Effects of Precursor, Temperature, and Illumination on Theanine Accumulation in Tea Callus

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The theanine (THE: γ -glutamylethylamide) accumulation level was greatly increased in tea callus when ethylamine hydrochloride ($\text{EtNH}_2 \cdot \text{HCl}$) was added to the medium. The optimum temperature for THE accumulation was the same temperature (25°C) as for growth of callus. Little THE was discharged into the medium. Dark culture was better than illumination for the accumulation of THE (201 mg/g dry wt.). These results suggested that THE synthetase had a positive activity, the decrease of THE content in callus was caused by the lowering of EtNH_2 biosynthesis level, and temperature and illumination strongly affected THE accumulation.

Theanine (THE), an amide that enhances the taste of infused green tea, was first found in shoot tips of tea plants (*Camellia sinensis*).¹⁾ *C. sasanqua* and *C. japonica* also contain THE, but its contents decrease during growth.²⁾ Sasaoka *et al.* have reported that THE was biosynthesized from glutamic acid (Glu) and EtNH_2 in tea roots, and the enzyme involved in this biosynthesis was THE synthetase (Glu: EtNH_2 ligase).^{3,4)} Takeo has reported that ammonium nitrogen acquired in tea roots served in the synthesis of Glu and alanine (Ala), and that Ala was then used in the synthesis of EtNH_2 .⁵⁾ In all seasons, 7–21 mg/g dry wt. of THE are contained in tea leaves.^{6,7)} However, the THE level in induced callus decreases greatly in comparison with the stem tips as explants,⁸⁾ while Glu, one of the precursors in THE biosynthesis, is accumulated in tea callus. Therefore, we supposed that the decrease of the THE level in callus is caused by the lowering of EtNH_2 level or the precursor level of EtNH_2 , or an inactivity of THE synthetase. Doi has reported that the optimum temperature was 32°C for growth of tea callus.⁹⁾ However, the optimum temperature for THE accumulation in callus is not known, and the effects of illumination on THE accumulation also are not known.

Therefore, we investigated the effects of the precursors, temperature, and illumination on THE accumulation in tea callus.

Materials and Methods

Callus induction and growth. Stem tips of *C. sinensis* cv. Yabukita obtained from a tea field in Haibara-gun, Shizuoka-ken, Japan, in July 1988 were used as explants for callus induction.

The explants were sterilized on the surface with 70% ethanol for 10 sec, 1% sodium hypochlorite solution (containing one drop of Tween 20 per 100 ml solution) for 20 min, then were rinsed 3 times with sterilized distilled water and cut about 5 mm long. Stem tips were cultured at 25°C under bright illumination (3,000 lux) for 16-hr photoperiods.

Induced callus was transplanted onto fresh medium every month. The basal media used in all of the experiments were the Murashige and Skoog salt solutions supplemented with 100 mg/l inositol, 0.5 mg/l pyridoxine and nicotinic acid, 1.3 mg/l thiamine, 2.0 mg/l glycine, and 30 g/l sucrose.¹⁰⁾

Media for callus induction and growth were supplemented with 4 mg/l benzyladenine (BA) and 2 mg/l indolebutyric acid (IBA) and were gelled with 8 g/l agar. All media were adjusted to $\text{pH } 5.85 \pm 0.05$ before autoclaving for 15 min at 121°C .

Effects of the precursor. Two g of callus cultured for 2 months at 25°C under the same conditions of bright illumination after callus induction was transplanted in 70 ml of medium for growth of callus without agar and

incubated for 10 days using the rotary culture (3.5 rpm), under the same conditions as for growth of callus. The media were supplemented with one of the precursors ($(\text{NH}_4)_2\text{SO}_4$, Ala or $\text{EtNH}_2 \cdot \text{HCl}$) in the biosynthesis of THE. THE in cultured callus were measured by HPLC.

Effective concentration of $\text{EtNH}_2 \cdot \text{HCl}$. Two g of callus grown for 7 months was cultured in 30 ml of media containing various concentrations of $\text{EtNH}_2 \cdot \text{HCl}$ for 30 days using the rotary shaker (70 rpm) at 25°C in the dark. The concentrations of $\text{EtNH}_2 \cdot \text{HCl}$ were 0, 0.1, 1, 5, 10, 25, 50, and 100 mM. Three samples of calli were cultured for each group treatment. THE in cultured callus was measured by HPLC.

Effects of temperature. Four g of callus grown for 5 months was cultured in 60 ml of medium containing 25 mM $\text{EtNH}_2 \cdot \text{HCl}$ for 5, 10, 20, and 30 days using the rotary shaker (70 rpm) in the dark. Temperatures tested were 10, 15, 20, and 25°C. Then about 1.5 g of callus grown for 7 months was cultured in 30 ml of medium containing 25 mM $\text{EtNH}_2 \cdot \text{HCl}$ for 10, 20, and 30 days using the rotary shaker (70 rpm) in the dark. Temperatures tested were 25, 28, 32, and 35°C. Three samples of calli for each group treatment were cultured in both experiments. THE in cultured callus was measured by HPLC.

Discharge of theanine from cultured callus into medium. Four g of callus grown for 5 months was cultured in 60 ml of medium containing 25 mM $\text{EtNH}_2 \cdot \text{HCl}$ for 30 days using a rotary shaker (70 rpm) at 25°C in the dark. Three samples of calli for each group treatment were cultured. THE in cultured callus and media was measured by HPLC.

Effects of illumination. One and a half g of callus grown for 6 months under bright illumination (3,000 lux) or in the dark was cultured in 30 ml of medium containing 25 mM $\text{EtNH}_2 \cdot \text{HCl}$ for 30 days using the rotary shaker (100 rpm) at 25°C under bright illumination (7,200 lux) or in the dark. Three samples of calli for each group treatment were cultured. THE in cultured callus was measured by HPLC.

Analytical method. Fifty mg of pulverized tea callus was extracted with 80°C of distilled water for 30 min and the extract was made up to 100 ml with distilled water. Tannins were removed from this extract with polyvinylpyrrolidone (0.2 g), then the extract was filtrated through a 0.45- μm membrane filter. The THE in the filtrate was measured by HPLC.

THE in tea callus was measured with a fluorescence detection system using *o*-phthalaldehyde (OPA) and 2-mercaptoethanol. THE purchased from Tokyo Kasei Kogyo Co. was used as a standard THE. The filtrate (0.5 ml) was mixed with 0.5 ml of OPA solution (OPA: 246 mg, 0.1 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (pH 10): 20 ml, 2-mercaptoethanol: 200 μl). The mixture (5 μl) was injected into HPLC.

HPLC conditions were: column, Shiseido Capcell Pak C_{18} AG (4.6 mm i.d. $\times$ 250 mm); guard column, Shiseido Capcell Pak C_{18} AG (4.6 mm i.d. $\times$ 35 mm); column temperature, 40°C; eluent A, 10 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (pH 6.0); eluent B, acetonitrile; gradient, A(90–75%)·B(10–25%); program, 60 min; flow rate, 1 ml/min; detection, Ex 335 nm, Em 450 nm; range, $\times 16$.

Results and Discussion

Effects of precursors on theanine formation in tea callus

The effects of the addition of $(\text{NH}_4)_2\text{SO}_4$, Ala, and $\text{EtNH}_2 \cdot \text{HCl}$ on the formation of THE are shown in Table I. THE was little biosynthesized in callus without treatment of precursors. THE content in callus was increased by the addition of $\text{EtNH}_2 \cdot \text{HCl}$. However, neither the addition of $(\text{NH}_4)_2\text{SO}_4$ nor Ala made the content increase. The optimum concentration of $\text{EtNH}_2 \cdot \text{HCl}$ was 25 mM for THE accumulation (Fig. 1). These

Table I. EFFECTS OF PRECURSORS ON THEANINE FORMATION IN TEA CALLUS

Calli were cultured for 10 days at 25°C using the rotary culture (3.5 rpm) under bright illumination (3,000 lux) for 16-hr photoperiods.

Treatment	THE (mg/g dry wt.)
Non-treatment	0.384
25 mM $(\text{NH}_4)_2\text{SO}_4$	0.350
25 mM Ala	0.343
25 mM $\text{EtNH}_2 \cdot \text{HCl}$	30.9

Fig. 1. Effects of Ethylamine Concentration on Theanine Accumulation in Tea Callus.

Calli were cultured for 30 days at 25°C using the rotary shaker (70 rpm) in the dark.

results suggested that THE synthetase had a positive activity and affected THE formation, and EtNH₂ was biotransformed into THE. Therefore, we supposed that the decrease of THE content in callus was caused by the lowering of the EtNH₂ biosynthesis level.

Effects of temperature on THE accumulation in tea callus

The optimum temperature for growth was 25°C (Fig. 2). Calli cultured at 32 and 35°C turned brown. This result agreed with the optimum temperature for growth of tea

Fig. 2. Effects of Temperature on Growth of Tea Callus. Calli were cultured in the medium containing 4 mg/l BA, 2 mg/l IBA and 25 mM EtNH₂·HCl for 10, 20 and 30 days using the rotary shaker (70 rpm) in the dark.

Temperatures tested were about 10 (□), 15 (▲), 20 (▽), 25 (○), 28 (▼), 32 (△), and 35 (■) °C.

Fig. 3. Effects of Temperature on Theanine Accumulation in Tea Callus.

Temperatures tested were about 10, 15, 20, and 25°C. The symbols of temperatures were the same as Fig. 2. For experimental procedures, see Fig. 1.

plants,¹¹⁾ but differed from the report by Doi.⁹⁾

Glu, glutamine (Gln), and Ala are involved in the biosynthesis of THE.⁵⁾ These free amino acids were affected by temperature. The contents of Gln and Ala greatly decreased in calli cultured at 20–35°C and linearly increased at lower than 20°C. However, the content of Glu showed only a small variation compared with the other free amino acids (data not shown).

THE in calli positively accumulated at 20–28°C (Fig. 3). The optimum temperature for THE accumulation was 25°C. The biosynthesis of THE lagged in spite of the accumulation of enough Glu as substrate at lower than 20°C. These results suggested that the temperature strongly affected THE synthetase activity.

Discharge of theanine from cultured callus into medium

The discharge levels of each total amounts of free amino acids (Glu, Gln, and Ala) into the medium were all approximately 4%, while the level of THE discharge was less than 1% (Table II). The cause of these results was not clear yet. We need to investigate the conditions of THE accumulation in cells and the culture conditions of the discharge of biosynthesized THE. Thirty percent of total caffeine (Caf) in callus was discharged into the medium. The discharge of Caf in tea callus had the same tendency as *Coffea arabica*,¹²⁾ but the total amount of Caf was small in comparison with cultured *Coffea arabica* cells.

Table II. DISCHARGE OF THEANINE FROM TEA CALLUS INTO MEDIUM

Calli were cultured in the medium containing 25 mM EtNH₂·HCl for 30 days at 25°C using the rotary shaker (70 rpm) in the dark.

Place	THE	
	mg	%
Callus	89.5	99.1
Medium	0.840	0.9
Total	90.3	100.

Table III. EFFECTS OF ILLUMINATION ON THEANINE ACCUMULATION IN TEA CALLUS

Calli were cultured in the medium containing 25 mM EtNH₂·HCl for 30 days at 25°C using the rotary shaker (100 rpm).

Treatment	THE content in callus (mg/g dry wt.)	Transformation rate of EtNH ₂ ^a (%)
Control (L) ^a	0.803	—
Control (D) ^b	1.23	—
L-l ^c	89.1	32.8
L-d ^d	144	44.6
D-l ^e	157	61.8
D-d ^f	201	72.8

^a Callus cultured under illumination (3,000 lux, 16-hr photoperiods) in the pre-culture.

^b Callus cultured in the dark in the pre-culture.

^c Callus cultured under illumination in the pre-culture (3,000 lux, 16-hr photoperiods) and after the pre-culture (7,200 lux, 16-hr photoperiods).

^d Callus cultured under illumination (3,000 lux, 16-hr photoperiods) in the pre-culture and in the dark after the pre-culture.

^e Callus cultured under dark condition in the pre-culture and illumination (7,200 lux, 16-hr photoperiods) after the pre-culture.

^f Callus cultured in the dark in the pre-culture and after the pre-culture.

^g (Amount of accumulated THE_(mol)/Added EtNH₂·HCl_(mol)) × 100.

Effects of illumination on THE accumulation in tea callus

Effects of illumination on THE accumulation in tea calli are shown in Table III. In the pre-culture, dark culture was better than illumination for the accumulation of THE. In addition, THE content greatly increased in calli cultured in the dark after the pre-culture (201 mg/g dry wt.). Dark culture caused a high transformation rate of EtNH₂ in callus.

We have reported that THE content in tea leaves greatly increased from November to January in wholeyear cultivation of tea plants.^{6,7)} From the results in this investigation, we supposed that the cause of this phenomenon was the change of photoperiod rather than temperature.

Gln is synthesized from Glu and NH₄⁺, and THE synthetase is concerned with the formation of Gln.⁴⁾ From this report, we conclude that the THE accumulation is disturbed by Gln formation in tea callus, and the ratio of NH₄⁺ and NO₃⁻ in the medium affects the growth of callus and the formation of THE and Gln. Therefore, we have to investigate the relationship THE and Gln formation, and the effects of growth on the biosynthesis of THE and Gln in further studies.

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