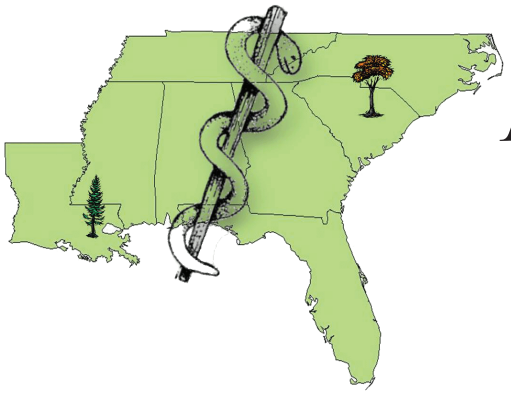


AUBURN UNIVERSITY
FOREST HEALTH COOPERATIVE

RESEARCH REPORT 16-04

RESPONSE OF ECTOMYCORRHIZAL FUNGI ASSOCIATED WITH *PINUS TAEDA* TO *IMPERATA CYLINDRICA* EXUDATE CONSTITUENTS

by
Adam M. Trautwig and Lori G. Eckhardt

ABSTRACT
Imperata cylindrica represents a significant threat to communities of native vegetation in its invaded range. As part of the invasion process *I. cylindrica* produces compounds that inhibit the germination and growth of some plant species, including *Pinus taeda*, a commercially important timber species. These compounds may also inhibit the growth of symbiotic microbial organisms associated with *P. taeda*. We applied treatments of individual components of *I. cylindrica* exudate to plates of modified Melkin-Norans (MMN) agar on which several species of mycorrhizal fungi were grown. We analyzed the effect of these treatments on mean area of individual fungi relative to a control, several times over the course of 8 weeks. In addition, we performed analyses to determine if two isolates from the same fungal species would react similarly to the exudate components. All fungi demonstrated a significant time by treatment interaction that did not have a consistent causal agent. There were also significant differences between how isolates of the same species reacted to the same treatment. This research demonstrates that several compounds consistent with *I. cylindrica* invasion will reduce area of mycorrhizal fungi at concentrations found in natural conditions.

INTRODUCTION
Cogongrass (*Imperata cylindrica* (L.) Beauv.) is a rhizome producing, C4, perennial grass with a multifaceted and dynamic invasion process (MacDonald 2004). *Imperata cylindrica* is a prolific seed producer and reproduces vegetatively from established plants, which makes it an effective disperser that thrives on disturbance (King & Grace 2000; MacDonald 2004). Once present *I. cylindrica* can outcompete native vegetation through several strategies such as shading out, altered fire regimes, and production of potentially allelopathic compounds (Lippencott 2000; Koger & Bryson 2009; Holzmueller & Jose 2011). These compounds were enumerated by Hagen et al. 2013 and demonstrated to be present in the soil in higher concentrations in *I. cylindrica* present soil than *I. cylindrica* absent soil.

During a 27 month study Daneshgar et al. 2008 showed that *Pinus taeda* L. seedlings grown in plots with dense *I. cylindrica* cover had lower rates of survival, displayed reduced growth and root collar diameter. Similarly, Koger & Bryson 2004 found that treatment with *I. cylindrica* extract resulted in reduced germination and growth of some grass and broadleaf species, concluding that *I. cylindrica* extract may contain allelochemicals. Phenolic acids, comparable to those found in *I. cylindrica* exudate were found to reduce growth of two common mycorrhizal fungi *Cenococcum geophilum* Fr. and *Laccaria laccata* (Scop. ex Fr.) Berk and Br., although the effect varied (Boufalis *et al.* 1994). Changes to soil-based ecosystem processes may establish positive feedback loops that further facilitate invasion; affecting native soil microbes, like mycorrhizal fungi (Ehrenfeld *et al.*

2001).

Ectomycorrhizal fungi (ECM) perform functions vital to healthy plant growth and development, in particular, exchange of N and P for excess plant C, increase nutrient uptake, heavy metal tolerance and disease resistance (Smith & Read 2010; Allen *et al.* 2003; Ingham 1988). Using individual species, without confounding factors, we established a study to measure individual components of *I. cylindrica* exudate on various mycorrhizal species in culture. We hypothesized that these compounds would have varying effects and severity on growth of individual species.

MATERIALS & METHODS

Cultures

Fungal specimens were collected from multiple points around Oxford, Mississippi. Specimens were preferentially selected if they were in an early developmental stage and less likely to have been contaminated by insect feeding. Individual fruiting bodies were split open and internal pieces of cap or stalk context tissue were excised and plated on modified Melin-Norkrans (MMN) agar under aseptic conditions. Identities of successfully cultured fungi were subsequently identified utilizing Sanger sequencing of the ITS regions 1 and 2 (as in Hoeksema et al. 2012). Cultures were maintained by sub-culturing when cultures neared plate edge. The following nine isolates of ectomycorrhizal fungi were cultured from fruiting bodies and used in this experiment: *Amanita muscaria* (L.: Fr.) Hooker, *Laccaria laccata*, *Lactarius paradoxus* Beardslee & Burlingham, *Rhizopogon roseolus* (Corda) T.M. Fries, *Suillus brevipes* (Pk.) Kuntze, *Suillus hirtellus* (Peck) Snell (2 isolates, A and B), *Suillus salmonicolor* (Frost) Halling (2 isolates, A and B).

Plate study

Modified Melin-Norkrans plates were inoculated with one of six components of *I. cylindrica* exudate (hereby referred to as treatments) found by Hagen *et al.* 2013 to be present in the soil in significantly higher abundances in the presence of *I. cylindrica* compared to control plots. Each treatment was mixed to the exact concentration reported in Hagen *et al.* 2013 in autoclaved deionized water using high purity chemicals. Treatments were applied in 500 µl increments with control plates receiving 500 µl of autoclaved deionized water. Treatments were applied evenly over the surface of the plate with a sterile implement and the autoclaved deionized water was allowed to evaporate. Each fungus was plated with each treatment 25 times and all fungi were plated within the span of 3 days. Control plates were inoculated the following week, also within 3 days. Plates were stored in complete darkness in an incubator at room temperature (25° C).

Measurement of growth was conducted every two weeks with a Lasico series 1281 Area/Length Meter (Lasico, Los Angeles, California). Measurements were taken and recorded in cm^2. Initial measurements were calculated for consistency. Fungal colonies that reached plate edge on all sides were also calculated for consistency. Plates contaminated by ambient microbes were marked and discarded if the contaminant physically interacted with the fungal growth. Measurements were taken until the first fungus reached the edge of any plate, to a maximum of eight weeks. Seven fungal isolates were measured for the full trial period, but the two isolates of *S. salmonicolor* reached the edge of their plates two weeks early during the six week measurement period.

Statistical analysis

SAS® version 9.3 (SAS Institute Inc. 2010) and STATISTICA® (StatSoft 2013) were used for all statistical analyses. Separate mixed models were constructed for each fungal isolate separately, to determine if differences in area existed across treatments. Pairwise Tukey HSD comparisons (α=0.05) were also carried out between every treatment and the corresponding control, when significant treatment effects were observed. In these models, meanarea was the response variable and treatment and time were predictor variables.

Factorial ANOVAs were utilized to determine if final area was significantly different between isolates of the same species. Pairwise Tukey comparisons (α=0.05) were also carried out to determine which treatments, if any, were significantly different.

RESULTS

Amanita muscaria demonstrated a significant time by treatment interaction ($F_{(6,608)}$=13.12, p<0.0001) (Figure 4.1A). Sinapinic acid elicited an increase in growth compared to control treatments ($F_{(2,174)}$=4.88, p=0.0002) in pairwise comparisons. *Laccaria laccata* also demonstrated a significant time by treatment interaction ($F_{(6,627)}$=11.35, p<0.0001) (Figure 4.1B). In *L. laccata* cultures treatment with gallic acid resulted increased growth ($F_{(2,169)}$=4.77, p<0.0001), while treatment with sinapinic acid resulted in significantly decreased growth ($F_{(2,169)}$=3.61, p=0.007) in pairwise comparison. *Lactarius paradoxus* demonstrated a significant time by treatment interaction ($F_{(6,630)}$=11.34, p<0.0001) (Figure 4.1C).

In *L. paradoxus* cultures treatment with caffeic acid ($F_{(2,169)}$=6.27, p<0.0001), salicylic acid ($F_{(2,169)}$=4.69, p=0.0001), sinapinic acid ($F_{(2,169)}$=4.99, p<0.0001) and emodin ($F_{(2,169)}$=4.88, p<0.0001) all resulted in negative growth according to pairwise comparisons. *Rhizopogon roseolus* likewise yielded a time by treatment interaction ($F_{(2,639)}$=11.63, p<0.0001) (Figure 4.1D), although no treatment differed significantly from the control. *Suillus brevipes* demonstrated a time by treatment interaction ($F_{(6,629)}$=5.19, p<0.0001) (Figure 4.1E). Gallic acid ($F_{(2,171)}$=3.55, p=0.009), sinapinic acid ($F_{(2,171)}$=3.73, p=0.005), cinnamic acid ($F_{(2,171)}$=3.12, p=0.034) and emodin ($F_{(2,171)}$=4.05, p=0.002) were correlated with decreased growth in pairwise comparisons.

Two isolates of *Suillus hirtellus* were examined. Specimen A (*S. hirtellus* A) didn't demonstrated a significant time by treatment interaction ($F_{(6,620)}$=1.20, p=0.307) (Figure 4.1F). No treatment decreased growth of *S. hirtellus* A but caffeic acid ($F_{(2,171)}$=3.49, p=0.011) was consistent with increased growth. Specimen B (*S. hirtellus* B) had a significant time by treatment interaction ($F_{(6,625)}$=6.81, p<0.0001) (Figure 4.8G). Again no treatment was consistent with decreased growth but salicylic acid ($F_{(6,170)}$=3.43, p=0.013) was consistent with increased growth. Final growth of *S. hirtellus* A and *S. hirtellus* B were compared and it was determined that a significant difference existed between isolate growth ($F_{(6,292)}$=4.29, p=0.0004) (Figure 4.2). Specifically, a difference was observed in *S. hirtellus* response to cinnamic acid (p=0.048) and emodin (p=0.006).

Two isolates of *Suillus salmonicolor* were also incorporated. Specimen A (*S. salmonicolor* A) displayed significant time by treatment interaction ($F_{(6,460)}$=11.21, p<0.0001) (Figure 4.1H). In this specimen all treatments resulted in significantly decreased growth: gallic acid ($F_{(2,177)}$=3.15, p=0.031), caffeic acid ($F_{(2,177)}$=3.48, p=0.011), salicylic acid ($F_{(2,177)}$=3.82, p=0.003), cinnamic

acid ($F_{(2,177)}$=5.95, p<0.0001) and emodin ($F_{(2,177)}$=3.15, p=0.031). Specimen B (*S. salmonicolor* B) had a time by treatment interaction ($F_{(6,476)}$=49.46, p<0.0001) (Figure 4.1I). Gallic acid ($F_{(2,170)}$=4.75, p<0.0001), caffeic acid ($F_{(2,170)}$=8.93, p<0.0001), salicylic acid ($F_{(2,170)}$=8.81, p<0.0001), cinnamic acid ($F_{(2,170)}$=7.75, p<0.0001) and emodin ($F_{(2,170)}$=5.95, p<0.0001) treatments all resulted in decreased growth relative to the control. Final growth of *S. salmonicolor* A and *S. salmonicolor* B was compared and a significant difference was present between similar treatments of different isolates ($F_{(6,286)}$=5.42, p<0.0001) (Figure 4.3). *Suillus salmonicolor* had two treatments that were significantly different between specimens of the same species: caffeic acid (p=0.003) and salicylic acid (p=0.010). In total gallic acid and emodin were the two most common sources of reduced area (Table 4.1)

DISCUSSION

Emodin most frequently resulted in reduced growth when applied to plates on which mycorrhizal fungi were grown (4 out of 9). Emodin is found across 17 families in a diversity of growth forms worldwide (Izhaki 2002). In experiments on a variety of media and taxa emodin has been found to reduce the growth of several plant species and soil bacteria, in some cases at minimal concentrations (Inoue *et al.* 1992, Hasan 1998, Izhaki 2002). Emodin has also been recorded to decrease availability of Mn^{2+} and increase the availability of Na^{+} and K^{+} (Inderjit & Nishimura 1999). The preponderance of these findings was consistent with conditions observed in other *I. cylindrica* invaded plots (See chapter 3).

All treatments but cinnamic acid elicited a statistically significant response of some kind in 4 out of 9 fungi. The effect we observed was not, however, consistent; some species increased in growth in the presence of an exudate constituent that decreased or had no effect on other species. From these results we determined that although almost every fungal species demonstrated a time by treatment interaction, no one compound was singularly responsible. Instead it is far more likely that compounds act in tandem to exclude some individual species of mycorrhizal fungi and facilitate others *in situ*.

In addition, within the confines of our limited replications (n=2) we observed a divide in the effect of compounds on growth of two isolates of the same species, although these results were also inconsistent. To this end we conclude that isolates within the same species responds similarly, within the confines of our experimental design, but not the same and that additional research is necessary to provide more strength to this trend.

Few other researchers have investigated the effects that invasive species utilizing allelopathic compounds may have on belowground mutualists. Those that have largely confirm our findings that invaders may disrupt some aspects of these mutualisms. Garlic mustard (*Allaria petiolata* [M. Bieb.] Cavara & Grande) may produce compounds that inhibit germination and reduce inoculum of arbuscular mycorrhizal fungi in the soil (Roberts & Anderson 2001, Stinson *et al.* 2006). Anderson *et al.* 2010 found that mycorrhizal fungi recovered from removal of *A. petiolata* more slowly than non-mycorrhizal species. Beyond the role of mycorrhizal fungi, disturbance to belowground communities may affect biogeochemical cycling, other soil microbial organisms that have direct effects on plant growth as well as soil structure (Wolfe & Klironomos 2005).

CONCLUSION

A significant time by treatment effect was observed in every mycorrhizal fungi. No single compound was the sole cause of decreased area. We therefore conclude that different compounds provoke different responses in mycorrhizal fungi and that almost all compounds produce both positive and negative effects to area. When multiple isolates of the same species were exposed to the same treatments a few treatments produced different responses. We attribute this to variations among a species and note that for neither of the two species with more than one specimen did the control treatments vary significantly.

REFERENCES

Allen, M.F., W. Swenson, J.I. Querejeta, L.M. Egerton-Warburton, and K.K. Treseder. 2003. "Ecology of Mycorrhizae: A Conceptual Framework for Complex Interactions Among Plants and Fungi." *Annual Review of Phytopathology* 41 (1): 271–303.

Anderson, R.C., M.R. Anderson, J.T. Bauer, M. Slater, J. Herold, P. Baumhardt, and V. Borowicz. 2010. "Effect of Removal of Garlic Mustard (Alliaria Petiolata, Brassicaeae) on Arbuscular Mycorrhizal Fungi Inoculum Potential in Forest Soils". *Open Ecology Journal* 3: 41–47.

Boufalis, A., F. Pellissier, and L. Trosset. 1994. "Responses of Mycorrhizal Fungi to Allelopathy: Cenococcum Geophilum and Laccaria Laccata Growth with Phenolic Acids." *Acta Botanica Gallica* 141 (4): 547–50.

Brundrett, M., N. Bougher, B. Dell, T. Grove, and N. Malajczuk. 1996. "Working with Mycorrhizas in Forestry and Agriculture."

Daneshgar, P., S. Jose, A. Collins, and C. Ramsey. 2008. "Cogongrass (Imperata Cylindrica), an Alien Invasive Grass, Reduces Survival and Productivity of an Establishing Pine Forest." *Forest Science* 54 (6): 579–87.

Ehrenfeld, J.G., P. Kourtev, and W. Huang. 2001. "Changes in Soil Functions Following Invasions of Exotic Understory Plants in Deciduous Forests." *Ecological Applications* 11 (5): 1287–1300.

Hagan, D.L., S. Jose, and C. Lin. 2013. "Allelopathic Exudates of Cogongrass (Imperata Cylindrica): Implications for the Performance of Native Pine Savanna Plant Species in the Southeastern US." *Journal of Chemical Ecology* 39 (2): 312–22.

Hasan, H.A.H. 1998. "Studies on toxigenic fungi in roasted foodstuff (Salted seed) and halotolerant activity of emodin-producingAspergillus wentii". *Folia microbiologica*, *43*(4), 383-391.

Holzmueller, E.J., and S. Jose. 2011. "Invasion Success of Cogongrass, an Alien C4 Perennial Grass, in the Southeastern United States: Exploration of the Ecological Basis." *Biological Invasions* 13 (2): 435–42.

Hoeksema, J. D., J.V. Hernandez, D.L. Rogers, L.L. Mendoza, and J.N. Thompson. 2012. "Geographic divergence in a species-rich symbiosis: interactions between Monterey pines and ectomycorrhizal fungi". *Ecology*,*93*(10), 2274-2285.

Horton, T.R., and T.D. Bruns. 2001. “The Molecular Revolution in Ectomycorrhizal Ecology: Peeking into the Black-Box.” *Molecular Ecology* 10 (8): 1855–71.

Inderjit., H. Nishimura. 1999. Effect of the anthraquinones emodin and physcion on availability of selected soil inorganic ions”. *Annals of applied biology*, *135*(1), 425-429.

Ingham, R.E. 1988. “Interactions Between Nematodes and Vesicular-Arbuscular Mycorrhizae.” *Agriculture, Ecosystems & Environment*, Proceedings of a Workshop on Interactions Between Soil-Inhabiting Invertebrates and Microorganisms in Relation to Plant Growth, 24 (1–3): 169–82.

Inoue, M., H. Nishimura, H.H. Li, and J. Mizutani. 1992. “Allelochemicals fromPolygonum sachalinense Fr. Schm.(Polygonaceae).” *Journal of Chemical Ecology*, *18*(10), 1833-1840.

Izhaki, I. 2002. “Emodin–a secondary metabolite with multiple ecological functions in higher plants”. *New Phytologist*, *155*(2), 205-217.

King, S.E., and J.B. Grace. 2000. “The Effects of Soil Flooding on the Establishment of Cogongrass (Imperata Cylindrica), a Nonindigenous Invader of the Southeastern United States.” *Wetlands* 20 (2): 300–306.

Koger, C.H., and C.T. Bryson. 2004. “Effect of Cogongrass (Imperata Cylindrica) Extracts on Germination and Seedling Growth of Selected Grass and Broadleaf Species.” *Weed Technology* 18 (2): 236–42.

Lippincott, C.L. 1997. “Ecological Consequences of Imperata Cylindrica (cogongrass) Invasion in Florida Sandhill.” Gainesville, FL: University of Florida. https://ia601702.us.archive.org/27/items/ecologicalconseq00lipp/ecologicalconseq00lipp.pdf.

MacDonald, G.E. 2004. “Cogongrass (Imperata cylindrica)—Biology, Ecology, and Management.” *Critical Reviews in Plant Sciences* 23 (5): 367–80.

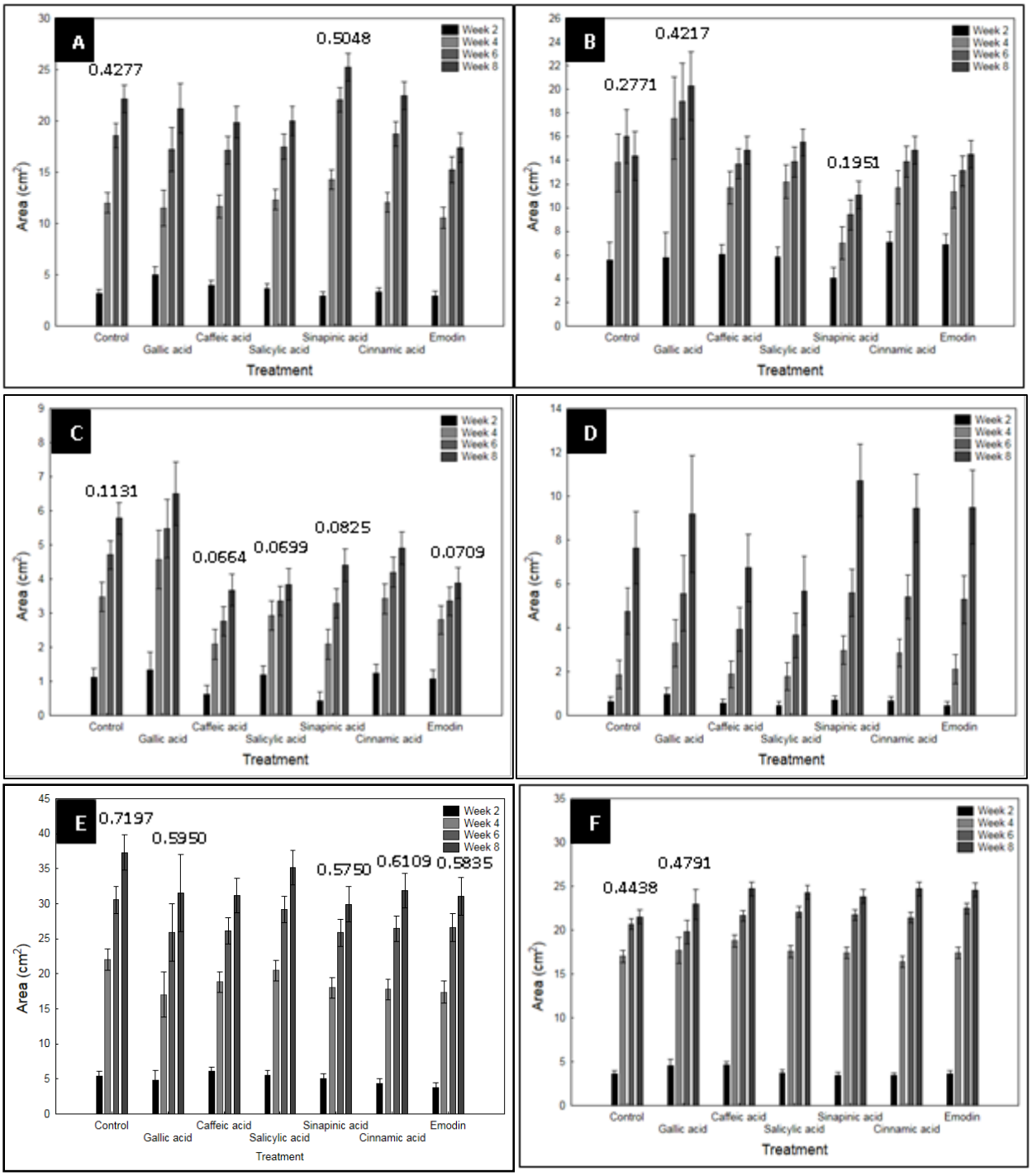
Peay, K.G., M. Garbelotto, and T.D. Bruns. 2010. “Evidence of Dispersal Limitation in Soil Microorganisms: Isolation Reduces Species Richness on Mycorrhizal Tree Islands.” *Ecology* 91 (12): 3631–40.

Peay, K.G., M.G. Schubert, N.H. Nguyen, and T.D. Bruns. 2012. “Measuring Ectomycorrhizal Fungal Dispersal: Macroecological Patterns Driven by Microscopic Propagules.” *Molecular Ecology* 21 (16): 4122–36.

Roberts, K.J., and R.C. Anderson. 2001. “Effect of Garlic Mustard [Alliaria Petiolata (Beib. Cavara & Grande)] Extracts on Plants and Arbuscular Mycorrhizal (AM) Fungi”. *The American Midland Naturalist* 146 (1): 146–52.

Smith, S.E., and D.J. Read. 2010. *Mycorrhizal Symbiosis*. Academic Press.

Stinson, K.A., S.A. Campbell, J.R. Powell, B.E. Wolfe, R.M. Callaway, G.C. Thelen, S.G. Hallett, D.Prati, and J.N. Klironomos. 2006. "Invasive plant suppresses the growth of native tree seedlings by disrupting belowground mutualisms." *PLoS biology*, *4(5), 727.*


A
0.4277
0.5048
B
0.4217
0.2771
0.1951
C
0.1131
0.0664
0.0699
0.0825
0.0709
D
E
0.7197
0.5950
0.5750
0.6109
0.5835
F
0.4791
0.4438
Week 2
Week 4
Week 6
Week 8
Area (cm²)
Control
Gallic acid
Caffeic acid
Salicylic acid
Sinapinic acid
Cinnamic acid
Emodin
Treatment

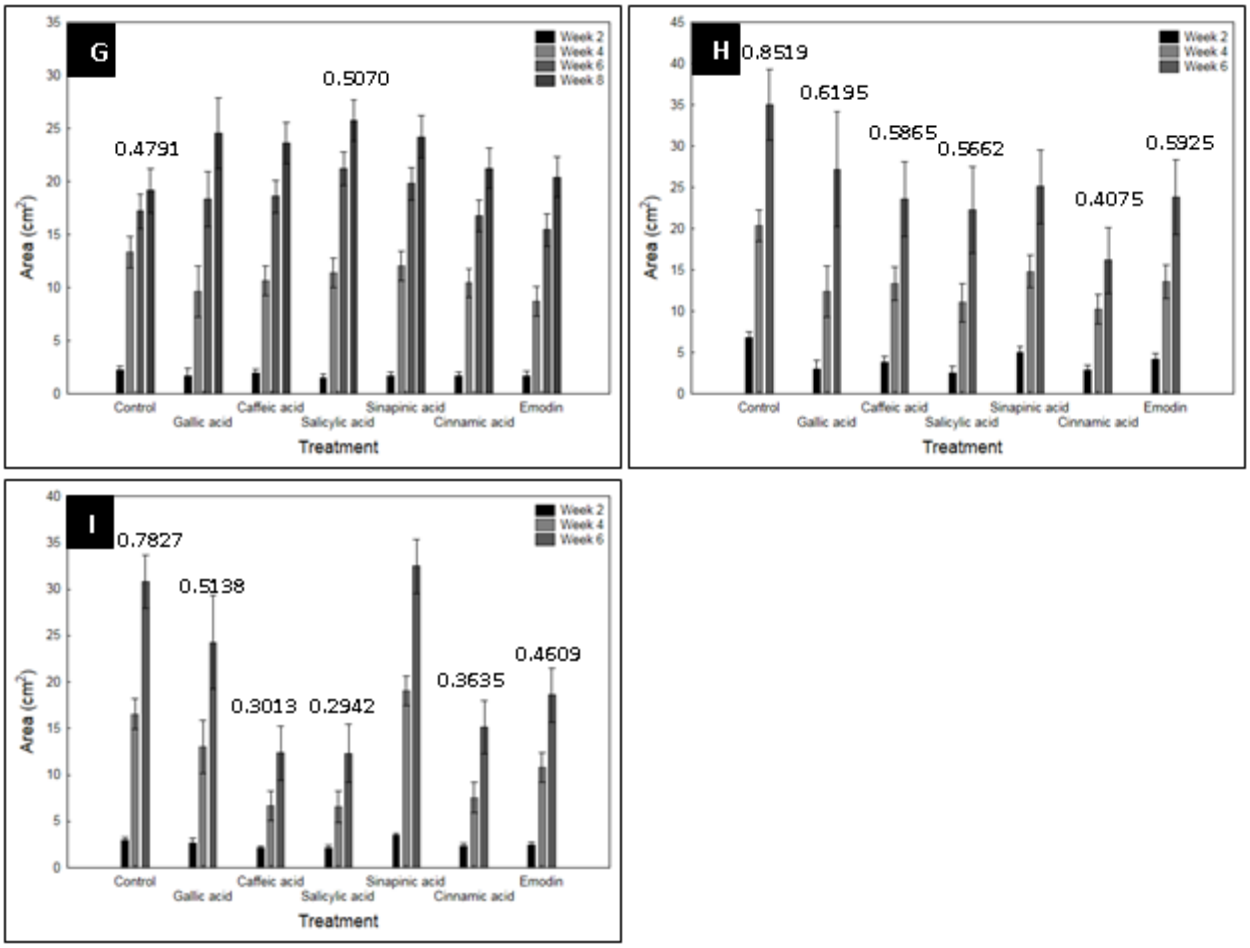


Figure 4.1. Mean values of fungal colony area measured across four collection periods ((a) *Amanita muscaria*, (b) *Laccaria laccata*, (c) *Lactarius paradoxus*, (d) *Rhizopogon roseolus*, (e) *Suillus brevipes*, (f) *Suillus hirtellus* A, (g) *Suillus hirtellus* B) or two collection periods ((h) *Suillus salmonicolor* A, (i) *Suillus salmonicolor* B), each two weeks apart. Pairwise comparisons (α=0.05) were made between individual treatments and controls in the same collection period, not among collection periods. Significant differences are denoted by growth rates (cm/day), bars denote 95 % standard error.

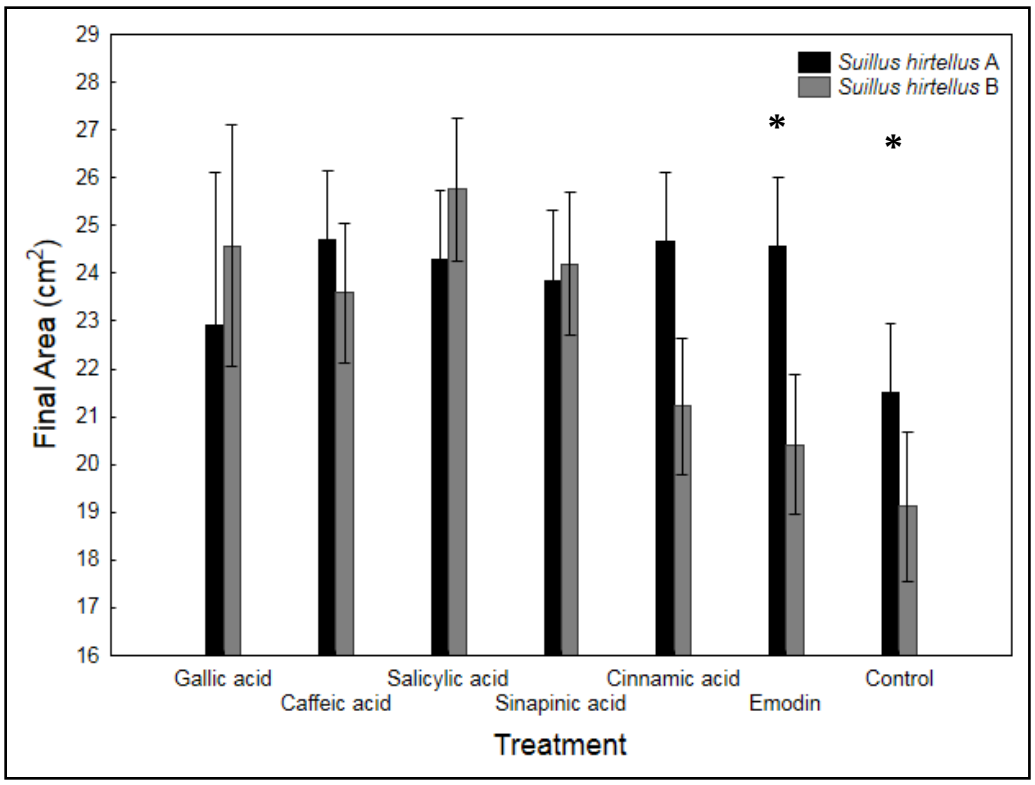


Figure 4.2. Mean final area of fungal colonies is compared across like treatments of different specimens of the same fungal species *Suillus hirtellus*. Significant differences (α=0.05) are denoted with asterisks.

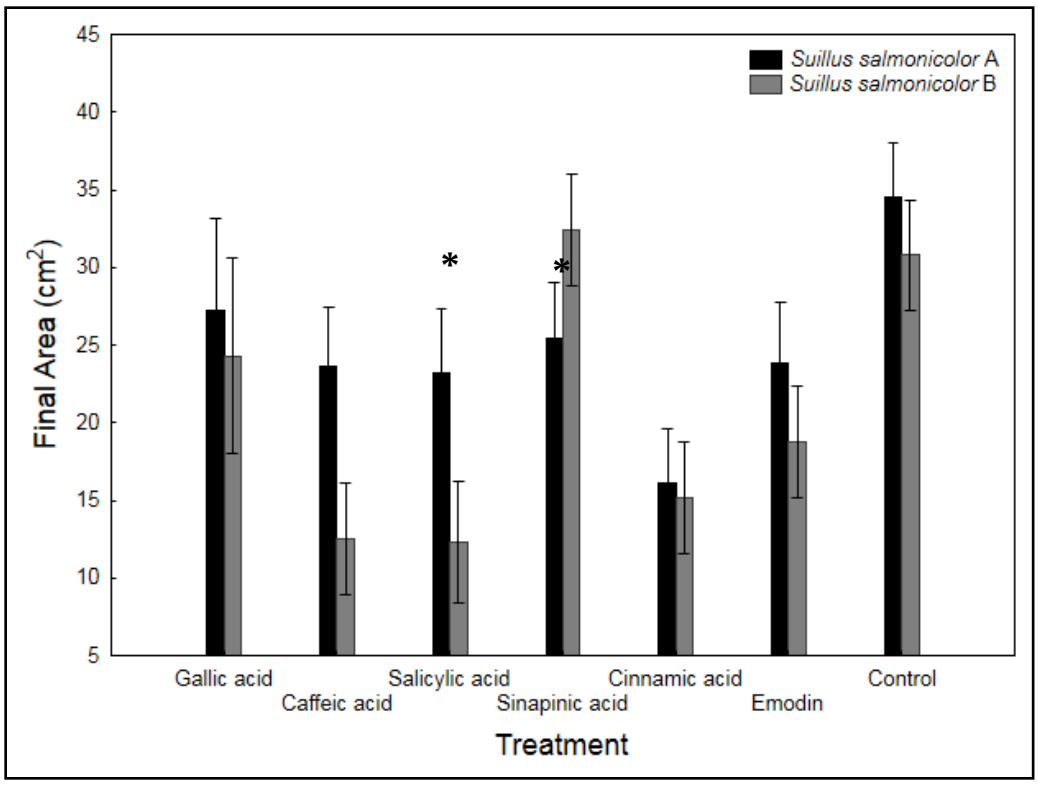


Figure 4.3. Mean final area of fungal colonies is compared across like treatments of different specimens of the same fungal species *Suillus salmonicolor*. Significant differences (α=0.05) are denoted with asterisks.

Table 4.1. Effects of individual components of *I. cylindrica* exudate are summarized across fungal species of isolates of the same species, differentiated with the variable "A" or "B." Cells with "+" indicate that mean area was greater than control. Cells with "-" indicate that growth was retarded compared to control. Cells with "0" denote no difference was observed between treatment and control.

	Gallic acid	Caffeic acid	Salicylic acid	Sinapinic acid	Cinnamic acid	Emodin
Amanita muscaria	0	0	0	+	0	0
Laccaria laccata	+	0	0	-	0	0
Lactarius paradoxus	0	-	-	-	0	-
Rhizopogon roseolus	0	0	0	0	0	0
Suillus brevipes	-	0	0	-	-	-
Suillus hirtellus A	0	+	0	0	0	0
Suillus hirtellus B	0	0	+	0	0	0
Suillus salmonicolor A	-	-	-	0	-	-
Suillus salmonicolor B	-	-	-	0	-	-