Nutritional profile and effects on fungal growth of yeast symbionts associated with North American spruce beetle (Dendroctonus rufipennis)

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Abstract

We characterized yeasts isolated from the integument of North America spruce beetle (Dendroctonus rufipennis Kirby) across four populations, using molecular methods to identify 59 representative isolates. Seven yeast species were detected; Wickerhamomyces canadensis (Wickerham) Kurtzman et al. (Saccharomycetales: Saccharomycetaceae) was the most common (74% of isolates) and found in all populations. Isolates of W. canadensis were subsequently tested for in vitro competitive interactions with symbiotic (Leptographium abietinum) and pathogenic (Beauvaria bassiana) fungi, and isolates were nutritionally profiled (N and P content). Three key findings emerged: (1) exposure to yeast headspace emissions had isolate-dependent effects on colony growth of symbiotic and pathogenic fungi; most isolates of W. canadensis slightly inhibited growth rates of symbiotic (L. abietinum) and entomopathogenic (B. bassiana) fungi. (2) A single volatile (2-phenylethanol) was produced by W. canadensis on growth media and was emitted at an average rate of 1.47 µg·mg⁻¹·yeast·min⁻¹. Exposure to vapors of synthetic 2-phenylethanol reduced growth rates of both L. abietinum and B. bassiana by 36% on average. (3) W. canadensis cultures were high in both protein (0.8%) and phosphorous (7.2%) in comparison to spruce phloem, but isolates varied in nutritional content with the most nutrient-rich strains isolated from populations in outbreak or post-outbreak population phases. We conclude that W. canadensis is a primary yeast symbiont of D. rufipennis in the southern Rocky Mountains and may serve as a source of volatile emissions that can affect growth of associated microbes. Although W. canadensis has higher nutritional content than spruce tree phloem, the concentration of limiting nutrients (protein and phosphorous) is less than reported for the symbiotic fungus L. abietinum, and variation in nutritional content may be associated with beetle population density.

Introduction

Yeasts are frequently associated with insects and play a variety of important functional roles in these associations (Ganter 2006). Yeasts can provide nutritional benefits (Sasaki et al. 1996), produce volatile compounds that act as insect infochemicals (Becher et al. 2018, Herrera et al. 2013) or fumigants with antibiotic properties (Davis et al. 2011), and yeasts may alter habitat suitability by regulating microbial interactions, substrate decomposition, and substrate chemistry (Ganter et al. 1986, Foster and Fogleman 1989). Through these effects, insect-symbiotic yeasts have consequences for insect demography and may influence vitality rates including fecundity, lifespan, and survivorship (Becher et al. 2012, May et al. 2015). Although insects and their feeding behaviors contribute to the dispersal and diversity of yeast communities (Stamps et al. 2012), many of these associations are somewhat incidental and related to the abundance of particular yeast species in the local environment. However, for some plant-feeding insect taxa that utilize nutritionally poor substrates (e.g., Homoptera, many Coleoptera), specific yeast taxa are associated with specialized anatomical structures, generally ‘mycetocytes’ (internal cells in the digestive tract; Douglas 1989) or ‘mycetangia’ (external secretory glands; Vega and Biedermann 2020), underscoring their important role in the survival of insect species with limited or poor diets. Yet, most insect-yeast associations remain uncharacterized, which may ultimately constrain our ability to understand the mechanisms driving important plant-insect interactions.

In temperate forests of western North America, widespread tree mortality resulting from interactions between mycophagous bark beetles (Coleoptera: Curculionidae) in the genus Dendroctonus and foundation conifer species are among the most conspicuous natural disturbances on the landscape. Dendroctonus beetles are keystone insects that periodically outbreak across large areas, with substantial consequences for ecosystem forest structure, function, and composition (Hicke et al. 2016). Dendroctonus beetles have a unique life history (Six and Bracewell 2015); although most species feed on and reproduce within the subcortical phloem environment, they also rely on associations with a variety of filamentous fungal and bacterial symbionts to provide nutrition (Ayres et al. 2000, Bentz and Six 2006, Bleiker and Six 2007), antibiotics and pathogen defense (Adams et al. 2008, Scott et al. 2008), and reduce concentrations of toxic secondary chemicals in the tree phloem environment (Adams et al. 2013). Although yeasts are well-known as ubiquitous associates of Dendroctonus beetles (Davis 2015) and were among the earliest fungal symbionts to be consistently isolated from bark beetles and their substrates (e.g., Grosmann 1930, Callaham and Shifrine 1960), their ecological functions have not been investigated to the same extent as for filamentous fungi and bacteria and remain mostly untested.

Here, our goal is to characterize the yeast species associated with Dendroctonus rufipennis Kirby, the North American spruce beetle, in the southern Rocky Mountains and test some possible ecological functions of beetle-associated yeasts. Over the last decade, an ongoing outbreak of D. rufipennis caused extensive mortality of alpine spruce forest in the southern Rocky Mountains, with millions of hectares of collective forest mortality occurring from 2010–2020 in the states of Colorado, Utah, and Wyoming (Jenkins et al. 2014).
Consequently *D. rufipennis* populations are a significant concern for ecosystem managers, and insight into factors impacting beetle success could potentially be exploited for integrated pest management.

Earlier studies aimed at describing fungal symbiont communities associated with *D. rufipennis* reported that yeasts were isolated with ~100% frequency (Six and Bentz 2003), but yeasts were not further considered during identification of fungal taxa. Here, we grow this body of work by isolating and identifying yeast associates. Our specific objectives are to (1) isolate and describe the primary yeast species associated with the *D. rufipennis* in the Southern Rocky Mountain region; (2) evaluate interactions between yeasts and beetle-associated filamentous fungi (including symbionts and pathogens); and (3) characterize the nutritional profiles of yeasts isolated from *D. rufipennis*. These findings expand our knowledge of holobiome function in an important forest insect, with potential consequences for understanding complex interactions among tree-killing bark beetles, microbial symbionts, and forest ecosystems.

**Materials And Methods**

**Isolation of yeasts from *D. rufipennis***

*Dendroctonus rufipennis* were collected from four populations in the southern Rocky Mountain region in 2016, including (1) Guanella Pass (Colorado, hereafter CO), (2) Cameron Pass (SB), (3) Togwotee Pass (Wyoming; WY), and (4) Soapstone Basin (Utah, UT; Fig. 1). At the time of sampling regions were in different stages of *D. rufipennis* outbreak; beetles in Guanella Pass (CO) were at an ‘incipient’ pre-outbreak population phase, Togwotee Pass (WY) and Soapstone Basin (UT) were both in an active outbreak, and Cameron Pass (CO) was at a post-outbreak phase, though populations were still relatively large. In each population the primary host tree of *D. rufipennis* is Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.), though in some locations Colorado blue spruce (*Picea pungens* Engelm.) is also present. Blue spruce is a resistant host (Ott et al. 2021) that often occurs in different micro-locations than Engelmann spruce (blue spruce is found mostly in drainages and mesic sites and is rarely colonized by *D. rufipennis*, whereas Engelmann spruce is more often found in mountain pass regions, at more xeric sites, and in contiguous stands). Accordingly, we only sampled *D. rufipennis* from Engelmann spruce in the present study.

We located trees with outward evidence of natural infestation, including visible pitch tubes, sawdust/frass, woodpecker flaking, and/or evidence of crown decline. A hatchet was used to remove bark and expose egg gallery sections; live adult *D. rufipennis* were collected from galleries and were immediately placed into individual 10 ml glass sample vials using sterile forceps (Aukema et al. 2005). Forceps were cleaned with ethanol between uses. Vials containing *D. rufipennis* were placed on ice and returned to the lab for isolation.

Yeasts were isolated from the *D. rufipennis* integument onto 2% malt extract agar (MEA) amended with gentamicin sulfate (MilliporeSigma, St. Louis, MO, USA) in a sterile laminar flow hood by pressing individual beetles into growth media; a single beetle was pressed 5–10 times in a dish. Dishes were inverted and incubated in the dark at 23°C for 72 h. After 72 h, a sterile 5 µl inoculating loop was used to collect yeast growth from the pressings and transfer to a new dish. All (100%) of sampled *D. rufipennis* yielded yeasts. Morphologically similar representative isolates were chosen at random from each population (n = 59 isolates total: Guanella Pass, CO, n = 13; Cameron Pass, SB, n = 15; Soapstone Basin, UT, n = 10; Togwotee Pass, WY, n = 21) for subsequent identification using molecular methods.

**Identification Of Yeast Isolates**

Yeast DNA was extracted using ZR Fungal/Bacterial DNA MiniPreps (Zymo Research Corporation, Irvine, CA). Extractions were performed following manufacturer's protocols. DNA was quantified using a Nanodrop 2000 (ThermoFischer, Inc., Waltham, MA), and was stored at -20°C until use. The D1/D2 domains of the rDNA large subunit were amplified with the NL-1 (forward) and NL-4 (reverse) primers following the protocols of O'Donnell (1993). PCR products were run on 1.5% agarose gels with 0.5X TBE buffer and stained with GelRed (Biotium, Fremont, CA), and bands were visualized using UV light to confirm amplification. PCR products were purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) following manufactures instructions and sequenced at Eurofins (MWG Operon USA, Louisville, KY).

Isolates were sequenced in both directions, and sequences were aligned and manually edited in Geneious Prime (Biomatters, Inc.). A single alignment with all 58 sequences was generated using MUSCLE 3.8.425 (Edgar 2004). Unique haplotypes were identified using DnaSP v5 (Rozas et al. 2003). Yeast species of these unique haplotypes were identified by using the BLAST function in the NCBI blastn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogeny generation, matching sequencing were added as reference sequences.
and a sequence of *Sakaguchia meli* (Genbank # NG 058080) and *Naohidea sebacea* (Genbank # NG 042442) were used as outgroups; a maximum likelihood phylogeny was generated using PhyML (Guindon et al. 2010) and a Bayesian phylogeny was generated using MrBayes 3.2.6 (Heuelsenbeck and Ronquist 2001), both implemented in Geneious Prime using the GTR substitution model. Support for clades in the maximum likelihood phylogeny was assessed using 1,000 bootstrap resamples. Bayesian inferences phylogeny was performed whereby the Markov chain Monte Carlo (MCMC) search was run with four chains for 3,000,000 generations from which 30,001 trees were drawn; the first 6,000 trees were discarded as "burn-in".

**Ecological Profiling Of Yeast Isolates**

We (1) tested effects of exposure to headspace volatiles and direct contact with yeast cells of multiple yeast isolates on growth of symbiotic and pathogenic filamentous fungi (*L. abietinum* and *B. bassiana*), (2) analyzed the headspace volatiles produced by yeast isolates, and (3) subsequently tested effects of exposure to synthetic volatiles identified from yeast headspace on growth of filamentous fungi. Only yeasts identified as matching the sequence profile of *W. canadensis* were used, and only a subset of these isolates were tested in order to manage the size of experiments.

**Test 1: Test of yeast headspace on growth of filamentous fungi.** An *in vitro* experiment was used to test effects of exposure to headspace volatiles of five *W. canadensis* isolates (CO1 and CO3 [Guanella Pass, CO], SB 13 [Cameron Pass, CO], and WY 8 and WT 15 [Togwotee Pass, WY]) on radial growth rates of five isolates of the symbiotic blue stain fungus *L. abietinum* (isolates: C01, C07, and CO11 [Guanella Pass, CO], and WY5 and WY7 [Togwotee Pass, WY]; Davis et al. 2018) and five isolates of the entomopathogen *B. bassiana* (isolates: 14B, 50C, 429DA, GHA, and Spruce 1; Mann and Davis 2020).

Partitioned petri dishes containing 2% MEA with a shared headspace were used for the experiment. On one side of the dish, 10 µl of yeast cell culture was streaked onto media using an inoculating loop; the other side of the dish was inoculated with a 1 x 1 mm plug of agar containing actively growing mycelia of *L. abietinum* or *B. bassiana* in the exponential growth phase. *Wickerhamomyces canadensis* isolates were allowed to establish for 48 h prior to inoculating dishes with filamentous fungi. Each *W. canadensis* isolate x filamentous fungal isolate combination was replicated three times (5 *W. canadensis* isolates x 5 fungal isolates x 2 species x 3 replications = 150 experimental units). Each isolate of *L. abietinum* and *B. bassiana* was also grown in partitioned dishes with no yeast as a control; controls were also replicated three times. Dishes containing *W. canadensis* + fungi (treatments) or fungi alone (controls) were placed in the dark at 23°C and new growth of fungal colonies was traced daily for 10 d or until hyphae reached the end of the dish. Radial growth of fungal colonies in response to yeast headspace was computed by dividing total growth distance by the number of days (mm/day). Growth rates of replicates in the ‘treatment’ category were standardized to the mean growth rate of isolates in the ‘control’ category such that colony growth was analyzed as a percent change relative to the control growth rate for each *L. abietinum* and *B. bassiana* isolate.

**Test 2: Test of contact between yeast and filamentous fungi.** A second experiment was performed to test (1) whether exposure to *W. canadensis* cultures arrest growth of *L. abietinum* via inhibition, and (2) whether *L. abietinum* was able to grow over media occupied by *W. canadensis*. In this test the same *W. canadensis* x *L. abietinum* combinations were used and replicated in triplicate, but the following conditions differed (1) both microorganisms were plated at the same time rather than allowing *W. canadensis* to establish first, and (2) petri dishes with no partition were used to allow physical contact among isolates as well as a shared headspace. *Wickerhamomyces canadensis* isolates were inoculated onto dishes by streaking 1/3rd of the dish, and *L. abietinum* isolates were inoculated as in the first test. Growth of *L. abietinum* was traced daily for 10 d, and the following categorial responses were recorded: (1) whether *L. abietinum* cultures reached *W. canadensis* cultures during to test period (scored as ‘yes’ or ‘no’), and (2) whether *L. abietinum* cultures grew over media occupied by *W. canadensis* (scored as ‘yes’, ‘no’, or ‘inhibited’ in the case that the first condition was scored as a ‘no’).

**Test 3: Collection and analysis of yeast headspace volatiles.** Headspace volatile emissions were sampled from ten isolates using a modified dynamic headspace system (Heath and Manukian 1992), as previously described for yeast-like microbial species (Davis et al. 2012). All isolates used in this analysis were from the same population (Guanella Pass, Colorado, USA). Briefly, isolates were prepared by streaking 10 µl of pure cell culture onto 8.5 cm diam petri dishes containing 2% MEA and incubated for 72 h in the dark. To trap headspace volatiles, individual petri dishes containing cultures were placed, open, inside of a 3-L glass chamber with an entry and exit port and charcoal-filtered nitrogen was passed through the chamber at a rate of 50 ml/min for 100 min. Nitrogen exiting the chamber passed through a collection trap consisting of a glass tube containing 10 mg of an adsorbent polymer (HayeSep Q). Traps
were eluted with 250 µl of GC-grade hexane (> 99%) and concentrated to a final volume of ~ 10 ml under a stream of nitrogen. System blanks were also collected using petri dishes containing 2% MEA only (no yeast culture) as a control.

A 1 µl aliquot of each (n = 10) concentrated eluant was manually injected onto a gas chromatograph (Agilent 7820A) coupled to a mass spectrometer (Agilent 5977B Mass Selective Detector) equipped with an HP5 ultra-inert column (dimensions: 30 m × 250 µm × 0.25 µm film thickness). The GC was operated in split mode (1:1) with helium as the carrier gas at a flow rate of 1.2 ml/min, and a front inlet temperature of 250°C and foreline pressure of 6.90 kPa. The temperature program was as follows: initial temperature 40°C then increasing by 10°C/min until 250°C and a final temperature hold time of 5 min. Total ion chromatograms were integrated using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA) and compounds in extracts were putatively identified by comparing mass spectra to those in the National Institute of Standards and Technology (NIST 2017) mass spectral library. Compounds of interest were subsequently confirmed and quantified by comparing peak areas in samples to authentic standards at known concentrations.

Test 4: Test of synthetic volatiles isolated from yeast headspace on growth of filamentous fungi. A second experiment was performed to evaluate effects of the primary volatile identified in yeast headspace (2-phenylethanol). Instead of inoculation with yeast, one side of the partitioned dish was left empty and a 2 ml vial containing 1 ml of 2-phenylethanol stoppered with ~ 0.1 g cotton (concentration in test arena: 1% v/v). Radial growth of each L. abietinum and B. bassiana isolate was also tested in triplicate in response to 2-phenylethanol.

Test 5: Nutritional Profiling of Yeast Isolates. A total of nine isolates were selected for nutritional profiling; isolates were selected based on species identifications, and only yeasts identified as matching the sequence profile of Wickerhamomyces canadensis (the dominant yeast in our sample) were used in nutritional analysis. The rationale was that this approach would yield information on interspecific variation in a common yeast symbiont, relative to beetle population phase.

Selected isolates were prepared for analysis by lysing. Yeast cells were collected from media and suspended in Y-PER (Thermo Fisher, Waltham, MA, USA) at a ratio of 5 µL reagent per mg culture. Lysate was collected per reagent protocol- suspensions were agitated at room temperature for 20 min before centrifugation at 14,000 × g for 10 min. Supernatant was reserved for analysis.

Phosphorous content. Total phosphorus was measured using an adaptation of the ascorbic acid method (Pisal 2010). In brief, reagent solutions of ammonium molybdate (6.00 g ammonium molybdate tetrahydrate, 0.24 g antimonyl potassium tartrate, 5.00 g ammonium sulfamate, 120 mL sulfuric acid diluted to 500 mL with deionized H2O), and ascorbic acid (7.2 g ascorbic acid in 100 mL dH2O) were combined at a ratio of 5:1. Samples were prepared by combining 7 µL of ammonium molybdate-ascorbic acid with 93 µL of sample. A standard curve was generated using a 2.0 mg/L phosphate ion solution. Samples were incubated for 15 min at room temperature before measurement of absorbance at 880 nm using a UV-Vis spectrophotometer (Jasco V-730, JASCO, Inc., Easton, MS, USA).

Protein content. Total protein content of collected lysates was measured using a commercially available bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher, Waltham, MA, USA) according to manufacturer’s instructions.

Data analysis

Test 1: Test of yeast headspace on growth of filamentous fungi. A two-way ANOVA model was used to analyze the partitioned petri dish experiment testing the fixed effects of ‘treatment’ (W. canadensis isolate), fungal isolate (L. abietinum or B. bassiana) and a treatment × fungal isolate interaction on the response of mean percent change in L. abietinum and B. bassiana radial growth rates.

Test 2: Test of contact between yeast and filamentous fungi. A chi-square test was used to analyze the conditional outcomes of direct physical exposure to W. canadensis colonies on the ability of L. abietinum to (1) reach yeast colonies during the growth period (yes or no) and (2) grow over yeast colonies (yes, no, or inhibited).

Test 3: Analysis of yeast headspace volatiles. The mean and standard error of volatile compounds collected from yeast isolates are reported in units of µg compound per mg yeast per minute (µg-1 mg-1 min-1).

Test 4: Test of synthetic volatiles isolated from yeast headspace on growth of filamentous fungi. To test effects of exposure to yeast headspace volatiles, the percent change in mean relative growth rates of L. abietinum and B. bassiana (i.e., relative to control growth rates) were pooled across replicates within each species and compared to zero using a two-tailed Student’s t-test.
Test 5: Nutritional profiling of yeast isolates. Two-way ANOVA models were used to test the fixed effects of isolate identity and source population on variation in the response of mean phosphorous and protein content of yeast tissues. Post-hoc tests (Tukey's HSD test) were applied to make all pairwise comparisons of means.

Results

Identification of Yeast Isolates

Seven unique haplotypes were identified out of the total 58 isolates that were sequenced at the rDNA large subunit. Each of the 7 haplotypes were identified as different species using NCBI BLAST and this was verified in the phylogenetic analyses (Table 1). Some BLAST results highlighted two species within the top 5 hits, therefore these two species were included in the phylogeny (Table 1; Fig. 2). Most sequences had 100% identity to a single yeast species which also clustered together in the phylogeny. However, from the combined BLAST and phylogenetic analyses, we found one haplotype that may represent an undescribed species (Haplotype 3). Sequence identity of Haplotype 3 was a close match to both *Peterozyma toletana* and *P. xylosa*. The phylogeny also showed that *P. toletana* and *P. xylosa* were more closely related to each other (Fig. 2).

<table>
<thead>
<tr>
<th>Yeast isolate IDs</th>
<th>D1/D2 haplotype</th>
<th>Blast Results (species: query coverage: e-value: percent identity)</th>
<th>GenBank #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1, CO2, CO3, CO4, CO7, CO8, CO9, CO10, CO11, CO12, CO13, CO16, SB1, SB2, SB3, SB7, SB8, SB9, SB10, SB11, SB13, SB14, SB15, UT3, UT7, UT8, UT13, UT15, UT16, UT18, UT23, WY1, WY3, WY6, WY8, WY9, WY11, WY12, WY15, WY16, WY17, WY18, WY19, WY22, WY23</td>
<td>Hap1 <em>Wickerhamomyces canadensis</em>: 100%: 7e-153: 98.12%</td>
<td>ON783877</td>
<td></td>
</tr>
<tr>
<td>CO5, SB5, SB6, SB12, UT17, WY9</td>
<td>Hap2 <em>Candida piceae</em>: 100%: 9e-148: 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ogataea cecidiorum</em>: 100%: 5e-159: 98.77%</td>
<td>ON783878</td>
<td></td>
</tr>
<tr>
<td>WY22</td>
<td>Hap3 <em>Peterozyma toletana</em>: 100%: 6e-161: 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Peterozyma xylosa</em>: 100%: 5e-159: 99.83%</td>
<td>ON783879</td>
<td></td>
</tr>
<tr>
<td>WY14</td>
<td>Hap4 <em>Kuraishia capsulata</em>: 100%: 9e-157: 99.12%</td>
<td>ON783880</td>
<td></td>
</tr>
<tr>
<td>UT2, WY9.2, WY13</td>
<td>Hap5 <em>Yamadazyma tenuis</em>: 99%: 5e-159: 100%</td>
<td>ON783881</td>
<td></td>
</tr>
<tr>
<td>WY5</td>
<td>Hap6 <em>Candida oregonensis</em>: 100%: 4e-180: 100%</td>
<td>ON783882</td>
<td></td>
</tr>
<tr>
<td>WY15.2, WY16.2</td>
<td>Hap7 <em>Cystobasidium minutum</em>: 100%: 5e-159: 100%</td>
<td>ON783883</td>
<td></td>
</tr>
</tbody>
</table>

The most commonly species identified was *Wickerhamomyces canadensis* (formerly *Pichia canadensis*) that represented 43/58 total isolates (74%). Only two species were found in all locations, *W. canadensis* and *Candida piceae*, though *C. piceae* was only represented 10% (6/58) of the total isolates. The five additional yeast species were found infrequently. One isolate of *Yamadazyma tenuis* was
collected each from Utah and Wyoming. Wyoming beetles has the greatest diversity of yeast species that included all 7 yeast species (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Candida oregonensis</th>
<th>Candida piceae</th>
<th>Yamaadzyma tenuis</th>
<th>Cystobasidium minutum</th>
<th>Kuraishia capsulata sp.</th>
<th>Peterozyma sp.</th>
<th>Wickerhamomyces canadensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanella Pass, CO</td>
<td>13</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Cameron Pass, CO</td>
<td>15</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Soapstone Basin, UT</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Togwotee Pass, WY</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>total</td>
<td>59</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>43</td>
</tr>
</tbody>
</table>

Test 1: Test of yeast headspace on growth of filamentous fungi. Exposure to yeast headspace had significant effects on the radial growth rate of *L. abietinum* colonies (whole model: $F_{24,50}=3.703, P<0.001$). There was evidence that the main effect *W. canadensis* isolate ($F_{4,50}=4.626, P=0.003$) and the main effect of *L. abietinum* isolate affected the response of mean (%) difference in *L. abietinum* radial growth rates (relative to control) ($F_{4,50}=10.845, P<0.001$). However, there was no evidence of a *W. canadensis* isolate × *L. abietinum* isolate interaction ($F_{16,50}=1.678, P=0.080$). On average, exposure to *W. canadensis* volatiles reduced *L. abietinum* colony growth by -3.0% (range: -29.8% to +88.6%), and this reduction differed from zero ($t_{74}=-1.687, P=0.047$). Headspace of two *W. canadensis* isolates (CO1 and WY15.1) had slight positive effects on mean radial growth of *L. abietinum*, whereas two had slight negative effects (CO13 and WY8), and one isolate had strong negative effects on mean radial growth rates (SB 13; Table 3).

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Population phase</th>
<th><em>Leptographium abietinum</em> isolate (blue stain fungi)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CO 1</td>
<td>CO 7</td>
</tr>
<tr>
<td><em>W. canadensis</em>, CO 1</td>
<td>incipient</td>
<td>-8.66 ± 4.09</td>
<td>12.80 ± 4.43</td>
</tr>
<tr>
<td><em>W. canadensis</em>, WY 8</td>
<td>outbreak</td>
<td>-2.99 ± 6.76</td>
<td>4.56 ± 1.09</td>
</tr>
<tr>
<td><em>W. canadensis</em>, WY 15</td>
<td>outbreak</td>
<td>-7.63 ± 4.92</td>
<td>2.02 ± 1.67</td>
</tr>
</tbody>
</table>

a approximate spruce beetle population phase at the time yeast isolates were collected

Exposure to yeast headspace also had significant effects on the radial growth rate of *B. bassiana* colonies (whole model: $F_{24,50}=8.280, P<0.001$). There was no evidence that *W. canadensis* isolates differentially affected *B. bassiana* growth rates ($F_{4,50}=2.003, P=0.108$); however, *B. bassiana* isolates varied substantially in their responses to *W. canadensis* headspace ($F_{4,50}=45.510, P<0.001$). There was no evidence of a *W. canadensis* isolate × *B. bassiana* isolate interaction ($F_{16,50}=0.542, P=0.910$). On average, exposure to *W. canadensis* headspace had significant effects on the radial growth rate of *B. bassiana* colonies (whole model: $F_{24,50}=8.280, P<0.001$). There was no evidence that *W. canadensis* isolates differentially affected *B. bassiana* growth rates ($F_{4,50}=2.003, P=0.108$); however, *B. bassiana* isolates varied substantially in their responses to *W. canadensis* headspace ($F_{4,50}=45.510, P<0.001$). There was no evidence of a *W. canadensis* isolate × *B. bassiana* isolate interaction ($F_{16,50}=0.542, P=0.910$). On average, exposure to *W. canadensis* headspace had significant effects on the radial growth rate of *B. bassiana* colonies (whole model: $F_{24,50}=8.280, P<0.001$). There was no evidence that *W. canadensis* isolates differentially affected *B. bassiana* growth rates ($F_{4,50}=2.003, P=0.108$); however, *B. bassiana* isolates varied substantially in their responses to *W. canadensis* headspace ($F_{4,50}=45.510, P<0.001$).
**Table 4**
Mean percent change (± standard error) in radial growth rate of *B. bassiana* isolates (entomopathogenic fungus; white muscardine disease) in response to volatiles from *W. canadensis* (yeast) headspace in a partitioned petri dish experiment. Percent changes are relative to the mean control growth rate for each *B. bassiana* isolate (i.e., grown in the absence of yeast volatiles). Lettering in the ‘mean’ row denotes Tukey’s HSD test for main effects of *B. bassiana* isolate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Population phase</th>
<th><em>Beauvaria bassiana</em> isolate (entomopathogen)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14B</td>
<td>50 C</td>
</tr>
<tr>
<td><em>W. canadensis</em>, CO 1</td>
<td>incipient</td>
<td>2.90 ± 1.45</td>
<td>-4.12 ± 1.47</td>
</tr>
<tr>
<td><em>W. canadensis</em>, CO 3</td>
<td>incipient</td>
<td>0.00 ± 2.51</td>
<td>-11.50 ± 2.55</td>
</tr>
<tr>
<td><em>W. canadensis</em>, WY 3</td>
<td>outbreak</td>
<td>6.12 ± 5.38</td>
<td>-14.45 ± 1.47</td>
</tr>
<tr>
<td><em>W. canadensis</em>, WY 8</td>
<td>outbreak</td>
<td>1.45 ± 1.41</td>
<td>-15.93 ± 5.10</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.96 ± 1.41</td>
<td>-10.91 ± 1.60</td>
</tr>
</tbody>
</table>

* a approximate spruce beetle population phase at the time yeast isolates were collected

* b commercially available strain

**Test 2: Test of contact between yeast and filamentous fungi.** In 84% of trials, *L. abietinum* cultures were able to grow to make contact with *W. canadensis* cultures, and there was no difference in the frequency with which fungal cultures grew to contact with yeast across the yeast isolates ($χ^2 = 7.540, P = 0.110, df = 4, N = 75$), indicating no evidence for a zone of inhibition among *W. canadensis* isolates. However, there was evidence that yeast isolates varied in their ability to maintain resource space from *L. abietinum* cultures and there was no difference in the frequency with which fungal cultures grew to make contact with yeast across the yeast isolates ($χ^2 = 26.379, P < 0.001, df = 8, N = 75$): *L. abietinum* grew over yeast cells in 49% of trials, was prevented from growing over yeast cells in 34% of trials, and never made contact with yeast cells in the remaining 16% of trials (Fig. 3).

**Test 3: Analysis of yeast headspace volatiles.** The single primary component of yeast headspace was 2-phenylethanol and was emitted by yeast isolates at an average rate of $1.47 ± 71$ SE mg$^{-1}$ per mg$^{-1}$ yeast per min$^{-1}$ (Fig. 4). Other minor components identified in headspace included styrene and butane, but comparison with system blanks revealed these products were off gasses from petri dishes or growth media. Accordingly, abundances of these compounds are not reported and were not directly tested.

**Test 4: Test of synthetic volatiles isolated from yeast headspace on growth of filamentous fungi.** Exposure to 2-phenylethanol at a concentration of 1% (v/v) uniformly reduced growth rate of *L. abietinum* by 36% on average, and this difference was different from zero ($t_{14} = -7.728, P < 0.001$). However, *L. abietinum* isolates varied in their responses and some were more tolerant to 2-phenylethanol than others, with the greatest negative effects on isolates from an active outbreak population (Togwotee Pass, WY). Similarly, exposure to 2-phenylethanol uniformly reduced growth rate of *B. bassiana* by 36% on average, and this difference was also different from zero ($t_{14} = -10.199, P < 0.001$; Fig. 5).

**Test 5: Nutritional profiling of yeast isolates.** Mean phosphorous and nitrogen content of *W. canadensis* was 7.2 and 0.8% per unit mass, respectively. There was evidence that mean phosphorous content of *W. canadensis* tissues varied significantly due to the effects of isolate identity ($F_{8,95} = 71.457, P < 0.001$) and population ($F_{2,101} = 24.355, P < 0.001$), with isolates from Togwotee Pass (WY) and Cameron Pass (CO) exhibiting higher mean phosphorous concentrations than isolates from the Guanella Pass (CO) region (Fig. 6a). Similarly, there was evidence that mean protein content of *W. canadensis* tissues varied significantly due to the effects of isolate identity ($F_{8,97} = 36.491, P < 0.001$) and population ($F_{2,103} = 51.019, P < 0.001$). All three populations differed from one another in terms of *W. canadensis* volatiles reduced *B. bassiana* colony growth rate by -6.1% (range: -35.4% to +16.5%), and this reduction differed from zero ($t_{14} = -4.263, P < 0.001$). Exposure to *W. canadensis* volatiles had slight positive effects on growth response of three *B. bassiana* isolates (14B, 429DA, and GHA), while two isolates (50C and Spruce 1) were strongly negatively affected (Table 4).
protein concentration, and isolates from Togwotee Pass (WY) had more protein on average than isolates from Cameron Pass (CO) and Guanella Pass (CO), respectively (Fig. 6b). Subsequent correlation analysis indicated that phosphorous and protein content were not correlated in W. canadensis isolates (Pearson’s $r = 0.416, P = 0.264$).

**Discussion**

Yeasts are extremely common associates of tree-feeding bark beetles; most yeast assemblages in the bark beetle holobiome are poorly understood but may have important ecological effects on beetles or microbial communities they interact with (Davis 2015). Earlier reports estimated that nearly 100% of sampled D. rufipennis were associated with yeasts and yeasts were even more common than symbiotic filamentous fungi (i.e., L. abietinum, Six and Bentz 2003), but until now these yeasts have not been further characterized. Here, seven yeast species were isolated from the integument of D. rufipennis. Wickerhamomyces canadensis was the most frequently encountered species (74% of isolates) and was collected from all sampled populations and across a range of population phases (i.e., incipient/low-density, outbreak/high-density, or post-outbreak), indicating that W. canadensis is the most abundant yeast on the beetle exoskeleton in the southern Rocky Mountains. Given the ubiquity of yeast-bark beetle associations and the prevalence of W. canadensis in the microbial assemblage of D. rufipennis, a better understanding of W. canadensis ecology could provide insight into the interactions that impact spruce beetle performance across regions and population phases.

Headspace volatiles produced by cultures of W. canadensis had inhibitory effects on both symbiotic (L. abietinum) and pathogenic (B. bassiana) fungi, but on average these effects were relatively minor (8 and 6% reduction to mean colony growth rates, respectively). Wickerhamomyces canadensis isolates collected from beetle populations in a post-outbreak phase (Cameron Pass, CO) had the most inhibitory effects on growth of symbiotic fungi (Table 3), though there was no evidence of variation among isolates in terms of their inhibitory effects on pathogenic fungi (Table 4). However, there was considerable variation among isolates of pathogenic fungi in their responses to W. canadensis, and an isolate collected from Englemann spruce (Spruce 1; Mann and Davis 2020) was strongly negatively affected by W. canadensis headspace volatiles. In a subsequent assay testing contact between W. canadensis and L. abietinum, we found little evidence for ‘contact zone inhibition’ by W. canadensis, but cultures were able to exclude L. abietinum from colonizing resource space in more than half of trials (Fig. 3). This outcome contrasts with the findings from earlier studies (e.g., Davis et al. 2011), where yeasts (Ogataea pini, formerly Pichia pini) associated with the southwestern pine beetle (Dendroctonus barbieri Hopkins) were strongly inhibitory to B. bassiana but readily grew in mixed culture with beetle-symbiotic fungi (Entomocorticium sp.).

A single compound (2-phenylethanol) was identified as the primary volatile metabolite produced by W. canadensis and was consistently emitted by all tested cultures and at a relatively high rate (Fig. 4). 2-phenylethanol is a common fermentation byproduct and has been characterized in the headspace of other ubiquitous yeasts and yeast-like fungi growing on carbon-rich substrates including Saccharomyces cerevisiae, Pichia anomala, and Aureobasidium pullulans (Eshkol et al. 2009, Davis et al. 2012, Hua et al. 2014, and others). Consistent with other studies (e.g., Hua et al. 2014), we report that exposure to 2-phenylethanol is inhibitory to filamentous fungi; here, isolates of both L. abietinum and B. bassiana were similarly negatively affected by synthetic 2-phenylethanol (~36% reduction in growth rate for both filamentous fungal species). In the subcortical gallery environment of a tree colonized by spruce beetles and associated microbes, both symbiotic and pathogenic fungi would also be exposed to a suite of tree secondary metabolites, particularly terpenoids, in addition to yeast metabolites. Terpenoids are extremely toxic to B. bassiana and strongly reduce radial growth (Mann and Davis 2020), while L. abietinum tolerates much higher concentrations (Davis et al. 2018), suggesting that tree secondary metabolites likely mediate D. rufipennis-pathogen and D. rufipennis-symbiont interactions in addition to volatiles produced by W. canadensis.

Aside from their interactions within the microbial assemblage of bark beetles, earlier studies report that associations with yeast broaden the diet breadth of bark beetles by providing access to limiting nutrients that are uncommon in tree phloem, and access to yeast may be a requirement for development (Safranyik et al. 1983). Specifically, nitrogen is a minor component of conifer phloem and in general limits the development of insects in many habitats; in some cases, nitrogen limitations faced by herbivorous insects are overcome via symbiotic associations (Hansen et al. 2020). Wickerhamomyces canadensis had relatively high nitrogen (protein) content (0.8%) in comparison to spruce phloem; for example, phloem nitrogen content of Picea abies (L.) H. Karst is approximately 0.4% by dry weight and may be as low as 0.05% in the sapwood (Lahr and Krokene 2013). The highest mean protein concentrations were found in W. canadensis isolated from D. rufipennis populations that were actively in an outbreak at the time of sampling (Togwotee Pass, WY; Fig. 6), suggesting a potential association between nutritional content of microbial symbionts and beetle population density. Nonetheless, protein content of W. canadensis was about 4-fold lower than reported for the symbiotic fungus L.
indicating that association with both *W. canadensis* and *L. abietinum* is likely more nutritionally beneficial to *D. rupennis* than association with *W. canadensis* alone.

We also detected isolate-to-isolate variation in all measured phenotypic traits of *W. canadensis* including production of 2-phenylethanol (Fig. 4), interactions with filamentous fungi (Table 3 and Fig. 3), and nutritional content (Fig. 6). The level of phylogenetic resolution in our analysis was insufficient to determine whether these differences were associated with genetic variation (Fig. 2), but it seems probable since all tests were done under nearly identical, controlled laboratory conditions (i.e., a constant environment). This phenotypic variation underscores the importance of analyzing microbial traits and interactions at an isolate level, and we expect that associations with specific genetic groups of *W. canadensis* could have consequences for beetle performance by mediating interactions with other symbionts or providing differing nutritional benefits. Although it is difficult to experimentally assess beetle-microbe associations under controlled or repeatable settings, such an approach would be extremely valuable for disentangling the relative contributions of symbionts from their genetic variation to bark beetle population dynamics and could advance basic theory in insect-symbiont ecology.

In summary, multiple saccharomycetous yeasts were isolated from the integument of the North American spruce beetle (*D. rupennis*), but *W. canadensis* was the by far the most frequent. *Wickerhamomycyes canadensis* is likely also present in other substrates (e.g., frass, beetle galleries) and life stages (larvae, pupae), but only adults (the primary life stage associated with movement of microorganisms across habitats) were evaluated here. *Wickerhamomycyes canadensis* had isolate-dependent interactions with the spruce beetle symbiont *L. abietinum* and the entomopathogen *B. bassiana*, and these interactions were likely mediated by production of the volatile compound 2-phenylethanol, a common fermentation byproduct. In addition, isolates likely provide access to nutritional resources (protein and phosphorous) that are extremely limiting in the phloem of coniferous trees, and there appears to be within-haplotype diversity in nutritional benefits provided by *W. canadensis* across beetle populations and population phases. Collectively, these findings add to our knowledge of the factors affecting interactions within the bark beetle microbiome, and may help to elucidate the mechanisms driving or contributing to beetle population dynamics across landscapes.

**Declarations**

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**Author Contributions**

TSD and JES designed the study. TSD collected isolates and performed interaction experiments. JES performed genetic identification of isolates. CVB and CC performed the nutritional analyses. TSD, JES, and CVB analyzed the data. TSD wrote the first draft of the manuscript and all authors contributed to editing the manuscript.

**Conflict of Interest**

The authors declare no conflict of interest

**Ethics Approval**

This work does not contain any studies with human participants or animals performed by any of the authors.

**References**


32. Ott DS, Davis TS, Mercado JE (2021) Interspecific variation in spruce constitutive and induced defenses in response to a bark beetle-fungal symbiont provides insight into traits associated with resistance. Tree Physiol 41:1109–1121

**Figures**
Figure 1

A map of the locations in the western United States where *Dendroctonus rupipennis* samples were collected for yeast isolation. Sampled populations are denoted with crosshatches; dark gray polygons denote areas of mapped forest mortality from *D. rupipennis* from 2012-2016.

Figure 2

Bayesian inference phylogeny based on the D1/D2 large subunit rDNA region of representative yeast haplotypes from cultures isolated from *Dendroctonus rupipennis*. All isolates are listed in Table 1 with corresponding haplotype identification numbers and/or reference accession numbers. Branch support includes posterior probability (PP) and bootstrap support (BS) and indicated as (PP/BS). Colored circles denote location of where species were collected.
Figure 3

The proportion of trials in which *W. canadensis* isolates prevented overgrowth, prevented contact with (zone inhibition), or were overgrown by the symbiotic fungus *L. abietinum* in tests allowing physical contact between the two species.

Figure 4

Gas chromatograph traces comparing headspace volatiles collected from four isolates (*Wickerhamomyces canadensis*) associated with *Dendroctonus rufipennis*. The x-axis shows retention time (RT) in minutes, and the y-axis shows total ion chromatograms (TIC). Traces are offset for ease of visual comparison. All tested isolates were from beetles collected in Guanella Pass, Colorado, USA.
Figure 5

Effects of *in vitro* exposure to vapors of synthetic 2-phenylethanol on change in mean radial growth rate (±SE) of isolates of *Leptographium abietinum* (symbiotic fungus) and *Beauvaria bassiana* (entomopathogenic fungus), relative to control growth rates. Isolate identity is shown on the x-axis.

Figure 6

The distribution of (A) phosphorous and (B) protein concentration for nine yeast isolates (*Wickerhamomyces canadensis*) associated with *Dendroctonus rufipennis* across three regions of Colorado and Wyoming. Lettering represents Tukey’s HSD test; boxplots in each panel not connected by the same letter differ significantly. Beetle populations were at different population densities at the time of microbial sampling with Guanella Pass in an incipient/growth phase, Guanella Pass in a post-outbreak phase, and Togwotee Pass in an active outbreak.