**Localized application of NH_4^+ -N plus P enhances zinc and iron
accumulation in maize via modifying root traits and rhizosphere
processes**

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Abstract

32 Micronutrient deficiency commonly occurs in calcareous soils. We hypothesized that
localized application of ammonium in the P-banding zone could improve
34 micronutrient uptake and thus grain yield through inducing rhizosphere acidification
and stimulating root proliferation. A 2-year field experimentation with maize growing
36 on a calcareous soil was conducted with localized application of P (superphosphate)
only (P), P plus ammonium as ammonium sulfate ($\text{NH}_4\text{-N+P}$) or urea plus P (Urea+P)
38 at sowing and jointing. Compared with localized Urea+P, localized $\text{NH}_4\text{-N+P}$
significantly improved root dry weight, whole root length and first-order lateral root
40 density/length at the seedling stage, as well as chlorophyll content and net
photosynthetic rate at flowering. Shoot Zn content was 30-50% greater at the seedling
42 stage and 22-36% higher at grain maturity in localized $\text{NH}_4\text{-N+P}$ compared with
localized P and Urea+P in 2012 and 2013. The Fe content at the seedling, flowering
44 and harvest stages in localized $\text{NH}_4\text{-N+P}$ was higher than that in localized P, and also
exhibited an increasing trend relative to localized Urea+P. Compared with localized
46 Urea+P, grain Zn concentration in the $\text{NH}_4\text{-N+P}$ treatment increased by 17% in 2012,
and exhibited an increasing trend in 2013. Grain Fe concentration in the $\text{NH}_4\text{-N+P}$
48 treatment increased in 2013 in comparison with the P and Urea+P treatments. Maize
grain yield in localized $\text{NH}_4\text{-N+P}$ increased by 10-14% in 2012 and 13-25% in 2013
50 compared with localized P and Urea+P. The results indicate that localized application
of $\text{NH}_4\text{-N+P}$ may be an efficient approach to improving maize growth, grain Zn and
52 Fe accumulation and yield via modifying root traits (such as root length, lateral root

proliferation and root length density), and intensifying rhizosphere acidification by

54 ammonium uptake.

56 *Keywords:* Ammonium; Phosphate; Zinc; Iron; Root growth; Micronutrient
biofortification

1. Introduction

Availability of micronutrients in soil is largely dependent on soil type and environmental factors, such as pH, redox potential and applied N sources (Rengel et al., 1999; Marschner, 2012). Zinc (Zn) and iron (Fe) deficiencies generally occur in alkaline and calcareous soils because of their high pH and high bicarbonate concentration that depress solubility of Zn and Fe in soil and decrease their uptake by roots (Zuo and Zhang, 2011). However, using ammonium-containing fertilizers may result in acidification of the rhizosphere soil; for every pH unit decrease there is an increase in solubility of Zn and ferric Fe by a factor of 10-1000 (Chen and Barak, 1982). Furthermore, the root traits such as root length, root production and root spatial distribution have a significant influence on nutrient uptake efficiency, especially for sparingly soluble nutrients i.e. Fe, Zn and P (Lynch, 1995). Therefore, modifying root growth traits and rhizosphere processes can be an effective strategy to improving micronutrient nutrition, particularly Fe and Zn for biofortification of grain crops in the field conditions.

Large quantities of N fertilizer are consumed to obtain high yield in many crops, with the application of N fertilizer in China accounting for 30% of the world total (Zhu and Chen, 2002). Urea is the preferred fertilizer throughout China due to high N content, low price and easy application. On the North China Plain, the proportional use of urea in Jilin, Heilongjiang and Hebei provinces exceeded 80% of all N fertilizers (Zhang et al., 2011). Most soils in North China are calcareous and alkaline (high pH and high carbonate content), which limits the availability of Zn and Fe and decreases nutritional quality of grain (Cakmak et al., 1999).

Rhizosphere processes such as root-induced changes in the rhizosphere pH and exudate release are of particular importance to mobilization and acquisition of sparingly soluble soil nutrients (Shen et al., 2011, 2013). Rhizosphere chemistry can be significantly changed depending on the forms of N taken up. Application of ammonium-N fertilizer, such as ammonium sulfate, decreases pH in the maize rhizosphere (Jing et al., 2012), leading to enhanced availability of Zn on calcareous soil (Rehman et al., 2012). By contrast, soil pH around a urea granule can be as high as 8-9 (Chien et al., 2011), which may have an inhibiting effect on Zn uptake due to precipitation of Zn as ZnCO_3 (Rengel et al., 1999).

Banding phosphorus and ammonium significantly improved shoot dry biomass and N and P accumulation by maize seedlings (Kaiser et al., 2005; Roth et al., 2006), accompanied by increased root proliferation and modified root spatial distribution

(Ma et al., 2013a). Lateral root proliferation was one of the most important root traits in response to N- and P-rich patches (Robinson, 2001). Because of greater total length of lateral roots (as much as 90% of the total root) (Pierret et al., 2006), the spatial distribution of lateral roots and their density in soil have a predominant role in nutrient uptake (Li et al., 2006). Due to a large root length and surface area, the fine roots are efficient in exploring a large volume of soil in order to absorb nutrients and water. Therefore, the magnitude of root proliferation in localized nutrient-rich patches was important not only for uptake of macronutrients, such as P and N (Ketterings et al., 2005; Jing et al., 2012; Ma et al., 2013b), but also may directly contribute to mobilization and capture of micronutrients (such as Zn and Fe).

The effect of localized supply of different N forms on micronutrient concentration and uptake in maize has seldom been studied, especially under field conditions. Recent studies found that N management could affect grain micronutrient status and production (Manzeke et al., 2012; Wang et al., 2012). Rehman et al. (2012) indicated that using ammonium-containing N source may be effective in improving soil Zn availability and Zn uptake by affecting the cation/anion uptake ratio and lowering the rhizosphere pH. Root proliferation can be stimulated under localized supply of P plus ammonium; in addition, ammonium application in the local zone can cause rhizosphere acidification by inducing proton release from roots (Jing et al., 2010, 2012; Ma et al. 2013a). These processes resulted in increased uptake of N and P, and enhanced grain yield (Jing et al., 2012; Ma et al., 2013b). Given that micronutrient deficiency generally occurs in calcareous soils because of high pH, acidification of the rhizosphere can increase solubility of micronutrients (Marschner, 2012). Therefore, we hypothesized that localized application of P plus ammonium could improve micronutrient uptake and thus grain yield via modifying root traits and intensifying rhizosphere acidification by ammonium uptake under field conditions.

Our previous study showed that localized application of P plus ammonium significantly enhanced N and P uptake in maize (Ma et al., 2013b), which was associated with ammonium-induced rhizosphere acidification and root proliferation (Jing et al., 2010 and 2012), as well as increased N and P uptake efficiency per unit of root in comparison to the treatment with P plus urea (Ma et al., 2012a). In the present study, the 2-year field experimentation on a calcareous soil was conducted to examine the effect of localized banding of P with different N forms on micronutrient (Zn and Fe) concentration and uptake by maize in different growth stages. The paper also discussed the possible mechanisms and agronomic implications of P banding with

different N forms on micronutrient status in maize.

2. Materials and methods

2.1. Site information and experimental design

The field experiments were conducted at the research station of China Agricultural University in Shangzhuang, Beijing, China (40°N, 116°E) in 2012 and 2013. Maize is a typical crop in the region. The weather data during maize growing season were listed in Table 1. The soil is calcareous and typical of the North Plain China, with bulk density 1.44 g cm⁻³. Initial soil test results were pH 8.3 (1:5 soil:water suspension), organic carbon 8.5 g kg⁻¹, total N 0.79 g kg⁻¹, Olsen-P 8.2 mg kg⁻¹ and available (NH₄OAc-extractable) K 84 mg kg⁻¹ in the topsoil layer (0-30 cm).

Maize (*Zea mays* L. cv. DH661) was planted on May 1, 2012 and May 15, 2013 at density of 75,000 plant ha⁻¹. Individual plot size was 5.0 m × 8.0 m. Plants were sown in alternating 30- and 50-cm-wide rows referred to as narrow and wide rows, respectively. There were six twin rows in each plot. The experiment was carried out in a completely randomized block design with four replicates.

The experiment consisted of three treatments in each of the two years: (1) localized supply of superphosphate as starter fertilizer at sowing and as side-dressing at jointing (P), (2) localized supply of urea plus superphosphate as starter fertilizer at sowing and as side-dressing at the jointing stage (Urea+P) and (3) localized supply of ammonium nitrogen (as ammonium sulfate) plus superphosphate as starter fertilizer at sowing and as side-dressing at jointing (NH₄-N+P). The application rates of nutrients were as follows: 165 kg N ha⁻¹ and 50 kg P ha⁻¹, of which 28% N and 65% P was used at sowing and then the rest was applied at the jointing stage 54 days after sowing (DAS). Superphosphate plus urea or superphosphate plus ammonium sulfate was locally banded in the narrow row 5 cm to the side and 5 cm below the seeds at sowing. To ensure the accuracy of fertilization site, two trenches were opened in the narrow row using a sharp hand-held furrow opener. The fertilizers were put into the trench, covered with the soil just removed. Then maize seeds were sown by a hand-held seeder. Side-dressing application of superphosphate (35% of 50 kg P ha⁻¹), Urea+P or NH₄-N+P (72% of 165 kg N ha⁻¹ and 35% of 50 kg P ha⁻¹) was banded in the wide row 10 cm to the side of plant and 15 cm below the soil surface at 54 days (jointing stage) using a hand-held furrow opener.

The basal dressing including 80 kg K₂O ha⁻¹ (as potassium chloride) and 15 kg Zn ha⁻¹ (as ZnSO₄·7H₂O) was broadcast before sowing. There was no irrigation in 2012

due to adequate rainfall during the growing season. In 2013, the top soil layer at 0-20 cm in all treatments was irrigated once (on May 17) to full field capacity by watering 460 m³ ha⁻¹ based on measurement and calculation of the content of water remaining in a soil after having been well supplied with water and free drainage having stopped (Jiang et al., 2006).

2.2. Plant sampling and measurements of leaf area, chlorophyll content, photosynthetic rate and nutrient uptake

The leaf area was measured at the following growth stages in 2012 (or 2013): seedling 30 (22), early jointing 41 (45), late jointing 51(53) and flowering 73 (75) DAS. The total green leaf area was calculated according to Sanderson et al. (1981): Leaf area = leaf length × maximum width × *k*, where *k* is a shape factor with the value of 0.5 for partially unfolded leaves and 0.75 for completely unfolded leaves. Each time five plants were selected for measurement. Leaf area index (LAI, projected leaf area per unit ground area) was calculated. At 10, 20 and 30 days after anthesis, chlorophyll content in the ear leaves was estimated using a chlorophyll meter (SPAD-502, Minolta, Osaka, Japan). The net photosynthetic rate (P_n) was measured with a portable Licor 6400 photosynthesis measurement system (Li-6400, Li-Cor, Lincoln, NE, USA) by using an internal light source. Five ear leaves per plot were selected for measuring P_n from 9 a.m. to 11 a.m.

Plants were sampled at the following growth stages in 2012 (or 2013): seedling 39 (35), jointing 51(53), flowering (73 or 75, separated into leaves, straw and ear) and grain maturity (140 or 145 DAS, separated into leaves, straw and grain). At each sampling time, 3-5 plants (depending on the specific growth conditions) in a plot were cut at the ground level to measure shoot dry weight and nutrient uptake. The term “shoot” refers to all above-ground parts, and “straw” to the shoot excluding the leaves and grain (i.e. just the stalk).

All plant samples were oven-dried at 105°C for 30 min, and then heated at 60°C for 3-7 days until constant weight. Dry samples were digested in a mixture of concentrated H₂SO₄ and H₂O₂ (3:7 volume ratio), and digests were analyzed for N (Kjeldahl method) by titration and for P (molybdo-vanadophosphate method) by a chromometer (Johnson and Ulrich, 1959).

The dried shoot samples were digested using a HNO₃–H₂O₂ mixture in a microwave-accelerated reaction system (CEM Corp., Matthews, NC, USA), and digests were analyzed for Zn and Fe concentration by ICP-AES (Optima 3300 DV). International Plant-Analytical Exchange (IPE) sample 556 (Wageningen University,

The Netherlands) was used as a reference material to assess reliability of the analyses.

2.3. Root sample collection and measurement of root traits

In order to estimate maize root development and lateral root production in the field, a monolith method (Böhm, 1979) was used at the seedling stage (22 and 35 DAS in 2013). In the same rows, entire root systems of three maize seedlings per plot were excavated to a depth of 40 cm. First-order lateral root length was measured, and lateral root density was determined by counting the branches on the radical roots (10-cm length beginning at the base) at 22 DAS and by counting the branches of axile roots in the nutrient-rich zone (in the narrow row) at 35 DAS in 2013.

An auger sampling method (Böhm, 1979) was used at the flowering stage to assess root length density (RLD) and root spatial distribution. Soil cylinder cores (8 cm diameter $\times$ 45 cm deep, separated into three 15-cm increments: 0-15, 15-30 and 30-45 cm) were taken from three sampling sites (narrow inter-row, plant row and wide inter-row) in every plot. The roots from each core were collected from soil by passing through a 2-mm-diameter mesh, placed in an icebox for transport to the lab and washed with running water in the lab. The roots were scanned by a scanner with a threshold setting of 174 and resolution of 400 dpi. The root images were analyzed using WinRhizo software (Regent Instruments Inc., Quebec, QC, Canada) to calculate root length. The RLD at the flowering stage was calculated from the root length and the soil core volume.

2.4. Grain yield and Zn and Fe harvest index

At maturity, an area of 8 m² for each plot was harvested by hand to determine the grain yield of maize. Grain was threshed from the ear, rinsed with distilled water and dried in an oven at 80°C to constant weight. Grain numbers per ear were counted on six ears per plot.

The Zn and Fe harvest indices were calculated by dividing the amount of grain Zn or Fe by the amount of total Zn or Fe (grain + shoot) at grain maturity.

2.5. Statistics

One-way ANOVA for completely randomized block design was conducted using the SAS statistical software (SAS Inst., 1999). Significant difference among means was determined by LSD at the $P \leq 0.05$ probability level.

3. Results

3.1. Plant growth

Localized supply of ammonium plus P as starter fertilizer and as side-dressing at the jointing stage had a significant influence on plant growth, shoot dry weight, total leaf area, chlorophyll content and net photosynthetic rate in different growth stages. Shoot dry weight in the treatment of localized $\text{NH}_4\text{-N+P}$ increased by 16-36% at seedling (39 and 35 DAS) and 12-27% at grain maturity (140 and 145 DAS) compared with that in the localized P and Urea+P treatments across the two years (Table 2). In comparison to localized P and Urea+P treatments, localized $\text{NH}_4\text{-N+P}$ increased shoot dry weight at 51 DAS in 2012 and at 75 DAS in 2013. There was no significant difference in shoot dry weight among the treatments at 53 DAS in 2013.

Total leaf area measured in 2012 and 2013 showed the same pattern as shoot dry weight. The LAI in the localized $\text{NH}_4\text{-N+P}$ treatment increased by 21-37% at 30 (22) DAS and 9-15% at 73 (75) DAS compared with the localized P and Urea+P treatments in both years (Table 3). The LAI was higher in the localized $\text{NH}_4\text{-N+P}$ treatment than the localized P and Urea+P treatments at 51 DAS in 2012 and 45 DAS in 2013, but there was no difference among the treatments at 53 DAS in 2013.

3.2. Root biomass, total root length, mean length and density of lateral roots at seedling stage as well as root length density at flowering stage

Localized supply of $\text{NH}_4\text{-N+P}$ as starter fertilizer had a significant effect on maize root development at seedling stage, resulting in modified root production, whole root length and lateral root proliferation (Fig. 1). Compared with localized P and Urea+P treatments, root dry weight in the localized $\text{NH}_4\text{-N+P}$ treatment increased by 21-23% and 32-36% at 22 and 35 DAS, respectively (Fig. 1a). Similarly, the total root length in the $\text{NH}_4\text{-N+P}$ treatment was higher (22-38% at 22 and 25-32% at 35 DAS) than that in the localized P and Urea+P treatments (Fig. 1b and e). The mean length and density of the first-order laterals on the radical roots (or axile roots) in the $\text{NH}_4\text{-N+P}$ treatment were greater than those in the Urea+P treatment at 22 or 35 DAS (Fig. 1c, d and f). By contrast, lateral root proliferation in the fertilized zone did not consistently differ between the $\text{NH}_4\text{-N+P}$ and the P treatment.

The spatial distribution pattern of RLD along the soil profile at the flowering stage was altered by localized supply of $\text{NH}_4\text{-N+P}$ as starter fertilizer at sowing and as side-dressing at jointing (Fig. 2). The RLD increased by 15-35% at the depth of 0-15 cm and by 131-169% at 15-30 cm in the plant row in the localized $\text{NH}_4\text{-N+P}$ treatment compared with the localized P and Urea+P treatments (Fig. 2a). Similarly, in

the wide inter-row, greater RLD at the depth of 0-15 cm was found in the localized treatment with $\text{NH}_4\text{-N+P}$ than localized P and Urea+P (Fig. 2b). In contrast, there was no difference in RLD in the narrow inter-row among the three treatments (data not shown).

3.3. Zn and Fe concentration and accumulation in shoot at different stages, and the relationship between root length density and Zn and Fe contents at flowering

Localized $\text{NH}_4\text{-N+P}$ supply significantly increased grain Zn concentration at maturity compared with localized Urea+P (increased by 17%) in 2012 and localized P (increased by 50%) in 2013 (Fig. 3a and c). Localized application of $\text{NH}_4\text{-N+P}$ also increased Zn concentration in leaves at flowering (73 DAS in 2012 and 75 DAS in 2013) compared with localized P supply. There was no difference among the treatments in Zn concentration in shoot from seedling to the jointing stage in either year.

Compared with localized P and Urea+P treatments, Fe concentration in localized $\text{NH}_4\text{-N+P}$ was increased by 13-38% in leaves at flowering (73 DAS) in 2012 and 18-27% in grain at maturity in 2013 (Fig. 3b and d). In comparison with localized P supply, localized $\text{NH}_4\text{-N+P}$ increased Fe concentration in shoot at 39 DAS and grain in 2012 and in leaves at 75 DAS in 2013.

The localized $\text{NH}_4\text{-N+P}$ treatment had a significant effect on Zn and Fe accumulation in shoot at different stages (Fig. 4). Compared with localized P and Urea+P treatments, Zn content in shoot was 30-50% greater at the seedling stage (39 DAS in 2012 and 35 DAS in 2013) and 22-36% higher at grain maturity (140 and 145 DAS) in localized application of $\text{NH}_4\text{-N+P}$ (Fig. 4a and c). There was also an increase in Zn content in shoot with localized $\text{NH}_4\text{-N+P}$ compared with localized P at 51 and 73 DAS in 2012. Shoot Zn content was greater in the treatment with localized $\text{NH}_4\text{-N+P}$ than localized P and Urea+P at flowering (75 DAS) in 2013.

Similarly to Zn, in comparison with localized P and Urea+P treatments, Fe content in localized $\text{NH}_4\text{-N+P}$ was higher by 41-57% at seedling (39 DAS) in 2012 and 24-52% at flowering stage (75 DAS) in 2013 (Fig. 4b and d). Iron content in the localized $\text{NH}_4\text{-N+P}$ was higher than that in localized P at 73 and 140 DAS in 2012 and 35 and 145 DAS in 2013. No difference was found in Zn and Fe content among the three treatments at jointing at 51 DAS in 2012 and 53 DAS in 2013.

At flowering, Zn and Fe contents were significantly higher in the treatment with localized $\text{NH}_4\text{-N+P}$ than with localized P and Urea+P, accompanied by higher RLD at the depth of 15-30 cm in the plant row (Fig. 5a and b), but there was no correlation

between Zn or Fe content and RLD at other depths.

3.4. Chlorophyll content and net photosynthetic rate

Localized $\text{NH}_4\text{-N+P}$ significantly improved the chlorophyll content and net photosynthetic rate at flowering stage in 2013 (Fig. 6a and b). Compared with localized P and Urea+P treatments, the chlorophyll content in the $\text{NH}_4\text{-N+P}$ treatment was increased by 4-12% at 10, 5-10% at 20 and 8-9% at 30 days after anthesis. Net photosynthesis was 15-17% and 8-10% greater, respectively, at 10 and 20 days after anthesis in the $\text{NH}_4\text{-N+P}$ than the P and Urea+P treatments.

3.5. Maize grain yield, kernel number per ear and harvest index of Zn and Fe

Significant differences in maize grain yield, kernel number per ear and harvest index of Zn and Fe were found among the treatments in 2012 and 2013 (Table 4). Maize grain yield in localized $\text{NH}_4\text{-N+P}$ increased by 10-14% in 2012 and 13-25% in 2013 compared with localized P and Urea+P. Kernel number per ear showed a similar pattern as maize grain yield, except no difference was found between the $\text{NH}_4\text{-N+P}$ and Urea+P treatments in 2013.

Zinc harvest index significantly increased in the $\text{NH}_4\text{-N+P}$ compared with the localized P treatment in 2013. Iron harvest index increased by 25-26% in localized $\text{NH}_4\text{-N+P}$ compared with localized P in both years. No difference was found in Zn and Fe harvest indices between the Urea+P and $\text{NH}_4\text{-N+P}$ treatments in the two years.

4. Discussion

4.1. Effect of localized application of ammonium plus P on root development and root distribution at seedling and flowering stages

Micronutrient availability (especially Fe and Zn) is relatively low in calcareous soils. In the present study, maize plants grown in the calcareous soil (DTPA-extractable Zn was 0.65 mg kg^{-1} , and DTPA-extractable Fe was 6.2 mg kg^{-1}) exhibited some Zn-deficiency and Fe-deficiency symptoms. The most important reasons for low availability of Zn and Fe could be high pH and high carbonate content in the calcareous soils, as well as seasonal drought in North China plain, leading to poor diffusion of micronutrients and hampering of root growth (Marschner, 2012). In the present study, localized application of $\text{NH}_4\text{-N+P}$ improved lateral root density and elongation, thus increasing root surface area and enhancing nutrient accumulation, especially of micronutrients with low spatial and biological availability. It has been suggested that using agronomic strategies to enhance appropriate root traits (such as

root length, lateral root production and root spatial distribution) can increase the acquisition of nutrients by roots, thereby improving the mineral nutrition of crops and grain yield (Jing et al., 2012; White et al., 2013). Indeed, in the present study, localized application of $\text{NH}_4\text{-N+P}$ proved to be an effective root management strategy to decrease the rhizosphere pH and increase nutrient concentration in the localized fertilizer zone, leading to root proliferation and improved crop growth.

Localized application of $\text{NH}_4\text{-N+P}$ as starter fertilizer significantly improved maize root biomass, the whole root length and lateral root proliferation at the seedling stage compared with localized P and Urea+P (Fig. 1). The enhanced root growth could greatly contribute to improved nutrient uptake and biomass accumulation, and thus grain yield (Fig. 3 and 4; Table 2, and 4). Compared with localized Urea+P, the root length density (RLD) was significantly higher in the localized zone with $\text{NH}_4\text{-N+P}$ due to fine root proliferation. These fine roots helped acquire soil nutrients efficiently through expanding root absorption surface and enhancing nutrient uptake (Jing et al., 2012, Ma et al., 2013a). The enhanced root exploration of soils via expanding root growth could partly account for the improved micronutrient uptake and grain production under localized supply of $\text{NH}_4\text{-N+P}$ in the present study.

The RLD at 0-15 and 15-30 cm in the plant row and the wide inter-row were significantly greater in the treatment with localized $\text{NH}_4\text{-N+P}$ compared with localized Urea+P (Fig. 2). Increased RLD in deep soil was considered beneficial for the acquisition of nutrients in subsoil and restricting nutrient leaching (White et al., 2013). In the present study, root proliferation in the localized fertilizer zone could increase root branching, thus resulting in relatively high RLD and root elongation downward to the 15-30 cm depth. Hence, the results suggested that root distribution in soil profile at the seedling and flowering stages could be manipulated by changing the nutrient supply in the localized root zone.

The difference in root growth and production between localized $\text{NH}_4\text{-N+P}$ and Urea+P can be mainly attributed to the N forms. Compared with ammonium, urea produces NH_4^+ through hydrolysis by urease enzyme, but this transformation of urea can be slowed down in calcareous soils because of a negative relationship between soil CaCO_3 content and the soil urease activity (Zantua et al., 1977). Moreover, urea hydrolysis can further increase soil pH at the localized sites. All these reactions could result in low availability of sparingly soluble nutrients, especially, Zn, Fe and P, as well as stunted root growth. In contrast, higher root proliferation in the fertilizer zone with localized $\text{NH}_4\text{-N+P}$ might contribute to increased root growth (elongation and

branching) and distribution in the soil profile, being associated with “acid-growth theory” due to intensive rhizosphere acidification in the localized area (Marschner, 2012; Ma et al., 2013b). It is suggested that ammonium-fed plants can excrete protons into the cell wall (apoplast) and the rhizosphere, resulting in a decrease in apoplastic pH, and activation of wall-loosening processes, leading to root cell elongation (Rayle and Cleland, 1992). In addition, increased availability and acquisition of Zn with localized $\text{NH}_4\text{-N+P}$ could likely help maintain the stability of root cell membranes because lack of Zn supply causes ion leakage from the roots (Welch and Shuman, 1995). Recent studies showed that ammonium supply could play an important role in stimulating root branching (Lima et al., 2010; Jing et al., 2012). Taken together, all these effects could account for the improved root growth and distribution, as well as the enhanced micronutrient uptake and maize yield with the localized application of $\text{NH}_4\text{-N+P}$ relative to other treatments.

4.2. Effect of localized application of ammonium plus P on shoot growth, Zn and Fe uptake and grain yield

In the present study, localized supply of $\text{NH}_4\text{-N+P}$ significantly increased maize shoot biomass, LAI, chlorophyll content and net photosynthetic rate compared with localized P or Urea+P supply. Our previous studies showed significant increases in shoot biomass, leaf area, chlorophyll content as well as N and P uptake in the treatment with localized $\text{NH}_4\text{-N+P}$ compared with Urea+P (Jing et al., 2012; Ma et al., 2013b). Leaves are the critical organs that act as the main photosynthetic structures of plants and convert various resources to biomass (Marschner, 2012). Therefore, the results indicated localized $\text{NH}_4\text{-N+P}$ supply not only increased leaf expansion but also enhanced the photosynthetic rate, which greatly contributed to the production of shoot biomass and grain yield. In addition, localized $\text{NH}_4\text{-N+P}$ supply significantly improved Zn and Fe accumulation in maize leaves and grain.

The Zn concentration in maize leaves/shoots at different stages was in the range of 25-34 mg kg^{-1} in both years. These values appear suitable for maize growth because an optimal range of Zn concentration in maize leaves is 21-70 mg kg^{-1} (Mengel et al., 2001). At the flowering stage, maize leaf Zn concentration under localized supply of $\text{NH}_4\text{-N+P}$ showed an increasing trend compared with localized P treatment (Fig. 3). Grain Zn concentration in localized $\text{NH}_4\text{-N+P}$ was significantly higher than localized Urea+P in 2012 and localized P supply in 2013. Wang et al. (2012) reported that application of Zn fertilizer significantly increased grain Zn concentration in maize (27-37%) growing on a calcareous soil with DTPA-extractable Zn in soil lower than a

critical range (0.48-0.63 mg kg⁻¹). However, in the present study, DTPA-extractable soil Zn (0.65 mg kg⁻¹) was just outside the critical range, and ammonium-induced rhizosphere acidification in the localized fertilizer zone significantly enhanced Zn availability and acquisition, resulting in improved maize growth and grain Zn nutrition. The Zn and Fe concentration in leaves and grain showed a similar pattern under localized application of NH₄-N+P. In the present study, grain Fe concentration was significantly higher in the localized NH₄-N+P compared with the localized P treatment in both years, and with Urea+P in 2013 (Fig. 3). These findings suggested an important role of root proliferation and rhizosphere acidification in mobilization and acquisition of Zn and Fe in the calcareous soils.

The Zn and Fe contents significantly increased in plants in the localized NH₄-N+P compared with the localized P and Urea+P treatments at seedling, flowering and grain harvest (Fig. 4). The mechanisms underlying increased micronutrient accumulation under localized NH₄-N+P supply could involve: (1) localized application of acid-forming N fertilizer, such as ammonium sulfate, enhancing availability of soil Zn and Fe to plants by affecting the cation/anion uptake ratio and lowering the rhizosphere pH on calcareous soils (Chen and Barak, 1982; Jing et al., 2010). In contrast, a depressing effect of urea on Zn uptake on calcareous soil was found to be due to interference by HCO₃⁻ and increased soil pH (Rengel et al., 1999). (2) the improved N status and growth potential of maize crops might play an important role in stimulating root Zn and Fe uptake and nutrient accumulation (Rehman et al., 2012), and (3) plants can increase their capacity to acquire nutrients by proliferating lateral roots in nutrient-rich patches and increasing root density in deep soil layers as reported before (White et al., 2013). In addition, auxin could also play a critical role in cell division and elongation, but auxin synthesis in plants can be affected by Zn deficiency (Mengel et al., 2001). Hence, increased Zn availability and accumulation in shoot in the localized NH₄-N+P treatment may increase auxin synthesis, leading to lateral root proliferation in the nutrient-rich zone. Our results suggested that localized NH₄-N+P supply was more effective than localized Urea+P for maize Zn and Fe accumulation, which not only increased root capacity (such as higher root length and surface area due to lateral root proliferation) and micronutrient availability in soil, but also contributed to root physiological modification, such as ammonium-induced acidification and potentially auxin production.

Localized NH₄-N+P supply on calcareous soil significantly improved maize grain yield in the present study. Similarly, ammonium sulfate decreased soil pH and

enhanced rice growth and grain yield compared with urea or ammonium nitrate on calcareous soils (Rahman et al., 2002). Combining N and Zn fertilization increased wheat yield (Holloway, 2010). In the present study, localized $\text{NH}_4\text{-N+P}$ significantly improved maize yield compared with localized Urea+P or P in both years. The higher yield in the $\text{NH}_4\text{-N+P}$ than Urea+P treatment was possibly due to increased nutrient uptake and a larger number of filled grains (Table 4). Taken together, positive effects of localized application of $\text{NH}_4\text{-N+P}$ on maize growth and Zn and Fe accumulation were associated with improved root proliferation, enhanced root distribution, and rhizosphere acidification, suggesting root/rhizosphere management as a potential approach for improving grain Zn and Fe concentration in maize and achieving micronutrient (Zn/Fe) biofortification on calcareous soils.

5. Conclusions

Localized supply of $\text{NH}_4\text{-N+P}$ improved maize shoot biomass, grain Zn and Fe accumulation and grain yield on the calcareous soils. The underlying mechanisms may be attributed to stimulated root proliferation, increased total root length and root length density, enhanced root distribution in deep soil, increased net photosynthetic rate, and decreased rhizosphere pH. In contrast, no significant effect was observed in treatments with localized application of Urea+P or P. It is suggested that localized supply of $\text{NH}_4\text{-N+P}$ is an effective approach to improving grain Zn and Fe accumulation and maize yield on calcareous soils via modifying root traits and rhizosphere processes. The molecular and physiological mechanisms behind the effect of localized $\text{NH}_4\text{-N+P}$ application on maize growth and Zn and Fe accumulation need to be further investigated.

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Table 1. The weather data during the two maize growing seasons at the research station of China Agricultural University, Shangzhuang, North China, near the Pacific Coast (40°N 116°E).

Month	Minimum temperature (°C)		Maximum temperature (°C)		Rainfall (mm)		
	2012	2013	2012	2013	2012	2013	Average (1950-2012)
May	12	10	34	33	12	2	15
June	15	15	38	34	118	72	82
July	21	20	35	37	310	226	199
August	16	16	34	36	30	80	153
September	10	10	29	31	118	48	50
October	2	2	27	24	63	22	20

Table 2. Shoot dry weight of maize (g plant⁻¹) at different growth stages in 2012 and 2013.

Days after sowing 2012 (2013)	2012			2013		
	P	Urea+P	NH ₄ -N+P	P	Urea+P	NH ₄ -N+P
39 (35)	14±0.2b	14±0.8b	19±0.9a	7.0±0.3b	7.0±0.2b	8.1±0.2a
51 (53)	42±1b	44±1b	50±3a	37±3a	38±1a	42±3a
73 (75)	105±2b	122±5ab	124±1a	88±6c	104±3b	114±5a
140 (145)	244±11b	273±5b	309±6a	188±9b	209±11b	234±4a

Each value is the mean of four replicates (±SE). Different letters in each row for each year denote significant difference among treatments at a specific growth stage ($P \leq 0.05$). Shoot dry weight at grain maturity at 140 (145) DAS refers to all above-ground including the grain. P: localized supply of superphosphate as starter fertilizer at sowing and as side-dressing at the jointing stage, Urea+P: localized supply of urea plus superphosphate as starter fertilizer at sowing and as side-dressing at the jointing stage, NH₄-N+P: localized supply of ammonium nitrogen (as ammonium sulfate) plus superphosphate as starter fertilizer at sowing and as side-dressing at the jointing stage. DAS: days after sowing for years 2012 (2013) [39 (35)=seedling; 51 (53)=jointing; 73 (75)=flowering; 140 (145)= grain maturity/harvest].

Table 3. The leaf area index (LAI, projected leaf area per unit of ground area) of maize plants at different growth stages in 2012 and 2013.

Days after sowing 2012 (2013)	2012			2013		
	P	Urea+P	NH ₄ -N+P	P	Urea+P	NH ₄ -N+P
30 (22)	0.31±0.01b	0.29±0.03b	0.39±0.02a	0.12±0.01b	0.12±0.01b	0.14±0.02a
41 (45)	1.21±0.01ab	1.12±0.07b	1.42±0.09a	1.14±0.02c	1.28±0.01b	1.44±0.06a
51 (53)	2.8±0.1b	2.7±0.2b	3.4±0.2a	2.7±0.2a	2.8±0.1a	3.0±0.1a
73 (75)	4.5±0.2b	4.4±0.2b	5.0±0.1a	4.5±0.1b	4.5±0.1b	5.0±0.1a

Each value is the mean of four replicates ($\pm$ SE). Different letters in each row for each year denote significant difference among treatments at a specific growth stage ($P \leq 0.05$). For explanations of the treatments, see Table 2. DAS: days after sowing for years 2012 (2013) [30 (22)=seedling; 41 (45)=early jointing; 51 (53)=later jointing; 73 (75)=flowering.

Table 4. Effects of different fertilizer supply on maize grain yield, kernel number per ear and harvest index of Zn and Fe in 2012 and 2013.

Treatments	2012				2013			
	Grain yield (t ha ⁻¹)	Kernel number (per ear)	Harvest index		Grain yield (t ha ⁻¹)	Kernel number (per ear)	Harvest index	
			ZnHI (%)	FeHI (%)			ZnHI (%)	FeHI (%)
P	9.9b	464b	53.4a	12.2b	6.5b	421b	50.2b	7.8b
Urea+P	10.3b	466b	51.9a	14.8a	7.2b	483ab	54.8ab	8.8ab
NH ₄ -N+P	11.3a	504a	52.8a	15.3a	8.1a	513a	61.5a	9.8a

Each value is the mean of four replicates. Different letters in each column denote significant difference among treatments ($P \leq 0.05$). For explanations of the treatments, see Table 2.

Figure legends

Fig. 1. Effects of different nutrient supplies on root dry weight (a), the whole root length (b), and the mean length (c) and density (d) of the first-order laterals at the seedling stage (22 and 35 DAS), and the whole root and laterals on radical roots at 22 DAS (e and f) in 2013. The first-order lateral root density represents root number per cm of radical root at 22 DAS or per cm of axile root at 35 DAS. Each value is the mean of four replicates (+SE). Different lower case letters denote significant difference ($P \leq 0.05$) among treatments at 22 DAS, and capital letters denote differences among treatments at 35 DAS. For explanation of the treatments, see Table 2. DAS: days after sowing.

Fig. 2. Effects of different fertilizer treatments on root length density of maize at different soil depths and different positions (a, plant row, and b, wide inter-row) at flowering stage (75 DAS) in 2013. The roots were separated into three depths at 15-cm increments (0-15, 15-30 and 30-45 cm). Each value is the mean of four replicates (+SE). Different lower case letters denote significant difference ($P \leq 0.05$) among treatments in individual graphs. For explanation of the treatments, see Table 2.

Fig. 3. Effects of different fertilizer supplies on Zn and Fe concentration in maize shoots at seedling [39 (35) DAS] and jointing [51 (53) DAS] and in leaves at flowering [73 (75) DAS] and grain at maturity [140 (145) DAS] in 2012 (a, b) and 2013 (c, d). Each value is the mean of four replicates (+SE). Different lower case letters for each DAS in each graph denote significant difference ($P \leq 0.05$) among treatments. For explanation of the treatments, see Table 2.

Fig. 4. Effects of different fertilizer supplies on Zn and Fe content by maize shoot at seedling [39 (35) DAS], jointing [51 (53) DAS], flowering [73 (75) DAS] and grain maturity [140 (145) DAS] in 2012 (a, b) and 2013 (c, d). Each value is the mean of four replicates (+SE). Different lower case letters denote significant difference ($P \leq 0.05$) among treatments. For explanation of the treatments, see Table 2.

Fig. 5. The relationship between root length density (RLD) in the 15-30 cm soil layer in the plant row and Zn (a) or Fe content (b) at the flowering stage (75 DAS) in 2013. For explanation of the treatments, see Table 2.

Fig. 6. The chlorophyll content (a) and net photosynthetic rate (b) of maize with

different nutrient supplies at 10, 20 and 30 days after anthesis in 2013. Each value is
the mean of four replicates (+SE). Different lower case letters for each DAS in each
graph denote significant difference ($P \leq 0.05$) among treatments. For explanation of the
treatments, see Table 2.

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