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Nitrogen Form Modulates Plant Responses to Heterogeneous Iron Availability in *Arabidopsis* and Wheat

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Abstract

Plants commonly encounter spatially heterogeneous distributions of nitrogen (N) forms and iron (Fe) in soils, but how N form modifies plant responses to heterogeneous Fe availability remains poorly understood. Using a split-root system, we examined the phenotypic responses of *Arabidopsis thaliana* and wheat to contrasting Fe availability combined with ammonium (NH_4^+) or nitrate (NO_3^-) supply. Plants were exposed to high (100 μM) or low (5 μM) Fe in combination with NH_4^+ or NO_3^-, either under homogeneous or spatially heterogeneous conditions. Within each species, NH_4^+ and NO_3^- treatments were supplied at equivalent total N concentrations. In both species, high Fe combined with sole NH_4^+ supply resulted in pronounced reductions in shoot and root growth, whereas spatial presence of NH_4^+ and NO_3^- between root compartments was associated with improved growth. Under high-Fe conditions, sole NH_4^+ supply to both root compartments (HAHA) resulted in pronounced reductions in shoot and root growth, whereas supplying NH_4^+ and NO_3^- to separate root compartments (HAHN) was associated with improved growth. Under these conditions, shoot fresh weight in HAHN was 178% higher than that in HAHA in *Arabidopsis* and 231% higher in wheat. In wheat, root fresh weight under HAHN was 471% higher than under HAHA. Wheat exhibited stronger spatial differentiation of root growth between contrasting nutrient compartments, whereas *Arabidopsis* showed greater variation in whole-plant growth in response to N form. Chlorophyll accumulation also showed species- and Fe-dependent responses to N form. Overall, these results indicate that N form modifies plant phenotypic responses to heterogeneous Fe–N supply and that wheat exhibits greater root plasticity under spatially heterogeneous Fe–N conditions. The roles of plant Fe and N status, rhizosphere pH, and long-distance signaling in these responses remain to be determined.

Keywords: iron heterogeneity; nitrogen forms; split-root system; root plasticity; ammonium; nitrate

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1. Introduction

Plants in natural ecosystems rarely experience uniform nutrient availability [1]. Instead, soil nutrients are distributed in highly heterogeneous patterns due to variations in soil properties, microbial activity, water movement, and organic matter decomposition [2]. This spatial heterogeneity occurs not only for macronutrients such as nitrogen (N), but also for micronutrients including iron (Fe), creating complex nutritional environments to which plants must adjust their nutrient acquisition strategies. Roots, as the primary interface between plants and soil [3], respond to local nutrient fluctuations and contribute to whole-plant growth [4].

Nitrogen is one of the most important limiting nutrients for plant growth and is mainly acquired in the forms of nitrate (NO_3^-) and ammonium (NH_4^+) [5]. These two inorganic N forms differ substantially in uptake mechanisms, metabolic costs, and effects on the rhizosphere environment [6]. NH_4^+ uptake is generally associated with proton release and rhizosphere acidification, whereas NO_3^- uptake can promote rhizosphere alkalization [6–9]. These N-form-dependent changes in rhizosphere pH may influence the availability of other nutrients, particularly Fe, whose solubility and mobility are strongly affected by soil chemical conditions [10].

Iron is an essential micronutrient involved in chlorophyll biosynthesis, photosynthetic electron transport, respiration, and numerous redox reactions [11]. Despite its abundance in soils, Fe availability is frequently limited because Fe is predominantly present in the form of poorly soluble ferric (Fe^{3+}) compounds, especially under neutral and alkaline conditions [12]. In contrast, acidic environments promote the reduction of Fe^{3+} to soluble Fe^{2+}, increasing Fe mobility and availability [13–16]. Therefore, plant Fe acquisition is strongly influenced by external soil conditions as well as plant physiological responses.

Recent studies have highlighted interactions between N form and Fe acquisition [17,18]. Previous studies suggest that N form can enhance Fe acquisition, with NH_4^+ supply potentially affecting Fe availability through rhizosphere acidification and NO_3^- supply influencing Fe acquisition through changes in rhizosphere conditions and plant metabolism [18,19]. However, excessive NH_4^+ supply can also cause ammonium toxicity, which may impair plant growth and root development and has been associated with disturbances in intracellular pH homeostasis, ion balance, and carbon metabolism [20]. Thus, plant responses to N form may depend on Fe availability and the spatial distribution of Fe and N. Understanding how plants respond to spatially heterogeneous Fe and N environments is therefore important for elucidating plant responses to nutrient heterogeneity.

Root systems possess remarkable plasticity that enables plants to exploit nutrient-rich patches while maintaining growth under nutrient limitations [21,22]. Previous studies using split-root systems have shown that plants can locally modify root growth and physiological activity in response to heterogeneous nutrient distributions [22–26]. These local responses can also be accompanied by whole-plant adjustments that may involve systemic signals, including hormones, peptides, and nutrient-related signals [25]. However, most previous studies have focused on single nutrient heterogeneity, particularly N or phosphorus, whereas the phenotypic responses to combined spatially Fe–N supply remain less well understood.

Moreover, plant species with distinct Fe-acquisition strategies may respond differently to heterogeneous Fe–N environments. Dicots such as *Arabidopsis thaliana* generally rely on Strategy I Fe acquisition, which involves rhizosphere acidification and reduction of Fe^{3+} prior to uptake [27]. By contrast, monocots such as wheat (*Triticum aestivum* L.) utilize Strategy II Fe acquisition, involving the secretion of phytosiderophores that chelate Fe and facilitate uptake [28]. These physiological differences may contribute to contrasting

responses to Fe and N heterogeneity. However, whether dicots and monocots exhibit contrasting root responses to spatially heterogeneous Fe–N supply remains unclear.

In this study, we established a split-root system using *Arabidopsis thaliana* and wheat to investigate plant responses to heterogeneous Fe–N environments. Plants were exposed to high and low Fe availability in combination with NH_4^+ or NO_3^- under homogeneous conditions, as well as to spatially heterogeneous Fe–N supply under split-root conditions. Specifically, we aimed to address three questions:

(1) How do Fe availability and N form affect plant growth, root development, and chlorophyll accumulation under homogeneous and heterogeneous conditions? (2) Does heterogeneous Fe–N supply induce differential root growth between spatially separated root compartments? (3) Do *Arabidopsis* and wheat exhibit contrasting phenotypic responses and patterns of root plasticity under heterogeneous Fe–N conditions? We hypothesized that (i) plant growth and chlorophyll accumulation would respond to the interaction between Fe availability and N form; (ii) sole NH_4^+ supply would cause stronger growth inhibition than spatially separated NH_4^+ and NO_3^- supply under unfavorable Fe–N combinations; (iii) heterogeneous Fe–N supply would induce asymmetric root growth between spatially separated nutrient compartments; and (iv) wheat would exhibit stronger spatial root plasticity than *Arabidopsis*.

By integrating root responses with whole-plant growth and chlorophyll traits, this study provides a phenotypic framework for understanding plant responses to spatially heterogeneous Fe–N conditions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The wild-type *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used in this study. Seeds were surface-sterilized and stratified at 4 °C for 48 h before sowing on standard growth medium. The composition of the growth medium was modified according to previous studies [29] and contained 2 mM KH_2PO_4, 5 mM $NaNO_3$, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 100 µM $FeSO_4$-EDTA, 50 µM H_3BO_3, 12 µM $MnSO_4$, 1 µM $ZnCl_2$, 1 µM $CuSO_4$, 0.2 µM Na_2MoO_4, 1% (*w*/*v*) sucrose, and 0.8% (*w*/*v*) agar. The pH of the medium was adjusted to 5.7 using 1 M NaOH.

Seeds were considered as day 0 after sowing. Seedlings were vertically grown on agar plates in a controlled growth chamber under a 16 h light/8 h dark photoperiod with a light intensity of 100 µmol m^{-2} s^{-1} at 23 ± 1 °C.

Wheat (*Triticum aestivum* L.), cultivar Jimai 22, was used in the experiments. Seeds were surface-sterilized and cold-treated at 4 °C for 48 h, followed by germination on moist gauze under dark conditions at 28 °C. After germination, uniform seedlings were transferred to hydroponic culture containing nutrient solution composed of 2 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 50 µM H_3BO_3, 12 µM $MnSO_4$, 1 µM $ZnCl_2$, 1 µM $CuSO_4$, and 0.2 µM Na_2MoO_4. The solution pH was adjusted to 5.7 using 1 M NaOH.

Plants were grown in a controlled-environment chamber under a 16 h light/8 h dark photoperiod with a light intensity of 400 µmol m^{-2} s^{-1}. The temperature was maintained at 25 °C during the day and 20 °C at night, with relative humidity maintained at approximately 60%.

2.2. Split-Root Experimental Design

A split-root system was established to investigate plant responses to spatially heterogeneous Fe and N availability (Figure 1). The split-root system was modified according to previous studies [30]. For the split-root treatment, seedlings were transferred to freshly prepared treatment media (without background N) containing the designated Fe and N

treatments. Fe was supplied as $FeSO_4$-EDTA (Fe:EDTA = 1:1). Fe-EDTA is commonly used as an Fe source in plant nutrient solutions under acidic to mildly acidic conditions [31,32].

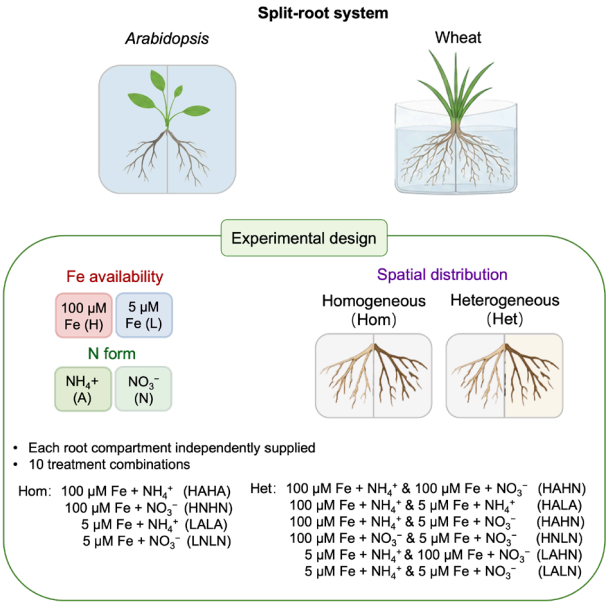

Figure 1. Experimental treatments and abbreviations.

For the *Arabidopsis* split-root system, three days after germination, seedlings were removed from the agar medium and the primary root was cut at the hypocotyl–root junction using sterile scissors. After an additional 3 days of regeneration, seedlings with two similarly developed lateral roots were selected and transferred to split-root plates containing two spatially separated nutrient compartments, allowing the two root systems to be independently exposed to different Fe and N treatments. Fe was supplied as $FeSO_4$-EDTA (Fe:EDTA = 1:1) at low (L, 5 μM) or high (H, 100 μM) concentration. Nitrogen was supplied at an equal elemental N concentration of 5 mM in all treatments, either as NH_4^+ or NO_3^-. Specifically, 5 mM N was supplied as 2.5 mM $(NH_4)_2SO_4$ in the NH_4^+ treatment (ammonium, A) or as 5 mM $NaNO_3$ in the NO_3^- treatment (nitrate, N). Thus, the total elemental N concentration was equivalent between the two N-form treatments. Homogeneous and heterogeneous Fe–N combinations were established by independently supplying the designated nutrient conditions to each root compartment. Plants were grown under these split-root conditions for 8 days before phenotypic measurements were taken. Ten independent biological replicates were included for each treatment.

For the wheat split-root system, approximately one week after germination, uniform seedlings with two similarly developed roots were selected and transferred to split-root hydroponic systems. Fe treatments were identical to those used for *Arabidopsis*: low Fe (L, 5 μM) and high Fe (H, 100 μM), supplied as $FeSO_4$-EDTA (Fe:EDTA = 1:1). Nitrogen was supplied at an equal elemental N concentration of 10 mM in all treatments, either as NH_4^+ or NO_3^-. Specifically, 10 mM N was supplied as 5 mM $(NH_4)_2SO_4$ in the NH_4^+ treatment (ammonium, A) or as 10 mM $NaNO_3$ in the NO_3^- treatment (nitrate, N). Thus, the total elemental N concentration was equivalent between the two N-form treatments. Each root compartment was independently supplied with different Fe–N combinations to generate homogeneous and heterogeneous nutrient environments. The hydroponic solutions were renewed every 2 days, and plants were grown for 12 days before phenotypic analysis. Six independent biological replicates were included for each treatment.

2.3. Measurements of Physiological Traits

Arabidopsis received an 8-day treatment, while wheat was subjected to a 12-day treatment. Afterwards, plants were collected for phenotypic measurement. Shoot and root fresh weights were measured using an analytical balance. Leaf area, plant height, and root length were determined using ImageJ software (version 1.53). Total chlorophyll content was measured using a spectrophotometric method.

2.4. Statistical Analysis

All statistical analyses were performed using SPSS version 25.0. Two-way ANOVA followed by Tukey's multiple-comparison test was applied for all treatment comparisons. Specifically for wheat, multiple *t*-tests were used for comparisons of root length between the two root compartments within the same plant. Statistical significance was indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****), and ns (not significant). Data are presented as means ± SD. Sample sizes for individual measurements are indicated in the corresponding figure legends. Figures were generated using GraphPad Prism version 10.

3. Results

3.1. Heterogeneous Fe–N Conditions Modify Shoot Growth and Leaf Expansion in Arabidopsis

Different combinations of Fe availability and N forms significantly affected shoot growth and leaf area in *Arabidopsis thaliana* (Figure 2A,B). Shoot fresh weight and leaf area showed broadly similar response patterns, indicating that the treatments produced coordinated changes in whole-plant growth. Under high-Fe conditions (100 μM Fe), plants supplied exclusively with NH_4^+ (HAHA and HALA) exhibited the lowest shoot fresh weight. Compared with the corresponding nitrate-only treatments, shoot fresh weight was reduced by 87.90% in HAHA relative to HNHN and by 86.25% in HALA relative to HNLN. These results indicate that the growth response to high Fe availability depended strongly on N form under the conditions tested.

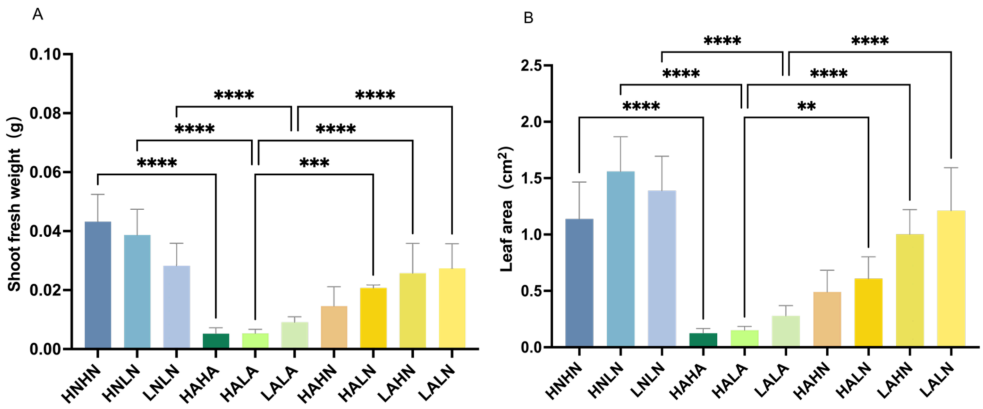

Figure 2. Effects of different iron concentrations and nitrogen forms on shoot fresh weight and leaf area of *Arabidopsis thaliana*. (**A**) Shoot fresh weight; (**B**) leaf area. Treatments represent combined iron concentrations and nitrogen forms under split-root systems. H = 100 μM Fe, L = 5 μM Fe; A = ammonium (NH_4^+), N = nitrate (NO_3^-). The first two letters indicate the left compartment, and the last two letters indicate the right compartment. For example, HALN indicates the left root side received 100 μM Fe plus ammonium, while the right side received 5 μM Fe plus nitrate. Blue bars denote sole nitrate supply, green bars denote sole ammonium supply, and yellow bars denote spatially separated NH_4^+/NO_3^- supply; the shade of each bar indicates high or low iron concentration. All data are presented as means ± SD, with n = 10. Significant comparisons among treatment means were performed by two-way ANOVA followed by Tukey's multiple-comparison test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

When NO_3^- was supplied to one root compartment under high-Fe conditions (HAHN), shoot fresh weight increased by 178.01% relative to HAHA. Under heterogeneous Fe conditions, supplying NO_3^- to one root compartment (HALN and LAHN) also significantly increased shoot fresh weight relative to HALA. Although shoot biomass remained lower than that observed under nitrate-only conditions, this response indicates that the presence of NO_3^- in one root compartment was associated with improved whole-plant growth compared to plants grown under high-Fe and sole-NH_4^+-dominated conditions.

Plants grown under heterogeneous Fe–N conditions (HAHN, HALN, and LALN) generally exhibited shoot biomass and leaf area that differed from those observed under uniformly high or low nutrient conditions, indicating that spatially heterogeneous Fe–N supply generated distinct whole-plant growth responses. Overall, N form strongly modified *Arabidopsis* growth responses across the tested Fe conditions.

3.2. Heterogeneous Fe–N Conditions Modify Root Growth in Arabidopsis

Root fresh weight showed pronounced differences among Fe–N treatments and generally followed a pattern similar to, but stronger than, that observed for shoot biomass (Figure 3). Regardless of Fe concentration, root fresh weight was consistently the lowest under sole-NH_4^+ conditions; moreover, root fresh weight was significantly reduced whenever NH_4^+ was supplied under high-Fe conditions. Under high-Fe conditions, the HAHA treatment resulted in the lowest root fresh weight among the tested treatments. Compared with HNHN, root fresh weight was 85.29% lower in HAHA and 82.07% lower in HAHN. These results indicate that sole NH_4^+ supply was associated with strong inhibition of root growth under high-Fe conditions. The reduction in root biomass was consistent with the decrease in shoot biomass observed under the same treatment, suggesting that root growth inhibition was an important component of the overall growth response to high Fe combined with sole-NH_4^+ conditions.

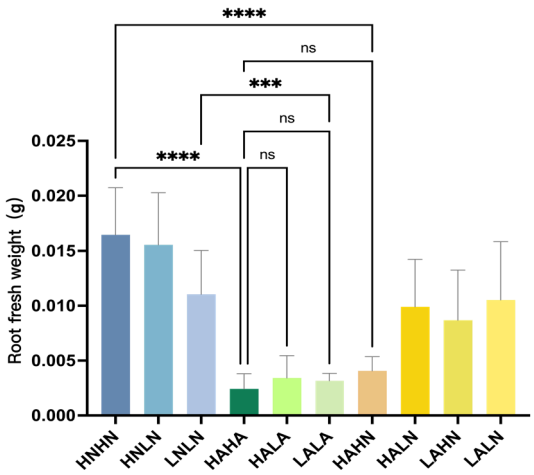

Figure 3. Effects of different iron concentrations and nitrogen forms on root fresh weight in *Arabidopsis thaliana*. Detailed information for each treatment is shown in Figure 2. All data are presented as means ± SD, with n = 8–10 (n varied because of exclusion of damaged/non-uniform seedlings). Significant comparisons among treatment means were performed by two-way ANOVA followed by Tukey's multiple-comparison test. *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

When NO_3^- was supplied to one root compartment under high-Fe conditions (HAHN), root fresh weight increased by 67.61% relative to HAHA. This result indicates that the presence of NO_3^- in one root compartment was associated with increased root biomass compared with sole NH_4^+ supply under high-Fe conditions.

Under low-Fe conditions, the LALA treatment also exhibited relatively low root fresh weight, indicating that low Fe availability combined with sole NH_4^+ supply also constrained root growth. However, the magnitude and pattern of root responses differed between low- and high-Fe conditions, suggesting that the effect of N form depended on Fe availability. Overall, these results show that *Arabidopsis* root growth was strongly influenced by the combination of Fe availability and N form.

3.3. Heterogeneous Fe–N Conditions Modify Chlorophyll Accumulation in Arabidopsis

Fe availability and N form significantly affected total chlorophyll content in *Arabidopsis* (Figure 4). Generally, high Fe availability promoted chlorophyll accumulation; however, this positive effect was strongly dependent on N form.

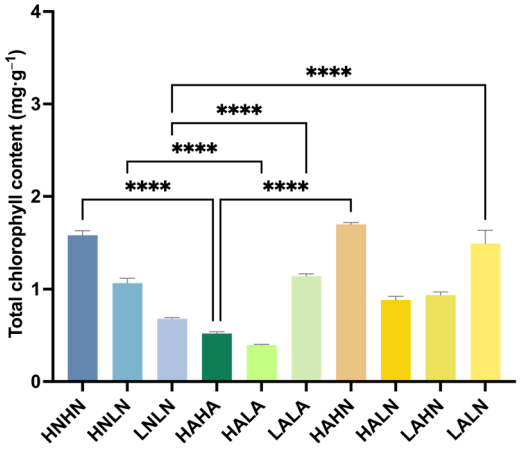

Figure 4. Effects of different iron concentrations and nitrogen forms on total chlorophyll content in *Arabidopsis thaliana*. Detailed information for each treatment is shown in Figure 2. Data are presented as means ± SD, n = 3. Significant comparisons among treatment means were performed by two-way ANOVA followed by Tukey's multiple-comparison test. **** $p < 0.0001$.

Under high-Fe conditions, plants receiving sole NH_4^+ supply (HAHA and HALA) exhibited lower total chlorophyll content than the corresponding nitrate-only treatments. Compared with HNHN and HNLN, total chlorophyll content was reduced by 67.14% in HAHA and 62.89% in HALA, respectively. Under high-Fe conditions, total chlorophyll content in HAHN was significantly higher than that in HAHA, increasing by 226.83%. This result shows that the presence of NO_3^- in one root compartment was associated with substantially greater chlorophyll accumulation than sole NH_4^+ supply under high-Fe conditions.

Under low-Fe conditions, the response pattern differed from that observed under high Fe. Compared with LNLN, total chlorophyll content was significantly higher in LALA, increasing by 68.10%. Similarly, LALN exhibited a 119.62% higher total chlorophyll content than LNLN. These results indicate that the effect of N form on chlorophyll accumulation depended on Fe availability. Overall, chlorophyll accumulation in *Arabidopsis* varied with both Fe availability and N form, and the direction and magnitude of the N-form effect differed between high- and low-Fe conditions.

3.4. Heterogeneous Fe–N Conditions Modify Shoot Growth in Wheat

Similar to *Arabidopsis*, wheat plants exhibited significant growth responses to Fe availability and N forms (Figure 5A,B). Shoot fresh weight and plant height showed comparable trends, although shoot biomass exhibited stronger differences among treatments.

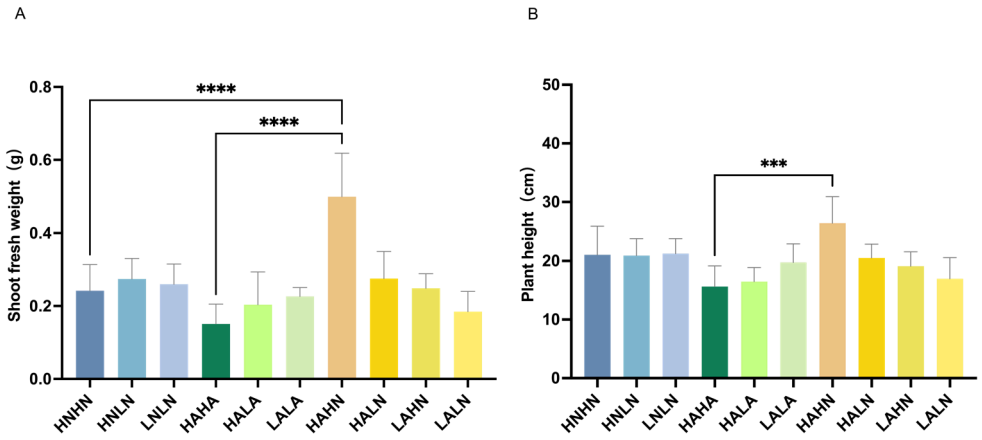

Figure 5. Effects of different iron concentrations and nitrogen forms on biomass and plant height of wheat. (**A**) Shoot fresh weight; (**B**) plant height. Detailed information of each treatment is provided in Figure 2. Data are shown as means ± SD, n = 5–6 (n varied because of the exclusion of damaged or non-uniform seedlings). Significant comparisons among treatment means were performed by two-way ANOVA followed by Tukey's multiple-comparison test. *** $p < 0.001$, **** $p < 0.0001$.

Under high-Fe conditions, plants receiving sole NH_4^+ supply (HAHA) exhibited the lowest shoot fresh weight. When NO_3^- was supplied to one root compartment (HAHN), shoot fresh weight was significantly higher than that under HAHA, increasing by 230.80%. Plant height showed a similar response pattern and increased by 69.09% under HAHN compared with HAHA.

Under heterogeneous nutrient conditions, wheat growth generally declined as Fe availability decreased. The HALN treatment exhibited intermediate shoot growth between the HAHN and LALN treatments. This pattern indicates that heterogeneous Fe–N supply generated whole-plant growth responses that differed from those observed under the corresponding more favorable or less favorable nutrient combinations.

3.5. Heterogeneous Fe–N Conditions Induce Differential Root Growth in Wheat

Compared with shoot traits, wheat roots showed stronger responses to heterogeneous Fe–N conditions (Figure 6). Root fresh weight was significantly reduced under HAHA, indicating a strong negative association between sole NH_4^+ supply and root growth under high-Fe conditions. When NO_3^- was supplied to one root compartment (HAHN), root fresh weight increased by 471.16% relative to HAHA. Thus, the presence of NO_3^- in one root compartment was associated with a substantial increase in root biomass under high-Fe conditions.

Spatially separated nutrient conditions induced clear differential root growth between the two compartments. Under homogeneous high-Fe conditions and under heterogeneous Fe conditions, wheat roots preferentially elongated toward nitrate-containing compartments, whereas under low-Fe conditions, greater root growth was observed toward NH_4^+-supplied regions. For example, under LALN, root elongation was significantly greater in the NH_4^+-supplied compartment than in the nitrate-supplied compartment. This pattern indicates that the direction of root growth depended on the local combination of Fe availability and N form.

These results indicate pronounced spatial root plasticity in wheat under heterogeneous Fe–N conditions. The direction of root growth varied with Fe availability and the N form present in each compartment, suggesting that wheat roots responded to the local combination of nutrient conditions rather than to a single nutrient factor alone. However, the present root-growth measurements do not directly demonstrate differences in Fe or N uptake or nutrient-use efficiency.

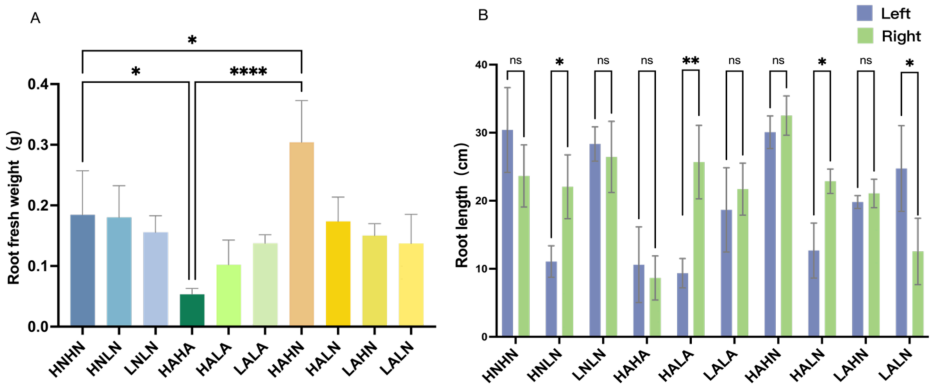

Figure 6. Effects of different iron concentrations and nitrogen forms on root fresh weight and split-root length in wheat. (**A**) Root fresh weight; (**B**) root length on the two sides of split-root system. Full details of all treatments are listed in Figure 2. The first and second letters of each treatment label correspond to nutrient conditions for the left and right root compartments, respectively. Taking HALN as an example, the left compartment HA supplied 100 μM Fe plus ammonium, while the right compartment LN supplied 5 μM Fe plus nitrate. Panel A was analyzed by two-way ANOVA followed by Tukey's multiple-comparison test, whereas panel B was evaluated by multiple *t*-tests. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ns = not significant. Data are presented as means ± SD, with n = 4–6 for panel A (n varied because of the exclusion of damaged or non-uniform seedlings) and n = 3 for panel B.

3.6. N-Form Effects on Chlorophyll Accumulation in Wheat Depend on Fe Availability

Unlike *Arabidopsis*, chlorophyll content generally increased as the number of NH_4^+-supplied root compartments increased (Figure 7).

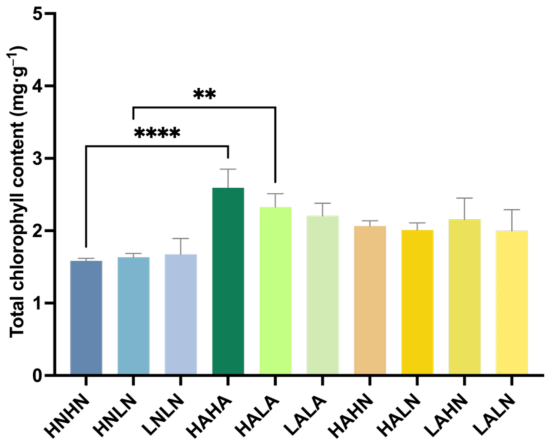

Figure 7. Effects of different iron concentrations and nitrogen forms on total chlorophyll content in wheat. Detailed information of each treatment is shown in Figure 2. Data are expressed as means ± SD, n = 3. Significant comparisons among treatment means were performed by two-way ANOVA followed by Tukey's multiple-comparison test. ** $p < 0.01$, **** $p < 0.0001$.

Under high-Fe and heterogeneous Fe conditions, total chlorophyll content was generally higher when NH_4^+ was supplied to at least one root compartment than under the corresponding nitrate-only treatments. Notably, under homogeneous high-Fe conditions, HAHA exhibited a 63.75% higher chlorophyll content than HNHN, whereas HALA exhibited a 42.51% higher chlorophyll content than HNLN.

These results indicate that the effect of N form on chlorophyll accumulation differed between *Arabidopsis* and wheat and depended on Fe availability. *Arabidopsis* showed stronger reductions in chlorophyll content under sole NH_4^+ supply at high Fe, whereas wheat generally maintained higher chlorophyll content under NH_4^+-containing treatments.

This interspecific contrast was accompanied by the stronger spatial root responses observed in wheat, highlighting differences in the phenotypic responses of the two species to heterogeneous Fe–N conditions.

4. Discussion

4.1. Plant Responses to Fe Availability Depend on N Form

Iron availability is an important determinant of plant growth and chlorophyll accumulation [33,34]. However, our results show that plant responses to Fe availability varied with N form. In both *Arabidopsis* and wheat, high Fe availability (100 μM Fe) combined with sole NH_4^+ supply was associated with marked reductions in biomass and root growth. Thus, sufficient Fe availability did not necessarily translate into improved plant growth when NH_4^+ was supplied as the sole N source. These findings indicate that the growth response to Fe availability should be considered in the context of N form rather than Fe supply alone.

Previous studies have shown that Fe status and N form can jointly influence plant growth and Fe-related physiological responses. NH_4^+ can promote rhizosphere acidification and affect Fe acquisition, whereas Fe status has also been reported to modify plant responses to NH_4^+ supply [18,35,36]. Conversely, excessive NH_4^+ supply has been associated with metabolic and physiological disturbances, including altered cellular pH, ion balance, carbon demand, and oxidative stress [20,37–40]. These processes may help explain the strong growth inhibition observed under high Fe combined with sole NH_4^+ supply in the present study. However, because N assimilation, cellular pH, ion balance, and tissue Fe status were not measured here, the present results should be interpreted primarily as a phenotypic interaction between Fe availability and N form.

In this study, the strongest growth inhibition occurred under high Fe combined with sole NH_4^+ supply rather than under low-Fe conditions. This finding suggests that high Fe availability does not necessarily promote plant growth when the N form is suboptimal. Importantly, under high-Fe conditions, shoot fresh weight in HAHN was 178.01% higher than that in HAHA in *Arabidopsis* and 230.80% higher in wheat. Thus, supplying NO_3^- to one root compartment was associated with substantially greater shoot biomass than supplying NH_4^+ alone under the same high Fe level. These responses are consistent with previous observations that Fe status and N form can interact to influence plant growth [18,35].

Our findings are consistent with previous observations in rice and lettuce [18,35] and further show that strong growth inhibition under high Fe combined with sole NH_4^+ supply can also occur in both a dicot (*Arabidopsis*) and a monocot (wheat). This cross-species response suggests that the interaction between Fe availability and N form is not restricted to a single plant species. More importantly, the split-root design indicates that this interaction can also be expressed under spatially heterogeneous Fe–N conditions, where different root compartments experience distinct nutrient combinations.

4.2. Root Plasticity Contributes to Responses to Heterogeneous Fe–N Conditions

Roots are directly exposed to spatial variation in nutrient availability and can respond through changes in growth and architecture [41]. In this study, *Arabidopsis* root fresh weight showed pronounced differences among Fe–N treatments, particularly under high Fe combined with sole NH_4^+ supply. Under high-Fe conditions, HAHA plants exhibited the lowest root biomass, whereas supplying NO_3^- to one root compartment (HAHN) was associated with a 67.61% increase in *Arabidopsis* root fresh weight. This pattern was consistent with the shoot growth responses observed under the same treatments, indicating that root growth was an important component of the whole-plant response to Fe–N conditions.

In wheat, spatially separated Fe–N conditions induced clear differences in root growth between the two root compartments. Under high-Fe conditions, roots showed greater growth toward nitrate-containing compartments, whereas under low-Fe conditions, greater root growth was observed toward NH_4^+-supplied compartments. This contrasting pattern indicates that the direction of root growth varied with the local Fe–N combination rather than being explained by N form alone. Such responses are consistent with root plasticity under heterogeneous nutrient conditions, in which plants alter root growth according to local resource availability.

The contrasting root responses also suggest that the direction of root growth cannot be explained by the concentration of a single nutrient alone. A nutrient form associated with greater root growth under one Fe condition was not necessarily associated with the same response under another Fe condition. Thus, root responses to nutrient heterogeneity appear to depend on the combination of locally available Fe and N forms.

4.3. Spatially Separated NH_4^+ and NO_3^- Supply Is Associated with Improved Plant Growth

Plants commonly encounter both NH_4^+ and NO_3^- in soil rather than a single inorganic N form [42]. In this present study, supplying NH_4^+ and NO_3^- to separate root compartments was generally associated with greater plant biomass than sole NH_4^+ supply, particularly under high-Fe conditions. In *Arabidopsis*, shoot fresh weight under HAHN was 178.01% higher than that under HAHA, while the corresponding increase in wheat was 230.80%. The improvement was also evident in root growth and chlorophyll accumulation under several Fe–N combinations. These results suggest that the presence of different N forms across root compartments can modify plant growth responses under heterogeneous Fe conditions.

Several mechanisms reported in previous studies could potentially contribute to these responses. The beneficial effects of spatially separated NH_4^+ and NO_3^- supply may result from complementary physiological functions of NH_4^+ and NO_3^- [43]. NH_4^+ assimilation can impose substantial carbon and proton-balancing demands, whereas NO_3^- uptake and metabolism can influence rhizosphere pH and carbon metabolism [6,40,44,45]. Nitrate also acts as a signaling molecule that can affect root development and root system architecture [22,23,46–48]. These processes provide plausible explanations for why the presence of NO_3^- in one root compartment was associated with greater growth under some Fe conditions. Accordingly, the observed growth differences may reflect the combined effects of N form on rhizosphere processes, carbon metabolism, and root developmental responses, although the relative contribution of these processes remains to be resolved.

4.4. Species-Specific Differences Shape Responses to Fe–N Heterogeneity

Although both *Arabidopsis* and wheat showed clear responses to Fe–N treatments, the magnitude and spatial pattern of their responses differed. *Arabidopsis* showed greater sensitivity of biomass and chlorophyll traits to N form under high-Fe conditions, whereas wheat exhibited stronger spatial differentiation in root growth. These differences may partly reflect variation in Fe acquisition strategy. Wheat, as a Strategy II species, acquires Fe predominantly through phytosiderophore-mediated mechanisms, whereas *Arabidopsis*, as a Strategy I species, relies primarily on rhizosphere acidification and ferric reduction [49]. However, because Fe uptake, Fe transporter expression, rhizosphere pH, and other signaling processes were not measured, the mechanisms responsible for the species-specific responses remain unresolved. Moreover, differences in growth rate, root architecture, developmental characteristics, and N demand could also contribute to the contrasting responses.

The stronger spatial differentiation of root growth observed in wheat is consistent with pronounced root plasticity under heterogeneous nutrient conditions. Previous studies have shown that localized nutrient availability can modify root development and that local nutrient responses may be coordinated with whole-plant responses [22,25,50,51]. Such processes could potentially contribute to the spatially differentiated root growth observed here. The present findings therefore highlight phenotypic differences between *Arabidopsis* and wheat while providing a basis for future studies that directly examine the physiological and molecular mechanisms underlying these responses.

4.5. Local Heterogeneity Is Reflected in Whole-Plant Growth Responses

The split-root system provides a useful framework for examining how spatially separated nutrient conditions are reflected in whole-plant growth. In the present study, plants exposed to heterogeneous Fe–N conditions generally showed whole-plant phenotypes that differed from those observed under homogeneous nutrient conditions. In wheat, for example, heterogeneous treatments produced distinct patterns of root growth between the two compartments, while shoot biomass showed a whole-plant response to the combined conditions experienced by the two root compartments. These observations indicate that local differences in Fe availability and N form can be expressed at both the root and whole-plant levels.

Nitrate-responsive pathways, hormonal signals, and changes in nutrient transport have all been proposed to contribute to local–systemic coordination [26,52–55]. These processes could potentially contribute to the whole-plant responses observed in the present study. Importantly, the responses observed here also indicate that plant responses to nutrient heterogeneity cannot be predicted from Fe availability or N form considered independently. In wheat, the direction of root growth varied with the local combination of Fe availability and N form, whereas shoot and chlorophyll responses differed among homogeneous and heterogeneous treatments. This suggests that Fe–N interactions should be considered when evaluating plant responses to spatially variable nutrient environments.

4.6. A Working Model of Plant Responses to Heterogeneous Fe–N Supply

Based on the phenotypic responses observed in this study, we propose a working model for plant responses to heterogeneous Fe–N supply. The main effects of Fe availability, N form, and their interaction on different physiological traits were analyzed using two-way ANOVA (Supplementary Table S1). Under high-Fe conditions, sole NH_4^+ supply was associated with reduced shoot and root growth in both *Arabidopsis* and wheat, whereas the presence of NO_3^- in one root compartment was associated with greater plant growth. Under heterogeneous conditions, wheat additionally showed spatially differentiated root growth, with the direction of root elongation depending on the local combination of Fe availability and N form. *Arabidopsis*, in contrast, showed stronger differences in whole-plant biomass and chlorophyll responses to N form.

Together, these observations support a phenotypic framework in which Fe availability and N form jointly shape plant growth responses, while spatial heterogeneity further modifies root growth patterns. The proposed model is intended as a working interpretation of the observed phenotypes rather than a mechanistic regulatory model. The physiological and molecular processes underlying these responses, including Fe and N uptake, rhizosphere pH regulation, hormone signaling, and long-distance nutrient signaling, remain to be tested directly.

5. Conclusions

This study examined plant responses to heterogeneous Fe availability and N forms using a split-root system with *Arabidopsis* thaliana and wheat (Figure 8). The results show that plant responses to Fe availability depended strongly on N form. Under high-Fe conditions, sole NH_4^+ supply was associated with pronounced reductions in shoot and root growth in both species, whereas supplying NO_3^- to one root compartment was associated with substantially greater biomass and chlorophyll accumulation. These responses indicate that Fe availability alone is insufficient to predict plant growth responses when different N forms are present.

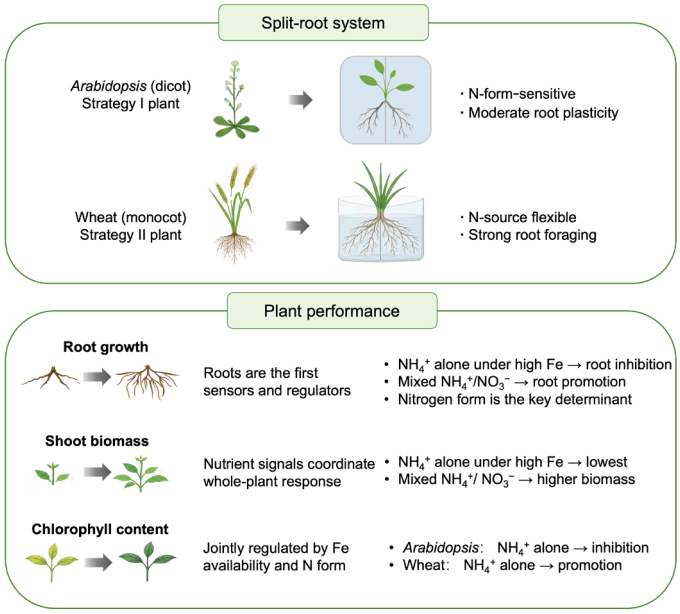

Figure 8. Summary schematic of a working model illustrating plant responses to heterogeneous Fe–N supply in *Arabidopsis* and wheat under split-root conditions.

The two species also differed in their responses to spatially heterogeneous Fe–N conditions. *Arabidopsis* showed pronounced changes in whole-plant growth and chlorophyll traits in response to N form, whereas wheat exhibited stronger spatial differentiation in root growth. In wheat, the direction of root growth varied with the local combination of Fe availability and N form, demonstrating phenotypic root plasticity under heterogeneous conditions.

Overall, our findings identify nitrogen form as an important modifier of plant responses to Fe availability and highlight the importance of considering Fe–N interactions at the root-zone scale. The present study provides phenotypic evidence that spatially heterogeneous Fe–N supply can influence both root growth patterns and whole-plant performance. However, the underlying physiological and molecular mechanisms, including Fe and N uptake, rhizosphere pH regulation, and systemic signaling, remain to be determined. These findings highlight that plant responses to Fe availability should be evaluated in the context of both N form and spatial nutrient heterogeneity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nitrogen7030102/s1, Supplementary Table S1. Two-way ANOVA results for homogeneous Fe-N treatments.

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