Differences in *Rhizopogon* Colonization on Pines in the Eastern Sierra Nevada

Theresa Hendrick

Abstract *Rhizopogon* is a genus of hypogeous fungi that forms mutualistic associations with many members of the *Pinaceae*. *Rhizopogon* maintains spore viability after large-scale disturbances such as stand-replacing fire and is abundant in soil after such disturbances. It provides much of the inoculum necessary for post-fire rehabilitation of pine seedlings and therefore is a key element encompassing fire ecology. Bioassaying pine seeds in soil can successfully capture Rhizopogon. *Pinus muricata* (Bishop pine) is the preferred pine to bioassay with because it is fast growing, able to capture *Rhizopogon* in high frequency, and requires little preparation time before planting. Preliminary data from this study shows that Bishop pine is more frequently colonized as compared to the other pines used. In this study Bishop pine was bioassayed to determine whether it could provide a set of similar fungi that colonize the root tips of the native tree populations in the Eastern Sierra Nevada. These native tree populations consist of *Pinus jeffreyi* (Jeffrey pine), *Pinus lambertiana* (Sugar pine), and *Pinus contorta* (Lodgepole pine). Samples of *Rhizopogon* were obtained directly from the roots of each individual pine in the bioassay containers and screened by ITS-RFLP and unknown *Rhizopogon* species were further sequenced. I anticipate that similar sets of *Rhizopogon* will be obtained from Bishop, Jeffrey, and Lodgepole pines while Sugar pine, because of its differences in lineage, may capture a slightly different set of *Rhizopogon*.

Introduction

Mycorrhizal fungi are extremely important for the growth and survival of many plant species because they increase the plant's ability to obtain water and nutrients such as phosphorus and calcium, which can be limiting in soil environments (Kernaghan *et. al.* 2003). These fungi can also protect roots from adverse conditions such as drought and high metal concentrations within the soil (Brownlee *et. al*. 1983, Burgess *et. al.* 1993). *Rhizopogon* is a genus of ectomycorrhizal fungi with a large number of species specifically associated with members of the *Pinaceae* (Cairney 1999). Recent work by Kjoller and Bruns (2003) has shown that *Rhizopogon* species form an abundant sporebank in pine-dominated forests in coastal California, and following severe disturbance, such as stand-replacing fire, this sporebank provides most of the mycorrhizal inoculum for newly establishing pine seedlings. *Rhizopogon* can be retrieved from soil by bioassaying the soil with pine seedlings.

The purpose of this study is to test the hypothesis that different pine species will yield different species of *Rhizopogon* under identical bioassay conditions. The pine species used in this study are: *P. muricata, P. jeffreyi, P. lambertiana, and P. contorta*. The different soil types were collected from five different areas in the Eastern Sierra Nevada. *P. muricata* is the foreign pine of the group because it is native to California coastal regions. It is readily available to us and is easy to germinate and grow. The other pine species are native to Sierra Nevada forests and involve a much longer germinating and growing process.

Therefore, if *P. muricata* (Bishop pine) yields similar *Rhizopogon* species, researchers would be able to bioassay it with soils from the Sierra Nevada and other mountainous regions. In turn, they would benefit in the faster germinating and growing process as well as have an accurate representation of the different *Rhizopogon* species within these areas. Little is known about the below ground community in the Eastern Sierras; therefore, gaining an understanding of this otherwise unknown mycorrhizal system benefits future research in this ecologically rich area. According to Kernaghan *et. al*. (2003) and many others diversity within mycorrhizae in natural ecosystems is yet to be clearly determined.

Materials and Methods

Plant material Sugar, Jeffrey, and Lodgepole pine seeds were ordered and shipped to us from the United States Forest Service. Bishop pinecones had previously been collected from Point Reyes National Seashore and the seeds were harvested directly from the cones. Each of the Sugar, Jeffrey, and Lodgepole seeds were surface sterilized in 30% hydrogen peroxide with a drop of Tween 20, which acts as a detergent, and placed on a shaker for 20 minutes. The seeds were then rinsed and placed in individual plastic bags with small holes punctured in the bottom and placed under running water for two days followed by a 30-day drip dry in the cold room (33°F). Moist paper towels surrounded the seeds in the plastic bags to retain moisture and they were checked every other day to make sure the moist environment was maintained**.**

After the 30-day drip dry the seeds were once again rinsed, dried, and placed in a new plastic bag and set to dry in the cold room for various periods of time following the suggested protocol obtained from the Forest Service. Jeffrey Pine remained in the cold room for 33 days, Sugar Pine for 61 days, and Lodgepole Pine for 15 days. The seeds require this process to simulate the Sierra Nevada winter conditions. The starting and stopping times for the seed preparation were arranged such that all the seeds would be ready to plant at approximately the same time. This process was started on 7/15/03 and ended on 10/14/03. Bishop Pine seeds were surface sterilized as the others, rinsed, and dried. The Bishop Pine seeds do not require any soaking or drying time and are ready to plant immediately after they are surface sterilized.

Soil Material The five different soil samples were obtained from Eastern Sierra pine sites near Mammoth Lakes area (Table 1). Each sample consisted of 10 small scoops of soil, approximately 10 cm into the mineral layer. Each scoop was collected 2 to 3 meters from the previous scoop along a line. The first four samples were under predominately Jeffrey pine and the last was under primarily Lodgepole Pine with a few Jeffery Pines.

Tag	Date	Location and Forest Type
4700	6/18/03	Obsidian Dome – under a small group of older Jeffery pine on the volcanic dome.
4696	6/18/03	Obsidian Dome forest – under the established forest adjacent to dome. Forest composed of JefferyPine, LodgepolePine,

		Western White pine, and a few white fir.
4697	6/19/03	In Devil's Postpile Natl. Mon. Under surviving and reestablishing forest of Jeffery and Lodgepole pine in the part of 1991 fire site near Rainbow Falls
4699	6/19/03	Next to Inyo Crater parking lot; Jeffery pine forest
4698	6/20/03	At Fern Lake trail head near Silver Lake. Forest predominantly lodgepole pine, with a few large Jeffery Pine

Table1. Sampling locations for soil and the pine species present at those sites.

Each of these five inoculum soils were sifted in a No. 10 sifting dish to remove large rocks, roots, and other debris from the samples. This process was carried out in a hood sterilized with a dilute bleach solution to control contamination between soils. All sifting equipment was soaked in a bleach solution between siftings for 10 minutes, rinsed in water for 5 minutes, and then dried in an oven. Each sifted inoculum was transferred to plastic bags and stored in the cold room until needed. Extra inoculum soil is archived at the Department of Plant and Microbial Biology, University of California at Berkeley.

Experimental Set-Up and Design Seventy quart-sized jars with lids removed and replaced with foil were autoclaved for 20 minutes. The foil ensures no spores get into the containers after they are removed from the autoclave. Approximately 900 bioassay tubes (RLC-4 Super "Stubby" Cell Container from Stuewe & Sons Inc., Corvallis Oregon) were soaked in bleach for 24 hours and then transferred to the dishwasher where they received two cycles of washing and rinsing. The tubes were then transferred to a hood in an adjacent room and allowed to drip dry completely over an eight-hour period. The tubes were then placed in autoclave bags and stored in a spore-free area to avoid contamination.

Soil collected from Tomales Point, in a region known to be devoid of *Rhizopogon*, was transferred from storage into a bleached metal pan, covered with foil and autoclaved for 60 minutes with a 10 minute drying cycle. Coarse sand was obtained from the greenhouse and treated exactly as the Tomales Point soil. In each of the sterilized quart jars, 300mL of sterile soil and 300mL of sterile sand was mixed with 4.5uL of the inoculum soil producing an approximate volume of 600mL per jar. Each jar was approximated to fill 12 bioassay tubes with a volume of 50mL per tube. A total of fourteen jars were needed for each of the five different soil types and each jar was shaken

thoroughly to ensure uniform mixing of the inoculum throughout. The remaining Tomales Point soil and coarse sand were used for the controls. In a laminar-flow hood each of the bioassay tubes were stuffed with polyester up to an approximate height of 2-3 in., a label was placed on each tube, and the tubes were placed back into the autoclave bags. The polyester was used to prevent any soil added from falling through the drainage holes in the bottoms of the tubes, as well as allow for sufficient drainage while maintaining a moist environment.

All planting material and equipment was taken to the greenhouse where the planting process was carried out. The bioassay tubes were placed in sterilized bioassay racks and each filled with approximately 50mL of soil from the appropriate jar as indicated by the labels on the tubes. The tubes were then watered to moisten the soil and 2-3 pine seeds were distributed with clean forceps to each tube. Approximately 1 in. of sterilized sand was added on top of the seeds and the tubes were watered again. This process was repeated until all 800 tubes were planted with seeds. As a control for possible contamination, seeds were planted in bioassay tubes containing only autoclaved soil/sand mixtures. From the planting area of the greenhouse, each rack was covered and transported to the insectory area of the greenhouse where they were maintained and watered everyday with tap water.

Isolation of Rhizopogon Bioassay tubes were harvested 5-6 months after planting. *Rhizopogon* ectomycorrhizal root tips were identified under a dissecting scope following identifiable characteristics such as, cottony white surfaces, often with rusty covered areas, and thick white-branched rhizomorphs as described by Kjoller & Bruns (2003) and Agerer (1994). The *Rhizopogon* species collected from each bioassay tube were recorded and placed in a 2ml epindorf screw top tube containing 3 glass beads, snap frozen in liquid nitrogen, and stored in the –80°C freezer until they were freeze-dried. Freeze-dried root tips were crushed in a bead beater and DNA extraction was performed using the Dneasy Tissue Kit™ (QIAGEN Inc., Valencia, California). PCR amplification was carried out using the internal transcribed spacer (ITS) of the fungal ribosomal DNA with the primers ITS1-F and ITS4 as described by Gardes & Bruns (1993). A gel of the PCR product was run after each complete PCR cycle to ensure that the PCR was carried out successfully, and the PCR product was then restricted with the enzymes *Hinf-I* and *Hha-I*

(purchased from New England Biolabs. Inc., Beverly Massachusetts). RFLP patterns were compared with those previously archived in the lab by matching band patterns and all unique patterns were further sequenced. Sequencing was preformed following instructions from the BIGDYE v3.1 chemistry + ABI 3100 sequencing kit, as well as referring to protocol from White *et. al*. (1990). Sequencing data was done on an ABI Model 377 DNA sequencer (Perkin-Elmer Corporation) and data obtained was processed using DNA sequence analysis (version 2.01) and Sequence Navigator software. (* A list of all taxonomic identification of *Rhizopogon* species identified by sequencing of the ITS region of ribosomal DNA will be included in a table within this section of the paper).

Statistical Analysis Formal statistics have not yet been performed on the sequenced data but will be included upon completion. The *Rhizopogon* species present in the contaminated controls will be disregarded. Only *Rhizopogon* species that were not in the controls will be considered in the analysis. T-tests will be used to analyze differences in *Rhizopogon* colonization on the four different pine seedlings. Also, frequency of colonization for the different pine seedlings will be shown in a histogram.

Discussion

Controls were contaminated with (currently) unknown species of *Rhizopogon*. The reason for the contamination is unknown and difficult to determine because of the number of steps involved in the planting and growing processes. Preliminary RFLP analysis shows that there are four different species in the contaminated controls. All of these RFLP patterns seemingly look like *Rhizopogon* based on the small number of base pairs from the gels ran (the number of base pairs for most *Rhizopogon* species are between 500 and 800). When the contaminated controls were processed and ran on a gel, the resulting RFLP patterns turned out to be more complex than previously expected. It was expected that there would only be one individual *Rhizopogon* contaminating the controls, but instead there were four unique patterns present indicating four unique *Rhizopogon* species. These exceptions were surprising because from past experiments with bioassay contamination in greenhouse settings there was only one fungus involved (source from unpublished findings).

I cannot make any definite conclusions as to what *Rhizopogon* species are present without sequencing these patterns and comparing them to the database of described fungal species (Bruns *et. al*. 1998). The sequencing run last attempted was unsuccessful due to complications with equipment and therefore no sequencing data has been obtained. Since the fungal species that were in the controls will most likely be present in the other bioassays, I will to have to disregard those present in the controls across the whole experiment. I will then be left with *Rhizopogon* species unique to each bioassay tube containing the different pines. I expect to obtain statistically significant results when quantifying the differences and similarities of colonization data because from the gels that were run thus far, most of the fungal species seen have colonized all four of the pine seedlings. I also expect to see overall similarities of colonization throughout the different pine species with possible differences in Jeffery pine according to RFLP data. For example, it was expected that because of differences in lineage, sugar pine would show differences in colonization, but in this case Jeffery pine showed different RFLP patterns as compared to the others. When you look at one of the gels you see a straight line (same base pairs, around 500) then a different line level (different base pairs, around 800) in some areas and those areas are the wells containing Jeffrey pine.

With regard to frequency of colonization, Bishop pine has contained the highest amounts of colonization on root systems so far (across all soil types). It has proven once again to be the best pine to use in bioassay experiments because of its ability to capture *Rhizopogon* much more efficiently. It will not be established until later whether or not Bishop pine will capture similar sets of *Rhizopogon* and in return be able to replace the native pines of the Sierra Nevada used for experiments in that area. The unfortunate result in the contamination of my controls ultimately means that it will not be possible to make any concrete conclusions as to what is happening in the below ground soil community of the Eastern Sierra Nevada. But I will be able to determine differences and similarities of colonization on the different pines. Thus far, I anticipate that similar sets of Rhizopogon will be found on Bishop pine, Lodgepole pine, and Sugar pine, while Jeffrey pine may have differences from RFLP patterns seen.

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