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Author(s)	Fujiishi, Manami; Maejima, Eriko; Watanabe, Toshihiro
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Effect of mixed cropping with lupin (*Lupinus albus* L.) on growth and nitrogen uptake in pasture grasses grown under manure application

Fujiishi, Manami^a, Maejima, Eriko^a, Watanabe, Toshihiro^{a,*}

^a *Research Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kitaku, Sapporo 0608589, Japan.*

* Corresponding author.

E-mail address: nabe@chem.agr.hokudai.ac.jp (T. Watanabe).

1 **Effect of mixed cropping with lupin (*Lupinus albus* L.) on growth**
2 **and nitrogen uptake in pasture grasses grown under manure**
3 **application**

4

5 **Abstract**

6 The use of organic fertilizer is essential to ensure sustainable agricultural production.
7 Because organic fertilizer normally acts as a slow-release fertilizer, improving its
8 nutrient-use efficiency is important, particularly in terms of nitrogen (N) nutrition. In
9 the present study, we attempted to increase the N-use efficiency of cattle farmyard
10 manure (CM) in the cultivation of pasture grasses by mixed cropping with white lupin
11 (*Lupinus albus*), which has been reported to decompose organic N in its rhizosphere.
12 Timothy (*Phleum pratense*) and orchard grass (*Dactylis glomerata*) were cultivated
13 with or without either lupin or soybean (*Glycine max*) in pots under three different N
14 treatments (CM, ammonium sulfate, or no N). In the CM treatment, growth was
15 higher in grasses cultivated with lupin than in those cultivated alone or with soybean.
16 Moreover, decomposition of soluble organic N and protease activity in the
17 rhizosphere soil of grasses with CM treatment were enhanced by mixed cropping with
18 lupin. Analyses of microbial activity and bacterial community structure using Biolog
19 EcoPlates suggested that the enhanced decomposition of soluble organic N was
20 facilitated by lupin roots rather than by rhizosphere microorganisms.

21

22 **Keywords:** *Lupinus albus*; mixed cropping; manure; pasture grass; rhizosphere

23

Introduction

The use of chemical fertilizers has contributed to the development of modern agriculture and to the dramatic increases in crop production. However, excessive application of chemical fertilizers, particularly nitrogen (N) fertilizers, often results in environmental pollution of agricultural land, grassland, and associated surface and ground water (Di & Cameron 2002). Moreover, excessive application of N fertilizer can produce crops with accumulated high levels of nitrate (NO_3^-), which can pose a serious risk to human health (Anjana & Iqbal 2007). Manure is used as a fertilizer as well as a soil amendment to improve the physical, chemical, and biological properties of soil (Drinkwater et al. 1995). However, because manure is a slow-release fertilizer, deficiency of nutrients, particularly N, often occurs during crop cultivation (Pang & Letey 2000). Therefore, improvement of the nutrient-use efficiency of manure is required.

N is one of the most essential nutrients for plant growth. Plants often contain 1%–7% N in their aboveground tissues (Mattson 1980). In soil environments, N occurs in both organic and inorganic forms; however, the majority of soil N is in the organic form. Plants primarily take up the inorganic forms of N such as NO_3^- and ammonium (NH_4^+). Therefore, the mineralization of organic N is important to acquire N in soils available for plant uptake in response to manure application. Organic N is normally mineralized by microorganisms in soils. However, it has been suggested that the roots of some plant species can directly decompose organic N in the rhizosphere (Godlewski & Adamczyk 2007; Paungfoo-Lonhienne et al. 2008). Moreover, plants can also absorb low-molecular-weight organic N compounds, including amino acids, peptides, and proteins (Rentsch et al. 2007; Näsholm et al. 2009), although this is highly dependent on the organic N types and the plant species (Chapin et al. 1993;

49 Kielland 1994; Weigelt et al. 2005). These differences are considered to be related to
50 different growth responses to organic N among different plant species (Okamoto &
51 Okada 2004).

52 Lupin (*Lupinus albus* L.) is an annual legume grown in temperate regions, and
53 its seeds are rich in nutritive value for livestock because of their high protein content
54 (Vicenti et al. 2009). Lupin is a member of the Fabaceae family and can carry out a
55 mutualistic symbiosis with root-colonizing rhizobial soil bacteria, to fix atmospheric
56 N₂. Lupin is known to have a high tolerance for phosphorus (P) deficiency (Gardner et
57 al. 1982). It has cluster roots, which are bottlebrush-like clusters of rootlets along the
58 lateral roots that are formed under conditions of nutrient deficiency, particularly P
59 deficiency. Cluster roots efficiently exude phosphatase and organic acids to
60 decompose organic P and to solubilize insoluble P, respectively, in the rhizosphere
61 (Neumann et al. 2000). This type of root is widely observed in the Proteaceae and is
62 known to possess similar mechanisms to lupin (Watt & Evans 1999). Moreover, in
63 *Hakea actites* (Proteaceae), N deficiency induces the formation of cluster roots, which
64 secrete peptidases or proteases (Schmidt et al. 2003). Because proteinaceous
65 compounds are the major form of organic N in soils (Schulten & Schnitzer 1997), the
66 secreted peptidases may decompose the proteinaceous compounds to a more available
67 N form (e.g., amino acids) for the plant roots. Similarly, the concentration of organic
68 N in the rhizosphere soil of lupin, following organic matter application, decreased in
69 comparison with that of the rhizosphere soil of soybean or unplanted soil (Watanabe
70 et al. 2006), suggesting that lupin can mineralize organic N in its rhizosphere.

71 Mixed cropping or intercropping is a common agricultural practice in which
72 two or more crop species are cultivated together. The effect of mixed cropping varies
73 depending on the combination of plants. For example, mixed cropping with chickpea

has been shown to increase the P uptake of wheat and maize by phosphatase released from the roots of chickpea (Li et al. 2003; 2004). In addition, mixed cropping with maize has been shown to improve the iron (Fe) nutrient status of peanuts, presumably due to the secretion of phytosiderophores from the roots of maize (Zuo et al. 2000). Mixed cropping with lupin has also exhibited positive effects on P and N uptake in other plants (Gardner & Boundy 1983; Cu et al. 2005; Watanabe et al. 2006), possibly due to the enhanced mineralization and/or solubilization of these nutrients in the rhizosphere, as described above. It is possible that the decomposition of organic N is stimulated in the rhizosphere of lupin, which supplies available N to other plant species sharing the rhizosphere with lupin. Protease activity is considered primarily responsible for the decomposition of organic N in soil. However, because soil microbes secrete proteases as well as plant roots do (Sakurai et al. 2007), it is also possible that lupin roots alter the rhizosphere environment, resulting in an increase in microbial activity and/or community structure to enhance their protease production.

As described above, improvement of the nutrient-use efficiency of manure is necessary to increase crop and pasture productivities with the application of manure to the extent comparable to that of chemical fertilizer. Therefore, the objective of the present study was to determine whether growth and N uptake of pasture grasses could be improved by mixed cropping with lupin, to enhance the decomposition of organic N in manure supplied to the soil. Furthermore, microbial activity and community structure, as well as protease activity in the rhizosphere, were analyzed to elucidate the primary route (plant- or microbial-derived) for organic N decomposition in soil. The N-fixing soybean, from the same N-fixing family, the Fabaceae, as lupin, was used as a control.

99 **Materials and methods**

100 ***Plant materials, treatments, and soil culture***

101 To examine the effect of mixed cropping with lupin on the growth of grasses, pot
102 experiments were conducted in a randomized complete block design with five
103 replicates in a greenhouse at Hokkaido University. Timothy (*Phleum pratense* L. cv.
104 Horizon) and orchard grass (*Dactylis glomerata* L. cv. Bacchus) were cultivated with
105 or without either lupin (*Lupinus albus* L. cv. Luxor) or soybean (*Glycine max* (L.)
106 Merr. cv. Wasemidori) in the same pots. These grass species were chosen because
107 they are widely used as pasture species in Hokkaido Prefecture, Japan. As controls,
108 pots with lupin alone or with soybean alone were also prepared.

109 The field soils (Gleyic Fluvisol) were collected from the 0–25 cm layer at the
110 experimental farm of Hokkaido University. The soil was air-dried and passed through
111 a 2.0-mm mesh screen followed by 1.75 L of soil and 0.75 L of perlite, which were
112 mixed and placed in each plastic pot (18 cm diameter at the top, 13 cm diameter at the
113 base, and 15 cm in height). Because the soil used in a preliminary experiment showed
114 a compacted layer and limited penetration of plant roots, we mixed the soil with
115 perlite to improve the soil physical conditions. The chemical properties of the field
116 soil are shown in Table 1. Soil pH was determined by using a 1:2.5 soil-to-water ratio
117 using a pH meter. Total carbon (C) and N concentrations were determined by CN
118 analyzer (Vario Max; Elementar Analysensysteme GmbH, Hanau, Germany).
119 Available P concentration was determined by the Truog method (Truog, 1930).
120 Exchangeable potassium (K) was determined by inductively coupled plasma-mass
121 spectrometry (ELAN DRC-e; Perkin Elmer, Waltham, MA, USA) after 1 M
122 ammonium acetate extraction. For N treatments, 350 mg of N (equivalent to 200 kg
123 ha⁻¹) was applied to a pot either as ammonium sulfate (AS) or cattle farmyard manure

(CM). Treatment without N fertilization (–N) was also performed. Inorganic P and K fertilizers were also applied to all pots as superphosphate and K sulfate, respectively (at 350 mg P₂O₅ pot^{–1} and 540 mg K₂O pot^{–1}). As CM contained 16.2 mg N g^{–1}, 15.6 mg P₂O₅ g^{–1}, and 25 mg K₂O g^{–1}, the amounts of P and K supplied by CM in a pot were 337 mg P₂O₅ and 540 mg K₂O, respectively. Therefore, inorganic P fertilizer (13 mg P₂O₅) was also supplied to the pot with CM treatment. Total N concentration in CM was analyzed using the CN analyzer. Total P and K concentrations in CM were analyzed by inductively coupled plasma-mass spectroscopy after wet digestion (Watanabe et al. 2015). After fertilization, all the pots were incubated in a greenhouse for 10 days.

Seeds of lupin or soybean were sown at the center of each pot (A), followed by 20 seeds of each pasture grass, which were sown in each pot (2 cm diameter, B₁ and B₂), as shown in Figure 1. Pots sown with seeds of only lupin, soybean, or one of the pasture grasses were also prepared. After germination, lupin and soybean were thinned to three and two per pot, respectively. During soil incubation and plant cultivation, soils were irrigated with deionized water daily to maintain the water content at approximately 60% of the maximum water-holding capacity.

Sampling

After growing for 2 months (at the flowering stage for each of the test plant species), seedlings with soil attached were gently taken from the pot, and the roots were carefully separated from the soil. The separated soil was mixed well and defined as the far-rhizosphere soil. The soil still adhering to the roots was then collected by vigorously shaking the roots in a plastic bag, and this soil was defined as the near-rhizosphere soil (Sakurai et al. 2007). The roots and shoots of each plant were

separated, washed with deionized water, and lyophilized. After weighing, the lyophilized samples were milled for further analysis. After sieving the soil samples, the first subsample of soil was weighed before and after drying at 105°C for 48 h to determine the soil moisture content. The second subsample of soil was stored at 4°C before determining protease activity and performing microbial community analysis. The third subsample of soil was dried for 2 weeks at room temperature to measure soil chemical properties.

Plant mineral analysis

For determining plant N concentration, each 70 mg sample was digested with 1.25 mL of concentrated H₂SO₄ in a test tube at 200°C. At 30-min intervals, 0.3 mL of H₂O₂ (semiconductor grade; Santoku Chemical, Tokyo, Japan) was added to each tube (eight times), following which the N concentration in the digests was determined by the micro-Kjeldahl method. To determine the P concentration in the shoot, each 60mg sample was digested in 2 mL of 61% (w/v) HNO₃ (EL grade; Kanto Chemical, Tokyo, Japan) at 110°C in a DigiPREP apparatus (SCP Science, QC, Canada) for approximately 2 h until the solution had almost disappeared. After the samples had cooled, 0.5 mL of H₂O₂ was added, and the samples were heated at 110°C for a further 20 min. Once digestion was complete, the tubes were allowed to cool, and a volume of 10 mL was obtained by adding 2% (w/v) solution of HNO₃ in Milli-Q water. Phosphorus concentration was measured using inductively coupled plasma-mass spectrometry.

Soil analysis

For the determination of phosphate buffer-extractable organic N concentration in the soils, each 5 g sample was extracted with 20 mL of 1/15 M phosphate buffer (pH7.0) by shaking for 1 h. The soil extracts were filtered through filter paper (No. 6; Advantec, Tokyo, Japan). The organic N concentration in the filtrate was determined as the difference between $\text{NH}_4\text{-N}$ concentrations before and after Kjeldahl digestion (Scheiner 1976; Matsumoto et al. 2000). Available P concentration was measured as described by Truog (1930). For determination of inorganic N (NH_4^+ and NO_3^-) concentration, soils were extracted with 2 M KCl, and inorganic N concentrations were determined with an autoanalyzer (AACS-3, Bran+Luebbe Norderstedt, Germany) following the manufacturer's instructions.

Protease activity in soil

Soil protease activity was measured according to Ladd & Butler (1972). In brief, 1.8 mL of 100 mM Tris buffer (pH 8.1) was added to the test tube containing 0.5 g of fresh soil, following which 2 mL of 2 mM benzyloxycarbonyl phenylalanyl leucine solution or Milli-Q water (control) was added to the tube and incubated for 1 h at 40°C in a water bath. After incubation, 0.2 mL of 5 M HCl was added to stop the enzyme reaction. After 10 min, 6.8 mL of deionized water was added and filtered using No. 6 filter paper. The amino acid concentration in the filtrate was determined by the ninhydrin reaction.

Biolog EcoPlate

To characterize the microbial community structure and activity in the rhizosphere soil of grass, lupin, and their mixed culture, the Biolog EcoPlate™ system (Biolog Inc., Hayward, CA, USA) was used. Each 96-well Biolog EcoPlate contained three

replicate wells of each of 31 carbon substrates and a water blank. For assessing microbial carbon utilization patterns, near-rhizosphere soils were aseptically extracted. Each 1 g sample of near-rhizosphere soil was suspended in 9 mL of 0.85% sterile saline solution and diluted 1,000 times with the same saline solution in a sterile laminar air-flow cabinet bench (Airtech Japan, Tokyo, Japan). A subsample (150 μ L) was added into each well of the Biolog EcoPlates, following which the plates were maintained at 25°C. The absorbance (at 595 nm) of each well was determined every 12 h using a microplate reader (Sunrise Remote, TECAN, Austria). The average well color development (AWCD) in each plate, which indicates microbial activity, was calculated as follows:

$$AWCD = [\Sigma(R_i - C)]/31$$

where R_i and C are the values of optical density (OD) at 595 nm of the response wells (containing a sole carbon source) and the control well (water), respectively. Due to the high experimental cost, only 3 replicates randomly selected from each treatment were used for the Biolog EcoPlate analysis.

Statistical analyses

All statistical analyses were performed using Sigmaplot 11.0 (Systat Software, Inc., San Jose, CA, USA) and Minitab 14 (Minitab Inc., State College, PA, USA). The differences among the treatments were compared with ANOVA with Tukey's multiple comparison test and considered significant at $P < 0.05$. Principal component analysis (PCA) was performed to compare microbial community structures from different soils based on the Biolog EcoPlate data using Minitab 14 software (Minitab Inc., USA). For PCA, the Biolog EcoPlate data at 96 h were transformed by dividing the difference in OD₅₉₅ relative to the control well by AWCD of the plate for each

substrate, i.e., $(R_i - C)/AWCD$. Three technical replications in each plate were averaged to achieve one biological replicate value. Relationships among samples were obtained by plotting scores of their first two principal components in two dimensions. Data from individual wells which had an average OD for all treatments of <0.25 were not used for PCA.

Results

Plant growth

The shoot dry weights of timothy and orchard grass after the treatment are shown in Figure 2. In both grasses treated with CM, the dry weights were significantly higher in plants cultivated with lupin than in those cultivated alone or with soybean. In AS treatment, the dry weights were lower in both grasses cultivated with soybean than in those cultivated with lupin or cultivated alone.

Plant mineral analysis

The N concentrations in shoots of grasses are shown in Figure 3. In timothy, the N concentration in the shoot of plants fertilized with CM was significantly higher in plants cultivated with lupin than in those cultivated alone or with soybean. In contrast, the N concentration in shoots of plants fertilized with AS was significantly lower in plants cultivated with lupin than in those cultivated alone. In orchard grass, the N concentration in the shoot was not significantly affected by the cropping method.

The P concentrations in shoots of grasses were analyzed (Figure S1). In both grass species, P concentrations in the shoot were not significantly affected by cropping with lupin, with the exception of orchard grass in the N treatment, and P

concentrations were significantly decreased by cropping with soybean, regardless of the N treatment.

Soil analysis

Phosphate buffer-extractable organic N concentrations in the far-rhizosphere and near-rhizosphere soils with CM treatment are shown in Figure 4. In both soils, the extractable organic N concentration was significantly lower in grass cultivated with lupin than in grass cultivated alone, particularly in the near-rhizosphere soil. Although the extractable organic N concentration in the near-rhizosphere soil of grass cultivated with soybean showed similar trends, the extent of the difference was much smaller than that of grass cultivated with lupin and was not statistically significant.

Available P (Truog-P) concentrations in the far-rhizosphere and near-rhizosphere soils with CM treatment were analyzed (Figure S2). No significant difference was observed in the far-rhizosphere soil; however, mixed cropping with both lupin and soybean increased available P concentrations in the near-rhizosphere soil.

No significant differences in inorganic N concentrations in near-rhizosphere soils were observed among grass cultivated alone with lupin or with soybean (Figure S3).

Protease activity in soil

Protease activities in the far-rhizosphere and near-rhizosphere soils with CM treatment are shown in Figure 5. In both the soils, soil protease activity was significantly higher in grass cultivated with lupin than in grass cultivated alone, particularly in the near-rhizosphere soil. There was no significant difference in soil

protease activity in near-rhizosphere soil between grass cultivated with soybean and grass cultivated alone.

Biolog EcoPlate

The microbial community structure in near-rhizosphere soil with CM treatment was compared using the Biolog EcoPlate. PCA was conducted to assess the utilization patterns of the different carbon sources. The total variance explained by the first two components (PC1 and PC2) was 56.6%. The score plot of PCA indicated a distinction between monocultured lupin and grass, irrespective of whether the grass was cultivated alone or with lupin (Figure 6; significant at $P < 0.05$ in PC2). Although substrate data with average OD less than 0.25 was not used in this PCA, the results showed the same trend even when the threshold of the average OD was set to 0.1 (18 substrates; data not shown). AWCD was used as an indicator of microbial activity in soil. The AWCD value showed no significant difference among monocultured grass, monocultured lupin, and their mixed cultures with CM treatment (Figure 7).

Discussion

The dry weight and N concentration of grasses were higher when cultivated with lupin than in grasses cultivated alone with CM treatment, whereas the dry weight of grasses cultivated with lupin was similar to that in grasses cultivated alone in AS or in the -N treatments (Figures 2 and 3). In contrast to lupin, mixed cropping with soybean did not increase the dry weight or N concentration in grasses. These results suggest that the roots of lupin supplied N that could be utilized by the roots of pasture grasses with CM treatment. Because the phosphate buffer-extractable soil organic N and soil protease activity in near-rhizosphere soil with CM treatment in grasses

decreased and increased significantly, respectively, as a result of mixed cropping with lupin, (Figures 4 and 5), proteinaceous N derived from CM appears to be efficiently decomposed by proteases in the rhizosphere soil of lupin, producing low-molecular-weight peptides and amino acids that are available for uptake by plant roots. Significant negative correlations between the phosphate buffer-extractable soil organic N and soil protease activity in near-rhizosphere soil with CM treatment also support this hypothesis (Figure 8). Different growth responses to mixed cropping with lupin between timothy and orchard grass may be due to different uptake characteristics of low-molecular-weight peptides and amino acids between these two grass species (Pirhofer-Walzl et al. 2012; Watanabe et al. 2012).

Lupin is well known to decompose organic P and to solubilize insoluble inorganic P in the rhizosphere (Gardner et al. 1983; Wasaki et al. 1997). In the present study, available P concentration in the near-rhizosphere soil of grass cultivated alone was lower than that in the far-rhizosphere soil, possibly due to P uptake by grass roots (Figure S2). By contrast, this decrease was not observed in the near-rhizosphere soil of grass mix-cropped with lupin, suggesting that lupin roots increased the amount of available P in the near-rhizosphere. However, in this study, the improvement of P nutrient status did not occur in pasture grasses cultivated with lupin in this study (Figure S1), possibly because of competition for P uptake between lupin and the grasses.

As described above, it has been suggested that growth increase of pasture grasses cultivated with lupin was due to the improvement of N nutrition of grasses by the increased decomposition of soluble organic N in the rhizosphere. It is generally considered that the decomposition of organic N in soils is mainly performed by soil microbes (Colman & Schimel 2013). Sakurai et al. (2007) also reported an increase in

soil protease activity in the rhizosphere of lettuce following organic matter application, an effect that was associated with changes in the composition of proteolytic bacterial communities in the rhizosphere. On the other hand, it has also been reported that the roots of plants have the ability to decompose organic N in soil by the release of proteases into the rhizosphere (Schmidt et al. 2003). Therefore, to examine whether changes in the soil microbial community structure and/or microbial activity were related to the increased decomposition of organic N in rhizosphere soils of grasses co-cultivated with lupin in the present study, the Biolog Ecoplate system was applied. Values of AWCD, an indicator of microbial activity in soil, showed no significant difference among grasses cultivated with lupin (mixed cropping), grasses cultivated alone, and lupin cultivated alone with CM treatment (Figure 7), whereas AWCD has been reported to exhibit a positive correlation with microbial protease activity in soil (Elfstrand et al. 2007). Furthermore, no significant correlation was observed between the utilization of carbon source in the Biolog Ecoplate (absorbance of each well) and either soil protease activity or phosphate buffer-extractable soil organic N (data not shown).

On the other hand, PCA of the EcoPlate data demonstrated that the microbial community structure in near-rhizosphere soils of grasses cultivated with lupin was more similar to that in near-rhizosphere soils of grasses cultivated alone rather than that in near-rhizosphere soils of lupin cultivated alone (Figure 6). These results imply that the increased activity of soil protease in the near-rhizosphere of grasses cultivated with lupin in the present study (Figure 5) is not related to changes in microbial activity or community structure but rather to the roots of the lupin itself. However, although culture-based methods such as Biolog EcoPlate have the advantage that can detect active microorganisms, non-culturable bacteria or bacteria which grow slowly

in the presence of the limited number of carbon substrates in the plates are neglected (Shrestha et al. 2015). In the future, results that are more reliable will be obtained by microbial community structure analysis using high-throughput sequencing, targeting bacterial DNA or RNA in the soil, particularly genes encoding microbial proteases. Furthermore, pot experiments using sterilized soil should test the hypothesis that the roots of lupin directly decompose organic N in soils. In addition, as nodules were observed in roots of both lupin and soybean in this study (data not shown), it is also necessary to examine N supply to the rhizosphere derived from atmospheric N₂ fixation by rhizobia in pure and mixed combinations.

Conclusions

The present study indicated that mixed cropping with lupin increased the biomass of both tested grass species and increased N concentration in timothy by the enhanced decomposition of organic N in soils following manure application. These results provide new insights into sustainable and more efficient grassland productivity using animal manure as a fertilizer in the cultivation of pasture grasses. However, the cultivation in this study was conducted under relatively high plant density conditions at a pot level. Since lupin and soybean have different shoot structures, and both of them have higher growth rates and taller habits than do pasture grasses, different levels of light competition could have occurred between grass mixed with lupin and that mixed with soybean in the current study. Further field experiments are required to elucidate whether mixed cropping with lupin actually increases the yield and nutritional value (protein concentration) of pasture grasses under manure application, and to determine the optimum cropping intensity of lupin. Moreover, it is also necessary to examine whether lupin roots release substantial amounts of protease into

the rhizosphere and whether pasture grasses absorb N derived from the decomposition of organic N in the rhizosphere when cultivated with lupin.

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Figure captions

Figure 1. Top view image of the pots used in this study.

Figure 2. Shoot dry weight of pasture grasses after treatment. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass × Lupin), with soybean (Grass × Soybean), or alone (Grass alone). CM, AS, and –N indicate cattle farmyard manure, ammonium sulfate, and no nitrogen treatments, respectively. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods in each fertilizer treatment at $P < 0.05$ using Tukey’s multiple-comparison test following one-way ANOVA.

Figure 3. Shoot N concentration of pasture grasses after treatment. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass × Lupin), with soybean (Grass × Soybean), or alone (Grass alone). CM, AS, and –N indicate cattle farmyard manure, ammonium sulfate, and no nitrogen treatments, respectively. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods in each fertilizer treatment at $P < 0.05$ using Tukey’s multiple-comparison test following one-way ANOVA.

Figure 4. Phosphate buffer-extractable organic N in far-rhizosphere and near-rhizosphere soils of cattle farmyard manure treatment after cultivation. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass × Lupin), with soybean (Grass × Soybean), or alone (Grass alone). Data for lupin alone and soybean alone were also presented. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods at $P < 0.05$ using Tukey’s multiple-comparison test following one-way ANOVA.

Figure 5. Protease activity in far-rhizosphere and near-rhizosphere soils of cattle farmyard manure treatment after cultivation. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass × Lupin), with soybean (Grass × Soybean), or alone (Grass alone). Data for lupin alone and soybean alone were also presented. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods at $P < 0.05$ using Tukey’s multiple-comparison test following one-way ANOVA.

Figure 6. Principal component analysis of carbon source utilization activity (Biolog EcoPlate) in near-rhizosphere soils of grasses cultivated with lupin (Grass × Lupin), grasses cultivated alone (Grass alone), and lupin cultivated alone (Lupin alone) in cattle farmyard manure treatment after cultivation. Data were obtained from three replicates in each cropping method. Data from the substrate which had an average OD for all treatments of <0.25 were not used. Left: scores on the first two components (PC1 and PC2). Right: loading plot for PC1 and PC2.

Figure 7. Average well color development (AWCD) values of near-rhizosphere soils of grasses cultivated with lupin (Grass × Lupin), grasses cultivated alone (Grass alone), and lupin cultivated alone (Lupin alone) in cattle farmyard manure treatment after cultivation. Data are means of three replicates (±standard error). Different letters indicate statistically significant differences between cropping methods at $P < 0.05$ using Tukey's multiple-comparison test following one-way ANOVA.

Figure 8. Correlation between phosphate buffer-extractable organic N and protease activity in near-rhizosphere soils of cattle farmyard manure treatment after cultivation. ***: significant at $P < 0.001$.

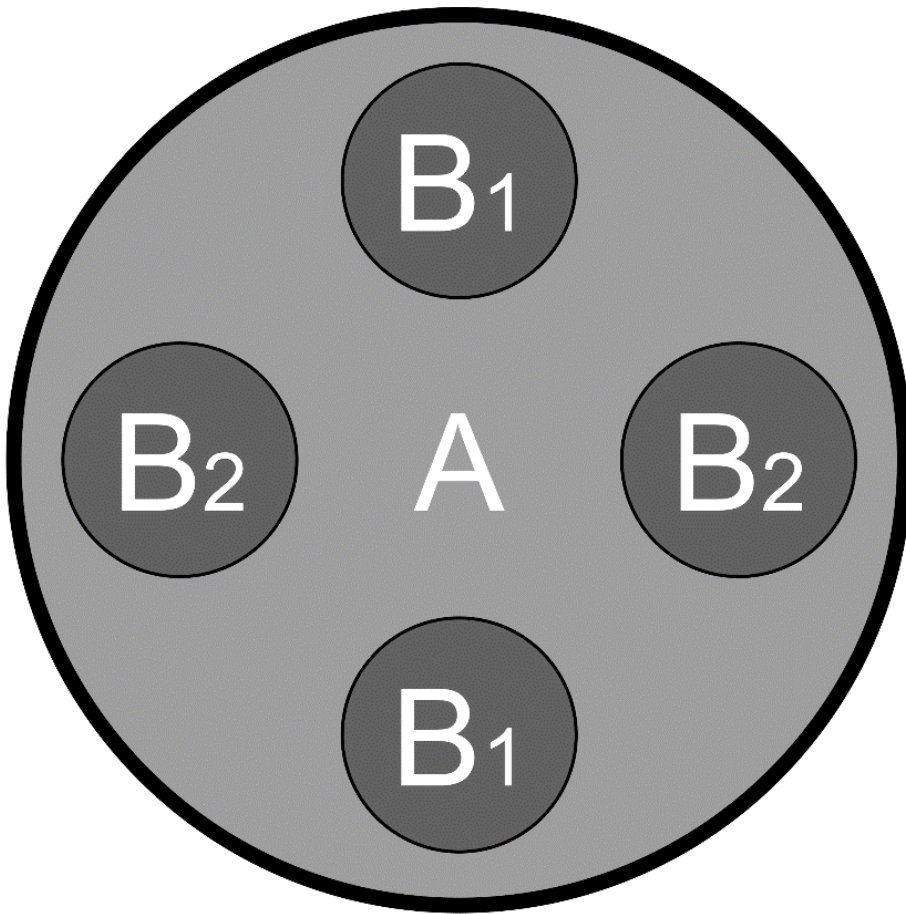
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Table 1

Chemical properties of the field soils. Data are means of three replicates ($\pm$ standard error).

pH (H₂O)	Total C	Total N	Truog-P	Exchangeable K
	(g kg⁻¹)	(g kg⁻¹)	(g kg⁻¹)	(g kg⁻¹)
6.03 $\pm$ 0.21	29.7 $\pm$ 0.3	2.00 $\pm$ 0.07	0.127 $\pm$ 0.002	0.324 $\pm$ 0.011

519



A: Lupin, soybean, or unplanted

B: Pasture grasses (B₁: timothy,
B₂: orchard grass) or unplanted

Fig. 1

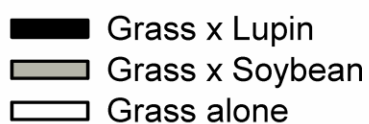
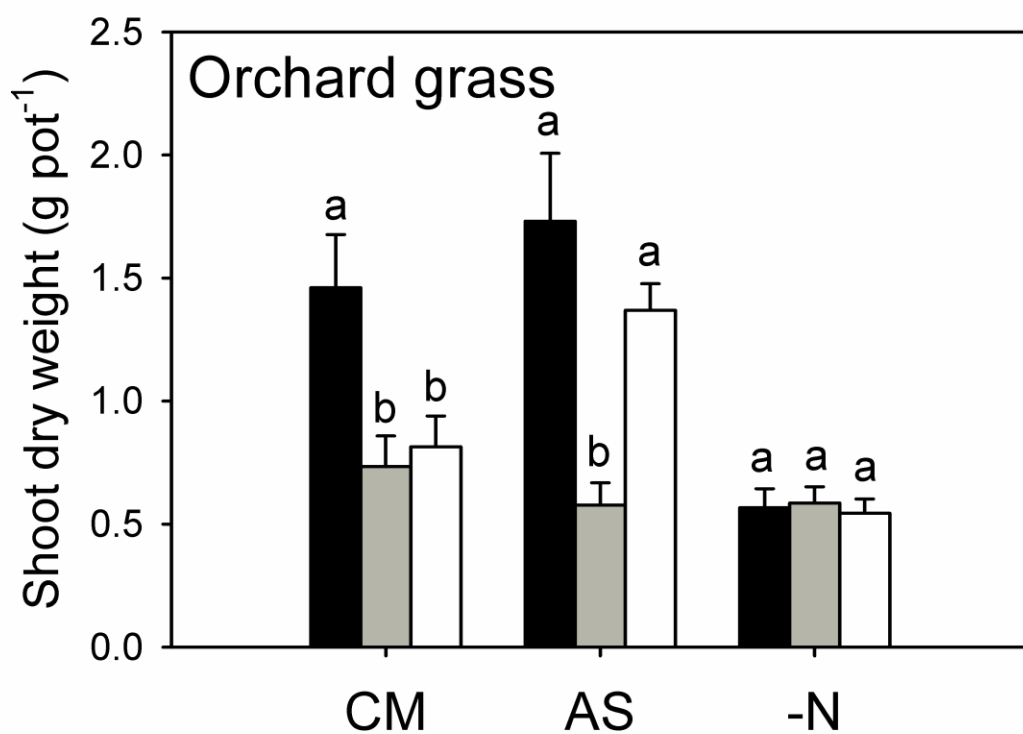
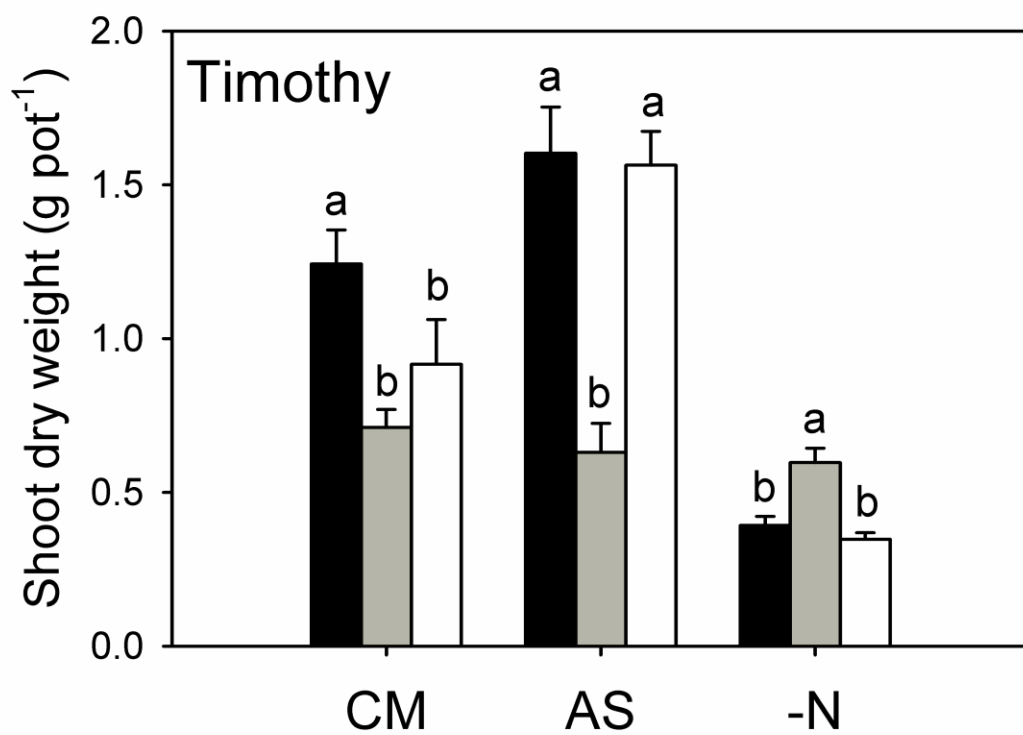
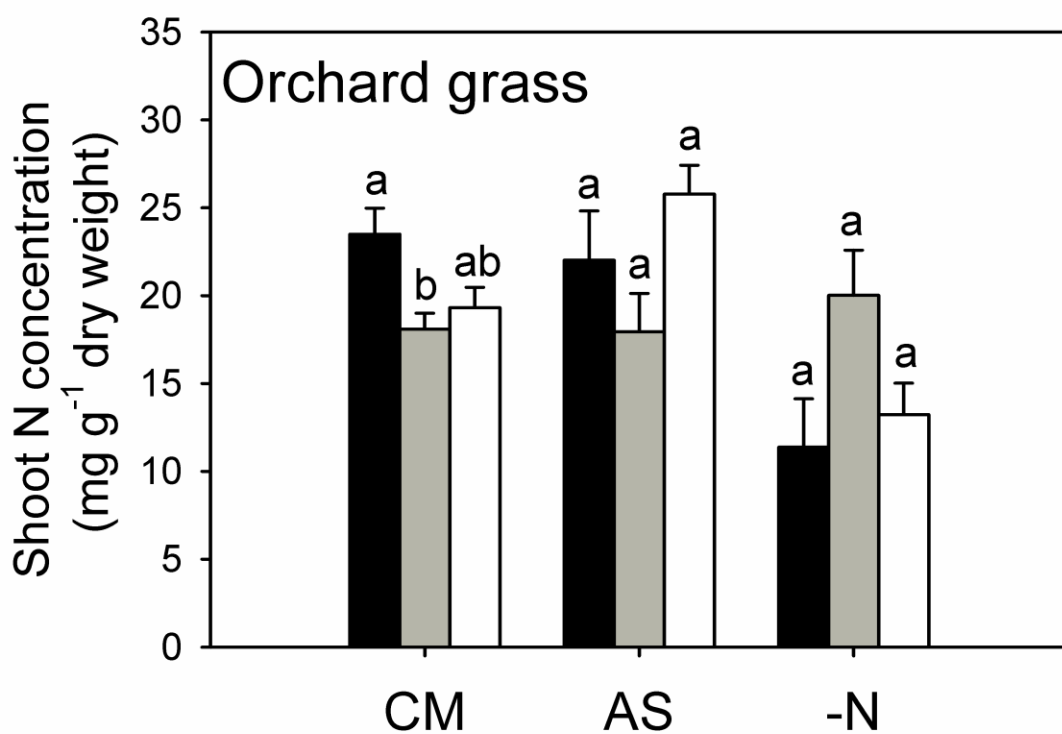
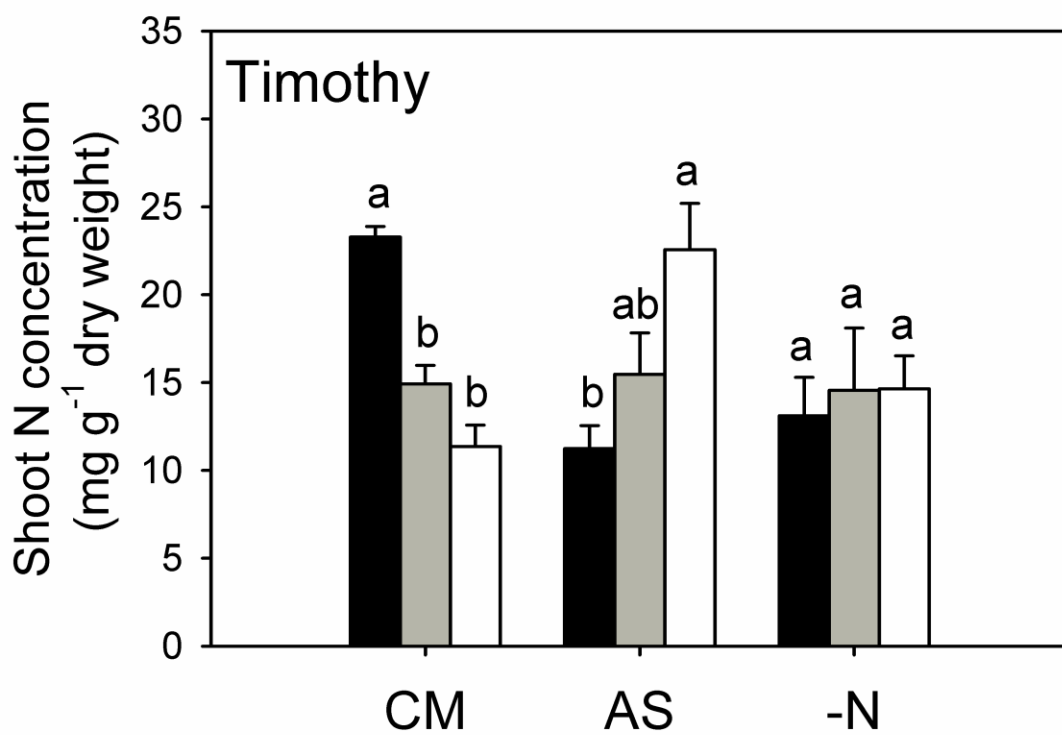


Fig. 2



■ Grass x Lupin
■ Grass x Soybean
□ Grass alone

Fig. 3

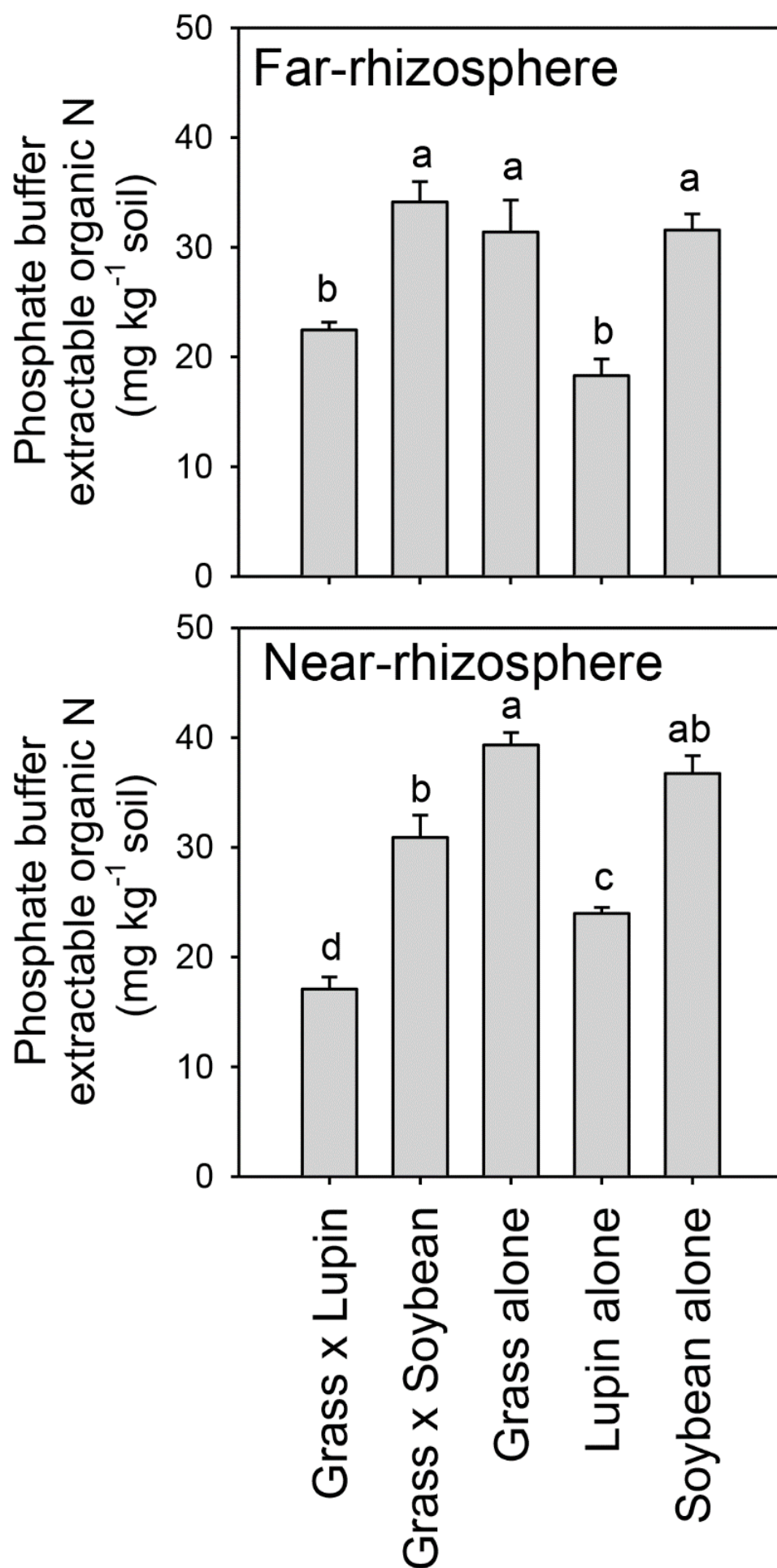


Fig. 4

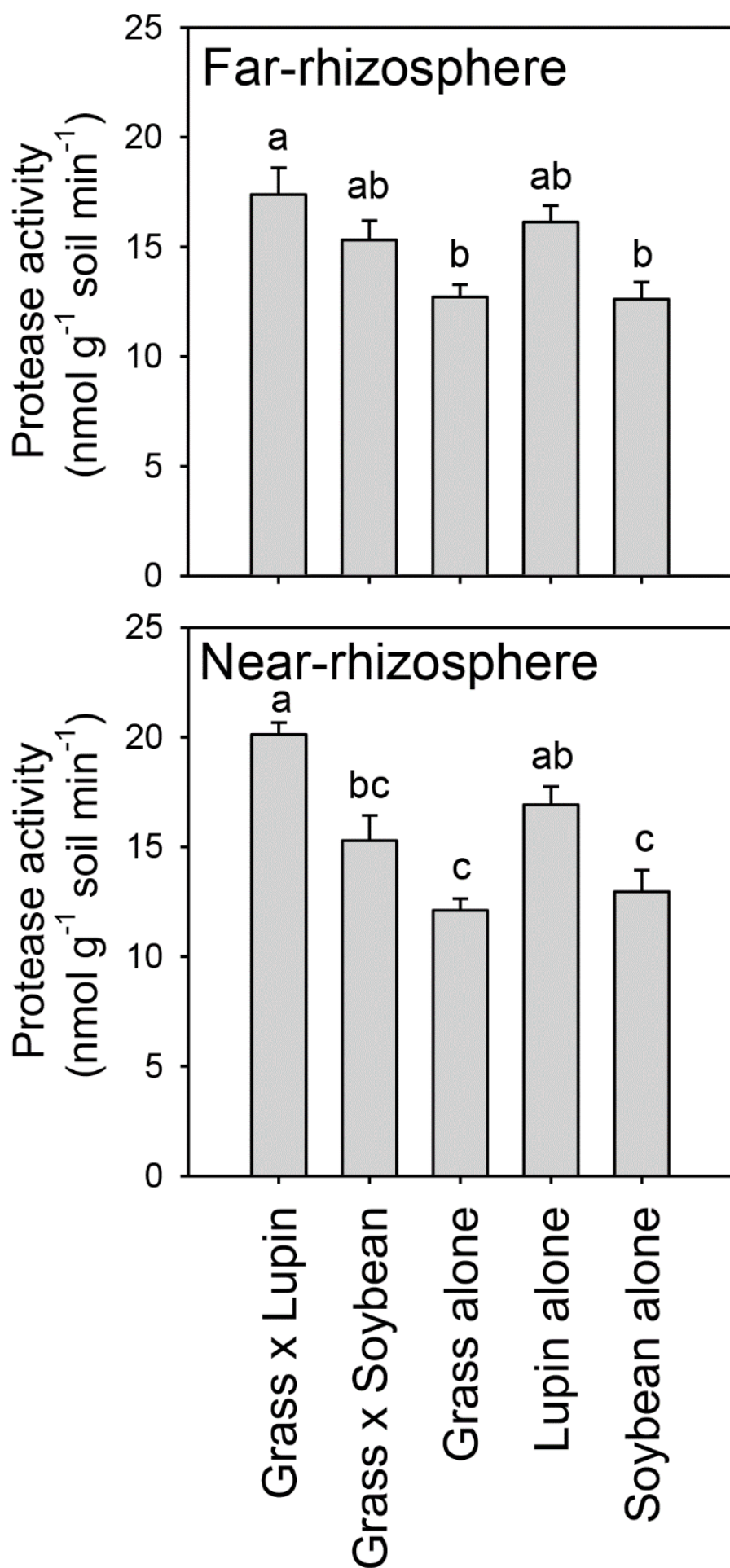


Fig. 5

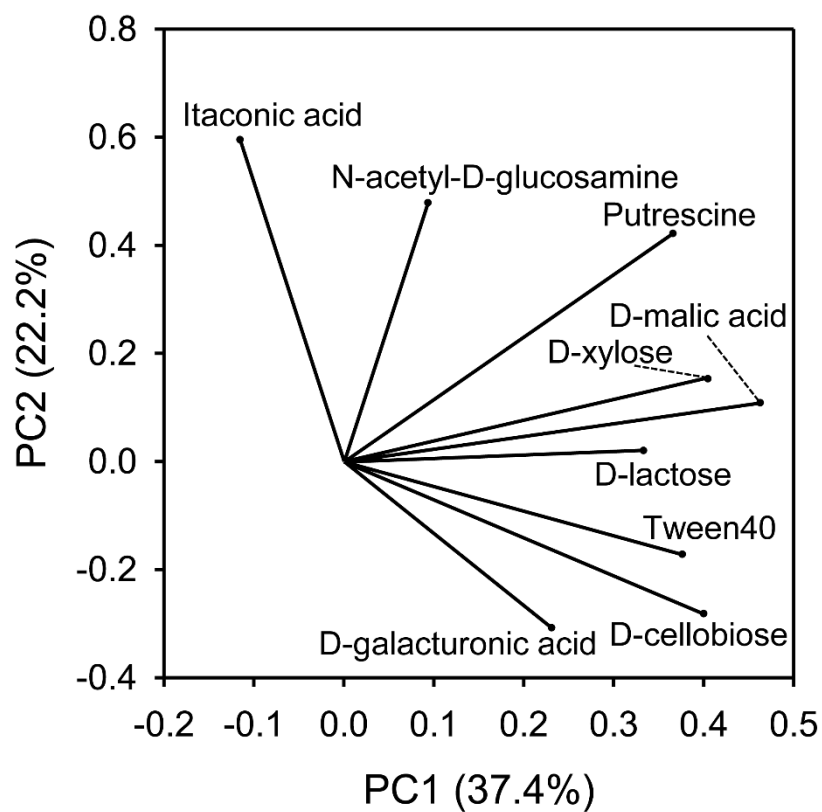
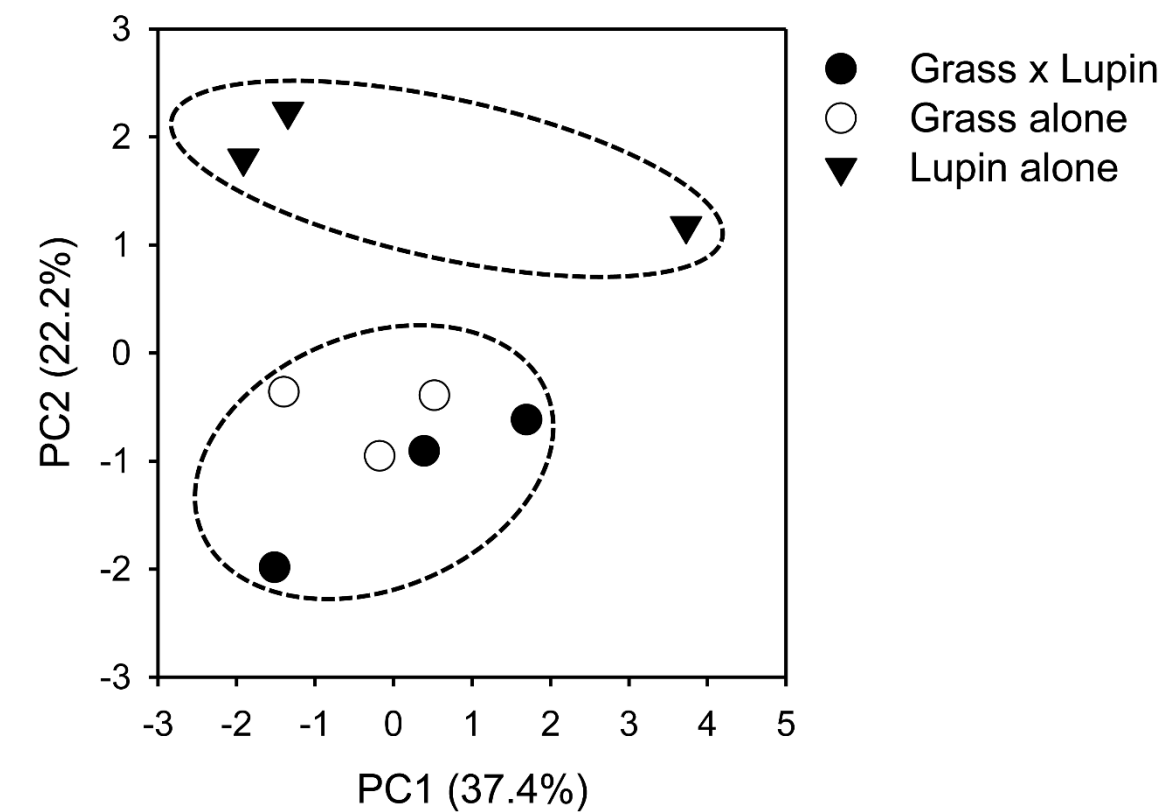


Fig. 6

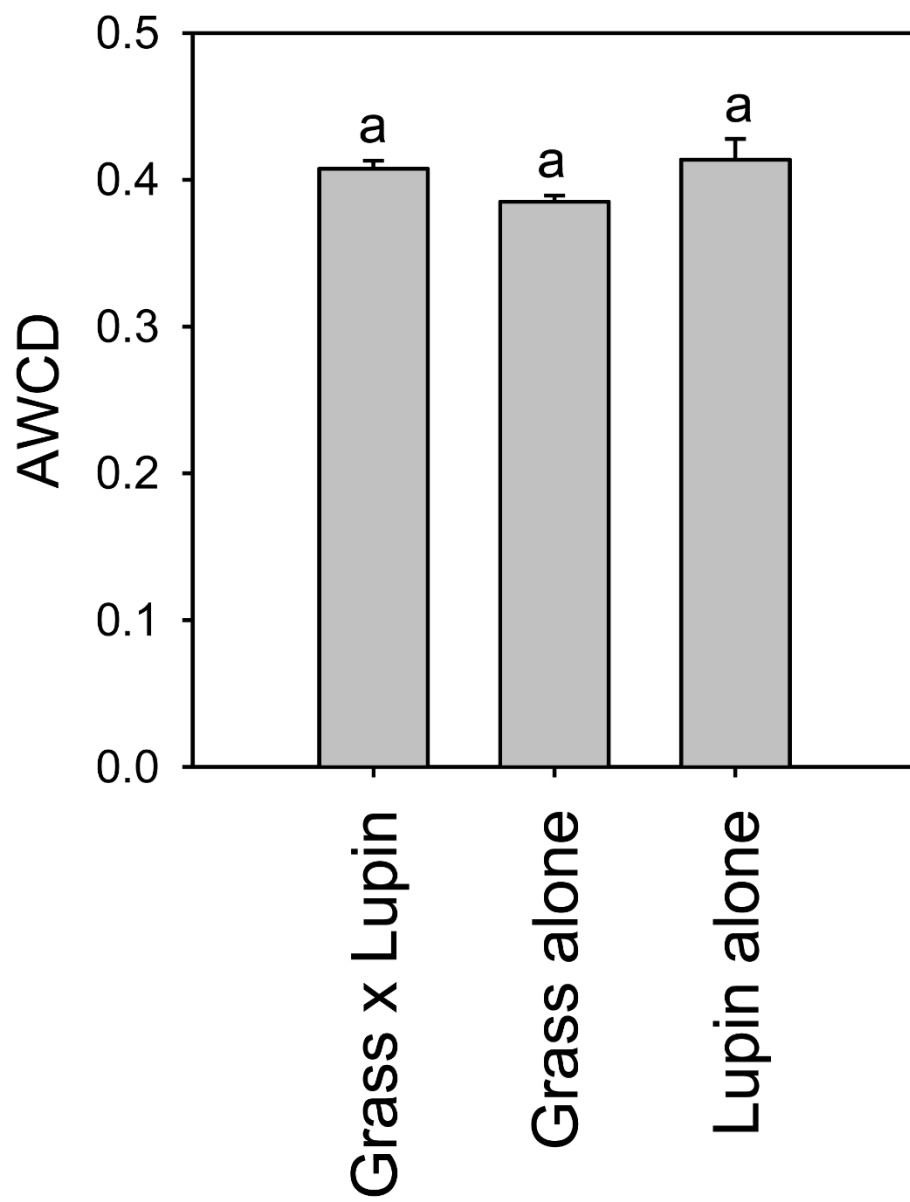


Fig. 7

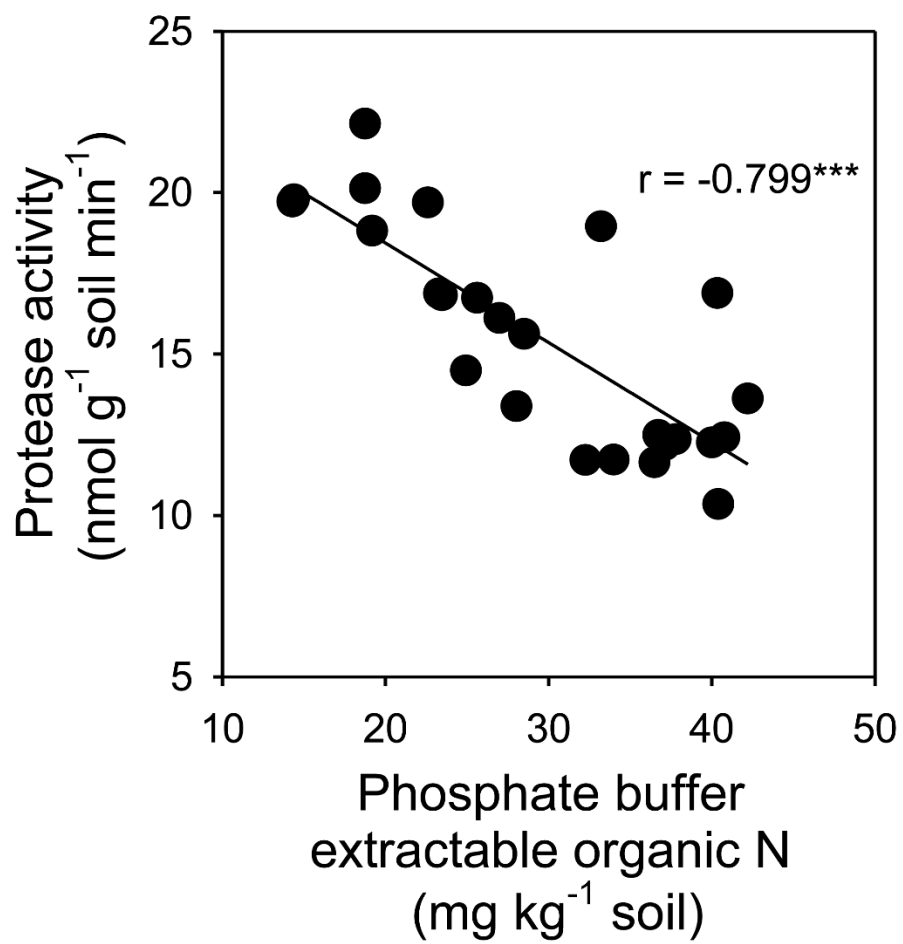


Fig. 8

Supplementary Materials

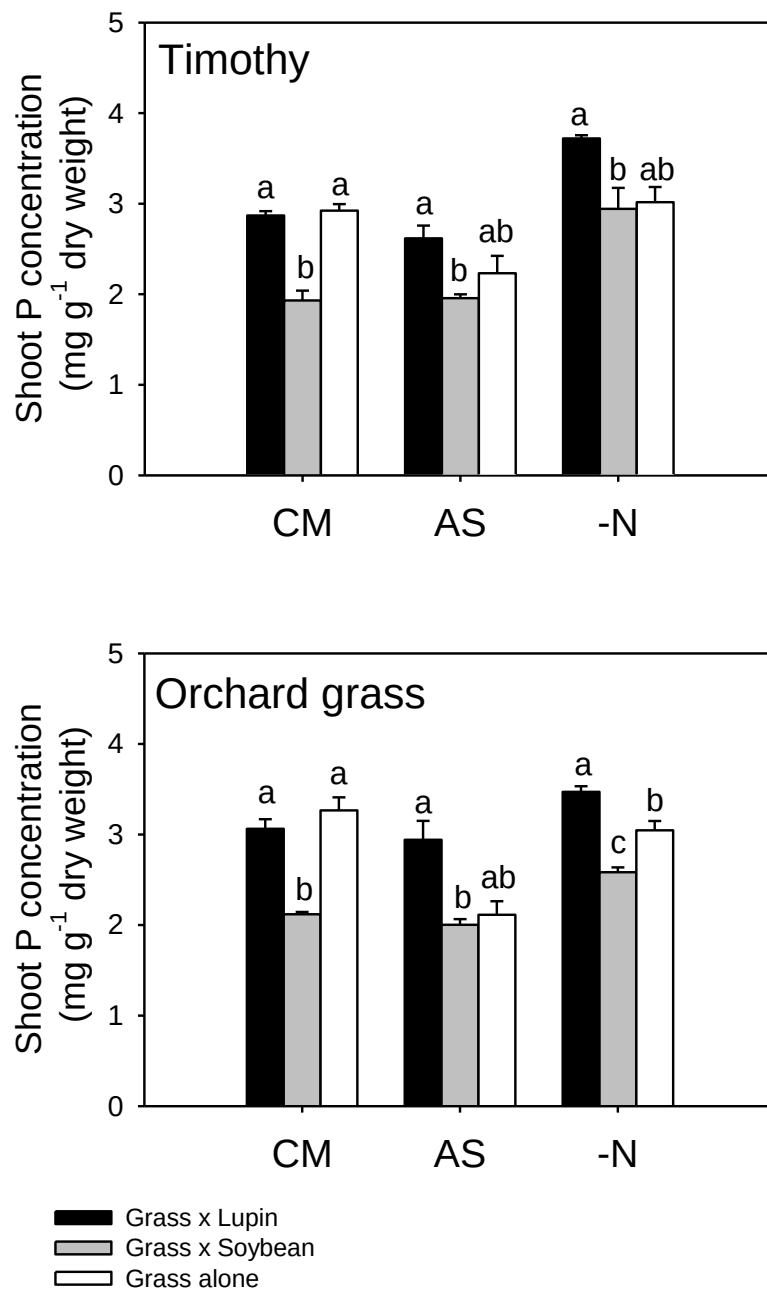


Figure S1. Shoot P concentration of pasture grasses after the treatment. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass x Lupin), with soybean (Grass x Soybean), or alone (Grass alone). CM, AS, and -N indicate cattle farmyard manure, ammonium sulfate, and no nitrogen treatments, respectively. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods in each fertilizer treatment at $P < 0.05$ using Tukey's multiple-comparison test following a one-way ANOVA.

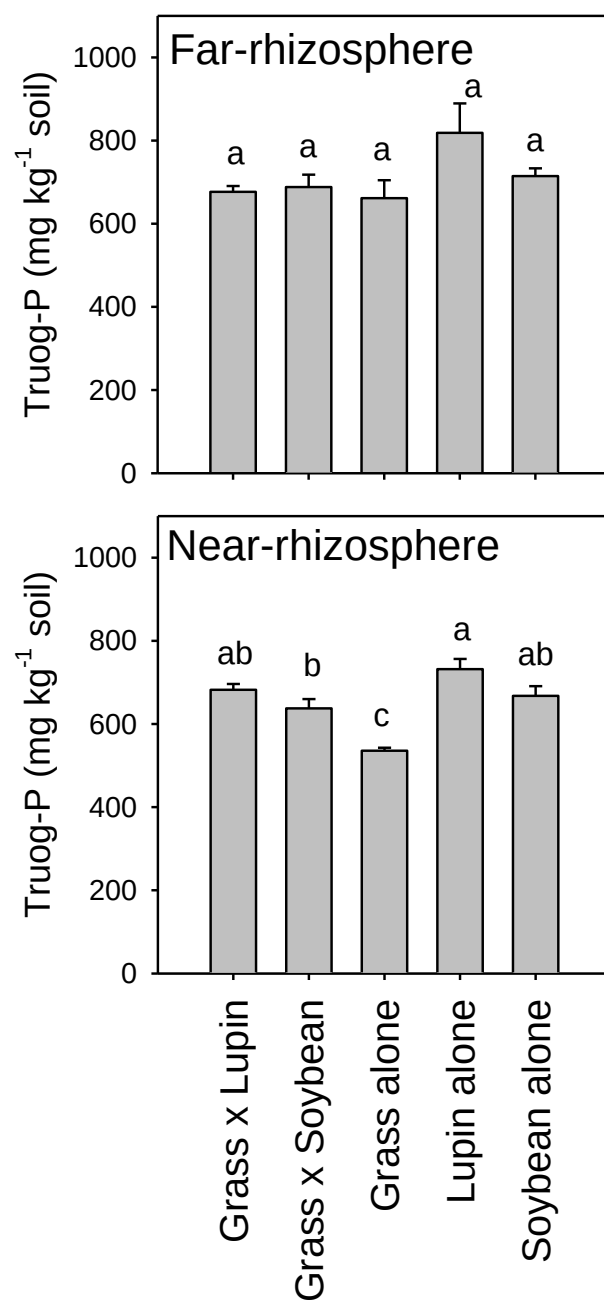


Figure S2. Truog-P in far-rhizosphere and near-rhizosphere soils of cattle farmyard manure treatment after the cultivation. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass x Lupin), with soybean (Grass x Soybean), or alone (Grass alone). Data for lupin alone and soybean alone were also presented. Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods at $P < 0.05$ using Tukey's multiple-comparison test following a one-way ANOVA.

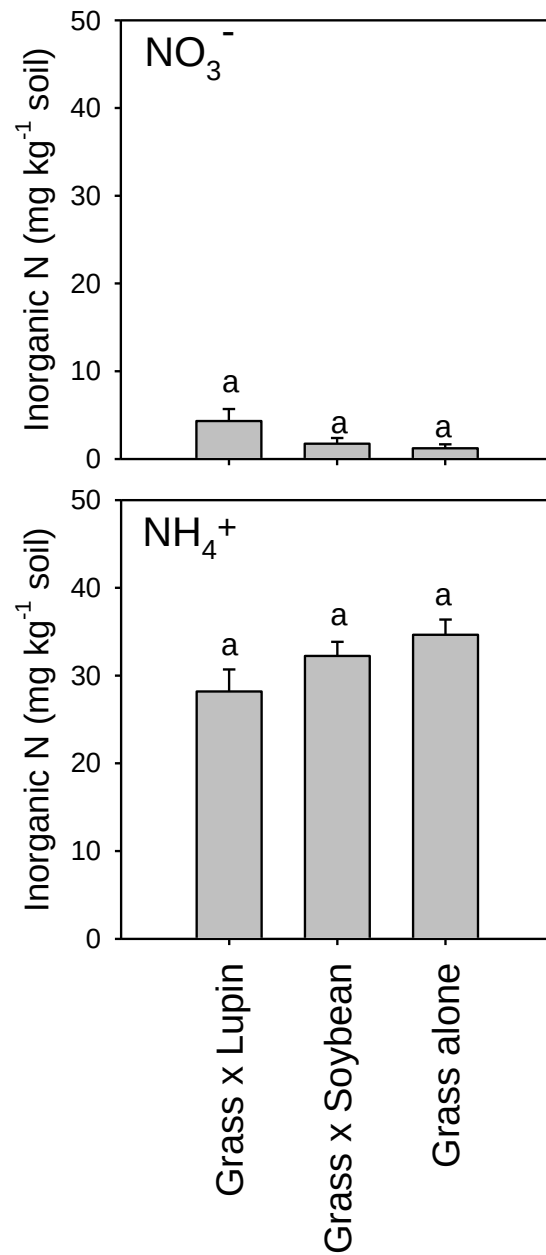


Figure S3. Inorganic N concentration in rhizosphere soils of cattle farmyard manure treatment after the cultivation. Pasture grasses (timothy and orchard grass) were cultivated with lupin (Grass x Lupin), with soybean (Grass x Soybean), or alone (Grass alone). Data are means of five replicates ($\pm$ standard error). Different letters indicate statistically significant differences between cropping methods at $P < 0.05$ using Tukey's multiple-comparison test following a one-way ANOVA.