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ORIGINAL ARTICLE

Net fluxes of ammonium and nitrate in association with H^+ fluxes in fine roots of *Populus popularis*

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Abstract Poplar plants are cultivated as woody crops, which are often fertilized by addition of ammonium (NH_4^+) and/or nitrate (NO_3^-) to improve yields. However, little is known about net NH_4^+/NO_3^- fluxes and their relation with H^+ fluxes in poplar roots. In this study, net NH_4^+/NO_3^- fluxes in association with H^+ fluxes were measured non-invasively using scanning ion-selective electrode technique in fine roots of *Populus popularis*. Spatial variability of NH_4^+ and NO_3^- fluxes was found along root tips of *P. popularis*. The maximal net uptake of NH_4^+ and NO_3^- occurred, respectively, at 10 and 15 mm from poplar root tips. Net NH_4^+ uptake was induced by ca. 48 % with provision of NO_3^- together, but net NO_3^- uptake was inhibited by ca. 39 % with the presence of NH_4^+ in poplar roots. Furthermore, inactivation of plasma membrane (PM) H^+-ATPases by orthovanadate markedly inhibited net NH_4^+/NO_3^- uptake and even led to net NH_4^+ release with NO_3^- co-provision. Linear correlations were observed between net NH_4^+/NO_3^- and H^+ fluxes in poplar roots except that no correlation was found between net NH_4^+ and H^+ fluxes in roots exposed to NH_4Cl and 0 mM vanadate. These results indicate that root tips play a key role in NH_4^+/NO_3^- uptake and that net NH_4^+/NO_3^- fluxes and the interaction of net fluxes of both ions are tightly associated with H^+ fluxes in poplar roots.

Keywords Efflux · Fertilizer · Influx · Ion uptake · Poplar · Proton

Abbreviations

PM	Plasma membrane
SIET	Scanning ion-selective electrode technique

Introduction

Nitrogen (N) is an essential component in proteins, nucleic acids, chlorophylls and many secondary metabolites of plants and therefore required as macronutrient for plant growth. Ammonium (NH_4^+) and nitrate (NO_3^-) are two main forms of inorganic N available for plants in soil. Plants can absorb and utilize both ions because root cells possess transport systems such as ammonium and nitrate transporters (Jackson et al. 2008). Although both ions can be utilized by plants, NH_4^+ and NO_3^- have different energetic and biochemical characteristics for assimilation, resulting in different net fluxes of both ions and NH_4^+/NO_3^- preference by plants (Patterson et al. 2010).

Fluxes of NH_4^+/NO_3^- in roots have been investigated in the past few decades. Spatial variability in uptake of NH_4^+

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and NO_3^- has been found along the roots in herbaceous and woody plants. The net NO_3^- uptake increased from the root apex to the basal regions of maize (*Zea mays* cv. Dekalb) and barley (*Hordeum vulgare* L. cv Prato) roots (Henriksen et al. 1992; Taylor and Bloom 1998), but the opposite pattern was observed in rice (*Oryza sativa* L.) and carob (*Ceratonia siliqua* L. cv. Mulata) roots (Cruz et al. 1995; Colmer and Bloom 1998). The highest NO_3^- influx occurred at 20–50 mm from the root tips in non-mycorrhizal roots of *Pinus pinaster* (Plassard et al. 2002). In Douglas fir (*Pseudotsuga menziesii*) and Lodgepole pine (*Pinus contorta*) the maximal net NO_3^- uptake appeared at 0–30 and 0–10 mm from the root tips, respectively, while the highest net NH_4^+ uptake occurred at 5–20 mm and 5 mm, respectively (Hawkins et al. 2008). Additionally, temporal dynamics of net ion fluxes and influences of other ions and environmental factors such as pH in the media on net ion fluxes have been reported for roots of maize, barley, rice, coniferous and *Eucalyptus* species (Henriksen et al. 1992; Colmer and Bloom 1998; Garnett et al. 2001, 2003; Hawkins et al. 2008; Hawkins and Robbins 2010; Sorgona et al. 2011).

The interaction of NH_4^+ and NO_3^- on fluxes of both ions and the underlying physiological mechanisms are yet unclear. For instance, net NH_4^+ uptake was unaffected in the presence or absence of NO_3^-, and vice versa, in roots of Douglas fir and lodgepole pine (Hawkins et al. 2008), but the net uptake of NO_3^- was markedly reduced when NH_4^+ was present simultaneously with NO_3^- in non-mycorrhizal roots of *Pinus pinaster* (Gobert and Plassard 2007). For most plants including woody plants, high concentrations of NH_4^+ are toxic, if supplied as a sole N resource and this toxicity disappears if both ions are provided together, whereas no detrimental effects are found in plants supplied with NO_3^- as a sole N fertilizer (Babourina et al. 2007; Ehlting et al. 2007). Although the underlying mechanisms of NH_4^+ toxicity are explored extensively (Patterson et al. 2010), little information is available on fluxes of both ions related to NH_4^+ toxicity in plant roots.

Fluxes of NH_4^+ and NO_3^- in plant roots are associated with plasma membrane (PM) H^+-ATPases which extrude protons from the cytosol to the outside at the expense of ATP (Britto and Kronzucker 2006). NH_4^+ may enter root cells passively through a potential uniporter system following the electrochemical potential gradient across the plasma membrane, but NH_4^+ must be actively transported out of root cells with the help of PM H^+-ATPases during NH_4^+ efflux (Britto et al. 2001; Britto and Kronzucker 2006). NO_3^- is transported into root cells via H^+-coupled symporters with involvement of PM H^+-ATPases and may leak back passively into the apoplast as efflux (Miller and Cramer 2004; Britto and Kronzucker 2006). Activities of PM H^+-ATPases and the expression of the corresponding genes are induced in response to NO_3^- in roots of maize and citrus plants (Santi et al. 1995; Sorgona et al. 2010, 2011). Thus, PM H^+-ATPases play an essential role in re-establishing the membrane potential and in maintaining the H^+ gradient across the PM during NH_4^+ and NO_3^- fluxes (Palmgren 2001; Palmgren and Nissen 2011). It is therefore important to consider PM H^+-ATPases when fluxes of NH_4^+ and NO_3^- are under investigation. To our knowledge, however, PM H^+-ATPases were not considered in previous studies dealing with fluxes of NH_4^+ and NO_3^- in plant roots.

Populus is a model for studies of woody plant biology with ca. 30–40 species mainly distributed in the temperate regions of the north-hemisphere (Polle and Douglas 2010). This genus contains many species adapted to riparian ecosystems in which the moving water table may bring high amounts of N to the roots due to run off from intensive N-fertilization application in agriculture (Rennenberg et al. 2010). In these riparian ecosystems, NH_4^+ may become rapidly oxidized to NO_3^-, rendering it as a major N source for plant roots (Rennenberg et al. 2010 and references therein). Thus, poplars may have different patterns of NH_4^+/NO_3^- fluxes compared with other herbaceous and woody species which require habitats enriched in reduced N forms such as NH_4^+. Moreover, most *Populus* species grow in flooded soils (at least during some period of the year), leading to periods with anoxia or hypoxia which can suppress the activity of PM H^+-ATPases via a decrease of oxygen and ATP provision. In this context, it also appears important to study the coupling between the inhibition of PM H^+-ATPases and the net fluxes of NH_4^+ and NO_3^-. Due to easy propagation and fast growth characteristics, poplar plantations are widely established in recent years and may play crucial roles in the pulp and paper industry, carbon mitigation and biomass production for biofuels (Luo et al. 2006, 2008; Luo and Polle 2009; Novaes et al. 2009; Studer et al. 2011). Poplar plantations are frequently established on marginal land where N is a constraining factor for maximal growth rates (Novaes et al. 2009; Li et al. 2012). NH_4^+ and NO_3^- as N fertilizers are often added to these plantations to maximize yields. Knowledge of NH_4^+ and NO_3^- fluxes in roots of *Populus* species will lead to improved N fertilizer management practices such as selection of N-forms (NH_4^+, NO_3^- or both) for application in plantations. However, to date, net fluxes of NH_4^+ and NO_3^- related to PM H^+-ATPases are unknown in roots of *Populus* species.

In this study, we employed scanning ion-selective electrode technique (SIET), a powerful tool to investigate ion fluxes in plant roots (Xu et al. 2006). Net NH_4^+ and NO_3^- fluxes associated with PM H^+-ATPases were measured non-invasively by SIET along fine roots of *Populus popularis*, which is a species widely distributed in north

China and often selected for afforestation in nutrient-poor soils in this region. The aims of this study are (1) to examine the spatial patterns of net NH_4^+ and NO_3^- fluxes and to determine the positions from the root tips where the maximal net NH_4^+ or NO_3^- uptake occurs; (2) to monitor net NH_4^+ or NO_3^- fluxes associated with PM H^+-ATPases and the interaction of both ions and (3) to find the possible correlations between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes.

Materials and methods

Plant cultivation

Cuttings (ca. 15 cm in length, 2 cm in diameter, one-year-old stem) of *Populus popularis* collected from a tree-breeding program (Cao et al. 2012) were rooted and planted in pots (10 L) filled with sandy soil. Each plant was irrigated daily with 50 mL Long-Ashton (LA) nutrient solution (Dluzniewska et al. 2007). Plants were cultivated for 6 weeks in a glass house (natural light; day/night temperate: 25/20 °C; relative humidity: 75 %). Subsequently, plants with similar height (ca. 60 cm) were selected and the root system for each selected plant was carefully washed by tap water. Washed plants were cultivated in hydroponics with modified LA solution (0.1 mM NH_4NO_3, 0.5 mM KCl, 0.9 mM $CaCl_2$, 0.3 mM $MgSO_4$, 0.6 mM KH_2PO_4, 42 μM K_2HPO_4, 10 μM Fe-EDTA, 2 μM $MnSO_4$, 10 μM H_3BO_3, 7 μM Na_2MoO_4, 0.05 μM $CoSO_4$, 0.2 μM $ZnSO_4$ and 0.2 μM $CuSO_4$, pH 5.5) for 2 weeks. Finally, plants were N starved for 2 days in modified LA solution without NH_4NO_3 prior to flux analysis.

Measurements of ion fluxes at root surface

To monitor net fluxes of NH_4^+, NO_3^- and H^+ in roots of *P. popularis*, three white fine roots (0.31 ± 0.01 mm in diameter, 50.0 ± 1.1 mm in length) were selected and excised from the root system (total volume: 3.56 ± 0.63 cm^3, total surface area: 861.63 ± 49.40 cm^2, total length: 274.26 ± 17.55 cm, total dry weight: 1.23 ± 0.09 g) of each plant (ca. 9-week-old). The excised roots were immersed in measuring solution. Six plants for NH_4^+ and another six plants for NO_3^- were used for ion flux analyses. The net ion fluxes were measured using scanning ion-selective electrode technique (SIET, system BIO-003A; Younger USA Science and Technology Corp.; Applicable Electronics Inc.; Science Wares Inc., Falmouth, MA, USA) at the company (Xuyue Science and Technology Co., Ltd. Beijing, China). The SIET system and its application in ion flux detection were described in detail (Xu et al. 2006; Li et al. 2010; He et al. 2011). Briefly, the ion-selective microelectrodes with 2–4 μm apertures were manufactured and silanized [for NH_4^+ electrode: 100 mM NH_4Cl as backfilling solution, followed by an NH_4^+-selective liquid ion-exchange cocktail (#09879, Sigma); for NO_3^- electrode: 10 mM KNO_3 as backfilling solution, followed by a NO_3^--selective liquid ion-exchange cocktail (#7254, Sigma); for H^+ electrode: 15 mM NaCl and 40 mM KH_2PO_4 as backfilling solution, followed by a H^+-selective liquid ion exchanger cocktail (#95293, Sigma)]. Prior to the flux measurements, the microelectrodes were calibrated [for NH_4^+: 0.05 and 0.5 mM NH_4Cl in addition to other compounds used in the measuring solution (see below); for NO_3^-: 0.05 and 0.5 mM KNO_3 in addition to other compounds used in the measuring solution; for H^+: pH 6.0 and pH 5.0 in addition to the compounds used in the measuring solution] and only electrodes were used with Nernstian slopes higher than 55 mV per tenfold concentration difference.

To determine the positions along the root where the maximal ion influxes of NH_4^+ and NO_3^- occur, a preliminary experiment was carried out with an initial measurement at the root tip followed by either 300 μm (in the region of 0–2,100 μm) or 5 mm (in the region of 5–30 mm) walk steps (Fig. 1a). Gradients of ions (NH_4^+ and NO_3^-) near to the root surface (ca. 5 μm above the root surface) were measured by moving the ion-selective microelectrode between two positions (with a distance of 30 μm) in perpendicular direction to the root axis. The recording rate for the ion flux was one reading per 6 s. The ion flux was recorded at each measurement point for 10 min. Acquisition of root images was performed with Mageflux software (version 1.0) attached to the SIET system.

To investigate net NH_4^+ and H^+ fluxes associated with PM H^+-ATPases and the interference of NO_3^- on net NH_4^+ fluxes, a white fine root was transferred to a Petri dish containing 10 mL of measuring solution (0.1 mM NH_4Cl, 1 mM KCl, 0.1 mM $CaCl_2$, pH 5.5) and equilibrated for 20 min. The equilibrated root was removed to another Petri dish containing fresh measuring solution and used to simultaneously record net NH_4^+ and H^+ fluxes for 10 min at the position from the root tip where the maximal net NH_4^+ uptake was found using NH_4^+ or H^+-selective microelectrodes, respectively. Subsequently, the root was transferred to a Petri dish containing 0.5 mM orthovanadate, which is a specific inhibitor for PM H^+-ATPases (Ramos et al. 2009) and incubated for 30 min. Then the root was equilibrated and used to record net NH_4^+ and H^+ fluxes as described above. To examine the interference of NO_3^- with net NH_4^+ fluxes, roots were analyzed for net NH_4^+ fluxes in the measuring solution containing NH_4NO_3 instead of NH_4Cl (0.1 mM NH_4NO_3, 1 mM KCl, 0.1 mM $CaCl_2$, pH 5.5) as above.

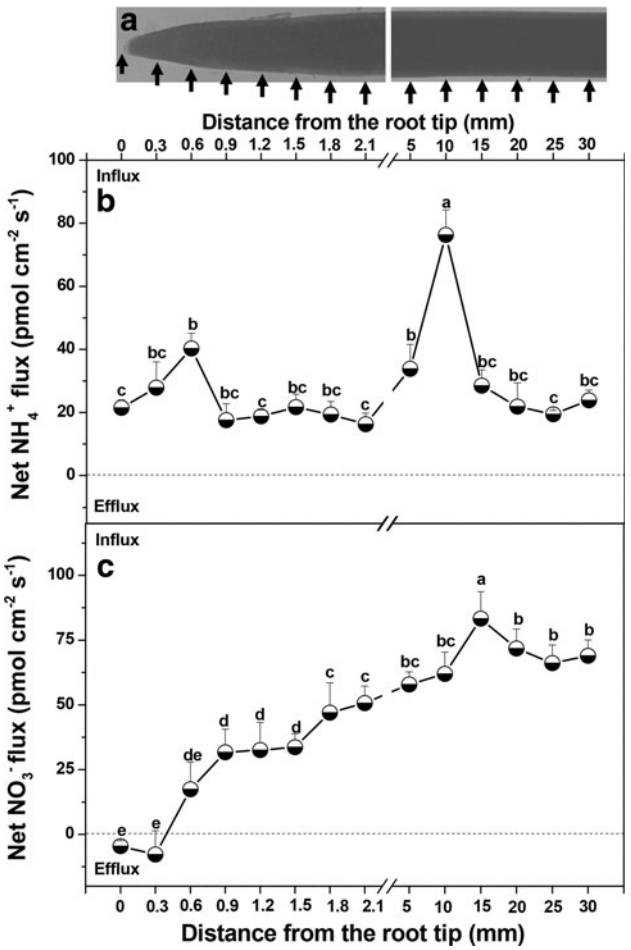

Fig. 1 Root tip (**a**), net NH_4^+ (**b**) and NO_3^- (**c**) fluxes along the root tip of *P. popularis*. Data indicate mean ± SE ($n = 6$). Different letters on the error bars in each panel indicate significant difference among the measured positions. Net influxes correspond to positive values and net effluxes indicate negative values, respectively. The measuring solution contained 1 mM KCl and 0.1 mM $CaCl_2$, pH 5.5, to which either 0.1 mM NH_4Cl for NH_4^+ or 0.1 mM KNO_3 for NO_3^- flux measurements were added

Similar to the measurements of net fluxes of NH_4^+ and H^+, net fluxes of NO_3^- and H^+ were determined at the distance from the root tip where the maximal net NO_3^- uptake was detected in white fine roots exposed to the measuring solution (1 mM KCl, 0.1 mM $CaCl_2$, pH 5.5) containing either 0.1 mM KNO_3 or 0.1 mM NH_4NO_3 using the ion (NO_3^- or H^+) selective microelectrodes. The roots were treated with orthovanadate as described above.

To examine whether there exist correlations between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes in roots of *P. popularis*, analyses of linear fit were performed using data from net NH_4^+ or NO_3^- fluxes and net H^+ fluxes before and after orthovanadate treatments.

Measurements of NH_4^+ and NO_3^- fluxes in the root xylem

To examine NH_4^+ and NO_3^- fluxes in the root xylem, the Petri dish system with two compartments was used as proposed by Sorgona et al. (2011). The measurement procedure for NH_4^+ and NO_3^- fluxes in the root xylem was the same as that for ion flux measurements described above. The fine roots (similar size as ion flux measurements by SIET) of *P. popularis* were selected for measurements of NH_4^+ and NO_3^- fluxes in the root xylem. The NH_4^+ or NO_3^- solution collected from one compartment of the Petri dish system was used for NH_4^+ or NO_3^- quantification spectrophotometrically. NH_4^+ concentration was measured based on the Berthelot reaction (Brautigam et al. 2007). About 100 µL NH_4^+ solution was mixed with 500 µl 1 % (w/v) phenol–0.005 % (w/v) sodium nitroprusside solution. Subsequently, 500 µL 1 % (v/v) sodium hypochlorite–0.5 % (w/v) sodium hydroxide solution was added. The mixture was incubated at 37 °C for 30 min and finally absorption was measured at 620 nm. NO_3^- concentration was measured at 210 nm as suggested by Sorgona et al. (2011). Finally, the NH_4^+ or NO_3^- flux in the root xylem was estimated using the NH_4^+ or NO_3^- amount, the area of root xylem on the cross section and the time which was used for collection of NH_4^+ or NO_3^- in one compartment of the Petri dish system.

Data processing and statistical analysis

Net ion flux data were calculated and exported with Mageflux software (version 1.0) attached to the SIET system (Xu et al. 2006). For determination of the positions along the root tip with the maximal net NH_4^+ or NO_3^- influx of the fine root, readings for net ion NH_4^+ or NO_3^- influxes within 10 min were averaged at each measuring point in each plant. One-way ANOVA was performed using the distance from the root tip as a factor. For analyses of net fluxes of NH_4^+, NO_3^- and H^+ at the distance from the root tip with the maximal fluxes, readings were averaged for net ion ($NH_4^+/NO_3^-/H^+$) fluxes within 10 min in each plant. The effects of vanadate and N source (NH_4Cl/KNO_3/NH_4NO_3) on net ion fluxes ($NH_4^+/NO_3^-/H^+$) were analyzed by two-way ANOVAs. All statistical tests were performed with STATGRAPHICS (STN, St. Louis, MO, USA). Data were tested for normality prior to the statistical analysis. Differences between means were considered to be significant when the *P* value of the ANOVA *F*-test was less than 0.05.

Results

Positions for the maximal net uptake of NH_4^+ and NO_3^-

To determine the positions where the maximal net uptake of NH_4^+ or NO_3^- into roots of *P. popularis* occurs, net NH_4^+ and NO_3^- fluxes were monitored along the root tip up to 30 mm from the apex (Fig. 1b, c). Net NH_4^+ influxes varied dramatically from 16 to 76 pmol cm^{-2} s^{-1} along the root tip (Fig. 1b). The maximal net NH_4^+ uptake was detected at 10 mm from the root tip (Fig. 1b). Net NO_3^- fluxes ranged from −8 (efflux) to 83 (influx) pmol cm^{-2} s^{-1} along the root apex (Fig. 1c). At 15 mm from the root tip, net NO_3^- flux displayed the maximum influx among the analyzed positions in the root apical region (Fig. 1c).

Net NH_4^+ and H^+ fluxes and their correlations

At 10 mm from the root tip of white fine root of *P. popularis* where the maximal net NH_4^+ uptake was detected, net NH_4^+ influxes were investigated in detail (Fig. 2a, b, e). At this position, net NH_4^+ influxes displayed little fluctuation in 10 min (Fig. 2a, b). The mean of net influx of NH_4^+ in 10 min was ca. 76.3 pmol cm^{-2} s^{-1} and the orthovanadate treatment significantly decreased net influx of NH_4^+ by 52 % in roots exposed to 0.1 mM NH_4Cl (Fig. 2e). Under 0 mM orthovanadate conditions, net influx of NH_4^+ was stimulated by 48 % in roots exposed to 0.1 mM NH_4NO_3 compared with that in roots exposed to 0.1 mM NH_4Cl (Fig. 2e). Under 0.1 mM NH_4NO_3 conditions, the orthovanadate treatment markedly decreased net influx of NH_4^+ and led to net efflux of NH_4^+ (Fig. 2e).

Accompanying net NH_4^+ flux measurement, net H^+ fluxes were also determined (Fig. 2c, d, f). The mean of net efflux of H^+ in 10 min was −6.9 pmol cm^{-2} s^{-1} in roots exposed to 0.1 mM NH_4Cl, and it was significantly decreased and showed a weak net influx after the orthovanadate treatment (Fig. 2f). Under 0 mM orthovanadate conditions, net efflux of H^+ was stimulated four folds in roots exposed to 0.1 mM NH_4NO_3 compared with that in roots exposed to 0.1 mM NH_4Cl (Fig. 2f). Under 0.1 mM NH_4NO_3 conditions, 0.5 mM orthovanadate exposure markedly decreased net efflux of H^+ and displayed net influx of H^+ compared with 0 mM orthovanadate (Fig. 2f).

The correlations were analyzed between net NH_4^+ fluxes and net H^+ fluxes in fine roots exposed to 0.1 mM NH_4Cl/NH_4NO_3 in the presence or absence of 0.5 mM orthovanadate (Fig. 3a–d). No correlation was found between net NH_4^+ influxes and net H^+ effluxes in the monitored period of time in roots exposed to 0.1 mM NH_4Cl (Fig. 3a). After inhibition of PM H^+-ATPases, net H^+ uptake decreased when net NH_4^+ influxes increased in the measuring period for roots in 0.1 mM NH_4Cl (Fig. 3b). Net H^+ effluxes increased with net NH_4^+ influxes when roots were exposed to 0.1 mM NH_4NO_3 (Fig. 3c). After 0.5 mM orthovanadate treatment, net H^+ influxes increased with net NH_4^+ effluxes (Fig. 3d).

Net NO_3^- and H^+ fluxes and their correlations

At 15 mm from the root tip of white fine root of *P. popularis* where the maximal net NO_3^- uptake was detected, net NO_3^- fluxes were investigated for more details (Fig. 4a, b, e). At this point, net NO_3^- influxes showed little fluctuation in 10 min (Fig. 4a, b). Under 0.1 mM KNO_3 conditions, net influx of NO_3^- in 10 min was ca. 83.3 pmol cm^{-2} s^{-1} in roots, but a significant decrease (ca. 32 %) was found after exposure to 0.5 mM orthovanadate (Fig. 4e). Under 0 mM orthovanadate conditions, net influx of NO_3^- was inhibited by 39 % in roots exposed to 0.1 mM NH_4NO_3 compared with that in roots exposed to 0.1 mM KNO_3 (Fig. 4e). Under 0.1 mM NH_4NO_3 conditions, the orthovanadate treatment decreased net influx of NO_3^- by 48 % (Fig. 4e).

Along with net NO_3^- influx measurement, net H^+ fluxes were also determined (Fig. 4c, d, f). The mean of net efflux of H^+ in 10 min was −27.8 pmol cm^{-2} s^{-1} in roots exposed to 0.1 mM KNO_3 and it was markedly reduced by 69 % after the orthovanadate treatment (Fig. 4f). Under 0 mM orthovanadate conditions, net efflux of H^+ was suppressed by 74 % in roots exposed to 0.1 mM NH_4NO_3 compared with that in roots exposed to 0.1 mM KNO_3 (Fig. 4f). Under 0.1 mM NH_4NO_3 conditions, 0.5 mM orthovanadate exposure markedly decreased net H^+ efflux and displayed net influx of H^+ compared with 0 mM orthovanadate (Fig. 4f).

The relationships between net NO_3^- and H^+ fluxes were also analyzed for roots in 0.1 mM KNO_3/NH_4NO_3 in the presence or absence of 0.5 mM orthovanadate (Fig. 5a–d). During the monitoring period, net H^+ effluxes increased with net NO_3^- influxes in roots exposed to 0.1 mM KNO_3, irrespective of orthovanadate treatments (Fig. 5a, b). Similarly, net H^+ effluxes increased with net NO_3^- influxes in roots exposed to 0.1 mM NH_4NO_3 and 0 mM orthovanadate (Fig. 5c), but the relationship turned to that net H^+ influxes declined with increases in net NO_3^- influxes after 0.5 mM orthovanadate exposure (Fig. 5d).

Fluxes of NH_4^+ and NO_3^- in the root xylem

The NH_4^+ and NO_3^- fluxes in the root xylem were monitored using the two-compartment Petri dish system (Fig. 6). Under 0.1 mM NH_4Cl conditions, NH_4^+ flux was ca. −7.6 nmol cm^{-2} s^{-1} in the root xylem, and it

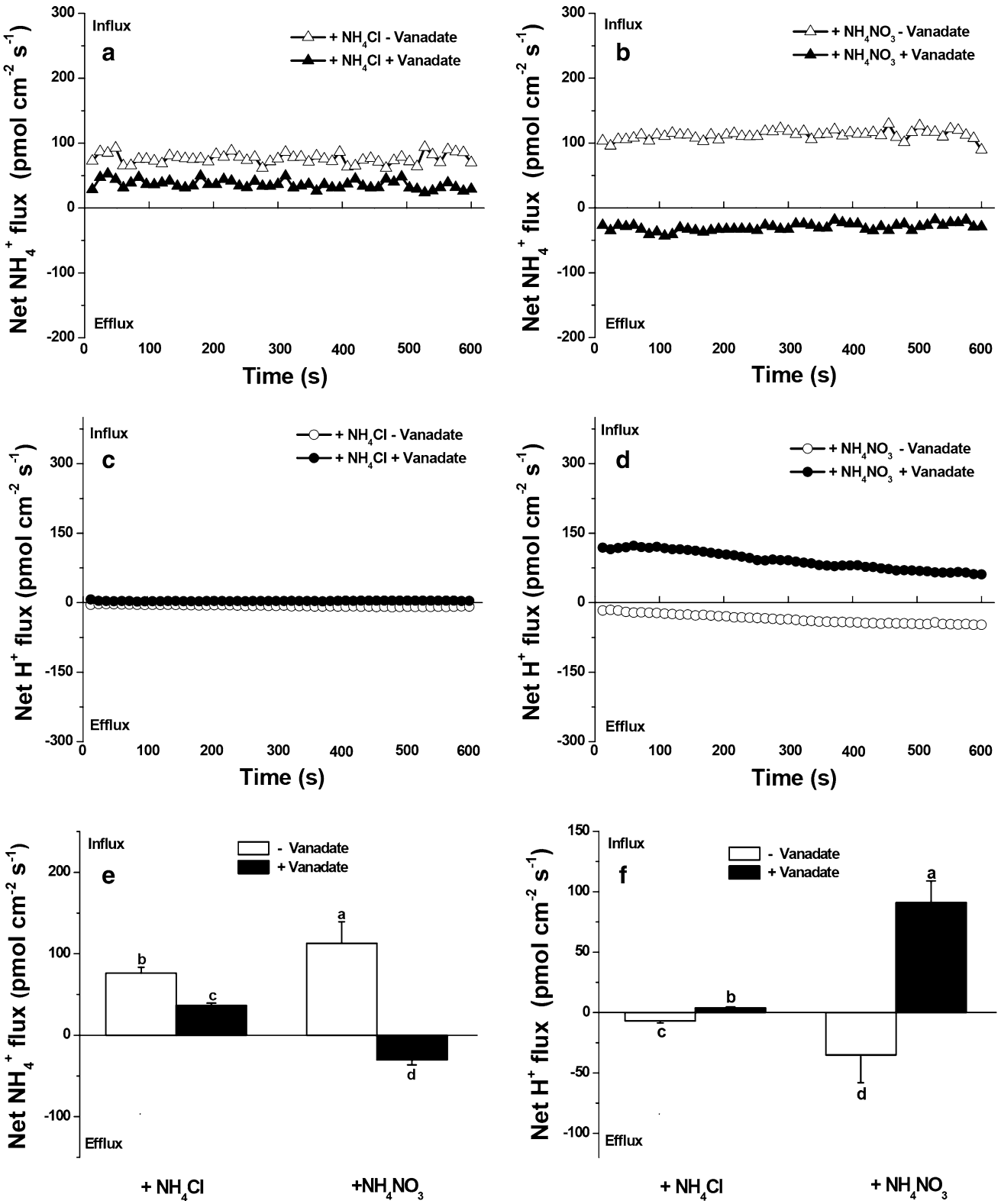

Fig. 2 Net NH_4^+ (**a**, **b**) and H^+ (**c**, **d**) fluxes in 10 min at 10 mm from the root tips of fine roots of *P. popularis*. The mean fluxes of NH_4^+ (**e**) and H^+ (**f**) within the measuring period are also shown. Data indicate mean ± SE ($n = 6$). The measuring solution (pH 5.5) contained 1 mM KCl and 0.1 mM $CaCl_2$ besides the following compounds: $+NH_4Cl$ − Vanadate, 0.1 mM NH_4Cl and no vanadate; $+NH_4Cl$ + Vanadate, 0.1 mM NH_4Cl and 0.5 mM vanadate; $+NH_4NO_3$ − Vanadate, 0.1 mM NH_4NO_3 and no vanadate; $+NH_4NO_3$ + Vanadate, 0.1 mM NH_4NO_3 and 0.5 mM vanadate. Bars labeled with different letters indicate significant difference between the treatments

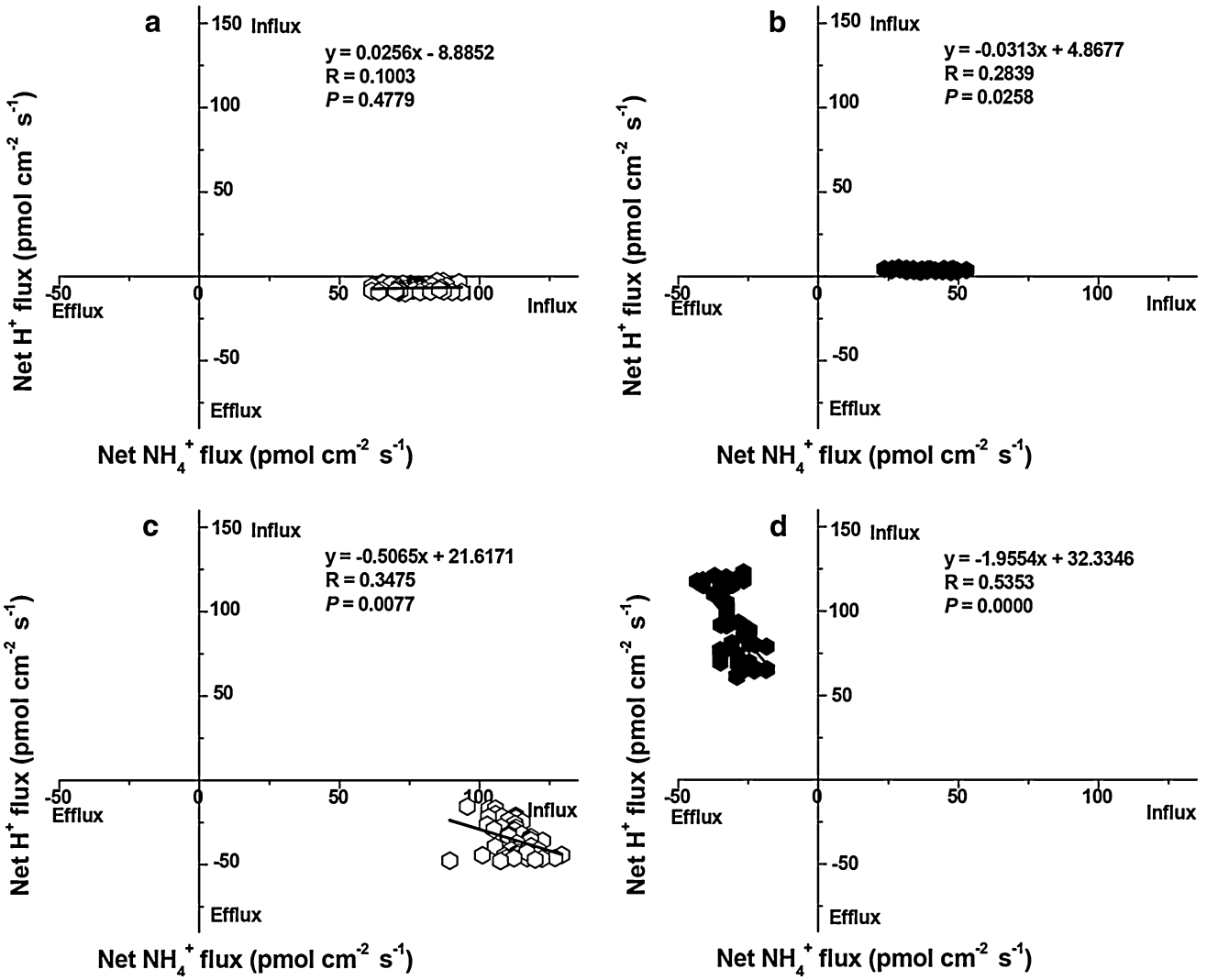

Fig. 3 The relationships between net NH_4^+ fluxes and net H^+ fluxes in fine roots of *P. popularis* exposed to the measuring solution (pH 5.5) contained 1 mM KCl and 0.1 mM $CaCl_2$ besides the following compounds: $+NH_4Cl$ − Vanadate, 0.1 mM NH_4Cl and no vanadate (**a**); $+NH_4Cl$ + Vanadate, 0.1 mM NH_4Cl and 0.5 mM vanadate (**b**); $+NH_4NO_3$ − Vanadate, 0.1 mM NH_4NO_3 and no vanadate (**c**); $+NH_4NO_3$ + Vanadate, 0.1 mM NH_4NO_3 and 0.5 mM vanadate (**d**)

significantly decreased after exposure to 0.5 mM orthovanadate (Fig. 6a). Under 0 mM orthovanadate conditions, NH_4^+ flux tended to reduce in the root xylem exposed to 0.1 mM NH_4NO_3 compared with that under 0.1 mM NH_4Cl (Fig. 6a). Under 0.1 mM NH_4NO_3 conditions, the orthovanadate treatment decreased NH_4^+ flux in the root xylem by 77 % (Fig. 6a). Under 0.1 mM KNO_3 conditions, NO_3^- flux was ca. −3.2 nmol cm^{-2} s^{-1} in the root xylem, and it tended to decrease after exposure to 0.5 mM orthovanadate (Fig. 6b). Under 0 mM orthovanadate conditions, NO_3^- flux tended to decline in the root xylem exposed to 0.1 mM NH_4NO_3 compared with that under 0.1 mM KNO_3 (Fig. 6b). Under 0.1 mM NH_4NO_3 conditions, the orthovanadate treatment decreased NO_3^- flux in the root xylem by 75 % (Fig. 6b).

Discussion

Spatial variability of net NH_4^+ and NO_3^- fluxes along the root tip of *P. popularis*

The apical region of the root is characterized by root cap, meristematic, elongation and maturation zones, which have distinct anatomical and functional features leading to different abilities to absorb nutrient ions (Enstone et al. 2001; Fang et al. 2007; Li et al. 2010). Previous studies suggest that different zones of root apical region have distinct net fluxes of NH_4^+ and/or NO_3^- (Fang et al. 2007; Li et al. 2010). The observation that spatial variability and net influxes of NH_4^+ and NO_3^- were the largest at 10 and 15 mm, respectively, from the root tips in fine roots of

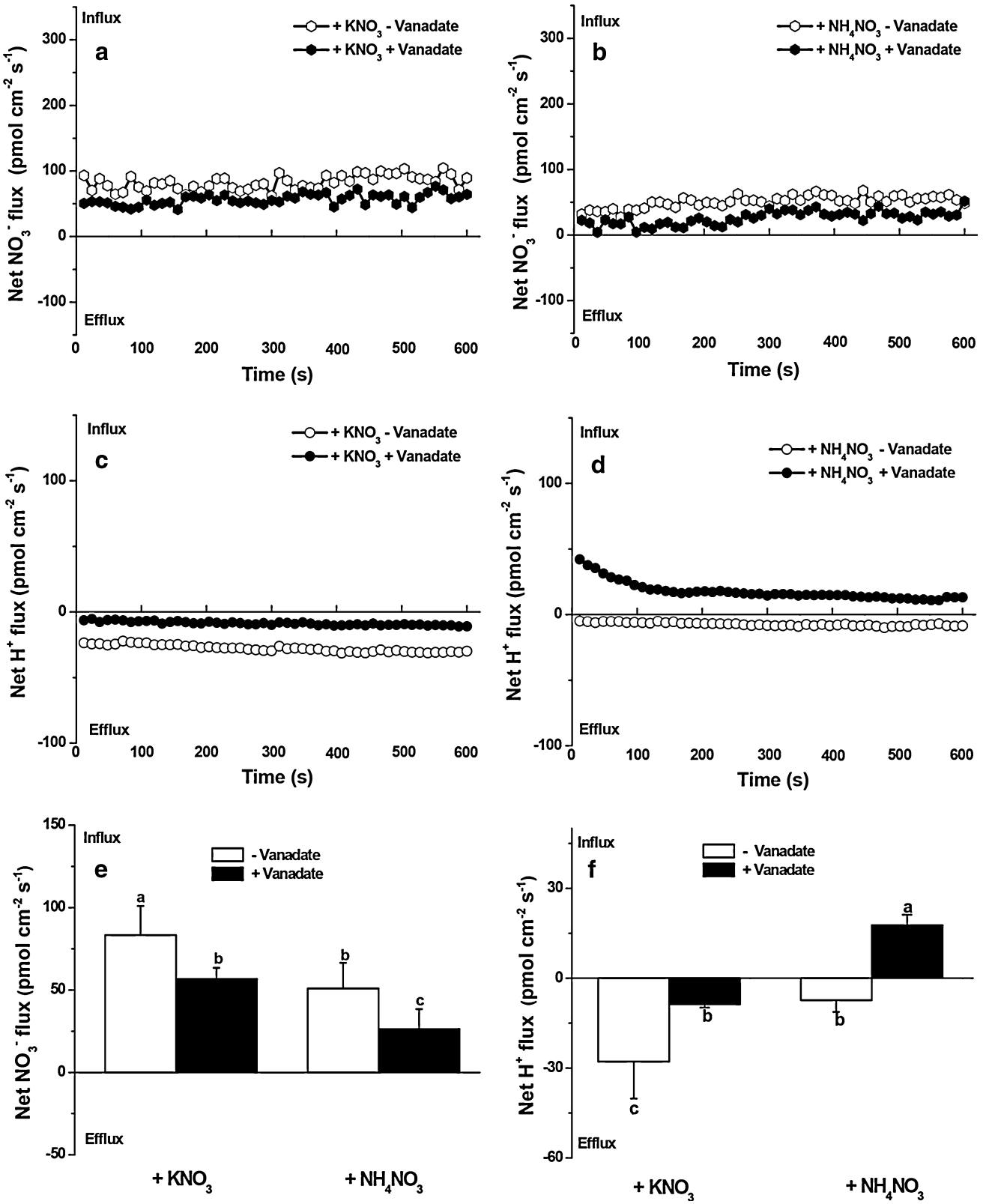

Fig. 4 Net NO_3^- (**a**, **b**) and H^+ (**c**, **d**) fluxes in 10 min at 15 mm from the root tips of fine roots of *P. popularis*. The mean net influxes of NO_3^- (**e**) and H^+ (**f**) within the measuring period are also shown. Data indicate mean ± SE ($n = 6$). The measuring solution (pH 5.5) contained 1 mM KCl and 0.1 mM $CaCl_2$ besides the following compounds: $+KNO_3$ − Vanadate, 0.1 mM KNO_3 and no vanadate; $+KNO_3$ + Vanadate, 0.1 mM KNO_3 and 0.5 mM vanadate; $+NH_4NO_3$ − Vanadate, 0.1 mM NH_4NO_3 and no vanadate; $+NH_4NO_3$ + Vanadate, 0.1 mM NH_4NO_3 and 0.5 mM vanadate. Bars labeled with different letters indicate significant difference between the treatments

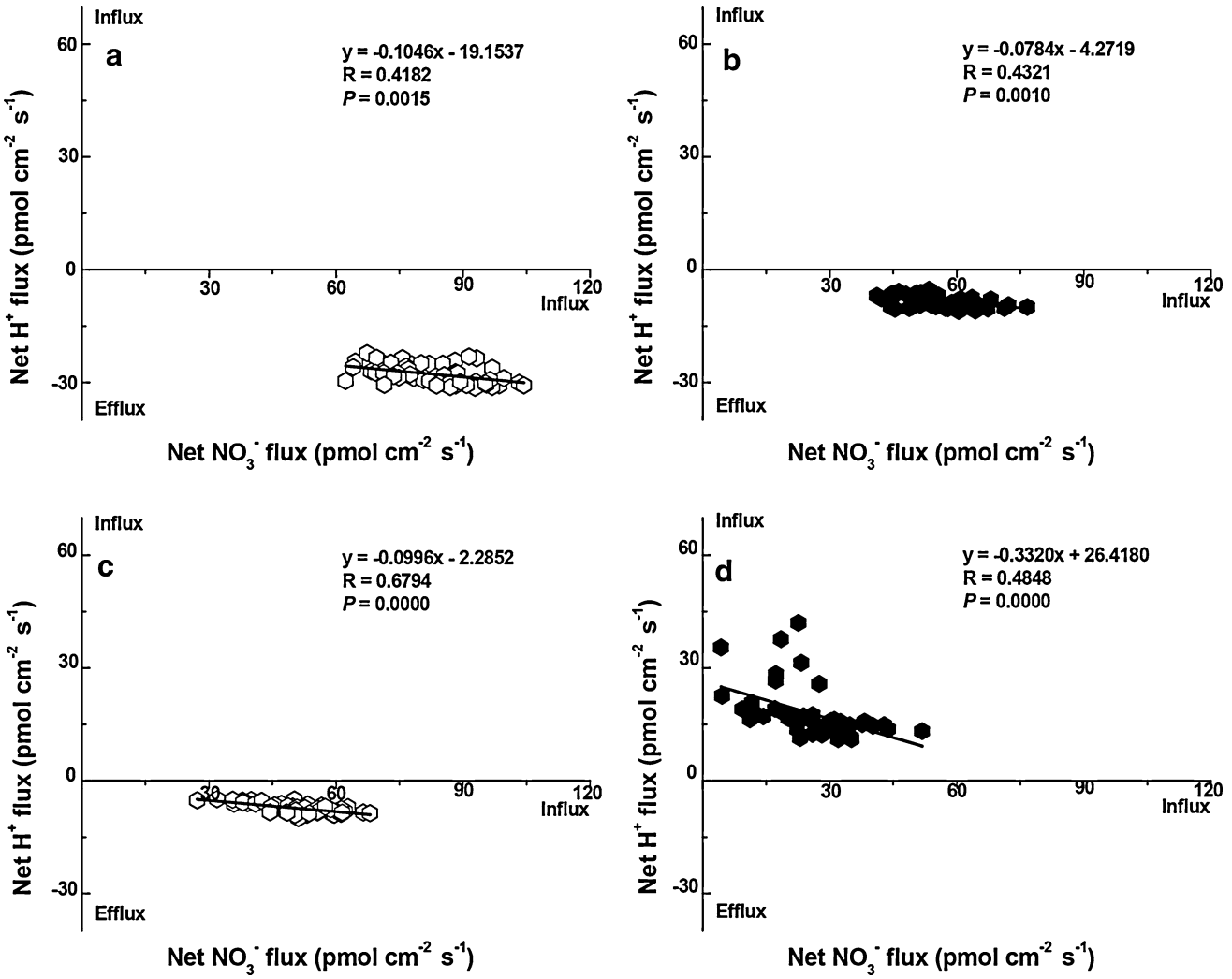

Fig. 5 The relationships between net NO_3^- influxes and net H^+ fluxes in fine roots of *P. popularis* exposed to the measuring solution (pH 5.5) contained 1 mM KCl and 0.1 mM $CaCl_2$ besides the following compounds: $+KNO_3$ − Vanadate, 0.1 mM KNO_3 and no vanadate (**a**); $+KNO_3$ + Vanadate, 0.1 mM KNO_3 and 0.5 mM vanadate (**b**); $+NH_4NO_3$ − Vanadate, 0.1 mM NH_4NO_3 and no vanadate (**c**); $+NH_4NO_3$ + Vanadate, 0.1 mM NH_4NO_3 and 0.5 mM vanadate (**d**)

P. popularis (Fig. 1b, c) is consistent with the results of previous studies in woody plants. Net fluxes of NH_4^+ and NO_3^- are the largest at 5–20 and 0–30 mm from the root tips in Douglas fir and at 5 and 0–10 mm from the root tips in lodgepole pine, respectively (Hawkins et al. 2008; Hawkins and Robbins 2010). In *Picea abies*, the maximal uptake rates of NO_3^- occur at 0–10 mm from the root tips and the NO_3^- fluxes behind 10 mm from the root tips are significantly decreased (Boukcim and Plassard 2003). Taken together, these results suggest that spatial variation in uptake of NH_4^+ and NO_3^- may be linked with different anatomical properties along the root and the root tip is of key importance for uptake of NH_4^+ and NO_3^- in roots of woody plants.

Net NH_4^+ and NO_3^- fluxes associated with PM H^+-ATPases and the interaction of both ions

Net fluxes of NH_4^+ and NO_3^- in plant roots are determined by the sum of influx and efflux of the respective ion. Net fluxes of NH_4^+ and NO_3^- reflect the results of N assimilation and uptake kinetics of these ions into root cells (Britto and Kronzucker 2006; Hawkins et al. 2008). The high net uptake of NH_4^+ and NO_3^- in fine roots of *P. popularis* (Figs. 2, 4) suggests that cytosolic concentrations of NH_4^+ and NO_3^- are lower than the thresholds which need to be maintained for N assimilation to support growth. As fast-growing species, poplars have strong demands for N (Luo et al. 2008; Rennenberg et al. 2010; Li

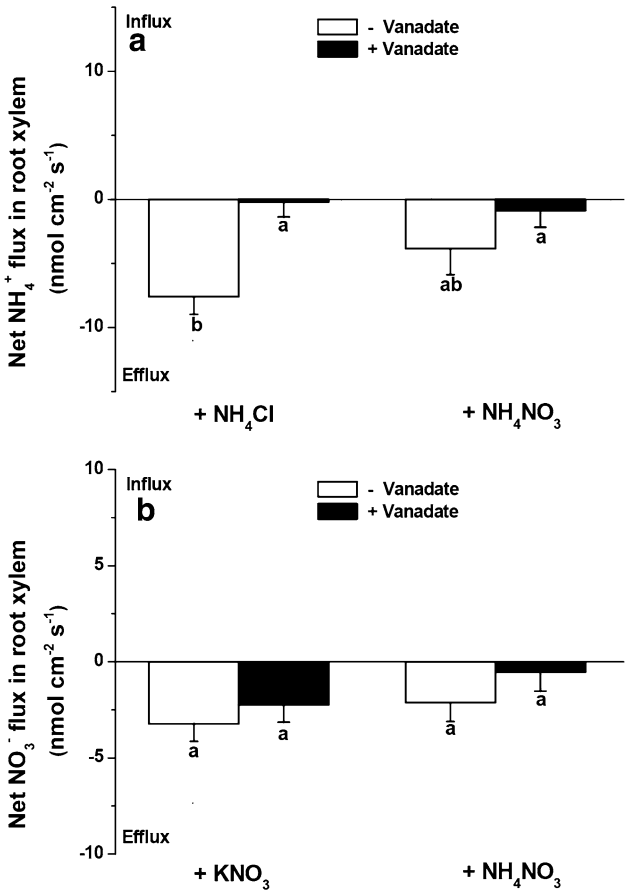

Fig. 6 Net fluxes of NH_4^+ (**a**) and NO_3^- (**b**) from xylem of fine roots of *P. popularis*. Data indicate mean ± SE ($n = 10$). The measuring solution (pH 5.5) contained 1 mM KCl and 0.1 mM $CaCl_2$ besides the following compounds: $+NH_4Cl$ − Vanadate, 0.1 mM NH_4Cl and no vanadate; $+NH_4Cl$ + Vanadate, 0.1 mM NH_4Cl and 0.5 mM vanadate; $+NH_4NO_3$ − Vanadate, 0.1 mM NH_4NO_3 and no vanadate; $+NH_4NO_3$ + Vanadate, 0.1 mM NH_4NO_3 and 0.5 mM vanadate; $+KNO_3$ − Vanadate, 0.1 mM KNO_3 and no vanadate; $+KNO_3$ + Vanadate, 0.1 mM KNO_3 and 0.5 mM vanadate. *Bars* labeled with different letters indicate significant difference between the treatments

et al. 2012). The poplar plants used here were cultivated with low NH_4^+ and NO_3^- in the nutrient solution. Therefore, strong net uptake of NH_4^+ and NO_3^- in fine roots of *P. popularis* was expected to occur when NH_4^+ or NO_3^- alone or together were present in the measuring solution.

The net uptake of NH_4^+ which ranged from 76 to 113 pmol cm^{-2} s^{-1} and of NO_3^- from 51 to 83 pmol cm^{-2} s^{-1} in fine roots of *P. popularis* (Figs. 2e, 4e) is relatively larger in comparison with those found in roots of other woody plants measured under similar conditions. For example, net NH_4^+ and NO_3^- uptake of ca. 40–80 and 28 nmol m^{-2} s^{-1} (i.e., 4–8 and 2.8 pmol cm^{-2} s^{-1}), respectively, was reported for tap roots of *Eucalyptus nitens* in 0.1 mM NH_4^+ or NO_3^- solutions at pH 6.1 (Garnett et al. 2001, 2003). The net NH_4^+ and NO_3^- uptake of seedlings of Douglas fir and lodgepole pine is ca. 8–11 and 11–19 nmol m^{-2} s^{-1} (i.e., 0.8–1.1 and 1.1–1.9 pmol cm^{-2} s^{-1}), respectively, in primary roots in solutions with 0.05 mM NH_4^+ or NO_3^- (Hawkins et al. 2008). N-starvation pretreatment had also been applied to the coniferous seedlings (Hawkins et al. 2008). Although fine roots derived from cuttings of *P. popularis* may be different from tap roots of seed-derived *E. nitens* and coniferous species, the higher net uptake of NH_4^+ and NO_3^- in fine roots of *P. popularis* underlines that poplar seedlings need higher uptake rates of NH_4^+ and/or NO_3^- to meet N demands for fast growth.

NO_3^- is co-transported with H^+ through a symporter into root cells, whereas NH_4^+ is transported via a uniporter and/or a symporter (co-transport with H^+) to the cytosol (Garnett et al. 2003; Britto and Kronzucker 2006; Miller et al. 2009). Thus, inactivation of PM H^+-ATPases may inhibit uptake of NH_4^+ and NO_3^- in plant roots because the proton motive force is blocked (Santi et al. 1995, 2003; Sorgona et al. 2010, 2011). In line with this presumption, application of a specific inhibitor for PM H^+-ATPases caused a significant decline in net uptake of NH_4^+ and NO_3^- or even resulted in net effluxes of NH_4^+ from poplar roots (Figs. 2e, 4e). These results demonstrate that PM H^+-ATPases play a critical role in uptake of NH_4^+ and NO_3^- in roots of *P. popularis*.

Uptake rates of NH_4^+ and NO_3^- in plant roots are affected by the presence or absence of both ions together in solutions. In this study, net NH_4^+ uptake was strongly stimulated in the presence of NO_3^-, whereas net NO_3^- uptake was inhibited by the presence of NH_4^+ under no vanadate exposure conditions (Figs. 2e, 4e). The induced effect of NO_3^- on net NH_4^+ uptake in poplar roots can be the results of induction of NH_4^+ uptake, suppression of NH_4^+ release or both processes. The induced effects of NO_3^- on net NH_4^+ uptake have been also observed in previous studies (Kronzucker et al. 1999b; Babourina et al. 2007). The synergistic effect of NO_3^- on net NH_4^+ uptake in poplar roots might be associated with higher activity of NH_4^+ transporters involved in homeostasis of cytosolic NH_4^+ (by a higher rate of NH_4^+ transfer into vacuole and/or plastids or further to the xylem) or with a more complicated feedback response via metabolism such as the increased activities of NH_4^+ assimilation enzymes (Babourina et al. 2007). All these processes may lead to a lower cytoplasmic NH_4^+ concentration in root cells, facilitating a higher NH_4^+ uptake from and/or a lower NH_4^+ release to the apoplast. However, net NH_4^+ flux in the xylem of fine roots of *P. popularis* tended to decrease in the presence of NO_3^- under no vanadate conditions (Fig. 6a), indicating that the synergistic effect of NO_3^- on net NH_4^+ uptake in poplar root surface may be mainly due to a higher rate of NH_4^+ transfer into vacuoles and/or plastids. The switch of

net NH_4^+ uptake to net release in the presence of NO_3^- in poplar roots after vanadate exposure (Fig. 2e) indicates that provision of NO_3^- probably stimulates the activity of NH_4^+ efflux systems under this condition. Since NO_3^- can act as a signal molecule in root tips (Walch-Liu and Forde 2008; Forde and Walch-Liu 2009), it may function as a messenger to activate NH_4^+ efflux systems in poplar roots. Although specific transporters for NH_4^+ efflux are unidentified yet, NH_4^+ efflux, e.g., via ammonium transporters, non-selective cation channels and NH_4^+/H^+ antiporters, is a critical step in the futile NH_4^+ cycling in root cells when NH_4^+ is present in excess (Britto et al. 2001; Loque and von Wiren 2004; Britto and Kronzucker 2006).

Although there exist inconsistent results about influence of NH_4^+ co-provision on net NO_3^- uptake in roots of woody plants (e.g., Garnett et al. 2003; Hawkins et al. 2008), most studies suggest that the presence of NH_4^+ in growth media may reduce net NO_3^- uptake as the result of decreased NO_3^- uptake, elevated NO_3^- release or both processes in roots of woody plants (Kronzucker et al. 1999a; Garnett et al. 2003; Gobert and Plassard 2007). Our results about decreases in net NO_3^- uptake by NH_4^+ co-provision in poplar roots (Fig. 4e) are in good agreement with those of previous studies. It is suggested that the increases in NH_4^+ in root cells are related to the decreases in gene expression of NO_3^- transporters involved in high-affinity transport process (Zhuo et al. 1999; Vidmar et al. 2000), which may lead to lower activity of NO_3^- transporters and to reduce NO_3^- uptake. This negative regulation process may be induced by NH_4^+ in poplar roots exposed to NH_4NO_3. Inhibition of NO_3^- influx and/or activation of NO_3^- efflux system such as aquaporins may also be involved (Ikeda et al. 2002). Since a significant amount of absorbed NO_3^- is loaded to the xylem in poplar roots (Min et al. 1998), the tendency to reduce NO_3^- flux to the xylem in fine roots of *P. popularis* (Fig. 6b) may also contribute to the lower net NO_3^- uptake in the presence of NH_4^+ in the measuring solution. Furthermore, since NO_3^- enters root cells via a symporter with H^+, inhibition effect of NH_4^+ on net H^+ release (Fig. 4f) may also lead to decreased H^+ availability for NO_3^- co-transport in poplar roots.

H^+ fluxes and correlations between net NH_4^+ or NO_3^- fluxes and net H^+ fluxes

Protons play a pivotal role in plant uptake of NH_4^+ and NO_3^- because the H^+ gradient maintained by PM H^+-ATPases facilitates absorption of these nutrient ions (Miller and Cramer 2004; Britto and Kronzucker 2006). Net H^+ effluxes are often observed during NH_4^+ and NO_3^- absorption in roots of woody plants (e.g., Hawkins et al. 2008; Hawkins and Robbins 2010). Consistently, net H^+ release was observed in poplar roots exposed to NH_4^+, NO_3^- or both ions containing solutions under 0 mM vanadate condition (Figs. 2f, 4f). Net H^+ efflux may be associated with uptake and/or assimilation of NH_4^+ or NO_3^- in fine roots of *P. popularis*. There is evidence that H^+ may be cotransported with cations such as NH_4^+ (Wang et al. 1994) or with anions such as NO_3^- through transporters (Marschner 2002; Hawkins and Robbins 2010). Additionally, roots of many species exhibit net H^+ efflux in apical regions to maintain the acid environment for cell wall expansion (Bloom et al. 2003). Since PM H^+-ATPases are inactivated under vanadate exposure, maintaining net H^+ fluxes in NH_4^+, NO_3^- or both ions containing solutions in poplar roots during the monitoring period are probably due to (1) the futile cycling of NH_4^+/NO_3^- via channels of these ions across root cell PM (Britto et al. 2001; Britto and Kronzucker 2003, 2006) and/or (2) loading cytoplasmic and vacuolar pools, assimilation and transport to xylem of NH_4^+ and NO_3^- in root cells (Garnett et al. 2003). In fact, our data showed that transport of NH_4^+ and NO_3^- to root xylem still occurred in root cells of *P. popularis* after vanadate exposure, although these ion fluxes to root xylem remained small (Fig. 6).

The correlation is expected between net H^+ flux and net NH_4^+/NO_3^- flux since H^+ is tightly associated with NH_4^+ or NO_3^- during the uptake and assimilation processes in plant roots under 0 mM vanadate conditions. Our data are consistent with this anticipation except that no correlation was found between net H^+ flux and net NH_4^+ flux in fine roots of *P. popularis* under 0.1 mM NH_4Cl and 0 mM vanadate condition (Figs. 3a, c, 5a, c). The correlations between net H^+ fluxes and net NH_4^+/NO_3^- fluxes have also been reported in roots of Douglas-fir, lodgepole pine and *Eucalyptus nitens* (Garnett et al. 2001; Hawkins et al. 2008). Moreover, the ratio between net H^+ flux and net NH_4^+ flux is reported to be −1.6 (efflux) to 1 (influx) and the flux stoichiometry for H^+ to NO_3^- is −0.8 (efflux) to 1 (influx) in roots of *E. nitens* (Garnett et al. 2003). In contrast to these stoichiometric relations in *E. nitens*, the ratios between net H^+ effluxes and net NH_4^+ or NO_3^- influxes were much smaller in fine roots of *P. popularis* (Figs. 3a, c, 5a, c). Although no correlation existed between net H^+ flux and net NH_4^+ flux in fine roots of *P. popularis* under 0.1 mM NH_4Cl and 0 mM vanadate condition (Fig. 3a), a significant correlation was detected after vanadate exposure (Fig. 3b). Similarly, the correlation between net H^+ fluxes and net NH_4^+ fluxes also markedly changed under 0.1 mM NH_4NO_3 after vanadate treatment (Fig. 3c, d). In the same line, the correlation between net H^+ fluxes and net NO_3^- fluxes altered under 0.1 mM NH_4NO_3 after vanadate exposure (Fig. 5c, d). Changes in these correlations may be largely due to the vanadate treatment which inactivates PM H^+-ATPases resulting in significant reductions in H^+ effluxes from root cells. In previous

studies, to our knowledge, no vanadate treatment is reported to link the correlations between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes. Thus, it is the first time for us to demonstrate changes in correlations between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes after inactivation of PM H^+-ATPases, highlighting the tight coupling between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes. Although the stoichiometric correlations have been explored between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes in previous studies, the biological meaning underlying these stoichiometric relations remains unclear (Newman 2001; Garnett et al. 2003; Fang et al. 2007). Since the SIET method used in this study just measures net fluxes of $NH_4^+/NO_3^-/H^+$, not the individual influx/efflux of these ions in the measuring solutions, in combination with possible confounding effects of N assimilation (H^+-consuming and -producing processes involved), it is a challenge to explain the biological meaning of stoichiometric relations between net H^+ fluxes and net NH_4^+ or NO_3^- fluxes based on the correlations in fine roots of *P. popularis* (Newman 2001; Garnett et al. 2003; Fang et al. 2007).

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