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# Stimulation of γ-Aminobutyric Acid Production in Vine-Ripe Tomato (*Lycopersicon esculentum* Mill.) Fruits under Modified Atmospheres

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Stimulation of γ-aminobutyric acid (GABA) production under low $O_2$ and high $CO_2$ conditions (adjusted aerobic atmosphere) under which ethanol fermentation could be avoided was studied. Vine-ripe tomato fruits were stored under hypoxia conditions and adjusted aerobic atmospheres as well as in the air at 15 °C for 13 days and at 30 °C for 6 days. At 30 °C tomato fruit GABA concentration under the adjusted aerobic atmosphere ($O_2$ 11%, $CO_2$ 9%) was significantly higher by 48% than that in air after 6 days from the start of storage. Increased accumulation of alanine under the adjusted aerobic atmosphere supports the observation that this atmosphere stimulates GABA production. The results demonstrate that the concentration of GABA as a beneficial substance for antihypertensive effects and so on can be increased by storing tomato fruits under adjusted aerobic atmospheres for the first time.

**KEYWORDS: Tomato; *Lycopersicon esculentum* Mill.; γ-aminobutyric acid; modified atmosphere packaging**

## INTRODUCTION

γ-Aminobutyric acid (GABA) is an inhibitory amino acid that is widely distributed throughout the biological world (*1*). It has been reported to have relaxation and immunity enhancement effects (*2*), as well as antihypertensive and natriuretic effects (*3*). Therefore, accumulation of GABA in several food types, including rice (*Oryza sativa* L.) kernel (*4*), tea [*Camellia sinensis* (L.) O. Kuntze] (*5*), and soybean [*Glycine max* (L.) Merrill] (*6*) has been studied.

GABA is mainly produced by glutamate decarboxylase (GAD, EC 4.1.1.15) reaction catalyzing decarboxylation from glutamate (*7*). This suggests that production of GABA is promoted in organisms with a high concentration of glutamate as a main substrate of the enzymatic reaction. In the current study, tomato (*Lycopersicon esculentum* Mill.) fruits were selected for stimulating GABA production because they contain high glutamate levels (*8*). Previous results have shown that high levels of GABA accumulate rapidly in plant tissues exposed to a variety of different stresses (*1*). Stress from anoxia is reported to be effective for stimulating the production of GABA (*1*); however, horticultural products stored under anoxic/hypoxic conditions generate off-odors during ethanol fermentation and lose their commercial value (*9*). The objective of this study was to accumulate GABA by storing tomato fruits using modified atmosphere packaging (MAP), a technique used to prolong the shelf life of horticultural products, in which ethanol fermentation could be avoided (*9*).

## MATERIALS AND METHODS

**Materials.** *Horticultural Product.* Vine-ripe tomato fruits (*L. esculentum* Mill., Kagome original brand, Round 1 cultivar) harvested November 23, 2006, at Iwaki Onahama Vegetable Farm Ltd. (Fukushima prefecture, Japan) were sampled. After the harvest, the samples were maintained at 10−16 °C and were transported to the laboratory within about 24 h and stored in a constant-temperature unit (15 °C) for another 24 h before being used. The average mass of the 114 pieces of fruit was 0.110 kg (SD ± 0.009; range = 0.092−0.133 kg). Intact fruits without any pretreatments were used for the following experiments.

*Pouches.* Three-layer laminated [polyethylene terephthalate (PET)/aluminum/polyethlene] high-barrier pouches Lamizip (AS ONE Corp., Tokyo, Japan) ($O_2$ permeance = $1.7 \times 10^{-5}$ mmol·m$^{-2}$·h$^{-1}$·kPa$^{-1}$, water vapor transmission rate = 1.0 mmol·m$^{-2}$·h$^{-1}$, film thickness = 110 μm; surface area = 0.056 m$^2$) were used to create hypoxic conditions with high $CO_2$ levels around tomato fruits. Microperforated PET/low-density polyethylene (LDPE) film pouches ($O_2$ permeance = 0.1 mmol·m$^{-2}$·h$^{-1}$·kPa$^{-1}$, water vapor transmission rate = 10.0 mmol·m$^{-2}$·h$^{-1}$, film thickness = 47 μm; surface area = 0.0588 m$^2$) and microperforated polypropylene film pouches ($O_2$ permeance = 0.25 mmol·m$^{-2}$·h$^{-1}$·kPa$^{-1}$, water vapor transmission rate = 12.5

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**Table 1.** Mean Values and Standard Deviations (SD) of Tomato Fruits[a] in Each of the Storage Methods

| | | storage time | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 days[b,c] ($n = 6$) | | 5 days[b] or 2 days[c] ($n = 6$) | | 9 days[b] or 4 days[c] ($n = 6$) | | 13 days[b] or 6 days[c] ($n = 6$) | |
| storage temperature (°C) | pouch | mean (g) | SD (g) | mean (g) | SD (g) | mean (g) | SD (g) | mean (g) | SD (g) |
| 15 | macro-perforated | 108.8 | 9.7 | 106.6 | 5.9 | 110.5 | 10.9 | 110.7 | 14.3 |
| | three-layer laminated high barrier | 108.8 | 9.7 | 107.1 | 7.3 | 109.3 | 7.2 | 108.4 | 10.0 |
| | microperforated | 108.8 | 9.7 | 111.7 | 8.7 | 108.3 | 5.5 | 107.4 | 8.1 |
| 30 | macro-perforated | 108.8 | 9.7 | 105.2 | 7.6 | 113.8 | 10.4 | 106.5 | 4.8 |
| | three-layer laminated high barrier | 108.8 | 9.7 | 113.7 | 10.2 | 109.4 | 7.1 | 111.9 | 10.0 |
| | microperforated | 108.8 | 9.7 | 119.4 | 5.7 | 111.4 | 4.9 | 109.7 | 10.9 |

[a] There was no significant difference ($P < 0.05$, Tukey's honestly significant difference test) among all mean values. [b] Storage days at 15 °C. [c] Storage days at 30 °C.

mmol·m$^{-2}$·h$^{-1}$, film thickness = 25 $\mu$m; surface area = 0.0588 m$^2$) were used to contain an aerobic controlled atmosphere ($O_2$ 11% and $CO_2$ 9%) available for storage of tomato fruits (*10*) at 15 and 30 °C, respectively. The atmospheric conditions were created as described previously (*11*). Macroperforated pouches (the same PET/LDPE film as the microperforated pouch with 5 mm o.d. × eight holes) were used as a control in which the in-package atmosphere was expected to be the same as the ambient air.

**Methods.** *Storage of Tomato Fruits.* Two tomato fruits were sealed in a pouch containing 10 g of $C_2H_4$ absorbent (*12*) using an impulse sealer. Fifty-four pouches including $C_2H_4$ absorbent were prepared and stored at 15 °C for 13 days and at 30 °C for 6 days (**Table 1**) in dark constant-temperature units. The storage temperature of 15 °C was selected as a level effective for the keeping quality of tomato fruits according to Nakhasi et al. (*13*) On the other hand, the storage temperature of 30 °C was selected to stimulate enzymatic reactions associated with GABA production. Storage periods at each temperature were determined subjectively with storage terminated when the fruit visually lost its commercial value in air caused by softening (*14*) or fungi (*15*). It was presumed that differences of relative humidity among the pouches did not affect the stimulation of GABA production because the humidity was kept at a level which could not stimulate GABA production caused by water stress (*16*).

*Analysis of In-Package Atmospheres.* Aliquots (300 $\mu$L) of in-package gas were sampled over time using a gastight syringe inserted through a silicone rubber septum stuck on the pouch. $O_2$, $CO_2$, and $C_2H_4$ concentrations in the sampled gas were measured by gas chromatography as reported by Makino et al. (*11, 17*)

*Analysis of Free Amino Acid Concentrations in Tomato Fruits.* All tomato samples were analyzed for free amino acid concentrations. A hull-less fruit, cooled with crushed ice, was homogenized, and the protein in an aliquot (0.5 g) of the homogenate was denatured by adding 0.5 mL of trichloroacetic acid. The mixed solution was centrifuged at 2300*g* for 10 min. An aliquot (0.04 mL) of supernatant was diluted by 0.96 mL of 0.02 M HCl (pH 2.2) and filtered using a membrane filter (0.2 $\mu$m pore size). Concentrations of free amino acids in the treated samples were analyzed using a JLC-500/V2 Amino Acid Analyzer (Japan Electron Optics Laboratory Ltd., Tokyo, Japan) at the Chemical Analysis Division of the Research Facility Center for Science and Technology of the University of Tsukuba (Tsukuba, Ibaraki, Japan). Amino acid concentrations were expressed on the basis of fresh weight.

*Statistical Analysis.* JMP7.0.1 software (SAS Institute Inc., Cary, NC) was used for statistical analysis. When between-class variation was significant at $P < 0.05$ using one-way analysis of variance (ANOVA) of the data, mean values were compared by the least significant difference (LSD) test ($P < 0.05$).

## RESULTS

Gas composition in the macroperforated pouch was the same as that in the surrounding air regardless of temperature. Changes in $O_2$ and $CO_2$ concentrations in the high-barrier and microperforated film pouches over time are shown in **Figure 1**. In the high-barrier pouch at 15 °C, the mean $O_2$ concentration decreased from approximately 21 to 3.4% after 120 h of storage

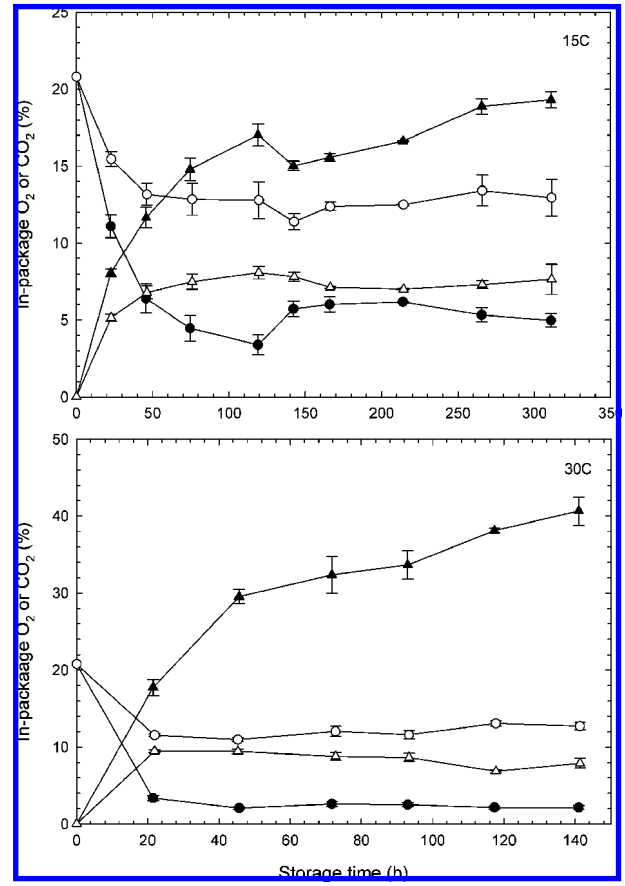


**Figure 1.** In-package atmosphere changes in pouches at 15 and 30 °C ($n = 3$). Open and solid symbols denote microperforated film and three-layer laminated high barrier pouches, respectively. Circles and triangles denote in-package $O_2$ and $CO_2$ concentrations, respectively. Mean ± SE of three observations has been plotted. In-package $O_2$ and $CO_2$ concentrations were significantly different ($P < 0.05$; one-way ANOVA with LSD test) among pouches in each temperature and in the same time interval.

and remained at about 5% until the end of storage (312 h). The mean $CO_2$ concentration increased from 0 to 12% after 46 h and gradually increased to 19% at the end of storage. At 30 °C, the mean $O_2$ concentration decreased to 3.4% after 20 h from the start of storage and remained at about 2% until the end of storage (144 h). The $CO_2$ concentration increased to 18% after 22 h of storage and continued to increase until reaching 41% at the end of storage. In the microperforated film pouches at 15 °C, a steady state was reached after 50 h from the start of storage. Mean $O_2$ and $CO_2$ concentrations of 12 and 8%, respectively, were maintained until the end of storage (312 h). At 30 °C, a steady state was reached after 20 h, and mean $O_2$

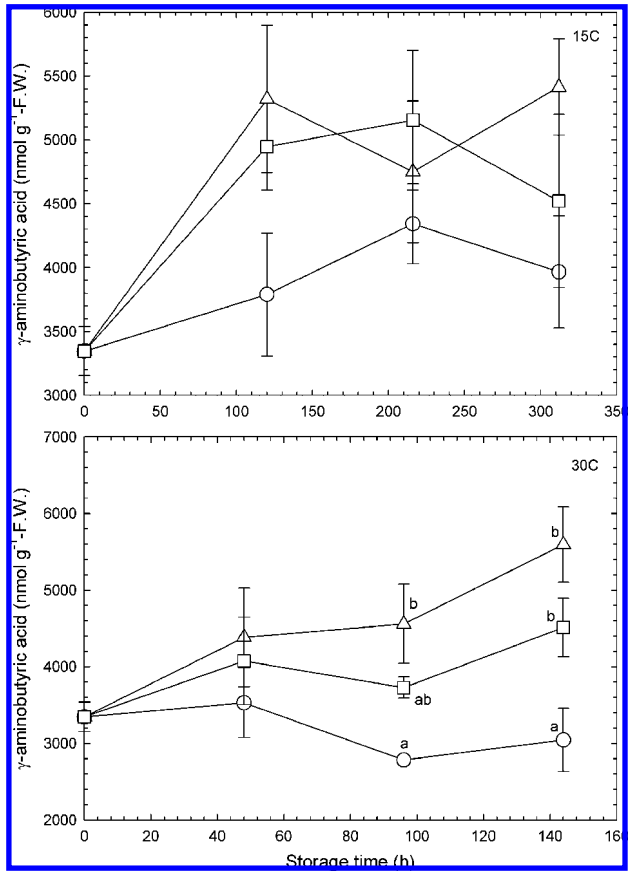


**Figure 2.** Changes in $\gamma$-aminobutyric acid in tomato fruit at 15 and 30 °C ($n = 6$). Circles, triangles, and squares denote values under ambient air, hypoxic conditions with high $CO_2$ levels, and adjusted aerobic (low $O_2$ and high $CO_2$ conditions) atmospheres, respectively. Mean ± SE of six observations has been plotted. Within the same time interval, symbols followed by the same letter are not significantly different ($P < 0.05$; one-way ANOVA with LSD test).

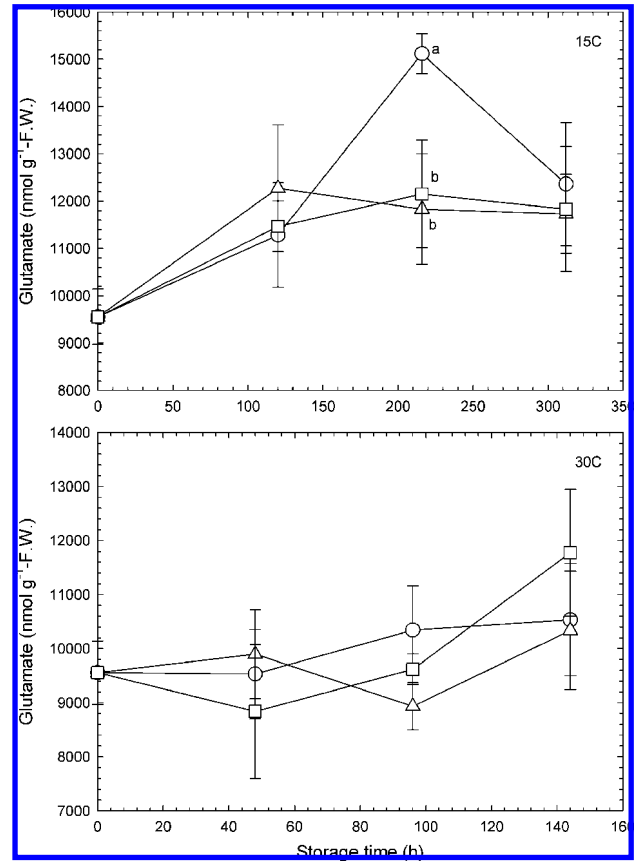


**Figure 3.** Changes in glutamate in tomato fruit at 15 and 30 °C ($n = 6$). Circles, triangles, and squares denote values under ambient air, hypoxic conditions with high $CO_2$ levels, and adjusted aerobic (low $O_2$ and high $CO_2$ conditions) atmospheres, respectively. Mean ± SE of six observations has been plotted. Within the same time interval, symbols followed by the same letter are not significantly different ($P > 0.05$; one-way ANOVA with LSD test).

and $CO_2$ concentrations of 11 and 9%, respectively, were maintained until the end of storage (144 h). The gas composition in the storage packaging was, regardless of storage temperatures, hypoxic in the high-barrier pouch and aerobic in the microperforated film pouch. $C_2H_4$ concentrations were kept at 0 ppm in all of the pouches. Therefore, in-package $C_2H_4$ did not affect the experimental data.

Changes in GABA concentrations over time are shown in **Figure 2**. At 30 °C, the GABA concentration was significantly higher in the high-barrier pouch than in the macroperforated pouch after 96 h of storage. In addition, GABA concentrations were significantly higher in both the high-barrier and microperforated film pouches than in the macroperforated pouches after 144 h. At 15 °C, no significant differences were observed among the three packaging methods. As the experiment durations differed for the 15 and 30 °C samples, the time available to measure GABA was markedly different. However, when GABA concentrations after 144 h at 30 °C, after 120 h at 15 °C, and after 216 h at 15 °C are compared, the influence of higher temperature on the stimulation of GABA production is apparent. On the other hand, even under hypoxic conditions with high $CO_2$ levels, GABA production was not stimulated at 15 °C. A decrease in storage $O_2$ and an increase in storage $CO_2$ concentrations are considered to promote GABA production, and our results indicate that the effect is influenced by storage temperature.

In the perforated pouches, glutamate concentrations were significantly higher at 216 h of storage at 15 °C (**Figure 3**). No other significant differences were observed among the different packaging pouches at either storage temperature.

After 96 h from the start of storage at 30 °C, the alanine concentration in the microperforated film pouch became significantly higher compared with other pouch types and reached 1134 mmol·g$^{-1}$ of FW at the end of storage (144 h) (**Figure 4**). This level was 2.2 times the value at the end of storage (312 h) at 15 °C. Thus, the alanine concentration was highest under the adjusted aerobic atmosphere, and its concentration was affected by storage temperature.

## DISCUSSION

Among the atmospheres tested in the current study, production of GABA in tomato fruit was most stimulated under the hypoxic condition created by the high-barrier pouches (**Figure 2**), similar to the observations in a study on tea (*5*). Under the adjusted aerobic atmospheres created in the current study, tomato fruits respired without ethanol fermentation as reported by Geeson et al. (*10*). Although no reports were found concerning stimulation of GABA production by weak $O_2$ stress under aerobic conditions, we observed that GABA concentration in the adjusted aerobic atmosphere was significantly higher than that observed in ambient air at 30 °C. Kinnersley and Turano (*1*) reported that the GAD reaction is stimulated by the acidification of plant tissue. GABA is predominantly produced by the GAD reaction,

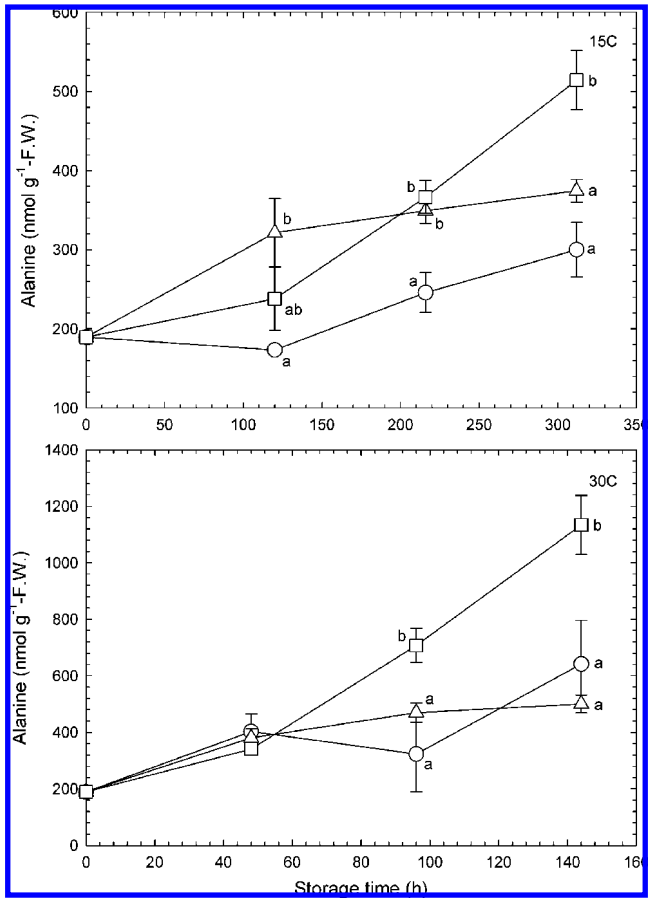


**Figure 4.** Changes in alanine in tomato fruit at 15 and 30 °C ($n = 6$). Circles, triangles, and squares denote values under ambient air, hypoxic conditions with high $CO_2$ levels, and adjusted aerobic (low $O_2$ and high $CO_2$ conditions) atmospheres, respectively. Mean ± SE of six observations has been plotted. Within the same time interval, symbols followed by the same letter are not significantly different ($P > 0.05$; one-way ANOVA with LSD test).

which is an irreversible reaction involving consumption of $H^+$. An accumulation of $H^+$ reduces the pH and stimulates the GAD reaction. Reduction of $O_2$ concentration around tomato fruits might result in decreased oxidation of $H^+$ on an active site of cytochrome *c* oxidase as the terminal enzyme in the respiratory chain (*18*).

Glutamate is a main substrate in the GAD reaction, which suggests that the GAD reaction may be stimulated with an increase of glutamate. This is supported by a study using rice kernel (*4*). If glutamate is consumed to produce GABA, the concentration of glutamate would decrease with GABA accumulation, as has been demonstrated using radish leaves (*19*). However, the relationship between the concentrations of GABA and glutamate in the current study remains unclear (**Figures 2** and **3**). Glutamate is produced from α-ketoglutarate by glutamate dehydrogenase (GDH, EC 1.4.1.2) and GABA transaminase (GABA-T, EC 2.6.1.19) reactions as well as being consumed by the GAD reaction (*7*). The obscurity of this relationship may be related to the complex metabolic pathways involved. Streeter and Thompson (*19*) demonstrated that glutamate might be provided by decomposition of proteins in the case of long-term incubation in their paper. Therefore, they conducted incubation of trimmed radish leaves for 6 h at room temperature to detect a decrease in glutamate with an increase in GABA. In the current study, intact tomato fruits were stored for 6 days at 30 °C and for 13 days at 15 °C. Liu and Luh (*8*) reported that concentrations of GABA and glutamate in tomato paste from harvest-delayed fruit were elevated with the delay of harvest. Glutamate may be increased by the reaction of nitrogen fixation, known as the glutamine synthetase (GS, EC 6.3.1.2)−glutamate synthase (GOGAT, EC 1.4.1.13) cycle, during cultivation. Differences in sample storage conditions appear to be the cause of the differences in the glutamate data mentioned above.

Streeter and Thompson (*19*) reported that alanine and GABA were accumulated in radish leaves under anoxic/hypoxic conditions. In the current study, alanine accumulated the most under the adjusted aerobic atmospheres (**Figure 4**). Miyashita et al. (*20*) reported that $O_2$ stress promoted the decomposition of accumulated alanine by alanine aminotransferase (AlaAT, EC 2.6.1.2) in *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.]. This suggests that alanine may be decomposed under hypoxic conditions through strong $O_2$ stress and be maintained under adjusted aerobic atmospheres through weak $O_2$ stress. Accumulation of alanine in tomato fruits under the adjusted aerobic atmosphere supports the observation that atmospheric conditions stimulate the production of GABA (*19*).

The results demonstrate that GABA production can be stimulated by storing tomato fruit under an adjusted aerobic atmosphere in which ethanol fermentation can be avoided. In general, freshness is an important quality of horticultural products, which is known to degrade with postharvest storage time. Cold storage, controlled atmosphere, MAP, and 1-methylcyclopropene are conventional techniques applied to slow the deterioration of horticultural products. In this paper, an unconventional approach to increase the concentration of GABA as a beneficial substance for maintaining postharvest horticultural product quality using MAP was demonstrated. According to the research reported by Inoue et al. (*21*), a dose (10−12 mg) of GABA for 12 weeks to people with mild hypertension was effective for lowering their systolic and diastolic blood pressures by 17.4 ± 4.3 and 7.2 ± 5.7 mmHg. The dose is equivalent to one-fifth of a fruit stored under adjusted aerobic atmosphere created by MAP or to one-third of a fruit stored in the air at 30 °C for 6 days in the current study. This suggests that MAP is useful for increasing the antihypertensive effect of tomato fruits. However, the temperature (30 °C) effective for stimulating GABA production may be unsuitable for the storage of tomato fruits. Therefore, other temperatures within the range of 15−30 °C, which may be effective for stimulating GABA production and maintaining product quality, should be researched.

GABA production in vine-ripe tomato fruits under low $O_2$ and high $CO_2$ conditions (adjusted aerobic atmosphere) was stimulated significantly more than in air at 30 °C. Accumulation of alanine under the adjusted aerobic atmosphere supports the observation that this atmosphere type is effective for stimulating GABA production. However, the relationship between glutamate, a main enzymatic substrate of GAD, and GABA concentrations remains unclear because of the complex metabolic pathways in the production of glutamate.

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