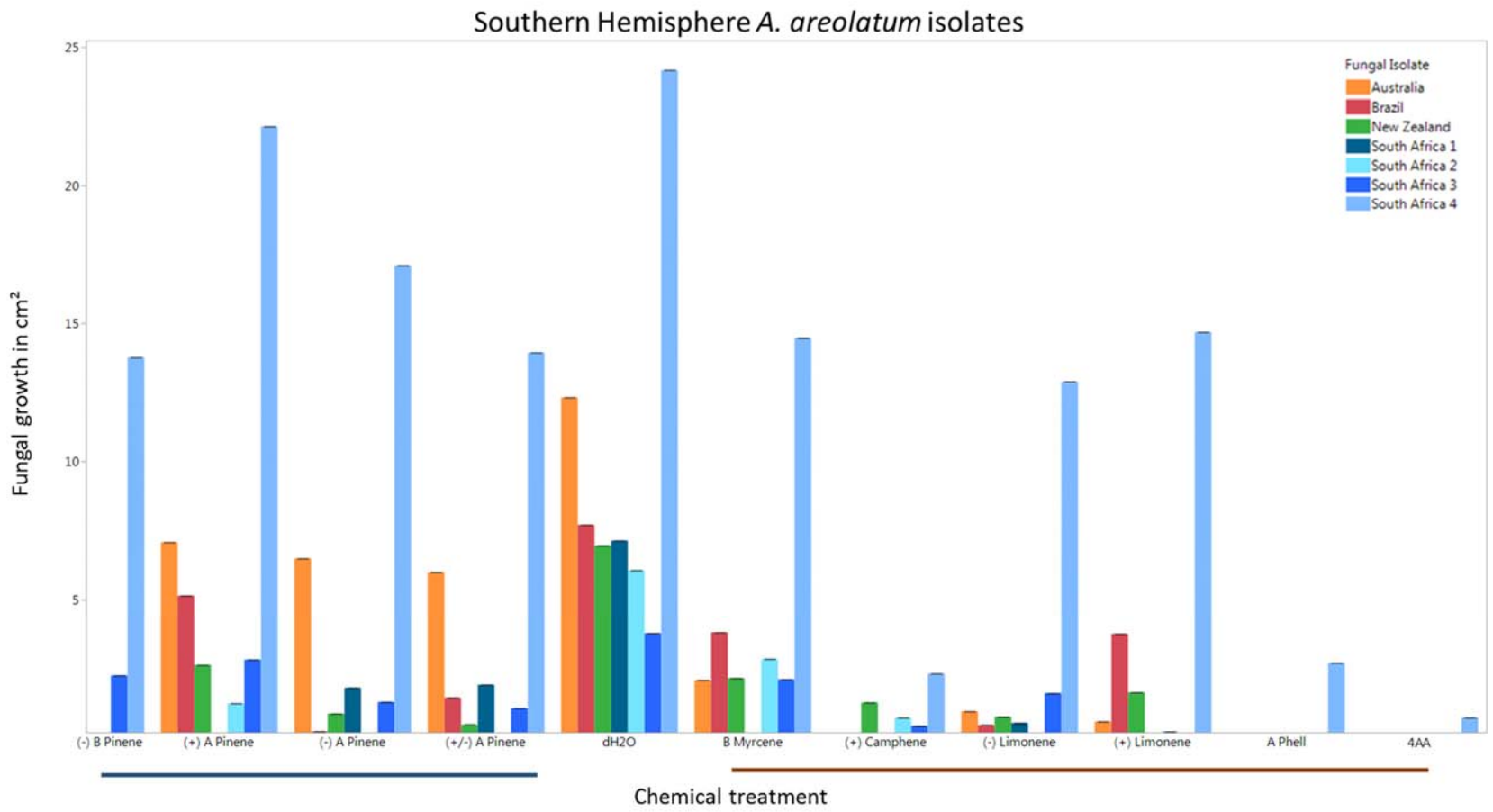

Figure 4.8. Effects of the tactile study of *A. areolatum* isolates from the Southern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. (+) Camphene, (+) Limonene, and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

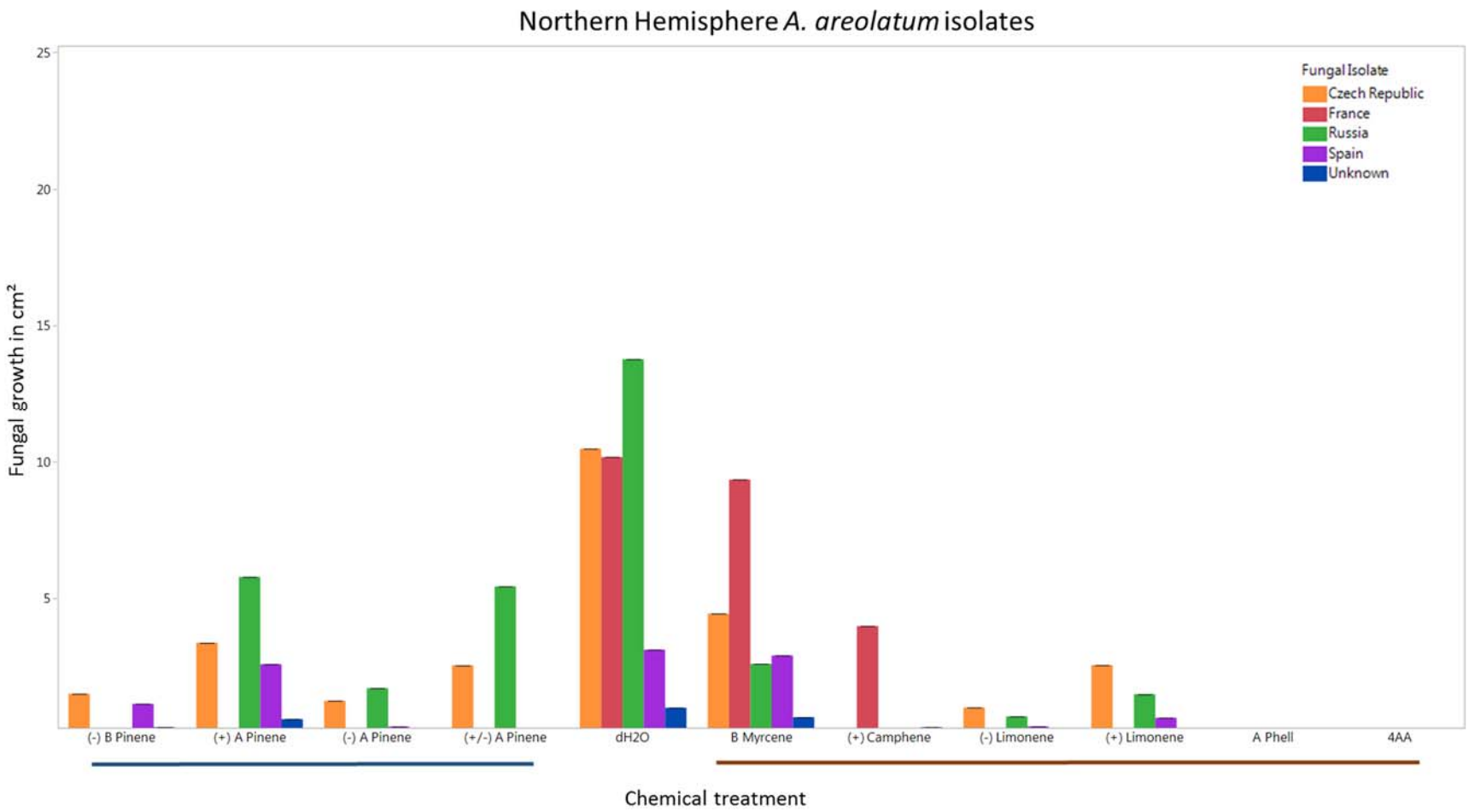

Figure 4.9. Effects of the tactile study on isolates of *A. areolatum* from the Northern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. (+) Camphene, (+) Limonene, α-Phellendrene, and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

Table 4.1 Sources of fungal isolates used in Tactile and Vapor Study, obtained from the culture collection at the FABI, University of Pretoria, South Africa.

Isolate Number	Species	Origin of isolate
CMW 3045	*Amylostereum ferreum*	Brazil
CMW 3309	*A. areolatum*	Unknown
CMW 3310	*A. areolatum*	France
CMW 6863	*A. areolatum*	Australia
CMW 8898	*A. areolatum*	Brazil
CMW 8902	*A. areolatum*	Russia
CMW 13827	*A. areolatum*	South Africa (1)
CMW 15102	*A. chailletii*	Sweden
CMW 15117	*A. laevigatum*	Sweden
CMW 27326	*A. chailletii*	Czech Republic
CMW 27371	*A. areolatum*	Czech Republic
CMW 37116	*A. areolatum*	New Zealand
CMW 37414	*A. areolatum*	South Africa (2)
CMW 37416	*A. areolatum*	South Africa (3)
CMW 40565	*A. areolatum*	Spain
CMW 40874	*A. areolatum*	South Africa (4)
S1	*A. chailletii*	Alabama, USA (1)
N1	*A. chailletii*	Alabama, USA (2)