Original Article

Different nitrogen acquirement and utilization strategies might determine the ecological competition between ferns and angiosperms

Running title: Differences in nutrient characteristics between ferns and angiosperms

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● **Background and Aims** The abundance or decline of fern populations in response to environmental change has been found to be largely dependent on specific physiological properties that distinguish ferns from angiosperms. Many studies have focused on water use efficiency and stomatal behaviors, but the effects of nutrition acquirement and utilization strategies on niche competition between ferns and flowering plants are rarely reported.

● **Methods** We collected 34 ferns and 42 angiosperms from the Botanic Garden of Hokkaido University for nitrogen (N), sulfur (S), NO_3^-, and SO_4^{2-} analysis. We then used a hydroponic system to compare the different N and S utilization strategies between ferns and angiosperms in N deficiency conditions.

● **Key Results** Ferns had a significantly higher NO_3^--N concentration and NO_3^--N/N ratio than angiosperms, although the total N concentration in ferns was remarkably lower than in the angiosperms. Meanwhile, a positive correlation between N and S was found, indicating that nutrient concentration is involved in assimilation. *Pteris cretica*, a fern species subjected to further study, maintained a slow growth rate and lower N requirement in response to low N stress, while the biomass and N concentration in wheat (*Triticum aestivum*) both quickly responded to N deficiency conditions.

● **Conclusions** The different nutritional strategies employed by ferns and angiosperms mainly depended on the effects of phylogenetic and evolutionary diversity. Ferns tend to adopt an opportunistic strategy of limiting growth rate to reduce N demand and store more pooled nitrate, whereas angiosperms likely utilize N nutrition to ensure as much development as possible under low N stress. Identifying the effects of mineral nutrition on the evolutionary results of ecological competition between plant species remains a challenge.

Key words: ferns, angiosperms, nitrogen, sulfur, ecological competition, malnourished stress, nitrogen deficiency.

INTRODUCTION

Ferns, as with vascular plants, likely appeared in the Devonian and were widespread during the Mesozoic before angiosperms came to dominate the terrestrial ecosystem by the end of the Cretaceous period (Kenrick and Crane, 1997). Many studies indicate that angiosperms likely replaced ferns due to the change in atmospheric conditions and the plants' different corresponding survival strategies (Brodribb and Cochard, 2009, Flexas and Keeley, 2012). Ferns emerged under an atmosphere with tenfold higher than present atmospheric CO_2 concentration and were eclipsed by angiosperms when atmospheric CO_2 concentration dropped to two- or threefold higher than the present (Haworth et al., 2011). Amatangelo and Vitousek (2008) indicated the plant groups could replace the ecological niche of other populations as habitat dominants in environmental change. Higher CO_2 concentrations undoubtedly increase the photosynthetic capacity and biomass of plants, but the effects of CO_2 on plant biomass can be restricted by many factors such as water and mineral nutrition (Kramer, 1981). In addition, uncontrolled photosynthesis and biomass accumulation under high CO_2 conditions can lead to malnutrition of over-grown plants. In a study by Carriquí et al., (2015), it was found that the photosynthetic capacity of ferns is about half that of angiosperms. Yang et al. (2021) show that ferns tend to maintain lower photosynthetic water use efficiency under water deficit conditions compared to seed plants. Therefore, ferns may conquer deficiencies in growth-limiting major nutrients by adopting lower nutrient assimilation efficiency. If ferns use this strategy, then this adaptation could be

a supplementary reason for why ferns were succeeded by angiosperms during ancient changes in atmospheric conditions.

Nitrogen (N) has been identified as the most abundant nutritional component of cellular metabolites in all living organisms and is a critical limiting factor in plant growth and nutritional status (Ågren and Weih, 2012, Wang et al., 2017). N is required for the synthesis of amino acids, proteins, chlorophyll, nucleic acids, and a variety of other metabolites. After nitrate is absorbed by plants, the assimilation of N requires the reduction of nitrate to ammonium, followed by ammonium assimilation into organic compounds (Hawkesford et al., 2012). Therefore, N utilization is one of the most important indexes to reflect the nutritional strategy of plants. Nitrogen-limiting conditions promote leaf senescence, lowering yield and biomass in plants (Kant et al., 2011). Crops, such as rice and wheat, are very sensitive to N deficiency, while growth of fern is proved rarely to be limited by N by a survey in montane forest (Wegner et al., 2003). To avoid N deficits in agriculture and their accompanying loss of yield, nitrogenous chemicals are used as fertilizer, with increasing dosages in recent decades (Tilman et al., 2002). However, the large application of N chemical fertilizer is facing a serious dilemma. More than 50% of the applied N is not accessible to the plants and leaches into the environment, but farmers have to apply excess N fertilizer to ensure yield (Coskun et al., 2017). Therefore, investigating N usage in ferns may add to our understanding of the effect of nutritional condition on species succession as well as provide a reference for developing N deficiency tolerance in plants. Sulfur (S) also plays a significant role in protein synthesis and metabolism, that is essential for plant

biomass accumulation. Meanwhile, S and its metabolites have function on the resistance to abiotic and biotic stress (Davidian and Kopriva, 2010). Therefore, we also considered S behavior when studying the nutritional response to N deficiency.

We consequently compared the differences in N and S uptake and assimilation among 34 fern and 42 angiosperm species cultivated in the Botanic Garden of Hokkaido University. Subsequently, two common and representative species were selected (see section Materials and Methods) and grown in N-deficient conditions to compare the different N utilization strategies between ferns and angiosperms, study the evolutionary result of niche competition and trade-offs between ferns and angiosperms, and gain insight into low N tolerance in plants.

MATERIALS AND METHODS

Fieldwork experiment: leaf sampling

The field investigation was conducted in the Botanic Garden of Hokkaido University, founded in 1886, covering *c.* 13.3 ha in the middle of Sapporo, Japan (43°03′N–43°06′N, 141°19′E–141°21′E). Contents (μg g^{-1} soil dry weight) of total N, NH_4-N, NO_3-N, and Truog-P in soil are 1980 ± 71, 28.9 ± 5.1, <5 (little detected), and 6066 ± 174, respectively, and additional chemical properties are listed in our previous study (Watanabe and Azuma, 2021). At least three mature leaf samples from living plant material were carefully collected using ceramic scissors, in the morning, on different dates of 2009 (June 26, July 2, August 12) and 2010 (July 7). Data on leaf longevity

and plant growth period were not available; we distinguished adult plants from young plants by the presence of senescent leaves in the former. Fully developed mature leaves of adult plants were considered to be those located in the middle and bottom of the phyllotaxis, while old leaves were on the ground (abscission), and young leaves were on the tips (smaller and lighter colored). The collection standard of leaf samples consequently guaranteed the optimal status of nutrient uptake and metabolism in different plant species in this survey.

In total, we have collected 76 leaf samples, representing 34 species from 21 families of ferns (Table S1) [Supplementary Information] and 42 species from 40 families of angiosperms (Table S2) [Supplementary Information]. The classifications and Latin names of the angiosperm species were obtained from the Angiosperm Phylogeny Website (http://www.mobot.org/MOBOT/research/APweb/), whereas fern classification was conducted following the classification of PPG I (PPG, 2016). At Hokkaido University, all leaf samples were packaged in kraft bags, labeled, dried in an oven at 70°C for 7 days, and ground for further quantitative analysis.

Hydroponic experiment: plant materials and treatments

To compare the differences in nutrient acquirement and utilization, we selected a common fern and angiosperm for a hydroponic experiment. The bracken *P. cretica* (*Pteris cretica* L. var. *Albolineata*) (A fern species with white-striped leaf, see Fig. S1) [Supplementary Information] was selected to represent ferns because this species is

easy to obtain and hydroponic cultivation systems have already been established (Watanabe et al., 2009). Seedlings of *P. cretica* were purchased from a gardening store. For angiosperms, wheat (*Triticum aestivum* (L.) Thell. cv. Haruyokoi), an artificially selected crop with a high demand for soil fertility, was selected. Seedlings of *P. cretica* were pre-cultured in a pot with mixed horticultural soil (Kanuma soil/Akadama soil = 1:1). Wheat seeds were surface disinfected with sodium hypochlorite (NaClO, 0.5%–1% available chlorine) for 10 min, washed with deionized water, and then germinated in trays filled with moist perlite for 24 h. After germination, the wheat seedlings were transplanted into a pot with a growth substrate (perlite/vermiculite = 1:1) for 7 d with daily watering by distilled water.

After pre-culture, *P. cretica* and wheat seedlings were transferred to a polypropylene container with 30 L of standard nutrient solution for 7 d so that plants could establish a healthy inner nutritional status and subsequently be measured for a well-response to N deficiency. The nutrient solution was set by following former formula (Watanabe et al., 2014): 2.14 mM N (NH_4NO_3), 0.32 mM P ($NaH_2PO_4 \cdot 4H_2O$), 0.77 mM K (K_2SO_4: KCl = 1:1), 1.25 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.82 mM Mg ($MgSO_4 \cdot 7H_2O$), 35 μM Fe (Fe-EDTA), 9.1 μM Mn ($MnSO_4 \cdot 5H_2O$), 46 μM B (H_3BO_4), 3.1 μM Zn ($ZnSO_4 \cdot 7H_2O$), 0.16 μM Cu ($CuSO_4 \cdot 5H_2O$), and 0.05 μM Mo [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]. The seedlings were subsequently transplanted to a lightproof plastic pot with a five-hole lid. Two plant seedlings per hole were fixed by sponge. Each pot contained 4 L of standard nutrient solution; in total, five different N treatments were performed in the hydroponic system: no N supply (N0), 1/80-strength N supply (N1),

1/20-strength N supply (N2), 1/4-strength N supply (N3), and full-strength N supply (CK). The solution was renewed every 7 d, and pH was adjusted to 5.5 ± 0.5 with 1 mM NaOH or 1 mM HCl every day. Three repetitions were performed, and the hydroponic system was continuously aerated. All experiments were conducted in a glasshouse with 14 h photo-duration (350-400 μmol m^{-2} s^{-1}) and day/night temperature of 25–28/18°C–22°C, respectively, at Hokkaido University, Sapporo, Japan. Due to their different growth rates, *P. cretica* and wheat were treated for 2 months and 14 d, respectively. Then, seedlings were separated into shoots and roots, lyophilized, weighed, and ground for further measurements.

Chemical analysis

50 mg of powdered plant samples was digested with 1.25 ml of 18 M sulfuric acid (H_2SO_4) and analyzed for total N using the Kjeldahl method (K-350 Distillation Unit, BUCHI Labortechnik AG, Switzerland). Another *c.* 50 mg of sample was weighed and added to 2 ml 61% HNO_3 (EL grade; Kanto Chemical, Tokyo, Japan) for S analysis by ICP-MS (Elan, DRC-e; PerkinElmer, Waltham, MA, USA). A *c.* 10 mg of powdered sample was weighed, suspended in 1.5 ml deionized water in a centrifuge tube and shaken for 30 min. The mixture was then centrifuged (12,000 rpm, 4°C) for 10 min. The supernatants were filtered using a 0.45 μm disposable membrane filter (Toyo Roshi Kaisha, Ltd., Japan) and diluted tenfold to determine the NO_3^--N and SO_4^{2-}-S concentrations by capillary electrophoresis (CE) (Quanta 4000 CE; Waters, Milford, MA).

Statistical analysis

ANOVA was performed to test the significant differences between fern and angiosperm, as well as different N treatments. The correlation coefficients were determined using the Pearson's correlation method. All statistical analyses and visualizations were performed using the Microsoft Office Excel 2010 and Minitab 19 program (Minitab Inc., State College, PA, USA).

RESULTS

Comparisons of leaf N and S characteristics between ferns and angiosperms in the field

Ferns and angiosperms differed significantly in their foliar accumulation of N, NO_3^--N, and SO_4^{2-}-S, but not for S (Fig. 1). Leaf N concentration in angiosperms was significantly higher than that of ferns (Fig. 1A), while foliar anion accumulation of both NO_3^--N and SO_4^{2-}-S was significantly less in angiosperms than in ferns (Figs. 1C–D). Accordingly, the proportion of NO_3^--N in total N in ferns is significantly higher than that observed in angiosperms, but there is no statistical difference in the ratio of SO_4^{2-}-S to S between ferns and angiosperms (Figs. 1E–F).

To identify the differences in interactions of N, S, SO_4^{2-}-S, and NO_3^--N between ferns and angiosperms, we performed Pearson's correlation analysis (Fig. 2). Generally, the correlations of these elements differed significantly between ferns and angiosperms, but a similar significant positive correlation between N and S of about 0.4 was observed in both phyla ($p < 0.05$). Unexpectedly, there was no significant correlation between N

and NO_3^--N or S and SO_4^{2-}-S in ferns, while N and S significantly positively correlated with their corresponding anions NO_3^--N and SO_4^{2-}-S, respectively, in angiosperms. Moreover, a positive significant correlation ($p < 0.01$) between S and NO_3^--N was detected in ferns.

Plant biomass in hydroponics

In the hydroponic experiment, the shoot biomass of both *P. cretica* and wheat decreased with the decline in N supply (Figs. 3A-B). Wheat shoot biomass was reduced by over 50% under N3 and 75% under other N treatments compared to normal N supply (CK), while shoot biomass of *P. cretica* was no different under N3 and decreased no more than 50% under other treatments. No significant difference between root biomass of *P. cretica* under different N treatments was observed, with the exception of N1 (Fig. 3C). However, wheat root biomass has markedly increased with the increase of N supply from N0 to CK, although there was a drop of 27% from N3 to CK (Fig. 3D). The root mass ratio between *P. cretica* and wheat was significantly different. As shown in Fig. 3e, the root mass ratios of wheat were higher than that of *P. cretica* under N0-N3, while the reverse was true in the CK treatment.

Concentrations of N, NO_3^--N, S, and SO_4^{2-}-S in hydroponics

Concentrations of N, NO_3^--N, S, and SO_4^{2-}-S in the shoots and roots of *P. cretica* and wheat are shown in Figs. 4a–d. N concentration in *P. cretica* and wheat in both shoot and root was noted to increase with the increase in N supply, from N0 to CK, except

for *P. cretica* roots in the N3 treatment, which had the highest concentration (Fig. 4A). Meanwhile, N concentrations in wheat shoots and roots were determined to be significantly higher than those of *P. cretica* under CK treatment, but N concentrations in *P. cretica* roots were higher than those of wheat under N0 to N3. Similar to N, the NO_3^--N concentrations generally showed an increasing trend with the rise in applied N, excluding the shoots of *P. cretica*. However, the NO_3^--N concentration in *P. cretica* roots was markedly lower under N3 relative to all other N treatments (Fig. 4B). S concentration in wheat shoots was highest under N3 treatment, while that of *P. cretica* was lowest under N1 and N2 (Fig. 4C). Meanwhile, S concentration in *P. cretica* roots did not change under different N treatments, but S concentration did increase in wheat roots exposed to higher N applications. As shown in Fig. 4d, the changes in SO_4^{2-}-S concentrations in wheat shoots and roots were similar to the S concentrations, and the highest value was observed under N3 treatment. The SO_4^{2-}-S concentrations in *P. cretica* shoots showed no significant difference among N supply treatments and in roots and were lowest under CK treatment.

Translocations of N, NO_3^--N, S, and SO_4^{2-}-S in hydroponics

The translocation abilities of N, NO_3^--N, S and SO_4^{2-}-S from roots to shoots were estimated using the translocation factor ($TF_{root/shoot}$), which was calculated by dividing the element concentration in shoots by that in roots (Fig. 5). Low N treatments had no effect on the $TF_{root/shoot}$ ratio of N in *P. cretica*, but an increase in wheat was observed (Fig. 5A). In contrast, the $TF_{root/shoot}$ ratio of NO_3^--N was the opposite; *P. cretica*

translocated more NO_3^--N from roots to shoots under low N treatment, while no change was observed for wheat among different N treatments (Fig. 5B). The profile of $TF_{root/shoot}$ of S was similar to that of N, i.e., there was no observed change in *P. cretica* and the ratio was significantly lowest under the CK treatment in wheat (Fig. 5C). However, the $TF_{root/shoot}$ ratio of N in *P. cretica* showed generally lower than that in wheat. The $TF_{root/shoot}$ ratio of SO_4^{2-}-S in wheat showed no change among different N treatments, while that in *P. cretica* increased with more N supply and was highest under CK (Fig. 5D).

DISCUSSION

Across all plant samples collected, we observed a marked difference ($p < 0.05$) in N, NO_3^--N, and SO_4^{2-}-S concentrations but no difference in S concentration between fern and angiosperm leaves (Fig. 1), indicating there may be different strategies for N nutrition and N-S homeostasis between ferns and angiosperms. Our results were similar to the N and S concentrations between ferns and angiosperms observed in a previous study (Zhang et al., 2012) (Fig. 1), where N concentration in angiosperms was determined to be significantly ($p < 0.001$) higher than in ferns, while there was no marked difference ($p = 0.898$) in S concentration. In addition, the dataset in Zhang et al., (2012) was obtained from a literature collection with different mean annual temperature and precipitation. Thus, the environment-induced differences in N acquirement strategies are likely confined to the phylogenetic differences between ferns

and angiosperms (Watanabe and Azuma, 2021, Watanabe et al., 2016, Zhang et al., 2021), although environmental factors are very important to leaf elemental composition (Roumet et al., 2016, Zhao et al., 2017). The data are consistent with a common notion that total N concentration in fern leaves is lower than in angiosperm plants (Richardson and Walker, 2010). Interestingly, the concentrations of NO_3^--N and SO_4^{2-}-S, especially the former, in ferns were significantly higher than in flowering plants (Fig. 1). After uptake, inorganic N and S are rapidly assimilated into amino acids by enzymes, and this process normally occurs in the leaf due to photosynthetic energy and the presence of several reductases in chloroplasts (Iqbal et al., 2019). When N and S nutrition exceed assimilation into proteins, excess N and S are stored as NO_3^--N and SO_4^{2-}-S, respectively (Millard, 1988, Rennenberg, 1984). Therefore, our data indicate that N requirements in ferns are lower than that of angiosperms. Moreover, the much higher ratio of NO_3^--N/N in ferns than that in angiosperms suggests that ferns tolerate more NO_3^--N accumulation to establish a N pool for when N deficiency conditions exist (Fig. 1E). Although there exists no significant difference in S concentration or the ratio of SO_4^{2-}-S/S between ferns and flowering plants, a much higher concentration of SO_4^{2-}-S in ferns is likely due to limitations in protein production associated with N (Rennenberg, 1984).

A significant positive correlation between leaf N and S in both ferns and angiosperms provided additional evidence that the synergistic accumulation effects on N and S were common within species (Fig. 2). However, in contrast to flowering plants, there was no correlation between N and NO_3^--N as well as S and SO_4^{2-}-S in fern leaves,

indicating that the transformation strategies of inorganic nutrients to organic compounds varied among fern species. Although fern diversity is lower than that of angiosperms, the diversification of ferns is likely an opportunistic response to angiosperm dominance in terrestrial ecosystems (Schneider et al., 2004, Schuettpelz et al., 2009), and can also affect development of gymnosperms (Coomes et al., 2005). Ferns may develop diverse nutrition utilization strategies to survive a symbiotic habitat with other plant species. Competition may even drive the evolution of many ferns toward traits of angiosperm (Jones et al., 2007). Therefore, the N concentration in some ferns were showed higher than that in some angiosperms. Due to potential lower N requirements and assimilation, as well as a more variable nutritional metabolic strategy, we hypothesized that ferns adapt to N-deficient environments more easily compared to angiosperms and provided a perspective of plant nutrition to explain why ferns are able to dominate the planet under high CO_2 levels with limited soil nutrients.

To test this, we conducted a hydroponics experiment to elucidate the interspecific diverse nutritional responses of *P. cretica* (fern) and wheat (angiosperm) to N deficiency. As most fern species live in the forest understory, they have on average a lower value of leaf mass per unit area (LMA) and lower photosynthetic capacity compared to flowering plants (Karst and Lechowicz, 2007). The photosynthetic effectiveness in *P. cretica* with white-striped leaf (see Fig. S1) may be limited. Therefore, we conclude that the comparison of the nutritional characteristics between photosynthetically limited fern and photosynthetically optimized monocot crop might be biased, and is not suitable for generalization between ferns and angiosperms, but we consider that these

differences are still important because ferns with white-striped leaf are likely forced to live in the shade by competing with angiosperms, perhaps as a strategy of reducing photosynthesis and nutrient utilization. In terms of plant biomass, wheat was more sensitive than *P. cretica* to low N conditions, especially in shoot growth (Figs. 3A–D). Plant shoots (especially leaves) require large amounts of N to synthesize the key photosynthetic enzyme ribose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco, which utilizes more than 25% of N input and accounts for 50% of the soluble protein in plant leaves, establishes a relationship between N and shoot biomass (Parry et al., 2013). Roots act as a signal acceptor and transmitter to alter plants' adaptation strategies to different levels of environmental nutritional stress (Gruber et al., 2013, Rellán-Álvarez et al., 2016). Therefore, the biomass trend in roots under different N deficiency conditions in both plant species confirmed that mild nutrient limitations can regulate root growth and root mass ratio. Root growth was stimulated under a mild N deficiency, whereas stunted growth occurred in very low N conditions (Gruber et al., 2013, Lambers et al., 2006, Giehl and von Wirén, 2014). In the hydroponic experiment, the root mass ratio showed almost no significant difference among low N treatments N0 to N3 due to the more sensitive decrease in shoot biomass (Fig. 3E). Therefore, *P. cretica* and wheat seemingly adopted similar survival strategies under low N conditions that prioritized root growth to better acquire nutrients (Alves Negrini et al., 2020). However, *P. cretica* was more tolerant to N deficiency than wheat. The slow growth rate likely explains the higher tolerance of ferns to N-poor conditions and thus may be a result of niche competition with rapid-growing flowering plants (Page, 2002, Meerts,

1997). Slow growth may also be related to the different N absorption and utilization strategies in ferns compared to angiosperms under N deficiency.

Wheat showed a much higher N concentration in shoots than *P. cretica* under CK, although the latter had been growing in nutrient solution for a longer time (Fig. 4A). Moreover, due to the large difference between N and NO_3^--N concentration, the N assimilation with normal N supply in wheat was much higher than in *P. cretica*, consistent with the conclusion in the field study (Fig. 1). The concentrations of N and NO_3^--N in *P. cretica* declined more smoothly than that in wheat under low N stress (Fig. 4A-B). Consistent with the biomass results (Fig. 3), *P. cretica* showed a higher tolerance to low N stress or a lower N requirement for growth. Roots are the major anchoring organ for resource acquirement and storage (Kramer-Walter et al., 2016, Weemstra et al., 2016), and they determine the nutrient composition and profile of the shoot. However, NO_3^--N concentrations in shoots of *P. cretica* were robust under different N treatments while drastic changes occurred in the roots (Fig. 4B). An important nitrate transporter, NRT1.1 (NITRATE TRANSPORTER 1), can be triggered as a sensor by an external nitrate signal (Ho et al., 2009, Vidal et al., 2020, Zhang et al., 2020) to stimulate nitrate-related gene expression and root growth in plants (Bouguyon et al., 2015, Bouguyon et al., 2016). Therefore, it is possible that the expression of NRT1.1 in wheat is more sensitive to changes of external N signal than that in *P. cretica* to rapidly adjust N homeostasis in shoots. Furthermore, a lower $TF_{root/shoot}$ ratio of N and NO_3^--N in *P. cretica* than that in wheat under all N treatments indicates that NO_3^--N transporter expression in *P. cretica* was generally lower than that in wheat, and this is consistent

with the minimal changes in root biomass observed in *P. cretica* (Fig. 3C). Moreover, more NO_3^--N was translocated into the shoot, but the $TF_{root/shoot}$ ratio of N was stable in *P. cretica* under low N treatments (Figs. 5A-B), confirming that *P. cretica* likely inhibited N assimilation in shoots to tackle low N stress, while wheat probably took an opposite strategy and utilized restricted external N for as much protein production as possible. In general, angiosperms, living in a dominant niche, adopt more aggressive nutrient acquisition strategies than ferns, which often fill ecological niche gaps.

A large number of studies have confirmed a strong, interactive link between uptake and assimilation of N and S in plants. For example, cysteine, as a S metabolite, is largely dependent on adequate N availability (Hesse et al., 2004, Koprivova et al., 2000, Davidian and Kopriva, 2010). In this present study, the S and SO_4^{2-}-S concentrations in wheat generally maintained a high value under N3 treatment, much higher even than under CK (Figs. 4C–D). As such, it is likely that the mild low N treatment in N3 stimulated wheat root growth and root mass ratio (Fig. 3D). Consequently, the effect of mild N deficiency on root growth stimulation is likely a crucial reason for S accumulation, not only the co-regulation of proteins between these two elements (Hesse et al., 2004). However, N deficiency increased SO_4^{2-}-S concentration in *P. cretica* roots, but the S concentrations in roots showed no change (Figs. 4C–D). Cysteine biosynthesis is a common junction of N and S assimilation, and the related enzymes interact in the regulation of N and SO_4^{2-}-S (Kopriva and Rennenberg, 2004). Excess cysteine can repress the uptake and assimilation of S under low N conditions (Zhao et al., 1999). *P. cretica* may have absorbed excess SO_4^{2-}-S and thus strengthening the NO_3^--N uptake,

but sulfide transformation seems inhibited. Therefore, *P. cretica* likely adopted the strategy of fixing more SO_4^{2-}-S in roots and limiting SO_4^{2-}-S transportation to shoots (Fig. 5D).

In conclusion, we found different strategies for N and S uptake and utilization between ferns and angiosperms, which depended on the effects of phylogenetic and evolutionary diversity, which covered environmental influence, resulting in the evolutionary result of ecological competition. *P. cretica* (fern), an opportunist living in ecosystems dominated by angiosperms, likely maintains a slower growth rate to reduce N demand per unit time, and store more NO_3^--N in shoot to tackle low nutrient environments. In contrast, as observed for wheat, angiosperms, the dominant plants in ecology, take up and utilize as much NO_3^--N as possible under low N stress for development. Therefore, when facing natural selection due to climate change, different nutrient strategies largely determined the current angiosperm-dominated and fern-marginalized eco-landscape. The responses of S absorption and assimilation to low N stress are closely associated with these different strategies, and the relevant molecular mechanisms need to be further studied. Therefore, this present study provided new prospects for further studies on the evolutionary results of niche competition and compromise between ferns and angiosperms as well as insight into the development of stress tolerance in ferns.

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Captions for figures

Fig. 1. Boxplot showing leaf concentrations of N (a), S (b), NO_3^--N (c), and SO_4^{2-}-S (d) as well as the ratios of NO_3^--N/N (e) and SO_4^{2-}-S/S (f) between ferns and angiosperms. The t-test of pairwise comparisons was performed: *ns*, *, and ** represent no, significant ($p < 0.05$), and extremely significant ($p < 0.01$) differences, respectively, between ferns and angiosperms.

Fig. 2. Correlations between leaf N, S, SO_4^{2-}-S, and NO_3^--N in fern and angiosperm. The gray and black dots represent ferns and angiosperms, and the gray solid line and black dashed line represent linear regression lines for ferns and angiosperms, respectively. Pearson's correlation analysis was performed, and asterisks represent a significant correlation: *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Fig. 3. Shoot biomass of *P. cretica* (a) and wheat (b), root biomass of *P. cretica* (c) and wheat (d), and root mass ratio (e), under five different N applications (N0, N1, N2, N3, and CK represent no N (0 mM), 1/80 of standard N (0.027 mM), 1/20 of standard N (0.107 mM), 1/4 of standard N (0.535 mM), and full-strength standard N (2.14 mM), respectively). Grey bars represent *P. cretica*, whereas white bars represent wheat, respectively. Error lines on bars were based on standard error (SE). A one-way ANOVA with Tukey method was performed, and different letters (capital letters represent *P.*

cretica, and lowercase letters represent wheat) represent significant differences at $p < 0.05$.

Fig. 4. Concentrations of N (a), NO_3^--N (b), S (c), and SO_4^{2-}-S (d) in shoot (black) of *P. cretica*, root (dark grey) of *P. cretica*, shoot (light grey) of wheat, and root (white) of wheat, under five different N applications (N0, N1, N2, N3, and CK represent no N (0 mM), 1/80 of standard N (0.027 mM), 1/20 of standard N (0.107 mM), 1/4 of standard N (0.535 mM), and full-strength standard N (2.14 mM), respectively), respectively. Error lines on bars were based on standard error (SE). A one-way ANOVA with Tukey method was performed, and different letters (capital letters represent *P. cretica*, and lowercase letters represent wheat) in the same plant organs represent significant differences among treatments at $p < 0.05$.

Fig. 5. Transport factors ($TF_{root/shoot}$) of N (a), NO_3^--N (b), S (c), and SO_4^{2-}-S (d) in *P. cretica* (grey) and wheat (white), under five different N applications (N0, N1, N2, N3, and CK represent no N (0 mM), 1/80 of standard N (0.027 mM), 1/20 of standard N (0.107 mM), 1/4 of standard N (0.535 mM), and full-strength standard N (2.14 mM), respectively). Error lines on bars were based on standard error (SE). A one-way ANOVA with Tukey method was performed, and different letters (capital letters represent *P. cretica*, and lowercase letters represent wheat) in the same plants represent significant differences among treatments at $p < 0.05$.

Captions for supplementary tables

Fig. S1. A photo of *P. cretica*

Table S1. Fern species analyzed.

Table S2. Angiosperm species analyzed.

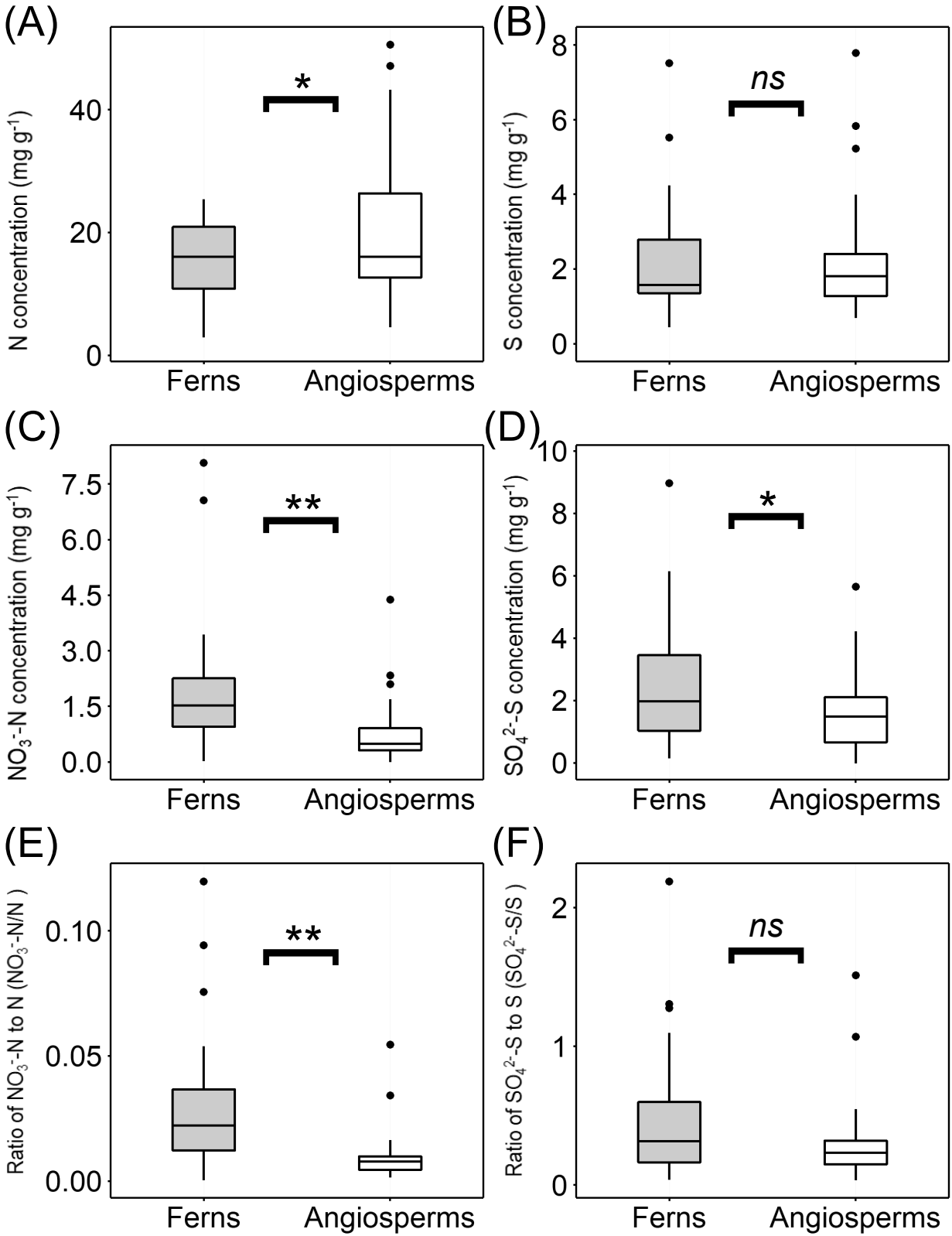

(A)
N concentration (mg g-1)
0
20
40
*
Ferns
Angiosperms
(B)
S concentration (mg g-1)
0
2
4
6
8
ns
Ferns
Angiosperms
(C)
NO3--N concentration (mg g-1)
0.0
1.5
3.0
4.5
6.0
7.5
**
Ferns
Angiosperms
(D)
SO42--S concentration (mg g-1)
0
2
4
6
8
10
*
Ferns
Angiosperms
(E)
Ratio of NO3--N to N (NO3--N/N)
0.00
0.05
0.10
**
Ferns
Angiosperms
(F)
Ratio of SO42--S to S (SO42--S/S)
0
1
2
ns
Ferns
Angiosperms

Fig. 1

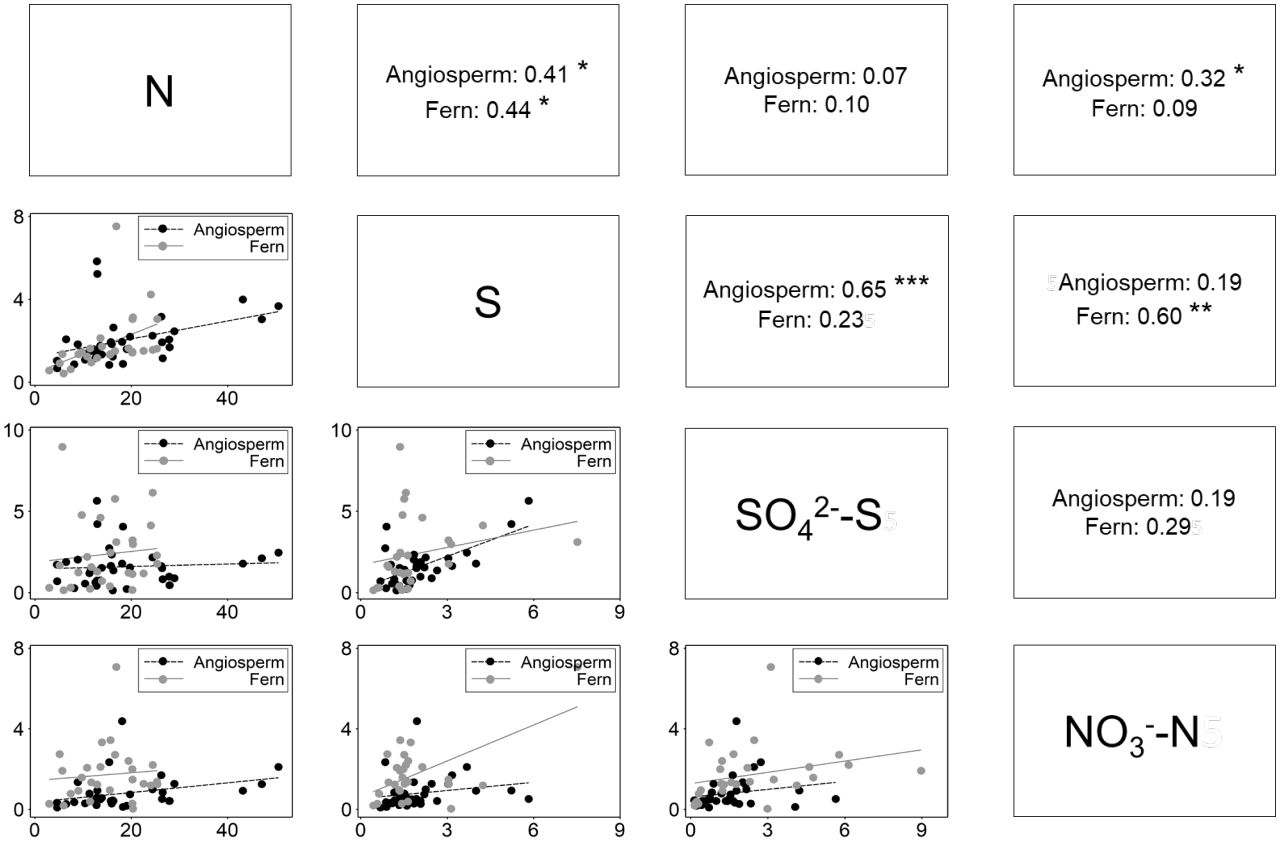
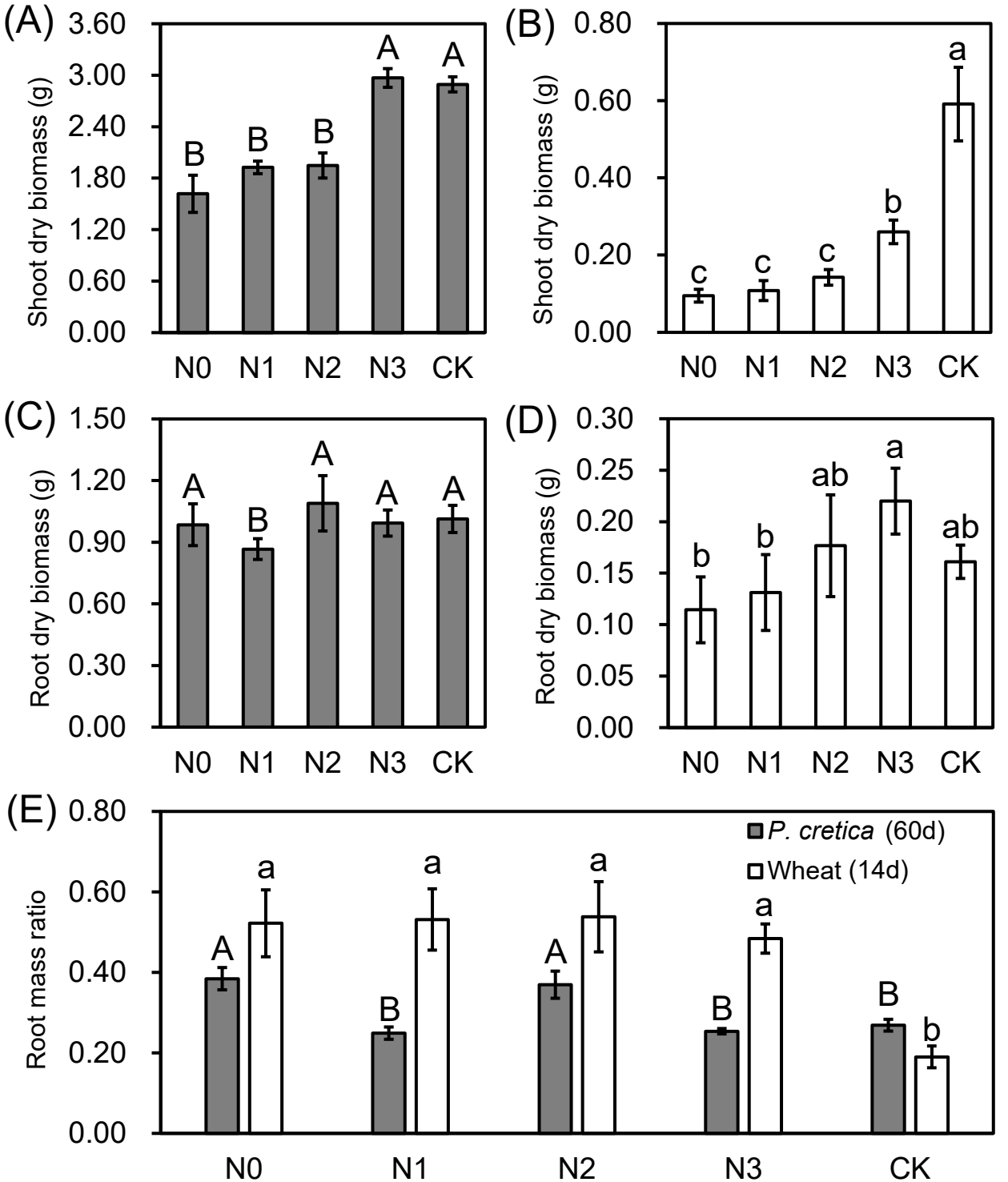
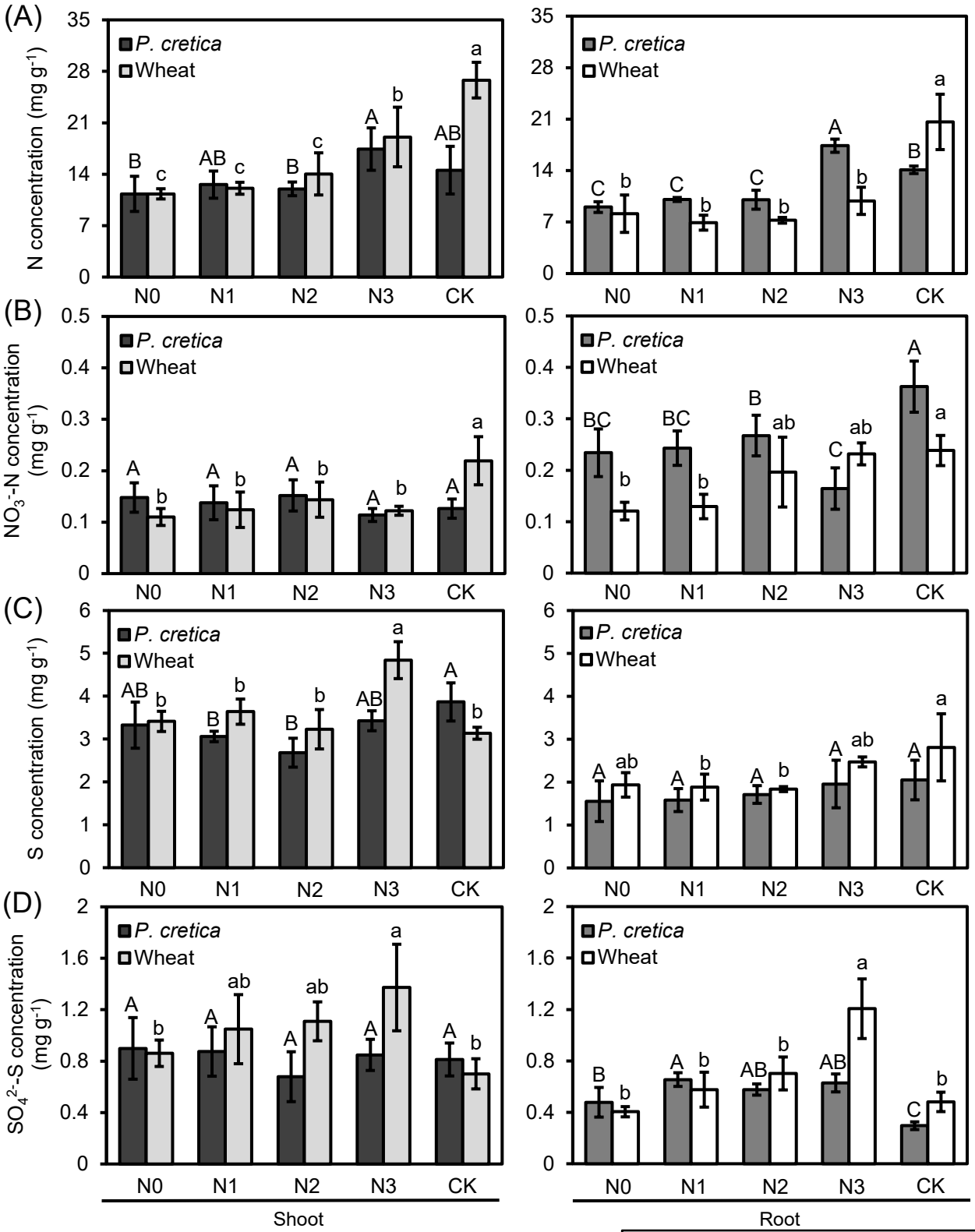

N
Angiosperm: 0.41 *
Fern: 0.44 *
Angiosperm: 0.07
Fern: 0.10
Angiosperm: 0.32 *
Fern: 0.09
S
Angiosperm: 0.65 ***
Fern: 0.23
Angiosperm: 0.19
Fern: 0.60 **
SO_4^{2-}-S
Angiosperm: 0.19
Fern: 0.29
NO_3^--N
Angiosperm
Fern

Fig. 2



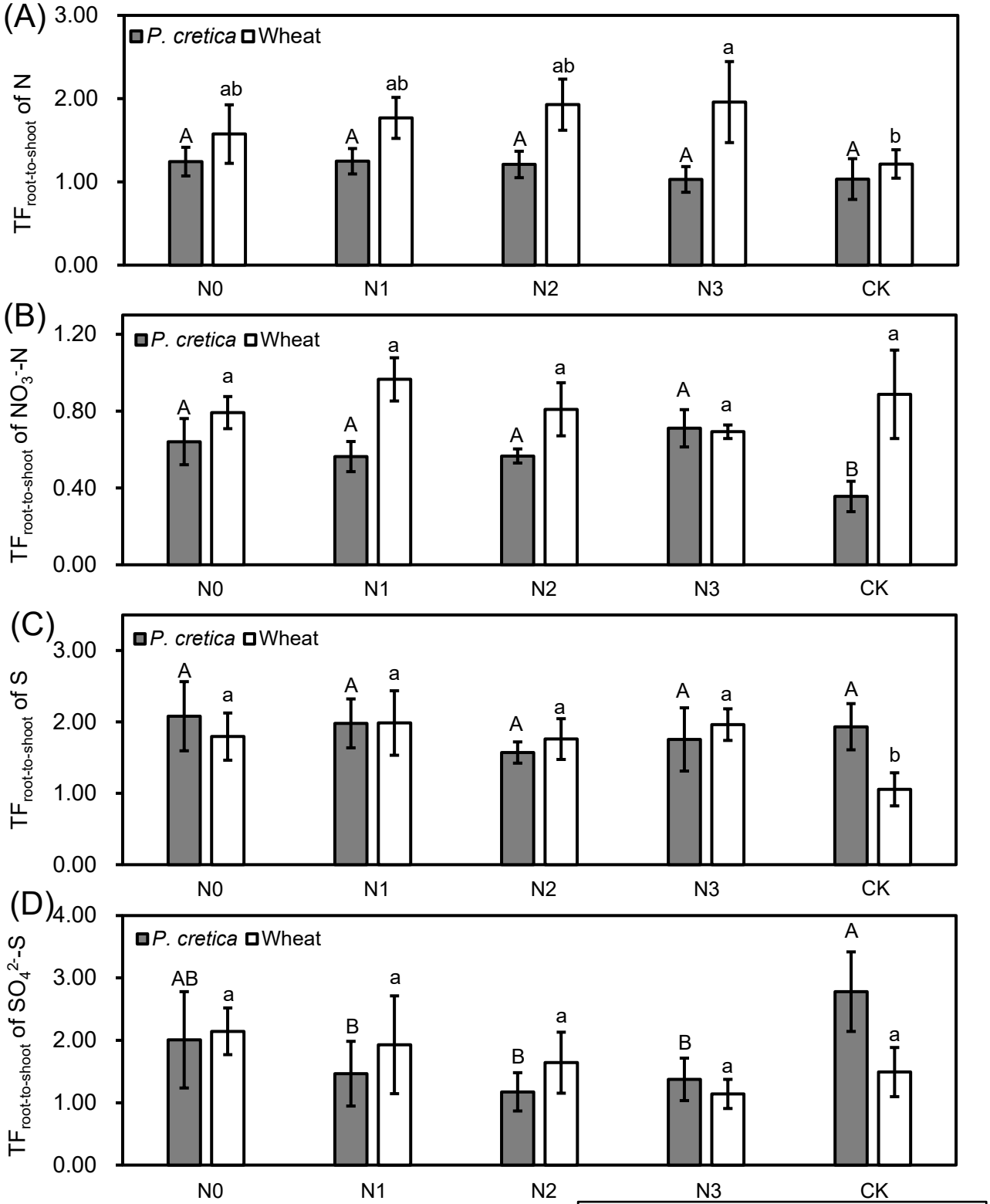
N0 = no N (0 mM)
N1 = 1/80 of standard N (0.027 mM)
N2 = 1/20 of standard N (0.107 mM)
N3 = 1/4 of standard N (0.535 mM)
CK = full-strength standard N (2.14 mM)

Fig. 3



N0 = no N (0 mM)
N1 = 1/80 of standard N (0.027 mM)
N2 = 1/20 of standard N (0.107 mM)
N3 = 1/4 of standard N (0.535 mM)
CK = full-strength standard N (2.14 mM)

Fig. 4



N0 = no N (0 mM)
N1 = 1/80 of standard N (0.027 mM)
N2 = 1/20 of standard N (0.107 mM)
N3 = 1/4 of standard N (0.535 mM)
CK = full-strength standard N (2.14 mM)

Fig. 5