Trees
DOI 10.1007/s00468-016-1394-5

ORIGINAL ARTICLE

Distinct effect of pH on N uptake and assimilation in two conifer species

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Received: 8 July 2015 / Accepted: 21 March 2016

Abstract

Key message **Prince Rupprecht's Larch from the Loess Plateau takes up and assimilates a greater proportion of N as NO_3^-, particularly at neutral pH, whereas Chinese Fir assimilates a greater proportion of N as NH_4^+, particularly at low pH levels.**

Abstract The effects of pH on nitrate and ammonium uptake and assimilation in two coniferous species were compared. Prince Rupprecht's Larch (*Larix principis-rupprechtii Mayr*) grows on the loess plateau in alkaline soils with low available nitrogen (N), whereas Chinese Fir (*Cunninghamia lanceolata*) grows in acidic soils. In the present study, the net fluxes in ammonium (NH_4^+) and nitrate (NO_3^-) were measured using a non-invasive microelectrode ion flux measurement system, and the expression of NH_4^+ and NO_3^- transporters (*AMTs* and *NRTs*, respectively) as well as H^+-ATPase was examined to provide insights into the N uptake mechanisms in Prince Rupprecht's Larch and Chinese Fir. The enzyme assays involved in N assimilation were also determined. For Prince Rupprecht's Larch, low pH (pH 4) resulted in a decrease in net ammonium uptake, which remained unchanged in Chinese Fir. Net nitrate uptake in Prince Rupprecht's Larch and Chinese Fir was much lower in soils with pH 4 relative to those with pH 7. Low pH significantly decreased the H^+-ATPase activity and the expression level of *NRT*s in roots of Prince Rupprecht's Larch. However, the expression level of *AMT*s in Prince Rupprecht's Larch was significantly higher at pH 7 than at pH 4. The H^+-ATPase activity in roots of Chinese Fir remained unaltered in response to changes in pH, and the transcript abundances of *AMT*s and *NRT*s were down-regulated by low pH. Low pH decreased N assimilation in both conifer species with the exception of NH_4^+ assimilation in Chinese Fir, which displayed higher glutamine synthetase (GS) and glutamate synthetase (GOGAT activities) at low pH. Prince Rupprecht's Larch from the Loess Plateau takes up and assimilates a greater proportion of N as NO_3^-, particularly at neutral pH, whereas Chinese Fir assimilates a greater proportion of N as NH_4^+, particularly at low pH levels. This study contributes to our understanding of nitrogen metabolism mechanisms in response to pH changes.

Keywords Prince Rupprecht's Larch · Chinese Fir · pH · Nitrogen uptake · Nitrogen assimilation · Nitrate transporters · Ammonium transporters · Ion flux

Introduction

Spatial and temporal variations in soil N availability have promoted plant adaptations from the molecular to the ecosystem level to address challenges of limited N

Communicated by H. Rennenberg.

Electronic supplementary material The online version of this article (doi:10.1007/s00468-016-1394-5) contains supplementary material, which is available to authorized users.

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Published online: 02 April 2016

availability (Nacry et al. 2013). Although both NH_4^+ and NO_3^- ions can be utilized by plants, they have different energetic and biochemical characteristics that affect assimilation, resulting in different net fluxes and different NH_4^+/NO_3^- preferences in specific plants (Luo et al. 2013a, b). N metabolism involves the uptake, transport, assimilation and utilization of N for amino acid biosynthesis and ultimately growth. NO_3^- is converted to NH_4^+ by NR and NiR. After direct uptake or conversion from NO_3^-, NH_4^+ is assimilated to glutamine and glutamate via GS and GOGAT, and the products of the GS/GOGAT pathway are required for the biosynthesis of other nitrogenous compounds. Each step of N metabolism can be influenced by species and environmental factors (soil type, water availability, salt and climate) (Cousins and Bloom 2003; Dong et al. 2001; Luo et al. 2013a, b; Zhang et al. 2014). For instance, some maize varieties displayed a higher capacity to absorb and utilize N than the others (Machado and Fernandes 2001)). *Populus simonii* took up more NH_4^+ after acclimation to moderate salinity (Zhang et al. 2014). However, little information is available on responses of N metabolism in woody plants to pH changes.

In soils, external pH influences the electrical potential difference between the plasma membrane and surrounding environment (Reid and Hayes 2003). In root cell membranes, pH can also influence the amount and activity (Zhu et al. 2009) of H^+-ATPase protein and proton permeability (Yan et al. 1998) and polarization state (Babourina et al. 2001) of the plasma membrane. These effects directly influence the uptake of inorganic N ions.

The influence of pH on N uptake and assimilation is complex; therefore, results vary among studies investigating this phenomenon. The optimum pH for maximum NO_3^- uptake ranges from 8.0 in *Arabidopsis* (Doddema and Telkamp 1979) and 5.0 in *Typha latifolia* (Brix et al. 2002) to 4.5–5.0 in soybean (*Glycine max*) (Vessey et al. 1990) and 4.0 in barley (*Hordeum vulgare*) (Rao and Rains 1976). The optimum pH required for NH_4^+ uptake ranges from 6.5 in *T. latifolia* (Brix et al. 2002) and 6.0 in soybean (Vessey et al. 1990) to 4.0 in *Eucalyptus nitens* (Garnett and Smethurst 1999). pH also affects N assimilation, with higher nitrate reductase (NR) in maize seedlings observed when the seedlings were grown at nutrient pH 6.3 than at pH 4.3 (Shankar et al. 2001). However, pH did not have a significant effect on glutamine synthetase (GS) in either rice or tomatoes grown in a medium with NH_4^+–N as the N source (Magalhäes and Huber 1989). These studies provide a starting point; nevertheless, the direct effects of pH on NH_4^+ and NO_3^- rate uptake and assimilation in conifer roots are poorly understood.

Prince Rupprecht's Larch grows in NO_3^--rich alkaline soils (pH 7–8) on the Loess Plateau, whereas Chinese Fir grows in southern China forests, where soils are typically acidic and rich in NH_4^+. Soils on the Loess Plateau in northwest China are alkaline and low in available N. $NH4^+$ levels are particularly low, making NO_3^- the most available form of N in this region. Moreover, timber production and forestry in North and South China rely on Prince Rupprecht's Larch and Chinese Fir, respectively, and conifers that grow in these regions are expected to exhibit different N uptake and assimilation strategies based on the soil pH.

The mechanisms of NH_4^+ and NO_3^- transport across the root membrane differ, and the optimum pH levels for the uptake of these two ions vary. NH_4^+ may enter root cells passively by traveling through a potential uniporter system and following the electrochemical potential gradient across the plasma membrane; however, NH_4^+ must be actively transported out of root cells and requires the help of PM H^+-ATPase during efflux (Britto and Kronzucker 2006). NO_3^- is transported into root cells via H^+-coupled symporters assisted by PM H^+-ATPase and may exit passively into the apoplast during efflux (Britto and Kronzucker 2006).

In this study, we employed the non-invasive micro-test technique (NMT), which measures ion fluxes by moving an ion-selective microelectrode between two positions near the live tissue in situ. This technique provides high spatial (<2 μm) and temporal resolution (approximately 5 s) (for theory, see Shabala and Bose (2012)) and is a powerful tool in the investigation of ion fluxes in plant roots. We also examined the expression of NH_4^+ and NO_3^- transporters (*AMTs* and *NRTs*, respectively) as well as H^+-ATPase to provide insights into the N uptake mechanisms in Prince Rupprecht's Larch and Chinese Fir. Our hypothesis stated that the response of N metabolism in Chinese Fir adapted to acidic forest soils would be less impacted by pH pretreatment relative to the uptake and assimilation of N in Prince Rupprecht's Larch. Our objectives were to (1) investigate the NH_4^+ and NO_3^- fluxes and the expression of NH_4^+ and NO_3^- transporters (*AMTs* and *NRTs*, respectively) in fine roots of two conifer species exposed to different pH levels and (2) compare the uptake and utilization of NH_4^+ and NO_3^- in two conifer species exposed to different pH levels. We expected that Prince Rupprecht's Larch would take up and assimilate a greater proportion of N as NO_3^-, particularly at neutral pH, whereas Chinese Fir would assimilate a greater proportion of N as NH_4^+, particularly at low pH levels. This study is valuable because it improves our understanding of N uptake and utilization mechanisms in response to pH changes in conifers.

Materials and methods

Plant cultivation

Seeds of Prince Rupprecht's Larch and Chinese Fir were sown in 500 ml pots filled with moistened vermiculite in a

climate-controlled growth chamber that provided a day/night temperature of 22/20 °C, relative humidity of 60/70 %, and irradiance of 300 μmol m^{-2} s^{-2}. Five seeds of each species were sown per pot, and the seeds germinated after approximately 10 days. The nutrient solutions were adjusted to pH 4 or 7 and contained 100 μM NH_4NO_3, 100 μM KH_2PO_4, 100 μM $MgSO_4$, 100 μM $CaCl_2$, 100 μM Na_2SO_4, 100 μM EDTA·FeNa, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 30 μM H_3BO_3, and 0.5 μM H_2MoO_4. Three pots of seedlings per species were allocated to each pH treatment, and nutrient solutions were applied every 2 days. All measurements were performed on healthy seedlings 8 weeks after germination.

Measurement of growth parameters

The net photosynthetic rate was measured from 9:00 to 11:00 h using a portable photosynthesis system (Li-Cor-6400; Li-Cor, Inc., Lincoln, NE, USA) with an attached LED light source (500 μmol photon m^{-2} s^{-1}). The CO_2 concentration in each chamber was 400 μmol mol^{-1}, and the air flow was 500 μmol s^{-1}. The height of the main shoot of each plant was measured with a ruler.

The roots of each plant were harvested, and the fresh weight was recorded. Roots were excised from the root system, scanned, and analyzed with a WinRHIZO root analysis system (WinRHIZO version 20007, Regent Instruments Canada, Montreal, Canada). Harvested roots and leaves were then dried for 72 h at 80 °C in an oven and cooled in a desiccator to calculate dry mass.

Measurements of NH_4^+ and NO_3^- fluxes at the root surface

To monitor the net fluxes of NH_4^+ and NO_3^- in the roots, six white fine roots (0.20 ± 0.01 mm in diameter, 50.0 ± 1.1 mm in length) were selected and excised from the root system of each plant (ca. 10 weeks). Measurements of ion fluxes along the root tips were performed non-invasively using the scanning ion-selective electrode technique (SIET, system BIO-IM; Younger USA, LLC., Amherst, MA, USA), and the work was conducted at Xuyue Science and Technology Co. Ltd. (Beijing, China). The method's principles and application are described in detail by Xu et al. (2006). Briefly, silanized glass micropipettes with 2–4 μm apertures were first filled with a backfilling solution (100 mM NH_4Cl for the NH_4^+ electrode; 10 mM KNO_3 for the NO_3^- electrode).The micropipettes were then front-filled with 15–50 μm columns of selective liquid ion-exchange cocktails (NH_4^+LIX, #09879, Sigma; NO_3^- LIX, #72549, Sigma). An Ag/AgCl wire electrode holder (XY-DJGD, Younger USA) was inserted into the back of the electrode to make electrical contact with the electrolyte solution. YG003-Y05 (Younger USA) was used as the reference electrode. The microelectrodes were calibrated (for NH_4^+: 0.05 and 0.50 mM NH_4Cl as well as other compounds used in the measuring solution (see below); for NO_3^-: 0.05 and 0.50 mM KNO_3 as well as other compounds used in the measuring solution), and only electrodes with Nernstian slopes higher than 55 mV per tenfold concentration difference were used.

An initial measurement was performed to monitor the net fluxes of NH_4^+ and NO_3^- along the roots. Six white fine roots were selected from the root system for each treatment. Fluxes were measured at the root tip and either 300 μm (approximately 0–3 mm) or 8 mm (approximately 5–30 mm) walk steps from the root tip. Ion gradients (NH_4^+ and NO_3^-) near the root surface (approximately 5 μm above the root surface) were measured by moving an ion-selective microelectrode between two positions that were 30 μm apart and perpendicular to the root axis. Ion flux readings were performed every 6 s for an average of 10 min at each location. Flux data and root images were acquired using the MageFlux software attached to the SIET system.

To investigate the net NH_4^+ flux and interference of NO_3^- with the net NH_4^+ flux, white fine roots were equilibrated for 30 min in the measuring solution (0.05 mM NH_4Cl, 1 mM KCl, 0.1 mM $CaCl_2$, pH 4 or 7). The net NH_4^+ flux was recorded for 10 min at each position. To examine the interference of NO_3^- with the net NH_4^+ flux, the net NH_4^+ flux was recorded in a measuring solution containing NH_4NO_3 instead of NH_4Cl (0.05 mM NH_4NO_3, 1 mM KCl, 0.1 mM $CaCl_2$, pH 4 or 7) and following the process described above.

As with the measurements of net NH_4^+ fluxes, the net NO_3^- fluxes were determined by exposing white fine roots to a measuring solution (1 mM KCl, 0.1 mM $CaCl_2$, pH 4 or 7) containing either 0.05 mM KNO_3 or 0.05 mM NH_4NO_3. NO_3^- selective microelectrodes were used to measure the net NO_3^- flux along the roots.

Activities of PM H^+-ATPases

The PM H^+-ATPase activity was determined spectrophotometrically at 700 nm as described by Sorgona et al. (2011). Briefly, the assays were conducted at 38 °C in 0.6 ml of medium containing 30 mM BTP/MES (pH 6.5), 5 mM $MgSO_4$, 5 mM ATP, 0.6 mM Na_2MoO_4, 100 mM KNO_3, 1.5 mM NaN_3, and 0.01 % (w/v) polyoxyethylene 20 cetyl ether with or without 100 μM vanadate (an inhibitor of P-type H^+-ATPase). The difference between these two activities was attributed to PM H^+-ATPases. Sodium azide and KNO_3 were used as selective inhibitors of mitochondrial and tonoplast H^+-ATPases, respectively.

The reaction was initiated by the addition of membrane vesicles (1 μg of membrane protein) and terminated after 30 min by the addition of a solution containing 0.6 M HCl, 3 % (w/v) SDS, 3 % ascorbic acid and 0.5 % ammonium molybdate at 2 °C. After solubilization of the membrane vesicles with 0.5 M NaOH, the total soluble protein was measured according to Bradford (1976).

Analysis of the transcript levels of representative genes involved in N uptake

To identify the NH_4^+ and NO_3^- transporters of Prince Rupprecht's Larch and Chinese Fir, transcriptome sequencing data obtained from the two conifers were downloaded from the NCBI website and analyzed (Supplementary Tables S1 and S2). The representative genes encoding proteins associated with N uptake were selected for transcript analysis by quantitative RT-PCR (qPCR). The total RNA from plant tissues was isolated and purified using a plant RNA extraction kit (R6827, Omega Bio-Tek, GA, USA), and trace genomic DNA was digested by DNase I (E1091, Omega Bio-Tek). Aliquots of 1 μg of total RNA were used for first-strand cDNA synthesis using a PrimeScript RT reagent kit (DRR037S, Takara, Dalian, China) in a 20 μl reaction volume, according to the manufacturer's instructions. PCR was performed in a 20 μl reaction volume using 10 μl of 2× SYBR Green Premix Ex Taq II, 2 μl of cDNA, and 1 μl of 20 mM primers (Supplementary Tables S1 and S2) in a Roche LightCycler 96 instrument. 18S rRNA was used as a reference gene. Three biological replicates with three technical replicates were assayed for each sample. The reference gene was included in each plate. The efficiencies of all PCR reactions were between 95 and 105 % (Supplementary Tables S1 and S2).

Enzymes involved in N assimilation

Enzyme assays for N assimilation were conducted on six replicate seedlings from each treatment.

NR activity was assayed using the method described by Natali et al. (2009). Approximately 0.5 g fresh weight frozen material was ground to a fine powder in an ice-bath. The powder was extracted in a 4 ml ice-cold extraction buffer consisting of 25 mM phosphate buffer (pH 7.5), 5 mM cysteine and 5 mM EDTA–Na_2. The extract was centrifuged at 4000 rpm for 15 min at 4 °C, and then 0.4 ml enzyme extract was added to 1.6 ml of the assay mixture (1.2 ml of 0.1 M KNO_3–phosphate buffer and 0.4 ml of 2.0 mg ml^{-1} NADH) and incubated at 25 °C for 30 min. For the control, 0.4 ml phosphate buffer was used instead of 0.4 ml NADH. We analyzed the incubation buffer's nitrite (NO_2^-) concentration by adding 1 ml each of 1 % (w/v) sulfanilamide in 3 N HCl and 0.02 % *N*-naphthylethylenediamine in water. After a 15 min reaction, all samples were centrifuged for 5 min at 4000 rpm, and the supernatant was analyzed in a spectrophotometer at 540 nm. The NO_2^- concentration was determined using a standard curve.

Nitrite reductase (NiR) activity was measured as the reduction in the amount of NO_2^- in the reaction mixture. The reaction mixture consisted of a 0.1 M potassium phosphate buffer (pH 6.8), 0.4 mM $NaNO_2$, 2.3 mM methyl viologen, enzyme extract and 4.3 mM sodium dithionite in 100 mM $NaHCO_3$, which initiated the reaction. After a 30 min incubation period at 27 °C, the reaction was stopped by spinning and boiling the mixture for 1 min. After the reaction, the amount of NO_2^- ions remaining in the reaction mixture was measured at 540 nm using the standard $NaNO_2$ curve.

For the GS activity assay, frozen tissues (approximately 1 g) were ground in 3.0 ml of a 100 mM Tris–HCI (pH 7.6) extraction buffer containing 1 mM EDTA, 1 mM $MgCl_2{\cdot}6\ H_20$ and 10 mM 2-mercaptoethanol using an ice-cold mortar and pestle. The homogenate was centrifuged at 13000 rpm for 25 min to clarify the solution, and the supernatant was used as the crude enzyme solution in the assay. A sample of 1.2 ml of the crude enzyme extract was added to a 1.6 ml assay mixture containing 0.6 ml imidazole-muriatic acid buffer (0.25 M, pH 7. 0), 0.4 ml glutamic acid-Na (0.30 M, pH 7.0), 0.4 ml ATP-Na (30 M, pH 7.0), 0.2 ml $MgSO_4$ (0.5 M) and 1.2 m1 crude GS solution. The mixture was incubated for 5 min at 25 °C, and then 0.2 ml of hydroxylamine hydrochloride (a mixture of 1 M hydroxylamine hydrochloride and 1 M HCl at a ratio of 1:1) was added and left to stand for 15 min. The reaction was stopped by adding 0.8 ml acidic $FeCl_3$ (2 % (W/V) TCA and 3.5 % (W/V) FeC_{l3} in 2 % HCl). The samples were centrifuged at 4000 rpm for 15 min, and the absorbance of the supernatant was measured at 540 nm. The amount of γ-glutamylhydroxamate formed was determined using a standard curve derived from authentic glutamylhydroxamate in the presence of all assay components.

Glutamate synthase (GOGAT) activity was measured following Singh and Srivastava's method (1986). Briefly, control and treated root and shoot samples (100 mg) were homogenized in 0.2 M sodium phosphate buffer (pH 7.5) containing 2 mM EDTA, 50 mM KCl, 0.1 % (v/v) mercaptoethanol and 0.5 % (v/v) Triton X 100. The homogenate was centrifuged at 6000 rpm for 15 min at 4 °C. The supernatant was used to estimate GOGAT activity. Three milliliters of the reaction mixture contained25 mM sodium phosphate buffer (pH 7.3), which consisted of 1 mM EDTA, 20 mM L-glutamine, 5 mM 2-oxoglutarate, 100 mM KCl, 1 mM NADH, and 0.3 ml enzyme extract. A decrease in absorbance was measured at 340 nm for 5 min.

Data processing and statistical analysis

Net ion flux data were calculated and exported using the MageFlux software (version 1.0) attached to the SIET system. Readings for the net NH_4^+ influxes and NO_3^- influxes over 10 min were averaged at each measuring point for each plant. All statistical tests were performed with SPSS (version 20.0, SPSS Inc., Chicago, IL, USA). A two-way ANOVA was used to examine the effects of pH and species on the experimental variables. The data were tested for normality prior to further analyses. Differences between the means were determined on the basis of least significant differences ($P = 0.05$).

Results

Growth parameters

pH had different effects on root morphology and photosynthesis within Prince Rupprecht's Larch and Chinese Fir (Table 1). Prince Rupprecht's Larch grown in a neutral pH (pH 7) nutrient solution had greater biomass, root surface area and net photosynthetic rates compared with those grown in pH 4 (Table 1). However, low pH increased root length and net photosynthetic rate in Chinese Fir, demonstrating the acclimation of this species to low pH (Table 1).

To determine the N uptake in fine roots, we monitored the net NH_4^+ and NO_3^- fluxes from the root tip to 30 mm from the apex using the SIET technique. The analyses of data from seedlings pre-treated with pH 4 or 7 showed that root position had a strong effect on the flux of both ions. In fine roots of Prince Rupprecht's Larch, the net NH_4^+ flux at pH 7 varied from 20 to 67 pmol cm^{-2} s^{-1} along the root tip, and the value was greater than that of roots at pH 4 (from −2 to 30 pmol cm^{-2} s^{-1}) (Fig. 1a). In roots of Chinese Fir, the net NH_4^+ flux ranged from 5 to 90 pmol cm^{-2} s^{-1} along the root apex (Fig. 1b). Both Prince Rupprecht's Larch and Chinese Fir had high NH_4^+ influxes near the root tip, and the net NH_4^+ uptake decreased slightly from the root tip to the basal regions (Fig. 1A, B); however, the decline was more abrupt in Chinese Fir.

The net NO_3^- flux in Prince Rupprecht's Larch was greatest at 1.5–2.1 mm from the root tip (Fig. 1c). However, the net NO_3^- flux in Chinese Fir was zero or negative (efflux) at the root tip, and the greatest flux rate occurred 13 mm from the root tip. In Chinese Fir roots, locations near the root tip (i.e., 0–0.3 mm) exhibited an NO_3^-efflux that was significantly lower than that observed in locations distal to the root tip. The net flux of NO_3^- in Chinese Fir ranged from −10 to 31 pmol cm^{-2} s^{-1} in pH 7 solution and from −5 to 17 pmol cm^{-2} s^{-1} in pH 4 solution (Fig. 1d).

NH_4^+ and NO_3^- uptake and H^+-ATPase activity

In roots of Prince Rupprecht's Larch, the mean net NH_4^+ uptake was significantly greater in the pH 7 solution relative to the pH 4 solution (Fig. 2a). The net uptake of NH_4^+ decreased by approximately 30 % when both NH_4^+ and NO_3^- were present in the pH 4 solution, whereas it remained unaltered in the pH 7solution (Fig. 2a). The NO_3^- flux in Prince Rupprecht's Larch was also greater in the pH 7 solution than in the pH 4 solution in the presence of KNO_3. pH did not have a significant effect on net NO_3^- flux when NH_4NO_3 was used as the N source (Fig. 2c).

pH did not have a significant effect on mean net uptake of NH_4^+ in Chinese Fir (Fig. 2b); however, the mean net

Table 1 Growth parameters and photosynthesis of Prince Rupprecht's Larch and Chinese Fir treated with solutions at pH 4 or 7

Species	pH	Biomass (mg DW)	Total root length (cm)	Total root surface area (cm^2)	Total root volume (cm^3)	Height (cm)	Net photosynthetic rate (mmol CO_2 m^{-2} s^{-1})
Prince Rupprecht's Larch	4	100.59 ± 2.35b	46.90 ± 4.33a	11.03 ± 1.04b	0.64 ± 0.02a	11.95 ± 0.38a	5.02 ± 0.26bc
	7	120.48 ± 4.91a	49.37 ± 2.65a	15.61 ± 1.19a	0.60 ± 0.03a	12.65 ± 0.52a	6.33 ± 0.12a
Chinese fir	4	108.21 ± 5.90ab	31.30 ± 1.68b	10.96 ± 1.12b	0.54 ± 0.04a	8.62 ± 0.27b	5.07 ± 0.30b
	7	115.96 ± 3.94ab	19.12 ± 1.71c	10.16 ± 0.69b	0.61 ± 0.03a	8.80 ± 0.19b	4.66 ± 0.18c
P values	Species	ns	***	*	ns	***	**
	pH	**	ns	ns	ns	ns	ns
	Species × pH	ns	*	*	ns	ns	***

Data indicate mean ± SE ($n = 6$). A two-way ANOVA was used to examine the effects of pH and species. Different letters in the same column indicate significant difference. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *ns* not significant

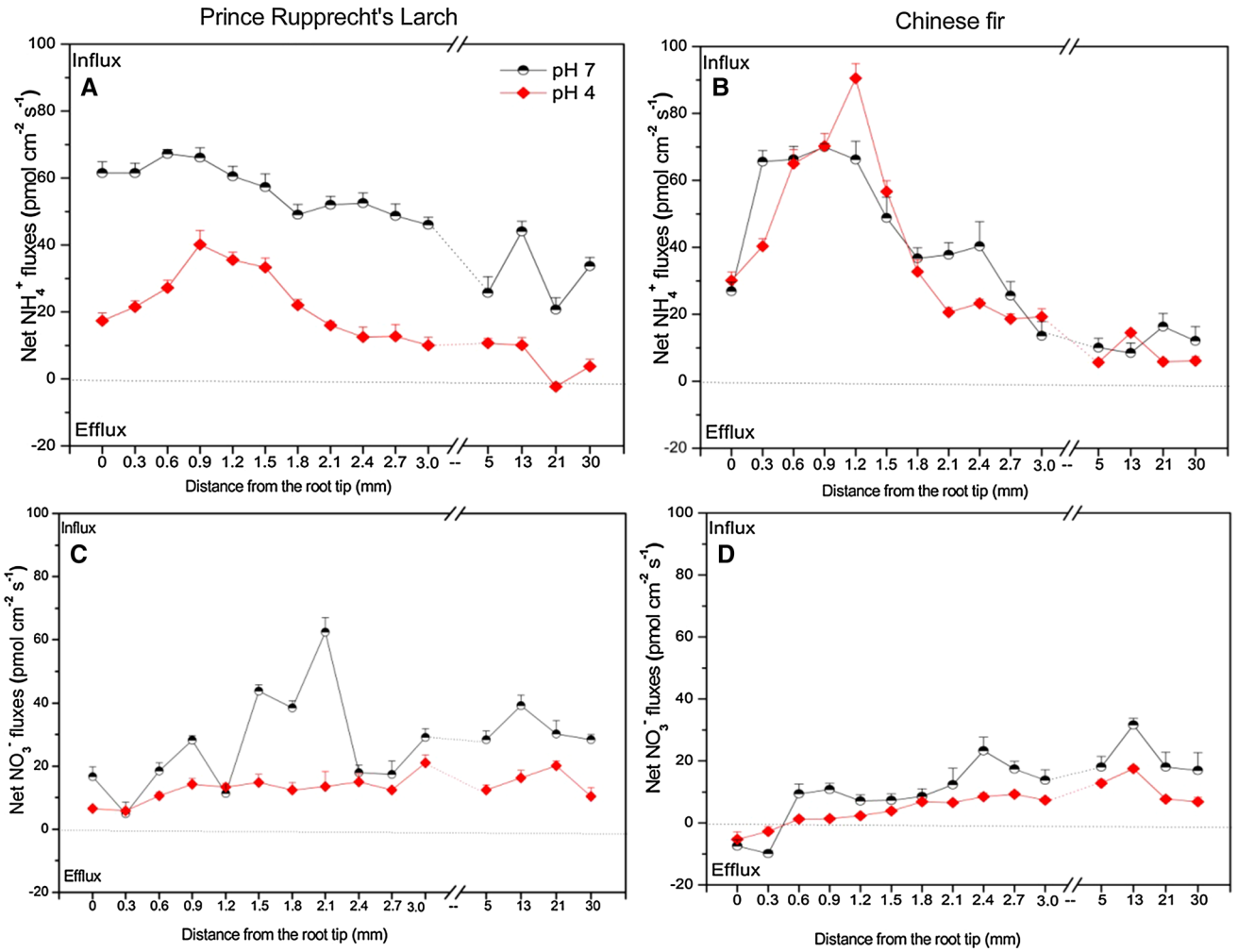

Fig. 1 Net fluxes of NH_4^+ (**a**, **b**) and NO_3^- (**c**, **d**) (pmol cm^{-2} s^{-1}) (Mean ± SE, $n = 6$) measured at specified distances from the tips of Prince Rupprecht's Larch and Chinese fir roots. Eight-week-old seedlings were incubated in a solution containing 1 mM KCl, 0.1 mM $CaCl_2$, pH 4 or 7, to which either 0.05 mM NH_4Cl for NH_4^+ or 0.05 mM KNO_3 for NO_3^- flux measurements was added

NO_3^- uptake was significantly greater at pH 7 than at pH 4 in both measurement solutions (Fig. 2d). Significant differences were not found in NH_4^+ and NO_3^- influx when NH_4Cl or NH_4NO_3 were used as the N source (Fig. 2b, d).

A comparison of the uptake of the two forms of nitrogen found in NH_4NO_3 showed that in Prince Rupprecht's Larch, the rate of NO_3^- uptake was 1.3 times higher than the rate of NH_4^+ uptake at pH 4, whereas the rate of NH_4^+ uptake was 1.7 times higher than the rate of NO_3^- uptake at pH 7. Moreover, the rate of NH_4^+ uptake in Chinese Fir was 5.6 (pH 4) and 2.6 (pH 7) higher than the rate of NO_3^- uptake in NH_4NO_3 measurement solutions.

Low pH significantly decreased the H^+-ATPase activity in roots of Prince Rupprecht's Larch by more than 50 % (Fig. 3). However, the H^+-ATPase activity in roots of Chinese Fir remained unaltered (Fig. 3).

Transcriptional regulation of genes involved in N uptake

In Prince Rupprecht's Larch, three *AMTs* and ten *NRTs* were selected and analyzed. Among the three *AMTs* expressed in the roots of Prince Rupprecht's Larch, *AMT1;1* exhibited high transcript abundance. The expression level of *AMT1;1* in Prince Rupprecht's Larch was significantly higher at pH 7 than at pH 4. Low pH also induced a significant down-regulation of all *NRT*s. In particular, the expression levels of *NRT1;1*, *NRT1;6* and *NRT1;8* were several-fold higher at pH 7 than at pH 4 (Fig. 4a). The observed down-regulation of the expression of *NRTs* in Prince Rupprecht's Larch after treatment with pH 4 solution was consistent with the decreases in the net uptake of NH_4^+ and NO_3^- (Fig. 4a).

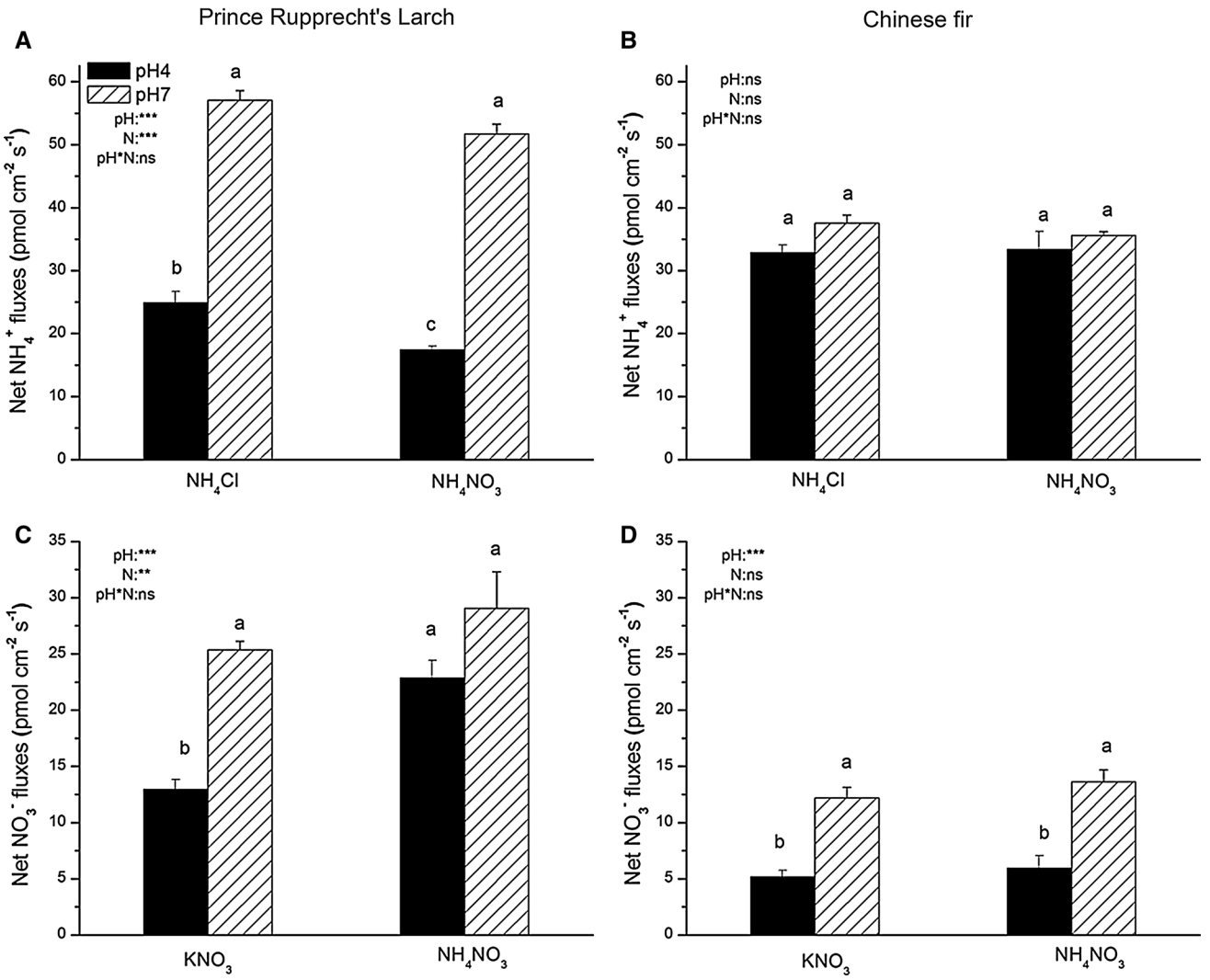

Fig. 2 Mean net fluxes of NH_4^+ (**a**, **b**) and NO_3^- (**c**, **d**) (Mean ± SE, $n = 6$) averaged over the apical 30 mm of roots of Prince Rupprecht's Larch and Chinese Fir. A two-way ANOVA was used to examine the effects of pH and measurement solution. *Bars* labeled with *different letters* indicate significant difference between the treatments

In Chinese Fir, five *AMTs* and ten *NRTs* were found in the transcriptome sequencing data and used for the analysis. The transcript abundances of *AMT2;1* and *AMT2;2* in pH 7 solution were significantly decreased compared with those in pH 4 solution (Fig. 4b). However, the remaining three *AMTs* (*AMT1;1*, *AMT1;2* and *AMT2;3*) were not significantly affected by the pH level. Low pH also decreased the expression levels of *NRTs*, such as *NRT1;8* and *NRT3;1*, in Chinese Fir (Fig. 4b).

N assimilation

The NR activity in Prince Rupprecht's Larch was reduced by 64 and 34 % (in roots and leaves, respectively) in the pH 4 solution compared to that in the pH 7 solution (Fig. 5a, b). The NiR activity in Prince Rupprecht's Larch was also inhibited when roots were exposed to pH 4 solution (Fig. 5c, d), and low pH also decreased NR activities and NiR activities by 28 and 25 % in roots of Chinese Fir, respectively (Fig. 5a, c). However, foliar NR activity and NiR activity in Chinese Fir were not influenced by pH (Fig. 5b, d).

In Prince Rupprecht's Larch, low pH had no significant effect on GS activity in roots, whereas it decreased GS activity in leaves (Fig. 6a, b). GOGAT activity was not influenced by pH in either location (Fig. 6c, d). In contrast, GS activity in Chinese Fir was 34 and 80 % higher in the roots and leaves in the pH 4 solution, respectively (Fig. 6a, b). Low pH also induced higher GOGAT activity in Chinese Fir roots (Fig. 6c), whereas significant difference were not found between pH 4 and pH 7 for foliar GOGAT activity (Fig. 6d).

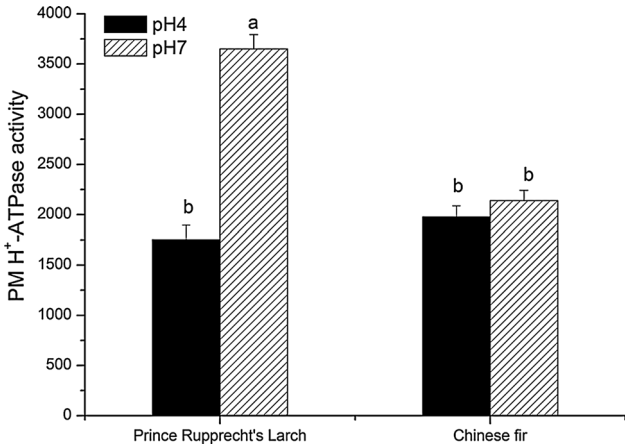

Fig. 3 Activities of H^+-ATPase activity [nkat (mg protein)$^{-1}$] in roots of Prince Rupprecht's Larch and Chinese Fir pretreated in solutions at pH 4 or 7. A two-way ANOVA was used to examine the effects of pH and species. *Bars* labeled with different *letters* indicate significant difference between the treatments

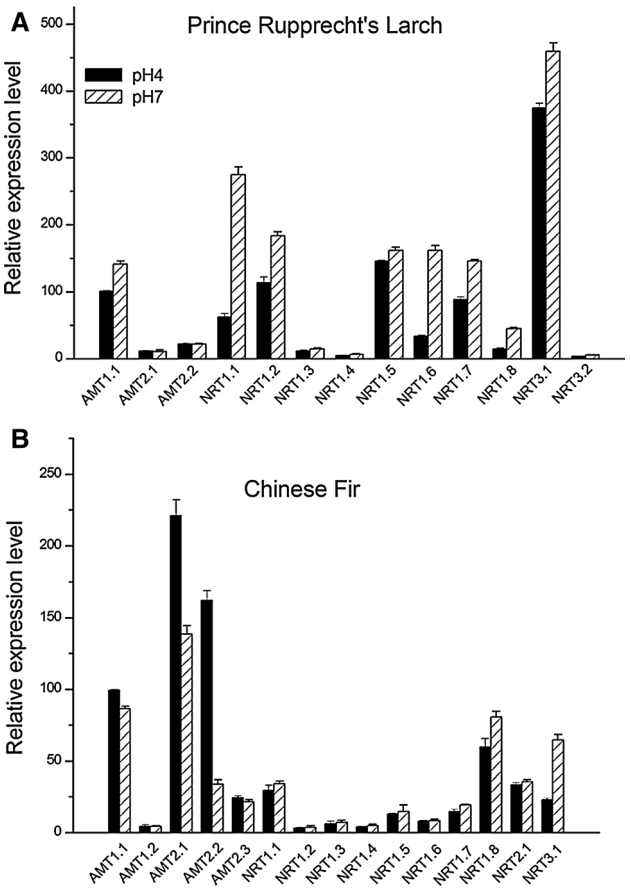

Fig. 4 Transcriptional fold-changes of key genes involved in N uptake in roots of Prince Rupprecht's Larch (**a**) and Chinese Fir (**b**) in solutions at pH 4 or 7. The signal intensities were calibrated based on a constitutively expressed Prince Rupprecht's Larch 18 s rRNA gene and Chinese Fir 18 s rRNA gene, respectively. The scales of the *y*-axis are different from each other

Discussion

Plant growth

Following forest disturbance, NH_4^+ is easy to utilize and a greater proportion of N may be available as NO_3^- (Hope et al. 2003). These altered soil conditions may negatively affect the growth of trees adapted to NH_4^+-rich soils (Kronzucker et al. 1997). In conifers that prefer NH_4^+, growth declines when pH changes from acidic to neutral (Van Den Driessche 1978). This is in agreement with the greater root length and net photosynthetic rates of Chinese Fir. However, Prince Rupprecht's Larch shows opposite pattern. These results indicate that pH may have different effects on N uptake and assimilation mechanisms in two conifers species.

Variation in NH_4^+ and NO_3^- fluxes along the root

Fine roots have four distinct regions: root cap and meristematic, elongation and maturation zones. Each area has different anatomical and functional characteristics that provide different nutrient ion uptake capacities (Enstone et al. 2001; Li et al. 2012). Previous studies have suggested that different zones of a root's apical region have distinct net fluxes of NH_4^+ and/or NO_3^- (Fang et al. 2007; Li et al. 2010). In this study, Prince Rupprecht's Larch and Chinese Fir both exhibited the highest net fluxes of NH_4^+ near the root tip, whereas NO_3^- fluxes near the root tip were significantly lower than those observed further away. The net NH_4^+ and NO_3^- fluxes were greatest at 5–20 and 0–30 mm from the root tips in Douglas Fir, respectively and 5 and 0–10 mm from the root tips in lodgepole pine, respectively (Hawkins et al. 2008). Axial scans have revealed a slight decline in net NH_4^+ flux with distance from the root apex in barley roots (Henriksen et al. 1992), and NO_3^- uptake near the root tips was slower compared with the uptake by the more basal regions in maize (Lazof et al. 1992), barley (Siebrecht et al. 1995) and poplar (Luo et al. 2012). Overall, these results indicate that spatial variation in the uptake of NH_4^+ and NO_3^- may be linked to the different anatomical properties of roots. Different ion uptake profiles likely reflect differences in root development rates and root anatomy at a given distance from the tip. The expression of N transporters or genes responsible for these transporters has been shown to vary with distance from the root tip (Okamoto et al. 2003). Further research is required to better understand the correlation between gene expression patterns and flux profiles along the roots.

NH_4^+ and NO_3^- uptake

Low pH decreased the net NH_4^+ uptake in Prince Rupprecht's Larch but had no effect on NH_4^+ uptake in

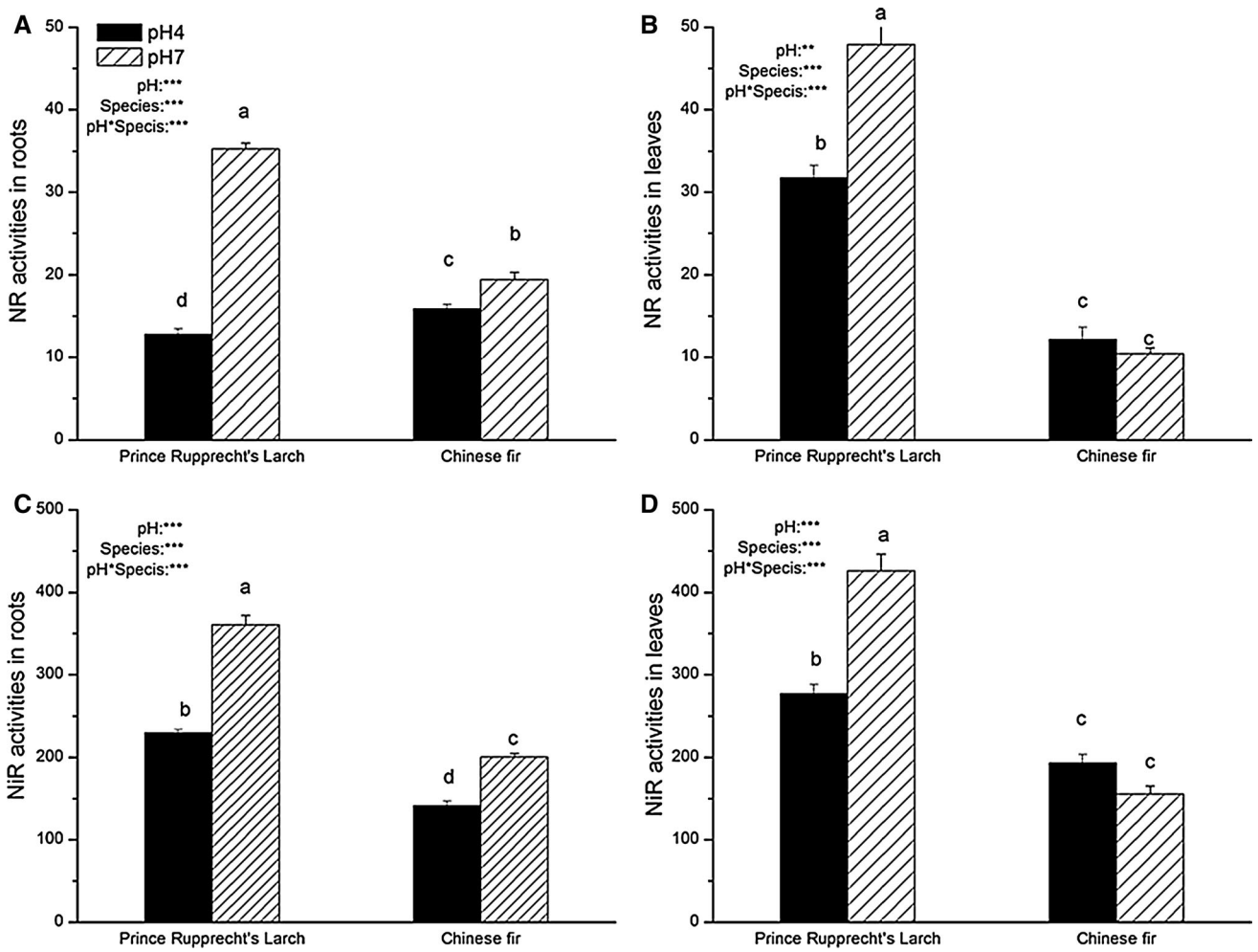

Fig. 5 Activities of nitrate reductase (NR, nkat [mg protein)$^{-1}$] (**a**, **b**) and nitrite reductase [NiR, nkat (mg protein)$^{-1}$] (**c**, **d**) in roots and leaves of Prince Rupprecht's Larch and Chinese fir pretreated in solutions at pH 4 or 7. A two-way ANOVA was used to examine the effects of pH and species. *Bars labeled with different letters* indicate significant difference between the treatments

Chinese Fir. We hypothesize that Chinese Fir is adapted to acidic soils of South China and can maintain N uptake, particularly in NH_4^+ form, under low pH conditions. However, Prince Rupprecht's Larch grows on the Loess Plateau in northwest China in neutral or alkaline soils. The differences in NH_4^+ uptake under different pH levels between the two species could be related to the ability of the species to maintain proton efflux associated with NH_4^+ assimilation at low pH or different uptake mechanisms in the species for NH_4^+ (Hawkins and Robbins 2010). Chinese Fir is likely capable of maintaining its H^+-ATPase activity and proton efflux at pH 4 and pH 7. In addition, the NH_4^+ transporters were also up-regulated at pH 4. Therefore, these trees have a high net uptake of NH_4^+ at both low and neutral pH conditions. In contrast, Prince Rupprecht's Larch from the Loess Plateau cannot maintain proton efflux and therefore exhibits inhibited NH_4^+ uptake. The down-regulation of the expression of *AMTs* is also consistent with the inhibition of NH_4^+ uptake.

Low net NO_3^- uptake from pH 4 soils was found in most of the treatments, which is inconsistent with existing theories (Fig. 2c, d). In anion transport across the root plasma membranes, proton-anion cotransport likely uses the steep electric potential gradients and pH as driving forces (Hawkins and Robbins 2010). NO_3^- uptake, therefore, is expected to increase as pH decreases. However, this result was not found in our research or in previous studies. For example,NO_3^- uptake in *Arabidopsis thaliana* was greatest at pH 8–9 when pH ranged from 3 to 10, and NO_3^- uptake in *Picea abies* was greatest at pH 5.5 when pH ranged from 2.5 to 6.5 (Doddema and Telkamp 1979). As demonstrated in the present study, at low pH, the PM H^+-ATPase activity was decreased in Prince Rupprecht's Larch and remained unchanged in Chinese fir.

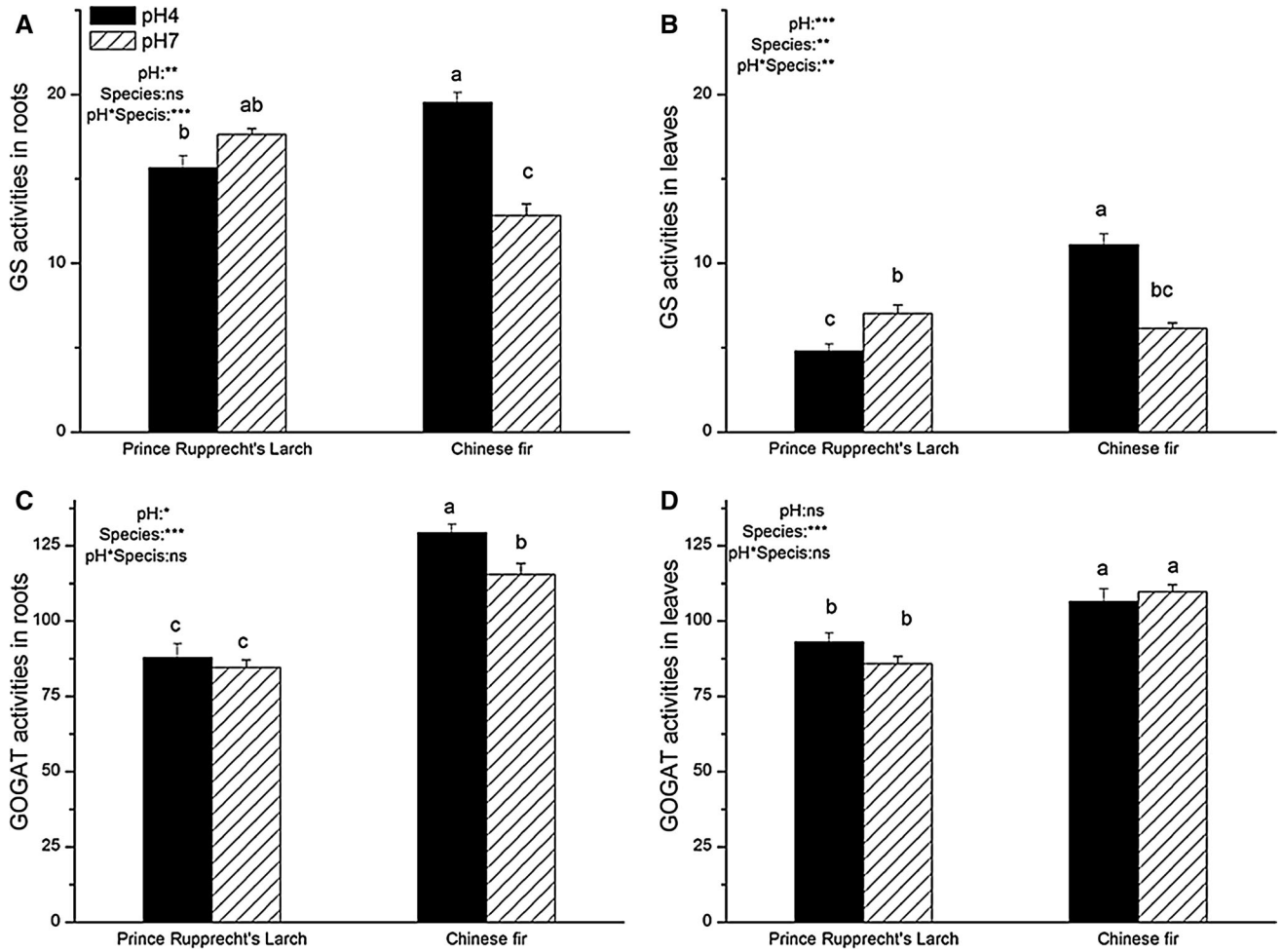

Fig. 6 Activities of glutamine synthetase (GS, nkat (mg protein) $^{-1}$) (**a**, **b**) and glutamate synthase [GOGAT, nkat (g protein)$^{-1}$] (**c**, **d**) in roots and leaves of Prince Rupprecht's Larch and Chinese fir pretreated in solutions at pH 4 or 7. A two-way ANOVA was used to examine the effects of pH and species. *Bars labeled with different letters* indicate significant difference between the treatments

Additionally, the transcript levels of *NRTs* were also decreased at low pH solution in the two conifers. Previous studies have attributed the low NO_3^- uptake of conifers under acidic conditions to reduced NO_3^- reductase activity, which is consistent with the results obtained in our study (Marschner et al. 1991). Reid and Hayes (2003) noted that the negative effect of high chloride ion concentrations on NO_3^- transporters could also result in low NO_3^- uptake because competition occurs between the two anions for the same anion transporter; however, further research is required to test this theory.

Our results revealed a significantly greater uptake of NH_4^+ compared with that of NO_3^- in most treatments, and this result is consistent with previous studies in which conifers have shown a preference for NH_4^+ (Lucash et al. 2005; Socci and Templer 2011). The greater capacity for NH_4^+ uptake may be an adaptation to the greater availability of NH_4^+ in forest soils (Lucash et al. 2005). Soils tend to possess an overall negative charge, which allows NO_3^- to move freely, and NO_3^- is also easily lost from the root zone through leaching because of its high diffusion coefficient in soil (Lambers and Colmer 2005).

N assimilation

A limited number of studies have focused on the effects of pH on N assimilation. The mechanism by which enzyme activity decreases or increases with pH is not well known, although the mechanisms of enzyme regulation associated with several environmental (salt, drought, heat) and nutritional factors are well understood (Lambers and Colmer 2005; Xu et al. 2012). In this study, low pH significantly reduced NR and NiR enzyme activities in both species. NR is a substrate-inducible enzyme; thus, lower NO_3^- availability at pH 4 may be a potential mechanism that lowers NR activity. This hypothesis supports the net NO_3^- flux results obtained in the pH 4 solution in this study. NR is also known to be regulated at the NR protein synthesis level and

through post-translational modification of the protein, which could be an area of further investigation (Kaiser et al. 1999). Low NiR activity at highly acidic pH may be caused by limited NO_2^- resulting from low NR activity at the same pH. pH has distinct effects on GS and GOGAT activities in Prince Rupprecht's Larch and Chinese Fir. Low pH induces higher GS and GOGAT activities in Chinese Fir but reduces these activities in Prince Rupprecht's Larch, which may be caused by the different habitats for these trees. Prince Rupprecht's Larch grows on the Loess Plateau in soils with low NH_4^+, whereas Chinese Fir grow in acidic soils rich in NH_4^+. In acidic forest soils, NO_3^- may become rapidly reduced to NH_4^+, which would render NH_4^+ a major N source for plant roots (Rennenberg et al. 2010 and references therein). Thus, Chinese Fir exhibits higher GS activity in response to high uptake of ammonium and are adapted to low pH levels in soil and to avoid the possible toxic effect of ammonium ion on cell metabolism.

Author contribution statement Conceived and designed the experiments: Sen Meng, Zhong Zhao. Performed the experiments: Sen Meng, Yang Cao, Li Su, Yiming Li. Analyzed the data: Li Su, Chunxia Zhang, Sen Meng. Wrote the paper: Sen Meng, Zhong Zhao.

Acknowledgments This research was supported by the State Key Basic Research Development Program (973 Program, Grant No. 2012CB416902). We thank the numerous students and laboratory staff from Northwest A&F University who assisted in the laboratory experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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