Behaviours of termite workers of *Odontotermes obesus* that maintain their crop of *Termitomyces*

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Certificate of Examination

This is to certify that the dissertation titled "**Behaviours of termite workers of *Odontotermes obesus* that maintain their crop of *Termitomyces***" submitted by **Mr. Raunak Dhar** (Reg. No. MS15120) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: June 2020

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rhitoban Raychoudhury at the Indian Institute of Science Education and Research Mohali.

The work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Raunak Dhar

(Candidate)

Dated: June 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Raychoudhury

(Supervisor)

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I would like to thank Dr. Rhitoban for taking me into his lab and being my PI. Most gratitude is due to Renuka Agarwal under whose aegis I have performed all my experiments and who has always been helpful and resourceful.

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Abstract:

This work is an inquiry into the nature of the obligate symbiosis that exists between fungus-growing termites and white-rot fungi—which is their crop. Some of these experiments were to look at how and what termites perceive, while other experiments were to see what their response is to certain stimuli. Other than this behavioural aspect, there was an attempt to test for a microbial candidate to see if it works to protect the fungal crop. Taken together, what was gathered from the experiments was that there are more than one ways in which termite workers fight invasive weeds and also that more than one senses are used to detect threats. It was noted that termite workers can bury an invasive weed, disintegrate it or even leave it be. And it was learned when can and cannot the termite workers control infection to their crops.

Introduction:

Life has been around for billions of years. Ever so often, in such a long stretch of time, organisms evolved new traits, small and large. Most of these new traits lead to the death of the organism. But, every now and then, some trait remains and isn't immediately weeded out by natural selection. Evolution of the life forms that roam the earth today is, in all probability, the biggest miracle in the universe. We ascribe this magical creation of life forms, broadly speaking, to two mechanisms: random mutation and natural selection. Natural selection is the sieve that filters what phenotypes live on and what phenotypes don't survive. It is a direction dependent mechanism in so far as a particular phenotype which doesn't survive in one environment might be useful in the future when environment changes, but if it has already been weeded out completely, that cannot be. Phenotypes that came into existence earlier have had more impact on life than those that came later, because these phenotypes formed the environment in which the newer traits were selected. The longer a phenotype has been around, the more robust it is—because time is a destroyer of any and all weakness: and longer time more so.

Animals evolved some 800 million years ago. After the evolution of animals, in this enormous stretch of time, two of the many major-league updates that were gathered by them, in their makeup, are:

a) evolution of colonies of organisms with sterile castes, and

b) evolution of agriculture from foraging animals. (Maynard Smith and Szathmary, 1997)

The former, which is called eusocial organisation, completely changed the focus of natural selection from the individual to the colony—in fact, the very concept of an individual organism (from a Darwinian perspective) doesn't makes sense for the sterile castes because they don't reproduce at all. We know that the discovery and utilization of agriculture in human societies fundamentally transformed every aspect of human life as division of labour, property rights and delayed-reward environment came into existence. In insects, however, before even apes had diverged from monkeys in the

lineage that eventually led to humans, agriculture had begun. (Chapela et al., 1994; Mueller et al., 1998; Currie et al. 1999)

Agriculture has evolved independently in multiple lineages of beetles and in ants and termites among the hymenopterans (Mueller et al., 2005; Aanen et al., 2002; Mueller et al., 2001; Farrell et al., 2001). In ants of the new world and termites of old world, there are many similarities in agriculture. I shall focus, in this dissertation, on the cultivation of fungi by termites.

Termites are insects classified in the cockroach order Blattodea that have descended from wood-roaches (Lo et al., 2000; Inward, Beccaloni & Eggleton, 2007). Termites feed on dead and decaying plant matter. Termites are divided into lower and higher termites based on how many derived and ancestral traits there are. There are seven taxonomic families of termites and of them, Termitidae is the only family that comprises the 'higher termites', which are the ones with the most number of derived traits (Inward, Vogler & Eggleton, 2007). One subfamily of higher termites is Macrotermitinae—the fungus growing termites that our study is about. This subfamily of termites is spread far and wide across the tropics of the Old World (Wood 1978). This study is done on *Odontotermes*—a very common genus of fungus-growing termites found in India. (Akhtar, 1972; Kambhampati & Eggleton, 2000)

Before I describe what I did as part of my Master's project, I will take the reader through a tour of what has been done here in the lab by others and what all is already known about fungus-growing termites. These termites live in mounds of soil (Darlington, 1997). The mounds of termites inside the campus of IISER Mohali had already been located by other lab members. The DNA sequencing of samples from these termites had also been done. There has also been done study on all the termite diversity on campus along with taking samples from different parts of the mound to see the mycodiversity present inside by plating onto culture media.

Now let's take a look at where the literature stands on the knowledge of the agriculture of fungus-growing termites. It is known that the termites on the campus that we study are divided into worker, soldier and reproductive castes. It is known that during the rainy season when the soil is wet, winged reproductives—called Alates—take to air and try to found a new colony somewhere else. Most of these alates die and only a small fraction of them are able to successfully establish new colonies. It is known that, except

for Microtermes, alates of all other genera of fungus-growing termites have a system of cultivation in which they don't take spores of their cultivar fungi along with them to inoculate the new colony; instead, they found a colony, give birth to workers who go out and collect spores of the cultivar fungi and then inoculate their newfound colony with those spores (Korb & Aanen, 2003; Johnson, 1981; Johnson et al., 1981). It is a horizontal mode of transmission and what that means is that the reproductive cycles of the termite colony and the reproductive cycle of their fungal cultivar are decoupled. And what that makes scientists to speculate is that there must be competition between the fungal crop and the termite farmers—after all, why would the crop fungus want the termites to produce so many alates that don't do the job of crop maintenance but rather fly off from the colony; and, much along the same lines, why would termites want their crop fungus to sprout out into mushrooms whose nutrition they can't use (Korb & Aanen, 2003; Aanen, 2006). Now, these, of course, are speculations because they don't describe the scenario in the way of how forces of natural selection work, but just give a simplistic anthropomorphic kind of an explanation. And we must remember always to be leery of all theories unless they are backed by solid empirical corroboration, because no matter how good a theory looks on paper, it could always be wrong on account of other variables that the theorist failed to take into account or didn't know the existence of—reality, and not proper-sounding logic, is the sole judge of any theory.

The termites that I worked on are called *Odontotermes obesus*—and how I know the species is through the work of other lab members who extracted DNA from the colony individuals, got it sequenced and did an NCBI Blast search. Even though the fungus-growing termites are a subfamily, it is interesting that their fungal crop consists only of species from just one particular genus of fungi called *Termitomyces*. (Katoh et al., 2002)

Figure 1: Termite mound in our study location at IISER Mohali

Figure 2: Fungus comb inside a mound

This agricultural symbiosis has been described in the literature—though the literature is in no way complete (or why would we be doing this work anyway?). From what all

is known, it seems that the primary job of *Termitomyces* in this symbiosis is to extract nutrition from the plant material collected by the workers. So it is as if the workers have part of their digestion outsourced to the fungus—much like we outsource much of our digestion to the elaborate art of cooking. These *Odontotermes* termites, like most animals, can't properly extract nutrition from dead and decaying plant material because of the inability of their intrinsic digestive enzymes to cut through lignin—which forms a mesh inside which cellulose is to be found. The use of *Termitomyces*, therefore, is to turn that hard-to-digest plant material into fungal structures which are very easy to digest. The farmer termite and the crop fungus are obligatorily dependent on each other; this is known already. And what does the fungus get out of this symbiosis? The answer is pretty simple: nowhere else will *Termitomyces* get such a perfect environment to grow as it gets inside a termite mound where there is so much surface area along with proper temperature and humidity, and where all weeds are kept suppressed by the termites. (Nobre et al., 2010)

An immediately striking feature of this symbiosis is that only *Termitomyces* visibly grows inside the termite mound and only one strain in a mound (Thomas, 1987). Now, the mechanism that is proposed for how this genetically uniform *Termitomyces* crop is present can be found in the way termites maintain this agriculture. Our model system is a higher termite and in higher termites, workers are a specialized sterile altruistic caste—in contrast to the Pseudergates in lower termites—that supply the food for the colony (Roisin & Korb, 2010). In *Odontotermes formosanus* (a different *Odontotermes* species) there has been done a study on how work is partitioned even among the workers based on age: younger workers do the tending of the garden while older workers do the foraging. Older workers go outside the mound for foraging and also bring in plant material for others. The younger workers eat this and then put their faeces on the comb which they also inoculate with spores from outside the mound (Li et al., 2015). These young workers feed on the nodules of the white-rot fungi *Termitomyces* which are premature mushrooms containing asexual spores (Licht et al., 2005) (In other basidiomycete fungi, such nodules do not contain asexual spores. It means that this particular feature is a special adaptation that *Termitomyces* has developed in this particular symbiosis). The asexual spores are so adapted that they survive the passage through the gut of these young workers. Then they get inoculated on the comb with their faeces (Leuthold et al., 1989). And the older workers feed on the older comb and,

therefore, there is a dynamic process of constant rebuilding and eating up of comb happening in a way that the newer layers are added on top and the older layers are scrapped off from beneath. The faeces of older workers is different from the younger workers and is called final faeces and is not put on the comb but is disposed of in another place and covered with soil (Li et al., 2015).

What this mechanism does is that it puts an artificial selection which the termites subject their crop fungus to in which only the nodule producing spores—and the fastest nodule producing ones only— are selected for to form the next layer of cultivar (Aanen, 2006). Even though termite workers eat the nodules and prevent them growing into mushrooms, it remains the case that whenever a new colony is founded, they have to go out and get spores from the environment—where they can only be present if some of the nodules successfully form mushrooms and release their spores in the air. To make sense of this conundrum, we need one additional piece of information: when the alates take flight to go establish their new colonies, almost 40 percent of the colony biomass is lost (Wood & Sands, 1978). And it has been seen that it is after a few weeks when the alates have taken off that mushrooms are seen on these mounds (Johnson et al., 1981; Darlington, 1994). A reasonable explanation of the proximate mechanism could be that during this period the colony members are not able to eat up all the nodules and, therefore, some of them turn into mushrooms.

It is interesting to note, however, that the fungal garden of termites is overrun by other weedy fungi very quickly if it is taken out of the mound (Thomas, 1987). When termites are not present, or even when termites are present but the comb is taken out of the mound, we see that that which earlier was a monoculture of *Termitomyces* has quickly *Pseudoxylaria*—which is a subgenus of Ascomycota fungi—overgrow it (Wood & Thomas, 1989). The whole enterprise of our work on this model system is to try to understand the underlying mechanism by which the termite agricultural system is maintained free of the weeds inside the mound. In this dissertation, I will describe the experiments that I have done to inquire about it.

Figure 3: Pseudoxylaria overgrows the fungus comb

Many of my experiments that I will describe here were to see the behavioural response of termite workers. We used termite workers only and not other castes because we have seen in other experiments that it is only the workers that do the weed control activity in this system. Now, there are a lot of questions that need to be solved in this system. Some of the questions that needed to be dealt with are:

- What cues, precisely, do the termite workers process to recognize their crop from the weeds?
- What are all the factors that play into the termites' maintenance of their system?
- What do the workers do behaviourally to rear and protect their crop?
- Are there other symbionts in this mutualism like in the agriculture of fungus-growing attine ants?
- Does the mechanism by which *Pseudoxylaria* and other weedy fungi are kept in check involve chemical secretions, mechanical processes or biological agents like other microbes?

And so I tried to look at some of these questions in the experiments that I did. What is interesting to note is that *Pseudoxylaria* isn't found commonly in soil but it immediately overgrows the fungus comb if termites are not present (Thomas, 1987). It seems that it is present in a dormant state in active combs in certain regions and

then when for some reason weeding mechanisms are not working properly, it overgrows the comb.

It must also be told the reader at this moment that termites are available for experimentation only in the months that they are active which is the period from April to early November. So most of the experiments on termites can only be done during this period.

Experiments on termite perception

Materials and methods:

A good many experiments of mine involve getting fungus combs from the termite mounds and many times I would have to get termites out of the comb.

The tools used to dig termite mounds and get the comb were: a hoe, a trowel, rubber gloves and a box to put the comb in.

It should be noted that even when I had no use for the comb and only needed termite workers, I would nonetheless still have to get a fungus comb from the mound because that is the only reliable way of getting termite workers in needed numbers—they are always found inside the combs.

It is imperative to mention here that in all of these experiments we have added water to the soil in order to prevent the termites from dying of desiccation—inside the mound, humidity is very high, something like 90 percent.

All these experiments that I list here were done in petri plates of glass.

Photos of experimental plates were taken every day.

Experiment 1:

We already had *Termitomyces* cultured in the lab (on special media that is made by mixing Potato Dextrose Agar with Yeast Malt Agar) and it is morphologically different (visibly) from the nodules that grow on comb. This experiment was a behavioural assay to see what behaviours would the termite workers show towards it. It was important to see this to find out if they recognize their crop by somehow recognizing the species or if the same species in a different morphological form would be treated differently.

Got fresh comb from the mound we shall call mound 2 and took out workers from it using simple paint brush, dividing them separately based on whether they were major or minor workers. The soil and water used were sterilized already by autoclaving.

Setup:

Test (n=3): 15g sterilized soil + Fresh comb on one side + Fresh comb that had its nodules dusted off by a brush with a plug of *Termitomyces* culture on it + 15 major workers + 15 minor workers + 2mL sterilized water

Control was same but without the workers.

Experiment 2:

We also have pieces of old autoclaved comb that we keep in the lab. I had seen in other experiments that termite workers do not treat it as a weed and it is somewhat of a neutral thing in their surroundings that doesn't elicit any response in them. So in this experiment I did what I had done in the previous one, only that this time the substrate on which the *Termitomyces* was kept was a piece of autoclaved comb instead of fresh comb. It was required because it needed to be seen if the reaction of termite workers to the culture plug was dependent on being on the fresh comb. The process of autoclaving fundamentally changes the constitution of a piece of comb in that it kills all the microbial life in it and essentially turns it from a little ecosystem to a piece of non-living matter. In this experiment, however, we took the termites from comb taken out of a mound that we call mound 1.

Test (n=2): 20g sterilized soil + 20 Major workers + Autoclaved comb with *Termitomyces* culture plug on one side + Only autoclaved comb on the other side

Control was same but didn't have *Termitomyces* culture on one of the autoclaved combs.

Experiment 3:

What I next tried to see was how nodules of *Termitomyces* would be treated when put on an autoclaved comb. Before this experiment I had tried to see how nodules of *Termitomyces* are treated by workers by putting the nodules directly on soil, but clear inferences could not be drawn from that one and so I tried this one.

Test (n=5): 15g sterilized soil + 2mL sterilized water + 30 workers (Major and Minor) + Nodules on autoclaved comb

Control was same but nodules were not present.

Experiment 4:

This experiment was an extension of the previous one. In this one, I put an autoclaved comb in the middle of the plate and put a fresh comb on the side of the plate from which nodules had been dusted off. On both of these, nodules of *Termitomyces* were put and termite workers were added. This experiment was done to see that even when the same nodules are put on two substrates, which are both neutral at the very least for termites, are put close to each other, would they respond differently towards nodules based on just which substrate they are put on. The fresh comb and nodules used in this experiment came from the mound 1.

Setup:

Test (n=3): 12g sterilized soil + 2mL autoclaved water + 20 Major workers + 5 Minor workers + Autoclaved comb with nodules put on it + Fresh comb with nodules on it

Control also had workers with only autoclaved comb having nodules put on it and no fresh comb, all other things being same.

Experiment 5:

This experiment was done to see whether only living things elicit a response from termite workers or non-living things also elicit some response. It was needed because

we had seen already that autoclaved comb was one substrate that did not elicit any response from the termite workers on its own. So what I did was I took metal foil, little wood chunks, and plastic caps of MCTs and sterilized them. Then I put these items on sterilized soil and put in termite workers which were taken from the mound 1.

Setup:

Test for foil (n=2): 12g sterilized soil + 2mL sterilized water + 20 Major workers + sterilized foil

Same was done for wood chunks and plastic cap.

Results and Discussion:

Experiment 1:

Figure 4a: Test Day 0

Figure 4b: Test Day 2

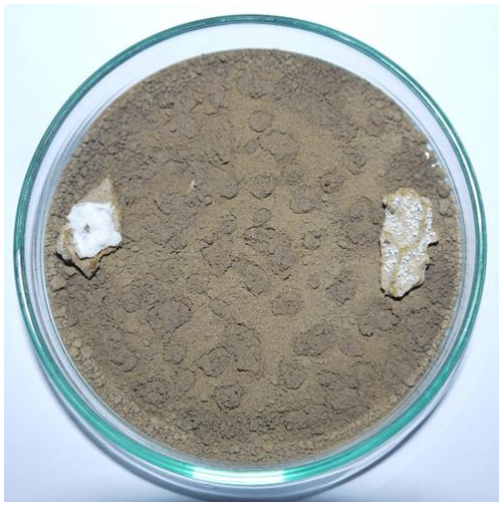

Figure 5a: Control Day 0

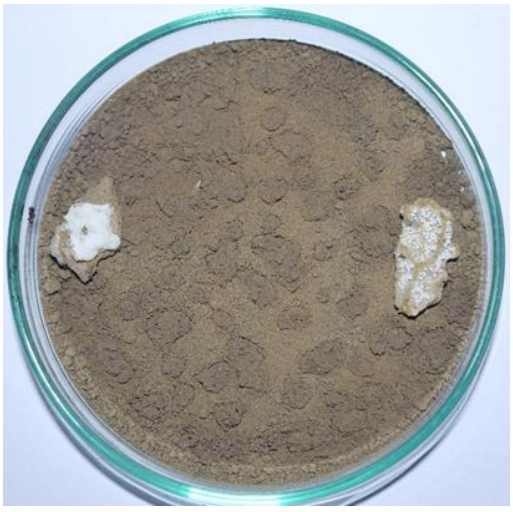

Figure 5b: Control Day 2

What we saw in this experiment was that termite workers did really recognize that this piece of culture was different from their crop even though it was on exactly the same

substrate and exactly the same species. What this experiment tells us is that termites can morphologically distinguish between what is and isn't their crop and they couldn't have done this in this case by olfaction because olfactory signals of both forms of *Termitomyces* should be the same because they are the same species. It is a curious phenomenon because in this case they did not eat the nodules on the fresh comb as far as we could see, but they disintegrated and spread the culture crumbs around.

But there is one question that this result begs: Why not treat even the culture morph of *Termitomyces* as food and what is the need to attack it like that?

Experiment 2:

Figure 6a: Test Day 0

Figure 6b: Test Day 3

What we see here is that only the culture is attacked by termite workers. This is in contrast to the result we will see in experiment 3 in which sometimes we will see that the whole of autoclaved comb is attacked.

From this and the previous experiment, we see that the crop of termites is considered crop only so long as it is in a certain morphology, and even a morphological change which is in no ways a chemical change is enough to make the termites treat it like a foreign object.

Figure 7a: Control Day 0

Figure 7b: Control Day 3

Experiment 3:

This one turned out somewhat of a tricky experiment because I got mixed results in this one. Here's what I got:

Figure 8a: Test Day 0

Figure 8b: Test Day 6

What was seen here was that nodules of *Termitomyces*—which is their food crop—on autoclaved comb—which is a neutral substrate on its own—elicits the same burial response from the termite workers which is their standard defence mechanism against infections like when a piece of comb infected by *Pseudoxylaria* is given to them, they bury it and the infection is contained that way.

However, what was also seen in this experiment was:

Figure 9a: Test Day 0

Figure 9b: Test Day 6

I also saw that sometimes the burial response to infection was not shown by termite workers even when fungal contamination was visibly seen on the autoclaved comb.

Now, that piece of information is a little hard to interpret. What that tell us is that just because there is contamination does not mean that the termites will show their standard burying defence. The next experiment that I have done will shed some more light on this somewhat recondite aspect of termite decision making; to bury or not to bury is the question!

Experiment 4:

Figure 10a: Test Day 0

Figure 10b: Test Day 6

We see in this experiment again that sometimes nodules on autoclaved comb make it some kind of a threat to be buried, but there was also one plate in which no burial was seen.

The interesting thing to see in this experiment is that fresh comb is never buried.

It is a very enlightening result because it tells us quite a few things:

First, it seems like a logical thing to conclude that maybe fresh comb contains some microbes/chemicals which keep the nodules in proper condition—we have also seen that the nodules take an orangish hue in a day or two on autoclaved comb but not on fresh comb. It is said that the nodules get oxidised and their colour changes.

Second, in experiment 1, I told you that the distinguishing factor should not be olfaction because it was the same species in two different morphologies. But here, it could be olfaction that the workers might be using to detect if the nodules are in proper chemical composition or not. It tells us that termites workers are probably using more than one of their senses to distinguish their crop from weeds.

This experiment also tells us how nodules and the comb are one and an inseparable system—the nodules are not their crop in isolation, the nodules on a healthy comb are.

Experiment 5:

Figure 11a: Day 0

Figure 11b: Day 1

Figure 11c: Day 5

What we saw in this experiment is that just because a substance is non-living does not mean that it is neutral and inert for the termite workers. Unlike how they just leave an autoclaved comb, they put soil on these non-living substances. This is interesting because it shows us that autoclaved comb is the best substrate to do assays that might involve interaction between weeds of termite garden and some microbial candidate that we may be suspecting might be acting as a defensive mutualist helping to protect the termite garden; I have done such an assay later which I write about in the third chapter.

Figure 12a: Day 0

Figure 12b: Day 1

Figure 12c: Day 5

Figure 13a: Day 0

Figure 13b: Day 5

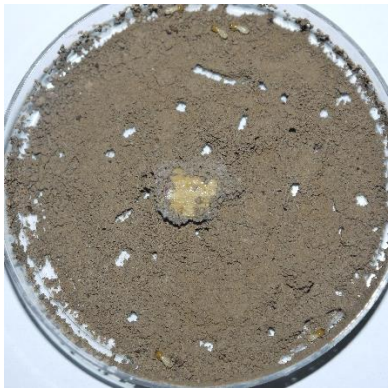

Figure 13c: Day 10

It is also important to note in this experiment that there is a great variation in the way the three substances elicited the burial response of termites.

Plastic was buried the fastest which is understandable given that there is zero probability that they might encounter plastic naturally in their dwelling place.

The metal foil was also put under soil fairly quickly but it was not so thoroughly buried as the plastic cap.

The wood chunk was a different case. It was hardly touched for the first few days. Only after a week or so they put some soil on it and even then very little. This is also understandable because wood is a fairly normal thing that they might encounter in their vicinity.

What we have to realize at this point is that in these petri plate experiments we are unable to differentiate between soil that is put on an object to bury it versus the natural mode of termites to build a mound over whatever is in the vicinity. And it is my intuition that this is the difference that we see in how they respond to plastic versus wood—they might be burying plastic but just building mound over wood.

How workers fight Pseudoxylaria

I have already described in the introduction how *Pseudoxylaria* is a preeminent weed of the fungal agriculture and that termites have to actively keep it from taking over the fungus comb. In this chapter I will detail the experiments I have done to investigate the behaviours of termite workers in dealing with the threat of *Pseudoxylaria* on their crop. Here I must mention that before me Abin Antony of MS14 had been doing this work and he showed that termite workers buried infected comb.

These experiments in this chapter were mostly done in plastic plates.

Photos were again taken every day.

Experiment 6:

As I mentioned in the previous chapter, autoclaved comb is a substrate which the workers don't show any burial behaviour toward and being very similar to their original substrate—only without the microbiome—it is a perfect choice to see how termite workers might treat *Pseudoxylaria*. So in this experiment I take some soil in a petri plate and put a piece of autoclaved comb on it and then placed a piece of *Pseudoxylaria* hyphae on it with some termite workers.

The workers used were from the mound 1.

The way we get *Pseudoxylaria* is we bring a comb to the lab and in just a couple of days it gets overgrown with *Pseudoxylaria*. Then we might use forceps to pluck a piece of hyphae and put it to our use. Additionally, we also maintain cultures of *Pseudoxylaria* on PDA plates and we have used culture plugs also in some experiments.

Setup:

Test (n=4): 12g sterilized soil + 2mL sterilized water + 15 Major workers + 9 Minor workers + *Pseudoxylaria* hyphae on autoclaved comb

Control was same but without the termite workers.

Experiment 7:

After seeing the defense that the termite workers mount against *Pseudoxylaria*, I thought that it might be that other than the active defence that the termite workers seem to carry out, it might also be the case that the mere presence of termites would hamper the growth of *Pseudoxylia* if the bodies of termites contained any chemicals that were antagonistic to the growth of *Pseudoxylaria*.

So in this experiment in experiment what I did was that I crushed fresh comb and I also crushed termite workers. In the test plates I put the powdered comb mixed with the mash of the bodies of termites. The controls had only the powder of comb but didn't have any mash of termite bodies.

The comb used was from a mound called mound 2.

Setup:

Test (n=6): Powdered fungus comb mixed with the mash of crushed bodies of termite workers

Control was same without the termite bodies—only the powdered comb.

Experiment 8:

As you might have noticed, the previous experiment was somewhat like shooting in the dark. This one, however, was a little less like that. Before I describe this experiment, it is important to tell you guys that from what other people have measured, it says in the literature that the pH of the fungus comb is just over 4—which is a somewhat acidic pH (Thomas, 1987). At this point I decided to see if diluting the acidic nature of the comb substrate would cause *Pseudoxylaria* to overgrow it faster. In other words, I wanted to see if the acidic pH is one of the factors that helps keep *Pseudoxylaria* away from the termite garden.

In this experiment, the number of workers used was extraordinarily high (with respect to most of our other behavioural assays): 50 workers were used in all plates, minor

workers in some plates and major workers in other plates but no less than 50 in any case.

The hypothesis with which I approached this experiment was a surety that 50 workers would definitely prevent *Pseudoxylarial* contamination. And so my test plates had powdered comb that had Sodium Hydroxide added to it along with 50 workers. The rationale with which I was approaching this experiment was to see if or not the 50 workers will be able to undo the damage to the defences of the comb that the increase in pH had caused (or so I assumed). This experiment had a flaw which I couldn't have foreseen and which I came to know only after doing the experiment (remember what I had said in the introduction about the difference between a theory sounding good and actually working on account of variables not known beforehand?). And the flaw was that I hadn't even considered the possibility that it might be that even 50 termites might be unable to stop *Pseudoxylarial* growth in the powdered comb. We knew from other experiments that 20 or even 15 workers are enough to stop *Pseudoxylarial* growth on a piece of comb when it is put on soil.

There were two types of controls in this experiment (You may have noticed in all experiments I have shown only one type of control—it isn't because I had only one kind of control but because in other experiments, other controls were unimportant but in this experiment, both of these controls are important so I am showing them). One kind of control was the same as the test but without the workers i.e. it had only powdered comb with NaOH in it. The second kind of control was just powder of comb without any NaOH added and without any termites.

Setup:

Test (n=12): 5g dry fresh comb powder + 2mL Sodium Hydroxide + 50 Workers

Control Basic: 5g dry fresh comb powder + 2mL Sodium Hydroxide

Control No Base: 5g dry fresh comb powder + 2mL sterilized water

Now there is some extra information that needs to be given here. The 12 test plates included pairs of plates with NaOH of pH 13.3, 12.6 and 11.6 added to them for both minor and major workers separately.

This pH was measured using a pH meter. And even though starting from pH 13.3 solution of NaOH, I tried diluting it 10X two times but the resulting pHs came out 12.6 and 11.6 instead of the expected 12.3 and 11.3 (These minor details would not matter in the end as you will see in the results section of this experiment).

What I was trying to see with these different pH solutions in which the difference in basic strength was supposed to be 100X (10*10) was to simply see if there is a threshold amount of basic stressor that the comb might be able to take without its immunity being too compromised. You will see in the results section that none of these machinations of mine came to much fruition.

Experiment 9:

This experiment was inspired after I came to know through the previous experiment that even 50 termites seemed unable to stop any *Pseudoxylarial* growth on any of the test plates (See the results section of experiment 8). In the previous experiment, there weren't any plates where those termite workers were put on powdered comb directly without any interference of any base. It could have been that the workers couldn't do their natural job of fighting *Pseudoxylaria* because the fumes of NaOH were messing with their system somehow. This experiment was my attempt to see, without any interference of any base, are the termite workers even able to stop *Pseudoxylaria*, even though they be in such a high number—something which I had just assumed before that they should be easily able to do.

Setup:

Test (n=3): 5g dry fresh comb powder + 2mL sterilized water + 25 Major workers + 25 Minor workers

Control was same but without the workers of course.

Results and discussion:

Experiment 6:

Figure 14a: Test Day 0

Figure 14b: Test Day 2

Figure 15a: Control Day 0

Figure 15b: Control Day 2

What we see in this experiment is how adroitly termite workers target the piece of *Pseudoxylaria* without burying the autoclaved comb. This is a response different from what nodules of *Termitomyces* sometimes elicit in which the whole of the autoclaved comb is buried. It is, however, hard to say what exactly do they do the piece of

Pseudoxylaria but one thing is clear, these termite workers have an almost surgical accuracy in detection and elimination of the threat of *Pseudoxylaria*.

Experiment 7:

Figure 16a: Test Day 0

Figure 16b: Test Day 3

Figure 17a: Control Day 0

Figure 17b: Control Day 3

What we saw from this experiment was what might have been expected; there was no inhibition of *Pseudoxylarial* growth on the surface of the powdered comb just by the addition of a mash of bodies of termite workers.

As we had suspected, the weeding process is a very much active and dynamic process rather than something that happens on its own by some chemicals that might be present in the bodies of workers.

Anyway, it could have been possible that it might have shown some inhibition. After these many experiments we seem to be getting more and more clue that the defence of the fungal garden of these termites might be fundamentally behavioural rather than chemical.

Still, we can't rule out all possibilities and say that no chemicals are used as weedicides; they may be used but in some other way.

Experiment 8:

Figure 18a: Test Day 0

Figure 18b: Test Day 3.5

Figure 19a: Control Basic Day 0

Figure 19b: Control Basic Day 3.5

Figure 20a: Control No Base Day 0

Figure 20b: Control No Base Day 3.5

The first thing I need to clarify is why aren't the days in whole numbers. It is because on the day when I was setting up the experiment, comb was brought in the morning and the work of taking out the termites was started after lunch in the afternoon. Since I decided to add 50 workers in each plate and there were 12 test plates, I had to get out 600 workers which took many hours. After this, setting up the rest of the experiment also took some hours and by the time I set the experiment it was midnight. And I clicked photos only during the day so the photos were clicked after 1.5, 2.5, 3.5 days and so on.

The result came out clear: change in pH of the comb made no difference in the growth of *Pseudoxylaria*. From this result, I think we can safely infer that the acidic pH of the fungus comb is not a likely factor in inhibiting weeds like *Pseudoxylaria*. So with this we have ruled out one more variable and are a step closer to understanding this agricultural system with one less variable to worry about.

What was astounding to me when I saw these results was that even 50 termites seemed powerless against the *Pseudoxylaria*. So, naturally, I was inclined to check as I did in my next experiment if, indeed, termites are capable at all of stopping the growth of *Pseudoxylaria* when comb is in powdered form.

In this experiment, we could say that maybe NaOH was messing with termites so the next experiment had to be done.

Experiment 9:

Figure 21a: Test Day 0

Figure 21b: Test Day 4

What we see in this experiment is a very new piece of information—termite workers which in even such low number as 15-20 can fightoff *Pseudoxylaria* on fresh comb (as in Abin's experiments) even when it is externally introduced cannot prevent the growth of *Pseudoxylaria* even when there are 50 if this powdered form is to be dealt with.

Figure 22a: Control Day 0

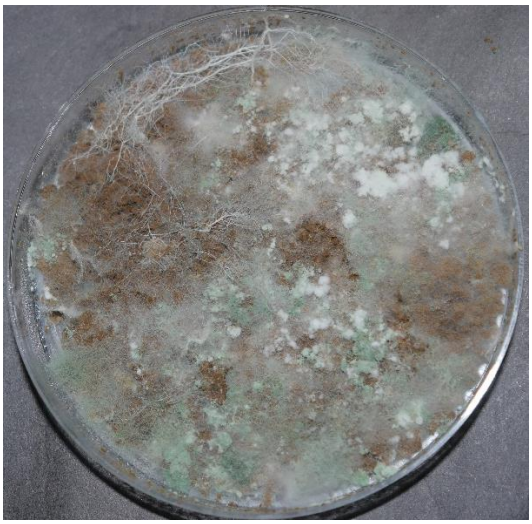

Figure 22b: Control Day 4

This could be because there was no soil here that they could use to bury the infection in. But we did see experiments previously like the one at the beginning of this chapter where *Pseudoxylaria* was externally introduced on autoclaved comb where termites had the opportunity to bury it but they dealt with it some other way, disintegrating it somehow. From other experiments by other lab members it is known that when termites bury infected comb, it isn't just the soil that stops *Pseudoxylaria*, it is what they add with their saliva that does the magic.

It begs the question of whether soil is or isn't important to fight the *Pseudoxylarial* infection? (Could some other powdered substance also work instead of soil? This is an experiment someone should do, perhaps the reader!)

An experiment which I would suggest to anyone who wishes to do it would be to take a petri plate and in half segment keep sterilized soil and in the other half keep powdered comb and put in termites. This might shed further light on whether termites can then fight the infection.

One more question that pops in one's mind is if the shape and structure of the termite comb plays a role in preventing *Pseudoxylarial* infection somehow. Crushing the comb and spreading it like this on the plate should make it easier to fight infection given that the surface area has been reduced. But this is just a guess, a wrong guess most probably.

Pseudomonas-Pseudoxylaria interaction assays

This section will briefly tell about the interaction assays that I did between *Pseudomonas*, which is a genus of gram-negative gammaproteobacteria, and *Pseudoxylaria*, the principal weed of the agricultural system of fungus growing termites.

Before I explain why these assays were done, I will tell you that I did five of these assays, some of which were slightly different and others were majorly different from others.

Here I will show only one of them and then briefly write about what the others were.

First, why these assays:

Another lab member, and my de facto guide, Renuka Agarwal found out in her culture assays that *Pseudomonas* was one bacterium that inhibits *Pseudoxylaria* in culture plates and simultaneously, does not inhibit *Termitomyces* in culture plate interaction assays (It is unpublished data at the moment and I am writing this section of my thesis only because these theses are published after a period of two years, in which time it will surely be published). So the next step after the culture assays was something more near to an *in vivo* experiment and excitedly we went into doing them.

Experiment 10:

As you might have understood by now that our favourite substrate to do experiments on is autoclaved comb. And here also we chose our very own autoclaved comb to do the interaction assay. This was chosen because the substrate is very near to the comb that we are studying but is inert and therefore we can do an interaction assay on it in isolation without having to worry about all the hundreds of living things which might

also interact with the assay if it were done on a fresh comb (Notwithstanding any of this, we also did this with fresh comb; more on that later).

Simply what was done in this experiment was that I dipped autoclaved comb in *Pseudomonas* solution that would have optical density of around 0.5 and then put *Pseudoxylarial* plug on it for interaction. This was our test. Control was same but the autoclaved comb was dipped in LB broth instead of *Pseudomonas* solution and then *Pseudoxylarial* plug was added. There were other controls but they are unimportant and so I will skip them.

These were done in plastic petri plates for we were running a little low on glass plates.

Setup:

Test (n=6): 12g sterilized soil + 2mL sterilized water + autoclaved comb dipped in *Pseudomonas* solution + *Pseudoxylarial* culture plug on the autoclaved comb

Control was same but the solution used for dipping the comb was LB broth.

Pictures were clicked every day.

Result and discussion:

Figure 23a: Test Day 0

Figure 23b: Test Day 6

If *Pseudomonas* were to show any inhibition of *Pseudoxylaria*, we would have seen it. But from our results we did not see any difference between test plates and control plates. It seems that *Pseudomonas* shows its magic only on culture plates.

Figure 24a: Control Day 0

Figure 24b: Control Day 6

This came out to be a result which is a little hard to interpret in that we would expect it to inhibit *Pseudoxylaria* even here but something else is going on (Again I will remind you about what I alluded to in Introduction about difference between theory and practice). Now, what is going on here and why did we not get inhibition of *Pseudoxylaria*? I don't know.

Anyway we did many variants of this experiment:

- One used hyphae of *Pseudoxylaria* instead of culture. Same result was gotten however.
- One was done on fresh comb. Fresh comb is somewhat liquid repellent so I made powder of it and added *Pseudomonas* solution to it. This gave me a paste which I coated on fresh comb and then did this same interaction assay on it. No inhibition there too.
- One was a setup in which instead of adding *Pseudoxylaria* over a comb that had *Pseudomonas*, we took combs infected with *Pseudoxylaria* and put them in *Pseudomonas*. Our little trick of changing the sequence could not accomplish anything though. Negative result again.
- Then we grew *Pseudomonas* and *Pseudoxylaria* together for a couple of days thinking that previous exposure might switch some genes which might make them more antagonistic toward each other. So after growing them together we did our assay again on fresh comb this time but to no avail. Inhibition of *Pseudoxylaria* remained elusive as ever. Note that this one hasn't been done on our pet autoclaved comb, but if you ask me I wouldn't be much optimistic about that either.

That concludes this tiny chapter.

Concluding remarks:

Finally, I would like to remind the reader that here and there in this dissertation I have suggested some experiments which I might have done if I were to continue working on this project. These:

- To check if termite workers can deal with *Pseudoxylaria* if instead of soil, they are given some other powdered substance.
- Put crushed fresh comb on half the petri plate and soil on the other half to see if workers can then fight off infection which they were unable to fight when only powdered fresh comb was present.
- And maybe doing the interaction of *Pseudoxylaria* and *Pseudomonas* on autoclaved comb which I am not very optimistic about.

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