Pure culture response of bryophilous fungi to matric-induced water stress

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Matric potential influences water availability for soil- and wood-inhabiting fungi because the more negative the matric potential, the harder the substrate holds onto water, making it less available for microbial growth. The objectives of this study were to isolate epiphytic and endophytic fungi from bryophytes and assay their tolerance to matric-induced water stresses, and isolate terrestrial (soil) fungi to compare optimal matric potential of soil fungi to that of endophytes. Twelve fungal endophytes were isolated from two species of liverworts (*Bazzania trilobata* and *Conocephalum conicum*) and two moss species (*Mnium punctatum* and *Polytrichum commune*) from three habitats in central Pennsylvania. All epiphytic and endophytic isolates were assayed for their ability to grow on matric modified media (PEG 8000) at ca. 0 MPa, -5 MPa, -10 MPa, -15 MPa and -20 MPa. Results for matrically modified media suggest that epiphytes/endophytes of *Bazzania trilobata*, *Conocephalum conicum*, and *Mnium punctatum* produced greater biomass (mg) from -10 MPa to -20 MPa, while epiphytes/endophytes of *Polytrichum commune* display more variation with greater biomass (dry weight) from -5 MPa to -20 MPa. Eleven out of twelve of the endophytes had limited biomass production (dry weight) at the weakest matric potential (ca. 0 MPa) which represented non-matrically modified media). All soil isolates in this study demonstrated the expected Type II response with diminished growth under increasing matric stress.

Key words – *Bazzania trilobata* – *Conocephalum conicum* – endophytes – epiphytes – ericaceous – Helotiales – *Mnium punctatum* – PEG 8000 –*Polytrichum commune*

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Introduction

Bryophytes are ubiquitous, occurring as the dominant vegetation of boreal forest floors, which constitute a terrestrial biome covering approximately 1.4×10^7 km^2 of the earth's surface (Kauserud et al 2008). Bryophytes differ from vascular plants in that the predominant stage of their life cycle is the gametophyte generation, having rhizoids or scales instead of true root structures, and lacking a true vascular system (Goffinet & Shaw 2009). Kauserud et al (2008) documented significant fungal biodiversity associated with bryophytes. From environmental sampling of bryophyte shoots from three different species; *Hylocomium splendens*, *Pleurozium schreberi*, and *Polytrichum commune*, Kauserud et al (2008), were able to obtain numerous sequences from several different fungal phyla. The highest percentage of bryophilous fungi were in the Ascomycota (Helotiales). The most common orders from the Basidiomycota were in the Agaricales, including ectomycorrhiza-forming basidiomycetes, and the Tremellales (Kauserud et al 2008). The least common fungi

identified from bryophyte shoots were the Glomeromycota, accounting for only 1.3% of the total estimated biodiversity (Kauserud et al 2008).

There are a wide range of bryophyte-fungal interactions, including specific associations with different host structures (Döbbeler 2005, Davey & Currah 2006, Ligrone et al 2007, Ptaszyńska et al 2009, Read et al 2000). Bryophilous fungi can be isolated from different regions of the hosts such as external surfaces of liverworts and mosses, internally in leaves of mosses, within the thalli of some liverworts, and in rhizoids of both liverworts and mosses (Davey & Currah 2006, Zhang & Guo 2007, Pressel et al 2008, Ptaszyńska et al 2009). Differences seen in liverwort bryophilous fungal communities appear to be related to geographic distance more than host specificity (Davis & Shaw 2008). Davis and Shaw (2008) established that repeatedly sampled host species did not share ITS sequence groups among regions but different regions of the host shared the same ITS sequence groups.

Water availability and its relationship to stratification of bryophilous fungi, especially with regard to location of fungi with respect to host structures and water availability, has not been well-documented. Bryophytes are poikilohydric (no mechanisms to prevent desiccation), and as such many bryophytes tolerate desiccation to -20 MPa to -40 MPa and desiccation tolerant species can survive desiccation to -300 MPa (Goffinet & Shaw 2009). Dilks & Proctor (1979) demonstrated that water in bryophyte shoots can be divided into external capillary water (at the surface), symplast water (within the cell), and apoplast water (within cell walls). Proctor et al (1998) further showed that external capillary water is held at near zero water potentials (-0.01 MPa to -0.5 MPa), symplast water declined over a range which is generally -0.5 MPa to -10 MPa, and that apoplast water remains at lower water potentials, specifically -21.5 MPa and lower. Stratification of bryophilous fungi along and within the host appears to mirror this relationship. Epiphytes are found along the surface where water is freely available, whereas epi-endophytes and endophytes can be found growing between cells walls or even penetrating the host cells (symplast) in both mosses and liverworts. Some mycothalli-derived fungi from liverworts have been shown to initiate ericoid-type mycorrhizae in vascular plants grown in axenic culture (Duckett & Read 1995).

Matric potential (the ability of the substrate to hold onto water) is an important part of the total water potential in tissues with low water content (Öpik et al 2005). Proctor et al (1998) established that most water in the apoplast of bryophytes is microcapillary + matric water. Concomitantly, much of the water within the symplast can reach very low water potentials (- 10 MPa) via osmoticum. However, most fungi tolerate osmotic-induced water stress better than matric-induced water stress (Adebayo & Harris 1971, Ramirez et al 2004, Jurado et al 2008), suggesting that tolerance to matric-induced water stress would be an important component when considering adaptations of bryophilous fungi to the host.

Hutton et al (1996) and Chen et al (2003) demonstrated that ericoid-type mycorrhizal associations of ericaceous plants are influenced by matric-induced water stress established using polyethylene glycol (PEG) amended media. This raises the question whether a similar physiological response occurs in ericoid-type mycorrhiza found in bryophilous associations. Hutton et al (1996) examined PEG-induced, matric water stress tolerance (-0.16 MPa to -2.96 MPa) for ericoid endophytes of Epacridaceae hosts and describe three growth response types: type I — overall minimal growth after 5 weeks, type II — maximum growth at control with decreased growth as matric water stress increased, and type III — maximum growth under some degree of matric water stress. Chen et al (2003) examined ericoid mycorrhizal (ERM) fungi for PEG-induced water stress tolerance (-0.05 MPa to -2.24 MPa) and concluded that ERM endophytes function under water stress conditions.

The most common response for arbuscular mycorrhiza (AM), most basidiomycetes, and soil fungi is an intolerance to PEG-induced water stress (Magan & Lynch 1986, Coleman et al 1989, Hutton et al 1996, Chen et al 2003, Ramirez et al 2004). Chen et al (2003) reported that most AM fungi had reduced growth when under water stress of less than -3.0 MPa. Most basidiomycetes and soil fungi are intolerant to

PEG-induced water stress, although there are exceptions that appear to be tolerant.

Several methods utilizing PEG exist for evaluating the response of fungi to matric-induced water stress. Polyethylene glycol has been reported to be nontoxic and not metabolized by fungi (Mexal & Reid 1973). Polyethylene glycol is also akin to soil colloids. As such it should be considered "as a matricum and not an osmoticum" (Steuter et al 1981, Coleman et al 1989).

Our hypothesis is that bryophilous epi-endophytes and endophytes that survive surface sterilization will have inter- and intra-specific variations in response to PEG-induced matric potential, demonstrating a type III response as proposed by Hutton et al (1996). Environmental sampling on rhizoids of each bryophyte are performed in order to establish known fungi of vascular plants, document rhizoid-associated fungi from the sampled bryophyte species of Pennsylvania, and to see if there is congruency been sterilization, isolation and environmental sampling of the rhizoids. Environmental sampling also allows for the detection of fungal species that are not amenable to culturing. There is a general lack of information about the water potential of bryophyte rhizoids in their natural environment, so the final objective is to use indirect means of measuring endophyte tolerances to PEG-induced matric potential to assess if similar water potential values occur as have been previously reported in bryophyte shoots.

Methods

Field sampling and endophyte isolation

The gametophytes of two species of liverworts (*Bazzania trilobata* and *Conocephalum conicum*) and two moss species (*Mnium punctatum* and *Polytrichum commune)* were collected during August through October 2009 from three central Pennsylvania habitats (hydric, mesic, xeric), rinsed three times in distilled water, sterilized according to the methods outlined by Petrini (1986), and plated on Acidified Potato Dextrose Agar (APDA; 39 g PDA (Difco) + 10 g Technical Agar (Difco) + 2 ml Lactic Acid (*Lactic Acid* 85% *Baker* Analyzed ACS Reagent) L^{-1} distilled water) and on Acidified Rose Bengal Agar (ARBA; 32 g RBA (Difco) + 10 g Technical Agar (Difco) + 2 ml Lactic Acid (*Lactic Acid* 85% *Baker* Analyzed ACS Reagent) L^{-1} distilled water). Three fungal isolates were obtained from each bryophyte species.

Field sampling and soil fungi isolation

Soil was collected August through October 2009 from each bryophyte site. A few grains of soil (≈ 0.5 g) from each site were plated on APDA or RBA and the first visible fungus growing out of the soil was isolated into pure culture.

Water stress assay SNA agar plug to SN amended broth at 35 days

SNA agar plug to SN amended broth is defined as the primary experiment. This combination reduces the impact of osmotic potential by utilizing chemically defined media, and provides time for fungi to adjust to the environment. Two 5 mm modified Spezieller Nährstoffarmer (SNA: 0.2 g sucrose + 0.2 g glucose + 1 g KNO_3 + 1 $KH2PO_4 \cdot 7H_2O$ + 0.5 g NaCl + 12 g agar L^{-1} distilled water) agar plugs containing actively growing, non-sporulating mycelium where placed into an autoclaved 250 ml Erlenmeyer flask that contained 50 ml of liquid SN media (SN; modified SNA media without agar) and the targeted amount of Polyethylene glycol (PEG 8000, Fisher Bio-Reagents) according to the protocol of Michel (1983): $\Psi = 1.29[PEG]^2T - 140[PEG]^2 - 4.0[PEG]$. Each isolate was assayed at control (SN without PEG ≈ -0.08 MPa), -5 MPa, -10 MPa, -15 MPa, and -20 MPa. The Erlenmeyer flask was covered with sterilized tin foil, sealed with Parafilm, placed in a shaker chamber (90 rpm at 25C) for 35 days. Whatman 8 filter paper was conditioned in a humidity chamber at 20% relative humidity (RH) for 48 hours and then weighed to four decimal places. The filter paper was used in vacuum filtration to separate the mycelium from the liquid. The filter paper was rinsed with 500 ml of distilled water while under vacuum to remove any residual broth residue. The washed filter paper with mycelium was then placed in a Deni food dehydrator for 12 hours, and reconditioned in the humidity chamber at 20% RH for 48 hours prior to obtaining the final weight. Reported fungal growth weight is the final weight of the mycelium plus filter paper at 20% RH minus the

original weight of the filter paper at 20% RH. Growth types were assessed as outlined in Hutton et al (1996).

Water stress assay PDA agar plug to SN amended broth at 35 days

This experiment was used as the first conformational test in order to verify that the minimal media was not a confounding variable. Two 5 mm Potato Dextrose Agar (PDA; 39 g PDA (Difco) L^{-1} distilled water) plugs containing actively growing, non-sporulating mycelium were placed in SN media as outlined above. Dry mycelial weight was calculated as previously mentioned and the growth types were assessed as outlined in Hutton et al (1996).

Water stress assay RBA agar plug to SN amended broth at 10 days

This experiment was used as a second conformational test to evaluate the growth response to water stress with time as the limiting factor. Two 5 mm Rose Bengal Agar (RBA; 32 g RBA (Difco) L^{-1} distilled water) plugs containing actively growing, non-sporulating mycelium were place into SN media as in the primary experiment. Dry mycelial weight was calculated as above and growth type assessed as outlined in Hutton et al (1996).

Isolate identification

Identification is based on morphological characteristics following Barnett & Hunter (1998).

Environmental Clone sampling

Bryophytes were collected in July 2010 from the same sites, washed three times with distilled water, rhizoids were removed, and densely packed into 5, 1.5 ml centrifuge tubes, and sent (cold packed) to the Illinois Natural History Survey. Environmental DNA was obtained using a protocol modified from Lindner & Banik (Lindner & Banik 2009). Rhizoids were pulverized with a sterile high speed rotary bit in a 1.5 mL microcentrifuge tube. 500 mL cetyltrimethyl ammonium bromide (CTAB) buffer solution (100 ml 1 M Tris HCl pH 8.0, 280 ml 5 M NaCl, 40 ml of 0.5 M EDTA, 20 g of CTAB, total volume to 1 L with ddH_2O) was added to each tube. Ground material was subjected to three freeze/thaw cycles and stored at -20C until ready for processing. Samples were thawed at room temperature, vortexed, and placed in a 65C water bath for 2 hours to extract genomic DNA. A series of wash steps and centrifugations were performed to produce a cell pellet which was then processed using the GeneKleen III kit (Qbiogene) according to the manufacturer's instructions. PCR of the extracted DNA was performed using Illustra Ready-To-Go™ PCR Beads (GE Healthcare) using the primer pair ITS1F and ITS4 (White et al 1990, Gardes & Bruns 1991,) on an MJ Research PTC-200 thermocycler using the following thermocycling parameters: initial denaturation at 95C for 5 min, followed by 40 cycles of 95C for 30 s, 50C for 15 s, 72C for 10 s with a final extension step of 72C for 10 minutes. Mixed ITS PCR products were ligated using the Promega pGEM T-Vector cloning kit according to manufacturer's instructions. Ligation reactions were transformed the following day into DH5α competent *E. coli* cells (University of Illinois at Urbana-Champaign Cell Media Center). Eight colonies were chosen at random from each plate through blue/white screening. White colonies were used for PCR using the primer pair ITS1F and ITS4. Successful reactions were purified using Exo-SAP-IT PCR Clean-up Kit (GE Healthcare). Unidirectional sequencing with primer ITS1F was performed at the W.M. Keck Center for Comparative and Functional Genomics. Sequences were aligned with DNA star and blast searched in GenBank.

Results

Water stress assay; SNA agar plug to SN amended broth at 35 days

All bryophilous isolates (Table 1) increased biomass at -5 MPa compared to the Control (≈ -0.08 MPa) which demonstrates a Type III growth response to matric water stress as defined by Hutton et al (1996) (Table 2). One half of bryophilous isolates demonstrated bryophyte location demonstrated a reduction in growth at -5 MPa compared to the control (≈ -0.08 MPa) indicating a Type II growth response (Table 3). In addition, the isolate LHU61 was isolated from an area that contains all the host bryophyte species and also demonstrated a

Table 1 Fungal isolates examined for PEG-induced water stress.

Host species	Location	Isolate	Genus[1]	Order	Type[2]
Conocephalum conicum	41°1.537'N, 77°13.364'W	A-1	*Leptosphaeria sp.*	Pleosporales	A
		A-2	unidentified		
		A-3	unidentified		
		A-soil	*Sordaria sp.*	Sordariales	A
Mnium punctatum	41°8.922'N, 77°17.820'W	B-1	*Mucor sp.*	Mucorales	Z
		B-2	*Pestalotiopsis sp*.	Xylariales	A
		B-3	*Trichoderma sp.*	Hypocreales	A
		B&C-soil	*Trichoderma sp.*	Hypocreales	A
Bazzania trilobata	41°8.920'N, 77°17.820'W	C-1	unidentified		
		C-2	unidentified		
		C-3	*Humicola sp.*	Sordariales	A
Polytrichum commune	41°3.604'N, 77°19.694'W	D-1	*Mucor sp.*	Mucorales	Z
		D-2	unidentified		
		D-3	unidentified		
		D-soil	*Rhizopus sp.*	Mucorales	Z

1) Identification based on morphological characteristics. 2) Z= Zygomycota, A=Ascomycota, B=Basidiomycota

Type II growth response a decrease in growth from -10 MPa to -15 MPa followed by increased growth at -20 MPa (Table 2). All soil fungal isolates from each.

Water stress assay; PDA agar plug to SN amended broth at 35 days

All bryophilous isolates demonstrated a Type III growth response when provided with an initial higher nutritional source. The most significant change was in the increase in overall growth, as determined by weight (Table 4).

Water stress assay; RBA agar plug to SN amended broth at 10 days

All bryophilous isolates produced greater biomass under matric induced water stress compared to the control, demonstrating a Type III growth response when time was a limiting factor (Table 5).

Environmental cloning

Environmental sampling produced a total of 128 ITS sequences, of which 13 pairs of sequences had at least 90-95% match. Only one clone sequence was obtained from *Conocephalum conicum* with a 93.5% max identity match to *Volutella ciliata* (Alb. & Schwein.) Fr. a species in the order *Hypocreales*. Five sequences were obtained for *Bazzania trilobata* clones with matches to three ascomycete orders: three matches to *Helotiales* (83.1% to 87.6%), one match to *Verrucariales* (89.5%), and one match to *Chaetothyriales* (88.2%). All clone sequence results for *Mnium punctatum* were from two orders: a 92.8% match to *Venturia hystrioides* (Dugan, R.G. Roberts & Hanlin) Grous & U. Braun within *Pleosporales* and three separate matches (76.9% to 84%) to *Trechisporales*. Clone sequence matches for *Polytrichum commune* resulted in a match in both the Basidiomycota and Ascomycota: two matches (90.5% and 92.9%) to *Rickenella mellea* (Singer & Clem.) and one match (94.9%) to *Trichoderma hamatum* (Bonord) Bainier.

Discussion

This paper represents the first study of water stress tolerance in bryophilous fungal endoph-ytes. Hutton et al (1996) examined the matric water stress tolerance of epacrid root-inhabiting fungal endophytes from Australian heaths using PEG-3350. They concluded that the epacrid endophytes displayed a more varied response to matric water stress than basidiomycetes. Chen et al (2003) examined matric water stress tolerance of helotialian fungal isolates (*Hymenoscyphus* complex) from epacrid roots using PEG-6000 and concluded that these endophtyes can potentially function under matric water stress conditions. Coleman et al (1989) examined basidiomycete ectomycorrhizal isolates from roots and that the basidiomata using PEG-3350 and concluded vast majority demonstrate a Type I growth

Table 2 Bryophilous isolate growth response to matric water stress at 35 days on shake culture. Inoculum on SNA agar transferred to SN broth.

Isolate	Type of Growth response	Average Dry weight (mg) at water potential of:				
		Control (-0.08 MPa)	-5 MPa	-10 MPa	-15 MPa	-20 MPa
A1	III	2.25±0.15	9.60±0.8	10.4±1.5	12.4±1.9	20.0±5.6
A2	III	0.635±0.065	1.35±0.45	5.70±1.3	3.45±1.6	8.10±1.1
A3	III	0.900±0.20	2.60±1.4	4.10±0.1	7.95±3.6	5.10±4.0
B1	III	4.85±1.6	7.80±0.40	9.70±1.1	10.6±1.1	11.1±3.8
B2	III	11.5±3.6	12.1±0.15	13.4±5.0	15.9±10.	24.9±5.8
B3	III	6.60±4.1	11.5±2.6	15.3±11.	11.6±6.6	23.0±7.3
C1	III	1.20±0.90	4.85±0.25	2.30±0.90	10.1±3.3	2.65±1.5
C2	III	0.90±0.40	3.90±2.4	1.35±0.050	6.20±0.60	9.35±0.35
C3	III	0.25±0.15	3.30±2.4	5.75±1.3	3.85±1.6	6.00±4.3
D1	III	4.60±0.90	7.30±2.9	8.30±2.1	7.70±2.4	10.9±1.8
D2	III	8.05±6.4	14.8±6.7	13.6±5.6	12.5±4.2	18.8±6.25
D3	III	5.55±0.85	10.2±2.2	8.30±3.4	5.45±1.4	13.5±7.3

Growth was replicated over time with N=2, data reported as Average ± SEM.

response with diminished growth with increasing matric stress, which differed strikingly from the ascomycete associations found in epicarids that were matric stress tolerant. Ramirez et al (2004) examined matric stress on two strains of *Fusarium graminearum* Schw. using PEG-8000 and found that matric water stress inhibits growth and germination more than osmotic stress. Growth rates of the two *F. graminearum* strains under matric stress closely match the Type II growth response; meaning that over a broad range of matric stress, there was a decline in growth rate from high water availability to low water availability. Based on the results of this study, bryophilous fungi have a Type III growth response to PEG-8000 induced water stress, while the site soil fungi had a Type II response. The site soil isolates' intolerance to matric induced water stress is congruent with previous published literature (Magan & Lynch 1986, Ramirez et al 2004). Concomitantly, bryophilous fungal associations seem to mirror those found in some epicarid associations, as well as those previously documented for helotialian fungal endophytes (Hutton et al 1996, Chen et al 2003) in their ability to cope with matric water stress.

There are variations in the test methods that previous researchers have used to evaluate the water stress tolerance of fungal isolates in culture. Mexal & Reid (1973) used liquid modified Melin-Norkrans media (MMN) and determined growth by dry weight. Both Coleman et al (1989) and Hutton et al (1996) grew isolates in liquid MMN media on nylon mesh supported by glass beads in Petri plates. Growth was determined by colony diameter and colony radius respectively. Ramirez et al (2004) utilized polycarbonate membranes supported by capillary matting soaked in ≈ 15mL of 2% wheat-flour suspension and determined growth rates by colony radius. Our design utilized a minimal, chemically defined, liquid media on shake culture with growth determination by dry weight at 20% RH. Growth on a shaker accounted for any possible oxygenation issues with PEG as reported by Mexal & Reid (1973). The use of dry weight measurements took into consideration fungal colony density and vertical growth patterns that could hinder accurate growth measurements when only considering colony diameter measurements (Garraway & Evens 1991).The minimal media, SN broth, was also utilized to minimize the media's osmotic contribution to total water stress as the isolates were being evaluated based on the matric component of water stress. Both nutrition and time allocated for growth are confounding factors that can influence the final growth results (Coleman et al 1989, Garraway & Evens 1991).

Two separate conformational experiments were performed to rule out time and nutrition as confounding variables. The first conformational experiment evaluated the

Table 3 Soil isolates growth response to matric water stress at 35 days on shake culture. Inoculum on SNA agar transferred to SN broth.

Isolate	Type of Growth response	Average Dry weight (mg) at water potential of:				
		Control (-0.08 MPa)	-5 MPa	-10 MPa	-15 MPa	-20 MPa
A-Soil	II	6.7	5.9	5.0	4.9	2.5
B&C-Soil	II	6.0	5.4	4.1	2.8	2.4
D-Soil	II	7.7	5.9	2.1	1.4	1.3
LHU 61	II	19.0	16.0	18.0	12.0	12.0

Growth report as N=1.

effects of starting nutrition. It was clear that that there was no change in the Type III growth response of the bryophilous isolates when the starting nutrition was increased, but there was an overall increase in the final amount of growth (Table 2 and Table 4). The second conformational experiment assessed the influence of growth time allotted from 35 days to 10 days. Coleman et al (1989) reported that experiments estimating growth rates over a short duration favor the control, while long term experiments estimating growth rates favor stress conditions. When measuring growth by dry weight, the results for this study remained consistent, demonstrating a Type III growth response for the bryophilous isolates regardless of the experimental time (Table 2 and Table 5). The consistency of our bryophilous isolates to generate a Type III growth response leads to the conclusion that they have the ability to tolerate matric induced water stress and that they may be physiologically adapted to the bryophyte host.

A tolerance to matric induced water stress would be a beneficial adaptation for bryophilous endophytes because when the bryophyte desiccates to < -21.5 MPa, all water is bound to the host tissue by matric potential (Dilks & Proctor 1979). All the isolates in this study were subjected to a larger range of water stress (-0.08 MPa to -20 MPa) than previous authors in order to relate the matric water stress tolerance of the isolates to the bryophytes' physiology (Fig. 1). Soil isolates exhibit greater growth when water availability is high, while bryophilous isolates have the lowest growth under the same condition (Fig. 1). This same response can be elicited (Fig. 2 A1, A-soil) on PDA, which has a reported water potential of -0.36 MPa. In Figure 2, the soil isolate completely covers the entire surface while the endophytic isolate (A1) has minimal growth. Under matric water stress, the bryophilous isolates continue to show more robust growth, while the soil isolates have diminished growth (Fig. 1). Interestingly, most of the endophytic isolates had increased growth at -10 to -20 MPa. One half of the bryophilous isolates had decreased growth at -15 MPa followed by an increase in growth at -20 MPa. There was pigment production at the -20 MPa treatments for some bryophilous isolates, while no pigmentation occurred at the -15 MPa treatments. Many fungi produce sugars (arabitol, mannitol, and trehalose) in response to matric water stress (Ramirez et al 2004). It is not clear whether pigment production plays a similar role to sugar alcohols in coping with water stress. Increased pigment production under water stress is a phenomenon which de-serves further study.

A *Trichoderma* anamorph referable to *Trichoderma* sect. *Trichoderma* Bissett was isolated from the soil near *Mnium punctatum* and *Bazzania trilobata* (Table 1). A bryophilous isolate (B3) also referable to *Trichoderma* sect. *Trichoderma* was isolated from the moss *Mnium punctatum*. *Hypocrea* cf. *leucopus* (LHU61) (P. Karst.) H. Chamb., was isolated from decomposing hickory nut husks from Clinton County State Game Lands #295 in Pennsylvania, where all four bryophytes in this study are found. LHU61 is a *Trichoderma* species referable to *Trichoderma* sect. *Hypocreanum* Bissett. Isolate LHU61 and the B/C soil isolate displayed a Type II growth response to matric induced water stress (Table 3), which contrasts with the Type III growth response found for the bryophilous *Trichoderma* isolate (B3). All soil isolates in this study demonstrated the expected Type II response with diminished growth under increasing matric stress. However, LHU61 appeared to be more tolerant to matric stress than the soil isolates (Table 3).

Table 4 Bryophilous isolate growth response to matric water stress at 35 days on shake culture. Inoculum on PDA agar transferred to SN broth.

Isolate	Type of Growth response	Average Dry weight (mg) at water potential of:				
		Control (-0.08 MPa)	-5 MPa	-10 MPa	-15 MPa	-20 MPa
A1	III	47.7	73.7	79.4	71.1	84.0
A2	III	48.8	61.7	48.4	41.2	57.0
A3	III	44.5	47.7	62.8	76.5	60.2
B1	III	37.0	42.0	42.0	42.0	39.0
B2	III	64.1	82.0	79.0	77.0	72.0
B3	III	45.9	56.2	61.9	54.4	59.8
C1	III	54.0	65.7	65	63.6	58.4
C2	III	56.1	61.0	63.7	71.7	75
C3	III	52.5	64.6	63.5	56.6	63.1
D1	III	34.1	47.0	53.0	56.0	51.0
D2	III	64.0	67.0	74.0	78.0	76.0
D3	III	47.0	54.1	60.2	67.5	83.9

Conformation experiment to study the impact of nutrition. Growth report as N=1.

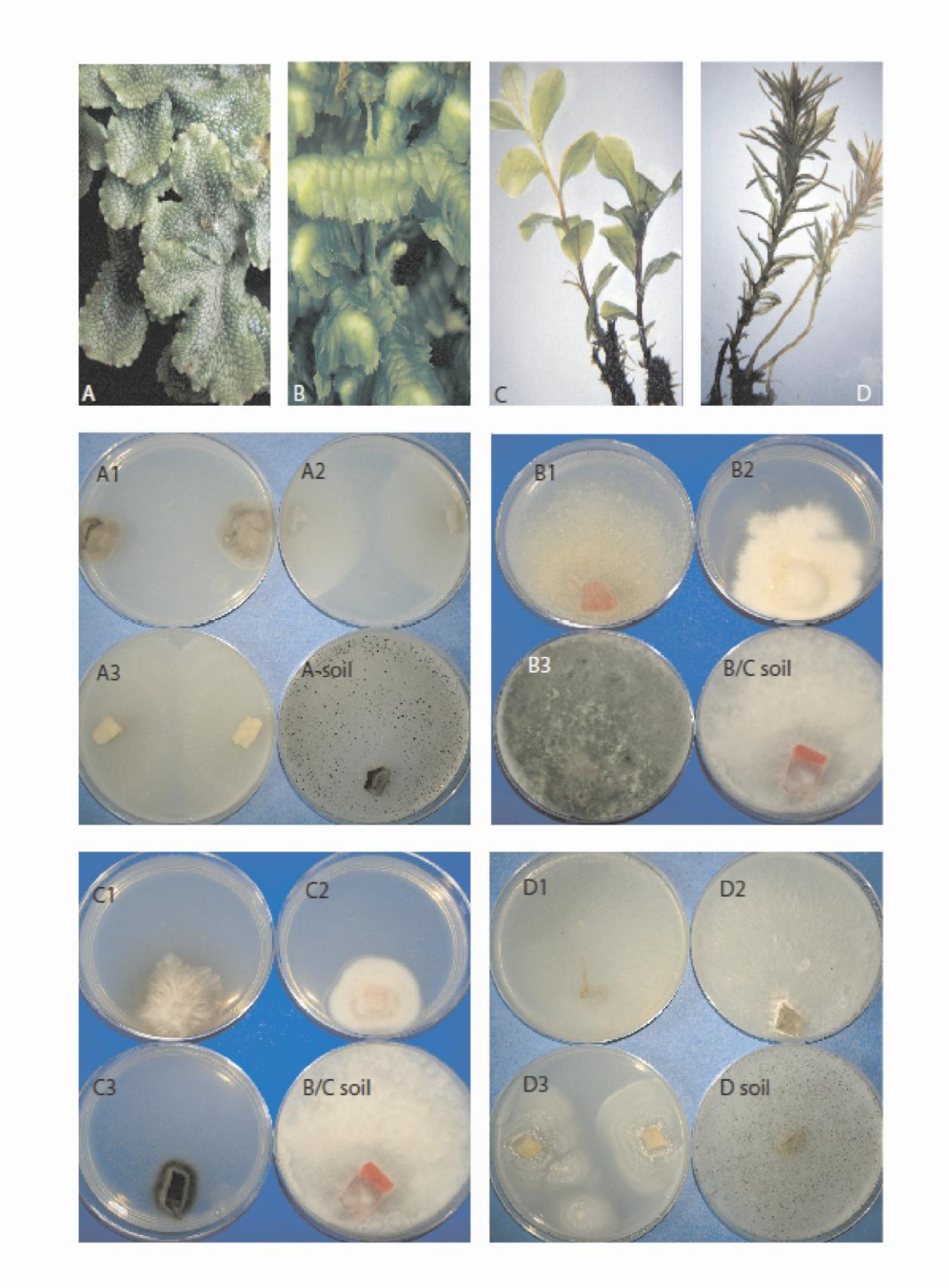
Table 5 Bryophilous isolate growth response to matric water stress at 10 days on shake culture. Inoculum on RBA agar transferred to SN broth.

Isolate	Type of Growth response	Average Dry weight (mg) at water potential of:				
		Control (-0.08 MPa)	-5 MPa	-10 MPa	-15 MPa	-20 MPa
A1	III	9.0	29.0	28.0	32.0	33.0
A2	III	9.0	25.	31.0	33.0	48.0
A3	III	28.0	34.0	36.0	35.0	25.0
B1	III	11.0	27.0	29.0	25.0	27.0
B2	III	16.0	23.0	23.0	30.0	28.0
B3	III	18.0	23.0	34.0	23.0	31.0
C1	III	3.0	4.0	10.0	3.0	4.0
C2	III	4.0	15.0	16.0	14.0	14.0
C3	III	1.0	5.0	16.0	3.0	1.0
D1	III	2.0	3.0	8.0	13.0	9.0
D2	III	8.0	37.0	10.0	13.0	14.0
D3	III	4.0	12.0	13.0	18.0	41.0

Conformational experiment to study the impact of limited time. Growth report as N=1.

Interestingly, GJ Samuels (pers. comm.) suggests the possibility, based on tropical studies and observations, that clavate species of *Hypocrea*, growing on decomposing fruits and nuts may have an endophytic association. The inter-mediate response found here, although anecdotal, suggests his observations may be correct. The evaluation of fungal species responses to matric potential may present a novel taxonomic character to identify fungi within species complexes; groups of fungi that are currently indistinguishable from one another without the use of DNA analysis. This could be especially important for *Trichoderma* anamorphs referable to the harizianum-complex that have both soil and endophytic associations. Environmental cloning found more ascomycete associations with liverworts as compared to the mosses. Ascomycetes ac-counted for 100% (N=6) of the clones of the liverwort rhizoids, while they consisted of only 29% (N=2) for the moss species rhizoids.

The remaining 71% (N=5) of the clones from the moss rhizoids were basidiomycetes. Addition-ally, several blast results from liverwort rhizoid-associated ascomycete taxa were found to be within the Chaetothyriales and Helotiales. The environmental cloning results generated in this study are similar to those previously reported by Kauserud et al (2008) from boreal forest bryophytes. Kauserud et al (2008) reported Agaricales, Chaetothyriales, Helotiales, and Tremellales to be the most common fungal orders found from their environmental sampling of bryophytes. The low % matching in GenBank also indicates that there are no sequences deposited for many of these current species. These species may be



Figs 1 (A-D) – A *Conocephalum conicum* (thalloid liverwort). A1-A3. Endophytic isolates from *C. conicum*. A soil. Site soil isolate. **B** *Mnium punctatum* (moss). B1-B3. Endophytic isolates from *M. punctatum.* B/C soil. Site soil isolate. **C** *Bazzania trilobata*, (leafy liverwort). C1-C3. Endophytic isolates from *B. trilobata*. B/C soil. Site soil isolate. **D** *Polytrichum commune* (moss). D1-D3. Endophytic isolates from *P. commune.* D soil. Site soil isolate.

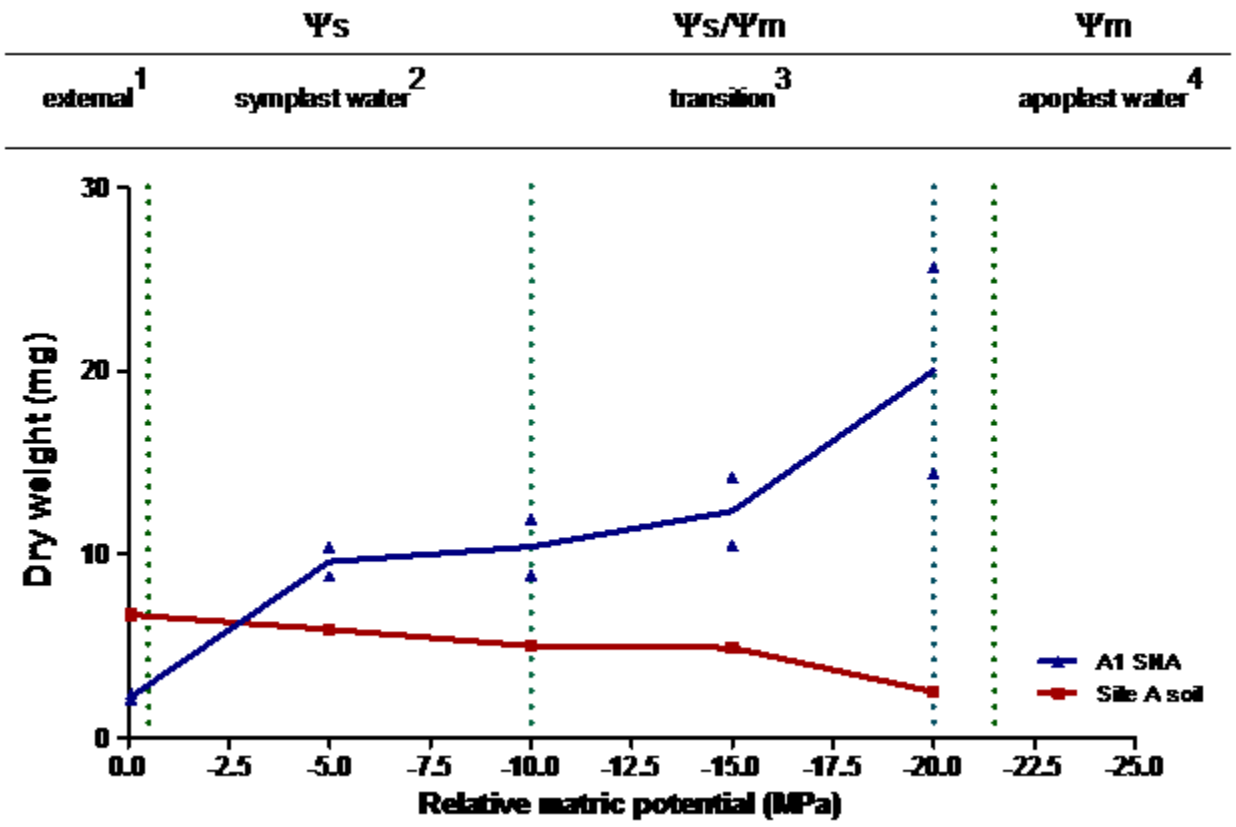


Fig 2 – Bryophyte water availability correlated to bryophilous and soil isolate responses to matric induced water stress. Fully hydrated bryophytes have high water availability at the external surfaces (> -0.5 MPa) which is available to epiphytic fungi. Initial desiccation (-0.5 to -10 MPa) requires endophytic fungi to obtain water by overcoming osmotic potential. Further desiccation (-10.0 to -21.5 MPa) acts as a transition for endophytic fungi whereby obtaining water requires overcoming either osmotic or matric potential. Below -21.5 MPa all water in the bryophyte is bound by matric force and endophytic fungi need to overcome matric potential to extract water from the host.

new and hence represent novel species as assessed using molecular data.

Pure culture isolates obtained by following the sterilization technique of Petrini (1986) suggests the possibility that rhizoid-associated endophytes may not survive the sterilization process (Table 1). Several pure-culture isolates identified based on morphology were incongruent with results obtained from environmental cloning. Additionally, two zygomycete isolates were obtained following the sterilization technique from the mosses. While some of the incongruence can be explained by the fact that pure-culture isolations were from the entire bryophyte (thallus, leaves, shoots, and rhizoids), the differences in fungal diversity between sterilization isolation techniques and environmental sampling were greater than expected. One specific hindrance for our research is that liverwort rhizoids are one cell thick and it is highly probable that surface sterilization killed the liverwort rhizoid-associated fungi. Ramirez et al (2004) showed that macro-conidial germination of *Fusarium graminearum* was suppressed by both osmotic and matric stress. Magan & Lynch (1986) also reported a noticeable decrease in growth below -2.8 MPa for most of the fungi in their study. Future research will evaluate matrically modified liquid media, as it has been shown to suppress fungal growth and conidia germ tube formation in soil fungi, suggesting a potential medium for the bio-amplification of liverwort rhizoid-associated fungi that may not survive surface sterilization.

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