BES Grant Report:

Time-dependent effects of plant-microbe interactions

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What was the overall aim of the work supported by this grant?

This project aims to understand the complexities of plant-microbe interactions over time: How does the length of plant growth (hereafter "conditioning time") change the strength of plant-microbe interactions? I used an aquatic plant-microbe community with two species of duckweed and a mixture of microbiomes collected from natural habitats (ponds). I grew the first duckweed species in the medium with or without microbiomes, then used this "conditioned" medium to grow the second species. I controlled for the length of conditioning time and the source of medium (whether previously inhabited by duckweeds and whether contains an environmental microbiome). Measuring the growth of the second species under different temporal and medium treatments should provide insight into the mechanisms that contribute to the potential temporal changes in the strength of plant-microbe interactions.

Have the overall aims of the project been met?

The experiments were completed successfully, with slight changes in focal species and the overall timeline. The presence of microbiomes led to the strongest difference among all treatments of the medium, while the conditioning time only played a secondary role. Duckweed response to conditioned medium depended on the treatment of the medium and was strongly species-specific. The growth rates of the CBS strain increased with the conditioning time with the presence of the conditioned microbiome and additional nutrients. The opposite was observed when the microbiome was absent during the conditioning phase, removed during the growth phase, or no additional nutrients were added. The growth rates of *Lemna japonica* were mostly consistent across temporal and medium treatments. The presence of microbiomes during the conditioning phase or the growth phase determined the calculated fitness difference between the CBS strain and *Lemna japonica*. Although conditioning time determined the stabilization potential between the two duckweed species, the direction was inconsistent and the effect was weak. Overall, we gained more mechanistic insight into the process behind plant-microbe feedback in this system, although time seemed to contribute weakly to the observed results. Nutrient and microbiome analyses are underway to provide further support to these results.

1. **Background and Rationale**

Plant-microbe interactions are often studied under the context of legacy effects: the previous plant resident "conditions" the habitat by altering the microbial community or chemical compositions (Bever et al. 1997; Crawford et al. 2019). Past studies often use a classic two-phase design: a conditioning phase that grows one species in the unconditioned medium, followed by a growth phase that grows the same or different species in the previously conditioned medium (Kandlikar et al. 2019; Yan et al. 2022). This method falls short on two aspects: (1) it ignores changes in microbial communities or the environment over time by fixing the length of the conditioning phase and (2) does not characterize the underlying mechanisms causing the feedback, e.g., microbial community composition, nutrient levels, or chemical characteristics. To address these gaps, I aim to answer the following questions:

(1) How does conditioning time affect the strength of plant-microbe interactions?

(2) Which mechanism(s) contribute to the temporal changes in the strength of plant-microbe interactions?

While (1) can be tackled by using different lengths of the conditioning phase, (2) is often infeasible in commonly studied terrestrial plants, because complete, non-destructive sampling of microbiomes in soil and on roots is challenging. Duckweeds are small, fast-reproducing aquatic plants that display plant-microbe interactions via their aquatic rhizosphere (Tan et al. 2021). The microbiome attached to duckweeds can be extracted by ultrasonic vibration and sampled from the liquid medium; conversely, the microbiome in the medium can be removed by filtration, leaving a conditioned medium without microbes. These characteristics allow me to decompose (2) into more specific sub-questions that directly address mechanisms of plant-microbe interactions:

(2) How does conditioning time change the (H1) microbial community, (H2) nutrient levels, (H3) overall environment other than the microbial community, or (H4) synergistic effects of all the above mechanisms?

2. Methods

2.1 Experimental Setup

We used axenic cultures of two duckweed species. Species 1 (CBS strain) was purchased from Carolina Biological Supplies (Burlington, North Carolina, USA); species 2 (*Lemna japonica*) was collected from field sites in Louisiana, USA. We used a mixture of environmental microbiomes isolated from freshwater ponds in Ohio, Pennsylvania, Louisiana, and Texas. All experiments were conducted in incubators under 25˚C and a 16-to-8 light-to-dark cycle.

We used sterile 1:5 standard Hoagland medium (Sigma Aldrich, St. Louis, Missouri, USA) as the base medium. For treatments with environmental microbiomes, we added a total of 12.5mL of the diluted microbiome culture suspended in sterile DI water into ~1200 mL of the base medium.

The experiment consisted of two phases. In the conditioning phase, we added one species (hereafter species *i*) in the unconditioned medium for 1, 5, and 10 days. In the growth phase, we added another species (species *j*) in the medium conditioned by species *i*, and observed its growth after 10 days. To test for the four mechanisms outlined in (2) above, we controlled the amount of nutrients or the presence of microbiomes in the conditioning or growth phases. Inoculating species *i* without the environmental microbiome tested for the effect of growing species *i* on nutrient levels (H2). To test for the other mechanisms, we inoculated species *i* with environmental microbiomes, then either kept the conditioned microbiome only while adding additional nutrients (H1), kept the conditioned medium without microbiome (H3), or kept both conditioned microbiome and medium without adding nutrients (H4). Species *i* and *j* can be the same or different species. *L. japonica* individuals have a larger surface area than the CBS strain. We therefore use two individuals of *L. japonica* and six CBS strains as the initial population for each phase.

We grew duckweed in 6-well culture plates (Corning, Corning, New York, USA) with 5mL of medium per well. Each plate represents one out of 48 of specific treatments (a factorial design of four conditioning-growth species pair by three conditioning time treatments by four medium treatments), and each well represents one replicate of the specific treatment.

We could not sample the medium at the end of the conditioning phase due to experimental design. Therefore, we set up two additional plates with environmental microbiomes and grew the two species for 1, 5, and 10 days, then sampled the medium in each well. We also sampled the medium

at the end of the growth phase. We took two 1.5mL samples from each well, and then analyzed the samples for nutrient content and microbial community composition. These analyses are still in progress.

We started the experiment on Feb 21, 2024 and ended the experiment on Mar 12, 2024.

2.2 Data Analysis

Out of the 288 wells in the conditioning phase, five were dropped for calculation in growth rates because the mortality reached 100% during the conditioning phase. All the dropped wells were in treatments with microbial addition (one in H1, one in H3, three in H4). Out of the 288 wells in the growth phase, two wells were dropped (one in H1, one in H3) because the wells dried up during the experiment, leading to 100% mortality. In addition, 14 wells, all in H4, had 100% mortality five days after the addition of duckweed (on day 6), and new duckweed fronds were replenished. The replenished duckweed grew well and was therefore used to calculate growth rates from day 6 to day 11.

We counted individuals in all wells at the end of the conditioning phase and growth phase. We calculated population growth rates as $\log(N_t/N_0)$, where N_t is the end population and N_0 is the initial population. A one-day, five-day, or 10-day growth rate was calculated for each well during the conditioning phase, except for the five dropped wells. Five-day growth rates were used for consistency with the five-day growth rates calculated from the growth phase. Two five-day growth rates were calculated in the growth phase, one from day 1 (first day of addition) to day 6, and one from day 6 to day 11. In addition, the growth rates from day 1 to day 11 (i.e., 10-day growth rates) were also calculated. Because some wells had 100% mortality from day 1 to day 6 (see above), 14 out of 288 wells only had the five-day growth rate from day 6 to day 11. For the completeness of data, we used the five-day growth rate from day 6 to day 11 in the growth phase and five-day growth rates in the conditioning phase for further analyses.

Using growth rates, we further calculated stabilization and fitness differences between the CBS strain (species 1) and *L. japonica* (species 2; personal communication, Xinyi Yan):

Stabilization $= -1/2(G_{1A} - G_{1B} - G_{2A} + G_{2B})$;

Fitness difference $= -1/2\left(G_{1A} + G_{1B} - 2G_{1,ref} - G_{2A} - G_{2B} + 2G_{2,ref}\right)$

In these equations, G_{1A} represents the log growth rate, averaged across wells, of species 1 in medium conditioned by species 1 (i.e., microbiome A), and $G_{1,ref}$ represents the average log growth rate of species 1 in the reference medium, calculated from the conditioning phase. Note that the fitness difference equation calculates the difference from 1 to 2; if this value is positive, species 1 is advantageous, and vice versa.

3. Preliminary Results and Discussion

Duckweed response to conditioned medium depended on the treatment of the medium and was strongly species-specific. The growth rates of the CBS strain during the growth phase increased with the conditioning time with the presence of the conditioned microbiome and additional nutrients (H1). The opposite was observed when the microbiome was absent during the conditioning phase (H2), removed during the growth phase (H3), or no additional nutrients were

added (H4; Figure 1). These results imply that the additional nutrients promoted the growth of duckweed in the growth phase, especially with the longer conditioning phase, which increasingly depleted the nutrients available during the growth phase and led to a decrease in growth rates in other treatments that have not received the additional nutrients. These patterns were mostly consistent among the two different conditioning species, except when the CBS strain was conditioned by *L. japonica* in a completely axenic medium (H2). This likely arose from a smaller demand for nutrients by *L. japonica* during the conditioning phase due to its smaller initial density. The other treatments that did not receive additional nutrients (H3, H4) showed a decreasing growth rate with a longer conditioning phase likely because of the presence of microbiomes in the conditioning phase, during which they depleted more nutrients. On the other hand, the growth rates of *L. japonica* were highly consistent among conditioning species, conditioning time, and medium treatments, and did not differ from the reference growth rates measured from the conditioning phase.

The presence of microbiomes during the conditioning phase or the growth phase determined the calculated fitness difference between the CBS strain and *L. japonica* (Figure 2), although data between treatments overlapped widely accounting for standard errors. Interestingly, the mean fitness difference values were mostly positive for H2 (no microbiomes in conditioning or growth phase), indicating a higher fitness of species 1, but mostly negative for all other treatments, indicating that adding microbiomes in either conditioning phase, growth phase, or both reversed the rank of species fitness. Among the other medium treatments, H4 (microbiome present for both phases and no nutrient addition) led to the highest fitness differences between the two species, while H1 (microbiome present for both phases with nutrient addition) and H3 (microbiome present for conditioning but not growth phases) led to similar magnitudes. On the other hand, the length of conditioning times mostly contributed to stabilization, but the effects were weak and inconsistent among treatments. These results implied that the presence, but not the temporal dynamics, of microbiomes during the conditioning and growth phases were the most important in determining the fitness difference and stabilization between the two species.

Overall, the presence of microbiome and the addition of nutrients seemed to contribute to the most patterns observed in the system, while temporal changes in microbiomes might have played a secondary role. However, the ongoing water analysis of nutrient consumption and microbial community composition may lend new insights into interpreting the results presented above.

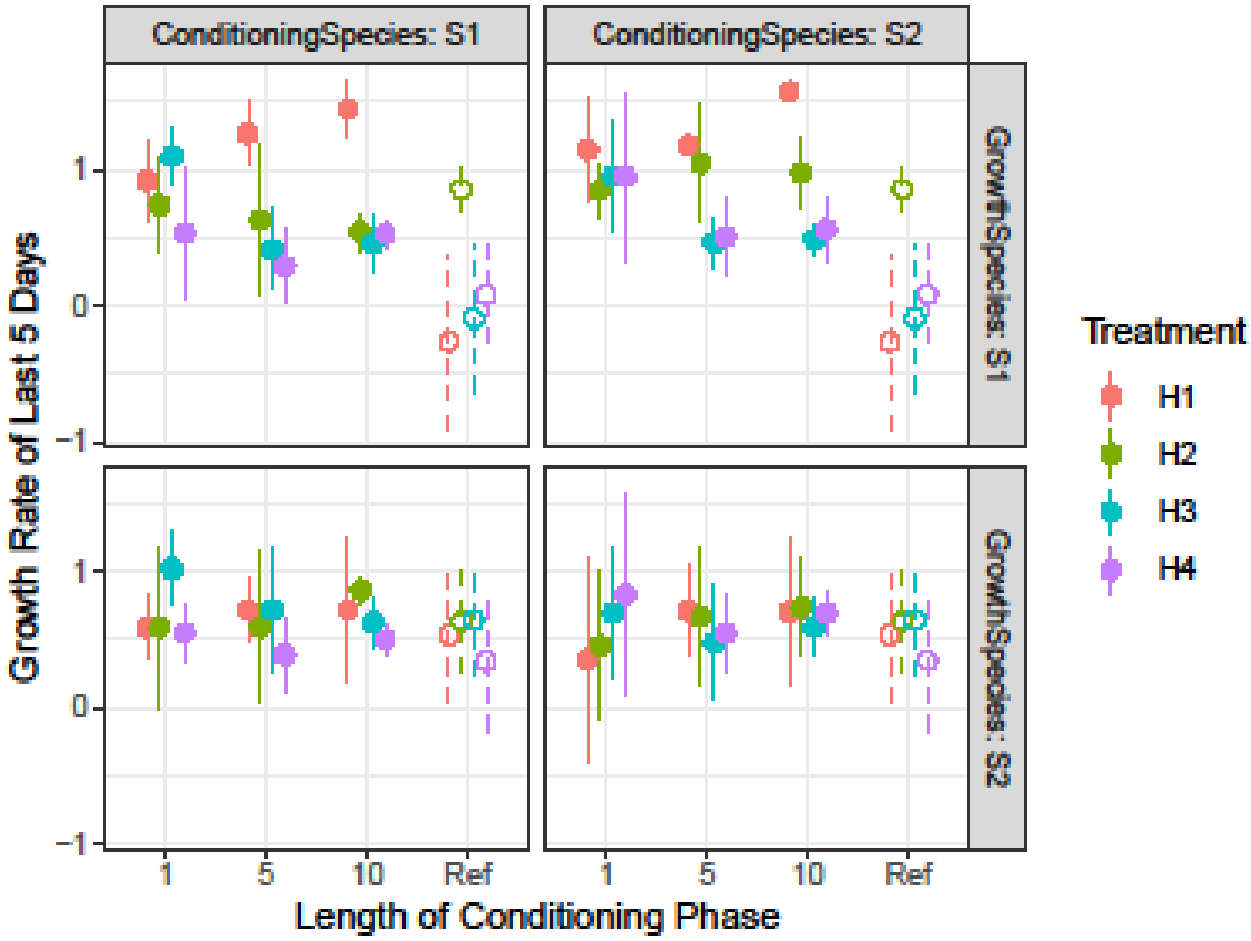

Figure 1. Five-day growth rates calculated from day 6 to day 11 of the growth phase (i.e., last five days). Each species was conditioned by species 1 or 2 for 1, 5, and 10 days. Empty dots and dashed lines are reference five-day growth rates calculated from the conditioning phase. S1 represents the CBS strain, and S2 represents *Lemna japonica*.

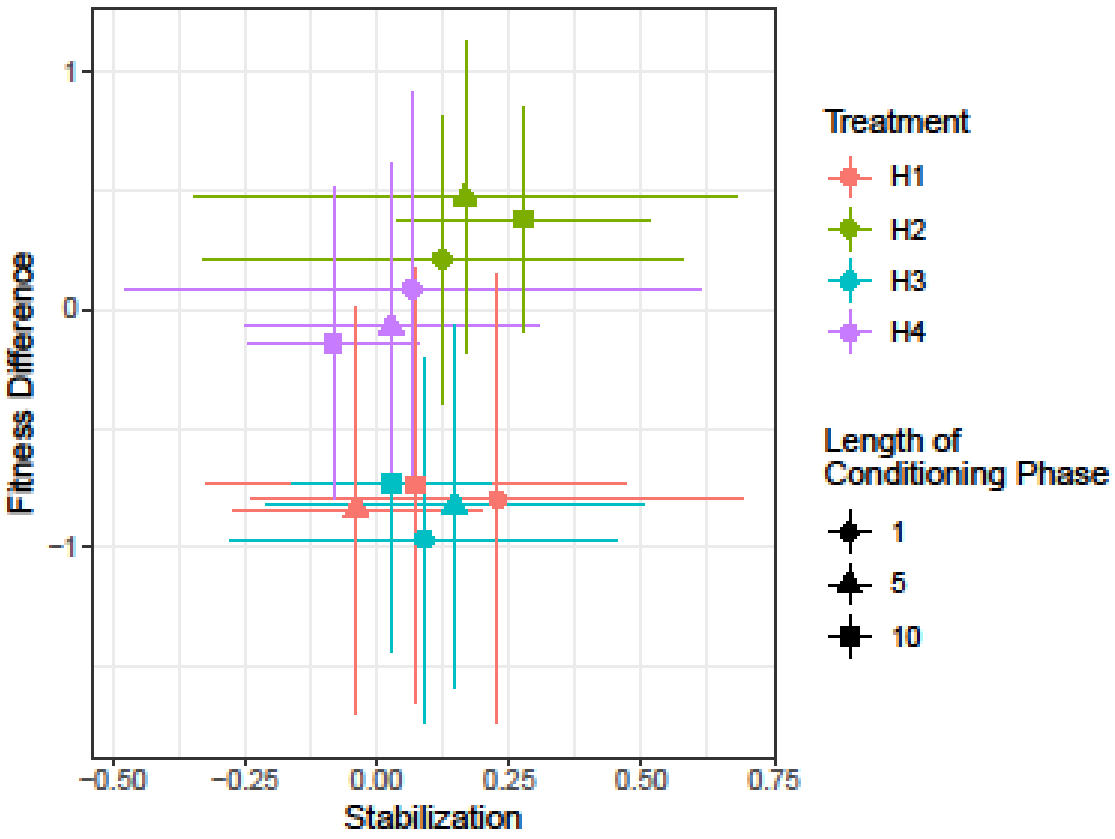

Figure 2. Stabilization and fitness differences between each species with different treatments and lengths of conditioning phase, calculated using the growth rates of last five days (day 6 to day 11).

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Section 2 - Monitoring and Evaluation

How much do you feel your BES award enhanced your career prospects? 5

How much do you feel your BES award contributed towards the science of ecology? 5

Regarding your BES award, which of the following do you feel to be true:

- Increased your knowledge of ecology
- Improved communication and collaboration among ecologists
- Improved your access to equipment
- Provided travel assistance

Please provide any specific feedback or comments that you would like to make on your award:

I am grateful for receiving the award and very satisfied with the application, judging, and funding acquisition process.

Section 3 - Impact Report

Project Description.

Please describe the work funded by the BES grant in terms that would be understood by a member of the public. Please do not use detailed scientific terms.

How does the interaction between plants and microbes change with time? My project aims to understand this temporal process by looking into a specific type of plant-microbe interaction. The environment previously occupied by one plant can exert certain feedback on the other plant. I intend to understand this feedback by controlling for several possible mechanisms. The simplest case is that the first plant can consume nutrients in the environment, making them less available to the second plant. Second, the first plant can change the microbiome in its environment over time, and the microbiome will in turn affect the second plant. Third, the microbiome of the first plant may generate some chemicals that affect the growth of the second plant. Finally, a combination of all the mechanisms above may determine this feedback. I tested these hypotheses with two duckweeds, which are tiny plants floating on the water. They compete readily for resources and can associate with microbes in the environment, making them ideal for my purpose. I grew the first duckweed for different lengths, treated the medium according to the four hypotheses, and then observed the growth of a second duckweed in those media. Unsurprisingly, duckweed growth was limited by nutrients and affected by the presence of microbes. However, the length during which the first species “conditioned” the medium seemed to only have a weak effect. Analyses of nutrient content and microbe community are ongoing to better explain these explanations.

Personal Impact.

What impact did receiving this grant have on you personally and the development of your career in ecology?

This is my first independent grant proposal. This is also my first time reaching out to a collaborator not through my advisor's connection. These "firsts" make this project special not only to the development of my career in ecology but also to my personal development. The application greatly helped me better understand the grant application process, and the discussions with my collaborator further gave me a taste of how collaborative science is done in academia. On the other hand, fund management, purchase of materials, and coordination of logistics for my visit to my collaborator's institution brought me a layer of realism under the grandeur and excitement of conducting research that I, as a graduate student, often had little exposure to. After the experiment was concluded, I found myself solely responsible for the progress instead of adhering to certain deadlines as the projects for my thesis required, and this improved my time management skills. Overall, receiving this grant and successfully conducting the experiment almost identically to my plans is an important experience in shaping the necessary skills for an independent researcher.

Scientific Impact.

What impact did receiving this grant have on the research community?

The project provides first understanding of how the interaction between duckweed and its environment is shaped by microbiomes and other factors, and how these mechanisms could change with time. The temporal aspect is especially important because most literature on plant-microbe interactions and plant-soil feedback do not consider the temporal dynamics of the environment, or more specifically, of the microbial community. Although current results show a weak effect of time on duckweed growth and interactions, further analyses of nutrient contents and microbial community may reveal temporal changes in the medium. Further investigation is needed if these temporal changes in the medium are not reflected by temporal changes in the duckweed population. On the other hand, Receiving this grant allows me to work on my project that contributes to the knowledge of plant-microbe interactions in ecology. As an international student in the United States, I am not eligible for many federal funding opportunities, so independent grants like the BES Small Research Grant are especially important to me. They allow me, or any other early career ecologists in similar positions, to explore ideas that could greatly advance our knowledge with no or less financial burdens.

Wider Impact and Outcomes.

What impact and outcomes do you think the work funded by the BES may have within fields outside of academia? Please take into account all wider implications e.g. society/policy/public.

Plant-microbe interaction is essential to shaping plant productivity and diversity, which is a central focus of conservation and agriculture. The temporal dynamics of plant-soil feedback can be important for conservation because the fitness of the planted seedling could depend on both the identity and the length of occupancy of the previous inhabitants. Similar to mechanisms proposed in my project, such feedback can arise from changes in microbiomes, accumulation of chemicals generated by the plants or their microbiomes (allelopathy), depletion of nutrients, or a combination of both. For instance, non-native plants could have negative feedback to native plants via conditioned soil, and sites that are occupied longer may be more difficult to restore with lower recruitment of native plants. These mechanisms are also important for agriculture as certain crop

rotation strategies (i.e., the order of planting crops on the same field) can greatly increase productivity. In addition, duckweed is often considered a promising candidate for water treatment and biofuel production. Therefore, the population- and community-level consequences of environmental microbiome on duckweed can help guide their production.

Publications and Outputs.

Please provide us with a summary of any key outputs and publications. Have you published/are you intending to publish any papers relating to this work? (e.g. Published/submitted/in preparation)

Yes, I intend to publish the output of the project partially funded by the grant. The manuscript is still in preparation because analyses are still in progress.

Your Shout.

We'd love you to provide us with a testimonial. Testimonials will be used on various BES channels for new applicants to view and to promote our grants to the ecological community.

The British Ecological Society's Small Research Grant Program funded my first independent experimental project that could provide new insights into plant-microbe interactions. Not only did I benefit scientifically, but this project also allowed me to get the full experience of independent research, from design, and funding acquisition, to execution. As a budding PhD student, this experience marks a fundamental step towards a fully-grown scientist. I am extremely grateful for the support of BES on this project and hope that they can continue to support early career scientists like me to grow and become an integral part of the ecological research community.