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Scorched Earth: *Suillus* colonization of *Pinus albicaulis* seedlings planted in wildfire-impacted soil affects seedling biomass, foliar nutrient content, and isotope signatures

Martha L. Jenkins · Cathy L. Cripps · Leslie Gains-Germain

Abstract

Aims In western North America ectomycorrhizal fungi are critical to establishment of conifers in low nitrogen soils. Fire can affect both ectomycorrhizal fungi and soil properties, and inoculation with ectomycorrhizal fungi is recommended when planting on burns for restoration. The aim of this study was to examine how *Suillus* species used in inoculation affect whitebark pine (*Pinus albicaulis* L.) seedlings planted in fire-impacted soil.

Methods In a greenhouse experiment, *Suillus*-colonized and uncolonized whitebark pine seedlings were planted in unsterilized and sterilized (control) soil from a recent burn. After 6 months, foliar nitrogen and carbon content, concentration, and stable isotope values were assessed, along with growth parameters.

Results When seedlings were colonized, biomass was 61% greater, foliar nitrogen content 25% higher, foliar nitrogen concentration 30–63% lower; needles had lower $\delta^{15}N$ and higher $\delta^{13}C$. Differences were more pronounced in sterilized soil where colonization was higher. Foliar N content was negatively correlated with $\delta^{15}N$ values.

Conclusions Colonization by host-specific fungi produced larger seedlings with higher foliar nitrogen content in both burn soils. The hypothesis that ectomycorrhizal fungi on roots fractionate nitrogen isotopes leading to lower $\delta^{15}N$ in needles is supported. This helps explain restoration outcomes, and bridges the gap between field and in vitro investigations.

Introduction

Ectomycorrhizal fungi are important mutualists that cover the fine roots of woody plants and enhance nutrient uptake in their hosts (Smith and Read 2008). In western North America these beneficial fungi are critical in the establishment and survival of conifers in low nitrogen soils (Mayor et al. 2015; Hawkins et al. 2015). Fire is an important feature of western conifer forests that impacts both ectomycorrhizal fungi and soil properties (Neary et al. 1999; Certini 2005; Cairney and Bastias 2007; Taudière et al. 2017). Fire can wipe out or alter ectomycorrhizal communities (Stendell et al. 1999; Grogan et al. 2000; Dahlberg et al. 2001; Treseder et al. 2004; Smith et al. 2005) and decrease fungal propagules in the soil (Bruns et al. 2002b; Izzo et al. 2006; Peay et al. 2009; Jones et al. 2010; Kipfer et al. 2010; Glassman et al. 2016). Impacts are strongly dependent on fire intensity, burn severity, and forest type, with hotter fires and repeated burning being the most destructive to microbial soil communities (Dahlberg et al. 2001; Cairney and Bastias 2007).

Fire impacts on ectomycorrhizal communities can persist for years (Treseder et al. 2004; Taudière et al. 2017), yet seedlings depend on ectomycorrhizal fungi for establishment (Ortega et al. 2004; Quoreshi et al. 2009). These beneficial fungi enhance nutrient uptake and access forms of nutrients otherwise unavailable to woody plants (Chalot and Brun 1998; Landeweert et al. 2001; Hobbie and Högberg 2012) which is of particular importance on burns. Fire also directly impacts soil properties by reducing organic matter, changing pH, and altering nutrient availability by releasing ammonium, retarding nitrogen mineralization, and increasing nitrate leaching (Covington and Sackett 1992; Certini 2005). This is why it is recommended that seedlings be inoculated with ectomycorrhizal fungi prior to being planted on severe burns for restoration purposes (Wiensczyk et al. 2002; Keane et al. 2012). However, there is little information on how ectomycorrhizal fungi actually affect seedlings on burns beyond simple monitoring for survival. Greenhouse experiments can tell us more about how ectomycorrhizal fungi affect the growth and physiology of conifer seedlings in fire-impacted soil and findings can be useful for understanding what happens on burn sites during restoration.

Few studies have examined ectomycorrhizal fungi in burned soil in the greenhouse, and most were bioassays used to detect viable spores as assessed by their ability to form mycorrhizae on pines (Baar et al. 1999; Kjøller and Bruns 2003; Buscardo et al. 2010; Kipfer et al. 2010; Glassman et al. 2016). To test spore viability after fire, greenhouse bioassays have simulated the effects of wildfire by using 'heat-treated' field soil (Izzo et al. 2006; Peay et al. 2009; Kipfer et al. 2010), adding ashes to field soil (Peay et al. 2009), blasting soil in a furnace (Sousa et al. 2011), and using blocks of soil cut from a burn (Vásquez-Gassibe et al. 2016); other studies have used soil from a burn diluted with sand, peat or vermiculite to improve drainage and to increase the probability of picking up less common fungi (Baar et al. 1999; Taylor and Bruns 1999; Kjøller and Bruns 2003). Most of these 'burn' bioassays examined *Pinus-Suillus/Rhizopogon* systems; these fungi are particularly important to pines because they have some level of host specificity, occur with both young and mature trees, and are involved in nitrogen uptake (Bruns et al. 2002a). There is evidence that hotter fires eliminate the propagules of these fungi, and indeed results from biossays and field studies suggest that *Suillus* species may be particularly sensitive to fire as mycelium or spores (Bruns et al. 2002b; Jones et al. 2010; Trusty and Cripps 2011; Glassman et al. 2016). However, the physiological effects of these ectomycorrhizal fungi on seedlings in burned soil have largely been ignored in bioassay studies.

Field studies have examined survival and growth of pines inoculated with *Suillus* species on burns; greenhouse studies have examined their effects in unburned soil (Heumader 1992; Colpaert et al. 1996; Hobbie and Colpaert 2003; Hobbie et al. 2008; Verma et al. 2014). However, we only found two studies that examined how *Suillus* species affect seedlings in burned soil in the greenhouse. Both used *P. pinaster* as the host because it is a Mediterranean species strongly impacted by wildfire and it is of interest in restoration (Sousa et al. 2011; Vásquez-Gassibe et al. 2016).

The physiological processes underlying enhanced growth from ectomycorrhizal colonization such as those that involve nutrient uptake, nutrient flow within seedlings, and transfer of compounds between organisms have not been well examined in the greenhouse. *Suillus* species are particularly involved in the uptake of nitrogen and make good candidates for this kind of study (Keller 1996; Nygren et al. 2008; Cripps and Antibus 2011; Antibus et al. 2018). *Suillus* mycorrhization has been shown to affect various measurements of nitrogen in needles or shoots of pine seedlings in the nursery and greenhouse (Heumader 1992; Colpaert et al. 1996; Kohzu et al. 2000; Rincón et al. 2007). However, results vary and are dependent on the level and form (ammonia or nitrate) of nitrogen in the soil (Hobbie and Colpaert 2003; Hobbie et al. 2008).

Stable isotope signatures of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) have been used to examine mycorrhizal systems in several ways: to suggest ecological status of macro-fungi (Hobbie et al. 2012), to trace nutrient flow in mature trees in the field (Dawson et al. 2002), and to examine how fungi access nitrogen sources unavailable to plants (Hobbie and Högberg 2012). Of interest here is that ectomycorrhizal fungi have been hypothesized to alter foliar $\delta^{15}N$ in host plants through isotopic fractionation against ^{15}N (Kohzu et al. 2000; Hobbie and Colpaert 2003; Hobbie et al. 2008). In the assimilation of nitrogen, mycorrhizal fungi preferentially retain ^{15}N and pass compounds containing fractionated nitrogen (with proportionately more ^{14}N) on to the host; therefore, seedlings colonized by mycorrhizal fungi should have foliage lower in ^{15}N in contrast to uncolonized seedlings, although only a few studies have

confirmed this under controlled conditions (Kohzu et al. 2000; Hobbie and Colpaert 2003; Hobbie et al. 2008), and none have been done in burned soil.

Foliar $\delta^{13}C$ values in plants also have been correlated with ectomycorrhizal status but the implications of this relationship are less clear. In one of the few studies to examine this in the greenhouse, foliar $\delta^{13}C$ of *P. sylvestris* seedlings colonized by *S. luteus* was higher in comparison to uncolonized seedlings at low but not high nitrogen concentrations under controlled conditions (Hobbie and Colpaert 2004). It was hypothesized that because ectomycorrhizal fungi create a carbon sink and stimulate photosynthesis in colonized host plants (Dosskey et al. 1990), and that their presence would increase foliar $\delta^{13}C$ (Hobbie and Colpaert 2004). Plant water use efficiency (WUE) is often correlated with foliar ^{13}C‰, but nursery plants are typically watered to saturation and factors such as the concentration of available nitrogen can also affect WUE (Farquhar et al. 1989; Hobbie and Colpaert 2004).

Currently thousands of whitebark pine (*Pinus albicaulis*) seedlings are being planted in areas where they have been devastated by mountain pine beetles and white pine blister rust (Tomback et al. 2001; Schwandt et al. 2010). Many are being planted on burns as there is some evidence that this and inoculation with *Suillus* species can enhance the survival of these difficult to establish pines (Cripps et al. 2014; Lonergan et al. 2014; Asebrook and Hintz 2015). Wildfire can reduce the diversity of fungi on roots of whitebark pine seedlings regenerating on burns (Trusty and Cripps 2011), and in areas where whitebark pine fails to recover after fire, inoculation of seedlings with *Suillus* species prior to out-planting is recommended (Keane et al. 2012).

In 2013, a large intense fire burned 6,000 acres in southwest Montana; currently, thousands of *P. albicaulis* seedlings, some colonized by *Suillus* species, are being planted and monitored on this burn site (Jenkins 2017). For this parallel greenhouse study, soil was collected from the burn site and a bioassay set up to determine if viable ectomycorrhizal fungi were present as assessed by their ability to form mycorrhizae on *P. albicaulis* seedlings. Results for uncolonized seedlings planted in unsterilized (test) and sterilized soil (control) were compared. The greenhouse experiment also examined how colonization by *Suillus* species affects *P. albicaulis* seedlings planted in soil from a severe burn in terms of seedling growth parameters, foliar nitrogen and carbon concentration, and stable isotope values; correlations of nitrogen and carbon isotope signatures with other parameters were also analyzed. We hypothesized that *Suillus* colonization of roots would increase nitrogen uptake as assessed by foliar nitrogen content, and that nitrogen isotope signatures in needles would be lower because of fungal fractionation. This greenhouse study is useful for examining the mycorrhizal process in burned soil (hereafter referred to as "burn soil" for this study) under semi-controlled conditions, for explaining field results, and for ultimately facilitating development of restoration strategies. It bridges the gap between field and in vitro culture studies.

Materials and methods

Burn site and soil collection

The Gravelly Range in southwestern Montana (Beaverhead-Deerlodge National Forest) is characterized by high valleys and steep mountain slopes, at elevations of 1,600 to 3,200 m with treeline ca. 2,900 m (Cooper et al. 1997). The average annual precipitation is 46 cm (NRCS 2017) and the soil is a gravelly loam derived from limestone, sandstone, and shale (Soil Survey Staff 2017). In 2013, the Eureka Basin fire burned over 2430 ha (6,000 acres) in the Gravelly Mountains, and 44% of the resulting burn was classified as moderate to highly severe. Much of the burned area was previously in mixed whitebark pine (*Pinus albicaulis*) and spruce-fir (*Picea engelmannii-Abies lasiocarpa*) that contained a significant component of dead beetle-killed whitebark pine (GYCC 2011). In 2015, a 107-acre planting unit (44°50′39" N, 111°53′19" W, elevation 2774 m) was designated as part of a 10-year plan for *P. albicaulis* restoration. Two years after the fire only scorched standing dead and minimal understory regeneration were observed on the burn site (fire data was provided by the Beaverhead-Deerlodge National Forest).

In June 2015, approximately 75 l of soil were collected from the burn along two 50 m transects representative of the planting unit. For each transect, 3–4 l of soil were collected every 5 m to a depth of 15–20 cm. Larger material was sifted out, and smaller root material retained to serve as a potential source of fungal inoculum. Samples were mixed and a subsample sent to Agvise Laboratories for elemental analysis. The burn soil had a pH of 5.9,

a cation exchange capacity (CEC) of 18.9 meq, organic matter content of 11.8%, phosphate level of 14 ppm, nitrate-N availability of 12 ppm, and a texture of coarse loam.

To improve drainage while maintaining pH and nitrogen levels, the burn soil was diluted 50:50 with a mixure of vermiculite, peat, sand, and loam (6:5:2:2) in an electric barrel mixer. This soil mix had a pH of 5.7, a phosphorus level of 13.3 ppm, and a nitrate availability of 15.2 ppm. Available nitrate in the 50:50 mixture of burn soil was estimated to be 13.6 ppm, calculated by averaging available nitrate (ppm) by soil proportions. Half of the diluted burn soil was sterilized in an autoclave on a 60-min solid materials cycle to kill any native ectomycorrhizal that might be present for the control treatment.

Seedlings and soil treatments

The one-and-a-half year old *Pinus albicaulis* seedlings obtained from the Coeur d'Alene Forest Service Nursery in Idaho were grown from seed collected in the same seed zone (Greater Yellowstone/Grand Tetons, GYGT) that includes the Gravelly Mountains (Mahalovich et al. 2006). At the Idaho nursery, seedlings are grown in Ray Leach SC10 Super Cone-tainers (3.8 cm diameter, 21 cm depth; Stuewe & Sons, Inc., Tangent, Oregon) in a medium of ground Canadian sphagnum peat moss and composted Douglas-fir bark (7:3) and seedlings are maintained under standard nursery practices (N. Robertson personal communication to M. Jenkins 2017). Seedlings were then vernalized for 5 months. At the time of this experiment the Coeur d'Alene Forest Service Nursery typically planted two stratified seeds per container to ensure germination, and consequently each container held two seedlings.

Seedling units (containers) were separated into colonized and uncolonized (<1% abundance) groups, after visual inspection for ectomycorrhizae on roots, and transplanted into D40 Large Deepots (6.4 cm diameter, 25.4 cm depth; Stuewe & Sons, Inc., Tangent, Oregon); half of each treatment group (colonized or not) was planted in the non-sterilized burn soil mix and half in the sterilized burn soil mix. In total, 82 seedling container pairs (164 seedlings) were used in the experiment: 43 (17 uncolonized, 26 colonized) were planted in the sterilized soil and 39 (19 uncolonized, 20 colonized) in the unsterilized burn soil mix. Seedlings were grown for approximately 6 months in the greenhouse under standard conditions. Seedlings were watered to saturation three times a week and no fertilizer was applied.

Identification of ectomycorrhizae

The single morphotype initially observed to be present on seedlings appeared to be a 'suilloid' type, characterized by a dingy white color, "hand like" to coralloid branching, white rhizomorphs, a plectenchymatous mantle, and absence of hyphal clamps (Treu 1990; Agerer 1997; Cripps and Grimme 2011; Lonergan and Cripps 2013). To confirm this and to further identify the fungi present, a total of 18 ectomycorrhizal root tips were sampled for direct sequencing of the internal transcribed spacer (ITS) region of the fungal ribosomal DNA (Menkis et al. 2005). Individual mycorrhizae were removed with sterilized fine tweezers, rinsed free of soil with deionized water, and placed directly in 1.5 mL screw-cap microcentrifuge tubes with 2–3 glass beads. Samples were disrupted in a MiniBeadbeaterTM (BioSpec Products, Inc.) for two 30-s cycles on the lowest speed. DNA was extracted following the Quick-Start Protocol for the DNeasy® Plant Mini Kit (Qiagen) with the single exception of adding 50 μl of Buffer AE for the final elution instead of the recommended 100 μl in order to increase the final DNA concentration.

PCR amplification of the ITS region was conducted using the fungal specific primers ITS1-F and ITS4 (IDT®; White et al. 1990; Gardes and Bruns 1993) and RedTaq® ReadyMixTM (Sigma-Aldrich Co.). PCR tubes were placed in a Thermocycler for amplification of the ITS region (Barge et al. 2016) and purification of PCR products was performed using a QIAquick® PCR Purification Kit (Qiagen). Manufacturer's instructions were followed utilizing 30 μl of molecular grade water for the final elution to increase the concentration of DNA which was measured using a NanoDropTM 2000 spectrophotometer (Thermo Scientific) for each sample.

For each sample, forward and reverse primers were used to make separate reaction mixtures, each with a total volume of 13 μl at a concentration of 100 ng/1000 bp DNA and 0.8 pmol/μl primer, and mixtures were submitted to the DNA Sequencing Facility at the University of California, Berkeley (http://mcb.berkeley.edu/barker/dnaseq/home). The resulting raw forward

and reverse sequences were aligned and edited using SeqTrace v. 0.9.0 (Stucky 2012), and compared to sequences in GenBank for best species matches using NCBI Standard Nucleotide BLAST (Basic Local Alignment Search Tool) (blast.ncbi.nlm.nih.gov).

Assessment of new root colonization in burn soil

After 6 months, the new roots growing into the two burn soil treatments (sterilized or not) were assessed for ectomycorrhizal colonization. Roots of the two seedlings in each container were assessed as a unit. The complete root system was washed of soil, and new root growth (easily distinguishable) was clipped from root-bound core roots. New roots growing into the burn soil were assessed for ectomycorrhizal colonization under a dissecting microscope (Nikon SMZ 1500, Meridian Instrument Company, Inc., Kent, Washington) (Brundrett et al. 1996; Lonergan and Cripps 2013). Frequency was assessed as the percentage of seedlings colonized in each treatment; a root system was considered 'colonized' if >1% of the new root growth was colonized (Marx and Cordell 1988). Abundance was assessed as the percentage of new roots colonized determined by visual estimates done with a dissecting scope. Importance values represent the overall significance of ectomycorrhizal colonization and were calculated by summing frequency and abundance percentages (Horton and Bruns 2001). Results were averaged for seedlings in each treatment.

Seedling growth parameters

Height and stem diameter were measured just before destructive harvesting, and were recorded for each seedling in the container. Height was measured from the cotyledon scar to the tip of the terminal bud (Haase 2008). Stem diameter was measured with a digital caliper just below the cotyledon scar (Mexal and Landis 1990; Haase 2008). Total seedling biomass was measured per container by combining results for the two seedlings in each container. Shoots were separated from root systems at the point of root differentiation and needles were removed from stems; new roots, core roots, needles, and stems were dried separately at 49 °C until stable dry weights were recorded. Total shoot biomass represents the combined dry weight of needles and stems. Total root biomass represents the combined weight of new and core root systems.

Foliar nutrient and isotope assessment

Dried needles of the two shoots (per container) were combined and subsamples were assessed for foliar nutrient content and $\delta^{15}N$ and $\delta^{13}C$ i. Approximately 0.1 g of dried needles from each container was placed in a 1.5 ml screw cap microcentrifuge tube with 3 glass beads and ground to a powder by placing sample tubes in a tissue homogenizer for two 30 s cycles. Powdered samples were weighed to 5–6 mg in 5 × 9 mm tin capsules (Costech Analytical Technologies, Inc., Valencia, California). Samples were processed at the University of California at Berkeley Center for Stable Isotope Biogeochemistry (https://nature.berkeley.edu/stableisotopelab/) using a CHNOS Elemental Analyzer linked to an IsoPrime100 mass spectrometer which yields measurements of %N, $\delta^{15}N$, %C, and $\delta^{13}C$. Total container foliar nitrogen and carbon content were calculated by multiplying foliar %N or %C results by total foliar dry weight (Landis et al. 2005).

Natural abundances of isotopes in a substance are measured as a ratio relative to an internationally designated standard. Units of measurement are in parts per mil (‰) because deviations from the standard are so small. For example, $\delta^{15}N$ is calculated using the following equation: $\delta^{15}N = (R_{sample} / R_{standard} - 1) \times 1000‰$, where R_{sample} represents the ratio of the heavier to lighter isotope, $^{15}N/^{14}N$ in this case, in the substance being sampled and $R_{standard}$ represents the ratio of the heavier to lighter isotope in the standard (Dawson et al. 2002; Hobbie and Högberg 2012). The standard for N is atmospheric N_2 (3.6765×10^{-3}; Dawson et al. 2002). For carbon, $^{13}C/^{12}C$, the standard has been designated by the International Atomic Energy Agency (IAEA) as "Vienna"-PDB (1.1237×10^{-2}; Dawson et al. 2002). A positive value of δ indicates that the sample has more of the heavier isotope and is enriched in ^{15}N and ^{13}C relative to the standard; and a negative value indicates that the sample is depleted in ^{15}N and ^{13}C relative to the standard.

Experimental design

This was an observational study due to the non-random application of the 'treatment' termed ectomycorrhizal colonization, i.e. seedlings from the Coeur d'Alene nursery were visually separated by initial conditions into uncolonized and colonized groups. Therefore, the

experimental design consists of two colonization groups (colonized or not) × two soil treatments (sterilized and unsterilized burn soil mixtures). Soil treatments were randomly applied in the planting of seedlings and seedling trays were rotated monthly in the greenhouse. Containers were considered independent after accounting for soil treatment and initial colonization.

Statistical analysis

For the analyses of biomass, foliar nutrient content, and isotope values, measurements are per container (two seedlings combined). For the analyses of height and stem diameter, the greater measurement of the two seedlings was used for each container. Statistical analyses were performed utilizing *R* 3.1.2 statistical software (R Core Team 2014). A few seedling pairs ($n = 8$) initially designated as uncolonized exhibited some colonization at the final mycorrhizal assessment likely because initial colonization was minimal and overlooked. This occurred randomly across sterilized and unsterilized soil and these seedlings were excluded from statistical analyses.

Analyses for the response variables of seedling height, stem diameter, biomass, shoot:root ratio, foliar C:N ratio, foliar %N, and foliar %C were performed by fitting standard regression models rather than by using analysis of variance (ANOVA) for the purpose of estimating specific contrasts of interest. The regression model for each growth response variable (biomass, height, and stem diameter) included initial colonization and soil treatment as the two main effects as well as a term for interaction between the two: $y_{ijk} = \beta_i + \eta_j + \gamma_{ij} + \epsilon_{ijk}$, where β_i represents the effect of initial colonization, ηj represents the effect of soil treatment, γ_{ij} represents the interaction between initial colonization and soil treatment, and ϵ_{ijk} represents the random error associated with each container. Box plots, which provide exploratory information on the spread of the data, and 95% confidence interval plots, which provide a visual representation of statistical significance, were utilized for data exploration (Krzywinski and Altman 2014).

For the analysis of foliar nitrogen content and foliar δ^{15}N, boxplots and scatterplots were used to explore the relationship between the two responses. To account for this relationship, a full linear model was fit with an additional model term to estimate the correlation between two responses on the same container. A linear mixed effects model was considered with a random effect for the "type of response" variable, but this option was too restrictive as it only allows for a positive relationship between the two responses. Foliar nitrogen content and foliar δ^{15}N exhibit a negative relationship, so the more flexible generalized least squares approach was used to estimate model parameters. The *gls()* function in the *nlme R* package (Pinheiro et al. 2017) was used to fit the model. The full model initially considered included an induced correlation structure between responses and a three-way interaction between the two main effects (initial colonization and soil treatment) and response type. After fitting the full model there was no evidence of a three-way interaction between burn soil treatment, colonization status, and type of response ($p = 0.44$). A second reduced model showed no evidence of an interaction between burn soil treatment and type of response ($p = 0.37$) or between burn soil treatment and colonization status ($p = 0.39$). A third reduced model did show strong evidence of an interaction between colonization status and type of response ($p = <0.001$) as indicated by the exploratory plots. A simplified final model was fit and is shown below.

$$y_i = \beta_0 + \beta_1{}^* I_{colB} + \beta_2{}^* I_{soil2} + \beta_3{}^* I_{N15} + \beta_4{}^* I_{colB} I_{N15} + b_{i[j]} + \epsilon_{i,}$$

where $I_{colB} = 1$ if container i is colonized and 0 if not; $I_{soil2} = 1$ if container i contains unsterilized soiland 0 if it contains sterilized burn soil; and $I_{N15} = 1$ if the response is foliar δ^{15}N, and 0 if the response is total nitrogen. $b_{i[j]}$ indicates the jth container corresponding to observation i. This term allows two responses measured on each container (nitrogen content and δ^{15}N) to be correlated.

The analysis of foliar carbon content and foliar δ^{13}C was similarly explored. After fitting the full model there was evidence of a three-way interaction between burn soil treatment, original colonization status, and type of response ($p = 0.016$). Therefore, the full model was fit and is shown below.

$$y_i = \beta_0 + \beta_1{}^* I_{colB} + \beta_2{}^* I_{soil2} + \beta_3{}^* I_{C13} + \beta_4{}^* I_{colB} I_{C13} + \beta_5{}^* I_{soil2} I_{C13} + \beta_6{}^* I_{colB} I_{soil2} I_{C13} + b_{i[j]} + \epsilon_i$$

where the terms are defined as in the nitrogen model.

Prior to fitting the model, the responses of foliar nitrogen content and δ^{15}N were centered and rescaled.

They were put on a standardized scale in order to account for the difference in units of measurement as the spread of both response variables are included in the estimation of residual standard deviation. Estimates for contrasts of interest were interpreted for total nitrogen content and foliar $\delta^{15}N$ after converting the estimates back to original units. To further analyze the relationship between foliar nitrogen content and $\delta^{15}N$, a multiple linear regression model was utilized, in which the correlation coefficient (r) between the two responses was calculated along with slope and a p-value for each treatment combination to describe the direction and strength of the linear relationship between the two responses. The same process was used for the analysis of foliar carbon content and $\delta^{13}C$.

In the analysis of seedling parameters, differences were estimated between colonized and uncolonized seedlings. If there was a significant interaction between soil treatment and colonization, estimates were made within each treatment (sterilized and unsterilized soil mixtures). If there was no significant interaction, seedling parameters were pooled across treatments.

Results

Identification of ectomycorrhizae

Of the 18 ectomycorrhizal root tips sampled from seedling root systems for direct sequencing of the internal transcribed spacer (ITS) region, amplification was successful in nine, and all matched with species in the genus *Suillus*; at 99% identity four matched *S. americanus* (Peck) Snell) (=*S. sibiricus* (Singer) Singer) and five matched *Suillus discolor* (A.H. Sm, Thiers & O.K. Miller) N.H. Nguyen. Over 99.9% of all ectomycorrhizae observed on colonized seedlings in this study were morphologically similar to the *Suillus* type. No other types were detected either morphologically or molecularly at the beginning or end of the experiment.

Ectomycorrhizal colonization of new roots in burn soil

The objective of the bioassay was to determine if viable ectomycorrhizal fungi could be detected in the burn soil. For this, uncolonized seedlings planted in unsterilized soil were examined for ectomycorrhizae after six months in the greenhouse; no ectomycorrhizal fungi considered to originate from the burn soil were detected on roots. Similarly, no additional nursery fungi were detected on seedlings planted in the sterilized soil.

At the final mycorrhizal assessment, the *Suillus* species on core root systems of originally colonized *Pinus albicaulis* seedlings had grown onto new roots in both sterilized and unsterilized soil, indicating that the experimental burn soil mix was conducive to the growth of these *Suillus* species. For seedlings originally designated as colonized and planted in sterilized burn soil mix, almost three-quarters (73% frequency) of the new root systems were colonized by *Suillus* species, and on average over a quarter of each root system (abundance of 28%) was covered with ectomycorrhizae. For seedlings originally designated as colonized and planted in the unsterilized burn soil mixture, 40% (frequency) of seedlings were colonized and on average 12% of each new root system (abundance) was ectomycorrhizal. Therefore, the importance value (% abundance + % occurrence) for new root colonization was twice as high for originally colonized seedlings when they were planted in sterilized burn soil (101%) rather than unsterilized burn soil (52%).

Seedling growth parameters

The total biomass of colonized seedlings planted in sterilized burn soil was 61% greater than that of uncolonized seedlings ($p = <0.001$) (Fig. 1, Table 1). In unsterilized burn soil, colonized seedlings had 27% greater biomass than uncolonized seedlings ($p = 0.007$). A similar pattern resulted when shoot biomass and root biomass were analyzed separately (Fig. 1, Table 1). There was no difference in the shoot:total root biomass ratio between colonized and uncolonized seedlings ($p = 0.98$); thus, roots and shoots increased proportionally with colonization. The average total shoot:root ratio for all treatments was 0.71, an approximate ratio of 1:1.4. The biomass of new roots produced on colonized seedlings was 20% greater than that of uncolonized seedlings when differences were averaged across soil treatments ($p = 0.008$) (Fig. 1, Table 1).

The height of colonized seedlings planted in sterilized burn soil was 37% greater than that of uncolonized seedlings ($p = <0.001$). The average stem diameter of colonized seedlings planted in sterilized burn soil was 12% greater than that of uncolonized seedlings ($p = 0.013$). In unsterilized burn soil, there was no significant difference in height ($p = 0.33$) or stem diameter ($p = 0.84$) between colonized and uncolonized seedlings;

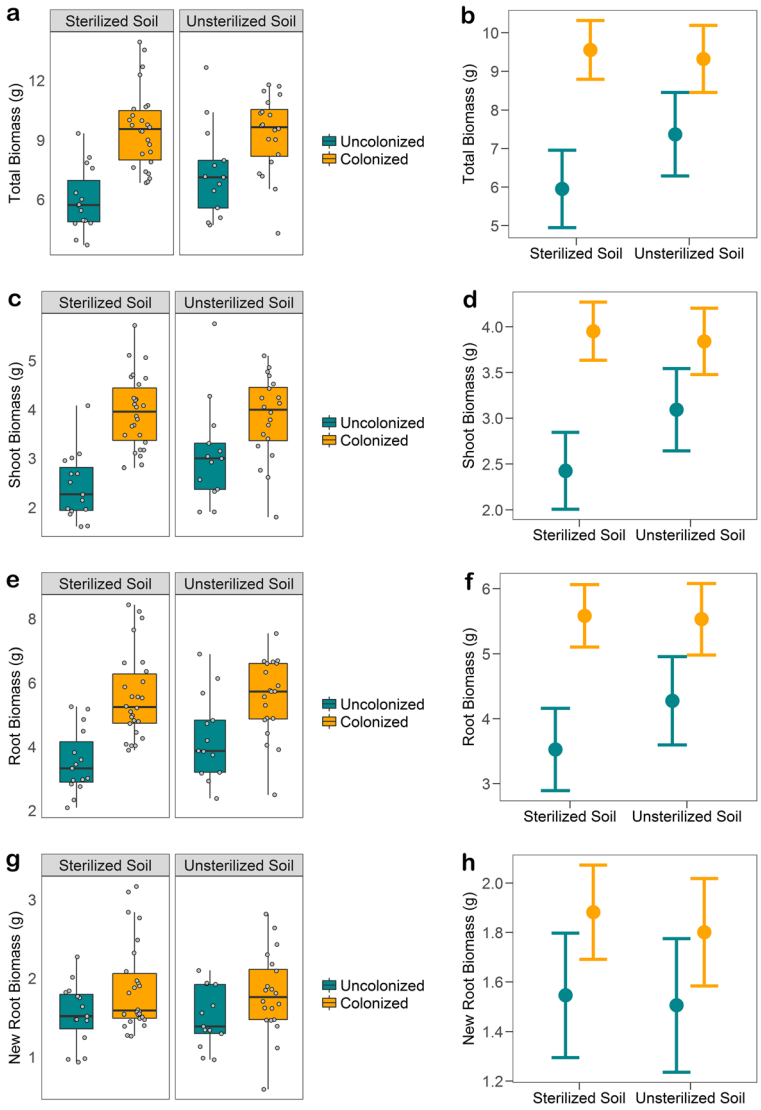

Fig. 1 Boxplots and 95% confidence interval plots for seedlings colonized or not by *Suillus* ectomycorrhizae and planted in sterilized or unsterilized soil for total biomass (**a**,**b**), shoot biomass (**c**,**d**), root biomass (**e**,**f**), and new root biomass (**g**,**h**)

although colonized seedlings tended to be slightly taller on average (Table 1).

Foliar nutrient and isotope analyses

The average foliar nitrogen concentration of colonized seedlings was 18% less than that of uncolonized seedlings (p = <0.001) in sterilized burn soil, and 8% less in unsterilized burn soil ($p = 0.044$). There was no difference in average carbon concentration between colonized and uncolonized seedlings when differences were averaged across soil treatments ($p = 0.104$) (Table 2).

In terms of nutrient content (concentration × biomass), needles of colonized seedlings on average contained 26% more foliar nitrogen than those of uncolonized seedlings (p = <0.001) (Fig. 2, Table 2); there was no interaction between soil treatment and original colonization status, and the colonization effect estimate was averaged across soil treatments. In the analysis of total foliar carbon content, there was a soil effect; colonized seedlings on average had 63% greater foliar carbon content than uncolonized seedlings (p = <0.001) when planted in sterilized burn soil and 30% greater foliar carbon content than uncolonized seedlings ($p = 0.005$) in unsterilized burn soil (Fig. 4, Table 2).

The foliar C:N ratio (content in grams) of colonized seedlings was greater than that of uncolonized seedlings. In sterilized burn soil the ratio was 33:1

Table 1 Growth parameters for colonized and uncolonized seedlings reported as means, standard errors, and differences ((colonized – uncolonized)/uncolonized, %) with t-values and statistical significance. In sterilized soil, $n = 26$ colonized and $n = 15$ uncolonized seedling pairs; in unsterilized soil, $n = 20$ colonized and $n = 13$ uncolonized seedling pairs

Seedling Parameter	Burn Soil Treatment	Uncolonized Mean ± SE	Colonized Mean ± SE	Difference (%)[a]	t-value	Significance
Height (cm)	sterilized	5.99 ± 0.49	8.18 ± 0.37	36.6	3.55	<0.001**
	unsterilized	7.22 ± 0.53	7.88 ± 0.43	8.9	0.98	0.33
Stem Diameter (mm)	sterilized	3.51 ± 0.13	3.92 ± 0.10	11.7	2.54	0.013*
	unsterilized	3.58 ± 0.14	3.54 ± 0.11	−1.1	−0.20	0.84
Total Biomass (g)	sterilized	5.95 ± 0.51	9.55 ± 0.39	60.5	5.64	<0.001**
	unsterilized	7.37 ± 0.55	9.32 ± 0.44	26.5	2.78	0.007*
Shoot Biomass (g)	sterilized	2.43 ± 0.21	3.95 ± 0.16	62.6	5.73	<0.001**
	unsterilized	3.09 ± 0.23	3.84 ± 0.18	24.3	2.56	0.013*
Total Root Biomass (g)	sterilized	3.53 ± 0.32	5.58 ± 0.24	58.1	5.11	<0.001**
	unsterilized	4.28 ± 0.34	5.53 ± 0.28	29.2	2.83	0.006*
New Root Biomass (g)	sterilized	1.56 ± 0.11	1.88 ± 0.09	20.3	2.74	0.008*
	unsterilized	1.49 ± 0.11	1.81 ± 0.10			
Shoot:Total Root Ratio	sterilized	0.71 ± 0.02	0.71 ± 0.02	0	−0.03	0.98
	unsterilized	0.71 ± 0.02	0.71 ± 0.02			

Significant at * $P < 0.05$ and ** $P < 0.001$

[a] For growth parameters, only one difference is reported if there was no evidence of an interaction ($p < 0.05$) between soil treatment and original colonization status

Table 2 Foliar parameters for colonized and uncolonized seedlings reported as means, standard errors, and differences ((colonized – uncolonized)/uncolonized, %) with t-values and statistical significance. Values for n are the same as in Table 1

Seedling Parameter	Burn Soil Treatment	Uncolonized Mean ± SE	Colonized Mean ± SE	Difference (%)[a]	t-value	Significance
Foliar N Concentration (%)	sterilized	1.86 ± 0.05	1.53 ± 0.04	−17.7	−5.31	<0.001**
	unsterilized	1.71 ± 0.05	1.57 ± 0.04	−8.2	−2.05	0.044*
Foliar N Content (g)	sterilized	0.11 ± 0.005	0.14 ± 0.004	26	4.88	<0.001**
	unsterilized	0.12 ± 0.005	0.15 ± 0.004			
Foliar $\delta^{15}N$ (‰)	sterilized	−1.15 ± 0.09	−1.56 ± 0.07	n.a.	−3.66	<0.001**
	unsterilized	−0.94 ± 0.09	−1.35 ± 0.08			
Foliar C Concentration (%)	sterilized	49.3 ± 0.48	50.2 ± 0.40	1.8	1.65	0.104
	unsterilized	50.0 ± 0.5	50.9 ± 0.44			
Foliar C Content (g)	sterilized	2.94 ± 0.28	4.80 ± 0.21	63.3	5.33	<0.001**
	unsterilized	3.67 ± 0.30	4.76 ± 0.24	29.7	2.84	0.0052*
Foliar $\delta^{13}C$ (‰)	sterilized	−31.7 ± 0.18	−31.1 ± 0.14	n.a.	2.88	0.0046*
	unsterilized	−31.6 ± 0.20	−30.5 ± 0.16	n.a.	4.49	<0.001**
Foliar C:N Ratio	sterilized	27.0 ± 1.08	33.3 ± 0.82	23.3	4.66	<0.001**
	unsterilized	29.4 ± 1.16	32.7 ± 0.94	11.2	2.25	0.028*

Significant at * $P < 0.05$ and ** $P < 0.001$

[a] For foliar parameters, only one difference is reported if there was no evidence of an interaction ($p < 0.05$) between soil treatment and original colonization status

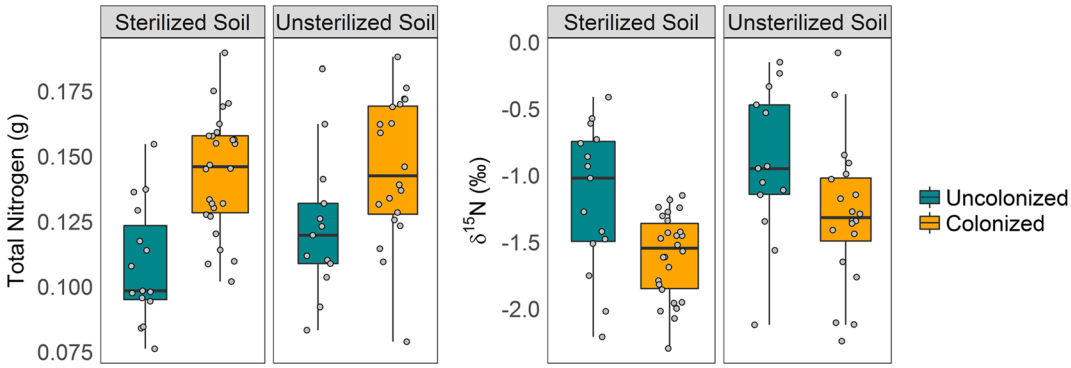

Fig. 2 Boxplots for seedlings colonized or not by *Suillus* ectomycorrhizae and planted in sterilized or unsterilized soil for foliar nitrogen content (left) and foliar $\delta^{15}N$ (right)

(colonized) and 27:1 (uncolonized) ($p < 0.001$); and in unsterilized burn soil the ratio was 33:1 (colonized) and 29:1 (uncolonized) ($p = 0.028$) (Table 2).

In the initial individual analysis of foliar $\delta^{15}N$, colonized seedlings had significantly lower foliar $\delta^{15}N$ ($p < 0.001$) than uncolonized seedlings when averaged across soil treatments. Boxplots and scatterplots indicate a negative relationship between foliar nitrogen content and foliar $\delta^{15}N$ in both colonized and uncolonized seedlings (Figs. 2 and 3). After testing for interactions, the reduced model was fit ($R^2 = 0.42$) and differences in foliar nitrogen content and $\delta^{15}N$ were estimated between colonized and uncolonized seedlings. As there was no evidence of an interaction between soil treatment and original colonization status, differences were averaged across soil treatments. In the analysis of total foliar nitrogen content, colonized seedlings had more foliar nitrogen than uncolonized seedlings ($p < 0.001$) (Table 2). In the analysis of foliar $\delta^{15}N$, colonized seedlings had significantly lower $\delta^{15}N$ than uncolonized seedlings ($p = <0.001$) (Table 2). Results from the multiple linear regression model, including a *p*-value, correlation coefficient, and slope for each treatment combination are presented in Fig. 3.

In the initial individual analyses of foliar $\delta^{13}C$, colonized seedlings had significantly higher foliar $\delta^{13}C$ ($p < 0.001$) than uncolonized seedlings when averaged across soil treatments. Boxplots and scatterplots indicate a positive relationship between foliar carbon content and foliar $\delta^{13}C$ for both colonized and uncolonized seedlings (Figs. 4 and 5). Due to evidence of a three-way interaction in the full model ($R^2 = 0.36$), differences between colonized and uncolonized seedlings were estimated within each soil treatment for each response. As mentioned previously, in the analysis of total foliar carbon content, colonized seedlings had on average greater foliar carbon content than uncolonized seedlings when planted in sterilized burn soil ($p = <0.001$), and in unsterilized burn soil ($p = 0.005$) (Table 2). The foliar $\delta^{13}C$ of colonized seedlings planted in sterilized burn soil was higher than that of uncolonized seedlings ($p = 0.005$). In unsterilized burn soil, the $\delta^{13}C$ of colonized seedlings was significantly greater than that of uncolonized seedlings ($p < 0.001$) (Table 2). Results from the multiple linear regression model, including a *p*-value, correlation coefficient, and slope for each treatment combination are presented in Fig. 5.

Discussion

Ectomycorrhizal fungi in burn soil

Greenhouse bioassays have been used to successfully detect viable ectomycorrhizal fungi in burn or simulated burn soil (Baar et al. 1999; Kjøller and Bruns 2003; Izzo et al. 2006; Peay et al. 2009; Buscardo et al. 2010; Kipfer et al. 2010; Sousa et al. 2011; Glassman et al. 2016; Vásquez-Gassibe et al. 2016). When uncolonized *P. albicaulis* seedlings were planted in unsterilized burn soil in the present greenhouse bioassay, no ectomycorrhizal fungi were detected. This was surprising although the burn was severe and the result of an intense fire; only standing dead and minimal understory regeneration were observed two years after the fire and soil was only collected to a depth of 20 cm. Dahlberg et al. (2001) found almost no mycorrhizas on roots 15 cm deep on a severe burn site in Sweden, and intense fire has been reported to decrease root colonization in a number of studies (Taudière et al. 2017). Site properties

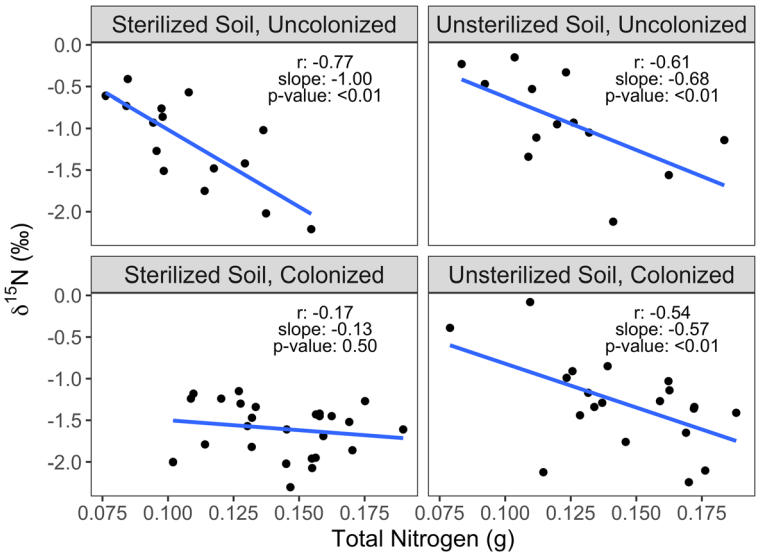

Fig. 3 Relationships between foliar nitrogen content and foliar $\delta^{15}N$ for seedlings colonized or not by *Suillus* ectomycorrhizae and planted in sterilized or unsterilized soil

can also impact the fungi (Kennedy et al. 2015) and many of the *P. albicaulis* trees in the area are dead, killed by mountain pine beetles in the last decade (MacFarlane et al. 2013). Treu et al. (2014) found that the species richness of the ectomycorrhizal community declined as much as 80% (from 20 to 3 species) in lodgepole pine stands with over 70% beetle kill, and *Suillus* species were not present in their infested stands. At least E-strain fungi (*Wilcoxina* species) were expected in our study as they are common after fire (Egger 1996; Baar et al. 1999; Fujimura et al. 2005), but none were detected. Thus, the beetle infestation followed by fire appears to have severely impacted the ectomycorrhizal community.

Alternatively, the ectomycorrhizal fungi may not have responded to bioassay conditions. However, a similar bioassay using *P. albicaulis* seedlings planted in diluted unsterilized burn soil (from a different burn) collected five years after the fire recovered several species of *Wilcoxina, Rhizopogon,* and *Suillus* in our greenhouse, although it did not pick up the additional taxa revealed by molecular analysis of roots from the field (*Pseudotomentella nigra, Amphinema byssoides, Cenococcum geophilum*, *Piloderma byssinum*, *Cortinarius* spp. and the Thelephoroid group) (Trusty 2009). Bioassays using other pine species also recovered only a portion of the species present (Baar et al. 1999; Bidartondo et al. 2001).

It was necessary to use seedlings and not seeds in the bioassay due to the long, unreliable stratification procedure necessary to germinate *P. albicaulis* seeds and the unavailability of stratified seeds. The dominant suilloid morphotypes (99.9%) that were molecularly confirmed (*Suillus americanus* and *S. discolor*) are known

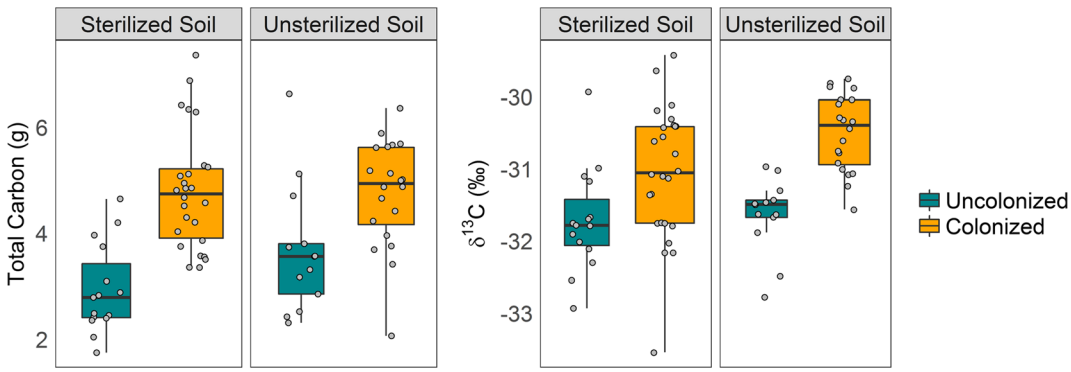

Fig. 4 Boxplots for seedlings colonized or not by *Suillus* ectomycorrhizae and planted in sterilized or unsterilized soil for foliar carbon content (left) and foliar $\delta^{13}C$ (right)

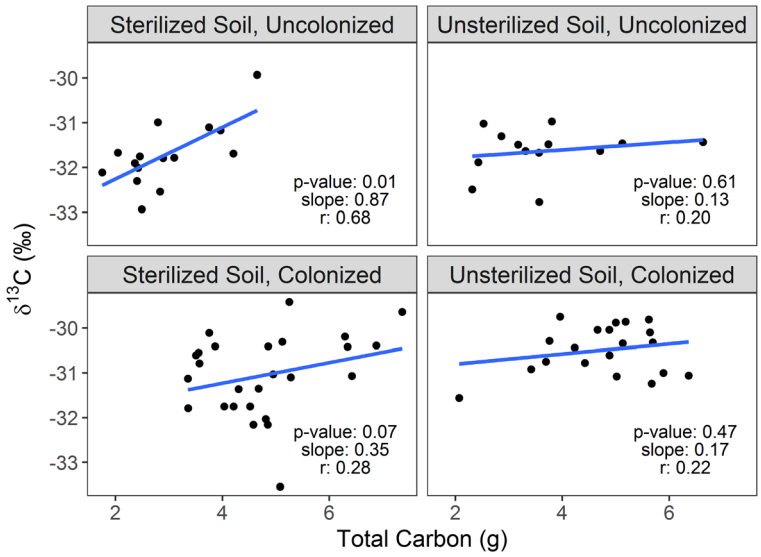

Fig. 5 Relationships between foliar carbon content and foliar $\delta^{13}C$ for seedlings colonized or not by *Suillus* ectomycorrhizae and planted in sterilized or unsterilized soil

associates of *P. albicaulis* (Mohatt et al. 2008); these originate from local sources at the Idaho nursery (Cripps and Jenkins 2015). E-strain and *Thelephora* can be present on nursery seedlings, but were not detected throughout the experiment. In a previous bioassay done in the same greenhouse using diluted sterilized burn soil from another source, *Wilcoxina* species were the most frequent and abundant nursery fungus on *P. albicaulis* seedling roots (Cripps and Trusty 2007), demonstrating that the method can detect these fungi as originating from nursery stock.

Effect of soil treatment

Sterilized soil was originally used as a control and to determine if any additional fungi in the study might originate from nursery seedlings. However, statistical analysis revealed a significant 'soil effect'; new roots were essentially colonized faster by the *Suillus* species in sterilized soil. This could have been due to microbial repression in the unsterilized soil or alteration of soil properties with sterilization. Microbial communities (bacterial) recover faster than fungal communities after fire (Hamman et al. 2007; Dooley and Treseder 2012) and mycelium can grow faster in sterile soil (Skinner and Bowen 1974; Fitter and Garbaye 1994). Uncolonized seedlings in the sterilized soil were smaller than those in unsterilized soil. This could also be due to altered nutrient availability i.e. volatilization of ammonia, toxicity due to autoclaving, or other factors. In any case, the mycorrhizal fungi were able to overcome the deficit in autoclaved soil as has been shown for other basidiomycete ectomycorrhizal fungi (Zak 1971). It might be preferable to sterilize soil by fumigation if autoclaving is problematic. We consider results for the unsterilized soil to be more realistic than those for the sterilized soil for explaining restoration outcomes.

Effect of *Suillus* species on seedlings in burn soil

In summary, colonization with *Suillus* species influenced most parameters of *P. albicaulis* seedlings in both burn soil treatments: biomass was greater, foliar nutrient content (nitrogen and carbon) increased, foliar nitrogen concentration decreased, needles had lower $\delta^{15}N$ and higher $\delta^{13}C$. Seedling height and stem diameter increased significantly with mycorrhizal colonization in sterilized but not in unsterilized soil. In general, differences in most parameters between colonized and uncolonized seedlings were more pronounced in the sterilized soil. This likely reflects greater root colonization (twice as many ectomycorrhizae) in the sterilized soil, the absence of other microbes, and altered soil properties.

The biomass increase in nursery seedlings was not expected since whitebark is a slow-growing pine and there is often an initial carbon drain from mycorrhizal colonization in the greenhouse (Dosskey et al. 1990; Hobbie and Wallander 2006), however seedlings were older and colonized over many months in the greenhouse. *Suillus* species can increase the size of pines in the greenhouse (Heumader 1992; Rincón et al. 2007;

Verma et al. 2014), but this is dependent on conditions. In the controlled studies of Hobbie and Colpaert (2003), Hobbie et al. (2008) and Colpaert et al. (1996), *P. sylvestris* seedlings inoculated with *S. bovinus* or *S. luteus* were smaller than uninoculated controls. Under their semi-hydroponic systems nutrient availability was held constant, and nitrogen and carbon retention by ectomycorrhizal fungi necessarily decreased plant productivity and nitrogen supply.

In our study, roots were one-and-a-half times larger than shoots, and the biomass of both increased with colonization by *Suillus* species, more so in the sterilized soil, but shoot:root ratios remained constant (0.7) across all treatments. Increased shoot biomass is indicative of increased photosynthetic and transpirational capacity, while proportionally more roots (ratios below 1.0) can imply greater drought tolerance in the field (Grossnickle 2005; Haase 2008). In contrast, in controlled experiments with younger pine seedlings, shoots increased more than roots with colonization of *P. sylvestris* by *S. bovinus,* presumably due to retention of much below-ground allocation by this fungal species (Colpaert et al. 1996).

In an experiment that used simulated (furnace-incinerated) burned soil, *S. bovinus* enhanced shoot biomass of *P. pinaster* seedlings; however nonmycorrhizal seedlings were larger in unburned soil where nutrient availability could have been higher (Sousa et al. 2011). In a second study, *Pinus pinaster* seedlings planted in blocks of burned soil in the greenhouse were larger than controls in unburned soil, but this could not be attributed to mycorrhization since this was confounded with soil properties; they suggested that a control of sterilized burn soil should have been added (Vásquez-Gassibe et al. 2016). In the field, growth and survival appear to increase when seedlings are both inoculated with *Suillus* and planted on burns (Lonergan et al. 2014; Asebrook and Hintz 2015).

Foliar nutrient and isotope analyses

Few studies have documented how ectomycorrhizal colonization affects nutrient content or isotope abundance in seedlings in the greenhouse. *Suillus* species are particularly involved in the uptake of nitrogen (Keller 1996; Nygren et al. 2008; Antibus et al. 2018), and are prime candidates for exploring the dynamics of N and C flow in ectomycorrhizal systems. In our experiment, the concentration of foliar nitrogen in whitebark pine seedlings colonized by *Suillus* was 18% lower in sterilized soil and 8% lower in unsterilized soil compared to uncolonized controls; the actual foliar nitrogen concentrations were around 1.53–1.57% after six months. In other studies using *Pinus-Suillus* systems, nitrogen concentrations were also lower than uncolonized controls, and levels were comparable to ours: 1.57–1.72% depending on nitrogen supply for *P. sylvestris* with *S. bovinus* (Hobbie et al. 2008); 0.95% after 12 weeks for *P. sylvestris* with *S. bovinus* (Colpaert et al. 1996); and half that of controls after two months for *P. densiflora* with *S. granulatus* (Kohzu et al. 2000). The nitrogen concentration in seedlings planted in simulated burn soil was lower in shoots of *P. pinaster* inoculated with *S. bovinus* compared to uninoculated controls in both simulated burn soil and unburned soil (Sousa et al. 2011). The consistently lower concentration of nitrogen in needles of colonized seedlings can be attributed to the mobility of nitrogen and its movement into growing tissues which produces a dilution effect if more biomass is produced (Landis et al. 2005; Jones et al. 2009) which is explained in the following pages.

Conversely, total foliar nitrogen content in our colonized seedlings was 26% higher than that of uncolonized seedlings averaged across soil treatments; actual N content ranged from 0.14 to 0.15 g for the colonized and 0.11 to 0.12 for the uncolonized seedlings and this supports results from other studies. *Pinus densiflora* seedlings inoculated with *S. granulatus* had greater foliar nitrogen content than uninoculated controls in a semi-hydroponic system (Kohzu et al. 2000). Nitrogen content was not calculated for *P. pinaster* seedlings in simulated burn soil, but *S. bovinus* inoculated seedlings were much larger than controls and nitrogen concentrations were significantly lower in controls, as in our study (Sousa et al. 2011). The higher total nitrogen content in our study primarily reflects the larger biomass of inoculated seedlings. Nitrogen content often is a more successful predictor of out-planting performance than nitrogen concentration (Larsen et al. 1988; Landis et al. 2005). Nitrogen was likely limiting in our burn soil since seedlings strongly benefitted in terms of increased nitrogen content and biomass when *Suillus* formed ectomycorrhizae on seedling roots. The burn soil mixture contained 13.6 mg kg^{-1} and no extra nitrogen (fertilizer) was added for the six month duration. Our previous study found that high nitrogen fertilizer inhibits *Suillus* colonization of *P. albicaulis* seedlings in the greenhouse (Lonergan and Cripps 2013).

In the current experiment, foliar carbon concentrations did not differ between colonized and uncolonized *P. albicaulis* seedlings. However, foliar carbon content was 63% higher in colonized seedlings in sterilized soil and 30% higher in unsterilized soil in comparison to uncolonized controls, reflecting the larger seedling biomass. Increased photosynthesis can increase plant growth, and this has also been linked to more effective nitrogen uptake (Jose et al. 2003); we conclude that this is what occurred via ectomycorrhizal fungi in our nitrogen-limited study.

Foliar stable isotope analysis can reveal information about physiological processes and movements of compounds, but there is sparse information about how stable isotope abundance in plants correlates with the ectomycorrhizal status of seedlings under controlled conditions. It has been hypothesized that ectomycorrhizal fungi preferentially retain the heavier isotope of nitrogen ^{15}N, passing fractionated nitrogen with relatively more ^{14}N to the host plant; therefore foliar $\delta^{15}N$ should be lower in plants colonized by ectomycorrhizal fungi (Högberg 1997). Several studies have provided evidence that this holds under controlled conditions, using pines colonized by *Suillus* species (Colpaert et al. 1996; Kohzu et al. 2000; Hobbie and Colpaert 2003; Hobbie et al. 2008). Here we add evidence to support the hypothesis with a less controlled greenhouse study using burn soil.

The foliar $\delta^{15}N$ values for needles of *P. albicaulis* seedlings ranged from −0.94‰ to −1.56‰, depending on treatment (Table 2). This is somewhat comparable to foliar $\delta^{15}N$ values for *Suillus* colonized *P. sylvestris* seedlings in other studies where values have ranged from −1.6‰ to −4.2‰ (Hobbie et al. 2008) and −0.84‰ to 1.62‰ (Hobbie and Colpaert 2003), depending on treatment. However, initial seedling $\delta^{15}N$, source and surrounding environmental $\delta^{15}N$ values were not measured in our experiment, making comparisons difficult.

The relationship between ectomycorrhizal fungi on roots and $\delta^{15}N$ in needles is dependent upon the availability and form of nitrogen in the system. In a controlled semi-hydroponic system, the foliar $\delta^{15}N$ of *Pinus sylvestris* seedlings colonized by *Suillus luteus* was 2.4‰ lower than that of uncolonized seedlings under a low supply of N (NH_4^+ and NO_3^-) and 0.7‰ lower under a high N supply (Hobbie and Colpaert 2003). Needles of *Pinus densiflora* seedlings colonized with *S. granulatus* under application of a low N (ammonium salt) fertilizer, had significantly lower $\delta^{15}N$ in comparison to uninoculated seedlings in a greenhouse setting (Kohzu et al. 2000). In another controlled system, the foliar $\delta^{15}N$ of *P. sylvestris* seedlings inoculated with *S. bovinus* was 1.7‰ lower than that of uncolonized seedlings under low rates of NH_4^+ application 3.5‰ lower under high NH_4^+, 1.3‰ lower under low NO_3^-, and 0.5‰ lower under high NO_3^- (Hobbie et al. 2008). Therefore it appears that fractionation was greatest when seedlings are colonized by *Suillus* species under low nitrogen conditions in many of the studies, but this is tempered by the source of nitrogen (Hobbie and Colpaert 2003; Hobbie et al. 2008). Results might also reflect the abundance of mycorrhizae on roots. In the same cited study, *Suillus* colonized roots to a greater extent when the main supply of nitrogen was ammonium (NH_4^+) rather than nitrate, and foliar $\delta^{15}N$ was lower with ammonium (Hobbie et al. 2008). *Suillus* species can metabolize a range of nitrogen sources including ammonium, nitrate, amino acids, dipeptides, and proteins, and this has been shown to be true for *S. sibiricus* (= *S. americanus*) as well (Keller 1996; Nygren et al. 2008; Antibus et al. 2018). While nitrogen levels were considered low in our *P. albicaulis*-*Suillus* system, $\delta^{15}N$ values were comparable to those of high nitrogen treatments in other studies. However, only total nitrate was measured in our study and other forms of nitrogen cannot be addressed.

In field studies, plant $\delta^{15}N$ has been correlated with soil availability, N form, and mycorrhizal status (Garten Jr and Miegroet 1994; Evans 2001; Craine et al. 2009), but results are difficult to interpret. In one large study across a 361-year fire-driven chronosequence of boreal forests in northern Sweden, foliage of eight dominant plant species declined in $\delta^{15}N$ given more time after fire. This suggests that as the chronosequence proceeds, plants become more dependent on nitrogen transferred from mycorrhizal fungi or from N deposition (Hyodo et al. 2013).

Total foliar nitrogen content has been negatively correlated with foliar $\delta^{15}N$ in pine seedlings colonized by *Suillus* (Kohzu et al. 2000), as was observed in our experiment. As *P. albicaulis* seedlings became more and more colonized by *Suillus* species, they received more nitrogen and thus had lower $\delta^{15}N$ because of fractionation of the nitrogen transfer compounds by the fungi. Basically, the nitrogen supply is improved by ectomycorrhizal colonization.

Foliar carbon isotope analysis

Ectomycorrhizal fungi affect host plants in ways that indirectly affect $\delta^{13}C$, but physiological links to the fungi are not well-tested, especially under controlled greenhouse conditions. Average foliar $\delta^{13}C$ for *P. albicaulis* seedlings ranged from −30.5‰ to −31.7‰ depending on soil treatment, and were higher in uncolonized seedlings. In the only reported study done under controlled conditions, shoots and roots of *P. sylvestris* also had higher $\delta^{13}C$ under low N conditions when seedlings were colonized by *Suillus luteus* in comparison to uncolonized controls; values ranged from −27.6‰ to −28.3‰ depending on treatment (Hobbie and Colpaert 2004). Thus, we add evidence that ectomycorrhizal colonization can result in greater $\delta^{13}C$ in needles.

Plants discriminate against the heavier $^{13}CO_2$ in the initial assimilation of carbon which is dependent on environmental conditions, plant physiology, morphology, and genetics. This initial selectivity sets up the baseline ^{13}C: ^{12}C ratio in the plant (Farquhar et al. 1989; Dawson et al. 2002). Subsequently there can be selectivity in the synthesis of carbon compounds such as lignin, lipids, and cellulose which are relatively depleted in ^{13}C for biochemical reasons (structural bias against using the heavier isotope ^{13}C), leaving sugars and starch relatively more enriched in ^{13}C; both are mobile compounds that move from shoots to roots (Gleixner et al. 1993; Hobbie and Werner 2006). Hobbie and Colpaert (2004) hypothesized that sugars transported down from needles become progressively more enriched in ^{13}C as ^{13}C–depleted compounds such as lignin are synthesized along the transit to roots. Indeed, Hobbie and Colpaert (2004) found higher ^{13}C in stems than in needles.

Summary and implications

The hypothesis that colonization of *P. albicaulis* seedlings by *Suillus* species would increase the nitrogen content of needles and decrease foliar $\delta^{15}N$ in burn soil is supported by the evidence. In addition to improved nutrition, the lower $\delta^{15}N$ in needles is evidence that this was due to the presence of the ectomycorrhizal fungi. Basically, *Suillus* species stimulated growth in seedlings by overcoming the nitrogen limitation in the burn soil. However, the burn soil of necessity was diluted, making it difficult to infer what might take place on the actual burn site. Results are for two *Suillus* species, *S. americanus* and *S. discolor*, and other ectomycorrhizal fungi may impact seedlings differently. Even within the genus, there are differences in nitrogen use. Some isolates of *Suillus* species associated with five-needle pines, including *S. plorans* and *S. sibiricus*, prefer ammonium over nitrate, and a few isolates of *S. placidus* prefer nitrate (Keller 1996; Antibus et al. 2018). Additionally, some *Suillus* species can use amino acids and dipeptides as forms of nitrogen which could relate to the habitat and soil type with which these fungi are often associated such as the harsh, high elevation sites where whitebark pine exists (Antibus et al. 2018). Results suggest that improved nitrogen uptake can help explain improved survival of whitebark pines planted on burns when they are colonized by *Suillus* species for restoration purposes. However, soil nutrient releases after fire still need to be addressed in conjunction with use of *Suillus* in the restoration process.

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