**The Effects of a Resuscitation Promoting Factor (Rpf) on Microbial Diversity and Plant Fitness**

**Peyton Thomas**

**Transylvania University**

**Jay Lennon and Venus Kuo**

**Evolution, Ecology & Behavior Graduate Program**

**Indiana University**

**Abstract**

Microbial communities have a significant impact on plant diversity and fitness through various plant-soil interactions. These interactions build a complex system of relationships, both mutualistic and competitive, that are vital to maintain the diversity of both microbes and plants. When microbial communities experience unfavorable conditions, they can enter a lowered metabolic state of dormancy to maintain their ability for reproduction and to preserve genetic diversity. A Resuscitation Promoting Factor (Rpf) through muralytic activity is able to resuscitate cells out of dormancy, restoring their growth. Rpf is not widely studied and there is little to no knowledge about how the resuscitation of dormant cells impacts the surrounding ecosystem of plants and organisms. Because of this, the study focused on how the addition of Rpf affects microbe activity, and how this shift in activity affects plant growth and fitness. We studied this by conducting a growth chamber experiment in which there were three varying factors: Rpf treatment (active and control), soil type (live and sterile), and plants (with or without). Rpf treatments were administered every week on the same day, and all samples were kept in the same constant conditions (humidity, temperature, and intensity of light). Although Rpf was predicted to greatly affect microbe activity, it was found that Rpf only significantly affected microbial activity in sterile soil during the first week. The repetition of treatments every week did not have a continuous resuscitation effect. In fact, the only significant factor that affected microbe activity was sterile soil (t = 6.4766, df = 462.37, p-value = 2.403e-10). On the other hand, Rpf and soil had significant effects on various plant fitness measures such as above and below ground biomass, and leaf biomass. While our predictions were not completely accurate, we are confident that Rpf has a resuscitation effect on dormant cells, and that these effects positively and negatively affect plant fitness.

**Background**

Microbial communities have a significant impact on the fitness and diversity of plants (Lau & Lennon, 2011). There are various interactions that can exist between microbial communities, the soil, and plants. In soil systems, soil microbes act as decomposers, giving plants the necessary nutrients they need, while plant matter is a source of fixed carbon for the decomposers. In this way, microbes and plants have a mutualistic relationship, but they also compete for the same soil nutrients making themselves competitors (Reynolds, Packer, Bever, & Clay, 2003). Therefore, there can be many interactions happening simultaneously in an environment.

These relationships build a complex system of interactions and dependence that is vital to maintain the fitness of both microbes and plants. Microbes not only provide nutrients in the soil for plants, but they aid in reducing stress on plants from abiotic environmental factors (Lau & Lennon, 2011; Rodriguez & Redman, 2008a). Empirical studies have shown that microbial communities influence plant growth, productivity, nutrient availability and ecosystem functioning (Marschner & Rumberger, 2004). For example, some strains of microbes are able to activate biochemical processes in plants, which promote an immune defense response that functions to fight off harmful pathogens when present (Rodriguez & Redman, 2008b). This mutualistic relationship is only one of many that convey the importance and variety of roles that microbes and plants play for one another’s success. Microbial community activity and composition affects individual plants, and thus indirectly affects the overall plant community as well. This complex system of interactions shows the vital role that microbial communities play in maintaining a diverse and healthy ecosystem.

In unfavorable environmental conditions, some microbes can enter a state of dormancy to preserve their fitness and maintain biodiversity. Dormancy is a reversible state in which microbes have lower metabolic activity and are reproductively inactive. Going into dormancy allows cells to survive unfavorable environmental conditions, and resume growth or division once the conditions have improved (Mukamolova et al., 2006). Dormant cells contribute to a seed bank, which is “a reservoir of dormant individuals that can potentially be resuscitated in the future under different environmental conditions” (Chesson & Warner, 1981; Lennon & Jones, 2011). Seed banks play a large role in preserving the genotypes of these populations, thus allowing the biodiversity of these ecosystems to be sustained (Chesson & Warner, 1981). Maintaining a diverse set of microbes is not only important for sustaining that population, but the ecosystem as a whole. The surrounding ecosystem greatly benefits from seed banks and the role they play in stabilizing populations in unfavorable conditions, maintaining fitness, and sustaining biodiversity (Lennon & Jones, 2011).

Some microbes are able to resuscitate from dormancy in the presence of a Resuscitation Promoting Factor (Rpf) (Mukamolova et al., 2002). Rpf is a protein that is able to resuscitate cells out of a dormant state through muralytic activity (Kana et al., 2008). Rpf originates from *Micrococcus luteus,* a nonsporulating bacteria that also exhibits a likelihood for dormancy and resuscitation (Kana et al., 2008).The Rpf taken from *M. luteus* is shown toresuscitate “non-culturable” cells, restoring growth and reintroducing those genotypes to the community as a whole (Kana et al., 2008; Mukamolova et al., 2006). The resuscitation of cells is easily initiated in the presence of Rpf. The research of Votyakova et al. supports that only a very small portion of cells in a community need to be active to initiate the production of Rpf in the microbial community, thus promoting the resuscitation of remaining cells (Votyakova, Kaprelyants, & Kell, 1994). Entering dormancy and then resuscitating with Rpf allows cells to successfully maintain viability for growth at later periods once environmental conditions have improved. The resuscitation of cells is essential to sustain the biodiversity of microbes, and without Rpf this would not be possible. Because of the various complex interactions between microbes and plants, it is essential to understand how this specific resuscitation protein (Rpf) can increase microbial activity in the soil, and how that increase in activity affects the fitness of the plants and thus the diversity of the ecosystem.

With the growing knowledge of Rpf and its function, we were interested in using it to test questions about dormancy and feedback systems within the microbial community. We used Rpf to resuscitate cells out of dormancy, and measure the amount of activity taking place. We aimed to determine how increasing the activity of microbial communities alters plant-soil feedbacks (positive or negative effects), and how that is seen through plant fitness. Improved plant growth helps contribute to a healthy and diverse ecosystem, and is essential to understand how the resuscitation of a microbial community can affect plant fitness and plant-soil interactions. This research will be able to give us a better understanding of how resuscitated microbial communities can influence plant fitness.

**Methods**

**Experimental Design**

To investigate how the addition of Rpf indirectly affects the composition of the soil microbial community and the fitness of plants, a soil mesocosm experiment in a growth chamber was conducted. Rpf promotes the resuscitation and growth of dormant cells, and this should result in an affect on the diversity and composition of the microbial community. Due to increased competition for resources, some taxa will excel and we would see a large increase in cells, while other taxa would be suppressed. It is this way that Rpf indirectly affects the composition and activity of the microbial community.

The design of the experiment consists of 52 phizan sterilized plastic pots (~50cm diameter by ~30cm diameter height), filled with autoclaved sterilized Metro mix, and vermiculite at one part each. To manipulate the microbial community structure, we inoculated half the pots with live soil (taken from Griffy Lake Forest Preserve, Bloomington, IN, 47404), and the remaining with three times autoclaved soil (three cycles, 90 min., over 72 hours). For this study, we used Wisconsin Fast Plants TM (Wisconsin Fast Plants Program, University of Wisconsin, Madison, WI, USA) due to its fast life cycle and its association with diverse soil microbial community. 32 pots were sown with *B. rapa* seeds; the remaining twenty pots were left exposed.

Two treatments were chosen to study the effects of Rpf on microbial community activity. Rpf addition treatment (Rpf+) was comprised of 150 uL of Rpf diluted with 850 uL of e-pure H2O, and the control treatment (Rpf-) was comprised of150 uL of Rpf buffer, and 850 uL of e-pure H2O. The Rpf buffer is inactive, or does not activate dormant cells. Rpf- was chosen as the control instead of water because it maintains the same level of protein, which is beneficial for the growth of bacteria. With the use of the Rpf- treatment as our control, we know that the change in activity is due to the active protein, not due to the presence of proteins in general. By having protein material in Rpf+ and Rpf-, we accurately measured the effect of Rpf on the activity of the microbial community. Rpf+ and Rpf- were both applied by pipetting 1 mL of the solution to four equidistant points of the pot (Figure 1.).

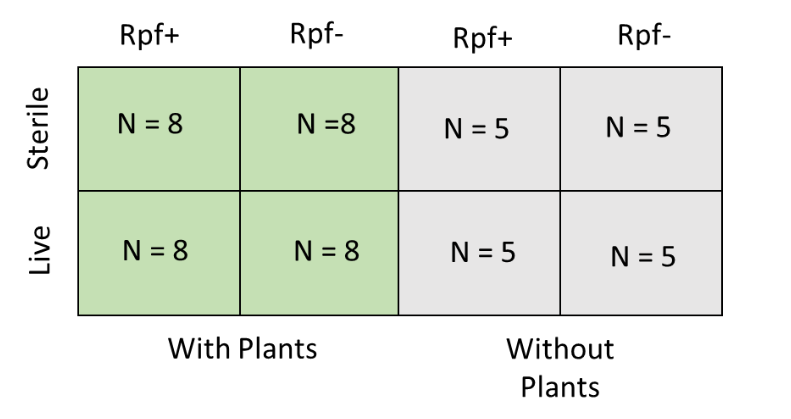


Figure 1. Experimental design focusing on the change in microbial activity due to the addition of Rpf to pots inoculated with live or sterile soil containing either plants or no plants. Rpf+ refers to the treatment containing the active protein Rpf, and Rpf- refers to the control treatment of Rpf buffer (inactive). Live and sterile refer to the type of soil inoculated to autoclaved sterilized Metro mix, and vermiculite at one part each. N is the number of replicates per treatment type.

All pots were watered by the addition of e-pure H2O in tins under the pots everyday to prevent splashing in between the pots, and gently watered on the soil surface every other day to maintain soil surface moisture.

**Microbial Activity**

To observe the shift in microbial activity following Rpf addition to pots, we measured the CO2 respiration and calculated the colony-forming units (CFU) of each sample. We chose these measures because they will both quantify the amount of active and reproducing cells in the samples. We would expect that increases in CO2 respiration and CFU, would correlate to higher microbial community activity in the soil.

For the soil sampling, we performed two destructive samplings (24 hr and 48 hr) with mesocosm vials. We used 104 mesocosm vials (two vials per sample (24 hr and 48 hr)) to measure the soil CO2 respiration. The soil sampling was done by extracting soil from three haphazardly chosen areas on each of the pots, homogenizing by mixing, and then weighing out 1g from that mixture to be placed into mesocosm vials. These samples were then stored at room temperature until the appropriate time for data collection. At hour 24 and 48 after the treatment of Rpf+ or Rpf-, the samples were taken for analysis where a milliliter of air was extracted from the vial headspace and then read using an Infrared Gas Analyzer (IRGA). This analysis was performed every week that the experiment was run and on the same days. After the CO2 readings had been read for the 24-hour soil samples, 5mL of 0.85% physiological saline solution was added in a 15mL Falcon centrifuge tube. They were then shaken on a vortex for 30 minutes to homogenize. A serial dilution was done to 10^8 with the homogenized soil mixture and the 0.85% saline solution. 100 uL of the final dilution was spread onto an R2A plate infused with 50uL/mL final concentration of cyclohexamide. Plates were then incubated at 25°C for four days. Afterwards, plates were removed and CFU was calculated using a plate reader.

The 48 hour soil samples were analyzed in the same manner as the 24 hour samples, but were transferred into Nasco Whirl-Paks TM to be stored in the -80°C freezer for later analysis.

**Plant Fitness**

The alterations in microbial community also have an effect on plant growth and fitness. Therefore, we measured plant germination time, biomass, flower number, lateral height, Surface Leaf Area (SLA), and seed production, which are all indicators of plant fitness as a result of plant-soil interactions.

After the *Brassica* *rapa* seeds were sown, the pots were checked daily for germination and the date recorded for each replicate. On day 17, *B. rapa* flower count was taken for each replicate and plants were cross-pollinated. We cross-pollinated plants by transferring pollen to the pistol of a separate flower with a paintbrush while sterilizing in between with 30% isopropyl alcohol to prevent bacterial transfer. The flower number was used to measure the potential reproductive fitness of the plant. On the 22nd day of the experiment, the approximate surface leaf area (SLA) and lateral height were calculated. The lateral height was measured by stretching a string from the soil surface to the tip of the plant and measuring its length. SLA was calculated by taking a digital picture and using ImageJ software to calculate area based on a scale of pixel/cm. On day 40, we destructively harvested the plants and allowed them to dry for two days, in a drying oven at 65°C. After the plants were completely dried, seeds were gently removed from the seedpods and counted. The above and belowground biomass was also determined at this time. These measurements allowed us to understand how the change in microbial activity was affecting plant growth.

**Statistical Analyses**

**Microbial Community Activity** To analyze the soil CO2 respiration, we used a three-way ANOVA with repeated measures to determine the significance of each main effect (rpf treatment, soil type, plants, week) and their interactions. All data was balanced and normal, and did not need to be transformed.

**Plant Fitness Measures** We used a two-way ANOVA followed by a two-sample t test to test how each response measure was affected by rpf treatment, soil type, or both. Response measures included: total plant biomass, above and below ground biomass, height, leaf weight (dry and wet), seed count, flower count, and Surface Leaf Area (SLA).

**Results**

**Microbial Community Activity**

Our previous predictions on how Rpf would affect microbial activity were not fully supported by our data. The statistical analyses showed that Rpf treatment, as a main effect does not play a significant role in increasing microbial activity. There was no significant difference between Rpf+ and Rpf- treatments on the mean of CO2 respiration (t = 0.35067, df = 605.88, p-value = 0.726). While Rpf treatment did not have a significant effect, it was shown that simple microbe type significantly affects microbial activity, seen by CO2 respiration (ANOVA, value= -12765.462 Std. Error=2739.810 Df=48 t-value=-4.659250 p-value=0.0000).

**Plant Fitness**

Both Rpf treatment and microbe type affected plant growth. It was shown that Rpf+ negatively affected total plant biomass (t = 3.1768, df = 30, p-value = 0.003438), belowground biomass (t = 2.5041, df = 18.353, p-value = 0.02191), and leaf weight (t = 2.182, df = 30, p-value = 0.03708). Plants in simple microbe systems were shown on average to have a higher aboveground biomass (t = 4.3051, df = 30, p-value = 0.0001641), and leaf weight (t = 3.015, df = 30, p-value = 0.00519). There were not any significant effects on Surface Leaf Area (SLA), height, flower count, and seed count due to Rpf treatment or microbe type (simple, complex).

**Discussion**

Our study focused on the interactions between dormant and active microbes, and how this interaction can affect plant growth. While Rpf was not significant in increasing microbial activity, it did have affects on multiple plant characteristics.

**Microbe Activity**

We know from previous studies that Rpf is very effective in resuscitating dormant cells when the abundance of active cells is low (Votyakova, Kaprelyants, & Kell, 1994). We predicted that the addition of Rpf would resuscitate dormant cells, increasing the active microbial population, and thus creating a higher output of CO2 from the soil. Our results did not show this, and reported that there was no significant difference between samples treated with active Rpf and those that were treated with the control solution (t = 0.35067, df = 605.88, p-value = 0.726). This could be due to multiple factors. One possible explanation for this result is that Rpf could be lysing a portion of the microbes in that community. Lysing would cause the number of actively respiring cells to decrease since they are no longer able to respire. Normally, one would expect to see a sharp increase of CO2 respiration, indicating the resuscitation of cells, but in this case, we would observe no significant increase. In other words, Rpf is active, but is not producing the effect that you would expect to see. It is by this manner, that there would be no significant difference between the control and Rpf treatment.

Another way in which we could explain the insignificance between treatments is that Rpf is resuscitating cells that favor less CO2 respiration. Rpf does not have control over which cells resuscitate and in this case, therefore it is possible that through chance, Rpf could be resuscitating microbes that don’t respire as much as others. Since the resuscitation of these cells wouldn’t have a large increase of respiration, it would account for the insignificant difference between the control and Rpf+ treatment.

While the two scenarios above are possible, it could also be that we did not see a significant difference between treatment type because CO2 is not a good measure for microbial activity. Microbes are not the only organisms that occupy the soil. Fungi, and spores among other things are present, which also affect the concentration of CO2 in the soil. Because of this, we know that it is likely that the reason we did not see a clearer result is due to the fact that the fungi, spore, and microbial community were all affecting respiration. In the future, or if we were to re run this experiment, we would want to reconsider the use of CO2 respiration as an appropriate measure for microbial activity.

**Microbe Composition**

The CO2 respiration was also higher in the simple systems when compared to the complex. This was contrary to our predictions, but can be explained by a possible effect of Rpf on the composition in the microbial community. Since the simple system included one-third sterilized soil (autoclaved three times within 72 hours), we know that at the beginning of the experiment, those samples contained less active bacteria, fungi and spores. Therefore when Rpf+ was added, it is possible that a completely different set of microbes were able to resuscitate compared to the complex soil systems. This would mean that the microbes being resuscitated and the already active community would be different. In other words, complex and simple systems would have significantly different microbial communities. If the complex and simple systems have different compositions, then we would expect them to have different CO2 respirations. We do see this trend with the study, so this could be a possible explanation for these results.

**Plant Fitness**

The positive treatment of Rpf+ negatively affected total plant biomass (t = 3.1768, df = 30, p-value = 0.003438), belowground biomass (t = 2.5041, df = 18.353, p-value = 0.02191), and leaf weight (t = 2.182, df = 30, p-value = 0.03708). Previous studies present that there are various interactions between microbes and plants, ranging from mutualistic to competitive relationships (Reynolds, Packer, Bever, & Clay, 2003). From previous studies, we would expect to see a positive relationship between Rpf+ and plant fitness because Rpf is thought to create a more diverse active microbial community. Here, we see that the opposite is true. In this case, it seems that the Rpf could have resuscitated cells that were pathogenic, or harmful to the fitness of plants. In this case, we would see that the control (Rpf-), would have a higher fitness due to a positive relationship and a negative relationship from the positive treatment (Rpf+). If this were true, it would explain why plants did not benefit, from the positive treatment of Rpf.

Plants in simple microbe systems were shown on average to have a higher aboveground biomass (t = 4.3051, df = 30, p-value = 0.0001641), and leaf weight (t = 3.015, df = 30, p-value = 0.00519). The simple microbial system was achieved by sterilizing live soil three times in an autoclave over 72 hours. This was to reduce the amount of live bacteria, spores, and fungi. The complex system was also from the same soil samples taken from Griffy Lake. It is possible that the complex system of microbes (live soil) contained a significant amount of microbes that were pathogenic or causing negative effects on plant growth. During the sterilization process, it is possible that these active pathogenic microbes were removed, thus allowing more opportunistic taxa to take over. These new taxa could be much more beneficial to plant growth, causing the plants in simple microbial samples to have a greater fitness. This is one possible explanation for the results found.

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