Impacts of Heat on the Spore Bank Community Structure in Mixed Conifer Forest Soil Using *Pinus jeffreyi* Seedlings as a Host Species.

Megan Canright

Abstract The effect of temperature on the presence and community composition of ectomycorrhizal fungi associated with *Pinus jeffreyi* seedlings was investigated in this study. We followed the colonization of ectomycorrhizal fungi in 8 month old *Pinus jeffreyi* seedlings whose soil had been subjected to one of four treatments: no heat, 40°C heating, 55°C heating and 70°C heating. Ectomycorrhizas were harvested by hand under dissecting microscope from 8 month old seedlings and subjected to PCR/RFLP analysis of the ITS region of the ribosomal DNA for identification to the species or genus level. Significant difference in species richness was analyzed using Wilcoxon's rank-sum test. Species diversity was measured using the Shannon-Weaver index and tested for significant difference among treatments using t-tests. Species richness was significantly higher in heated treatments as compared to non-heated treatments, however no significant difference in species richness was found between seedlings heated at different temperatures. Species diversity was also significantly different between heated and non-heated treatments, but was not different across heated treatments. Preliminary results indicate that species composition experiences a shift across treatments. Results may show that dominant species include *Rhizopogon subcaerulescens* in non heat-treated seedlings, *Russula amoenolens* in 40°C seedlings, *Suillus brevipes* in 55°C seedlings and *Cenoccocum geophilium* and *Tomentella sublilicina* in 70°C treatments. *Thelephoraceae* sp. may be seen equally across all treatments. If preliminary results reflect actual results, species seen in heat treatments may be either the most heat-resistant ectomycorrhizae or are stimulated by heat and may play a role in the re-establishment of forest ecosystems directly following fire as other less heat-resistant mycorrhizae rebuild their communities. In short, the overall effect of increased temperature seems to be increased species richness and diversity accompanied by a shift in species composition from basidiomycetes to ascomycetes.

Introduction

Historically, fire has been a common disturbance in California pine stands, especially before the onset of modern fire prevention techniques (Biswell 1989). Studies of historical and pre-historical forest fire regimes have shown that moderate intensity fires typically afflicted pine stands every 3 to 11 years (Brown et al. 2001). Prescribed fire has been utilized in pine stands as a management technique for over 50 years in an attempt to encourage the success of the pine (Biswell 1989). Fire reduces tree density and prevents succession by more shade-tolerant species such as white fir, and protects against disease, pest infestation and stand replacing wildfire (Choromanska et al. 2001; Fule et al. 1997).

Moderate intensity prescribed burning has proven to be a useful management tool in *Pinus jeffreyi* (Jeffrey pine) stands for the prevention of bark beetle infestation. In addition, *Pinus jeffreyi* responds well to prescribed fire in terms of overall stand health; stand health and density of *P. jeffreyi* have been shown to increase with prescribed fire treatments (Roy & Vankat 1999).

Mycorrhizae commonly associate symbiotically with various *Pineaceae* species, including *Pinus jeffreyi*. Walker and Kane (1997) found that after 6 months, mycorrhizal Jeffrey pine seedlings had greater root dry weight, increased shoot dimensions and overall weight. Mycorrhizal hyphae penetrate soils, branching into fine thread-like extensions called hyphae, which are much finer than root hairs. The hyphae are very absorptive and are more efficient than plant roots in taking up nutrients. Mycorrhizae associate with pine roots, helping the tree to take up minerals, namely nitrogen, and nutrients in exchange for sugars produced by the pine during photosynthesis. The increased nitrogen nutrition due to mycorrhizae allows mycorrhizal plants to grow much more quickly than non-mycorrhizal plants (Varma and Hock 1999).

The role of mycorrhizal fungi in the re-establishment of pine forests following fire disturbance is unknown. Some research has been conducted looking at the affect of fire on mycorrhizal numbers and biomass. Stendell *et al*. (1999) found that one year following prescribed fire, ectomycorrhizal biomass was not significantly reduced in comparison to pre-burn quantities, indicating that mycorrhizae are not adversely affected by fire. Vazquez *et al.* (1993) found that hyphal length post-fire exceeded pre-burn values. However, little research has attempted to tackle the question of how mycorrhizal species composition is altered in response to fire.

The goal of this study is to take the first step towards this understanding by addressing the relationship between mycorrhizal community structure and soil temperature. A mixed conifer ecosystem with a historically naturally occurring fire regime was chosen to conduct this experiment because it can be assumed that organisms in this type of environment will respond to temperature disturbance due to the natural fire regime. Three main questions will be addressed by this study in the forest system: What does the spore bank look like for the soil taken from the site? Does temperature have a significant effect on the species composition of mycorrhizae in this soil? If so, which species are more resistant to heat disturbance or which species may be stimulated by heat?

The study was conducted using Jeffrey pine seedlings set up in bioassays as a host which mycorrhizae were expected to infect. Jeffrey pine was used because pine species are the dominant post-fire regeneration species and thus would be colonized more readily by mycorrhizae following temperature disturbance. Thus, Jeffrey pine gives a more accurate picture of the post-heat spore bank community as these species are more likely to colonize the dominant post-fire tree species. The seeds were planted in treated soil taken from the site. Analysis of these bioassays without heat treatment is expected to give a picture of the soil spore bank in Teakettle, the Sierran mixed conifer forest where the soil was collected. This information can be used to determine which species are out-competed in fire-disturbed settings. If species changes do occur with temperature treatments, dominant species found can be looked at and studied in order to reach further understanding of their roles in the re-establishment of healthy pine ecosystems following prescribed fire. This knowledge can be used to improve prescribed fire as a management tool in pine forests.

Materials and Methods

Description of the study site The study site was at 1980 m above sea level in north drainage of Kings River in the Sierra National Forest, California (36°52’15”N, 119°07’05”W). Teakettle experimental forest consists of 1300 hectares of old-growth-mixed-conifer and red fir (*Abies magnifica*) forest. Annual precipitation in Teakettle averages 110 cm and falls mostly as snow between November and April. Teakettle grades from a mix of white fir (*Abies concolor*), sugar pine (*Pinus lambertiana*), Jeffrey pine (*Pinus jeffreyi*), and incense cedar (*Calocedrus decurrens*) and red fir at lower elevations to red fir, lodgepole pine (*Pinus monticola*) and western white

pine (*Pinus contorta*) at higher elevations. Soils are generally Xerumbrepts and Xeropsamments typical of the southwestern slopes of the Sierra Nevada.

Soil Extraction Soils from Teakettle were extracted randomly from eight arbitrarily chosen white fir stands. Stands were selected for ecological and environmental similarity to ensure a more accurate comparison. Plots within the white fir stands were selected randomly and soil was removed by brushing away the litter layer and extracting 18 x 18 x 6 inch deep cubes of mineral layer soil. White fir stands were chosen because white fir is the dominant overstory in the parts of the forest being studied.

After collection, soils were filtered through a 0.35 mm sieve to remove debris and root material from the soil. The goal was to remove any living material to ensure that results seen after treatment were coming primarily from the spore bank in the soil. Soils were then pooled, mixed uniformly, and divided into five equal parts, each receiving a different treatment. Soils were treated as follows: sterilization (control), no heat treatment, and heated at 40°C, 55°C and 70°C. Controls were set up using the same soils that had been autoclaved for 30 minutes in a wet cycle at 250°C and 30 minutes in a dry cycle at 250°C.

Temperature treated soils were placed in metal pans to a depth of 1-2 cm to equalize heating throughout the soil. Soil was then heated in the metal pans in a 150°C oven until they reached their designated temperature. Temperature was monitored by a thermocouple placed in the middle of the soil. The quick increase in temperature from the oven (Figure 1) was used to simulate a typical temperature increase during an actual prescribed fire (Preisler *et al*. 2000). As dry soil cools very quickly, upon reaching their target temperature (40°C, 55°C, 70°C), soil pans were transferred to a 37°C oven to allow soil cooling rates that closely mimicked natural soil cooling after fire (Figure 1). This was done to avoid quick drops in soil temperature not characteristic of soil cooling patterns following natural fire. (Preisler *et al*. 2000) During heating, the treated soil was mixed every five minutes in attempt to avoid pocketing of uneven temperature.

Following treatment, soils were mixed 50/50 with sterilized sand to increase filtration and placed in 200 mL conical tubes. Bioassays were planted with a single *Pinus jeffreyi* (Jeffrey Pine). Seed was obtained from the Placerville forest outplanting station, which keeps large collection of Sierra seed. The seeds were surface sterilized with hydrogen peroxide for five minutes, soaked in running water for 2 days and air dried to reduce general saprophobic fungal

growth. The seeds were then refrigerated for 3 months to simulate the Sierran winter conditions needed for the pine to germinate (Flannigan and Woodward 1993).

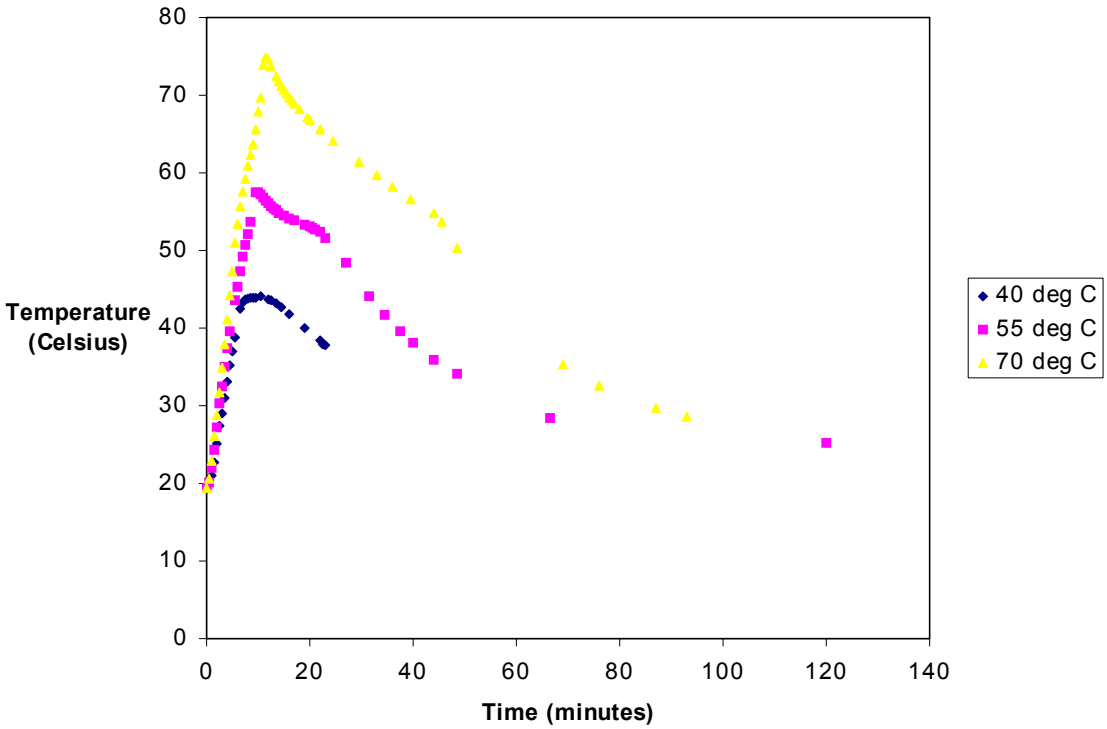


Figure 1. Soil Heating Rates as measured by thermocouples during heating process of temperature treated soil.

After seed planting, heat bioassays were monitored and seeds were left to grow for 8 months. Seedlings were grown in relatively low light conditions roughly equal to that of a forest with thick overstory based on both visual comparison and light meter data to Teakettle forest. Seedlings were watered twice a week and bioassays were rotated once a week to prevent uneven lighting.

Collecting of ectomycorrhizas After 8 months of growth, Jeffrey pine seedlings were harvested from the heat bioassays; seedling health and percent coverage by ectomycorrhizae was estimated by random sample of root tips and recorded. Root systems were observed under a dissecting microscope for the collection of mycorrhizal tips. All viable lateral short roots covered by a fungal mantle were classified as ectomycorrhizal. Viability of the root tips was based on color and turgidity (Harvey *et al.* 1976). Ectomycorrhizas were sorted into morphological types

based on color, size, and type of ramification as described in Agerer (1994). Representative samples from each morphotype were selected for PCR amplification. To increase the likelihood of detecting morphologically similar species, two tips from each morphotype were selected to represent the maximum species variation within that morphotype. Seedlings were sorted randomly across treatments to avoid bias and effect of time on data collection. Root tips were then lyophilized (freeze dried) for two days to maintain DNA structure and viability. They were then stored at room temperature until amplification.

Molecular Techniques & Fungal Identification DNA was extracted individually from one to two root tips as described by Gardes & Bruns (1993). Several tips were extracted together only when part of a single morphologically uniform clump.

The reagents, protocols, and cycling parameters used in Polymerase Chain Reaction (PCR) followed Gardes & Bruns (1993). Identifications of ectomycorrhizal fungal symbionts were based on PCR amplification of the ITS region of the 5.8S nuclear rRNA gene. Amplification was performed using ITS1-F and ITS-4 as primer pairs (White *et al.* 1990; Gardes & Bruns, 1993). Both primer pairs preferentially amplify specific fragments of fungal DNA from mixtures of plant (i.e. root) and fungal DNA. The ITS region was characterized by Restriction Fragment Length Polymerization (RFLP) analysis, using the enzymes *Hinf*I and *Alu*I, which was used to match ectomycorrhizas to one another (Table 1). This region was used because it has been shown to distinguish at roughly the species level (Karen *et. al.* 1997). Species-level identification was determined by identically matching DNA sequences to sequences of previously identified species or by identical RFLP pattern match.

Fungal family placement was conducted using the mitochondrial large subunit rRNA gene database (Bruns *et al.* 1998). PCR amplification was done with primer pairs ML-5 and ML-6. Determination of the fungal division was accomplished with a database of the 5.8S nuclear rRNA gene (Cullings & Vogler 1998). Sequencing was done with the same primer pair by the cyclic reaction termination method using florescence labeled dideoxyribonucleotide triphosphates. The processing of templates for sequencing was performed by following the instructions for the sequencing kit (PRISM Ready Reaction Dideoxy Terminator Cycle sequencing kit, Perkin-Elmer Corporation). Electrophoresis and data collection were done on an ABI Model 377 DNA sequencer (Perkin-Elmer Corporation). DNA sequencing analysis (version 2.01) and Sequence Navigator software were used to process raw data. Sequences were aligned

by visual estimation using a matrix created in PAUP 3.1.1 (Swofford 1993) and the heuristic search option. If no ITS-RFLP match was found for ectomycorrhizas identified to family, the fungus was labeled by its family with the ending replaced with –oid followed by a number for that group (i.e. Cenoccocoid 5).

Taxonomic identification	**AluI**	**HinfI**
Thelephoraceae sp.	548/195	344/230/195/124
R. subcaerulescens *	471/334/87	252/133/75
R. ochraceorubens *	453/345/106/87	355/254/164/140/77
Russula amoenolens *	335/253/191/90	321/252/205
A. gemmata *	405/254/106/93	354/236
Hebeloma sp.	340/257/214	398/356/145
Suillus brevipes *	671/97/83	284/238
C. geophilius *	550/440/200	260/180/120/90
Tomentella sublilicina *	458/111/86	366/230/195/124
Tomentella sp.	458/111/86	359/230/147/129
Tuber sp.	600	232/179/158
Wilcoxina mikolae *	645	295/193/167
Wilcoxina sp.	421/200	278/210/135
Cortinarius sp.	500/440	360/340

Table 1. Mycorrhizal fungal taxa on *P. jeffreyi* identified by PCR-RFLP with restriction enzymes *Alu*I and *Hinf*I. Numbers in columns for each restriction enzyme are the DNA fragment sizes (in base pairs) after the PCR product was cut with enzyme. Fungi were amplified with primers ITS1-F and ITS4. *Indicates taxa identified by nucleotide sequencing of ITS region of ribosomal DNA. All other taxa identified through RFLP matching.

Statistical Analysis Species Richness was calculated by counting the total number of species that occurred on each seedling within all treatments. The Wilcoxon rank-sum test (Zar 1996) was used to test for significant difference in species richness between treatments, as data was not normally distributed. Mycorrhizal species diversity in each treatment was measured using the Shannon/Weaver index (Zar 1996); t-tests were used to analyze differences (Zar 1996).

Results

In total, 256 individual ectomycorrhizae were collected and morphotyped. Of the 256 ectomycorrhizae processed by PCR-RFLP, 229 were successfully amplified (89%). RFLP patterns were obtained from 223 mycorrhizas (87%). Across the treatments, 25 RFLP-taxa were found.

The results of the Wilcoxon rank-sum test show that species richness in the non-treated seedlings was significantly lower than that of the heat-treated seedlings. Species richness was significantly higher in the 40°C treatment (p=.0001, df=3), 55°C treatment (p=.0001, df=3) and 70°C treatment (p=.0001, df=3) (Figure 2). However, none of the heated treatments were statistically significant from each other in terms of species richness.

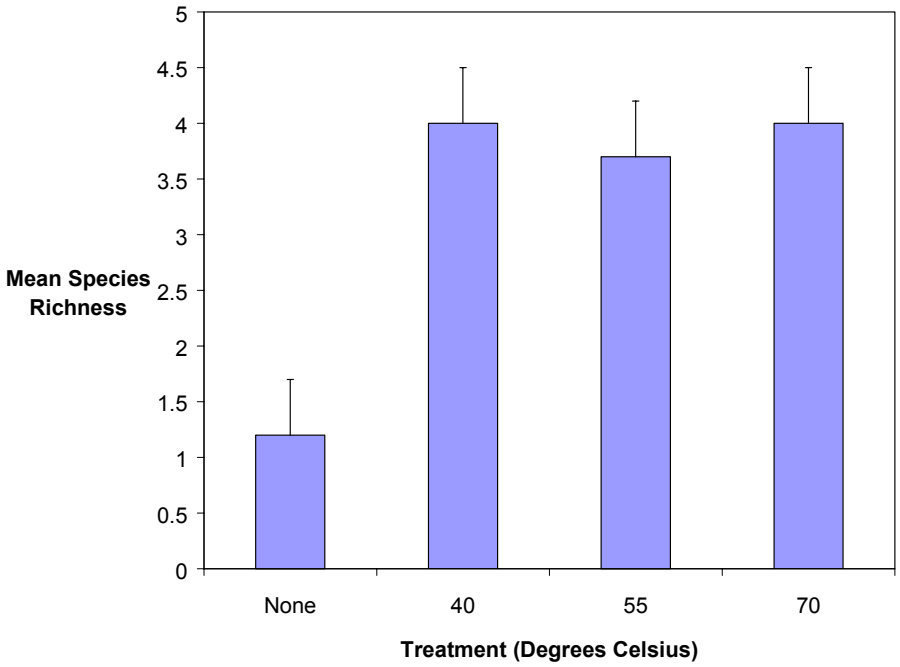
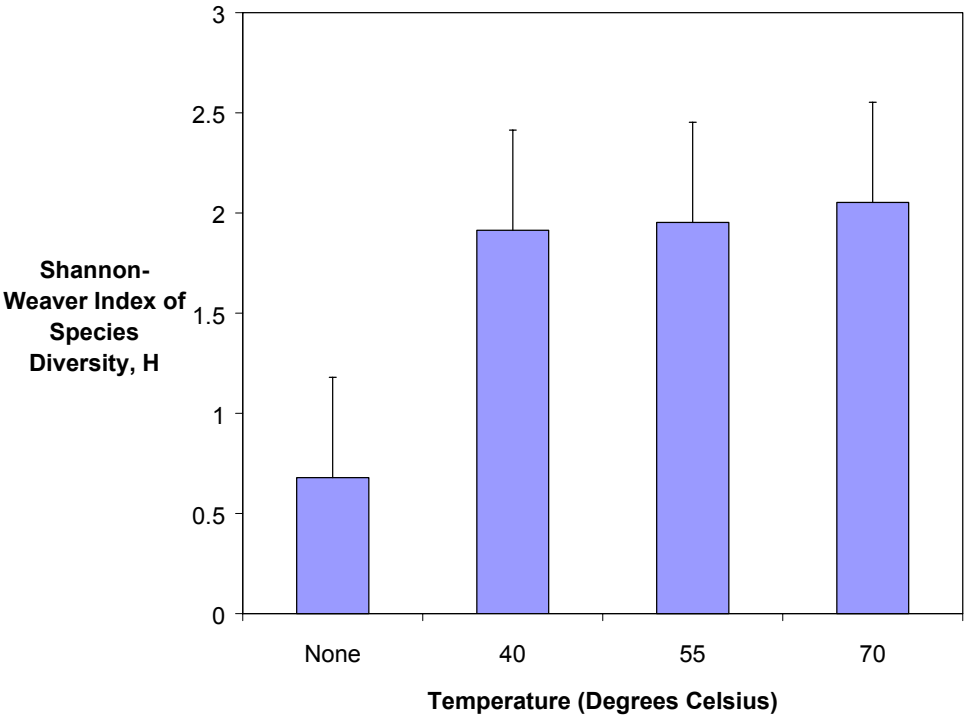


Figure 2. Ectomycorrhizal species richness for heat treatments. Data are the mean ±1 s.e.

The Shannon-Weaver index for species diversity was calculated and tested for significant difference between treatments using t-tests. It was found that species diversity was significantly lower in the non heat-treated seedlings. Species diversity was significantly different from non-heated seedlings in the 40°C seedlings ($t_{.001}$=3.7842, df=486), 55°C seedlings ($t_{.001}$=3.74766, df=416), and 70°C seedlings ($t_{.001}$=4.052055). Species diversity was not statistically significant between heated treatments (Figure 3).

Figure 3. Ectomycorrhizal species diversity using the Shannon-Weaver index.



Data are the calculated index, H, ± 1 s.e.

Sequence data was collected for all individual species identified by unique RFLP pattern. Multivariate statistics using principle components (Zar 1996) will be calculated in order to make statements about changes in mycorrhizal community structure with heat treatment. Preliminary results show that the most common species found within non-heated treatments may be *R. subcaerulescens*. Common ectomycorrhizal species which could be found on 40°C seedlings might include *A. gemmata*, *Russula amoenolens*, *R. ochraceorubens* and *Hebeloma* sp. 55°C treatments seem to be dominated by *Suillus* sp., *A. gemmata*, *R. ochraceorubens*, and *Hebeloma* sp. Finally, species that could occur most frequently in the 70°C seedlings might include *C. geophilius*, *Tomentella* sp., *Tuber* sp., *Tomentella sublilicina*, *W. mikolae*, *Wilcoxina* sp., and

Cortinarius sp. (Figure 4). Formal statistics have not yet been performed on these data, but the results of the principle components analysis will be reported upon completion.

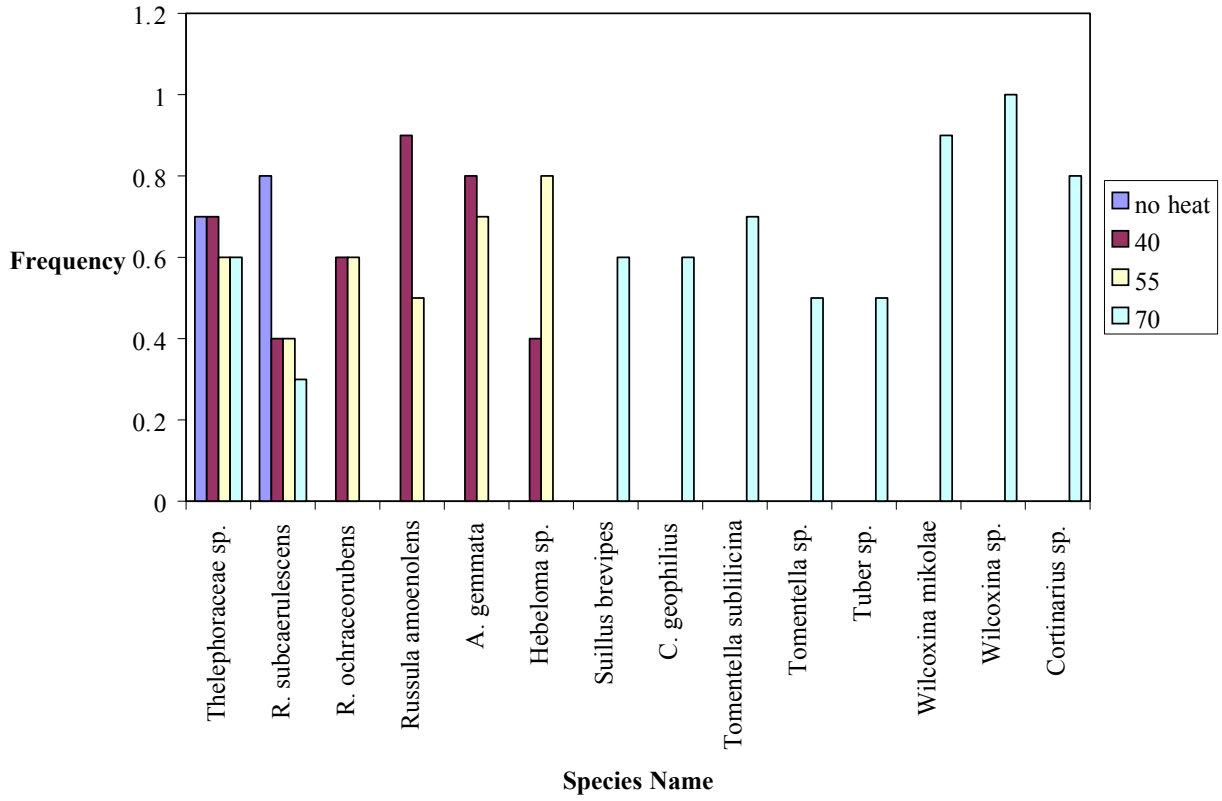


Figure 4 Composition of mycorrhizal taxa on P. jeffreyi seedlings in heat bioassays.

Discussion

These preliminary results show that a shift may be seen in ectomycorrhizal community structure from basidiomycetous ectomycorrhizae to ascomycetous ectomycorrhizae. This is consistent with the findings of (Torres & Honrubia, 1997). Those species prevalent in the temperature treatments are either resistant to heat or are stimulated by it. If resistant to heat, these species are most likely out-competed by more aggressive species in normal conditions. Yet when these species are killed off with temperature, the resistant species excel because they are not kept in check by competitors. On the contrary, these species may be stimulated by heat, and thus must have a mechanism that causes growth when heat is inputted. If this is the case, this mechanism will need further study in order to achieve understanding of its function. It is apparent that spores from these species lie dormant in the soil until stimulated by temperature.

These results are consistent with the results of Baar *et al.* (1999) and their conclusion that mycorrhizae colonize *Pinus muricata* seedlings from propagules following fire. *Thelephoraceae* sp. seems to be present throughout treatments, indicating that this species may be resistant to temperature change in the soil.

The results of this study indicate that temperature stimulates mycorrhizal richness and diversity and is an important aspect in the maintenance of the health and prominence of certain species. In conclusion, the study suggests that certain species of ectomycorrhizae are either stimulated by temperature or are resistant to it. This may also be indicative of the behavior of these species during low to moderate intensity wildfire. It seems that species richness is enhanced by temperature and in fact may also be enhanced following fire. Further studies should take on the role of ash in ectomycorrhizal community structure. The addition of ash as a variable will give a better picture of how these species might behave following natural fire conditions. Eventually, studies should take place in the field, using actual low intensity fire, to gain a picture of how the mycorrhizae behave under natural conditions.

References

Agerer, R. 1994. Characterization of ectomycorrhizae. Pp. 25-73 *In* Techniques for Mycorrhizal Research. Norris, J.R., Read, D & Varma, A.K., eds. Academic Press, London.

Baar, J., Horton, T.R., Kretzer, A.M., Bruns, T.D. 1999. Mycorrhizal Colonization of *Pinus muricata* from resistant propagules following stand-replacing wildfire. New Phytology 143: 409-418.

Biswell, Harold. 1989. Prescribed Burning in California Wildlands Vegetation Management. University of California Press, Berkeley. 345 pp.

Brown, Peter M.; Kaye, Margot W.; Huckaby, Laurie S.; Baisan, Christopher H. 2001. Fire history along environmental gradients in the Sacramento Mountains, New Mexico: Influences of local patterns and regional processes. Ecoscience. 8 (1): 115-126.

Bruns, T.D., Szaro, T. M., Gardes, M., Cullings, K.W., Pan, J.J., Taylor, D.L., Horton, T.R., Kretzer, K., Garbelotto, M & Li, Y. 1998. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Molecular Ecology 7: 257-272.

Choromanska, U.; DeLuca, T. H. 2001. Prescribed fire alters the impact of wildfire on soil biochemical properties in a ponderosa pine forest. Soil Science Society of America Journal. 65 (1): 232-238.

Cullings, K.W. & Vogler, D.R. 1998. A 5.8S nuclear ribosomal RNA gene sequence database: applications to ecology and evolution. Molecular Ecology 7: 919-923.

Flannigan, M.D., Woodward, F.I. 1993. A laboratory study of the effect of temperature on red pine seed germination. Forest Ecology & Management. 62(1-4): 145-156.

Fule, Peter Z.; Covington, W. Wallace; Moore, Margaret M. 1997. Determining reference conditions for ecosystem management of southwestern ponderosa pine forests. Ecological Applications. 7 (3): 895-908.

Gardes, M. & Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Harvey, A.E., Larsen, M.J., & Jurgensen, M.F. 1976. Distribution of ectomycorrhizae in a mature Douglas-fir/larch forest soil in Western Montana. Forest Science 22: 393-398

Karen, O., Hogberg, N., Dahlberg, A., Jonsson, L., & Nylund, J. 1997. Inter- and Intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytology 136: 313-325.

Preisler, H.K., Haase, S.M., & Sackett, S.S. 2000. Modeling and risk assessment for soil temperatures beneath prescribed forest fires. Environmental and Ecological Statistics 7: 239-254.

Roy, D, Gramn; Vankat, John L. 1999. Reversal of human-induced vegetation changes in Sequoia National Park, California. Canadian Journal of Forest Research. 29 (4): 399-412.

Stendell, Eric R.; Horton, Thomas R.; Bruns, Thomas D. 1999. Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest. Mycological Research. 103 (10): 1353-1359.

Swofford, D.L. 1993. PAUP: Phylogenetic analysis using parsimony, version 3.1. Illinois Natural History Survey: Champaign, Illinois. 102 pp.

Torres, P., Honrubia, M. 1997. Changes and effects of natural fire on ectomycorrhizal innoculum potential of soil in a *Pinus halepensis* forest. Forest Ecology and Management. 96: 189-196.

Varma, A. and Hock, B. 1999. Mycorrhiza: Structure, Function, Molecular Biology, and Biotechnology. Springer, New York.

Vazquez, Francisco J.; Acea, Maria J.; Carballas, Tarsy. 1993. Soil microbial populations after wildfire. FEMS (Federation of European Microbiological Societies) Microbiology Ecology. 13 (2): 93-104.

Walker, R. F.; Kane, L.M. 1997. Containerized Jeffrey Pine growth and nutrient uptake in response to mycorrhizal inoculation and controlled release fertilization. Western Journal of Applied Forestry. 12 (2): 33-40.

White, T.J., Bruns, T.D., Lee, S. & Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322 *In* PCR protocols: a Guide to Methods and Applications. M.A. Innis, D.H. Gelfand, J.J. Sninsky, & J.J. White, eds. Academic Press, London.

Zar, Jerrold H. 1996. Biostatistical Analysis. Prentice Hall, Inc., New Jersey. 920 pp.