# An Investigation into Mycorrhizal Associations amongst American Plum (*Prunus americana*) and Chokecherry (*Prunus virginiana*) populations on Boulder County Open Space Restoration Sites

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## Abstract

Mycorrhizal fungi form a symbiotic association with living plants, assisting the plant with drought resistance and nutrient uptake via expansive networks of hyphae. Research has consistently demonstrated that the presence of mycorrhizal fungi is associated with a higher stress tolerance in plants. This study is an investigation into mycorrhizal associations found amongst populations of two herbaceous shrubs native to Boulder County - *Prunus virginiana* (Chokecherry) and *Prunus americana* (American Plum). The purpose of the study was to analyze the relative presence and abundance of mycorrhizal fungi in both wild and introduced populations of both *Prunus* species. This comparison intends to discern if there exists a deficit in fungal partnerships in plant communities introduced by restoration efforts. The study did not produce data indicating a reliably higher or lower concentration of mycorrhizal fungi in wild plant communities at the study sites, however it did demonstrate a consistently higher level of adaptability to localized environmental conditions in soil microbial populations amongst wild plant communities than of those in planted areas. Significant field observations were also made regarding the possibility of an ectomycorrhizal association between a native species of *Entoloma* fungus and *Prunus virginiana,* an observation which should be the subject of further study. This investigation serves as a starting point for understanding the communities of soil microflora associated with *Prunus* species introduced in a riparian restoration setting on Boulder County Open Space properties.

## 1. Introduction

Understanding the community dynamics of soil microflora is of significant importance to ecosystem restoration efforts. Within these diverse communities, one of the important categories of soil microorganisms for plant establishment and healthy plant growth is mycorrhizal fungi. Mycorrhizal fungi assist plant roots with nutrient and water uptake, as well as provide increased drought resistance through moisture retention within fungal networks (van der Heijden et al., 2015). The presence of mycorrhizal partnerships with host plants are associated with an increase in whole plant hydration, as evidenced by relative leaf water content (Barros et al., 2018). This is of particular importance for plantings in a restoration setting, where existing soils may have a poor composition due to a history of ecological disturbance. Poor soil conditions found in restoration areas may present lower moisture retention capacities and therefore lead to higher levels of stress in introduced plantings, especially during months with low natural precipitation. In the presence of difficult environmental conditions, plants initiate a direct correspondence with mycorrhizal fungi to help mitigate the negative effects of environmental stressors. Upon the perception of drought stress, most plants immediately send a signal to their associated mycorrhizal fungi by secreting a class of phytohormone called a “strigolactone” which asks the mycorrhizae for help (Oldoroyd et al., 2013). In one Chinese study, researchers demonstrated that seedlings of *Pinus sylvestris* which were inoculated by the ectomycorrhizal fungus *Suillus*

*luteus* had greater growth in water stressed conditions than non-inoculated seedlings, and that colonization of seedling roots by the fungus also improved rhizosphere soil-enzymatic activity and rhizosphere soil nutrition (Yin et al., 2018). When conducting site planning for restoration projects which involve plantings of native species, it is important to consider not only what species of plants are being introduced for the project, but also the existing soil conditions into which those plants are expected to establish and grow. If biological treatments such as mycorrhizal fungi exist to improve plant stress tolerance, they may serve as an asset to a project when poor soil conditions are present. When assessing soil health of a particular restoration site prior to planting efforts, conducting both a nutritional and biological assessment, such as by using a PLFA test, can help provide the most complete set of information to inform which treatment methods lead to the best rate of plant establishment and growth.

This investigation is motivated by various observations from restoration efforts which have taken place on Boulder County Open Space land. Firstly, according to open space staff, amongst prior restoration efforts, on multiple sites across multiple years, the two species of *Prunus* which are the focus of this study have historically shown poor performance when intentionally introduced to an ecosystem as container plants. While these plants have been introduced into areas to which they would naturally exist, and with native stands on the same sites, the introduced plantings have demonstrated slow establishment and growth rate and a higher mortality than expected from a native species. For this reason, it is of interest to understand if there are deficiencies in the soil which may negatively impact the growth of the plants. One such deficiency could be related to a reduced population of available mycorrhizal fungi, which could present a challenge for plant establishment in restoration areas where soil quality may be poor due to past ecological disturbance due to excavation, grading work, and compaction from heavy equipment.

A second observation which has motivated this study is a prior effort demonstrating the successful colonization of *Pinus ponderosa* seedlings by a slurry of the ectomycorrhizal fungus *Suillus kaibabensis,* produced by Boulder Mushroom for Boulder County Parks and Open Space. This is a native species of fruiting fungus which forms an ectomycorrhizal association with Ponderosa Pine trees. In October 2022, open space staff attempted an experimental inoculation of approximately 500 Ponderosa pine seedlings to be planted into the burn scar of the 2020 Calwood Fire as part of a postfire reforestation effort at the Heil Ranch property. Each of the 500 seedlings was watered once with a liquid slurry of *S. kaibabensis* a few weeks prior to being planted in the field. By the time the seedlings were brought to the site for planting, most of the saplings had attained full colonization of the root system by the fungus. The roots had taken on a distinctly fuzzy texture and yellow coloration which was clearly indicative of the sheath-like growth of ectomycorrhizal fungi. These observations together created the motivation to research mycorrhizal associations amongst *Prunus* species in hopes that this could generate an opportunity to utilize mycorrhizal inoculation to improve planting success in a restoration setting.

**Fig 1.1 - *P. ponderosus* roots with ectomycorrhizal *S. kaibabensis* mycelium.**

## 2. Methods

### 2.1 Site Selection

Three Parks and Open Space properties where past stream restoration projects have been completed were selected as sampling locations for the investigation based off of the following criteria:

- Location contains a population of both species of *Prunus* planted by BCPOS in riparian restoration projects which serve as primary habitat for species of interest.
- Location contains a population of both species of *Prunus* naturally occurring and growing in abundance.
- Naturally occurring populations are healthy and do not show a sign of significant disease or rot.

**Site #1: South Saint Vrain Creek Restoration Area at Hall Ranch Property (Map 2.1)**
Restoration project completion year: 2017
Sampling Date: June 30, 2023


**Map 2.1 -** South St. Vrain Creek Sampling Area at Hall Ranch Property

**Site # 2: Longmont Supply Ditch Restoration Area at Western Mobile Property (Map 2.2)**

Restoration project completion year: 2020
Sampling Dates: June 30, 2023 and October 17, 2023


**Map 2.2 -** Longmont Supply Sampling Area at Western Mobile Property

**Site # 3: Bielins-Hock Restoration Area at Bielins-Hock Property (Map 2.3)**
Restoration project completion year: 2017
Sampling Dates: June 30, 2023 and October 11, 2023


**Map 2.3** - Bielins-Hock Sampling Area at Bielins-Hock Property

## 2.2 Sampling Methods

The soil samples were collected from within 12” of the root crown of each plant. To collect the sample, one shovelful of soil was unearthed, and the surface vegetation was removed. The remaining soil was placed into a plastic bag. Three soil samples were generated from each site. For each sampling area, one soil sample was collected from under the base of one naturally occurring Chokecherry, and one soil sample from under one naturally occurring American Plum to produce 2 total samples from naturally occurring plants. Additionally, one amalgamated sample was created for the planted specimens by mixing together samples from three Chokecherry and three American Plum plants planted in BCPOS restoration projects. In future

research, separating planted samples by species can provide more granularity of data for each plant species.

### 2.3 PLFA Soil Microbial Analysis

Soil samples were submitted to Ward Laboratories (4007 Cherry Ave. Kearney, NE 68848) to perform a third-party analysis of the microbial composition of each sample. A phospholipid fatty acid (PLFA) analysis was conducted for each sample to produce the microbial diversity results. The PLFA layer is a major structural constituent of biological membranes, and the fatty acids components vary among taxa (Lewe et al., 2021). PLFA analysis utilizes biomarkers of known soil microorganisms to generate valuations of relative abundance of bacteria, fungi, and protozoa through microbial biomass measurements. The test is effective at indicating the relative abundance of these microbial classes within a sample, however each report also contains an 'undifferentiated' organism category, creating a degree of ambiguity which is accounted for in the interpretation of final results.

### 2.4 Fungal Culturing and Isolation

Fungal cultures were generated on petri dishes filled with malt extract agar nutritive media (MEA) from tissue samples from above ground mushroom fruiting bodies collected in well-established chokecherry stands along Coal Creek in the OSMP system as a part of the 2023 macrofungal survey. Fruiting bodies which exhibited growth patterns indicating high probability of mycorrhizal association were used for tissue culture. The mushroom fruiting bodies collected from the area were repeatedly observed growing extremely close to the bases of healthy chokecherry shrubs, and on numerous occasions, growing directly from the root crown of the plants. Fungal cultures were also generated from soil samples from each site. 5 grams of soil were added into 500mL of nutrient broth and left to incubate for 72 hours at room temperature. Subsequent to incubation, the soil broth mixture was thoroughly agitated and a small quantity was applied to the MEA. The petri dishes were then incubated at room temperature for microbial activity to be exhibited on the MEA petri plates.

## 3. Results

### 3.1 PLFA Soil Microbial Analysis Results

Numerous data points were collected using the PLFA soil microbial analysis performed by Ward Laboratories. For each of the three sites, a full PLFA report was generated for 1) the amalgamated sample of planted specimens, 2) one wild plum, and 3) one wild chokecherry. These reports create a microbial profile for each sample. The first important result to analyze is

the total fungal biomass (TFB), which is further split into 2 categories: arbuscular mycorrhizal fungi (AMF) and saprophytic fungi (SF) (Fig 3.1). Saprophyte fungi are responsible for the enzymatic digestion of organic matter and feed on various forms of biomass. AMF form a symbiotic partnership with living plant root systems and can assist stress tolerance and nutrient uptake (van der Heijden et al., 2015). For the analysis of these results, it is important to note that the PLFA test does not provide a specific reading for the presence of ectomycorrhizal fungi. While arbuscular mycorrhizae play an important role in ecosystem function, ectomycorrhizal associations are of particular importance with respect to forest ecosystem health, and for this reason should be considered as a topic for investigation in further research. Of the nine samples submitted, the sample with the highest TFB reading was WC3, the wild chokecherry from Beilins-Hock property. WC3 tested 985.82 ng/g of TFB, of which 216.27 ng/g is AMF and 769.54 ng/g is SF. At Beilins-Hock the amalgamated planted sample 3P was not far behind, with 943.98 ng/g TFB, with 257.59 ng/g AMF and 686.39 ng/g SF. For the other two sites, total fungal biomass was notably higher in the amalgamated planted samples than either of the two wild samples. There is a possibility that if certain fungal species in the sample are species specific to either chokecherry or wild plum, creating an amalgamated sample of those two species together could produce a higher TFB reading than when testing them by individual species. However, if this was the case on would assume a higher TMB reading for wild specimens, which was not the case (Fig 3.2).

| Sample ID | Total Fungal Biomass (ng/g) | Arbuscular Mycorrhizal Fungi (ng/g) | Saprophytic Fungi (ng/g) |
|---|---|---|---|
| **Planted 1 - Hall Ranch** | 713.56 | 195.61 | 517.96 |
| **Wild Plum 1 - Hall Ranch** | 431.84 | 100.29 | 331.56 |
| **Wild Chokecherry 1 - Hall Ranch** | 460.99 | 96.43 | 364.56 |
| **Planted 2 - Western Mobile** | 827.43 | 152.29 | 675.14 |
| **Wild Plum 2 - Western Mobile** | 683.67 | 159.46 | 524.2 |
| **Wild Chokecherry 2 - Western Mobile** | 668.61 | 168.18 | 500.44 |
| **Planted 3 - Bielins-Hock** | 943.98 | 257.59 | 686.39 |
| **Wild Plum 3 - Bielins-Hock** | 783.15 | 218.22 | 564.92 |
| **Wild Chokecherry 3 - Bielins Hock** | 985.82 | 216.27 | 769.54 |

**Table 3.1**: Total Fungal Biomass (ng/g)

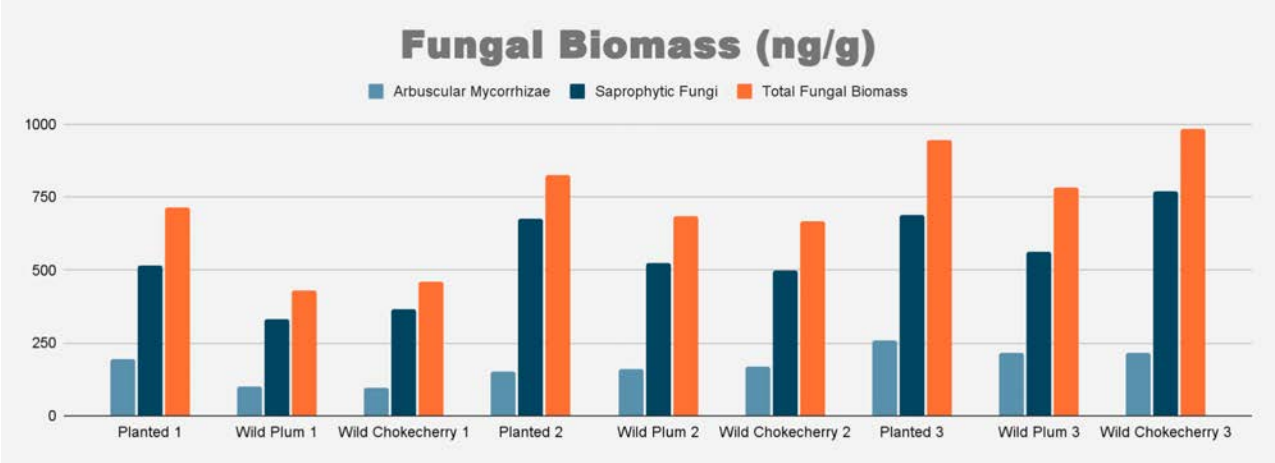


**Fig 3.1:** Total Fungal Biomass (ng/g)

Total microbial biomass (TMB) was also measured, subdivided into total fungal biomass (TFB) and total bacterial biomass (TBB). The highest TMB reading also came from WC3, at 4296.88 ng/g. At the Longmont Supply restoration site, the wild American plum produced a TMB value significantly higher than the other two samples at 3744.85 ng/g. This sample also produced a noticeably high TBB value of 683.67 ng/g. At the Hall property, the highest TMB value was 2968.67 ng/g, which came from the amalgamated planted sample. (Fig 3.2). The TMB did not present any significant trends between planted and wild stands.

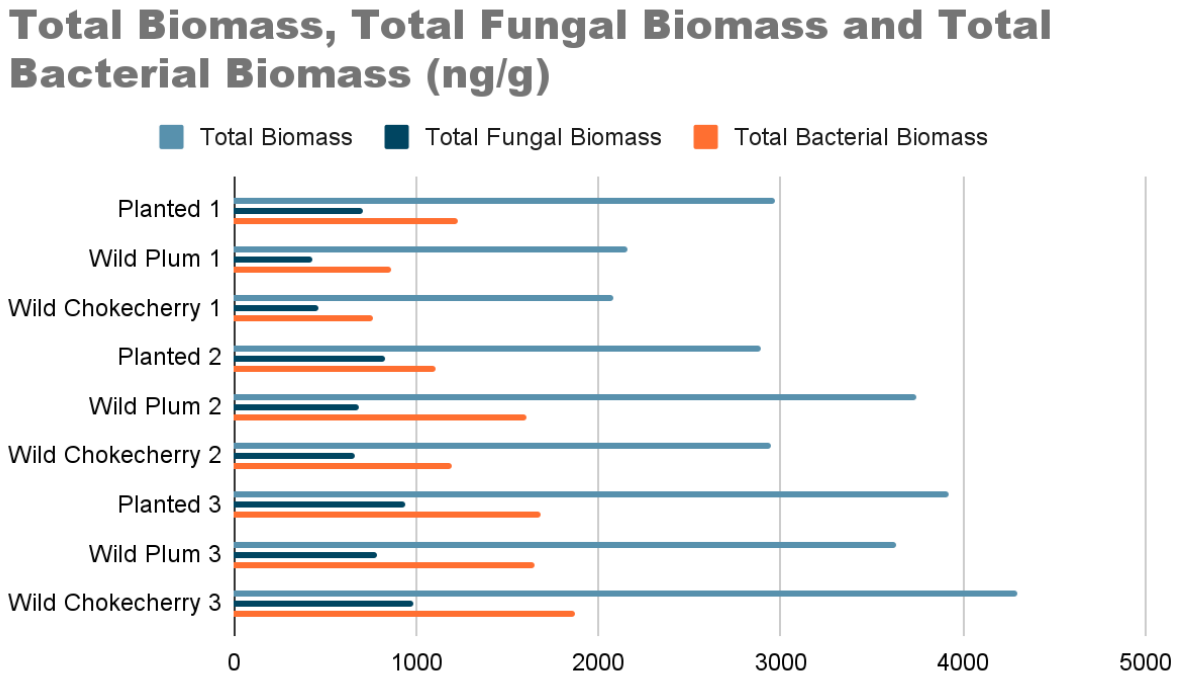


**Fig 3.2:** Total Microbial Biomass (ng/g)

Finally, the PLFA report generated the ratio of saturated fatty acids to unsaturated fatty acids (SFA:UFA) in the sample. Saturated fatty acids may indicate a microbial community better adapted to its environment, whereas unsaturated fatty acids are indicators of a community under stressed conditions. Therefore, a higher SFA:UFA ratio is generally a sign of a healthier microbial population. This is the only data point within the PLFA report which reported a consistently higher value for both wild specimens than for the amalgamated planted sample (Fig 3.3).There is no trend across age of sites, with Bielins-Hock and Hall Ranch being completed the same year and presenting both the highest and lowest ratios.

| **Sample ID** | **Saturated Fatty Acids : Unsaturated Fatty Acids** |
|---|---|
| Planted 1 | 0.8937 |
| Wild Plum 1 | 1.1388 |
| Wild Chokecherry 1 | 1.1321 |
| Planted 2 | 0.8345 |
| Wild Plum 2 | 1.0442 |
| Wild Chokecherry 2 | 0.9788 |
| Planted 3 | 0.8528 |
| Wild Plum 3 | 0.9426 |
| Wild Chokecherry 3 | 0.938 |

**Table 3.3**: Saturated to Unsaturated Fatty Acids Ratio

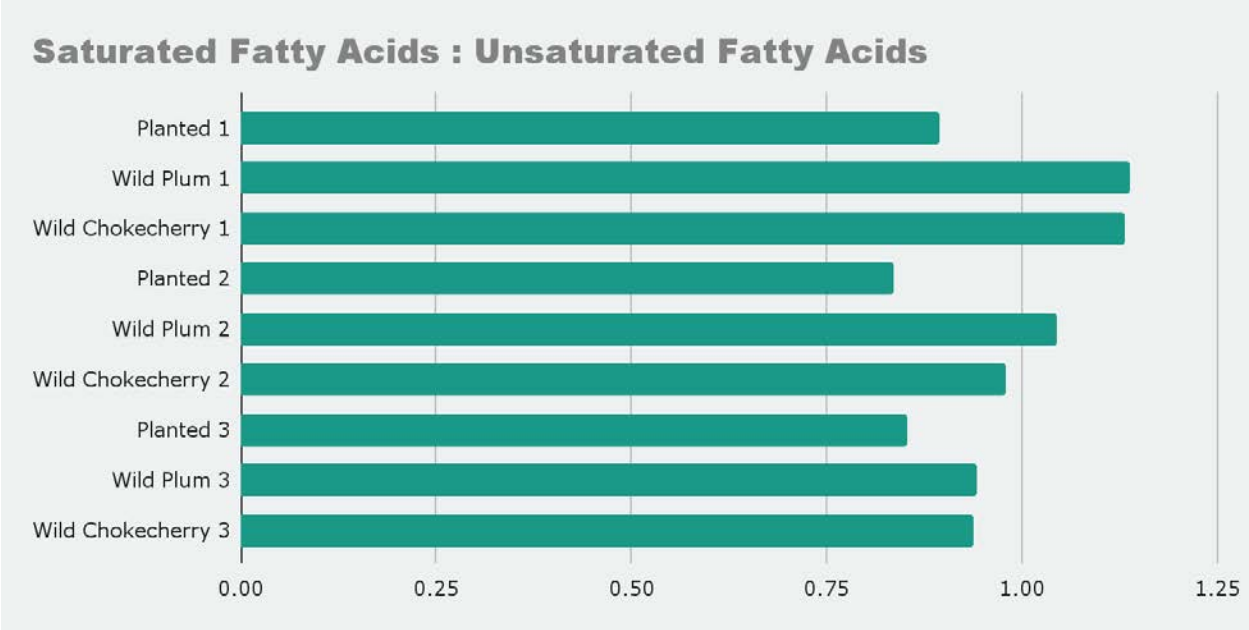


**Fig 3.4:** Saturated:Unsaturated Fatty Acid Ratio

## 3.4 Fungal Culturing and Isolation Results

The most significant result generated by the use of MEA to culture and propagate wild mycelia was produced by creating tissue cultures from a group of fruiting bodies identified as belonging to the *Entoloma lividoalbum* group (Fig 3.4). These fruiting bodies were collected on OSMP property in the upper Coal Creek riparian area, a landscape dominated by miles of prolific and well-established wild chokecherry stands. While generally considered saprophytic, the genus *Entoloma* is also known to occasionally form ectomycorrhizal relationships, often with plants in the family Rosaceae. According to Arora, in Europe several species of *Entoloma* are known to associate exclusively with trees and shrubs in the Rose family (Arora, 1986). In the Rocky Mountains, *Dryas octopetala* is an alpine shrub in the family Rosaceae which forms an ectomycorrhizal relationship with *Entoloma alpicola* (Cripps et al., 2005). The mushroom fruiting bodies collected from the upper Coal Creek area were repeatedly observed growing extremely close to the bases of healthy chokecherry shrubs, and on numerous occasions, growing directly from the root crown of the plants (Fig 3.5, 3.6). In one instance, digging into the soil exposed the base of the mushrooms wrapped around the roots of the plant and mycelium was visible growing on the surface of the root itself (Fig 3.7). This observation and behavior of *E. lividoalbum* is not well documented and creates an opportunity for further research into the possibility of its mycorrhizal association with *P. virginiana* in Colorado.

Growing cultures directly from the soil samples by utilizing a nutrient broth did not successfully yield any notable results in this investigation. When the soil/nutrient broth mixture was added to MEA for culturing, the resulting plates produced a significant diversity of growth which was very difficult to isolate. Various bacteria and molds were observed after transferring the slurry onto MEA, however any mycelium potentially indicative of a mycorrhizal relationship with sampled plants was not easily separated from the other organisms present. One potential solution to this issue for future efforts could involve the use of an antibiotic media, which would limit the effects of bacterial competition and provide a higher likelihood of promoting mycorrhizal fungi within the petri dish cultures.

**Fig 3.4, 3.5:** *Entoloma lividoalbum* fruiting bodies growing directly from the root crown of *Prunus virginiana* at upper Coal Creek - above ground view. (Photo: Zach Hedstrom)

**Fig 3.6:** *Entoloma lividoalbum* fruiting bodies growing directly from root crown of *Prunus virginiana* at upper Coal Creek - below ground view. (Photo: Zach Hedstrom)

**Fig. 3.7** - MEA tissue culture of *Entoloma lividoalbum* in process of isolation. Visible is pinkish white mycelia of *E. lividoalbum*, alongside green Trichoderma mold and unidentified bacteria. (Photo: Zach Hedstrom)

## 4. Discussion and Next Steps

This investigation has served as a starting point for understanding the biological soil profiles and possible mycorrhizal associations found amongst *P. virginiana* and *P. americana* in the front range riparian zones of Boulder County. A primary study objective for this research was to lend insight into the existing soil conditions amongst completed restoration projects where these two plant species were introduced, and to provide guidance for the success of future plantings with respect to soil treatments which could be useful in restoration efforts. The PLFA biological soil analysis for the three study sites has provided data which, by and large, does not demonstrate verifiable trends in any particular data category except for the saturated : unsaturated fatty acid ratio. In this regard, PLFA testing verified that wild plant communities are consistently associated with a higher SFA:UFA ratio, indicating a more adequately adapted microbial population to the localized environmental conditions. No verifiable correlation between mycorrhizal fungi and wild versus established plantings were verified by a PLFA analysis of the soil samples by TMB or TFB. AMF:SF ratios were variable, though SF was higher than AMF across all samples. A larger sample size for data collection would help discern if any statistically significant trends exist.

Another important point of further research is related to the presence and concentration of ectomycorrhizal fungi in established populations of these *Prunus* species. Due to the fact that ectomycorrhizal fungi can be site specific and have significant effects on plant performance and stress tolerance, further research could help understand where ectomycorrhizal associations may occur within our region and how to replicate these relationships in restoration efforts. The observation at upper Coal Creek of *Entoloma lividoalbum* associating closely with chokecherries in an area where chokecherry is extremely abundant and vigorous could be a significant discovery. No observations of *Entoloma* fruiting bodies were observed amongst sampled plants from the three study sites, however it is possible that they could have been present at a time when samples were not being collected.

The data and observations made during this study can help guide further research efforts in order to gain further clarification and build on data gathered from this research. An investigation into the possible ectomycorrhizal association between *Entoloma lividoalbum* and *Prunus* species in the Colorado front range using the mycelial cultures generated from the fruiting bodies collected from Coal Creek is a recommended next step of this research. This can be attained by generating a liquid slurry and treating *P. virginiana* and *P. americana* plants with the slurry in a nursery setting. Conducting an analysis of the plant performance side-by-side with a control, and analyzing roots of treated plants for indications of an ectomycorrhizal association taking place can help further an understanding if these species are ectomycorrhizal.

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