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RESEARCH ARTICLE

The impact of Thiourea on Tea (*Camellia sinensis*) callus proliferation and secondary metabolites content

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ABSTRACT: In the present experiment, the in vitro callus production from tea leaf explants was induced on three different basal media. Then, the efficiency of tea callus proliferation was enhanced through application of thiourea to the proliferation medium. Furthermore, the caffeine and Epigallocatechin Gallate content of the callus and leaf tissues were estimated through HPLC method. The calli mass volume in WPM (woody plant medium) supplemented with thiourea (either 0.1 or 1.0 mM) was significantly higher than other basal media. The callus tissue collected from media enriched with thiourea had significantly more caffeine content but the level of Epigallocatechin Gallate was not statistically affected by thiourea treatments. The amount of these two compounds was also estimated in young and old leaves of the tea mother plants and it was found that the callus tissue had little amount of caffeine and Epigallocatechin Gallate as compared to maternal tissues. It is concluded that, in species with low rate callus proliferation, addition of thiourea to the medium may be followed as an effective and low cost option for callus proliferation improvement.

KEYWORDS: Catechin, Callus culture, Polyphenols, Tea, Thiourea

INTRODUCTION

A plant tissue culture medium is composed of necessary and optional components required for plant growth, which vary according to the plant species, cultivar or explant type that is used and must be experimentally defined for each particular case. Moreover, all the nutrients in a medium should be present in optimum concentrations to ensure the best possible growth of explants (George, 1993). Under *in vitro* conditions, an intact plant requires macronutrients, micronutrients, plant growth regulators, vitamins, amino acids and other nitrogen supplements and sugars (Gamborg, 1991). As far as *in vitro* studies are concerned, an efficient protocol would be required for rapid callus induction, profuse proliferation and likely high amount of potential secondary metabolites. Number of studies recorded the callus induction from various tea explants such as leaf blade (Sarathchanira et al., 1988; Nikolaeva, 2009) petiole (Sarwar, 1985), cotyledon explants (Sarwar, 1985; Kaviani, 2013) and even anther (Chevala et al. 2016). But they all have reported a reluctant and sluggish response in callus proliferation of tea explants as compared to some other plant species.

Thiourea is an organo-sulfur compound, structurally similar to urea except that oxygen atom is replaced by a sulfur atom. Low concentration of thiourea was previously used as a seed dormancy breaking agent. Addition of thiourea to the plant callus medium may also enhance the rate of callus proliferation and also improve the content of secondary metabolites.

Tea (*Camellia sinensis*) is the manufactured drink most widely consumed aromatic beverage in the world, ranks

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after drink water and is especially popular in Asian countries (Chang, 2015; Chevala et al. 2016). China and India are the world largest tea producers, respectively (Chang, 2015). There are two major kinds of tea, black tea and green tea, and they both contain caffeine (1–5%) with small amounts of other alkaloids. Tea also contains large amounts of tannins or phenolic substances (5–27%) consisting of catechin (flavanol) and gallic acid units, with those in green tea being higher than those in black tea (Leung. 1996). The previous studies reported high medicinal properties of tea due to presence of nearly 4000 bioactive compounds in green tea (Chevala et al. 2016). Among all the chemical constitutes major polyphenolic / flavonol compounds in tea are the flavan-3-ols (*i.e.,* catechins), which include Catechin (C), Epicatechin (EC), Epigallocatechin (EGC), Epigallocatechin Gallate (EGCG), Gallocatechins (GC), Epicatechin Gallate (ECG), and Gallocatechin Gallate (GCG) (Goenka, 2013; Kerio, 2013).

Many of the plant species that provide medicinal herbs have been scientifically evaluated for their possible medical applications (Mulabagal, 2004). For example, number of studies recorded the beneficial effects of tea, which include antioxidant, anticarcinoma, anti-inflammatory, antidiabetic and antimicrobial properties (Chevala et al. 2016). It has been mentioned that natural habitats for medicinal plants are disappearing fast and together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into *in vitro* cultures as an alternative supply for the production of plant pharmaceuticals (Chevala et al. 2016). It means, the capacity for plant cell, tissue, and organ culture to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology (Muthaiya et al. 2013). So, there has been considerable interest in *in vitro* techniques as a potential alternative to traditional agriculture for the industrial production of secondary metabolites (Dicosmo et al. 1995). Furthermore, the objectives of many industries are to develop plant cell culture techniques to the stage where they yield secondary products cheaper than extracting either the whole plant grown under natural conditions or synthesizing the product (Mulabagal, 2004). The plant callus proliferation may be enhanced using different organic and inorganic compounds to the induction or proliferation media (George, 1993). In the present study, the *in vitro* callus formation from tea leaf explants was induced on three different culture media. Then, the efficiency of callus proliferation was enhanced through application of thiourea as an adjoining experiment. Lastly, the calli mass was collected from *in vitro* conditions and was analyzed for epicatechin and caffeine content as compared to intact leaves of the mother plants.

MATERIALS AND METHODS

Plant materials

The north of Iran is considered as important region for tea growing and production. The five healthy tea shrubs were procured from tea commercial farms of Lahijan, Gilan. These mother plants were transplanted in desirable pots and transferred to a private laboratory (Hirkan Plant Tissue Culture Laboratory, Golestan, Gorgan). The mother plants were kept in a semi-shady area and irrigated regularly with distilled water.

In vitro studies

The healthy and green leaves were harvested. Samples were wrapped immediately in moist cloth and transferred to the laboratory for the *in vitro* studies. The leaves were then prewashed in a solution contained 0.1-0.2 % commercial detergent (Pril Co., Iran) followed by stirring in Carbendazim (2 g l^{-1}) solution for 2 h. The fungicide was drained and the leaves were then surface disinfected inside the laminar flow hood following a procedure previously standardized for a range of plant species in Hirkan Plant Tissue Culture Laboratory (unpublished data). The leaves were subjected to 60 % (v/v) NaOC1 solution (commercial bleach with 5 % available chlorine) for 25 min followed by several times rinsing with autoclaved distilled water. These were then agitated in 70 % ethanol for 25 sec followed by 4-5 rinses in sterile distilled water. Each leaf blade was dissected into 3-4 segments and were inoculated in glassware containing 20 ml MS (Murashige and Skoog, 1962), SH (Schenk R, Hildebrandt, 1972) or WPM (Lloyd G, McCown, 1980) media supplemented with 6-benzyl aminopurine (BAP) and α-naphthalene acetic acid (NAA) and 200 mg l^{-1} activated charcoal (AC) (The concentration of PGRs were shown in Table 1). The pH was adjusted to 5.8 prior to the addition of 0.8 % agar, and the media were autoclaved at 121 °C and 15 PSI for 15 min.The cultures were incubated at 25 ± 2 °C under continuous light (50 µmol·m^{-2} s^{-1}).

Table 1. Initial screening of some media for callus induction in tea leaf segment explants.

S. No.	Basal Medium	PGR composition (mg/l)	Callus induction (%)
1	MS	BA (5.0), NAA (0.2)	85.50 [a]
2	MS	BA (0.05), NAA (2.0)	91.75 [a]
3	MS	BA (2.0), NAA (2.0)	75.00 [a]
4	WPM	BA (1.0), 2,4-D (2.0)	91.75 [a]
5	WPM	BA (2.0), 2,4-D (0.05)	73.00 [a]
7	WPM	BA (5.0), NAA (0.2)	95.00 [a]
6	WPM	No PGRs	8.25 [b]

In order to stimulate callus proliferation, the thiourea at the rate of 0, 0.1 and 1.0 mM (encoded as T0, T1 and T2 respectively) were added to different media. The proliferated cultures were evaluated for callus volume, firmness, color as per procedures already explained by Alizadeh (2011) *i.e.* the callus firmness and volume were determined as observative scoring method (1, 2, 3 represents low, moderate and high level of firmness or callus volume).The pooled callus mass from each medium was collected and evaluated for secondary metabolites (caffeine and EGCG) through HPLC technique.

Extraction and HPLC analysis of EGCG and Caffeine

The callus tissues were subjected to HPLC analysis to determine their secondary metabolites. However, to compare the level of these metabolites with those of maternal mature tissues, the HPLC analysis also were performed with samples of both young and old leaves of mother plants. The method described by (INA method 11.002) as used by Zokti et al. (2016) for the extraction of tea phenolic compounds was applied to EGCG extraction with slight modifications with regard to callus samples. The samples were grinded and homogenized when used. Then, 250 mg of the powdered samples was weighted into a 25 ml Erlenmeyer flask and mixed with 5 ml methanol 70% and stirred on a magnetic stirrer for 30 min. The extraction mix was centrifuged at 3500 rpm for 10 min, and carefully decanted the supernatant into a 10 ml volumetric flask. The steps of extraction were repeated and two extractions were combined. Finally the extraction volume was reached to mark with the extraction solvent. The extract stored at 4°C until further analysis (The extract is stable for at least 24 h if stored at 4 °C). The EGCG and Caffeine content were determined in the samples. Analyses were performed in a high performance liquid chromatography (Knauer, Germany) equipped a HPLC pump K-1001 and a UV detector K-2600 and software EZ chrome. Separation was carried out using an uncleosil-100, ODS C18 column (250×4.6 mm, 3-5 micrometer). The mobile phase was acetonitrile and water containing 0.1% v/v of phosphoric acid as a gradient program (acetonitrile 8% to 22% in 35 min, hold for 1 min, acetonitrile 22% to 8% in 9 min and hold for 5 min) at a flow rate of 1 ml/min. The compounds were detected at wavelength of 280 nm. Sample aliquots were filtered through a 0.45 µm nylon membrane and injected a 20 µl of solution. The HPLC graph for EGCG standard, callus tissue and tea old leaves were shown in Fig 1.

Data collection and analysis

The experiment was conducted as completely randomized design with 10 replications in callus culture experiment and at least three replications with respect to HPLC analysis. The data were analyzed using SAS software and means were comapared with LSD test.

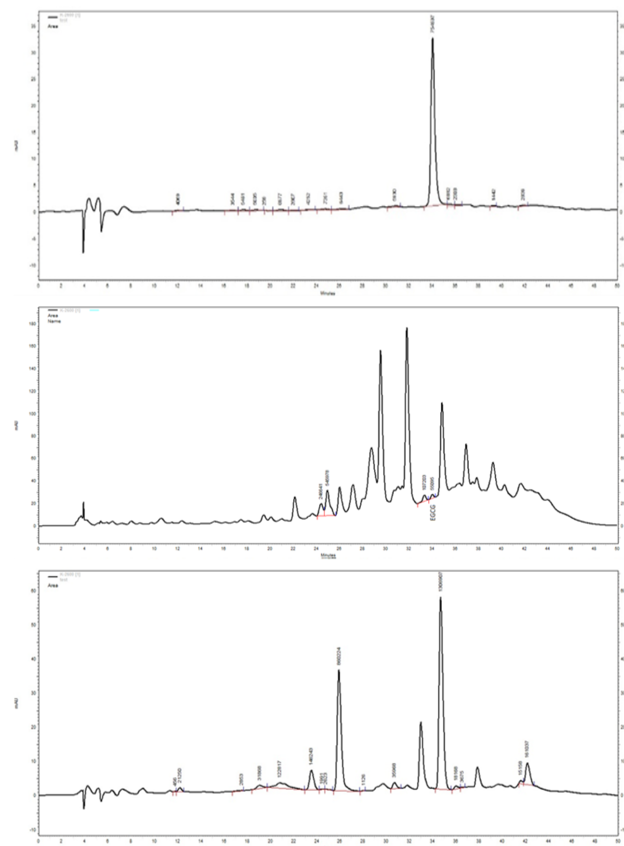

Fig 1. The HPLC chromatogram of EGCG standard (top), callus tissue sample (middle) and tea old leaves (bottom).

RESULTS AND DISCUSSION

In the present study, firstly a screening experiment was performed with regard to callus initiation from young leaves of tea plants using either MS, SH or WPM media comprising 20 different PGRs combinations (data not shown), however, the most responsive media were shown in Table 1. This screening experiment was revealed that BA in combination with an auxin (NAA or 2,4-D) are essential for callus initiation. In any growth regulator free medium the initiated caulls was significantly decreased for example only 8.25% callus initiation was recorded in WPM hormone free medium (Table 1). The efficacy of callus induction by application of cytokinin and auxins was already reported by George (1993) for many species and also by Nikolaeva et al. (2009) and Chevala et al. (2016) for tea explants.

In order to assess the role of thiourea on callus proliferation three basal medium *i.e.* MS, SH and WPM was supplemented with either 0.0, 0.1 or 1.0 mM thiourea. Addition of thiourea to the media significantly enhanced callus proliferation (Fig 2 and 3). Furthermore, the callus firmness as well as volume were increased in thiourea supplemented media (Table 3). The basal medium itself has not any effect on these parameters. The callus mass initiated in 7 different media represented in Table 1 was regularly sub-cultured in the same basal media but fortified with BA (2.0 mg l^{-1}), 2,4-D (0.5 mg l^{-1}) and AC (200 mg l^{-1}) in 4 week intervals. However, it was found that application of little amount of thiourea to these media may enhance the rate of callus proliferation (Fig 1 and 2). According to Fig 1, the callus volume in WPM media supplemented with thiourea (either 0.1 or 1.0 mM) was significantly higher (1.6 times more) than that of WPM without thiourea. This rate of proliferation was superior to other basal media as well. The encouraging role of thiourea on callus proliferation was depicted in Fig 2 as well. Thiourea is an organo-sulfur compound with formula $SC(NH_2)_2$. It is structurally similar to urea except that oxygen atom is replaced by a sulfur atom. The properties of urea and thiourea differ significantly. Thiourea has a wide range of applications. In plants, low concentration of thiourea was first used as a dormancy breaking agent (Hartmann et al. 2007). Use of thiourea significantly improved plant growth in terms of root and shoot weight, height and number and leaf area (Perveen et al. 2015). Thiourea not only improved growth at whole plant level but it also enhances growth at cellular level under stress (Ikram, 2015). The accelerated cell divisions is a function of thiourea already reported by Sanaullah et al. (2016) in callus tissues of maize hybrids under *in vitro* conditions which is corroborated with our results for positive role of thiourea in tea callus proliferation (Fig. 2 and 3).Therefore, it is clear that, in plant species with slow *in vitro* callus proliferation, addition of thiourea to the medium may be followed as an effective and low cost option for callus proliferation improvement.

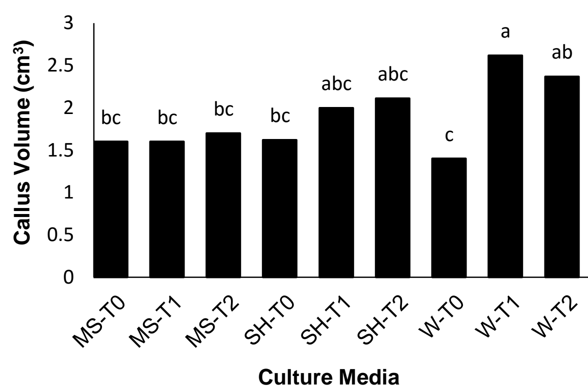

Figure 2. The comparative growth of tea callus in MS, SH and WPM basal media 4 weeks after sub-culture. The basal medium was supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA and 200 mg l^{-1} AC. The T_0, T_1 and T_2 denote 0, 0.1 and 1.0 mM thiourea supplemented to the basal media.

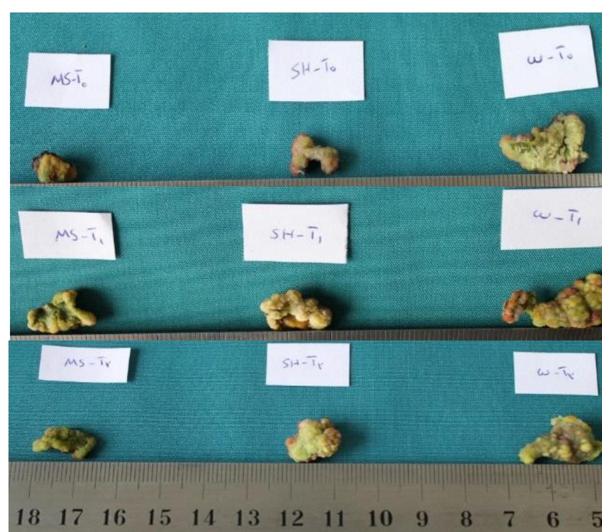

Figure 3. The comparative growth of tea callus in MS (left), SH (middle) and WPM (right) basal media 4 weeks after sub-culture. The basal medium was supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA and 200 mg l^{-1} AC. The T_0, T_1 and T_2 denote 0, 0.1 and 1.0 mM thiourea supplemented to the basal media.

Table 2. The callus attributes procured from different media enriched with thiourea.

Media code	Basal Medium**	Thiourea (mM)	Callus traits*		
			Firmness	Volume	Color
MS-T0	MS	0	1.66 bc	1.6 b	G
MS-T1	MS	0.1	1.83 abc	1.8 b	G
MS-T2	MS	1.0	1.83 abc	2.0 ab	G
SH-T0	SH	0	1.83 abc	1.8 b	G/R
SH-T1	SH	0.1	2.00 abc	2.0 ab	G/R
SH-T2	SH	1.0	2.00 abc	2.1 ab	G/R
W-T0	WPM	0	1.33 c	1.4 b	G/R
W-T1	WPM	0.1	2.50 a	2.6 a	G/R
W-T2	WPM	1.0	2.16 ab	2.1 ab	G/R

* The G and R means Green and Red colors, respectively.
** All the media were supplemented with BA (2.0 mg l^{-1}) and (2,4-D mg l^{-1}).

Table 3. The amount of EGCG and Caffeine content of tea callus tissues procured from different media with or without thiourea.

S. No.	Media code	Basal Medium	Thiourea (mM)	EGCG (mg/g dw)	Caffeine (mg/g dw)
1	MS-T0	MS	0	0.067 a	0.52 g
2	MS-T1	MS	0.1	-	0.88 d
3	MS-T2	MS	1.0	-	1.23 b
4	W-T0	WPM	0	0.128 a	0.72 f
5	W-T1	WPM	0.1	0.122 a	1.05 c
6	W-T2	WPM	1.0	0.298 a	1.05 c
7	SH-T0	SH	0	0.072 a	0.84 e
8	SH-T1	SH	0.1	0.070 a	0.87 d
9	SH-T2	SH	1.0	0.067 a	1.36 a

Analysis of variance (ANOVA)

Sources of variation	df	EGCG	Caffeine
Treatment	8	0.024 ns	0.198 **
Error	18	0.027	0.00004
CV		13.8	0.7

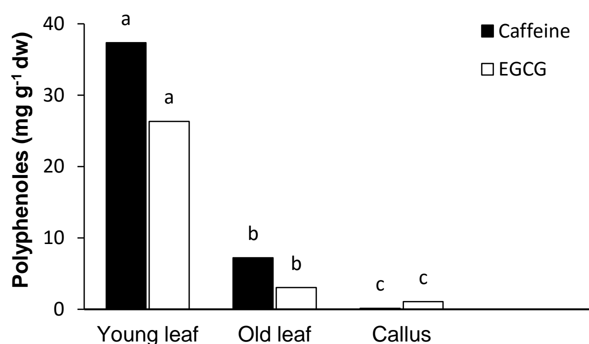

Fig 4. The amount of caffeine and EGCG of tea callus tissues as compared to young and old leaves of the mother plants.

Plant cell and tissue cultures hold great promise for controlled production of myriad of useful secondary metabolites. Exploration of the biosynthetic capabilities of various cell cultures has been carried out by a group of plant scientists and microbiologists in several countries during the last decade. A number of medicinally important alkaloids, anticancer drugs, recombinant proteins and food additives are produced in various cultures of plant cell and tissues (Filova, 2014). Medicinal plants produce a wealth of secondary metabolites also known as natural products, which are small molecular weight compounds with enormous structural diversity and show various biological activities (Harvey, 2015). The phenolic compounds are nowadays among the moststudied families of natural products, for their bioactive properties, being naturallyproduced by plants and showing an immense structural and chemical diversity. The phenoliccompounds became desirable targets for *in vitro* culture, which provides the necessarystress conditions for their production and increases the excretion of these secondarymetabolites by the plant tissues (Matkowski, 2008). Around sixty plant species are known to contain caffeineand it has long been reported that tea callus tissues are also capable of caffeine formation (Ogutuga and Northcote, 1970; Hegazi and El-Lamey, 2012).

The calli mass collected from different media with or without thiourea was analyzed with HPLC for levels of caffeine and EGCG (Table 2). The results revealed that the caffeine levels were significantly different among media. All the media enriched with thiourea had considerably higher caffeine as compared to other treatments. The highest amount of caffeine (1.36 mg g^{-1} dw) was estimated in SH basal medium supplemented with 1.0 mM thiourea. However, thiourea had no remarkable effect on EGCG accumulation in tea callus tissues and the highest level (0.298 mg g^{-1} dw) was recorded in WPM supplemented with 1.0 mM thiourea that was not statistically different with other treatments (Table 2).

The amount of caffeine and EGCG young and old leaves of the tea mother plants as compared to callus tissues were showed in Fig. 4. It is clear that tea callus had little amount of caffeine and EGCG as compared to maternal tissues. The young leaves exhibited maximum accumulation of these polyphenols. In the present research work caffeine and EGCG were estimated as an adjoining experiment in callus as well as tea leaves (Fig. 4). It was found that these compounds were synthesized

in callus cells as well. However, their amount were very low as compared to tea leaf content (about 300 times lower than young leaves). So, the callus itself may not be a reliable source of extraction of these metabolites. However, these metabolites sharply reduced with the leaf aging and it was clear that old leaves had about quintuple lower concentrations of both caffeine and EGCG (Fig. 4). So, where there is unavailability of young leaves materials the application of *in vitro* techniques may be considered in this regard. On the other hand, application of thiourea to the medium increased the caffeine levels of the callus samples (Table 3). The EGCG in callus tissues treated by thiourea was also increased by the changes were not statistically different. Similarly, casein hydrolysate and L-phenylalanine have also been used to induce the production of phenolics in *calli* of *Ephedra alata* Decne.(Anand, 2010). He noticed a higher accumulation of chlorogenic acid, rutin, catechin, quercetin and coumaric acid in *calli* elicitated with casein hydrolysate.

CONCLUSION

In overall, it can be concluded that callus can be successfully induced from young leaf segments of tea plants on MS, WPM, SH basal media supplemented with BA (0.02 – 1.0 mg l^{-1}) in combination with NAA (0.2-2.0 mg l^{-1}) or 2,4-D (0.2- 2.0 mg l^{-1}). The calli mass may be sub-cultured on the same basal media supplemented with 2.0 mg/l BAP and 0.5 mg l^{-1} 2,4-D and 200 mg l^{-1} AC. Furthermore, addition of thiourea to the medium may enhance the rate of callus proliferation and also improve the content of caffeine and EGCG. Furthermore, as noted from the changes in other callus attributes (volume, firmness) in this research, there is a need to explore the anticipated signaling properties of thiourea in gene expression of tea callus cells. Furthermore, *in vitro* application of thiourea to callus proliferation may be applied in other plants with low rate of callus proliferation.

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مطالعه اهمیت تیوره بر میزان تکثیر کالوس و محتوی متابولیت‌های ثانویه گیاه چای(*Camellia sinensis*)

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چکیده

در پژوهش حاضر، تولید بافت کالوس از قطعات برگ چای در سه محیط کشت پایه مختلف القا شد. سپس، کارآیی تکثیر کالوس گیاه چای با استفاده از افزودن تیوره در محیط تکثیر بررسی شد. علاوه بر این، میزان کافئین و اپی‌گالوکاتچین‌گالات بافت کالوس و برگ به روش HPLC ارزیابی شد. حجم توده کالوس در محیط کشت (WPM محیط کشت گیاهان چوبی) به همراه تیوره (۰/۱ یا ۱/۰ میلی‌مولار) به طور معنی‌داری بیشتر از سایر محیط کشت‌ها بود. بافت کالوس جمع‌آوری شده از محیط غنی شده با تیوره از نظر دارا بودن میزان کافئین به طور معنی‌داری بیشتر بود، اما سطح اپی‌گالوکاتچین‌گالات از نظر آماری تحت تأثیر تیمارها قرار نگرفت. مقدار این دو ترکیب در برگ‌های جوان و مسن گیاهان مادری چای نیز تخمین زده شد و مشخص شد که بافت کالوس نسبت به بافت‌های مادری دارای مقدار کافئین و اپی‌گالوکاتچین‌گالات کمتری است. در نتیجه‌گیری کلی پیشنهاد می‌شود که، در گیاهانی که تکثیر کالوس آن‌ها با سرعت کم پیش می‌رود، ممکن است افزودن تیوره به محیط کشت به عنوان گزینه‌ای موثر و کم‌هزینه برای بهبود تکثیر کالوس آنها دنبال شود.

واژهای کلیدی: کاتچین، کشت کالوس، پلی فنول، چای، تیوره