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Full Length Research Paper

Effects of *Camellia sinensis* L. extract and cysteine on browning, growth and paclitaxel production of subcultured *Taxus brevifolia* L. calli

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So far huge studies have been reported on antioxidant effects of *Camellia sinensis* L. in animal/human cells, however little is known about its impacts in plant cells/tissue culture. In this investigation, we evaluated the biological effects of *C. sinensis* extract and cysteine on production of paclitaxel using tissue cultures of *Taxus brevifolia* L. Various calli and its subcultures were cultivated using B5 media in presence or absence of designated amount of *C. sinensis* extract and cysteine. Activities of polyphenol oxidase (PPO) and peroxidase (PO), browning rate, callus growth and paclitaxel production were examined in the treated cultures of *T. brevifolia*. PPO and PO enzymes were found to be activated in the calli subcultures upon treatment with the *C. sinensis* extract. Subcultured callus of *T. brevifolia* treated with cysteine (100 mg/L) showed lower activity of PPO, while those treated with high amounts of cysteine (>200 mg/L) displayed higher activities of these enzymes. Use of *C. sinensis* extract and cysteine failed to suppress the browning phenomenon, however tissues treated with these compounds showed significant increase in paclitaxel production (794 mg/kg dry weight of calli). Based on our findings, the production of paclitaxel can be improved in subculture or suspension cell culture of *T. brevifolia* upon treatment with *C. sinensis* extract, which may be considered as a cost-beneficial elicitor.

Key words: Antioxidant, callus browning, tissue culture, cysteine, paclitaxe, polyphenol oxidase, proxidase, phenolics.

INTRODUCTION

Paclitaxel is a natural compound isolated from the bark of *Taxus brevifolia* L., which is known as a potent anticancer agent (Wani et al., 1971). The extraction of paclitaxel from natural sources has been restricted due to paucity of *Taxus* species that are in danger of extinction. These drawbacks can be exacerbated with extremely low yielding of this secondary metabolite in the bark of *Taxus* trees. While paclitaxel use appears to be an increasing demands in clinic, its production needs to be improved through alternative biotechnological approaches. We have previously reported that suspension cultures of *Taxus baccata* treated with some elicitors can result in considerably high amount of paclitaxel (Khosroushahi et al., 2006). However, for commercial large scale production of paclitaxel, it is a prerequisite to obtain healthy calli and accordingly cell line, which has been found to be a very difficult process because of browning phenomenon during subculture of callus. Basically, browning phenomenon is considered as a limiting obstacle associated with the cell culture approach, perhaps due to production of phenolic compounds and their oxidative impacts on tissues (Banerjee et al., 1996; Bhardwaj and Ramawat, 1993; Tang et al., 2004; Vaishnav et al., 2006). The phenolic materials can be converted to pestilent compounds by enzymes such as PPO and PO (Murata et al., 2001; Wu and Lin, 2002). Thus, the biosynthesis of these compounds must be regulated during calli induction or cell suspension of *Taxus sp.* Cysteine can inhibit the enzymatic browning

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and acts as an antioxidant. It has been previously reported that cysteine can act as an effective suppressor of PPO in *Lactuca sativa* L. (Altunkaya and Gkmen, 2008) and apple (Richard et al., 1991). Having showed antioxidant activities, cysteine can prevent the browning and necrosis in *Cocculus pendulus* L. callus cultures (Bhardwaj and Ramawat, 1993). Green tea (the so called *Camellia sinensis)* represents potent antioxidant activity (Liang et al., 2004; Ohmori et al., 2005; Park et al., 2006), free radical scavenging potential (Lee et al., 2008; Sawai et al., 2005) and peroxidation inhibiting activity (Babu et al., 2006; Hamden et al., 2008; Skrzydlewska et al., 2002).

In this investigation, we hypothesized that *C. sinensis* extract and cysteine can inhibit the browning phenomenon through suppression of polyphenol oxidase and peroxidase that may increase the paclitaxel production in subcultured calli of *T. brevifolia*.

MATERIALS AND METHODS

The 1,1-diphenyl-2-picrylhydrazyl (DPPH), tripyridyl-triazine (TPTZ), ciocalteu (F-C), 2,6-dimethoxyphenol and gallic acid were purchased from Sigma Aldrich Chemical Co. (Poole, UK). The 4-aminoantipyrine, phenol and sodium dodecyl sulfate (SDS) were purchased from Merck (Darmstadt, Germany). All other materials used in culture medium (that is, salts, sugars, vitamins, growth regulators and phyto-hormons) were purchased from either Sigma Aldrich Chemical Co. (Poole, UK) or Merck (Darmstadt, Germany).

Tissue culture and maintenance

All explants were taken from one *T. brevifolia* tree located in the botany garden of the University of Tehran. Juvenile stem was used as explants after sterilization cultured using a modified B5 medium supplemented with 2 mg/L naphthaleneacetic acid (NAA), 1.5 mg/L 3-indoleacetic acid (IAA), 0.2 mg/L 6-benzyl aminopurine (BA) and 20 g/L sucrose at pH=5.7. After callus induction (20 days), subcultures were performed onto the media that were designed for every section of this investigation.

C. sinensis extraction and experiment design

To extract the total phenolics compounds of *C. sinensis* and to perform the antioxidant activity assay, 1 g dry leaves of C*. sinensis* were ground, suspended in 20 ml of methanol (80%), sonicated for 20 min, and centrifuged at 15000 × *g* for 20 min. The supernatant was dried with vacuum evaporator and resolved in 1 ml deionized water, which was then subjected to an antioxidant activity assay (Hong et al., 2008). Scavenging effect assay was carried out using a previously reported method (Oliveira et al., 2008). Briefly, 0.3 ml of concentrate of *C. sinensis* extract was mixed with 2.7 ml methanol solution of DPPH. The mixture was vigorously shaken and left to stand for 30 min in the dark. The DPPH scavenging effect was calculated as a percentage of decreasing of absorption in 517 nm. Ascorbic acid was used as the reference compound. This provided the exact amounts (100 µl) of extract used for experiments. Such amount of the extract showed antioxidant activity of 5-fold greater than 0.5 g/L ascorbic acid. The experiment was planned based on completely random design (CRD) with 5 treatments and 3 replications per treatment. The media were supplemented with 100, 200, 300, 400 and 500 µl/L extract of *C. sinensis* and considered as T1, T2, T3, T4, T5 groups, respectively.

Cysteine effects

To assess the effect of cysteine on callus subculture, the experiment was planned based on CRD with 10 treatments and 3 replications per treatment. The treatments of the cysteine experiment were conducted using 100-1000 mg/L with 100 mg interval (that is, as T1-T10 groups, respectively).

Polyphenol oxidase extraction and enzyme activity assay

Fresh callus (1 g) was extracted using 1 ml extraction buffer [buffer phosphate (Na_2HPO_4 and NaH_2PO_4) 100 mM, pH = 7.2; 0.1% (w/v) SDS; 3 mM ascorbic acid] (Tang et al., 2004) by sonication at 4°C for 15 min. The extractions were centrifuged at 15000×*g*, 4°C for 20 min, and supernatants were subjected to assay. The assay condition was the mixture of 300 µl buffer phosphate (pH=7.4), 400 µl ethanol solution of 2, 6-dimethoxyphenol (0.5 mM) and 300 µl fresh callus extracts. The absorbance of samples was measured at 468 nm by UNICAM UV4 UV/VIS spectrophotometer and the change of 0.01 in absorbance defined as a unit (U).

Peroxidase extraction and enzyme activity assay

Fresh callus (1 g) was homogenized in the 10 ml acetone -20°C (Chisari et al., 2008) and then centrifuged at 15000×*g*, 4°C for 20 min. The supernatant was dried by vacuum evaporator, and the attained powder was resuspended in 1 ml buffer phosphate (200 mM), kept at 4°C over the night then recentrifuged at 15000×*g*, 4°C for 20 min. To assay the crude peroxidase activity, 100 µl crude extract was dissolved in the mixture of 700 µl H_2O_2 and 200 µl of buffer phosphate containing solvent 1.6 g phenol and 50 mg 4-Aminoantipyrine in 50 ml of buffer phosphate. The absorbance of samples was measured at 510 nm by UNICAM UV4 UV/VIS spectrophotometer and the change of 0.01 in absorbance defined as a unit.

Total phenolic extraction and assay

Fresh callus (1 g) was suspended in the 20 ml aqueous methanol (80%) (Velioglu et al., 1998) and sonicated at 25°C for 20 min then centrifuged at 15000×*g*, 4°C for 20 min. The supernatant was dried, then resolved in the 1 ml methanol and used for determining of total phenolic concentration. For total phenolic assay, the Folin Ciocalteu method was applied (Ainsworth and Gillespie, 2007). The solution of Folin Ciocalteu (200 µl) was mixed with 100 µl of the concentrated extract and vortex thoroughly. It was then mixed with 700 µl of Na_2CO_3 (700 mM) and maintained at 25°C for 2 h. The absorbance of samples was read at 765 nm by UNICAM UV4 UV/VIS spectrophotometer, for which the gallic acid was considered as a reference (Ainsworth and Gillespie, 2007).

Paclitaxel extraction and determination

Paclitaxel was extracted from dried callus with some modifications as described in our previous report (Khosroushahi et al., 2006). Briefly, 0.2 g of dried callus was ground in pestle and mortar then suspended in 20 ml methanol and sonicated for 15 min. The samples were centrifuged at 15000×*g* and supernatants were collected and evaporated by vacuum evaporator. The obtained dried extract was resolved in 1 ml of methanol and used for HPLC analysis using Pharmacia Biotech system equipped with C18

Table 1. Effects of *Camellia sinensis* extract on subcultured *Taxus brevifolia* calli.

Camellia sinensis extract	PPO activity (U)	PO activity (U)	Total phenolics (GAE/gFW)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
100(µl)	0.045±0.000	0.257±0.011	157.407±6.481*	428.037±18.115*	0.831±0.111*	2.66±0.577
200(µl)	0.082±0.005	0.313±0.009*	184.907±9.062*	469.361±16.319*	0.693±0.040*	3.132±1.012*
300(µl)	0.107±0.002*	0.361±0.014*	200.808±2.577*	600.494±22.375*	0.573±0.066*	3.333±0.577*
400(µl)	0.149±0.001*	0.393±0.004*	218.555±12.807*	713.322±47.656*	0.336±0.050*	3.666±1.154*
500(µl)	0.186±0.004*	0.370±0.012*	197.777±8.053*	794.354±22.008*	0.152±0.05*	4.023±1.213*
Control	0.053±0.002	0.231±0.006	82.0987±2.918	190.062±9.802	1.466±0.450	1.666±0.577

GAE: Gallic acid equivalent; DW: Dry weight; FW: Fresh weight. *Significant ($P < 0.05$) differences compared to other treatments.

Column (4.6×250 mm, 5 µm, Philips). The mobile phase was acetonitrile/H2O (60:40). The flow rate was fixed at 1.0 ml/min and injection volume was set at 20 µl. Detection of paclitaxel was accomplished at 227 nm.

Growth measurement

The induced callus was weighed under the sterile condition prior to its subculture. After 30 days, they were weighed and the weight difference was considered as the growth rates of calli.

Cell morphology study

To study the morphological changes in browned and yellow-white callus cells of *T. brevifolia*, the inverted microscope (Olympus, IX81) equipped with a digital camera (Olympus, 72 DPI), super plan apochromat lenses and WIB and WIG mirrors was exploited.

Browning estimation

Upon the intensity of browning in cultured callus, five classes of calli based on the intensity of browning were ranked morphologically. To rank the browning, 1 to 5 scores were used representing the intensity from the lowest to the highest levels.

Data analysis

Variance analyses were carried out using one way ANOVA with Duncan's multiple comparison test and also orthogonal comparisons ($p<0.05$) for all data by utilizing Minitab version 15 and MSTATC version 1.2. A p value less than 0.05 was considered to represent the statistical difference.

RESULTS

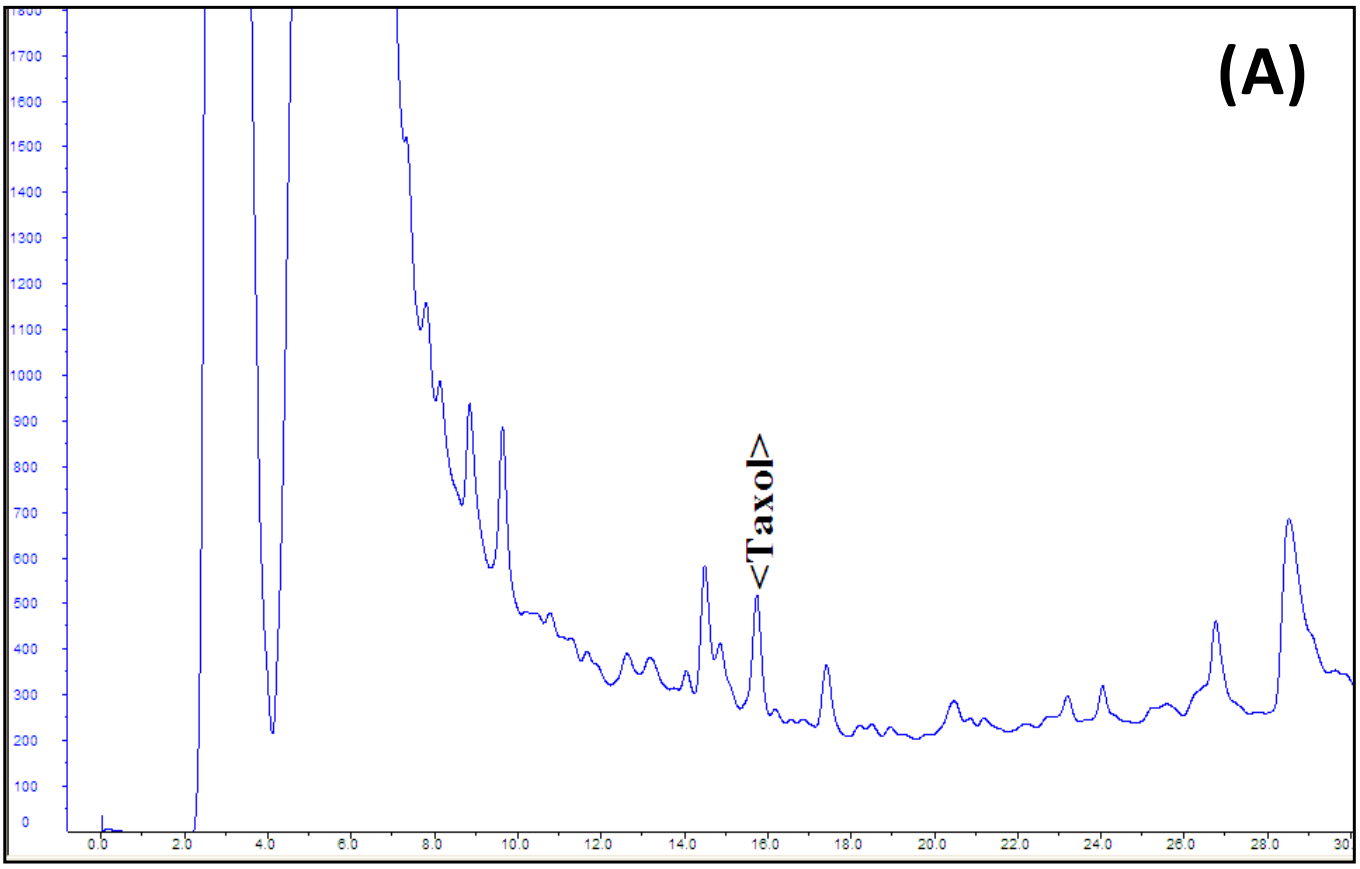
Effects of *C. sinensis* extract

The callus of *T. brevifolia* treated with *C. sinensis* extract revealed significantly enhanced enzymatic activities of the polyphenol oxidase and the peroxidase compared to the untreated control. The activity of PO was greater than PPO's. By increasing the amount of *C. sinensis* extract from 100 µl (T1) to 500 µl (T5), significant enhancements were observed for the enzymatic activities of PO and PPO. The maximum activities of PPO (0.19 U) and PO (0.39 U) enzymes were observed in callus treated with 500 µl (T5) and 400 µl (T4) as shown in Table 1. The addition of *C. sinensis* extract to culture media increased production of total phenolics and paclitaxel and decreased the rate of *T. brevifolia* callus growth. The maximum production of total phenolics (218.55 GAE/gFW) and paclitaxel (794.35 mg/kgDW) happened in the callus treated with 400 and 500 µl, respectively (Table 1). Figure 1, Panel (C), shows HPLC chromatogram of paclitaxel analysis. The minimum rate of growth (0.15 g) was observed in the callus treated with 500 µl (Table 1). In spite of potent antioxidant activity of *C. sinensis*, these findings in relation with control of browning in *T. brevifolia* callus subcultures and utilizing of *C. sinensis* extract significantly intensified the browning phenomenon compared to the untreated control (Table 1).

Cysteine effects

The orthogonal comparisons showed significant differences between all treated and control groups ($p<0.05$). Cysteine significantly increased the enzymatic activities of PPO and PO, as well as the amount of total phenolics in subcultured callus of *T. brevifolia* as compared with control. In callus treated with 100 mg/L cysteine, however, enzyme activity of PPO showed slight decrease. Cysteine significantly induced total phenolics production in all treated groups, in which the groups were treated with 200 (mg/L) cysteine resulted in 165.28 ± 5.56 (GAE/gFW) phenolic compounds. Table 2 illustrates the enzymatic activities of PPO, PO and the amount of total phenolic compounds.

Calli treated with cysteine showed significant increase in production of paclitaxel in comparison with untreated control. The maximum amount of paclitaxel (444.46 mg/kgDW) was produced in the callus treated with 300 mg/L cysteine (Figure 1A and B), while the untreated control callus produced 190.06 mg/kgDW (Table 2). Besides, treatment with the cysteine significantly prevented growth of subcultured callus and enhanced


(A)
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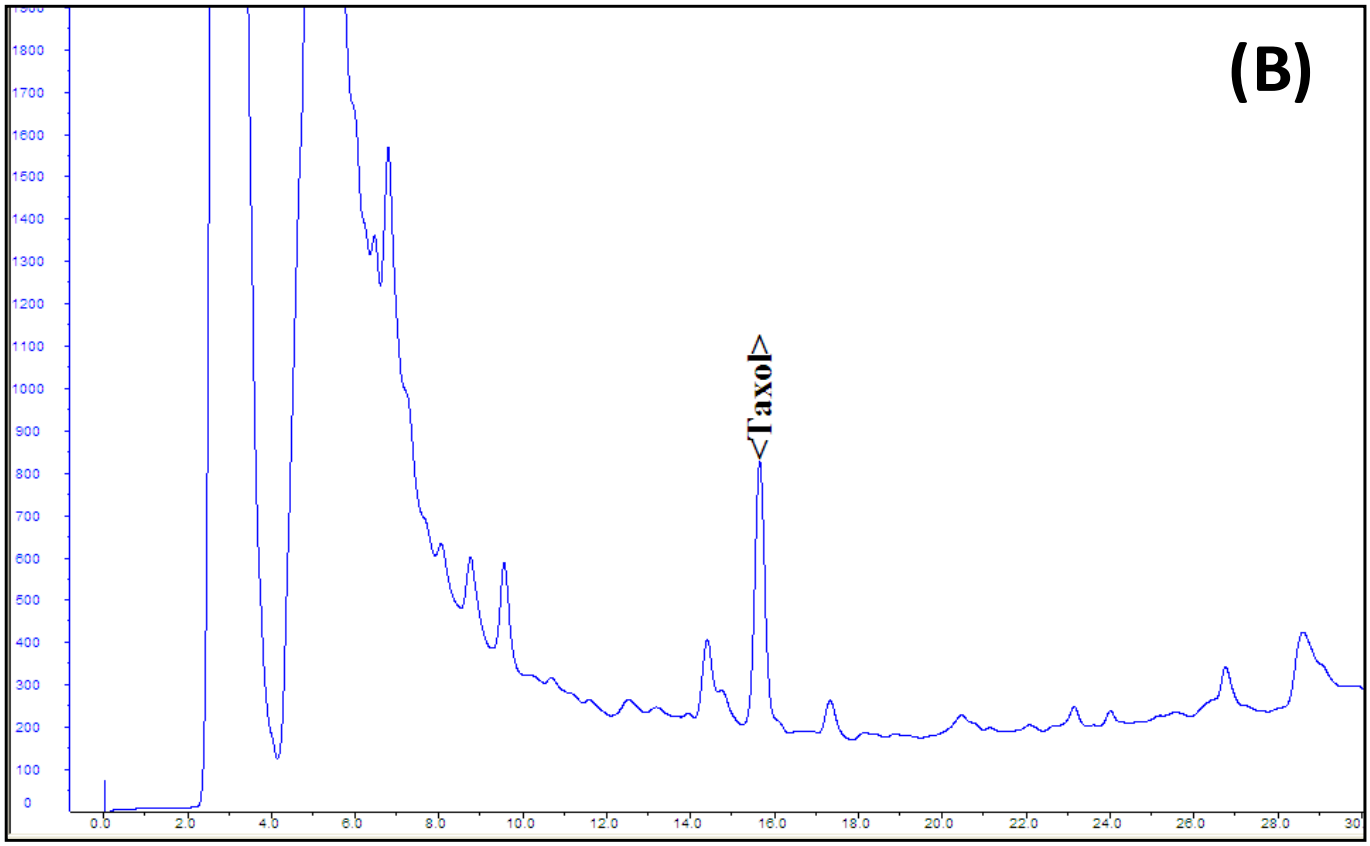
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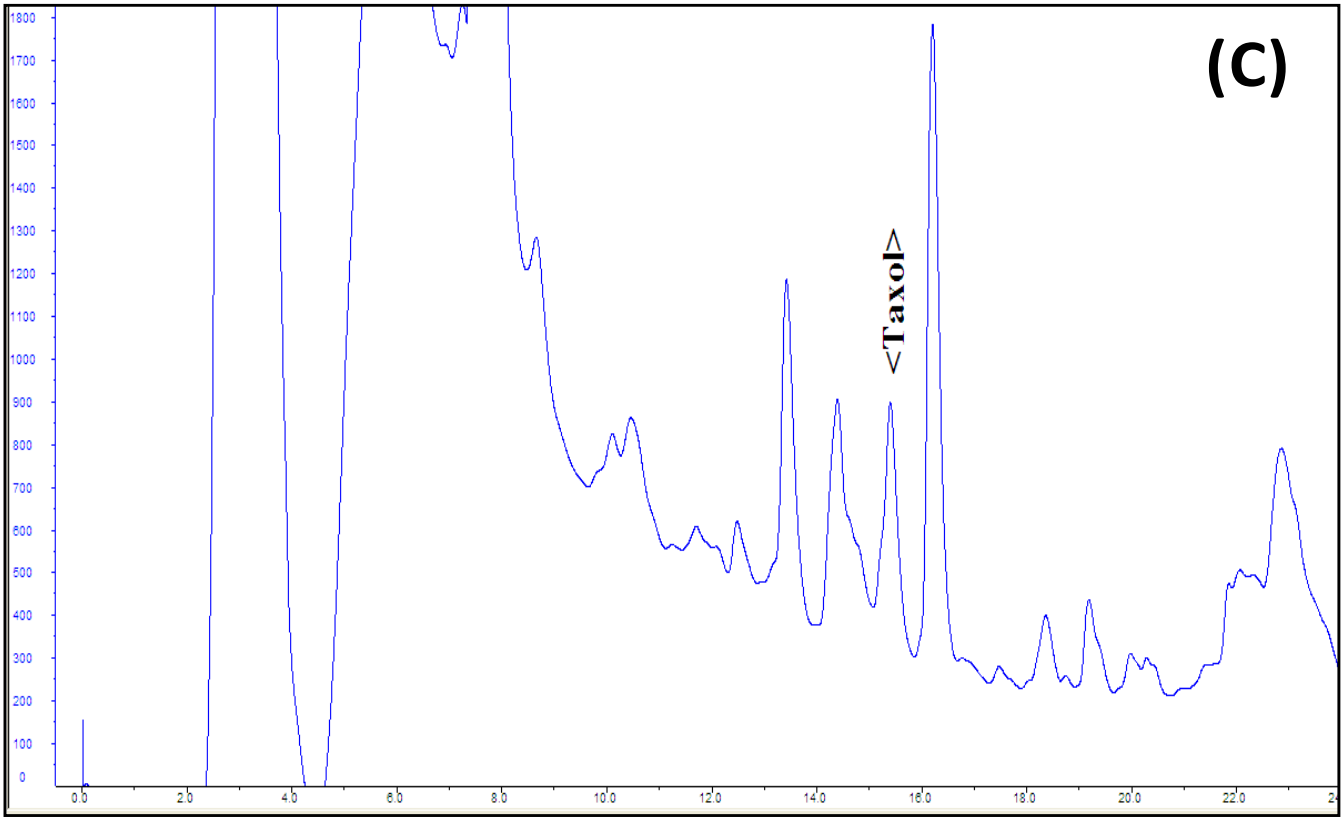

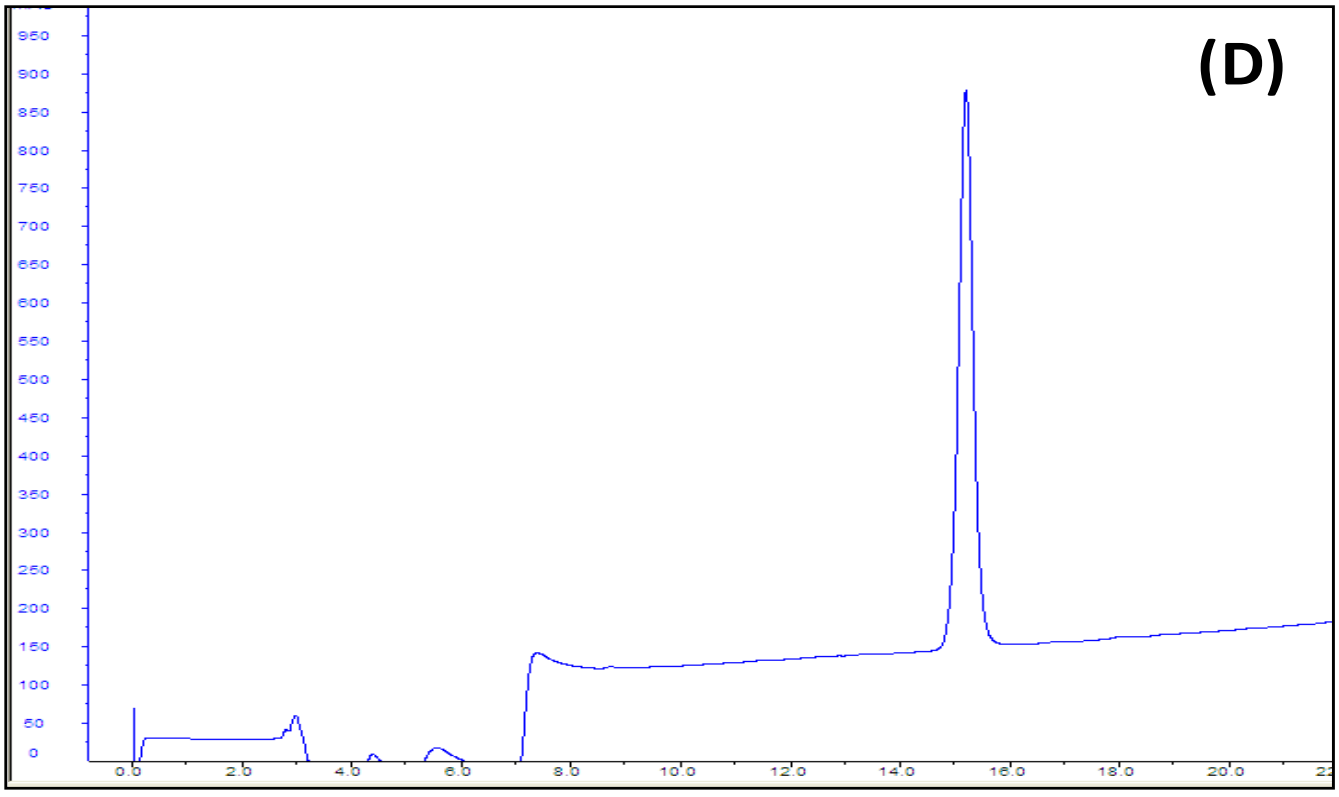

Figure 1. HPLC chromatograms for paclitaxel analysis. Panels A and B represent the highest amount of paclitaxel production by treated group with cysteine (T3) and spiked paclitaxel pick, respectively. Panel C shows the highest amount of paclitaxel production by treated group with *C. sinensis* extract (T5). Panel D illustrates standard pick of paclitaxel.

Table 2. Effects of L-cystein in subcultured calli of *T. brevifolia*.

	PPO activity (U)	PO activity (U)	Total phenolics (GAE/g)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
Cystein (100 mg/L)	0.022* ± 0.000	0.113 ± 0.014	143.889 ± 5.9	313.185 ± 27.6	0.787 ± 0.07	2.333 ± 0.5
Cystein (200 mg/L)	0.073 ± 0.002	0.212 ± 0.021	165.278* ± 5.5	427.741 ± 19.9	0.757 ± 0.06	2.667 ± 0.5
Cystein (300 mg/L)	0.077 ± 0.004	0.210 ± 0.015	162.479 ± 11.1	444.460* ± 15.4	0.657 ± 0.09	3.333 ± 0.5
Control	0.054 ± 0.002	0.051 ± 0.001	82.099 ± 2.9	190.062 ± 9.8	1.467 ± 0.45	1.667 ±0.5

GAE: Gallic acid equivalent; DW: Dry weight; FW: Fresh weight. *Significant ($P < 0.05$) differences compared to other treatments.

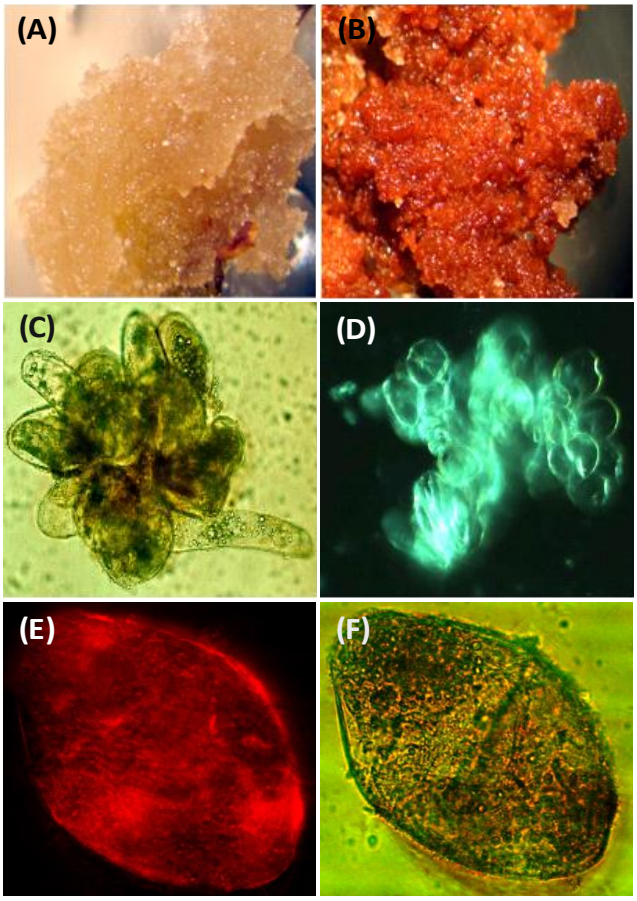

Figure 2. Effects of *Camellia sinensis* extract and cysteine on browning and morphology of subcultured *Taxus brevifolia* calli. Panels A and B represent browning intensity; the lowest and the highest, respectively. Panels C and D represent clamps of yellow-white callus cells with light and dark filed microscopy, respectively. Panels E and F represent fluorescent images from *T. brevifolia* cell in browned calli with WIG mirror and light microscopy, respectively.

their browning intensity compared to the control.

Cell morphology

The morphological evaluation of callus cells illustrates dispensable differences between the shape of cells in browned and yellow-white calli. Figure 2C and D shows the yellow-white callus cells imaged by light and dark field microscopy respectively, whereas these cells illustrated the minimum fluorescence with WIG mirror. Since the fluorescent image's lucidity of the yellow-white callus cells were not high enough the results are not presented. However, the browned callus cells exhibited high intensity of fluorescence with WIG mirror. Figure 2E and F represent a fluorescent and light microscopy of a single browned cell.

DISCUSSION

To date, huge efforts have been devoted to investigate the antioxidant effects of *C. sinensis* extract on animal/human cells. For example, it has been reported that the potent antioxidant compound (that is, epigalocatechin-3-gallate) obtained from *C. sinensis* extract has potential to improve viability of skin fibroblasts (Borawska et al., 2009; Hsu, 2005; Wei et al., 2009). Besides, its protective effects against skin cancer has also been shown (Katiyar et al., 2007). The *C. sinensis* extract was shown to elevate the activity of xanthine oxidase and reduces the activity of adenosine deaminase, whose activities appear to be very important upon inhibition of carcinogenesis (Erguder et al., 2008). All these findings together with its inhibitory impacts on the activity of phospholipase A, proteases, hyaluronidase and L-amino acid oxidase (Pithayanukul et al., 2010) clearly highlight the antioxidant effects of *C. sinensis* in mammalian cells/tissue. To the best of our knowledge, no substantial information is available about the biological effects of *C. sinensis* extract on plant cell/tissue culture. Thus, we aimed to evaluate its impacts as well as cysteine effects on the activity of PPO and PO enzymes, callus growth, phenolics and paclitaxel production. It should be evoked that cysteine has been previously used as an antioxidant to control the browning phenomenon in *Cocculus pendulus* callus cultures (Bhardwaj and Ramawat, 1993). Besides, N-acetyl-cysteine and reduced glutathione were shown to impose inhibitory effects on browning of potatoes (Friedman et al., 1992).

In our study, we found induced activities of PPO and

PO in the calli subcultures of *T. brevifolia* treated with the *C. sinensis* extract, even though it is known as a potent antioxidant. While decreased PPO enzyme activity was observed in subcultured callus of *T. brevifolia* treated with 100 mg/L of cysteine, higher amounts increased the activities of both PPO and PO. The positive correlation between the increase of cysteine concentration in culture media and activity of both PPO and PO were observed. In terms of the relationship between *C. sinensis* extract and PPO and PO enzymes activity in subcultured calli, the positive correlation was observed with the activity of PPO but not with PO. The employed antioxidants displayed limited effects on inhibition of the activities of PPO and PO enzymes; however, these bioactive agents raised production of paclitaxel in treated groups in comparison with the untreated group of subcultured calli. Surprisingly, the growth of the treated *T. brevifolia* calli with *C. sinensis* extract and cysteine was decreased. By increasing concentration of *C. sinensis* extract and cysteine in subculture media, the growth of callus more decreased. Thus, our findings show the negative correlation between growth and utilizing of these bioactive agents. We speculate that these bioactive agents progressively trigger the inhibition of growth of subculture calli through excess production of phenolic compounds and accordingly induction of browning. Upon our observations, the browning intensity was positively correlated with the activity of PPO and PO regardless of *C. sinensis* extract and cysteine potent antioxidant activity. These agents may also suppress the proliferation, while inducing differentiation towards higher production of paclitaxel. Based on our findings this investigation is the first report on improved production of paclitaxel elicited by *C. sinensis* and cysteine in subcultured calli (Table 1). In fact, *C. sinensis* extract in combination with cysteine not only failed to control the activities of the enzyme and browning phenomenon, but also intensified their activities in some cases. The mechanism by which *C. sinensis* extract imposed such biological impacts is not fully understood, however our speculation is that its components may act as an elicitor in subcultured calli of *T. brevifolia*. No direct relationship was observed between PPO and PO activities and paclitaxel production. However, the positive correlation was observed between the *C. sinensis* extract concentration in subculture media and paclitaxel production while at higher concentrations of cysteine (>300 mg/L), the decrease of paclitaxel production was perceived.

Conclusions

In the current investigation, we studied the effects of *C. sinensis* extract and cysteine on the activity of PPO and PO enzymes, callus growth, total phenolics and paclitaxel production. The extract of *C. sinensis* and cysteine were found to raise the amount of total phenolics in the subcultured calli, and accordingly increased the activities of PO and PPO enzymes and browning. Despite decrement of callus growth, *T. brevifolia* subcultures treated with these compounds resulted in profound production of paclitaxel. Based on these findings, for improvement of paclitaxel production after stationary phase of growth, we suggest supplementation of *C. sinensis* extract in subculture or suspension cell culture of *T. brevifolia*.

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