Forgotten fungi that could be used to control the spread of the Spotted Lanternfly (Hemiptera: Fulgoridae)

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Introduction

The Spotted Lanternfly (*Lycorma delicatula*) is an invasive species of plant hopper insect that belongs to the order Hemiptera first found in Berks County, Pennsylvania in 2014 (Harper et al., 2019; Urban, 2020). Since that time, *L. delicatula* has quickly spread through the northeastern part of the United States, including six other states: Virginia, New Jersey, West Virginia, Delaware, New York, and Maryland (Harper et al., 2019). Fourteen counties in Pennsylvania have been quarantined to inhibit the spread of the spotted lanternfly across the state (Harper, et al., 2019). The Spotted Lanternfly is a threat to many agricultural industries across the state, particularly grapes and hops production as the insects can commonly be found feeding on grape vines (*Vitis* sp.) and hops (*Humulus* sp.). In addition to this, the insects will also feed on black walnut trees (*Juglans nigra*) damaging the state's lumber industry (Murman et al., 2020; Urban, 2020). The plant species that are fed on by the spotted lanternfly from its native range include the tree-of-heaven (*Ailanthus altissima*) and oriental bittersweet (*Celastrus orbiculatus*), both are invasive plant species in Pennsylvania (Murman et al., 2020; Xin et al., 2021; Urban, 2020).

The Spotted Lanternfly goes through four life stages after hatching from an egg structure on the sides of trees, called the first, second and third instar and then the flying adult (Barringer and Caifré, 2020; Murman et al., 2020; Urban, 2020). As they progress through their life cycle, the insects will always seek to be in the highest possible location, usually on the trees that they are feeding from. While the first instars are hard to see, the adults are frequently seen coating the sides of tree trunks and feeding from them (Barringer and Caifré, 2020; Murman et al., 2020; Urban, 2020). Spotted Lanternflies also produce a substance called "honeydew" when they feed because they do not process all the phloem of the plants they consume, and the excess is excreted in a sticky substance (Barringer and Caifré, 2020; Murman et al., 2020; Urban, 2020). When it gets onto a person's skin, honeydew is uncomfortable, sticky and is very hard remove from skin and hair. Aside from the general annoyance that the honeydew produced by lanternflies causes, it also promotes the growth of sooty mold which comes from the genera *Capnodium*, *Fumago*, and *Scorias* that inhibit photosynthesis of the plants it grows on (Barringer and Caifré, 2020; Murman et al., 2020; Windbiel-Rojas and Messenger-Sikes, 2020; Urban, 2020).

The quarantine of 14 counties in the state of Pennsylvania has been established since their arrival in 2014 (Harper et al., 2019; Urban, 2020). The quarantines have been maintained by using various biocontrol and chemical agents in order to control the spread of the insects across the state. A popular biocontrol option used for this task is the fungus *Beauveria bassiana* (Sordariomycetes: Hypocreales), which is a cosmopolitan species. The use of fungi as a biopesticide in order to control the population of pest insects has become a common occurrence in many areas of the world (Doolotkelvieva et al., 2019; El Kichaoui et al., 2016; Harper et al., 2019; Borisade and Magan, 2014; Mariam et al., 2016; Aw and Hue, 2017; Clifton et al., 2019; Bustamante et al., 2019; Gallou et al., 2015; Zimmermann, 2008). Currently, *B. bassiana* is being used in Egypt to control the pest *Tuta absoluta* (Lepidoptera: Gelechiidae), commonly known as the Tomato Leafminer (El-Kichaoui et al., 2016). The goal is to increase the amount of biopesticide (*B. bassina*) in the environment to control the population of invasive insects (Clifton et al., 2019).

In our study, the objective was to isolate fungi from dead Spotted Lanternflies and to determine if some may be an option for controlling this serious pest across the East Coast. Specifically, saprotrophic fungi were initially isolated, and then entomopathogenic fungi selected from those and then, using Koch's postulate, were inoculated onto healthy host insects to confirm pathogenicity.

Methods and Materials

Fungal Isolates: In order to isolate fungi from the dead Spotted Lanternflies, several moribund specimens were collected from the wild. One insect was placed onto a plate of Sabouraud Dextrose agar (SAB) to allow any fungi to grow out onto the media. Each of the fungi that grew from the insects was then isolated onto separate plates of SAB in order to have the fungi grow in pure cultures. All isolates from SAB media were identified by the letter S followed by the isolate number.

A dead lanternfly was placed into 9 milliliter peptone broth and ground up. The solution was dilute plated in a 1 to 100 solution onto DOC2 media. DOC2 media is specifically designed to be selective for fungi that are insect pathogens (Abdullah et al., 2015; Shimazu and Sato, 1996). After the fungal isolates grew on the DOC2 media, the new fungal isolates were subcultured onto Acidified Rose Bengal agar (ARBA) to grow pure cultures of the isolated fungi. All isolates from DOC2

media were identified by the letter D followed by the isolate number.

The fungal isolates that were grown on SAB from a dead Spotted Lanternfly were visually identified to the genus. The isolates from DOC2 were sequenced using PCR to the species after growing on ARBA media.

PCR: DNA sequence analysis was conducted using three gene sequences: ITS rDNA barcode region (ITS). ITS sequences were generated following the protocol and primers described in Overton et al. (2006). A 50 µL polymerase chain reaction (PCR) for ITS was performed following the conditions outlined by Chaverri et al. (2001) and Overton et al. (2006) using the following primer pairs: for ITS, the primers utilized were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') from White et al. (1990) and O'Donnell et al. (1998). Two percent dimethyl sulfoxide (DMSO) from AMRESCO® was added to each 50 µL PCR reaction. PCR products were purified and sequenced following the protocol in Overton et al. (2006). Samples were cleaned using ChargeSwitch®-Pro PCR Clean-up Kit from Invitrogen.

After the samples were cleaned, four isolates of the fungi found to grow on the DOC2 media (labeled D6, D8, D10, and D12, respectively) were sent for individual Sanger Sequencing at the Penn State Core Facility for Genomics I lab in concentrations of 5 µL of 1 micromolar concentration with 35 µL of the ITS 1 and ITS 4 primers. The results of this sequencing were received from the Penn State Core Facility for Genomics I lab.

Mealworm Experiment: Koch's fourth postulate according to Segre (2013) is as follows: an infectious agent will be isolated from an organism that has been infected with and died because of said infectious agent. Mealworms (*Tenebrio molitor*) were used as a test organism to determine if Koch's fourth and third postulates were fulfilled as the other two postulates were already fulfilled. The fulfilled postulates included the first postulate that an infectious agent will be found on a diseased organism and not a healthy one and Koch's second postulate, stating the causal agent must be cultured from a diseased individual both of which were fulfilled by the isolation of the fungi from the moribund lanternfly (Segre, 2013). Koch's third postulate states that a healthy individual inoculated with the microorganism believed to be the causal agent and the microorganism must reinfect the inoculated specimen (Segre, 2013). Fulfilling Koch's third and fourth postulate would allow us to see if the isolated fungi on DOC2 were capable of infecting the mealworms as this would indicate that the isolated fungi were capable of reinfecting insects after being cultured. Mealworms were used as the test animal for this experiment due to their easy availability at local pet shops.

Thirty grams of oat flakes and two apple slices were placed into four Tupperware containers. Twenty-five mealworms were then placed into each of these Tupperware containers. In preparation for this experiment, four tubes of 10 mL of peptone broth blanks were prepared. One of these blanks were kept as a control. Using the next tube of peptone broth, a plate of isolate D4 (determined to also be *Isaria farinosa* by Sanger Sequencing) from the DOC2 media was scraped into a tube of peptone broth each. The tubes were then vortexed using a Vortex Genie 2 from Scientific Instruments. Using a micropipette, 1 mL of the control peptone broth was added to one of the containers containing the mealworms and was designated as a control group. 1 mL of the isolate D4 peptone broth was added to the second Tupperware container and 1 mL of the isolate D12, (determined to be *Cladosporium cladosporioides* by Sanger Sequencing) peptone broth was added to the third Tupperware container. Lastly, in the fourth Tupperware container 0.5 mL of the isolate D4 peptone broth and 0.5 mL of the isolate D12 broth were both added to the container. This was done to see if the different species of isolated fungi synergized with or hindered each other when exposed to the mealworms at the same time. These inoculations were repeated four days later in order to make sure there were enough spores from the solution within each of the Tupperware containers. Thus, properly exposing the mealworms to the infectious agents. The experiment was carried out over the course of two months, running from the 15th of February to the 16th of March of 2021. Every week, the pieces of apple were replaced in order to prevent saprotrophic mold growing on the apples.

If mealworms were found to be moribund, the insect was placed into a glass humidity chamber with a wet paper towel in order to stimulate fungal growth. When the mealworms molted into a pupa or beetle, they were left in the Tupperware container to see if the fungi could infect the later stages of the mealworm's life cycle. A separate humidity chamber was prepared for the pupa to be placed in when found coordinated to each fungi treatment. On the 12th of March, the still living beetles and mealworms were placed into sealed Petri dishes with breathing holes melted into the top and 5 grams of oat flakes to see if they would continue to survive.

When the moribund mealworms began to have various fungi grow on them, they were placed onto DOC2 media to grow out the fungi that was found growing on the mealworm. This allowed for the fulfillment of Koch's postulates.

At the end of the experiment, the contents of each Tupperware container were poured into an autoclave bag and placed into an autoclave for 20 minutes to kill any living fungi and insects. The autoclave bag and its contents were then disposed of following university procedures.

Results and Conclusions

Gene Sequencing: The isolates from the moribund Spotted Lanternfly that were isolated from and grown on SAB were identified visually using *The Illustrated Guide to Imperfect Fungi*, Fourth Edition (Barnett and Hunter, 1998) (Table 1). All of the fungi found in this manner were saprotrophic according to Barnett and Hunter (1998).

The species of four of the isolates from the DOC2 media that were sequenced were identified as the following: Isolates D6 and D8 were both found to be *Isaria farinosa*, isolate D10 was found to be *Fusarium lateritium* and isolate D12 was found to be *Cladosporium cladosporioides*. The three species are all known insect pathogens and *F. lateritium* is also a known plant pathogen (AlMatar and Makky, 2016; Borisade and Magan, 2014; Clark, 1994; Clark et al., 1990; Ekan and Hayat, 2009; Gallou et al., 2015; Habashy et al., 2016; Köhl et al. 2015; Kwon-Chung et al., 1975; Mariam et al., 2016; Shaker et al.,

Table 1. Genera of fungi isolated from moribund *L. delicatula* on Sabouraud Dextrose agar (SAB).

Isolate	Family
S2	*Fusarium*
S4	*Pithomyces*
S5	*Pestalotia*
S9	*Fusarium*
S11	*Penicillium*
S12	*Fusarium*
S13	*Fusarium*
S17	*Cladosporium*

*SAB isolated fungi were all identified by morphological examination.
**Used the *Illustrated Genera of Imperfect Fungi* Fourth Edition (Barnett and Hunter, 1998) to identify these isolates to genus.

Table 2. PCR Results of isolates from *L. delicatula* on DOC2 media.

Isolate	Scientific Name
D4	*Isaria farinosa*
D6	*Isaria farinosa*
D8	*Isaria farinosa*
D10	*Fusarium lateritium*
D12	*Cladosporium cladosporioides*

*DOC2 isolated fungi were all identified by DNA sequencing.

2019; Teetor-Barsch and Roberts, 1983; Zimmermann, 2008.

As shown in Table 2, isolates D4, D6, and D8 were determined by Sanger searching and BLAST sequencing to be *Isaria farinosa*, specifically identified by Gallou et al. (2015) and Luangsa-Ard et al. (2005). This species has a complicated phylogenetic history, both Gallou et al. (2015) and Luangsa-Ard et al. (2005) have *I. farinosa* on different parts of a consensus phylogram of *I. farinosa* using ITS regions. Using the *I. farinosa* isolated on DOC2 media from our dead Spotted Lanternfly, we generated a phylogenetic tree (Fig. 1). A bootstrap consensus tree was generated by using several other members of the genus *Isaria* using the ITS gene shown on Genbank for these other members of the genus. Each species is listed in Table 3 with their CBS numbers acquired from

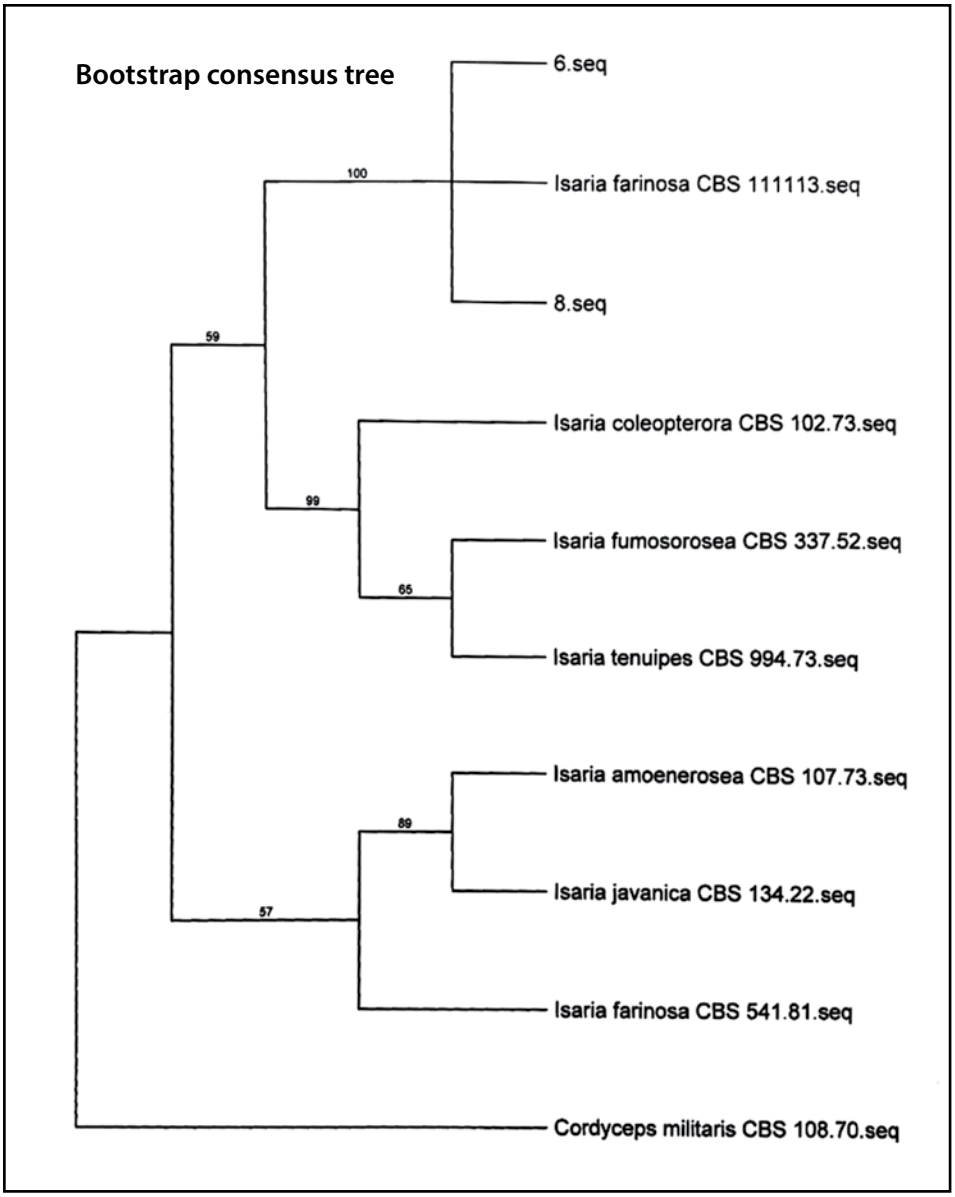
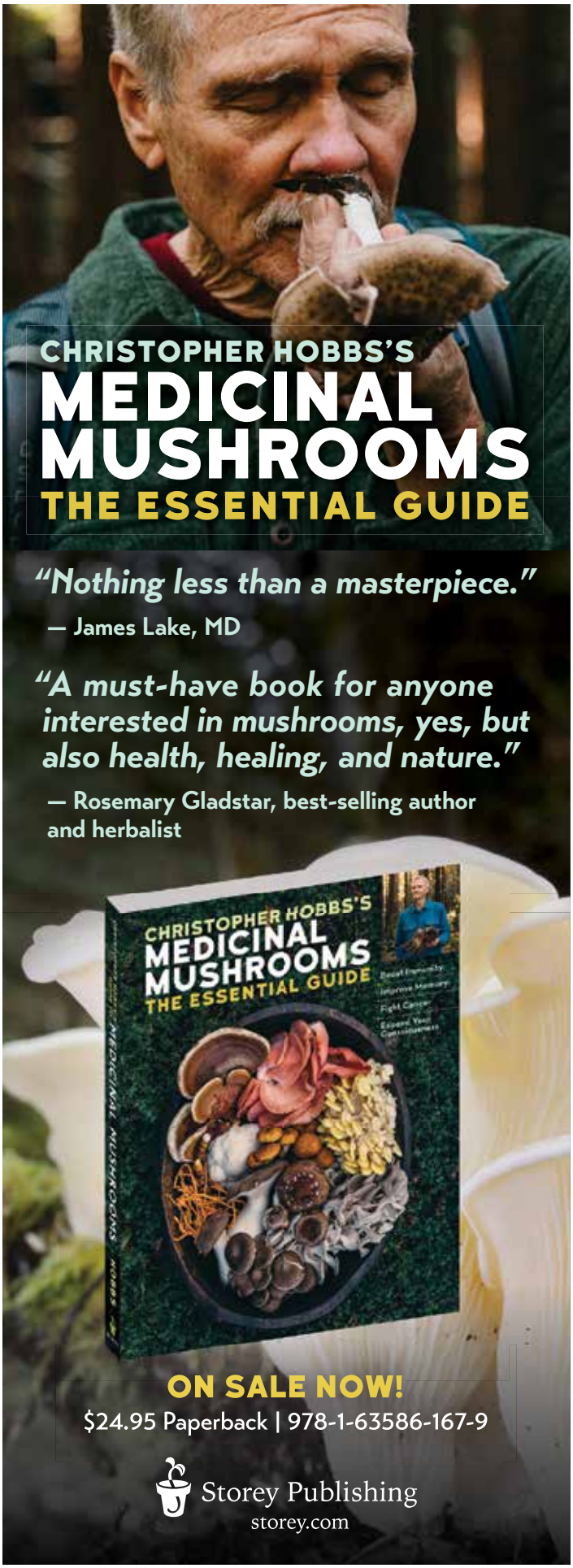


Figure 1. Bootstrap consensus tree of Isaria *species. This was done to determine how the* Isaria farinosa *isolated from the moribund Spotted Lanternfly were related to other species of* Isaria. *DNA sequences were found in Genbank for species that were reported in Gallou et al. (2015).* Isaria *isolated from the dead Spotted Lanternfly in this study were most closely related to the* Isaria farinosa *CBS 111113 (Gallou et al., 2015).*

Genbank (Gallou et al., 2015; Luangsa-Ard et al., 2005). Figure 1 shows the bootstrap consensus tree generated using the gene sequences obtained for isolates D6 and D8. Figure 1 compares the *Isaria* gene sequences from Gallou



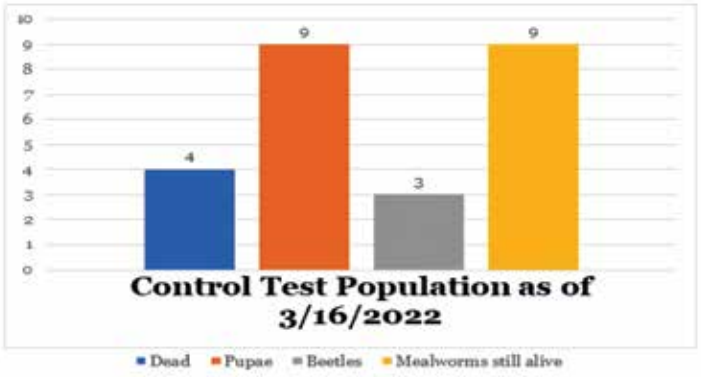


Figure 2. Graphical representation of the mealworm population of the control test at the end of one month. These results were used for our Expected Values in our χ^2 Analysis.

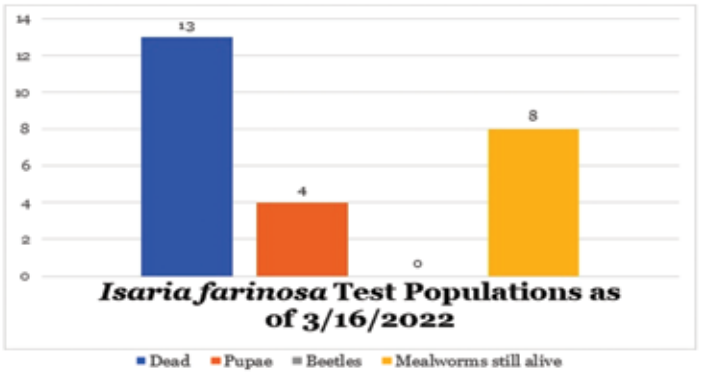


Figure 3: Graphical representation of the mealworm population of the Isaria farinosa *test at the end of one month.*

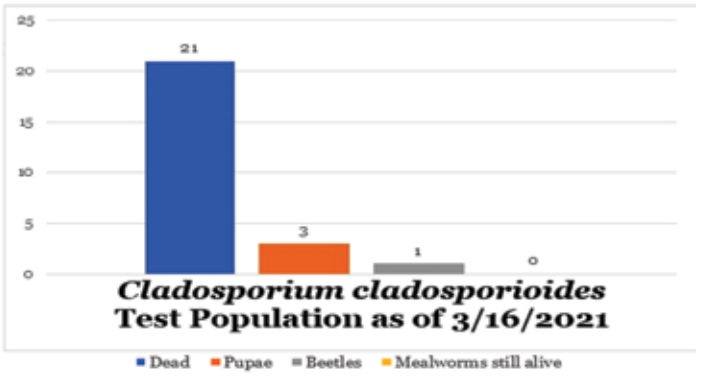


Figure 4: Graphical representation of the mealworm population of the Cladosporium cladosporioides *test at the end of one month.*

et al. (2015) and Luangsa-Ard et al. (2005) to the gene sequences for isolates D6 and D8. The consensus tree shows that both isolates D6 and D8 conform favorably with *I. farinosa* CBS 111113 with a 100% bootstrap consensus. In Luangsa-Ard et al. (2005) the isolate with the CBS number 111113 is listed as *Paecilomyces farinosus* while the specimen with the same CBS number is listed as *I. farinosa* in Gallou et al. (2015). Due to this, it is unclear where exactly *I. farinosa* lands in the clade *Clavicipitacae.* The *I. farinosa* species is being used in Mexico as a way to control pest insect species and is being used to control the population of white flies (*Trialeurodes vaporariorum*) throughout warm areas of the world (Gallou et al., 2015; Zimmermann, 2008). Thus, *I. farinosa* being used as a biocontrol agent for

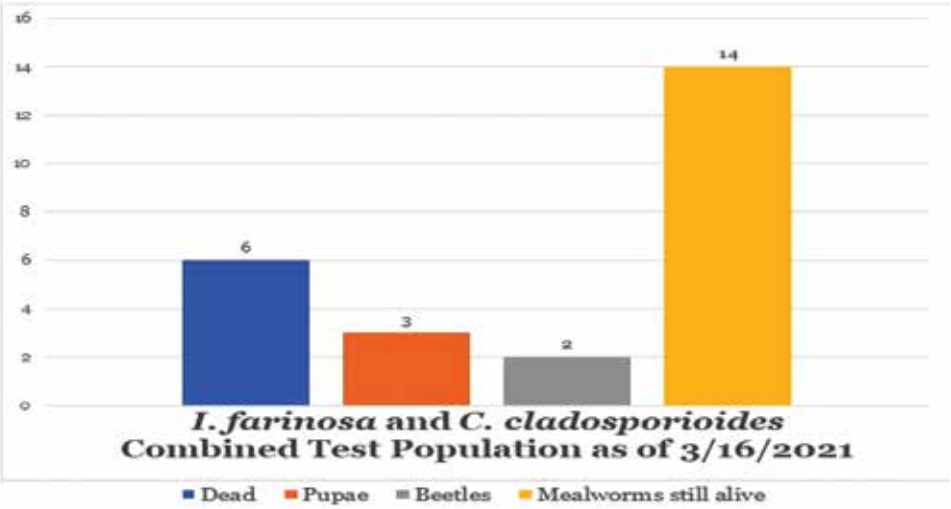


Figure 5. Graphical representation of the mealworm population of the combined I. farinosa *and* C. cladosporioides *test at the end of one month*

Table 3. ITS Sequences of the genus *Isaria* used to generate bootstrap consensus of isolated *I. farinosa* species.

Species	CBS Number for Sequence
Isaria tenuipes	994.73
Isaria coleopterora	102.73
Isaria fumosorosea	337.52
Isaria farinosa	111113
Isaria amoenerosea	107.73
Isaria javanica	134.22
Isaria farinosa	541.81
*Cordyceps militaris**	108.70

*Used as outlier group in bootstrap consensus tree to determine how isolates 6 and 8 were related to other members of their genus.

spotted lanternflies is possible as the fungi has been found to infect species within many orders of insects. These orders include but are not limited to: Lepidoptera, Coleoptera, Diptera, and Hemiptera (Zimmermann, 2008).

The primary fungus used as a biocontrol for Spotted Lanternflies, *Beauveria bassiana*, was not isolated from the mycelium-covered lanternflies in this study on SAB or DOC2 media. However, other entomopathogenic fungi, *Isaria farinosa*, *Cladosporium cladosporioides*, and *Fusarium lateritium* were isolated from the dead lanternflies on DOC2 media. Due to this, it is imperative that other fungi are considered as biocontrol agents for the spotted lanternfly as the invasive insect spreads through the eastern United States.

Mealworm Experiment: The work done by Gallou et al. (2015) indicates that *Isaria farinosa* can be used as a biocontrol as it is being used to control the spread of Asian Citrus Psyllid (*Diaphorina citri*). This presented the opportunity to test two of the three fungi isolated from DOC2 to see which of the isolates were lethal to insects and thus viable biocontrol agents.

In order to determine if the fungi isolated on the DOC2 were capable of killing insects, mealworms were inoculated with the fungi isolated from DOC2 media. The mealworms were placed into 4 Tupperware containers containing oat flakes and apple slices and each were treated with a different peptone solution. The control mealworms were treated with peptone broth. The Test 1 mealworms were treated with peptone broth containing isolate D4 (*I. farinosa*). The Test 2 mealworms were treated with peptone broth containing isolate D12 (*C. cladosporioides*). The Test 3 mealworms were treated with peptone broth containing both isolate D4 and isolate D12. The use of *I. farinosa* and *C. cladosporioides* in this experiment was done to avoid the use of plant pathogens, like *Fusarium lateritium*. While *F. lateritium* was the third entomopathogenic fungi that was isolated from the dead lanternflies on DOC2 media, it is considered a plant pathogen in addition to being entomopathogenic (Clark, 1994; Clark et al., 1990). This was done in order to determine if there were other fungi that could be used as a biocontrol for insects that would not have a negative effect on the environment that was treated (Clark, 1994 and Clark et al., 1990).

Table 4. End results of the mealworm experiment.	
Date Experiment Started:	2/15/2021
Date Experiment Ended:	3/16/2021
Control	**Number of Mealworms:**
Dead	4
Pupae	9
Beetles	3
Mealworms still alive	9
Test 1 (Using Isolate 4)	**Number of Mealworms:**
Dead	13
Pupae	4
Beetles	0
Mealworms still alive	8
Test 2 (Using Isolate 12)	**Number of Mealworms:**
Dead	21
Pupae	3
Beetles	1
Mealworms still alive	8
Test 3 (Isolate 4 and 12 Combined)	**Number of Mealworms:**
Dead	6
Pupae	3
Beetles	2
Mealworms still alive	14

The experiment was ended on the 16th of March 2021 after starting a month prior on the 15th of February 2021. In the control Tupperware container, there were 4 dead mealworms, 9 pupae, and 3 beetles. There was a total of 9 mealworms that were still alive at the end of the experiment in the control container (Fig. 2).

In the Test 1 Tupperware container where the mealworms were exposed to isolate D4, there were 13 dead mealworms, 4 pupae, and 0 beetles

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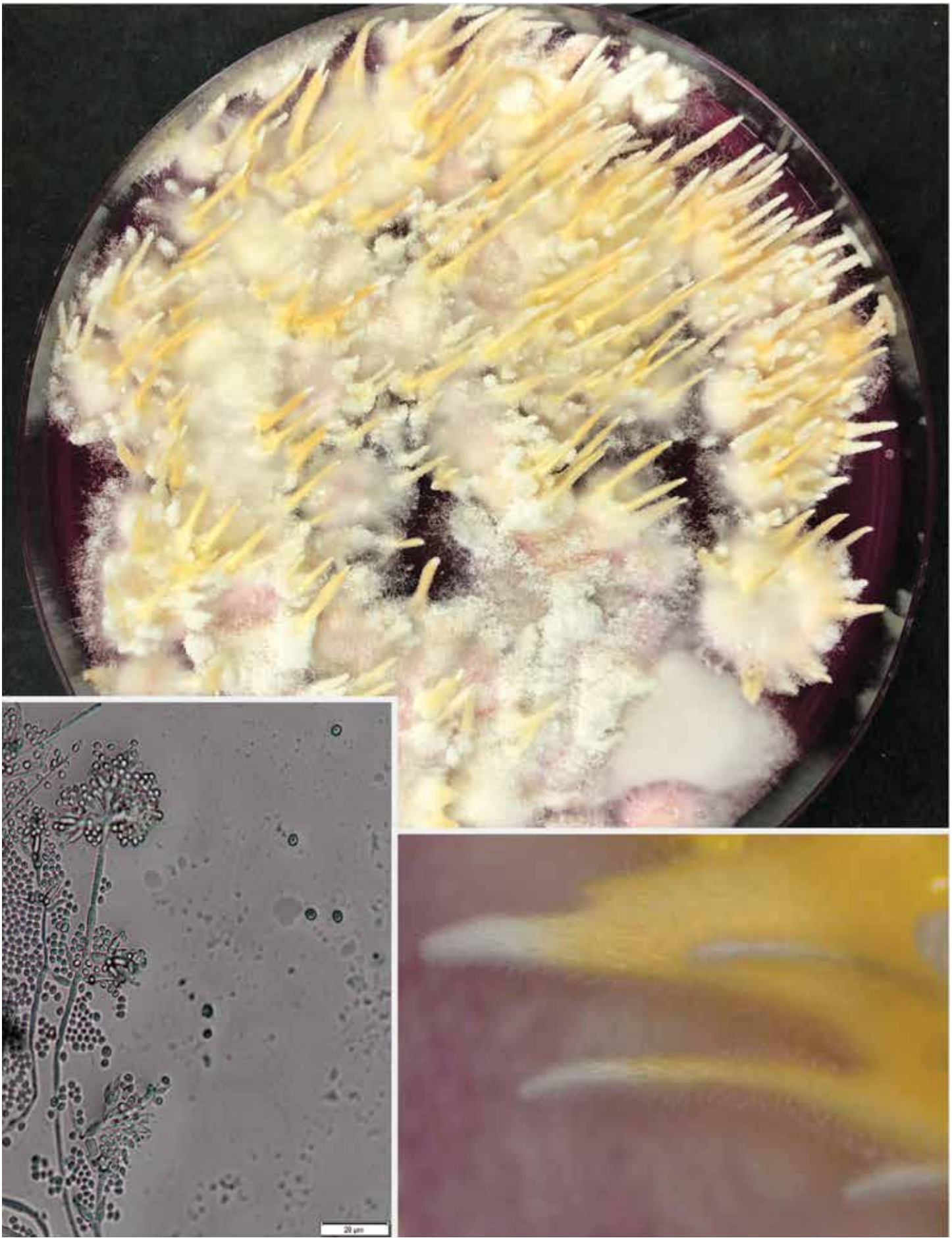

Table 5. Test 1, χ^2 Analysis.			
Expected	**Observed**	**$(O-E)^2$**	**$(O-E)^2/E$**
4	13	81	20.25
9	4	25	2.77777778
3	0	3	9
9	8	1	0.11
df=3	**x^2=7.18**	**x2 calc=**	**26.1377778**

Table 6. Test 2, χ^2 Analysis.			
Expected	**Observed**	**$(O-E)^2$**	**$(O-E)^2/E$**
4	21	289	72.25
9	3	36	4
3	1	4	1.33333333
9	0	81	9
df=3	**d=7.18**	**x2 calc=**	**86.5833333**

Table 7. Test 3, χ^2 Analysis.			
Expected	**Observed**	**$(O-E)^2$**	**$(O-E)^2/E$**
4	6	4	1
9	3	36	4
3	2	1	0.33333333
9	14	25	2.77777778
df=3	**d=7.18**	**x2 calc=**	**8.1077778**

on the 16th of March. There were 8 mealworms that were still alive in the Test 1 Tupperware when the experiment ended (Fig. 3). In the Test 2 Tupperware container where the mealworms were exposed to isolate D12, there were 21 dead mealworms, 3 pupae, and 1 beetle. When the experiment was ended, there were no mealworms alive in the Test 2 Tupperware container (Fig. 4). In the Test 3 Tupperware container where the mealworms were exposed to a combination of isolate D4 and isolate D12, there were 6 dead mealworms, 3 pupae, and 2 beetles. There were 14 mealworms still alive in the Test 3 Tupperware container (Fig. 5).

Only one trial of this experiment was performed at this time due to time constraints. Because of this, when performing the χ^2 analysis, the control variables were used to as the expected numbers of the dead mealworms, pupae, beetles and living mealworms respectively (Tables 6–8). The null hypothesis for this experiment would then be that the ratios are the same in each test as they are in the control. The alternate hypothesis would be the ratios are different than the control ratio in each test. The degrees of freedom for each of the tests was 3 and the d-value was 7.18.

For Test 1, the χ^2_{calc} value was 26.14. The null hypothesis was rejected by this value and the alternate hypothesis was adopted for Test 1, showing that the

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data collected was statistically significant. For Test 2, the χ^2_{calc} value was 86.58. The null hypothesis was rejected by this value and the alternate hypothesis was adopted for Test 2, showing that the data collected was statistically significant. For Test 3, the χ^2_{calc} value was 8.11. The null hypothesis was rejected by this value and the alternate hypothesis was adopted for Test 3, showing that the data collected was statistically significant.

The impact of the fungi in each of the tests is shown physically as well as statistically. There was one mealworm that made it to the adult stage in its life cycle found in the Test 2 Tupperware container at the end of the experiment. The χ^2_{calc} value for this test was 86.58, which rejected the null hypothesis and showed that there was a statistical significance between the Test 2 values and the control. This was not the case with *I. farinosa*, as there were still mealworms alive in the Test 1 container at the end of the experiment, a total of 8 mealworms alive and 13 dead, but no beetles were present. The χ^2_{calc} value for this test was 26.14, which rejected the null hypothesis as well, indicating a statistical significance between the Test 1 values and the control. In the Test 3 container, the mealworms were exposed to a combination of both *I. farinosa* and *C. cladosporioides*. In this container, there were 14 mealworms still alive at the end of the experiment as well as 2 beetles with 6 dead mealworms found in the container. The χ^2_{calc} value for this test was 8.1, this also rejected the null hypothesis and indicated a statistical significance between the Test 3 values and the control. The d-value for this experiment was 7.18 and is extremely close to the chi-squared-value of Test 3. This is significant as the number of still living mealworms is larger than that of Test 1 or Test 2, which indicates that there may be some inhibition taking place between the two species of fungi due to the relative closeness in between the d-value and the χ^2 value in Test 3. This would require further testing to see if inhibition was the cause of the decreased significance of values in Test 3.

The re-isolation of *Isaria farinosa* and *Cladosporium cladosporioides* on DOC2 media from the dead mealworms indicates that the cause of death was the fungi the mealworms were exposed to over the month-long period. However, the numbers of the mealworms that died in each test container indicates that *C. cladosporioides* was better at rendering the mealworms moribund in the container for Test 2 with none being left alive in the container at the end of the experiment, with a total of 21 dead while there were some mealworms still left alive in the containers for Test 1 and 2.

Conclusions

This work shows that there are some species of fungi that have been used in other countries to control pest insects, and that these can be used to control the spread of the Spotted Lanternflies across the East Coast of the USA which have been overlooked in recent years. *Cladosporium cladosporioides* and *Fusarium lateritium* have been isolated from Spotted Lanternflies both on specialized media and unspecialized media; *Isaria farinosa* was only found on the DOC2 media. With only one test of both *I. farinosa* and *C. cladosporioides* on mealworms, there is more work that needs to be done and this experiment needs to be repeated in the future. Use of *L. delicatula* in an experiment such as the above would prove to beneficial to see if the target insects are as susceptible to infection from *I. farinosa, C. cladosporioides* and *F. lateritium*. This is difficult due to the quarantining of several counties and would need to

be performed at an institution within the currently quarantined counties to prevent the spread of the invasive species of insect. In addition to this, seeing how widespread *I. farinosa* is throughout the state of Pennsylvania can also be used to determine if the fungi are common enough to be used as a biocontrol agent as the use of *B. bassiana* is tied to the prevalence of the fungi within the state (Clifton et al., 2019; Urban, 2020; El-Kichaoui et al., 2016). Sampling soil and moribund insects across the state would allow for a better understanding of the microbiome of the state and to see if the Spotted Lanternfly is found in places with a high or low content of entomopathogenic fungi.

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Springtime in the Rockies, Lichen

All the years I overlooked them in the
racket of the rest, this
symbiotic splash of plant and fungus feeding
on rock, on sun, a little moisture, air–
tiny acid-factories, dissolving
salt from living rocks and
eating them.

Here they are blooming!
Trail rock, talus and scree, all dusted with it:
rust, ivory, brilliant yellow-green, and
cliffs like murals!
Huge panels streaked and patched, quietly
with shooting-stars and lupine at the base.

Closer, with the glass, a city of cups!
Clumps of mushrooms and where do the
plants begin? Why are they doing this?
In this big sky and all around me peaks &
the melting glaciers, why am I made to
kneel and peer at Tiny?

These are the stamps on the final envelope.
How can the poisons reach them?
In such thin air, how can they care for the
loss of a million breaths?
What, possibly, could make their ground more bare?

Let it all die.

The hushed globe will wait and wait for
what is now so small and slow to
open it again.

As now, indeed, it opens it again, this
scentless velvet,
crumbler-of-the-rocks,

this Lichen!

Lew Welch

From *Ring of Bone: Collected Poems 1950-1971.*
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