Increase of Taxol Production in *Taxus globosa* Shoot Callus by Chlorocholine Chloride

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Abstract: Calluses from seasonal mature shoots of *Taxus globosa* Schltdl. were subcultured in new medium containing 1 mg l^{-1} NAA, 0.5 mg l^{-1} 2, 4-D, 0.025 mg l^{-1} BA. Calluses growing for 30 and 40 days in a medium added with 0.1-5.0 mg l^{-1} of chlorocholine chloride (CCC) were harvested for determination of taxol, phenolic compounds, and for polyphenol oxidase (PPO) activity. The results showed that by increasing the CCC concentration, callus growth was decreased, phenolic level was raised up, but taxol content and PPO activity changed differently. The highest taxol production (0.0269 mg g^{-1} dry weight callus) and PPO activity (4.53 U g^{-1} fresh weight callus) were achieved at day 40 with a medium containing 0.5 mg l^{-1} CCC. Possible reason of higher taxol level stimulated by CCC is discussed in the text.

Keywords: *Taxus globosa*, callus, taxol, phenolics, polyphenol oxidase.

INTRODUCTION

Taxol contents in *Taxus spp* are very variable, for instance, *Taxus globosa* produces as low as 0.0013%-0.0082% of taxol in dried bark [1] when *Taxus brevifolia* has higher rate such as 0.01-0.03% of taxol in the same extract [2], and *T. cuspidate* does 0.022-0.035% of that in the dried young stems or needles [3]. Because of limited natural resource, callus and cell cultures become a promising way to produce taxol. *T. globosa* as a native yew in Mexico is another natural source of taxol, callus culture of Mexican yew *in vitro* is valuable to approach high production of taxol. Xiao *et al*. (2000) and Zhang *et al*. (2003) reported culture from its shoot callus, but its taxol production *in vitro* has not been yet reported [4, 5].

Nowadays, some methods have been used for the improvement of taxol production *in vitro*: selection of callus or cell line [6, 7], modification of the medium [8] and use of elicitors [9-11]. Among them, elicitors are used frequently for promotion of taxol yield, which include methyl jasmonate, abscisic acid, chlorocholine chloride (CCC), salicylic acid, phenylalanine, as well as osmotic regulators such as sucrose, mannitol, sorbitol and polyethylene glycol [8, 12-18]. CCC is one of the plant growth retardants, which stimulates increase of taxol production in yew logs [9] and mature roots [19] of intact plants. Furthermore, Veeresham *et al*. (2003) reported that CCC significantly improved the taxol production of *T. wallichiana* leaf callus [16], Wang *et al*. (2003) found that among the metabolic inhibitors such as CCC, mevastatin, sodium pyrophosphate and D,L-glyceraldehyde, only CCC increased the total content of taxanes in cell suspension [14].

In order to improve taxol production of *T. globosa* callus *in vitro*, we investigated a fresh callus line of *T. globosa*, for taxol levels, phenolic contents and PPO activities under treatments with CCC.

MATERIALS AND METHODS

Induction and Culture of Callus

Seasonal mature shoots of *Taxus globosa* Sch. adult trees were taken from natural forest in Hidalgo State mountain of Pachuca City in México Country. After surface sterilization, shoot explants were cultured in a callus induction medium (CIM), i.e. SH basic medium [20] supplemented with 2.0 mg l^{-1} NAA, 0.1 mg l^{-1} BA, 3% sucrose and 0.6% agar (pH 5.6). After callus induction, cells were subcultured in a SH callus subculture medium (CSM), containing 1mg l^{-1} NAA, 0.5 mg l^{-1} 2,4-D, 0.025 mg l^{-1} BA and 3% sucrose, and kept in the darkness at 25 ± 1˚C during a 4-6 week period. After several subcultures, a bright yellow callus line was selected to evaluate any improvement of taxol production. For the purpose, cultures in the CSM added with 0.1, 0.5, 1.0 or 5.0 mg l^{-1} of CCC, respectively, were harvested at day 30 and day 40 for assaying of taxol level, phenolic content and PPO activity.

Estimation of Callus Growth

Calluses were weekly analyzed from the beginning and up to 49 days after culture including five repetitions for each experiment. Mean increment of fresh weight of callus from five replications was used to plot a kinetic curve of callus growth.

Determination of Taxol

Calluses from day 30 and day 40 were dried at constant temperature (50˚C for 48 h) and pounded thoroughly into a glass mortar, 0.8 g of the callus powder was extracted with methanol (20 ml for 48 h) and then filtered and centrifuged at 4000 × g for 3 min. Supernatant was evaporated under reduced pressure at 30˚C and the residual extracted with 60 ml of equal-volume dichloromethane-distilled water for 48 h. The organic phase, collected through a separatory funnel was

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evaporated to dryness and the residue was dissolved in 0.6 ml of HPLC grade methanol (Fisher Corporation, USA).

Analyses were performed by a previously reported method [21] to examine taxol by use of a chromatograph Waters model 510 with a Waters 490E programmable multiwave UV-detector (227 nm), and a C_{18} reverse phase column (250 mm×4.60 mm) LUNA ^{TM} 5µ from Waters Corporation, with a mobile phase of MeOH-H_2O 30:70 v/v. The flow rate was 1.0 ml min^{-1} and the volume in all injections was 10 µl.

Taxol's 99.9% purity used as standard was provided by the Institute of Materia Medica, Chinese Academy of Medical Sciences, and was dissolved in HPLC grade methanol. All samples and extractions were filtered through hydrophilic polyethylene filter (φ=0.45 µm, PALL Gelman Laboratory) prior to use in HPLC analysis. Extractions of *T. globosa* callus and HPLC analyses were repeated 3 times and those of the callus treated by CCC were done in two replicates.

According to the UV absorption peak area and its concentrations of standard taxol, the following regression equation is made out:

$$y = -6180.3398644 + 4.91592\times10^{7}\,x$$

(*y*, peak area; *x*, concentration of taxol in solvents (g l^{-1}))

and

$$x = \frac{y + 6180.33986}{4.91592\times10^{7}}$$

then, the equation for taxol content in callus is

$$W = x \times V_1 \times d / m$$

(W, mg g^{-1} Dry Weight (DW); *x*, concentration of taxol in solvents (g l^{-1}); V_1, 0.6 ml of HPLC grade methanol; *d*, dilution fold of extraction for injection; *m*, a dried callus weight).

Extraction and Estimation of Total Phenolics

Callus from a 40-day culture (0.1g) was homogenized in 1 ml of boiling water for 2 min, and then kept in boiling water bath for 5 min. After cooling at room temperature, centrifugation at 4000 × g for 3 min left a supernatant that was used for analysis of total phenolics within a 6 h period.

The procedure of Prussian blue assay (Price and Butler, 1977) [22], with a small modification was employed to detect phenolics. It was mixed with 50 µl extraction, 500 µl $FeCl_3$ (0.1 mol), 500 µl $K_3Fe(CN)_6$ (8 µmol) and 500 µl HCl (0.1 mol) in 10 ml reaction solution. After 30 min of reaction, the absorbance was measured at 685 nm. Tannic acid was used as a standard sample and a normalized curve was made out on tannin acid concentration and its absorbance value basis. Three experiments were repeated for each sample and the results were expressed as tannic acid equivalents (mg g^{-1} Fresh Weight (FW).

Determination of Polyphenol Oxidase Activity

According to Anosike and Ojimelukwe (1982) [23], calluses (0.5 g) from a 40-day culture were homogenized in 5 ml of phosphate buffer pH 6.8 in an ice bath. After the mixture was centrifuged at 4000 × g for 5 min, supernatant was stored at 4˚C for analysis of PPO activity within 6 h period.

Following the method of PPO assay (Benjamin and Montgomery, 1973) [24], a 3.8 ml mixture consisting of 50 µmol phosphate buffer (pH 6.8), 1 ml of 100 µmol catechol and 0.2 ml of supernatant from the enzyme extract was incubated at 35˚C in a water bath for 20 min, and rapidly cooled at 0˚C in an ice bath. Absorbance of the reacted solution was detected at 398 nm. One unit of PPO activity was defined as increasing absorbance by 0.01 within one min, and expressed as an enzyme specific activity (U g^{-1} FW). PPO activity is calculated as the following equation.

$$Y = \frac{\Delta D_{398}}{0.01mt} \times N$$

(*Y*, enzyme specific activity; ΔD_{398}, absorbance increase in a period of reaction; *m*, fresh weight of sample (g); *t*, reaction time; *N*, diluting fold of enzyme extract).

RESULTS AND DISCUSSION

Callus Growth and its Taxol Content

After cell proliferation from shoot explants in the CIM for about 14 days, calluses with different colors were in-

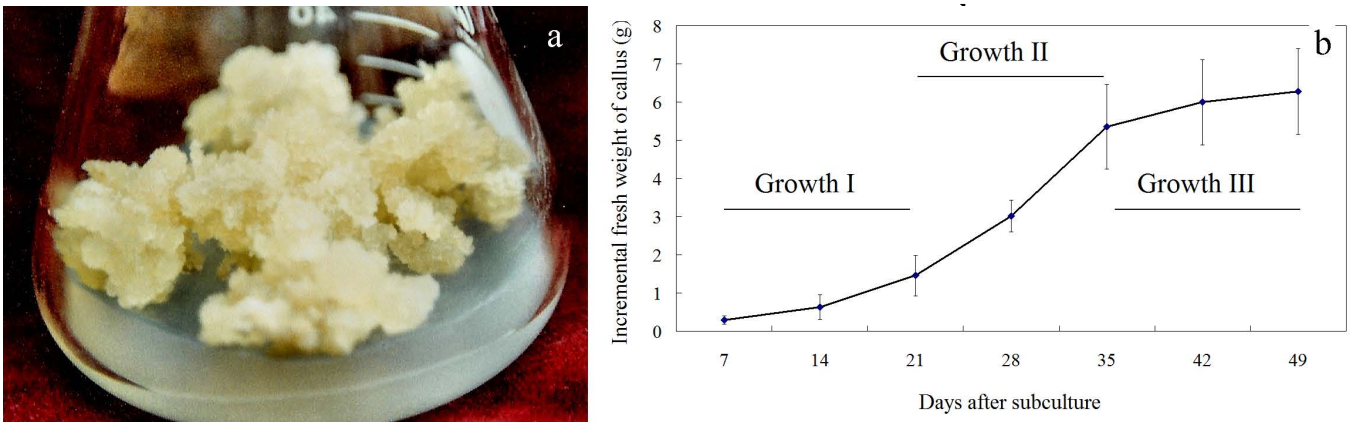

Fig. (1). (**a**) Callus from *Taxus globosa*. (**b**) Its growth kinetic curve. Each data points represent the mean of five replications ± standard error.

duced at a rate of 31.6%. When they were transferred into the CSM in terms of color, a bright yellow cell line was initiated (Fig. **1a**), which presented the higher cumulus size when compared to others. As shown in Fig. (**1b**), a growth kinetic curve of the cell line exhibits three phases in a period of 49-day culture. During the phase I from day 7 to day 21 after subculture, callus grew slowly and its fresh weight increased by 1.248 g (0.089 g per day); In growth II period from day 21 to day 35, a fast rate of growth was observed with a fresh weight of 3.823 g (0.273 g per day); during the last period from day 35 to day 49, calluses grew slowly again and its fresh weight increased only by 1.364 g, (0.09 g per day). At the end, fresh weight of callus had a net increase of 6.72 ± 1.17 g (Fig. **1b**). The doubling time of Mexican yew callus was 14 days, similar to those of *T. × media* habituation callus and *T. baccata* one, whose doubling times were 13-14 days and 13-23 days, respectively [6, 25].

Analysis of taxol content by HPLC shew that *T. globosa* callus cultured in the CSM yielded 0.0028 mg g^{-1} or 0.00028% on a dry weight basis. In comparison with previous report [1], taxol level production by calluses is less than the one in bark of the same species. Similarly, Witherup *et al.* (1990) reported that taxol content in *T. brevifolia* dried callus was 0.0007% [26], less than the one in its dried bark (0.01-0.03%); Fett-Neto *et al.* (1993) found that callus culture of *T. cuspidate* produces 0.020% of taxol on an extracted dry weight basis, which means less than those from needles (0.035%) and young stems (0.022%) [27]. It is a fact that taxol production *in vitro* would be a process of two steps, i.e. increase in biomass and accumulation in taxol. When callus was cultured in a growing medium such as the CSM, cell proliferation was vigorously up but taxol synthesis was limited. If the calluses were cultured in a production medium supplemented with some elicitors such as plant growth retardants, physiological state of callus would be changed with an enhanced secondary metabolism involving taxol synthesis enhancement.

Effect of CCC on Callus Growth and Taxol Production

When callus was cultured in the CSM containing 0.1, 0.5, 1.0 and 5.0 mg l^{-1} of CCC, its proliferation was inhibited and the fresh weight of callus at day 40 was decreased by 11.26%, 24.82%, 18.62% and 47.82%, respectively. This callus at a lower growth rate resulted in a great accumulation of taxol.

As shown in Fig. (**2**), it is obvious that CCC stimulated taxol production in callus cultures. Growing in media supplemented with 0.1, 0.5, 1.0 or 5.0 mg l^{-1} of CCC, callus produced 0.00417, 0.02695, 0.00536 and 0.00361 mg g^{-1} taxol on a dry weight basis, respectively, more than the control (0.0028 mg g^{-1} DW). Among these treatments the one with 0.5 mg l^{-1} (about 3 μmol) CCC was the most effective and taxol level in dried callus was elevated up to 0.0027%, which represent 9.6 fold as high as the control, and more than the one in dried bark (0.0013%) of the species [1]. However, higher concentration of CCC is required to improve the taxol production in bark of *T. brevifolia* plants (0.1 mmol CCC) [9], in roots of *Taxus × media* plants (1000 μmol CCC) [19] and in *T. wallichiana* leaf callus (1 mmol CCC) [16]. Because *T. globosa* callus needs lower concentration of CCC for the highest taxol production than other yew species, it might be more sensitive to CCC in taxol biosynthesis than the others.

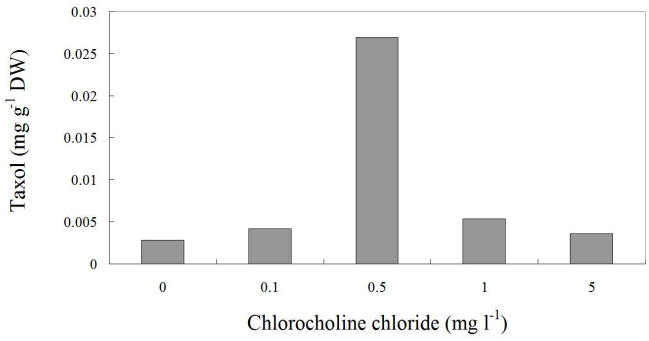

Fig. (2). Taxol levels in callus treated with CCC.

Inhibition of callus growth is important to taxol production, even during the 2nd step of taxol production. Under the same treatment such as 0.5 mg l^{-1} CCC, taxol level in 40-day callus (in phase of growth III) was higher (0.0226 mg g^{-1} DW) than in the 30-day one (in phase of growth II) (Fig. **3**). However, under treatments of different concentration of CCC, increase of taxol content in callus was not related with an increase of CCC concentration, e.g. callus treated by 1.0 or 5.0 mg l^{-1} CCC produced less taxol than with 0.5 mg l^{-1} CCC although fresh weight of the former was reduced more than the latter. Hence, it is suggested that lower growth rate of callus would not only be one factor to influence the biosynthesis of taxol; other unknown physiological factors might still play more important roles in taxol production.

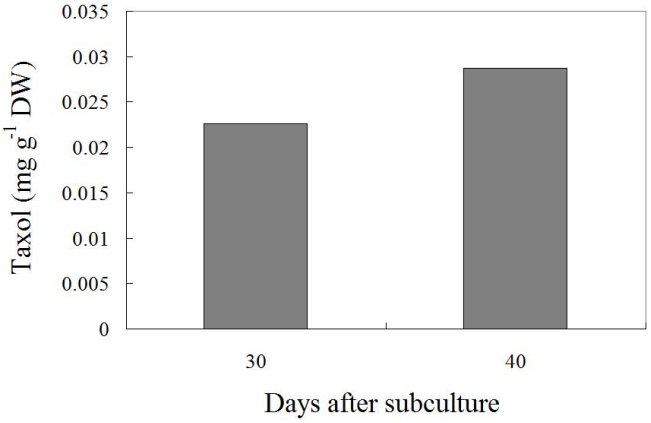

Fig. (3). Taxol levels in callus cultured in the CSM containing 0.5 mg l^{-1} CCC.

Effect of CCC on Phenolic Content and PPO Activity in Callus

In order to approach physiological factors related to biosynthesis of taxol under treatment of CCC, we assayed phenolic contents and PPO activities in callus. As a result, phenolic level in callus kept up with the increase of CCC concentration (Fig. **4a**), from 0.46 mg g^{-1} of phenolics in the control to 0.49 and 0.55 mg g^{-1} of phenolics in callus cul-

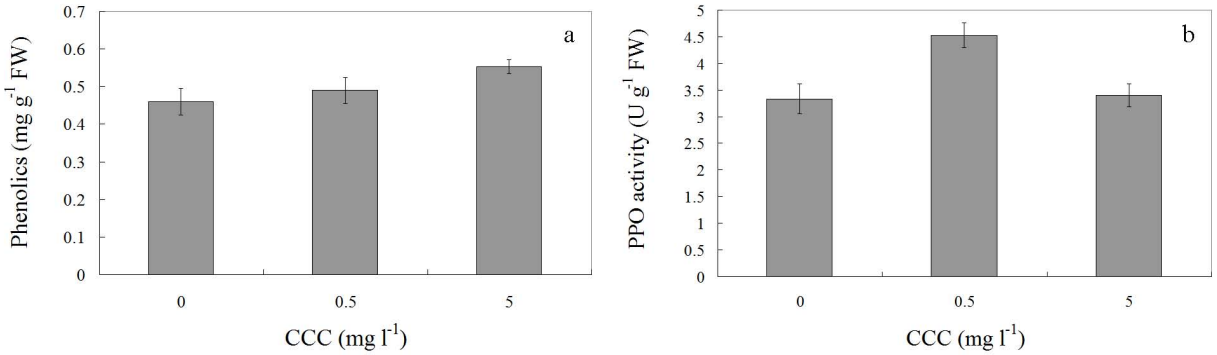

Fig. (4). Total phenolic levels (left) and Polyphenol oxidase activities (right) of *T. globosa* callus in medium containing CCC. Each data points represent the mean of three-replication ± standard error.

tured in medium added with 0.5 and 5.0 mg l^{-1} CCC, respectively. However, PPO activity in callus growing in medium supplemented with 0.5 mg l^{-1} CCC (4.53 U g^{-1} FW) was higher than with 0 or 5.0 mg l^{-1} of CCC (3.34 or 3.40 U g^{-1} FW) Fig. (**4b**). Phenolics are the secondary metabolic products in phenylpropanoid pathway as a result of the response of plant cells to environmental stress [28]. Higher phenolic content is accompanied with an increase of PPO activity, proposed that PPO activity may regulate the redox state of phenolic compounds and become involved in the phenylpropanoid pathway [29]. Yuan *et al*. (2002) found that the oxidative burst signals in *Taxus* cells induced by oligosaccharide not only altered the redox state of cells, but also affected the secondary metabolism [30]. In our experiment, the highest activity of PPO and the highest accumulated level of taxol occurred simultaneously in callus treated with 0.5 mg l^{-1} CCC, which implicated that PPO might be involved in taxol production *in vitro*.

Based on the above analysis, it is presumed that CCC stimulates taxol production in callus of *T. globosa* by trigging plant defense reaction, during which PPO not only catalyzed the one- and two- electron oxidation of elevated phenols to quinones, but also took part in secondary metabolism that deals with taxol biosynthesis. Hence, PPO would be one of the important physiological factors responsive to taxol production. However, how PPO affects secondary metabolism or taxol biosynthesis, still remains to be researched.

CONCLUSIONS

Cultured in the CSM containing a range from 0 to 5.0 mg l^{-1} CCC, shoot callus of *T. globosa* accumulated the highest level of taxol under treatment with 0.5 mg l^{-1} CCC, meaning 9.6 fold more than the control. Meanwhile, the highest PPO activity in the callus appeared with the same concentration of CCC. As similar trend changes in taxol contents and PPO activities, it is suggested that PPO might be involved in a way of taxol biosynthesis *in vitro*.

ABBREVIATIONS

BA = 6-benzylaminopurine

CCC = Chlorocholine chloride

2,4-D = 2,4-dichlorophenoxyacetic acid

HPLC = High performance liquid chromatography

NAA = α-naphthaleneacetic acid

SH = Schenk and Hildebrandt medium (Schenk, R.U. and Hildebrandt, A.C., 1972)

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