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Article

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Growth characteristics and pathogenic consequences of predominant entomopathogenic Yukon soil fungi *Mortierella alpina* and *Penicillium expansum*, and effectiveness of Met52®, against larvae of the winter tick, *Dermacentor albipictus*

Yoder JA[1], Krieger M[2], Oakley M[3], Trotter J[3], Schmelzer P[1], Niksic A[1], Rodell BM[1], Klever LA[1]

[1] *Department of Biology, Wittenberg University, Springfield, OH, 45501, USA*
[2] *Champagne and Aishihik First Nations, Haines Junction, YT, Y0B 1L0, CAN*
[3] *Oakley Veterinary Services, Haines, AK, 99827, USA*

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Abstract

Water activity-temperature relationships are reported for Yukon soil-isolated strains of *Mortierella alpina* and *Penicillium expansum* that are natural enemies to larvae (infestation stage) of the winter tick *Dermacentor albipictus*. Both fungal strains are psychrotrophs, characterized by low thermal growth ranges, consistent with their occurrence in Yukon soil. In contrast to *P. expansum*, *M. alpina* is more temperature sensitive, has a higher requirement, and is more virulent to tick larvae. Researchers and Biological Control Officers should note that for tick population control these *M. alpina* and *P. expansum* strains grow and sporulate well under moderate temperature and humid conditions. Our experiments used Met52® Bioinsecticide as a positive control, and this is the first study that demonstrates the effectiveness of this commercial product against winter tick larvae.

Key words – Alaska – biological control – Canada – elk tick – horse tick – moose tick – temperature – water activity

Introduction

Mortierella alpina and *Penicillium expansum* were isolated from soil samples collected from Yukon, co-occurring, and appearing in high frequency at multiple different field sites. The importance of these fungi pertains to their entomopathogenic activity against larvae of the winter tick, *Dermacentor albipictus* (sole infestation stage of this parasite), and their potential to act as natural regulators of populations of this tick species in more northern climates. The most virulent fungal strains of natural enemies against ticks are isolated from soils where the tick is established (Greengarten et al. 2011). The purpose of this paper is to report growth characteristics for these Yukon isolates of *M. alpina* and *P. expansum*. Such information is urgently needed.

The problem is that the range of *D. albipictus* is expanding into colder climates. This tick species is broadly distributed throughout Canada, United States, Central America and Mexico

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Corresponding Author: Jay Yoder – e-mail – jyoder@wittenberg.edu

(Durden et al. 2016). This tick appears to be prevalent and carried by mule deer (*Odocoileus hemionus*) (pers. observ. of authors Trotter J & M. Oakley M, pers. comm. Beckmen K, Alaska Department of Fish and Game, Fairbanks, AK), for dispersal through the Yukon, and possibly into Alaska (Zarnke et al. 1990, Leo et al. 2014, Durden et al. 2016). If *D. albipictus* were to become established in Alaska, then this tick could potentially threaten moose (*Alces alces*) populations by debilitating cows (i.e., "ghost moose") and killing calves (Samuel 2004). The importance of this study pertains to providing basic biological information on the habitat requirements of these natural tick regulators that can be used in future studies for examining (i.e., modeling) population dynamics of *D. albipictus*, focusing on the primary ecologic factors that limit fungus growth: temperature and water activity (Carlile et al. 2001).

Ticks become infected by these fungi as a result of spore exposure. Upon contact, spores stick to the outside of the tick, germinate into hyphae, and gain access to inside the tick by penetrating through the mouth, anus, and soft cuticle between leg segments (Fernandes et al. 2012). As the fungus takes over, the tick dies, becomes enveloped within the fungus mycelium, and dries, producing a dried-up tick mummy (Yoder et al. 2017). The tick mummy subsequently serves as a secondary source of spores that can infect ticks that are co-occurring within the same region.

Temperature and water requirements (as defined by water activity, a_w) for growth and sporulation, to be infective to ticks, of these *M. alpina* and *P. expansum* Yukon strains have not been determined. This paper seeks to make a contribution to this area. Our experiments also seek to examine whether dehydration is the mode of action, as a pathogenic consequence of infection of these particular fungi. We are testing the hypothesis that because these fungi originated from the Yukon that these soil isolates of *M. alpina* and *P. expansum* may be modified (i.e., low water activity, low optimal temperature) for low temperature survival.

Materials & Methods

Fungi, ticks, and experimental materials

Mortierella alpina and *Penicillium expansum*, original entomopathogenic Yukon soil isolates are archived at the Department of Biology, Wittenberg University, Springfield, OH (specimen lot WU-6-XI2018), and the University of Cincinnati Microfungus Collection, University of Cincinnati, Cincinnati, OH (specimen lot UC112018). Identification of these fungus isolates was by macroscopic/microscopic characteristics, culture comparison to authentic standards, and molecular identification (University of Alberta Microfungus Collection and Herbarium, Toronto, ON; University of Cincinnati Microfungus Collection, University of Cincinnati, Cincinnati, OH; Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, United States Department of Agriculture, Ithaca, NY). Each isolate was plated on Potato Dextrose Agar (PDA, acidified with lactic acid to pH 5.5), 25 ± 0.5ºC, and darkness for experimentation. *Metarhizium anisopliae* was isolated from Met52® (Novozymes Biologicals, Salem, VA) and was used as the source of *Metarhizium anisopliae* as a control. Each fungus was purified by two rounds of subculturing hyphal tips on fresh media.

An aqueous fungus spore inoculum was prepared using 2% malt agar, scraping spores from 1 month old sporing cultures (n = 4 each) into phosphate buffered saline (PBS, pH 7.5) + 0.05% Tween 20, and adjusting spore concentration to 1.6×10^7 spores/ml (0.05% Trypan blue exclusion; AO Spencer Bright-Line hemocytometer, St. Louis, MO) (Fernandes et al. 2012, Tuininga et al. 2009, Greengarten et al. 2011, Yoder et al. 2017). Control inoculum was PBS (pH 7.5) + 0.05% Tween 20.

Fungus pathogenicity tests were conducted using unfed larvae of *Dermacentor albipictus*. These larvae were identified using keys (Lindquist et al. 2016) and were obtained from mated, fed females (Pekins P, coll., under permit, University of New Hampshire, NH) that had been stored at 93% RH, 10h:14h (Light: Dark, L: D), and 20ºC in mesh-covered 50 ml sterile polypropylene centrifuge tubes for oviposition and hatching (Yoder et al. 2016). Larvae were used at five months of age, consistent with their age in the field during questing (Samuel 2004). Larvae were handled

with an aspirator (polypropylene pipette tip fixed to a piece of Tygon tubing) and soft forceps (Featherweight narrow tip forceps, DR Instruments, Bridgeview, IL). Larvae were in healthy condition, defined by the ability to self-right and crawl five body lengths.

A standard aseptic protocol was used throughout this investigation. All materials and instruments were purchased sterile from the manufacturer (Fisher Scientific, Pittsburgh, PA, unless otherwise noted) or autoclave- (121ºC, 19 psi, 15 min), 95% ethanol- or flame-sterilized before use. All fungus culturing was performed using 100 x 15 mm Petri plates in a laminar flow hood (Cole-Palmer, Vernon Hills, IL).

Growth characteristics

Water activity (a_w) was adjusted using PDA + [variable] glycerol (> 99.5 pure, unopened bottle, Sigma Chemicals, St. Louis, MO) as described by Rousseau & Donèche (2001). Water activity was measured with a Thomas hygrometer (SD ± 0.005% a_w, Philadelphia, PA). A 0.5 cm^2 plug of fungus was taken from the white advancing edge of a 1 week old mycelium. The 0.5 cm^2 plug was placed at the center of the Petri plate on solidified media, over top the intersection of four lines that had been scored as a "+" across the bottom of the plate, dividing the plate into quadrants. As the mycelium spread over the agar surface, five measurements were taken on each of the four lines as a function of time. The experiment was stopped after ten days according to standard practice (Tuininga et al. 2009).

The equation $K_r = (R_1 - R_0)/(t_1 - t_0)$ was used to calculate the radial growth rate, where K_r is the radial growth rate, R_1 and R_0 are colony radii at the beginnings of the linear (t_0) and stationary (t_1) phases of growth (Baldrian & Gabriel 2002). This experiment was replicated three times, and the radial growth rate was expressed as mm/day (n = 3 replicates 60 measurements each). Data are the mean ± SE. Radial growth rates were determined at various temperatures (Percival programmable incubator, SD < ± 0.5ºC). Radial growth rates were compared with an analysis of covariance (ANCOVA, p = 0.05), and optimization effects were analyzed by response surface methodology (JMP, SAS Institute, Cary, NC).

Impact on survivorship

Larvae were treated, ten at a time, with 1 ml inoculum in a 1.5 ml polypropylene microcentrifuge tube for 1 min (with gentle agitation), and then each larva was transferred to an individual, clean, mesh-covered microcentrifuge tube and stored at 80% RH (Winston & Bates 1960), 10h:14h L:D, and 20ºC to allow for individual monitoring. Larvae that could not self-right and crawl five body lengths (checked under the microscope at 40/45x) after treatment were not used in the experiment. Every two days, each larva was examined (40/45x) to determine whether it had died, based on appendage immobility, legs curled (namely legs I), and opisthosoma deflated.

Dead larvae were subsequently analyzed by internal fungus culturing for evidence of the treatment fungus (i.e., Koch's postulates, Brown 2007, Tuininga et al. 2009, Greengarten et al. 2011, Fernandes et al. 2012, Yoder et al. 2017). Briefly, the dead larvae were rinsed in a mild bleach solution (18:1:1 v/v/v deionized water: ethanol: 5.25% NaOCl) followed by fresh deionized water, twice for 1 minute each, and a scalpel was used to cut the body into halves. Each body section was embedded into PDA, incubated at 20ºC, darkness, and observed daily (40/45x). Tips of hyphae that could be traced to having originated internally from within tissues of the larvae were subcultured on fresh PDA and incubated (20ºC, darkness) until characteristics appeared for identification (colony obverse/reverse, spore and phialide characteristics at 1000x oil) and pure culture comparison (PDA, 2% malt agar, Czapek Dox agar, cornmeal agar; Samson et al. 1988, Barnett & Hunter 2003).

The total sample size for this experiment was 100 larvae per treatment (n = 10 replicates of 10 larvae each). PBS + 0.05% Tween acted as the control. Data were expressed as the mean ± SE. An ANCOVA (p = 0.05) was used to compare data, using a log-it transformation in the case of percentages and an Abbott correction for mortality. Student's t test ($p < 0.05$) was used to compare

survival times, based on the log rank test and Kaplan-Meier estimate (JMP, SAS Institute, Cary, NC).

Impact on water loss

Larvae were treated with a spore inoculum, or PBS + 0.05% Tween control, as described above, stored individually (80% RH, 10h:14h L:D, 20ºC), and used for this experiment two days later post-treatment. Each larva was then weighed by placing the larva directly (no enclosure) onto the weighing pan of a microbalance (Cahn, SD ± 0.2 μg precision, ± 6 μg accuracy at 1 mg, Ventron Co., Cerritos, CA). This initial mass measurement was taken as the fresh mass (*f*). The larva was then transferred to 0% RH (an. $CaSO_4$, Drierite, W.A. Hammond Drierite Co., Xenia, OH; only % RH where whole organism water loss, respiratory + integumental, is exponential, Wharton 1985), in a 3000 ml glass desiccator at 10h:14h L:D, and 20ºC. Each larva was re-weighed at hourly intervals for a total of five mass readings.

After this series of consecutive mass measurements, the larva was transferred to a 90 ± 0.5ºC drying oven (Blue M. Electric Co., Chicago, IL) and dried until a constant mass was obtained; this mass measurement was taken as the dry mass (*d*). Each mass measurement was represented as a water mass (*m*, amount of body water), by the conversion of subtracting the dry mass. Percentage body water content was calculated by 100% $(f - d)/f$, and the water loss rate ($-kt$) was calculated by $m_t = m_0 \exp(-kt)$, where m_t, is the water mass at any time *t*, and m_0 is the initial water mass (Wharton 1985).

The total sample size was 100 larvae for each water balance characteristic (n = 10 replicates to 10 larvae each/treatment), using an ANCOVA (p = 0.05) to compare data, and a log-it transformation in the case of percentages (JMP, SAS Institute, Cary, NC).

Results

Growth characteristics

The thermal growth range for *M. alpina* was 5-30ºC, with peak growth at 25ºC and 0.997 a_w, and there was no detectable growth at 35ºC (Fig. 1, $p < 0.05$ for each pairwise comparison between two different temperatures per a_w). Spores for *M. alpina* were only observed at 0.997 a_w. There was no detectable growth at 0.96, 0.95, 0.90, 0.85, and 0.80 a_ws (i.e., K_r = 0.00 mm/day).

P. expansum had a 5ºC–35ºC thermal growth range, with no growth detected at 40ºC, and peak growth occurred at 25ºC and 0.98 a_w (Fig. 2, $p < 0.05$ for each pairwise comparison between two different temperatures per a_w). Under all conditions where there was measurable growth, *P. expansum* produced spores. At higher temperatures, sporulation occurred over a range of 0.95-0.997 a_ws, whereas growth, and sporulation, at 5ºC and 10ºC was limited to 0.997 and 0.98 a_ws. No growth occurred at 0.90, 0.85, and 0.80 a_ws; i.e., K_r = 0.00 mm/day.

Impact on survivorship

Survival time was shorter as the result of *M. alpina* treatment (7.1 ± 0.8 days), *P. expansum* treatment (9.2 ± 0.6 days), and *M. anisopliae* treatment (3.7 ± 1.4 days) for 50% of larvae when compared to the saline-treated control (11.4 ± 1.1 days) (Fig. 3, $p < 0.05$ comparing each to the control). *M. alpina* was more virulent than *P. expansum*, based on an increased number of larvae that were killed as well as shorter kill times at and after day 4 post-treatment ($p < 0.05$ for each pairwise comparison between same day). Positive control treatment *M. anisopliae* (commercial product Met52®) produced the greatest number of killed larvae and in the shortest amount of time ($p < 0.05$ for each pairwise comparison among other fungus treatment per day).

Upon internal fungus culture, 81.6 ± 3.1% dead larvae tested positive for *M. alpina* in the *M. alpina* treatment group, 76.2 ± 2.7% tested positive for *P. expansum* having received the *P. expansum* treatment, and 94.4 ± 1.8% tested positive for *M. anisopliae* that were treated with *M. anisopliae*. Any of these fungi were isolated in 9.7 ± 1.3% dead larvae (*M. alpina*, *P. expansum*, combined; no *M. anisopliae* was isolated) in the saline-treated control, and this control value was

significantly lower in frequency than in the *M. alpina*, *P. expansum*, and *M. anisopliae* treatment groups ($p < 0.05$ comparing each fungus treatment to control).

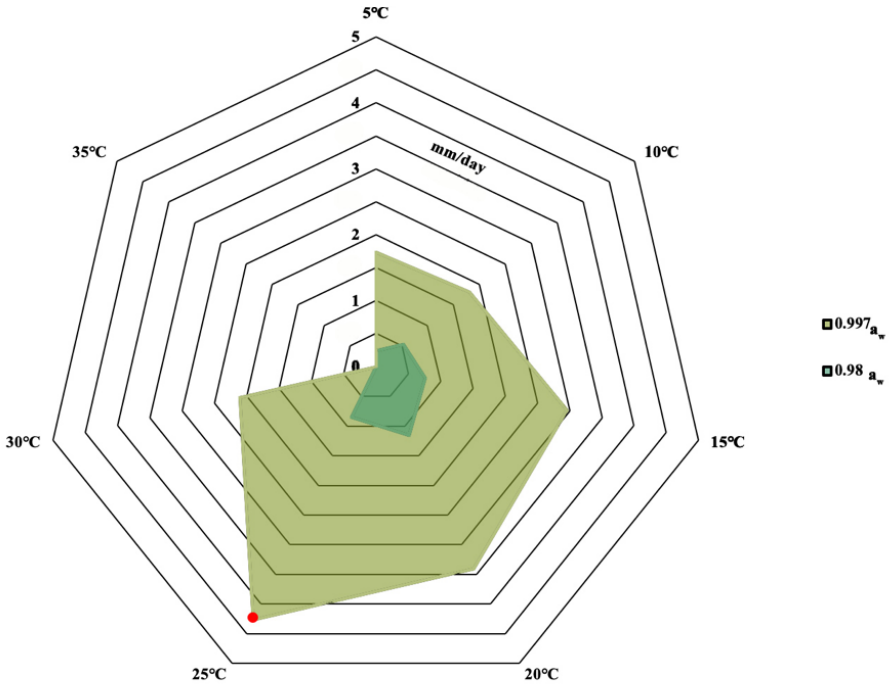

Fig. 1 – Radial growth rate (K_r, mm/day) of *Mortierella alpina* (Yukon strain) as a function of temperature (ºC) and water activity (a_w). The red point on the plot is the optimum growth rate. $n = 3$ replicates of 20 measurements each (the mean ± SE ≤ 0.34).

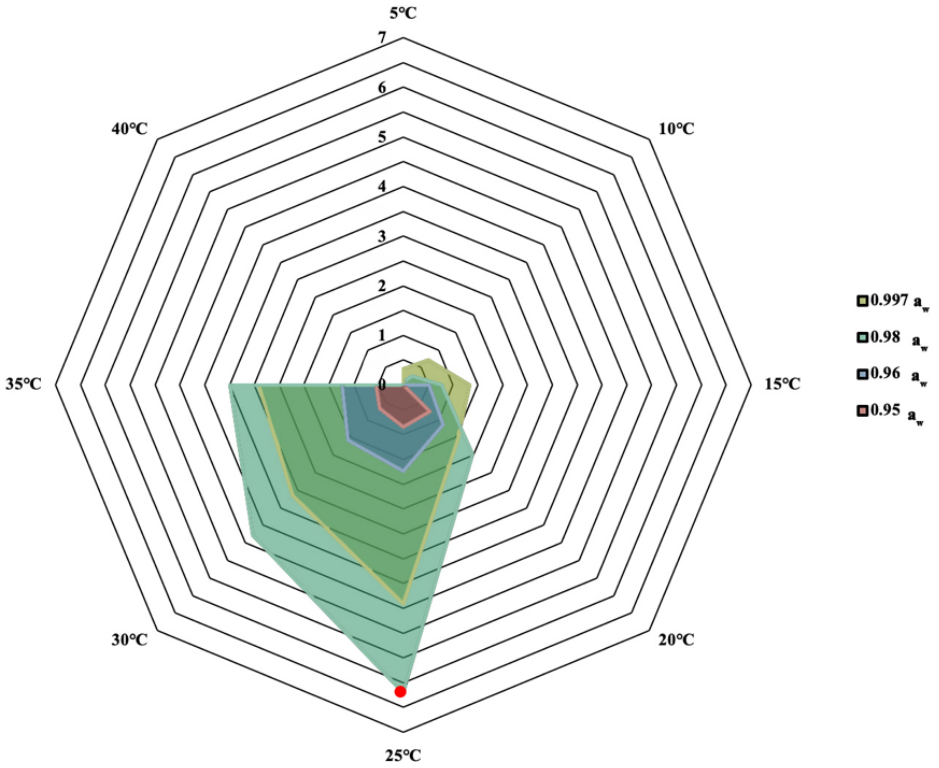

Fig. 2 – Radial growth rate (K_r, mm/day) of *Penicillium expansum* (Yukon strain) as a function of temperature (ºC) and water activity (a_w). The red point on the plot is the optimum growth rate. $n = 3$ replicates of 20 measurements each (the mean ± SE ≤ 0.51).

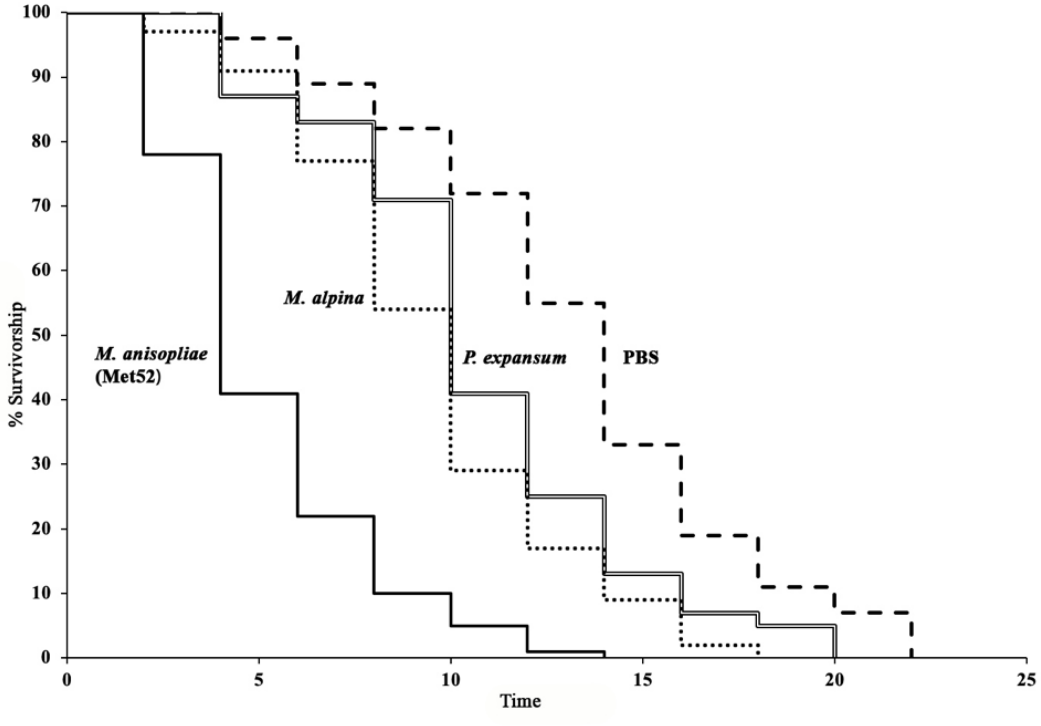

Fig. 3 – Larval survival of *Dermacentor albipictus* post-treatment with *Metarhizium anisopliae* (Met52®), *Mortierella alpina* (Yukon strain), *Penicillium expansum* (Yukon strain), and saline (PBS, phosphate buffered saline + Tween). Each point is the mean of 100 larvae (± SE ≤ 3.2).

Impact on water loss

Treatment groups of larvae had similar body water content and corresponding water:dry mass ratios (Table 1). In all cases, the water mass correlated positively with the dry mass: $R \geq 0.92$ (saline control treatment), $R \geq 0.89$ (*M. anisopliae* treatment), $R \geq 0.93$ (*M. alpina* treatment), and $R \geq 0.89$ (*P. expansum* treatment) ($p < 0.001$).

Significantly higher water loss rates were measured as the result of *M. alpina* treatment compared to the saline-treated control: 3.23 ± 0.03%/h versus 2.06 ± 0.04%/h, respectively ($p < 0.05$, Fig. 4). Similarly, treatment with *P. expansum* (water loss rate 2.98 ± 0.03%/h) and *M. anisopliae* (water loss rate 3.86 ± 0.05%/h) produced significantly higher water loss rates than the saline control ($p < 0.05$ for comparing each fungus treatment). Larvae had the highest water loss as the result of the commercial product *M. anisopliae* (Met52®) treatment compared to the *M. alpina* and *P. expansum* treatments ($p < 0.05$ for comparing each fungus treatment to each other).

Table 1 Water content of unfed larvae of *Dermacentor albipictus* that were used for the various fungus treatment groups, *Metarhizium anisopliae* (Met52®), *Mortierella alpina* (Yukon strain) *Penicillium expansum* (Yukon strain) and phosphate buffered saline (PBS + Tween). Data (the mean ± SE) followed by the same superscript letter within a column do not differ significantly. *n* = 10 replicates of 10 larvae each/ treatment group.

	Larval water balance characteristic, 2 days post-treatment:				
Treatment	**Fresh mass, *f* (mg)**	**Dry mass, *d* (mg)**	**Water content, *m* (mg)**	***m*/*d***	**(%)**
PBS (saline)	0.049 ± 0.006[a]	0.018 ± 0.004[a]	0.031 ± 0.004[a]	1.7	63.27 ± 1.6[a]
M. anisopliae	0.046 ± 0.008[a]	0.019 ± 0.003[a]	0.027 ± 0.007	1.4	58.70 ± 1.8[a]
M. alpina	0.056 ± 0.005[a]	0.023 ± 0.004[a]	0.033 ± 0.004[a]	1.4	58.92 ± 1.3[a]
P. expansum	0.054 ± 0.006[a]	0.021 ± 0.004[a]	0.033 ± 0.005[a]	1.6	61.11 ± 1.5[a]

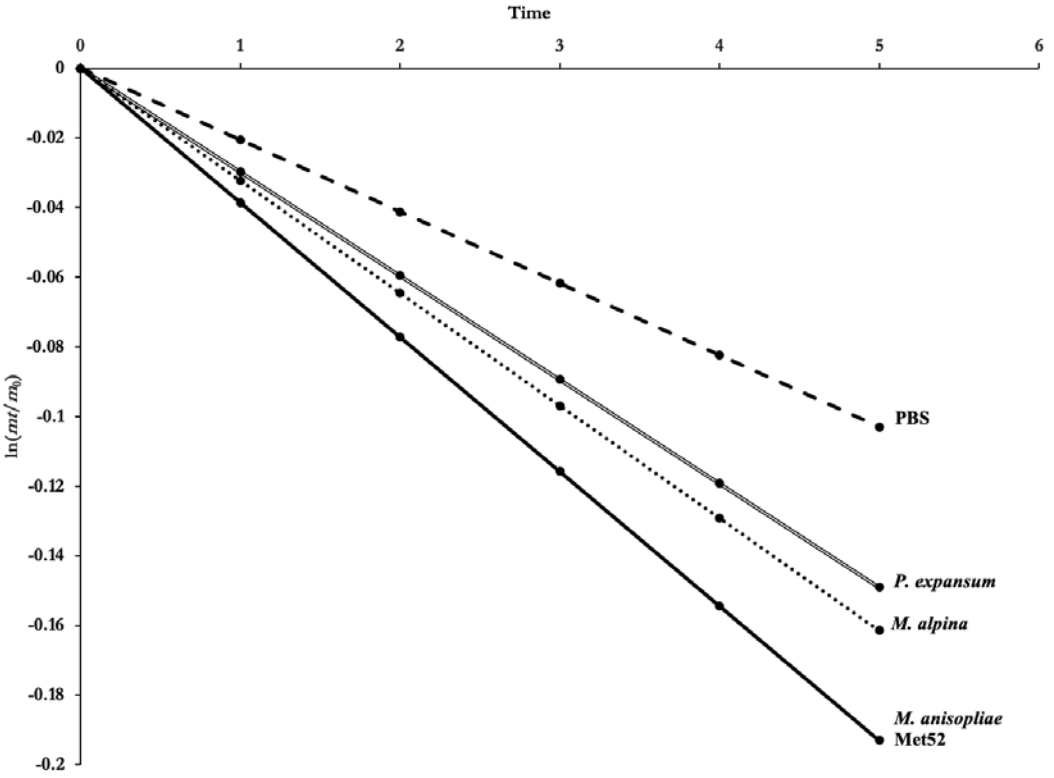

Fig. 4 – Larval water stress on *Dermacentor albipictus* post-treatment with *Metarhizium anisopliae* (Met52®), *Mortierella alpina* (Yukon strain), *Penicillium expansum* (Yukon strain), and saline (PBS, phosphate buffered saline + Tween). m_t, water mass at any time t; $m_{0,}$ initial water mass. Each point is the mean of 100 larvae, and the slope of the regression is the water loss rate.

Discussion

These Yukon strains of *M. alpina* and *P. expansum* showed explosive growth on agar media and produced measurable mycelia within 1-2 days on PDA; the highly prolific hyphal growth and spread of *M. alpina* (Zygomycete) along with the copious spore production by *P. expansum* (Mitosporic Fungus) were both especially noteworthy macroscopic culture characteristics. *M. alpina* and *P. expansum* demonstrated peak growth on PDA at 25ºC, narrow thermal growth ranges, growth as low as 5ºC, radial growth rates, all consistent with a psychrotroph and previous studies (criteria in Bergero et al. 1999, Robinson 2001, Kurek et al. 2007, Timling & Taylor 2012, Edgington et al. 2014, Tannous et al. 2016). *M. alpina* is low temperature-adapted and even capable of ice nucleating activity (Fröhlich-Nowoisky et al. 2015), and *P. expansum* is commonly referred to as the psychrophilic blue mold rot of plants (Morales et al. 2010). It is unsurprising that *M. alpina* and *P. expansum* co-occur in high frequency in Yukon soils given that these fungi are characteristically psychrotropic. For these Yukon strains, radial growth of *M. alpina* stops at 35ºC and the growth of *P. expansum* stops at 40ºC. Thus, the new information is that for these Yukon strains, *M. alpina* is more temperature sensitive than *P. expansum*.

A minimum of 0.997 a_w is required by *M. alpina* to grow and produce spores, whereas *P. expansum* requires a minimum, lower water activity of 0.95 a_w for growth and spore production. The water activity of *P. expansum* has been reported as low as 0.83-0.89 a_w (Lahlali et al. 2005, Tannous et al. 2016), and the water activity is similarly low for *Mortierella* spp. at 0.80-0.90 a_w (Khot et al. 2018); to our knowledge, the data presented herein are the first reports of water activity for *M. alpina*. Applying Christian's (1980) interpretations, a water activity = 1.00 a_w corresponds to pure water (rain and dew), water activities ≥ 0.90 a_w are considered "wet", and the water activities 0.65-0.85 a_w are considered "damp". Thus, both Yukon strains of *M. alpina* and *P. expansum* require contact with a "wet" surface for growth and sporulation. There is also a distinctive hygric-thermal correlation: fungi that have higher water requirements (i.e., higher a_w) tend to be more

temperature sensitive (Grant et al. 1989). *M. alpina* has a higher water activity and narrower water activity range, than *P. expansum*, and, indeed, *M. alpina* is more temperature sensitive. Of interest is that the water activity conditions for both *M. alpina* and *P. expansum* to function optimally are unusually high and close to water saturation (1.00 a_w), thus implying that these Yukon strains of *P. expansum*, and especially *M. alpina*, are particularly wet-adapted.

From a host-parasite perspective, these Yukon strains of *M. alpina* and *P. expansum* are essentially isosmotic with the activity of the tick's body water (= 0.99 a_w, Wharton 1985). As such, the body water activity of the tick larva satisfies the absolute water requirement for both *M. alpina* and *P. expansum* to grow, establish, and spore (transmission stage to infect ticks; this study, Fernandes et al. 2012). Secondary spore production from tick larval cadavers is a key mechanism for transmission and spread of infection, especially given the tendency for larvae of *D. albipictus* to form dense aggregations, typically numbering in the thousands of individuals, after hatching (Yoder et al. 2016). Thus, dead, sporulating larvae of *D. albipictus* will subsequently infect living larvae that cluster around it. The ability to penetrate, survive, and reproduce within the body of the tick (i.e., virulence factors, Fernandes et al. 2012) is considerably enhanced given high water activity that permits *M. alpina* and *P. expansum* to function as entomopathogens that naturally regulate populations of the winter tick *D. albipictus*.

This is the first study that allows for the pathogenic effects against *D. albipictus* larvae by these Yukon strains of *M. alpina* and *P. expansum* to be separated. Our results conform to the regular "Dehydration Hypothesis" of mycoses, where infection and death are related to an increased water loss as the primary mode of action (Cradock & Needham 2011, Willis et al. 2011). *M. alpina* resulted in a higher water loss rate in *D. albipictus* larvae post-treatment by a large spore inoculum than *P. expansum*. The greater water stress imposed by *M. alpina* than *P. expansum* treatment is reflected by more rapid death and greater tick kill in larval survivorship curves as a product of artificial infection. High frequency of re-isolation from larval cadavers (i.e., Koch's postulates) confirmed that *M. alpina* and *P. expansum* were responsible for the increased water loss rate and tick death due to water stress. Although both are effective as entomopathogens, the evidence now indicates that *M. alpina* is more virulent than *P. expansum*.

This study showing water and temperature requirements indicate these Yukon soil isolates of *M. alpina* and *P. expansum* would grow and sporulate well under conditions of moderate temperature at more humid, wet times of the year (criteria of Carlile et al. 2001). The importance of this study is that it highlights differences in properties of the two major Yukon soil isolates: we now report that *M. alpina* is more thermo/hygrosensitive, as well as more virulent to larvae of *D. albipictus*, than *P. expansum*. Low temperature tends to select for *Mortierella* spp. (Zygomycete) more so than *Penicillium* spp. (Mitosporic Fungi, Deuteromycetes), and the composition of the soil mycoflora is known to vary seasonally and with animal activity (Carreiro & Koske 1992, Kumpula et al. 2000, Ali et al. 2013). Whether such changes in fungal composition are reflected by pathogenicity for control of *D. albipictus* larvae (sole infestation of this moose parasite), and the overall population of winter ticks, is not known, and we are investigating this.

As a note for Biological Control Officers, *M. alpina* and *P. expansum*, the fungal isolates themselves or their products, are not approved for use as biological control agents against ticks. Fernandes et al. (2012) should be consulted as a guide for suitable agents. Information regarding the storage of fungus cultures, mass rearing (i.e., spores, transmission stage) for small/large-scale application, and release strategy (climatic adaptation, first release, timing of application) for biocontrol of ticks are found in Fernandes et al. (2012) or on label instructions provided by the manufacturers.

For *D. albipictus* control, we are not recommending any particular mycoinsecticide product or formulation at this time. In our experimental design, we used *Metarhizium anisopliae*, a well-known entomopathogenic fungus to ticks (Fernandes et al. 2012), as a positive control. Our results demonstrated positive acaricidal effects in the laboratory by *D. albipictus* larval bioassays (Figs 3, 4) with the commercial product Met52® EC Bioinsecticide that uses *M. anisopliae* as the active ingredient. Whether Met52® EC Bioinsecticide will be effective in the field for control of winter

ticks *D. albipictus* has not yet been examined. For future directions, some papers provide good results of mix fungus for pest control (Gouli et al. 2008). It has been suggested to us to test tank mixtures of the two species (*M. alpina* and *P. expansum*), perhaps including *M. anisopliae* as well, for *D. albipictus* control.

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