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Seed-associated fungi in the alpine tundra: Both mutualists and pathogens could impact plant recruitment

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ABSTRACT

Seed-borne microbes are important pathogens and mutualists in agricultural crops but are understudied in natural systems. To understand the diversity and function of seed-borne fungi in alpine tundra, we cultured fungi from seeds of six dominant plant species prior to seed dispersal and evaluated their function using germination experiments in *Zea mays*. A total of 55 fungal cultures (9 species) were isolated with up to 4 genera per plant species. Dominant orders included Pleosporales and Hypocreales. Sixty-six percent of the isolates showed pathogenic effects. The most common genus was *Alternaria* which had a negative effect on both seed germination and plant growth. *Cladosporium* was only isolated from the two dominant plant species and showed positive effects on germination and plant growth. The high number of pathogenic fungi found coupled with the variation in seed endophytic communities among plant species suggests that seed-associated fungi could affect community composition through differential seedling recruitment.

1. Introduction

Fungal communities are important drivers of plant diversity and community structure (Reynolds et al., 2003). Most studies on plant-fungal interactions in natural systems in the last four decades have focused on root and leaf endophytes, rhizosphere and bulk soil communities, and soil-borne pathogens (Van Der Putten and Peters, 1997; Higgins et al., 2007; Hoffman and Arnold, 2008; Porras-Alfaro and Bayman, 2011); however, a potentially diverse and important group of microorganisms inhabit seeds. Seed-borne microbes are important pathogens and mutualists in agricultural crops (Newton et al., 2010), but they are understudied in natural systems (Clay, 1990; Ravel et al., 1997; Dalling et al., 2011). Most studies in natural systems have focused on fungi acquired in post-dispersal events when the seeds come in contact with fungal soil communities (Gallery et al., 2007; U'Ren et al., 2009).

Seed-borne fungi colonize internal tissues (endophytes) and can show mutualistic or pathogenic activity (Baker and Smith, 1966; Stone et al., 2000; Rodriguez et al., 2009). They are highly ubiquitous and abundant: many cultivated crops and natural plants have some form of seed-borne fungi (e.g., Clay, 1990; Malone and Muskett, 1997; Dhingra et al., 2002; Ganley and Newcombe, 2006). Pathogenic seed-borne fungi have been extensively studied causing infectious diseases. Common species include *Tilletia caries, Colletotrichum lindemuthianum, Botrytis cinerea,* and several *Fusarium* species (Tillet, 1937; Noble et al., 1958; Malone and Muskett, 1997; Anderson et al., 2004). *Epichloë typhina* in *Lolium* spp. improves embryo development (McLennan, 1920), but in adult plants, it also causes fescue toxicity as a way to control herbivory (Leuchtmann, 1992). There are also some saprotrophic fungal species that produce toxic secondary metabolites that help control plant pathogens. For example, *Chaetomium* spp. have been reported as seed endophytes that can provide protection against Fusarium blight in oat seedlings (Tveit and Wood, 1955; Soytong et al., 2001).

This study focuses on seed-borne fungi associated with

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dominant plants in the alpine tundra. The alpine tundra is characterized by harsh environmental conditions, including low temperatures, frost events, short growing season and high winds and solar radiation which lead to desiccation (Greenland, 1989). Diverse communities of fungi have been reported to be associated with plants in cold habitats (Dean et al., 2014; Tedersoo et al., 2014; Timling et al., 2014). In the alpine tundra, vertical transmission of fungi (via seeds) may be an important mechanism by which plants can pass on beneficial fungi to their offspring. Seed-borne fungi may also play an important role in the alpine environment by reducing dormancy or improving germination. Contrasting evidence is available about the role of microbial communities in breaking dormancy (Baskin and Baskin, 2000) but most of the studies have focused on external microbial fungi (e.g. Sanchez-Delgado et al., 2011) or seed-associated fungi acquired after dispersal (Baskin and Baskin, 2000; Zalamea et al., 2014). It is well known that alpine seeds can remain viable after many years in freezing conditions and have the ability to germinate and grow rapidly when the conditions become favorable (Billings and Mooney, 1968); seed-borne fungi may contribute to these processes.

The main goal of this study was to describe the culturable fungal communities in alpine seeds and their potential contribution to plant growth. Fungi were isolated from surface sterilized seeds of six plant species and identified by sequencing the internal transcribed spacer (ITS) rRNA region. Potential roles of the fungal isolates were tested in germination and plant growth experiments using a model crop (maize; *Zea mays*) as well as one of the alpine tundra plants, *Deschampsia cespitosa*. All the isolates closely related to the genus *Cladosporium* showed positive results for seed germination as well as plant growth, so further phylogenetic analysis was performed on the genus to determine the placement of isolates with respect to known species.

2. Materials and methods

2.1. Seed collection and isolation of fungi

Seeds of *Trisetum spicatum, Artemisia scopulorum, Erigeron simplex, Polygonum bistortoides, Geum rossii* and *D. cespitosa* were collected in 2011 and 2012. Both *Deschampsia* and *Trisetum* belong to the family Poaceae. *Erigeron* and *Artemisia* are members of the Asteraceae family while *Polygonum* belongs to Polygonaceae and *Geum* belongs to Rosaceae. Each of these seed families are reported to share common physiological dormancy (Baskin and Baskin, 2004). Preliminary germination trials suggest that all species, with the exception of *P. bistortoides*, exhibit germination rates between 15 and 52% without the use of any dormancy-breaking treatment (after a few months of dry, room-temperature storage) (Farrer, unpublished data). *P. bistortoides* requires 3–4 weeks of cold, moist stratification for germination (germination rate was 0% if not cold, moist stratified) (Farrer, unpublished data). All the seeds with the exception of *A. scopulorum* were kept refrigerated before the experiment; however our seeds were not stored moist in the refrigerator, which is likely why we observed 0% germination for *P. bistortoides*.

The seeds were collected from plants in a moist meadow mainly composed of grasses and forbes in the alpine tundra at the Niwot Ridge Long Term Ecological Research (LTER) site located 35 km west of Boulder, Colorado. The site is located between 3297 and 3544 m above sea level, with a mean temperature for the winter of −8 °C and the mean temperature for the summer of 13 °C. In addition, the site remains under snow cover 9–10 months each year (Dean et al., 2014).

Seeds were removed from infructescences and the lemma and palea were removed prior to surface sterilization. Sixty seeds of each species in small batches were surface sterilized in microcentrifuge tubes with 1 mL of 95% ethanol. Tubes were vortexed briefly to disperse seeds evenly and left to stand for 5 min. Ethanol was removed and 1 mL of 0.5% Clorox bleach (5.25% NaOCl; Clorox Company, Oakland, CA) was added for 5 min. This was followed by three rinses in 1 mL of distilled autoclaved water. Seeds were allowed to stand in sterile water for three additional minutes. To determine if the surface sterilization procedure was effective, seeds were tapped briefly on sterile malt extract agar (MEA) (Difco Laboratories, Detroit, MI) and incubated at room temperature. No fungi grew on these MEA plates.

Five sterilized seeds were plated on MEA with streptomycin and tetracycline (50 mg mL^{-1}) to limit bacteria contamination. A total of 60 seeds per plant species were plated (5 seeds/12 plates). The seed lemma and palea were removed prior to sterilization but entire seeds were incubated for the isolation of putative endophytes. Plates were incubated at 25 °C and evaluated daily for hyphal growth for 68 d. Fungal colonies were transferred to new MEA plates to obtain pure cultures.

2.2. Seed viability

Alpine tundra seed viability was measured using tetrazolium (MP Biomedicals LLC. Solon, OH) following a protocol by Elias et al. (2006). Fifty seeds of each plant species (*T. spicatum, A. scopulorum, E. simplex, G. rossii, P. bistortoides, D. cespitosa*) were placed in microcentrifuge tubes with 1 mL of dH_2O and allowed to soak overnight to hydrate. *T. spicatum, P. bistortoides, D. cespitosa* seeds were punctured with a needle and dissected longitudinally to allow exposure to the tetrazolium solution. Filter paper was soaked with a 1% (m/v) tetrazolium solution, seeds were placed on paper and incubated at 35 °C for 3 h. After incubation, seeds were examined under a dissecting microscope to evaluate viability. Seeds stained red were counted as viable and the colorless seeds were considered dead.

2.3. DNA extraction, PCR, and sequencing

Fungal DNA was extracted from pure cultures grown on MEA using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's protocols. DNA was amplified using ITS rRNA fungal specific primers, ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC-3′) (Gardes and Bruns, 1993; White et al., 1990). PCR was conducted in 25 μL reactions containing the following: 6.5 μL of nuclease free water, 1 μL of ITS1F (5 μM), 1 μL ITS4 (5 μM), 3 μL of 1% BSA (Sigma-Aldrich, St. Louis, MO), 12.5 μL of PCR Master Mix (Promega, Madison, WI), and 1 μL of the DNA sample. Reactions were amplified under the following conditions: 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, then annealing at 50 °C for 30 s with an extension at 72 °C for 45 s, and a final extension of 72 °C for 7 min. Gel electrophoresis with 1% agarose in Tris Acetate EDTA was used to verify the PCR reaction, PCR samples (3 μL DNA and 2 μL dye) were compared to a low DNA mass ladder (Invitrogen Corporation, Carlsbad CA) to determine fragment size and concentration for sequencing reactions.

Prior to sequencing, PCR products were cleaned using ExoSAP-IT (Affymetrix, Cleveland, OH) following the manufacture's protocol. Sequences were prepared using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with ITS rRNA fungal specific primers for forward (ITS1F) and reverse sequences (ITS4). Each sequencing reaction contained the following: 5.5 μL of nuclease free water, 1.5 μL of BigDye sequencing buffer (Applied Biosystems, Foster City, CA.), 1 μL of ITS1F (3 μM), 1 μL of ITS4