Nitorogen Metabolism Pertaining to Biosynthesis of Theanine in Tea Plants

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Introduction

Many reports explaining the pathway of ammonia-nitrogen assimilation in plants have been presented so far.[1] However, there are only a very few reports on that subject in perennial plants.

The tea plant, a perennial plant, shows a distinct nitrogen metabolism related to ammonia-nitrogen assimilation, such as the synthesis and accumulation of theanine which is a characteristic amide found in the tea plant. It is also a remarkable property of the tea plant that it is highly adaptable to heavy application of ammonia-type fertilizer.

In Japanese green tea, theanine is one of the important chemical components effecting the taste of green tea infusion.

High grade teas show a high level of theanine contents. Especially, the Gyokuro, the highest grade green teas plucked from shaded fields, contains about 2% of theanine on dry weight basis.

Furthermore, yields of new shoots increase under the application of high dosage of ammonia-type fertilizer.

Therefore, problems pertaining to application of ammonia-type fertilizer are very important for the cultivation of tea plants in Japan.

Biosynthesis of theanine

Theanine (N^5-ethyl-L-glutamine) was first found in green tea by Sakato (1950)[2]. Then Sasaoka (1965) reported that theanine is biosynthesized in tea roots from L-glutamic acid and ethylamine, and the enzyme involved in this biosynthesis is theanine synthetase (L-glutamic acid: ethylamine ligase)[3].

However, the route of biosynthesis of ethylamine, one of the precursors from which theanine is formed, has not yet made clear. Therefore, the present author investigated the biosynthesis route of ethylamine in tea plants by using ^{14}C-compounds[4].

Previously, some compounds, such as acetoaldehyde and L-alanine, were speculated as the precusors of ethylamine in plants.

After L-alanine U-^{14}C) and acetoaldehyde (1.2^{14}C) were absorbed by roots of tea plant, 20–30% of the total radioactivity recovered in the ethanol extract was found to be present in amino acids. The main components containing ^{14}C were L-alanine, L-glutamic acid, L-aspartic acid and theanine, indicating that the absorbed alanine and acetoaldehyde were transformed to glutamic acid and theanine. The incorporation of ^{14}C into the ethylamine moiety of theanine was much more higher from ^{14}C-alanine than from ^{14}C-acetoaldehyde, as shown in Table 1.

The incorporation of ^{14}C into the ethylamine fraction of theanine from ^{14}C-alanine in roots was effectively interrupted by adding excess amounts of ethylamine to the culture solution. As shown in Table 2, the incorporation rate of ^{14}C into the ethylamine fraction of theanine was reduced by about 50% in the ethylamine-fed roots as compared with the non-fed roots. Furthermore, ratios of the radioactivity of the ethylamine fraction of theanine to that of theanine showed 13.2 and 76.9% in both treatments, respectively. On the other hand, the

Table 1. Incorporation of ^{14}C derived from ^{14}C-alanine and ^{14}C-acetoaldehyde into amino acids of tea seedlings

^{14}C-recovered	^{14}C-alanine		^{14}C-acetoaldehyde	
	Root	Leaf	Root	Leaf
Total ^{14}C absorbed	2.6×10^6 cpm		7.7×10^6 cpm	
Alcohol-extract	345,000	107,200	367,000	195,200
Amino acid fraction	70,000	10,000	113,000	11,300
Alanine	24,900	1,050	52,100	1,000
Glutamic and aspartic acids	3,100	1,750	11,600	3,000
Theanine	9,550	800	26,300	2,650
Ethylamine fraction of theanine	3,810	544	975	800
	(40%)*	(68%)*	(4%)*	(30%)*

*: Ratio of cpm of ethylamine fraction of theanine to that of theanine.

Each experiment: 10 seedlings (5 g). Values are means of duplicate lots.
Treatment with labelled compounds: The roots of 10 cotyledonfree seedlings (green or etiolated) were dipped in 150 ml of either a soln. containing 0.01 M phosphate buffer (pH 6), 50 μmol L-alanine and 5 μCi L-alanineU-^{14}C(130 mCi/μmol) [^{14}C-L-alanine plot] or a phosphate buffer soln. containing 50 μmol MeCHO, 5 μCi of MeCHO 1-$2^{14}C$ (23 μCi/mg) and 250 ppm of $(NH_4)_2SO_4$, [AC Me CHO plot]. The seedlings were then exposed to continuous illumination at an intensity of approx. 10,000 lx at 30°C for 24 hr.

Table 2. Conversion of ^{14}C-alanine into ethylamine fraction of theanine

Radioactivity recovered	Treatment	
	Ethylamine-fed roots[1]	Control roots[2]
Radioactivity in theanine	2340 cpm/g[3]	695 cpm/g
Sp. activity of theanine	49.9 cpm/μmol	47.9 cpm/μmol
Radioactivity in ethylamine fraction	296 cpm/g	534 cpm/g
Ratio of cpm of ethylamine fraction of theanine of that of theanine	13.2 %	76.9 %
Ratio of incorporation into ethylamine from ^{14}C-alanine	0.55	1

1) Ten roots were dipped in 150 ml of a soln. containing 50 μmol mol of L-alanine, 5 μCi of ^{14}C-alanine with 50 μmol of ethylamine, pH 6.
2) Ten roots were treated as above but without ethylamine.
3) Fresh weight. Values are means of duplicate lots.

incorporation of ^{14}C into theanine of the ethylamine-fed roots was 3 times greater than that of the non-fed roots. However, the specific activities of $^{14}C/\mu$mol of theanine showed about the same value in both treatments. Therefore, it was considered that the conversion of L-alanine to ethylamine in roots was inhibited by excess ethylamine in the culture solution, and fed ^{14}C-L-alanine was transformed into glutamic acid through the Krebs cycle after deamination, and then incorporated into theanine.

From the above results, it was thought that L-alanine might be a precursor of ethylamine and L-alanine decarboxylase might play a role in the synthesis route of ethylamine.

After that, L-alanine decarboxylase was extracted from the roots of tea plant. The enzyme was proved to form ethylamine from L-alanine by decarboxylation[5].

On the basis of these results, a scheme

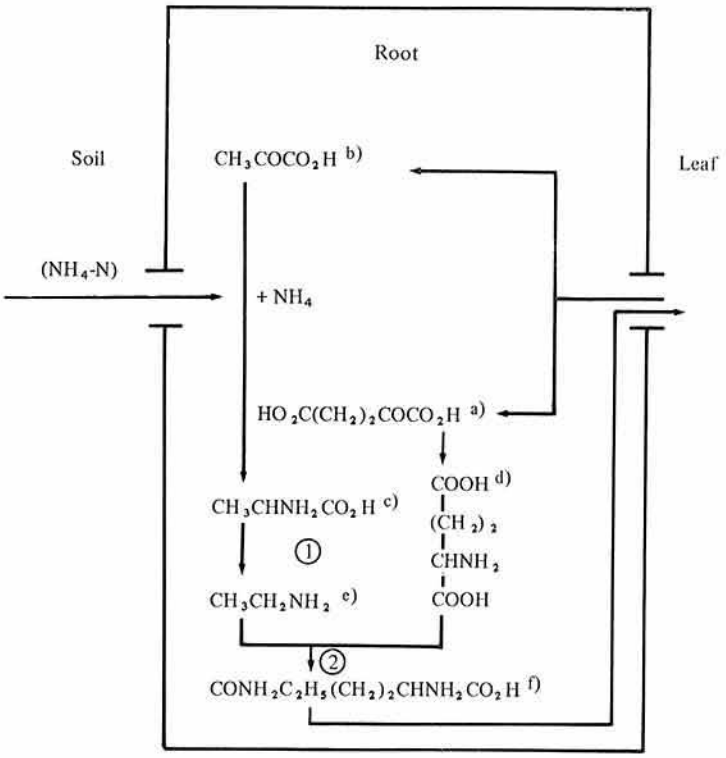

Fig. 1. Synthesis route of theanine in tea root

①: L-alanine decarboxylase
②: Theanine synthetase
a): 2-oxo-glutarate b): aceto-acetic acid
c): L-alanine d): L-glutamic acid
e): ethylamine f): L-theanine

shown in Fig. 1 is proposed for the theanine biosynthesis route in roots of tea plants.

Utilization of ammonia-nitrogen in roots of tea plants

Recently, the glutamine synthetase and glutamine- 2 -oxo - glutarate-aminotransferase system (GS/GOGAT) and the glutamate dehydrogenase system (GDH) have been proposed as the enzyme systems in plants for the entry of ammonia into amino acids[6].

Therefore, the author investigated the roles of the GDH and the GS/GOGAT systems on ammonia-nitrogen assimilation of tea plants.

1) Glutamate dehydrogenase system[7]

The glutamate dehydrogenase (GDH) (1.4.1.2.) prepared from tea roots requires NADH as the coenzyme for reductive amination of 2-oxo-glutarate to glutamic acid.

Opt. pH of GDH was ca. pH 8.0.

The activity of reductive amination of GDH in tea roots was remarkably depressed when the roots were cultured in a medium containing glutamate, as shown in Fig. 2.

When the roots cultured in glutamate medium were transferred to ammonia-nitrogen medium, the activity of GDH was reactivated. However, nitrate-nitrogen medium was not effective in reactivating GDH. Furthermore, the activity of GDH was maintained at a high level in the medium containing the high con-

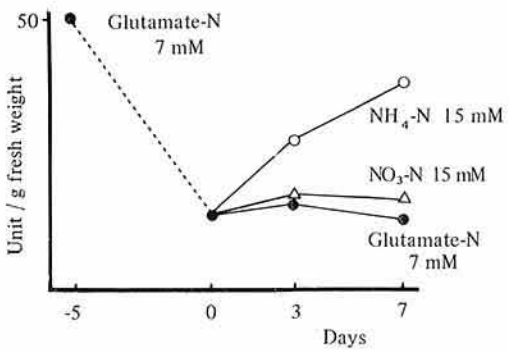

Fig. 2. Increase in NADH-dependent GDH activities in extracts of tea rootlets which were transferred from glutamate to ammonia- or nitrate-nitrogen medium

Treatment The rootlets of cuttings were grown for 5 days in glutamate (7 mM) medium and then transferred to either ammonia-nitrogen (15 mM), nitrate-nitrogen (15 mM) or glutamate (7 mM) medium, respectively.

Enzyme assay The activity of GDH was determined by the NADH assay measuring the reductive amination of 2-oxo-glutarate contained in 3 ml of reaction mixture composed of Tris-HCl (pH 8.0), 300 μmol; 2-oxo-glutarate (neutralized), 15 μmol; ammonium chloride, 300 μmol; and NADH, 0.3 μmol; in silica cells of 1 cm light path. The reaction was started by adding NADH, followed by recording oxidation of NADH at 340 nm at 30°C. The unit of GDH activity was defined as a 0.001 absorbance unit change per min.

centration of ammonia-nitrogen, as shown in Fig. 3.

The activity of GDH was not inhibited by glutamine and theanine which were transformed from glutamate.

Then, it was thought that the activity of GDH system was kept high in the ammonia-nitrogen medium when glutamate, an amination product, was transformed smoothly to other amino compounds, such as glutamine and theanine, but it was depressed in case when glutamate was accumulated in roots by absorption of excess glutamate or by inhibited transformation of glutamate to amides.

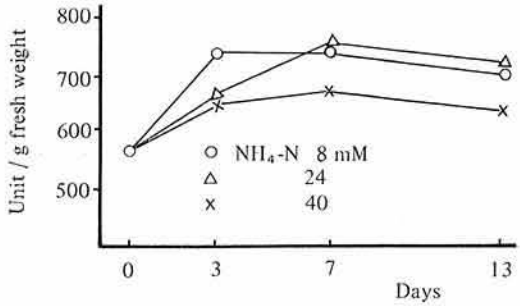

Fig. 3. Increase in NADH-dependent GDH activities in extracts of tea rootlets incubated in ammonia-nitrogen mediums of various concentrations.

Treatment Tea rootlets of cuttings were grown for 6 days in glutamate (N-2 mM) medium and then incubated in ammonia-nitrogen mediums of various concentrations.

2) Glutamine synthetase and glutamine-2-oxo-glutarate aminotransferase system (GS/GOGAT)[8]

Glutamine-2-oxo-glutarate aminotransferase (GOGAT or glutamate synthase (1.4.1.14)) extracted from tea roots formed glutamate from 2-oxo-glutarate and L-glutamine by using NADH as a coenzyme.

Opt. pH of glutamate synthase was ca. pH 8.0.

The activity of GOGAT in tea roots was effectively inhibited by L-methionine-DL-sulfoximine (MSO), a specific inhibitor of glutamine synthetase. On the contrary, GDH was not inhibited by MSO, as shown in Table 3.

When ammonia-nitrogen was supplied to the roots of tea plants treated with MSO, the contents of amino compounds in tea plant were decreased. Especially, the theanine content was remarkably reduced, as shown in Table 4.

Then, it was thought that although the ammonia-nitrogen assimilation in tea roots was sustained by both the GS/GOGAT system and the GDH system, the former system might be predominant in supporting smooth ammonia-nitrogen uptake in tea plants.

From the results shown in *1)* and *2)*, it is considered that although both of the GS/GOGAT system and the GDH system are

Table 3. Glutamate dehydrogenase activity and glutamate synthase activity in tea rootlets treated with or without L-methionine-DL-sulfoximine (MSO)

	Glutamate dehydrogenase	Glutamate synthase
Treated with MSO	0.15	0.016
Not treated with MSO	0.17	0.027

Treatment with L-methionine-DL-sulfoximine Tea seedlings were dipped in a medium containing 3.3 mA ATP and 6.6 mM $MgSO_4$ in 0.03 M Tris-HCl buffer (pH 7.4) for 24 hr. The seedlings were divided into two parts and each part was incubated in either a medium with or without MSO (5 mM) for another 24 hr. Then, each part was dipped in a 200 ppm N-$(NH_4)_2SO_4$ medium (pH 5.5) for 24 hr. The experiments were done under continuous illumination at 15,000 lx at 25°C.

Enzyme assay The activity of glutamate dehydrogenase in tea rootlets was determined by the NADH assay (NADH oxidation mol/N mg/min.), which measures the reductive amination, as shown in Fig. 2. The activity of glutamate synthase in tea rootlets was assayed in a 3 ml reaction mixture containing 300 μmol of Tris-HCl (pH 8.0), 0.3 μmol of NADH, 30 μmol of 2-oxo-glutarate, 300 μmol of L-glutamine and enzyme solution. The activity was monitored by measuring NADH oxidation at 340 nm at 30°C.

Table 4. Contents of amino acids and amides in tea plants treated with or without L-methionine-DL-sulfoximine

	Roots		Leaves	
	Treated with MSO	Control	Treated with MSO	Control
Total-Nitrogen	2.7	2.8	3.3	3.4
Aspartic acid	4.8	11.1	8.7	19.6
Threonine	4.3	4.9	3.7	5.6
Serine	21.0	37.3	7.6	16.2
Glutamic acid	13.3	24.9	1.0	40.9
Alanine	15.0	37.4	6.2	9.9
Arginine	108.0	154.1	4.0	8.0
Glutamine	0.0	5.5	0.0	13.3
Theanine	516.4	836.9	94.9	125.6

Total-N, % of dry weight.
Amino acids and amides, mmol in g dry weight.

present in tea roots, the former system is the regular route for the entry of ammonia-nitrogen and the GDH system is a supplementary one reacting under the heavy application of ammonia-nitrogen.

Amino acid content in tea shoots[9,10]

Aspartic acid, threonine, serine, glutamic acid, glycine, alanine, arginine, glutamine and theanine were recognized as main components of tea new shoots. Especially, theanine accounts for about 60–70% of the total content of amino compounds.

In Japan, we have three crops of tea (plucked in May, late June and early August, respectively.) in a year. Generally, the spring crop plucked in May has the high content of amino compounds and the other two crops show the low contents.

In the winter, it is observed that theanine, glutamine and arginine, which are synthesized from the fertilizer applied in the late autumn, accumulate in roots, stalks and matured leaves of tea plants (Fig. 4). These stored amino compounds are translocated specifically into new shoots in the spring and accumulated there. Therefore, the spring shoots show high contents of the amino compounds.

During the period from spring to summer, growth rate of tea plants in high. Consequently, the content of the stored amino compounds in the tea plants is decreased due to a rapid consumption of them for growth. Therefore, the amounts of amino compounds translocated into new shoots in the summer crops (plucked in June and August) are less.

This is one of the reasons for the differences in amino compound content observed between the spring crop and the summer crops.

Recently, it was found that heavy application of ammonia-nitrogen to tea plants after plucking in May is effective not only in increasing contents of amino compounds in new shoots sprouted in late June (Table 5), but also in improving the quality of green tea made from these tea shoots.

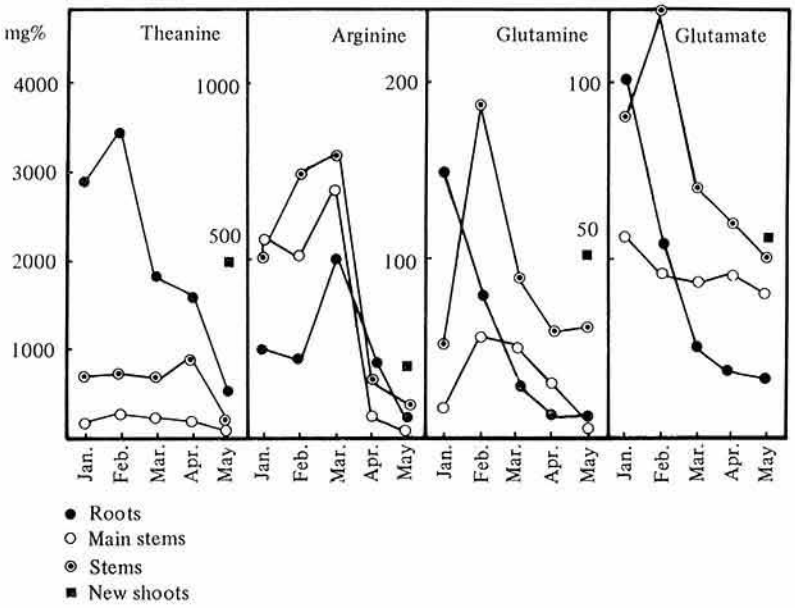

Fig. 4. Amino acids contents in tea plants in winter season

Table 5. Nitrogen components of spring and summer teas

	T-N	S-N	AA.	Thr.	GA.	Arg.	Gln.	The.
1st crop (May)	4.3%	1.3%	174	50	203	120	139	1491
2nd crop (June)								
Standard application	3.4	0.8	50	29	93	6	14	292
Heavy application	4.0	1.0	152	47	143	64	44	893
3rd crop (August)								
Standard application	3.6	1.1	93	20	93	6	21	232
Heavy application	3.9	1.1	66	27	102	86	27	476

Standard application: 100 kg N/ha of $(NH_4)_2SO_4$ applied each after plucking the 1st crop (late May) and the 2nd crop (early July).
Heavy application: 400 kg N/ha and 320 kg N/ha of $(NH_4)_2SO_4$ applied after plucking the 1st crop and the 2nd crop, respectively.
T-N: Total nitrogen, S-N: Water soluble nitrogen, AA.: Aspartic acid, Thr.: Threonine, GA.: Glutamic acid, Arg.: Arginine, Gln.: Glutamine, The.: Theanine.

References

1) Mieflin, B. J. & Lea, P. J.: Amino acid metabolism. *Ann. Rev. Plant Physiol.*, 28, 299 (1977).
2) Sakato, Y.: Studies on the chemical constituents of tea. III. On a new amide Theanine. *J. Agr. Chem. Soc., Japan*, 23, 262 (1950) [In Japanese with English summary].
3) Sasaoka, K., Kito, M. & Onishi, Y.: Some properties of the theanine synthesizing enzyme in tea seedlings. *Agr. Biol. Chem.*, 29, 984 (1965).
4) Takeo, T.: L-Alanine as a precursor of theanine in *Camellia sinensis*. *Phytochem.*, 13, 1401 (1974).
5) Takeo, T.: L-Alanine decarboxylase in *Camellia sinensis*. *Phytochem.*, 17, 313 (1978).
6) Lea, P. J. & Mieflin, B. J.: Alternative route for nitrogen assimilation in higher plants. *Nature*, 251, 614 (1974).
7) Takeo, T.: Glutamate dehydrogenase from tea rootlet. *Agr. Biol. Chem.*, 43, 2257 (1979).
8) Takeo, T.: Ammonia-type nitrogen assimila-

tion in tea plants. *Agr. Biol. Chem.*, 44, 2007 (1980).
9) Takeo, T.: Formation of amino acids induced by ammonia application and seasonal level fluctuation of amino acids contents in tea plant. *Study of Tea*, No. 56, 70 (1979) [In Japanese with English summary].
10) Takeo, T.: Effect of ammonia application on the amino acid concentration of tea new shoots in summer season. *Study of Tea*, No. 56, 78 (1979) [In Japanese with English summary].

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