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Tea tissue culture (B)
Theanine and polyphenols accumulation in tea calli

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ABSTRACT: *Camellia sinensis* (L.) Kuntze is the crop plant used as the source for the popular beverage 'Tea'. The main active component in tea responsible for its stimulating 'pick-me-up' effect on the brain is a unique non-protein amino acid called Theanine. In the present study, the effect of theanine precursors - i.e., ethylamine and the ethylamine precursor alanine - on theanine accumulation in tea callus was studied. The precursors were used both singly as well as in combination to observe a potential synergistic effect. At a 15 day interval, callus cultured with 25 mM Ethylamine. HCl showed maximum increase in theanine, 34.64 mg theanine/g dry weight of callus. However, at a 30 day interval, a synergy between ethylamine and alanine was seen with maximum theanine accumulation in the callus grown on 25 mM Ethylamine. HCl + 25 mM L-alanine, 29.93 mg theanine/g dry weight of callus. At the same time, at the 30 day interval a decrease in theanine accumulation was observed in callus grown on 25 mM Ethylamine. HCl with the theanine content falling to 23.14 mg theanine/g dry weight of callus. The theanine content of the tea calli was also found to vary almost inversely with the polyphenol content of the tea calli over the duration of the 60 day culture cycle.

KEYWORDS: Camellia sinensis, precursor, alanine, ethylamine, theanine

Introduction

Camellia sinensis (L.) Kuntze, commonly known as Tea, is one of the most widely cultivated beverage plants in the world. While tea contains more than 700 active constituents[1], the component of particular interest is the unique amino acid Theanine. L-theanine is a non-protein amino acid found only in the genus *Camellia*, *Ilex guayusa* and the fungus *Boletus badius*. It is the primary amino acid present in tea. Theanine has a sedative, relaxing effect on the central nervous system and can cross the blood-brain barrier[2]. Therefore, increasing the amount of theanine content in tea has been a long researched area[2,1]. The maximum biosynthesis of theanine in the tea plant occurs in the roots. The theanine biosynthesis pathway utilizes glutamic acid and ethylamine derived from the amino acid alanine[4]. While glutamic acid levels are high in *in vitro* cultures, it is supposed that the levels of ethylamine are the limiting factor in theanine synthesis[5]. In the present investigation, effect of theanine precursors - ethylamine and alanine - on callus cultures was studied. It has been reported that degradation products of theanine supply the ethyl group (derived from ethylamine) required for the biosynthesis of catechin and related polyphenols[3]. In order to substantiate this hypothesis, a comparison of the relative concentrations of theanine and polyphenols in the tea calli over the duration of the culture cycle was made.

Methodology

Explant source

Explant used for callus induction were leaves from *in vitro* cultured plantlets. Nodal stem segments from UPASI-9 were cultured on modified Murashige and Skoog (MS) medium[6] supplemented with 0.5 mg L^{-1} 6-furfuryl aminopurine (Kinetin) and gelled with 8 g L^{-1} agar (HiMedia, India). The pH of the medium as adjusted to pH 5.7±0.05 prior to autoclaving for 15 min at 121 °C and 15 lb psi. Cultures were maintained under a photoperiod regime of 16/8 hours light/dark using 3000 lux cool white fluorescent tubes. Temperature was maintained at 25±2 °C.

Callus induction

Leaf explant of approximately 1 cm^2 were inoculated on modified MS medium supplemented with 0.5 mg L^{-1} -naphthalene acetic acid (NAA) + 0.5 mg L^{-1}

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2,4-dichlorophenoxyacetic acid (2,4-D) (M_C medium), and gelled with 8% (w/v) agar (HiMedia, India). The pH of the medium was adjusted to pH 5.7±0.05 prior to autoclaving for 15 min at 121 °C and 15 lb psi. The callus was allowed to grow on this medium for 60 days before utilizing it for further experiments. Cultures were maintained under a photoperiod regime of 16/8 hours light/dark using 3000 lux cool white fluorescent tubes. Temperature was maintained at 25±2 °C.

Effect of precursor on theanine formation

Ethylamine in the form of Ethylamine.HCl (Sigma-Aldrich Pvt. Ltd., Germany) ($EtNH_2$·HCl) and L-alanine (HiMedia, India) (Ala) was supplemented in the M_C medium in varying concentrations - 5, 10, 25 and 50 mM. Synergistic effect of both the precursors was investigated. The pH of the medium as adjusted to pH 5.7±0.05 prior to autoclaving for 15 min at 121°C and 15 lb psi. In each concentration, 0.10 g of callus was inoculated and observed for 30 days. Triplicates were maintained in each treatment set.

Estimation of theanine and polyphenol content

Callus samples of 0.1 g were subjected to 24 hour extraction in 1 ml of 50% (v/v) Ethanol. The samples, along with standard L-theanine (1.0 mg ml^{-1}), were then subjected to HPTLC analysis with 70% (v/v) n-Propanol as the solvent system and 2% (w/v) Ninhydrin in Acetone was used for visualization and derivatization. Plates were observed under visible light. Theanine content in the samples was estimated densitometrically by comparing with the standard of known concentration. The estimation of polyphenols in terms of catechin equivalents was performed using the method described by Slinkard and Singleton (1977).

Results

The maximum accumulation of L-theanine in the calli was observed on day 30 of the culture cycle. Increasing culture duration further up to 60 days, resulted in a decrease in the theanine content of the calli (Fig 1). L-theanine is known to serve as a reserve of raw material for the biosynthesis of catechin and catechin-like polyphenols[3]. It has also been reported that the polyphenol content of in vitro cultures increases with the time of incubation[7]. Hence, it is likely that increasing the culture duration of the callus beyond 30 days, causes the accumulated theanine to be channeled into polyphenol synthesis, thereby decreasing the theanine content of the callus while simultaneously increasing polyphenol content.

When ethylamine.HCl was used as supplement, the calli grown on modified MS (P) medium accumulated lower amounts of L-theanine than those grown on modified MS supplemented with 0.5 mg L^{-1} each of NAA and 2,4,-D (M_c) (Fig 2). The roots are known to be the primary site of L-theanine synthesis in the tea plant. The PGRs NAA and 2,4-D are auxins and promote root formation in the calli. Induction of rhizogenesis is observed in some calli if the callus is maintained on M_c medium for more than 90 days. It is likely that the synthesis of theanine occurs in the calli, since they have been grown on auxin containing medium. Auxins can induce the callus to adopt a developmental pathway that favors root formation and hence the cells of the callus show the ability to produce theanine like the roots of the tea plant.

In the L-theanine biosynthesis pathway, ethylamine is the immediate precursor of L-theanine[3]. Ethylamine is also believed to be a limiting factor for theanine synthesis[5]. Hence adding ethylamine as a supplement to the growth medium was expected to increase the theanine content of the calli. Our findings showed that on day 30 of the culture cycle, the accumulation of theanine was highest in calli supplied with 25 mM ethylamine (Fig. 2). Supplementation of growth medium with 25 mM

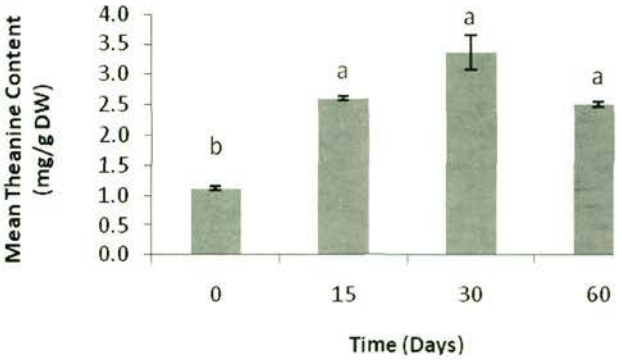

Fig. 1: Theanine accumulation over time in *C. sinensis* callus grown on optimum media.

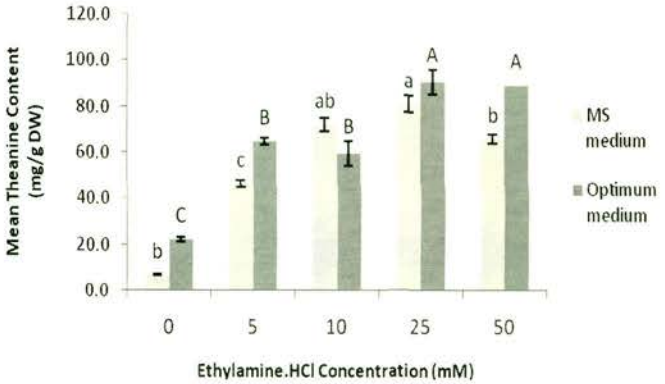

Fig. 2: Theanine content in *C. sinensis* calli grown for 30 days in media supplemented with varying concentrations of Ethylamine.HCl.

ethylamine.HCl resulted in an average theanine content of 90.20 mg g^{-1} dry weight of callus on M_c medium and 80.72 mg/g dry weight of callus on MS (P) medium. These results were similar to those of an earlier investigation of the effect of ethylamine on theanine content in callus cultures of tea[5]. M_c medium was considered to be the optimum growth medium for the calli and was used as growth medium for studies with L-alanine and a combination of ethylamine.HCl and L-alanine.

Alanine is the starting material for the biosynthesis of ethylamine[3]. Supplying L-alanine to the growth medium was expected to enhance theanine synthesis since it may provide additional ethylamine which could be channeled into theanine synthesis. The calli that were grown in optimum medium supplemented with L-alanine did not show significantly different theanine content as compared to the control. The theanine content of the calli which were grown with different concentrations of L-alanine for 15 and 30 days was found to be lower than that of the respective controls (Fig. 3). Among the calli treated with L-alanine, the highest content of theanine was found in calli treated with 25 mM L-alanine, both at 15 days (2.67 mg g^{-1} dry weight) and 30 days (3.28 mg g^{-1} dry weight). However, it appears that adding L-alanine as a precursor does not positively affect theanine content of C. sinensis calli.

The combination of the two precursors was also added to the optimum growth medium as a supplement. Supplying ethylamine and alanine in the growth medium was expected to enhance theanine synthesis since ethylamine would no longer be a limiting factor. The concentration of ethylamine.HCl was maintained at 25 mM while the concentration of L-alanine was varied as 5, 10 and 25 mM. At 15 days, the theanine content of the calli treated with a combination of both precursors was significantly higher than the control and all tested concentrations of L-alanine (Fig. 4). The calli grown with a

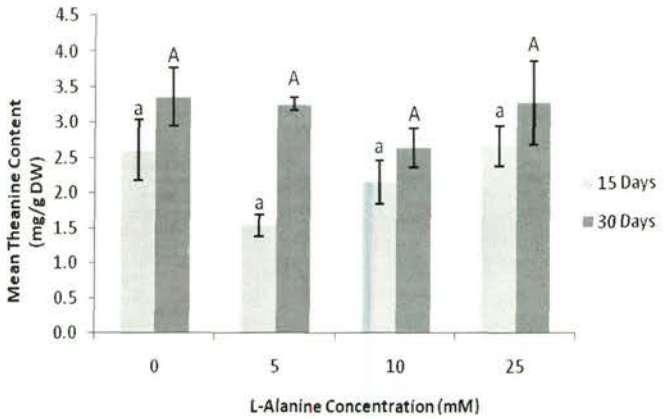

Fig. 3: Theanine content in *C. sinensis* callus grown for on optimum medium supplemented with L-Alanine.

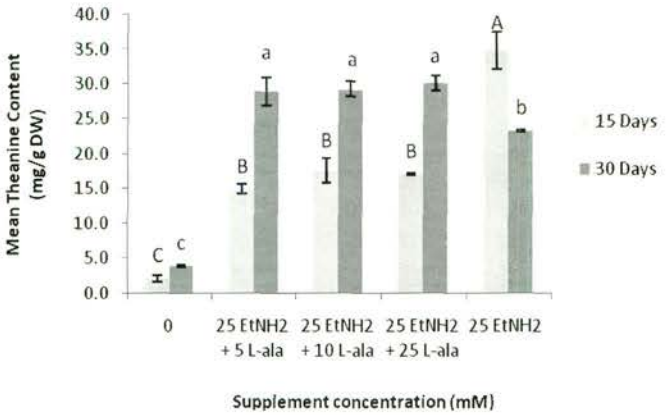

Fig. 4: Theanine content in *C. sinensis* callus grown for 15 days and 30 days on optimum medium supplemented with L-Alanine and 25 mM Ethylamine.HCl.

combination of 25 mM ethylamine and 10 mM L-alanine showed the highest theanine content at 15 days (17.50 mg g^{-1} dry weight). The calli grown with a combination of 25 mM each of ethylamine.HCl and L-alanine showed the highest theanine content (29.93 mg g^{-1} dry weight) at 30 days.

A comparison was made of the most effective concentrations of each of the precursors singly and in combination on the theanine content of *C. sinensis* calli over 15 and 30 days. It was seen that at 15 days, the calli treated with 25 mM ethylamine.HCl alone was significantly higher (34.64 mg g^{-1} dry weight) than that of the calli treated with only 25 mM L-alanine (2.67 mg g^{-1} dry weight) as well as that of the calli treated with a combination of 10 mM L-alanine and 25 mM ethylamine.HCl (17.50 mg g^{-1} dry weight).

At 30 days, the theanine content of the calli treated with a combination of 25 mM each of ethylamine.HCl and L-alanine (29.93 mg g^{-1} dry weight) was significantly higher than either 25 mM ethylamine.HCl (23.14 mg g^{-1} dry weight) or 25 mM L-alanine (3.28 mg g^{-1} dry weight) treated calli. From the above findings, it appears the theanine content of the calli grown with 25 mM ethylamine. HCl as supplement is higher on day 15 than on day 30 of the culture cycle. The synergistic action of the two precursors is apparent on day 30 of the culture cycle.

The productivity of theanine defined as the product of theanine content (mg g^{-1} dry weight of callus) and increase in biomass (g) of callus was calculated for each of the different treatments over 15 days and 30 days. The productivity of calli treated with 25 mM ethylamine.HCl (0.142 mg) was highest at 15 days and was significantly higher than the theanine productivity of calli given any other treatment and the control (0.009 mg). At 30 days, the productivity of calli treated with a combination of

25 mM ethylamine.HCl and 25 mM L-alanine (0.476 mg) was significantly higher than that of the calli treated with 25 mM ethylamine.HCl (0.245 mg) and control (0.046 mg). Hence supplementing the growth medium of the *C. sinensis* calli with a combination of 25 mM ethylamine.HCl and 25 mM L-alanine significantly improves the theanine content of the calli over the culture duration of 30 days.

The theanine content was found to be highest in the tea calli after 30 days. The polyphenol content of the tea calli during the culture cycle appeared to follow a trend opposite to that of theanine, with respect to time (Fig. 5). The polyphenol content of the tea callus increases from day 0 (13.92 mg/g DW) up to day 30 (10.79 mg/g DW). Also, as time progresses from day 30 to day 60, the polyphenol content increases (12.40 mg/g DW). This observation supports the hypothesis that as the culture duration of tea calli increases beyond 30 days, the accumulated theanine begins to get channelized towards the synthesis of polyohenols. It has also been observed that the polyphenol content of cultures incubated for longer durations is greater than that of cultures incubated for shorter durations[7]. The findings of the present study suggest that the calli harvesting stages to obtain highest concentrations of polyphenols and theanine do not coincide. The stage at which the calli cultures are to be harvested will depend upon the required product i.e. theanine or polyphenol.

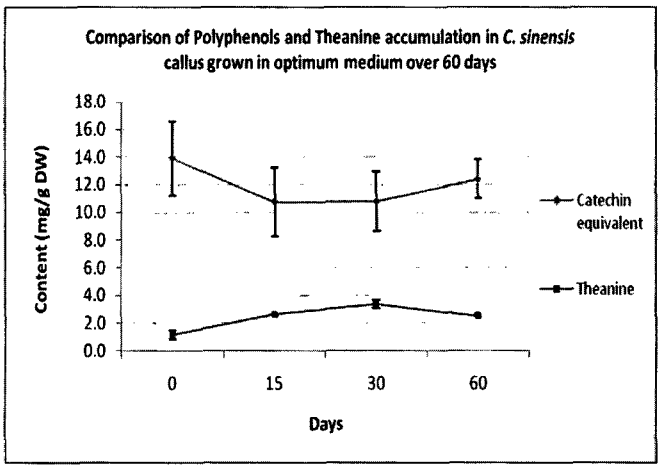

Fig. 5: Accumulation of Polyphenols and Theanine in callus of *C. sinensis* as a function of time.

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Table 1: Effect of supplements on mean theanine productivity in callus cultured on modified MS medium supplemented with 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} 2,4-D

Supplement	Mean theanine productivity (mg) ±SE	
	15 days	30 days
Control	0.009 ±0.001	0.046 ±0.001
25 mM $EtNH_2$.HCl	0.143 ±0.021	0.245 ±0.024
5 mM L-alanine	0.008 ±0.000	0.048 ±0.002
10 mM L-alanine	0.008 ±0.001	0.021 ±0.008
25 mM L-alanine	0.016 ±0.003	0.064 ±0.010
25 mM $EtNH_2$.HCl + 5 mM L-alanine	0.063 ±0.001	0.353 ±0.015
25 mM $EtNH_2$.HCl + 10 mM L-alanine	0.077 ±0.010	0.318 ±0.011
25 mM $EtNH_2$.HCl + 25 mM L-alanine	0.068 ±0.005	0.476 ±0.008