



Full Length Article

Effects of Nitrogen Forms and Osmotic Stress on Root Aerenchyma Formation of Japonica Rice and Its Relationship with Water Absorption

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Abstract

In order to study the effects of different nitrogen forms and osmotic stress on aerenchyma formation and the xylem sap in rice root, hydroponic experiment was conducted in a greenhouse. The forms of nitrogen were ammonium nutrition and nitrate nutrition, with an identical nitrogen concentration of 40 mg L⁻¹. The water conditions were non-osmotic stress and osmotic stress [100 g L⁻¹ polyethylene glycol 6000 (PEG 6000) added to the nutrients solution and the osmotic potential was equivalent to -0.15 MPa]. Two rice genotypes (86you 8# and Wuyunjing 7#) were utilized in current experiments. The results showed that osmotic stress promoted the formation of aerenchyma in the roots of japonica rice for nitrate nutrition supply, but no significant effect was observed on japonica rice with ammonium nutrition supply. Under osmotic stress condition, the flow rate of root bleeding sap in the plants supplied with nitrate nutrition was significantly decreased, and there was a significant linear negative correlation between the flow rate and the root porosity ($P < 0.01$). However, no significant difference was observed with the ammonium treatment under the osmotic stress condition. Under osmotic stress condition, the water absorption capacity of japonica rice root can be effectively improved by the supply of ammonium nutrition. By calculating the decreasing ratio of water absorption in rice root after adding HgCl₂, we could speculate that the supply of ammonium nutrition can maintain a higher aquaporin activity of japonica rice roots under osmotic stress conditions. Therefore, the increased root porosity might be a key factor for the decreased water absorption in the plants supplied with nitrate nutrition under osmotic stress condition.

Keywords: Aerenchyma; Nitrogen form; Water absorption; Osmotic stress; Xylem sap; Japonica rice

Introduction

Water stress is one of the most important restriction factors to crop production (Tuberosa *et al.*, 2007; Arum *et al.*, 2018). Many agricultural practices have been advocated to increase drought resistance, primarily by improving the nutrients and water status (Shangguan *et al.*, 2000; Saneoka *et al.*, 2004). In particular, some studies mainly focused on the effects of ammonium and nitrate nutrition on plant growth and water uptake under water stress conditions (Steudle, 2000b, Cabuslay *et al.*, 2002; Guo *et al.*, 2007b). Generally, rice (*Oryza sativa* L.) plants have a stronger ammonium assimilation capacity than other crops particularly under water stress conditions (Guo *et al.*, 2007a). In recent years, some researchers suggested that the tolerance of rice seedlings to water stress can be promoted by ammonium nutrition (Guo *et al.*, 2007a; Guo *et al.*, 2008, Li *et al.*, 2009).

Root is an important organ in rice which absorb water and its form and anatomical structure are closely related to water and nutrients (Wang *et al.*, 2017). The root's cortex aerenchyma is the main obstacle for root's water absorption

and transportation, and its degree of development is closely related to the water conductance of plants. Earlier studies showed that availability of water and nitrogen forms have significant effect on root aerenchyma formation. In well ventilated cultures, developed aerenchyma can be observed in adventitious roots of rice when nitrogen or phosphorus are deficient in the solution (He *et al.*, 1992; Lu and Neumann, 1999). Zhu *et al.* (2010) reported that the root aerenchyma is beneficial for drought resistance in maize because root respiration can be reduced by converting cortical tissue to aerenchyma. Yang *et al.* (2012) discovered that the supply of ammonium nutrient after osmotic stress can speed up the development of aerenchyma in rice roots, thereby improving its drought resistance and maintaining high water absorption capacity.

Indica rice and japonica rice are two important rice subspecies, and the varieties of japonica rice are mainly grown in northern regions of China where drought stress is serious environmental stress. Nonetheless, few studies were conducted on the relationship between root aerenchyma and water uptake supplied with different forms of nitrogen under

water stress. In this study, we hypothesized that supplying of ammonium nitrogen under osmotic stress conditions can increase the development degree and water absorption ability of aerenchyma in japonica rice. Therefore, the effects of different nitrogen forms and water conditions on the formation of root aerenchyma and water uptake of japonica rice, and its relationship with the root hydraulic conductivity in rice seedlings, were investigated by simulating osmotic stress in current study. The objectives of this study are to provide strong theoretical basis for water-saving irrigation of japonica rice.

Materials and Methods

Test Materials and Design

In this study, two nitrogen forms and two water conditions were set. The forms of nitrogen were ammonium nutrition [$\text{NH}_4^+\text{-N}$, supplied in the form of $(\text{NH}_4)_2\text{SO}_4$] and nitrate nutrition [$\text{NO}_3^-\text{-N}$, supplied in the form of $\text{Ca}(\text{NO}_3)_2$], with an identical nitrogen concentration of 40 mg L^{-1} . The water conditions were non-osmotic stress and osmotic stress osmotic stress [100 g L^{-1} polyethylene glycol 6000 (PEG 6000) added to the nutrients solution and the osmotic potential was equivalent to -0.15 MPa]. There was a total of four treatments, namely ammonium nitrogen (A) and nitrate nitrogen (N) under no osmotic stress, as well as ammonium stress (AP) and nitrate nitrogen (NP) under osmotic stress, and each treatment was repeated 5 times. The nutrients solution used the conventional nutrient solution formula of the International Rice Research Institute (IRRI) (Lu and Neumann, 1998). In addition, Na_2SiO_3 was added to maintain the concentration of SiO_2 in the nutrients solution at 0.1 mmol L^{-1} , and 1 mg L^{-1} dicyandiamide (DCD) was added as a nitrification inhibitor. The nutrients solution was changed every 3 days, and the pH of the nutrient solution was adjusted to 5.50 ± 0.05 with 1 mol L^{-1} HCl and NaOH every day.

The japonica rice varieties tested in this study were 86 you 8# and Wuyunjing 7#. The rice seeds were first immersed in 10% hydrogen peroxide (H_2O_2) to disinfect for 30 min, then rinsed repeatedly with deionized water. Rice seeds of the same size were placed in a nursery basket containing deionized water to culture in a constant temperature incubator at 30°C . When the seedlings had developed an average of 2.5 visible leaves, they were transplanted to 3.5 liter buckets and into a full-strength mixture of nutrients solution (including nitrogen form as ammonium nitrogen: nitrate nitrogen = 1:1). One week later, they were cultured with different forms of nitrogen nutrition alone for 6 days, and then treated with PEG6000 as osmotic stress for another 1-2 weeks. Nutrient solutions were updated every 3 d, and pH was adjusted to 5.50 ± 0.05 every day with 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl. There were six replicates within a completely random design for all of the treatments. The different treatments were placed randomly in a greenhouse to avoid edge effects.

Measurement Items

Collection of Bleeding Sap

It started from 19:00 and ended at 7:00 the next day. The overground part 2 cm from the root was cut, and the stem base was wrapped with degreasing cotton covered with plastic wrap for accurate collection for 12 h. The flow rate of bleeding sap was calculated with subtraction weighing method (Li, 2001).

Observation of Root Aerenchyma

After rinsing the roots with deionized water, a complete adventitious root about 10 cm long was selected, the part 30 to 40 mm from the root tip was cut into small pieces in 1 mm . For the sample preparation method for indica rice root scanning electron microscope (SEM), it refers to David and Olga (2000). Finally, the prepared root sample was observed with SEM (S-3000N, Hitachi Inc., Japan) for imaging.

Determination of Root Porosity

Three rice plants were randomly selected from each treatment group, then washed and dried, and the fresh root weight (W_r) was weighed. Distilled water put in vacuum for 12 h was poured into a 50-mL pycnometer and the water and pycnometer (W_{wb}) were weighed at room temperature, then the weighed sample was placed in a water-filled pycnometer and weighed again (W_{wbr}). Then it was placed in a vacuum oven and evacuated until there is no bubble. The evacuated roots were then transferred back to a pycnometer filled with water and weighed again (W_{wbra}). The porosity was calculated according to the following formula:

$$\text{Porosity}\% = [(W_{wbra} - W_{wbr}) / (W_{wb} + W_r - W_{wbr})] \times 100$$

Unit Root Water Absorption

After 10% PEG simulated osmotic stress treatment was completed, the water absorption of japonica rice within 2 h (9:00-11:00) was determined with subtraction weighing method and the fresh root was weighed after that. At the same time, pre-made 1 mM HgCl_2 solution was added to another batch of corresponding nutrients solution (the final concentration of HgCl_2 in the nutrient solution is 0.1 mM), and the water absorption rate of the rice seedlings within 2 h was measured with subtraction weighing method.

Plant Samples Collecting

The rice plants were harvested and separated into root and shoot fractions when the experiments were completed. All fractions were kept in an oven at 105°C for 30 min and then at 75°C until constant weight.

Data Processing

All data was statistically analyzed with JMP10.0 (SAS Institute, Cary, NC, USA, 2011) and Microsoft Excel 2016. All data was compared with LSD method.

Results

Effect of Nitrogen Form and Osmotic Stress on Rice Morphology

Under osmotic stress condition, rice showed pronounced growth depressions in shoot biomass with ammonium or nitrate nutrition (AP, NP) (Table 1). No significant differences in root biomass were observed between non-osmotic stressed and osmotic stressed plants using ammonium nutrition. However, under a nitrate nutrition supply, rice showed pronounced increases in root biomass with osmotic stress (Table 1). Osmotic stress obviously enhanced the root/shoot ratio under ammonium and nitrate nutrition for the two japonica rice cultivars. Compared with no-osmotic stress, tillers were greatly decreased in Wuyunjing 7# under osmotic stress and nitrate nutrition, whereas no significant depression was found in plants using ammonium nutrition (Table 1).

Effect of Nitrogen Forms and Water Conditions on the Flow Rate of Root Bleeding Sap

Under non-osmotic stress conditions, the flow rate of root bleeding sap with N treatment significantly higher than that with A treatment (Fig. 1). Under osmotic stress conditions, the flow rate of root bleeding sap with NP treatment was significantly decreased (the flow rates of bleeding sap were reduced by 60% and 70% for 86 you 8# and Wuyunjing 7#, respectively, compared with the normal water conditions). However, no significant difference was observed with the AP treatment under non-osmotic stress conditions. Therefore, the supply of ammonium nutrition was conducive to maintaining a high rice root activity and water absorption capacity under osmotic stress conditions.

Effects of Nitrogen Forms and Osmotic Stress on Aeration Formation in Japonica Rice Roots

The development of aerenchyma in japonica rice roots was affected by different water conditions and nitrogen forms (Fig. 2). Under non-osmotic stress conditions, air cavity can be observed between the radially developed through stele and sclerenchyma on the cross section 30-40 mm from the roots on the cortex parenchyma cells of different japonica varieties with A treatment. Only different degrees of collapse occurred, and no air cavity was formed between the radially developed through stele and sclerenchyma with A treatment. Compared with non-osmotic stress conditions, both AP-treated and NP-treated rice roots form air cavity between the through stele and sclerenchyma after osmotic stress.

Table 1: Effects of nitrogen form and osmotic stress on shoot and root dry weight, the root/shoot ratio and tillers of rice plants (cv. 86you 8# and cv. Wuyunjing 7#)

| Rice cultivars | Treatment | Shoots (g plant ⁻¹) | Roots (g plant ⁻¹) | Root/shoot ratio | Tillers (No. plant ⁻¹) |
|----------------|-----------|---------------------------------|--------------------------------|------------------|------------------------------------|
| 86you 8# | A | 1.40±0.07a | 0.30±0.07ab | 0.19±0.02c | 7.5±0.6a |
| | N | 1.07±0.06b | 0.24±0.02b | 0.23±0.01b | 5.3±0.3b |
| | AP | 1.11±0.06b | 0.26±0.03b | 0.24±0.01b | 7.0±0.8a |
| | NP | 0.81±0.08c | 0.35±0.05a | 0.40±0.02a | 4.8±1.0b |
| Wuyunjing 7# | A | 1.27±0.13a | 0.28±0.02a | 0.21±0.02d | 7.0±1.0 a |
| | N | 0.78±0.02c | 0.19±0.01b | 0.24±0.01c | 6.3±1.2ab |
| | AP | 0.94±0.04b | 0.25±0.03a | 0.28±0.01b | 6.8±0.6 a |
| | NP | 0.54±0.04d | 0.26±0.05a | 0.44±0.07a | 4.3±1.5 b |

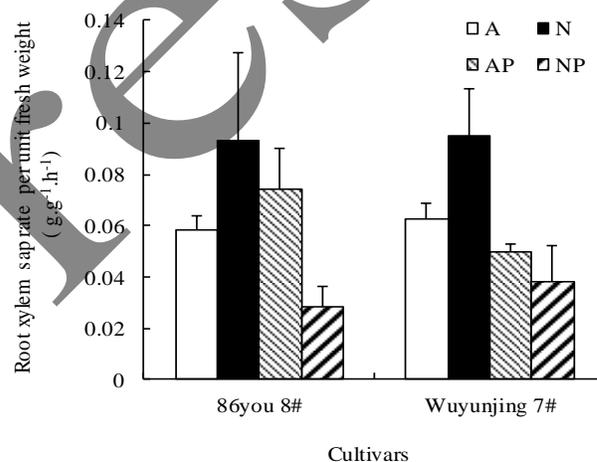


Fig. 1: Effects of nitrogen form and osmotic stress on root xylem sap flow rate per unit fresh weight of rice plants (cv. 86 you 8# and cv. Wuyunjing 7#). Data represent means of three replicates. Bars indicate the SD. Rice plants were

Thus, osmotic stress promoted the formation of aerenchyma in the roots of japonica rice for nitrate nutrition supply, but no significant effect was observed on japonica rice with ammonium nutrition supply.

Effects of Nitrogen Forms and Osmotic Stress on Root Porosity of Japonica Rice

Under non-osmotic stress conditions, the porosity of japonica rice root with A treatment was significantly higher than that with N treatment (Fig. 3). Under osmotic stress conditions, there was no statistical increase of the root porosity of AP treated rice, while the porosity of rice roots increased significantly with NP treatment. Therefore, the formation of root aerenchyma was stimulated by osmotic stress under the condition of nitrate nutrition.

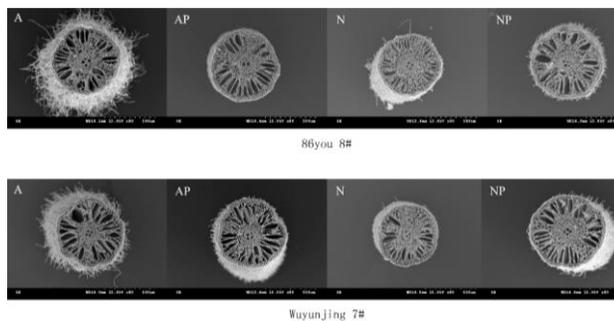


Fig. 2: Scanning electron micrographs of transverse sections from newly formed adventitious roots of rice plants. Rice plants were supplied with ammonium (A) or nitrate (N) under either normal or osmotic stress simulated by adding 10% PEG6000 (NH_4^+ + PEG as AP; NO_3^- + PEG as NP)

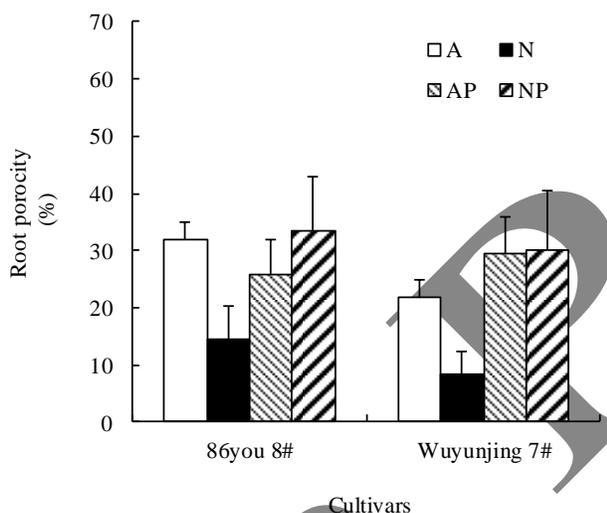


Fig. 3: Effects of nitrogen form and osmotic stress on root porosity in rice plants (cv. 86you 8# and cv. Wuyunjing 7#). Data represent means of three replicates. Bars indicate the SD. Rice plants were supplied with ammonium (A) or nitrate (N) under either normal or osmotic stress simulated by adding 10% PEG6000 (NH_4^+ + PEG as AP; NO_3^- + PEG as NP)

Under the condition of ammonium nutrition, the flow rate of the bleeding sap in unit fresh weight of rice roots does not change significantly with the increase of root porosity, and there was no correlation between the flow rate and root porosity ($P > 0.05$, Fig. 4). Under the condition of nitrate nutrition, the flow rate of root bleeding sap decreased due to osmotic stress, and there was a significant linear negative correlation between the flow rate and root porosity ($P < 0.01$).

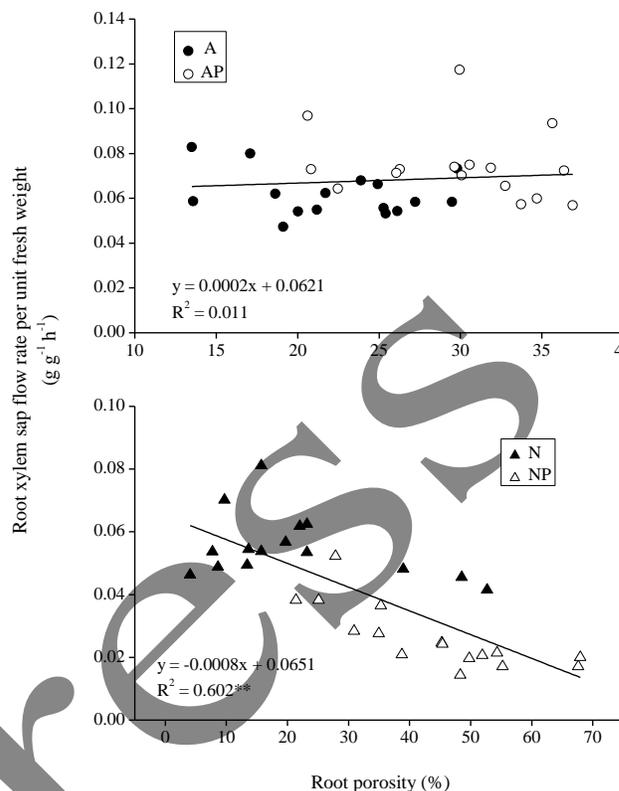


Fig. 4: Relationship between root porosity and root xylem sap rate per unit fresh weight. Rice plants were supplied with ammonium (A) or nitrate (N) under either normal or osmotic stress simulated by adding 10% PEG6000 (NH_4^+ + PEG as AP; NO_3^- + PEG as NP). Data from 86you 8# and Wuyunjing 7#. Lines represent linear regressions, and ** represent the significant at $P < 0.01$

Effects of Nitrogen Forms and Osmotic stress on Water Absorption of Rice Roots

Under non-osmotic stress conditions, there was no significant difference in water absorption per unit root of japonica rice with A and N treatments. Under osmotic stress conditions, water absorption of AP- and NP-treated japonica rice roots decreased significantly, but the reductions were correlated to the different nitrogen supply forms. Under the supply of ammonium nutrition, the water absorption of roots was decreased by 22% and 26% for the varieties of 86 you 8# and Wuyunjing 7#, respectively, while they were decreased by 35% and 40% under the condition of nitrate nutrition. Therefore, under osmotic stress condition, the supply of ammonium nutrition can effectively improve the water absorption capacity of japonica rice root.

After addition of $0.1 \text{ mmol L}^{-1} \text{ HgCl}_2$ to the nutrient solution, the water absorption per unit root of the japonica rice per unit time showed a downward trend under different nitrogen forms and water treatments (Table 2).

Table 2: Effects of nitrogen form and osmotic stress on water uptake rate (without HgCl₂ as control and with HgCl₂) based on per unit root fresh weight for 2 h [gH₂O (g root fresh weight)⁻¹] after addition of 0.1 mmol l⁻¹ HgCl₂ to rice plants (cv. 86you 8# and cv. Wuyunjing 7#)

| Rice Cultivars | Treatment | Water uptake rate | |
|----------------|-----------|-------------------|--|
| | | Control | 0.1 mmol l ⁻¹ HgCl ₂ |
| 86you 8# | A | 2.50±0.45 a | 0.70±0.36 b |
| | N | 2.47±0.09 a | 1.11±0.14 ab |
| | AP | 1.96±0.71 b | 0.65±0.20 b |
| | NP | 1.61±0.16 bc | 1.15±0.16 ab |
| Wuyunjing 7# | A | 2.28±0.06 a | 1.01±0.35 bc |
| | N | 2.60±0.15 a | 1.11±0.17 abc |
| | AP | 1.69±0.47 b | 0.66±0.16 c |
| | NP | 1.56±0.37 bc | 1.14±0.13 ab |

Notes: Different letters on the same column indicate significant difference at 5% level by LSD test. Rice plants were supplied with ammonium (A) or nitrate (N) under either non-osmotic stress or osmotic stress simulated by adding 10% PEG 6000 (NH₄⁺-N+PEG as AP; NO₃⁻-N +PEG as NP)

By calculating the decreasing ratio of water absorption in rice root after adding HgCl₂, we could estimate the ratio of water absorption by rice root through aquaporin transmembrane transport pathway under different conditions. It can be seen that, under non-osmotic stress conditions, the ratio of water absorption showed decreased after adding HgCl₂ in rice root with A and N treatments. Similarly, the decreasing ratio of water absorption was observed in rice root with AP treatment after adding HgCl₂ under osmotic stress conditions. Nonetheless, there was a significant reduced ratio of water absorption of rice root with NP treatment. Therefore, it can be speculated that, under osmotic stress conditions, the supply of ammonium nutrition can maintain a higher aquaporin activity of japonica rice roots.

Discussion

A Stronger Capacity of Water Absorption for Japonica Rice with Ammonium Nutrition under Osmotic Stress

The water absorption capacity of the root was closely related to the flow rate of root xylem's bleeding sap (Mu *et al.*, 2006). Our experiment showed that, compared with the non-osmotic stress conditions, the flow rate of the root bleeding sap of japonica rice treated with nitrate nutrition decreased significantly under osmotic stress conditions, while there was no significant difference in the japonica rice treated with ammonium nitrogen.

Root water absorption was regulated by the adjustment of physical and physiological properties of the root itself (Kong *et al.*, 2016). It can be known from the composite transport model of roots that water enters the root canal mainly through the following three routes, namely the apoplast pathway, the plastid pathway and the transcellular pathway, and the latter two pathways were collectively referred to as the cell-cell pathway. Some earlier researchers investigated that the cell-cell pathway was significantly

affected by aquaporin activity on the plasma membrane of roots (Steudle and Peterson, 1998; Steudle, 2000a, 2001). As a specific inhibitor of aquaporins, the water absorption of mercury decreased after mercury treatment (Song *et al.*, 2007). Under the experimental conditions, compared with the control, the water absorption of japonica rice root with ammonium and nitrate nutrition declined in both the non-osmotic stress conditions and the osmotic stress conditions. In particular, the water absorption of the japonica rice with ammonium nutrition was lower than that of the rice with nitrate nutrition. It can be speculated that the water in the japonica rice root with ammonium nutrition under osmotic stress conditions may be mainly transported through the mercury-sensitive aquaporins (cell-cell pathway), while the water in japonica rice with nitrate nutrition was mainly through apoplast pathway. The reason for the difference in root water absorption of rice plants with ammonium and nitrate nutrition may be the difference in activity of root aquaporins.

Effects of Osmotic Stress and Nitrogen Forms on Aerenchyma of Rice and Their Relationship with Water Absorption

The formation of aerenchyma was closely related to the environmental conditions in which the roots grow. In addition to hypoxia stress, other environmental stresses such as nutrient stress, mechanical disorders and even drought can induce the formation of plant aerenchyma (Eshel *et al.*, 1996; Fan *et al.*, 2005; Dong *et al.*, 2016). This study showed that under non-osmotic stress conditions, the development of root aerenchyma of rice treated with ammonium nutrition was higher than that with nitrate nutrition treatment in different genotypes. However, there was no significant difference between the two treatments under osmotic stress conditions. The aerenchyma of japonica rice treated with nitrate nutrition was significantly increased, and the root porosity was increased by 1 to 2 times compared with that under non-osmotic stress conditions. Osmotic stress significantly increased the formation of aerenchyma of japonica rice root treated with nitrate nutrition.

The form and structure of roots are important factors affecting the root water fluidity (Miyamoto *et al.*, 2001). It was found in this study that under the conditions of nitrate nutrition, there was a significant negative correlation between the root xylem sap flow rate and the root porosity of japonica rice varieties, while there was no correlation under ammonium nutrition (Fig. 4). This indicated that the development of aerenchyma in rice roots did not inhibit root's water absorption and transport capacity under ammonium nutrient conditions, but significantly inhibited the moisture and transportation of japonica rice root. The reason might be related to the change in water transport pathways of japonica rice under the two nutrition supply forms. Radial transport of water in japonica rice root with ammonium nutrients may be mainly through aquaporins that are sensitive to Hg reaction.

The formation of aerenchyma did not affect the water transport, while the water in japonica rice with nitrate nutrition was mainly transported through apoplast pathway. The mechanism for the root forming aerenchyma under different nitrogen forms and osmotic stress conditions is unclear yet and needs further study. The results in our study provide an important information for understanding the physiological significance of root aerenchyma under osmotic stress in rice. In addition, these results provided a strong theoretical basis for water-saving irrigation of japonica rice.

Conclusion

Under osmotic stress condition, supply of ammonium nutrition was beneficial to maintain the higher water absorption capacity and xylem sap flow rates of japonica rice varieties than those in plants supply of nitrate nutrition. Under the stress condition, the aerenchyma of the rice root treated with nitrate nutrition was significantly increased; however, no significant changes were observed in the plants treated with ammonium nutrition. Moreover, the supply of ammonium nutrition can maintain a higher aquaporin activity of rice roots. As a result, the lower root porosity and relatively higher aquaporin activity might be the key factor for the increased water absorption in the rice supplied with ammonium nutrition under osmotic stress condition.

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