Mycoscience xxx (2017) 1–9

Contents lists available at ScienceDirect

Mycoscience

journal homepage: www.elsevier.com/locate/myc

Full paper

Sporocarp δ^{15}N and use of inorganic and organic nitrogen *in vitro* differ among host-specific suilloid fungi associated with high elevation five-needle pines

Robert K. Antibus [a,*], Erik A. Hobbie [b], Cathy L. Cripps [c]

[a] Department of Biology, Bluffton University, 1 University Drive, Bluffton, OH, 45817, USA
[b] Earth Systems Research Center, University of New Hampshire, Durham, NH, 03824, USA
[c] Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA

ARTICLE INFO

Article history:
Received 30 October 2016
Received in revised form
17 November 2017
Accepted 24 November 2017
Available online xxx

Keywords:
Amino acids
Pinus albicaulis
Pinus flexilis

ABSTRACT

Widespread decline of whitebark and limber pines in the northern Rocky Mountains (USA) has created an imperative to understand functional diversity in their ectomycorrhizal associates. Because suilloid fungi are likely important in successful reestablishment of pines the nitrogen-related functional traits of 28 high-elevation suilloid isolates were examined. Radial growth, mass accumulation and mycelial density were measured for isolates on six different nitrogen sources. The δ^{15}N values of suilloid sporocarps used as sources for pure cultures were compared against growth parameters to investigate a possible link between these N-related functional traits. Isolates grew poorly on nitrate and BSA and grew well on glutamine, alanyl-glutamine and ammonium phosphate, with somewhat slower growth on alanine. Isolates and species varied considerably in their growth response to different nitrogen sources. Effective use of nitrate and BSA was uncommon and associated with isolates with high inherent growth rates. Sporocarp δ^{15}N was negatively correlated with relative growth on alanine of the corresponding isolates. Our results suggest strong similarities in N source use patterns of suilloid fungi of whitebark pine origin and those of another high-elevation five-needle stone pine, the Swiss stone pine.

1. Introduction

In conifer-dominated ecosystems, nitrogen often limits growth (Hawkins, Jones, & Kranabetter, 2015), and ectomycorrhizal (ECM) fungi play an essential role in accessing forms of nitrogen considered unavailable to nonmycorrhizal hosts (Smith & Read, 2008). Mutualistic ECM fungi may be of particular benefit at higher latitudes and elevations where nitrogen is predominantly present in organic forms (Read & Perez-Moreno, 2003). Under such conditions of short growing seasons and low soil temperatures, fungi are well suited to circumvent the mineralization-immobilization-turnover route traditionally considered to precede N uptake (Polacco & Todd, 2011; Schimel & Bennett, 2004).

Five-needle pines are a prevalent and functionally significant life form at the alpine-subalpine ecotone from a global perspective (Arno & Hammerly, 1984). In the northern Rocky Mountains of North America, *Pinus albicaulis* Engelm. (whitebark pine = WBP) and *Pinus flexilis* E. James (limber pine = LP), often grow in pure stands on rocky soils at high elevations where they associate with a unique set of host-selected or host-specific suilloid fungi (Cripps & Antibus, 2011; Mohatt, Cripps, & Lavin, 2008). These suilloid fungi, including species such as *Suillus sibiricus* (Singer) Singer studied herein, are of importance because they are specific to five-needle pines (Bruns, Bidartondo, & Taylor, 2002), associate with both seedlings and mature trees (Rainer et al., 2015), and play a role in nitrogen uptake (Cripps & Jenkins, 2015). These suilloid fungi are also important to restoration of WBP and LP as these pines are currently suffering range-wide die-offs from blister rust and mountain pine beetles (Keane et al., 2012, p. 106; Lonergan, Cripps, & Smith, 2014; Tomback, Achuff, Schoettle, Schwandt, & Mastrogiuseppe, 2011). In a recent review, Karst, Randall, and Gehring (2014) noted that loss of host pines at the landscape level, in this case WBP or LP, can lead to loss of host-specialist ECM fungi. The potential loss of suilloid species or genotypes and its effects create a critical need for studies aimed at understanding the

* Corresponding author.
E-mail addresses: antibusr@bluffton.edu (R.K. Antibus), Erik.Hobbie@unh.edu (E.A. Hobbie), ccripps@montana.edu (C.L. Cripps).

https://doi.org/10.1016/j.myc.2017.11.007

extent and variation in functional traits of host-associated ECM (Karst et al., 2014).

Generally, suilloid fungi have played an essential part in studies characterizing N-related functional traits of ECM fungi. Pioneering studies employing *Suillus bovinus* (L.) Roussel and *Rhizopogon roseolus* (Corda) Th. Fr., associated with two-needle *P. contorta* Douglas ex Loudon, demonstrated *in vitro* the ability of these species to use simple organic N sources and demonstrated their resynthesized ECM to provide N to hosts from bovine serum albumin (BSA) (Abuzinadah & Read, 1986a, b). Subsequent studies and field observations led to functional classifications into nitrophilic, protein, and nonprotein ECM fungi (Hawkins et al., 2015), and fostered suggestions that fungi from sites with lower or higher N availability differ in their use of different N sources (Lilleskov, Hobbie, & Horton, 2011; Talbot & Treseder, 2010). Most studies however have focused on a few species or employed only a small number of isolates (Cairney, 1999). Intraspecies trait variation is high in fungi (Crowther et al., 2014) and is expected to be critical in a community dominated by a small number of keystone species (Johnson, Martin, Cairney, & Anderson, 2012), as is true of WBP and LP stands. Intraspecies variation allows greater species coexistence and more complete resource use (Albert et al., 2010), with Kranabetter (2014) suggesting that site-adapted ECM fungi and non-redundant functional diversity are key elements to forest fitness and productivity. Intra- and interspecific variation both enhanced nitrogen use and ECM fungal community production in microcosm studies (Wilkinson, Solan, Taylor, Alexander, & Johnson, 2010; 2012). Similarly, intra- and interspecific diversity yielded complex but generally positive impacts on plant and ECM production with *Pinus sylvestris* L. seedlings (Hazard, Kruitbos, Davidson, Taylor, & Johnson, 2017). In the most comprehensive study of *Suillus* isolates to date Rineau et al. (2016) demonstrated widespread protease production *in vitro* with a range of species and species isolates. Most species demonstrated enhanced protease activity when sourced from sites with increased soil organic N availability, however exceptions were observed. In contrast, Keller (1996) studied three high elevation *Suillus* species and was unable to link protein use with stand age or soil development.

Genomic methods currently provide a broad-spectrum analysis of fungal communities and functional traits, however physiological studies are needed to confirm results (Branco et al., 2015). In pure culture studies, fungal traits are examined under standard conditions, allowing cross study comparison and illuminating patterns that foster hypothesis generation (Aguilar-Trigueros et al., 2015). Crowther et al. (2014) noted that isolated fungi are necessary to match traits to individuals or ecotypes. Here we evaluate both inter- and intraspecific variation in N-related functional traits of suilloid fungi tissue-cultured from sporocarps collected in WBP and LP forests and grown *in vitro* on different N sources.

An ongoing challenge has been relating differences in ECM fungal functions *in vitro* with ECM activity in soils. Studies of the natural abundance of ^{15}N provide time-integrated measures on the functional roles of ECM (Mayor et al., 2015). The examination of nitrogen stable isotope ratios (^{15}N:^{14}N, expressed as $\delta^{15}N$ values) of ECM sporocarps may provide complimentary insight into nutritional differences among these fungi (Hobbie et al., 2014). For example, work on Alaskan ECM fungi by Lilleskov, Hobbie, and Fahey (2002) demonstrated that protein use by pure cultures was positively correlated with high $\delta^{15}N$ in sporocarps, however few studies have tested the generality of this observation. A better understanding of how isolate physiology and sporocarp $\delta^{15}N$ link functionally for whitebark and limber pine ECM fungi will enhance our knowledge of their ecology and could provide an additional tool for identifying fungal species or strains of high conservation interest.

Pellitier and Zak (2017) recently highlighted a need for greater biogeographic coverage in studies dealing with nitrogen physiology of ECM fungi. To date Keller's study (Keller, 1996) on the European five-needle pine Swiss stone pine (*Pinus cembra* L.) remains the only such work on high-elevation ECM forests. In that study, inorganic and organic nitrogen use by *P. cembra* associates varied among species and individual strains, including several suilloid fungi. However, several of these isolates had been in culture for more than two decades, which can complicate interpretation (Anderson, Chambers, & Cairney, 2001). Here we examine *in vitro* inorganic and organic nitrogen use and further examine $\delta^{15}N$ of sporocarps from which the cultures were obtained. Our aim is to probe the linkage of these traits as well as the diversity in N-related functional traits of high-elevation suilloid fungi. Pure cultures of newly isolated suilloid fungi were used to test *in vitro* the following hypotheses: 1) ammonium is the preferred nitrogen source of these fungi; 2) protein use will be well-developed and widespread among these fungi; 3) interspecific differences among *Suillus* species will be robust to high levels of intraspecific variation; 4) protein use of pure cultures and $\delta^{15}N$ of the corresponding sporocarp will positively correlate.

2. Materials and methods

2.1. Collections and sites

The fungal isolates employed in the present study, with three exceptions, were obtained during the 2009 field season from stands dominated by either *P. albicaulis* or *P. flexilis* primarily in Montana (Table 1). Stands varied in tree age, slope, aspect and parent materials. Although all stands were on rocky soils; soil development varied and understory species when present included primarily grasses or ericaceous shrubs. Additional stand details for select sites are given in Mohatt (2006) and Mohatt et al. (2008). Fungal sporocarps were collected, identified and dried following standard practices accepted for macrofungi (Lodge, Ammirati, O'Dell, & Mueller, 2004). Voucher specimens of dried sporocarps were deposited in the MONT Herbarium fungal collection at Montana State University, Bozeman, Montana.

2.2. Culture and experiment

Isolations of fungi into pure culture were attempted within 24–36 h of sporocarp collection. Tissue was removed aseptically from sporocarp context tissue and placed on sterile MMN (modified Melin-Norkrans) agar (Molina & Palmer, 1982) supplemented with ampicillin and tetracycline at 50 mg l^{-1}. Parafilm-sealed petri dishes were incubated at room temperature (22–25 °C) until visible signs of growth appeared at which point they were transferred to modified MMN medium lacking antibiotics and malt extract while containing biotin at 1.0 μg per liter. Stock cultures were maintained on slants of this medium at 4 °C. A list of fungi employed in nitrogen source and stable isotopes analysis studies along with site of origin is given in Table 2.

Nitrogen source experiments were conducted within months of initial isolation. In addition to controls (no N source) treatments included, ammonium phosphate [$(NH_4)_2PO_4$], potassium nitrate (KNO_3), amino acids (L-alanine and L-glutamine), dipeptide (alanyl-glutamine) and protein (bovine serum albumin). All organic N sources exceeded 96% purity. The base media were MMN salts with glucose reduced to 5 g l^{-1} and ammonium phosphate removed from all but the ammonium treatment. Phosphorus for treatments lacking ammonium phosphate was supplied at equimolar concentrations with KH_2PO_4. For all treatments except nitrate the potassium concentrations were held constant by addition of KCl. A trace

Please cite this article in press as: Antibus, R. K., et al., Sporocarp $\delta^{15}N$ and use of inorganic and organic nitrogen *in vitro* differ among host-specific suilloid fungi associated with high elevation five-needle pines, Mycoscience (2017), https://doi.org/10.1016/j.myc.2017.11.007