Relations between intracellular pH and γ-aminobutyric acid levels in tea (*Camellia sinensis*) leaves, after two types of anaerobic treatment

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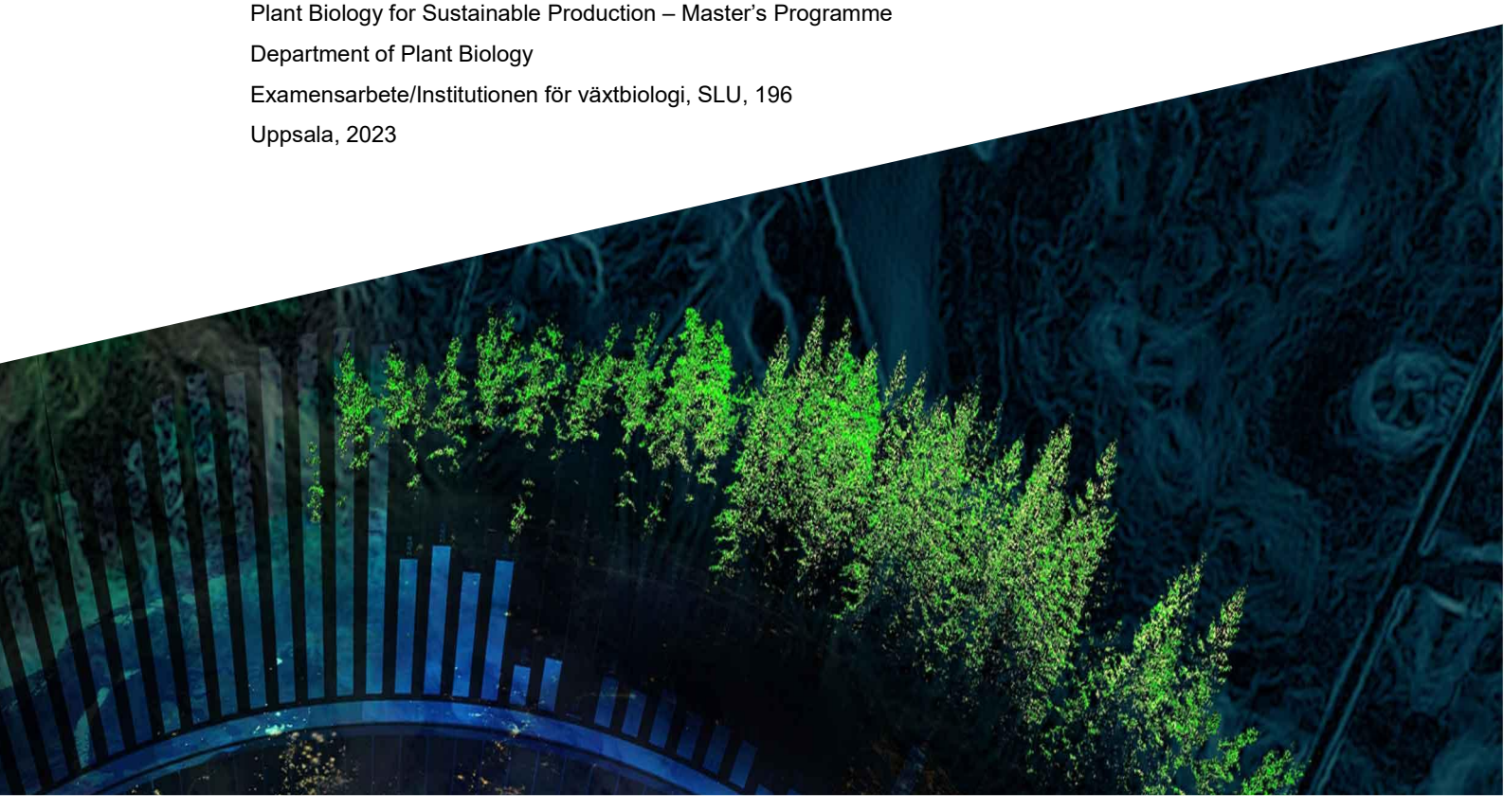

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Abstract

Tea (*Camellia sinensis*) is one of the three major non-alcoholic beverages in the world. In Gabaron tea, the levels of γ-aminobutyric acid (GABA) are more than 1.3 mg/g. In the human body, GABA lowers blood pressure and cholesterol levels. GABA in plants is mainly produced by two pathways: the glutamate (Glu) decarboxylation pathway (dominated by Glu decarboxylase; GAD), and the polyamine oxidation pathway (dominated by polyamine oxidase; PAO). In previous studies, it was demonstrated that GABA in tea tissue originates mainly from Glu decarboxylation. Intracellular pH is an important regulator of cell physiological activities. Intracellular pH influences enzyme activity and important metabolic processes, including DNA replication, RNA and protein synthesis, as well as cell growth.

The purpose of this project was to develop a detection method for intracellular pH of tea leaves, use the method to analyze the relationship between GABA enrichment and pH change during the production of Gabaron tea.

To this end, fresh tea leaves (*Longjin 43*) were picked and exposed to two kinds of anaerobic treatment (N_2, CO_2). The intracellular pH of the upper and lower mesophyll cells of the two kinds of anaerobically treated tea leaves was measured by fluorescent dye (BCECF/AM). Combined with the changes of pH and the levels of two amino acids (Glu, GABA), the relationship between N_2 and CO_2 and intracellular pH during GABA enrichment was followed.

The results showed that after different anaerobic treatments (N_2 or CO_2), the pH value of cells in the upper and lower mesophyll of tea leaves varied depending on treatment.

For tea leaves treated with CO_2, the pH in the upper and lower mesophyll cells increased after 1 h, and returned to normal after 6 h. Combined with the levels changes of the two amino acids, it is speculated that under high concentration of CO_2, the stomata of tea leaves were still open, and anaerobic respiration was carried out at this time. Excessive CO_2 causes the internal environment of cells to become acidic. At this time, GAD is activated. GAD consumes a lot of protons and produces GABA, which reduces damage of the cell membrane under acidic conditions.

For tea leaves treated with N_2, the intracellular pH of the upper mesophyll first increased and then decreased, while the intracellular pH of the lower mesophyll first decreased and then increased. It is speculated that in the high concentration of N_2, the stomata of the lower mesophyll are closed, the tricarboxylic acid cycle is inhibited, and protons cannot be consumed, resulting in the acidic cytoplasm. After 6 h of treatment, GAD is activated, consuming protons and producing GABA.

Future work should focus on pH determination after short-term anaerobic treatment. The GAD activity during anaerobic treatment and the detection of stomatal opening and closing of upper and lower epidermis during different anaerobic treatments are also necessary experiments to verify the hypothesis.

Keywords: Tea leaves, intracellular pH, γ-Aminobutyric acid, glutamate, decarboxylase, glutamate, fluorescent dye

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Abbreviations

ABA	Abscisic acid
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine triphosphate
BCECF	2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein
CDCF	5-(and-6-) carboxy-2',7"-dichlorofluorescein
CF	5-(and-6-) carboxyfluorescein
CO_2	Carbon dioxide
cSNARF-1	Carboxy seminaphtorhodfluor
CTK	Cytokinin
DAO	Diamine oxidase
DMO	5,5-dimethyl-oxazolidine dione
DMSO	Anhydrous dimethyl sulfone
GA	Gibberellic acid
GABA	γ-aminobutyric acid
GABA-T	GABA aminotransferase
GAD	L-glutamate decarboxylase
Gln	Glutamine
Glu	Glutamate
His	Histidine
IAA	Indoleacetic acid
KADH	α-ketoacid dehydrogenase complexes
Leu	Leucine
MeJA	Methyl jasmonate
N_2	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
PAO	Polyamine oxidase
PEP	Phosphoenolpyruvate
pH_i	Intracellular pH
SSA	Succinic acid semialdehyde
SSADH	Succinate-semialdehyde dehydrogenase
Trp	Tryptophan

1. Introduction

1.1 Introduction of γ-aminobutyric acid

1.1.1 Properties and characteristics of GABA

γ-aminobutyric acid (GABA) is a four carbon non-protein amino acid that is present in prokaryotes and eukaryotes. Its molecular formula is $NH_2CH_2CH_2CH_2COOH$ ($C_2H_2NO_2$), and the molecular weight is 103.12. There is an amino group on the γ-carbon, which exists in the form of uncombined state. It is very soluble in water, but insoluble in alcohol, ether and benzene. It has several configurations in the cytosol and can form a ring structure similar to proline (Christensen *et al*., 1994). GABA is an amphoteric ion with positive and negative charges at physiological pH (4.03 ~ 10.56).

GABA is a major inhibitory neurotransmitter in the central nervous system of mammals, and mainly in the brain and GABAergic nerves. It mainly interacts with other neurotransmitters through three receptors ($GABA_A$, $GABA_B$ and $GABA_C$) in the postsynaptic membrane to participate in the information transmission of neural pathways and the regulation of multiple physiological functions in animals (Bormann et al., 1995).

GABA widely exists in plants and fungi and participates in stress metabolism, seed vitality and cell development (Bown *et al*., 2016). In plant cells, it is an important part of the free amino acid pool.

1.1.2 GABA and human health

GABA is a non-essential amino acid for humans, but it can be used for treatment of diseases or for improving human function from many ways. It mediates more than 40% of the inhibitory nerve conductions in human brain. In animals, presynaptic nerve terminals synthesize and secrete GABA into synaptic space, activate postsynaptic GABA receptors, lead to the opening of Cl^- channels, cause

postsynaptic membrane hyperpolarization, hindering neuronal overdischarge and postsynaptic polarization (Olsen *et al*., 1991). Therefore, GABA is of great significance in regulating the nervous system. It has also been found that GABA has significant effects on reducing blood pressure (Hata *et al*., 1994; Unger *et al*., 1984; Lin *et al*., 2002), treating epilepsy (Hang *et al*., 1994) and hepatic encephalopathy (Zhang *et al*., 2005), improving sleep and enhancing memory (Olsen *et al*., 1991).

1.1.3 Metabolism of GABA in higher plants

It has been shown that GABA is mainly distributed in seeds, roots and stems of plants. It widely exists in various plants and plant tissues. The levels of GABA increase under various environmental conditions such as hypoxia, cold injury, thermal stimulation, mechanical stimulation, drought and salt stress (Perez Alfocea *et al*., 1994; Bown *et al*., 2016; Narayan *et al*., 1990). The mechanism of GABA accumulation under stress, and its effect on plants to alleviate stress, have attracted people's attention. Under normal conditions, the levels of GABA in plants is about 0.03-32.5 μmol/g, and when plants are experiencing stress, the levels of GABA can increase several to dozens of times (Song *et al*., 2010).

GABA biosynthesis in plants is mainly catalyzed by L-glutamate decarboxylase (GAD) with synthesis by decarboxylation at the α position, and the reaction is irreversible. L-Glu forms GABA under the catalysis by L-GAD, and then GABA undergoes transamination with pyruvate under the action of GABA aminotransferase (GABA-T) to produce succinic acid semialdehyde (SSA) and alanine (Ala). Finally, SSA is catalyzed by succinic semialdehyde dehydrogenase (SSADH) to produce succinic acid, which enters the tricarboxylic acid cycle. This metabolic pathway is called the GABA shunt (Figure 1).

Glu is an important precursor for the synthesis of GABA. The amount of Glu and transformation ability are important factors for the levels of GABA in plants. After soybean and asparagus were fed with Glu and CO_2, which both contained ^{14}C, the synthesized GABA contained 78% of the radioactivity of ^{14}C from Glu and CO_2 (Chung *et al*., 1992; Tuin *et al*., 1994). This indicates that most GABA synthesized in plants comes from Glu. GAD widely exists in plants and specifically uses L-Glu as substrate. The optimum pH is about 5.8 (Bown *et al*., 2016).

Another way to synthesize GABA is the polyamine oxidation pathway dominated by polyamine oxidase (PAO) and diamine oxidase (DAO). DAO catalyzes putrescine to form pyrroline, H_2O_2 and H^+, while PAO catalyzes spermidine and spermine to form pyrroline, H_2O_2, diaminopropane and 1 - (3-propylamino) 2-

pyrroline. Pyrroline can form GABA under the catalysis of pyrroline dehydrogenase (Tiburcio *et al*., 1997; Bhatnagar *et al*., 2001). In previous studies (Chen et al., 2018), we found that GABA synthesis in tea plants is mainly formed by GAD- dominated Glu decarboxylation rather than by the PAO- and DAO-dominated polyammoxidation pathways.

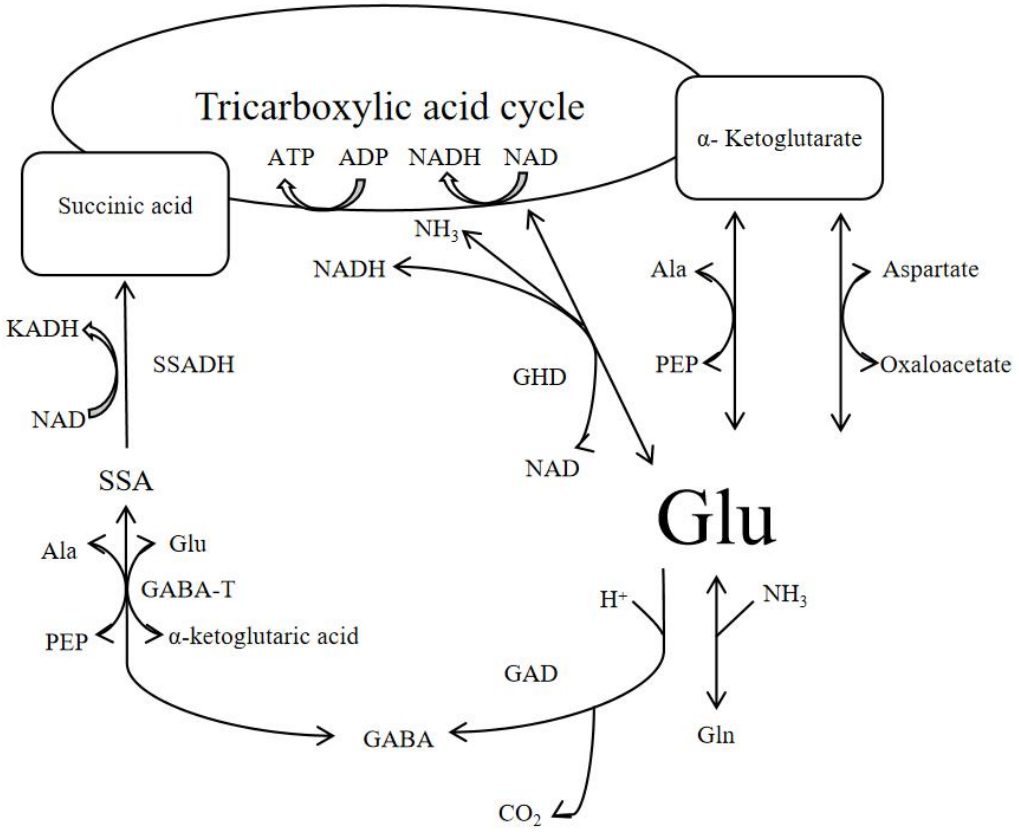

Figure 1. Relationship between the GABA shunt and the tricarboxylic acid cycle, the metabolic pathway from α-ketoglutarate to succinate through Glu, GABA and SSA is called GABA Shunt.

1.2 Gabaron tea

1.2.1 Research progress of gabaron tea

Tea (*Camellia sinensis*) is one of the three major non-alcoholic beverages in the world, and many active ingredients in tea are good for human health. The levels of GABA in ordinary tea is usually 0.002-0.206 mg/g, but due to the different varieties and picking time of tea plants, the content of GABA in tea leaves varies greatly. In 1987, Tojiro Tsushida found that a large amount of GABA will be produced after anaerobic treatment of freshly picked tea leaves. He named this tea

Gabaron tea (Tsushida *et al*., 1987). A large number of animal experiments and clinical experiments have proven that this tea has a good effect of reducing blood pressure.

Animal experiments have proven that the antihypertensive effect of gabaron tea on spontaneously hypertensive rats is better than that of ordinary green tea (Qian *et al*., 2008). Clinical trials also show that gabaron tea has a good effect on insomnia patients (Cheng *et al*., 2009).

The increase of GABA levels in tea is mainly realized in two ways. The first way is to find tea tree species with GABA-rich leaves, but the cost of this method is high. The second method is to increase the GABA levels in fresh tea through a special production process, where anaerobic treatment is the most common method in industrial production (Wang *et al*., 2009).

1.2.2 Enrichment of GABA in tea leaves by anaerobic treatment

There are usually three kinds of anaerobic treatment of tea leaves, namely CO_2, N_2 and vacuum. After fresh leaves were treated with N_2 for 6 h, the levels of GABA in tea increased from 0.3 mg/g to 2.0 mg/g; After treatment with N_2 or CO_2, at 5 h, the GABA levels were 1.7 mg/g and 1.8 mg/g, respectively (Ling *et al*., 2004; Sawai *et al*., 2001). The temperature during anaerobic treatment had little effect on the level of GABA, but had a great effect on tea quality. When GABA tea is produced in factories, N_2 or CO_2 is usually used for 5-10 h.

1.3 Plant intracellular pH

1.3.1 Effect of intracellular pH

Intracellular pH (pH_i) is an important regulatory factor of cell physiological activities. Intracellular pH can not only regulate enzyme activity and metabolic processes, eig., ATP synthesis, DNA replication, RNA and protein synthesis, but also physiological processes such as cell growth (Fukuda *et al*., 2004; Molina-Gutierrez *et al*., 2002).

Plant vacuoles are usually weakly acidic and occupy more than 90% of the space of plant cells. They regulate the pH of cytoplasm and the dynamic balance of ions through the ion transport over the vacuole membrane (Taiz *et al*., 1992). More and more experiments show that the change of intracellular pH value acts as a secondary messenger and plays a signal role in physiological activities such as apical growth, root tropism, nodulation, plant defence, induction of phytoalexin,

and the responses to plant hormones (such as GA and ABA) (Scott *et al*., 2004). Generally, the pH value in the cytoplasm of most plants is close to neutral, and the pH value in the vacuole is maintained in the range of 5.0 ~ 6.5. There will be some changes of pH value under special physiological conditions or under the action of external environmental factors, but plant cells can maintain the steady state of intracellular pH through plasma membrane reverse transporters (Na^+/H^+ or K^+/H^+), proton pumps, anion exchange and the production or consumption of organic acids (Fukuda *et al*., 2004). When studying cell functions, the intracellular pH is an important parameter. Therefore, how to accurately detect the pH value in plant cells is of great significance.

1.3.2 Method for detecting pH in plant cells

For plant cells, the regulation of intracellular pH is an important physiological parameter. There are five main methods for measuring intracellular pH in plants: weak acid and base distribution, the commonly used weak acid is 5,5-dimethyl-oxazolidine Dione (DMO); nuclear magnetic resonance (NMR) spectroscopy; microelectrodes; fluorescence dyes, the most widely used ones are 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and carboxy seminaphtorhodfluer (carboxy SNARF-1); the least commonly used method is cell sap extract (Table 1). These five methods have their own advantages and disadvantages, so it is necessary to choose an appropriate method to determine the pH in plant cells.

The most popular method for determining pH is the use of fluorescent dyes. The optimal spectra of different fluorescent dyes differ (Table 2).

Table 1. Intracellular pH of different plant materials and their detection methods used

Plant species	Method	Cytoplasmic pH**	Vacuole pH
Higher plant cell			
Suspension culture			
Acer pseudoplatanus[a]	^{31}P NMR	7.5	5.7
Acer pseudoplatanus[a]	^{31}P NMR	6.6*	6.3*
Eschscholzia californica[b]	SNARF	7.1~7.4	5.5~6.3
Organs			
Root			
Zea mays[c]	^{31}P NMR	7.0~7.2	5.6
Sinapis alba[d]	Microelectrodes	7.3	4.6
Raphanus sativus[e]	Microelectrodes	7.2	6.0
Arabidopsis thaliana[f]	BCECF	7.22	-
Leaf			
Zea mays[g]	SNARF	6.6	-
Elodea densa[h]	DMO	7.5	5.3
Algal cell			
Prorocentrum micans[i]	SNARF	7.7	-

* indicates that plant cells have been treated anaerobically for more than 6 h.

**Cytoplasmic pH refers to the pH of cytoplasm (including vacuoles), and vacuole pH refers to the pH of cell fluid

(*a*: Gout *et al*., 2004; *b*: Roos *et al*., 1998; *c*: Gerendás *et al*., 2000; *d*: Felle *et al*., 1987; *e*: Strack *et al*., 1987; *f*: Scott *et al*., 1999; *g*: Johannes *et al*., 2001; *h*: Marrè *et al*., 1987; *i*: Nimer *et al*., 1999)

Table 2. Optimum spectra of different fluorescent dyes

Dye name	pK_a *	Best fit spectrum	
		Excitation wavelength (nm)	Emission wavelength (nm)
Carboxy SNAFL-1 (cSNAFL-1)	7.8	508/540	543/632
SNARF calcein	7.2	522/574	535/625
BCECF	7.0	482/503	520
5-(and-6-) carboxyfluorescein (CF)	6.4	475/492	517
5-(and-6-) carboxy-2',7''-dichlorofluorescein (CDCF)	4.8	488/504	529
Oregon green	4.7	478/492	518

*: Acidity coefficient, also known as acid dissociation constant, in chemistry and biochemistry, refers to a specific equilibrium constant to represent the ability of an acid to dissociate hydrogen ions

1.4 Research Progress on the relationship between GABA and intracellular pH in plants

GAD catalyzes the decarboxylation of Glu to form GABA, and this process requires H^+. Therefore, it is considered that stress-induced formation of GABA plays a role in the regulation of pH, and GABA synthesis can improve the cytoplasmic acidic environment (Perez *et al*., 1994). Early NMR studies confirmed that the decrease in cytoplasmic pH in maize root tips under anaerobic conditions is related to the instantaneous production of lactic acid (Roberts *et al*., 1994). However, it has also been hypothesized that the main acid production reaction stops after a large amount of GABA has accumulated, and two different studies support the accumulation of GABA in response to cytoplasmic acidification. First, the changes of cytoplasmic pH and GABA accumulation in photosynthetic cells of asparagus plants exposed to weak acids were detected by fluorescent pH probe and GABA enzymatic analysis. It was found that the cytoplasmic pH decreased by 0.6 within 1 second, while the GABA level increased by 200% - 300% within 15 seconds. After 45 seconds of weak acid treatment, GABA synthesis consumed about 50% of the acid (Crawford *et al*., 1994). Second, when ^{31}P and ^{15}N NMR spectroscopy was used to detect the cytoplasmic pH and GABA level of carrot tissue culture cells *in vivo*, it was found that the initiation of ammonia assimilation reaction could reduce the cytoplasmic pH by 0.5% (Carroll *et al*., 1994). The activity of GAD increased with the decrease in pH. The acid promoted the synthesis of GABA, which did not involve the flow of Ca^{2+} caused by acidification.

1.5 Thesis projects

There are many available methods for measuring intracellular pH in different plant species, but there are few reports on measuring the intracellular pH specifically of tea. The preliminary aim of this thesis project was:

1. To explore a method to determine accurately the pH in tea cells, so as to further understand the metabolic pathways of tea plants under stress (such as GABA synthesis).

In previous studies, we showed that GABA in tea leaves mainly came from the Glu decarboxylation pathway rather than the polyammoxidation pathway, when Gabaron tea was produced in a factory. In order to further explore the relationship between the GABA enrichment process and the change of Glu level, our further aim was:

2. To accurately detect the changes of intracellular pH in the process of GABA enrichment in tea leaves by anaerobic treatment, and to discuss the relationship between GABA enrichment in tea leaves and pH changes in combination with the changes of Glu and GABA levels.

The results of this project will provide new insights into the GABA enrichment process of tea leaves in the production of Gabaron tea, and also provide direction for studies on the relationship between pH and plant stress in the future.

2. Materials and methods

2.1 Plant materials

Tea leaves (*Longjin 43*) were picked in the autumn of 2021 in the national high tech agricultural park of Anhui Agricultural University, Anhui Province, China (Figure 2).

Longjing 43 is a clonal national variety selected from *Longjing* population by Tea Research Institute of Chinese Academy of Agricultural Sciences. In 1987, it was recognized as a national variety by China Crop Variety Approval Committee. It is a national excellent variety with tall and straight appearance, flat and smooth, light green colour, lasting fragrance and refreshing taste. It is suitable for making both black tea and green tea, especially flat famous green tea. It is suitable for planting in the tea areas in the middle and lower reaches of the Yangtze River. The most obvious features of *Longjing 43* are the strong ability of bud cultivation, early appearance of new leaves and high density (Li L *et al*., 2007).

Figure 2. Longjing 43 tea garden. Photo: Tao Mingmin. High tech Industrial Park, Anhui Agricultural University, Anhui Province, China

2.2 Experimental instruments and materials

All the experimental materials of this project were provided by the State Key Lab of Tea Plant Biology and Utilization, Anhui Agricultural University.

For detecting pH in plant cells (see section 1.3.2), we choose BCECF as the fluorescent dye. The specific machine (e.g, Zeiss L510 meta fluorescence confocal microscope) was first set up to give a good linear relationship between the fluorescence intensity ratio of the BCECF (Thermo Fisher, 99.99%) and pH. Then pH was measured by using BCECF/AM (Thermo Fisher, 99.99%) and the same settings. In this part of the experiment, anhydrous dimethyl sulfone (DMSO) (Thermo Fisher, 100%) that dissolves fluorescent dyes, Pluronic F-127 (Sigma, 100%) that increases the solubility of fluorescent dyes, and 1mol/L phosphate buffer PBS that dissolves protective reagents are used

The experimental instruments were provided by the State Key Lab of Tea Plant Biology and Utilization, Anhui Agricultural University. The model and company of experimental instruments are Laser confocal microscope (L510 Zeiss AG, Germany), Lyophilizer (Fd-2 / Beijing Boyikang Experimental Instrument Co., Ltd, China), Amino acid analyzer (1260 infinity II GPC/SEC / Agilent Technologies Inc，Germany).

2.3 Carbon dioxide or nitrogen treatment of fresh tea samples

In order to balance experiment and factory production (see section 1.2.2), the fresh tea leaves were divided into group A, B and C, each consisting of about 50 g tea leaves. Group C was placed in normal temperature air as control (NT). The leaves were put into sealed glassware, the air was extracted and either carbon dioxide (anaerobic treatment with CO_2, ATC) as group A, or nitrogen (anaerobic treatment with N_2, ATN) as group B, was injected into the glassware until the container was full (Figure 3). After treatment for 1 h or 6 h, the tea was transferred to a 50 ml sampling tube, which was sealed and stored at - 80 ℃ to keep amino acid content and intracellular pH stable.

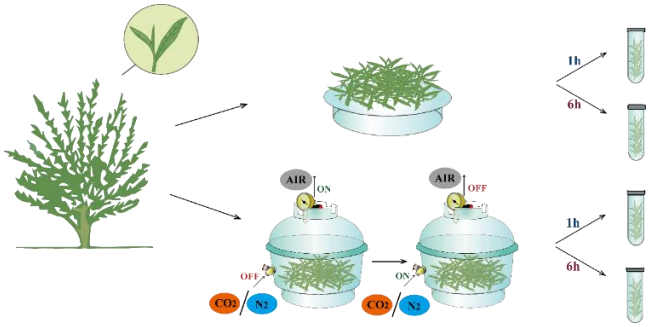

Figure 3. Tea leaf samples were treated using special vent valve and glassware. There was a vent valve connected by three glass tubes above each glassware, which was respectively connected with gas tank (carbon dioxide or nitrogen), vacuum pump and glassware. The vent valve was used to regulate the inlet and outlet of different gases. The gas tank connected to group A was carbon dioxide, while the gas tank connected to group B was nitrogen.

2.4 Detection of Glu and GABA in tea leaves

The tea leaves treated with N_2 and CO_2 were freeze-dried in Petri dishes and ground with liquid nitrogen and PVPP in grinding dishes. Sulphosalicylic acid (4 g) was dissolved in 100 ml of pure water and the ground tea powder (0.04 g of sample) was added to 4 ml of 4 % sulphosalicylic acid, the leaves extracts were further homogenised by ultrasonication for more than 30 min followed by, centrifugation at 12,000 rpm for 30 min, and filtering through an aqueous phase membrane. The filtered supernatant (1 ml) was placed in a brown liquid bottle and the treated sample was assayed by an amino acid analyser (1260 infinity II GPC/SEC / Agilent Technologies Inc，Germany).

The experimental conditions of amino acid analyzer were: separation column, 26 mm × 150 mm stainless steel column; Exchange resin, model, No.2619; Column temperature, 53 ℃; Pump flow rate, 0.225 ml/min; Pump pressure, 8.8 MPa; Injection volume, 50 μL.

The amino acid levels were plotted by GraphPad Prism 5 software (follow GraphPad Prism 5 user manual), and statistically analyzed by Statistical Product and Service Solutions (SPSS) (following the Concise Guide of IBM SPSS Statistics 26). The "Tukey" method was used for multiple comparison, and the significance level was selected as 0.05.

2.5 Standard curve making of BCECF

To determine a method suitable for detecting pH in tea leaf cells, the method requirements are: simple operation, high repeatability, more common experimental instruments, short experimental time (to avoid the impact of long time on the experimental results), which can eliminate the error caused by spontaneous fluorescence. According to the laboratory conditions and previously published methods (Table 3), we determined to use the fluorescent dye BCECF as the experimental method.

Table 3. Comparison of advantages and disadvantages of five methods for measuring intracellular pH in plants

Method	Advantage	Disadvantage
Weak acid and base distribution	Simple operation [a,b]	The sample processing time is long and the sample tissue needs to be damaged [a]
Nuclear magnetic resonance spectroscopy (NMR)	No external molecular probe is required and there is no damage to the sample [c,d]	Low sensitivity and a large amount of biomaterials required [e]
Microelectrodes	Suitable for continuous recording and study of transient changes in pH [a]	The operation is difficult, and errors may be caused by the microelectrode entering organelles by mistake [c,f]
Fluorescence dye	Suitable for cells of various sizes, with good repeatability and simple operation [g]	The dyes easily chelate with organelles, resulting in errors [h]
Cell sap extract	Suitable for tissue culture materials	Cells need to be broken before measuring pH, resulting in easy contamination of extracted cytosol

(*a*: Kurkdjian *et al*., 1989; *b*: De Michelis *et al*., 1979; *c*: Schwiening *et al*., 1999; *d*: Guern *et al*., 1991; *e*: Biagini *et al*., 2001; *f*: Miller *et al*., 2001; *g*: Roos *et al*., 2000; *h*: Parton *et al*., 2000)

To prepare a fluorescent reagent, 0.1 mg BCECF or BCECF/AM was dissolved in 10 mg dimethyl sulfoxide giving a concentration of 10 mg/L. BCECF solution was added to phosphoric acid buffer with different pH values (5.0, 5.4, 5.8, 6.2, 6.6, 7.0). The excitation wavelength of the laser scanning confocal microscope (Zeiss L510) was set as 488 nm, green and red fluorescence channels were chosen, in which the green channel band was 510 ~ 530 nm and the red channel band was

630 ~ 660 nm. The fluorescence intensity at the emission peak wavelengths 520 nm and 640 nm was detected and the fluorescence intensity ratio $F_{640/520}$ was calculated. The parameter setting of the laser scanning confocal microscope was: scanning zoom: 1; Pixels: 512 × 512, the excitation light intensity was 90.

2.6 Determination of intracellular pH of tea leaves

Tea samples from 2.3 were cut perpendicular to the main leaf veins and then placed on slides (Figure 4).

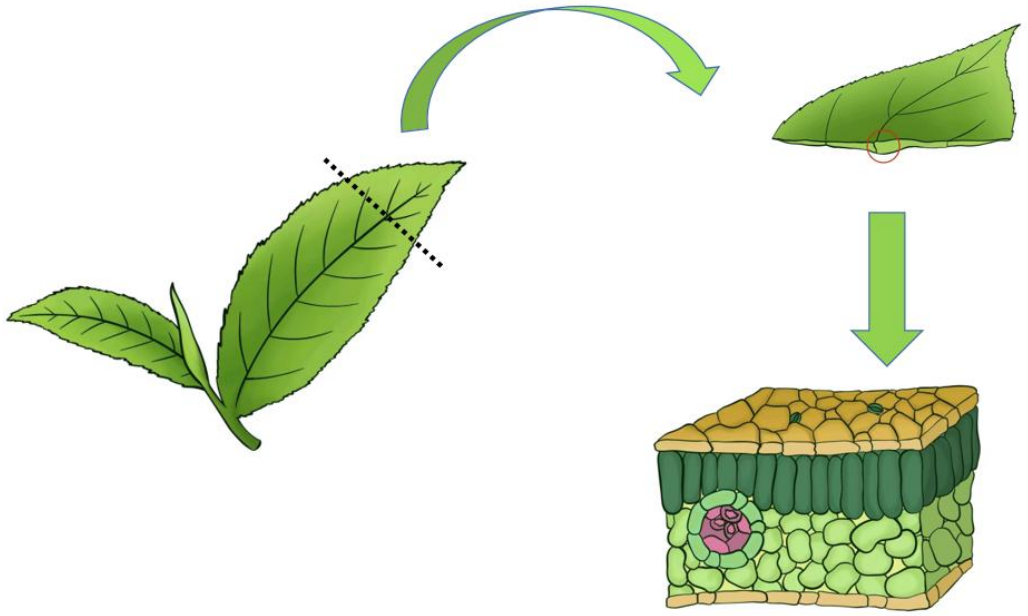

Figure 4. Schematic diagram of tea leaf sample sectioning. In the experiment, we chose to use a sharp blade to cut the tea leaves. In order to better load the BCECF into the cells, we did not cut vertically, but had some angles with the leaf surface, so that the sample had a greater contact area with the fluorescent dye.

BCECF/AM (2 ml of 1 mg/L) and 1 drop of Pluronic F-127 surfactant were added into a Petri dish which was shaken well. The slides with leaf sections were placed into the dish followed by incubation at room temperature of 28 ℃ away from light for 10 min. Then, the slices were washed twice with PBS solution, and subsequently placed at 28 ℃ for 30 min.

The slices were placed on the stage of a meta laser scanning confocal (Zeiss L510) and the operation followed Software Guide ZEN 2 (blue edition). The ratio of fluorescence intensity $F_{640/520}$ was measured at the same distance (100 μm) from

the upper or lower epidermis (Figure 5). The $F_{640/520}$ ratio was brought into the standard curve and the pH in the cell was calculated. The intracellular pH value was plotted by GraphPad Prism 5 software according to the manual, and the results were statistically analyzed by SPSS software following The Concise Guide of IBM SPSS Statistics 26. The "Tukey" method was used for multiple comparison, and the significance level was selected as 0.05.

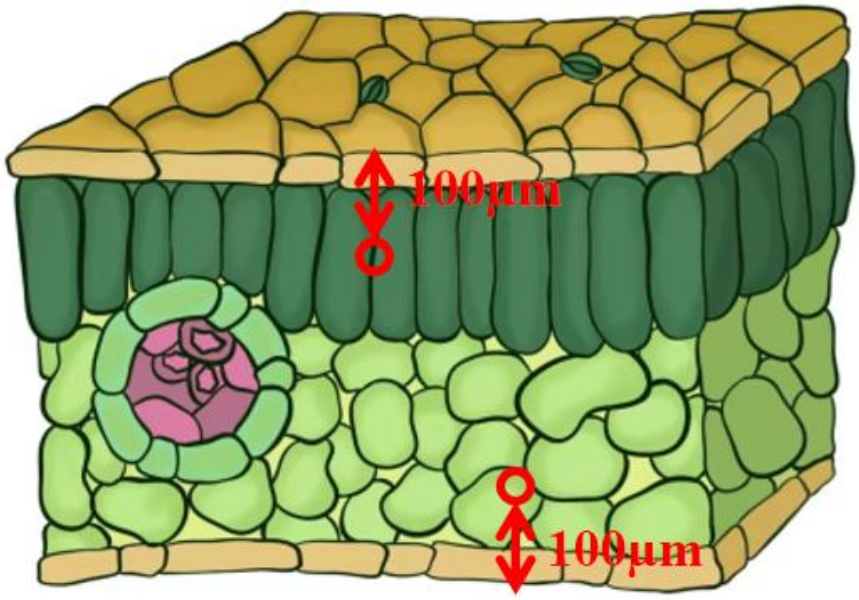

Figure 5. The intracellular pH of tea leaves were calculated from cells of mesophyll which are equidistant from epidermis.

3. Results

3.1 Changes in Glu and GABA levels in tea leaves after anaerobic treatment

In the process of determining the amino acid levels of tea samples after anaerobic treatment, we detected 38 different amino acids. The amino acids that changed more significantly in levels after anaerobic treatment were aspartic acid (Asp), glutamic acid, alanine (Ala), leucine (Leu), γ-aminobutyric acid, tryptophan (Trp), histidine (His) and arginine (Arg). For this study, the results for two key amino acids, Glu and GABA, are shown.

Compared with the control (NT), the level of GABA increased significantly after the two kinds of anaerobic treatment. The GABA level reached 5.3 mg/g after 6 h of CO_2 treatment, and 4.6 mg/g after 6 h of N_2 treatment (Figure 6). This is 50 and 40 times, respectively, higher levels compared to the level of GABA before the treatments. During the same period, the level of Glu decreased significantly from 13.5 mg/g at 0 h to 0.2 mg/g after 6 h of CO_2 treatment, and 0.9 mg/g after 6 h of N_2 treatment. Compared with 1 h after CO_2 treatment, the level of Glu after N_2 treatment decreased less, but Glu was almost consumed at 6 h. For the control (NT), the level of Glu, although significantly reduced at 1 h, did not change significantly between 1 h and 6 h, and the level of GABA did not show a significant change over time.

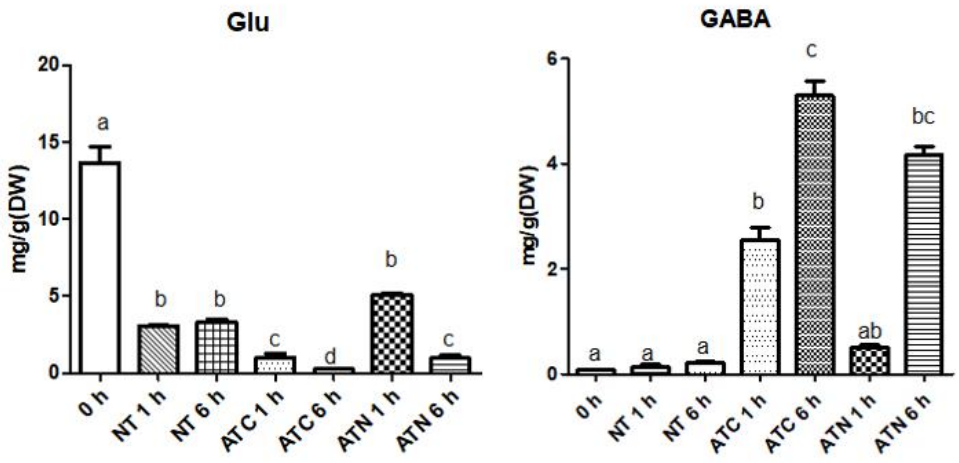

Figure 6. Changes in Glu and GABA levels at different times under different anaerobic treatments. NT is group C without treatment at room temperature as control, ATC is group A injected with CO_2, and ATN is group B injected with N_2. The values of different letters differ significantly ("Tukey's range test", $P < 0.05$).

3.2 Fluorescence of leaf cells without and loaded with fluorescent dyes

BCECF is the most popular fluorescent dye for measuring pH in plant cells, and it is suitable for cells of variable sizes, with good repeatability and simple operation (Roos *et al*., 1998). It is a ratio fluorescence probe with single wavelength excitation (488 nm) and dual wavelength emission (520 nm, 640 nm). Its fluorescence intensity ratio $F_{640\,/\,520}$ is different from the environment (solution or intracellular) and it is related to the pH value. However, it is not easy for BCECF to enter the cell through the cell membrane, but non-fluorescent lipophilic BCECF/acetoxymethyl (BCECF/AM) molecules can diffuse freely across the membrane into the cell. Under the action of cellolactonase, acetoxymethyl groups are hydrolyzed to form hydrophilic and fluorescent BCECF molecules, and its fluorescence intensity ratio depends on the cell pH.

Tea leaf cells will produce spontaneous fluorescence (Yao *et al*., 2009), and it is necessary to eliminate the interference of spontaneous fluorescence in actual experiments. In the observed cells, there was a weak autofluorescence in the cell wall, cuticle, wax layer and villi of tea leaves without BCECF/AM fluorescent probe, but there was no fluorescence in the cytoplasm. However, under the same observation conditions, there was a bright fluorescence in tea leaf cells incubated

with the pH-dependent fluorescent probe BCECF/AM at 28 ℃ for 10 min (Figure 7).

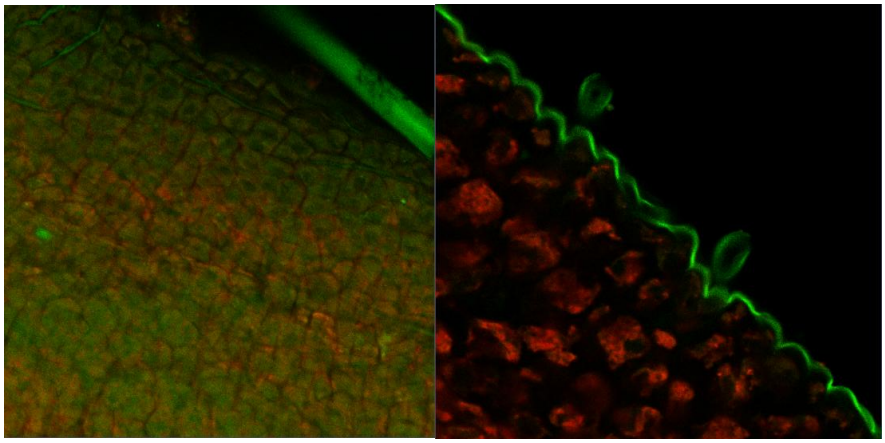

Figure 7. Imaging comparison of tea leaves (left) without BCECF/AM and tea leaves (right) with BCECF/AM. Strong fluorescence indicates that fluorescent dyes have been successfully loaded into leaf cells.

3.3 Relationship between fluorescence intensity ratio $F_{640/520}$ of BCECF and pH value

The fluorescence intensity of BCECF in different pH buffers was measured by a single excitation/double emission method. It was found that the fluorescence intensity of BCECF had a good linear relationship with pH (Figure 8), the correlation coefficient was 0.9781, and the linear regression equation was y = 0.6172x - 1.2052, where X is pH value and Y is fluorescence intensity ratio $F_{640/520}$. The R^2 value of the standard curve of 0.9781 is high, why intracellular pH values of tea leaves calculated from the standard curve are credible.

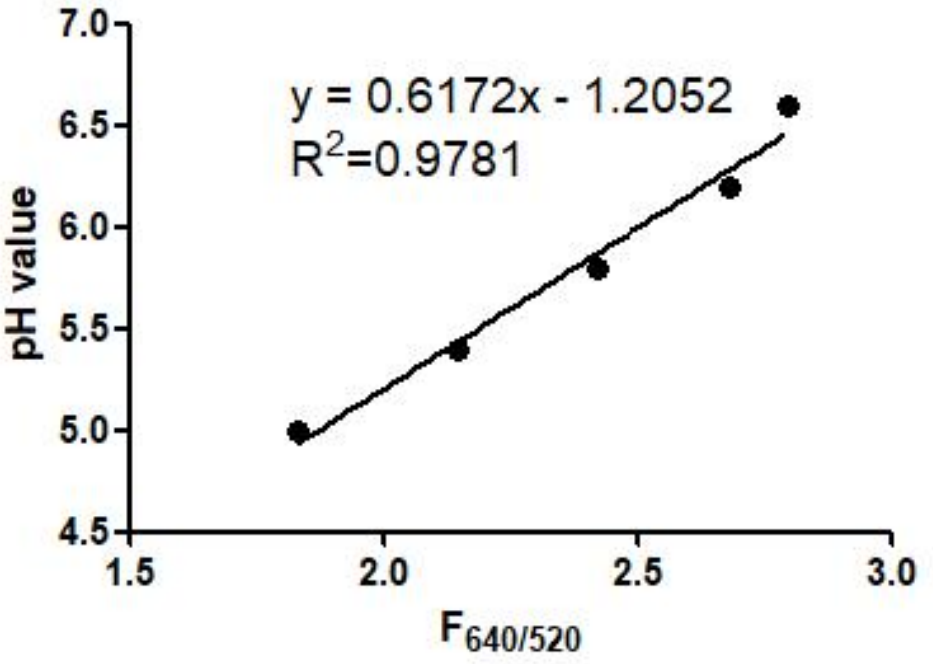

Figure 8. Relation of fluorescence intensity of BCECF and pH. Because the ratio of $F_{640/520}$ has a good linear relationship with different pH, we can use this method to accurately measure the pH in tea plant cells under anaerobic stress. At the same time, it also provides a reference for future experiments on the pH in tea plant cells.

3.4 Determination of intracellular pH in tea leaves after different anaerobic treatments

The fluorescence intensity ratio measured by laser scanning confocal microscope was included in the regression equation of the standard curve to obtain the pH value of the leaves. In the experiment, it was found that the pH values of upper and lower mesophyll of tea leaves are different depending on the treatment. The pH values in the upper mesophyll and lower mesophyll of leaves during CO_2 treatment (ATC) increased first and then decreased, but the time and value of pH change of upper mesophyll and lower mesophyll were different (Figure 9). However, the pH value of the lower mesophyll leaves during N_2 treatment (ATN) showed a trend of decreasing first and then increasing, indicating that different anaerobic treatment methods resulted in different stress response strategies of leaves.

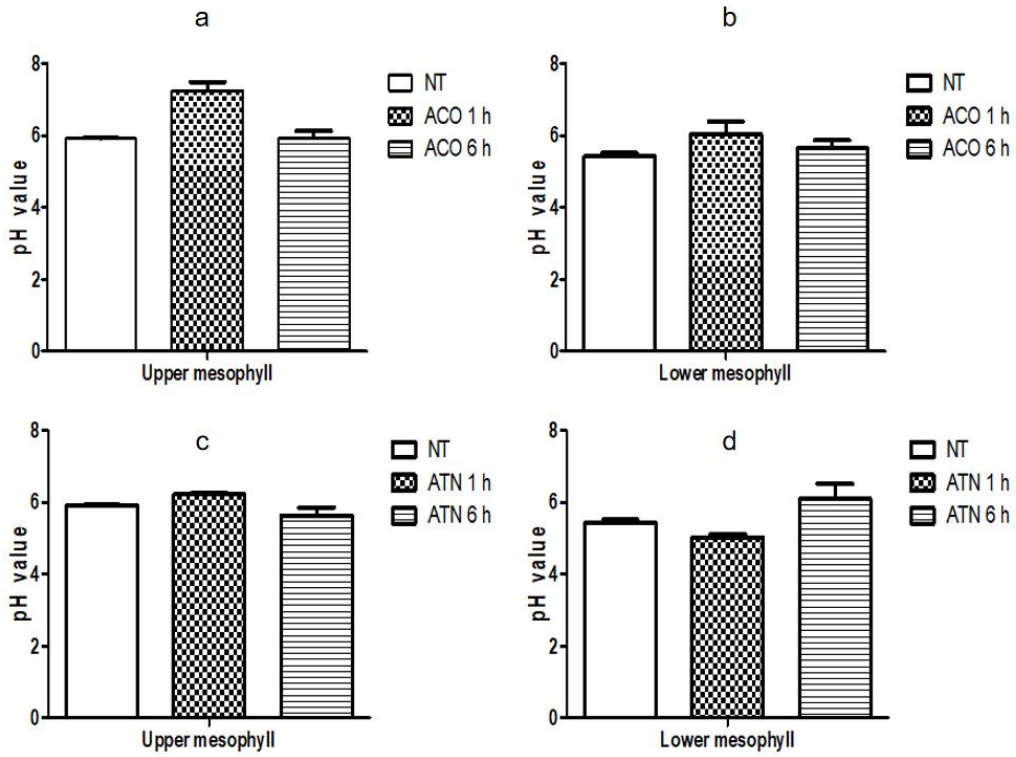

Figure 9. a: Comparison of intracellular pH in upper mesophyll cells of tea leaves after CO_2 treatments for 1 h and 6 h. b: Comparison of intracellular pH in lower mesophyll cells of tea leaves after CO_2 treatments for 1 h and 6 h. c: Comparison of intracellular pH in upper mesophyll cells of tea leaves after N_2 treatments for 1 h and 6 h. d: Comparison of intracellular pH in lower mesophyll cells of tea leaves after N_2 treatments for 1 h and 6 h. NT is group C without treatment at room temperature as control, ATC is group A injected with CO_2, and ATN is group B injected with N_2. (by "Tukey", $P < 0.05$).

4. Discussion

4.1 Determination of pH in tea leaf cells

For the determination of pH in tea leaf cells, an enzyme-linked immunosorbent assay (ELISA) was considered during the experimental design phase. However, this method will damage the tissue and also cause the hairs on tea leaves to mix with the sample (as shown in figure 7, the hairs on the tea leaves exhibit strong spontaneous fluorescence), affecting the accuracy of the results. With the use of laser confocal it is possible to measure accurately the pH of the different parts of tea leaves (upper and lower mesophyll), while still preserving the integrity of the leaves. Therefore, the method developed in this thesis can accurately measure the intracellular pH in tea leaves while maintaining leaf integrity, providing support for the studies on GABA metabolism in tea leaves.

Tea leaves have two kinds of spontaneous fluorescence, chlorophyll fluorescence and yellow green spontaneous fluorescence, and these two kinds of fluorescence may interfere with the determination of fluorescence ratio (Zhang *et al*., 2005). The interference can be minimized when the green channel band was 510~530 nm and the red channel band was 630~660 nm. By detecting the fluorescence intensity at 520 nm and 640 nm of the emission peak wavelength, calculate the fluorescence intensity $F_{640/520}$, and substitute it into the standard curve, the intracellular pH of tea tree can be effectively calculated.

The establishment of a pH measurement method for tea cells from this thesis not only provides evidence for observing the self-regulation of intracellular pH in the process of GABA production, but also provides a method for subsequent experiments related to tea pathology. For example, fluorescent green spot of tea tree is a common physiological disease of mature leaves. Its symptoms are: the lower mesophyll of mature leaves is partially concave, showing dark green particles or patches. Under light, the diseased spot can emit yellow green fluorescence (Yao *et al*., 2009; Zhang *et al*., 2005). The detection of the intracellular pH of the tea leaves at the lesion site would be helpful to explore the physiological changes of the lesion cells of great significance to clarify the pathogenesis of the lesion.

4.2 Linkage analysis of intracellular pH and amino acid changes in tea leaves after anaerobic treatment

In order to explore the effect of different anaerobic gas treatments on the pH in the cytoplasm of leaves and the effect of this process on GABA accumulation, the fluorescent dye BCECF/AM was used to detect the pH changes in the upper and lower mesophyll of leaves. The pH values of the upper and lower mesophyll were different (Figure 9), presumably due to their differences in structure and function, where the lower epidermis of a leaf usually has more stomata than the upper epidermis. When tea leaves were in an anaerobic environment, the intracellular pH would change dramatically due to the inhibition of gas exchange and the change of proton level.

Under normal temperature and anaerobic conditions, the level of Glu in tea leaves decreased sharply within 1 h. However, only a small amount of Glu was converted into GABA, whereas the main part participated in the synthesis or consumption of other amino acids. Glu under different gas treatments (CO_2 or N_2) decreased more sharply than without gas treatment and most of it was converted to GABA. According to a previous study, different anaerobic treatments have different effects on the synthesis of GABA (Chen *et al*., 2018).

It can be seen that during CO_2 treatment, GABA levels rapidly rose in 1 h and continued to accumulate up to 6 h at the end of experiment (Figure 6), whereas the increase in GABA caused by N_2 treatment is lagging. It is speculated that this is because the leaf stomata remain open during CO_2 treatment, which will maintain the respiration of tea to a certain extent and form an acidic environment in the cytoplasm. In a high concentration of carbon dioxide, leaves still carry out anaerobic respiration, keep stomata open, consume carbon dioxide and produce ATP. When the leaves were treated with carbon dioxide for 1 hour, the GAD enzyme was activated under acidic conditions, consuming Glu to produce a large amount of GABA, which consumed protons and reduced the cell membrane damage caused by acidic conditions. The regulating mechanism of this process is as follows. The F-type ATPase on mitochondria and chloroplast membranes synthesizes ATP through proton transmembrane transport. The V-type ATPase in the vacuole membrane transports H^+ from the cytoplasm into the vacuole, and the P-type ATPase in the cell membrane transports protons from the cytoplasm out of the cell. These processes regulate intracellular pH to achieve balance (Mulkidjanian *et al*., 2007; Axelsen *et al*., 1998; Fukuda *et al*., 2004). In the

present study, the pH of upper and lower mesophyll increased after 1 h of CO_2 treatment, and the pH increase of upper mesophyll was more obvious than of the lower mesophyll. It is speculated that this is due to the large consumption of protons to produce GABA. However, after 6 h, Glu is basically consumed, and the pH gradually falls back to the normal level, indicating that it is difficult to regulate protons in plants under a longer period of CO_2 treatment.

Different changes in pH values were observed during N_2 treatment. The pH of the lower mesophyll decreased first and then increased, while the GABA level first increased slowly after 1 h and then increased sharply after 6 h. It is speculated that this is because during N_2 treatment, the stomata of leaves are closed under hypoxia, the cells slowly enter the anaerobic breathing state, the tricarboxylic acid cycle is inhibited, the GABA shunt in the leaves of tea plants is activated, and begins to produce GABA. Succinate semialdehyde, the downstream product of GABA, participates in and completes the tricarboxylic acid cycle (Fait *et al*., 2008). However, after N_2 treatment for a long time, the intracellular acidic environment is enhanced, GAD is activated, and a large amount of protons are consumed to maintain cell homeostasis (Shelp *et al*., 1995). Therefore, the plant treated with N_2 showed a large increase in GABA and an increase in pH level after 6 h.

This result confirms the view mentioned in section 1.4 that GABA accumulates in response to acidification. However, due to the use of different gases in anaerobic treatment, the respiratory efficiency of tea leaves depends on whether the stomata are open or closed, thereby, the degree of cytoplasmic acidification differs between anaerobic treatments and treatment times. During CO_2 treatment, stomata open, plants absorb a large amount of CO_2, cytoplasmic acidification is relatively rapid, and GAD enzyme is activated faster. Therefore, after 1 h treatment, the pH value of the upper and lower mesophyll is higher than that of the control, because the GAD enzyme has been activated, consumes protons and generates GABA. Although samples have not been analysed during the treatment time of 0 ~ 1 h, it can be speculated that the cytoplasmic pH has decreased until a certain time point after the start of the CO_2 treatment, and the GAD enzyme has been activated and consumes protons. At the critical point, there is a balance between proton consumption and production, and the pH begins to increase with proton consumption (Figure 10). Under N_2 treatment, the stomata of tea leaves were closed, and weak respiration led to slow cytoplasmic acidification. It can be observed that the lower mesophyll was still acidic relative to the control at 1 h. In 1 ~ 6 h, the pH gradually returned to normal with the consumption of protons.

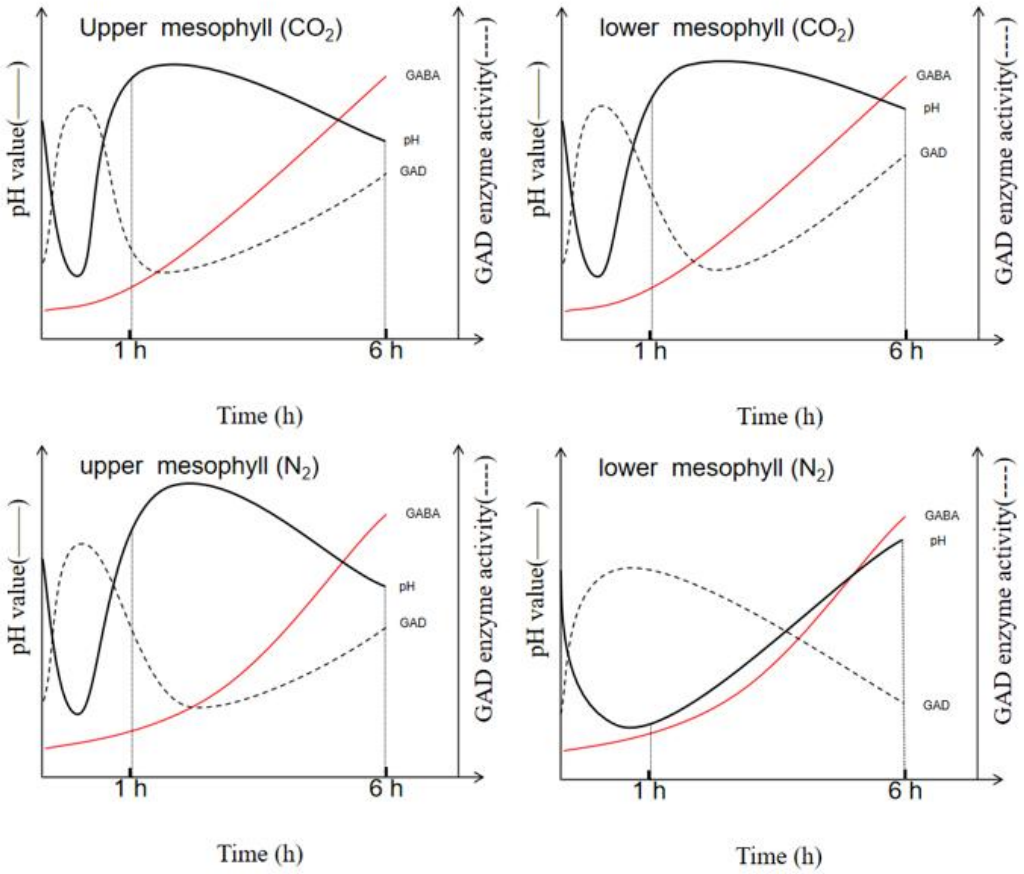

Figure 10. Hypothetical diagram of GAD enzyme activity and pH change. The solid line indicates the change of pH value, which first decreases and then increases due to the accumulation and consumption of protons. The dotted line indicates the change in GAD enzyme activity, which increases first and then decreases due to the acidity and alkalinity of the environment and substrate concentration.

It is speculated that there is a relationship between pH in plant cells, opening and closing of stomata and hormones related to plant stress resistance. Fluorescent dyes are the key to study these relationships. With the results in this study, the two aims of this project have been achieved. There are few studies on the intracellular pH of tea plants, but there are many studies on stress. Considering the relationship between the cytoplasmic pH and the regulation of various plant hormones, the use of fluorescent dyes can well be a useful method when studying the cellular response to stress in tea plants. Fluorescent dyes can also be used to observe the opening and closing of plant stomata. Stomatal opening is regulated by many factors, including plant hormones such as abscisic acid (ABA) (Herde *et al*., 1997; Pei *et al*., 2000), cytokinin (CTK) (Morsucci *et al*., 1991; Chen *et al*., 2014), indoleacetic acid (IAA) (Pemadasa *et al*., 1982; Xiao *et al*., 2006) and methyl jasmonate (MeJA) (Munemasa *et al*., 2007; Yan *et al*., 2015). Moreover, H_2O_2 in guard cells (Murata *et al*., 2001; Zhang *et al*., 2001), NO in cells (Bright *et al*.

2006), Ca^{2+} (MacRobbie *et al*., 2000) are also involved in the regulation of stomatal movement. Experiments have proven that cytoplasmic pH is involved in stomatal movement regulated by ABA and IAA (Suhita *et al*., 2004; Zhu et al., 2014).

As a very popular beverage in the world, the health value of tea has always attracted much attention. Gabaron tea is rich in GABA, which is not only an inhibitory neurotransmitter with positive effects on human health, but also a key amino acid for plants under stress, and further research on GABA is important for both humans and plants.

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Popular science summary

GABA is a non-essential amino acid for the human body. It can be used to treat some diseases and improve human function through metabolic mechanisms or other ways. GABA is an inhibitory neurotransmitter, which mediates more than 40% of the inhibitory nerve conduction. In medicine, it has been found that GABA can reduce blood pressure, treat epilepsy, treat hepatic encephalopathy, promote reproductive function, promote sleep, enhance memory, anti-convulsion, anti-anxiety, inhibit the response of the lung to various inflammatory mediators and so on. When animals are given GABA orally, GABA is highly utilized in the process of metabolism.

GABA also plays an important role in plants. GABA accumulation can alleviate the damage to plants under stress conditions such as hypoxia, chilling injury, thermal stimulation, mechanical stimulation, drought and salt stress. The main role of GABA in plants is to regulate cytoplasmic pH, nitrogen reserve, signal transduction and defense response. It is mainly produced by two pathways in plants, Glu decarboxylation or polyamine oxidation. Previous studies have proven that in tea plants, the production of GABA mainly comes from Glu decarboxylation rather than polyamine oxidation.

As a new tea product developed in 1987, Gabaron tea has attracted much attention because it is rich in GABA and has good health effects on the human body. At present, the industrialized production of Gabaron tea is mainly through three kinds of anaerobic treatment to enrich GABA in tea. The treatment time is between 5 ~ 10 hours. If the treatment time is short, the GABA levels is low. If the treatment time is too long, the quality and taste of tea will be seriously reduced.

In this project, a method for detecting the pH in tea leaf cells was developed by using the fluorescent dye BCECF/AM, and the pH in tea leaf cells treated with CO_2 or N_2 was determined. It was found that the process of the GABA enrichment in tea leaves was different under the two gas treatments. After the CO_2 treatment, tea leaves enriched GABA faster and produced more GABA. It is speculated that when the cells took up CO_2 without oxygen, it resulted in an acidic environment

of the cytoplasm and activation of the GAD enzyme. Compared with the CO_2-treated tea leaves, the N_2-treated tea leaves enriched GABA more slowly and produced less GABA. It is speculated that the cells entered anaerobic respiration and accumulated protons more slowly. The accumulation of GABA in the N_2-treated tea leaves had an obvious lag effect compared with the CO_2 treated tea leaves.

In conclusion, although large amounts of GABA accumulated in tea leaves treated with either N_2 or CO_2 from the decarboxylation reaction of Glu, the timing of GABA synthesis differed. There was a significant lag effect in the enrichment of GABA in N_2-treated tea leaves compared to CO_2-treated tea leaves.

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