Effects of N application methods on cotton yield and fertilizer N recovery efficiency in salinity fields with drip irrigation under mulch film using $^{15}$N tracing technique

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Abstract

Background

Drip irrigation under mulch film promotes a non-uniform salinity distribution in salt fields. To study the effect of different nitrogen (N) application methods on cotton plant growth, yield and fertilizer N recovery efficiency (FNRE) using drip irrigation under mulch film conditions, three N application methods were assigned: N applied under mulch film (low-salinity area; UM), N applied between mulch films (high-salinity area; BM), and half N applied under mulch film and half between mulch films (HUHB).

Results

Plant height, photosynthesis, Chl content, boll load, biomass, boll weight and boll density under UM were all significantly higher than those under the other two treatments. The N absorption of UM was higher than in the other two treatments, which might be attributed to the expression of *GHNRT1.5* and *GHNRT2.1*. The net NO$_3^-$ influx in the roots in UM increased significantly compared with that in BM. The yield and FNRE of UM were 3.9% and 9.1%, respectively, and were 26.52% and 90.36% higher than under HUHB and BM treatments.

Conclusions

UM not only improved cotton yield but also alleviated the pollution of N residue on drip irrigation under mulch film conditions in salt areas.

Background

Cotton (*Gossypium hirustum* L.), a major cash crop planted globally, is often used as a pioneer crop in saline lands because its salinity threshold is 7.7 dS/m (Maas and Hoffman, 1977; Rocha-Munive et al., 2018; Zhang et al., 2021). However, the negative impacts of salinity on cotton germination, nutrient absorption, photosynthesis and yield reduction have been reported in previous studies (Ashraf and Ahmad, 2000; Sikder et al., 2020). Soil salinity is a major abiotic stressor and a strict factor limiting plant productivity (Wang et al., 2003). Over half of all irrigated soils and about 20% of the world’s cultivated lands are affected by salinity (Arzani, 2008). Therefore, it is of great significance to increase the yield of cotton in saline-alkali soil for the sustainable development of the cotton industry.

The response of plants to salt stress varies in different soil environments. The salinity in salt fields is often heterogeneous, and a number of research studies have confirmed that non-uniform salinity in the root zone can alleviate salt injury and promote plant growth, which were mainly attributed to more water absorption by roots from the low salinity zone, increased Na$^+$ efflux of the low-salinity roots by the plasma membrane Na$^+$/H$^+$ antiporter, and decreased Na$^+$ concentration in leaves through transporting...
excessive $\text{Na}^+$ from leaves to the low-salinity roots (Bazihizina et al., 2009, 2012; Kong et al., 2012, 2016). Our previous studies have shown that nutrient uptake, such as nitrogen uptake, increases on the non-saline root side, and cotton growth, photosynthesis and transpiration are improved under non-uniform salinity compared with that under uniform salinity (Kong et al., 2016, 2017).

Drip irrigation technology under mulch film, which can save water and prevent salt accumulation, succeeded and was popularized extensively in Xinjiang in northwest inland China in 1996 (Zhang et al., 2020). A study on water and salt migration in cotton fields with drip irrigation under mulch film showed that salt migration is mainly divided into the leaching process of salt during irrigation and the redistribution process of salt with water after irrigation (Wang et al., 2000; Lu et al., 2001). Qi et al. (2007) reported that salinity migrated to the lower soil layer from the Earth's surface during drip irrigation; furthermore, soil water evaporation was restrained by plastic film, and salt accumulation on the surface was inhibited. Accordingly, a complete desalination region was formed in the root area, which was beneficial for cotton growth and development (Tan et al., 2008; Fu et al., 2013). In other words, the low salinity region was formed around the drip tube under the mulch film and other regions had high salinity (Supplemental Fig. 1). Therefore, drip irrigation under mulch film promoted the non-uniform distribution of salinity in saline cotton fields and then alleviated salt injury of the plants and promoted plant growth and cotton yield.

In conclusion, nutrient uptake and plant growth of cotton were inhibited under salinity stress; however, nutrient uptake increased on the non-saline root side under non-uniform salinity compared with that under uniform salinity (Kong et al., 2017). The non-uniform distribution of salinity in salt cotton fields was promoted by drip irrigation under mulch film. Accordingly, the uptake of $\text{N}$, a vital macronutrient, is increased in saline cotton fields by drip irrigation under mulch film conditions with proper $\text{N}$ application methods. The enhancement of $\text{N}$ supplementation can not only improve the water status of cotton plants but also alleviate salt damage, such as a decline in the generation of lethal oxidative stress biomarkers and an increase in the accumulation of osmolytes (Singh et al., 2014, 2019; Sikder et al., 2020). Both the northwest inland and eastern coastal saline-alkali land are currently the main cotton-producing regions in China. Due to the different climates, natural conditions and economic development, drip irrigation technology under mulch film has been extensively popularized in the northwest inland but not in the eastern coastal saline-alkali cotton land. Therefore, we hypothesized that the different $\text{N}$ application methods for drip irrigation under mulch film conditions would affect the growth and yield of cotton in eastern coastal saline-alkali land in China. The objectives of the present study were to determine: a) the effect of $\text{N}$ application methods on cotton growth and yield and b) the effect of $\text{N}$ application methods on $\text{N}$ uptake and FNRE using a $^{15}\text{N}$ tracing technique in saline fields on drip irrigation under mulch film conditions.

**Results**

**Changes in soil salinity during the growing season**
The soil salinity at 0, 30, 60, 90, 120 and 150 d after sowing are shown in Fig. 1. There were no differences in the soil salinity under mulch film or between mulch films among the different N application methods. Therefore, the data on the soil salinity under mulch film or between mulch films were an average of the three application methods. The soil salinity under mulch film first decreased and then increased and ranged from 1.5 to 2.0 g kg$^{-1}$ from 30 to 150 d after sowing. In contrast, the soil salinity between mulch films first increased and then decreased and ranged from 2.6 to 3.2 g kg$^{-1}$ from 30 to 150 d after sowing. The soil salinity between mulch films was 62.1%, 98.7%, 52.2%, 24.9% and 37.0% higher than that under mulch film on 30, 60, 90, 120 and 150 d after sowing, respectively.

**Effect of different N application methods on plant growth**

The effects of different N application methods on plant growth at the peak bolling stage are shown in Fig. 2. The highest plant height, net photosynthetic (Pn) rate, chlorophyll (Chl) content and boll load were found under N applied under the mulch film (UM) treatment. The lowest plant height, Pn rate, Chl content and boll load were found under N applied between the mulch films (BM) treatment. The plant height, Pn rate, Chl content and boll load under half N applied under the mulch film and half N applied between the mulch films (HUHB) were between that under UM and BM treatment. The plant height, Pn rate, Chl content and boll load under UM were 7.3%, 19.8%, 8.2% and 18.2% higher than that under BM treatment.

**Effect of different N application methods on dry matter and N accumulation**

Dry matter and N accumulation in vegetative organs and reproductive organs were measured on 0, 30, 60, 90, 120 and 150 d after sowing (Fig. 3). There was a rapid accumulation period of dry matter in the vegetative organs from 30 to 120 d after sowing (Fig. 3A). The dry matter accumulation in the vegetative organs of UM was significantly higher than that of HUHB and BM from 60 to 150 d after sowing. Similarly, dry matter accumulation in reproductive organs entered a rapid accumulation period 60 d after sowing (Fig. 3B). However, there were no significant differences among the three treatments until 120 d after sowing. The dry matter accumulation in the reproductive organs of UM increased 4.9% and 12.3% compared with that of HUHB and BM, respectively, 150 d after sowing. The dry matter ratio of reproductive to vegetative organs increased rapidly from 60 to 120 d after sowing (Fig. 3C). The dry matter ratio of the reproductive to vegetative organs of BM treatment increased 4.7% and 8.6%, compared with that of HUHB and UM treatments, respectively, 150 d after sowing.

The curve of N accumulation was similar to that of dry matter accumulation in cotton plants (Fig. 3A, B, D and E). N accumulation in the vegetative organs of UM and HUHB was significantly higher than that of BM from 30 to 150 d after sowing (Fig. 3D). N accumulation in vegetative organs of UM was 12.1%, 10.7% and 11.8% higher than that of HUHB 90, 120 and 150 d after sowing, respectively. N accumulation in reproductive organs maintained a higher accumulation speed from 60 to 150 d after sowing (Fig. 3E). N accumulation in the reproductive organs of UM increased 7.4% and 11.0% more than that of HUHB and BM 150 d after sowing. The N accumulation ratio of reproductive to vegetative organs maintained a high increase speed from 30 to 150 d after sowing (Fig. 3F). The N accumulation ratio of reproductive to
vegetative organs of BM treatment increased 6.5% and 10.9% compared with that of HUHB and UM treatment, respectively, 150 d after sowing.

**Effect of different N application methods on Ndff and $^{15}$N accumulation**

The Ndff and $^{15}$N accumulation effect mediated by different N application methods are shown in Table 1. The highest Ndff in roots, stems, leaves, boll shells and seedcotton was found under UM treatment, and the lowest Ndff in roots, stems, leaves, boll shells and seedcotton was found under BM treatment. Ndff in roots, stems, leaves, boll shells and seedcotton increased 53.85%, 50.00%, 57.69%, 54.17% and 50.00% under UM treatment compared with BM treatment. Similarly, the highest $^{15}$N accumulation in roots, stems, leaves, boll shells and seedcotton was found under UM treatment, followed by HUHB and BM. $^{15}$N accumulation in roots, stems, leaves, boll shells and seedcotton were 95.76%, 65.09%, 80.70%, 92.49% and 105.78% higher, respectively, under UM treatment than under BM treatment.

**Effect of different N application methods on yield, harvest index and FNRE**

The yield, yield components, harvest index and FNRE under different N application methods are shown in Table 2. The highest yield and biological yield were found under UM treatment, followed by HUHB and BM treatments. The yield of the UM treatment was 3.9% and 9.1% higher than that of the HUHB and BM treatments, respectively. The biomass of the UM treatment was 6.4% and 16.3% higher than that of the HUHB and BM treatments, respectively. Similarly, the highest boll density and boll weight were found under UM treatment. Boll density and boll weight increased by 2.3% and 6.9% under UM treatment compared with that under BM treatment. However, the highest harvest index was found under BM treatment, and there was no difference between HUHB and UM treatments. The highest FNRE was found under UM treatment. FNRE under UM treatment was 26.52% and 90.32% higher than that under HUHB and BM treatments, respectively.

**Effect of non-uniform root zone salinity on the expression level of the GHNRT gene and NO$_3^-$ flux**

The expression levels of *GHNRT1.1, GHNRT1.5* and *GHNRT2.1* in cotton roots of the laboratory experiment were measured 7 days after treatment (Fig. 4A–C). The expression level of the three *GHNRT*s decreased under uniform salt stress. The expression of *GHNRT1.1, GHNRT1.5* and *GHNRT2.1* under 1.5/1.5 treatment decreased 41.6%, 28.9% and 65.0%, respectively, compared with that under the control (0/0). The expression levels of *GHNRT1.5* and *GHNRT2.1* in the roots from the low salinity area of the non-uniform salt treatment (1.5/3.0–1.5) were comparable with those of the control (0/0). The expression levels of *GHNRT1.5* and *GHNRT2.1* of the roots in the low salinity area of the non-uniform salt treatment (1.5/3.0–1.5) were 102% and 467% higher than that of the uniform salt treatment (1.5/1.5).

NO$_3^-$ flux was also inhibited by salt stress (Fig. 4D). The NO$_3^-$ flux under 1.5/1.5 treatment decreased 28.7% compared with that under the control (0/0). However, the NO$_3^-$ flux of the roots in the low salinity
area of the non-uniform salt treatment (1.5/3.0–1.5) was comparable with that of the control (0/0) and was 34.0% higher than that of the uniform salt treatment (1.5/1.5).

**Discussion**

Nutrient uptake decreases drastically under salinity stress, which may inhibit nutrient migration and ultimately lead to yield reduction (Pessarakli, 2001). Various technical means have been used to alleviate salt damage to crop growth (Guo, 2020). The results of our previous studies indicated that nutrient uptake increases in the low-saline root side under a non-uniform salinity distribution (Kong et al., 2017). Moreover, a non-uniform salinity distribution is formed when using drip irrigation under mulch film (Qi et al., 2007; Fu et al., 2013). To determine the effects of different N application methods on cotton, plant growth, cotton yield, nitrogen uptake and FNRE were measured using drip irrigation under mulch film conditions in salinity cotton fields with the $^{15}$N tracing technique.

**Effect of drip irrigation under mulch film on the distribution of soil salinity**

Water is the solvent and carrier of fertilizer, salt and other particles; for this reason, salt follows the water in the soil. Previous research has shown that salt accumulated in the regions between the mulch films and to the lower soil layer (Tan et al., 2008; Fu et al., 2013) on drip irrigation under mulch film conditions. The soil salinity under the mulch films was significantly lower than that between the mulch films, especially in the 60 days after sowing (seedling stage) in the present study (Fig. 1). Although the rainy season arrived at 80 days after sowing and the distribution of salt in the soil was affected by rainfall, the soil salinity under the mulch films was significantly lower than that between the mulch films until 150 days after sowing. The soil salinity was redistributed and the root regions under the mulch films were desalinated to low salinity areas, while the regions between the films changed to high salinity areas. Therefore, the non-uniform salinity distribution in the cotton root area was maintained throughout the whole growing season in the present study.

**Effects of different application methods on the growth of cotton plants**

Salt stress can cause many adverse physiological and biochemical effects in cotton plants (Munawar et al., 2021), such as plant height, fresh and dry weights, leaf area, photosynthesis (Pn) and yield were decreased significantly by salinity stress (Loka et al., 2011). The decrease in photosynthesis was attributed to the reduction of total chlorophyll content and the distortion of chlorophyll ultrastructure under salinity stress (Meng et al., 2011). In the present study, the plant height, photosynthesis, Chl content and boll load under UM treatment were significantly higher than that under BM and HUHB treatments (Fig. 2). The UM treatment alleviated the salt damage to the cotton plant compared with the BM and HUHB treatments. In fact, recent studies have shown that increasing N supply could lessen the
salinity stress of *Brassica* genotypes (Siddiqui et al., 2010), tomato plants (Singh et al., 2019), wheat seedlings (Ahanger et al., 2019) and cotton plants (Sikder et al., 2020). Accordingly, the alleviated salinity stress under the UM treatment might be attributed to the increased N uptake compared with the other two treatments.

**Effect of different N application methods on dry matter and N accumulation**

Higher dry matter accumulation guarantees a high cotton yield. It was conducive to maintaining a balance between vegetative and reproductive growth and establishing a reasonable population basis for a high cotton yield so that the dry matter of the population was maintained in an appropriate range (Ye et al., 2004; Zhu et al., 2011). However, the dry matter of cotton decreased significantly under salt stress (Luo et al., 2015; Guo et al., 2022). The leaf and root dry weights decreased gradually as the NaCl concentration increased (Zhang et al., 2014). Although salt stress reduces the accumulation of dry matter in cotton, it might be more conducive to the transport of nutrients to the reproductive organs and increase the dry matter ratio of reproductive to vegetative organs (Feng et al., 2022). In the present study, dry matter accumulation in the reproductive organs of UM was significantly higher than that of BM and HUHB (Fig. 3A and B). The dry matter ratio of the reproductive to vegetative organs of UM obviously decreased compared with that of BM and HUHB (Fig. 3C). The results indicated that UM treatment could alleviate salinity stress in cotton.

N uptake is the basis for dry matter accumulation in cotton (Yin et al., 2016). Many studies have demonstrated a linear positive correlation between dry matter and N accumulation in cotton (Xue et al., 2006; Zheng et al., 2016). The present study indicated that the trend of dry matter accumulation was consistent with N accumulation in cotton across different N application methods using drip irrigation under mulch film in a saline field (Fig. 3A–D). Although N accumulation in cotton was inhibited by salinity stress (Ma et al., 2013; Luo et al., 2015), the N uptake in the non-saline root side increased significantly under non-uniform salinity conditions in our previous study, which was simulated using a split-root system (Kong et al., 2017). In the present study, non-uniform salinity conditions were formed in the cotton field using drip irrigation under film conditions. The N accumulation of the treatment that N applied in the low-salinity regions (UM) was significantly higher than that of the other two treatments (Fig. 3D and E). The results indicate that the increased N accumulation of UM treatment might be attributed to the increased N uptake from the low-salinity regions. N accumulation in different cotton organs has been affected by many management practices, such as N application rates and soil and environmental conditions (Shah et al., 2022). The transport of N to reproductive organs was inhibited, and the N accumulation ratio of reproductive to vegetative organs decreased with excessive N application rates (Ma et al., 2013; Luo et al., 2022). Similar results were found in the present study (Fig. 3F). This further indicates that the effect of increasing the N fertilizer rate can be achieved by changing N application methods using drip irrigation under mulch film in the saline field.
**Effect of different N application methods on Ndff, $^{15}$N accumulation, FNRE and yield**

The N uptake from different sources and the distribution of fertilizer N in different parts of crops can be directly detected by $^{15}$N tracer technology. Our previous study showed that the N absorbed from $^{15}$N-labeled urea (Ndff) increased with an increase in the N application rate (Luo et al., 2022). In the present study, Ndff and $^{15}$N accumulation in the cotton plants of the UM treatment were significantly higher than that of the other two treatments (Table 1). This indicates that N applied under mulch film (low-salinity area) could increase fertilizer N uptake of the cotton plant in the saline field. The fate of fertilizer N applied to the field includes uptake by crops (FNRE), left in the soil, or lost in various methods, such as denitrification, runoff, volatilization and leaching. FNRE is affected by the N application method, soil fertility and climate conditions (Fritschi et al., 2004, Roberts et al., 2016). The FNRE of UM treatment was 26.52% and 90.36% higher than that of HUHB and BM treatment, respectively, in the present study (Table 2). This might be the result of increased Ndff and $^{15}$N accumulation in cotton plants from the UM treatment. A higher FNRE means a lower N loss and N left in the soil. Consequently, N applied under mulch film in the saline field can reduce the environmental pollution caused by nitrogen residue and loss.

Boll weight and boll density are two major parameters that contribute to yield. Cotton yield decreased under salinity stress should be attributed to the reduction of boll weight and density with an increase in salinity (Sharif et al., 2019). In the present study, both the highest boll weight and density were found under UM treatment. This further confirmed that UM treatment could alleviate salinity stress using drip irrigation under mulch film in saline fields. However, cotton yield was determined by the total dry matter production and harvest index, which is the ratio of reproductive tissues to the total biomass (Bange and Milroy, 2004; Luo et al., 2018). High cotton yield depends on adequate total biomass and appropriate harvest index (Ma et al., 2013). In the present study, the lowest harvest index was found under the UM treatment; however, due to the highest total biomass, the highest yield was found under the UM treatment. This showed that total matter production and the balance between vegetative and reproductive growth synergistically determine cotton yield under salinity stress.

**Expression level of the GHNRT gene and NO$_3^-$ flux**

Our previous study indicated that the expression of nitrate uptake-related genes ($NRT$) and the net NO$_3^-$ influx on the non-saline root side increased significantly compared with those on the high-saline root side under non-uniform salinity and either root side under uniform salinity (Kong et al., 2017). To more accurately simulate the non-uniform salinity distribution using drip irrigation under mulch film in the saline field, the low-salinity area and the high-salinity area were set at 0.15% and 0.3% with a longitudinal salinity difference distribution device in the lab in the present study. The expression of $GHNRT1.5$ and $GHNRT2.1$, as well as the NO$_3^-$ influx in the roots of the low-salinity area, were significantly higher than those of the high-salinity area. This indicates that the increased N accumulation in the cotton plants of the UM treatment might be attributed to the increase in the N absorption from the low-salinity area.
Conclusion

The non-uniform salinity distribution was formed by the technology of drip irrigation under a mulch film in the eastern coastal saline-alkali cotton field in China. The soil salinity under the mulch films was significantly lower than that between the mulch films throughout the growing season. In the laboratory experiment, the expression of GHNRT1.5 and GHNRT2.1 and the net NO$_3^-$ influx in the roots of the low-saline area were significantly higher than those in the high-saline area under non-uniform salinity and roots in either area under uniform salinity. More N was absorbed under the UM treatment than under the other two treatments. Unsurprisingly, the increased N accumulation under the UM treatment alleviates the salt damage of the cotton plant. The plant height, photosynthesis, Chl content, boll load, dry matter accumulation, boll weight and density under UM treatment were significantly higher than those under BM and HUHB treatments. The yield and FNRE of the UM treatment were 3.9% and 9.1%, respectively, which were 26.52% and 90.36% higher than those under the HUHB and BM treatments. Accordingly, N applied under mulch film (low-salinity area) not only increased yield but also reduced the environmental pollution caused by nitrogen residue and loss in saline areas.

Materials and methods

Field experiment

Experimental sites

The field experiments were conducted in Wudi county (117°61′ E, 37°74′ N), Shandong, China. The climate in this region is warm, temperate and semi-humid monsoon. The average annual temperature is 12.7°C, and the annual effective accumulated temperature is 4300–4400°C. The annual average precipitation is 564.8 mm, and the annual average humidity is 66%. The annual average evaporation is 1806 mm, and the average annual duration of sunshine is 2632 h, with a 205-day frost-free season for crop growth. Daily weather variables of the experimental site of the growth season in 2021 and 2022 are shown in Fig. 5. Wudi County is a part of the Yellow River Delta. The soil in this area is coastal saline soil, and the top 20 cm of the soil contains organic matter (9.25 g kg$^{-1}$), alkali hydrolysable N (39.58 mg kg$^{-1}$), available P (12.53 mg kg$^{-1}$), available K (491.31 mg kg$^{-1}$), soil salinity (0.28%) and pH of 8.10.

Experimental design and field management

A randomized complete block design was adopted in the experiment, and each treatment was repeated three times. Three N application methods were assigned as follows: all N applied under the mulch film (UM), all N applied between the mulch films (BM), half N applied under the mulch film and half N applied between the mulch films (HUHB) (Supplemental Fig. 2). Each plot was 66 m$^2$ in area (14.5 m × 4.56 m), consisting of 6 rows with 2 rows under each mulch film, which were equally separated with 76 cm spacing. The drip irrigation tube was placed in the middle of the two rows under the mulch film. Plots with good drainage and irrigation conditions were selected as the experiment sites. The experiment was
performed in 2021 and repeated in 2022. The main local cotton cultivar ‘SCRC37’, whose male and female parent both are Asian cotton lines, was cultivated by the cotton breeding team of institute of industrial crops, shandong academy of agricultural sciences and used in this experiment. The cotton was sown on 17 April 2021 and 20 April 2022 and harvested on 8 October 2021 and 10 October 2022. To promote the non-uniform distribution of salt, drip irrigation was conducted three or four times in 2021 and 2022 according to the rainfall in that year. The volume of drip irrigation was 450 m$^3$ hm$^{-1}$. Irrigation occurred on 18 April, 3 June, 2 July and 21 August in 2021 and 21 April, 5 June and 25 August in 2022. Throughout the growing season, about 80% of the soil surface was covered with mulch film. Each plot was fertilized with 150 kg hm$^{-1}$ of N (in the form of urea), 75 kg hm$^{-1}$ of P$_2$O$_5$ and 60 kg/hm$^{-1}$ of K$_2$O. All fertilizers were applied as basal fertilizers when sowing.

To prevent surface runoff pollution and lateral infiltration, $^{15}$N-labeled urea was applied in the microplots (1 × 1 m), which were enclosed with hard plastic squares. The height of the hard plastic square was 0.45 m, of which 0.40 cm was buried in the soil and 0.05 m of the square remained above the soil surface. Three microplots were set aside in each plot. Urea, used in the experiment, was enriched with 10.2% atom $^{15}$N (provided by the Institute of Chemical Industry, Shanghai, China). To ensure normal growth of the cotton, all experimental plots were managed uniformly according to local agronomic practices.

**Data collection**

Data were collected for net photosynthetic (Pn) rate, chlorophyll (Chl) content, boll load, biological yield, seedcotton yield, harvest index, N uptake in vegetative organs and reproductive organs, $^{15}$N uptake in vegetative organs, and FNRE.

**Net photosynthetic (Pn) rate, chlorophyll (Chl) content and boll load**

The net Pn rate of the 3rd youngest fully expanded leaf from the main stem terminal was determined from 09:00 to 11:00 h on cloudless days when ambient photosynthetic photon flux density exceeded 1500 µM·m$^{-2}$·s$^{-1}$, using an LI-6800 portable photosynthesis system (Li-Cor, USA). In the meantime, the Chl content of the 3rd leaf was measured by SPAD 502DL plus (Spectrum, USA). The mean value was calculated from three plants per replicate. The leaf area was determined using an LI-3100 leaf area meter (Li-Cor, USA), and the leaf area index (LAI) was determined according to the ground area. All reproductive organs (squares, flowers, green and mature bolls) were dried at 105°C for 30 min, dried at 80°C for 48 h and weighed. The boll load (dry weight of reproductive organs per unit leaf area) was then determined.

**Biological yield, seedcotton yield, yield components and harvest index**

To determine boll weight, 50 bolls were randomly selected and harvested in the four central rows from each plot. After 10 days of sun drying, the seedcotton was weighed. Seedcotton yield (kg·ha$^{-1}$), the
average boll weight and boll density were determined for each plot. Seedcotton in the microplots was harvested and stored separately. Ten plants were sampled randomly from each microplot, pulled out naturally, and divided into leaves (previous fallen leaves included), stems and roots after harvest. All plant samples were dried at 105°C for 30 min, dried at 80°C for 48 h and weighed. Plant biomass, dry matter distribution and harvest index (seedcotton yield/ biological yield) were then determined.

**Soil salinity, N uptake, $^{15}$N accumulation and FNRE**

Soil samples in each plot under mulch film or between mulch films were collected from 0 to 20 cm depth using a 5 cm i.d. auger at 0, 30, 60, 90, 120 and 150 d after sowing. The soil salinity of the water-saturated soil paste extract was measured using an electrical conductivity meter, S230 (Mettler Toledo, Switzerland).

Two plants from each microplot were selected randomly, pulled out naturally, and divided into leaves (including previously fallen leaves), stems, roots, boll shells and seedcotton. Each part of the plant from the microplots was dried at 105°C for 30 min and then dried at 80°C to a stable weight before measuring the dry weight. All materials were ground and then sieved through a 0.25-mm sieve, and 5 g powder was collected randomly for total N and $^{15}$N isotope analysis. The total N concentration and $^{15}$N abundance were analyzed using an isotope mass spectrometer (Delta V Advantage, Thermo Fisher, Waltham, MA).

The amount of plant N derived from fertilizer (Ndff) was calculated following Eq. (1) (atom% $^{15}$N of naturally occurring N is 0.3663%):

$$Ndff = (\text{the atom}^\% \text{ $^{15}$N of crops ample} - 0.3663) / (\text{the atom}^\% \text{ $^{15}$N of $^{15}$N-labeled urea} - 0.3663) \times 100\%$$

(1)

$^{15}$N accumulation in different organs of cotton plants and FNRE was calculated following Eqs. (2 and 3):

$$^{15}\text{N accumulation} = \text{total N accumulation in cotton plant} \times \text{Ndff}$$

(2)

$$\text{FNRE} = \frac{^{15}\text{N accumulation in cotton plant}}{N_{appl}} \times 100\%$$

(3)

**Laboratory experiment**

**Experimental treatments**

To determine the expression of $GHNRT$ genes and the flow rate of $\text{NO}_3^-$ in cotton roots, the non-uniform distribution of salinity was simulated using a longitudinal salt difference distribution device (Supplemental Fig. 3 and Fig. 4). The device consisted of the upper and lower parts. To more accurately simulate the non-uniform salinity distribution of drip irrigation under mulch film in the saline field, treatments with the same NaCl content (1.5 g·kg$^{-1}$) in the soil of the two parts represented the uniform distribution salinity stress treatments, denoted as 1.5/1.5. The treatment with the different NaCl content
(1.5 or 3.0 g·kg$^{-1}$) in the two parts represented the non-uniform distribution salinity stress, denoted as 1.5/3.0. As the control, the two parts of the treatment were free of NaCl.

**Real-time PCR (RT-PCR) analysis**

The expression of $GHNRT$ genes was determined using quantitative real-time PCR (RT-qPCR). After 7 days of treatment, root tissues from both sides of all four treatments were harvested and extracted for total RNA using TRizol reagent (Invitrogen). Primer Premier 5.0 (Premier Biosoft International) was used to design the gene-specific primers according to the gene sequences and was then synthesized commercially (Shanghai Sangon Biological Engineering Technology & Services). The primers and gene identifiers are listed in Supplemental Table 1.

The PCR program was performed according to Kong et al. (2016). Each treatment was analyzed with three biological replicates. The expression level of $actin$ was used to normalize the RT-qPCR results relative to the NaCl-free control. SAS software was used to calculate the Pearson correlation coefficients of the expression patterns of the selected genes.

**Measurements of net NO3- flux with NMT**

Non-Invasive Micro-Test Technology (NMT) [NMT System BIOIM; Younger USA, LLC.] was used to measure the net flux of NO$_3^-$ according to Kong et al. (2012, 2017). After 7 days of treatment, 2–3 cm root segments from the apices were cut down and washed with redistilled water and then incubated in the measuring solution immediately to equilibrate for 30 min, and then put into the measuring chamber containing 10–15 ml fresh measuring solution. The measuring solution consisted of 0.1 mM NH$_4$NO$_3$, 0.1 mM CaCl$_2$ and 0.3 mM MES, and the pH was adjusted to 6.5 with choline and HCl. A site 5 mm from the root apex was used to measure the net flux of NO$_3^-$. Two-dimensional ionic fluxes were calculated using MageFlux developed by Yue Xu (http://xuyue.net/mageflux).

**Statistical analysis**

Analysis of variance (ANOVA) was performed using the randomized complete block design function of the DPS Data Processing System (Version 11.50; Tang and Feng, 2002). The combined data showed that there were no interactions with the years. Therefore, the data were pooled across the two years. Means were separated using Duncan’s multiple range tests at P < 0.05 for significant differences.

**Declarations**

**Ethics approval and consent to participate**

There was no requirement to seek ethical approval to carry out the work described above. The authors declare that the collection of plant material complies with Chinese and international guidelines and legislation.

**Consent for publication**
Not applicable.

**Availability of data and materials**

The datasets in this study are available from the corresponding author on reasonable request.

**Competing interest**

The authors declare that they have no conflict of interest.

**Author contributions**

X.K. and Z.L. conceived the research plans and designed the experiments; W.T., Z.L., X.W., and L.C. performed most of the experiments and analyzed the data; Q. H. and J.L. performed partial experiments; Z. L. and X.K. conceived the project and wrote the article with contributions from all the authors; X.K. supervised and complemented the writing.

**Acknowledgement**

Not applicable.

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13. Kong X, Luo Z, Dong H, Li W, Chen Y. Non-uniform salinity in the root zone alleviates salt damage by increasing sodium, water and nutrient transport genes expression in cotton. Scientific Reports. 2017; 1, 2879; doi: 10.1038/s41598-017-03302.


Tables

Table. 1 Effect of different N application methods on Ndff and $^{15}$N accumulation of cotton on drip irrigation under mulch film condition in salt field.
<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root</th>
<th>Stem</th>
<th>Leaf</th>
<th>Boll shell</th>
<th>Seedcotton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ndff</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUHB</td>
<td>0.35b*</td>
<td>0.34b</td>
<td>0.34b</td>
<td>0.33b</td>
<td>0.34b</td>
</tr>
<tr>
<td>UM</td>
<td>0.40a</td>
<td>0.42a</td>
<td>0.41a</td>
<td>0.37a</td>
<td>0.39a</td>
</tr>
<tr>
<td>BM</td>
<td>0.26c</td>
<td>0.28c</td>
<td>0.26c</td>
<td>0.24c</td>
<td>0.26c</td>
</tr>
<tr>
<td>$^{15}$N accumulation (kg ha$^{-2}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HUHB</td>
<td>1.33b</td>
<td>6.85b</td>
<td>17.44b</td>
<td>9.13b</td>
<td>24.60b</td>
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<tr>
<td>UM</td>
<td>1.70a</td>
<td>8.55a</td>
<td>22.38a</td>
<td>11.34a</td>
<td>31.13a</td>
</tr>
<tr>
<td>BM</td>
<td>0.87c</td>
<td>5.18c</td>
<td>12.39c</td>
<td>5.89c</td>
<td>15.13c</td>
</tr>
</tbody>
</table>

*Means within a column followed by same letters are not significantly different at p < 0.05 after ANOVA and the LSD tests.

Table. 2 Effect of different N application methods on yield, yield components, harvest index and FNRE of cotton on drip irrigation under mulch film condition in salt field.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Boll density</th>
<th>Boll weight</th>
<th>Yield (Kg ha$^{-2}$)</th>
<th>Biomass (Kg ha$^{-2}$)</th>
<th>Harvest index</th>
<th>FNRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUHB</td>
<td>602ab</td>
<td>4.87b</td>
<td>2932.81a</td>
<td>8668.98b</td>
<td>0.335b</td>
<td>39.57b</td>
</tr>
<tr>
<td>UM</td>
<td>612a</td>
<td>4.99a</td>
<td>3047.15a</td>
<td>9227.10a</td>
<td>0.330b</td>
<td>50.67a</td>
</tr>
<tr>
<td>BM</td>
<td>598b</td>
<td>4.67c</td>
<td>2792.38b</td>
<td>7932.14c</td>
<td>0.344a</td>
<td>26.31c</td>
</tr>
</tbody>
</table>

*Means within a column followed by same letters are not significantly different at p < 0.05 after ANOVA and the LSD tests.

**Figures**
The changes of the soil salinity at 0, 30, 60, 90, 120 and 150 days after sowing in the salt field. Different letters in the figure indicate statistically significant differences (P < 0.05) after ANOVA and the LSD tests.
Figure 2

Effect of different N application methods on plant height (A), Pn (B), Chl content (C) and Boll load (D) of cotton on drip irrigation under mulch film condition in salt field. Different letters in A, B, C and D indicate statistically significant differences (P < 0.05) after ANOVA and the LSD tests.
Figure 3

Effect of different N application methods on dry matter and N accumulation of cotton on drip irrigation under mulch film condition in salt field. Dry matter of vegetative organs (A) and reproductive organs (B), the dry matter ratio of reproductive to vegetative organs (C), N accumulation of vegetative organs (D) and reproductive organs (E), the N accumulation ratio of reproductive to vegetative organs (F).
**Figure 4**

Effect of non-uniform root zone salinity on the expression level of *GHNRT1.1* (A), *GHNRT1.5* (B) and *GHNRT2.1* (C) and NO$_3$- flux (D) in cotton root. Different letters in A, B, C and D indicate statistically significant differences (P < 0.05) after ANOVA and the LSD tests.

**Figure 5**

Daily weather variables of experimental site in 2021 (A) and 2022 (B).
Supplementary Files

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- SupplementalFiguresS1S4TableS1.pdf