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ARTICLE

# Metabolite profiling and mineral nutrient analysis from the leaves and roots of bell pepper (*Capsicum annuum* L. var. *angulosum*) grown under macronutrient mineral deficiency

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**Abstract** We analyzed the contents of 38 primary metabolites and 9 minerals in the leaves and roots of bell pepper (*Capsicum annuum* L. var. *angulosum*) to study metabolic responses to deficiency in nitrogen, phosphorus, potassium, calcium, or magnesium. Induced deficiencies of individual cations reduced the abundance of the other cations in both leaves and roots. Each nutrient-deficient condition was clearly grouped by principal component analysis, which also showed that leaves under cation-deficiency treatments were separated from those under non-cation-deficiency treatments. This was consistent with that a single cation deficiency decreased the levels of the other cations in leaves. Specifically, N deficiency reduced amino acids and organic acids in both tissues. The common response to P-, K-, Ca- or Mg-deficient conditions showed significant increases in the levels of amino acids in both tissues and organic acids in the roots. In the leaves, P- or Mg-deficient conditions reduced organic acids. Soluble carbohydrates were significantly increased under N-, K-, Ca- or Mg-deficient conditions in the leaves, whereas in roots under K deficiency. Notably, the level of γ-aminobutyric acid, an amino acid that helps protect against biotic and abiotic stresses, was increased threefold in leaves under K-deficient conditions and sixfold in roots under P-, K-, Ca-, or Mg-deficient conditions. These findings provide additional information about variations in metabolite and mineral abundance in bell pepper leaves and roots in response to mineral shortage.

**Keywords** Bell pepper · Mineral content · Mineral deficiency · Primary metabolite

## Introduction

Macronutrient mineral elements are consumed in large quantities during plant growth and development. N, P, or K deficiency often limits crop production in low-input agriculture, but Ca or Mg deficiency usually does not [1]. Understanding crop plant responses to nutrient deficiencies is important for selecting varieties with improved mineral acquisition and use, and thus, such selection will be facilitated by metabolic profiling [2].

N or P deficiency affects carbon partitioning, resulting in larger roots compared to shoots, whereas K or Mg deficiency causes sugar accumulation in shoots and reduces sugar transport to roots, inhibiting root growth [2]. Sung et al. [3] previously observed nutrient-specific responses to deficiency in the metabolic profiles of tomato roots and leaves: N deficiency increased soluble sugars and decreased organic acids and amino acids in leaves and roots; P deficiency decreased soluble sugars in leaves and

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increased soluble sugars, organic acids, and amino acids in roots; and K deficiency increased soluble sugars and decreased organic acids and amino acids in leaves and increased soluble sugars and amino acids in roots. Other reports indicate that Ca plays a variety of roles regulating metabolism [4] and that Mg deficiency concentrates carbohydrates in the leaf, which inhibits photosynthesis and hinders $CO_2$ fixation [5–8].

This wide variety of effects suggests that primary metabolism might be influenced by supplies of mineral nutrients from the growth medium, and we hypothesize that changes in metabolite concentrations in leaves or roots are related to mineral element concentrations in those tissues. To better understand these effects, we analyzed both mineral element and primary metabolite levels in the leaves and roots of bell pepper plants grown under macronutrient mineral deficiency. We focused on changed levels of mineral elements and metabolites of glycolysis, the trichloroacetic acid (TCA) cycle, and amino acid biosynthesis in both leaves and roots.

## Materials and methods

### Plant material and growth condition

Bell pepper (*Capsicum annuum* L. var. *angulosum*) seeds were germinated in perlite soaked with deionized water. After 14 days, seedlings were transferred to a hydroponic container. The 6 hydroponic containers containing 6 seedlings were filled with nutrient solution consisting of 2.5 mM $Ca(NO_3)_2$, 2.5 mM $KNO_3$, 1 mM $MgSO_4$, 0.25 mM $KH_2PO_4$, 0.03 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 $H_2MoO_4$. The light intensity at midday was 800–1200 μmol $m^{-2}$ $s^{-1}$. Plants were grown at 25 ± 3 °C during the day and 15 ± 3 °C at night with continuous aeration of the containers. Plants were allowed to grow for 14 days before nutrient deficiency was initiated by replacing the nutrient solution in each container with either N-, P-, K-, Ca-, or Mg-deficient solution, produced by omitting the targeted nutrient from the solution and compensating for the omitted non-targeted nutrients—$Ca(NO_3)_2$, $KNO_3$, 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, and $MgSO_4$ by $CaSO_4$ for Mg deficiency. Plant leaves and roots were harvested at 10:00–12:00 on 15th day from the initiation of the treatment. Samples were either oven-dried at 80 °C in order to analyze mineral elements or stored at − 80 °C in order to analyze the metabolites.

### Analysis of mineral element contents

Shoots and roots were dried in an 80 °C oven for 2–3 days and then ground in a blender. About 0.5 g of the resulting powder was mixed in a flask with a solution containing 10 mL of 60% $HClO_4$ and 200 mL of $H_2SO_4$. This mixture was allowed to digest inside an air-circulating hood on a hot plate whose temperature was increased from 100 to 240 °C by 30–40 °C increment over 2.5 h, and then kept at 240 °C for several hours until a white powder was obtained. After the flask had cooled down, warm deionized water was added to dissolve the powder, the solution was passed through filter paper, and the volume was adjusted to 100 mL with deionized water. This solution was used for mineral nutrient analysis. N content was analyzed with an automatic flow injection analyzer (QuAAtro, Bran + Luebbe, Norderstedt, Germany), P content by UV–visible light spectrophotometry (UV-2600, Shimadzu, Kyoto, Japan), and K, Ca, Mg, Fe, Cu, Mn, and Zn content by inductively coupled plasma optical emission spectrometry (ICP-OES; Integra XL, GBC, Braeside, Australia).

### Metabolic profiling

Primary metabolites were extracted as described by Kim et al. [9]. Powdered sample that was freeze-dried and ground (100 mg) was mixed with 1 mL of a mixture containing methanol, water, and chloroform (2.5:1:1 volume ratio) and 60 μL of ribitol as an internal standard (0.2 mg/mL) at 37 °C for 30 min with a mixing frequency of 1200 rpm using a Thermomixer Compact (Eppendorf AG, Hamburg, Germany). The mixed solution was centrifuged for 3 min at 16,000 × g. The methanol/water phase (0.8 mL) was transferred to a new tube and mixed with 0.4 mL of deionized water. The solution was centrifuged for 3 min at 16,000 × g. The methanol/water phase was dried for 2 h in a centrifugal concentrator (C-105, TOMY, Tokyo, Japan) and for another 16 h in a freeze dryer. Methoxyamine hydrochloride (80 μL, 20 mg/mL) in pyridine was added and the solution shaken at 30 °C for 90 min, and then 80 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide was added and the solution shaken at 37 °C for 30 min. An Agilent 7890A gas chromatograph (Agilent, Atlanta, USA) coupled to a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, USA) was used for gas chromatography–time-of-flight mass spectrometry. The sample was separated with a fused-silica capillary column coated with 0.25-μm film (CP-SIL 8 CB; 30 m × 0.25 mm; Varian Inc., Palo Alto, CA, USA). The split ratio, injector temperature, and helium gas flow rate through the column were 1:25, 230 °C, and 1.0 mL/min, respectively. The temperature was set at 80 °C for the first 2 min, increased to 320 °C at a rate of 15 °C/min, and

then kept at 320 °C for 10 min. The temperatures of the transfer line and ion source were 250 °C and 200 °C, respectively. The mass scan range was 85–600 m/z. Peak identification was based on the US National Institute of Standards and Technology mass spectral library and an in-house library. In addition, relative retention time, which was previously reported by Park et al. [10], was used to identify individual metabolites (Table S1). Concentrations of all metabolites were calculated from the relative peak areas relative to those of the internal standard.

### Statistical analysis

All analyses were performed at least three times. Principal component analysis (PCA; SIMCA-P version 13.0; Umetrics, Umeå, Sweden) was conducted using the mineral element data and the metabolite data as in Kim et al. [9]. The analysis was set to produce score plots to illustrate the dissimilarity between samples treated with different nutrient deficiencies and loading plots to show mineral elements and metabolites explaining the cluster separation. The PCA was performed based on the calculated eigenvector and eigenvalues. The principal components whose eigenvalues were more than 2.0 were extracted. Normalization of all the data process was performed with unit variance scaling. Pearson's correlation analysis was conducted with SAS 9.4 (SAS Institute, Cary, NC, USA). Hierarchical cluster analysis (HCA) and heat mapping were conducted in the MultiExperiment Viewer, version 4.4.0 (http://www.tm4.org/mev/). The heat map visualization of all the correlation coefficients with Pearson's correlation analysis was performed for metabolite–metabolite correlation.

## Results and discussion

### Analysis of mineral element content of bell pepper leaves and roots

We analyzed the effects of mineral deficiency on the concentrations of other mineral elements in leaves and roots (Fig. 1). The ratios of the abundances of other mineral elements to the abundances of the deficient mineral (as compared to non-mineral-deficient control plants) revealed the effects of individual mineral deficiencies on other minerals. Lower ratios indicate that mineral element contents decreased more. Ratios smaller than one (value of control plants) indicated that the mineral content was reduced further than the standard ratio in the control plants. In the leaves and roots, each cation deficiency (the K-, Ca-, and Mg-deficient conditions) reduced the other cation contents as well (Table S2). This was consistent with the results for PCA of primary metabolites in the present study showing that cation-deficiency treatments were separated from non-cation-deficiency treatments in leaves (see metabolic profiling results). In the roots, N and P deficiencies greatly reduced the Mg content (Fig. 1B). The effect of P deficiency on Mg content in the roots was in line with results from tomato plants reported by Gunes et al. [11]. These authors [11] explained their results by the antagonistic and synergistic effects between mineral nutrients.

### Primary metabolite profiling of bell pepper leaves and roots (PCA, HCA, and Pearson's correlations)

The relative abundances of 38 primary metabolites and 9 mineral elements were measured separately for the leaves and roots of bell pepper. A PCA score plot showed clusters for each deficiency (N, P, K, Ca, or Mg; Fig. 2A). Leaves were clearly separated on the basis of mineral-deficient condition (cation group and non-cation group) in principal component 1 (PC1; 39%; Fig. 2A). A loading plot was analyzed to determine which mineral and metabolite components contributed to the leaf differences by mineral-deficient condition. The loading plot for the leaves showed that phosphoric acid, sinapinic acid, P, K, Ca, Mg, Fe, Mn, and Zn had negative loading values and the other minerals and metabolites had positive loading in PC1 (Fig. 2B). We analyzed correlations between the concentrations of 47 components, including carbohydrates, organic acids, amino acids, and mineral elements, in pepper leaves and applied HCA (Fig. 3). There were two clusters: cluster I consisted of phosphoric acid, sinapinic acid, lactic acid, glyceric acid, citric acid, P, K, Ca, Mg, Fe, Mn, and Zn, and cluster II consisted of other minerals, soluble sugars, organic acids, and amino acids. In the leaf, cations (K, Ca, and Mg) had positive correlation with cations and negative correlation with amino acids and soluble sugars. N had positive correlation with organic acids and amino acids. P had positive correlation with organic acids. In the root PCA score plot, Ca and Mg deficiencies were separated from N, P, and K deficiencies, as well as the control in PC1 (49.3%; Fig. 4A). In the root, most soluble sugars, pyruvic acid, lactic acid, K, Ca, Mg, Cu, and Zn showed negative loading values, while the other minerals and metabolites showed positive loading in PC1 (Fig. 4B). In the root HCA (Fig. 5), there were also two clusters: cluster I included K, Ca, Mg, Cu, Zn, and soluble sugars except for sucrose, and cluster II consisted of other minerals, sucrose, organic acids, and amino acids. In the root, K, Ca, and Mg had negative correlation with organic acids and amino acids. N had positive correlation with organic acids and amino acids. P had negative correlation with organic acids.

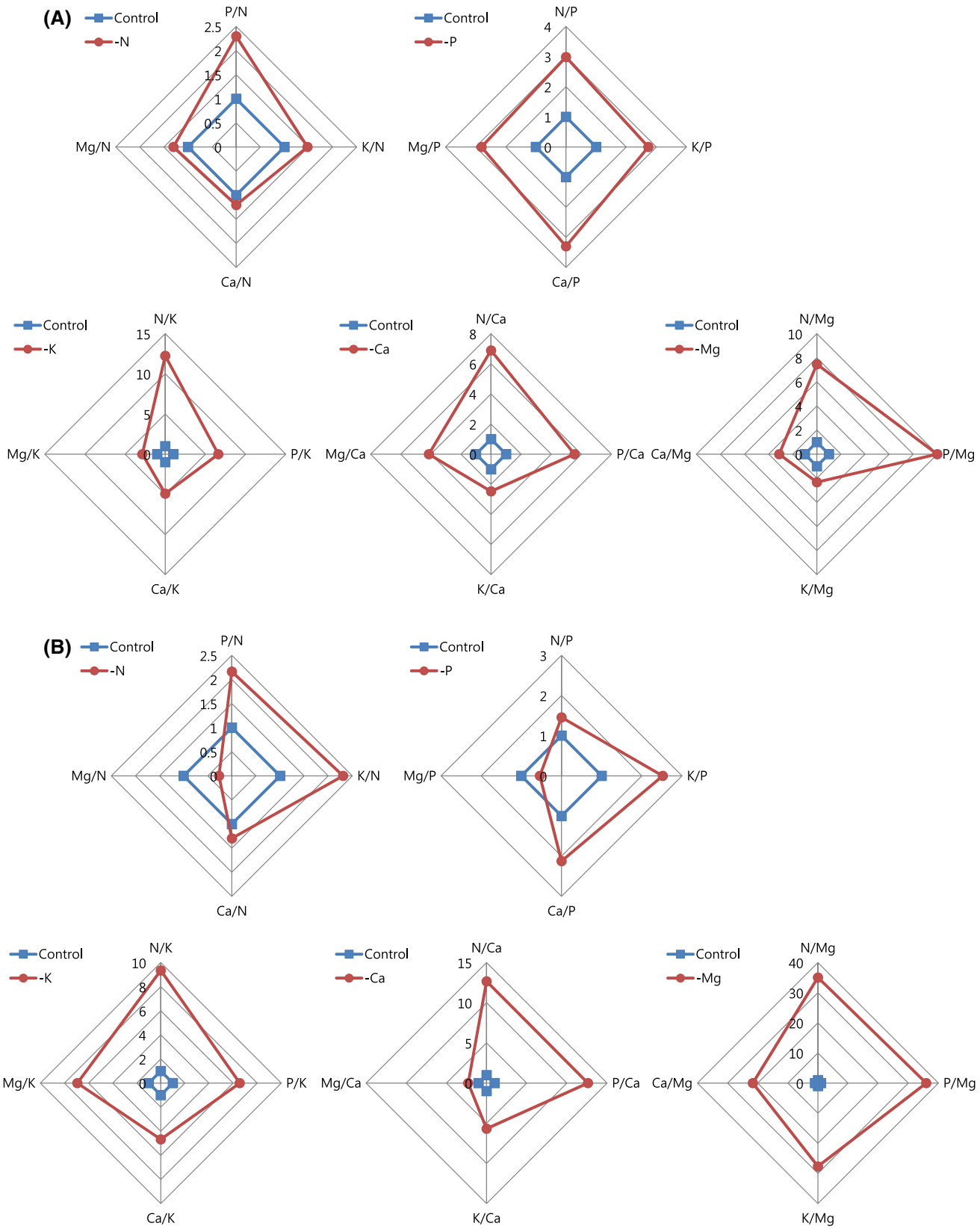



**Fig. 1** Relative ratios of other mineral elements to a deficient mineral in bell pepper organs grown under mineral-deficient conditions for 15 days (red circles). The reference was the value for the control conditions, in which plants were grown without nutrient deficiency (blue squares). Data are given for bell pepper (**A**) leaf and (**B**) root tissues

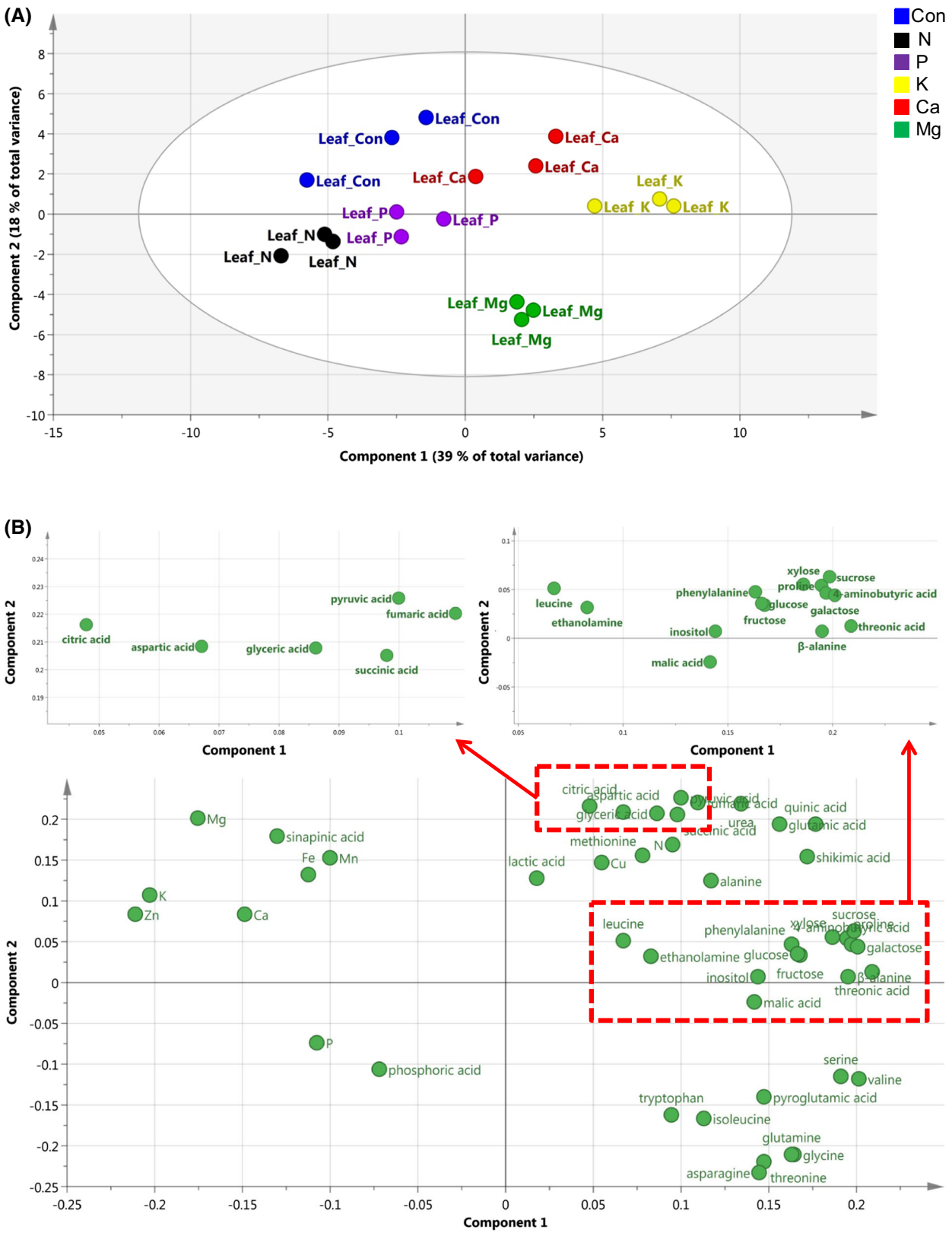



**Fig. 2** Principal component analysis (PCA) of 38 primary metabolites and 9 mineral elements in the leaf tissue of bell pepper plants grown either under mineral-deficient conditions for 15 days or without nutrient deficiency. (**A**) PCA score plot. Metabolite deficiencies are indicated by colors (see key at top right). (**B**) Loading plot

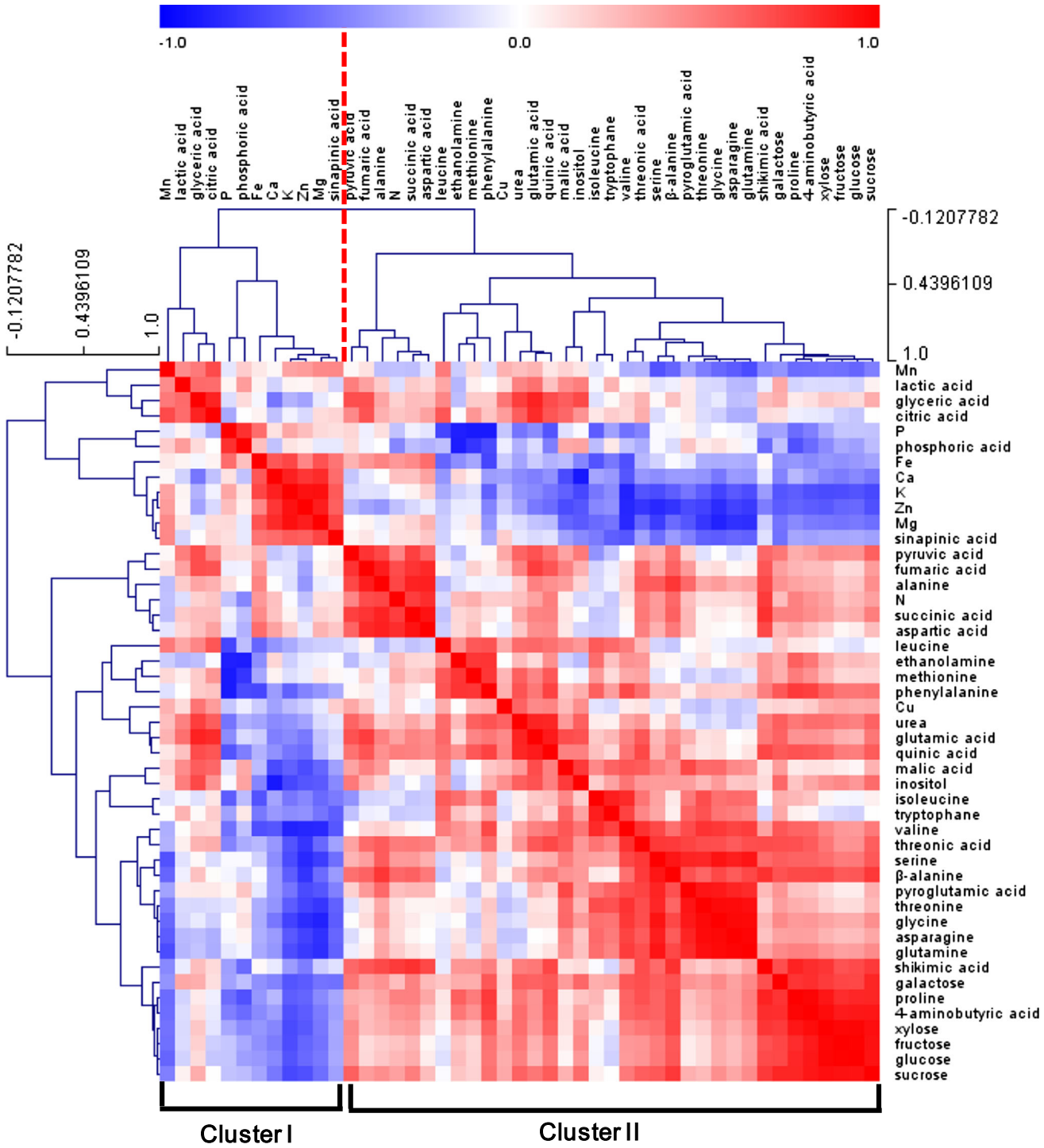



**Fig. 3** Correlation matrix of 38 primary metabolites and 9 mineral elements in leaves from bell pepper plants grown either under mineral-deficient conditions for 15 days or without nutrient deficiency. Each square of heat map shows the Pearson's correlation coefficient on a color scale ($n = 3$)

### Changes in soluble sugars of bell pepper leaves and roots in response to nutrient deficiency

The amount of each metabolite after nutrient deficiency is represented in Fig. 6 as a fold difference, and it is relative to that in the control plants grown without nutrient deficiency. Figure 6 shows that N deficiency increased glucose and fructose in the leaves but decreased them in the roots. In the leaves, glucose and fructose levels were increased by N, K, Ca, or Mg deficiency, and in the roots they were

decreased by N, P, Ca, or Mg deficiency but slightly increased by K deficiency. P deficiency led to decreases in glucose and fructose in both leaves and roots. Sucrose was increased by K or Ca deficiency in the leaves and by P, K, or Mg deficiency in the roots.

In the leaves, glucose and fructose increased in response to nutrient deficiency, except for P deficiency, and sucrose increased in response to K and Ca deficiency (Fig. 6). In the roots, glucose and fructose decreased as a result of each nutrient deficiency except K deficiency, and sucrose increased in response to each nutrient deficiency except N and Ca (Fig. 6).

Bell pepper showed a tendency toward increased soluble sugars in leaves upon N, K, Ca, or Mg deficiency and in roots upon K deficiency. The leaf sugar increase with N deficiency is in line with previous results [2, 3], but the root sugar decrease from N deficiency seen here diverges from the general observation that N deficiency increases sugar in roots [2, 3]. The leaf sugar decrease in response to P deficiency treatment is in agreement with results for tomato leaves [3], although the sugar levels in leaves are generally reported to increase as a result of P deficiency [2]. The leaf sugar increases we observed due to Mg or K deficiency are in line with other results for leaves [2–4, 12–14], and the root sugar increases from K deficiency are in line with results from tomato roots [3, 13] and Arabidopsis roots [12]. The root sugar increases resulting from K deficiency in the present study deviated from the general observation that K deficiency impairs sugar transport from shoots to roots and does not increase root sugar level [2]. For P deficiency, shoot-derived soluble sugars are transported to roots to increase root size and are thought to act as both signal molecules and metabolites [15]. For N deficiency, sugar accumulation acts as a metabolite feedback to reduce photosynthesis [2]. Overall, soluble carbohydrates increased in the leaf under N-, K-, Ca- or Mg-deficient conditions and in the root upon K-deficient condition in bell pepper.

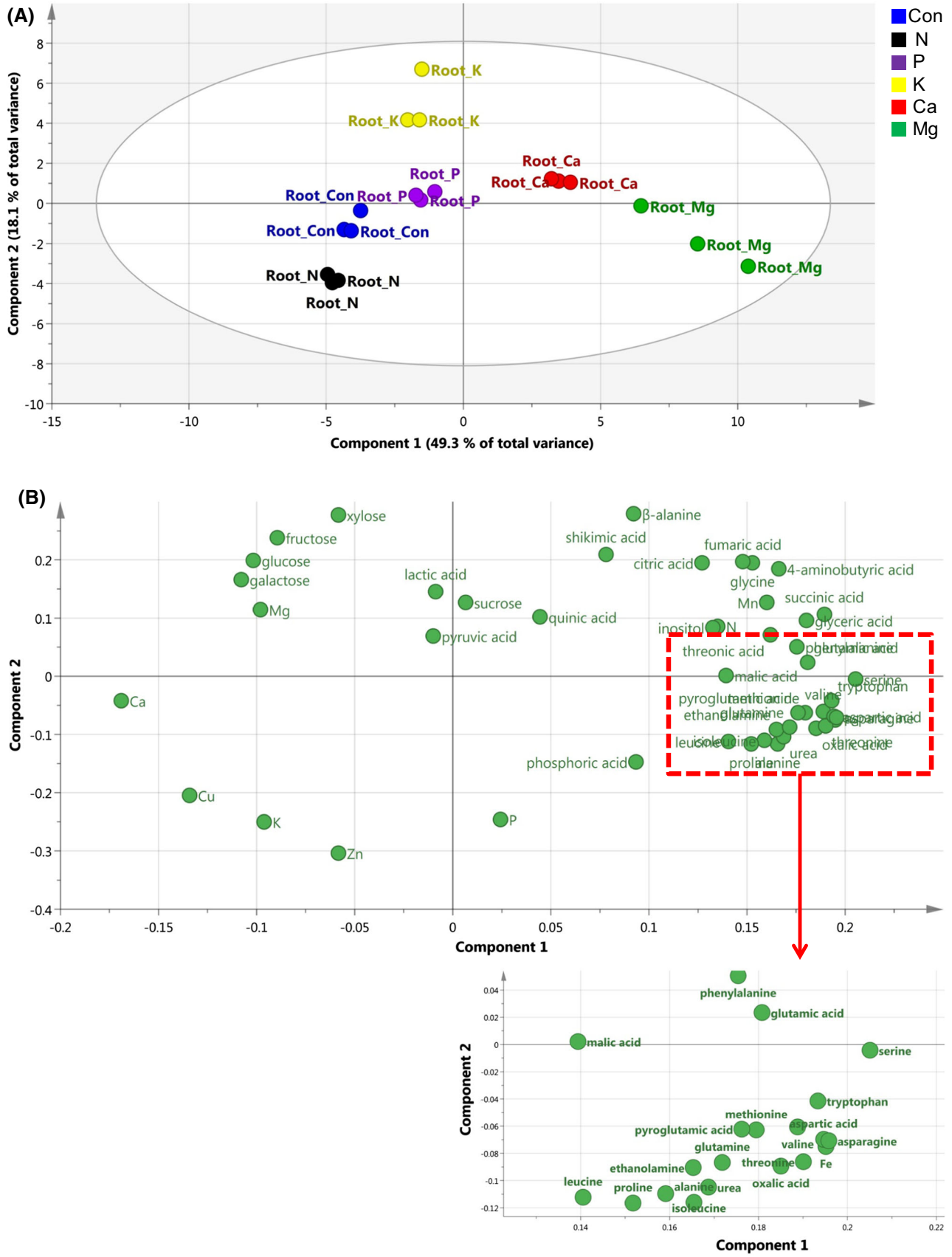
### Changes in organic acids, amino acids, and their precursors in bell pepper leaves and roots in response to nutrient deficiency

Figure 6 shows that N deficiency reduced the abundance of both organic acids and amino acids, except for slight increases for a few compounds. P, K, Ca, or Mg deficiency increased TCA-cycle organic acids in the root but decreased them in the leaves, with some exceptions, and also generally increased amino acids in both leaves and roots. Specifically, P deficiency increased amino acids, with a few exceptions, in both leaves and roots; K deficiency increased most of amino acids in the leaves and several amino acids in the roots; and Ca or Mg deficiency increased several amino acids in leaves and most amino acids in roots.

In the roots, TCA-cycle organic acids that increased in response to nutrient deficiency included citrate, succinate, fumarate, and malate, except for N deficiency, which slightly increased malate but decreased citrate, succinate, and fumarate (Fig. 6).

Among amino acids, glutamate in the roots and glutamine and γ-aminobutyric acid (GABA) in both leaves and roots increased in response to nutrient deficiency, except for N deficiency, which decreased glutamate, glutamine, and GABA in the leaves and caused no change in the roots. GABA increased by a factor of 3 in the leaves in response to K deficiency and by a factor of 3–6 in the roots in response to P, K, Ca, or Mg deficiency. The response of GABA in bell pepper was different from that seen in tomato by Sung et al. [3], who showed that P deficiency decreased GABA in both leaves and roots, but K deficiency did not change GABA abundance in the leaves and increased it in the roots. GABA is a functional metabolite that has a positive effect on immune cells and cancer cells [16, 17]. Growing bell peppers in K-deficient condition can increase the GABA content of the leaves and roots. Quinate and phenylalanine increased in bell pepper roots in response to nutrient deficiency, except for N deficiency. In the leaves, quinate increased in response to K or Ca deficiency, phenylalanine increased in response to P, K, or Ca deficiency, and tryptophan increased in response to each nutrient deficiency.

In TCA cycle, bell pepper showed a tendency for increases in metabolites in the roots upon nutrient deficiency, except for N deficiency, which decreased metabolites; in leaves, there was a tendency for decreases in metabolites resulting from each nutrient deficiency treatment. For amino acids, bell pepper showed a tendency for increases in the leaves and roots upon nutrient deficiency treatment, except for N deficiency, which decreased amino acids. The response of bell pepper leaves to N deficiency was in line with that of tomato leaves, which showed reduced TCA-cycle organic acids and amino acids [3, 18]. For N deficiency, it is known that photosynthesis is repressed in response to sugar accumulation [2]. In the leaves of bell pepper, the pattern of metabolites in response to P, K, Ca, or Mg deficiency—increases in soluble sugars (except with P), decreases in TCA-cycle organic acids, increases in amino acids—suggests that amino acid abundances are increased not through their production via glycolysis and the TCA cycle but through protein degradation. In the roots of bell pepper the pattern of metabolite changes induced by P, K, Ca, or Mg deficiency—increases in soluble sugars (except with Ca), TCA-cycle organic acids, and amino acids—suggests that amino acid abundances are increased by amino acid production through glycolysis and


(A)
Con
N
P
K
Ca
Mg
Component 2 (18.1 % of total variance)
Component 1 (49.3 % of total variance)
Root_K
Root_K
Root_K
Root_Ca
Root_Ca
Root_Ca
Root_P
Root_Con
Root_P
Root_P
Root_Mg
Root_Con
Root_Con
Root_Mg
Root_Mg
Root_N
Root_N
Root_N
(B)
Component 2
Component 1
xylose
fructose
glucose
galactose
Mg
Ca
Cu
K
Zn
P
lactic acid
sucrose
quinic acid
pyruvic acid
β-alanine
shikimic acid
fumaric acid
citric acid
4-aminobutyric acid
glycine
Mn
succinic acid
glyceric acid
threonic acid
malic acid
serine
tryptophan
valine
asparagine
oxalic acid
urea
phosphoric acid
phenylalanine
glutamic acid
malic acid
serine
tryptophan
methionine
pyroglutamic acid
glutamine
valine
asparagine
ethanolamine
Fe
leucine
proline
urea
oxalic acid
isoleucine

◀**Fig. 4** Principal component analysis (PCA) of 38 primary metabolites and 9 mineral elements in the root tissue of bell pepper plants grown either under mineral-deficient conditions for 15 days or without nutrient deficiency. (**A**) PCA score plot. (**B**) Loading plot

the TCA cycle. The decrease in TCA-cycle organic acids in bell pepper leaves in response to K deficiency was consistent with data on tomato leaves from Sung et al. [3] but contrasted with data on Arabidopsis shoots from

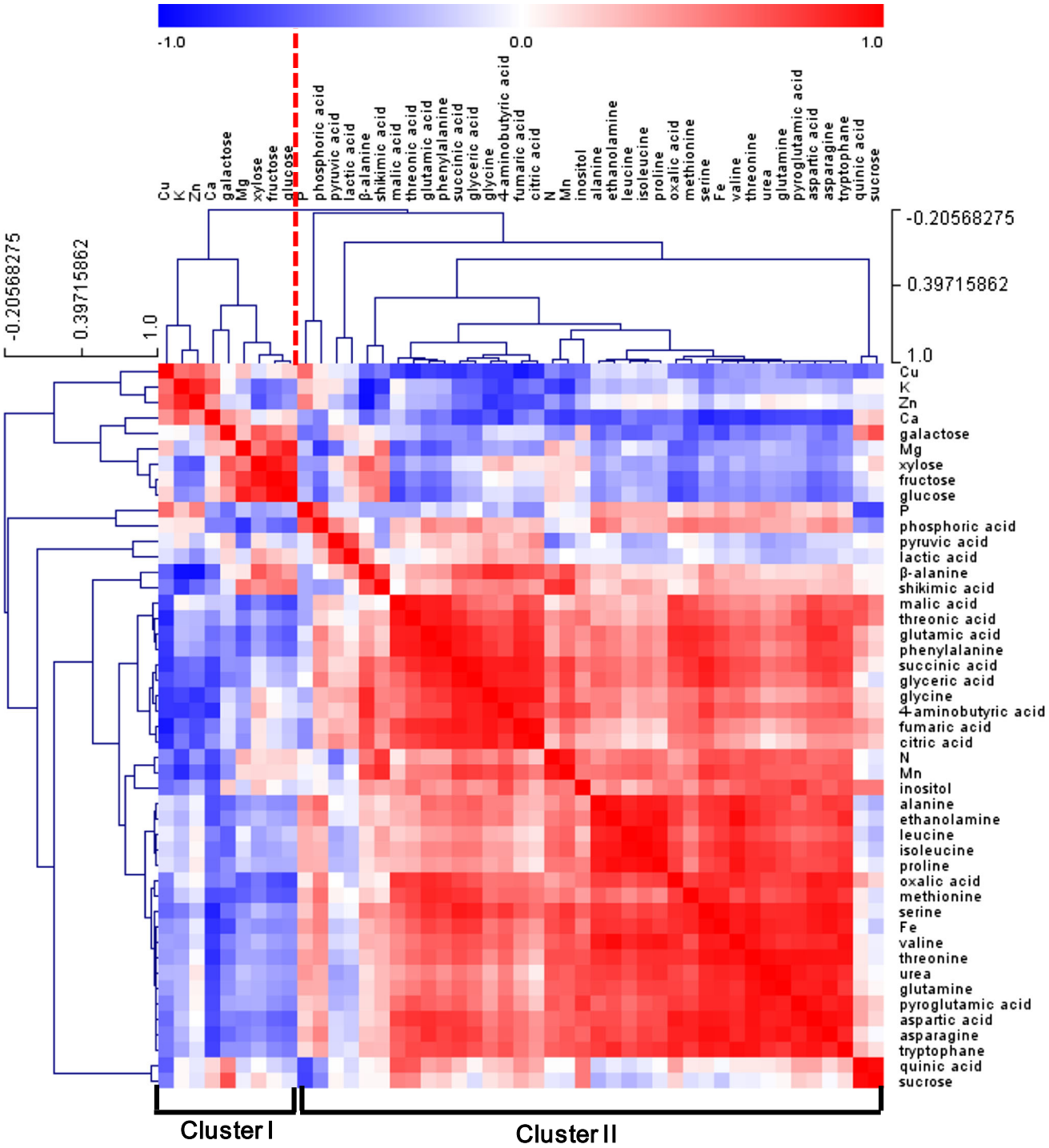



**Fig. 5** Correlation matrix of 38 primary metabolites and 9 mineral elements in roots from bell pepper plants grown either under mineral-deficient conditions for 15 days or without nutrient deficiency. Each square of the heat map shows the Pearson's correlation coefficient on to a color scale ($n = 3$)

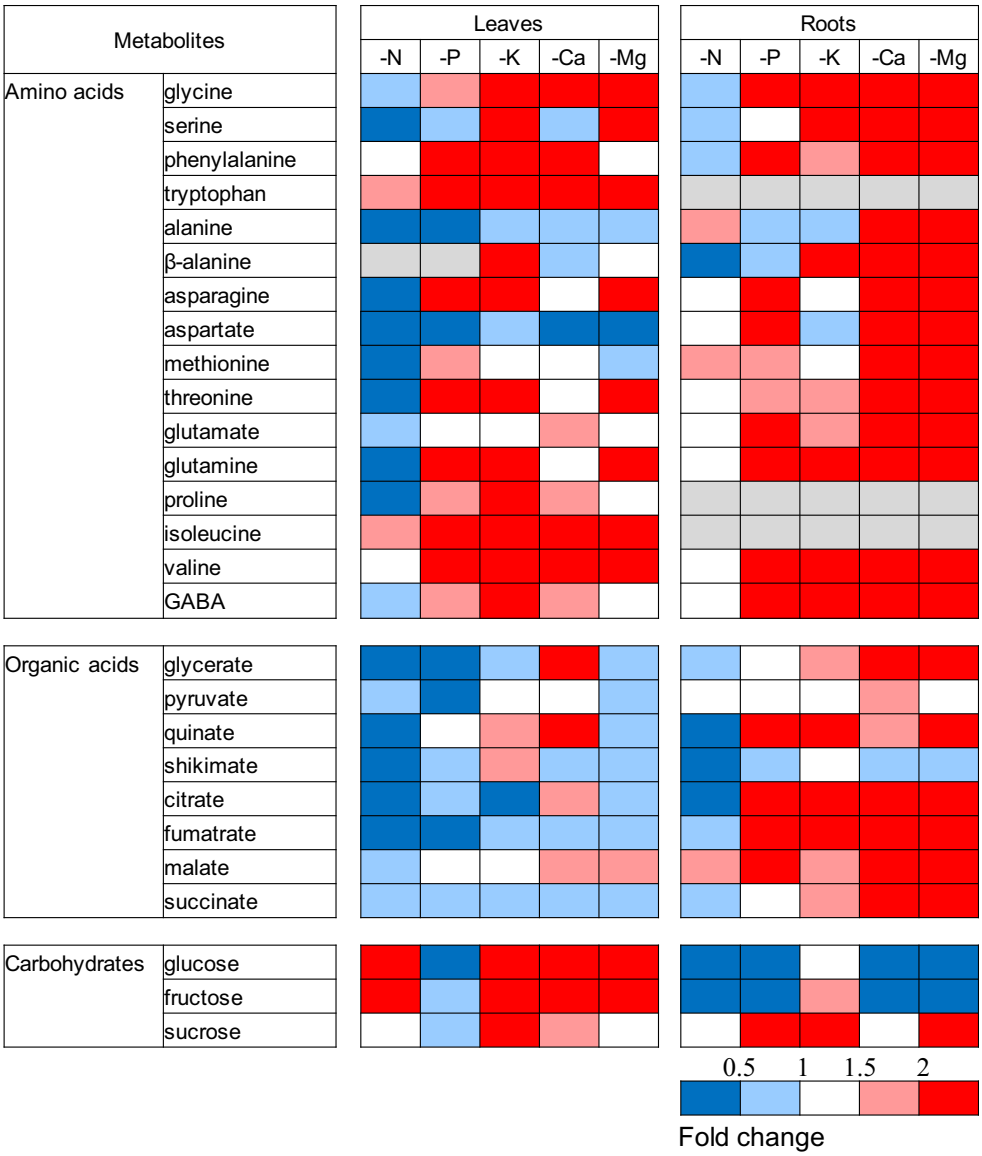



**Fig. 6** Differences in the concentrations of metabolites, including carbohydrates, organic acids, and amino acids, in glycolysis/TCA cycle and amino acid biosynthesis in the leaves and roots of bell pepper grown under mineral-deficient conditions for 15 days. The amount of each metabolite after nutrient deficiency, relative to that in the control plants grown without nutrient deficiency, is represented as a fold difference

Armengaud et al. [12], who showed increases in organic acids upon K deficiency. The increase in TCA-cycle organic acids in bell pepper roots resulting from K deficiency contrasted with the reductions in organic acids reported in tomato roots by Sung et al. [3] and in Arabidopsis roots by Armengaud et al. [12]. Overall, a notable observation in the levels of amino acids was that GABA increased in leaves under the K-deficient condition and in roots under P-, K-, Ca-, or Mg-deficient conditions.

In conclusion, the abundances of metabolite and mineral varied in both leaves and roots of bell peppers grown under macronutrient deficiency. We found some responses that were specific to specific nutrients and others that were common to cations. N deficiency reduced amino acids and organic acids in both tissues. P-, K-, Ca- or Mg-deficient conditions caused significant increases in the levels of amino acids in both tissues and organic acids in the roots. Soluble carbohydrates were significantly increased under N-, K-, Ca- or Mg-deficient conditions in leaves, whereas in roots under K deficiency. Leaves under cation-deficiency treatments were clearly separated from those under non-cation-deficiency treatments in the PCA, which explained the difference patterns in primary metabolites, and this was consistent with that a single cation deficiency decreased the levels of the other cations in leaves. These findings provide additional information about variations in metabolite and mineral levels at the tissue level in bell pepper in response to mineral shortage, and this will facilitate efforts to understand the mechanisms of plant responses to nutrient deficiency.

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