Effect of Antioxidants and Carbohydrates in Callus Cultures of *Taxus brevifolia*: Evaluation of Browning, Callus Growth, Total Phenolics and Paclitaxel Production

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A R T I C L E   I N F O

A B S T R A C T

Introduction: To control the tissue browning phenomenon, callus growth, total phenolics and paclitaxel production, in the current investigation, we evaluated the effects of citric acid and ascorbic acid (as antioxidants) and glucose, fructose and sucrose in callus cultures of *Taxus brevifolia*. *Methods:* To obtain healthy callus/cell lines of *Taxus brevifolia*, the effects of two antioxidants ascorbic acid (100-1000 mg/L) and citric acid (50-500 mg/L), and three carbohydrates (glucose, fructose and sucrose (5-10 g/L)) were studied evaluating activities of polyphenol oxidase (PPO) and peroxidase (PO) enzymes, callus growth/browning, total phenolics and paclitaxel production. *Results:* These antioxidants (ascorbic acid and citric acid) failed to show significant effects on callus growth, browning intensity or paclitaxel production. However, the carbohydrates imposed significant effects on the parameters studied. High concentrations of both glucose and sucrose increased the browning intensity, thus decreased callus growth. Glucose increased paclitaxel production, but sucrose decreased it. *Conclusion:* These results revealed that the browning phenomenon can be controlled through supplementation of the growth media with glucose, sucrose (5 g/L) and fructose (10 g/L), while increased paclitaxel production can be obtain by the optimized media supplemented with glucose (10 g/L), sucrose and fructose (5 g/L).

Introduction

Paclitaxel as a natural anti-cancer agent was first isolated from bark of *Taxus brevifolia* (Wani *et al.* 1971). Extraction of paclitaxel from natural resources has been shown to impose important ecological influences including the extinction of *Taxus* species. Moreover, this approach demands large amounts of bark thus it is not cost-beneficial. Given the fact that malignancies are increasingly growing, the demand to such a potent anti-cancer medicament is also enhancing. Thus, it appears to be a crucial need to improve the production of paclitaxel through an implementation of an alternative cost-effective approach and various ways are pursued such alternative hosts like yeast and moss (Simonsen *et al.* 2009) or tissue cultures of various *Taxus* species

(Khosroushahi *et al.* 2006). The tissue/cell culture of *Taxus* is considered to be a very promising manner among the other techniques (Khosroushahi *et al.* 2006). However, there exist some pitfalls that may dramatically affect the bioprocess engineering of paclitaxel through the culture systems. For example, one of the most interfering phenomena with this approach is the production of the plethora of phenolic compounds, which results in a particular phenomenon called "tissue browning". In fact, it is considered as a serious impediment during mass production of natural secondary metabolites via bioprocessing since the tissue browning event involves many toxic compounds through the phenolization process eventually resulting in the necrosis of cells (Banerjee *et al.* 1996; Murata *et al.* 2001; Wu and Lin 2002). Some previously published studies have

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highlighted the association of different biomolecules/enzymes with tissue browning phenomenon. It is related to the enhanced phenolic compounds production that in return causes augmentation of activities of some important enzymes (Banerjee *et al.* 1996) such as polyphenol oxidase (Murata *et al.* 2001) and peroxidase that convert phenolics to pestilent compounds (Wu and Lin 2002). In addition, several amyloplast containing 3-5 starch grains were observed at the vicinity of the cell nucleus, at which the amyloplast could be taken into the tonoplast resulting in browning in the aged cells (Kishiko Okada *et al.* 2004); these observations reveal that the browning phenomenon may be related to the metabolism of carbohydrates within cultured cells.

Thus, in this investigation, we aimed to study the effects of two antioxidants (citric acid and ascorbic acid) and three carbohydrates (glucose, fructose and sucrose) on tissue browning phenomenon, callus growth, total phenolics content and paclitaxel production in callus cultures of *Taxus brevifolia.*

Materials and methods

Materials

The Folin–Ciocalteu (F-C), 2,6-dimethoxyphenol, paclitaxel 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 (i.e., salts, sugars, vitamins, growth regulators and phytohormones) were purchased from either Sigma–Aldrich Chemical Co. (Poole, UK) or Merck (Darmstadt, Germany).

Tissue culture and maintenance

All explants were taken from one *Taxus brevifolia* tree located in the botany garden of the University of Tehran. Juvenile stems, 1cm size (5 explants in one Petri dish and 150 cultured Petri dish), were used as explants after sterilization with sodium hypochlorite (3%) and were cultured using a modified B5 medium (including 2-fold vitamins) supplemented with 2 mg/L naphthalene acetic acid (NAA), 1.5 mg/L 3-indoleacetic acid (IAA), 0.2 mg/L 6-benzyl aminopurine (BA) and 20 g/L sucrose at dark condition, 24˚C, and pH 5.7. After callus induction (in 20 days), subcultures were performed using the B5 media that were supplemented with aforementioned antioxidants and carbohydrates to obtain the desired objectives of this investigation.

Ascorbic acid and citric acid experiment design

Two experiments were planned based on completely random design (CRD) with 10 treatments and 3 replications per each treatment (i.e., 3 petri dishes with 5 calli each). For treatments, ascorbic acid (100-1000 mg/L), or citric acid (50-500 mg/L) were added to callus induction media. The cultures were maintained for 30 days at the same physical conditions described before (i.e., at dark, 24˚C, and pH 5.7).

Glucose, fructose and sucrose experiment design

The factorial design based on CRD was utilized to examine the effects of three factors (i.e., glucose, fructose and sucrose). For each factor, 2 different concentration (i.e., 5 and 10 g/L) were considered. The physical conditions were the same described above.

Polyphenol oxidase extraction and enzyme activity assay

Thirty days after callus subculture, samples of one gram of fresh calli (i.e., 5 mixed calli from each Petri dish) were 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 and Newton 2004) followed by sonication at 4˚C for 15 min. After centrifugation (15000×g, 4˚C for 20 min), the supernatants was collected and subjected to the polyphenol oxidase enzyme activity assay. Briefly, 300 µl of callus extracts were mixture with 300 µl of buffer phosphate (pH 7.4) and 400 µl ethanolic solution of 2,6-dimethoxyphenol (0.5 mM). The absorbance was measured at 468 nm using Unicam UV4 spectrophotometer (Unicam Co., Cambridge, UK) and the change of 0.01 in absorbance was defined as a unit (U).

Peroxidase extraction and enzyme activity assay

Thirty days after callus subculture, samples of one gram of fresh calli (i.e., 5 mixed calli from each Petri dish) were homogenized in 10 ml cold acetone (-20˚C) and the suspension was centrifuged at 15000×g, 4˚C for 20 min (Chisari *et al.* 2008). The supernatant was dried by vacuum evaporator. The powder was resuspended in 1 ml buffer phosphate (200 mM), kept at 4ºC over night and then recentrifuged at 15000×g, 4˚C for 20 min. To determine the peroxidase activity, 100 µl crude extract was dissolved in the mixture of 700 µl H_2O_2 and 200 µl of buffer phosphate containing 1.6 g phenol and 50 mg 4-aminoantipyrine in 50 ml of buffer phosphate. The absorbance of samples was measured at 510 nm using Unicam UV4 spectrophotometer.

Total phenolic extraction and assay

Thirty days after callus subculture, samples of one gram of fresh calli (i.e., 5 mixed calli from each Petri dish) were homogenized in 20 ml aqueous methanol (80%), sonicated at 25˚C for 20 min and then centrifuged at 15000×g, 4˚C for 20 min (Velioglu *et al.* 1998). The supernatant was dried by vacuum evaporator and resolved in 1 ml methanol. The Folin–Ciocalteu method

was applied to determine the total phenolic content (Ainsworth and Gillespie 2007). Briefly, the solution of Folin–Ciocalteu (200 µl) was mixed with 100 µl of concentrated extract. The mixture was then commixed with 700 µl of Na_2CO_3 (700 mM) and was maintained at 25°C for 2 hr. The absorbance of samples was measured at 765 nm using Unicam UV4 spectrophotometer. The gallic acid was considered as a standard reference (Ainsworth and Gillespie 2007). The results were expressed as µg of gallic acid equivalent per gram of fresh weight of calli (GAE/gFW).

Paclitaxel extraction and quantification

Paclitaxel was extracted from dried calli as previously described (Khosroushahi *et al.* 2006) with some modifications. Briefly, 0.2 g of dried callus was grounded and suspended in 20 ml methanol, followed by sonication for 15 min. After centrifugation at $15000 \times g$, the supernatant was collected and evaporated using vacuum evaporator (Heidolph, Schwabach, Germany). The obtained dried extract was resolved in 1 ml of methanol and used for HPLC analysis performed with a Pharmacia Biotech (San Francisco, CA, USA) system equipped with C18 Column (4.6×250 mm, 5 µm). The mobile phase was acetonitrile/H_2O (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. The UV spectra of paclitaxel were confirmed using standard paclitaxel (T7402, Sigma, USA).

Growth measurement

After 20 days in culture, the calli were weighed under sterile conditions prior to subculture. After 30 days, the calli were weighed again and the weight difference was considered as growth rates of calli.

Cell morphology study

The morphological changes in callus cultures of *T. brevifolia* were evaluated using an inverted microscope, Olympus IX81 (Olympus Optical, Tokyo, Japan) equipped with a digital camera (Olympus, 72 DPI), super plan apochromat lenses and WIB and WIG mirrors.

Browning estimation

To evaluate the browning phenomenon, calli were ranked in 5 classes based upon their browning intensity, after 30 days in culture. The ranking was established from the lowest (1) to the highest (5) browning intensity.

Data analyzing

One way ANOVA with an appropriate multiple comparison test was employed for statistical analysis using Minitab version 15, or MSTATC version 1.2. A p value less than 0.05 was considered to represent the statistical differences.

Results

Ascorbic acid effects

Based on the orthogonal comparison between the treated groups and control, it was found that ascorbic acid in subculture media imposed partial effects. It apparently altered most of the studied factors (i.e., the activities of PPO and PO enzymes, total phenolics production, paclitaxel production, and growth of calli) in callus cultures of *T. Brevifolia* as shown in Table 1.

Table 1. Effects of ascorbic acid on sub-cultured calli of *Taxus brevifolia*

Treatment Ascorbic acid	PPO activity (U)	PO activity (U)	Total phenolics (GAE/gFW)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
100 (mg/L)	0.017±0.001	0.015±0.005	302.00±5.171*	161.12±26.621	1.65±0.022	1.67±0.612
200 (mg/L)	0.009±0.002*	0.015±0.002	300.67±2.523	127.01±16.212	1.78±0.038	1.67±0.623
300 (mg/L)	0.024±0.002	0.014±0.002*	282.44±8.371	110.93±15.334	1.13±0.036	2.33±0.621
400 (mg/L)	0.069±0.008	0.028±0.001	275.44±5.424	152.86±39.743	0.96±0.035	2.67±0.634
500 (mg/L)	0.056±0.007	0.028±0.003	273.00±7.551	159.98±14.012	0.75±0.093	3.00±1.015
600 (mg/L)	0.045±0.008	0.034±0.003	258.11±7.532	188.11±12.553	0.85±0.061	3.33±0.614
700 (mg/L)	0.043±0.002	0.034±0.002	253.22±7.534	193.80±18.432	0.66±0.066	3.67±0.622
800 (mg/L)	0.054±0.003	0.041±0.005	255.44±11.501	185.19±13.424	0.63±0.116	3.67±0.624
900 (mg/L)	0.087±0.012	0.047±0.006	262.22±9.482	225.09±29.931	0.62±0.074	4.33±0.642
1000 (mg/L)	0.092±0.003	0.050±0.008	257.78±9.261	232.75±30.842	0.57±0.053	4.67±0.625
Control	0.145±0.005	0.051±0.005	221.67±7.881	190.06±9.803	1.47±0.451	1.67±0.616

U): activity unit, GAE: Gallic acid equivalent, DW: dry weight, FW: fresh weight. *Significant ($P < 0.05$) differences compared to other treatments.

The activity of PPO and PO enzymes was lessened in treated groups as compared to untreated control. The minimum activity of PPO (0.01U) and PO (0.014U) enzymes were respectively observed by addition of 200 mg/L and 300 mg/L of ascorbic acid into subculture media, while use of higher amounts of ascorbic acid resulted in greater enzymes activities. Supplementation of the subculture media with ascorbic acid caused a significant increase in total phenolics production in *T. brevifolia* callus in comparison to untreated control. Use of 100-300 mg/L of ascorbic acid resulted in decreased production of paclitaxel. However, higher concentrations (>300mg/L) caused significant (p<0.05) increase in paclitaxel production as compared with treated groups (<300 mg/L). The maximum growth of callus was detected by addition of 200 mg/L of ascorbic acid in media. The use of ascorbic acid in subculture media showed no substantial effects on the browning phenomenon, even its higher amounts (>200mg/L) exacerbated the browning phenomenon.

Citric acid effects

As shown in Table 2, the supplementation of citric acid to culture media resulted in subtractive effects on PPO and PO enzymes activities.

Table 2. Effects of citric acid on sub-cultured calli of *Taxus brevifolia*

Treatment Citric acid	PPO activity (U)	PO activity (U)	Total Phenolics(GAE/gFW)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
50 (mg/L)	0.066±0.004	0.037±0.001	273.22±31.371	209.10±25.652	1.56±0.162	2.00±1.001
100 (mg/L)	0.023±0.002*	0.024±0.002	270.67±37.023	191.73±17.821	1.58±0.171	1.67±0.582
150 (mg/L)	0.031±0.001	0.026±0.002	282.33±36.682	220.14±18.294	1.63±0.115	2.00±1.004
200 (mg/L)	0.029±0.003	0.022±0.001*	282.67±23.905	215.33±21.842	1.59±0.081	2.67±0.582
250 (mg/L)	0.029±0.002	0.028±0.002	257.67±39.343	203.76±23.082	1.60±0.153	2.33±1.153
300 (mg/L)	0.027±0.001	0.031±0.002	284.89±35.582*	186.67±16.314	1.55±0.121	2.67±0.584
350 (mg/L)	0.038±0.003	0.042±0.001	276.33±33.054	210.79±18.002	1.44±0.072	2.67±0.585
400 (mg/L)	0.048±0.006	0.056±0.004	281.44± 20.621	206.20±25.473	1.37±0.133	3.33±0.582
450 (mg/L)	0.053±0.005	0.052±0.003	282.78±23.943	172.63±10.251	1.35±0.094	3.33±0.584
500 (mg/L)	0.053±0.009	0.052±0.003	284.56±39.192	200.13±19.345	1.26±0.053	3.67±0.581
Control	0.145±0.006	0.051±0.001	221.67±7.881	190.06±9.805	1.47±0.454	1.67±0.585

(U): activity unit, GAE: Gallic acid equivalent, DW: dry weight, FW: fresh weight. *Significant (P < 0.05) differences compared to other treatments.

Table 3. Main effects of carbohydrates on sub-cultured calli of *Taxus brevifolia*

	PPO activity (U)	PO activity (U)	Total Phenolics(GAE/gFW)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
Glucose (5g/L)	0.109±0.014	0.069±0.002	220.81±4.433	233.10±6.513	1.72±0.027*	1.58±0.195*
Glucose (10g/L)	0.146±0.014	0.082±0.002	289.69±4.433*	263.85±6.513*	1.53±0.027	2.33±0.195
Fructose (5g/L)	0.129±0.014	0.075±0.002	239.72±4.433	257.73±6.513	1.52±0.027	2.08±0.195
Fructose (10g/L)	0.098±0.014*	0.066±0.002	270.78±4.433	238.09±6.513	1.74±0.027*	1.83±0.195
Sucrose (5g/L)	0.095±0.014*	0.065±0.002*	261.47±4.433	251.88±6.513	1.65±0.027	1.83±0.195
Sucrose (10g/L)	0.133±0.014	0.081±0.002	249.03±4.433	242.71±6.513	1.61±0.027	2.08±0.195

(U): activity unit, GAE: Gallic acid equivalent, DW: dry weight, FW: fresh weight. *Significant (P < 0.05) differences compared to other treatments.

The minimum activities of PPO (0.023U) and PO (0.022U) enzymes occurred respectively at 100 and 200 (mg/L) of citric acid supplementations. Considering the total phenolics production, all treated groups showed significant differences in comparison to untreated control, but within the treated groups the differences were insignificant. The utilization of citric acid in subculture media had no beneficial effect on paclitaxel

production and growth of callus even though citric acid induces the browning phenomenon when it is used in

concentrations higher than 100 mg/L.

Table 4. Effects of two and three interplay effects of glucose, fructose and sucrose in factorial experiment with two level of each factor (5 and 10 g/L) and control

|	PPO activity (U)	PO activity (U)	Total phenolics (GAE/gFW)	Paclitaxel (mg/kgDW)	Growth (gFW)	Browning intensity
G5F10	0.095±0.002	0.054±0.002	249.88±4.433	219.29±9.163	1.815±0.039	1.5±0.283
F10S5	0.060±0.021	0.042±0.002	295.11±4.431	226.75±9.164	1.815±0.039	1.33±0.281
G10F5S5	0.277±0.022	0.106±0.003	256.44±8.855	367.64±12.971*	1.353±0.054	2.66±0.395
G10F10S5	0.031±0.020*	0.013±0.003*	323.11±8.854*	323.11±12.973*	1.74±0.054	1.66±0.392
G5F10S5	0.115±0.023	0.037±0.003	267.11±8.851	243.30±12.975*	1.89±0.054*	1.00±0.393*
Control	0.145±0.005	0.051±0.001	221.67±7.884	190.06±9.805	1.47±0.451	1.67±0.634

GAE: Gallic acid equivalent; DW: dry weight; FW: fresh weight; G5: glucose 5g/L; G10: glucose 10g/L; F5: fructose 5g/L; F10: fructose 10g/L; S5: sucrose 5g/L; S10: sucrose 10g/L. *Significant ($P < 0.05$) differences compared to other treatments.

Glucose, fructose and sucrose effects

The effects of carbohydrates revealed significant (p<0.05) differences between the treated groups and untreated control (i.e., callus induction and maintenance media). As shown in Table 3, the glucose concentration 10 g/L with respect to 5 g/L increased PPO and PO enzymes activity as well as total phenolics and paclitaxel production, which also intensified browning phenomenon and hence decreased callus growth. In terms of fructose (10 as compared to 5 g/L) effects on studied attributes, the results were in reverse order with glucose except total phenolics production. Two concentrations of sucrose (10 and 5 g/L) acted similar to glucose in PPO and PO enzymes activities as well total phenolics productions. Among the two and three interplay effects of glucose, fructose and sucrose, we just presented data that showed noticeably important findings on studied characteristics in this research as shown in Table 4. Two interplay effects of these sugars (addition of 10 g/L fructose and 5 g/L sucrose) on PPO and PO enzymes activities resulted in their minimum activities (i.e., 0.06U and 0.04U, respectively) while among three interplay effects, the supplementation of 10 g/L fructose, 10 g/L glucose, and 5 g/L sucrose to subculture media yielded 0.03U and 0.013U for PPO and PO enzymes, respectively (Table 4). High amounts of glucose and fructose (10 g/L), and also low concentration of sucrose (5g/L) produced 289.63, 270.77 and 261.41(GAE/gFW) of total phenolics, respectively. As shown in Table 4, three interplay effects were greater than the main effect of carbohydrates (i.e., 323.11GAE/gFW of total phenolics). In terms of Paclitaxel production, 10 g/L glucose induced its production, but the same amount of fructose and sucrose decreased Paclitaxel production. The highest amount of Paclitaxel (367.65 mg/kgDW) was observed in subcultures grown in the media

supplemented with 10g/L glucose and 5 g/L fructose and 5 g/L sucrose (Table 4). The media containing high amounts of glucose and sucrose prevented the growth of calli, while fructose at a high concentration (10g/L) stimulated the growth of calli. Maximum growth of callus (1.89g/FW) occurred in cultures maintained on media contained 5 g/L glucose, 10 g/L fructose and 5 g/L sucrose (Table 4).

Cell morphology
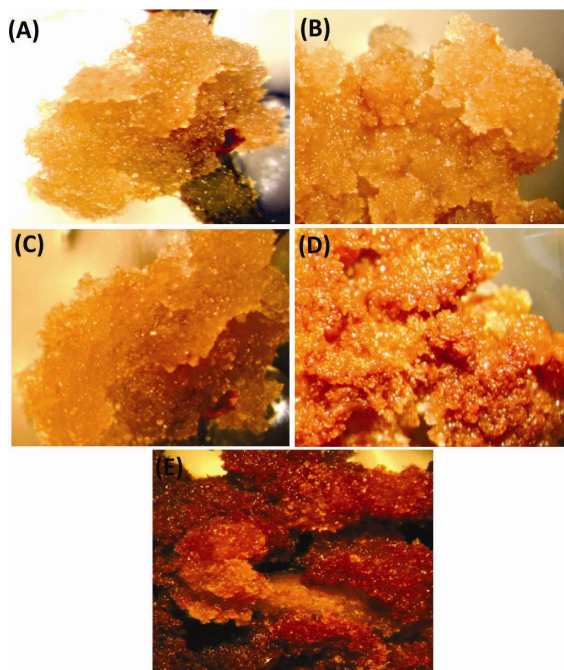
Fig. 1. Effects of three sugars and two antioxidants on browning in sub-cultured *Taxus brevifolia* calli. Panels A-E represent browning intensity ranking from 1 to 5, respectively.

The lowest and the highest browning intensities are shown in panels A - E, respectively (Fig. 1).

The morphological evaluation using light and fluorescence microscopy of callus cells illustrated differences between the shape of cells in browned and yellow-white calli as shown in Fig.2. Clamps of yellow-white callus are shown in panels A-B, while the morphology of a single healthy cell (yellow-white) and an unhealthy cell (brown cell) are presented in panels C-D and E-F, respectively.
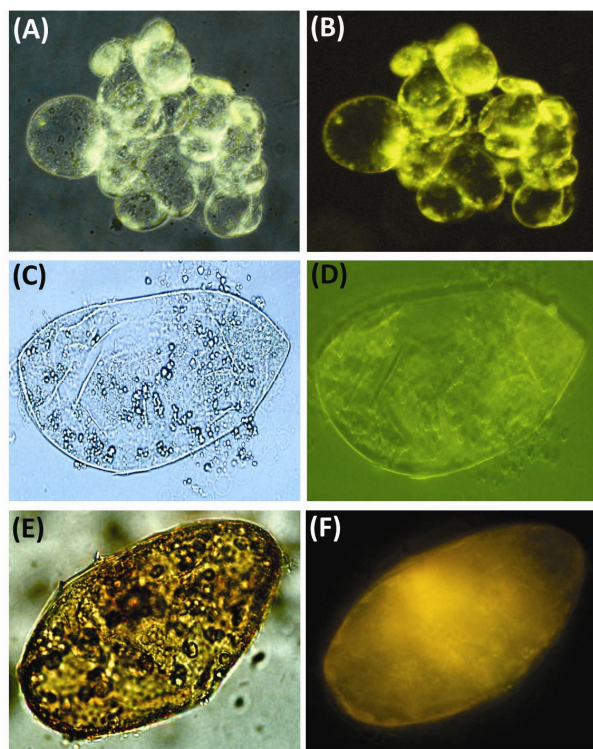
Fig. 2. Effects of three sugars and two antioxidants on browning in callus cultures of *Taxus brevifolia*. Panels A and B represent clamps of yellow-white callus cells with light and fluorescence microscopy, respectively. Panels C and D represent light and fluorescent (WIB mirror) microscopy images from *T. brevifolia* cell in yellow-white calli, respectively. Panels E and F shows the browned cell with light and fluorescent (WIB mirror) microscopy, respectively.

Discussion

The tissue browning phenomenon, callus growth, total phenolics content and paclitaxel production, as well as PPO, PO activities in callus of *Taxus brevifolia* were evaluated in culture media supplemented with ascorbic acid and citric acid (as antioxidants) alsoglucose, fructose and sucrose.

Based our findings, there was no correlation between total phenolics concentration and PPO and PO enzymes activity in subcultured calli (Table 1). Similar to these results, the browning development in potato revealed no significant correlation between rate/degree of browning and PPO, PO activities, ascorbic acid content and total phenolics accumulation (Cantos *et al.* 2002), while browning was partially correlated to phenylalanine ammonia-lyase activity. Nevertheless, in *Panax ginseng* cells, it was previously reported that the much higher PPO activity and rate of phenolics production in the cell cultures were in association with enzymatic browning (Wu and Lin 2002). In addition, the pericarp browning in *Dimocarpus longan* fruits was previously shown due to an increase in activity of PPO and PO enzymes because of water loss from the pericarp which can lead to rapid pericarp browning (Luna-Palencia *et al.* 2005). This result adapts with our observations because water loss rarely occurred in culture media. Therefore, phenolics accumulation in subcultured cells did not result in enhanced activity of PPO and PO enzymes. As shown in table 1, the total phenolics concentration, paclitaxel production and callus growth within the treated groups revealed a negative correlation between phenolics contents and paclitaxel production, but positive correlation between phenolics amounts and callus growth. This can be explained by utilizing the phenolics in the phenomenon that lead to cell proliferation, and thus increase in callus growth instead of secondary metabolite production such as paclitaxel.

A large number of investigations reported involvement of phenolic compounds in the plant cell wall (Maier *et al.* 1995; von *et al.* 1998). This may imply that an increase of phenolics might facilitate cell wall generation and subsequently enhance cell proliferation. The use of ascorbic acid in subculture media showed no substantial effects on the browning phenomenon, even its higher amounts (>200mg/L) exacerbated the browning phenomenon. Similar findings were reported for the transgenic tobacco (*Nicotiana tabacum* L.) BY-2 cells exposed to oxidative stress (Tokunaga *et al.* 2005). Addition of ascorbic acid to Murashige and Skoog's medium was shown to reduce significantly the browning of initiated callus (Banerjee *et al.* 1996). Similar results have been reported in the embryogenic cultures of *Themedaqu adrivalvis* upon treatment with ascorbic acid (Habibi *et al.* 2009). However, in our study, no notable differences were observed for the browning phenomenon between treated groups and untreated control.

Addition of citric acid to the subculture media resulted in significant differences in PPO and PO enzymes activities as well as total phenolics production, based on orthogonal comparison between the treated groups and untreated control. There was no significant difference between the treated groups and untreated control in terms of paclitaxel production, browning intensity and subcultured callus growth. Based on these findings, the

citric acid restrained the activity of PPO enzyme more than PO enzyme. Similarly, PPO enzyme activity was notably reduced by citric acid treatment and accordingly remarkable decrease was observed in the skin browning of santol fruit (Benjawan and Chutichudet 2009).The citric acid supplementation to culture media resulted in augmentation of total phenolics as compared with untreated control; however there was no positive correlation between activities of PPO/PO enzymes and enhancement of total phenolics (Table 2).Likewise, doubling the concentration of the nutrients and citric acid was not affected the cell growth or viability in *Taxus cuspidata* cell cultures (Naill and Roberts 2005). Considering the role of total phenolics on cell wall generation (Fry 1982; Kang *et al.* 2008; Wallace and Fry 1994), we speculate that high amounts of phenolics impose a stimulating effect on cell proliferation, by which the growth of calli can be improved. However, no quantitative information is available for the relationship between the growth rate and amounts of total phenolics in cells treated with citric acid. Low concentrations of total phenolics can favour cellular growth, but high concentrations can impede growth. The addition of citric acid partially increased total phenolics in all treated groups in comparison to control, but no changes were observed in the growth of calli. This may support our suggestion about quantitative correlation of the growth with total phenolics which depends on the nature and quantity of chemicals used to alter such a phenomenon. This means that cells modulate their biological activities in correspondence with the intensity and nature of the stimulation/elicitation during culture. It has been shown that the addition of citric acid to culture medium can control the toxic effects of harmful ions in the tobacco callus (Szoke *et al.* 1992). Other researchers have shown that treatment of fresh-cut mangoes with citric acid can delay the carotenoid formation and browning reactions during storage (Chiumarelli *et al.* 2010). However, we found that citric acid failed to control the browning phenomenon and even at higher concentrations exacerbated the browning in subcultured calli.

Based on our results, to improve of paclitaxel productivity in two stage cell culture systems, use of fructose may favour augmentation of cell population via enhancing of growth at the first stage of culture (exponential phase). Besides, feeding of culture media at the second stage (stationary phase) with glucose can lead to increased production of paclitaxel via involvement of some related pathways. Similarly, it was reported that supplementation of high concentration of glucose in *Fragaria*x *ananassa* culture media prevented the callus growth (Lopez *et al.* 2001), while glucose participates at ring C formation of the taxane ring (Shi *et al.* 2003). Furthermore, glucose was shown to be incorporated into both taxane ring and acetyl groups in *Taxus* cell

suspension culture (Eisenreich *et al.* 1996). The minimum activity of PPO and PO enzymes was observed at 10 and 5 (g/L) for fructose and sucrose, respectively. To the best of our knowledge, there is no previously reported information for alteration of the activities of these enzymes with supplementation of culture media with some carbohydrates. Addition of fructose, glucose, and sucrose synchronically in subculture media (as two and three interplay) was significantly affected the browning phenomenon. Subcultures treated with 5 g/L glucose, 10 g/L fructose and 5 g/L sucrose showed the least browning intensity (Table 4). Notably, glucosylation reactions may play a more general role in plant defenses, especially against toxin-mediated disease development in yew species (Strobel and Hess 1997).

Moreover, high amount of sucrose was reported to induce the production of paclitaxel through a metabolic process (Ellis *et al.* 1996). These evidences clearly imply that addition of various carbohydrates into culture media of plant cells/tissues may control the browning phenomenon which often occurs inevitably during callus induction or during its subculture. We found that high amounts of glucose could impose insignificant alterations in the enzymatic activity of PPO, while remarkable enhancements were observed in the PO enzyme activity, total phenolics and paclitaxel production. However, addition of glucose reduced the growth rate of calli and intensified browning in callus cultures of *T. brevifolia*. Recently, it was reported that addition of glucose to the culture media could lead to an increase of secondary metabolites production (Cloutier *et al.* 2009).

In addition, it has been shown that replacing sucrose with fructose in the sugar composition of micro-propagation media of GF305 peach, can cause a significant effect on the maintenance of long-term in vitro cultures (Kalinina and Brown 2007). On the other hand, sucrose increased the PPO and PO enzymes' activities and also browning phenomenon, while decreasing the production of total phenolics and paclitaxel and also the callus growth rate. According to previous studies, exposure of tissue/cell culture to high concentration of sucrose may increase the paclitaxel release to the suspension cell culture medium of *Taxus chinensis* (Wang *et al.* 2001). Nevertheless, higher sucrose contents (>5%) was shown to have a negative influence on callus growth despite the accumulation of rosmarinic acid in cell suspension culture of *Coleus blumei* (Qian *et al.* 2009), and a high sucrose concentration stimulated monoterpenoid oxindole alkaloid production in the maintenance medium of *Uncaria tomentosa* cell suspension cultures (Luna-Palencia *et al.* 2005).

Conclusion

Based on our findings, the inhibitory impacts of carbohydrate metabolic pathways on callus browning appeared to be higher than that of antioxidants in subcultures. High amounts of glucose increased paclitaxel production, but it also intensified the browning phenomenon. Thus, to modulate such effects, various assessments were carried out and we found that a medium containing 5 g/L glucose, 5 g/L sucrose and 10 g/L fructose can significantly control the callus browning, while the best media to increase the paclitaxel production was a medium contained 10 g/L glucose, 5 g/L fructose, and 5 g/L sucrose. This clearly means that a two steps cultivation of cells/tissues is essential for growth optimization and higher production of paclitaxel.

Ethical issues

None to be declared.

Conflict of interests

The authors declare no conflict of interests.

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