May 2017

Fungal growth inhibition by Quercus cortesii galls and the presence of gall-invaders

Samantha E. Simon

Fungal Growth Inhibition by *Quercus cortesii* Galls and the Presence of Gall-invaders

Samantha E. Simon
Department of Biological Sciences
University of California, Santa Barbara
EAP Tropical Biology and Conservation Program, Spring 2017
9 June 2017

ABSTRACT

Galls are abnormal plant growths induced by highly specialized arthropods and are used as a food source and for protection for the developing larvae. The chemical composition within galls is known for containing high levels of tannins, which have anti-fungal and anti-herbivory properties. This study focuses on the effect of tannins and other chemicals extracted from *Quercus cortesii* galls on the inhibition of the fungi *Mycena citricolor*. This study also explores the presence of gall-invaders such as arthropods and vertebrates. Two methods of treatment were used to determine the effect that tannins and other chemicals extracted from cynipid wasp galls found on the tree *Quercus cortesii,* have on fungal growth. One method, denoted as tannin agar, tested a tannin-infused substrate and showed that fungal growth is inhibited by the presence of gall extracts. The other method, denoted as tannin droplets, tested the effect of tannins after the fungus had already been established and showed no effect on the fungal growth. All of the data was analyzed using a one-way ANOVA and found that the tannin agar treatment had significantly less fungal growth and slower growth rate than the control treatment. The tannin droplet treatment showed no difference in fungal growth and growth rate when compared to the control treatment. Twelve galls were also collected and dissected to determine whether arthropods invade and use the gall after the cynipid wasps have left. At least thirty morpho-species of arthropods use the galls for feeding and protection. Additionally, it was observed that as the gall becomes wet, the diversity of morpho-species decreases. Lastly, camera traps revealed coatis could also be using the fallen galls to feed from.

Inhibición de Crecimiento de Hongo por Agallas de *Quercus cortesii* y la Presencia de Invasores de Agallas

RESUMEN

Las agallas son crecimientos anormales en las plantas inducidos por artrópodos altamente especializados. Las larvas en desarrollo utilizan las agallas como fuente de alimento y protección. Las agallas contienen altos niveles de taninos, los cuales tienen propiedades anti-fúngicas y anti-herbivoría. Este estudio se enfoca en el efecto de los taninos y otras sustancias químicas extraídas de las agallas de *Quercus cortesii* sobre la inhibición del hongo Ojo de Gallo *Mycena citricolor*. Este estudio también explora la presencia de invasores de las agallas tales como artrópodos y vertebrados. Se utilizaron dos métodos de tratamiento para determinar si los taninos u otros químicos presentes en las agallas afectan el crecimiento de hongos. El primer método (Agar con taninos) probó con un sustrato de agar mezclado con tanino que estos compuestos inhiben el crecimiento de hongos. El segundo método (Gotas de tanino) probó el efecto de los taninos después de que el hongo ya se había establecido y no mostró ningún efecto sobre el crecimiento de hongos. El tratamiento con agar de tanino tuvo un crecimiento de hongos significativamente menor y una tasa de crecimiento más lenta que el tratamiento de control. También colecté y disecté 12 agallas para determinar si habían otros artrópodos invasores de agallas luego de que la agalla había caído del árbol. Al menos 30 morfo-especies usan estas agallas para alimentación o protección. Además, se observó que a medida que la agalla está más húmeda, la diversidad de morfo-especies disminuye. Por último, las trampas de cámara revelaron que los pizotes también podrían estar utilizando las agallas para alimentarse.

Within the tropics, the world of insect-plant interactions is quite fascinating due to the high abundance of species, high diversity of species, and the complex interactions among them. The close host-pest interaction that I will be focusing on is the formation of galls. Galls are an abnormal plant growth, with an inner layer of nutritious tissue surrounding the gall-inducing larva (Nishida and Hanson, 2016). The insect is able to redirect normal plant metabolism and physiology in order for a gall to form solely for the insect's benefit (Oates et al. 2016). The female wasp uses her ovipositor to lay her eggs on the branch of the tree, which then signals the plant to send gall nutrients to that area-as if it were a seed (Nishida et al. 2016). This provides protection and nutritious food for the larva. The actual mechanism as to how the insect hijacks the plant is still poorly understood although it is hypothesized that effector proteins and induced phytohormonal imbalances are likely mechanism involved in gall formation (Oates et al, 2016). Gall formation is a form of parasitism in which the arthropod had to have evolved and adapted closely with the plant (Oates et al. 2016). Additionally, the gall-inducer is reliant on the host plant to form the gall in order for the arthropod's offspring to survive.

Galls come in a variety of colors, shapes, and sizes. Not only do they vary visually, but also the location on which they occupy the host plant, such as on a leaf or branch, can differ among species. Each gall-inducer is specialized to parasitize on a specific host plant, therefore the gall is species specific. The plant-insect interaction that I focused on is between Oak trees, *Quercus cortesii*, and is induced by wasps from the family Cynipidae. The Oak tree is found in Monteverde near the Biological Station and forms an especially large gall, about 10 cm in diameter.

Although the complete chemical properties of galls are somewhat mysterious, it is known that galls contain high levels of tannins, water-soluble chemicals found throughout the plant kingdom. These tannins serve as a defense against fungal invasion and herbivory, which in turn protects the developing cynipid wasp (Taper et al. 1986). On the other hand, galls are also highly nutritious and are comprised of high concentrations of lipids, proteins, and carbohydrates to provide nutrition to the developing wasp (Oates et al. 2016). The presence of anti-fungal properties, anti-herbivory properties, and nutrients provide the perfect conditions for wasp development, yet it is unclear what happens to these chemicals once the galls fall to the ground. Therefore, my first question addresses whether fallen galls inhibit, promote, or are neutral for the growth of fungi, specifically *Mycena citricolor*. I hypothesized that the tannins would inhibit fungal growth and kill pre-existing fungus.

Cynipid wasps are very specialized in manipulating their host plant into making them a personal home within each gall (Nishida et al. 2016). Given this information I studied whether there is a presence of gall-invaders, such as arthropods, that cannot form the gall but take advantage of its formation. I hypothesized that there would be a presence of gall invaders but was unsure of the kind and abundance of specific morpho-species. My final question was whether vertebrates, such as birds and mammals, feed from the galls after they have fallen to the ground regardless of anti-herbivore properties within the gall. I hypothesized that there would be a significant presence of vertebrates feeding on fallen galls because I had previously observed most of the galls were broken into pieces once on the ground.

MATERIALS AND METHODS

Fungal Inhibition

I collected the fungus *Mycena citricolor,* commonly known as Ojo de Gallo, from the leaves of coffee plants (*Coffea arabica*) located on the Life Monteverde farm on 10 May 2017. To isolate the Ojo de Gallo fungus from other fungi on the leaves, I first incubated the fungi from the coffee leaves on three plates of potato dextrose agar and let it grow for three days at 22°C. With the help of Dr. Obregon from the Laboratorio de Asesoramiento Fitosanitario, we identified *Mycena citricolor* (Ojo de Gallo) and inoculated it for the experimental treatments and control. I used 15 glass petri dishes in total, 5 per treatment, and all were incubated at 22°C.

The first of three treatments was done to study if there are gall properties that inhibit fungal growth even after the gall falls from the tree onto the forest floor. This treatment, denoted as tannin agar, involved altering the substrate on which the fungus could grow. To do so, I collected Oak galls from the ground near an oak tree at the Biological Station. I extracted the water-soluble tannins and other natural chemicals from two different galls by soaking them in about 300 mL of boiling water. Next, I strained the galls using coffee filters and combined 125 mL of gall tannin extract with 5 g of potato dextrose agar. Normally, to make agar you combine 125 mL of water with 5 g of potato dextrose agar but in my case I substituted the water for gall tannin extract in order to alter the substrate on which fungi would be inoculated. . I prepared five plates of this altered agar substrate treatment in which the Ojo de Gallo was later inoculated on.

The second treatment, denoted as tannin droplets, had Ojo de Gallo inoculated on pure potato dextrose for two days before the treatment was applied. On the third day of growth I used a pipette to place three 50 µL drops of gall tannin extract onto the fungus. The droplets were separate from one another and placed in the center of similarly sized colonies.

The last five plates were control plates and consisted of Ojo de Gallo grown on pure potato dextrose agar. In order to determine fungal growth I photographed each plate every day and the used ImageJ to determine the area (cm^2) of fungal growth and treatment inhibition of growth on each plate. The outcomes of each treatment group were analyzed through ANOVA.

Gall Invader Survey

I dissected 12 galls in order to determine the abundance and diversity of insects and arthropods present in the gall. I recorded the condition of each gall based on its percentage intact (50%-75%) and its water level on a scale from 1-3, with 1 being the lowest water content and 3 being the highest water content. I used a dissecting microscope and forceps to isolate the organisms from the gall and then identified the morpho-species with help from Emilia Triana.

Lastly, I set up a camera trap near the Oak tree in order to monitor whether invertebrates, such as birds and mammals, feed from the fallen galls. Another camera trap was set up about 300 m away, where galls are not present as a control area. The traps collected data from 16 May to 20 May and were checked every couple days.

RESULTS

Fungal Inhibition

The tannin agar treatment showed significantly less fungal growth than both the control treatment and the tannin droplet treatment (Fig.1). This shows that the tannin agar treatment was able to inhibit fungal growth whereas the tannin droplets were unable to kill pre-existing fungus or inhibit further growth. The tannin agar treatment showed significantly slower growth rate than the control treatment and tannin droplet treatment (Fig.2). It is likely that growth rate was less for the tannin agar treatment because the fungus has a hard to establishing itself in the presence of a tannin extract substrate. On the other hand, the tan nin droplet treatment showed very similar fungi growth rate to the control treatment, indicating that the droplets were not able to prevent pre-existing fungi from growing (Fig.2). The size of the droplet of treatment decreased slightly because the fungi were growing over it but this was not found to be significant (Fig.3).

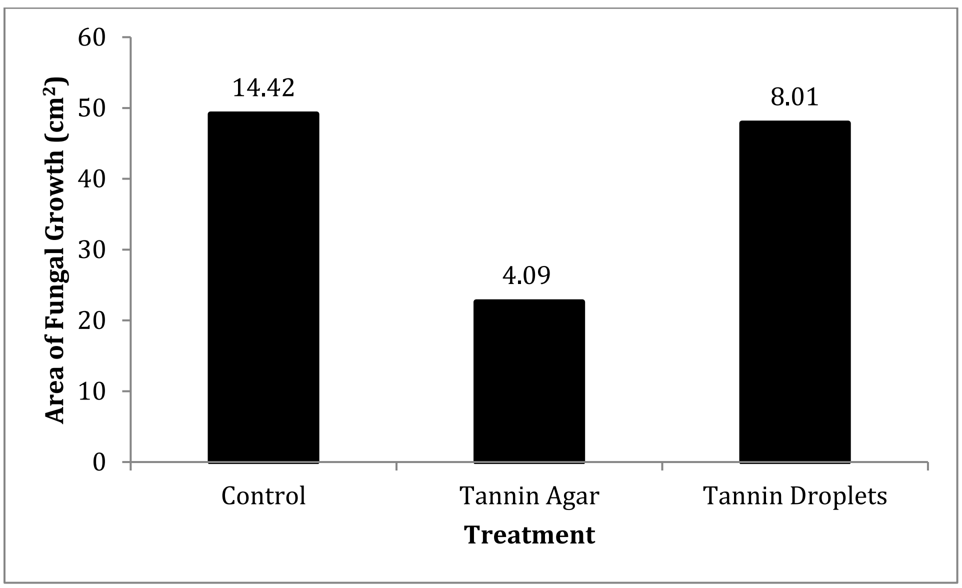

Fig.1. Summary of the total average fungal growth (cm^2) among three different treatments over a three-day period. The standard deviations are shown above each bar. ($F=13.43$, $df=2$, $p<0.002$).

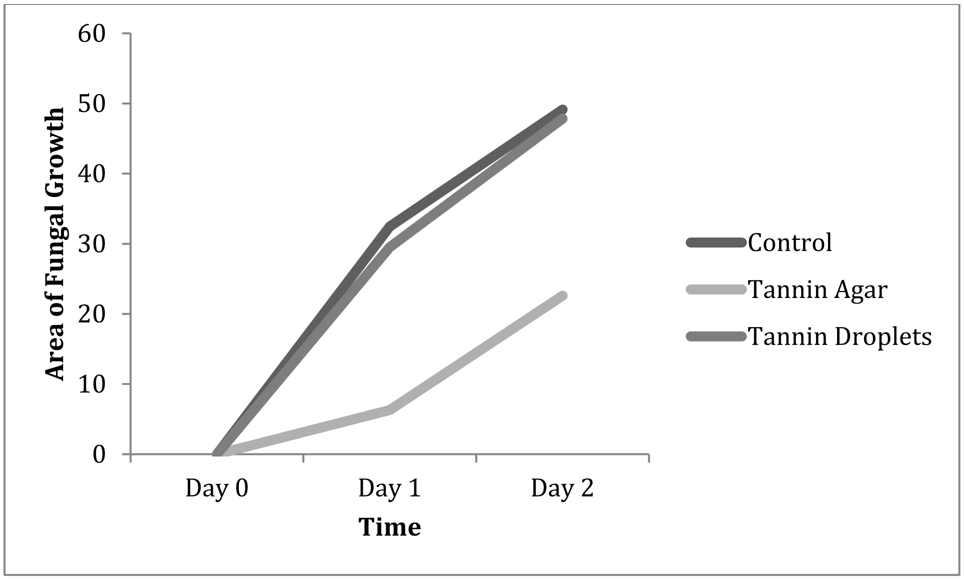

Fig.2. Average fungal growth (mm^2) over a two-day period. ($P<0.58$)

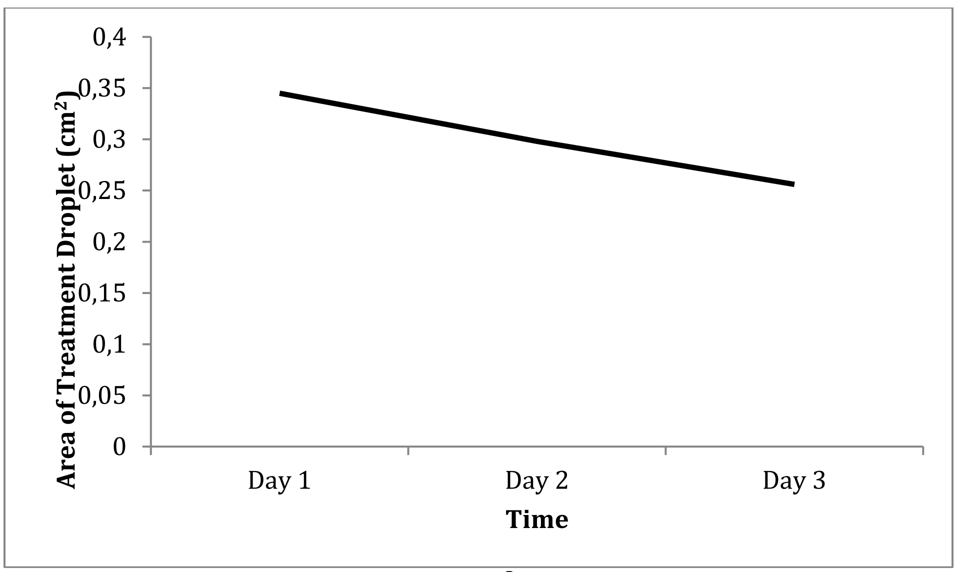

Fig.3. Summary of the size of tannin droplets (mm^2) on fungus on the tannin droplet plates over a three-day period.

Gall Invader Survey

Upon dissecting 12 galls, thirty different morpho-species were collected and placed into 9 categories (Fig.4). Overall, the most abundantly diverse gall invader was the mite (class Acari), with 14 different morpho-species present. Other arthropods present included fly larvae (order Diptera), pupas (order Diptera), beetle larvae (order Coleoptera), springtails(class Entognatha), adult beetles (order Coleoptera), wasps (order Hymenoptera), a worm (order Megadrilacea), and a hemiptera molt. I observed many of these organisms feeding from the gall or simply just living among the gall tissue. This is a record of the abundance of morpho-species found (Fig.4). As the water level inside of the gall increased, the morpho-species diversity decreased (Table 1). Not all arthropods are able to survive in wet environments so as a gall becomes too wet it is likely that the arthropods flee in search for a drier environment or risk drowning. The camera traps revealed similar results in the control area and gall tree area with the exception of coati abundance (Table 2). Coatis were found much more frequently near the gall tree area than near the control area. Although it was unclear whether the coatis were eating the gall tissue it was clear that they were foraging around the area. These results did not account for the same individual returning to the site multiple times.

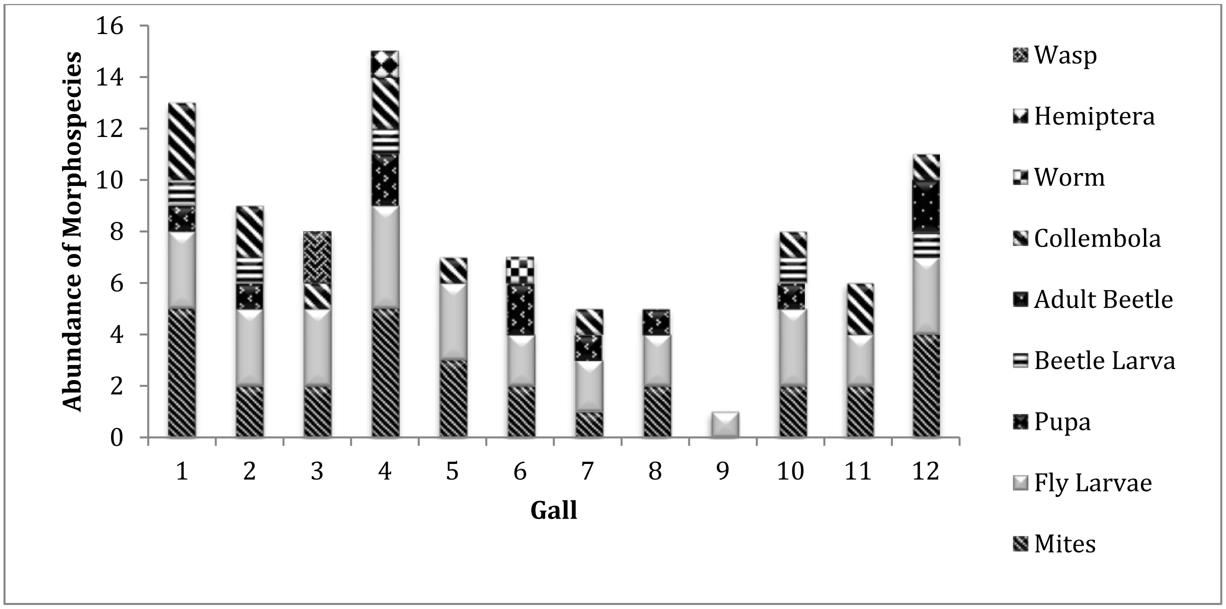

Fig. 4. The diversity of arthropod morpho-species found in 12 different galls.

Table 1. The water level of a gall affects the diversity of species present inside the gall.

Water Level	Average Morpho-Species Diversity
1	5
2	4
3	3.5

Table 2. Summary of the abundance of animal sightings in an area with and without galls on the ground. This is observational data collected from 16 May to 20 May

	Control Area	**Near Gall Tree**
Agouti	**3**	**3**
Bird	**2**	**2**
Coati	**2**	**16**
Mouse	**2**	**0**
Opossum	**1**	**1**
Squirrel	**0**	**1**

DISCUSSION

Fungal Inhibition

It was observed that tannin extract from the Oak galls were effective at inhibiting fungal growth. This result matched my prediction because oak galls contain high levels of tannins, which contain anti-fungal properties (Abrahamson et al 2003). The tannin droplet was not affected by the tannin treatment, which contradicts previous knowledge that soil high in tannin concentrations lowers the fungal biomass of many fungal species (Chang and Yu 1989). It is possible that pre-existing Ojo de Gallo in particular was not susceptible to the tannins but that further research could kill a different species of fungi. I hypothesize that the tannins and other chemicals within the gall are strong enough to slow down fungal invasion but not strong to kill pre-existing fungi. This result is further supported by the growth rate of fungi on the two different treatments. Growth rate increased on day 1 for the tannin agar plates once fungi had a chance to establish, further supporting my hypothesis. (Fig.2). It would be interesting to conduct further research using the tannin treatment as a fungicide on *Coffea arabica* as an alternative to unnatural chemicals polluting land.

A previous study addressing the gall as a secondary shelter for arthropods after the wasp emerges was supported with my evidence of morpho-species identified among the 12 galls (Wetzel et al. 2014). In the future, research would need to be done to identify the presence of arthropods while the wasps are still present inside the gall. The cynipid wasps need protection while inside the gall so I would expect a higher tannin concentration when the gall is still on the tree than on the ground. This higher tannin concentration may also be a deterrent for gall invaders. In turn, I would expect less arthropod in the galls on the tree because they are harder to access than when they are exposed on the ground. Morpho-species diversity decreased as water level within the gall increased. I hypothesize that arthropods living within the galls are not suited to survive in wet conditions. Future studies are necessary to identify each morpho-species by species name in order to know more about their environmental preference and whether water presence is a definite reason for fleeing an environment.

Coatis were documented in the area near the Oak tree more frequently than near the control area. Coati’s diet consists of arthropods, vertebrates, and fruits, which explain why the coatis were more abundant near the arthropod-rich galls (Alves-Costa et al. 2004). Additionally, I observed that the majority of galls on the ground were broken into pieces or rarely whole. I believe this is because the coatis are not actually eating the gall tissue but instead tearing the galls apart to eat the arthropods inside.

In conclusion, Oak galls contain tannins and other chemicals, which are capable of inhibiting fungal growth and slowing its growth rate yet it is not capable of killing pre-existing fungus. Additionally, the arthropod diversity found in Oak galls gives evidence of the gall as a secondary shelter for non-gall-inducing arthropods. Arthropods were less frequent in galls with a higher level of water indicating that gall-invading arthropods prefer a dry environment. Lastly, it was observed that coatis are more abundant near an Oak gall tree due to their diet of arthropods.

ACKNOWLEDGEMENTS

I would like to thank Emilia Triana for guiding me with this project from start to finish, helping me identify 30 morpho-species of arthropods, and spreading her enthusiasm for insects onto me. I would also like to thank Sofí Arce Flores and Federico Chinchilla for being amazing support systems and always making me laugh. I would also like to thank Life Monteverde for supplying fungus from their farm. Additionally, I could not have done this project without the hospitable people at Monteverde Institute and their constant supply of free coffee. Lastly, thank you to the Biological Station for providing a wonderful home away from home and giving me the opportunity to study tropical ecology and conservation in Costa Rica.

LITERATURE CITED

Abrahamson, W.G., et al., 2003. Cynipid gall-wasp communities correlate with oak chemistry. Jou. Chem. Ecol. 29:1

Alves-Costa, Cecilia P., et al. “Variations in the Diet of Brown-Nosed Coati (Nasua Nasua) in Southeastern Brazil. “Journal of Mammalogy, vol. 85, no. 3, June 2004, pp. 478-82. EBSCO *host,* search.ebscohost.com/login.aspx?direct=true=a9h&AN=13373919&site=ehost-live.

Chang, Shu Ting, Yu, Marilyn. 1989, Tolerance of tannin by the shiitake mushroom, Lentinus edodes. M1RCENJournal, 1989, 5,375-37

Hanson, Paul E., and Kenji Nishida. “The Fascinating Phenomenon of Galls; Gall Wasps.” *Insects and Other Arthropods of Tropical America.* Ithaca: Comstock Associates, a Division of Cornell UP, 2016. N. pag. Print.

Oates, C., Denby, K., Myburg, A., Slippers, B., Naidoo, S., 18 November 2016, Insect Gallers and Their Plant Hosts: From Omics Data to Systems Biology, *International Journal of Molecular Sciences,* doi: 10.3390/ijms17111891.

Taper, M.L., E.M. Zimmerman, T.J. Case, 1986. Sources of mortality for a cynipid gall-wasp (*Dryocosmus dubiosus* (Hymenoptera:Cynipidae)): The importance of the Tannin/Fungus interaction. Oecologia. 68:437-445.

Weitz,H. J., Campbell, C. D. and Killham, K. (2002), Development of a novel, Bioluminescence-based, fungal bioassay for toxicity testing. Environmental Microbiology, 4: 422-429. Doi:10.1046/j. 1462-2920.2002.00315.x

Wetzel et al. 2014. Ecosystem engineering by a gall-forming wasp indirectly suppresses diversity and density on oak trees. (manuscript)