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REGULAR ARTICLE

Hebeloma crustuliniforme modifies root hydraulic responses of trembling aspen (*Populus tremuloides*) seedlings to changes in external pH

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Abstract The main objective of the study was to compare the effects of short-duration pH treatments on root hydraulic properties in trembling aspen (*Populus tremuloides*) seedlings that were either inoculated with the ectomycorrhizal fungus *Hebeloma crustuliniforme* or remained non-inoculated (control). Inoculated and non-inoculated plants were exposed in solution culture to the root zone pH ranging from 4 to 9 and their root hydraulic conductivity was examined using the hydrostatic method and after subjecting the plants to treatments with 100 μM $HgCl_2$ (aquaporin blocker) and 0.02% trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (apoplastic transport tracer). In a separate experiment, pure cultures of *H. crustuliniforme* were also grown on a slid medium with the pH ranging from 4 to 9 to determine their pH growth optimum and changes in medium pH over time in the presence and absence of 8 mM NH_4NO_3. When grown in pure culture, *H. crustuliniforme* demonstrated maximum growth at pH 7–8 and was capable of modifying the pH of its growth media, especially in the presence of NH_4NO_3. The plants that were inoculated with *H. crustuliniforme* had a maximum root hydraulic conductivity at pH 7. At this pH, root hydraulic conductivity was significantly higher compared with non-inoculated plants and showed greater sensitivity of root water transport to pH changes relative to non-inoculated seedlings. Relative apoplastic flux was largely unaffected by pH in inoculated seedlings. Fungal inoculation modified the response of root hydraulic conductivity to pH. The increased root hydraulic conductivity in inoculated seedlings was likely due to an increase in aquaporin-mediated cell-to-cell water transport, particularly at the higher pH. A possible role of fungal aquaporins in the root hydraulic conductivity responses of mycorrhizzal plants should be examined.

Keywords *Hebeloma crustuliniforme* · pH · *Populus tremuloides* · Root hydraulic properties

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Introduction

Soil pH can profoundly affect tree function and forest health (Skousen et al. 1994; Westbrook et al. 2006). Although it has been well documented that soil pH affects the uptake of nutrients and heavy metals (Rygiewicz et al. 1984; Smith 1994), the effects of root zone pH on other physiological processes have received less attention. It has been demonstrated that soil pH can affect root hydraulic properties (Tang et al. 1993; Kamaluddin and Zwiazek 2004), probably due to effects on the

function of aquaporins (Kamaluddin and Zwiazek 2004). Cytosolic pH regulates aquaporin activity through protonation of a highly conserved histidine residue (Tournaire-Roux et al. 2003; Törnroth-Horsefield et al. 2006).

In most of the forest tree species, the roots of trees are colonized by ectomycorrhizal fungi which help the trees survive and grow in adverse environments (Smith and Read 1997). Mycorrhizal fungi have been reported to assist their host plants in mitigating pH changes (Aggangan et al. 1996; Wallander 2002; Trocha et al. 2007; Calvo-Polanco et al. 2009) and often enhance root water transport (Landhäusser et al. 2002; Muhsin and Zwiazek 2002a, b; Marjanović et al. 2005), partly due the induction of aquaporin expression (Marjanović et al. 2005) and resulting increase in cell hydraulic conductivity (Lee et al. 2010). However, no changes (Nardini et al. 2000; Yi et al. 2008) and decreases (Nardini et al. 2000) in root hydraulic conductivity by mycorrhizal fungi have also been reported. The reasons for the differences in responses of root hydraulic properties to mycorrhizas are unclear.

Since the reported increases in root hydraulic conductivity of mycorrhizal plants are thought to be largely due to an increase in aquaporin-mediated water flow and since the function of aquaporins is known to be sensitive to pH, it is possible that root hydraulic properties may be differently affected by mycorrhizal associations depending on the soil pH. The effect could vary depending on the pH tolerance of mycorrhizal fungus and, in the longer term, on its ability to modify the pH of the rhizosphere (Hung and Trappe 1983; Barros et al. 2006). Root water transport is often a limiting factor in cold (Wan et al. 2001), poorly aerated (Zhang and Tyerman 1991) and polluted (Kamaluddin and Zwiazek 2002) soils. Therefore, a possible modification of root responses to soil pH by mycorrhizal fungi may be especially important for temperate and boreal plants that are affected by soil pH changes in urban and reclamation areas (Renault et al. 2000; Landhäusser et al. 2002; Calvo-Polanco et al. 2008).

Mycorrhizal fungi may be capable of modifying pH of their growth substrate, possibly via extrusion of protons and organic acids (Arvieu et al. 2003). Therefore, in the present study, we grew *H. crustuliniforme* in pure culture to demonstrate the effects of fungal mycelia on pH of the growth medium since this could potentially have a significant impact on the soil pH tolerance of the host plants. We also inoculated trembling aspen (*Populus tremuloides*) seedlings with *H. crustuliniforme* and compared the responses of root hydraulic properties to the root zone pH in inoculated and non-inoculated plants. *H. crustuliniforme* is a ubiquitous early successional fungus with broad host specificity (Smith and Read 1997). Since it has been demonstrated that the growth of pure cultures of *H. crustuliniforme* increases with increasing pH (Kernaghan et al. 2002), we hypothesized that the effectiveness of *H. crustuliniforme* to facilitate water movement in ectomycorrhizal roots of trembling aspen would also increase at the higher pH levels.

Materials and methods

Fungal culture

Hebeloma crustuliniforme (Bull.) Quél. (University of Alberta, Devonian Microfungus Collection, UAMH 5247) was sub-cultured on Melin Norkrans Media (MNM) agar (Mason 1980). Plugs of MNM agar-grown fungus were aseptically sub-cultured in MNM liquid media for 4 weeks. Mycelia grown in liquid culture was homogenized under sterile conditions using a blender to produce liquid inoculum for seedling roots.

For the pure culture experiment, plugs of MNM agar-grown fungi were sub-cultured on plates of Murashige and Skoog (MS) agar media (Murashige and Skoog 1962), modified by decreasing the sucrose concentration to 3 gL^{-1} (low-sucrose MS, LSMS media) for pure culture fungal growth. *H. crustuliniforme* (Hc) was grown on LSMS for approximately 4 weeks prior to transfer to pH treatment plates.

Plant culture

Populus tremuloides Michx. seeds were collected from the North Saskatchewan river valley (Edmonton, AB, Canada). Seeds were surface-sterilized with 5% sodium hypochlorite for 5 min, and rinsed thoroughly with deionized water. Seeds were germinated on washed, sterile silica sand in Petri dishes and germinants transplanted to styroblocks™ (superblock

160/60, Beaver Plastics Ltd., Edmonton, AB, Canada) filled with sterilized 1:1 peat:sand mixture. The plants were placed in a controlled-environment growth chamber (60% RH, 18-h photoperiod, 22°C/18°C (day/night), 300 μmol m^{-2} s^{-1} photosynthetic photon flux density). They were bottom-watered every 2 days with deionized water, and fertilized weekly with 0.1% 20-20-20 fertilizer (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc., Brantford, ON, Canada).

After 6 weeks, seedlings were transferred to 12-cm diameter pots containing a 2:1:1 peat:perlite:sand mixture. The bottoms of the pots were lined with aluminum foil and placed inside a second pot to prevent flow-through during watering. Following transplantation, fertilizer solution was omitted from the watering schedule a minimum of 3 weeks prior to inoculation (Muhsin and Zwiazek 2002a, b).

Seedling inoculation

Half of the seedlings were inoculated with 5 ml of *H. crustuliniforme* homogenized liquid medium as previously described (Siemens and Zwiazek 2008). The medium was injected with a pipette at least 3 cm below the soil surface in two locations near the seedlings (inoculated group, MYCO). The other half of the seedlings was designated as the non-inoculated control (CTRL) group and 5 ml of autoclaved, fungal-free, liquid MNM was applied to them in the same manner as for fungal inoculation. Inoculated and non-inoculated seedlings were separated from each other in the growth chamber to prevent contamination of non-inoculated seedlings with the fungal inoculum. Following inoculation, seedlings were watered with a small amount of water every 2 days for 2 weeks. At the start of the third week, 0.025% 20-20-20 fertilizer solution (Plant Prod® Water-Soluble Fertilizer, Spectrum Brands Inc.) was applied twice per week as part of the regular watering schedule. Seedlings were grown for an additional 9 weeks following inoculation prior to the start of pH treatments.

pH treatments

Seedlings with intact root systems were removed from pots and gently rinsed with deionized water. Seedling roots were excised with a razor blade, leaving approximately 2 cm of stem attached to the root system. Roots were immersed in an aqueous root bathing medium of 0.02% trisodium 3-hydroxy-5,8,10-pyrenetrisulfonic acid (PTS_3), a fluorescent tracer dye restricted to the apoplastic pathway of water movement (Steudle and Peterson 1998; Siemens and Zwiazek 2003; Schaider et al. 2006). The pH of the solution was adjusted to 4, 5, 6, 7, 8, and 9 with either 1 M H_2SO_4 or KOH, while continuously stirred with a magnetic stirrer.

Root hydraulic properties and xylem exudate measurements of aspen seedlings

Roots in their pH-treatment solutions were placed in Scholander pressure chambers (PMS Instruments, Corvallis, OR). The solutions were continuously aerated during measurements with a magnetic stirrer and a magnetic stirring plate was placed underneath each pressure chamber. Root water flow (Q_v, m^3 s^{-1}) was measured for a minimum of 20 min at hydrostatic pressures of 0.4, 0.6, 0.8, and 1.0 MPa (Siemens and Zwiazek 2003). Minimum intervals of 10-min. were maintained between Q_v measurements at each increasing pressure to stabilize Q_v values. Once initial Q_v measurements had been made, $HgCl_2$ solution was added to the solutions in the pressure chambers to reach a final concentration of 100 μM and the solutions were readjusted to their target pH. Root systems were re-pressurized for 30 min at 0.4 MPa, and a second set of Q_v values was collected at the above four hydrostatic pressures.

Xylem exudates were collected from all roots under pressure following both sets of Q_v measurements to determine PTS_3 concentrations. Samples were measured against pH-adjusted PTS_3 standard curves using a Sequoia-Turner 450 spectrofluorometer (Apple Scientific, Chesterland, OH, USA) with a 405 nm excitation and 515 nm emission spectrum (Skinner and Radin 1994).

To determine root hydraulic conductivity (L_{pr}, s^{-1} MPa^{-1}), root volumes were measured for each root system using the water volume displacement method (Voicu and Zwiazek 2004). Following the volume measurements, approximately 15 root tips were excised from inoculated (MYCO) and non-inoculated (CTRL) plants for microscopic examination of root colonization (Siemens and Zwiazek 2008).

pH treatments of *H. crustuliniforme* in plate culture

Modified MS media (Murashige and Skoog 1962) was used for the six pH treatments (4, 5, 6, 7, 8, and 9) of *H. crustuliniforme* with either 0 or 8 mM NH_4NO_3 added to the treatment medium. This resulted in a total of 12 pH*nitrogen treatment combinations. MS media was modified as follows. Sucrose concentrations were supplied to ensure an optimal 20:1 C:N ratio for the N treatment (4.7 g sucrose L^{-1}). A minimal amount of sucrose (0.12 g L^{-1}) was supplied for the 0 mM NH_4NO_3 media, resulting in a C:N ratio of 37:1. To minimize all forms of available carbon and nitrogen in MS media, casein enzyme hydrolysate was omitted from the media preparation, however, a minute amount of N-containing vitamins that are part of the MS formula was also present in the media. Purified agar (Sigma-Aldrich Canada, < 1% trace element concentrations) was used as the gelling agent for all plates. Pre- and post-autoclaving pH values are listed in Table 1.

The amount of pH adjustment that was required prior to autoclaving and the amount of agar that needed to be added to each pH treatment to solidify the media varied from 8 gL^{-1} (pH 9) to 22 gL^{-1} (pH 4), and was pre-determined in a preliminary experiment. The adjustment of pH prior to autoclaving was also affected by the nitrogen treatment. All pH measurements were taken with a flat-surface electrode (Fisher Scientific Co., Ottawa, Canada).

A minimum of eight (n=8) 100 mm diameter Petri dishes, each containing approximately 30 ml of autoclaved modified MS media, were poured for each pH*nitrogen treatment combination. Pieces of porous cellophane (Fisher Scientific Co., Ottawa, Canada) were trimmed, autoclaved separately, and transferred to the autoclaved, solidified plates of media. Cellophane circles were laid on top of the solidified media of each dish to facilitate harvesting of intact colonies at the end of the experiment. Eight-mm diameter plugs of low sucrose MS-grown *H. crustuliniforme* were cut from the periphery of an existing colony. One plug was transferred to each Petri dish with the pH*nitrogen treatments and centered in the middle of each piece of cellophane. Petri dishes were then sealed with Parafilm and stored upright. Two days after completion of mycelial transfers to all pH*nitrogen treatment plates, the dishes were inverted and incubated at room temperature for 6 weeks.

Table 1 Target pH for each of the six pH treatments and pH of modified MS media before and after autoclaving. Modified MS media were without added inorganic nitrogen (O) or with 8 mM NH_4NO_3 (N)

Target pH	pH before autoclaving		pH after autoclaving	
	O	N	O	N
4	3.95	3.97	3.92	4.11
5	5.10	5.05	5.10	4.83
6	6.60	7.20	6.25	5.82
7	7.60	8.00	7.05	7.27
8	9.46	9.86	7.96	8.00
9	11.2	11.45	9.25	9.10

Growth measurements of *H. crustuliniforme* colonies

Colony size was determined by taking the diameter measurements through the center and at the intersecting 90° angle and the mean of the two measurements taken. Then, the colonies and their underlying cellophane circles were removed and fresh weight of each colony was determined.

Once the colonies were removed, post-growth pH measurements were taken of the agar media on which fungal colonies had been growing. For each plate, two pH measurements were taken: one at the outer edge of the agar where no mycelia were present; and in the center of the agar which was directly underneath the center of the colony. Both pH values for each plate were averaged and used as the final post-growth pH value of the agar.

Statistical analysis

All data were analyzed with SAS 9.1. (SAS Institute, North Carolina, USA) to determine statistically significant ($p \leq 0.05$) differences between treatment combinations of pH level and inoculation for aspen, and for differences between treatment combinations of pH level and nitrogen treatment for *H. crustuliniforme*. Root hydraulic properties and xylem exudates measurements (n=10) from inoculated and non-inoculated seedlings were analyzed using a MIXED analysis of variance (ANOVA) randomized complete block design (block=day of measurement, with one seedling per treatment per day) with a 6 (pH)×2

(inoculation) factorial model to statistically compare differences in the means for each inoculation and pH treatment combination.

Measurements (n=8) from pure culture plates of *H. crustuliniforme* were analyzed using a 2 (nitrogen)×6 (pH) factorial complete randomized design ANOVA model.

Comparisons from all the ANOVA tests were conducted using least-squares means. For MIXED ANOVA, corresponding Tukey-adjusted p-values were used to further examine differences among the treatment combinations using pre-planned comparisons (α=0.05). The slice function was used to analyze and interpret interactions between main effects that were statistically significant (α=0.05) for multi-factor MIXED ANOVA.

Results

Root hydraulic conductivity (L_{pr})

Initial L_{pr} values of non-inoculated (CTRL) seedlings prior to the addition of $HgCl_2$ varied between 1.24×10^{-4} $s^{-1}MPa^{-1}$ (pH 4) and 1.65×10^{-4} $s^{-1}MPa^{-1}$ (pH 5), and did not significantly change in response to pH (Fig. 1A). In contrast, L_{pr} of inoculated (MYCO) seedlings significantly increased (p<0.05) with increasing pH from 4 to 7 (Fig. 1A). At pH 7, mean L_{pr} of MYCO seedlings was significantly higher compared with CTRL seedlings at their respective pH (Fig. 1A).

Following the application of 100 μM $HgCl_2$ to roots, normalized L_{pr} decreased for all of the pH treatments in CTRL seedlings and there were no significant differences between the different pH (Fig. 1B). In MYCO seedlings, normalized L_{pr} showed the largest decrease at pH 4. Normalized L_{pr} in MYCO seedlings was also reduced at other pH values with the exception of pH 8 where L_{pr} was not affected by the 100 μM $HgCl_2$ treatment (Fig. 1B). Also, at pH 8, normalized L_{pr} of MYCO seedlings was significantly higher than that of the CTRL seedlings at the same pH (Fig. 1B).

Although there were no significant statistical interaction effects (inoculation*pH) for L_{pr} or normalized L_{pr}, the main effects of pH (p<0.001) and inoculation (p<0.05) were significant for normalized L_{pr} (Table 2).

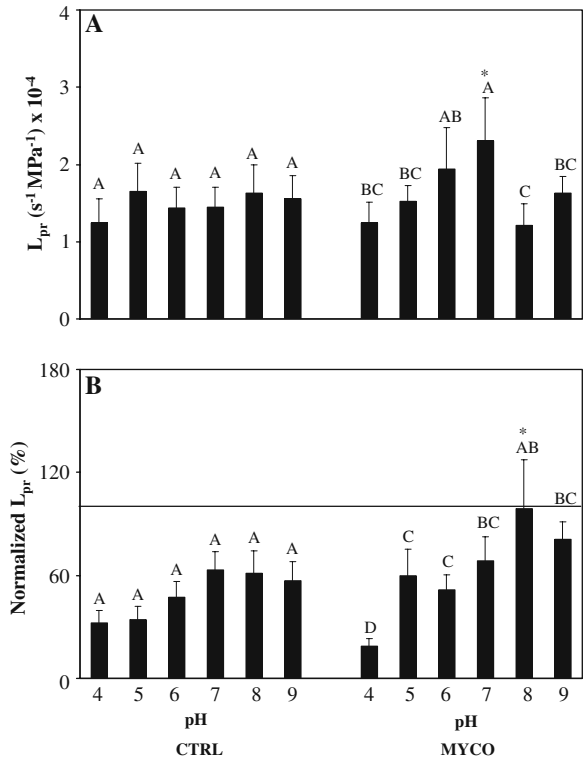

Fig. 1 **A** Root hydraulic conductivity (L_{pr}) measured prior to the addition of $HgCl_2$. **B** Normalized L_{pr} measured after the addition of 100 μM $HgCl_2$ and calculated as a percentage of the untreated L_{pr} values (represented by the *line* at 100%). Significant ($p\leq0.05$) differences indicated by uppercase letters (between pH treatments within CTRL or MYCO treatments) and *asterisks* (between CTRL and MYCO treatments within each pH treatment)

PTS_3 concentrations in xylem exudates

The pH treatments significantly affected PTS_3 concentrations in xylem exudates in CTRL, but not in

Table 2 ANOVA table with p-values for main effects and interactions for growth and pH measurements of *Hebeloma crustuliniforme* in response to nitrogen (no added inorganic nitrogen, 8 mM NH_4NO_3) and pH (4–9) treatment combinations. Effects were tested for significance at α=0.05 using PROC MIXED

Response variables	Effects		
	Inoculation	pH	Inoculation*pH
L_{pr}	n.s.	n.s.	n.s.
Normalized L_{pr}	p<0.05	p<0.001	n.s.
[PTS_3]	n.s.	n.s.	n.s.
Normalized [PTS_3]	p<0.05	p<0.001	n.s.

MYCO seedlings (Fig. 2A). Means for CTRL seedlings ranged from 2.64×10^{-3}% (pH 6) to 3.72×10^{-3}% (pH 4), with higher PTS_3 concentrations at pH 4 and 9, and significantly lower values for the intermediate pH. At pH 9, mean PTS_3 concentration was significantly higher for CTRL than for MYCO seedlings.

Following the application of 100 μM $HgCl_2$ to roots, normalized PTS_3 concentrations increased in low, but not high pH treatments and the increase was greater in CTRL compared with MYCO seedlings (Fig. 2B). Maximum normalized PTS_3 concentrations occurred at pH 4 (Fig. 2B).

There were no statistically significant interaction effects (pH*inoculation) for PTS_3 or normalized PTS_3 measurements, although the main effects of pH ($p<0.001$) and inoculation ($p<0.05$) were significant for normalized PTS_3 (Table 3).

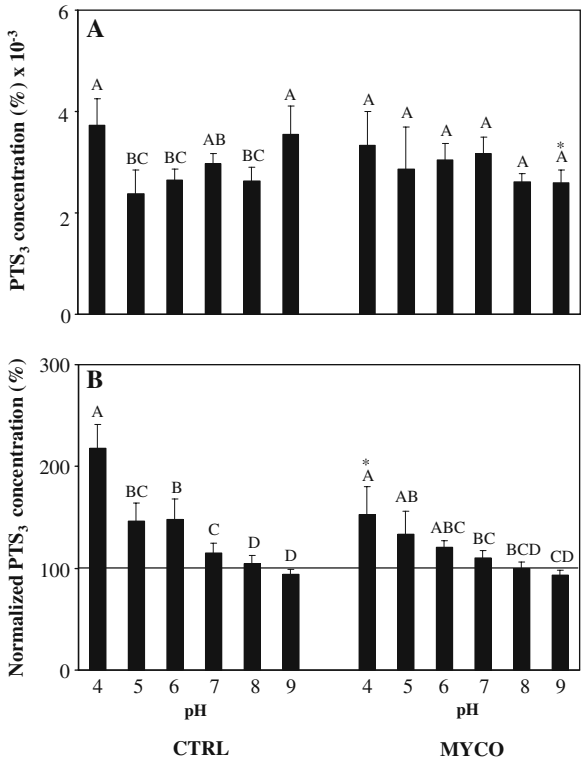

Fig. 2 **A** PTS_3 concentration in xylem exudate measured prior to the addition of $HgCl_2$. **B** Normalized PTS_3 concentration measured after the addition of 100 μM $HgCl_2$ and calculated as a percentage of the untreated PTS_3 values (represented by the *line* at 100%). in non-inoculated (CTRL) or inoculated (MYCO) aspen seedlings receiving a short-term pH treatment (pH 4–9). Least-*squares* means±SE are shown ($n=10$). Significant ($p\leq0.05$) differences indicated by uppercase letters (between pH treatments within CTRL or MYCO treatments) and *asterisks* (between CTRL and MYCO treatments within each pH treatment)

Growth of *Hebeloma crustuliniforme* colonies

Fresh weights (FWs) of *H. crustuliniforme* colonies significantly increased with increasing pH for the 0 mM NH_4NO_3 (O) and 8 mM NH_4NO_3 (N) treatments, with minimum fresh weights at pH 4, although the increases were greater for O compared with N (Fig. 3A). For all pH treatments, colony FWs were significantly higher with O media compared to N media. Maximum FWs were at pH 9 with O media, and at pH 8 with N media.

Colony diameters were significantly greater at pH values between 5 and 8, with lower diameters at pH 4 and 9, for both O and N treatments (Fig. 3B). Mean diameters of N media were significantly higher at pH 4 and pH 6–8 compared with O media (Fig. 3B).

For the colony FW and diameter, the main effects (nitrogen, pH) and interaction effects (nitrogen*pH) were highly significant ($p<0.0001$) (Table 3).

Growth medium pH changes by *H. crustuliniforme*

The medium pH changed significantly for many of the pH treatments, and the extent of the change differed between pH treatments and between the O and N treatment (Table 4). There was a decrease in medium pH for all of the N-containing pH treatments, but for only two of the O pH treatments. The greatest pH changes were at pH 9 for O (−0.621) and at pH 8 for N (−0.278). The statistical main effects (nitrogen,

Table 3 ANOVA table with p-values for main effects and interactions for root hydraulic conductivity (L_{pr}) and PTS_3 concentrations in xylem exudate prior to the addition of $HgCl_2$, and normalized L_{pr} and PTS_3 concentrations following the addition of $HgCl_2$. Effects were tested for significance at $\alpha=0.05$ using PROC MIXED

Response variables	Effects		
	Nitrogen	pH	Nitrogen*pH
Colony FW	$p<0.0001$	$p<0.0001$	$p<0.0001$
Colony Dia.	$p<0.0001$	$p<0.0001$	$p<0.0001$
Δ pH	$p<0.0001$	$p<0.0001$	$p<0.0001$

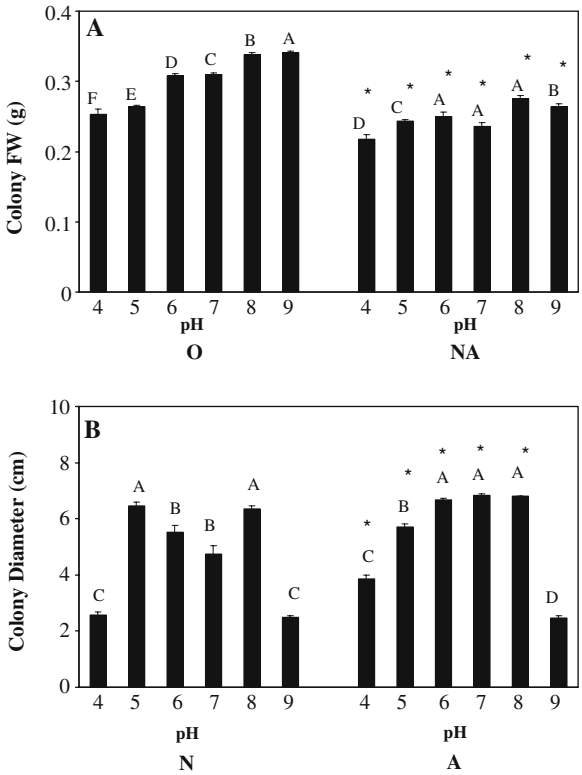

Fig. 3 **A** Colony fresh weight and **B** colony diameter of *Hebeloma crustuliniforme* pure culture grown on modified MS agar media adjusted to pH treatments of pH 4–9. Nitrogen treatments were 0 mM NH_4NO_3 (O) and 8 mM NH_4NO_3 (N). Least-squares means±SE are shown (n=8). Significant ($p \leq 0.05$) differences indicated by *uppercase letters* (between pH treatments within O or N treatments), and *asterisks* (between O and N treatments within each pH treatment)

pH) and interactions (nitrogen*pH) for mean pH changes were highly significant ($p<0.0001$) (Table 4).

Discussion

The present study demonstrated that root water flux in trembling aspen responded differently to changes in the root zone pH in seedlings that had been inoculated with *Hebeloma crustuliniforme* compared with non-inoculated seedlings. Inoculated seedlings had significantly higher L_{pr} values than non-inoculated plants at pH 7. The effect of pH on root hydraulic responses combined with other environmental factors could be partly responsible for the reported no effects of ectomycorrhizal fungi on root hydraulic conductivity in some studies (Nardini et al. 2000; Yi et al. 2008).

Root hydraulic responses of the host plant may differ depending on the mycorrhizal fungus (Siemens and Zwiazek 2008; Yi et al. 2008). Since different species of ectomycorrhizal fungi vary in their pH tolerance, soil pH could affect the effectiveness of an ectomycorrhizal association in producing physiological responses of the host plant. In our study, *H. crustuliniforme* demonstrated a pH growth optimum of 7–8 in pure culture, as indicated by its colony diameter and fresh weight (FW) with and without the addition of inorganic nitrogen. This confirms earlier reports that *H. crustuliniforme* is an alkaliphilic fungus (Hung and Trappe 1983; Kernaghan et al. 2002) and, therefore, we expected the mycorrhizal association to be functional at high soil pH. In addition to the short-term, immediate, responses of mycorrhizal plants to pH changes, the longer-term acclimation to soil pH conditions could occur since the growth of external mycelium could likely have consequences to plant water uptake from the soil.

It was apparent that *H. crustuliniforme* could modify the pH of its growth media in pure culture, resulting in pH decreases for most of the treatments. This could potentially have a significant impact on pH sensitive processes, including water transport, in roots. These findings are consistent with previous studies which demonstrated that mycorrhizal fungi were capable of modifying pH of their growth substrate, possibly via extrusion of protons and organic acids (Arvieu et al. 2003). Since fungal-induced increases or decreases in pH may be dependent on nitrogen form supplied (Zhu et al. 1994; Quoreshi et al. 1995), we tested the media containing either 0 or 8 mM NH_4NO_3. Most of the changes occurred in the medium containing 8 mM NH_4NO_3 where the pH of the medium declined suggesting a possible effect of a preferential uptake of NH_4^+ by the fungal mycelium.

In the present study, we used 100 μM $HgCl_2$ as an aquaporin blocker (Maggio and Joly 1995; Wan and Zwiazek 1999) and an apoplastic tracer dye, PTS_3 (Steudle and Peterson 1998; Siemens and Zwiazek 2003; Schaider et al. 2006) to examine possible changes in root water flow pathways as a result of fungal inoculation and pH treatments. Higher PTS_3 concentrations in xylem exudates indicate that the relative contribution of apoplastic water transport was higher at pH extremes (pH 4 and 9) in non-inoculated (CTRL) seedlings. However, there was no significant

Table 4 Initial pH and post-growth pH changes of modified MS media on which H. *crustuliniforme* was grown for 6 weeks. Modified MS media either contained 0 mM NH_4NO_3 (O) or 8 mM NH_4NO_3 (N). O control and N treatment growth media had pH-adjusted to 4, 5, 6, 7, 8, and 9. Values indicate adjusted (n=8) pre-growth media pH of each pH*nitrogen treatment combination, and the mean relative post-growth pH change following fungal growth on the media. Least-squares means± SE are shown. Significant ($p \leq 0.05$) changes in pH due to fungal growth are indicated by uppercase letters (between different pH treatments within either the O or the N treatment), and by asterisks (between O and N treatments for each pH treatment)

O			N		
Initial pH	Final pH (Mean±SE)	Δ pH	Initial pH	Final pH (Mean±SE)	Δ pH
3.92	4.06±0.012	$+0.138^{C}$	3.97	3.95±0.034	-0.024^{A*}
5.10	5.31±0.016	$+0.206^{D}$	4.98	4.73±0.013	-0.246^{D*}
6.25	6.21±0.012	-0.044^{B}	6.08	5.97±0.016	-0.113^{B*}
7.05	7.08±0.013	$+0.028^{A}$	7.28	7.11±0.013	-0.166^{C*}
7.96	7.97±0.078	$+0.009^{A}$	8.14	7.86±0.035	-0.278^{D*}
9.25	8.63±0.011	-0.621^{E}	8.96	8.74±0.018	-0.223^{D*}

difference across the studied pH range in the inoculated (MYCO) group suggesting that *H. crustuliniforme* modified the effects of both high and low pH on water transport. Although the predicted overall L_{pr} shift in MYCO seedlings at high pH was not detected with PTS_3, our data indicate that cell-to-cell transport was significantly greater at pH 9 in MYCO seedlings compared with CTRL plants suggesting that MYCO seedling roots had a greater ability to maintain cell-to-cell water transport at higher pH. The fact that CTRL seedling L_{pr} was not affected significantly by the short duration pH treatments indicates that longer-term changes in root properties, soil nutrient availability as a function of pH, or the natural presence of mycorrhizal associations may play an important role in the ecological pH preferences of aspen.

Cell-to-cell root water transport is largely mediated by aquaporins (AQPs) (Maurel and Chrispeels 2001; Javot and Maurel 2002). Some AQPs are blocked by mercurial compounds, which bind to sulfur-containing amino acids near the pore (Martre et al. 2001; Javot and Maurel 2002). In the present study, AQP-mediated root water flux was affected by both the inoculation with *H crustuliniforme* and by external pH. In CTRL seedlings, L_{pr} was reduced by the $HgCl_2$ treatment to between about 50% (pH 4 and 5) and 60% (pH 7–9) of the pre-exposure level. In MYCO seedlings, the greatest Hg sensitivity of L_{pr} was measured at pH 4 (less than 30% of the pre-exposure value) while at pH 8, there was no reduction in L_{pr} by $HgCl_2$. In the absence of Hg sensitivity in MYCO plants at pH 8, the significant decrease in L_{pr} at pH 8 compared with lower pH in MYCO plants could be interpreted as an inhibition of Hg-sensitive aquaporin transport. However, we cannot exclude the possibility that the mycorrhizal hyphae differently affected Hg uptake by the roots at different pH and that this affected L_{pr} responses in plants.

The movement of water and ions through mycorrhizal fungal tissue and between the fungus and root is currently little understood, especially in angiosperms (Peterson et al. 2004; Lehto and Zwiazek 2011). It is possible that structural modification, such as a looser network of mantle construction, could result in altered proportions of apoplastic and symplastic flux (Bogeat-Triboulot et al. 2004). Increases in L_{pr} in inoculated roots could be the result of increased cell-to-cell (Marjanović et al. 2005; Lee et al. 2010) or apoplastic (Muhsin and Zwiazek 2002a) water flux, enhanced by hyphal extensions (Smith and Read 1997). Also, fungal mycelia contain their own AQPs, however, little is known about their contribution to the water transport of mycorrhizal plants (Aroca et al. 2009; Lehto and Zwiazek 2011).

Previous studies demonstrated that acidic pH decreased both root water flux and AQP-mediated flux (Tournaire-Roux et al. 2003; Alleva et al. 2006; Pettersson et al. 2006). AQP function is regulated by cytosolic pH changes (Tournaire-Roux et al. 2003) and by a pH-sensitive gating mechanism involving the protonation of a histidine (His) residue (pKa ~6.5,

Zelenina et al. 2003) located within a highly-conserved PIP region (Fischer and Kaldenhoff 2008). Acidic conditions result in a conformational change with closure of the pore on the cytosolic side (Törnroth-Horsefield et al. 2006). Animal AQP pH studies suggest that the position and number of multiple His residues can result in greater sensitivity to acid or alkaline (Németh-Cahalan et al. 2004). Extracellular pH and the involvement of both His and serine (Ser) residues have resulted in reduced AQP activity in human cells (Zelenina et al. 2003). Some studies have also reported optimal AQP activity at pH 4 and minimal activity at pH 7 (Yasui et al. 1999; Németh-Cahalan and Hall 2000). Although the role of AQPs in water transport of the fungal remains to be clarified, it is possible that the fungal AQPs could also be involved in the L_{pr} responses of MYCO roots and be partly responsible for the greater sensitivity to pH observed in the MYCO seedlings. In an ongoing study with *Laccaria bicolor* (unpublished data) we have identified several pH-sensitive fungal aquaporins which may have a significant impact on root water transport in ectomycorrhizal plants.

In conclusion, our study demonstrated that inoculation of trembling aspen with *H. crustuliniforme* modified the response of root hydraulic conductivity to pH. The increased root hydraulic conductivity in inoculated seedlings was likely due to an increase in cell-to-cell water transport.

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