Theanine Biosynthesis during Tea Seed Germination

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ABSTRACT: Theanine content was measured in tea (*Camellia sinensis* L.) seed during different stages of germination and seedling development. Theanine content increased from 0.1% to 2% during germination. Theanine content progressively increased till 60 days of germination and decreased thereafter. Theanine analysis in different organs revealed that the emerging radicle and plumule contained 10–12% and 8–10% of theanine, respectively. Theanine content in the cotyledon decreased progressively over time during seedling development. The increase in theanine content during germination was found to be associated with increased theanine synthetase gene expression. Further, application of gibberellic acid during germination lead to dramatic increase in theanine content.

KEYWORDS: Germination; tea seed; Theanine; tea seedling

Introduction

L-Theanine (N_5-ethyl-glutamine), the predominant free amino acid in tea is so far observed only in a few species of *Camellia*[1,2,3] and a mushroom[4] *Xerocomus badius*. Theanine has been extensively studied in relation to potential health benefits such as, relaxation, immunity, mood, *etc.* in humans.[5,6] It has been reported that theanine is synthesized in the root and subsequently translocated to the shoot.[8,9,10] Glutamic acid and ethylamine are the likely key precursors for theanine biosynthesis, and the reaction is reported to be catalyzed by L-glutamate:ethylamine ligase.[8,9] The physiological significance of theanine in tea plant is not yet understood. It is speculated that theanine possibly functions as a reserve for storage and transport of organic nitrogen.[11]

Theanine content in tea seed has been studied by several authors.[2] Tsushida and Takeo have quantified theanine in root, shoot and cotyledon of 40 days germinating seedlings in *Camellia sinensis*, *Camellia japonica* and *Camellia sasanqua*.[2,11,12] Higher theanine content was observed in young seedlings. Theanine content decreased progressively during seedling maturation.[2,11] Deng *et al.*[12] have quantified theanine in root, shoot and cotyledons obtained from three weeks germinated seedlings. All these observations suggest an increase in theanine content during seed germination and also presence of theanine in shoot, root and cotyledons of germinating seedlings. However, no systematic studies have been reported on changes in theanine content in cotyledon, radicle and plumule during germination. Therefore, a systematic study was envisaged with three biclonal seed stocks (BSS-1, BSS-2 and BSS-3) to investigate the relationship between theanine content and seedling development. The key findings from these studies are reported in this study.

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Materials and Methods

Germination of Tea Seeds

Three biclonal seed stocks such as BSS-1, BSS-2 and BSS-3 of *Camellia sinensis* L. were procured from UPASI Tea Research Foundation, Valparai, Tamil Nadu, India. Seeds were soaked in water for 24 hrs and spread on a sand bed in a plastic tray. These trays were kept in growth chamber under 12 hrs light and 12 hrs dark cycle at 18°C with 90% relative humidity. First set of samples were collected at 15 days after sowing, and subsequent samples were collected at 5 days interval for 60 days. About 30 seeds were freeze-dried for theanine analysis.

Tea Seedling

Tea seeds of BSS-1 were sowed in pots containing vermiculate. Germination was carried out in plant growth chamber maintained at 18°C with relative humidity with 12 hrs of light and 12 hrs of dark cycle. Shoots, roots and cotyledons were separated from 70, 80 and 90 days seedlings and freeze-dried for theanine analysis.

Theanine Analysis

Freeze-dried samples were ground in mortar and pestle.

Freeze dried sample (1 g) was suspended in 100 ml of 70°C water and allowed to cool to ambient temperature. One ml of suspension was centrifuged at 12,000 × *g* for 15 min and the supernatant was used for analysis. Theanine was detected fluorimetrically following post-column derivatization with *O*-pthalaldehyde. A three pump HPLC system from Shimadzu with fluorescence detector containing auto-sampler was used for analysis. Perfluorooctanoic acid (5 mM) in water was used as a mobile phase 'A' while 5 mM perfluorooctanoic acid in acetonitrile was used as mobile phase 'B'. Borate buffer (1 M, pH 10) containing 0.1% (*w/v O*-pthalaldehyde) was used as buffer 'C' for post-column derivatization with constant flow of 1 ml/min. The column used was Hypersil HyPURITY C18, 5µ, 15 cm × 4.6 cm (Phenomnex Cat. No CH0-4258) along with guard column from Phenomenex. Separation of theanine was achieved using gradient elution of mobile phase A and B as follows. First 0–8 min, concentration of mobile phase 'B' was 15%, subsequently increased to 30% in the next 1 min and kept at 30% for further 11 min. At the 12th min, concentration of mobile phase 'B' was increased from 30% to 90% and maintained up to 15 min. Finally, the gradient was brought to initial concentration within next 2 min. Subsequently column was equilibrated with mobile phase 'B' with concentration of 15% for 15 min prior to next injection. Total flow of mobile phase 'A' and 'B' during gradient elution was 1 ml/min. The eluant from the column was fed into a low dead-volume three-way junction and mixed with *O*-pthalaldehyde reagent in a 1:1 ratio. Derivatized theanine was detected in fluorescence detector set at excitation wavelength of 340 nm and emission as 425 nm. Pure theanine obtained from Taiyo Kagaku Company from Japan was used as standard.

Determination of transcript levels of theanine synthetase

The RNA isolation was carried out from non-germinated and germinated tea seeds as per the instruction manual of the manufacturer (Ambion RNA AqueousTM Cat No. 1912). The RNA was reverse transcribed to cDNA using the reverse transcriptase. The prepared cDNA samples were used for quantitative PCR analysis using primers specific to theanine synthetase (TS). The primer sequence specific to TS used was:

Forward 5' – TTCGGACACTACGGAGAAGG – 3'

Reverse 5' – GAGCCACCCACAATTCATCT – 3'

Initial denaturation was performed at 94°C for 5 minutes. Followed by a short denaturation at 94°C for 45 sec, annealing was carried out at 58°C for 30 sec. This process was continued for 39 cycles. The quantitative analysis was performed by $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen.[13]

Plant Growth Regulators Application

Tea seeds (50 g) were incubated in 10, 100 and 1000 ppm of napthylacetic acid (NAA), gibberellic acid (GA) and benzyl adenine (BA). Samples were aliquoted at 5th, 10th, 15th, 20th, 30th, 45th and 60th day. Samples were freeze-dried for theanine analysis.

Results and Discussion

Theanine Profiling during Tea Seed Germination

Seeds from three biclonal seed stock were collected at various stages of germination for theanine analysis. Phenotypic differences at different stages of germination are shown in Figure 1.

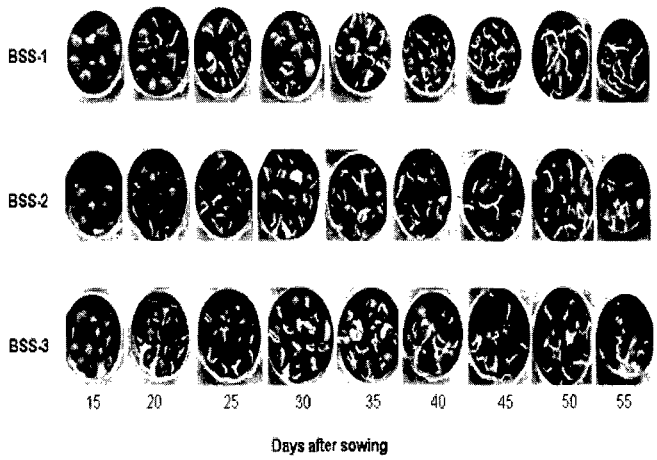

Figure 1. Phenotypic characteristics of tea seeds at various stages of germination.

Non-germinated tea seeds of BSS-1, BSS-2 and BSS-3 biclonal seed stocks found to contain 0.1-0.3% (w/w) theanine. Theanine content gradually increased up to ~2% around 60 days during germination (Table 1).

These results confirm the earlier observations by Feldheim *et al.*,[11] in wider phenotypes (young *vs.* matured) of tea seedlings. Around the time of 30–35 days during germination, theanine content of seeds was found to be around 0.88–1.24% in all three biclonal seed stocks. Since theanine content of tea seed increases prior to radicle emergence, it is likely that cotyledon or embryo have ability to synthesise theanine. In our experiments, where we have separated cotyledon from embryo and incubat-

ed in water at room temperature, we found, continuous increase in theanine content up to 1.3–1.6% on the day 25 (data not shown). These results indicate that tea seed cotyledon is capable of synthesising theanine and possibly translocating to the emerging radicle during germination and seedling development. The physiological significance of elevated theanine during germination and differentiation is unclear and needs further investigation.

Theanine Distribution in Cotyledon and Radicle during Tea Seed Germination

The phenotypic characteristics of 40 days germinated tea seed is depicted in Figure 2A. Theanine content was monitored in emerging radicle and cotyledon during germination of three biclonal seed stocks. On fresh weight basis the radicle and cotyledon contain 1.48–2.65% and 0.34–0.55% (Fig. 2B), respectively. On dry weight basis, theanine content in radicle and cotyledon was about 10–12% and 1–1.5%, respectively. It is important to note that the cotyledon contain ~80–85% of total theanine (Fig. 2C) despite lower theanine content per unit weight.

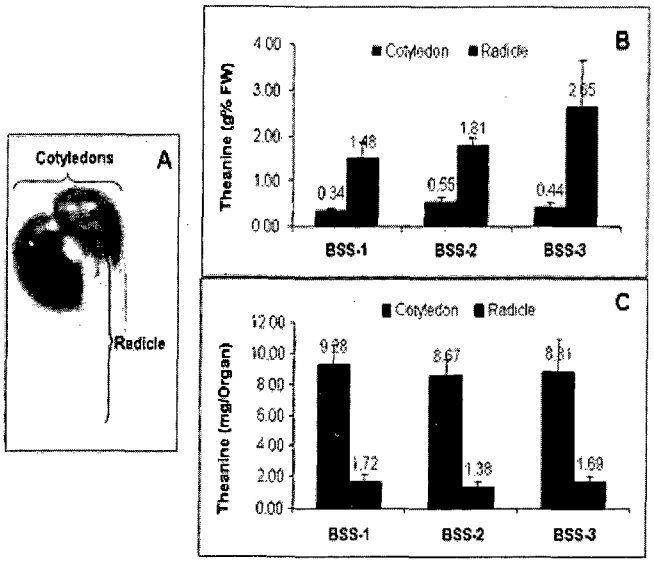

Figure 2. **(A)** 40 days germinating tea seed. **(B)** Theanine content in cotyledon and radicle. **(C)** Theanine distribution in cotyledon and radicle.

Theanine Distribution in Root, Cotyledon and Shoot during Seedling Development

Theanine content in well-differentiated germinated seedlings was monitored in BSS-1 biclonal seed stock from day 60 to day 90. The overall theanine content in seedlings decreased during this period from 0.75% to 0.38% on fresh weight basis (data not shown). These results confirm earlier observation by Feldheim *et al.*,[11] where they reported significant reduction of theanine in shoots and roots of 45 to 90 days seedlings. However, no theanine was estimated in the cotyledon. At around 60 days, both radicle and plumule get well established in germinating tea seed. The phenotypic characteristics of 60, 70, 80 and 90 days tea seedlings are depicted in Figure 3A–D. Root, shoot and cotyledon from 60 days tea seedlings contained 11.63%, 11.49% and 1.16% of theanine, respectively (Fig. 3E).

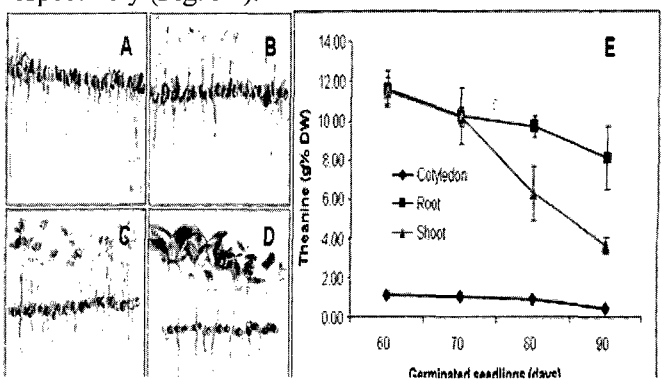

Figure 3. Phenotypic characteristics of germinated tea seedlings: **(A)** 60 days, **(B)** 70 days, **(C)** 80 days, **(D)** 90 days. **(E)** Theanine content in cotyledon, root and shoot of 60, 70, 80 and 90 days seedlings.

Theanine content gradually declined thereafter to reach 8.12%, 3.66% and 0.45% in root, shoot and cotyledon on day 90. Theanine content reduced by 30%, 68% and 61% in root, shoot and cotyledon of 60–90 days seedlings. A decrease in theanine content in the shoot and cotyledon was prominent during seedling maturation. This is an interesting observation and demands further detailed scientific investigation.

Role of Theanine Synthetase

High levels of theanine during germination may arise due to *de-nova* synthesis or through breakdown of proteins. Theanine has not been reported to be present in proteins.[14,15] However, to rule out the possibility, tea seed proteins were hydrolyzed according to the method described by Marconi et al.,[16] and the hydrolyasate was analysed for theanine and its breakdown product ethylamine. Non-hydrolyzed tea seed sample contained 60 µmoles of theanine. However, upon hydrolysis no theanine was detected, but a concomitant increase in ethylamine (57 µmoles) was observed. An increase of equimolar concentration of ethylamine upon acid hydrolysis indicates that no additional theanine was liberated due to protein hydrolysis. Therefore, we examined the pos-

sibility of *de-nova* synthesis by monitoring the levels of theanine synthetase (TS) transcripts levels. Theanine synthetase is one of the key enzymes known to be involved in the synthesis of theanine in tea.[8,9,12] Expression of TS was studied in non germinated and 35 days germinated tea seeds from three biclonal stocks (BSS-1, BSS-2 and BSS-3) using the quantitative PCR. Transcripts analysis for TS gene was carried out by $2^{-\Delta\Delta CT}$ according to the method described by Livak and Schmittgen.[13] Thirty five days germinated tea seeds from BSS1, BSS2 and BSS3 showed 13–26-fold higher TS gene expression over non-germinated tea seed (Table 2). These results indicate that an increase in theanine content in tea seed during germination is associated with increased expression of TS.

Effect of Plant Growth Regulators on Theanine Content in Tea Seed during Germination

Plant growth regulators play important role in seed dormancy and germination.[17,18] Tea cotyledons (50 g) were incubated in 10, 100 and 1000 ppm of different plant growth regulators namely, NAA, GA and cytokinin. Samples were aliquoted at 5th, 10th, 15th and 20th day after incubation and freeze-dried. Theanine analysis in these samples indicate 40%, 45% and 58% increase in theanine content in treatments of 10, 100 and 1000 ppm of gibberellic acid, respectively over control. In addition, 1000 ppm of benzyl adenine treatment also showed increase in theanine content. No significant difference in theanine content was observed in response to NAA treatment (Table 3).

Therefore, the dramatic increase in theanine content in tea seed during germination in response to GA treatment possibly could be attributed to either GA-induced progressive germination process or independent effects of GA on theanine synthesizing machinery. In order to validate this hypothesis, tea seeds were incubated in 100 ppm of GA as a positive control and in 2-chloroethyltrimethylammonium chloride (CCC), which is known to be GA biosynthetic inhibitor,[19] at the concentrations of 1500 ppm and 3000 ppm for 10 days. About 22% reduction in theanine content was observed in response to CCC treatment as compared to control, whereas ~58% reduction in theanine content as compared to GA treatment (Fig. 4).

These results clearly indicate that GA-mediated germination mechanisms appear to play important role on theanine enhancement in tea seed during germination.

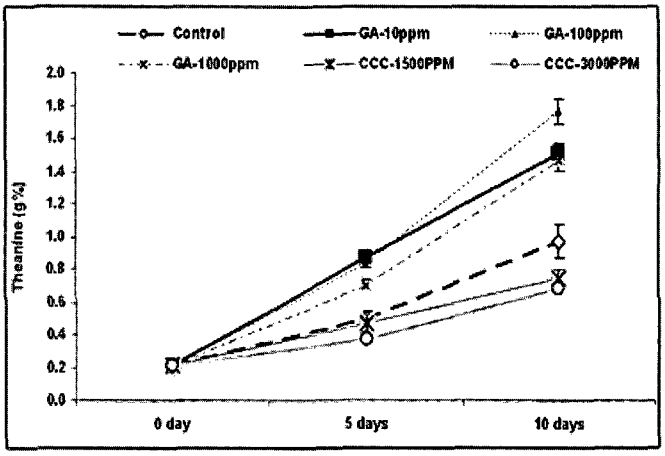

Figure 4. Effect of 2-chloroethyltrimethylammonium chloride (CCC) on theanine content in tea seed.

Conclusions

In summary, a significant increase in theanine content was observed during tea seed germination in three biclonal seed stocks. Theanine content progressively increased till 60 days during seed germination and decreased thereafter during seedling development. Theanine could possibly be synthesised in the cotyledon and translocated to emerging radicle and plumule during seedling development. In addition, external supply of GA during germination leads to further enhancement as compared to auxin and cytokinin. A significant increase in theanine content along with its spatial and temporal variation during tea seed germination needs to be explored further to understand its physiological significance and developmental role in tea.

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