



Tissue region-dependent differential responses of fruit quality attributes, mineral nutrients, and targeted major metabolites in blossom-end rot of paprika fruit

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Abstract

Paprika fruits are susceptible to blossom-end rot (BER) during fruit growth and development. This study investigated the tissue-specific differential responses in quality attributes, mineral nutrients, and targeted major metabolites of healthy and BER-affected ‘Volidano’ yellow paprika fruits. Fruit fresh weight, fruit length and diameter, titratable acidity (TA), and peel yellowness were lower in BER fruits than in healthy fruits. K, P, and Mg contents were highest at the blossom-end tissue region of BER fruits, while the Ca, Mn, and B contents were lowest compared with the other tissues. The contents of glucose, fructose, citric acid, and malic acid were highest at the blossom-end tissue region of BER fruits. The levels of glutamine, asparagine, aspartic acid, serine, γ -aminobutyric acid (GABA), threonine, and citrulline were highest at the blossom-end tissue region of BER fruits, thereby contributing to having the highest content of total amino acids. The results of variable importance in projection scores and volcano plots indicated that oleic acid, asparagine, GABA, aspartic acid, glutamine, citrulline, and citric acid were upregulated at the blossom-end tissue region of BER fruits. The results suggest that the BER symptom could be associated not only with mineral nutrients but also with targeted major metabolites in paprika fruits.

Keywords Fatty acids · Free amino acids · Mineral nutrients · Organic acids · Physiological disorder · Soluble carbohydrates

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1 Introduction

Paprika (*Capsicum annuum* L.) fruits are highly susceptible to blossom-end rot (BER) during fruit growth and development (Kabir et al. 2021; Topcu et al. 2022). The incidence of BER is highly related to environmental factors such as the level of irrigation (Jovicich et al. 2007; Kabir et al. 2021), shade (Díaz-Pérez 2014), temperature (Ara-kawa et al. 2021), and irradiance (Ho et al. 1993; Jovicich et al. 2007) in the Solanaceae family (de Freitas et al. 2011; Karni et al. 2000; Topcu et al. 2022). Thus, the incidence of BER is widely detected throughout the whole growth period in paprika under a protected cultivation system, such as a greenhouse. However, there is a seasonal fluctuation in the incidence of BER (Aktas et al. 2005; Bar-Tal et al. 2006; Silber et al. 2005), although the fruits were grown in protected cultivation system. In addition, the symptoms and responses of BER are fairly diverse depending on the cultivars or varieties of each commodity (Díaz-Pérez 2014; Indeche et al. 2020; Kye et al. 2022; Oh et al. 2021). The symptoms of BER begin as a light green or pale yellow-coloured sunken tissues at the blossom-end region of the fruit, which gradually expands into a larger collapsed tissues that becomes highly susceptible to secondary fungal and bacterial infections (Topcu et al. 2022). In turn, the development and incidence of BER are major contributors to significant losses in the fruit yield and quality of paprika.

The BER is known to result from calcium (Ca) imbalance or deficiency during the fruit growth and enlargement stage, especially at rapidly expanding periods in the paprika fruit (Aktas et al. 2005; Díaz-Pérez and Hook 2017). The contents of Ca^{2+} depend highly on the fruit tissue region during its development and ripening (Bar-Tal et al. 2001; Mayorga-Gómez et al. 2020). However, the application of Ca fertiliser has not effectively reduced the incidence of BER in open-field-grown paprika fruits (Díaz-Pérez and Hook 2017). The contents of Mg, B, Mn, and Zn vary depending on the tissue location, from the proximal to distal sections, in marketable paprika fruits. However, the levels of most mineral nutrients, except for Na, are higher in the distal region compared to the proximal region during ripening (Mayorga-Gómez et al. 2020). In addition, root pruning treatment causes different accumulations of mineral nutrients, such as Ca, Mg, K, and K/Ca, depending on the fruit region, from the stem-end region to the blossom-end region in paprika fruits (Kabir et al. 2021). Previous study reported that the incidence rate of BER is inversely related to Mn levels (Silber et al. 2005). Furthermore, the incidence and severity of BER in paprika fruit gradually decline with an increase in the levels of phosphorus fertiliser application (Ghoname et al. 2012). Thus, BER symptoms are affected by the Ca level, the complex

of mineral nutrients, and the ratio of mineral nutrient levels to Ca levels.

In addition to the responses of mineral nutrients, ascorbic acid levels are lower in BER fruits than in asymptomatic healthy fruits, regardless of apoplastic and symplastic ascorbic acids (Aloni et al. 2008). The application of phosphorous and amino acid fertiliser highly affects soluble solids content (SSC) and titratable acidity (TA) in paprika, with a similar response of ascorbic acid (Ghoname et al. 2012). Meanwhile, the production of reactive oxygen species is higher in BER-affected fruits than in healthy paprika fruits (Aktas et al. 2005). Furthermore, under water-stressed tomato plants, the levels of aspartic acid, glutamic acid, proline, and alanine are higher in BER fruits than in asymptomatic healthy fruits, which contributes to an overall increase in total amino acid content (Franco et al. 1999). The 24-epibrassinolide treatment reduces the incidence of BER by mediating the accumulation of ascorbic acid and enhancing the antioxidant enzyme activities involved in the reduction of H_2O_2 levels in tomato fruits (Riboldi et al. 2019). Additionally, the results of the proteomic approach have enabled the identification of differentially expressed proteins from BER tomato fruits, which indicates the induction of proteins involved in antioxidant scavenging and the pentose phosphate pathway (Casado-Vela et al. 2005). Nevertheless, there has been a lack of information on understanding major metabolites, including soluble carbohydrates, organic acids, amino acids, and fatty acids in BER-symptomatic fruits of paprika. Thus, we tested the hypothesis that the incidence of BER would be associated with alterations in major metabolites and mineral nutrients, with the relative ratios of major mineral nutrients to Ca levels. Therefore, this study aimed to evaluate and compare the physiological and physicochemical responses of asymptomatic and BER-symptomatic yellow paprika, as well as stem-end tissues and blossom-end tissues.

2 Materials and methods

2.1 Fruit materials and sampling

A blocky type of yellow paprika (*Capsicum annuum* L.) fruit from the ‘Volidano’ cultivar was harvested on February 6, 2023, from a commercial farm located in Yeongam, Jeollanam-do, Republic of Korea (34°44′ 19.35″N and 126°28′ 59.20″E). The fruits were cultivated following the standard agronomic conventional practices and were randomly harvested as asymptomatic control fruits and BER fruits simultaneously upon reaching commercial maturity (RDA 2024). Then, the fruits were immediately transported to the postharvest physiology laboratory at Chung-Ang

University in Anseong, Republic of Korea (37°00'13.60"N and 127°13'55.20"E). Fruits free from decay and phenotypic damage except BER symptoms were randomly sorted based on the fruit size, shape, peel pigmentation, and so on. The asymptomatic control and BER fruits were separately displayed in trays for further fruit quality measurements. On the BER fruit, the lesions of BER symptoms in fruit usually resulted from dry and sunken leathery spots without decay with a maximum 2 cm diameter lesion (Fig. 1A). The fruit quality attributes were evaluated in thirty fruits in which each treatment had three replicates ($n=3$), comprising ten fruits per one replicate.

2.2 Fruit quality attributes

Each fruit was evaluated for fruit quality attributes, including fruit fresh weight, length, diameter, ethylene production rate, respiration rate by CO_2 production, peel colour variables, pericarp firmness, soluble solids content (SSC), and titratable acidity (TA), based on a previous study (Lwin and Lee 2021). The fresh weight of the fruits ($n=30$) was measured using an analytical balance (WZ-3 A, CAS Corp., Yangju, Republic of Korea) at harvest. Peel colour variables expressed in terms of lightness (L^*), chroma (C^*), hue angle (h°), redness (a^*), and yellowness (b^*) values were determined using a chromameter (CR-200, Minolta Co., Osaka, Japan) following as previous study (Byeon et al. 2023).

The incidence of BER was visually evaluated by checking for the presence or absence of the symptom, mainly in the blossom-end tissue region of paprika fruits at the time of harvest. To measure the respiration (CO_2) and ethylene production (C_2H_4) rates of paprika fruit, six fruits were selected and used from both the control and BER symptom groups, respectively. After the fruits were incubated individually in airtight containers (1.9 L) for 2 h, 1 mL of gas sample was collected twice from the headspace of each container, as described in a previous study (Byeon et al. 2023). One gas sample was directly injected into a gas chromatograph (YL6500GC, YL Instrument Co., Ltd., Anyang, Republic of Korea) to evaluate the respiration rate by CO_2 production (Byeon et al. 2023), and the other was injected into another gas chromatograph (Agilent 8890 GC, Agilent Technologies Inc., Santa Clara, CA, USA) to measure the ethylene production rate, following methods described in the previous studies (Byeon et al. 2022, 2023; Latt et al. 2023).

Fruit pericarp hardness was measured with a 2-mm plunger on the opposite sides of each fruit using a Brookfield texture analyser (CT3 4500, AMETEK Brookfield, Inc., Middleborough, MA, USA) with 2 mm s^{-1} for pre-test and 10 mm s^{-1} for post-test, respectively. The results of pericarp firmness (or hardness) were expressed in Newton (N). Subsequently, the fruits were sliced horizontally 2 cm from

the stem-end towards the equator and again 2 cm from the blossom-end towards the equator, resulting in the separation of two distinct tissue regions (stem-end vs. blossom-end) for the analyses of mineral nutrients and targeted major metabolites. Afterwards, fruit flesh juice, which was squeezed using a household juicer, was used to determine the SSC using a refractometer (PAL-1, Atago Co., Ltd., Tokyo, Japan) and expressed as a percentage. TA was measured and expressed as a percentage of citric acid using an automatic titration system (EasyPlus Titrator, Mettler-Toledo, LLC, Columbus, OH, USA), where a 0.1 N NaOH solution was used to titrate until pH 8.2 with the juice (Park et al. 2024).

2.3 The contents of mineral nutrients

The process of fruit extraction for the amount of mineral nutrients was based on the protocol of Wang et al. (2018) with some modifications. The contents of mineral nutrients were determined using the inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 5300 DV, PerkinElmer Inc., Wellesley, MA, USA). The 0.1 g of the freeze-dried sample was digested with 4 mL of HNO_3 , and the mixture was maintained at 22°C for 24 h. After incubation, the mixture was boiled at 200°C ; immediately after, 3 mL of H_2O_2 was added to the mixture to get a clear solution. Afterwards, 1 N HCl with 1,000 ppm of La^+ was added to adjust final volume of 10 mL. The mixture was 50-fold diluted with deionised water to analyse mineral nutrients. The plasm, auxiliary, and nebuliser flow rates were 15.0 L min^{-1} , 0.2 L min^{-1} , and 0.8 L min^{-1} , respectively. The results were expressed in g kg^{-1} or mg kg^{-1} on a dry weight basis (Lwin et al. 2023c).

2.4 Soluble carbohydrates, organic acids, and free amino acids

The process of fruit extraction for the measurement of soluble carbohydrates, organic acids, and free amino acids was referred to the previous study with some modifications (Lwin et al. 2022). The ground frozen samples (5 g) were mixed with deionised water (25 mL) before homogenising. Then, the mixture was sonicated for 15 min in ice-cold water. The mixture was centrifuged at 3,000 rpm for 15 min. After centrifuge, the supernatant was filtered through a $0.45 \mu\text{m}$ hydrophilic filter for analysis performed with the Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Sunnyvale, CA, USA) used in a previous study (Latt et al. 2023).

For soluble carbohydrates, the oven temperature in a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Sunnyvale, CA, USA) set up at 70°C , equipped with a $300 \times 6.5 \text{ mm}$ Sugar-Pak column (Waters Co., Milford, MA,

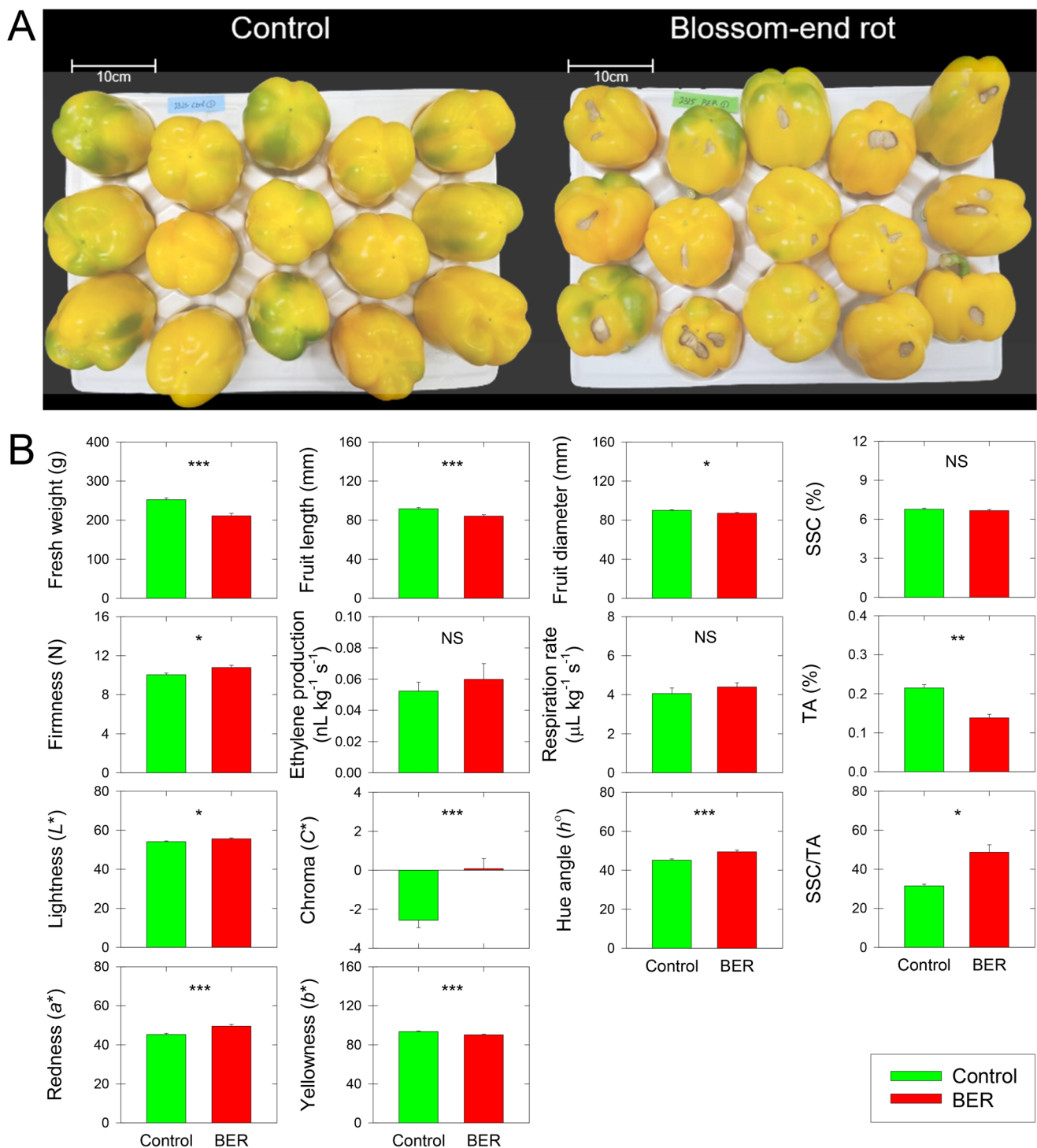


Fig. 1 Phenotypic difference (A) and physiological responses (B) of fruit fresh weight, fruit length, fruit diameter, pericarp firmness, ethylene production rate, respiration rate, soluble solids content (SSC), titratable acidity (TA), ratio of SSC to TA (SSC/TA), peel lightness (L^*), peel chroma (C^*), peel hue angle (h°), peel greenness to redness (a^*), and peel blueness to yellowness (b^*) in asymptomatic control and blossom-end rot (BER) fruits of ‘Volidano’ paprika cultivar at harvest. Fresh weight, pericarp firmness, fruit length, fruit diameter,

and peel colour variable data points are expressed as the mean of 30 paprika fruits as replicates ($n=30$) $\pm$ standard error (SE). However, ethylene production and respiration rate data points indicate the mean of six paprika fruits as replicates ($n=6$) $\pm$ SE. SSC, TA, and SSC/TA data points indicate the mean of three replicates ($n=3$) $\pm$ SE. Statistical difference is derived from the results of a Student’s t -test at $p < 0.05$. NS, *, **, or *** = not significant or significant difference at $p < 0.05$, 0.01, or 0.001, respectively

USA) (Lwin and Lee 2022). A 10 μL extract was injected into a Dionex UltiMate 3000 HPLC system, which applied HPLC grade water as a mobile phase with 0.5 mL min^{-1} flow rate. The Chromeleon version 6.0 software was used to analyse data, and the results were expressed on g kg^{-1} fresh weight basis (Lwin et al. 2023a).

Similarly, to measure the contents of organic acids, HPLC (Dionex Ultimate 3000) equipped with an Aminex 87 H column ($300 \times 10\text{ mm}$, Bio-Rad Co., Hercules, CA, USA) and an RI detector (RefractoMAX520, ERC Inc., Saitama, Japan) at 210 nm was applied. The oven temperature of $40\text{ }^{\circ}\text{C}$, and a flow rate of 0.5 mL min^{-1} was used. A 10 μL aliquot solution was used to determine organic acid contents, with $0.01\text{ N H}_2\text{SO}_4$ as the mobile phase. The results were described on mg kg^{-1} fresh weight basis (Lwin et al. 2023a).

The free amino acid content was determined following the protocol described in a previous study (Lwin and Lee 2020). HPLC (Dionex Ultimate 3000) equipped with an Inno C₁₈ column ($4.6\text{ mm} \times 150\text{ mm}$, $5\text{ }\mu\text{m}$, Youngjin Biochrom Co., Seongnam, Republic of Korea), an Agilent 1260 Infinity fluorescence detector (Agilent Technologies Inc., Santa Clara, CA, USA), and a UV detector at 338 nm were applied. The oven temperature was $40\text{ }^{\circ}\text{C}$ and the flow rate was 1.5 mL min^{-1} . A $0.5\text{ }\mu\text{L}$ aliquot of the extract was used with gradient elution, in which 40 mM sodium phosphate with pH 7.0 was used as solution A and distilled water, acetonitrile, and methanol (10:45:45, v/v %) as solvent B. The data were described as mg kg^{-1} on a fresh weight basis.

2.5 Chlorophyll and carotenoid contents

Ground frozen samples (1 g) were mixed with 10 mL of 80% acetone and stirred for 2 h in the dark at $22\text{ }^{\circ}\text{C}$. The mixture was centrifuged (1580R, Labogene, Gyrozen Co., Ltd., Gimpo, Republic of Korea) at 3,000 rpm for 15 min at $4\text{ }^{\circ}\text{C}$, and the extract solution was filtered using a No.51 Whatman filter paper (Merck KGaA, Darmstadt, Germany). The absorbance measurement of the extract was used by a UV spectrophotometer (Epoch 2, BioTek Instruments, Inc., Winooski, VT, USA) at wavelengths of 663, 646, and 470 nm. The contents of chlorophyll *a*, chlorophyll *b*, total chlorophylls, and total carotenoids were calculated using equations reported by Arnon (1949) with minor modifications (Latt et al. 2023). The results were expressed in mg kg^{-1} on a fresh weight basis.

2.6 Total antioxidant scavenging activities

Total antioxidant scavenging activity levels based on the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays

were measured using the method of Re (1999) with some modifications (Latt et al. 2024). To extract the sample, a frozen ground sample (1 g) was mixed with 80% methanol (10 mL). The mixture was centrifuged at 3,000 rpm for 15 min at $4\text{ }^{\circ}\text{C}$. Ten microliters of the extract were gently mixed with 190 μL of each working solution on a 96-well microplate. The absorbance of total antioxidant scavenging activities of ABTS and DPPH was read at 734 and 517 nm, respectively, using a spectrophotometer (Epoch 2, BioTek Instruments, Inc., Winooski, VT, USA). The activities of ABTS and DPPH were calculated based on a previous study (Latt et al. 2024).

2.7 Fatty acids

The method from Garces and Mancha (1993) was used for fatty acid analysis with some modifications (Lwin et al. 2022, 2023b). One hundred milligrams of the freeze-dried sample were added to a methylation mixture of methanol, benzene, 2,2-dimethoxy propane, and sulfuric acid with a ratio of 39:20:5:2 (v/v %), and after adding 1 mL heptane, it was gently shaken. After the mixture was shaken, the extraction was conducted at $80\text{ }^{\circ}\text{C}$ for 2 h, the samples were cooled at $22\text{ }^{\circ}\text{C}$, the collected supernatant was transferred to a vial, and pentadecanoic acid was added as the internal standard. The gas chromatograph (Agilent 7890 A, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with DB-23 column ($0.25\text{ mm} \times 60\text{ mm}$, $0.25\text{ }\mu\text{m}$, Agilent Technologies, Inc., Santa Clara, CA, USA) was used for the analysis of fatty acids. Helium was provided as the carrier gas at a constant flow rate of 4 mL and 35 mL min^{-1} for the column and flame ionisation detector, respectively. The identification of fatty acid compounds was confirmed using a fatty acid methyl ester mixture (Supelco™ 37 Component FAME Mix, Supelco Inc., Bellefonte, PA, USA) as a standard, and the results were expressed as mg kg^{-1} on a dry weight basis.

2.8 Statistical analyses

The complete randomised design was applied to evaluate the fruit and tissue regional differences of mineral nutrients and targeted major metabolites between the asymptomatic fruits and the BER fruits. The experiment was conducted in three replicates ($n=3$), with BER and asymptomatic control fruit samples collected simultaneously from the same commercial greenhouse. For each replicate, ten fruits were selected per treatment, resulting in a total of thirty fruits per group. The physiological fruit quality data were expressed as mean $\pm$ SE for both BER and asymptomatic control fruits. Statistical difference was derived from the results of an analysis of variance (ANOVA) along with a Student's *t*-test at $p<0.05$ (SAS version 9.3, SAS Institute Inc., Cary, NC,

USA) to evaluate the effect of only BER symptoms on fruit quality attributes. Afterwards, the least significant difference (LSD) test was applied to determine the tissue-region differential effects between asymptomatic control and BER fruits on mineral nutrients and targeted major metabolites at $p < 0.05$. The Pearson's correlation coefficient heatmap matrix with a hierarchical cluster analysis was generated using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>), and the correlation coefficient-based network of all the variables was visualised using the MetScape (Version 3.1.3) plugin available on Cytoscape (Version 3.10.1) (Latt et al. 2024; Lwin et al. 2023a). Volcano plot analysis was performed using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca/>) to evaluate the effects of BER incidence and tissue-regional effects of BER fruits on fruit quality attributes, mineral nutrients, and targeted major metabolites (Latt et al. 2024).

3 Results

3.1 Physiological responses of fruit quality attributes to BER symptoms

Among the fruit quality attributes, fruit fresh weight, length and diameter, TA, and peel yellowness (b^*) were lower in BER fruits than in asymptomatic control fruits. The firmness, SSC/TA, peel L^* , peel C^* , peel h° , and peel a^* were higher in BER fruit than in asymptomatic control fruits. However, ethylene production rate, fruit respiration rate, and SSC were not significantly different between the two contrast fruits (Fig. 1B).

3.2 Mineral nutrient responses to tissue regions of BER fruits

To evaluate the responses of mineral nutrients to tissue regions in control and BER fruits, pericarp tissues of stem- and blossom-end regions were used to determine the contents of major mineral nutrients and the ratio of mineral nutrient levels to Ca levels, as shown in Fig. 2. The K content in the stem-end tissue region was lower in BER fruits than in asymptomatic control fruits, but that in the blossom-end tissue region was higher in BER fruits than in control fruits. The contents of P and Mg did not differ between the control and BER fruits at the stem-end tissue region, but were higher in BER fruits than in control fruits at the blossom-end tissue region. Ca content was lower in the blossom-end tissue region than in the stem-end tissue region, regardless of BER symptoms. Na, Mn, and B contents were highest in the stem-end tissue region in control fruits, compared with the other tissues (Fig. 2).

The relative ratio of mineral nutrients to Ca, including K/Ca, Mg/Ca, Na/Ca, P/Ca, (K+Mg)/Ca, and (K+Mg+P)/Ca, was higher in the blossom-end tissue region than in the stem-end tissue region, irrespective of BER fruits. However, some of these observations were not consistent. The relative ratio to B/Ca was highest in the stem-end tissue region in asymptomatic control fruits, compared with BER fruits and the blossom-end tissue region (Fig. 2).

3.3 Differential responses of targeted major metabolites to BER fruits

Among the individual targeted major metabolites, glucose and fructose contents were highest in BER fruits at the blossom-end tissue region, compared with the other tissue regions, but lowest in control fruits at the stem-end tissue region. However, the sucrose content did not differ in both asymptomatic control and BER fruits, and the tissue regions (Fig. 3). The contents of citric and malic acids were highest in BER fruits at the blossom-end tissue region but the lowest in control fruits at the stem-end tissue region. Meanwhile, the levels of shikimic and fumaric acids were less influenced by BER fruits and tissue regions (Fig. 3). The content of chlorophyll *b* was highest in control fruits at the stem-end tissue region but the content of total carotenoids was highest in BER fruits at the stem-end tissue region, compared with the other tissues, while the levels of chlorophyll *a*, total chlorophyll, and total phenolic compounds did not differ at all. The ABTS antioxidant scavenging activity level was lowest at the blossom-end tissue region of BER fruits, compared with other tissues. The DPPH antioxidant scavenging activity level was higher in BER fruits than in control fruits, irrespective of tissue region (Fig. 3). The contents of palmitic, oleic, and linoleic acids were highest at the stem-end tissue region of BER fruits, compared with the other tissue regions, but lowest at the blossom-end tissue region of control fruits. However, the levels of stearic, arachidic, and α -linolenic acids were unaffected by the BER symptom or tissue regions. Nevertheless, the levels of unsaturated fatty acid (UFA)/saturated fatty acid (SFA) and double bond index (DBI) were highest at the stem-end tissue region of BER fruits but lowest at the blossom-end tissue region of control fruits, compared with the other tissue region (Fig. 3).

The contents of glutamine, asparagine, aspartic acid, serine, γ -aminobutyric acid (GABA), threonine, citrulline, and total amino acids were highest at the blossom-end tissue region compared with the other tissue regions in both BER and control fruits. However, the contents of valine, leucine, phenylalanine, histidine, tyrosine, glycine, and methionine were highest at the stem-end tissue region of BER fruits. The glutamic acid content was highest at the stem-end tissue

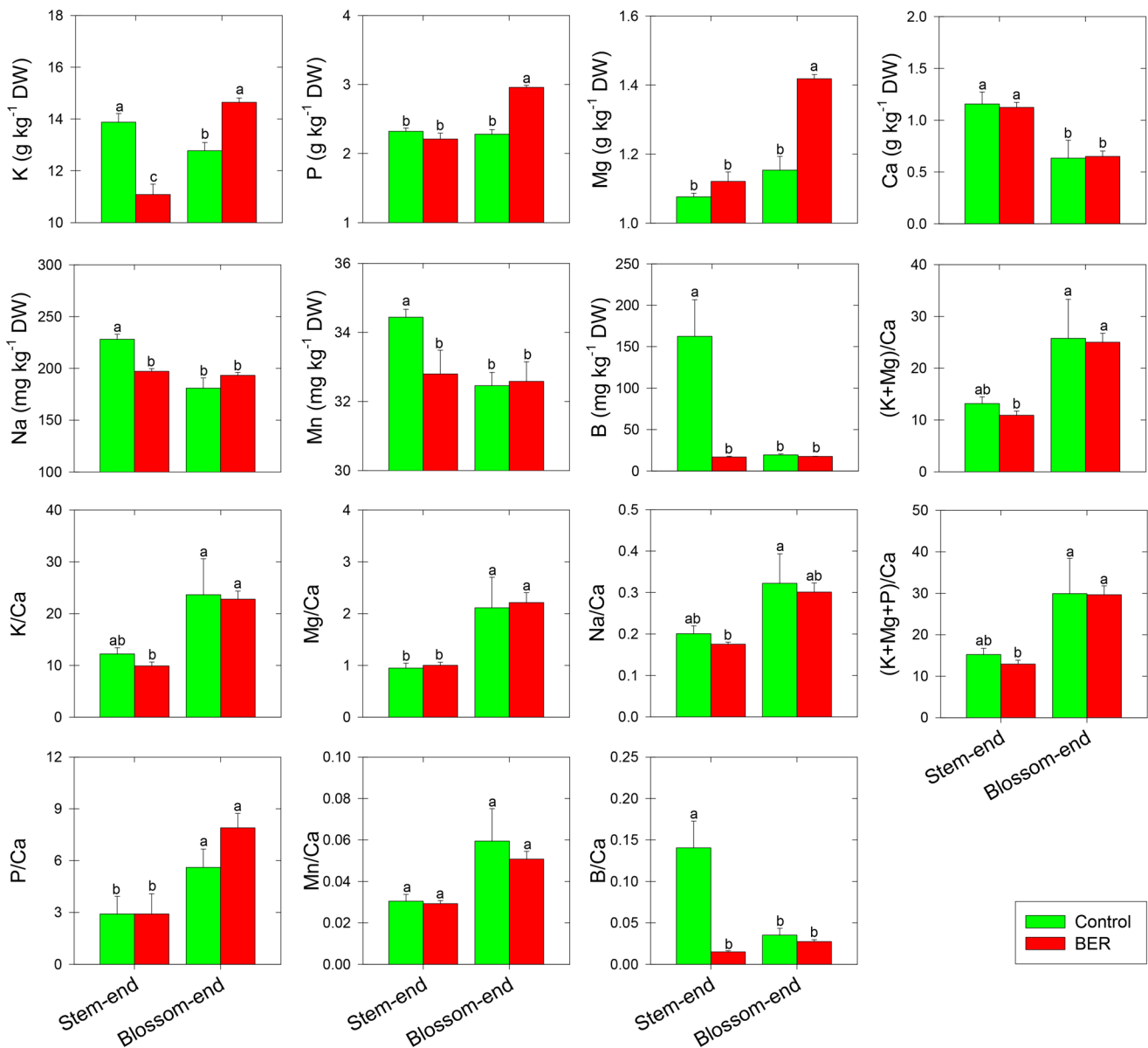


Fig. 2 Mineral nutrient contents and relative ratios of mineral nutrient levels to Ca level from stem-end and blossom-end tissue regions in asymptomatic control and blossom-end rot (BER) fruits of 'Voliano' paprika cultivar at harvest. Each data point is the mean of three replicates ($n=3$) $\pm$ standard error (SE). The least significant difference (LSD) test is applied to examine the significant difference at $p=0.05$

region of control fruits, compared with the other tissue regions. Nevertheless, the levels of arginine, alanine, lysine, proline, and tryptophan were not differentially affected by BER fruits or the tissue region. Additionally, although the level of total amino acids was not affected by BER fruits, it was higher at the blossom-end tissue region than at the stem-end tissue region (Fig. 4).

dano' paprika cultivar at harvest. Each data point is the mean of three replicates ($n=3$) $\pm$ standard error (SE). The least significant difference (LSD) test is applied to examine the significant difference at $p=0.05$

3.4 Overall relationship among fruit quality attributes, mineral nutrients, and targeted metabolites

To evaluate the overall responses or relationship among fruit quality attributes, mineral nutrients, and targeted major metabolites, a correlation coefficient heatmap matrix system with a hierarchical cluster analysis was constructed, as shown in Fig. 5. Based on the results, two major clusters, three minor clusters, and two small clusters were detected. In the first positive cluster placed at the upper-right region of the overall correlation coefficient heatmap matrix,

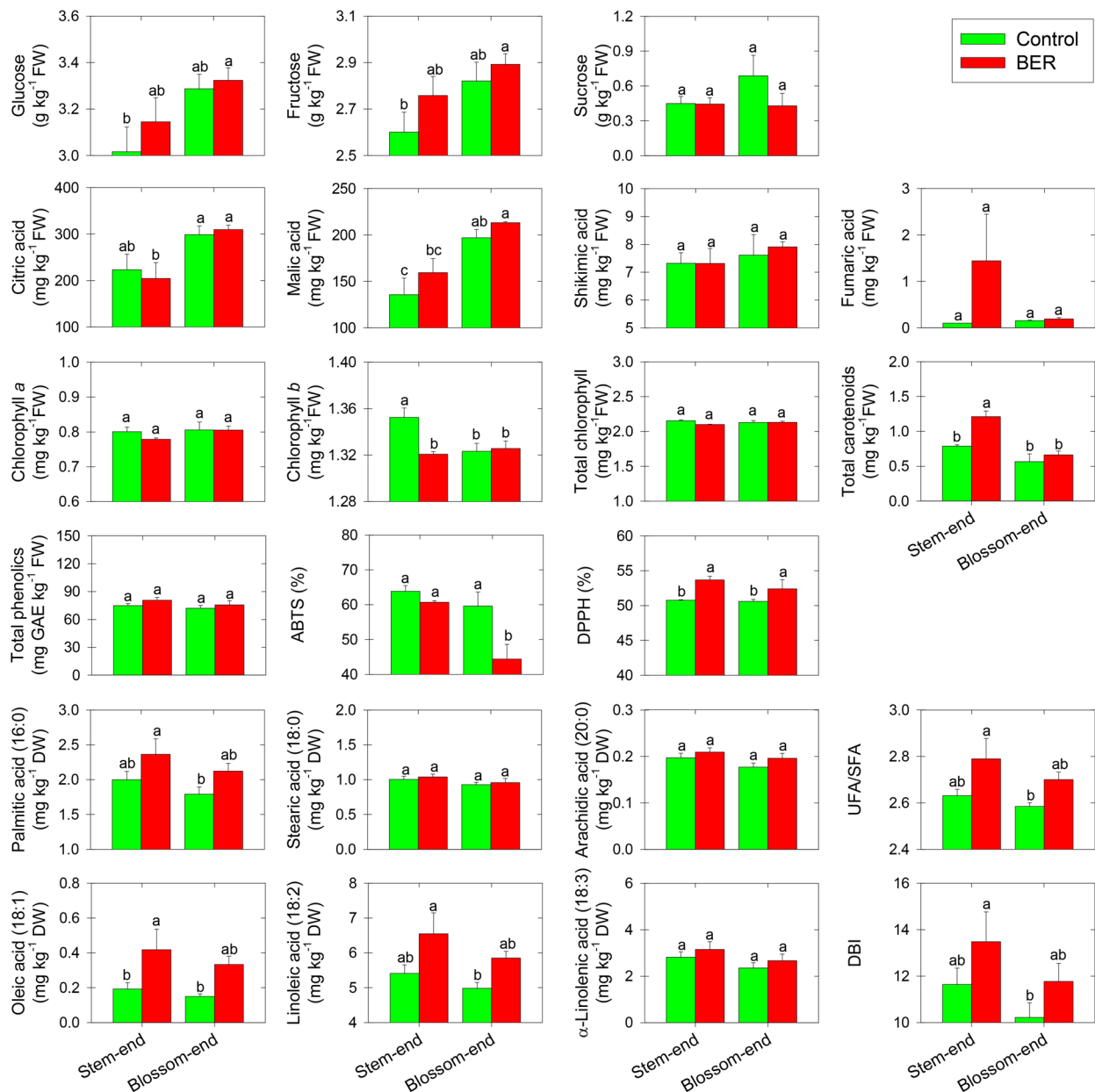


Fig. 3 The contents of soluble carbohydrates, organic acids, chlorophylls, total carotenoids, total phenolic compounds, fatty acids, and total antioxidant capacities (ABTS and DPPH assays) from stem-end and blossom-end tissue regions in asymptomatic control and blossom-end rot (BER) fruits of 'Volidano' paprika cultivar at harvest. Each data point is the mean of three replicates ($n=3$) $\pm$ standard error (SE).

methionine, tyrosine, and total carotenoids were positively correlated. Next to the first positive cluster, one small positive cluster was detected in which peel C^* , BER, and oleic acid were positively correlated. ABTS, Na, B/Ca, B, total phenolic compounds, arachidic acid, stearic acid, Ca, and glutamic acid were positively correlated in one of the minor positive clusters. Next to the minor positive clusters, a

The least significant difference (LSD) test is applied to examine the significant difference at $p=0.05$. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; SFA: saturated fatty acids; UFA: unsaturated fatty acids; DBI: double bond index

strong positive correlation cluster was detected, including DBI, α -linolenic acid, SFA, UFA, total fatty acid (TFA), linoleic acid, and palmitic acid. The first positive minor cluster was tightly correlated with the second positive minor cluster. Simultaneously, another minor cluster was detected in which leucine, glycine, histidine, ethylene, proline, sucrose, valine, threonine, isoleucine, arginine, fruit respiration,

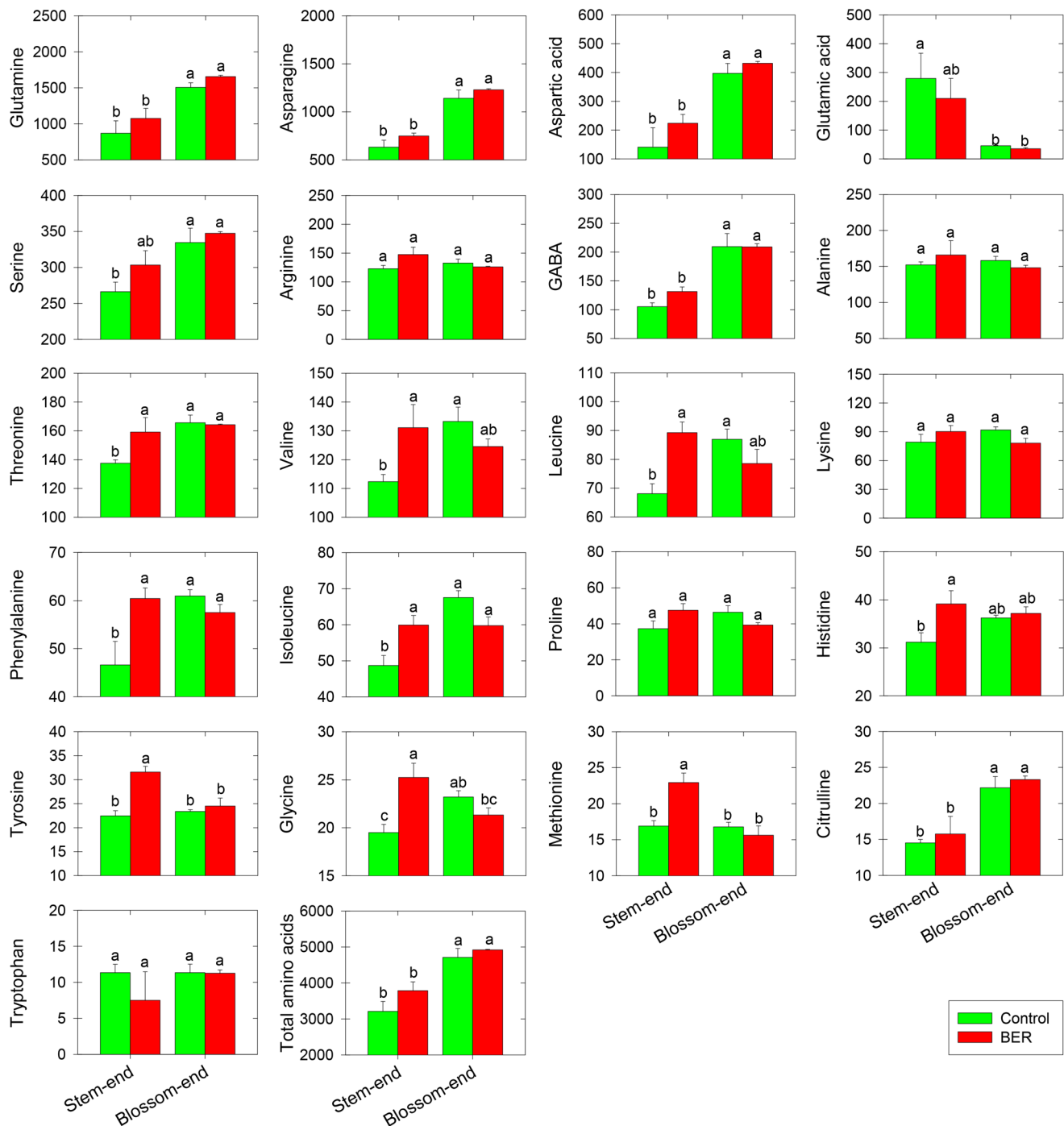


Fig. 4 The contents (mg kg⁻¹ FW) of free amino acids from stem-end and blossom-end tissue regions in asymptomatic control and blossom-end rot (BER) fruits of 'Volidano' paprika cultivar at harvest. Each

data point is the mean of three replicates ($n=3$) ± standard error (SE). The least significant difference (LSD) test is applied to examine significant differences at $p=0.05$. GABA: γ -aminobutyric acid

lysine, and alanine were positively correlated with each other. In one of the major positive correlation clusters, alanine, peel b^* , fruit length, fruit L/D, chlorophyll a , chlorophyll b , UFA/TFA, total chlorophyll, fruit diameter, peel L^* , fruit thickness, fruit fresh weight, UFA/SFA, firmness, peel a^* , peel hue angle, fructose, glucose, K, tryptophan, P, Mg,

and DPPH were positively correlated with each other. In the last major positive correlation cluster, GABA, aspartic acid, phenylalanine, citrulline, serine, citric acid, total amino acids, asparagine, glutamine, malic acid, (K+Mg)/Ca, K/Ca, (K+Mg+P)/Ca, Na/Ca, Mn/Ca, P/Ca, and Mg/Ca were positively correlated with themselves. Meanwhile, the ratios

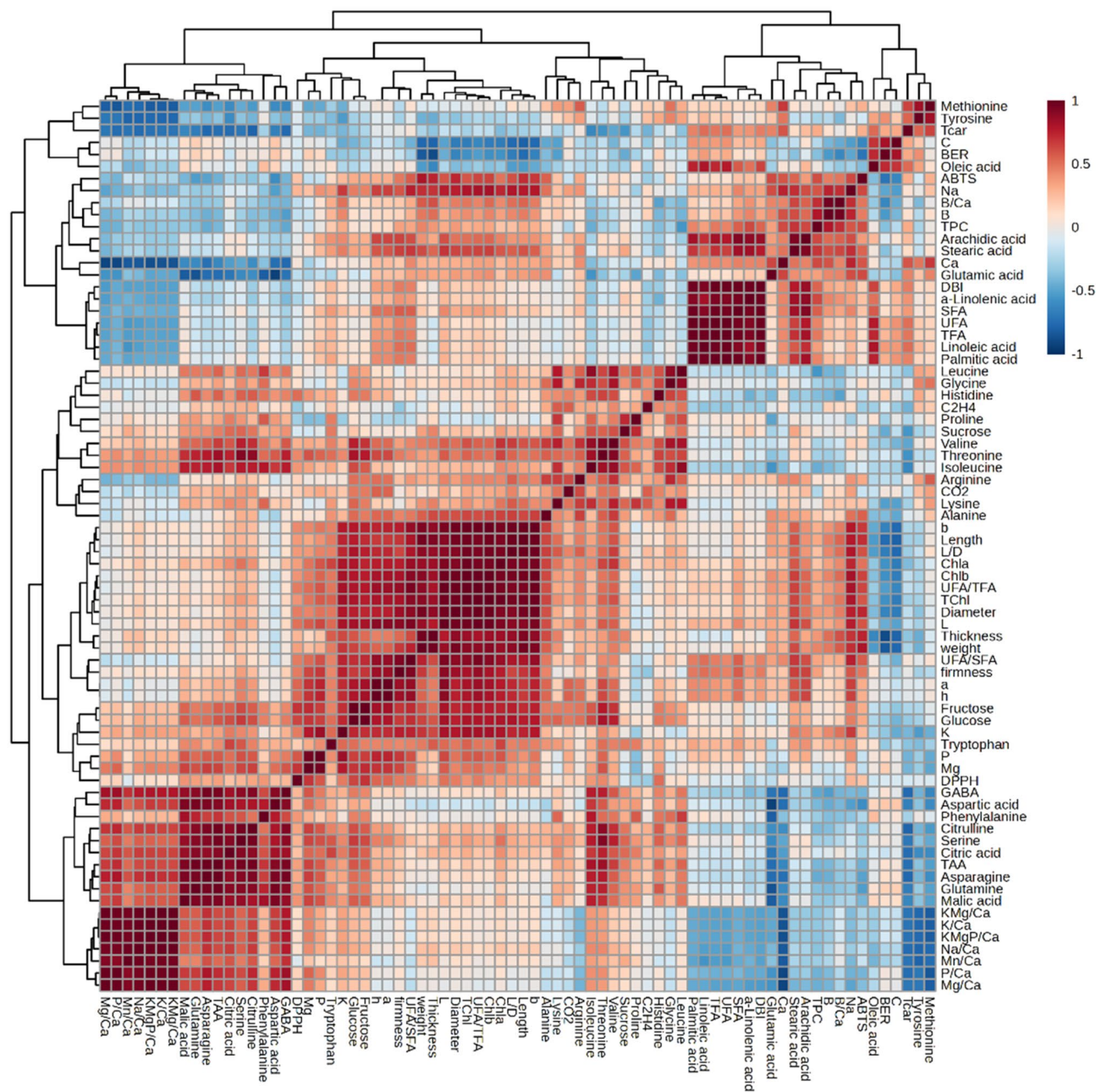


Fig. 5 Pearson's correlation coefficient heatmap matrix with a hierarchical cluster analysis among physicochemical fruit quality attributes, mineral nutrients, major targeted metabolites, and total antioxidant scavenging activities from asymptomatic control fruits to blossom-end

of mineral nutrient contents were strongly and negatively correlated with some amino acids, such as methionine, tyrosine, and total carotenoids, resulting in the lowest left side of the heatmap. Conversely, Mg/Ca, P/Ca, Mn/Ca, Na/Ca, (K+Mg+P)/Ca, K/Ca, and (K+Mg)/Ca were negatively correlated with methionine, tyrosine, total carotenoids, and Ca. GABA, aspartic acid, phenylalanine, citrulline, serine, citric acid, total amino acids (TAA), asparagine, glutamine, and malic acid were negatively correlated with methionine,

rot (BER) fruits of the 'Volidano' paprika cultivar at harvest. The figure legend indicates the results of the correlation coefficient (r). Red and blue colors indicate positive and negative correlation, respectively. Abbreviations are listed in Supplementary Table S1

tyrosine, total carotenoids, Ca, and glutamic acid. Peel C* and BER were negatively correlated with peel b*, fruit length, L/D, chlorophyll a, chlorophyll b, UFA/TFA, total chlorophyll, fruit diameter, peel L*, pericarp thickness, and fruit fresh weight (Fig. 5).

To focus mainly on the overall correlation among fruit quality attributes, mineral nutrients, and targeted metabolites from only BER fruits, a Pearson's correlation coefficient network system was applied, as shown in Fig. 6. BER

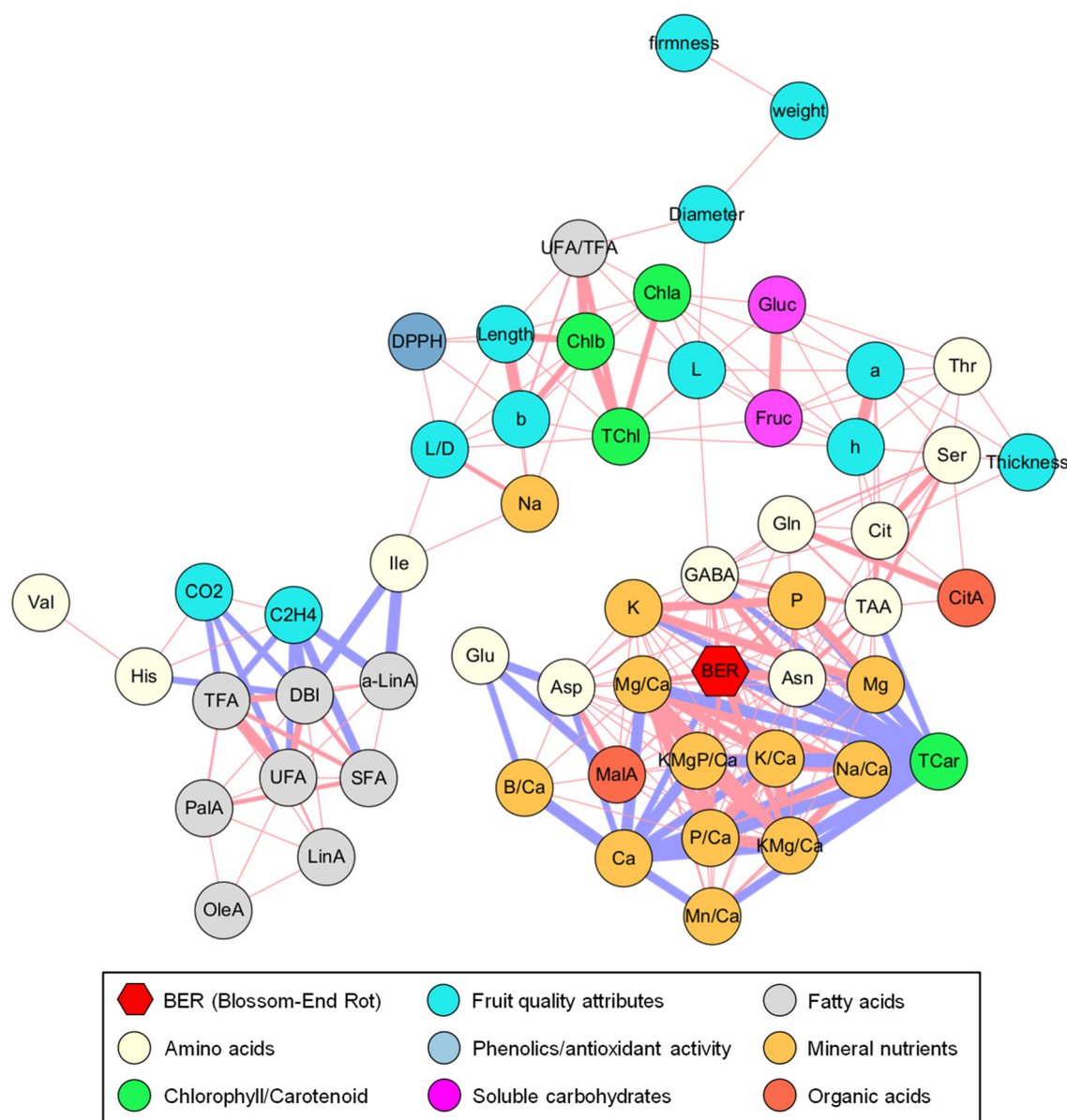


Fig. 6 Pearson's correlation coefficient ($r > |0.90|$) network among physicochemical fruit quality attributes, mineral nutrients, major targeted metabolites, and total antioxidant scavenging activities from only blossom-end rot (BER) fruits of the 'Volidano' paprika cultivar at harvest. Red and blue lines indicate positive and negative correla-

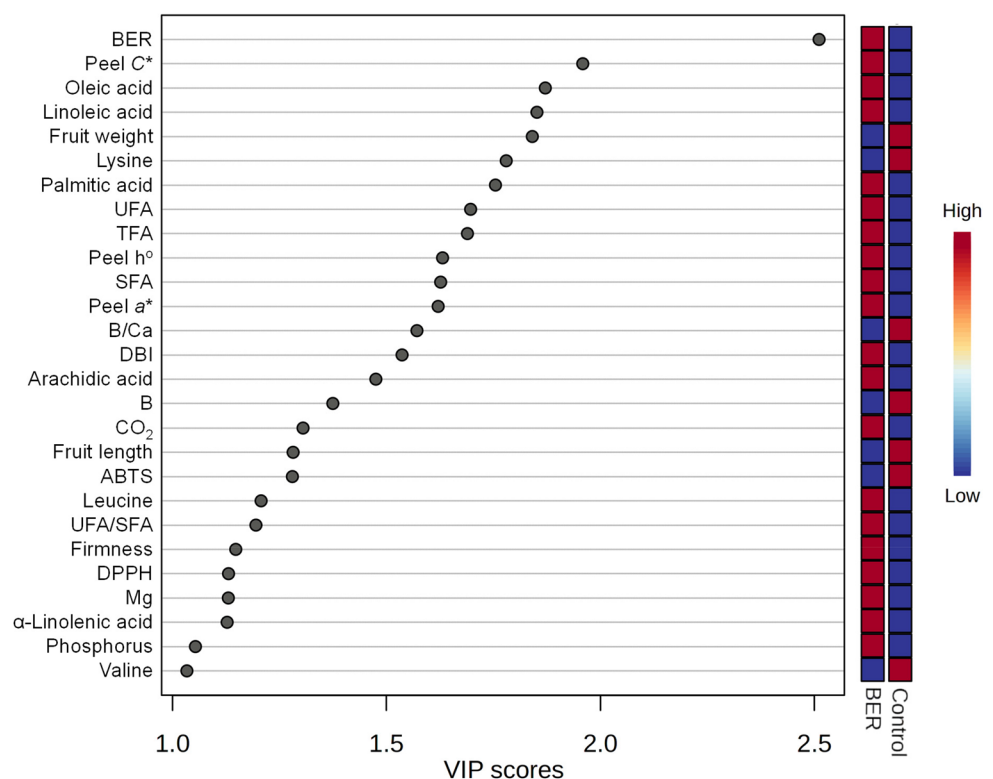
symptoms correlate with most mineral nutrients and the relative ratio of major mineral nutrient levels to Ca levels. Ca and total carotenoid levels were negatively correlated with the relative ratio of major nutrient levels to Ca levels. In contrast, chlorophyll variables were positively correlated with peel colour variables. Glucose and fructose were positively correlated with peel colour variables. Furthermore, fatty acid variables were mostly positively correlated with each other but negatively correlated with histidine, fruit respiration, ethylene production, and isoleucine. In turn, isoleucine was positively correlated with L/D and Na (Fig. 6).

tions among variables, respectively. The thickness of the correlation coefficient network lines provides the significance of the correlation coefficient (r) between variables. Abbreviations are listed in Supplementary Table S1

3.5 Differential responses to BER fruits and tissue regions

Variable importance in projection (VIP) scores, generated from a partial least-squares discriminant analysis model, were used to examine the variables most influenced by BER symptoms. The variables with VIP scores higher than 1 are generally considered essential distinctive indicators (Fig. 7). Notably, the VIP scores of fruit fresh weight, lysine, B/Ca, B, fruit length, ABTS, and valine were higher than 1.0

Fig. 7 Partial least squares-discriminant analysis (PLS-DA) variable importance in projection (VIP) scores plot among physico-chemical fruit quality attributes, mineral nutrients, major targeted metabolites, and total antioxidant scavenging activities from asymptomatic control fruits to blossom-end rot (BER) fruits of the ‘Volidano’ paprika cultivar at harvest. The individual variables provided have a higher VIP score than 1.0. Abbreviations are listed in Supplementary Table S1



in asymptomatic control fruits. On the other hand, peel C*, oleic acid, linoleic acid, palmitic acid, UFA, TFA, peel h°, SFA, peel a*, DBI, arachidic acid, respiration rate, leucine, UFA/SFA, firmness, DPPH, Mg, α-linolenic acid, and P were higher than 1.0 in BER symptomatic fruits (Fig. 7).

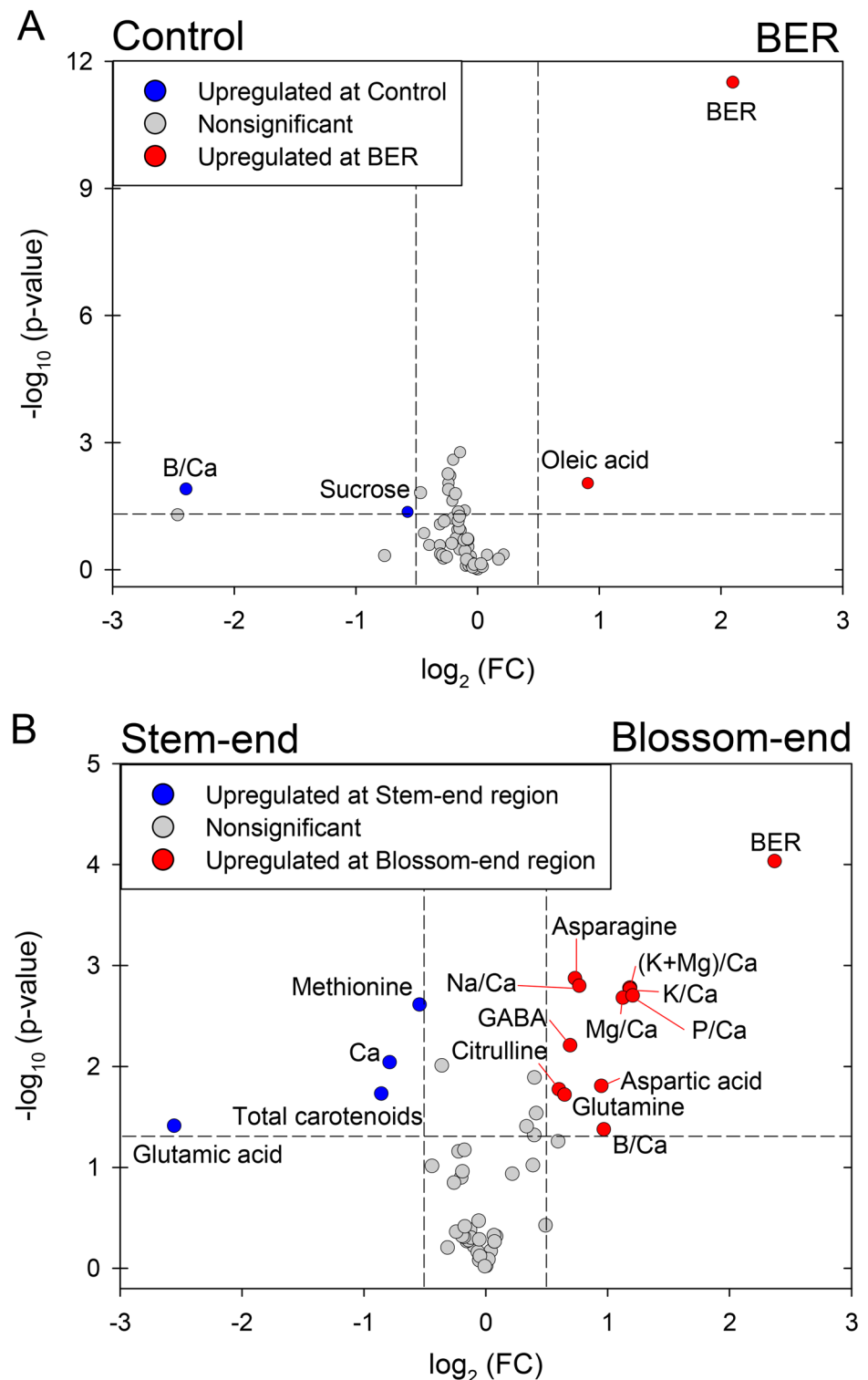
To determine the differential fruit physiological and metabolic parameters between BER symptom and tissue regions, the volcano plot was applied (Fig. 8). In the volcano plot, blue and red dots represent fold change values of 1.41 ($\log_2 1.41$ equates to ± 0.50) and a p -value of 0.05 cut-off ($-\log_{10} 0.05$ equates to 1.30) between BER fruits and asymptomatic control fruits and between stem-end and blossom-end tissue regions. B/Ca and sucrose were upregulated in control fruits but oleic acid and BER symptoms were upregulated in BER fruits (Fig. 8A). Glutamic acid, methionine, Ca, and total carotenoids were upregulated at the stem-end tissue region of BER fruits, while BER symptoms, asparagine, Na/Ca, (K+Mg)/Ca, K/Ca, Mg/Ca, P/Ca, GABA, aspartic acid, citrulline, glutamine, and B/Ca were upregulated at the blossom-end tissue region of BER fruits (Fig. 8B).

4 Discussion

During the fruit growth and developmental stages, the incidence of BER can occur due to several technical management issues, such as irrigation (Aktas et al. 2003), air temperature (Bar-Tal et al. 2006; Ho et al. 1993), ambient

vapour pressure deficit (Adams and Ho 1993), and irradiance (Ho et al. 1993) in paprika fruits. Although cultivated under the relevant technical management system, the imbalance or deficiency of mineral nutrients can potentially lead to the development of BER in paprika fruits (Bar-Tal et al. 2001; Kabir et al. 2021), along with a subsequent alteration in fruit quality attributes at harvest. In this study, the incidence of BER caused a reduction in fruit fresh weight, fruit length, and fruit diameter, and also affected peel colour variables (Fig. 1B). In tomato fruits, the incidence of BER reduced fruit fresh weight and dry weight at harvest (Van Ieperen 1996) and thus reduced fruit yield (Willumsen et al. 1996). Additionally, the incidence rate of BER was gradually increased with the increase in fruit size of sweet peppers during the fruit growth and developmental stages, regardless of cultivars (Marcelis and Ho 1999). This indicates that BER development is strongly connected to a higher rate of fruit growth and development compared to fruit cell expansion. In addition, the phenotypic responses of peel colour variables differed between the asymptomatic control and BER fruits at harvest (Fig. 1B). Of those colour variables, the results of peel yellowness (b^*) indicated that BER fruits would be relatively less mature or delayed in fruit maturation than control fruits for yellow bell pepper fruits. The peel colour variables of immature tomato fruits were influenced by the decrease in the L^* value and the increase in the a^* value, while the fruits were developing into BER fruits (Reitz and Mitcham 2021).

Fig. 8 Volcano plots of physicochemical fruit quality attributes, soluble carbohydrates, organic acids, free amino acids, fatty acids, total chlorophyll, total carotenoid, total phenolic compounds, and total antioxidant scavenging activities from the asymptomatic control fruits and blossom-end rot (BER) fruits (A) and from the stem-end tissue region and blossom-end tissue region in only BER fruits (B) of the ‘Volidano’ paprika cultivar at harvest. The X-axis indicates $\log_2$ fold changes (FC) between two treatment groups and the Y-axis indicates $-\log_{10} p$ -value of variation. The horizontal dot line indicates p -values of the 0.05 cut-off position and the vertical dot lines indicate an FC value of 1.41, respectively. The upregulated variables in asymptomatic control fruit or stem-end tissue region are shown as blue dots, while the upregulated variables in BER fruits or blossom-end tissue region are shown as red dots, respectively. Grey dots indicate non-significant variables in either treatment. GABA: γ -aminobutyric acid



K, P, and Mg contents were highest at the blossom-end tissue region of BER fruits compared with the other fruits, but the levels of Ca, Na, Mn, and B were lowest. To identify the physiological role of Ca in the development of BER, the ratios of the corresponding mineral nutrient levels to Ca levels were also determined. The level of Ca was lower at

the blossom-end tissue region than at the stem-end tissue region, irrespective of the BER symptom. Thus, the ratios of the corresponding mineral nutrients to Ca were inversely responded. The ratios were higher at the blossom-end tissue region than at the stem-end tissue region, regardless of BER fruits, except for the ratio of B level to Ca level

(Fig. 2). Typically, the severity of BER symptoms was positively correlated with the lower level of Ca in paprika fruits, and the contents of Ca were higher in the dorsal part of tissues than at the blossom-end tissue regions (Bar-Tal et al. 2001). The Ca level was gradually declined from the proximal to the distal region of mature paprika fruits (Karni et al. 2000; Mayorga-Gómez et al. 2020; Oh et al. 2021). Additionally, the Ca content was proportionally increased with the increase in fruit width and fresh weight (Marcelis and Ho 1999), although the level of Ca was still lower in BER fruits than in asymptomatic fruits of paprika (Oh et al. 2021). However, there was no significant difference in response to the Ca content between BER and asymptomatic fruits, regardless of tissue region, although there was still a substantial difference in the Ca content between the stem-end and blossom-end tissue regions (Fig. 2). The controversial results of the Ca levels between BER and asymptomatic fruits might lead by the size of the BER lesions in fruit samples used for this study. That is, the size of the BER lesions was mostly less than 1 cm in diameter in BER fruits. The increase in BER severity based on the BER lesion diameter from 2 to 20 mm wide was not strongly related to the apoplastic pH in the pericarp of paprika fruits (Aloni et al. 2008). Therefore, the results indicated that the difference in Ca content between BER and asymptomatic fruits is not influenced by the presence of BER but by the severity of BER. Nevertheless, BER symptom was tightly correlated with certain mineral nutrients and the ratios of mineral nutrient levels to Ca levels in BER fruits (Fig. 6). Ca level was only increased at the stem-end tissue region and not at the blossom-end tissue region, but the ratios of mineral nutrient levels to Ca level were greatly increased at the blossom-end tissue region in BER fruits (Fig. 8). As mentioned by Topcu et al. (2022) and Saure (2001), the development of BER symptoms is linked more to the abnormal balance of all mineral nutrients rather than to Ca deficiency during fruit growth and development. Overall, the results of the mineral nutrients indicated that BER symptoms are highly associated with the overall balance of mineral nutrients and the relative ratios of mineral nutrient levels to Ca, rather than by any single mineral nutrient.

TA was lower in BER fruits than in asymptomatic fruits for the whole fruit, but SSC was not affected (Fig. 1). In contrast, among the fruit tissue regions, the levels of glucose, fructose, and citric and malic acids were highest at the blossom-end tissue region of BER fruits, compared with the other tissue regions (Fig. 3). That is, although SSC was not affected by BER in terms of the whole fruit, the levels of major soluble carbohydrates were responded differently to BER symptoms. Meanwhile, the TA and individual organic acids responded differently. That is, the relationship of TA and individual organic acids was not substantially affected

by BER fruits but by tissue regions on the same fruit. Even in the Ponkan fruit, SSC and TA did not differ based on the entire fruit segment but were higher at the stylar end (blossom-end) tissue region than at the stem-end tissue region (Yao et al. 2018). The levels of major soluble carbohydrates were differentially distributed from the central tissue region to the outer tissue region at the equator tissue region of watermelon fruits at harvest (Fukuoka et al. 2009) or gradually localised from the inner mesocarp tissue region to the hypodermal tissue region in mature orange-fleshed honeydew melon fruits (Lester 2008). Overall, the differential results between the whole fruit and fruit tissue regions were derived from the dilution effect using the whole fruit, although there were differential and gradual concentration gradations of individual major soluble carbohydrates and organic acids from the proximal tissue region to the distal tissue region during the late stage of the fruit growth and development periods.

The contents of chlorophyll *a* and total chlorophyll were affected by tissue region and BER symptoms, but the total carotenoid content was higher in the stem-end tissue region of BER fruits than at the blossom-end tissue region of control fruits (Fig. 3). In turn, the total carotenoid content was negatively correlated with most variables of the ratio of mineral nutrient levels to Ca level. In contrast, chlorophyll variables were positively correlated with fruit quality and colour variables in BER fruits (Fig. 6). Based on the results of the volcano plot, the total carotenoid content was only greatly increased at the stem-end tissue region of BER fruits (Fig. 8). That is, the differential responses of chlorophyll and carotenoids to BER fruits and fruit tissue regions is strongly involved with pigment reorganisation, along with chromoplast differentiation (Rödiger et al. 2021). Thus, the results indicated that fruit ripening is less likely to progress in the symptomatic region of BER fruits but is enhanced at the stem-end tissue region, even in BER fruits. Nevertheless, the overall metabolic responses of chlorophyll and carotenoids could differentially appear during fruit ripening. That is, it would be assumed that the carotenoid anabolic pathway would be much more intensive and highly characterised than the chlorophyll catabolic pathway during the last stage of the fruit ripening period. Additionally, although the levels of individual fatty acids did not consistently respond to BER symptoms, the ratio of UFA to SFA and DBI were higher at the stem-end tissue region of BER fruits than at the blossom-end tissue region of control fruit (Fig. 3). In turn, most fatty acid variables were tightly correlated with themselves but negatively correlated with fruit respiration and ethylene production rates in BER fruits (Fig. 6). Nevertheless, the oleic acid level was only increased in BER fruits, compared with asymptomatic fruits, but no elevated fatty acid levels were detected in the comparison between the

stem-end and blossom-end regions of BER fruits (Fig. 8). The bitter pit symptom was strongly correlated not only with any individual fatty acids but also with the ratio of UFA to SFA levels and malondialdehyde levels in cold-stored ‘Golden Delicious’ apple fruits (Antunes et al. 2022). Thus, the results suggested that fatty acids neither play a pivotal role in the incidence of BER symptoms nor result from the development of BER.

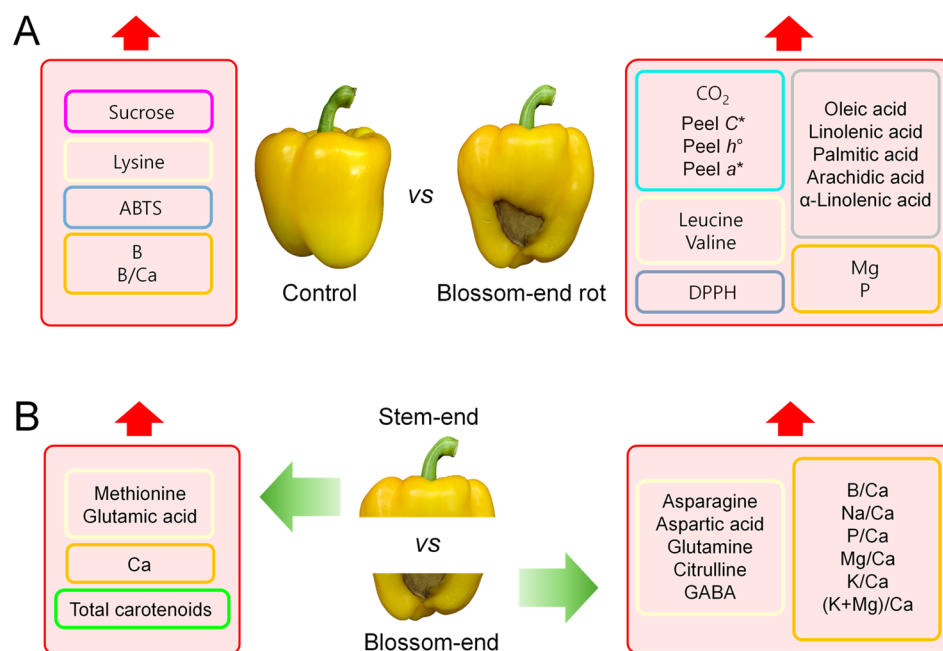
Among the free amino acids, the contents of glutamine, asparagine, aspartic acid, serine, GABA, threonine, and citrulline were highest at the blossom-end tissue region, compared with the other tissues, thereby contributing to enhancing the total amino acid content (Fig. 4). Thus, these individual amino acids were closely correlated with BER symptoms in the correlation coefficient network results of only BER fruits (Fig. 6). Additionally, the levels of most amino acids were only increased at the blossom-end tissue region of BER fruits (Fig. 8B). The profile and contents of amino acids were differentially responded to water stress-induced BER fruits of mature tomatoes, compared with well-irrigated healthy fruits. Aspartic acid, glutamic acid, proline, and alanine levels were higher in water stress-induced BER fruits from the first truss, thereby increasing the total amino acid levels (Franco et al. 1999). The increase in the levels of these bound amino acids was strongly linked with abiotic stress-induced BER symptoms of mature tomato fruits (Franco et al. 1999). That is, the accumulation of negative-charge and low-molecular-weight amino acids was fairly associated with the phenotypic responses of abiotically stressed fruits (Jones et al. 1980; Morgan 1984). Meanwhile, in this study, the levels of amino acids with a polar uncharged side chain, glutamine, asparagine, serine, and threonine, were greatly increased at the blossom-end tissue region of BER fruits. Glutamine plays a fundamental role in a wide range of metabolic reactions, such as redox homeostasis, the synthesis of tricarboxylic acid cycle metabolism, antioxidant metabolism, and the synthesis of nonessential amino acids and macromolecules (News-holme et al. 2003). Glutamine and glutamic acid metabolism based on the glutamine synthetase (GS) and glutamate synthase (GOGAT) pathways are mainly involved in C- and N-metabolisms to make energy sources in plant cells (Lancien et al. 2000). Based on the metabolic reaction of GS/GOGAT, numerous amino acids are produced and consecutively chain-reacted in plants (Hodges 2002). Thus, the increase in glutamine levels may directly or indirectly contribute to the accumulation of GABA, aspartic acid, citrulline, and serine in this study. That is, the increase in the levels of these amino acids is related to the results of the imbalance of C- and N-metabolisms during the rapid fruit growth period. Therefore, the results indicate that the incidence of BER could be directly or indirectly involved in

the abnormal metabolism of amino acids, especially in polar uncharged side-chain amino acids. Additionally, GABA, a stress metabolite, could play a fundamental role in numerous biochemical metabolisms associated with C: N balance, cytosolic pH regulation, protection against oxidative stress, defence against biotic stress, osmoregulation, and signalling roles (Bouché and Fromm 2004). Thus, the higher level of GABA would be more than enough to explain how BER fruits are exposed to abiotic stress during the second half of the fruit development. BER can be understood as a stress response induced by Ca^{2+} disequilibrium and the accumulation of reactive oxygen species. In this process, primary metabolism, focusing on amino acids and organic acids, has undergone extensive reprogramming (de Freitas et al. 2018). In fruit tissues, the non-protein amino acid, such as GABA, increased via Ca^{2+} /calmodulin-dependent activation of glutamate decarboxylase, engaging the GABA shunt that supports pH-stat regulation, maintenance of the C: N balance, anaplerosis of the TCA cycle, and buffering of oxidative stress (Aghdam et al. 2012; Bouché and Fromm 2004; Li et al. 2020). Accordingly, elevated GABA in the context of BER is better interpreted as a stress response in metabolic adaptation rather than a mere ripening effect. In turn, chilling stress-induced physiological disorders, such as flesh browning and internal breakdown, are highly associated with the increase in GABA levels (Lee et al. 2012; Mattheis et al. 2022). Additionally, the accumulation rate of GABA was higher in wilted fruits than in modified atmosphere packaging (MAP)-treated paprika fruits during shelf life (Lwin et al. 2022). Overall, the alteration of profiles and contents of amino acids could have resulted from the abnormal metabolism of amino acids during the rapid fruit-growing period. That is, the metabolic alteration of amino acids could occur more severely at the blossom-end tissue region than at the stem-end tissue region of paprika fruits during the rapid fruit-growing period. These amino acid changes have been observed repeatedly under foliar and soil treatments and across diverse environmental stresses. When it is associated with reduced Ca availability and to destabilization of the cell wall and plasma membrane at the symptomatic region of BER, they act as stress physiological indicators regardless of fruit maturation (Ghoname et al. 2012; Saure 2001).

5 Conclusion

BER symptoms caused a reduction in fruit size and affected fruit colour development in paprika fruit. Regarding mineral nutrients, the incidence of BER enhanced the K, P, and Mg levels but reduced the levels of Ca and Mn at the blossom-end tissue region, compared with the stem-end tissues. However, the ratios of mineral nutrient levels to Ca level were

Fig. 9 Schematic illustrating among physicochemical fruit quality attributes, mineral nutrients, major targeted metabolites, and total antioxidant scavenging activities from the asymptomatic control fruits and blossom-end rot (BER) fruits (A) and from the stem-end tissue region and blossom-end tissue region in only BER fruits (B) of ‘Volidano’ paprika cultivar at harvest. The red arrows indicate an increase. C*: peel chroma; h°: peel hue angle; a*: peel redness; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; GABA: γ -aminobutyric acid



high at the blossom-end tissue region of BER fruits, compared with the other tissues excluding B/Ca. Among the targeted major metabolites, the levels of glucose, fructose, and citric and malic acids were highest at the blossom-end tissue regions of BER fruits, compared with the other tissues, but those of total carotenoids, palmitic acid, oleic acid, linoleic acid, UFA/SFA, and DBI were higher at the stem-end tissue region of BER fruits than at the blossom-end tissue region of healthy fruits. In addition, the contents of glutamine, asparagine, aspartic acid, serine, GABA, threonine, and citrulline were highest at the blossom-end tissue region of BER fruits, thereby resulting in the highest level of total amino acids. However, the contents of valine, leucine, phenylalanine, histidine, tyrosine, glycine, and methionine were highest at the stem-end tissue region of BER fruits. Thus, the BER symptom was strongly correlated with most of mineral nutrients, glutamic acid, aspartic acid, asparagine, GABA, glutamine, and total carotenoids. Additionally, based on the results of the VIP scores and volcano plots, sucrose, lysine, ABTS activity, B, and B/Ca were highly upregulated in asymptomatic healthy fruit rather than in BER fruit but the fruit respiration, peel colour variables, leucine, valine, DPPH activity, and oleic, linolenic, palmitic, arachidic, and α -linolenic acids were highly upregulated in BER fruits (Fig. 9A). In addition, in terms of the tissue region of BER fruits, the levels of methionine, glutamic acid, Ca, and total carotenoids were highly upregulated at the stem-end tissue regions in BER fruit, but the levels of asparagine, aspartic acid, glutamine, citrulline, GABA, B/Ca, Na/Ca, P/Ca, Mg/Ca, K/Ca, and (K+Mg)/Ca were greatly upregulated at the blossom-end tissue region rather than at the stem-end tissue region in BER fruits (Fig. 9B).

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Data availability Data will be made available on request.

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