Title	Study on dynamics of ionome and nitrogen in plant and soil
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Citation	北海道大学. 博士(農学) 甲第12247号
Issue Date	2016-03-24
DOI	10.14943/doctoral.k12247
Doc URL	http://hdl.handle.net/2115/64862
Type	theses (doctoral)
File Information	Chu_Qingnan.pdf

Study on dynamics of ionome and nitrogen in plant and soil

（植物と土壌におけるイオノームおよび窒素の動態に関する研究）

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Chapter 1 General introduction

Plant ionome is termed as the inorganic component in plant tissues, which is a consequence of complex plant processes and plant-environment interactions, including 17 mineral elements which plant required to complete their life-cycles (Marschner, 2012), and some non-essential elements such as cadmium (Cd), aluminum (Al) and cesium (Cs) when these minerals are present in the environment (Salt et al., 2008). Many studies indicate that the concentrations of mineral elements in shoot differ among plant species and plant genotypes because of their own physiological factors and affected by widely environment factors such as temperature, soil properties, and fertilization (Chen et al., 2009; Quadir et al., 2011; Watanabe et al., 2007; White et al., 2012). Vice versa, plant ionome could be used as a multivariable system to detect a plant's physiological status and reflect plant adaptions to the environment (Baxter et al., 2008; Baxter and Dilkes, 2012). To date, ionomics approach has been applied extensively for forward and reverse genetics, and screening diversity panels in plant, including model plant *Arabidopsis thaliana* (Lahner et al., 2003), *Lotus japonicus* (Chen et al., 2009) and food crops rice (Norton et al., 2009), soybean (Sha et al., 2012) and maize (Menkir et al., 2008). Plants have to evolve to grow in widely environments by altering their elemental composition of their tissues, both of the wild genotypes of *A. thaliana* which show specific response to certain environment (Baxter et al., 2012), and *A. thaliana* mutants that have high abilities to accumulate certain element (Lahner et al., 2013), can help to identify loci or genes to understand their genetic and environmental regulations for certain elements (Baxter, 2009; Williams and Salt, 2009). In the field-collected 2000 plant leaves, the ionome of 42 elements has revealed that over 25% of the total variation in the element composition attributed to the evolutionary factors, and multiple interactions among the elements were found in many studies (Baxter et al., 2012). It is a dynamic network of elements that are controlled by the physiology and biochemistry of the plant, which are ultimately controlled by the genome, in response to the environment. With the advantages of low cost, high-through output compare to the proteomics and metabolomics (Baxter, 2010), ionomics became an excellent tool for detecting alterations in a plant's physiology or its environment (Baxter, 2009).

Most mineral elements found in plant tissues come exclusively from the soil, necessitating that plants adapt to highly variable soil compositions to survive and thrive. Profiling element concentrations in genetically diverse plant populations is providing insights into the plant-environment interactions that control elemental accumulation, as well as identifying the underlying genes (Baxter and Dilkes, 2012). Ionomic profiling of locally adapted plant species could help explain how certain species thrive on soils with radically different chemistries, such as serpentines (low calcium.magnesium ratio; low nitrogen, phosphorus, and potassium) (Brady et al., 2005), heavily contaminated soils in Litavka (high As, Cd, Pb, and Zn) (Vondráčková et al., 2014). Ionomic study of these forms of adaptation has the potential to uncover molecular mechanisms of adaptation and speciation. Moreover, the concentrations of distinct elements are interdependent and covary between genetic backgrounds and environment conditions. Elemental species and compounds that are sufficiently similar in size and charge can be bound, metabolized, and transported by some of the same proteins, chelators, and pathways. This results in the coordinated accumulation of these chemically similar elemental species when the shared membrane transport proteins or chelating metabolites are regulated at the same time. Examples include transport of both K^+ and Cs^+ entering root cells via the same molecular mechanism (White and Broadley, 2000), Fe^{2+} and Zn^{2+} by an iron transporter (Palmer et al., 2009), AsO_4^{3-} and PO_4^{3-} by phosphate transporters (Zhao et al., 2009). Similarly, the nonspecific chelator nicotianamine affects both Fe and Zn accumulation in shoot vacuoles and Fe limitation increases leaf concentrations of zinc in *A. thaliana* (Haydon, 2012). Fe limatation also alters levels of molybdenum (Mo), which indictes that this covariation can cover chemically dissimilar elements. On the other hand, after the explosion of nuclear power plant in Fukushima prefecture, the radionuclides ^{134}Cs and ^{137}Cs received particular concerns due to long half lives (2.06 years for ^{134}Cs and 30.2 years for ^{137}Cs) and high energy emissions of beta and gamma radiation (Ohmori et al., 2014). Consumption of food containing radioactive Cs including plant material and products from animals that have ingested contaminated plant matter represents the principal route for human exposure to the risk element (Zhu et al., 2000). Under such circumstances, ionomic profiling of the particular plant populations is useful for identifying the specific mechanism for Cs uptake. As ^{137}Cs is taken up by Amaranths in the similar manner with ^{133}Cs if the soil is cultivated (Shinano et al., 2014), it could be expected that the chemical behavior of ^{137}Cs is similar to that of potassium (K), sodium (Na), rubidium (Rb) and stable Cs, all of which have highly similar valence and ionic radii. For strontium (Sr), calcium (Ca) is an analogue to ^{90}Sr and becomes the competing ions in the soil solution with Sr. So a large number of studies were conducted to interpret the interactions between Ca and Sr in soil-plant system (Broadley et al., 2012; Camps et al., 2004; Guillaume et al., 2012; Roca et al., 1995). Knowledge of the systematic variation in shoot concentrations of mineral elements can be used in agriculture to optimize the application of fertilizers on crop and repress the delivery of risk elements to the diets of humans and livestock (White and Broadley, 2005, 2009).

For crop, the ionome has predominantly been studied in soil, plant seeds or shoots to determine the nutritional status of plants (Sha et al., 2012; Vohland et al., 2009; Vondráčková et al., 2014), or in the mutants to screen the species with fine elemental composition (Waters and Grusak, 2008; Ziegler et al., 2012), but inadequate information has been available on the element distribution in the whole plant.

Moreover, among the nutrients required by plants, nitrogen (N) is the most essential nutrient in terms of quantity. Study on interaction between N and ionome in plant and soil is important to develop strategies for better nutrient management, but this interaction has not been understood very well. Specially for legume plants, which can make the biological fixation of N_2 (BNF), 15 of the 17 essential elements of plants are also considered essential for rhizobia as well, showing in the list: carbon (C), calcium (Ca), copper (Cu), iron (Fe), hydrogen (H), K, magnesium (Mg), manganese (Mn), molybdenum (Mo), N, nickel (Ni), oxygen (O), phosphorus (P), sulfur (S), and zinc (Zn). These elements play various roles in the symbiosis between legumes and rhizobium, acting as part of the nitrogenase (Fe, Co, and Mo) (Mendel et al., 2011), cofactors to participate in N fixation and stabilize cell walls for N uptake (Mn, Zn, Fe, Cu, S, and Ni) (Mendel et al., 2011; Fester et al., 2014), crucial signals for bacteroid differentiation into the N_2-fixing form of rhizobium (Ca and B) (Will et al., 2012; Zobiole et al., 201*2*), or satisfying energy supply in the form of ATP for nitrogenase function (P) (Rubio et al., 2012). In addition, via BNF legumes take up more cations than anions because uncharged N_2 enters the roots, and the consequent high ratio of cation/anion uptake results in net release of protons, thus lowering the soil pH (White and Broadley, 2009). The induced rhizosphere acidification may increase the availabilities of toxic elements such as Cd and Ba. Moreover, the transport proteins in the plasma membrane of root cells are not able to differentiate effectively essential and nonessential elements that are chemically similar, such as K^+ and Cs^+, Ca^{2+}, Sr^{2+}, and Ba^{2+} (Baxter et al., 2008). Nevertheless, little information is available to describe the distribution of elements in the legume plant, particularly for micro- and toxic elements.

Furthermore, the study on the ionomic interaction between plant and soil with emphasis on rhizosphere has never been conducted previously. The interactions between the effect of N sources on N, ionome mobility, and microbial activity in the rhizosphere are still unclear. Plant roots take up N from the soil as nitrate (NO_3^-), ammonium (NH_4^+), or low molecular organic nitrogenous compounds, such as amino acid (Watanabe et al., 2000). Fertilization with various N forms could induce the change of nutrient availability and microbial activity around the root system, and this change is closely connected with the plant species. As mentioned above, soybean plant can make BNF with rhizobium. This symbiosis between plant and microbes not only increases the N supply but also potentially affected the availability of ionome in the soil due to the change on soil physical and chemical properties (White and Broadley, 2009). Also, soybean root has been demonstrated to take up glycine at low efficiency (Watanabe et al., 2006), and sorghum was able to utilize organic N, usually in the form of amino acid, with high efficiency (Okamoto and Okada, 2004). The decomposition of organic N in the soil induced by root exudate or microbial activity would possibly cause the change on ionome mobility in the rhizosphere. In addition, plant roots are often not highly efficient competitors for amino acids compared with microbes because of microorganisms' rapid growth rates and high-surface area-to-volume ratios. This raised questions: what is the interaction between soil microbial activity or diversity, mobility of N and ionome, and plant growth in response to various N sources? And what is the difference of such interaction between soybean and sorghum? Under such circumstances, a micro-scale mapping of inorganic N, amino acids, ionome and substrate utilization by microorganism will provide a better understanding on the interaction between N and ionome in plant and soil.

Extending the ionmics study from greenhouse to the field-grown crops is important for understanding the ionome of plant and environmental control. Moreover, nitrogen (N) is a major nutrient for plant growth and plays primary role as an essential constituent of proteins, nucleic acids, and many secondary products; plants typically contain up to 4% of their dry weight in the form of N (Glass, 2009). Study on interaction between N and ionome in plant and soil is important for improving the nutrient utilization and producing nutritious food. In addition, nobody has ever studied the ionomic interaction between plant and soil with emphasis on rhizosphere. As a result, three independent experiments were carried out to reveal the dynamics of ionome and nitrogen in plant and soil.

Firstly, we present a phylogenetic analysis of interspecies difference in ionome concentrations in amaranth shoots among 33 varieties of *Amaranthus* species. In the second experiment, we assess the relative distribution of 22 mineral elements in the root, nodule and shoot of the soybean at vegetative and reproductive growth stage in response to ammonium and manure N treatment. In the third experiment, we profile the inorganic N, amino acids and ionome movement, and microbial metabolism in the rhizosphere of soybean and sorghum.

This study clarifies the ionome information in plant in response to variety effect, the dynamic variation of N and ionome in the plant as plant develops from juvenile to ripening, as well as the interactions between N, ionome in plant and soil in response to various N sources. These achievements contribute greatly to interpret the connection among the ionomic study, N fertilization, and plant nutrition.

CHAPTER 2

Interactions between Cs, Sr, and other nutrients and trace elements accumulation in *Amaranthus* shoot in response to variety effect

2.1 Introduction

Amaranth is a dicotyledonous plant that is considered a pseudocereal due to its high production of seeds. The seed is of importance for nutrition supply in that it has peptides with several biological functions (Silva-Sanchez et al., 2008), and a better balanced content of the essential amino acids than that of cereals and legumes (Bressani, 1994). Also, Amaranth has capacity to bioaccumulate metals in contaminated soils. Broadley (1999) summarized the data of shoot Cs concentration covering 136 taxa and found that Caryophllidae had the highest relative shoot Cs concentration (Broadley et al., 1999). And Amaranths exhibited the highest contribution within Caryophllidae to Cs accumulation among 273 taxa of flowering plants and it was referred as a potential genus for Cs phytoextraction (Willey et al., 2005). On the other hand, after the explosion of nuclear power plant in Fukushima prefecture, the radionuclides ^{134}Cs and ^{137}Cs received particular concerns due to long half lives (2.06 years for ^{134}Cs and 30.2 years for ^{137}Cs) and high energy emissions of beta and gamma radiation (Ohmori et al., 2011). Consumption of food containing radioactive Cs including plant material and products from animals that have ingested contaminated plant matter represents the principal route for human exposure to the risk element (Zhu and Smolder, 2000). Under such circumstance, Amaranthaceae is of a particular concern as many previous studies demonstrated that some plants species of Amaranthaceae show the preferential uptake of Cs and there may exist some specific mechanism for Cs accumulation in shoot (Broadley et al., 1999; Dushenkov et al., 1999; Willey et al., 2005) In addition, though until now the radioisotope of strontium (^{90}Sr) has not been detected in the soil of Fukushima prefecture yet, prevention against the transfer of Sr to food should be prepared in advance because ^{90}Sr was a serious problem in the case of Mayak and Chernobyl accident (Balanov et al., 2007; Ivanov et al., 1997).

Notably, although Cs and Sr are not required by plants they can still be taken up by plants because they share the same transporting protein or channels, or competing for the same binding sites in cell wall, with some essential elements, mainly with K and Ca (Kanter et al., 2010; Smolders and Tsukada, 2011; Tsukada et al., 2005) Therefore, the elemental uptake mechanism for Cs and Sr by plants is of utmost importance to support the development of countermeasures to reduce the translocation of radioactive Cs (and potentially Sr) towards the crops. As we previously reported that ^{137}Cs is taken up by Amaranths in the similar manner with ^{133}Cs if the soil is cultivated (Shinano et al., 2014), it could be expected that the chemical behavior of ^{137}Cs is similar to that of K, Na, Rb and stable Cs, all of which have highly similar valence and ionic radii. For Sr, Ca is an analogue to ^{90}Sr and becomes the competing ions in the soil solution with Sr. So a large number of studies were conducted to interpret the interactions between Ca and Sr in soil-plant system (Broadley and White, 2012; Camos et al., 2004; Guilaume et al., 2012; Roca and Vallejo, 1995).

Besides these target analysis for Cs, Sr and their analogous elements, non-target analysis for multiple elements including nonessential elements has been conducted as ionomic analysis (Agular et al., 2011; Maiga et al, 2005; Sha et al., 2012), and the ionomics is termed as linking multi-element analysis of molecularly characterized diverse germplasm of given species (Salt et al., 2008). In the same ecological context, species differences can be reflected in shoot mineral concentrations and a great number of experiments have been done to know the phylogenetic variation in the shoot mineral accumulations (Baxter et al., 2012; Buescher et al., 2010; Shtangeeva et al., 2009). Knowledge of the systematic variation in shoot concentrations of mineral elements can be used in agriculture to optimize the application of fertilizers on crop and repress the delivery of risk elements to the diets of humans and livestock

(White and Broadley, 2005, 2006).

In Chernobyl, a large number of field trials have been conducted to understand the mechanisms responsible for reducing the transfer of radionuclides (Konoplev et al., 1993; Rigol et al., 1999; Vidal et al., 2001). But Cs transportability between soil and crop is probably different between Fukushima and Chernobyl because Japan has many young volcanic soils classified as andosols, lacking of weathered micas, which are dominant in Chernobyl field (Smolders and Tsukada, 2011). This means ^{137}Cs fixation reactions with clay may have slower rate than that in Europe. The past studies about decreasing Cs accumulation in crops in Fukushima dominantly focused on the effect of K fertilization on Cs mobility in soil-plant system (Fujimura et al., 2013; Kubo et al., 2011; Ohmori et al., 2014). However, more studies should be set out to establish the elemental network to associate the Cs (and Sr) with other essential and nonessential elements, not only K and Ca, to clarify how regulatory mechanisms of Cs, Sr and other elements interact, because the regulation of plant on given ion uptake is not only decided by competitive ions but the balance of multiple ions.

In previous study we concluded that the varietal effect of genus *Amaranthus* brings about the significant difference in radiocesium uptake and transfer from soil. In present study we further present a phylogenetic analysis of inter-species difference in nutrient and trace element concentrations among 33 varieties of *Amaranthus* species grown on the field of Fukushima Prefecture, Japan. This effort allows us to compare the varietal variation for concentrations of mineral elements, and to establish ionomic interactions between Cs, Sr and other nutrient and trace elements in Amaranths shoot.

2.2 Materials and methods

Plant materials and growth conditions

Field experiment was conducted at Iino town located in the suburbs of Fukushima City (37°39 N, 140°32 E). The sites were 50.9 km northwest far from the Fukushima 1 Nuclear Power Plant and the field was cultivated in 2011. Soil type was gray lowland soil and the contamination level of the soil by ^{134}Cs and ^{137}Cs was 2769 ± 141 Bq/kg (average ± SE). Thirty-three Amaranthus species were adopted including 10 local varieties, showing in Table 1 in Shinano T et al. 2014.[13] All the seeds were sown in vermiculite on June 30th, 2012 and then transplanted to the field on July 14th, 2012. The plot area was 120 m^2 (12 m x 10 m) and the soil was sampled for the chemical analysis before transplanting and the data for soil characteristics of the field was shown in Table 2 in Shinano et al 2014. To avoid possible interaction between ions during radiocesium transport by root, only nitrogen fertilizer in the form of ammonium sulfate was applied at a rate of 100 kg N ha^{-1}, and the plants were then cultivated at a 15 cm depth. Twenty plants for each examined plant variety or species were transplanted at 70 cm x 50 cm planting density.

Sampling and Analysis

Plant samples were harvested on October 6th 2012, with three replications (two to three individuals in each replication) for each variety or species, and then bottom of the stem was washed by the tap water to remove any contamination with soil. Plant samples were dried at 70˚C for one week and then mercerized by a stainless grinder. After drying and milling, 50 mg shoot samples were digested with 2 ml of 61% HNO_3 (EL grade; Kanto Chemical, Tokyo) in a tube at 110˚C in a DigiPREP apparatus (SCP Science, Quebec, Canada) for 3 h, and 0.5ml of hydrogen peroxide (semiconductor grade; Santoku Chemical, Tokyo) was added two times at 30 min interval as an oxidant, and heated at 110˚C until the solution became clear (Quadir et al., 2011). After cooling down to the room temperature, the tubes were filled up to 15 ml with 2% HNO_3 and concentrations of 23 elements (P, K, Mg, Ca, Mn, Fe, Cu, Zn, B, Mo, Ni, Sr, Cr, Al, Ba, Cd, As, Na, Co, Se, Li, Rb, Cs) in the digests were analyzed by an inductively coupled plasma mass spectrometer (ICP-MS) (Elan, DRC-e; Perkin-Elmer, Waltham, MA, USA). The ICP multi-element standard solution **IV** (Merck, Tokyo) was used as standard for ionomic determination.

Statistical Analysis

To visualize the differences of the 23 mineral elements in thirty-three Amaranthus varieties, descriptive statistics, one-way variance (ANOVA), correlation and principle component analysis (PCA) were performed using SPSS 18.0. For statistical differences between elemental compositions for the cultivars ANOVA was conducted and the ratio of variance between groups to variance within groups was used to estimate the variation caused by varieties. For correlation analysis Pearson correlation coefficients and student's test were applied to identify the significant correlations between mineral elements. A conservative p value limit of 0.001 was used to testify whether the correlation is significant. The overall accumulations of 23 elements were profiled by PCA. The varimax with Kaiser Normalization was used as rotation method.

2.3 Result and discussion

Statistics and variation of mineral elements contents in Amaranths shoot among thirty-three different varieties

In this study, shoot element concentrations varied by six orders of magnitude from 0.073 µg g^{-1} for Cs to 34.15 mg g^{-1} for K, in the order K > Ca > P > Mg > Mn > Rb > Al > B > Fe > Na > Sr > Ba > Zn > Ni > Cu > Co > Cr > Cd > Li > As > Mo > Se > Cs (Fig 2.1). Cs accumulation was the lowest among 23 elements, which is probably related to the high affinity of Cs to the clays (Smolders E and Tsukada H 2011). The affinity of cations to clays in soil follows the order $Cs^+ > NH_4^+ > K^+ > Na^+ > Ca^{2+}$.[32]

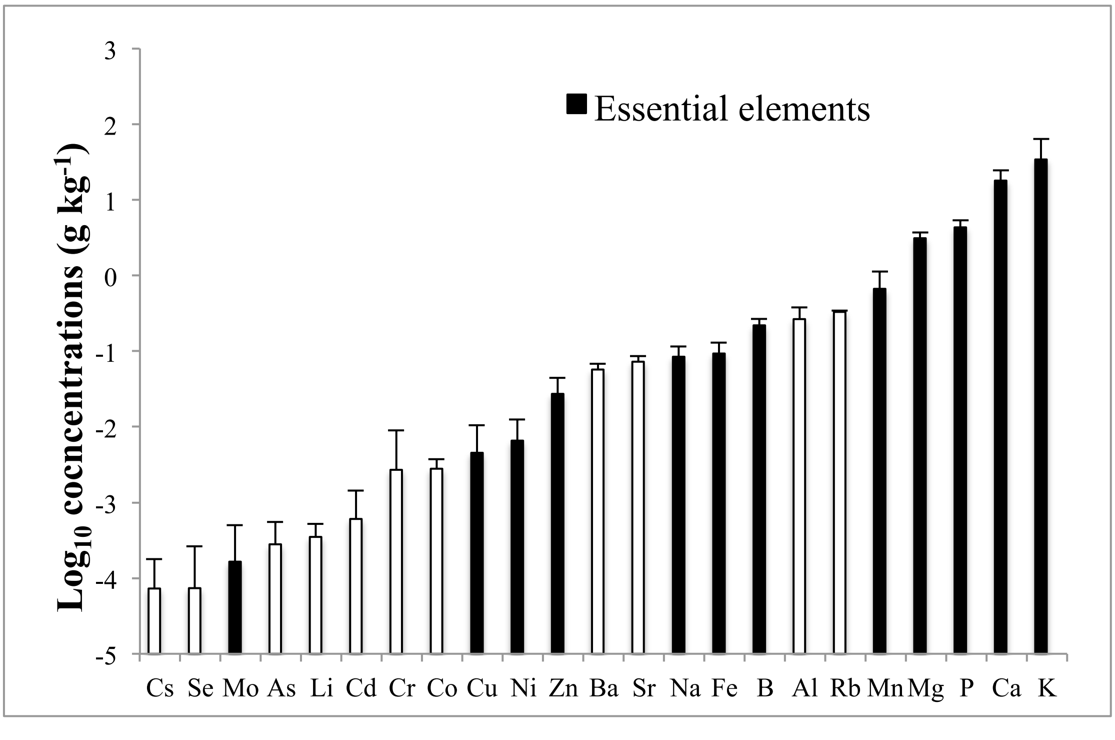


Fig 2.1 Average concentration of 23 mineral elements in shoots of 33 varieties of Amaranthus (n=99). Columns filled with black represent the essential elements and columns without filling represent nonessential elements. (Na is also regarded as essential element in this research as Na is an indispensible part of growth of C4 plants including Amaranths.

The results of varietal difference contributed to the variation of elements, and descriptive statistics were presented in Table 1. Significant difference ($p<0.001$) between different varieties was detected in all of the elements excluding Se. The relatively smaller phylogenetic variation in shoot Se was concordant with the previous studies (Watanabe et al., 2007; White et al., 2004). The remaining variation for given element corresponded to differences outside species within genus was likely to be caused by soil and climatic factors, or sampling techniques (Watanabe et al., 2007). In order to quantify the varietal variance affecting each mineral element, the ratio of variance between groups to total variance was calculated and ranged from 53.64% for Se to 99.14% for Cr. K, Ni, Na and Cr presented relatively larger variety difference among 23 elements and Cr revealed the largest one, approached to 100%, and the largest ratio of maximum to minimum as well as CV (coefficient of variation, ratio of standard deviation of all cultivars to mean of all cultivars) was also observed in Cr. As no metabolic activity involved by Cr is known in plant, the reason for such high phylogenetic variation within genus is unclear. Environment factor may have important effect, such as the specific soil characteristics and uneven distribution of nonessential elements in the field. While there is the variation associated with genotypes across entire elemental composition, the varied degree as measured by the ratio of the maximum to the minimum concentration or by the CV is highly element-dependent (Table 2.1, Fig 2.2). The CV in response to the variety effect, increased in the order Mg < P < Sr < Zn < B < Ca < Cu < Cd < Ba < Cs < Mn < Rb < K < Li < Se < Co < As < Fe < Al < Mo < Ni < Na < Cr. The CV of macronutrients including P, K, Ca and Mg varied less than 50%, while Na and Cr had C.V.s higher than 100%. The pattern of C.V. of shoot minerals is consistent with the Stability of Limiting Elements Hypothesis: the C.V.s of Mg, Ca, P, B, Zn and Cu (the essential elements) are lower than 25% while the C.V.s of As, Cr, Co and Al were higher than 50% and Cr approximated to 200%, becoming the most sensitive element of Amaranths grown on the field subject to radionuclides contamination.

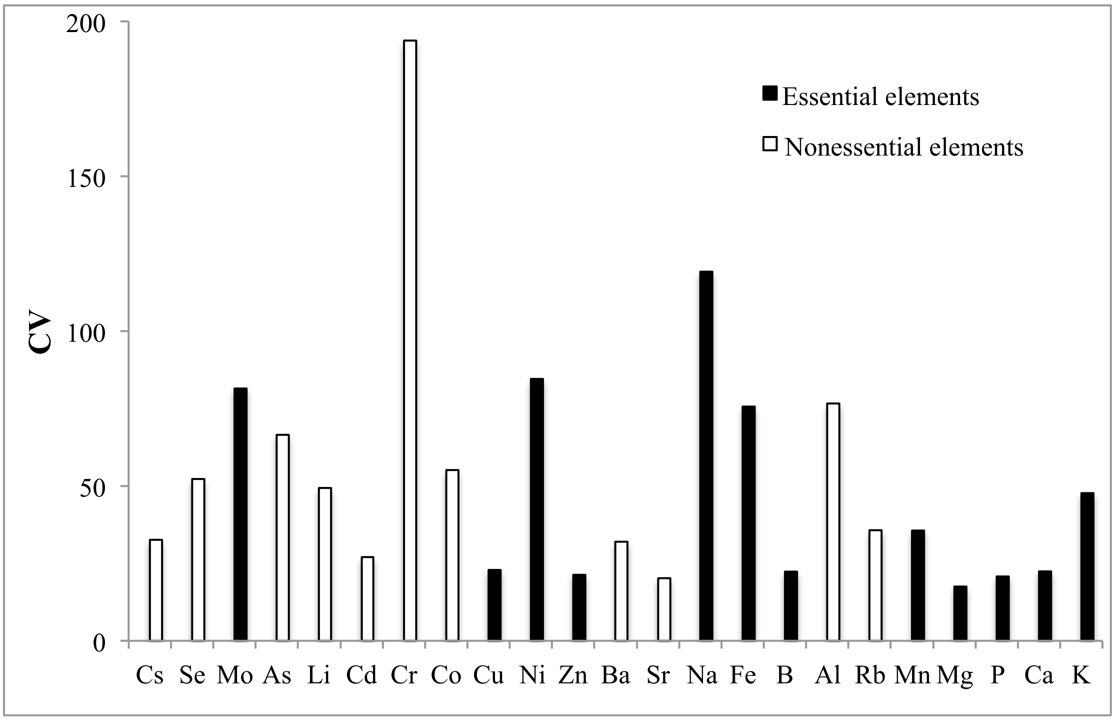


Fig 2.2 Relationship between mean concentration of elements in plants and their coefficient of variations (CV)

Table 2.1 Varietal variation in element concentrations in shoot among thirty-three cultivars[a] (n=99)

	Variety difference[a]	F	Max	Min	Ratio	Mean	SD
Cs	65.53%	3.92*	0.00015	0.00002	8.87	0.000073	0.000024
As	86.66%	13.4*	0.00089	0.00002	38.76	0.00028	0.00019
Rb	81.29%	8.96*	0.11	0.02	5.15	0.057	0.020
Al	86.41%	13.11*	1.16	0.10	11.32	0.33	0.25
Fe	78.89%	7.71*	1.08	0.021	51.46	0.27	0.20
Ba	68.55%	4.50*	0.15	0.016	9.38	0.086	0.027
Ni	93.34%	28.90*	0.021	0.0017	13.00	0.0047	0.0039
P	80.19%	8.35*	7.26	2.67	2.72	4.35	0.90
K	91.10%	21.11*	81.99	6.36	12.90	34.15	16.28
Ca	83.99%	10.82*	29.09	9.02	3.22	17.79	3.99
Mg	81.07%	8.834*	5.33	2.16	2.47	3.11	0.54
Mn	79.13%	7.82*	1.34	0.23	5.70	0.66	0.24
Cu	76.12%	6.58*	0.012	0.0032	3.76	0.0066	0.0015
Zn	85.25%	11.92*	0.12	0.041	2.82	0.073	0.015
Mo	79.04%	7.78*	0.00069	0.000052	13.30	0.00017	0.00014
B	89.56%	17.69*	0.040	0.013	2.98	0.027	0.0061
Na	92.69%	26.14*	1.35	0.064	20.94	0.22	0.26
Co	89.03%	16.74*	0.008	0.0008	10.02	0.0027	0.0015
Se	53.64%	2.39	0.00022	0.00001	31.52	0.000074	0.000039
Sr	75.49%	6.35*	0.15	0.045	3.24	0.094	0.019
Li	83.24%	10.24*	0.00077	0.00004	17.81	0.00035	0.00017
Cr	99.14%	238.04*	0.031	0.00013	251.40	0.0028	0.0054
Cd	87.32%	14.20*	0.00114	0.00026	4.45	0.00061	0.00016

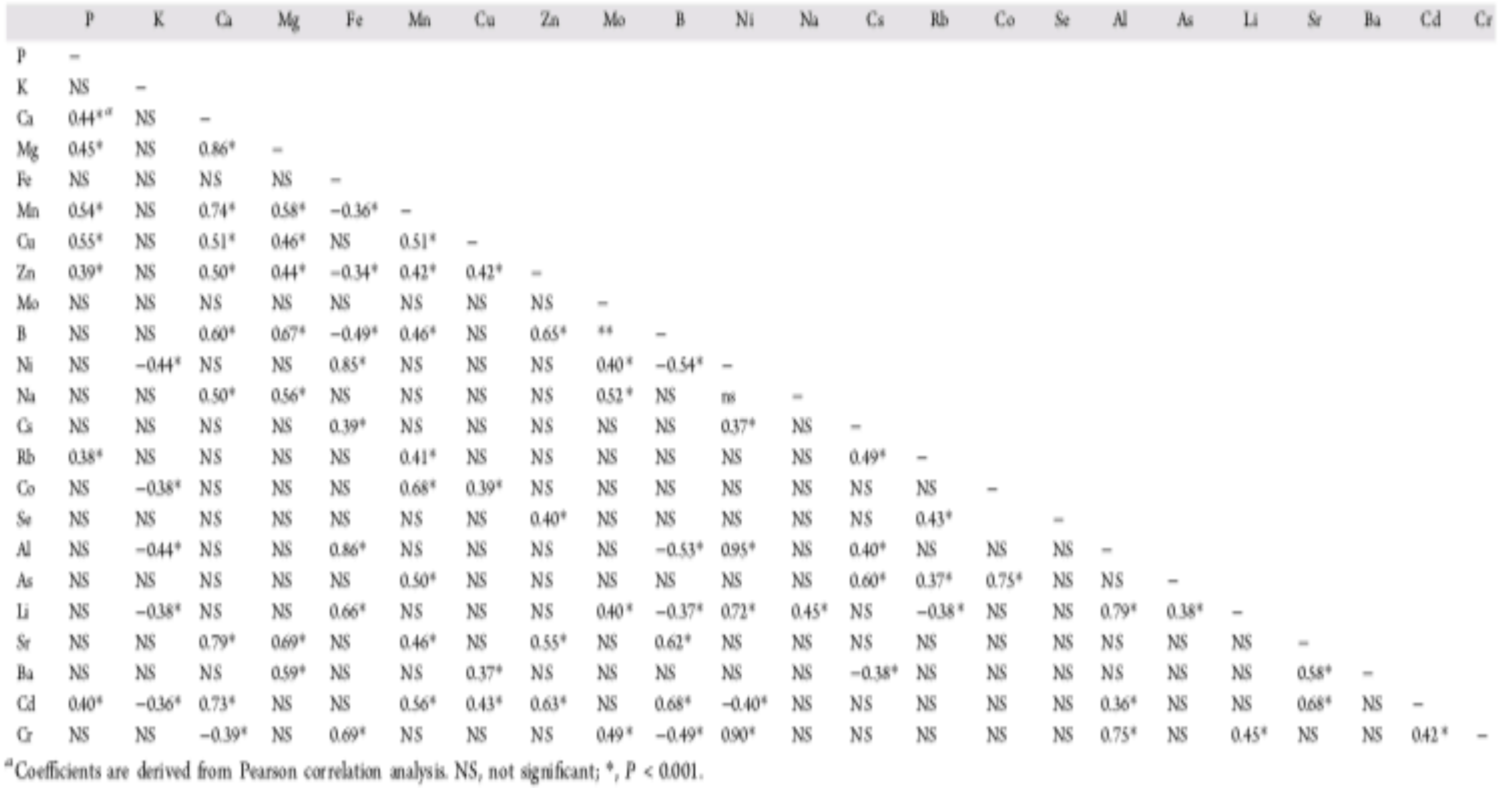
[a] Values reported are the partition of variance for the cultivar difference (ratio % of variance between groups to total variance); F values generated from the ANOVA with Tukey test; maximum (Max), minimum (Min), mean with the unit of μg g^{-1}; max/min (Ratio) and standard deviation (SD) of elemental accumulation in shoot for mineral elements. [b] *$P < 0.001$

On the other hand, the CVs of seven elements (Mg, Ca, Mg, B, Sr, Zn and Cu) were lower than 25% but only Sr belongs to nonessential elements. Both the CV and variety variance of Sr was only higher than Cs, Ba and Se. Meanwhile, variety difference of Cs was lower than all elements excluding Se. These results indicate that the Amaranths are less sensitive to the accumulation of Cs and Sr than most of other mineral elements to the species level. In contrast with Cs, the analogue K and Na showed relatively higher variety difference and CV. These results illustrate K and Na was unstably distributed in shoot of different varieties of Amaranthus. Compared to the alkali metal elements, the variety diversity was more consistent for alkali earth metals and Ca, Mg, Sr and Ba that all showed relatively lower CV (<50%). The relatively stable inter-varietal alterations for Cs and Sr within the Amaranthus genus coordinate with the larger range of investigations. According to meta-analysis for 27 plant species, Penrose B et al (2015) concluded that the inter-varietal variation at the ratio of the maximum to minimum of concentrations for a given crop species ranged from 1.0-6.3 for Cs and from 1.0-4.5 for Sr, respectively, which is similar with the ratio within the genus of Amaranthus in this study and relatively lower than that of many other mineral elements. In conclusion, phylogenetic variation for Cs and Sr within genus was lower than the essential elements of their analogues, which suggests the essential elements of group Ⅰ and Ⅱ in periodic table are more sensitive to genetic difference than their alternative nonessential elements. Moreover, within a

Amaranthus genus the lower inter-species difference of alkali earth metals (Ca, Mg, Sr and Ba) than some alkali metals (K and Na) suggests the elemental accumulation in shoot is independent on the mother rock composition of minerals. But Ca and Mg have higher phylogenetic variation above family than K and Na (Watanabe et al., 2007).

Among 23 elements, Rb concentration was higher than all elements excluding P, K, Ca, Mg and Mn, but there is unknown role for Rb in plant nutrition. As the analogue of Cs, the Rb concentration was also relatively constant across species with lower C.V and ratio of the maximum to the minimum concentration. This stable distribution in different varieties in Amaranths was similar with Cs, but dissimilar with K, which coincides with the previous study that Rb concentration in shoot mainly depends on soil chemical and physical property, rather than variety difference (Watanabe et al., 2007). In addition, Cs^+ and Rb^+ uptake are mediated by K transport systems because K^+, Rb^+ and Cs^+ have comparable physio-chemical characteristics owing to similar radii of their hydrated ions (Penrose et al., 2015; Taylor and Gillis, 1994).

Table 2.2 Correlation coefficients for mineral elements in 33 varieties of Amaranths shoot.（NS= not significant. *P < 0.001）



	P	K	Ca	Mg	Fe	Mn	Cu	Zn	Mo	B	Ni	Na	Cs	Rb	Co	Se	Al	As	Li	Sr	Ba	Cd	Cr
P	–																						
K	NS	–																					
Ca	0.44*[a]	NS	–																				
Mg	0.45*	NS	0.86*	–																			
Fe	NS	NS	NS	NS	–																		
Mn	0.54*	NS	0.74*	0.58*	−0.36*	–																	
Cu	0.55*	NS	0.51*	0.46*	NS	0.51*	–																
Zn	0.39*	NS	0.50*	0.44*	−0.34*	0.42*	0.42*	–															
Mo	NS	NS	NS	NS	NS	NS	NS	NS	–														
B	NS	NS	0.60*	0.67*	−0.49*	0.46*	NS	0.65*	**	–													
Ni	NS	−0.44*	NS	NS	0.85*	NS	NS	NS	0.40*	−0.54*	–												
Na	NS	NS	0.50*	0.56*	NS	NS	NS	NS	0.52*	NS	ns	–											
Cs	NS	NS	NS	NS	0.39*	NS	NS	NS	NS	NS	0.37*	NS	–										
Rb	0.38*	NS	NS	NS	NS	0.41*	NS	NS	NS	NS	NS	NS	0.49*	–									
Co	NS	−0.38*	NS	NS	NS	0.68*	0.39*	NS	NS	NS	NS	NS	NS	NS	–								
Se	NS	NS	NS	NS	NS	NS	NS	0.40*	NS	NS	NS	NS	NS	0.43*		–							
Al	NS	−0.44*	NS	NS	0.86*	NS	NS	NS	NS	−0.53*	0.95*	NS	0.40*	NS	NS	NS	–						
As	NS	NS	NS	NS	NS	0.50*	NS	NS	NS	NS	NS	NS	0.60*	0.37*	0.75*	NS	NS	–					
Li	NS	−0.38*	NS	NS	0.66*	NS	NS	NS	0.40*	−0.37*	0.72*	0.45*	NS	−0.38*	NS	NS	0.79*	0.38*	–				
Sr	NS	NS	0.79*	0.69*	NS	0.46*	NS	0.55*	NS	0.62*	NS	NS	NS	NS	NS	NS	NS	NS	NS	–			
Ba	NS	NS	NS	0.59*	NS	NS	0.37*	NS	NS	NS	NS	NS	−0.38*	NS	NS	NS	NS	NS	NS	0.58*	–		
Cd	0.40*	−0.36*	0.73*	NS	NS	0.56*	0.43*	0.63*	NS	0.68*	−0.40*	NS	NS	NS	NS	NS	0.36*	NS	NS	0.68*	NS	–	
Cr	NS	NS	−0.39*	NS	0.69*	NS	NS	NS	0.49*	−0.49*	0.90*	NS	NS	NS	NS	NS	0.75*	NS	0.45*	NS	NS	0.42*	–

[a] Coefficients are derived from Pearson correlation analysis. NS, not significant; *, $P < 0.001$.

Interactions between mineral elements

The homeostatic mechanisms that control the levels of different elements are reported interconnected, and certain interconnections could be detected across species (Baxter et al., 2012; White et al., 2012) Pearson rank correlation analysis of large number of ionomic data from shoot samples of different species makes it possible to reduce the random connections of elements in a specie and emphasize the relationships among those elements strongly interconnected through metal-homeostasis. In the present study, correlations among elements in 33 varieties of Amaranthus were investigated (Table 2.2).

In this study, positive correlations were identified between K, Na and Li, Cs and Rb in the first group, Mg, Ca and Sr in the second group. Fe, Ni, Ba, Al, As, Rb showed significant correlations with Cs but among them only Fe and Ni are of essential role in plant nutrition, which suggested the Cs uptake was more readily affected by some nonessential elements. The absence of significant correlations between K and Cs coordinates with the previous study (Broadley and White 2012; Vinichuk et al., 2013). While it has been reported K and Cs enter root cells through the same molecular mechanisms and Cs uptake could be effectively strengthened in K-deficient solution (Isaure et al., 2006; Qi et al., 2008), the correlation between K and Cs was still insignificant. It has been proposed that the lack of this correlation is consistent with the constitutive expression of some selective K^+ transporters such as AKT1, which does not contribute significantly to Cs^+ uptake under typical soil conditions (Broadley et al., 2001) and the expression of other proteins catalyzing the non-specific absorption of Cs^+ and K^+ are regulated by varietal factors including the K^+ status of the plant (Hampton et al., 2004; Qi et al., 2008). Tsukada H et al. 2002 also reported that the internal translocation rate of Cs is slower than that of K in rice shoot and the distribution of Cs in grain and non-edible parts also diverges from that of K. Therefore, the physiological reasons for well-known agronomic countermeasures of K fertilization on the transfer of Cs from soil should be separated from the transport inside plant, but should associate with the block effect from cation exchangeable capacity of K on root uptake of Cs from soil, because K can occupy the absorption site of Cs in root. But meanwhile K helps Cs desorb from clay and mobilize to root so a large dose of K fertilization on K-deficient soil is probably effective for Cs retention.

Moreover, there was a recent report that Cs accumulation in yeast and plants could be specifically inhibited by the SNARE protein without interrupting K homeostasis in *Saccharomyces cerevisiae* and the mechanism may be caused by raising non-selective cations storage to vacuole (Draxi et al., 2013). In the evaluation of taxonomic variation in shoot Cs concentration among flowering plants, it was speculated that the plants with Cs hyperaccumulation, including Amaranths, were all of halophytes or their crop derivatives with less selectivity to meet their cations demands (Broadley et al., 1999). In addition, the partition of luxury quantities of cations in cell vacuole is an effective energy-efficient method for generating an osmotic potential for rapid cell expansion and growth (Ahmad and Maathuis, 2014). Therefore, the interactions between Cs and its remarkably correlated cationic elements in vacuole are likely to be responsible for the higher Cs concentration in Amaranths rather than only by cutting off the K supply.

In addition, among elements significantly correlated with Cs, As was the sole anion and displayed the highest coefficient with Cs (Table 2.2). It has been reported *Athyrium yokoscense* not only displayed the highest TF (translocation factor) value of radiocesium (0.703) among 99 herbs grown on radiocesium-contaminated field but also hyperaccumulated As from mine soil with elevated concentrations of As and heavy metallic ions (Brown and Cummings, 2001; Yamashita et al., 2014). These results indicate that the mutual promotion may exist for taking up As and Cs into plants. The findings from some previous studies may be helpful to explain the regulation of As on Cs that the significant decline in essential elements was associated with increasing As in rice grain (Norton et al., 2010; Williams et al., 2009). Furthermore, Ba was the unique element showing significantly negative association with Cs. This correlation is highly probably resulted from the block of Ba^{2+} on the channel for Cs^+ xylem loading to aboveground tissues. The termed

K^+-selective outward-rectifying channels (KORC) facilitate K^+ efflux from root cells and are permeable to Cs^+, but are inhibited by extracellular Ba^{2+} because the gene encoding the typical KORC is down-regulated by Ba^{2+} at millimolar concentrations (White and Broadley, 2000). This negative correlation between Ba and Cs also suggests the importance of long-distance transport for Cs accumulation in shoot of Amaranths. The countermeasures on Cs transport from soil to plant may be also beneficial for blocking influx of Al and As into shoot, but the interaction between Cs and Ba is necessary to be taken into account.

The significant correlations between Ca, Mg, Mn, Zn, B, Ba, Cd and Sr were detected and all of the coefficients were positive. Compared with the relationship between K and Cs, the relationship between Ca and Sr were much more pronounced (Ca presented the highest coefficient with Sr), which concurs with many previous findings in other plant species (Ariyama et al., 2006; Penrose et al., 2015; Watanabe et al., 2007). Broadley MR and White PJ (2000) pointed that the interaction between Ca and Sr concentration in shoot is consistent with reduced transport selectivity due to a greater contribution of apoplastic movement of these elements in plants. But if the countermeasures to decrease Sr uptake are considered based on this this directly proportional correlation between Ca and Sr, the intake of Ca by humans may be compromised in varieties with low Sr accumulation. The positive correlation between Sr^{2+} and many bivalent ions possibly depends on the shared non-selective cation channels, including Ca^{2+}-ATPase, ZIP, YSL family of transport proteins, in xylem transport (Song et al., 2010; White and Broadley, 2009). Besides the bivalent cationic ions, B was the unique anion to significantly correlate with Sr. Bivalent cations are often transported in the xylem by complexation with organic acids but the relationship between B and Sr was little reported (White and Broadley, 2009). It is probably related to the cation-anion balance but the exact mechanism requires more studies.

Identification of extreme varieties

Table 2.3 Lists of the significantly highest and lowest accumulating varieties for each element [a]

	P	K	Ca	Mg	Fe	Mn	Cu	Zn	Mo	B	Ni	Na
Sig.H[b]	7[a]	1	24	23	17	12	10	10	17	27	17	23
Sig.L	17 20	9	17	20	NS	20	20	3	10 11	17	19	NS

	Cs	Rb	Co	Se	Al	As	Li	Sr	Ba	Cd	Cr
Sig.H	12 17	6	12	30	17	12	16	24 28	16	28	17
Sig.L	25	16 17 20	2	17	NS	23 25 26	4	3 17	3	17	14

[a] Numbers represent the variety exhibited in Table1 of reference 13. Based on ANOVA with Tukey's test significantly highest/lowest varieties for each element were selected no more than three and if there were over five varieties showing significant extreme value the NS would be showed in this table. [b] Sig.H means significantly highest and Sig.L significantly lowest.

On the account of large genetic variance observed in a majority of elements and the fold-ranges of C.V.s for certain elements, potential candidate varieties for accumulating extremely high or low status of elements are prone to be identified. As shown in Table 2.3 (see the serial number of varieties in Table 1 of Shinano et al. 2014), varieties were identified with the significant highest value for all elements and significant lowest value for all elements excluding Fe, Al and Na. Particularly some varieties showed up the same extreme for more than one element with the significantly highest or lowest concentration, which relates to the complicated elemental correlations. The variety 12 accumulated the significantly highest Cs, Mn, As and Co in shoot. The variety 25 displayed the strongest resistance for Cs accumulation in shoot among 33 varieties and it also restricted the As accumulation with the highest efficiency, which possibly associates with the result that As was the element with the highest correlation coefficient with Cs. Therefore, the countermeasures to prevent As uptake may be also beneficial for Cs resistance, such as increasing P fertilization. Variety 17 showed extreme promotion for the accumulations of Cs, Fe, Ni, Al, Cr, Mo, and the extreme inhibition for accumulations of Ca, Sr, Cd, Se, P, Rb and B. Interestingly, Cs and Rb exhibited significant positive correlation but in variety 17 the accumulations of Cs and Rb diverged. This result implies that although the positive correlation between Rb and Cs was demonstrated, the up-regulation on Rb^+ uptake may be still beneficial for restricting more Cs^+ influx into shoot. The accumulation of Rb^+, rather than Cs^+, was remarkably high in Amaranths shoot since it was ranked $K^+ > Rb^+ > Cs^+$, with a relative ratio 468386: 7818: 1, which coincides with the previous study on the mobility of K, Cs and Rb within soil-ectomycorrhizal system (Vinichuk et al., 2013). In that study, the fungi showed a high preference for uptake of Rb and K compared with Cs and it was reported that the mechanism of Cs uptake by fungi was similar to Rb, whereas that the K uptake mechanism may be different. But the research on relationship between Rb and Cs accumulation in shoot was quite scarce. Based on the inference from the extreme accumulation of variety 17, the much larger preference of Amaranths on Rb may provide the new insight into the regulation on Cs uptake. In addition, the significantly highest Cs and lowest Sr both appeared in variety 17. In the present study, Sr and Cs did not show significant correlation, which coordinates with a large proportion of past studies (Ariyama et al., 2006; Penrose et al., 2015). This specific accumulation in variety 17 probably resulted from the greatly different uptake mechanisms and functions of their analogous elements K and Ca. This result poses a challenge for selection of low-accumulating varieties owing to the exhibition of low accumulation of one radionuclide but not necessarily of the other. Variety 17 may be a potential plant material for studying on relationship between Cs and Sr accumulation in shoot.

The significantly highest concentrations of Sr and Ca were both detected in variety

24, which showed the role of Ca on Sr accumulation in shoot again. The significantly highest concentrations of Sr and Cd were both detected in variety 28. The significantly lowest concentrations of Sr, Ba and Zn were all observed in variety 3. All of the varieties, excluding variety 17, showed that the extreme accumulation for Sr must associate with other bivalent cationic elements, and Ca was not the only choice.

Profiling the ionome in thirty-three Amaranthus varieties

PCA succeeded to reduce the number of variables by clustering the elements with close interactions into the novel principle component and explained their contributions to the genetic variance, respectively (Table 2.4 and 2.5). A total of 23 elements of 33 varieties were subjected to PCA and these 23 original variables were converted to a set of linearly uncorrelated six PCs (principle components) that totally contributed to 83.7% total variance of elements among species (Table 2.4). The PC where the elements exposed the highest loading score can define the according elements. In this study, PC1 accounted for 23.2% of the highest total variance and was mainly affected by Ni, Al, Fe, Cr, Li, Mo and Ca. Notably, PC2 explained 20.3% of total variance and was dominantly incorporated by Sr and many of its significantly correlated elements, excluding Na. PC3 revealed 12.4% of the total variance and summed up the set of Cs and its significantly correlated elements, Rb, As and Se.

Table 2.4 Rotated component matrix of different elements loading score in different PC.s [a]

	PC1 (23.2%)	PC2 (20.3%)	PC3 (12.4%)	PC4 (10.1%)	PC5 (9.1%)	PC6 (8.6%)
Ni	0.96	-0.20	-0.02	-0.05	-0.06	0.09
Al	0.94	-0.18	0.05	0.05	-0.15	0.12
Fe	0.88	-0.18	0.05	-0.01	-0.22	-0.08
Cr	0.86	-0.25	-0.06	-0.21	0.11	-0.02
Li	0.76	0.25	0.02	-0.05	-0.41	0.30
Mo	0.53	0.40	-0.06	-0.49	0.08	-0.23
Ca	-0.23	0.88	0.04	0.06	0.22	0.21
Mg	-0.17	0.85	-0.06	-0.02	0.31	0.00
Sr	-0.07	0.84	-0.11	0.48	-0.04	0.02
Na	0.22	0.76	-0.17	-0.45	-0.17	0.00
Cd	-0.30	0.75	-0.04	0.26	0.25	-0.09
B	-0.51	0.59	0.02	0.30	0.16	-0.19
Mn	-0.30	0.55	0.37	0.00	0.35	0.47
Cs	0.35	-0.20	0.82	-0.27	0.04	-0.03
Rb	-0.41	0.07	0.74	0.05	0.20	-0.06
As	0.13	-0.01	0.78	-0.02	-0.01	0.53
Se	-0.35	0.18	0.53	0.41	0.34	0.04
Ba	0.08	0.10	-0.20	0.81	0.09	-0.27
Zn	-0.29	0.33	0.03	0.58	0.48	0.12
P	-0.33	0.17	0.13	0.05	0.78	0.04
Cu	0.10	0.43	0.20	0.13	0.69	0.16
Co	-0.04	0.06	0.41	-0.07	0.23	0.83
K	-0.41	0.01	0.26	0.14	0.07	-0.71

a. Shading areas represent the most dominant socre of each element in the according PC.

In Table 2.5, component score coefficient matrix of different varieties shows the relative accumulations of different elements in plants. The mean value of factor loading score is 0 and the positive value means the score is higher than the average level in the according PC. It is noted that variety 12 not only accumulated the significantly highest Cs in shoot but also obtained the highest loading score in PC3 and PC6, second lowest score in PC4. Besides variety 12, the variety with loading score of PC3 higher than 1 was only detected in variety 4 and 5. On the other hand, the variety 25 accumulated the lowest Cs and the loading scores in PC3 were the lowest among 33 varieties. Additionally, the variety 17 exhibited the highest loading score in PC1 and higher than variety 13 at the second place in the ranking of loading score in PC1 by 1.4-fold. The variety 9 presented uniquely positive values in all PCs. Conversely, the variety 3, with the lowest concentration of Sr in shoot, showed negative loading score in all PCs, which means any of the loading scores in PCs was lower than the mean value in the according PC.

Table 2.5 Factor loading score of 33 varieties in different PCs

Variety	PC1	PC2	PC3	PC4	PC5	PC6	Variety	PC1	PC2	PC3	PC4	PC5	PC6
1	-0.44	-0.34	-0.12	0.27	0.36	-1.39	18	1.74	-0.41	-0.50	-0.22	-0.44	0.07
2	-0.29	-0.77	0.91	-0.91	0.92	-1.86	19	-0.90	-0.95	-0.33	-0.67	-0.27	-0.03
3	-0.70	-1.66	-0.11	-2.38	-1.11	-0.09	20	-0.88	-1.73	-1.51	-0.88	-1.84	0.40
4	-0.89	-0.67	0.75	-0.11	0.92	-0.97	21	0.24	1.94	0.12	-1.41	0.04	-0.69
5	-0.29	-0.20	1.77	0.00	-0.67	-1.48	22	-0.18	0.91	-1.21	0.02	-0.20	0.78
6	0.50	-0.64	1.79	0.18	0.30	-0.32	23	-0.06	2.36	-1.06	-1.49	0.61	-0.15
7	-0.50	-0.41	0.98	0.10	1.95	0.33	24	0.71	2.82	-0.10	-1.32	-0.48	-0.72
8	-0.02	0.51	0.82	0.54	-2.36	0.93	25	-0.44	-0.61	-2.30	0.39	1.45	0.53
9	-0.06	-0.03	0.31	-0.36	-0.86	2.37	26	-0.15	-0.14	-1.39	0.39	1.35	-0.05
10	0.56	0.21	0.58	1.11	1.52	1.99	27	-0.82	-0.15	-0.02	-0.02	-0.70	-0.67
11	1.40	-0.42	0.75	1.28	-0.30	0.56	28	-0.17	1.47	0.11	0.99	-0.94	-0.26
12	-0.53	0.12	2.29	-1.51	0.83	2.52	29	-0.32	0.33	0.28	1.26	0.37	-0.57
13	-0.83	0.08	-0.86	-0.21	0.82	1.36	30	-0.44	0.09	0.52	1.14	0.11	-0.43

14	-0.63	-0.52	-1.21	0.33	0.01	-0.15	31	-0.18	-0.02	0.15	0.90	-0.35	-0.61
15	-0.04	0.30	-0.91	1.29	0.38	-0.41	32	-0.26	0.18	0.22	1.22	0.27	-0.52
16	1.66	-0.29	-0.42	1.53	-1.74	0.25	33	-0.81	-0.10	0.12	-0.06	-0.80	-0.18
17	4.04	-1.68	-0.44	-1.72	0.85	-0.52							

PC3 is important with respect to Cs accumulation because it collected Cs and the significantly correlated elements As, Rb and Se in the same set. The variety 12 obtained the highest loading score both in PC3 and in PC6 dominated by positive Co and negative K quantification, the second lowest loading score in PC4 dominated by Ba and Zn quantifications. As a result, more Cs deposited in shoot of variety 12 may be achieved by increasing the absorption of Co, Cs, Rb, As and other elements positively correlated with Cs, and diminishing the K, Zn and Ba, the potentially competitive or antagonistic ions flux into plants. It has been reported that lacking of K supply would result in the decrease of vacuolar K^+ concentrations and further lower osmotic potential (Van et al., 2006; Vinichuk et al., 2013), which is beneficial for non-selectively assimilation of minerals, especially for Rb^+ and Cs^+ owing to their chemical similarity. It could be speculated that K^+ starvation in the vacuole could probably push the flux of cations including Rb^+ and Cs^+ to vacuole to maintain the osmotic potential and limit the absorption of Ba^{2+} to be unobstructed in xylem transport, which is consistent with the loading score of variety 12 in PC3, PC4 and PC6.

The variety 17 revealed another similar mechanism for efficiently taking up Cs. It gained the highest loading score in PC1 that accounted for 23.2% of the highest total variance and was mainly affected by Ni, Al, Fe, Cr, Li and Mo, among which Ni, Al and Fe were significantly correlated with Cs. Meanwhile, the lowest loading score in PC2 and secondly lowest loading score in PC4 were achieved by the variety 17. PC2 and PC4 were dominantly affected by B, Na, Ca, Mg, Sr, Ba, Zn, Cd and Mn. These elements, excluding Na, were all significantly correlated with Sr and played indicative role for Sr accumulation. There is no competitive relationship between monovalent ion and bivalent ion so this pattern of elemental composition possibly derives from the regulation of plant on overall ionomic uptake. It was demonstrated that the inward-rectifying potassium channels (KIR) in the plasma membranes, also permeable for Cs^+, are inhibited by millimolar concentrations of Ba^{2+} and Ca^{2+}(White and Broadley, 2000). Although significant correlation was not detected between Cs^+ and any of bivalent cations, the particular ionomic characteristic in variety 17 may be of importance to associate Cs and Sr. The variety 12 and 17 both took up more Cs by promoting the influx of elements positively correlated with Cs into shoot, but the former decreased the K and Ba uptake and the latter inhibited the accumulation of Rb, Sr and significantly correlated elements of Sr in shoot.

Based on these results, K fertilization may be a feasible countermeasure on radiocesium uptake in K-deficient soils in Fukushima. In addition, vermiculates, zeolites and bentonites may be splendid selection as well because many cations including K^+, Ca^{2+}, Mg^{2+} are contained in them. The application of these minerals to achieve the radiocesium retention has been demonstrated successful in some previous studies (Atun and Bodur, 2002; Fujimura et al., 2013; Valke et al., 1997). Sha ZM et al. 2012 proved the manure application led to the inhibition on the upward translocation of Cs to soybean seed, and improved the accumulation of Cd, Ba, Co and Zn, which coordinates with the inference in this study. In addition, because among elements the highest positive coefficient for Cs was detected between Cs and As and the variety 33 accumulated both the lowest Cs and As, the countermeasures to inhibit As uptake may be beneficial for resistance of Cs translocation to shoot.

CHAPTER 3

The dynamic state of ionome in the root nodule and shoot of soybean under different nitrogen status and at different growth stages

3.1 Introduction

The ionome is defined as all the mineral nutrients and trace elements found in an organism (Lahner et al., 2003). For crop, the ionome has predominantly been studied in soil, plant seeds or shoots to determine the nutritional status of plants (Chu et al., 2015; Sha et al., 2012; Vohland et al., 2009), or in the mutants to screen the species with fine elemental composition (Waters and Grusak, 2008; Ziegler et al., 2012), but inadequate information has been available on the elemental composition of belowground parts, especially for legume crops with nodules. The biological fixation of N_2 (BNF), which occurs in nodules of legume plants inoculated with rhizobium, has been shown to meet 50-60% of soybean N demand (Salvagiotti et al., 2008). In fact, 15 of the 17 essential elements of plants are also considered essential for rhizobia as well, showing in the list: C, Ca, Cu, Fe, H, K, Mg, Mn, Mo, N, Ni, O, P, S, and Zn. These elements play various roles in the symbiosis between legumes and rhizobium, acting as part of the nitrogenase (Fe, Co, and Mo) (Mendel et al., 2011), cofactors to participate in N fixation and stabilize cell walls for N uptake (Mn, Zn, Fe, Cu, S, and Ni) (Mendel et al., 2011; Fester et al., 2014), crucial signals for bacteroid differentiation into the N_2-fixing form of rhizobium (Ca and B) (Will et al., 2012; Zobiole et al., *2012*), or satisfying energy supply in the form of ATP for nitrogenase function (P) (Rubio et al., 2012). In addition, via BNF legumes take up more cations than anions because uncharged N_2 enters the roots, and the consequent high ratio of cation/anion uptake results in net release of protons, thus lowering the soil pH (White and Broadley, 2009). The induced rhizosphere acidification may increase the availabilities of toxic elements such as Cd and Ba. Moreover, the transport proteins in the plasma membrane of root cells are not able to differentiate effectively essential and nonessential elements that are chemically similar, such as K^+ and Cs^+, Ca^{2+}, Sr^{2+}, and Ba^{2+} (Baxter et al., 2008). Nevertheless, little information is available to describe the distribution of elements in the soybean plant, particularly for micro- and toxic elements.

In addition, the patterns of ionomic uptake and delivery in different tissues have been studied at one growth stage (Vondráčková et al., 2014), but variations in the ionomic mobilizations from the soil to the root and shoot at different growth stages have not yet been considered. Nodule senescence is a programmed process that is coupled to flowering, the entry into the reproductive stage of the host plant life cycle (Puppo et al., 2005). Thus, anthesis not only divides the vegetative and reproductive stages but also signifies the start of nodule senescence. This work examines the metal efflux from nodules after anthesis. For instance, Fe is remobilized out of nodules and recycled to the flowering and embryogenesis upon flowering (Burton et al., 1998; Rodríguez-Haas et al., 2013). Based on this result, Zn, Cu, Mn and other chemically similar elements have been hypothesized to be moved in a Fe-like fashion due to their similar transport mechanisms (González et al., 2013), but few studies have tested this hypothesis by profiling the mobilization of elements between different growth periods.

The present study attempted to characterize the influence of ammonium and manure N source on (1) ionome accumulation, (2) the dynamic variability of the ionome in the root, nodule and shoot from R1 to R7. Moreover, we aimed to test whether essential metal elements were exported out of nodules upon nodule aging.

3.2 Materials and methods

Design of the experiment

Soybeans (*Glycine max* L. Merr.cv. Tsurumusume) were cultivated in pots in a greenhouse at the National Agricultural Research Centre (NARO/HARC), Sapporo, Japan. Volcanic ash soil, which is classified as a Melanudands according to the Classification of U.S Soil Taxonomy, was used in the pot experiment. The basic characteristic of the experimental soils was: pH 5.3, 4.3 g kg^{-1} total N, 189 mg kg^{-1} P_2O_5-availability (Truog P), 38.4 cmol kg^{-1} CEC (cation exchange capacity), 0.4 cmol kg^{-1} exchangeable K, 9.8 $cmol_c$ kg^{-1} exchangeable Ca, 1.3 $cmol_c$ kg^{-1} exchangeable Mg, and BS (base saturation) 29%.

Ten kilograms of air-dried soil was sieved to pass through a 2 mm mesh and then filled in a 12 L, free-draining pottery pot. In each pot, the soil pH was adjusted to 6.0 using $CaCO_3$. Each experiment consisted of three fertilizer treatments (four replicates per treatment): chemical fertilization with adequate N, P and K (CF), cattle farmyard manure application (M), and chemical fertilization without N (-N). For the CF treatment, fertilizers were applied at concentrations 1.6 g N pot^{-1} of ammonium sulfate, 2.0 g P_2O_5 pot^{-1} of calcium superphosphate and 3.12 g K_2O pot^{-1} of potassium sulfate. For the M treatment, cattle farmyard manure was applied at rates equivalent to the N, P_2O_5 and K_2O rates used in the CF treatment. The mineral elements concentration in the manure is shown in Table 3.1. For the -N treatment, the rates of P and K fertilizer application were the same as those used for the CF treatment, without N fertilizer. The manure was mixed with soil to incubate for 2 weeks before sowing. Five soybean seeds were sowed in a pot and thinned to one seedling after germination. Deionized water was added daily to maintain the 60% (w/w) of the water-holding capacity of the disturbed soil. Two same experiments were conducted at the same time in the same place.

Table 3.1 Concentrations of elements in Manure (DM: dry matter)

P	K	Mg	Ca	Mn	Fe	Cu	Zn	B
g kg^{-1} DM	g kg^{-1} DM	g kg^{-1} DM	g kg^{-1} DM	g kg^{-1} DM	g kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM
7.72	25.87	10.85	17.55	0.4	3.7	65.6	200.04	13.84
Co	Na	Sr	Ba	Ni	Cd	Cr	Se	Cs
mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM
3.76	5700	62.09	62.72	48.31	0.19	9.13	0.71	0.17

Sampling and chemical analysis for plant and soil

Soybean seedlings were harvested twice, 35 days or 68 days after sowing. The nodules, roots and shoots (includes all aboveground tissues) were rinsed with milli-Q water, and then dried at 70°C for 72 h, weighed and milled. For the elements analysis, the concentrations of 21 elements (P, K, Mg, Ca, Mn, Fe, Cu, Zn, B, Mo, Sr, Cr, Al, Ba, Cd, As, Na, Co, Se, Li, and Cs) in various tissues were determined as described in 2.2. For the N analysis, the samples were digested with [H_2SO_4 (98%) -H_2O_2] and the N concentrations were determined using the Kjeldahl method (Page et al., 1982). Fresh soils were collected from planted and unplanted pots at the same time of plant sampling. The rhizosphere soil (RS) was obtained by vigorously shaking the roots to which soil adhered in a paper bag (140×100×0.04 mm, length×width×thickness). The other soils sampled from planted pots were considered to be near-rhizosphere soil (NRS). The soil sampled from unplanted pot was regarded as bulk soil (BS). Soil samples were air-dried at 25°C and sieved to pass through a 2 mm plastic mesh. The soil pH and available concentrations of elements were analyzed. The soil pH was measured in a suspension of soil and Milli-Q water (1:2.5, w/v) using a pH-EC meter (MP220, Mettler Toledo, Japan). The concentrations of available elements in the soil were determined by extracting 2 g of air-dried soil with 40 ml of 1 M ammonium acetate. The mixture was shaken for 1h and then filtered with filter paper (No. 5C). Subsequently, 2 ml of 61% HNO_3 was added to 5 ml of filtered extract and concentrated in a DigiPREP apparatus until the solution had almost completely evaporated, using the same procedure as was used for the plant. Finally, the tube was filled to 10 ml with 2% HNO_3 for the ICP-MS analysis. The concentrations of 21 elements excluding N were determined as described for the plant analysis.

Statistical analysis

All statistical analyses were performed using the SPSS 18.0 (SPSS Inc. Chicago, IL, USA) programs. A one-way ANOVA with Bonferroni test and adjustment was applied to analyze the effect of the N treatments on the ionomic concentrations, and a conservative *P* value limit of 0.01 was used as a multiple testing correction for significant differences of high-throughput detection on 22 elements status. A principle component analysis (PCA) was adopted to profile the concentration of mineral elements in all parts of the soybean at R1 and R7. A varimax rotation with Kaiser normalization was used as the rotation method.

3.3 Results

Biomass in different organs

The effect of various N sources on the biomass of root, nodule and shoot is shown in Table 3.2. CF treatment significantly decreased the biomass production of root and nodule at R1 and R7. The shoot biomass was higher at R7 than at R1 independent of the N sources, whereas the changes in the root and nodule biomasses depended on the N sources.

Table 3.2 Effect of different N sources on the biomass of roots, nodules and shoots at R1 and R7.

Growth stage	Treatment	Dry weight (g plant^{-1})			Growth stage	Treatment	Dry weight (g plant^{-1})		
		Root	Nodule	Shoot			Root	Nodule	Shoot
R1	CF	1.30b	0.30b	6.89a	R7	CF	1.14b	0.29b	15.07a
	M	1.51b	0.53a	5.68a		M	1.62b	0.70a	16.10a
	–N	1.85a	0.59a	7.00a		–N	1.99a	0.83a	15.18a

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.Differences between treatments were evaluated using an ANOVA with Tukey test ($p<0.05$).

Available concentrations of mineral elements in the RS

The concentrations of available minerals in the RS in response to N treatments are displayed in Table 3.3. The N fertilizer treatment did not significantly affect the Mg, Fe, Zn, B, Ba, Sr, Se, Li and As levels in the RS. CF treatment markedly improved the available concentrations of Mn and Co at R1, reduced the levels of P at R1 and R7. M treatment significantly increased the availabilities of K, Na, Cr and decreased the levels of Ca, Cd and Cs at R1 and R7. -N treatment significantly enhanced the available concentration of Mo at R1. Moreover, the influence of N treatments on the Cu availability in the RS differed between R1 and R7. At R1, the available Cu concentration in the RS was higher for the M treatment than for the -N treatment by 0.86-fold. However, the Cu concentration for the M treatment at R7 was lower than that for -N treatment by 0.75-fold and lower than that for the CF treatment by 1.20-fold.

Table 3.3 Concentrations of available mineral elements (extracted by 1 M ammonium acetate) in rhizosphere soil at R1 and R7.

		mg kg^{-1}										
Stage	Treatment	P	K	Mg	Ca	Mn	Fe	Zn	Na	Al	Ba	Sr
R1	CF	**116.18b**	**734.36b**	253.63	**916.73a**	**14.74a**	8.81	0.61	**23.59b**	**6.20a**	42.30	10.94
	M	**159.42a**	**933.33a**	242.34	**769.78b**	**10.51b**	7.53	0.54	**33.00a**	**3.92b**	39.84	9.72
	–N	**161.21a**	**746.45b**	250.58	**944.35a**	**10.23b**	5.62	0.50	**26.44ab**	**3.24b**	50.24	11.70
R7	CF	**124.85b**	**732.63b**	246.87	**946.30a**	13.92	6.81	1.00	**30.32ab**	4.08	33.04	10.41
	M	**188.66a**	**965.04a**	281.98	**844.14b**	12.21	8.45	0.85	**37.87a**	5.22	39.44	11.26
	–N	**153.36ab**	**849.97ab**	269.96	**995.33a**	12.92	7.43	0.98	**28.51b**	5.03	41.87	11.60

		µg kg^{-1}									
Stage	Treatment	B	Cu	Mo	Co	Se	Li	As	Cr	Cd	Cs
R1	CF	496.65	**95.34ab**	**2.19b**	**26.60a**	12.89	10.91	14.51	**17.29b**	**19.50a**	**42.79ab**
	M	523.35	**134.40a**	**2.31b**	**18.56b**	9.60	10.02	13.21	**31.07a**	**14.83b**	**34.40b**
	–N	489.76	**72.28b**	**4.76a**	**19.68b**	11.89	7.73	15.05	**13.48b**	**18.65a**	**46.47a**
R7	CF	395.48	**117.16a**	2.91	24.35	11.91	6.33	15.93	**15.49b**	**19.52a**	**45.57ab**
	M	463.54	**67.05b**	3.21	20.66	10.87	8.92	15.89	**117.02a**	**15.98b**	**37.65b**
	–N	519.76	**147.47a**	3.49	23.51	12.25	9.51	16.46	**22.73b**	**19.63a**	**51.80a**

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M:

manure application; -N: chemical fertilization without N; R1: beginning of flowering stage; R7: beginning of ripening stage. Calculated by ANOVA with Bonferroni test ($p<0.01$), differences between treatments were significant representing by boldfaced entries on the mean ranks with letters (a-c).

Macroelements concentrations in the root, nodule and shoot

The concentrations of macroelements in various parts of soybean plant in response to N treatments are exhibited in Table 3.4. In the root, CF and M treatment significantly increased the N concentration at R1 and R7 compared with -N treatment but this difference was only observed in the nodule. The N concentration in the nodules affected by -N treatment at R1 was 1.90- and 1.77-fold higher than by CF and M treatment. In the shoot, M treatment improved the N accumulation at R1. CF treatment significantly reduced the P concentration in the root, nodule and shoot as well as the K concentration in the nodule and shoot at R1. Nevertheless, it enhanced the Ca concentration in the root and shoot at R1 and R7. CF and M treatment significantly enhanced the Ca and Mg concentration in the root and shoot, and reduced the Ca concentration in the nodule at R1 and R7.

Table 3.4 Effect of N sources on the macroelements in the root, nodule and shoot at R1 and R7.

	R1			**R7**		
	CF	**M**	**–N**	**CF**	**M**	**–N**
	Macroelement concentration in root (g kg^{-1} DM)					
N	**33.69a**	**35.87a**	**25.08b**	**32.47a**	**27.53b**	**22.29c**
P	**4.67b**	**5.72a**	**5.11ab**	**4.63a**	**4.66a**	**2.09b**
K	**14.41a**	**14.06a**	**13.80a**	**9.37b**	**8.02b**	**14.11a**
Ca	**9.62a**	**5.15b**	**5.47b**	**13.99a**	**5.52b**	**4.80b**
Mg	5.64	4.58	3.01	6.47	2.83	2.28

Macroelement concentration nodule (mg kg^{-1} DM)						
N	**42.95b**	**46.02b**	**81.62a**	**20.47c**	**34.29b**	**39.53a**
P	6.77	7.81	8.19	3.44	4.35	3.70
K	**17.71b**	**32.53a**	**39.32a**	**6.88b**	**13.04a**	**7.81b**
Ca	**5.67b**	**6.02b**	**11.55a**	**3.46c**	**6.69b**	**11.19a**
Mg	3.61	5.23	4.77	1.25	1.07	1.02
Macroelement concentration in shoot (g kg^{-1} DM)						
N	27.08	36.51	30.07	45.00	47.51	48.70
P	**6.77b**	**7.81a**	**8.19a**	4.61	3.44	4.35
K	**17.71b**	**32.53a**	**39.32a**	**6.81b**	**6.88b**	**13.04a**
Ca	**20.01a**	**17.84b**	**15.67b**	**19.19a**	**14.25a**	**11.07b**
Mg	4.65	4.19	3.88	4.08	3.84	2.88

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: beginning of flowering stage; R7: beginning of ripening stage. Calculated by ANOVA with Bonferroni test ($p<0.01$), differences between treatments were significant representing by boldfaced entries on the mean ranks with letters (a-c).

Microelements concentrations in the root, nodule and shoot

The concentrations of microelements in various parts of the soybean plant in response to N treatments are detailed in Table 3.5. Because Co is essential for the symbiosis between soybeans and rhizobium, it is considered as an essential microelement in the present study. In the root, the CF treatment resulted in significantly higher Mn concentration at R1 and R7, Mo and Co concentrations at R1, and Fe and Cu concentrations at R7. The N treatment significantly increased the Fe and Na concentrations at R1 and the Mo concentration at R7 but did not significantly affect the Zn or B concentration at either of the stages. In the nodules, the CF treatment significantly improved the Mn and Co concentrations at R1 and R7, and the Fe concentration at R7. The CF and M treatments significantly decreased the Mo concentration at R1 and R7, and the Cu concentration at R1. In the shoot, the CF and M treatments significantly decreased the Mn concentration at R1 and R7, the Zn concentration at R1 and the Fe concentration at R7. The CF treatment significantly increased the Cu concentration at R1. The Mo, B and Co concentrations in the shoot were not significantly affected by the N treatments at either stage.

Table 3.5 Effect of N sources on the microelement concentrations in the root, nodule and shoot at R1 and R7.

	R1			**R7**		
	CF	**M**	**–N**	**CF**	**M**	**–N**
			Microelement concentration in root (mg kg^{-1} DM)			
Mn	**470.81a**	**349.32b**	**144.25c**	**1367.66a**	**363.58b**	**197.34b**
Fe	**800.44b**	**900.81b**	**1591.79a**	1470.17	1367.53	1199.96
Cu	**8.0a**	**8.11a**	**4.88b**	**12.01a**	**6.83b**	**7.69b**
Zn	28.04	28.49	26.98	42.60	32.93	38.07
Mo	**1.30a**	**0.45b**	**0.57b**	**0.13c**	**0.87b**	**1.18a**
B	3.69	4.78	4.27	9.80	9.70	10.09
Co	1.13	1.08	1.28	2.81	1.43	1.75
			Microelement concentration in nodule (mg kg^{-1} DM)			
Mn	**645.01a**	**366.79b**	**259.65b**	**418.79a**	**247.79b**	**171.68b**
Fe	1132.34	824.24	919.86	**818.62a**	**423.11b**	**467.69b**
Cu	**3.64c**	**9.87b**	**10.42a**	3.02	4.38	4.63
Zn	36.64	27.44	30.83	22.74	22.53	22.21
Mo	**0.63c**	**3.81b**	**5.47a**	**0.22c**	**0.40b**	**0.90a**
B	10.31	10.42	9.21	8.34	7.71	7.65
Co	3.78	2.09	1.82	**5.11a**	**2.35b**	**2.02b**
			Microelement concentration in shoot (mg kg^{-1} DM)			
Mn	196.18	164.21	103.98	244.38	260.69	134.95
Fe	80.47	81.37	82.95	103.10	99.32	78.49
Cu	5.00	3.98	3.88	7.42	5.30	5.05

Zn	32.85	30.86	24.96	33.26	39.04	43.54
Mo	0.11	0.12	0.12	0.20	0.30	0.23
B	12.83	10.78	12.21	26.84	27.76	28.30
Co	0.057	0.051	0.057	0.09	0.11	0.10

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: beginning of flowering stage; R7: beginning of ripening stage. Calculated by ANOVA with Bonferroni test ($p<0.01$), differences between treatments were significant representing by boldfaced entries on the mean ranks with letters (a-c).

Nonessential elements concentrations in the root, nodule and shoot

The concentrations of nonessential elements in various parts of the soybean plant in response to N treatments are listed in Table 3.6. The CF treatment significantly increased the Al concentration in the root and nodule at R1 and R7, increased the As concentration in the root at R1 and in the nodule at R7. M treatment significantly improved the Al, Ba, Sr, Cd and Cr concentrations in the shoot as well as the Na and Se concentrations in the nodule at R7, but reduced the Cd concentration in the nodule at R1 and R7, the As, Sr, Cr and Ba concentrations in the root at R7, and the Cs concentration in the shoot at R1 and R7. The -N treatment significantly increased the Na and Se concentrations in the root at R1 and in the nodule at R7, whereas it decreased the Na concentration and increased the Se concentration in the shoot at R1 and R7. The N treatments did not significantly affect the Li concentrations in the nodule, root or shoot at either stage.

Table 3.6 Effect of N sources on the nonessential elements of the soybean shoot in the root, nodule and shoot at R1 and R7.

	R1			**R7**		
	CF	**M**	**–N**	**CF**	**M**	**–N**
	Nonessential element concentration in root (mg kg^{-1} DM)					
Na	**858.31b**	**934.22b**	**2271.55a**	290.94	480.05	328.38
Se	**0.13b**	**0.15b**	**0.26a**	**0.27a**	**0.11b**	**0.22a**
Al	**2750.33a**	**1466.67b**	**1909.15b**	**5671.63a**	**2377.85b**	**3430.18b**
Li	0.16	0.14	0.18	1.13	1.04	1.06
As	0.53	0.45	0.50	1.038	0.60	1.00
Ba	2.34	2.93	2.25	60.56	50.42	56.00
Cs	0.085	0.093	0.11	0.17	0.12	0.16
Sr	35.45	41.36	36.91	**39.82a**	**30.19b**	**36.22a**
Cd	1.22	1.38	0.73	**5.66a**	**2.56b**	**4.77a**
Cr	**40.55a**	**27.23b**	**45.42a**	**17.14a**	**11.38b**	**19.82a**
	Nonessential concentration in nodule (mg kg^{-1} DM)					
Na	249.56	266.86	308.38	**611.89b**	**775.84b**	**1219.24a**
Se	0.14	0.14	0.14	0.32	0.20	0.34
Al	687.58	566.67	477.29	**630.18a**	**464.21b**	**364.63c**
Li	0.036	0.036	0.032	0.10	0.083	0.090
As	0.31	0.32	0.35	0.83	0.53	0.67
Ba	1.67	2.09	1.61	2.36	2.83	2.25
Cs	0.067	0.072	0.099	0.15	0.11	0.14
Sr	28.13	24.44	24.77	32.25	29.34	33.40
Cd	0.098	0.11	0.010	**0.81ab**	**1.36a**	**0.36b**
Cr	20.96a	32.04a	35.23a	12.34a	8.19a	15.27a
	Nonessential concentration in shoot (mg kg^{-1} DM)					
Na	86.83	73.57	54.95	37.02	32.35	25.04
Se	0.061c	0.12b	0.18a	**0.11b**	**0.12b**	**0.21a**
Al	23.57	18.18	17.40	**54.71b**	**124.25a**	**37.57b**
Li	0.018	0.0055	0.018	0.029	0.0197	0.019
As	0.066	0.051	0.068	0.048	0.019	0.043
Ba	1.15	0.6	0.68	**1.66b**	**3.78a**	**2.38ab**
Cs	**0.022a**	**0.012b**	**0.021a**	**0.024a**	**0.013b**	**0.019a**
Sr	60.09	77.80	53.15	**76.10b**	**106.42a**	**71.43b**
Cd	0.26	0.22	0.13	**0.22b**	**0.41a**	**0.28b**
Cr	**2.56b**	**28.58a**	**5.52b**	**2.24b**	**16.09a**	**2.64b**

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: beginning of flowering stage; R7: beginning of ripening stage. Calculated by ANOVA with Bonferroni test

($p<0.01$), differences between treatments were significant representing by boldfaced entries on the mean ranks with letters (a-c).

Discussions

Effect of ammonium-N source on the ionome concentrations in different tissues of soybean plant

None of the N fertilizer significantly affected the N concentration in the shoot at either of the stages (Table 3.4), which implies that CF treatment reduced N utilization efficiency (NUE). By calculation based on the method of Devkota et al., (2013), the CF treatment reduced the NUE by 14.93 % and 38.20 % relative to -N treatment at R1 and R7, relatively. Moreover, the CF treatment not only decreased NUE but also hampered the root and nodule development (Table 3.2), which coincides with previous reports that adding inorganic N fertilizer inhibited nodulation and nodule biomass (Namvar et al., 2011; Otieno et al., 2009), as well as cell division in the cortex and nodulation of *Alns incana* at the early growth stage (Gentili et al., 2006).

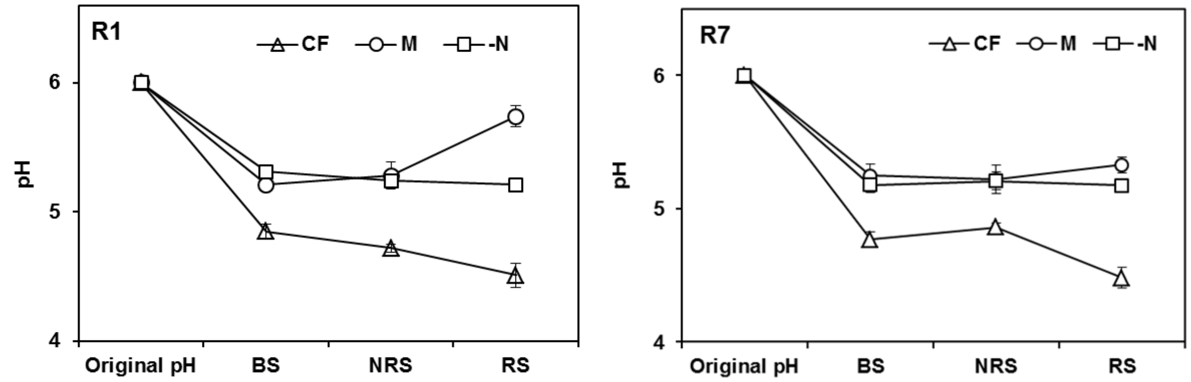


Fig 3.1 Variation in the soil water-extracted pH in the BS, NRS and RS in response

to different treatments at R1 and R. Error bars represents the SD. Abbreviation: BS: bulk soil; NRS: near-rhizosphere soil; RS: rhizosphere soil; CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.

The lower NUE, nodule and root biomass may be related to the soil acidification caused by CF treatment (Fig 3.1). CF treatment resulted in lower pH values in different soil fractions than did M or -N treatment, irrespective of the growth stages. This acidification is likely attributed to protons release into the soil that resulted from hydrolysis of ammonium sulfate fertilization. Increased protons release acidified the soil, which could constrain root elongation and bacteria recognition to further inhibit root and nodule growth (Table 3.2). Additionally, low pH affected the availability of many elements and influx into plant. Lower P availability in the RS (Table 3.3) may relate to soil acidification caused by CF treatment, because under low pH the available P was rapidly transformed into immobile forms via precipitation reactions with metal ions (Gyaneshwar et al., 2002), which may further caused the lower P concentration in the shoot (Table 3.4). The lower plant P uptake was likely related to the lower root biomass (Table 3.2) because root development has also been demonstrated to affect the P availability in the soil (Ma et al., 2013). Lower pH may also result in Al and Mn toxicity. The Mn and Al concentration in the root and nodule induced by CF treatment exceeded the threshold of toxicity (> 314 mg Mn kg^{-1}) (> 250 mg Al kg^{-1}) (Bethlenfalvay and Franson, 1989) for plant growth, at R1 and R7

(Table 3.5 and 3.6). The increase of Mn concentration in the root and nodule was consistent with the change in the RS in response to the CF treatment (Table 3.3, 3.5). The decreased nodule and root biomass may also concern Mn and Al toxicity because legume nodulation has been reported to be particularly sensitive to Mn excesses in the rooting medium (Rengel et al., 1993), and Al toxicities inhibited root elongation by reducing polar auxin transport by ethylene, which bounds Al to the cell wall to increase cell wall rigidity and decrease elasticity (Page et al., 2006). In addition, the same varying trend of Mn and Al in soil and root, higher Mn and Al availability in the soil and Mn, Al toxicity in the root, may be due to the soil contamination and surface-adhered elements on the root, as the protocol used at present study could not completely remove the soil particles from the root. Moreover, the CF treatment resulted in pronounced decrease of K concentration in the shoot at R1 and R7. The effect of N sources on the K accumulation in the shoot may be due to the competition in the internal transport between NH_4^+ and K^+ because ammonium ions could permeate K^+ channels due to the similarity of the ionic radius and size of the hydration shell (ten Hoopen et al., 2010). Furthermore, the ammonium-N source increased the Ca concentration in the root and shoot at R1 and R7 (Table 4). The lowest availability of Ca in the RS in response to the M treatment was also reflected on the lowest concentration of Ca in the root. However, similar as Mn and Al, the same varying trend for metal elements between soil and root, including Ca^{2+}, may be subjected to the soil contamination.

In the root and nodule, CF treatment significantly affected not only the Mn level but also the Mo, Fe, and Cu concentrations relative to the -N control (Table 3.5). Covalent metal ions are usually transported together in the phloem with the same transporters and chelating compounds, including *At*YSL1 (Morrissey and Guerinot, 2009), nicotianamine and deoxymugineic acid (Kaiser et al., 2005). The CF treatment significantly increased the Mo concentration in the root but reduced the level of this metal in the nodule at R1 (Table 3.5). This low accumulation of Mo in the nodule may be due to the low availability of this metal in the RS (Table 3.4), which probably resulted from rhizosphere acidification and the competition between sulfate from the ammonium sulfate supply and molybdate. Sulphate and molybdate enter root cells via the same proton-coupled symporters SHST1, and the molybdate uptake strongly decreases upon increases in the sulfate concentration in external substrates (Fitzpatrick et al., 2008). Because the S concentration in soil and soybean was not assessed in the present study, additional studies are needed to elucidate the interaction between Mo mobility and the sulphate supply. In addition to a low Mo influx, the inefficiency of Mo accumulation in nodules may be coupled to the decreased nodule weight, because the Mo-Fe protein, which contains Mo, is indispensable for nodule production and N_2 fixation (González and Jesús Gonzalez-López, 2010). Moreover, the CF treatment significantly reduced the Se concentration in the root and shoot at

R1 compared with the -N treatment, which may be due to sulfate application to the soil because Se competes with S in plants (Sun et al., 2010). Although at present study the S concentration was not determined in plant and soil, the S status may be speculated based on the Se status due to their chemical similarity. The competition between S and Se for the same assimilation pathway may make S status show an opposite allocation in the root and shoot with Se status.

In addition, the CF and -N treatments resulted in contrasting accumulation patterns of Mo and Fe in the root between R1 and R7: the increased ammonium supply enhanced the root Mo concentration and reduced Fe concentration at R1, but these relationships were reversed at R7. The change in the Mo accumulation between R1 and R7 induced by CF treatment may be associated with the decrease in the amount of Mo required by the nodule at R7 as nodule ages (Table 3.7). The increase of Fe concentration in the root at R7 induced by CF treatment was also reflected in the nodule. This change may relate to the variations of nodule biomass, N uptake and metabolism due to the interconnection between Fe and N (Rodríguez-Haas et al., 2013; Shen et al., 2014).

4.2 Effect of manure-N source on the ionome concentrations in different tissues of soybean plant

The effect of M treatment on the distribution of mineral elements in soybean plant was mainly reflected on the enhanced nonessential elements concentrations in the shoot (Table 3.6). M treatment, which induced the highest concentrations of Ba, Sr, Cd and Cr in the shoot observed in this study (Table 3.6), also produced the lowest concentrations of Ba, Sr and Cr in the root and the lowest concentration of Cd in the RS. Although a biological function in plants has not been demonstrated for these nonessential metal elements, they are taken up via essential metal uptake systems (Verbruggen et al., 2009). M treatment significantly enhanced the Cr availability in RS (Table 3.3), but conversely, manure application significantly reduced the available Cd concentration in the RS (Table 3.3). Moreover, the Sr and Ba concentration in the RS and BS did not differ by treatments at either stages, but the shoot concentrations of Ba, Sr, Cd and Cr were all increased. Thus, the increases in the low-molecular-weight organic acid released from manure and secreted by the root are hypothesized to have promoted the direct uptake and upward translocation of heavy metal elements because organic acids can increase the chelation and solubility of heavy metal ions to ameliorate long-distance translocation rate (Senten et al., 1990). Furthermore, we previously reported that M treatment could increase the Cd and Ba concentration in soybean seed (Sha et al., 2012). The increases in the Ba and Sr concentrations in the shoot were likely related to the Ca and Mg metabolism because Mg, Ca, Sr and Ba are chemically similar and share a translocation pathway

(Watmough et al., 2014).

In contrast to Sr, Ba, Cd and Cr, the accumulation of Cs in the shoot was significantly decreased at R1 and R7 in response to M treatment (Tables 3.4 and 3.7). This accumulation was reflected in the lowest Cs availability in the soil observed in this study, which may be attributed to the highest concentration of available K in the soil, because Cs generally enters root cells via the same molecular mechanism as K (Qi et al., 2008). This finding agreed with the results of a previous study, which showed that manure application reduced the Cs availability and restricted the K availability in the soil as well as the Cs concentration in soybean seeds (Sha et al., 2012). The improvement in the available Cs concentration in the soil in response to M treatment may have been due to differences in the affinity of cations for the clays: $Cs^+>NH_4^+>K^+>Na^+>Ca^{2+}$ (Staunton and Roubaud, 1997). The lower Cs concentration in the shoot may also be attributed to the enhanced Ba^{2+} level because Ba^{2+} has been shown to significantly correlate with Cs^+ in soybean seeds and Amaranth shoots (Chu et al., 2015).

4.3 Dynamic variations in ionomic concentration from vegetative to reproductive growth stage

A total of 22 elements in the nodules, roots and shoots that were affected by three types of N sources were subjected to a PCA (Fig 3.2). These 22 original variables were converted to a set of linearly uncorrelated 3 PCs that totally contributed 76.74%

and 81.55% of the total variance for R1 and R7, respectively. The PCA loading scores of elements are shown in Table 7. PC1 accounted for 42.86% of the total variance and discriminated in the ionomic concentrations between the root and nodule, shoot. As at R1 PC1 was primarily influenced by Fe, Ca and all 11 nonessential elements measured in the present study (Table 3.7), the ionomic difference between the root and nodule, shoot was mainly reflected in the root regulation on Fe, Ca and nonessential elements translocation to other organs. The concentrations of elements that are primarily part of PC1 were higher in the root than in the shoot at either stage (Tables 3.5, 3.6). This result partly corroborates a previous study that showed high concentrations of nonessential elements can be immobilized in the vacuoles of root cells to restrict their transport from the root to shoot (Vondráčková, 2014). However, at R7 fewer elements could discriminate between the root and shoot, nodule. These elements consisted of essential and nonessential elements at similar number, including Li, Al, Ba, Cu, Fe, P, Mn, Cd, Mg and Cr (Table 3.7, Fig 3.2). This variation between R1 and R7 illustrates that the capacity of plant declined to allocate the essential and nonessential elements into the suitable organs as plant aged, which may stem from the senesce of vegetative organs and decreased root activity.

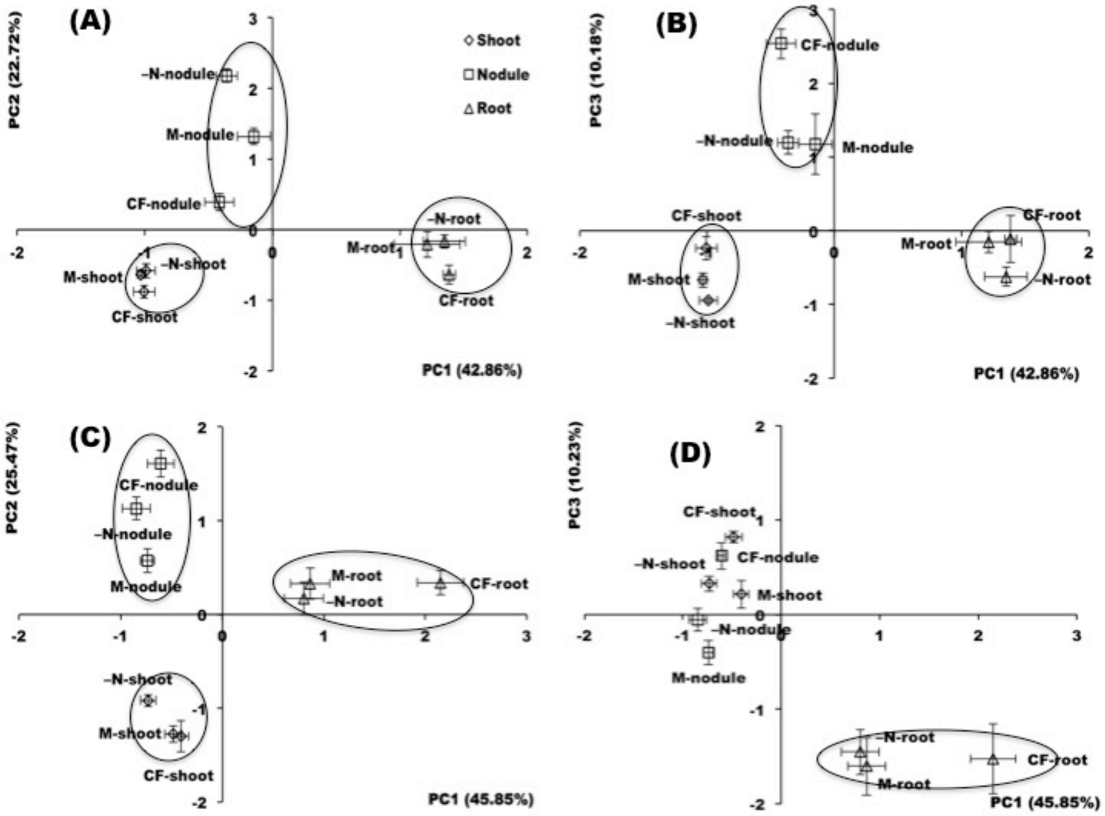


Fig 3.3 Sample scores for the first (PC1), second (PC2) and third principle components (PC3) provided by PCA identified in the root, nodule and shoot in response to different N sources at R1 and R7. (A) the samples scores for PC1 and PC2 at R1; (B) the samples scores for PC1 and PC3 at R1; (C) the sample scores for PC1 and PC2 at R7; (D) the sample scores for PC1 and PC3 and R7. Each group is represented by the average value of four replicates. Error bars are shown in each group. Abbreviation: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.

At R1, PC2 and PC3 together accounted for 32.9% of the total variance and discriminated the difference in mineral elements accumulation in the nodule between CF and M, -N treatment (Fig 3.2). This set of elements consisted of N, P, K, Mo, Cu, Co, Mn, Zn, Mg and Se, all of which are essential elements, except Se (Table 3.7).

This result may derive from the reduced nodule biomass observed in response to CF treatment, because decreases in the dry matter of nodules may delay the allocation of essential elements into nodules. As the soybean developed from R1 to R7, PC3 differentiated the ionomic concentrations in the nodule between CF and M, -N treatment but only accounted for 10.23% of the total variance with only two indicative elements: Mo and K. This change between the various stages illustrates that the effect of the original N treatments on the integrated variation of the nodule ionome was gradually became irregular as the nodules senesced after R1.

Table 3.7 PCA loading scores of elements with individual principal component in distinct soybean parts affected by N treatments at R1 and R7. CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.

Elements	R1			Elements	R7		
	PC1	PC2	PC3		PC1	PC2	PC3
Li	0.96	-0.13	-0.08	Al	0.96	0.18	0.02
Al	0.94	-0.11	0.08	Ba	0.94	0.05	-0.30
B	-0.92	0.02	0.05	Li	0.92	0.11	-0.34
As	0.89	0.24	0.17	Cu	0.84	-0.22	0.24
Cd	0.85	-0.33	-0.12	Fe	0.82	0.43	-0.26
Na	0.82	-0.06	-0.16	P	-0.82	-0.37	0.20
Ba	0.81	0.20	0.20	Mn	0.77	0.19	0.53
Cs	0.78	0.46	0.17	Cd	0.73	0.04	-0.15
Fe	0.72	0.34	0.38	Mg	0.69	-0.49	0.51
Ca	-0.70	-0.31	-0.44	Cr	0.47	0.37	-0.23
Cr	0.69	0.44	-0.03	Co	0.15	0.89	0.14
Sr	-0.61	-0.44	-0.45	Sr	-0.16	-0.87	0.28
Mo	0.00	0.94	0.05	B	-0.31	-0.85	0.30
K	0.28	0.88	-0.02	Cs	0.49	0.80	-0.18
N	-0.17	0.86	0.16	Na	-0.22	0.79	-0.22
Cu	0.33	0.76	-0.10	Se	-0.05	0.78	0.30

P	-0.49	0.75	0.26	As	0.59	0.74	-0.14
Co	0.18	0.86	0.38	N	-0.40	-0.69	0.40
Mn	0.28	0.05	0.88	Ca	-0.02	-0.62	0.59
Zn	-0.28	-0.08	0.70	Zn	0.48	-0.61	0.13
Mg	0.12	0.22	0.86	Mo	0.12	0.21	-0.84
Se	0.44	0.08	-0.72	K	0.55	-0.02	-0.72

The PC where the elements exposed the highest loading score can define the according elements. Grey-filled entries represent the most dominant score of each element in the PC.

Table 3.8 Effect of N sources on the R7/R1 ratio of mineral concentrations in the nodule. The data that is more than 1 was shaded with grey.

Treatment	N	P	K	Mg	Ca	Mn	Fe	Cu	Zn	Mo	B
CF	0.48	0.51	0.4	0.34	0.61	0.65	0.81	0.78	0.54	0.36	0.77
M	0.75	0.56	0.41	0.21	0.74	0.72	0.5	0.47	0.58	0.11	0.77
–N	0.48	0.45	0.2	0.52	0.97	0.66	0.63	0.5	0.63	0.17	0.81

	Na	Co	Se	Al	Li	Cs	Sr	Ba	As	Cd	Cr
CF	2.43	1.18	2.28	0.92	3.42	2.31	1.17	1.43	2.79	8.32	0.59
M	2.79	1.14	1.46	0.82	2.32	1.66	1.03	1.35	1.81	11.99	0.26
–N	3.74	0.64	2.43	0.78	2.92	1.47	1.29	1.4	2.00	34.57	0.44

Abbreviations: CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: beginning of flowering stage; R7: beginning of ripening stage.

The R7/R1 ratios for all mineral concentrations in the nodule in response to N treatments are shown in Table 8. Corresponding graphs that describe the changes in the concentration of each element in the root and nodule are shown in Fig 3.3 and 3.4. Notably, the R7/R1 ratio for the concentrations of nonessential elements in nodule exceeded 1 except for those of Cr and Al, irrespective of treatments. Conversely, this ratio was less than 1 for all essential elements. This result verified the hypothesis stated above: upon nodule senesce, Zn, Cu, Mn and other chemically similar elements can be remobilized out of nodules in an Fe-like fashion because of similar transport mechanisms (Burton et al., 1998; González et al., 2013). Moreover, in addition to the metallome that remobilized to other tissues after playing vital role in metalloproteins (González-Guerrero et al., 2014), P and B were shown to be recycled out of nodules. Conversely, the higher concentration of nonessential elements observed in the nodule at R7 than at R1 suggests that the import of nonessential elements into the nodule directly correlated with the efflux of essential elements out of nodule cells. To the best of our knowledge, this study is the first to show that the accumulation of nonessential elements in the nodule increases as the soybean matures and does not depend on the N source. Previous studies have not suggested a mechanism for this effect, but we speculate that the abundance of nonessential elements in that nodule maintains the ion balance after the essential elements are exported from the nodule cells. On the other hand, the differences in the concentrations of some essential elements were distinct between the root and nodule (Table 3.8). The concentrations of Mn, Zn, B and Al decreased in root but increased in the nodule from R1 to R7, and these changes were independent of the N treatment. The Na concentration in the root increased from R1 to R7 but decreased in the nodule, and this change did not depend on the N treatment. However, the N treatments produced complex changes in the Ca, Fe, Cu, Mo and Se concentrations in the nodules and roots from R1 to R7. Namely, the allocation of Ca, Fe, Cu, Mo and Se between the nodules and roots before and after anthesis is strongly regulated by various regimes of N fertilization. For instance, Ca concentration enhanced in root but decreased in nodule in response to CF treatment from R1 to R7, but both increased in root and nodule in response to M treatment, and both decreased in response to -N treatment. Fe concentration increased in root but decreased in nodule in response to CF and M treatment from R1 to R7, but both decreased in root and nodule in response to -N treatment. Therefore, the nodule senesce may have a complicated influence on elements mobility between nodule and root and more metallo-transporters may be involved in the mobilization of metals in belowground tissues than previously thought (Baxter et al., 2009; González-Guerrero, 2014; Shen et al., 2014;).

4 Conclusions

In conclusion, this study clarifies the ionome composition in soybean plant in response to ammonium and manure - N source, and the dynamic variation of N and ionome in the plant as soybean developed from juvenile to ripening. Given the negative effect of sole application of ammonium or manure, reducing the amount of ammonium-N application or incorporating ammonium and manure together may benefit soybean growth and development, because M treatment could avoid soil acidification and CF treatment promoted the accumulation of essential covalent metals in the shoot. The dynamic state of metal elements in nodule and root before and after nodule senesces was complicated. Additional studies of the effect of N and the metal availability in the growth medium on the transcriptome of nodulating and non-nodulaiting roots may help identify this mechanism.

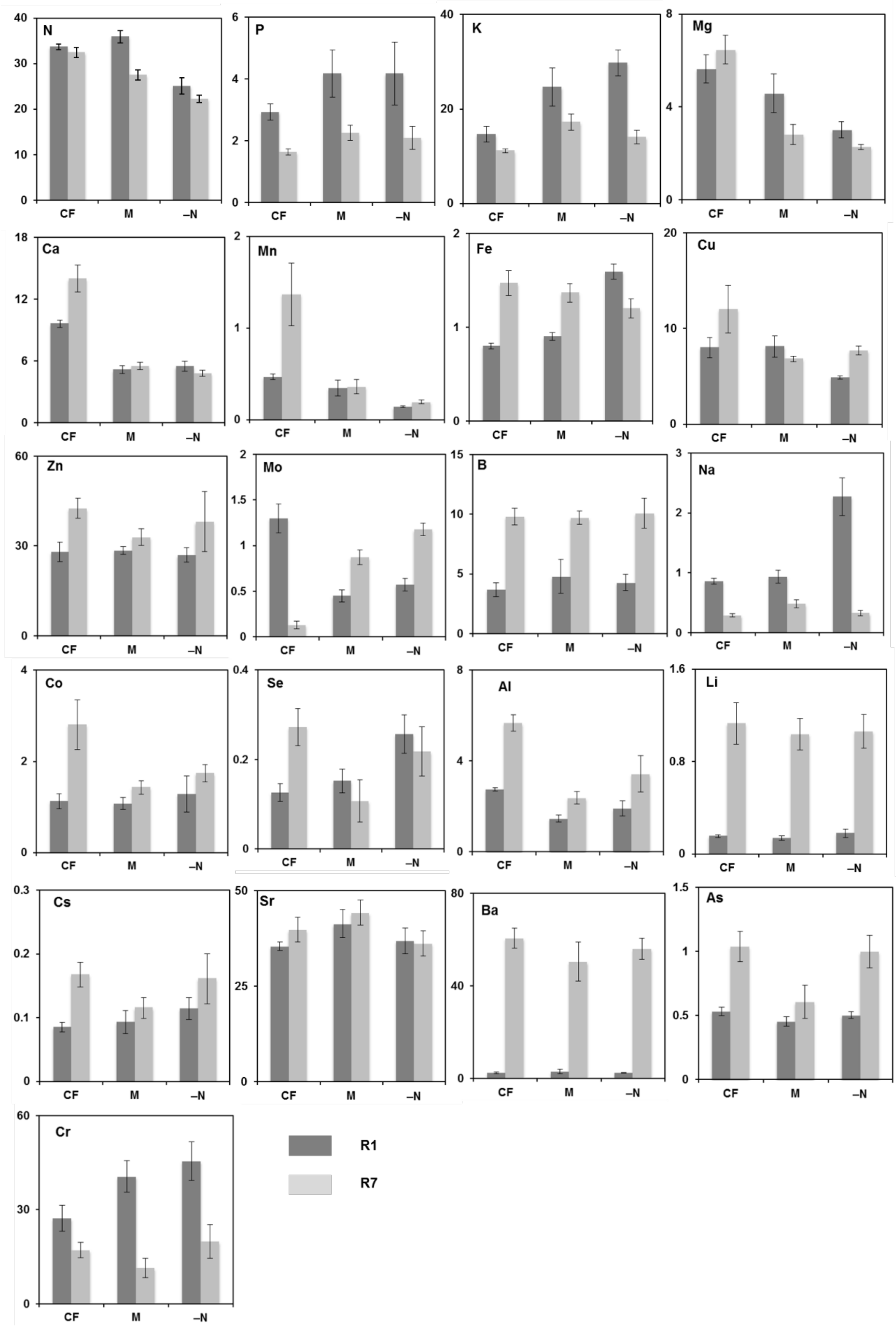

N
0
10
20
30
40
P
0
2
4
6
K
0
20
40
Mg
0
4
8
Ca
0
6
12
18
Mn
0
1
2
Fe
0
1
2
Cu
0
10
20
Zn
0
30
60
Mo
0
0.5
1
1.5
2
B
0
5
10
15
Na
0
1
2
3
Co
0
2
4
Se
0
0.2
0.4
Al
0
4
8
Li
0
0.8
1.6
Cs
0
0.1
0.2
0.3
Sr
0
25
50
Ba
0
40
80
As
0
0.5
1
1.5
Cr
0
30
60
CF
M
–N
R1
R7

Fig 3.3 Dynamic variability of ionome in root from R1 to R7 under the influence of various N regimes. Error bars represents SE. The unit of macroelements, Mn, Fe, Na, Al is g kg^{-1}, of other elements is mg kg^{-1}. CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.

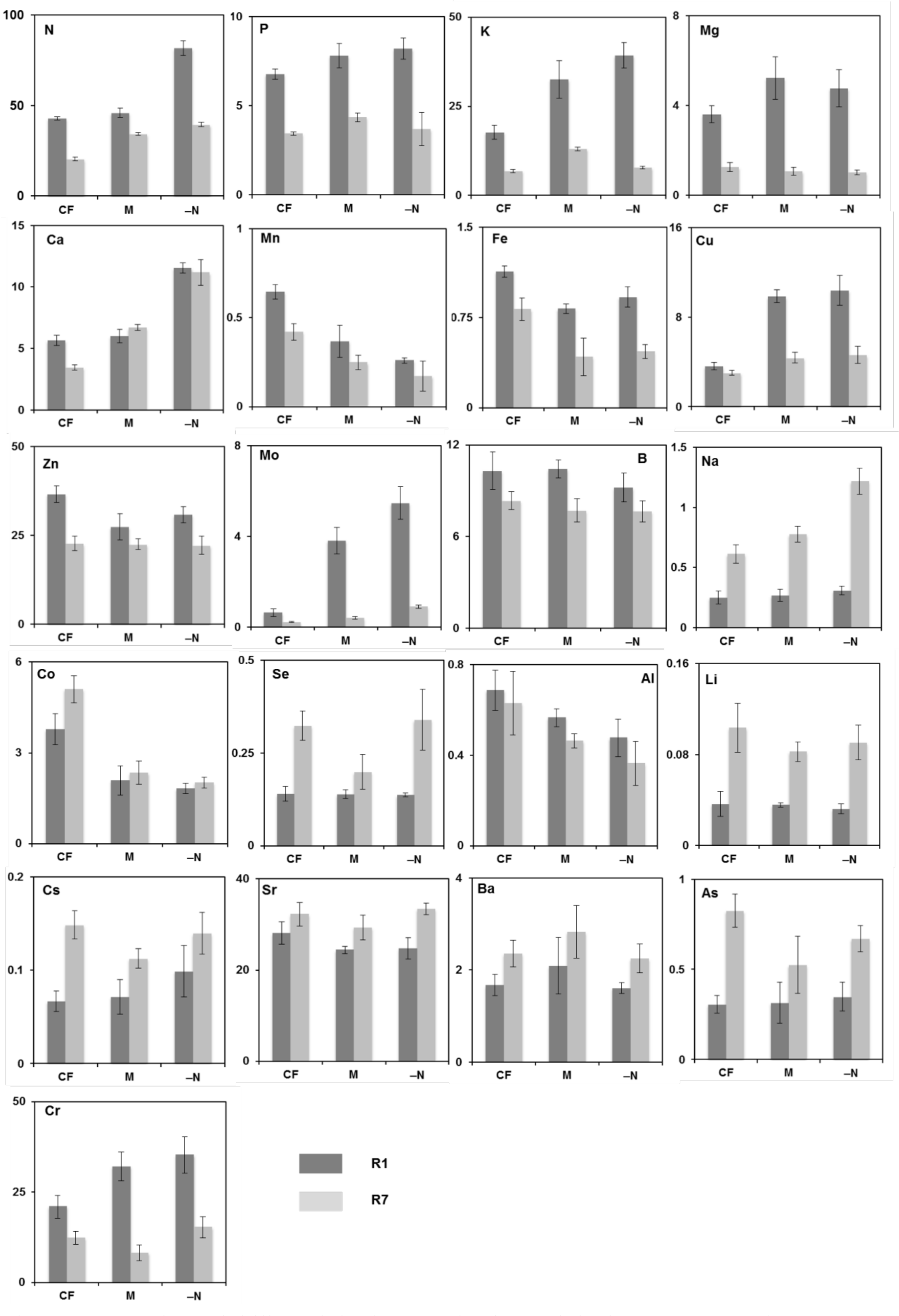


Fig 3.4 Dynamic variability of the ionome in the nodule from R1 to R7 in response to various N regimens. Error bars represents the SE. The concentration of

macroelements, i.e., Mn, Fe, Na, Al is g kg^{-1}. The concentrations of all other elements are give in mg kg^{-1}. CF: chemical fertilization with ammonium sulfate and P, K; M: manure application; -N: chemical fertilization without N; R1: the stage of beginning of flowering; R7: the stage of beginning of maturity.

CHAPTER 4

Manure application caused the divergent nitrogen utilization, ionome, and microbial metabolism in the rhizosphere between soybean and sorghum: a rhizobox study

4.1 Introduction

Plants typically contain N up to 4% of their dry weight (Glass 2009), but most higher plants cannot directly get access to the large pool of N tied up in soil organic matter (Jones et al., 2005). Comprehending the utilization of organic N from the plant's N budget is important since chemical N fertilizers, applied as inorganic N or those that are rapidly converted to inorganic nitrogen, can be easily lost from soil and cause environment problems (Erisman et al., 2008; Gruber and Galloway, 2008). For soybean producers, additional N fertilizer applications usually result in the inefficient use of synthetic N-fertilizers with average N-fertilizer efficiencies less than 50% (Salvagiotti et al., 2008). Under such circumstance, using organic materials as a nitrogen source for crop production is an important concept of sustainable agriculture

(Amiri and Fallahi, 2011; Cakmak, 2002). Organic N supplied to the soil undergoes a stepwise mineralization including proteolysis, ammonification and nitrification in which proteinous compounds are converted to amino acid, ammonium (NH_4^+) and nitrat (NO_3^-) by soil bacteria and fungi. Nitrate and ammonium are the most abundant forms of dissolved N in agricultural soils and their concentrations in the soil solution vary by orders of magnitude from μM to mM (Glass, 2003). In contrast, as a form of readily utilizable organic N, amino acid concentrations in soil solution ranged from 1 to 100 μM (Owen and Jones, 2001). Amino acids may account for a relatively small proportion of the dissolved N pool in fertilized soil but constitute a relatively larger proportion in non-fertilized soil.

Considerable work has been carried out to study N utilization by different plant species (Li et al., 2007; Okamoto and Okada, 2004; Song et al., 2011; Watanabe et al., 2012). Importantly, the capacity to utilize different forms of N varies by different plant species (Okamoto and Okada, 2004; Watanabe et al., 2012), factors affecting these characteristics have not yet been globally elucidated. Soybean plant can make symbiotic nitrogen fixation with rhizobium. This symbiosis between plant and microbes not only increases the N supply but also potentially affected the availability of ionome in the soil due to the change on soil physical and chemical properties (Liang et al., 2014). Also, researches in our group demonstrated that soybean root took up glycine at low efficiency in comparison to lupin under hydroponic cultivation (Watanabe et al., 2006). In contrast, sorghum was able to utilize organic N, usually in the form of amino acid, with high efficiency (Okamoto and Okada, 2004). Therefore, comparing the difference of utilizing pattern on N between soybean and sorghum in response to organic N addition is important to understand the soil N cycle and plant N nutrition.

Once organic materials are applied to the soil, they will be decomposed by soil microbes, converting proteins into amino acids and, finally, into the ammonium and nitrate. The alkaline metalloprotease, neutral metalloprotease, and serine protease are the primay microbial extracellular protease in soils encoded by *apr*, *npr* and *sub* genes, respectively (Fuka et al., 2008; Sakurai et al., 2007). Quantifying plant utilization on various N sources remains challenging due to simultaneous uptake, decomposition and conversion of N in the soil-microbe-plant system. The interactions of plant roots and microbes in the rhizosphere have been extensively researched because of their importance in nutrient management and soil C dynamics (Anderson et al., 2013; Bissett et al., 2013; Herman et al., 2006; Richardson et al., 2009). Microorganisms are less limited by organic N pool than plants because they can release the enzymes to utilize macromolecular soil organic N (Marschner, 2012). Plant roots are often not highly efficient competitors for amino acids compared with microbes because of microorganisms' rapid growth rates and high surface area-to-volume ratios. In addition, most plant roots provide soil microbes C while the microorganisms decompose soil organic N and make this N available to the roots (Herman et al., 2006; Kinzig and Harte, 1998). Moreover, the microbial diversity, and some types of microbial activity have also been demonstrated to be higher in rhizosphere than bulk soil (Herman et al., 2006; Tu et al., 2006; Yang and Crowley, 2006). The microbial responses to the organic matter addition are likely to increase the rates of N mineralization and bacterial numbers, change the microbial-root interactions to accelerate the flux of N from organic sources to the plant-available N pool (Herman et al., 2005, Yuan et al., 2011).

The ionome is termed as all the mineral nutrients and trace elements found in an organism (Lahner et al., 2003), including 17 mineral elements which plant required to complete their life-cycles (Marschner, 2012), and some non-essential elements such as cadmium (Cd), aluminum (Al) and cesium (Cs) when these minerals are present in the environment (Salt et al., 2008). With organic matter added into the soil, soil ionome is subjected to great change due to the release of root exudate, enhancement of microbial activity, and alteration of soil physical and chemical properties, such as pH, soil organic C pool. The availabilities of N, nutrients or trace elements have also been found to be higher in rhizosphere soil than bulk soil after adding organic matter into the soil (Li et al., 2007; Sha et al., 2012; Tian et al., 2015; Yuan et al., 2011). Nevertheless, little study has been done to interpret the interaction between soil microbial activity or diversity, availability of N and ionome, and plant growth in response to various N sources.

The rhizosphere can be defined as the zone of soil around plant roots within which complex interactions takes place between the plant roots, soil microorganisms and soil matrix. A micro-scale mapping of inorganic N, amino acids, nutrient, trace elements and substrate utilization by microorganism will provide a better understanding on the effect of manure application on N utilization and trace element accumulation in the rhizosphere. Youssef and Chino (1988) established a rhizobox system to study the nutrient status in the rhizosphere. After that, rhizobox has been extensively used to map the N mineralization and nitrification (Herman et al., 2006; Li et al., 2007; Yuan et al., 2011), concentration of N, nutrient or even heavy metal elements in the different micro-sections of rhizosphere and bulk soil (Huang et al., 2015; Kim et al., 2010; Moritsuka et al., 2013); therefore, we designed this rhizobox experiment to study the rhizosphere of soybean and sorghum receiving inorganic and organic N fertilizer. In this study, we aim to elucidate the different N utilizing pattern between soybean and sorghum by analyzing the inorganic N, amino acids and mineral elements movement, and microbial metabolism in the rhizosphere.

4.2 Materials and methods

Experimental soil

The soil, which is classified as a Haplic Fluvisols under the Classification of U.S Soil Taxonomy, was collected from agricultural fields in Hokkaido University and used in this rhizobox experiment. The basic properties of the experimental soils were: pH 6.05, total nitrogen 4.5 g kg^{-1}, 4.05 mg kg^{-1} ammonium-N, 13.4 mg kg^{-1} nitrate-N, available P (Truog-P) 194 mg kg^{-1}, 0.36 cmol kg^{-1} exchangeable K, 37.5 cmol kg^{-1} cation exchange capacity，30% base saturation.

Plant cultivation in rhizobox and the treatments

The experiment was carried out in a greenhouse. Rhizobox culture chambers (20×10×15 cm, Fig 4.1) were used to separate the plant root soil (root zone soil) from the rhizospheric and bulk soil away from the root zone, according to the method of Youssef and Chino (1988), Li et al. (2007). The rhizobox has two compartments: root compartments (RC, 2×10×15 cm, Fig 1) and two rhizosphere compartments at each side of the root compartment (Fig 1). The root compartment was made of a perspex frame and nylon cloth (pore radius less than 30 μm). This material enables water, nutrient and root exudates to pass through but prevents plant roots from penetrating. The soil was air-dried and ground, passed through 1-mm sieve and mixed homogeneously with perlite at the ratio of 1:1. Experiment contains three fertilizer treatments (Table 1): chemical fertilizer (CF), cattle farmyard manure (M), at the equivalent rate for N, P_2O_5 and K_2O as in the CF treatment (P_2O_5 is applied in the form of superphosphate calcium and K_2O is KCl), and a control treatment (-N) without N addition. There are three replicates in each treatment. The mineral elements concentrations in the manure are shown in Table 4.2. The cattle farmyard manure was mixed with soil to incubate 2 weeks before sowing.

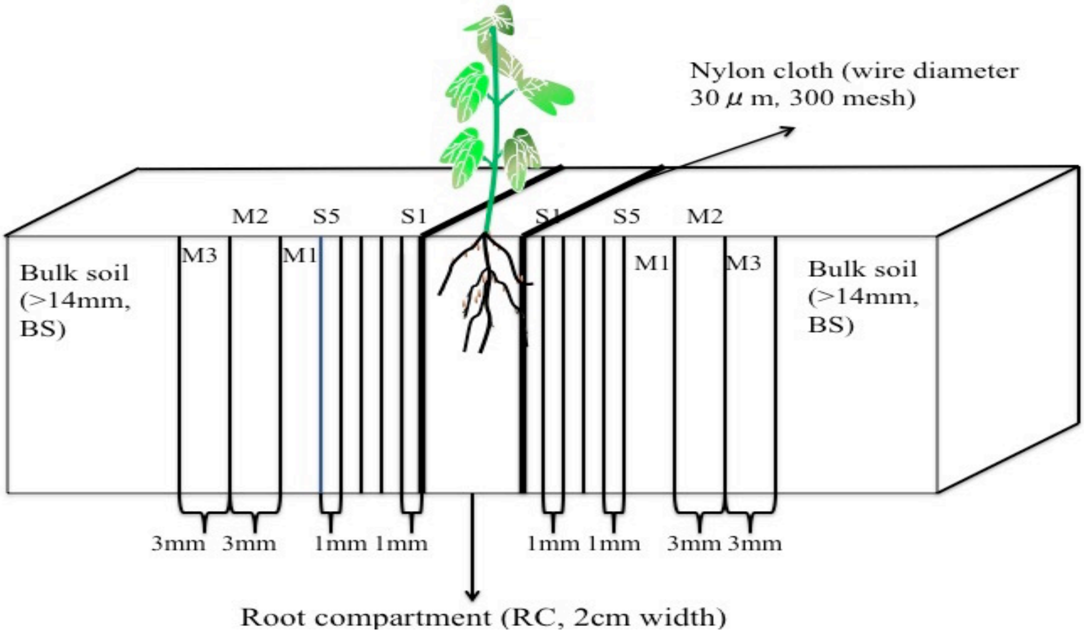


Fig 4.1 The structure of rhizobox and different soil fractions sampled in this experiment

Table 4.1 Treatments with various N sources.

Treatment	Manure	$(NH_4)_2SO_4$	P_2O_5	K_2O
	(g rhizobox^{-1})			
CF	—	2	0.47	0.4
M	17.3	2	0.47	0.4
-N	—	—	0.47	0.4

CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Table 4.2 Concentrations of elements in the cattle farmyard manure (DM: dry matter)

P	K	Mg	Ca	Mn	Fe	Cu	Zn	B
g kg^{-1} DM						mg kg^{-1} DM		
7.72	25.87	10.85	17.55	0.40	3.7	65.6	200.04	13.84
Co	Na	Sr	Ba	Ni	Cd	Cr	Se	Cs
mg kg^{-1} DM								
3.76	5700	62.09	62.72	48.31	0.19	9.13	0.71	0.17

Approximately 2 kg soil-fertilizer mixture was placed into each rhizobox, and deionized water was added immediately. Ten seeds of soybean (*Glycine max* L. Merr.cv.Hoyoharuka) or sorghum (*Sorghum bicolor* L. Moench) were sown in the RC and thinned to two after one week sowing. During the culture period, deionized water was added daily to maintain the 60% (w/w) of the field moisture capacity. Pots were arranged in a completely randomized design and positions were changed daily to avoid growth difference by lighting conditions. Unplanted pots were also prepared to compare the soil mineral availabilities with or without planting. Chemical properties in the unplanted pot after harvest are shown in Table 4.3.

Table 4.3 Concentrations of available mineral elements in unplanted pot after harvest.

					Exchangeable		
Treatment	pH (H_2O)	NH_4^+-N	NO_3^--N	Truog-P	K	Mg	Ca
				mg kg^{-1}			
CF	4.84	27.59	298.82	144.37	373.66	241.04	2097.91
M	5.22	27.23	187.91	129.48	578.79	416.08	2282.78
–N	5.18	19.99	52.93	154.92	347.85	211.67	1722.06

CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Harvest of the plants and sampling of the soils

Soybean and sorghum seedlings were harvested 35 days after sowing, just before anthesis. Plant samples are separated into shoot (aboveground plant parts) and root. The separated fresh samples were immediately frozen in liquid nitrogen, and then lyophilized, weighed and ground for mineral elements analysis.

For soil sampling in the rhizobox, different soil fractions are divided in the rhizobox and sampled, 0 (RC), 0-1 (S1), 1-2 (S2), 2-3 (S3), 3-4 (S4), 4-5 (S5), 5-8 (M1), 8-11 (M2), 11-14 (M3) and >14 mm (bulk soil, BS) distance from the root compartment (Fig 4.1). The two soil slices at the same distance from the root plane at either side were mixed together. The soil collected in the middle root compartment was defined as RC and the soils collected on the left and right compartments were divided into rhizospheric soil (RS, 0-14 mm away from the root zone) and BS (>14 mm away from the root zone). After slicing the soil samples were rapidly sieved to pass through a 2 mm sieve. Then some fresh soil samples were placed at 4 °C incubator waiting for Biolog Ecoplate^{TM} analysis; some samples were rapidly frozen by liquid nitrogen, stored at -80 °C, and then lyophilized for amino acid, inorganic N, and mineral elements analysis.

Analysis of inorganic N, mineral elements and TAA in soil samples, and mineral elements in plant samples

The concentrations of mineral elements in samples of plant and soil were determined as described in 2.2 and 3.2. The concentrations of inorganic N, TAA, and available mineral elements in soil were analyzed. The inorganic N (NH_4^+-N and NO_3^--N) concentrations in soil were measured by colorimetric assay at 630 nm and 538 nm according to Page (1982). Total amino acid concentrations in soil were determined by colorimetric assay at 570 nm in Ninhydrin method (Jones, 2002).

CLPP analysis

Community level physiological profiles (CLPPs) were assessed by the Biolog Ecoplate^{TM} system (Biology Inc., CA, USA). Each Ecoplate^{TM} is a triplicate combination of 32 wells containing 31 various carbon compounds (carbohydrates, carboxylic acids, amino acids, amines, polymers and miscellaneous) and a control (water). A tetrazolium dye is combined to each substrate and liberated by microbial breakdown of the individual carbon compound. Soil suspensions (soil 1g, 0.85% sterile saline solution 10 ml) were shaken and then pre-inoculated for 24h before inoculation to allow microbial utilization of any soluble organic compound from the soil. Hundredfold dilution was performed and aliquot of 150 μL of this dilution was added to each well of Biolog Ecoplate. The plates were incubated at 25 °C, and color development in each well was recorded as optical density (OD) at 595 nm with a plate reader at regular 12 h-intervals (from 12 to 120 h). Between measurements, the plates were incubated in the dark at 25 °C.

Normalization of the blank well (control) of each replicate was carried out and average well color development (AWCD) was determined by calculating the mean of every well's absorbance value (Abs) at 595 nm at each reading time. S. Shannon diversity index ($DQ = -\Sigma p_i \log_2 p_i$) (Magurran. 2004), where pi = the ratio of the absorbance of a particular well to the sum of absorbances of all microplate wells, was calculated considering absorbance values at each well as equivalent to abundance of individuals in each species (Zak et al., 1994). DQ represents the microbial diversity. The plates corresponding to an incubation time of 72 h were chosen for further statistics analysis, since it was the shortest incubation time that allowed the best resolution among treatments.

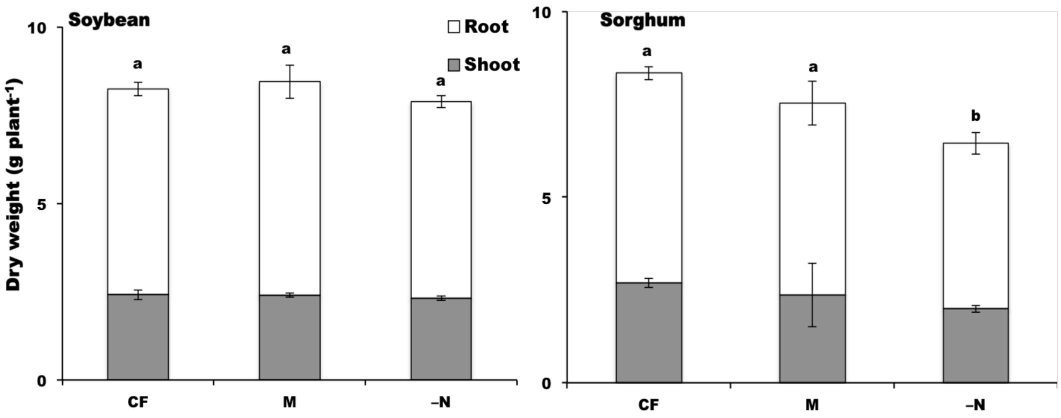
Statistics analysis

All statistical analyses were performed using the SPSS version 18.0 of Statistical Software Package (SPSS Inc. Chicago, IL, USA) programs. A one-way ANOVA with Tukey test was applied to analyze the effect of the N treatments on the concentrations of TAA, inorganic N, mineral elements and microbial carbon sources consumption. A probability (*P* value) below 0.05 was considered to indicate significant differences. The correlation between variables was calculated with Spearman nonparametric test to establish the interconnections between nitrogen, mineral elements and microbial activity. A principle component analysis (PCA) was adopted to profile the normalized data from N, TAA and elements concentrations in the RC and BS growing soybean and sorghum. A varimax rotation with Kaiser normalization was used as the rotation method.

4.3 Results

N and biomass accumulation in the soybean and sorghum plant

The dry weight and N concentration in the shoot affected by various N treatments are presented in Fig 4.2. The growth and N accumulation in the shoot of soybean was similar among the CF, M and -N treatments. However, the shoot biomass of sorghum was significantly decreased by the -N treatment compared with the other N treatments. The variation for N concentration in the sorghum shoot affected by various N sources was the same as the variation in shoot dry weight. The dry weight and N concentration in the sorghum root was similar among different CF, M and -N treatments.



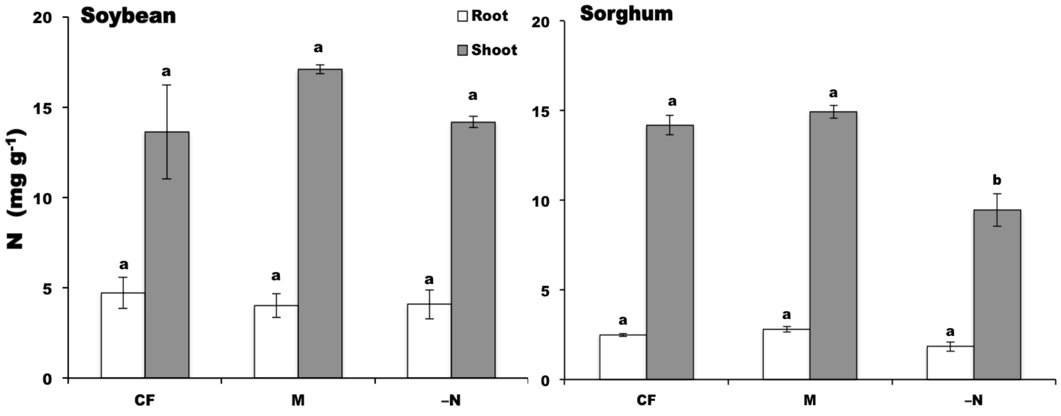


Fig 4.2 Dry weight and concentration of N accumulated in the soybean and sorghum plants. Values are the means of three replicates. Values followed by the same letter do not differ significantly at $P < 0.05$ for comparisons made within a crop by N treatments. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

The relative change of elemental concentrations in the soybean and sorghum shoot in CF and M treatments compared to -N treatment

For soybean, the ammonium application significantly increased the Mn and Zn concentrations in the shoot but decreased the Ba and Cr concentration; the manure application significantly increased the P, K, Cu, Mo, Na, Ba, Cr and As concentrations in the shoot in comparison with -N treatment (Fig 4.3). For sorghum, CF and M treatment both significantly increased the N, P, K, Mg, Fe, Cu, Zn, B, Ni, Co Al, As and Rb concentrations in the shoot in comparison with -N treatment (Fig 4.3). However, CF treatment also increased the concentration of Ca and Mn in the shoot but M treatment did not; significantly decreased the Ba concentration but M treatment increased, which is the same as in soybean. M treatment significantly increased the Mo, Na, Cr and Cs concentration in the shoot but CF treatment did not.

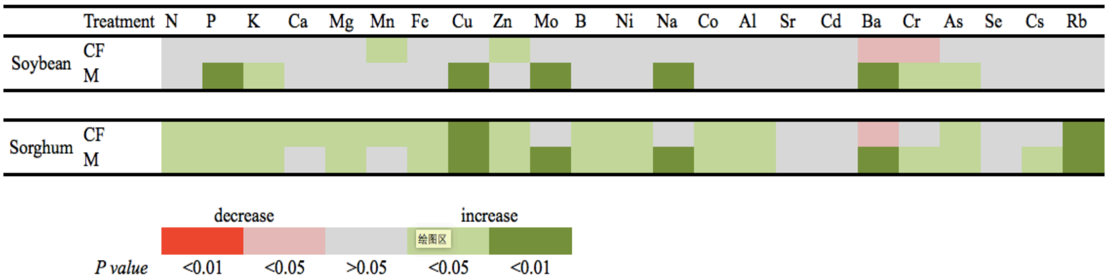


Fig 4.3 Heatmap showing relative differences in concentrations of elements in shoot of soybean and sorghum in CF and M treatments compared to -N treatment. The red color represents the relative increase and green color the relative decrease. P-value (*P* < 0.05, 0.01) gives the intensity of the blue or red color. CF: chemical fertilizer application; M: manure application.

NH_4^+ and NO_3^- and TAA concentration

The significant distribution characteristic of the NH_4^+ concentration of the soils at different distances from the RC was that there was almost no concentration gradient, irrespective of plant species or N sources, except that the NH_4^+ concentration in RC was obviously higher than in the soil 5 mm (or further) far from the RC growing soybean (Fig 4.4). For NO_3^- concentration, the concentration gradient was also hardly detected in the distribution in the soils at different distances from the RC growing soybean, irrespective of N sources, except that the concentration at BS was markedly higher than in RC and RS by receiving CF treatment. Nevertheless, the concentration of NO_3^- associated with sorghum increased with increasing distance from the sorghum roots by receiving CF treatment. For instance, the NO_3^- concentration at 5 mm far from the root was 2.54-fold higher than in the RC and at 20 mm far was 4.81-fold higher than in the RC of sorghum receiving CF treatment (Fig 4.4).

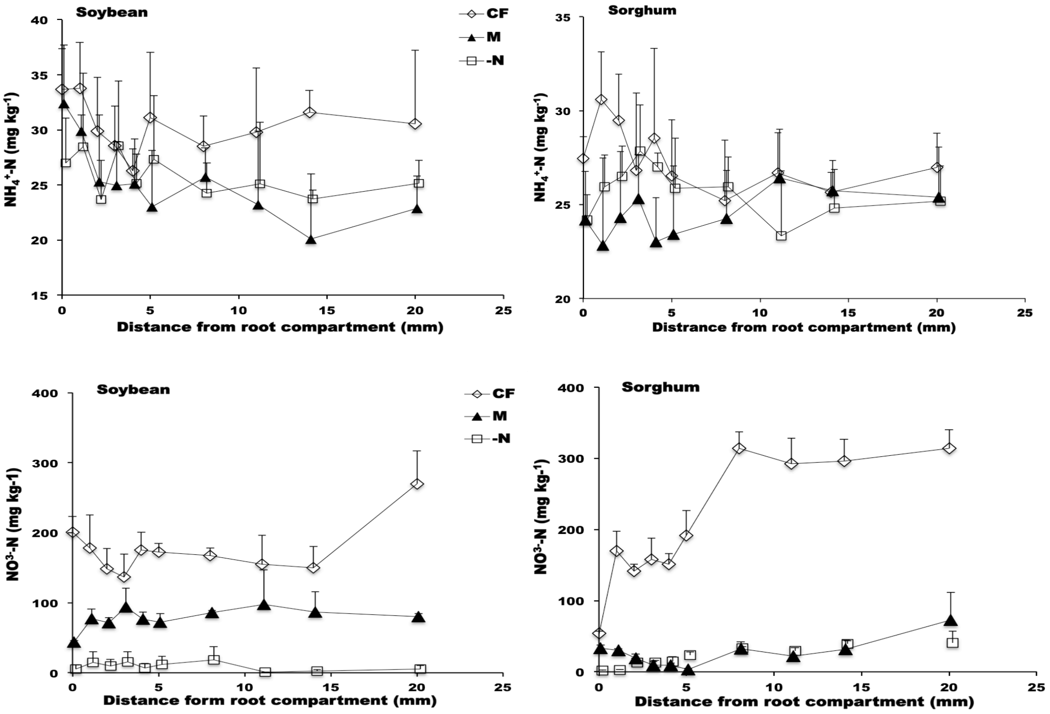


Fig 4.4 NH_4^+ and NO_3^- concentrations measured at different distances from the root of soybean and sorghum grown in the rhizobox by receiving various N treatments. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

For TAA concentration, the concentration gradient was almost constant in the distribution in the soils at different distances from the RC growing soybean, irrespective of N treatments. Nevertheless, the M treatment caused the decreased TAA concentration near the sorghum roots. The TAA concentration in RC was increased by 1.48-fold in comparison to in BS affected by M treatment (Fig 4.5).

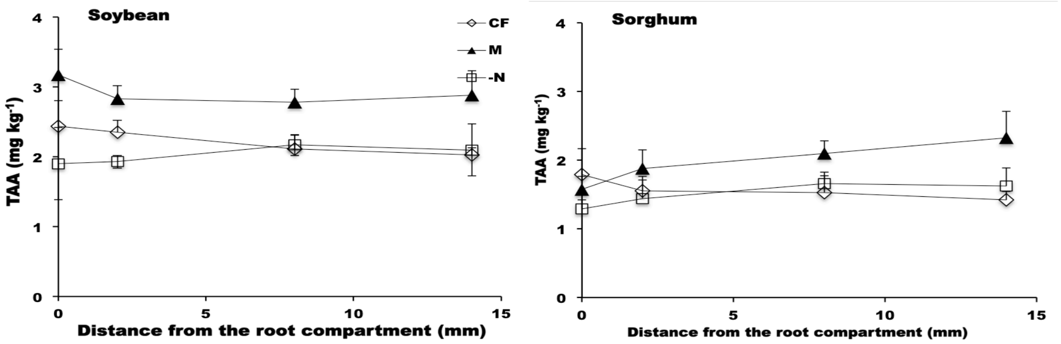


Fig 4.5 TAA concentrations measured at different distances from the root of soybean and sorghum grown in the rhizobox by receiving various N treatments. Abbreviations: CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

CLPP analysis

Fig 4.6 shows the AWCD and DQ curves obtained with the Biolog Ecoplate^{TM}. In this figure, for soybean, the value of AWCD and DQ of RC affected by M treatment was always higher than that affected by CF and -N treatment. For sorghum, the value of AWCD and DQ of RC affected by CF and M treatment was always higher than that affected by -N treatment, but the difference between CF and M treatment began to be observed at approximately an incubation time of 72 h; the value by M treatment was higher. That is why we chose the plates corresponding to an incubation time of 72 h for further statistics analysis.

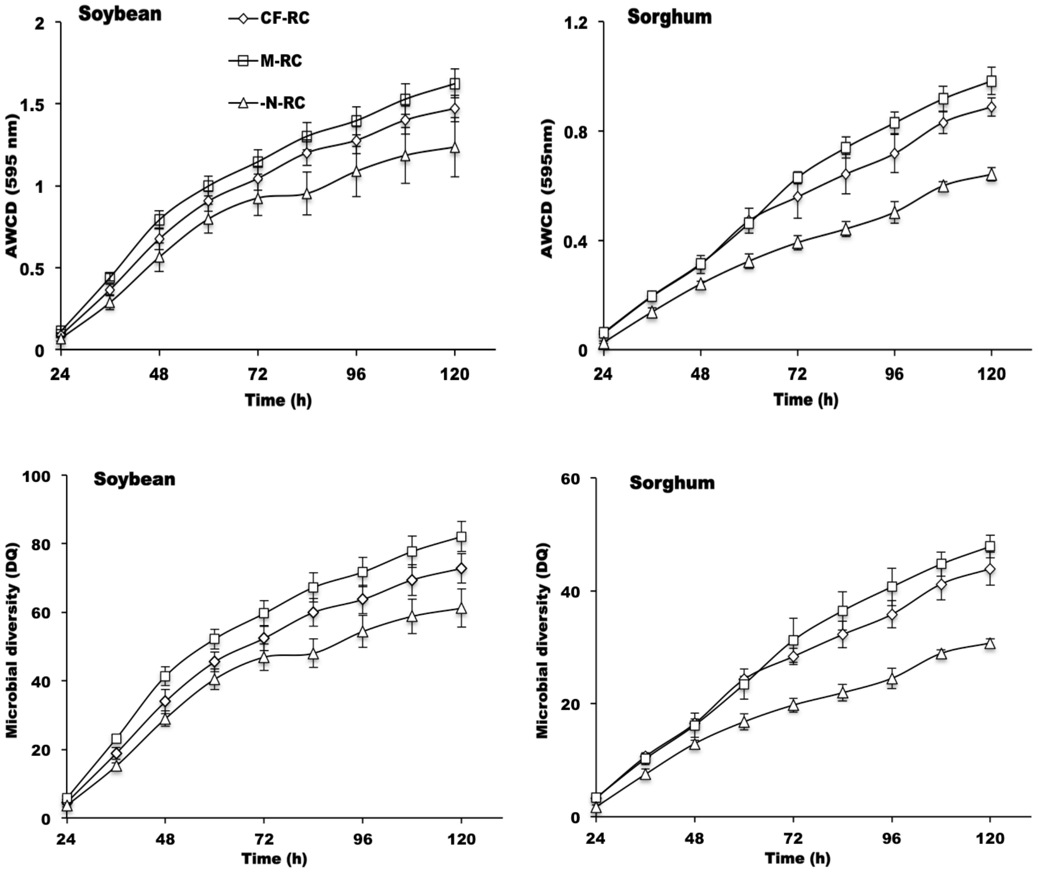
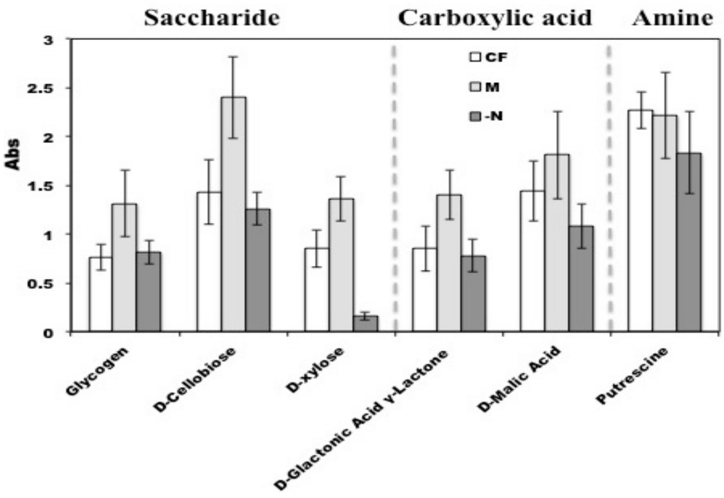


Fig 4.6 The average well colour development (AWCD) and microbial diversity curves obtained with the Biolog Ecoplates^{TM} in the root compartment. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Fig 4.7 shows the substrate utilization patterns in Biolog Ecoplate^{TM} obtained with the soil microorganisms of RC affected by CF, M, and -N treatments (only those substrates showing significant differences among treatments are presented). For the RC growing soybean, glycogen, D-cellobiose, D-xylose, D-galactonic acid-lactone, D-malic acid and putrescine were utilized to a significant content ($P < 0.05$) in response to various N treatments. There are 31 diverse carbon compounds in the Biolog Ecoplates^{TM}, including carbohydrates, carboxylic acids, amino acids, amines, polymers and miscellaneous, but only three types of saccharides (carbohydrates), two types of carboxylic acids and one type of amine presented significant difference. M treatment caused significant higher level of utilization on three types of saccharides and two types of carboxylic acids. For RC growing sorghum, two types of carboxylic acid (pyruvic acid methyl, D-galacturonic acid), two types of saccharide (D-cellobiose, N-acetyl-D-glucosamine), one type of alcohol (D-mannitol), one type of amine (putrescine) and four types of amino acids (L-asparagine, L-phenylalananine, L-serine and glycyl-L-glutamic acid) were utilized to a significant content. M treatment caused significantly higher level of utilization on all these ten carbon sources except D-galacturonic acid.



(A)

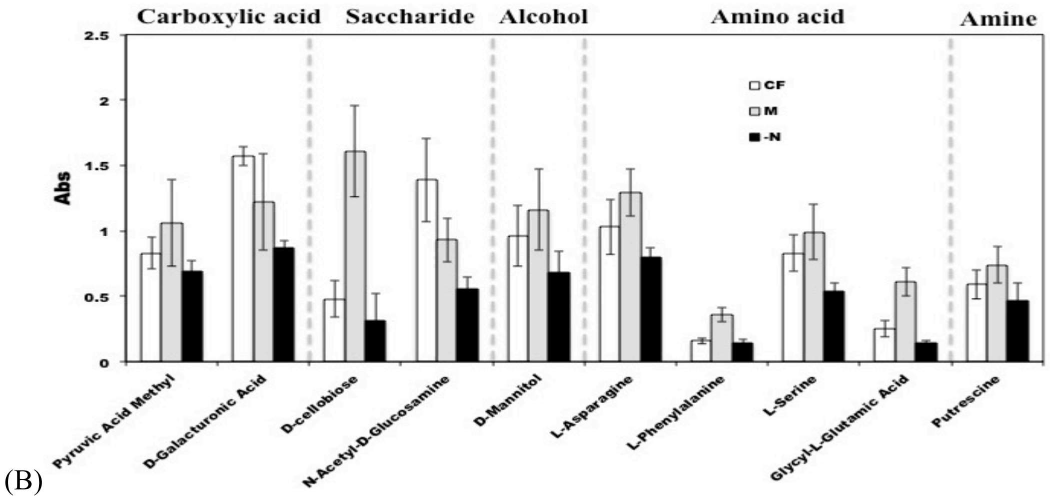
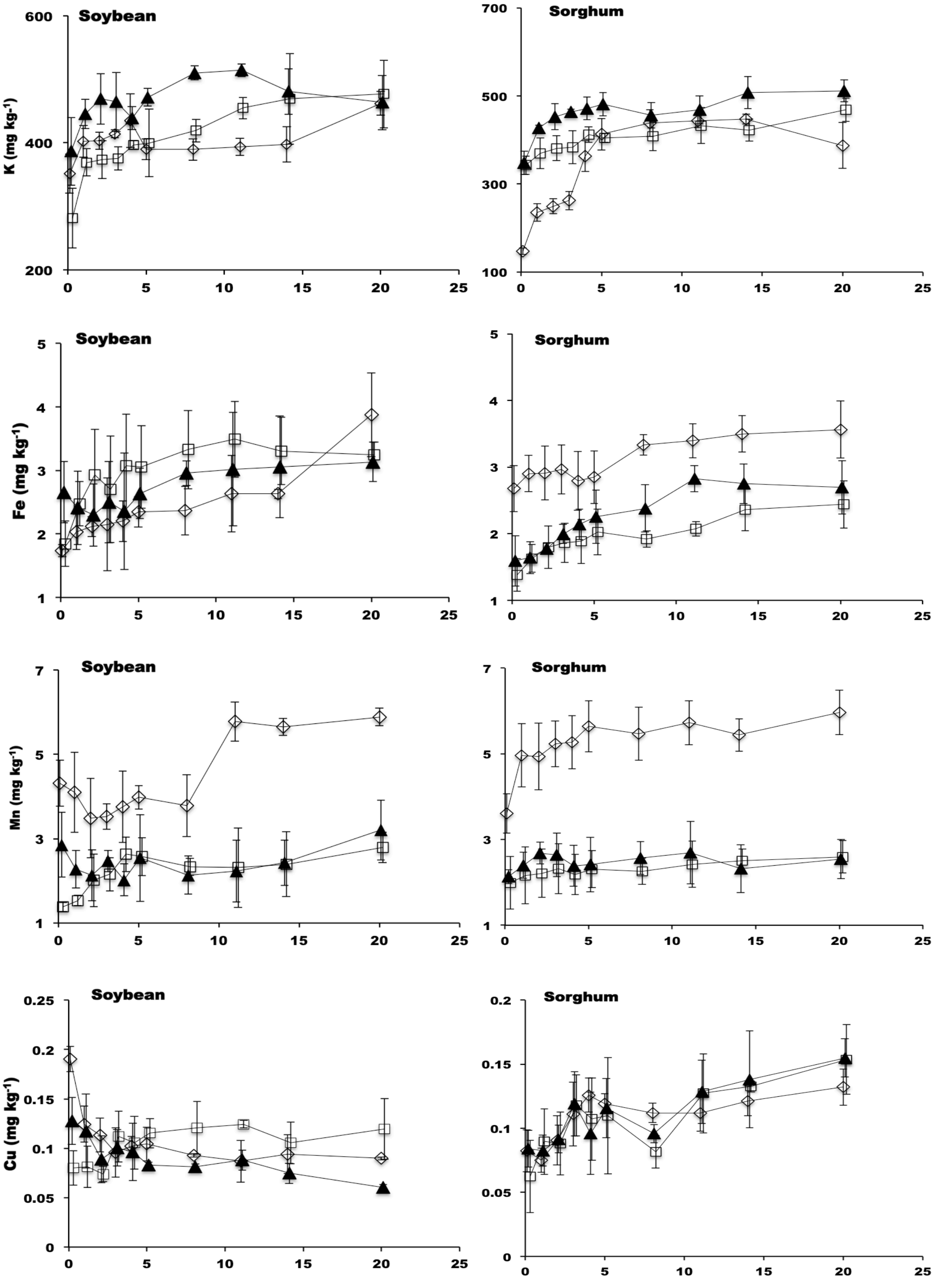
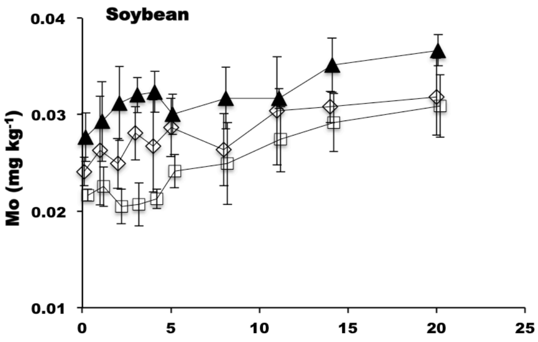


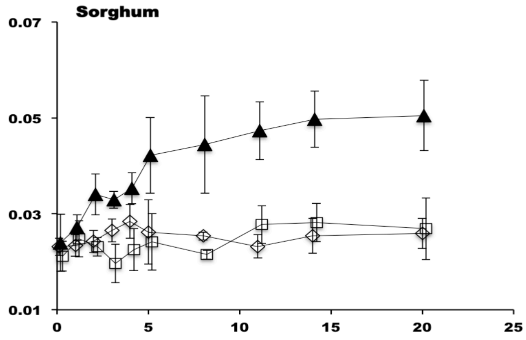
Fig 4.7 Metabolic fingerprints of substrate utilization patterns obtained with the Biolog Ecoplates^{TM} at an incubation time of 72 h [only those substrates showing significant differences ($P < 0.05$) among treatments, according to ANOVA, are presented]. (A): soybean; (B): sorghum. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

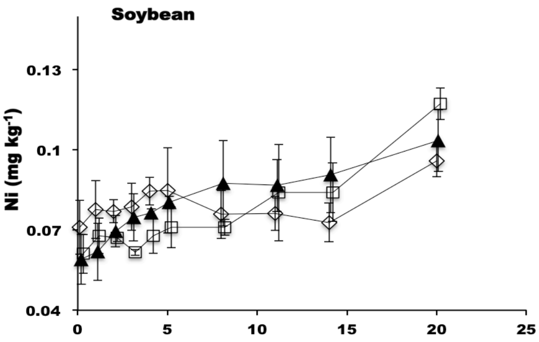
Microdistribution and depletion of mineral elements in the rhizosphere

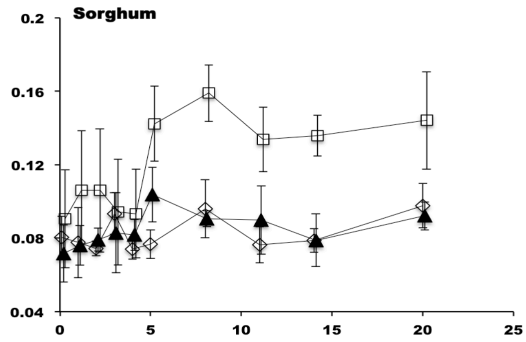
The microdistributions of elemental concentrations among different sites inside and outside the RC were examined (Fig 4.8). The elements showing significant difference in the soybean and sorghum shoot by various N treatments were used in this analysis. In both plant species, the concentration of P and K increased with increasing distance from the plant roots, irrespective of N treatments, but the degree of increase varied depending on the N treatments. For instance, the M treatment increased the P and K concentration at almost all of the soil sites compared to CF and -N treatment. The species difference of elemental microdistribution in response to various N sources was mainly reflected on micro- and trace elements. The Fe concentration was lower in the soil nearer the RC, but was decreased by CF treatments in almost all soil sites growing soybean, increased by CF treatments growing sorghum. For Mn, the soils at different distances from the RC was that there was almost no concentration gradient by M and -N treatments, but the Mn concentration enhanced with increasing distance from the RC. For Cu, the concentration decreased with increasing distance from the RC by CF and M treatments, was almost at the same level at different soil sites by -N treatments growing soybean. However, all N treatments increased the Cu concentration with increasing distance from the RC. The manure application elevated the Mo, Ba and Cr concentration with increasing distance from the plant roots, irrespective of sorghum and soybean, but there was almost no concentration gradient with or without ammonium sulfate application. For Na, the concentration was obviously higher in the RC than in other soil sites and there was almost no concentration gradient in other soils with different distance from plant roots, irrespective of plant species. The Na concentration in the RC varies depending on N treatments. For Rb and Cs, there was almost no concentration gradient or regular variation along the different soil sites with different distances from RC growing soybean, but by CF treatment the Rb concentration decreased with increasing distance from RC; by M treatment the Rb concentration increased with increasing distance from RC.

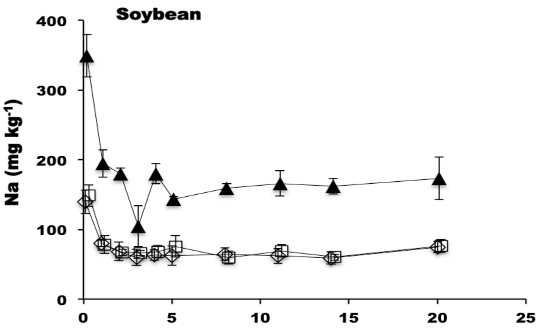

Soybean
K (mg kg-1)
600
400
200
Sorghum
700
500
300
100
Soybean
Fe (mg kg-1)
5
4
3
2
1
Sorghum
Soybean
Mn (mg kg-1)
7
5
3
1
Sorghum
Soybean
Cu (mg kg-1)
0.25
0.2
0.15
0.1
0.05
0
Sorghum
0 5 10 15 20 25

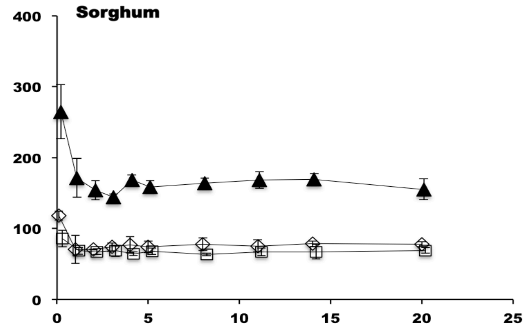

Soybean
Mo (mg kg-1)
0.04
0.03
0.02
0.01
0
5
10
15
20
25

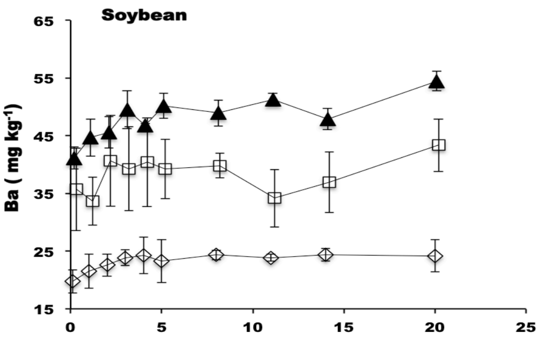

Sorghum
0.07
0.05
0.03
0.01
0
5
10
15
20
25

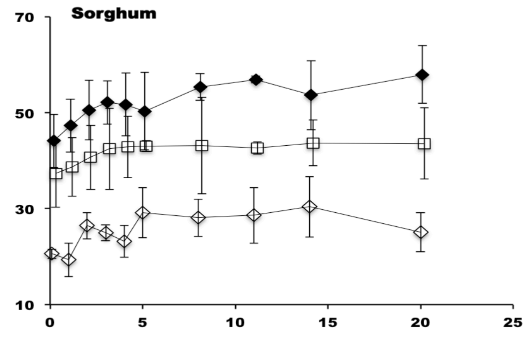

Soybean
Ni (mg kg-1)
0.13
0.1
0.07
0.04
0
5
10
15
20
25


Sorghum
0.2
0.16
0.12
0.08
0.04
0
5
10
15
20
25


Soybean
Na (mg kg-1)
400
300
200
100
0
0
5
10
15
20
25


Sorghum
400
300
200
100
0
0
5
10
15
20
25


Soybean
Ba (mg kg-1)
65
55
45
35
25
15
0
5
10
15
20
25


Sorghum
70
50
30
10
0
5
10
15
20
25

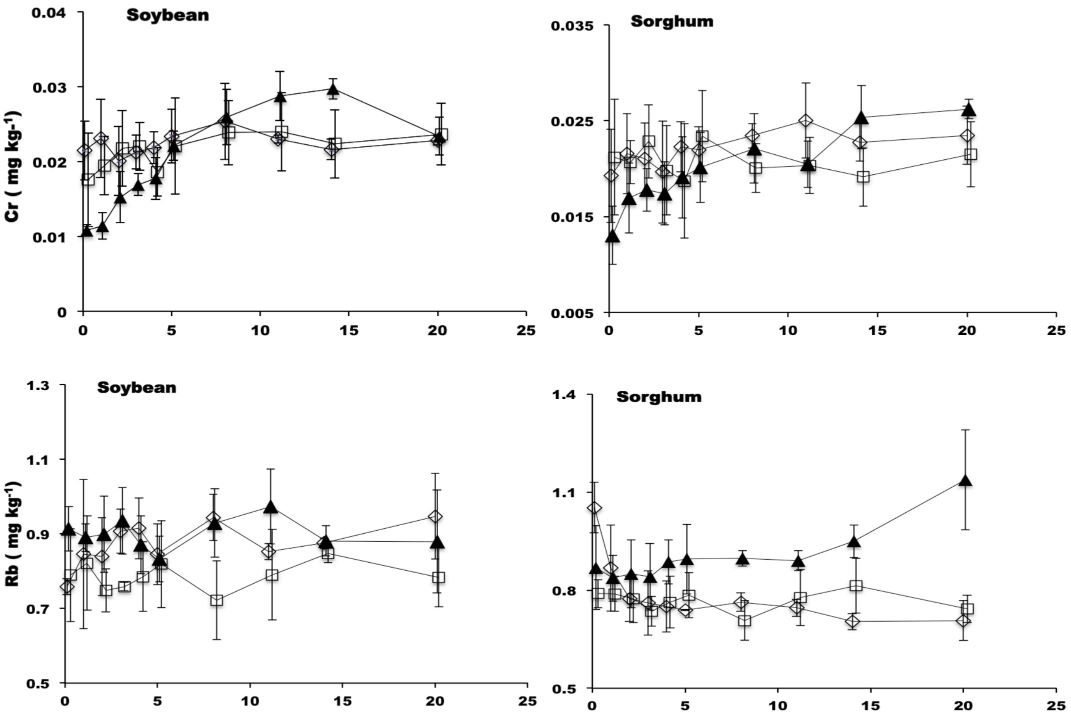
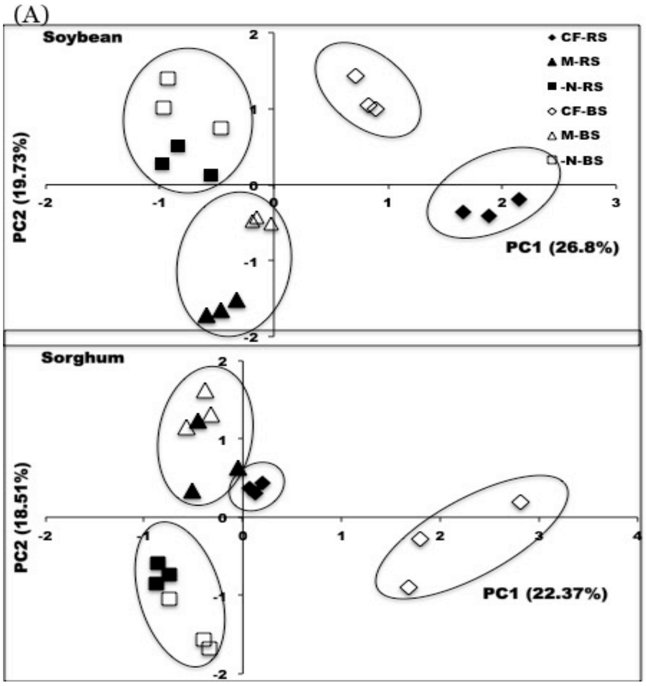


Fig 4.8 Elemental concentration profile along the root zone, rhizospheric soil and bulk soil growing soybean and sorghum receiving various N sources. *X*-axis scale defines distance in micrometres from the inner root. *Y*-axis scale defines the concentration of different elements. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Profiling ionomic difference between the RS and BS

A total of 21 elements, inorganic N, and TAA concentrations in the RC and BS that were affected by three types of N sources were subjected to PCA (Fig 4.9). These 22 original variables were converted to a set of linearly uncorrelated 2 PCs that toally contributed 46.53% and 40.88% of the total variances for soybean and sorghum, respectively. For soybean, the inorganic N, TAA and elemental difference between RC and BS affected by CF treatment, and the difference between M and -N treatment, irrespective of RC and BS, can be clearly separated by PC1. The difference between CF and M, -N treatment can be clearly divided by PC2, irrespectively RS and BS. The PC1 was positively and significantly correlated with NO_3^-, Ca, Mn, Cu, Zn, Mo, Cs. The PC2 was positively and significantly correlated with B, negatively and significantly correlated with TAA, Mg, Na, Cr and Ba. For sorghum, the separation by PCs on inorganic N, TAA and elemental difference affected by treatments or soil sites was similar with that of soybean. The PC1 was positively and significantly correlated with NO_3^-, Mg, Mn, Cu, Zn, Co, Se, Al and Cd, negatively and significantly correlated with Ba. The PC2 was positively and significantly correlated with TAA, Mg, Na and RB, negatively and significantly correlated with Zn and Ni.



(B) Soybean

	PC1		PC2
NO_3^-	0.733**	TAA	–0.711**
Ca	0.522*	Mg	–0.819***
Mn	0.828***	B	0.643**
Cu	0.504*	Na	–0.933***
Zn	0.65**	Cr	–0.579**
Mo	0.867***	Ba	–0.77***
Cs	0.756**		

Sorghum

	PC1		PC2
NO_3^-	0.929***	TAA	0.757***
Mg	0.949***	Mg	0.819***
Mn	0.884***	Zn	–0.512*
Cu	0.473*	Na	0.77***
Zn	0.493*	Rb	0.76**
Co	0.884***	Ni	–0.721**
Se	0.647**		
Al	0.971***		
Ba	–0.612**		
Cd	0.771***		

Fig 4.9 Principal component analysis (PCA) of the inorganic N, TAA, and elemental concentrations in the RC and BS (bulk soil, 14 mm far from the plant roots). (A)

Sample scores for the first (PC1), second (PC2) provided by the PCA identified in the elemental concentrations in the RC and BS. Each treatment contains three replicates. (B) Significant correlation coefficient between the inorganic N, TAA, and elemental concentrations and principal component. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Discussion

The interaction between microbial metabolism, N availability in the rhizosphere, and plant growth in response to manure application

By CLPP analysis we demonstrated that the M treatment improved the microbial activity and diversity in the rhizosphere of soybean and sorghum (Fig 4.6). The improvement of organic fertilization on microbial activity has been considerably reported in previous studies, and may be attributed to the build-up of a larger microbial biomass, increased proteolysis and mineralization of organic matter, or enhanced release of root exudates (Anderson et al., 2013; Gomez et al., 2006; Tian et al., 2015; Tu et al., 2006). The concentration gradient for inorganic N (NH_4^+ and NO_3^-) or TAA in the soils at different distance far from RC associated with soybean was almost constant, irrespective of the N sources (Fig 4.4&4.5). The NO_3^- released by proteolysis or mineralization may be assimilated by soil microorganisms more quickly, because the significant and negative correlation between NO_3^- concentration and AWCD was detected in different soil compartments at different distance far from RC (Fig 4.10). This significant and negative correlation ($R^2 = 0.713$, $P < 0.01$) illustrated that with higher microbial activity the NO_3^- concentration became lower near the soybean root. Moreover, AWCD presented significant and positive correlation with NH_4^+ for soybean (Fig 4.10). That is, nearer the soybean root, the

NH_4^+ concentration was also higher with higher microbial activity. As the transportation way of NH_4^+ in soil is mainly by diffusion (Li et al., 2007; Marschner, 2012), this result may suggest that the increase of microbial activity did not result in more NH_4^+ influx into the root. On the other hand, a drastic decline of NO_3^- near the sorghum root was created by CF treatment (Fig 4.4), which suggests that under the effect of CF treatment the sorghum root took up NO_3^- highly quickly. In fact, although NO_3^- is the main N source for plants in cropping systems as it is the most soluble and easily accessible N form (Glass, 2009), in peatland and paddy soil NH_4^+ often comprises the majority of inorganic nitrogen pool (Anderson et al., 2013； Li et al., 2007). In this experiment, the NH_4^+ concentration was much lower than the NO_3^- in the soils affected by CF treatment, irrespective of plant species, but the depleted zone of NO_3^- was only detected in the RC and RS growing sorghum (Fig 4.4). Increased NO_3^- uptake by sorghum correlated well with the total N and dry matter accumulation in the sorghum shoot (Harada et al., 2000). The ammonium sulfate application did not cause the pronounced increase of NH_4^+ concentration in the soil but NO_3^-, which may suggest the nitrification from NH_4^+ to NO_3^- is highly active in the soil. In addition, the depletion of NO_3^- around root correlated positively and significantly with soil pH (data now shown, $R^2 = 0.658$, $P < 0.05$). Although with the addition of ammonium sulfate into the soil, soil acidification around root of soybean or sorghum was detected (for soybean the acidification was severe, pH = 4.52 in RC, Fig 4.11), there was no significant correlation between pH and N, or AWCD.

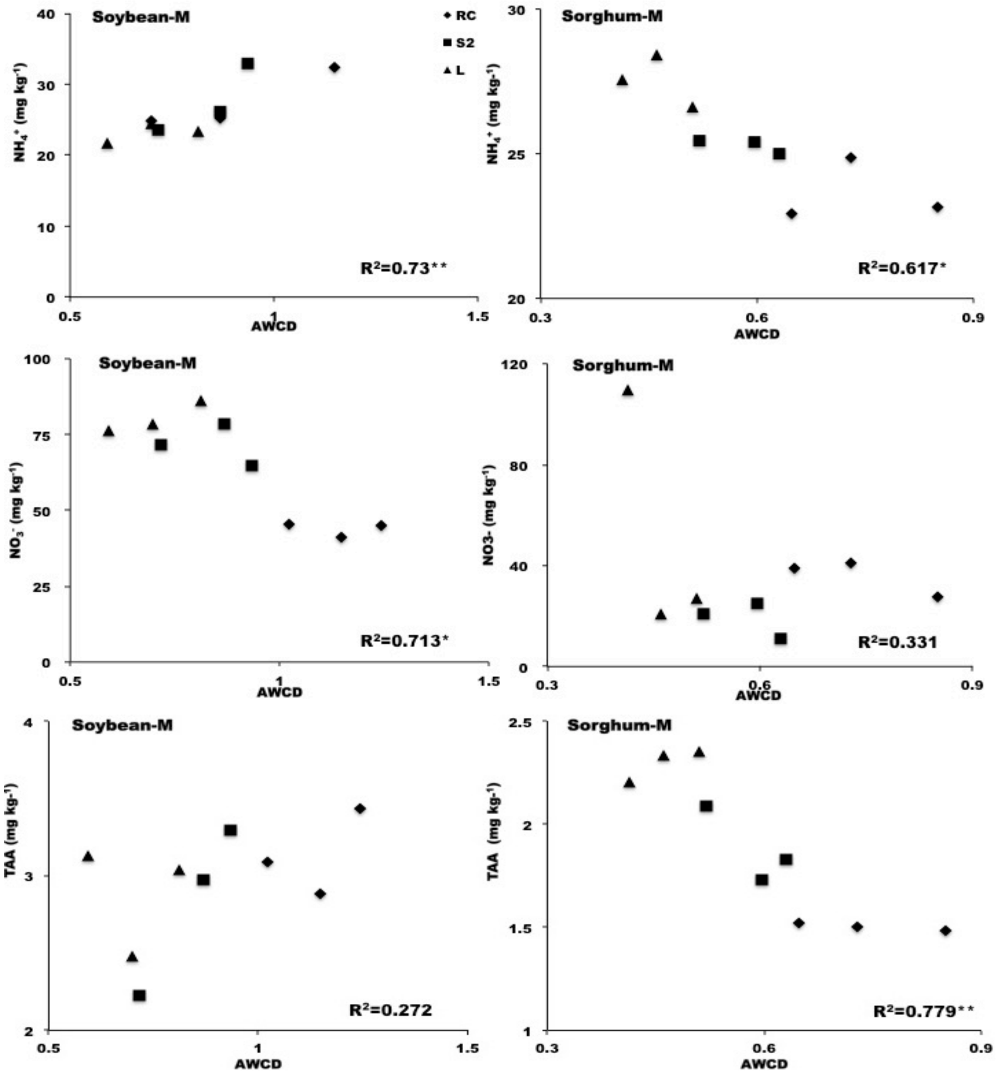


Fig 4.10 Significant correlation between the AWCD and N (inorganic N or TAA) in soil growing soybean and sorghum with the decreasing distance far from RC (14, 2, 0) affected by M treatment. The AWCD was analyzed at 72h after incubating Biolog Ecoplate. ***Significant correlation at $P < 0.001$. RC: root compartment; S2: 2 mm far from RC, L: 14 mm far from RC.

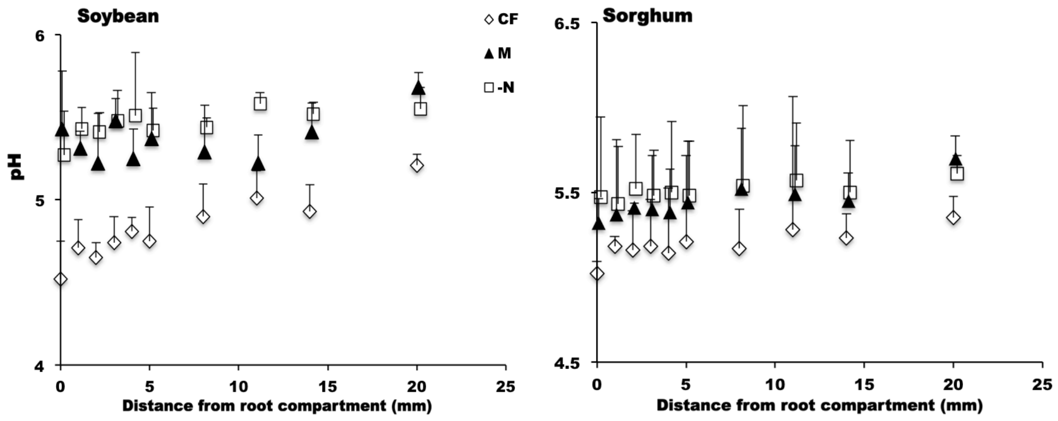


Fig 4.11 Effect of various N treatments on pH determined in the rhizosphere of soybean and sorghum. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

Moreover, the different response of growth and N accumulation in shoot to the M treatment between soybean and sorghum (Fig 4.2) may also relate to the soil microorganism metabolism. In the extracts from rhizospheric soil of soybean receiving M treatment, six types of carbon sources were utilized to a significantly higher level in Ecoplate^{TM} by microorganisms compared to -N treatment (Fig 4.7). Among these carbon sources, glycogen, D-cellobiose, D-xylose, D-galactonic acid-lactone, and putrescine were significantly and positively correlated with TAA concentration in the rhizosphere of soybean with the decreasing distance far from RC (Fig 4.12), which illustrates that with higher capacity of soil microorganisms to utilize these carbon sources near the root system, more amino acids were released into the soil. Glycogen, D-cellobiose, D-galactonic acid lactone all belong to the saccharide and the consumption of saccharide by the microorganism was beneficial for providing reduced C skeleton for formation of amino acids (Holst et al., 2012; Vinall et al., 2012). To increase the availability of organic N in the soil, plants need to release root exudates that include enzymes or organic acids. The exudates release could increase the activity of proteolytic bacteria in rhizosphere (Strickland et al., 2015), which may result in enhancement of N acquisition and decomposition of amino acids from the organic N pool in soil (Dakor and Phillips, 2000; Doornbos et al., 2012). But as there was almost no concentration gradient for TAA between different soil fractions by M treatment, the significance for these correlations needs to be verified in the future study. Maybe it is just because the uptake of amino acids by soybean root was highly fast, or the increased TAA concentration and microbial activity around root have significant effect on soybean plant at reproductive stage.

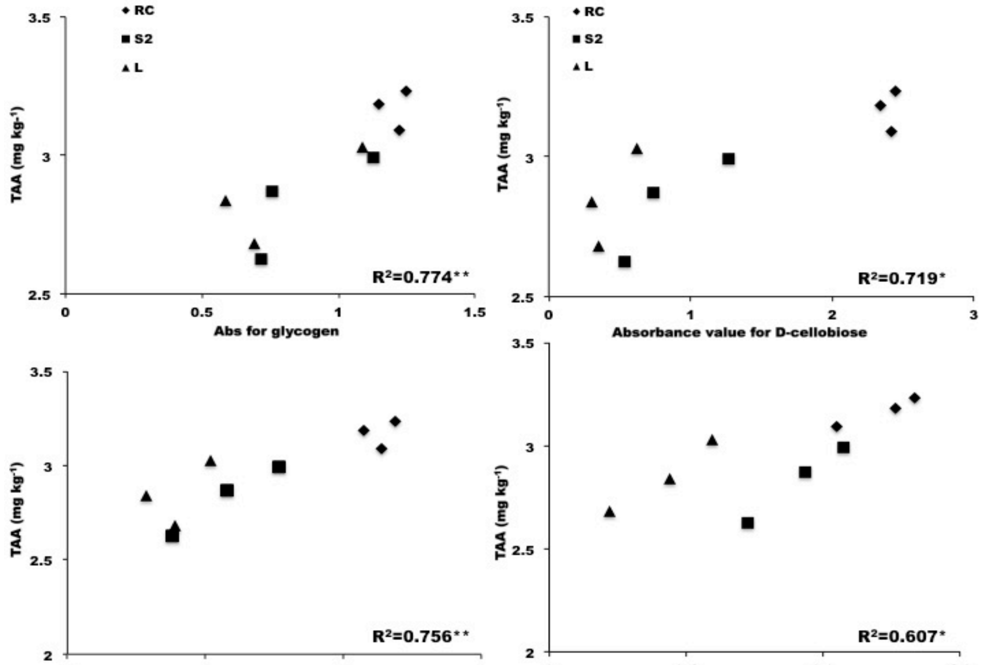


Fig 4.11 Significant correlation between the Abs for some microbial carbon source consumption and TAA concentration in soil growing soybean with the decreasing

distance far from RC (14, 2, 0) in response to M treatment. The Abs was analyzed at 72h after incubating Biolog Ecoplate. *Significant correlation at $P < 0.05$; **Significant correlation at $P < 0.01$. ***Significant correlation at $P < 0.001$. RC: root compartment; S2: 2 mm far from RC, L: 14 mm far from RC.

For sorghum, M treatment significantly increased the microbial utilization on ten types of carbon sources in Ecoplate^{TM} (Fig 4.7). Interestingly, only consumption of three types of amino acids presented significant and negative correlation with TAA concentration in the rhizosphere of sorghum with the decreasing distance far from RC (Fig 4.13). This result may concern with the competition for amino acids between sorghum roots and rhizosphere microorganisms. The turnover of amino acids by soil microorganisms was rapid relative to the movement of amino acids toward the root in the rhizosphere, which results in the poor competitive ability of plant roots to capture free amino acids from the soil solution (Owen and Jones, 2006; Reeve et al., 2012). These three types of amino acids may be involved in more competition between the sorghum root and rhizospheric microbes. When provided with glutamine as N source in uptake studies, plants are better to use it than minor ones such as phenylalanine or histidine, because glutamine was central to N metabolism (Näsholm et al., 2009). Asparagine and glutamine has also been demonstrated as the dominant amino acids in the sugarcane roots across inorganic or organic N treatments (Vinall et al., 2012). In *Arabidopsis thaliana*, the pathway for phosphorylating threonine was also discovered and dominated by AHK2 and AHK3 (Dautel et al., 2015).

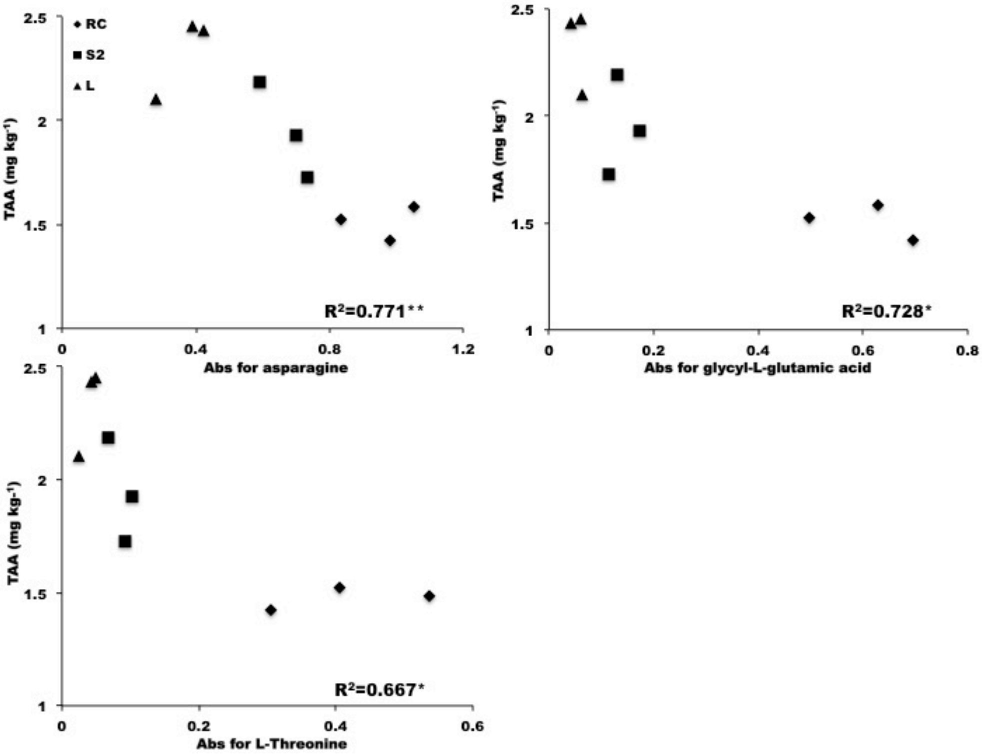


Fig 4.13 Significant correlation between the Abs for some microbial carbon source consumption and TAA concentration in soil growing sorghum with the decreasing distance far from RC (14, 2, 0) in response to M treatment. The Abs was analyzed at 72h after incubating Biolog Ecoplate.*Significant correlation at $P < 0.05$; **Significant correlation at $P < 0.01$. RC: root compartment; S2: 2 mm far from RC, L: 14 mm far from RC.

The difference for plant growth can be reflected on the N, ionome and microbial activity in the soil. N treatments hardly affected the dry weight and N concentrations in the root and shoot of soybean (Fig 4.2). This result partly accorded with the previous study in our research group (Watanabe et al., 2006). But the -N treatment significantly reduced the dry weight and N concentration in the shoot of sorghum.

The difference between soybean and sorghum may derive from the N_2 fixation by the soybean plant. The 50-60% of soybean N demand was met by biological N_2 fixation (Salvagiotti et al., 2006). In some previous study, they also found the difference for biomass production and N accumulation in the seedling shoot of soybean was not significant between N and -N treatment (Barker and Sawyer, 2005; Hungria et al., 2005). If the treatment period was prolonged, the decrease in N concentration and shoot biomass may be observed in the soybean plant because the maximum N_2 fixation occurs after anthesis and at reproductive stage the gaps between plant N demand and N supply by N_2 fixation must be met by N uptake from the fertilizer or other sources (Zapata et al., 1987). At present study, the movement of amino acid from the BS to the RC of soybean was slow relative to inorganic N due to the lower concentration of free amino acids, but the difference of N uptake by sorghum between M and -N treatment can be reflected on the TAA depletion in the soil. There is considerable evidence that plants can acquire and metabolize organic nitrogen in the form of amino acids (Okamoto and Okada, 2004; Persson et al., 2006; Soper et al., 2011; Vinall et al., 2012). Furthermore, given that the shoot N concentration of sorghum was significantly increased by M treatment (Fig 4.2), and significantly and positively correlated with the TAA concentration around root system ($R^2 = 0.783$, $P < 0.01$. Fig 4.14), the rapid consumption on amino acids by microorganisms may have negative influence on the acquisition of root on N source. Additionally, AWCD significantly and positively correlated with TAA in the soil growing sorghum (Fig 4.10). Because of the distinct correlation between microbial activity and TAA concentration in the rhizosphere, the utilization of soybean and sorghum on amino acids was different, which supports the previous report that the use of specific amino acids varied with species (Näsholm et al., 2009). This study is the first time to show the decreased TAA concentration gradient near sorghum root with manure application and demonstrate the relationship between TAA concentration in rhizopsheric soil and shoot N accumulation, which coincides with the previous report that sorghum roots have higher capacities to absorb organic N (Okamoto and Okada, 2004).

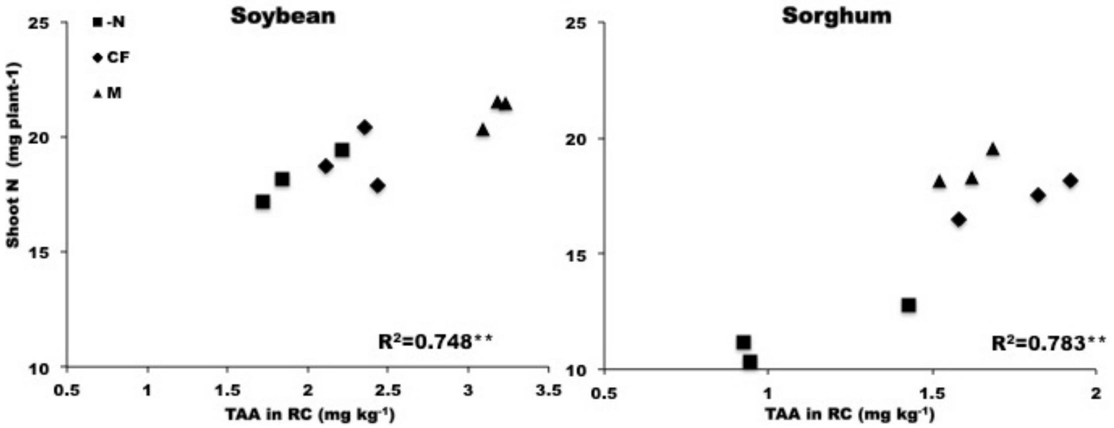
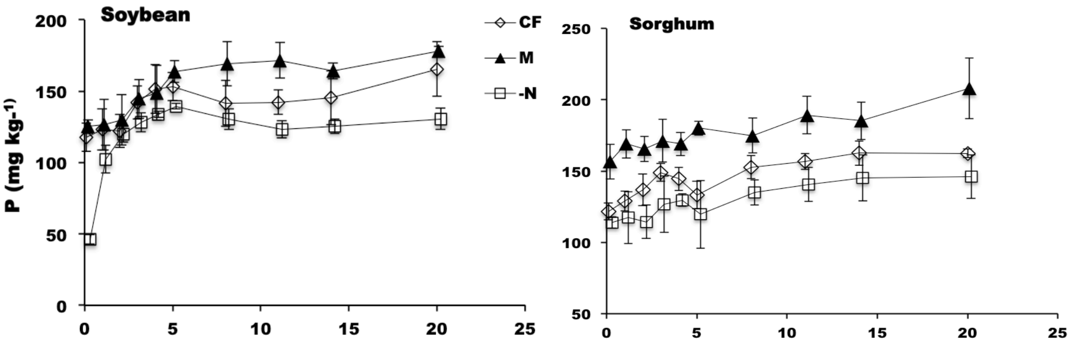


Fig 4.14 Relationship between N concentration in the plant shoot and TAA concentration in the RC growing soybean and sorghum affected by various N treatments. **Significant correlation at $P < 0.01$. CF: chemical fertilizer application; M: manure application; -N: chemical fertilizer application without N.

The interaction between microbial metabolism, N, ionome availability in the rhizosphere, and plant ionome in response to manure application

The manure application significantly altered the ionome accumulation in the shoot of soybean and sorghum (Fig 4.3). The significant increase of some elements affected by various N sources could also be reflected on the profile of elemental depletion in the rhizosphere (Fig 4.8). Moreover, the increased nutrient availability in the soil and accumulation in the shoot under manure application was closely related to enhanced microbial activity (Fig 4.15&4.16). It has been extensively reported that higher microbial activity often lead to higher nutrient availability to crops through enhancing both the microbial biomass turnover and the decomposition of non-microbial organic materials (Liang et al., 2014; Tu et al., 2006; Wang et al., 2004). For soybean, P and Mo mobility in the rhizospheric soil was negatively and significantly correlated with AWCD with the decreasing distance far from RC (Fig 4.15). This result may relate to the activity of rhizobium because P satisfies the energy supply for symbiotic N fixation in the form of ATP (Rubio et al., 2012), and Mo-Fe protein, containing Mo, is indispensible for production and N_2 fixation (Wakeel et al., 2010). In addition, the increased microbial utilization on three types of saccharides (Fig 4.7) may be beneficial for providing reduced C to synthesize N compounds after symbiotic N fixation (Marschner, 2012; Vinall et al., 2012), and on two types of organic acids may be beneficial for increasing availability of some metal elements, such as Ni, Cu (Epelde et al., 2008; Tolrá et al., 1996). For sorghum, P and Fe mobility in the rhizospheric soil was negatively and significantly correlated with AWCD with the decreasing distance far from RC (Fig 4.16). P mobilization in rhizopshere depends on many microorganisms, such as mycorrhizal fungi. M treatment also significantly increased nine types of carbon source consumption by microorganisms in the Ecoplates (Fig 4.7). The enhanced microbial degradation of some carboxylic acids and sacharrides can promote the release of organic anions to facilitate the P mobility through reduced sorption of P, desorption of orthophosphate from absorption sites or through chelation of cations (e.g. Al and Mn in acidic soils or Ca in alkaline soils) that are commonly associated with orthophosphate in soil (Nuruzzaman et al., 2006; Richardson et al., 2009).



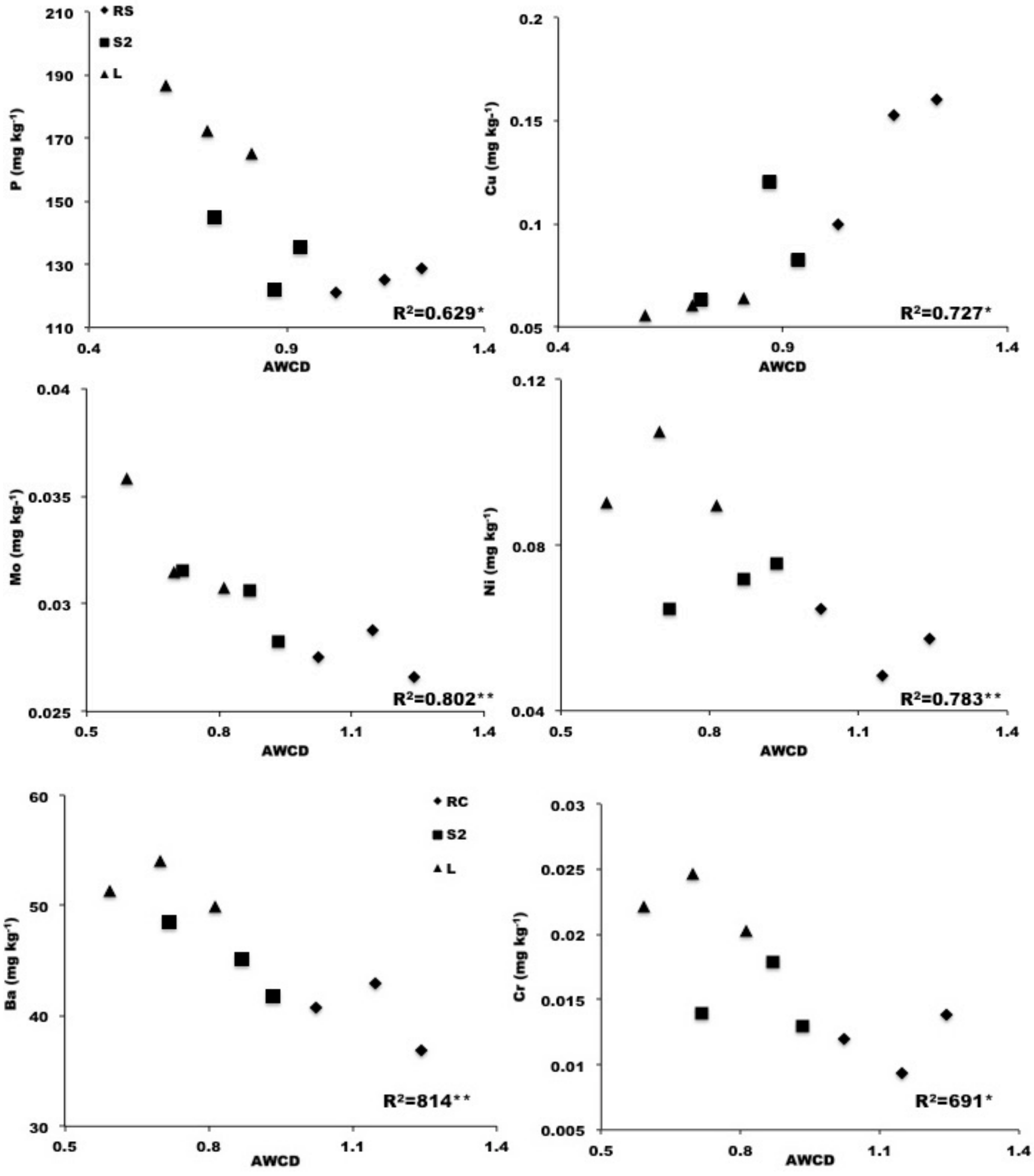


Fig 4.15 Significant correlation between AWCD and available elements in soil growing soybean with the decreasing distance far from RC (14, 2, 0) in response to M treatment. *Significant correlation at *P* < 0.05; **Significant correlation at *P* < 0.01. RC: root compartment; S2: 2 mm far from RC, L: 14 mm far from RC.

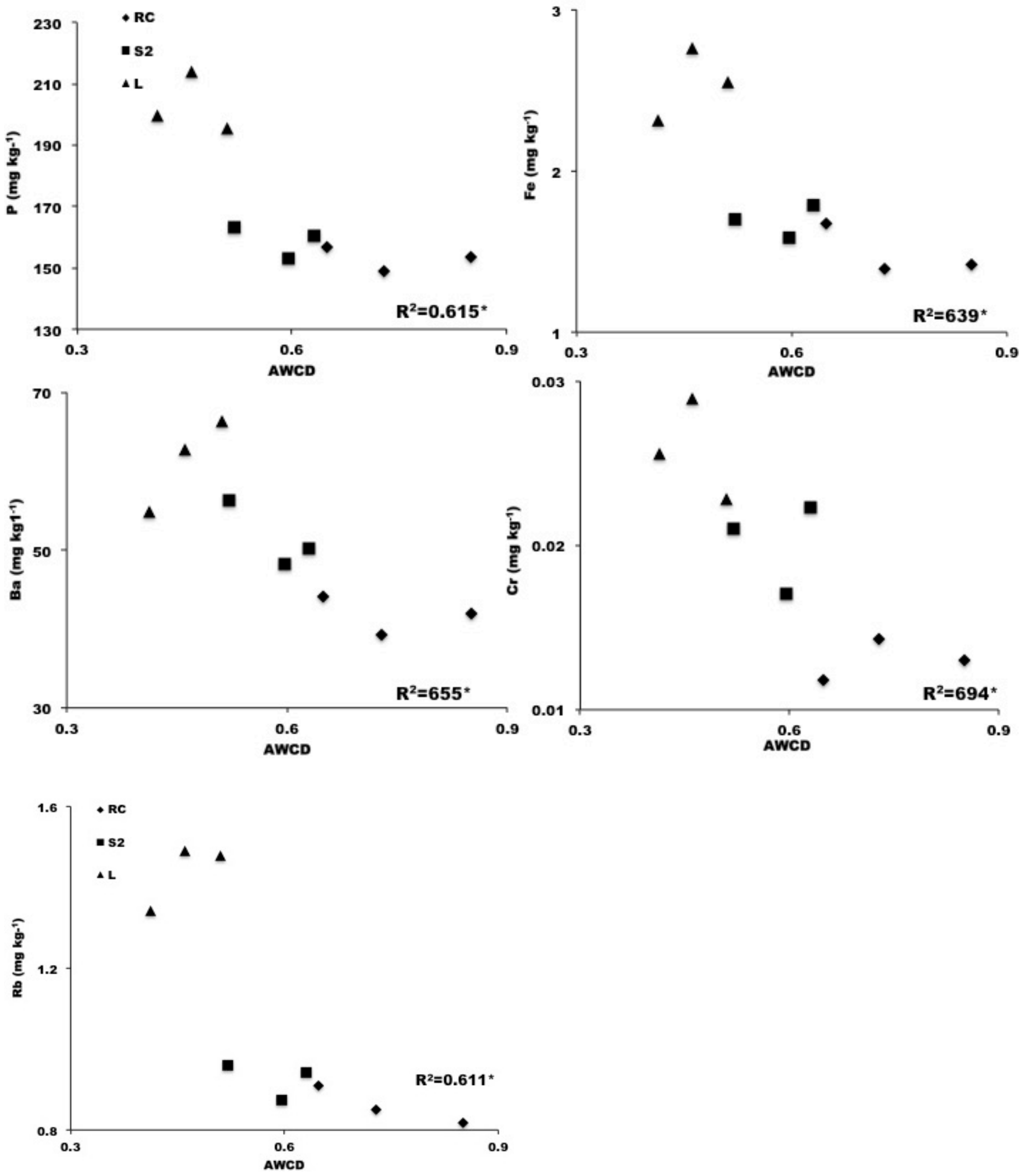


Fig 4.16 Significant correlation between AWCD and available elements in soil growing sorghum with the decreasing distance far from RC (14, 2, 0) in response to M treatment. *Significant correlation at $P < 0.05$; **Significant correlation at $P < 0.01$. RC: root compartment; S2: 2 mm far from RC, L: 14 mm far from RC.

By PCA, the species difference for element concentration in shoot and availability in rhizopshere receiving M treatment was mainly reflected on the micro- and nonessential elements, especially for the metal elements (Fig 4.9). In sorghum, the concentrations of Fe and Cu increased with increasing distance from the roots affected by M treatment, but there was almost no concentration gradient for Fe, and an opposite distribution characteristic for Cu in the rhizosphere of soybean. The Cu concentration in BS of soybean was lower by 2.11-fold than in RC but in BS growing sorghum was higher by 1.84-fold than in RC (Fig 4.8). For soybean, Fe and Cu both act as cofactors to participate in N fixation and stabilize cell walls for N uptake (Hänsch and Mendel, 2009). The relatively even distribution of Fe may illustrate the quick absorption by soybean roots to confirm the robustness of N_2 fixation, but this did not cause the significant increase of Fe accumulation in the soybean shoot. Conversely, the strong gradients of Fe concentration within RC and RS was reflected on the significant increase of Fe concentration in the sorghum shoot. Interestingly, the contrasting distribution of Cu in the rhizosphere both caused the significant increase of Cu concentration in the shoot. The distinct depletion profile for Ni concentration around soybean root affected by M treatment was observed in the rhizosphere of soybean, but this did not further enhance the Ni concentration in the shoot. This may derive from the characteristic of Ni to participate in degrading the urease in the root and nodule of soybean seedlings (Kutman et al., 2013; Marschner, 2012). Although a biological function in plants has not been demonstrated for nonessential elements, they are taken up via essential metal uptake system (Page et al., 2006). The M treatment significantly increased Ba and Cr concentration both in the shoot of soybean and sorghum (Fig 4.3), and the distinct depletion was detected both in the rhizosphere of soybean and sorghum (Fig 4.8). This result suggests that the transportation way of Ba^{2+} and Cr^{2+} in soil may be mainly by diffusion. This result is consistent with results reported previously (Sha et al., 2012; Tian et al., 2015). Moreover, Ba and Cr showed significant and negative correlation with AWCD with the decreasing distance far from RC growing soybean (Fig 4.15). Ba, Cr and Rb showed significant and positive correlation with AWCD with the decreasing distance far from RC growing sorghum. This result supported the previous reports with respect to the enhanced microbial biomass and activity as well as heavy metal availability in the soil with the addition of manure (Sha et al., 2012; Tian et al., 2015; Zhou et al., 2015). Interestingly, the distinct depletion profile for Rb was only detected in the rhizosphere of sorghum affected by M treatment, and this further resulted in the significant enhancement of Rb concentration in the sorghum shoot. Moreover, AWCD displayed negative and significant correlation with Rb concentration in the rhizosphere at different distances far from RC (Fig 4.16). These results suggest that the manure application could promote the soil microbial activity and Rb uptake by sorghum root at the same time. Also, this is a different characteristic from soybean. In our previous study, there was a highly significant and positive correlation between Cs and Rb accumulation in the *Amaranthus* plant grown on the ^{137}Cs-contaminated field (Chu et al., 2015). At present study, the Cs and Rb concentrations in the sorghum shoot were both significantly increased by the manure application. Therefore, more research on the interaction between Cs, Rb, organic amendment and microbial activity may be beneficial for removal of Cs from the contaminated field.

Conclusion

Seedlings of soybean and sorghum were grown in a rhizobox across three N input treatments. The capacity of microorganisms in using different carbon sources was highest at the rhizospheric soil receiving manure application, irrespective of plant species. For soybean, manure application significantly stimulated the microbial utilization on D-xylose, D-malic acid, glycogen, D-cellobiose, D-galactonic acid-lactone and putrescene in the substrate of Biolog Ecoplate$^{\mathrm{TM}}$. Microbial consumption on D-cellobiose, galactonic acid-lactone and putrescine presented significant and positive correlation with the deletion of total amino acids (TAA) in the rhizosphere. Moreover, clear depletion of P, K, Fe, Mo, Ni, Ba and Cr near the soybean root was detected and further resulted in the enhancement of corresponding elements concentration in the shoot. Also, depletion of P, Mo, Ni, Ba and Cr exhibited significant correlation with microbial activity. For sorghum, microorganisms utilized nine types of carbon sources to a significant level in

Ecoplate^{TM}, and among them the consumption of three types of amino acids was increased with lower TAA concentration near the root. The deletion of TAA was positively correlated with plant N content. In addition, clear depletion of TAA, P, K, Fe, Cu, Mo, Ba, Cr, and Rb was detected near the sorghum root and further induced the increased concentration of these elements in the shoot. Also, depletion of TAA, P, Fe, Ba, Cr and Rb was significantly correlated with microbial activity. The increased concentration of N and other elements in the shoot promoted the elevation of shoot biomass of sorghum. This research for the first time compared the difference between soybean and sorghum to utilize N under manure application by analyzing the profile of N, ionome and microbial metabolism in the microdistribution of rhizopshere soil, and N, ionome accumulation in the plant.

General conclusion

Ionome is a profile of elements in an organism at the organ, tissue or even cell level. The plant ionome represents all the mineral nutrients and trace elements of the plant, which is a dynamic network of elements that are controlled by the physiology and biochemistry of the plant, and ultimately controlled by the genome, in response to the various environmental factors. But in past studies, the ionmics study in plant under field conditions was rare. Moreover, among the nutrients required by plants, nitrogen (N) is the most essential nutrient in terms of quantity. Study on interaction between N and ionome in plant and soil is important to develop strategies for better nutrient management, but this interaction has not been understood very well. In addition, nobody has ever studied the ionomic interaction between plant and soil with emphasis on rhizosphere. As a result, three independent experiments were carried out. Firstly, a field study was conducted to investigate the ionomic information, particularly Cs and Sr, under thirty-three varieties of *Amaranthus*. Secondly, a pot experiment was designed to comprehend the regulation of various N sources on the interaction between N and ionome in soybean at different growth stages. Finally, a rhizobox experiment was conducted to profile the micro-distribution of N, ionome in the soil growing different plant species, which is helpful for expounding the N utilization by various plant species.

Firstly, the field study adopted 33 different varieties of *Amaranthus* and investigated the concentrations of 23 mineral elements in shoots grown in the fields of Iino in Fukushima prefecture. Among 23 elements, *Amaranthus* were less sensitive to the accumulation of Cs and Sr than most other mineral elements to the species level. There are six elements showing significant correlation with Cs, positive correlations between As, Rb, Al, Fe, Ni, and Cs, and negative correlation between Ba and Cs. Significant correlations between Ca, Mg, Mn, Zn, B, Ba, Cd, and Sr were detected, and all of the coefficients were positive. Cs and Sr did not present significant correlation, but they were both significantly correlated with Ba. By principal component analysis (PCA), the first and second principal components (PC1 and PC2) accounted for 23.2 and 20.3% of the total variance and associated with Cs and Sr, respectively.

Secondly, we assessed the relative distribution of 22 mineral elements in the root, nodule and shoot of the soybean (*Glycine max* L. Merr.cv. Tsurumusume) at R1 (beginning of flowering stage) and R7 (beginning of ripening stage) in response to ammonium and manure N treatment. Although the addition of ammonium sulfate to the soil significantly reduced the biomass production of nodule and root, the accumulation of Ca, Mg, Fe, Mn, Cu and Zn significantly improved in the shoot, and that of Mo and Co improved in the nodule. The addition of manure to the soil significantly enhanced the levels of Sr, Ba, Cr and Cd in the shoot, whereas the shoot Cs concentration was inhibited at R7. Moreover, although the nodule cells are part of the root, the variation of concentrations of many elements was not consistent between nodule and root when soybean developed from juvenile to maturation. The variation of Mn, Zn, B and Al concentrations was independent on N treatments but Ca, Fe, Cu, Mo and Se strongly regulated by N treatments.

Lastly, seedlings of soybean and sorghum were grown in a rhizobox across three N input treatments (without N, ammonium sulfate or cattle farmyard manure). The preferable C sources varied between the soils growing various plant species and were significantly affected by various N treatments. The manure treatment on soybean altered belowground processes by stimulating the microbial utilization on some saccharide and amine, and increasing the uptake of NO_3^-, P, and some metal elements, although these processes did not cause significant variation on shoot biomass. On the other hand, addition of manure to the soil altered the sorghum growth by stimulating the microbial utilization on several types of amino acids, increasing the uptake of total amino acid, P, B and many metal elements, leading to a higher accumulation of shoot N and biomass. Our results indicate that the correlation between microbial activity and N, ionome differed between species grown with various N sources.

In conclusion, this study clarifies the ionome information in plant in response to variety effect, the dynamic variation of N and ionome in the plant as plant develops from juvenile to ripening, as well as the interactions between N, ionome in the plant and soil in response to various N sources. These achievements contribute greatly to interpret the connection among the ionome study, N fertilization, and plant nutrition.

Acknowledgements

The research summarized in this thesis has been carried out at the Laboratory of Plant Nutrition, Graduate School of Agriculture, Hokkaido University during the years of 2012–2015 under the Ph. D course of “Special Postgraduate Programe in Bio-Systems Sustainability (SPPBSS)”.

First and foremost I would like to express my deepest gratitude to my supervisor Prof. Mitsuru Osaki and associate professor Toshihiro Watanabe for giving me the opportunity to study in this lab. It is my honor to be their student. Without their consistent trust and encouragement, I could not start and complete my doctoral career in the best in the most beautiful palace – Hokkaido University. I usually made careless mistakes during the process of experiments or paper-writing, but Dr. Toshihiro Watanabe usually instructed me highly patiently with his active scientific ideas. Also, when he reviewed my manuscript, he can provide lots of valuable suggestions for how to organize a story logically in order to accentuate the key points of our research, which is significant for a doctor student to understand how to design the experiments to testify the scientific hypothesis.

I also express my sincere gratitude to Dr. Takuro Shinano. Although he only supervised me less than one year, he really contributed much to my experiments and paper-writing. His wide knowledge, active scientific ideas and valuable discussions accompanied me through the doctor career.

I’ d like to extend my thanks to Dr. Tatsuhiro Ezawa, Dr. Norikuni Oka, Dr. Takuji Nakamura, for their thoughtful suggestion and kind cooperation through all the

period of my study.

I also want to express my heartfelt thanks to Dr. Jun Wasaki for kindly providing experiment materials to support my research. Although he is not my academic supervisor, still provide many valuable suggestions on my future research.

All the members in the Lab of Plant Nutrition of Hokkudai and in the Lab of soil group have contributed immensely to my personal and professional time in Sapporo. They are a good source of friendships in my daily life and good advice and collaboration in the experiment and seminars. In addition, I want to express my particular gratitude on Dr Zhimin Sha, Masako Owadano, and Eriko Maejima. Dr Sha treated me like his biological brother and instructed me much about the operation of instrumentation and paper-writing. Masako Owadano and Eriko Maejima always tolerated my careless mistakes in the experiment and supplied guidance with smile. Thank you all for accompanying me in the past three years.

I also want to thank all the faculty staff for their help to make my work easier.

I also appreciate the patient guidance and instruction on my Japanese-learning from the teachers in “ Intensive Japanese Course ”.

Great acknowledgement is given to my parents for all their love, understanding and encouragement throughout my life. Special appreciation is expressed to the friends in Sappro. Almost all my spare time were occupied by Miao Zheng, JunLong Chen, Xi Li and Yi Wang, and the figure of Miao Zheng could be seen in almost all the parties I attended and photos I was photographed, but I feel genuinely happy.

At last, I am very grateful to the financial support of the China Scholarship Committee, which made it possible for me to study here and complete this thesis.

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