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Growing *Trichoderma asperellum*, a natural fungicide, on coffee pulp and husks

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ABSTRACT

Coffee farms often rely on chemical fungicides to control fungal crop diseases such as ojo de gallo (*Mycena citricolor*) and red rust (*Hemileia vastatrix*). *Trichoderma* is a genus of fungus known to parasitize other fungi and it can be used to treat these crop diseases. Research has already proved that *Trichoderma spp.* can be cultivated on various organic substrates. Our goal was to help coffee farmers discover which coffee waste products could grow *Trichoderma asperellum* best. To do this, we collected three coffee by-products from Café de Monteverde: "young" coffee pulp, "old" coffee pulp, and coffee husks. We mixed these coffee waste byproducts into different ratios to create eight different coffee material test substrates. In addition to these test substrates, we chose goat waste and rice as test substrates. The three test substrates that had the highest percent colonization of *T. asperellum*, in order, were: (1) 50% old pulp and 50% coffee husk, (2) 75% old pulp and 25% coffee husk, and (3) 50% young pulp and 50% coffee husk. We saw conidial pigmentation indicating a more mature stage of *Trichoderma* growth in rice only. The pH had an optimal range of percent colonization of *T. asperellum* from about 5.5-7.5. Meanwhile, moisture had no observed correlation with percent colonization. Due to the colonization success of our *T. asperellum*, we know it is possible for *T. asperellum* to grow on coffee byproducts in a non-laboratory setting. Future research should study how long the *T. asperellum* needs to grow on the test substrates discussed in this experiment before it is concentrated enough for use as a fungicide and also how to make this procedure more effective for use on a larger scale.

Cultivo de *Trichoderma asperellum*, un fungicida natural, sobre pulpa y cáscaras de café

RESUMEN

Las fincas de café dependen a menudo de fungicidas químicos para controlar enfermedades fúngicas de cultivos como ojo de gallo (*Mycena citricolor*) y roya (*Hemileia vastatrix*). *Trichoderma* es un género de hongo conocido por parasitar otros hongos y puede usarse para tratar estas enfermedades en los cultivos. Estudios previos han demostrado que *Trichoderma* puede ser cultivada en diferentes sustratos orgánicos. Nuestro objetivo era ayudar a caficultores a descubrir

qué desechos de café podrían servir para cultivar *Trichoderma asperellum*. Con este fin, colectamos tres subproductos de café de Café de Monteverde: pulpa de café "joven", pulpa de café "vieja", y cáscaras de café. Mezclamos estos subproductos de desechos de café en proporciones diferentes para crear ocho sustratos. Además de estos sustratos, utilizamos boñiga de cabra y arroz. Los tres sustratos que tuvieron la colonización más alta de *T. asperellum*, en orden, fueron: (1) 50% pulpa vieja y 50% cáscara de café, (2) 75% pulpa vieja y 25% cáscara de café, y (3) 50% pulpa joven y 50% cáscara de café. Observamos pigmentación conidial en arroz solamente. La pigmentación indicaba una etapa más madura de crecimiento de *Trichoderma*. El pH tenía un rango óptimo del porcentaje de colonización de *T. asperellum* de aproximadamente 5.5-7.5. El porcentaje de humedad no tuvo correlación con el porcentaje de colonización. Debido al éxito de colonización de nuestro *T. asperellum*, sabemos que es posible que crezca en subproductos de café sin tener que ser cultivado en laboratorio. Estudios futuros podrían determinar cuánto tiempo necesita *T. asperellum* para crecer en los sustratos analizados en este experimento antes de que sea lo suficientemente concentrado para usarse como un fungicida y cómo hacer este procedimiento más eficaz para un uso a gran escala.

Coffee is the second most traded commodity in the world, only falling behind petroleum (Pushpa, 2012). Cultivation of this crop is not easy; coffee plants are susceptible to the fatal fungal diseases ojo de gallo (*Mycena citricolor*) and red rust (*Hemileia vastatrix*). These diseases wreak the most havoc in moist conditions. For countries that have intense wet periods and export significant amounts of coffee, such as Costa Rica, this poses a significant problem. In order to fight these fungal diseases and save their crops, many farms employ synthetic chemical fungicides that have the potential to harm the environment and human health (Martinez-Toledo, 1998, Yüzbaşıoğlu, 2006). However, for farms dedicated to producing organic crops, there is another option. *Trichoderma* is a genus of fungi that has evolved to parasitize other fungi (Harman, 2017). Many species in the genus *Trichoderma* possess fungicidal properties, especially *Trichoderma asperellum*, which can parasitize 19 genera of other fungi (Harman, 2004 & Wu, 2017). Coffee farms such as Café de Monteverde, a sustainable coffee farm in Monteverde, Costa Rica, use *Trichoderma* spp. to eliminate risk of ojo de gallo and red rust, but many do not. According to staff at Café de Monteverde, there are two limiting factors preventing otherwise organic farms from replacing chemical fungicide use with this biocontrol method. These are its cost of $10 per kilogram, expensive for a coffee farm of many hectares, and its lack of availability (S. Barrios, pers. comm.). *Trichoderma* spp. sold for agricultural biocontrol use are usually grown in tubes and plates of potato dextrose agar (PDA) before they are transferred to rice, the substrate on which they are distributed (Dr. M. Obregón, pers. comm.). This method of growth is highly specific to a laboratory setting and inaccessible to farms.

However, *Trichoderma* spp. that are useful for biocontrol have been successfully cultured on various organic materials, such as maize meal, rice husk, sawdust, and wheat bran (Singh, 2007), although a method specific to utilizing coffee byproducts has yet to be simplified and made accessible to small farms. Fungi outside of the genus *Trichoderma* have been grown using coffee pulp (Rathinavelu, 2005 & Martínez-Carrera, 2000), an organic waste that is readily available at Café de Monteverde and other coffee farms. After growing the *Trichoderma* sp. cheaply on these

alternate materials, it can be dispersed throughout a farm's coffee fields as a fungicide. Our goal is to provide a method for small-scale organic farms to produce their own affordable supply of *Trichoderma* for use as a biocontrol agent. By working with several easily accessible organic substrates we hope to answer the question: what mixes of coffee waste substrates work best to grow *Trichoderma asperellum*?

MATERIALS AND METHODS

We collected three coffee byproducts from Café de Monteverde: young coffee pulp, old coffee pulp, and coffee husks. The young coffee pulp was one week to two months old and the old coffee pulp was nine months to one year old. The young coffee pulp was more acidic and contained more liquid than the dry old coffee pulp. We collected fresh goat waste from Café de Monteverde. We chose goat waste as an additional test substrate because it is a nutrient rich waste material. Lastly, we chose rice as an additional test substrate because *Trichoderma* spp. are known to grow on cooked rice (Cavalcante, 2008). We chose ten different mixes of substrates (Table 1, Figure 1).

Table 1: Composition of the ten test substrates used in this experiment and their substrate codes. In this paper, test substrates are referred to by their substrate code. All percentages determined by volume.

Substrate code	**Substrate composition**
YP	young coffee pulp
75 YP	75% young coffee pulp + 25% coffee husk
50 YP	50% young coffee pulp + 50% coffee husk
25 YP	25% young coffee pulp + 75% coffee husk
OP	old coffee pulp
75 OP	75% old coffee pulp + 25% coffee husk
50 OP	50% old coffee pulp + 50% coffee husk
25 OP	25% old coffee pulp + 75% coffee husk
Rice	cooked rice
Feces	goat waste

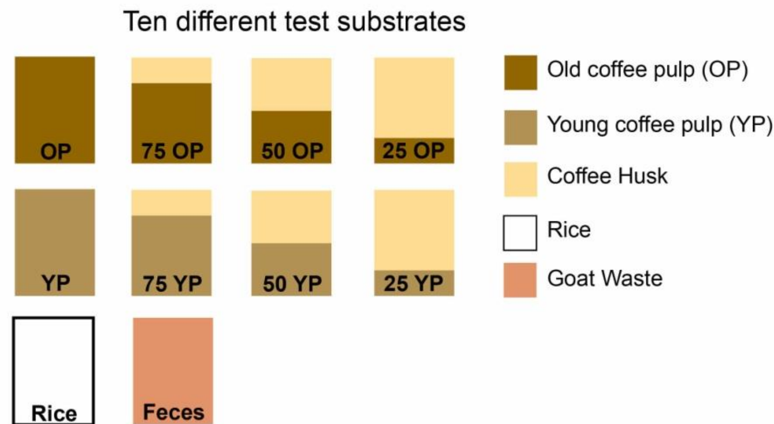

Figure 1: Composition of ten test substrates and their corresponding substrate codes.

We placed each substrate type into four separate bags with 750 mL of substrate each. The four bags were referred to as replicate 1 (R1), replicate 2 (R2), and replicate 3 (R3), and control. Controls were treated the same as the three replicates, but no *T. asperellum* was added.

Pasteurization and inoculation of test substrates

To pasteurize these materials before inoculating them with *T. asperellum*, we floated plastic bags of 750 mL of each substrate mix in water kept at 70°C for 20 minutes to complete the pasteurization process (van Loenen, 2003). We used this pasteurization to prevent other microorganisms already present in the substrate from outcompeting the *Trichoderma asperellum* for growth. After pasteurization, we labeled each bag with the type of substrate and the replicate number and allowed them to cool at room temperature overnight.

We sterilized PVC pipes 10 cm long and 2 cm in diameter. We did this by either (1) boiling for 10 minutes or (2) by washing with first 10% bleach, then 70% ethanol, and finally rinsing with distilled water.

We ordered one kilogram of *T. asperellum* from Laboratorios Dr. Obregón (San Francisco, Heredia Province, Costa Rica). The fungi came as dark green spores on rice. Once the bags had cooled after pasteurization, we used this *T. asperellum* to inoculate the substrates, adding 10 grams of the rice with *T. asperellum* spores to each of the sterilized bags. We inserted the sterilized PVC pipe into the opening of each bag. We blocked the inside of the PVC pipe with cotton to allow airflow into the bag with less risk of contamination. Lastly, we used a rubber band to tightly seal the plastic bag around the PVC pipe (Figure 2).

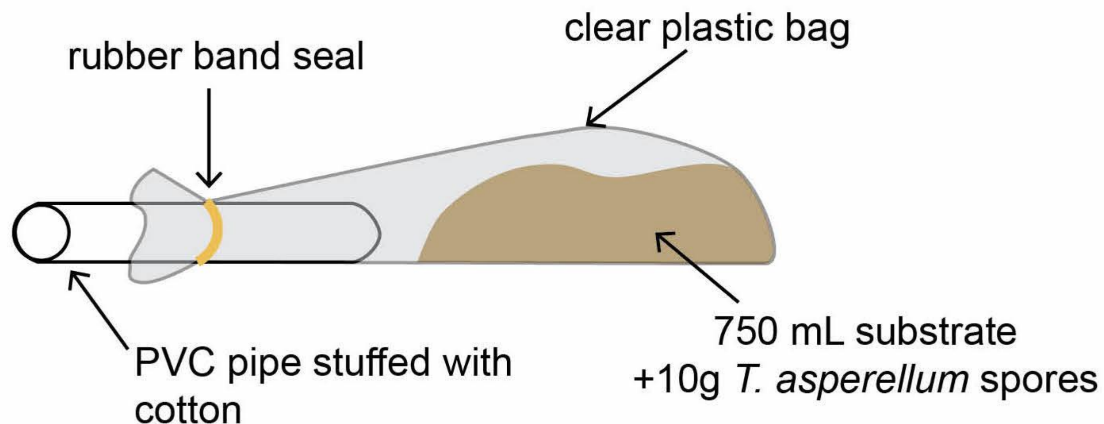

Figure 2: Experimental setup used in this experiment. Note that we pasteurized clear plastic bags and substrates and sterilized the PVC pipes before we added any *T. asperellum* to the mixes.

Measurements

On the first day of growth, we measured the pH and percent moisture of each bag. We took these measurements using a soil moisture meter and a pH meter with temperature capacities. After the first day, we only measured R1 of each test substrate every other day to decrease the risk of possible contamination for most of the bags. After one week of growth at outside temperature (approximately 20℃), we moved the bags into an incubator set to 28℃ to increase the growth rate.

On the eleventh and final day of growth, we spread the contents of each individual bag out in a box and visually approximated the percentage of the substrate that had been colonized by *T. asperellum*. We also took a picture of each spread. By measuring the approximate percent colonization per replicate of each substrate, we were able to qualitatively rank which substrates are best for T. asperellum proliferation.

This method of estimating the amount of Trichoderma was used as an alternative to the Neubauer counting chamber, which did not work for this experiment. We would have used a Neubauer chamber to view a dilution of our substrate under the microscope and count the mycelium and spores in each sample. Because each grid on the Neubauer chamber is an exact volume, this mycelium and spore count would have allowed us to quantitatively determine the exact amount of mycelium/mL of substrate. However, the complexity of the substrates and large, diverse amounts of microscopic material in each substrate made it difficult to distinguish the *T. asperellum* from other microscopic materials.

Plating for Microscopic Confirmation of *T. asperellum*

We sterilized plates and potato dextrose agar (PDA) in a pressure cooker used as an autoclave at 121℃ for 20 minutes. We poured PDA while it was hot into sterilized glass petri dishes with 25 mL PDA per plate. On day 9 of growth, we removed 1 mL of mycelium from replicate 1 of 75 YP and diluted it in 9 mL of water. We also diluted 1 mL of *T. asperellum* from Laboratorios Dr. Obregón. in 9 mL of water. We plated 25 µL of each solution on opposite ends

of the same plate. We left the plate in an incubator at 28°C for two days. We then used a metal spatula to remove a small amount of both laboratory and experimental *T. asperellum* from the plate onto a glass slide. We looked at these slides with a compound microscope.

Statistical analysis of results

We compared amount of growth of fungi (from percent colonization) by substrate using Kruskal-Wallis to see if substrate growth differed significantly by substrate. We also used the Tukey-Kramer test to group the substrates into statistically significant ($\alpha = 0.05$) groups as a way to rank our various substrates by success of *T. asperellum* growth. This ranking is intended as a tool for coffee farms to use when deciding how to grow *T. asperellum*.

RESULTS

Growth of *Trichoderma* by substrate

T. asperellum grew visible mycelium on 50 OP, 75 OP, and 50 YP significantly better than on other substrates (Figure 3, Tukey-Kramer, $p<0.05$,). We recorded the growth of all substrates as measured by percent colonization with the initial pH and moisture of each test substrate (Table 2).

The difference in the controls and experimental bags was clear after three days of growth. While on the second day of growth, we observed white mycelial growth on all replicates of 75 YP and 50 YP, there was no mycelial growth on any of the controls, even by the third day. We observed this qualitatively and did not open the bag to avoid introducing contamination. Because of this, although there were eventually small amounts of growth in some of the controls when opened (Table 2), the large amount of growth in the experimental replicates observed after three days and that grew thereafter was likely *Trichoderma*.

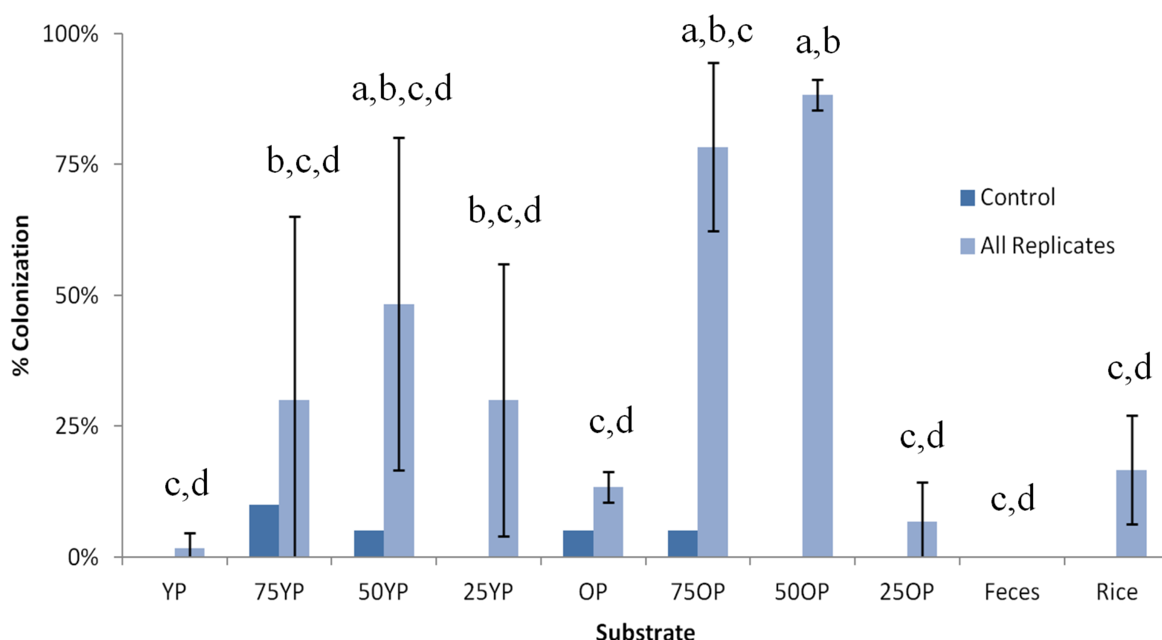

Figure 3: Average percent colonization of each substrate type and respective controls. Error bars represent one standard deviation. Letters indicate significant groupings from Kruskal-Wallis and Tukey-Kramer analysis. For substrate codes, see table 1.

Table 2: Substrates listed in order of decreasing average percent colonization. A control was not performed for goat feces because no colonization was observed on any of the replicates. For substrate codes, see table 1.

Substrate	**Average percent colonization**	**Percent colonization of control**	**Initial pH**	**Initial percent moisture**
50 OP	88.3±2.9%	0%	5.44	3.13%
75 OP	78.3±16.1%	5%	6.80	9.53%
50 YP	48.3±31.8%	5%	5.56	>50%
25 YP	30.0±26.0%	0%	5.33	>50%
75 YP	30.0±35.0%	10%	6.50	>50%
Rice	16.7±10.4%	0%	6.34	12.90%
OP	13.3±2.9%	5%	8.47	1.27%
25 OP	6.7±7.6%	0%	4.98	22.93%
YP	1.7±2.9%	0%	8.08	>50%
Feces	0.0±0.0%	NA	8.36	>50%

Substrate characteristics contributing to *T. asperellum* growth: pH and moisture

The growth of *T. asperellum* as measured by percent colonization was the highest within the optimal pH range of 5.5-7.5 (Singh, 2014). The four substrates with the lowest average approximate percent colonization all had pH's outside of this optimal pH range for *Trichoderma* spp. (Figure 4). The growth of *T. asperellum* was not affected by percent moisture of the substrate (Figure 5).

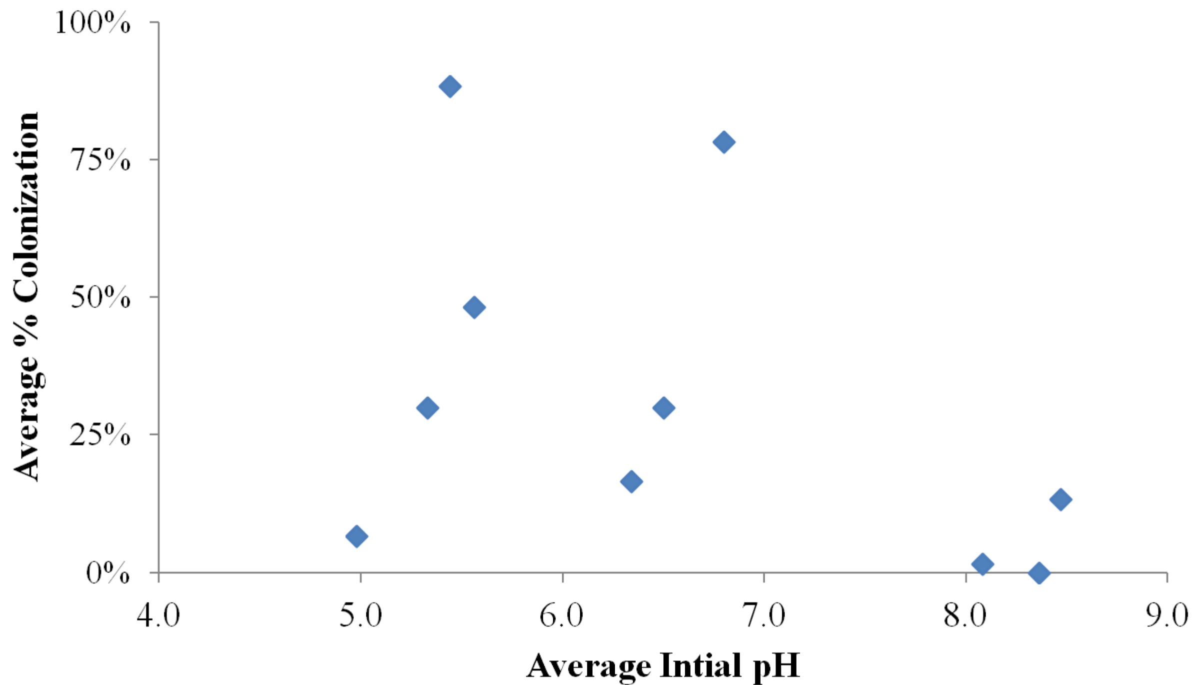

Figure 4: Average initial pH of each substrate affects the final average percent colonization. The three substrates with the highest percent colonization were all within about 1 pH unit of each other.

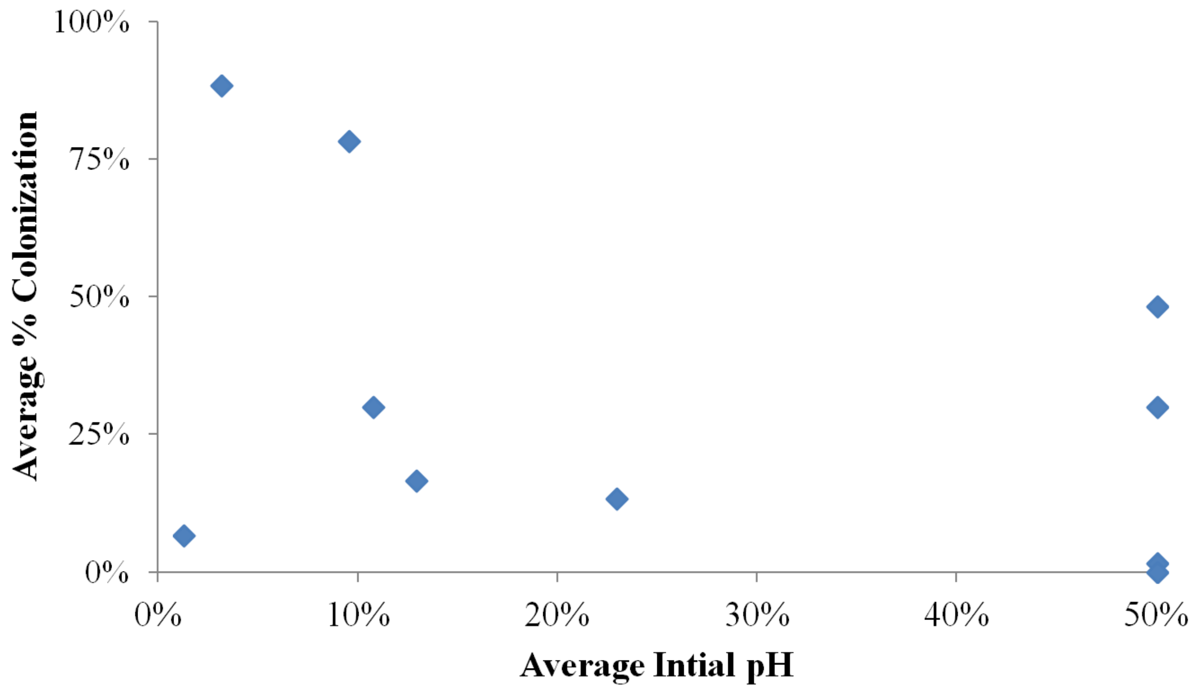

Figure 5: Initial percent moisture did not have a clear effect on average percent colonization. The moisture meter did not measure above 50%. Substrates measured as at least 50% moisture are graphed as 50% moisture.

Identification of experimental mycelium as *Trichoderma*

We compared the laboratory *T. asperellum* to our experimental *T. asperellum* and found conidial mycelium in both. Dr. Obregón confirmed both samples in these photos are *T. asperellum*.

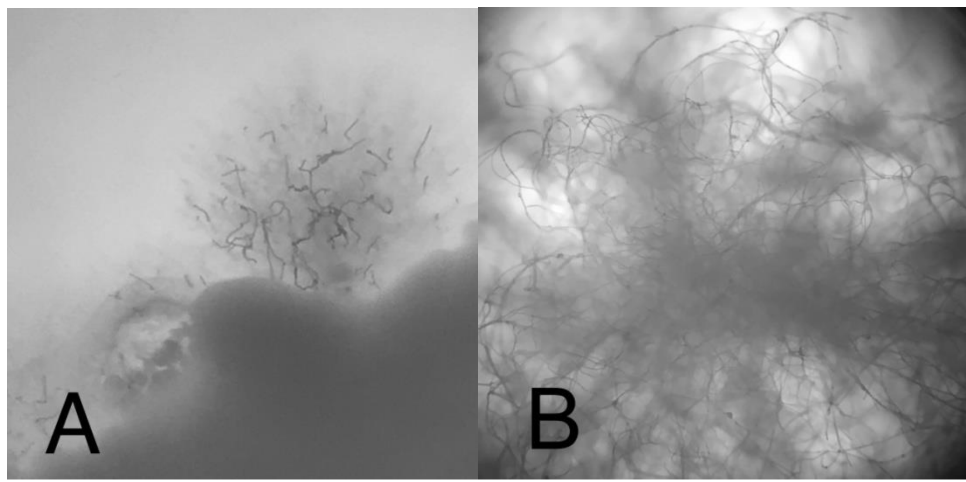

Figure 6A: Photo of laboratory *T. asperellum* conidial mycelium at 4x magnification.

Figure 6B: Photo of experimental *T. asperellum* conidial mycelium at 10x magnification. Identification confirmed by Dr. M. Obregón (pers. comm.).

DISCUSSION

T. asperellum grew best in 50 OP (50% old coffee pulp, 50% coffee husk), 75 OP (75% old coffee pulp, 25% coffee husk), and 50 YP (50% young coffee pulp, 50% coffee husk) (Figure 3). In this section, we consider the factors that made these three mixtures suitable test substrates as well as important points from this experiment for farms that intend to cultivate their own *Trichoderma asperellum*.

pH's role in measured fungal growth

Trichoderma spp. grow from a wide range of pH, from pHs of 4-8 (Singh, 2014). Maximum growth occurs between pH 5.5-7.5 (Singh, 2014). Our most successful test substrates had pH's at the more acidic end of the known maximum growth range. The four substrates with the least growth were more than 0.5 pH units away from known growth range (Figure 4). We did not find a single optimal pH value. This and the fact that we did not find successful substrates at the more basic end of the optimal pH range is likely due to other factors that affect *Trichoderma* growth such light, calcium, moisture, and oxygen (Kubicek, 1998 & Steyaert, 2010).

Moisture and its effect on fungal growth

The best three test substrates had a wide range of moisture (Figure 5). However, it seems as if excessively high or low moisture content can negatively affect *T. asperellum* growth. The 25 OP showed minimum amounts of growth and had a 1.27% moisture content. Meanwhile, YP and feces also had minimal growth, but had moisture content above 50%. All three of these test substrates that grew the least amount of *T. asperellum* had extreme moisture contents in addition to pH's outside of the known growth range, so lack of growth could be attributed to either parameter: pH or moisture content.

Relatively dry (moisture content of approximately 3-15%) substrates could have each had a higher percent colonization due to the ease of mixing the sample. When we incorporated *T. asperellum* into the old pulp and coffee husk mixtures (the two driest coffee byproducts), it was much easier to evenly distribute the spores throughout the substrate. In the wetter young pulp mixtures, many clumps had very obvious *T. asperellum* growth, but it was not evenly distributed, leaving patches without any *T. asperellum*. This patchy distribution may have contributed to the lower and more varied percent colonization of the best young pulp mixtures. Besides this, it was likely that moisture by itself could have affected growth of *Trichoderma asperellum*, since moisture has been shown to affect spore germination times and rates of growth in other fungi (Ayerst, 1969).

Conidial pigmentation and percent colonization in rice

When measuring percent colonization on the last day of growth, we noticed that *T. asperellum* on rice had patches of dark green that no other test substrates showed. This coloration indicates that *Trichoderma* sp. has entered a more mature part of its life cycle and is about to sporulate (Kubicek, 1998 & Steyaert, 2010). Nutritional factors, like the ratio of C:N in a substrate significantly affect conidiation of *Trichoderma* spp. (Steyaert, 2010). It is possible that the nutritional profile of rice promotes conidiation more than the nutritional profiles of coffee byproducts, which is why characteristic dark green was not seen on any of the coffee byproduct test substrates. The *T. asperellum* observed in the rice test substrate was more mature, as shown

by its green coloration, than the *T. asperellum* observed in other test substrates, such as 50 YP, that were more visibly colonized than rice. Although *T. asperellum* grown in rice may reach mature stages of growth faster, using coffee byproducts to grow the fungus is recommended because of the sheer amount of coffee waste that farms produce. For every two tons of coffee produced, one ton of coffee pulp and 0.36 tons of coffee husks are generated: waste that could be used to grow *T. asperellum* (Roussos, 1995 & Adams, 1981).

Our visual method of quantifying growth introduced some bias. The greater visibility of green in rice is due to its color contrast. This may be why we did not see dark conidial pigmentation in our dark coffee byproduct substrates. By contrast, the mycelium was white and thus difficult to see in the rice. Thus, the recorded *T. asperellum* growth in rice was only what we could see: the mature green sections. This may have accounted for the relatively small percent colonization measured in rice despite the maturity of the green *T. asperellum*.

Use of experiment for future farms

We attempted to grow *T. asperellum* in a manner that would be possible for farms to imitate. However, temperature was a limit. *T. asperellum* mycelium growth is low at 20°C and peaks around 27°C (Domingues, 2016). Because of the constant 20°C climate throughout the experiment, we had to move our test substrates from the outdoors into an incubator to promote more growth within a limited time period. This method of using an incubator would be inaccessible to most farms. The method we reported will work best during hot months or inside of an insulated space.

Future research must also determine how long *Trichoderma asperellum* needs to grow before it is concentrated enough for use as a fungicide. Farms would also benefit from further study on how to increase the efficiency of this procedure for use on a larger scale, making it more practical for farms to begin enough cultivation to replace chemical fungicides.

Possible contamination

Because a diverse and large community of fungal spores are present in large quantities in the air (Fröhlich-Nowoisky, 2009 & Bauer, 2008), it is inevitable that the test substrate samples we had taken outdoors from Café de Monteverde contained fungal spores. Some fungal spores can withstand temperatures above 70°C for longer than 20 minutes (Jesenská, 1993). Our pasteurization process did not guarantee that all fungal competition was eliminated from our test substrates. However, because of the aggressive nature of *T. asperellum* and its ability to parasitize other fungi (Harman, 2004 & Wu, 2017), small amounts of contamination were likely to have been overtaken by the fungicidal fungus, and all mycelium we observed was T. asperellum (Dr. M. Obregón, pers. comm.).

Future research including cost analysis

We have confirmed that it is possible to effectively grow *T. asperellum* on coffee byproducts. However, the effectiveness of the experimental *T. asperellum* as a fungicide has not been tested. One way the experimental *T. asperellum* differs from the lab-grown *T. asperellum* is that the lab-grown *T. asperellum* comes to farms in spores and the bulk of the experimental T. asperellum was still mycelium by the end of the growth period. Although the experimental Trichoderma would likely have sporulated with more time, after 10 days of growth it was still

mostly in the mycelial form. Thus, future research should also compare the effectiveness of experimental mycelium to laboratory spores as a fungicide. This would give coffee farms more information to aid in deciding whether to purchase *T. asperellum* or to grow it themselves.

A goal of our experiment was to make *T. asperellum* cultivation possible for farms as a cheaper alternative to buying Trichoderma from laboratories. In order to know if the methods in this experiment have succeeded in making this process effective and cheaper, the quantity of spores grown must exceed the 10g of *T. asperellum* spores originally added, increasing in amount and concentration as the *T. asperellum* colonizes the substrates. Because of the limited timeframe of our experiment, we were not able to wait and see sporulation in all test substrates. If the amount of spores grown with these methods shows a significant increase in the amount of *T. asperellum* present, this method is excellent for farms who wish to use a biocontrol fungicide but have a limitation due to cost.

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