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## Characteristics of $\text{NH}_4^+$ and $\text{NO}_3^-$ fluxes in tea (*Camellia sinensis*) roots measured by scanning ion-selective electrode technique

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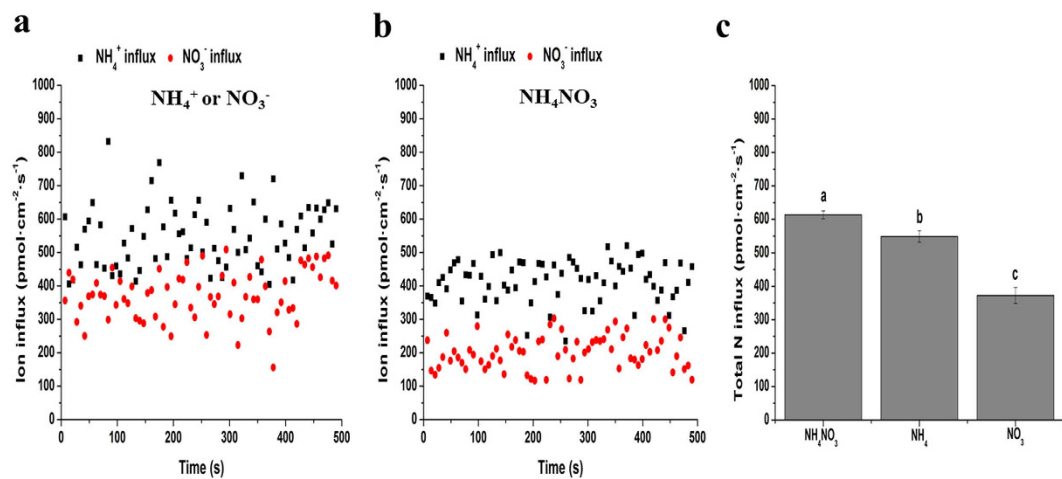
As a vital beverage crop, tea has been extensively planted in tropical and subtropical regions. Nitrogen (N) levels and forms are closely related to tea quality. Based on different N levels and forms, we studied changes in  $\text{NO}_3^-$  and  $\text{NH}_4^+$  fluxes in tea roots utilizing scanning ion-selective electrode technique. Our results showed that under both single and mixed N forms, influx rates of  $\text{NO}_3^-$  were much lower than those of  $\text{NH}_4^+$ , suggesting a preference for  $\text{NH}_4^+$  in tea. With the increase in N concentration, the influx rate of  $\text{NO}_3^-$  increased more than that of  $\text{NH}_4^+$ . The  $\text{NH}_4^+$  influx rates in a solution without  $\text{NO}_3^-$  were much higher than those in a solution with  $\text{NO}_3^-$ , while the  $\text{NO}_3^-$  influx rates in a solution without  $\text{NH}_4^+$  were much lower than those in a solution with  $\text{NH}_4^+$ . We concluded that (1) tea roots showed a preference for  $\text{NH}_4^+$ , (2) presence of  $\text{NO}_3^-$  had a negative effect on  $\text{NH}_4^+$  influx, and (3)  $\text{NH}_4^+$  had a positive effect on  $\text{NO}_3^-$  influx. Our findings not only may help advance hydroponic tea experiments but also may be used to develop efficient fertilization protocols for soil-grown tea in the future.

As a crucial component of chlorophylls, nucleic acids, proteins and a great number of secondary plant metabolites, nitrogen (N) is essential for the growth of plants. Nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) are two major inorganic N forms for plants in soils. Due to various factors (e.g., root interference, soil moisture, soil microorganisms, etc.), the reciprocal transformation between ammonium and nitrate is very common in soils<sup>1</sup>. Thus, roots are always ready to absorb both forms of nitrogen in soils. Both ions can be absorbed and used by plants because root cells possess transport systems such as nitrate and ammonium transporters<sup>2</sup>.  $\text{NO}_3^-$  and  $\text{NH}_4^+$  have different biochemical and energetic features for assimilation, leading to various net fluxes of  $\text{NO}_3^-/\text{NH}_4^+$  and ion preferences of plants, although both ions can be used by plants<sup>3</sup>.

Comparative studies on net fluxes of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  have been conducted in different plants, and the preference for  $\text{NH}_4^+$  or  $\text{NO}_3^-$  is usually associated with the physiological needs of plants in various ecosystems<sup>4</sup>. Tea is an important beverage crop that has been extensively planted in tropical and subtropical regions. In tea plants, N levels and forms, especially in young shoots, are associated with the quality of tea. Previous research has demonstrated that tea plants have a higher absorption of  $\text{NH}_4^+$  compared to  $\text{NO}_3^-$ <sup>5</sup>. However, high concentrations of  $\text{NH}_4^+$  are toxic in a majority of plants, including woody plants. If only  $\text{NO}_3^-$  or both ions are provided, no detrimental influences can be detected in plants<sup>6,7</sup>. Little information can be found on the interactions between  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes in tea roots, although the uptake of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  in tea has been explored extensively<sup>4,5,8</sup>. Moreover, most previous studies on N uptake were carried out using an <sup>15</sup>N labeling method, which was unable to interpret the dynamic processes of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes<sup>9,10</sup>.

Taking an electrophysiological approach, scanning ion-selective electrode technique (SIET) can evaluate ion/molecule-specific activities non-invasively<sup>11</sup>. To date,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{H}^+$ ,  $\text{Cd}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ,  $\text{O}_2$  and  $\text{Al}^{3+}$  have been identified utilizing SIET; however, the application of SIET for the examinations of net  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes in tea roots has not yet been reported.

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**Figure 1.** Net fluxes of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> on surfaces of tea roots under different N forms. Net fluxes of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> on tea root surfaces under single (a) and mixed N forms (b). Total N influx rates under the different N forms (c). The mean ± SE (n = 6) is shown in the data. In order to eliminate the “noise” caused by the oscillation, not only 6 biological repetitions, but also 70 measurement time points in each repetition were considered. Thus, SE = SD/√420. The different letters indicate differences between means at *P* < 0.05.

In this study, the fluxes of net NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in absorbing tea roots exposed to various N forms were evaluated with SIET non-invasively. This research had the following objectives: (1) to monitor any alterations in net NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes in tea roots under different N forms, and (2) to assess the interaction between fluxes of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in tea roots. This research is the first attempt to identify fluxes of net NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in the presence of different N forms and interactions between NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes in tea roots utilizing SIET. Our findings may help advance hydroponic tea experiments and effective fertilization protocols for future soil-grown tea plants.

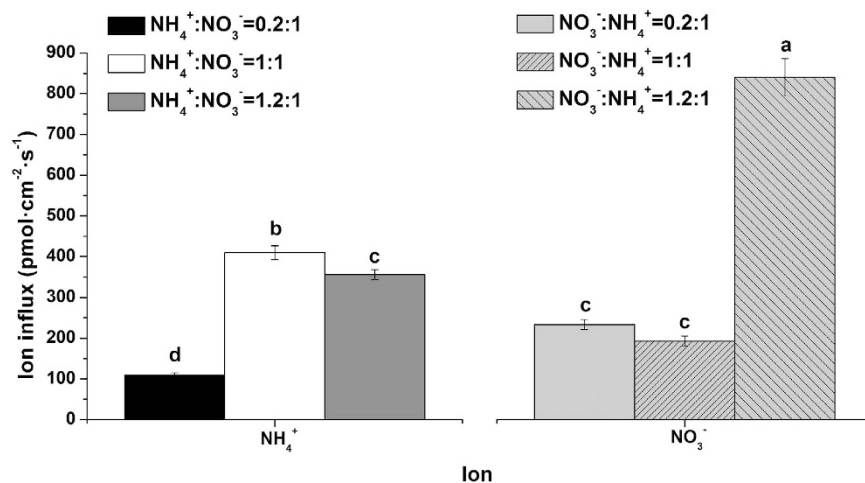
## Results

**Net fluxes of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> under different N forms and levels.** Tea roots were immersed in measuring solutions with different N forms (1 mM NH<sub>4</sub>NO<sub>3</sub>, 2 mM KNO<sub>3</sub> or 1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) to monitor the net fluxes of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> under various N forms. Net flux curves of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> are shown in Fig. 1. After the 7 d N starvation treatment, both NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> presented influx states on the root surface when different N forms were given. In addition, the influx rates of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> improved gradually. In comparison to the treatments of a single N form, the influx rates of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> under the NH<sub>4</sub>NO<sub>3</sub> treatment were more stable (Fig. 1a,b). The influx rates of NO<sub>3</sub><sup>-</sup> were lower than those of NH<sub>4</sub><sup>+</sup> under both single and mixed N form treatments, which suggested that tea roots had a preference for NH<sub>4</sub><sup>+</sup> (Fig. 1a,b). When N levels were the same, the total N influx rate of the NH<sub>4</sub>NO<sub>3</sub> treatment was considerably higher than that of the single N form treatments (Fig. 1c).

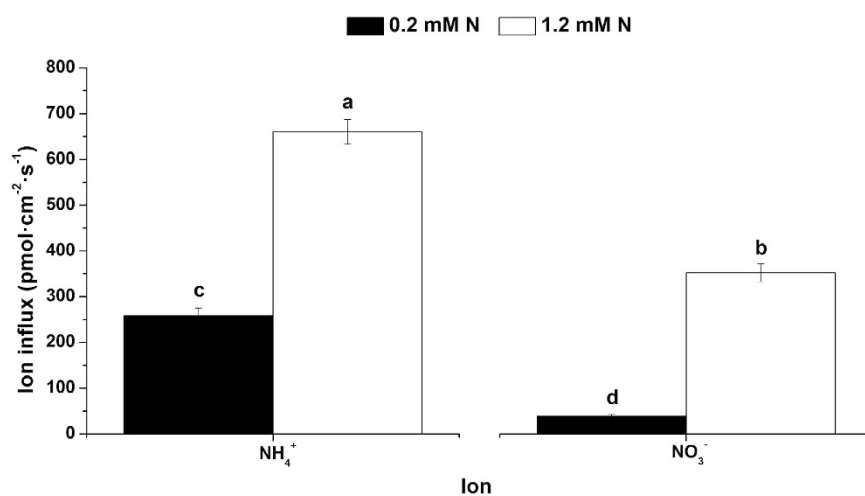
The influx rates of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> under various proportions of N sources are shown in Fig. 2. With an increase in the NH<sub>4</sub><sup>+</sup> concentration, the influx rates of NH<sub>4</sub><sup>+</sup> first increased and then decreased under the same concentration of NO<sub>3</sub><sup>-</sup> (Fig. 2). The highest influx rate of NH<sub>4</sub><sup>+</sup> appeared when the ratio of NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> was 1:1. For the influx rates of NO<sub>3</sub><sup>-</sup>, the highest influx rate of NO<sub>3</sub><sup>-</sup> appeared when the ratio of NO<sub>3</sub><sup>-</sup>:NH<sub>4</sub><sup>+</sup> was 1.2:1. This suggested that NH<sub>4</sub><sup>+</sup>:NO<sub>3</sub><sup>-</sup> at 1:1 was the critical point and that the absorption rate of NH<sub>4</sub><sup>+</sup> might not improve with an increase of NH<sub>4</sub><sup>+</sup>; meanwhile, the variation of the NO<sub>3</sub><sup>-</sup> influx rate was extremely different.

The influx rates of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> under different N levels are shown in Fig. 3. The influx rates of NH<sub>4</sub><sup>+</sup> were 6.69 and 1.87 times higher compared to NO<sub>3</sub><sup>-</sup> at 0.2 and 1.2 mM N levels, respectively. With increasing N concentration, the influx rates of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> improved significantly. In addition, the influx rate of NO<sub>3</sub><sup>-</sup> improved more than NH<sub>4</sub><sup>+</sup> with the increase in N concentration. Although a high concentration of ammonium N is toxic for a majority of plants (including woody plants), the high concentration of NH<sub>4</sub><sup>+</sup> (1.2 mM) did not affect tea tree growth in this study (Supplementary Figures S1 and 2). From phenotyping data, biomass and N contents of root, stem and leaf were higher with the supply of NH<sub>4</sub><sup>+</sup>-N compared to the supply of NO<sub>3</sub><sup>-</sup>-N. Thus, tea trees had better growth with the supply of NH<sub>4</sub><sup>+</sup>-N compared to the supply of NO<sub>3</sub><sup>-</sup>-N (Supplementary Figure S1). In addition, the content of total free amino acid in the supply of NH<sub>4</sub><sup>+</sup>-N was higher compared to the supply of NO<sub>3</sub><sup>-</sup>-N (Supplementary Figure S2). The main amino acids of the tea tree (such as aspartic acid, glutamic acid and theanine) were higher with the supply of NH<sub>4</sub><sup>+</sup>-N compared to the supply of NO<sub>3</sub><sup>-</sup>-N (Supplementary Figure S2). This suggested that tea roots had a stronger NH<sub>4</sub><sup>+</sup> uptake ability, especially under low N conditions.

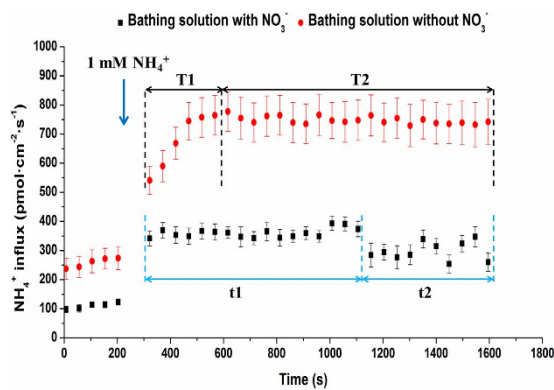
**Interactions between NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> fluxes in tea roots.** Changes in NH<sub>4</sub><sup>+</sup> flux are shown in Fig. 4 after adding NH<sub>4</sub><sup>+</sup> to the bathing solution either with or without NO<sub>3</sub><sup>-</sup>. NH<sub>4</sub><sup>+</sup> presented influx states on the root surface regardless of whether the bathing solution had NO<sub>3</sub><sup>-</sup>. The NH<sub>4</sub><sup>+</sup> influx rates in the bathing solution without NO<sub>3</sub><sup>-</sup> were much higher compared with those in a solution with NO<sub>3</sub><sup>-</sup>. In plants without NO<sub>3</sub><sup>-</sup> supply, the influx rates of NH<sub>4</sub><sup>+</sup> increased and peaked approximately 200 s after NH<sub>4</sub><sup>+</sup> addition (T1 stage), suggesting a



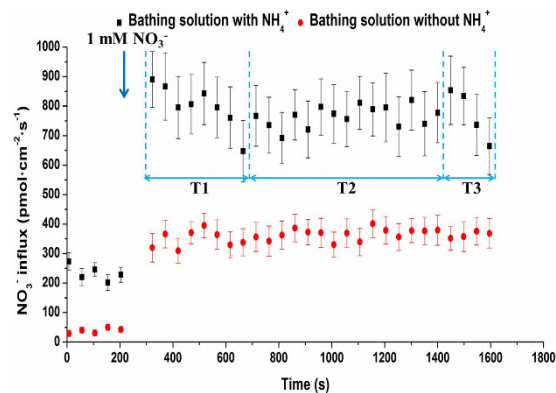
**Figure 2. Influx rates of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  on tea root surfaces under different proportion of N sources.** The mean  $\pm$  SE ( $n = 6$ ) is shown in the data. The different letters indicate differences between means at  $P < 0.05$ .



**Figure 3. Influx rates of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  on tea root surfaces under different N levels.** The mean  $\pm$  SE ( $n = 6$ ) is shown in the data. The different letters indicate differences between means at  $P < 0.05$ .



**Figure 4. Influence of  $\text{NO}_3^-$  on  $\text{NH}_4^+$  net fluxes on tea root surfaces.** After adding  $(\text{NH}_4)_2\text{SO}_4$  to the bathing solution without or with 1 mM  $\text{KNO}_3$ , the changes in tea root  $\text{NH}_4^+$  net fluxes (averaged over 49 s) are presented. The mean  $\pm$  SE of  $\text{NH}_4^+$  influxes during the measurement period are shown ( $n = 6$ ).  $(\text{NH}_4)_2\text{SO}_4$  was added at the vertical arrows.



**Figure 5. Influence of  $\text{NH}_4^+$  on  $\text{NO}_3^-$  net fluxes on tea root surfaces.** Variations of  $\text{NO}_3^-$  net fluxes in tea roots (averaged over 49 s) after adding  $\text{KNO}_3$  to the bathing solution without or with 0.5 mM  $(\text{NH}_4)_2\text{SO}_4$  are presented. The mean  $\pm$  SE of  $\text{NO}_3^-$  influxes during the measurement period are shown ( $n = 6$ ).  $\text{KNO}_3$  was added at the vertical arrows.

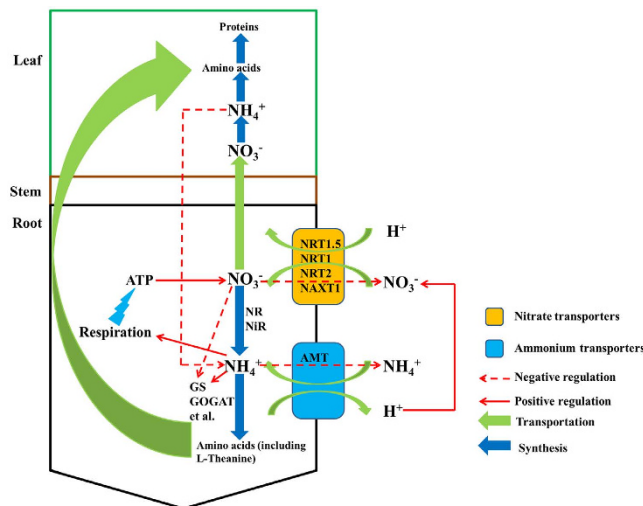
vibrant status of the influx system without the  $\text{NO}_3^-$  supply and the ability to retain cytoplasmic  $\text{NH}_4^+$  to a specific degree in tea roots. The influx rates of  $\text{NH}_4^+$  remained stable (T2 stage), demonstrating that the  $\text{NH}_4^+$  influx and efflux systems had reached a balance and that  $\text{NH}_4^+$  influx was dominant. In plants with the  $\text{NO}_3^-$  supply, the influx rates of  $\text{NH}_4^+$  did not increase; however, they stabilized quickly and maintained the rate (t1 stage). Approximately 800 s after  $\text{NH}_4^+$  addition, the influx rates of  $\text{NH}_4^+$  began to decrease (t2 stage). The  $\text{NH}_4^+$  influx rates in the bathing solution with or without  $\text{K}^+$  are shown in Supplementary Figure S3. There was little difference between the  $\text{NH}_4^+$  influx rates in the bathing solution with or without  $\text{K}^+$ , indicating that adding  $\text{K}^+$  had little effect on the net flux of  $\text{NH}_4^+$  in this study.

Changes in  $\text{NO}_3^-$  flux are shown in Fig. 5 after adding  $\text{NO}_3^-$  to the bathing solution without or with  $\text{NH}_4^+$ .  $\text{NO}_3^-$  presented influx states on the root surface regardless of whether the bathing solution had  $\text{NH}_4^+$ . The  $\text{NO}_3^-$  influx rates in the bathing solution without  $\text{NH}_4^+$  were much lower compared to those in a solution with  $\text{NO}_3^-$ , which were just the opposite of the  $\text{NH}_4^+$  influxes above. In plants without the  $\text{NH}_4^+$  supply, the influx rates of  $\text{NO}_3^-$  remained stable. However, with the  $\text{NH}_4^+$  supply, the influx rates of  $\text{NO}_3^-$  could be divided into three stages: (1) a decrease with the lowest point approximately 400 s after  $\text{NH}_4^+$  addition (T1 stage); (2) a spiral increase from approximately 400 to 1200 s after  $\text{NH}_4^+$  addition (T2 stage); and (3) a slope decrease (T3 stage). The influx rates of  $\text{NO}_3^-$  were more unstable compared to  $\text{NH}_4^+$ , especially in the bathing solution with  $\text{NH}_4^+$ .

## Discussion

In all treatments, tea roots showed absorption states of  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , showing that the thresholds for plant development were higher than the cytosolic concentrations of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  after a 7 d N deprivation. Tea roots needed to maintain a certain level of  $\text{NH}_4^+$  or  $\text{NO}_3^-$  in the cytoplasm<sup>7</sup>. Additionally, tea roots showed a preference for  $\text{NH}_4^+$  when  $\text{NH}_4^+$  and  $\text{NO}_3^-$  existed at the same time. Greater net uptake of  $\text{NH}_4^+$  compared to net uptake of  $\text{NO}_3^-$  were reported in maize, rice and wheat roots when  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were supplied simultaneously<sup>12–14</sup>. In comparison to  $\text{NO}_3^-$  influx, there were some possible reasons for the observed preference for  $\text{NH}_4^+$  influx. As various root tissues needed various amounts of  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , one reason might involve root morphology. A higher concentration of  $\text{NH}_4^+$  was needed for protein synthesis in the meristem zone<sup>12</sup>. In addition,  $\text{NH}_4^+$  absorbed by plants was transformed to amino acids directly in the roots, which required less energy and reducing equivalents for assimilation and transportation in most species<sup>15,16</sup>. For tea plants, ammonia supplied to the tea roots was quickly stored as theanine, glutamine and arginine in the roots and leaves before the sprouting new shoots<sup>17</sup>. Previous research<sup>18</sup> has reported that after tea plants were fed with  $^{15}\text{N}\text{-NO}_3^-$  and  $^{15}\text{N}\text{-NH}_4^+$ , the amount of total amino acid in the xylem sap significantly increased, and those fed with  $^{15}\text{N}\text{-NH}_4^+$  had a greater increase compared to those fed  $^{15}\text{N}\text{-NO}_3^-$ . Different from other plants, tea can turn redundant glutamic acid into theanine, which was a peculiar amino acid in tea<sup>19</sup>. Moreover,  $\text{NH}_4^+$  was more readily assimilated than  $\text{NO}_3^-$  into theanine<sup>20</sup>. This process might have eliminated  $\text{NH}_4^+$  toxicity in tea roots and have created a  $\text{NH}_4^+$  preference in tea<sup>5</sup>.

Previous studies demonstrated that crop growth and yield were significantly improved when two forms of nitrogen were supplied at the same time. In this study, the highest total N influx rates were observed with the  $\text{NH}_4\text{NO}_3$  treatment when N levels were the same, which suggested that tea roots had the highest nitrogen absorption efficiency when two forms of nitrogen were supplied simultaneously. This result was consistent with other plants reported<sup>14,21,22</sup>. For most plants, roots released  $\text{H}^+$  after absorption of  $\text{NH}_4^+$ , leading to a decreased pH in the growth medium, while roots released  $\text{OH}^-$  after absorption of  $\text{NO}_3^-$ , leading to the increased pH in the growth medium<sup>23–25</sup>. A mixed application of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  at a 1:1 ratio encouraged higher foliar N content and glutamine synthetase (GS) and glutamate synthase (GOGAT) activity in tea<sup>26</sup>. Although tea roots preferred  $\text{NH}_4^+$ , a single application of ammonium nitrogen could aggravate soil acidification in tea gardens<sup>27</sup>, and could significantly raise the amount of aluminum taken up by tea plants, leading to a decrease in tea quality<sup>28</sup>. Therefore, to get a higher N absorption efficiency in tea and reduce soil acidification in tea gardens, two forms of nitrogen should be supplied simultaneously.



**Figure 6.** Proposed mechanisms of interaction between  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes in tea roots. The absorption and transformation processes of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  are shown. Some influence factors of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  absorption are listed to explain possible mechanisms of interaction between the  $\text{NH}_4^+$  and  $\text{NO}_3^-$  fluxes in tea roots.

With the increase in N concentration, the influx rate of  $\text{NO}_3^-$  was improved more than  $\text{NH}_4^+$ , which might be contributed to differences in activities and expressions of the transport systems between the two ions. Net  $\text{NO}_3^-$  and  $\text{NH}_4^+$  absorption can be regulated by low-affinity (LATS) and high-affinity transporters (HATS). When the exterior  $\text{NH}_4^+$  concentration was below 1 mM, HATS played a leading role in the uptake of  $\text{NH}_4^+$  absorption, and when the exterior  $\text{NH}_4^+$  concentration was above 1 mM, LATS were activated<sup>29</sup>. While HATS played a main role in regulating  $\text{NO}_3^-$  uptake when the external  $\text{NO}_3^-$  concentration was below 1 mM, LATS started to work when the external  $\text{NO}_3^-$  concentration was above 0.5 mM<sup>30</sup>. According to previous studies, with the increase in N concentration, the LATS for  $\text{NO}_3^-$  were stimulated much earlier than the LATS for  $\text{NH}_4^+$ . In addition, Glass *et al.*<sup>31</sup> and Britto *et al.*<sup>32</sup> reported that transport through the low-affinity systems were poorly regulated when the high-affinity  $\text{NH}_4^+$  fluxes were effectively regulated. This might lead to the massive vain cycling of  $\text{NH}_4^+$  across the plasma membrane and toxic effects of superfluous  $\text{NH}_4^+$  accumulation. Thus, the influx rate of  $\text{NO}_3^-$  was improved more than  $\text{NH}_4^+$  with the increase in N concentration. The present data showed that the influx rate of  $\text{NO}_3^-$  was significantly lower than the influx rate of  $\text{NH}_4^+$  under low N conditions (0.2 mM N), which might be contributed to a lower energy cost for both transport and assimilation of  $\text{NH}_4^+$ <sup>16</sup>.

The present data demonstrates that the presence of  $\text{NO}_3^-$  had a negative effect on net  $\text{NH}_4^+$  uptake. Before being assimilated by plants,  $\text{NO}_3^-$  was restored as  $\text{NH}_4^+$ , leading to the increase  $\text{NH}_4^+$  concentration in the cytoplasm. The HATS of  $\text{NH}_4^+$  were influenced by the negative feedback regulations and an increased cytosolic  $\text{NH}_4^+$  concentration suppressed the root influx of  $\text{NH}_4^+$ <sup>31</sup>. As widely acknowledged,  $\text{NO}_3^-$  is a mobile ion and can be restored both in the roots and leaf. Nitrate in tea roots can be directly transported to the xylem sap and then to the leaf<sup>18</sup>. When  $\text{NO}_3^-$  was restored as  $\text{NH}_4^+$  in the leaf, the concentration of  $\text{NH}_4^+$  in the leaf would increase. A shoot-to-root signal might be regarded as the effect of the local N status that controls the influx of  $\text{NH}_4^+$ <sup>33</sup>. In addition,  $\text{NO}_3^-$  had an inhibitory influence on GS enzyme activity, which might also a reason for the negative effect of  $\text{NO}_3^-$  on  $\text{NH}_4^+$  influx<sup>34,35</sup> (Fig. 6).

In contrast, the presence of  $\text{NH}_4^+$  had a positive effect on net  $\text{NO}_3^-$  uptake, which was consistent with previous studies performed in other species<sup>14,33,36</sup>. There were several reasons to support this result. First,  $\text{NH}_4^+$  has been reported to increase the respiration rate of plants, which can provide energy for  $\text{NO}_3^-$  uptake<sup>37</sup>. Second, the balance of  $\text{H}^+$ ,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  could be used to explain this result. Tea roots took up a large amount of  $\text{NH}_4^+$  during growth and later released  $\text{H}^+$  to maintain the charge balance in the plant body<sup>38</sup>. According to our current understanding of  $\text{NO}_3^-$  transportation,  $\text{NO}_3^-$  influx occurs with one  $\text{H}^+$  symport, and two possible  $\text{H}^+$  ions promote the inward transportation of one  $\text{NO}_3^-$  ion, while the efflux of  $\text{H}^+$  is meant to balance the influx of  $\text{NH}_4^+$ . According to previous studies, due to  $\text{NH}_4^+$  stimulation of  $\text{H}^+$  efflux, a stimulation of  $\text{NO}_3^-$  absorption by  $\text{NH}_4^+$  might increase the availability of  $\text{H}^+$  for co-transport<sup>39,40</sup>. Moreover,  $\text{NH}_4^+$  could significantly increase the activities of the GS and GOGAT enzymes, which plays an important role in nitrate reduction and nitrogen assimilation, providing material bases for  $\text{NO}_3^-$  absorption<sup>34</sup>. In our study, the  $\text{NO}_3^-$  influx rates were more irregular in the various treatments. This was because  $\text{NO}_3^-$  could develop the functions of a mobile ion and an osmoticum<sup>14</sup> (Fig. 6).

In conclusion, the elucidation of the mechanisms related to N transport is difficult when assessing net N flux. Net N flux is established as the total of N efflux and influx. Additionally, net N flux is affected by transportation and assimilation rates. The findings showed that tea roots presented influx states of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  after a 7 d N-starvation. The uptake rates of  $\text{NH}_4^+$  in tea plants were higher than those of  $\text{NO}_3^-$ .  $\text{NH}_4^+$ -N can make tea trees grow better when only one single N source can be provided. Furthermore, the presence of  $\text{NO}_3^-$  had a negative effect on net  $\text{NH}_4^+$  influx, while  $\text{NH}_4^+$  had a positive influence on net  $\text{NO}_3^-$  influx. These findings may not only help guide further hydroponic experiments with tea but also help in developing efficient fertilization protocols for field-grown tea.

## Methods

**Plant materials and cultivation.** *Camellia sinensis* var. Longjing 43 was used in this study. Annual cutting seedlings of Longjing 43 were transplanted to a full-strength nutrient solution for 75 d. The full-strength nutrient solution contained macronutrients (mmol L<sup>-1</sup>) NH<sub>4</sub>NO<sub>3</sub> (1), KH<sub>2</sub>PO<sub>4</sub> (0.07), K<sub>2</sub>SO<sub>4</sub> (0.3), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.67), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.53), and Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·18H<sub>2</sub>O (0.035) and micronutrients (μmol L<sup>-1</sup>) H<sub>3</sub>BO<sub>4</sub> (7), MnSO<sub>4</sub>·H<sub>2</sub>O (1), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.67), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.13), (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (0.047) and EDTA-Fe (4.2) at pH 5.0. The nutrient solution was circulated by pumps for 24 h every day and replaced every 3 days. Next, an N starvation treatment was carried out for 7 d. The N starvation treatment was conducted using the following nutrient solution which contained macronutrients (mmol L<sup>-1</sup>) KH<sub>2</sub>PO<sub>4</sub> (0.07), K<sub>2</sub>SO<sub>4</sub> (0.3), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.67), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.53), and Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·18H<sub>2</sub>O (0.035) and micronutrients (μmol L<sup>-1</sup>) H<sub>3</sub>BO<sub>4</sub> (7), MnSO<sub>4</sub>·H<sub>2</sub>O (1), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.67), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.13), (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O (0.047) and EDTA-Fe (4.2) at pH 5.0<sup>20</sup>. The nutrient solution was circulated by pumps for 24 h every day and replaced every three days. After the 7 d N starvation treatment, the seedlings were harvested to measure ion fluxes.

**Determinations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> fluxes at the root surface.** The absorbing tea roots were chosen and cut off from the root system of every plant in every treatment group to evaluate the net fluxes of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> in tea roots under various N forms. For the different nitrogen form treatments, tea roots were immersed in measuring solutions with different N forms (NH<sub>4</sub>NO<sub>3</sub>-N: 0.1 mM CaSO<sub>4</sub>, 1 mM NH<sub>4</sub>NO<sub>3</sub> and 0.3 mM MES; NH<sub>4</sub><sup>+</sup>-N: 0.1 mM CaSO<sub>4</sub>, 1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.3 mM MES; and NO<sub>3</sub><sup>-</sup>-N: 0.1 mM CaSO<sub>4</sub>, 2 mM KNO<sub>3</sub> and 0.3 mM MES). MES is 2-(N-morpholino)ethanesulfonic acid hydrate buffer. For different N level treatments, tea roots were soaked in measuring solutions with different N levels (0.2 mM NH<sub>4</sub><sup>+</sup>-N: 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 0.1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1.2 mM NH<sub>4</sub><sup>+</sup>-N: 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 0.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.2 mM NO<sub>3</sub><sup>-</sup>-N: 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 0.2 mM KNO<sub>3</sub>; and 1.2 mM NO<sub>3</sub><sup>-</sup>-N: 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 1.2 mM KNO<sub>3</sub>). Before analysis, tea roots were transferred to Petri dishes containing 10 mL of measuring solution and equilibrated for 10 min to reduce possible transition effects due to changes in the environmental conditions. Next, the equilibrated root was moved to another Petri dish containing fresh measuring solution and either NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> flux was measured utilizing the SIET technique. The coefficient of variation under different balance times were shown in Supplementary Figure S4. When the pretreatment time was 10 min, the coefficient of variations under the different treatments was lower and more stable. Therefore, a 10-min pretreatment time was enough and suitable for our study. Six repetitions were established for each treatment. In order to determine the area along the root axis corresponding with maximal net NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> influx, the net fluxes of both ions were measured along the root tips to an area located 40 mm from the apex (Figure S5). The maximum net NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> influxes occurred in area between 15 and 25 mm from the root apex. Thus, we chose area between 15 and 25 mm from the root apex as the measurement site. The measuring time of each root was 10 min. The SIET technique was used to measure the net ion flux (NMT-NRP-00A00 system, Younger USA Science and Technology Corporation). The SIET system and the corresponding application process have been previously described in detail for ion flux detection<sup>14</sup>. Briefly, ion-selective microelectrodes designed with 2–4 μm apertures were manufactured and silanized (for the NH<sub>4</sub><sup>+</sup> electrode, 100 mM NH<sub>4</sub>Cl was used as a backfilling solution, followed by a NH<sub>4</sub><sup>+</sup> selective liquid ion exchange cocktail (#09879, Sigma); for the NO<sub>3</sub><sup>-</sup> electrode, 10 mM KNO<sub>3</sub> was used as the backfilling solution, followed by a NO<sub>3</sub><sup>-</sup> selective liquid ion exchange cocktail (#72549, Sigma)). Prior to performing the flux measurements, the microelectrodes were calibrated<sup>14</sup>.

The absorbing roots of tea were soaked in a test solution and excised from the root system to evaluate the effect of NO<sub>3</sub><sup>-</sup> on NH<sub>4</sub><sup>+</sup> flux (D (with NO<sub>3</sub><sup>-</sup>): 0.3 mM MES, 0.1 mM CaSO<sub>4</sub>, 0.1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 1 mM KNO<sub>3</sub>; E (without NO<sub>3</sub><sup>-</sup>): 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 0.1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). NH<sub>4</sub><sup>+</sup> flux was measured utilizing the SIET technique for 5 min after a 10-min balance in the measuring solution. Next, 0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the measuring solution. After each addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, during the first 1–2 min, the measuring solution was mixed thoroughly by expelling and sucking it into a pipette 10 times. NH<sub>4</sub><sup>+</sup> flux was measured using the SIET technique for another 25 min. The unstable data during the early stage were removed to gain ion flux curves.

The absorbing roots of tea were immersed in a measuring solution and excised from the root system to study the effect of NH<sub>4</sub><sup>+</sup> on NO<sub>3</sub><sup>-</sup> flux (F (with NH<sub>4</sub><sup>+</sup>): 0.3 mM MES, 0.1 mM CaSO<sub>4</sub>, 0.2 mM KNO<sub>3</sub>, and 0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; G (without NH<sub>4</sub><sup>+</sup>): 0.3 mM MES, 0.1 mM CaSO<sub>4</sub> and 0.2 mM KNO<sub>3</sub>). NO<sub>3</sub><sup>-</sup> flux was measured utilizing the SIET technique for 5 min after a 10-min balance in the measuring solution. Next, 1.0 mM KNO<sub>3</sub> was added to the measuring solution. The test process was the same as above.

**Statistical analysis.** To verify the importance of differences between treatments, one-way ANOVA was performed. Microsoft Excel (Microsoft Corporation, USA) and SPSS Window version 17 (SPSS Incorporation, Chicago, USA) were used to analyze data. To draw figures for the data, OriginPro 8.1 (Origin Incorporation, Chicago, USA) was utilized.

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## Author Contributions

L.Y.W. and H.C. designed the experiment. L.R. conducted the measurements, data analysis and wrote the manuscript. K.W., F.Z., L.Y.W. and P.X.B. assisted with the data analysis. C.C.Z. assisted with the experiment. These authors reviewed the manuscript before the submission.

## Additional Information

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