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https://doi.org/10.5109/2558887

出版情報：九州大学大学院農学研究院紀要. 65 (1), pp.25-34, 2020-02. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **65** (1), 25–34 (2020)

Nutrient–Specific Variation of C–N Metabolism in the Leaves and Roots of Bell Pepper (*Capsicum annunm*. L) in Response to Macronutrient Deficiency

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(*Received October 24, 2019 and accepted November 14, 2019*)

Mineral nutrients as an essential element for agricultural crops are absorbed by the roots, transported in the xylem to the shoots, and assimilated into organic molecules or involved in a large number of metabolism. Visual symptoms such as growth retardation, reduced crop production and resistance against disease and pests are strongly connected with a result of metabolic disturbance by mineral deficiency. Of essential mineral nutrients, we looked into mineral–mineral interactions (expressed by synergism and antagonism) and subsequent metabolic changes in the leaves and roots of bell pepper during macronutrient deficiency. The deficiency of cationic nutrients (K, Ca and Mg) and S responded generally antagonistically each other in terms of uptake, and these blockages resulted in significant changes in metabolite levels which are able to be caused by restricted shoots–roots communication of phytosynthates. Each nutrient affected differently to the type and amount of metabolites and plant organs. Interesting finding was significant increase in amino acids in both organs by cations deficiency, and, of them, glutamine and asparagine were more than 10–fold accumulated, which could be considered as a potential indicator of cation deficiency. Furthermore, it was carefully assumed that a limited uptake of sulfur accompanied by cations deficiency could be direct cause of disturbance in primary metabolism rather than cations itself. In view of this, on the premise of further study to verify what happens between cations and sulfur in plants, our study might help to make clear the complicated mechanisms of metabolic networks in responses to individual and multiple nutrient stresses.

Key words: Bell pepper, C–N metabolism, Macronutrient deficiency, Mineral–mineral interaction, Shoot–root communication

INTRODUCTION

As essential mineral nutrients for plant growth and development, nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) are classified as macronutrients due to relatively large requirements of these minerals. Accordingly, in case any one of those minerals is limited on plant growth and development, most of plants are not only to be encountered with unfavorable plant growth and yields but also to be vulnerable to pathogen and pest attack (Laegreid *et al*., 1999; Epstein and Bloom, 2005). Moreover, mutual influence between minerals varies not only in organs and species but the status of mineral nutrients in plant growth environments (e.g. soil). A suboptimal nutrient supply can influence on the absorption of other nutrients or limit their physiological functions (Bergman, 1992). Furthermore, a starvation or surplus of mineral nutrients can lead to any synergistic or antagonistic impact due to pH variability. Therefore, elucidating the behaviors (expressed as uptake and partitioning) of mineral nutrients is necessary for better nutrient management in crops which are suffering a deficiency of specific minerals.

Many studies have reported mineral nutrient–dependent metabolic changes in a variety of plant species; 1) N deficiency represents in decreases the levels of amino acids, proteins and nucleotides, and increases in the amounts of carbohydrate (starch) and secondary metabolites (flavonoids and phenylpropanoids) (Fritz *et al*., 2006), 2) P deficiency use a phosphorus ion from organic molecules to preferentially produce cellular energy (ATP) (Plaxton, 2004; Amtmann *et al*., 2006; Yuan and Liu, 2008), and thus leads to a striking decrease in P–containing metabolites like ATP, hexose phosphate and several TCA intermediates (Morcuende *et al*., 2007), 3) K deficiency greatly accumulates soluble carbohydrates and amino acids (basic– and neutral–forms) (Armengaud *et al*., 2009; Amtmann, 2008), 4) Little information is available how Ca or Mg deficiency regulates metabolic responses in plants. Apart from well–known, Ca directly and indirectly involves in regulating cellular metabolism. Mg is a crucial mineral in the transportation of photosynthates from source to sink tis-

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sue, and thus its starvation leads to substantial accumulation of carbohydrates in source organs which implements photosynthesis (Cakmak *et al*., 1994a; Cakmak *et al*., 1994b; Marschner *et al*., 1996; Hermans *et al*., 2004), and 5) S deficiency is closely linked to glucosinolates breakdown (Hirai *et al*., 2005; Hirai and Saito, 2008) and the subsequently long–term starvation decreases in the levels of lipids, proteins, RNA and chlorophyll, accompanied by decreased photosynthesis and increased photorespiration (Nikiforova *et al*., 2005).

Considering a quantity of mineral–dependent metabolic impacts, it is undoubtable that plant metabolism is greatly influenced by the status of mineral nutrient conditions. In the present study, we looked into changes in the levels of primary metabolites deeper to better understand an adaptation to macro mineral nutrients deficiency. Mutual influence between other macro mineral nutrients and deficient one was also investigated in terms of antagonistic or synergistic view. Our previous study with cabbage plant revealed some interesting changes against macro mineral deficiency (Sung *et al*., 2018), nevertheless, mineral nutrient–specific changes in metabolism are still somewhat debatable due to the differences in period of stress, plant tissue and species, experimental conditions and so on.

The present study, we supposed that primary metabolism is likely obviously affected by mineral nutrients which could result in unfavorable metabolic and ionic communications between leaves and roots as considering homeostasis of mineral nutrients in whole plant level. To verify these questions, we analyzed the levels of mineral nutrients and primary metabolites in the leaves and roots of bell pepper plants grown under individual macro nutrient–free condition. We emphasized on depicting mineral–specific changes in mineral nutrients and metabolites. We describe the results from the viewpoints of mineral nutrients–primary metabolism with current models.

MATERIALS AND METHODS

Plant materials and growth conditions

Bell pepper (*Capsicum annunm* L. cv. Superior) seeds were germinated on perlite fed with de–ionized water, and uniformly growing seedlings were carefully transplanted into aerated 20 L hydroponic containers containing 1/2–strength Hoagland solution and grown for an additional 2 weeks prior to the initiation of treatment. Seedlings were grown at 25 ± 3°C during the day and 15 ± 3°C at night with continuous aeration. The photosynthetic photon flux density at mid–day was 800–1200 μmol m^{-2} s^{-1}. The nutrient solution was replaced every 3 days. The composition of the nutrient solution (control) was as follows: 2.5 mM Ca $(NO_3)_2$, 2.5 mM KNO_3, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.75 mM Fe–EDTA, 0.5 mM NH_4NO_3, 2 μM H_3BO_3, 0.2 μM $MnCl_2$, 0.19 μM $ZnSO_4$, 0.01 μM $CuSO_4$ and 0.03 μM H_2MoO_4. To generate individual macronutrient–deficient conditions, Ca $(NO_3)_2$, KNO_3 and NH_4NO_3 were replaced by $CaCl_2$ and KCl for N deficiency, KH_2PO_4 by KCl for P deficiency, KNO_3 by NaH_2PO_4 for K deficiency, Ca $(NO_3)_2$ by NH_4NO_3 for Ca deficiency, $MgSO_4$ by $CaSO_4$ for Mg deficiency and $MgSO_4$ by $MgCl_2$ for S deficiency. To minimize any temporal effects on mineral nutrient and metabolite levels at 15 days after the onset of treatment, the experimental samples of bell pepper plants from each treatment group were carefully taken between 10:00 and 12:00, were rinsed briefly in deionized water, immediately frozen in liquid nitrogen and stored at –80°C prior to metabolite analysis or oven–dried at 80°C for 48 h prior to mineral nutrient analysis.

Mineral nutrients analysis

Dried samples were powdered, and 0.2 g was mixed with 5 mL of 368 $mmol^{-1}$ L salicylic acid in 84.7% sulfuric acid (H_2SO_4, v/v) for 24 h and wet–digested at 300°C for 6 h, followed by little addition of hydrogen peroxide (H_2O_2). The extract was diluted to 100 mL with deionized water for the analysis of mineral nutrient concentration. N was colorimetrically determined using an automatic flow injection analyzer (Bran + Luebbe, Germany). P was analyzed using the molybdate–blue colorimetry method (UV–2450, Shimadzu, Japan), and K, Ca, Mg and S were measured using an ICP–OES machine (INTEGRA XMP, GBC, Australia) according to manufacturer's manual.

Metabolites analysis

Polar metabolites were extracted as described previously (Kim *et al*., 2016). The metabolites were extracted from powdered tissue (100 mg) by adding 1 mL of 2.5:1:1 (v/v/v) methanol: water: chloroform. Ribitol (60 μL, 0.2 mg/mL) was used an as internal standard (IS). Extraction was performed at 37°C at a mixing frequency of 1200 rpm for 30 min using a Thermomixer Compact (Eppendorf AG, Germany). The solutions were centrifuged at 16,000 × g for 3 min. The polar phase (0.8 mL) was transferred into a new tube and combined with 0.4 mL water, mixed and centrifuged at 16,000 × g for 3 min. The methanol/water phase was dried in a centrifugal concentrator (CC–105, TOMY, Tokyo, Japan) for 2 h, followed by a freeze dryer for 16 h. MO–derivatization was performed by adding 80 μL of methoxyamine hydrochloride (20 mg/mL) in pyridine and shaking at 30°C for 90 min. TMS–esterification was performed by adding 80 μL of MSTFA, followed by incubation at 37°C for 30 min. GC–TOFMS was performed using an Agilent 7890A gas chromatograph (Agilent, Atlanta, GA, USA) coupled to a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI). Each derivatized sample (1 μL) was separated on a 30–m × 0.25–mm I.D. fused–silica capillary column coated with 0.25–μm CP–SIL 8 CB low bleed (Varian Inc., Palo Alto, CA, USA). The split ratio was set to 1:25. The injector temperature was 230°C. The helium gas flow rate through the column was 1.0 mL/min. The temperature program was as follows: Initial temperature of 80°C for 2 min, followed by an increase to 320°C at 15°C/min and a 10 min hold at 320°C. The transfer line temperature and ion–source temperature were 250 and 200°C, respectively. The

scanned mass range was 85–600 m/z, and the detector voltage was set to 1700 V. ChromaTOF software was used to support peak findings prior to quantitative analysis and for automated deconvolution of the reference mass spectra. NIST and in–house libraries for standard chemicals were utilized for compound identification. The calculations used to quantify the concentrations of all analytes were based on the peak area ratios for each compound relative to the peak area of the IS.

Statistical analysis

Statistical analysis was performed using SAS software (version 9. 4). Data were subjected to one–way ANOVA. If the ANOVA yielded a significant F value ($P < 0.05$), the differences among treatments were compared using Tukey's range test. The relative quantification data acquired from GC–TOFMS were subjected to PCA (SIMCA–P version 13.0; Umetrics, Umeå, Sweden) to evaluate the relationships in terms of similarity or dissimilarity between groups of multivariate data (Kim *et al*., 2017). The PCA output depicted with score plots for visualizing the contrast between different samples and loading plots to explain the cluster separation. The data file was scaled with unit variance scaling before all variables were subjected to PCA. Pearson's correlation analysis and t–tests were performed using the SAS 9.4 software package (SAS Institute, Cary, NC, USA). Correlation analysis among relative metabolite levels was performed using standardization pre–processing. HCA and heatmap visualization of the correlation coefficient were performed using MultiExperiment Viewer software version 4.4.0 (http://www.tm4.org/mev/).

RESULTS

Plant growth and mineral uptake

Four–week–old bell pepper plants were subjected to macronutrient (N, P, K, Ca, Mg or S) deficiency for 15 d. Figure 1 represents the relative growth comparison affected by each nutrient deficiency to the control (optimal supply) at 15 d after treatment. Overall, plant growth were clearly divided into three groups; severe (–N and –S), moderate (–P and –Mg) and insensitive (–K and –Ca). Compared to the control (6.1 g plant^{-1}, DW), the relative growth rate was highest under –K conditions (97%, 5.99 g), followed by –Ca (91, 5.63 g), –Mg (72, 4.47 g), –P (66, 4.09 g), –S (54, 3.33 g) and –N (48, 2.98 g).

We analyzed an antagonistic or synergistic effect to better understand the relation between nutrients. High score indicates a small causality of mineral nutrient deficiency. The uptake (based on the concentration) of macronutrients differently responded by the type of a deficient nutrient and plant organ (Fig. 2). In the leaves, N and S deficiency did not affect P uptake, P deficiency did not influence the concentration of N and cations (K,

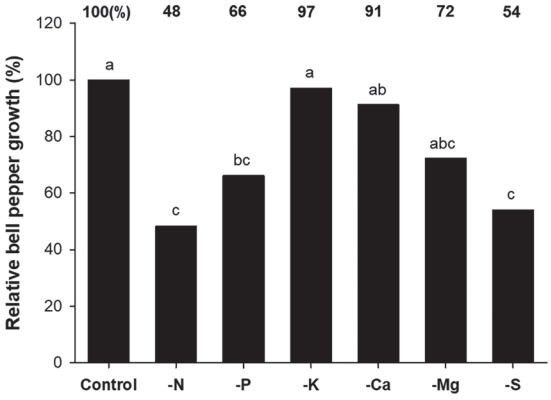


Fig. 1. Comparison of growth of bell pepper plants under deficient conditions of individual macronutrients for 15 days. See the *Materials and Methods* for details.

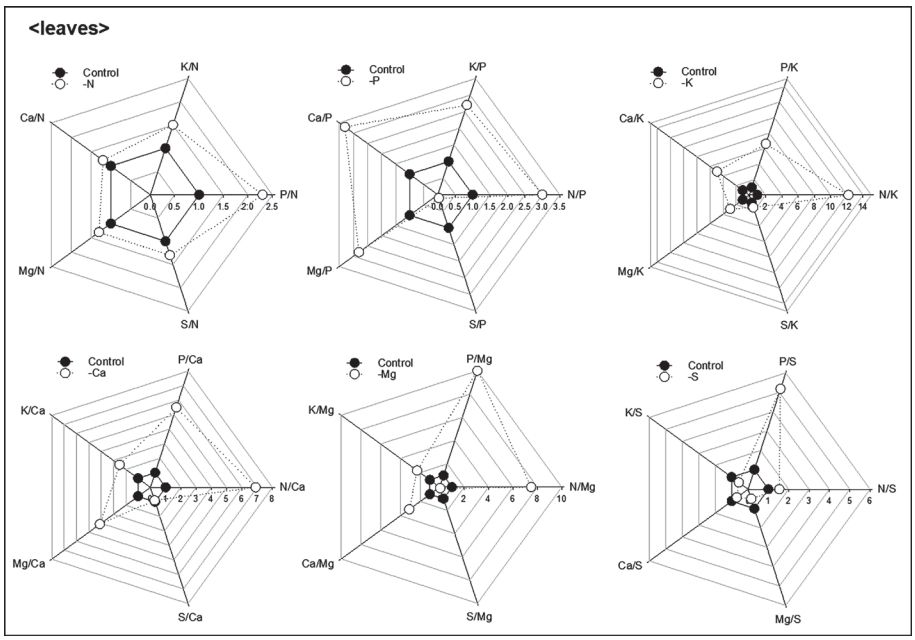
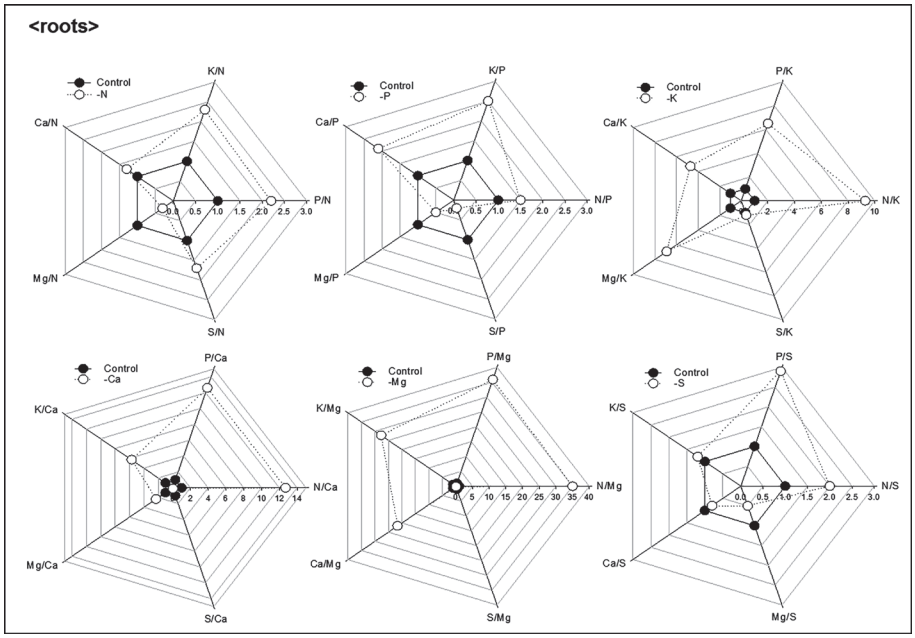


Fig. 2. Synergistic and antagonistic effects of other mineral nutrients by deprived nutrient in bell pepper leaves (top) and roots (bottom).

Ca and Mg), and the starvation of cations just showed the small effect on N and P accumulation. These cause–and–effect relationships between nutrients represented somewhat different results in the roots. The uptake of P and K was not influenced by all kinds of mineral deficiency. On the contrary, S uptake was noticeably limited by all mineral deficient conditions except for N deficiency.

Multivariate analysis

Since macro mineral nutrient deficiency can affect plant metabolic pathway and thus lead to a bunch of metabolic changes, we performed metabolite profiling of the leaves and roots of bell pepper plants. To characterize mineral–specific changes in primary metabolism, we measured the relative level of metabolites in the leaves and roots of bell pepper plants exposed to mineral–deficient conditions. We measured 42 metabolites including carbohydrates, organic acids, amino acids and others, and, in the present study, focused on discussing primary metabolism. Metabolite profiling was subjected to PCA and major differences among nutrient conditions were identified using a PCA score plot. The PCA revealed two principal components, PC1 and PC2 that explained 34.6 and 25.6% of the total variance for the leaves (Fig. 3–a) and 54.3 and 15.4% for the roots (Fig. 4–a), respectively.



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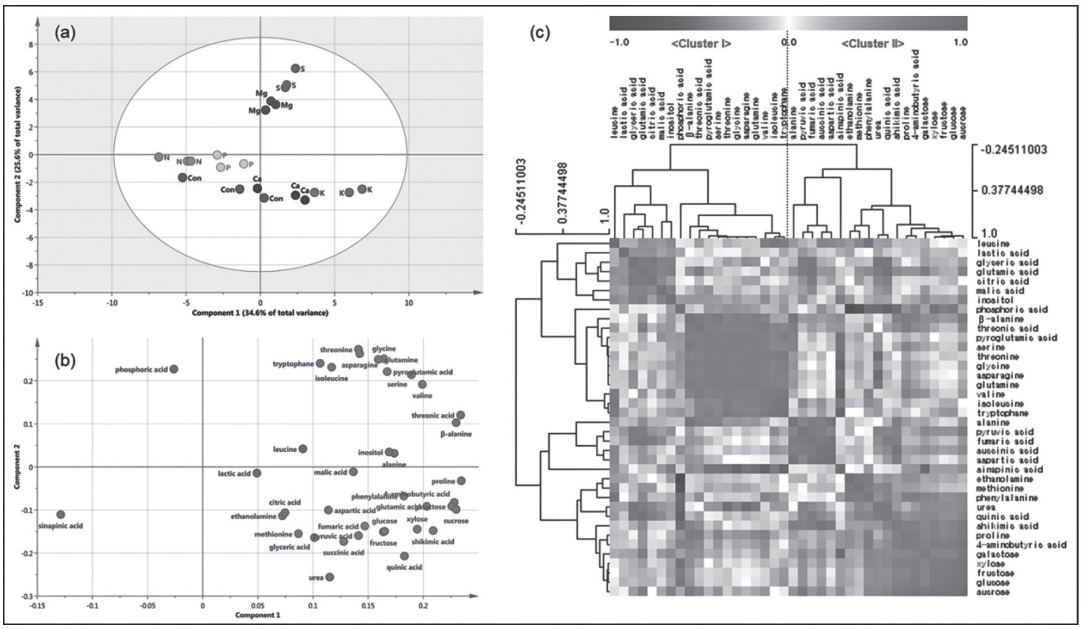

Fig. 3. PCA score plot (a), loading plots (b) and correlation matrix and cluster analysis (c) based on the abundance of polar metabolites from the leaves of bell pepper plants grown under macronutrient deficiency for 15 days with 3 plants. Each square in the heatmap indicates the Pearson's correlation coefficient for a pair of compounds. The degree of the coefficient expressed by the intensity of the color indicates blue (negative) or red (positive).

PC1 distinguished –K, –Ca, –Mg and –S with a positive coefficient from control, –N and –P in the leaves (Fig. 3–a), and control, –N, –P and –K from –Ca, –Mg and –S in the roots (Fig. 4–a). PC2 separated –Mg and –S with a positive coefficient from control, –N, –P, –K and –Ca in the leaves (Fig. 3–a), and control, –P, –K and –Ca from control, –N and –S in the roots (Fig. 4–a). Interestingly, a majority of metabolites was located on positive side by PC1 in the leaves (Fig. 3–b) whereas on negative side by PC1 in the roots (Fig. 4–b). PC2 also clearly separated most of amino acids with positive score from carbohydrates and organic acids in the leaves (Fig. 3–b), and, in the roots, represented opposite trend indicating most of amino acids with negative score from other metabolites (Fig. 4–b). We also subjected the metabolite to HCA (Fig. 3 and 4–c), which divided the components into two major clusters. In the leaves (Fig. 3–c), cluster I generally consisted of most of amino acids, and metabolites displaying a decreasing trend and carbohydrates were classified into cluster II. Cluster in the roots was somewhat differently classified compared to the leaves (Fig. 4–c). A majority of metabolites including amino acids and organic acids was classified into cluster I whereas carbohydrates and secondary metabolism–intermediates were placed in cluster II.

Metabolite profiles of macronutrient deficiency

An examination of the metabolite profiles revealed that mineral deficiency had profound effects on the levels of most primary metabolites in both leaves and roots of bell pepper plants (Fig. 5 and 6). Soluble sugars showed a completely different tendency between both tissues. The relative levels (expressed as a ratio of each mineral deficiency to the control) of soluble sugars in the leaves represented a tendency of noticeable increase except for –P, and were the highest in –K, and followed by –Ca, –N, and –Mg. Most remarkable changes in abundance were observed in –K that increasing more than 18.0–fold for glucose, fructose and xylose compared to the control, and –P that reducing 30 to 50% for those. On the contrary, their relative abundance in the roots decreased in general except for sucrose. In particular, the levels of glucose and fructose just existed 10 to 40% compared to the control, although –K caused a slight increase in the levels of both soluble sugars.

An abundance of organic acids (glycolysis and TCA intermediates) represented an inverse tendency compared to that of soluble sugars, and their responses seemed to be mostly larger tissue–specific rather than mineral–dependent. Most of organic acids including secondary metabolism intermediates, quinate, shikimate and sinapate in the leaves, was substantially reduced by macro mineral deficiency, whereas, in the roots, their levels showed somewhat increase in general. To get some prominent results, N deficiency in the leaves led to a significant decrease in the levels of glycerate, citrate, fumarate, quinate and shikimate which ranged less than 30% compared to control, and TCA intermediates, citrate, succinate, fumarate, and malate, were to be hugely accumulated in the roots by Ca or Mg deficiency.

A lager change in the amino acids occurred in both leaves and roots under macro mineral deficiency. Bell pepper leaves accumulated a substantial levels of amino acids in both tissues except for N deficiency. A striking interest was observed in two major amino acids, glutamine and asparagine, which revealed a huge increase (more than 10.0 times) by –K, –Mg and –S in the leaves and –P, –Ca, –Mg and –S in the roots. Besides this result, the deficiency of K, Mg or S led to higher accumulation of a variety of amino acids, Gly, Thr, Ile and Val in the leaves and Ser, Gly, Thr, Ala, Var, and GABA in the roots. Interestingly, aspartate was to represent dramatic

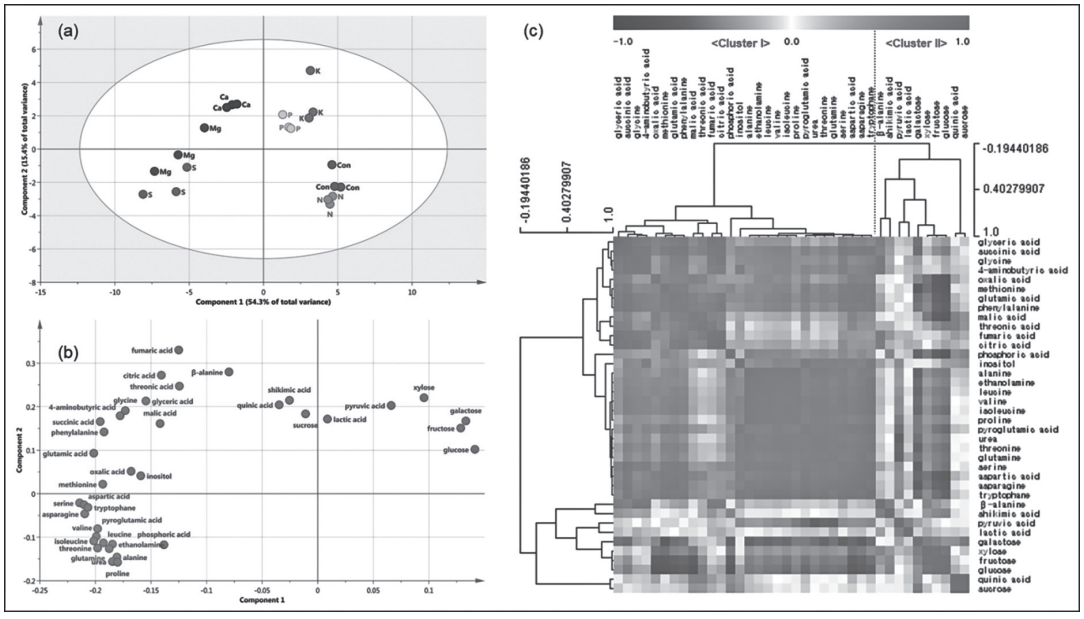

Fig. 4. Metabolic changes in primary C–N metabolism in the leaves of bell pepper plants (n = 3). Each color indicates the relative ratio to the control plants (red, increase; blue, decrease). Bell pepper was grown under individual macro nutrient–deficient conditions for 15 days, and separated into leaves and roots for targeted polar metabolite analysis.

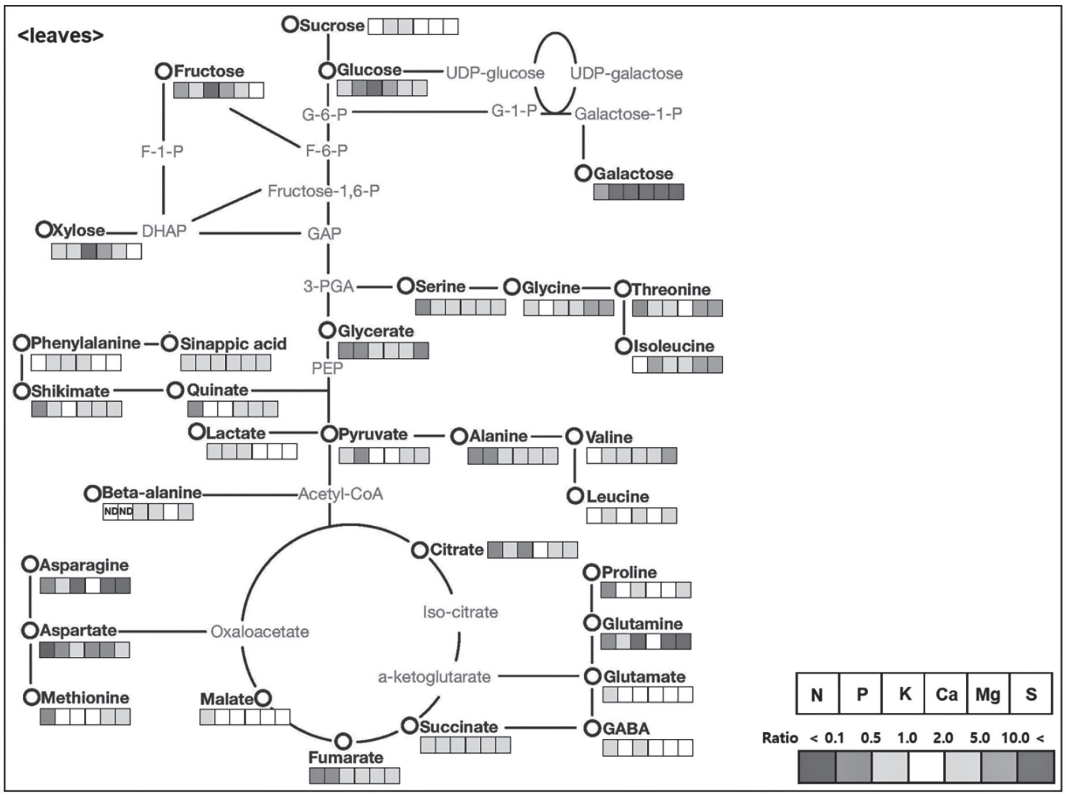

Fig. 5. Metabolic changes in primary C–N metabolism in the leaves of bell pepper plants (n = 3). Each color indicates the relative ratio to the control plants (red, increase; blue, decrease). Bell pepper was grown under individual macro nutrient–deficient conditions for 15 days, and separated into leaves and roots for targeted polar metabolite analysis.

decrease in the leaves whereas significant increase in the roots. These strongly imply that nutrients–deficient stress affects the quantity and composition of primary metabolites, and thus metabolic responses are closely networked between shoot and root or separately operated.

DISCUSSION

Deficiency of mineral nutrients, especially classified with macro elements, strongly affects the type and amount of metabolites produced by plants, and somewhat differently influences to plant tissues. A wide range of an application of high–throughput analytical instruments has led to remarkable achievements in field of plant metabolomics. Many studies have reported that a limited mineral supply leads to considerable variations in the levels of metabolites involved in C–N metabolism (Lavon *et al*., 1995; Scheible *et al*., 1997; Hirai *et al*., 2005; Huang *et al*., 2008; Okazaki *et al*., 2008; Takahashi *et al*., 2012; Sung *et al*., 2015; Sung *et al*., 2018), although diverse metabolic changes have been observed depending on the plant species and mineral stress conditions used. These mineral–deficient metabolic changes were thoroughly reviewed by Amtmann and Armengaud (2009). Therefore, our objective in this study was to improve an integrative knowledge on the leaves–roots communication of C–N metabolism derived from macro mineral deficiency, and, furthermore, to enhance our insights between metabolites and mineral nutrients in terms of synergistic and antagonistic aspects.

Prior to discussing the communication between metabolites and mineral nutrients, the growth of bell pepper plant displayed a large difference by charged state of ions like cation or anion. An inhibitory effect of mineral deficiency was greater in anions than cations in terms of the type of predominant absorption of minerals.

To explore mineral–mineral responses like synergistic or antagonistic effects of various nutrient regimes, we investigated the relative ratio (based on mineral uptake) of mineral nutrient in the leaves and roots of bell pepper plant (Fig. 2). An individual mineral deficiency revealed a strong effect on the absorption of other minerals, and noticeable findings were that sulfur uptake was greatly restricted by other macro minerals and sulfur deficiency was negatively interacted with the absorption of cations although there were negative effects between those. The positive (synergistic) and negative (antagonistic) effects between mineral nutrients observed in the present study are in line with previous observations (Mengel and Kirkby, 1987; Jones *et al*., 1991; Gunes, 1998; Sung *et al*., 2018). In overall point of view, the concentrations of all macro nutrients in both tissues were somewhat decreased, and thus it is reasonably expected that the deficiency of a specific nutrient leads to a shortened accumulation of other macro nutrient, and absorbed nutrients are dominantly accumulated in the roots to avoid any kinds of ion toxicity in the leaves through maintaining ion–homeostasis. Also, from our present study, it is carefully suggested that sulfur is one

of negatively affecting minerals in a large manner, and thus further study is required to clearly understand an interaction between sulfur and other mineral.

Much evidence in relation to mineral–deficient metabolic changes has been provided for N (Sung *et al*., 2015; Rufty *et al*., 1998), P (Huang *et al*, 2008; Chu *et al*, 1992; Ciereszko and Barbachowska, 2000), K (Amtmann and Armengaud, 2009; Sung *et al*., 2015), Ca and Mg (Lavon, 1995), and S (Nikiforova *et al*., 2005) deficiencies, strongly indicating that mineral stress has both direct and indirect effects on photosynthesis and C–N metabolism. We already observed C–N metabolic changes from cabbage plants grown under macro mineral–deficient conditions (Sung *et al*., 2018). To evaluate coordination in metabolic changes under macronutrient–deficiency, the measured metabolite concentrations were mapped on to plant biosynthetic pathways (Fig. 5 and 6) and nutrient–dependent responses both organs were summarized (Table 1). Two–fold higher accumulation of soluble sugars by N deficiency in leaves limited TCA reaction in a manner and thus caused reduced amino acid production. Also, lower conversion rates from disaccharide (sucrose) to monosaccharides (glucose, fructose) resulted in similar limitation in TCA reaction in roots although phloem loading was not affected by N deficiency (on the basis of sucrose concentration in both tissues). Huge accumulation of major soluble sugars including starch by N deficiency (Unrbanczyk–Wochniak and Fernie, 2005; Rufty *et al*., 1988; Sung *et al*., 2015) was well documented to lead to negative TCA reaction and amino acid synthesis (Unrbanczyk–Wochniak and Fernie, 2005; Scheible *et al*., 1997). The C–N metabolism by P deficiency in this study also represented similar or in accordance with previous reports (Huang *et al*., 2008; Morcuende *et al*., 2007; Sung *et al*., 2015). Despite of being controvertible evidences there is no doubtful that mineral nutrients play an essential role to operate metabolic process. However, there is little information how cations and sulfur are involved in C–N metabolism, and thus our concerns are to understand it deeper. In a current study, we have tried to address the interaction between mineral uptake and metabolite profiles from bell pepper grown under cations– and sulfur–deficient conditions. This carefully raises a simple question what plays as a trigger for higher accumulation of amino acids; interdependent or independent between mineral nutrients. Certainly, an analysis of plant ionomic data has demonstrated strong correlations between cations (Watanabe *et al*., 2007) and negative relation to sulfur by cationic Ca and Mg (Pii *et al*., 2015). As observed in our previous study (Sung *et al*., 2018), it has been demonstrated that the deficiency of cationic ions, especially K and Mg, not only induces marked accumulation in amino acids in both organs (Lavon *et al*., 1999; Jin *et al*., 2016; Gupta *et al*., 2017) but also restricts sulfur uptake. Sulfur deficiency also highly accumulated amino acids (Thomas *et al*., 2000; Nikiforova *et al*., 2005; Sung *et al*., 2018). Therefore, macronutrient deficiency is most likely to induce an accumulation of amino acids, and this tendency was also observed a current study. Interesting observation was a large accumulation of glutamine and asparagine under macronutrient deficiency except N with strong support (Thomas *et al*., 2000; Nikiforova *et al*., 2005; Amtmann and Armengaud, 2009), and it implies that both amino acids could be a potential indicator of macronutrient deficiency. With combining current knowledge and our study about the effects of macronutrient deficiency on metabolism, we depict an assumed model of nutrient deficiency–induced shoots–roots metabolic changes, and it might be provide better conceptual insights (Fig. 7). Prior to our current suggestion, Amtmann and Armengaud (2009) panoptically summarized huge changes in primary metabolism under macronutrients deficiency, and it is well documented that nutrient deficiency leads to reversible or sometimes irreversible impacts on plant metabolism entirely including metabolite levels, photosynthate transportation, and enzymatic performance (Dietz and Heilos, 1990; Lavon *et al*., 1995; Marschner *et al*., 1996; Lavon *et al*., 1999; Thomas *et al*., 2000; Nikiforova *et al*., 2004; summarized by Nikiforova *et al*., 2005; Hoefgen and Nikiforova, 2008; Takahashi *et al*., 2012; Sung *et al*., 2015; Guo *et al*., 2016; Sung *et al*., 2018). The data presented do not fully support our initial assumption, however some possibilities to generate mineral–mineral interaction–dependent metabolic changes, especially amino acid metabolism, are carefully suggested that a large accumulation could be a direct result by K or Mg deficiency itself or an influence by partly K or Mg–triggered S deficiency. In conclusion, as essential elements for plant life, mineral nutrients are closely linked with plant metabolism, and thus, in an agricultural point of view, their deficiency entirely and adversely affects growth and production of crops owing to the multitude of biochemical reactions. Here, we examined metabolic changes and shoots–roots commu-

Table 1. Summary of the behavior of mineral nutrients and metabolites in the leaves and roots of bell pepper plants affected by macronutrient deficiency

Mineral	Leaves	Roots
N	↑ (SSs), ↓ (Ca, Mg, S, OAs, AAs)	↓ (Ca, Mg, SSs, OAs, AAs), Unchanged (AAs)
P	↓ (S, SSs, OAs), Unchanged (AAs)	↑ (AAs), ↓ (Mg, S, SSs, OAs), Unchanged (OAs)
K	↑ (SSs, AAs), ↓ (Ca, Mg, S, OAs, AAs), Unchanged (AAs)	↑ (AAs), ↓ (S), Unchanged (SSs, OAs, AAs)
Ca	↑ (SSs, AAs), ↓ (K, S), Unchanged (OAs, AAs)	↑ (OAs, AAs), ↓ (Mg, S, SSs)
Mg	↑ (SSs, AAs), ↓ (K, S, OAs), Unchanged (OAs)	↑ (OAs, AAs), ↓ (S, SSs), Unchanged (OAs)
S	↓ (N, K, Ca, Mg, OAs), Unchanged (SSs, OAs), Uncharacterized (AAs)	↑ (OAs, AAs), ↓ (K, Ca, Mg, SSs), Unchanged (OAs)

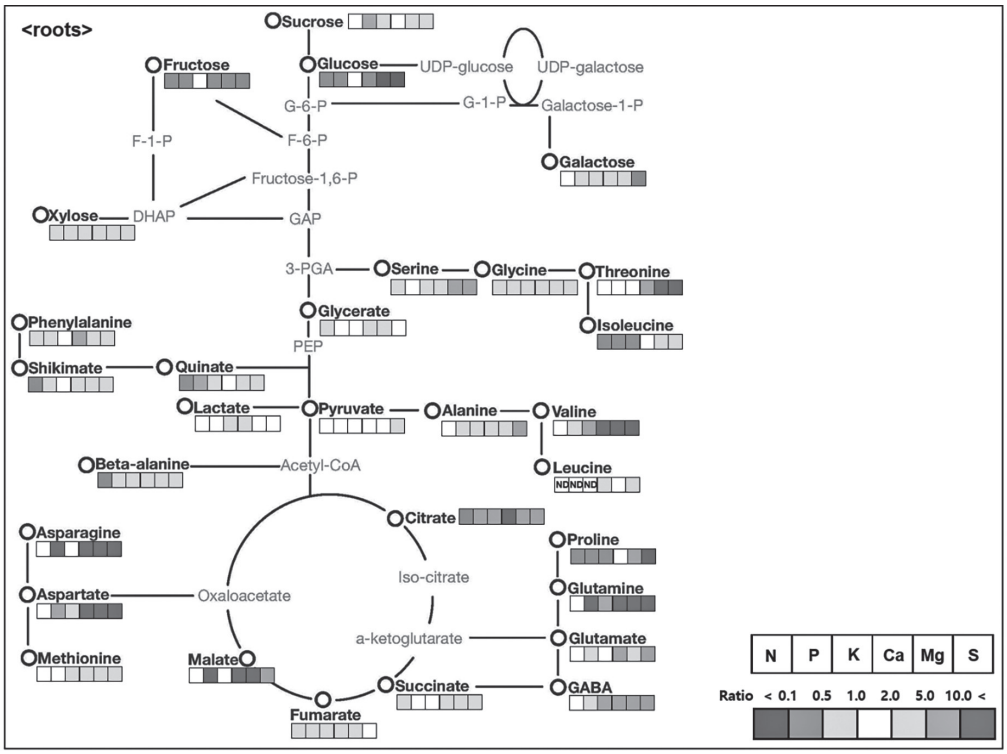

Fig. 6. Sentence Ts come with Fig. 5 with changing leaves to roots and an order is better to be placed Prior to Table 1.

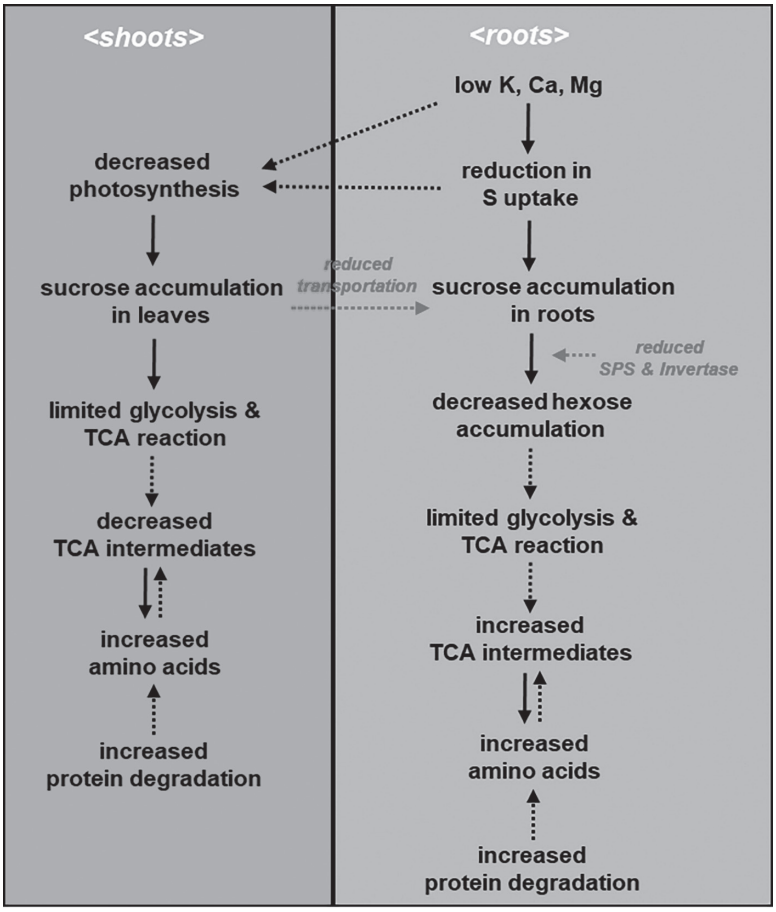

Fig. 7. Predicted model of shoot–root communication in primary metabolism in response to some macronutrient deficiency. Lined arrows indicate an observation in current study and dashed ones depict assumption– and confirmation–based responses (Dietz and Heilos, 1990; Lavon *et al.*, 1995; Marschner *et al.*, 1996; Lavon *et al.*, 1999; Thomas *et al.*, 2000; Nikiforova *et al.*, 2004; reviewed by Nikiforova *et al.*, 2005; Hoefgen and Nikiforova, 2008; Takahashi *et al.*, 2012; Sung *et al.*, 2015; Guo *et al.*, 2016; Sung *et al.*, 2018).

nication by macronutrient deficiency, and some interesting findings were observed. The deficiency of cationic nutrients (K, Ca and Mg) and S represented generally antagonistic relationships each other in terms of nutrient uptake, and these blockages resulted in unfavorable metabolite levels and restricted shoots–roots communication of phytosynthates. Interestingly, two major amino acids, glutamine and asparagine, was significantly accumulated by cations deficiency, and it makes us to dig into a curiosity. Besides, on the premise of further study to verify what happens between cations and sulfur in plants, we carefully suggest that a limited uptake of sulfur accompanied by cations deficiency could be direct cause of disturbance in primary metabolism rather than cations itself. In view of this, a current study provides a starting point to elucidate the complex mechanisms involved in the metabolic networks affected by individual and multiple nutrient stresses.

AUTHOR CONTRIBUTIONS

T. Y. HWANG, and S. LEE designed the experiment. Y. LEE analyzed the data. Y. SHINOGI commented on the manuscript. T. K. OH and J. SUNG supervised the work. All authors assisted in editing the manuscript and approved the final version. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACKNOWLEDGEMENT

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ010899), Rural Development Administration (RDA), Republic of Korea.

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