Plant Biotechnol Rep (2013) 7:357–369
DOI 10.1007/s11816-012-0270-z

ORIGINAL ARTICLE

Culture medium optimization for camptothecin production in cell suspension cultures of *Nothapodytes nimmoniana* (J. Grah.) Mabberley

Vijai Singh Karwasara · Vinod Kumar Dixit

Received: 31 August 2012 / Accepted: 13 December 2012 / Published online: 29 December 2012

Abstract The effect of culture medium nutrients on growth and alkaloid production by plant cell cultures of *Nothapodytes nimmoniana* (J. Grah.) Mabberley (Icacinaceae) was studied with a view to increasing the production of the alkaloid camptothecin, a key therapeutic drug used for its anticancer properties. Amongst the various sugars tested with Murashige and Skoog (MS) medium, such as glucose, fructose, maltose, and sucrose, maximum accumulation of camptothecin was observed with sucrose. High nitrate in the media supports the biomass, while high ammonium enhances the camptothecin content. Selective feeding of 60 mM total nitrogen with a NH_4^+/NO_3^- balance of 5/1 on day 15 of the culture cycle results in a 2.4-fold enhancement in the camptothecin content over the control culture (28.5 μg/g DW). Furthermore, the sucrose feeding strategy greatly stimulated cell biomass and camptothecin production. A modified MS medium was developed in the present study, which contained 0.5 mM phosphate, a nitrogen source feeding ratio of 50/10 mM NH_4^+/NO_3^- and 3 % sucrose with additional 2 % sucrose feeding (added on day 12 of the cell culture cycle) with 10.74 μM naphthaleneacetic acid and 0.93 μM kinetin. Finally, the selective medium has 1.7- and 2.3-fold higher intracellular and extracellular camptothecin content over the control culture (29.2 and 8.2 μg/g DW), respectively.

Keywords Cell suspension culture · Camptothecin · *Nothapodytes* · Sucrose

V. S. Karwasara (✉) · V. K. Dixit
Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar 470 003, Madhya Pradesh, India
e-mail: vijaipharma@gmail.com

Introduction

Camptothecin (CPT), a modified monoterpene indole alkaloid, was isolated for the first time from *Camptotheca acuminata* (Nyssaceae) by Wall et al. (1966), and was further detected in *Nothapodytes nimmoniana* (Icacinaceae) (Govindachari and Viswanathan 1972), some species of the genus *Ophiorrhiza* (Rubiaceae), *Ervatamia heyneana* (Apocynaceae), and *Merrilliodendron megacarpum* (Icacinaceae) (Gunasekera et al. 1979; Arisawa et al. 1981). The CPT derivatives, irinotecan and topotecan, are used throughout the world for the treatment of various cancers (Takimoto et al. 1998). Foetidine, one of the CPT analogues, has been shown to exhibit potent anti-HIV activity (Priel et al. 1991). *Nothapodytes nimmoniana* (Syn: *Nothapodytes foetida*, *Mappia foetida*) is an endangered species (Kumar and Ved 2000), which is native to the western ghats of India and a rich source of CPT and 9-methoxycamptothecin (9-MCPT). It represents the most convenient source for large-scale isolation of CPT, and has been reported to contain 0.06–0.1 % of CPT and 0.001–0.02 % of 9-MCPT (Govindachari and Viswanathan 1972). Fulzele and Satdive (2005) examined various morphological parts of *N. foetida*, and noted maximum alkaloid concentration in bark (0.27 % CPT and 0.11 % 9-MCPT).

Earlier, CPT was isolated from *C. acuminata* (0.005 %) (Wall et al. 1966). However, its bark accumulates a lower concentration of CPT, but young leaves produce 50 and 250 % higher CPT (up to 0.40 % DW) than fruits and bark, respectively (Lopez-Meyer et al. 1994). Weidenfeld et al. (1997) reported CPT content in different parts of *C. acuminata*, i.e. bark (0.012 %), roots (0.02 %), wood (0.05 %), and fruits (0.02 %). Puri et al. (1999) demonstrated that *N. foetida* trees cultivated in the north-western area of Jammu (India) accumulated 0.1 % DW CPT in

roots and seeds, whereas the bark produced lower concentrations of CPT. Among the various tissues of *N. nimmoniana* analyzed, the root bark (0.33 % DW) yielded the highest CPT content followed by the stem bark (0.24 % DW). The root wood and the stem wood had 0.18 and 0.14 % CPT, respectively. The leaves yielded the lowest CPT content of 0.08 % (Padmanabha et al. 2006).

In spite of the fast growth of the pharmaceutical market, CPT is still obtained by extraction from intact plants of both *C. acuminata* and *N. nimmoniana*. In the last decade, due to overexploitation and habitat destruction, over 50–80 % of the population of *N. nimmoniana* has been lost from the western ghats of India (Anonymous 2001). To sustain a stable production of CPT, a number of attempts have been made for the production of CPT by dedifferentiated cell cultures of *N. foetida* (Roja and Heble 1994; Ciddi and Shuler 2000; Fulzele et al. 2001; Thengane et al. 2003; Namdeo et al. 2010), *C. acuminata* (Weidenfeld et al. 1997), *Ophiorrhiza* sp. (Saito et al. 2001; Ya-ut et al. 2011), and *Chonemorpha grandiflora* (Kulkarni et al. 2010). Fulzele et al. (2001) established the suspension cultures of *N. foetida*, and found the maximum concentrations of CPT (0.035 mg/ml) and 9-MCPT (0.026 mg/ml) in the medium. The untransformed root cultures developed from immature zygotic embryos on Murashige and Skoog (MS) medium had maximum 0.01 % CPT and 0.0016 % 9-MCPT content (Fulzele et al. 2002). Fulzele and Satdive (2003) also reported somatic embryogenesis and plant regeneration in *N. foetida*, and both CPT and 9-MCPT were detected in somatic embryos. Two-year-old plantlets germinated from somatic embryos had higher alkaloid content in roots followed by stem and leaves. Recently, Namdeo et al. (2010) reported the comparative analysis of CPT content from different morphological parts and callus culture of *N. foetida* by HPTLC.

Cell suspension cultures of higher plants may provide a viable option for the production of high-value secondary metabolites in vitro (Weathers et al. 2010). The biosynthetic potential of cultured cells has been exploited for production of a number of metabolites but with limited commercial success. When plant cell cultures are developed in vitro as suspension cultures, the yield of metabolites is generally low, as secondary metabolite production is often associated with tissue differentiation. Other factors, such as a poor understanding of the controlling factors of the underlying biosynthetic pathways and genetic instability of the cell lines, contributed to low product yield. Various approaches have been used to increase the yield of metabolites from cell cultures (Ramachandra and Ravishankar 2002) including manipulation of the nutrient medium (Satdive et al. 2007; Zha et al. 2007; Karwasara and Dixit 2011), application of exogenous growth stimulators (Pasqua et al. 2005; Karwasara et al. 2011a), chemical treatment (Zhao et al. 2001; Jain and Dixit 2010), precursor feeding, elicitation (Karwasara et al. 2010; Bhambhani et al. 2011), transformed cultures (Sharma and Dixit 2006; Karwasara et al. 2011b), metabolic engineering, micropropagation, and bioreactor cultures (Sarin 2005), etc. The development of favorable culture conditions is of prime significance to the growth and biosynthetic capacity of the in vitro-cultured cells. Slight changes in culture conditions including carbon source, nitrogen source, nitrate-to-ammonia ratio, phosphorus, micronutrients, growth regulators, precursors, etc. causes great variations in biological and biosynthetic behavior (Merillon et al. 1984; Trejo-Tapia et al. 2003).

As the effect of concentrations of individual nutrients for better cell growth and CPT accumulation has not been studied in earlier work, the objective of present work was to establish in vitro shake flask cultures of *N. nimmoniana* and to check the different concentrations of essential nutrients for better biomass accumulation and CPT production.

Materials and methods

Plant material

Young plantlets (2-month-old) of *N. nimmoniana* were collected from Kolhapur, Maharashtra (India) during November–December and maintained at medicinal plant garden, Department of Pharmaceutical Sciences, Dr. H.S. Gour University, Sagar, Madhya Pradesh (India), for the experimental work. The plant specimen was authenticated from the Agharkar Research Institute, Pune (Authentification letter no. 9-14/3-386/2009) and a herbarium specimen no. VSK/NF/1504 was submitted to the Department of Pharmaceutical Sciences, Dr. H.S. Gour University, Sagar.

N. nimmoniana cell culture establishment

The leaf and stem explants of *N. nimmoniana* were first washed with running tap water and mild liquid detergent. The explants were soaked in 1 % fungicide solution (Danzole; Cheminova, Denmark) for 3 min. Further surface disinfestation was undertaken by treatment of the explants with 4 % (v/v) sodium hypochlorite for 10 min followed by dipping in 70 % ethanol for 30 s. After washing with sterile distilled water several times, the explants were incised into small pieces of 8–10 mm and aseptically cultured on MS medium (Murashige and Skoog 1962) supplemented with 10.74 μM α-naphthaleneacetic acid (NAA), 0.93 μM kinetin (Kn), and 3.0 % (w/v) sucrose. The pH of the medium was adjusted to 5.8 ± 0.2 and 8 g/l of agar (HiMedia, Mumbai, India) was added. It

was than autoclaved at 1.2 kg/cm^2 for 20 min. The cultures were kept in an incubator at 26 ± 2 °C under a 16-h photoperiod. The callus cultures were maintained on the medium of same composition under conditions as stated above by subculturing every 4 weeks and used as base cultures for the initiation of suspension cultures.

Suspension cultures were initiated by transferring high yielding 4-week-old friable fractions of leaf-derived callus (ca. 3 g/l DW) into 250-ml Erlenmeyer flasks containing 50 ml of liquid MS medium supplemented with 2 mg/l ascorbic acid and 1 mg/l polyvinyl pyrrolidone (PVP). The plant growth regulator and sucrose concentrations were the same as in the callus maintenance media. The pH of the medium was adjusted to 5.8 ± 0.2 before autoclaving. Cultures were incubated on a gyratory shaker (REMI, Mumbai, India) at 110 rpm at 26 ± 2 °C under a 16-h photoperiod and subcultured after every 3 weeks. Suspension cultures were harvested from shake flasks every 4th day to determine the packed cell volume (PCV), dry weight (DW), residual sugar concentration, and CPT content. For studying the influence of media constituents, independent experiments were conducted by manipulating carbohydrate, nitrogen, and phosphate levels.

Determination of culture parameters

Details on the determination of cell dry weight have been described by Karwasara et al. (2010). PCV was determined by centrifuging 10 ml of the culture medium in a 15-ml graduated conical centrifuge tube at 2,500 rpm for 5 min. It was expressed as per cent of the pellet to the entire culture volume. Residual sucrose was estimated by the phenol sulfuric acid method (Dubois et al. 1956). All the experiments were carried out in triplicate to check the reproducibility of the results.

Manipulation of culture medium constituents

The growth and CPT production in cell suspension cultures of *N. nimmoniana* was studied with different standard culture media (HiMedia) viz., MS (Murashige and Skoog 1962), Nitsch (Nitsch and Nitsch 1969), B5 (Gamborg et al. 1968), and White (White 1963). MS medium was found to be the best in this experiment and, therefore, it was taken up for the optimization of the constituents in the culture media. The culture growth and secondary metabolite production were observed initially by adding 3 % of either of the filter sterilized solutions of sucrose, glucose, fructose, and maltose (HiMedia) to the MS medium. In later experiments, different concentrations of the best carbon source (sucrose), i.e. 2, 3, 5, and 7 % (w/v) were added to the MS media. Sucrose feeding on the 12th day of the culture cycle was also studied and 1, 2, and 3 % (w/v) solutions of sucrose (filter-sterilized) were added. Cultures were incubated for 28 days and harvested at an interval of 3 days (after sucrose feeding) to monitor growth and production.

The effect of nitrogen source was studied by altering the concentration balance of NO_3^- and NH_4^+ as well as the total amount of initial nitrogen in the culture medium. NH_4Cl and KNO_3 (CDH, New Delhi, India) were used to provide sufficient concentrations of ammonia and nitrate. Different NO_3^-/NH_4^+ balances, such as 60/0, 50/10, 40/20, 30/30, 20/40, 10/50, and 00/60 mM were tested. The best NH_4^+/NO_3^- (50/10) balance was used for the nitrogen feeding experiment; it was supplemented on day 15 of the culture cycle and the culture were harvested 3 days after nitrogen feeding, while the control culture was devoid of nitrogen feeding. The phosphate effect was studied by supplementing 0.25, 0.50, 1.25, and 2.5 mM KH_2PO_4 (CDH) in the MS medium, with 1.25 mM KH_2PO_4 as a control. Finally, all the best results were tested to check their combined effect on the biomass and CPT production in *N. nimmoniana* cell cultures.

All the experiments were carried out in 250-ml Erlenmeyer flasks containing 50 ml MS medium. About 25 % (v/v) of fresh cells were transferred to each flask, and all experiments were performed in triplicate. Culture sampling was done at an interval of 5 days except the feeding (sucrose and nitrogen) experiments, and the cell dry weight (DW) and CPT content were determined.

Extraction and analysis of camptothecin

N. nimmoniana suspension cells and culture media were separated through filtration and used for chemical analysis. Approximately 100 mg DW of cells were oven-dried at 50 °C for 4 h and powdered by pestle and mortar. The dried homogenized cells were sonicated (Soniweld; Imeco Ultrasonics, Mumbai, India) with 5 ml of methanol (Spectrochem, Mumbai, India) for 10 min and further extracted in 5 ml methanol for 12 h at room temperature with intermittent shaking. The same process was repeated twice with fresh 5 ml methanol. The methanolic extract was combined and evaporated to dryness at 50 °C under reduced pressure to yield the residue. The residue was mixed with HPLC-grade methanol (1 ml), vortexed (Spinix, India) for 20 s and centrifuged (Remi, Mumbai, India) at 6,000 rpm for 5 min. The clear supernatants obtained were filtered through 0.22 µm injection filter (Millipore, Billerica, MA, USA) and applied directly to HPLC analysis. The filtrate from the culture medium (20 ml) was extracted twice with 20 ml of chloroform, which was separated and evaporated to obtain a concentrate. For HPLC analysis, the concentrate was treated as described above.

Quantitative estimation of CPT was carried out on a Schimadzu liquid chromatograph (Model SPD-M20A, Japan) equipped with a Schimadzu LC-20AT dual pump with a 25-µl loop and a photodiode array detector (Model SPD-M20A, Japan). Data collection and integration were accomplished using LC-Schimadzu software. Separations were performed on a Luna 5 µm RP-C18 100 Å LC (250 × 4.6 mm i.d.; Phenomenex, USA) column. The CPT content was determined by using isocratic solvent acetonitrile/water (45:55 v/v) as a mobile phase, at a flow rate of 0.8 ml/min. CPT was detected at 254 nm with a retention time of 5.6 ± 0.14 min. Quantitative method was validated with analysis of five samples and the analytical operation could be completed in a period of 10 min. A standard curve was drawn using authentic samples of CPT. Quantitation was achieved by the correlation between concentrations of CPT and respective peak area. A linear dependence of peak area on concentration was observed over the entire concentration range tested (0.001–0.050 mg/ml). Data was analyzed by one-way analysis of variance using the statistical software GraphPad Instat software (La Jolla, CA, USA).

Results and discussion

Establishment of *N. nimmoniana* cell cultures

The callus initiated from leaf explants of *N. nimmoniana* was cream to light yellow colored, after 5 subcultures, yellowish friable callus was obtained which was used for initiation of suspension cultures (Fig. 1). The growth curve for suspension culture with sucrose consumption and CPT production profiles were established (Fig. 2). A lag phase of 8 days was observed in batch kinetics. Maximum biomass (14.6 g/l DW) with 62.6 % PCV was obtained on the 20th day of cell suspension culture. Maximum intracellular CPT accumulation (29.8 µg/g DW) was also noted on day 20 of the growth cycle, which was the onset of stationary phase. These results showed that the production of CPT was well linked with the cell growth. Trace amounts of CPT were leached in culture medium with the onset of stationary growth phase and attained its maximum on day 28 (8.8 µg/ml) (Fig. 2). The first detection of CPT in culture medium was noticed on day 10 ± 2. A speedy decline in sugar concentration (about 80 %) was observed between

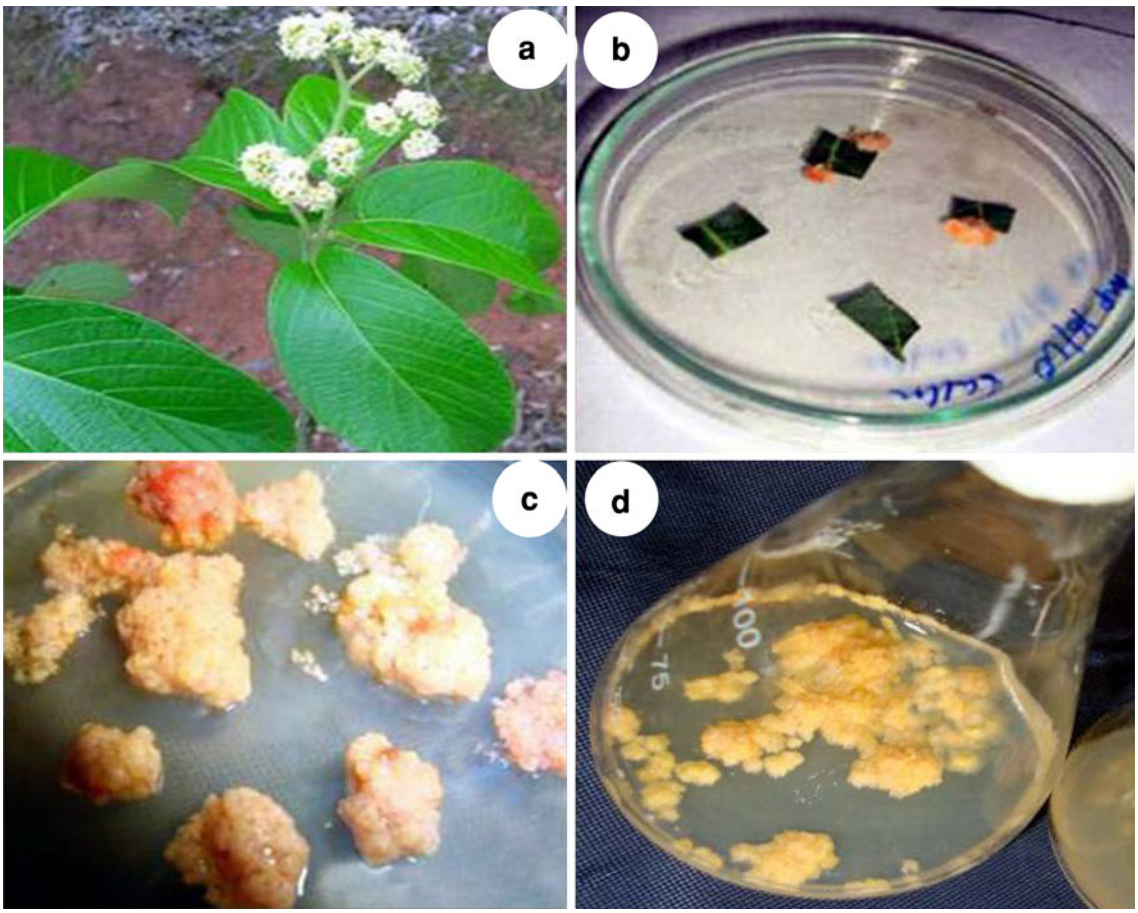

Fig. 1 Field-grown *N. nimmoniana* plant (**a**), callus initiation (**b**) from leaf explant of *N. nimmoniana* on MS medium with 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} Kn and 1.0 % agar, light yellow to cream colored friable callus culture (**c**, **d**) after II and V subculture, respectively

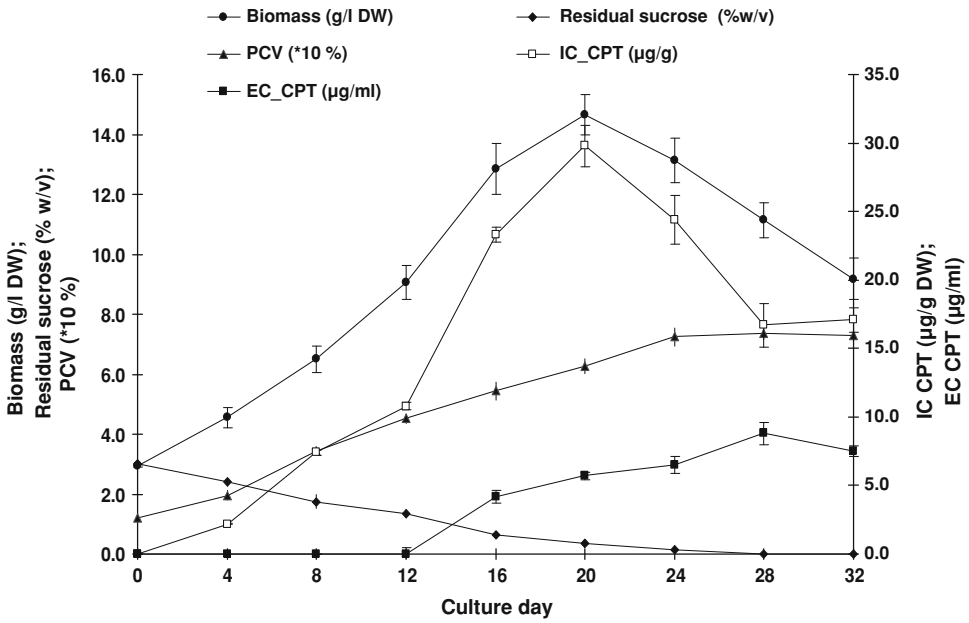

Fig. 2 *N. nimmoniana* cell suspension culture growth curve (average values are given; *error bars* are represented as *vertical lines*)

8th to 16th day of cell growth cycle, which represented the logarithmic growth phase of cell culture system. Only 10 % of sucrose remained unutilized on 20th day of cycle, suggesting stationary phase of growth cycle. It may be deduced that sucrose was the limiting substrate for biomass production because decreased sucrose concentration resulted in the start of stationary phase in suspension cultures. The browning of culture medium, possibly due to phenolics that cause cell death, was efficiently checked by the addition of 2.0 mg/l ascorbic acid and 1.0 mg/l PVP to the suspension culture medium.

Effect of culture media composition

To determine the effect of specific nutrients on CPT biosynthesis, *N. nimmoniana* cells were cultivated in four standard media commonly used in plant cell culture, viz., MS, Nitsch, B5, and White's media. These four media were chosen because of the difference in concentration and nature of major nutrients. All the media were supplemented with the same concentrations of growth regulators, i.e. 2.0 mg/l NAA and 0.5 mg/l Kn and the media were harvested on 20th day of cell cycle to determine the biomass and CPT content. As shown in Fig. 3, there were considerable differences in cell dry weight and CPT content between these media. On the basis of the nature and concentration of nutrients present in these media, high NH_4^+/$N0_3^-$ ratio, in combination with low phosphate concentration seemed to be favorable for CPT biosynthesis in *N. nimmoniana* cell culture. The MS medium supported better biomass production as well as CPT production; the B5 medium fostered comparable biomass production, but induced less CPT (Fig. 3). However, the present studies were restricted to the effect of major nutrients on cell cultures. Since cell growth and CPT production were the best in the MS medium, it was used in subsequent studies dealing with the optimization of the major medium constituents for improved CPT production.

Effect of carbohydrate source and concentration

Among the different carbohydrate sources tried, sucrose was the best, followed by maltose in promoting *N. nimmoniana* cell growth. Figure 4a, b shows the time course of cultivation of suspension cultures containing different carbon sources at 3 % (w/v) as initial concentration. The highest cell biomass (11.2 g/l DW) was recorded with sucrose. However, up-to day 10, the glucose and maltose addition showed higher biomass production but biomass production with sucrose was better from day 15 onwards until the end of growth cycle in *N. nimmoniana* cell culture (Fig. 4a).

The effect of change of carbon source on CPT content is shown in Fig. 4b. Maltose showed significant changes in CPT production at early stages of culture with highest production (25.4 µg/g DW) on the day 20 of the culture cycle followed by gradual reduction with further cultivation. In contrast, a steady increase in CPT production was obtained in cell cultures supplemented with sucrose and maximum of 28.6 µg/g of CPT was recorded on day 20. Medium made with fructose or glucose did not support the CPT accumulation and showed low production rate over the entire cultivation period. HPLC analysis showed that CPT was released in medium in small amounts with sucrose treatment only (described under the sucrose treatment).

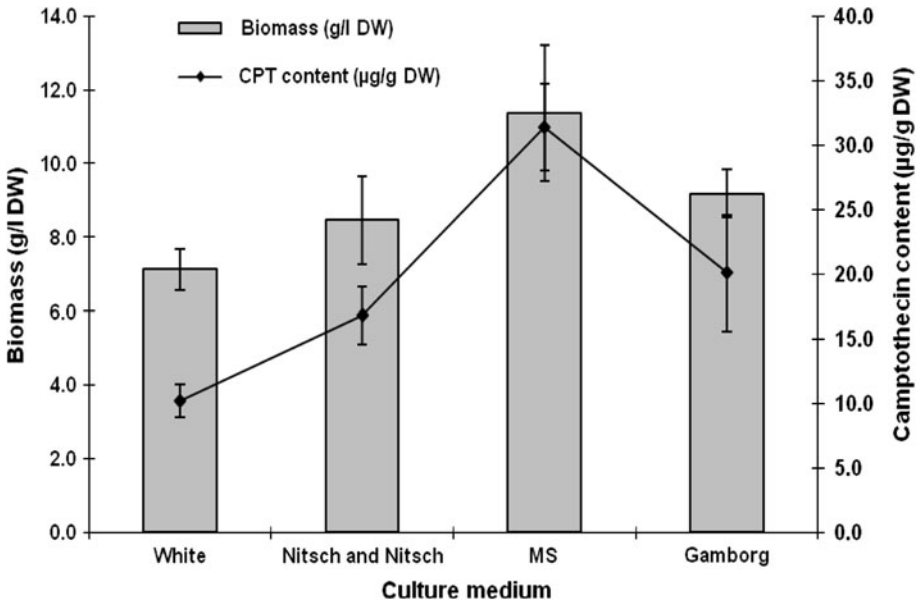

Fig. 3 Effect of different culture medium on growth and camptothecin content in *N. nimmoniana* cell cultures. The cultures were harvested on 20th day. The data shown are means of three replicates, and ±SD values are presented as *error bars*

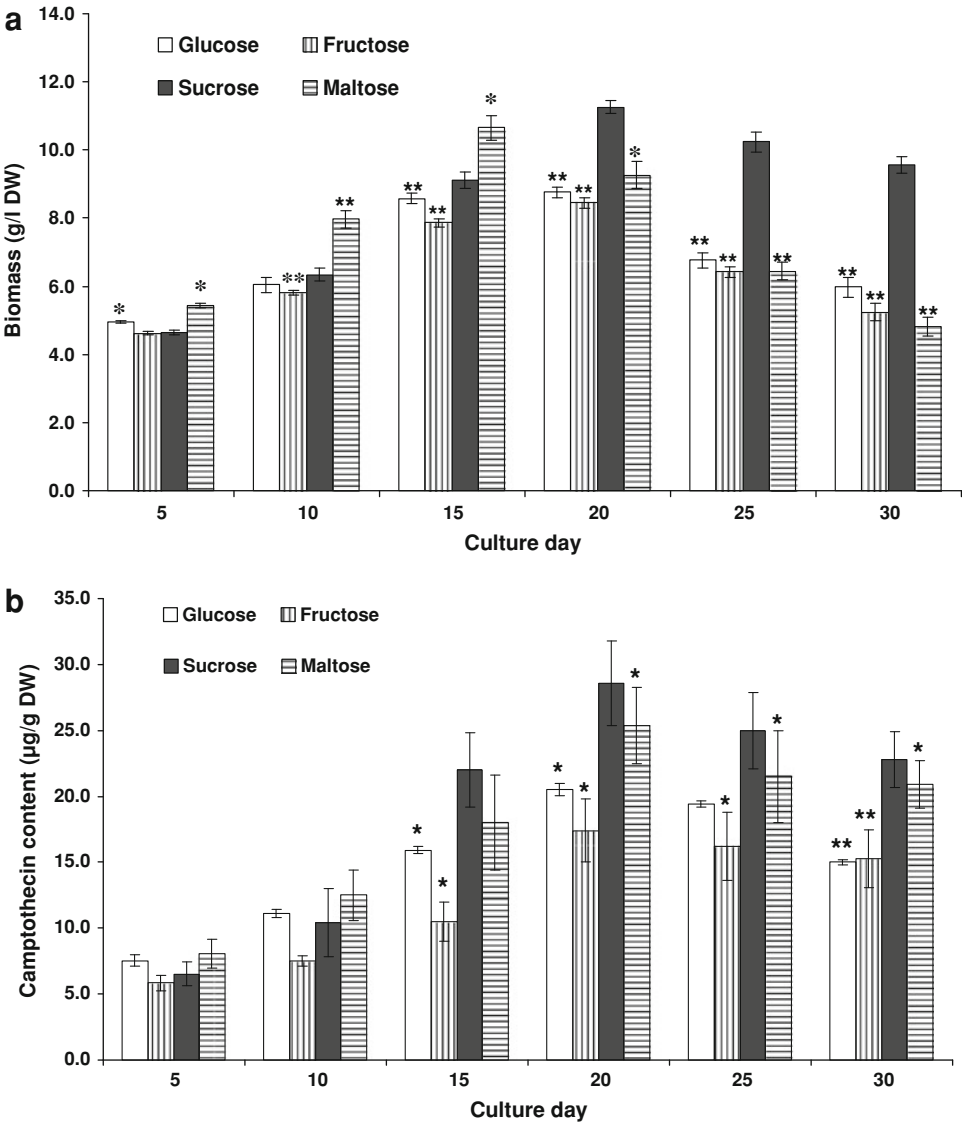

Fig. 4 Effect of different carbon source (3 %, w/v) on cell growth (**a**) and CPT production (**b**) by cell suspension cultures of *N. nimmoniana*. Values labeled *single asterisk*) and *double asterisks* were significant at $p < 0.05$ and $p < 0.01$, respectively, when compared with sucrose. Data were analyzed by one-way ANOVA followed by Dunnett's test. The data shown are mean of three replicates and ±SD values presented as *error bars*

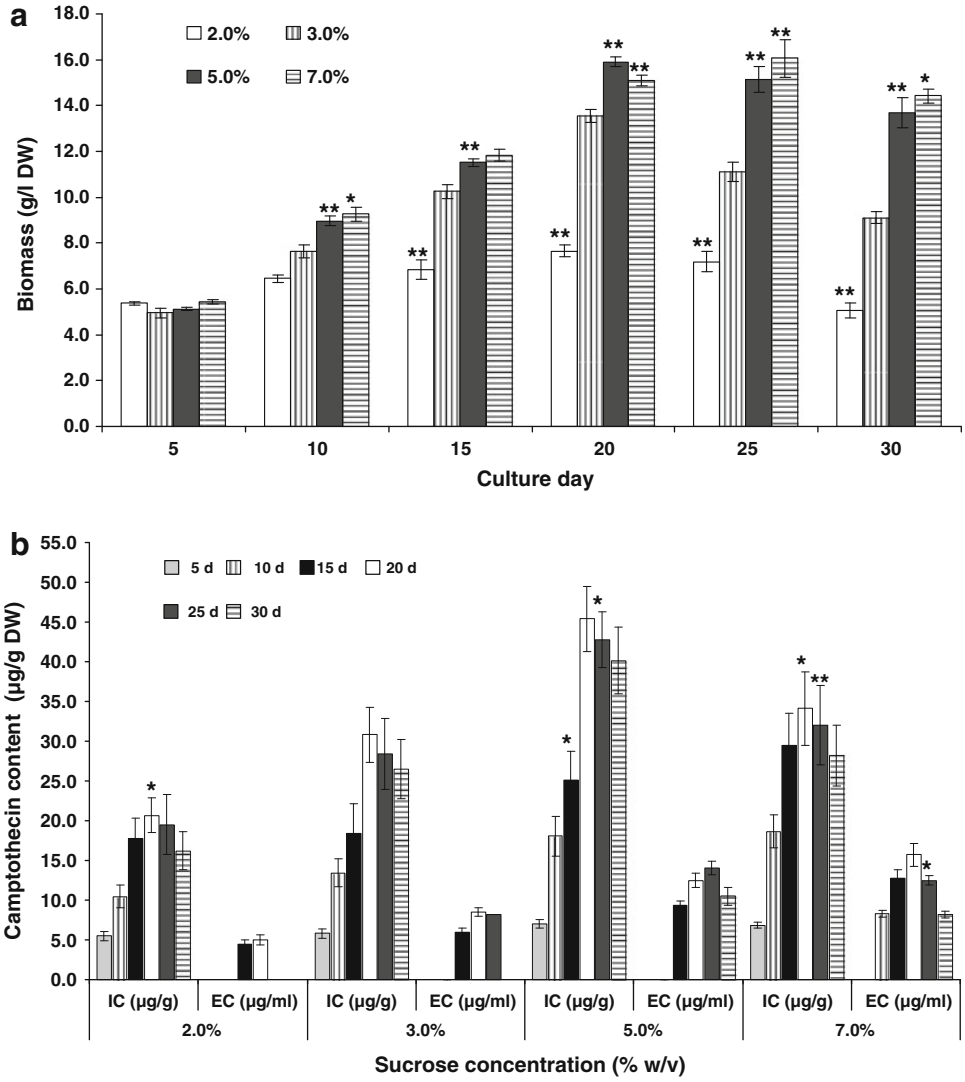

Fig. 5 Effect of different sucrose concentrations (%, w/v) on cell growth (**a**) and CPT (**b**) production by cell suspension cultures of *N. nimmoniana*. IC and EC correspond to intracellular (µg g^{-1} DW) and extracellular (µg ml^{-1}) CPT content, respectively. Values labeled *single asterisk* and *double asterisks* were significant at $p < 0.05$ and $p < 0.01$, respectively, when compared with 3 % sucrose. The data shown are mean of three replicates and ±SD values presented as *error bars*

The present results show that medium enriched with sucrose is efficient in inducing high growth as well as CPT production as compared to other carbon sources tested (Fig. 4a, b). It might be because sucrose provides a balanced carbon source for the cell growth. Sucrose was thus, selected as a carbon source to carry out further studies in cell suspension cultures. A sucrose concentration above 2 % (w/v) has been found to enhance the yield of secondary metabolites by not only stimulating growth but also increasing the rate of product synthesis (Merillon et al. 1984). A direct correlation in sugar concentration and biomass accumulation was shown in cultures grown on different concentrations of sucrose (Fig. 5a). Medium supplemented with high sucrose concentration of 7.0 % showed long linear growth phases until day 30, while in 3 % sucrose it lasts up to 20 days, followed by a stationary phase. The present results show that the linear growth phase was extended with increased concentrations of sucrose. In contrast, medium supplemented with lower concentration of sucrose (2 %) had an extended lag phase and reduced cell growth. Increased concentrations of sucrose (5.0 %) enhanced the growth rate of suspension cell cultures and achieved 15.91 g/l DW cell biomass which was nearly 1.2-fold greater than the control (3 %, w/v sucrose) on 20th day of culture cycle.

The alteration of sucrose (2–7 %, w/v) concentration has noteworthy effect on the production of CPT and culture biomass in the suspension cultures of *N. nimmoniana* (Fig. 5a, b). A rapid increase in CPT accumulation occurred after 20 days of culture, in medium with high concentrations of sucrose (5.0 and 7.0 %). Maximum accumulation of intracellular CPT 47.4 µg/g DW was achieved at 5.0 % concentration of sucrose on 20th day of culture cycle (Fig. 5b). The present results show that 5 % of sucrose is optimal for CPT production. Suppression of cell growth as well as CPT synthesis at high sucrose concentrations (7 %) could be due to osmotic shock or repression of biosynthetic pathways. Leaching of CPT in

culture media was noted at 7 % sucrose concentration and maximum of 15.7 μg/ml concentration reached on day 20 of culture cycle (Fig. 5b).

Effect of sucrose feeding

The experimental data obtained shows that a steady growth and CPT production was achieved with sucrose at 3 % (w/v) initial concentration and CPT production augments with higher sucrose concentration. Intermittent sucrose feeding experiment was thus designed in which additional quantities of 1, 2, and 3 % sucrose were added on day 12 of *N. nimmoniana* cell culture grown on MS medium with 3 % sucrose. This additional sucrose feeding had marked influence on cell growth. Culture fed with 2 % sucrose had a maximum biomass accumulation of 17.85 g/l DW on day 21 of cultivation (Fig. 6a). Similarly, the highest CPT content of 35.3, 42.5, and 39.1 μg/g DW were noted on 1, 2, and 3 % sucrose feeding on day 21, respectively (Fig. 6b). It is interesting to note that 2 % feeding of sucrose has favored CPT production more than 3 % sucrose suggesting that 2 % feeding is best for CPT production. Chromatographic analysis demonstrated that sucrose feeding at higher concentration leached little amount of CPT into culture medium which might be due to the cellular lysis.

The present work shows that essential nutrient components such as carbon influence the growth and production of CPT. Glucose, maltose, fructose, and sucrose are most efficacious energy sources studied for plant cell growth and secondary metabolites production. Sucrose is of special significance among the nutritional source, in cell growth and secondary product formation (Tal et al. 1982; Gertlowski and Petersen 1993). It is well reported that sugars are probable acting not only as carbon sources but also as signaling molecules to directly affect the production of secondary metabolites (Wang and Weathers 2007). Our results show that increasing sucrose (2–7 %, w/v)

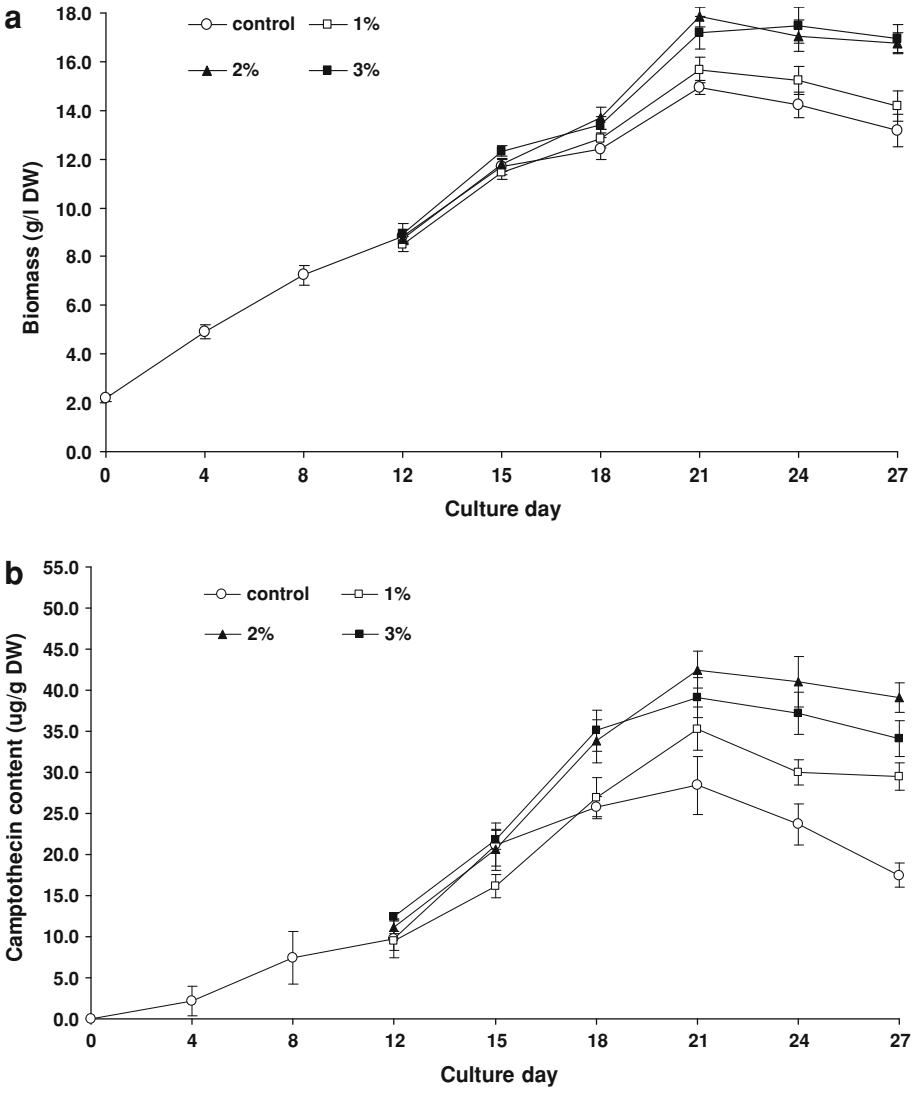

Fig. 6 Effect of sucrose feeding (%, w/v) on cell growth (**a**) and CPT (**b**) production by cell suspension cultures of *N. nimmoniana*. The sterilized sucrose solution was added on 12th day of cell culture. The data shown are mean of three replicates and ±SD values presented as *error bars*

concentration had beneficial effect on the biomass as well the CPT content while higher sucrose (7.0 %, w/v) concentration suppressed the cell growth as well the CPT content. This is in agreement with the finding of Thanh et al. (2007) who reported that sucrose at a concentration of 2–5 % enhances biomass and ginsenoside accumulation in the suspension culture of *Panax vietnamensis* and higher sucrose concentration of 6–7 % inhibits the ginsenoside accumulation. Similarly, Xu et al. (1999) reported that media containing 4–5 % sucrose had the highest salidroside content and further higher concentrations of sucrose (more than 5 %) reduced salidroside formation in liquid cultivated compact callus aggregates of *Rhodiola sachalinensis*. Present results have shown the efficacy of sucrose as a balanced carbon source for growth and CPT production compared to glucose, maltose, and fructose, which again is in agreement with the finding of Zha et al. (2007). Stimulatory effect of high sucrose concentration on anthocyanin production was observed in cell cultures of *C. acuminata* cell cultures (Pasqua et al. 2005). The influence of high sucrose concentration on *Perilla frutescens* and *Panax ginseng* secondary metabolite production has also been reported (Zhong and Yoshida 1995; Akalezi et al. 1999). Sugars have important functions in signal transduction, gene regulation, and development (Rolland et al. 2002; Gibson 2005). A 100- to 200-fold higher sucrose concentrations than those naturally occurring in *Vitis vinifera* berries, promoted polyphenol biosynthesis, and increased the intracellular accumulation and/or release in the media of anthocyanins, catechins, and stilbenes in the *V. vinifera* suspension culture. It was hypothesized that high sucrose concentrations could play a role in plant defense via the induction of secondary metabolites, such as stilbenes (Ferri et al. 2011).

Komaraiah et al. (2005) achieved a synergistic effect that increased the anthraquinones production by fourfold in suspension cultures of *Morinda citrifolia* by controlled addition of sucrose. Wang et al. (1999) established efficient sucrose feeding strategy to improve taxane productivity in the cell suspension cultures of *Taxus chinensis*. The present study shows that sucrose feeding during the start of exponential phase effectively improved alkaloid (CPT) production. Similar results on other plant cell cultures showed the sucrose feeding increased secondary metabolite production in suspension cultures of *Lithospermum erythrorhizon* (Shrinivasan and Ryn 1993) and *Perilla frutescens* (Zhong and Yoshida 1995).

Effect of phosphate

The increased phosphate concentration (provided as KH_2PO_4) was related with increased cellular growth and resulted in highest biomass (11.9 g/l DW) at a 1.25 mM-concentration (Fig. 7a). It is supposed that reduced phosphate level resulted in a delayed onset of log phase which adversely affected the cellular biomass production. A reduction in intracellular CPT content (12.5 μg/g DW) was recorded at higher phosphate levels (2.5 mM) while maximum CPT content (31.6 μg/g DW) was recorded with the 0.5 mM phosphate which is 1.1-fold higher over the control culture. The lower concentration of phosphate negatively affects the cell biomass (Fig. 7a). Trace amounts of extracellular CPT was detected with altered phosphate treatment.

There was no profound effect on the CPT production in *N. nimmoniana* cell suspension cultures by the varying concentration of phosphate, whereas high phosphate concentrations in suspension cultures impeded the CPT production as well as biomass accumulation. No direct link was established with the phosphate concentrations and CPT production. Our experimental data are in agreement with the earlier report by Liu and Zhong (1998), where low phosphate levels in medium enhanced the alkaloids and saponins production in *P. ginseng* and *P. quinquefolium*, and higher phosphate concentration suppressed product biosynthesis.

Effect of nitrogen and nitrogen feeding

Nitrogen is supplied as NH_4^+ or NO_3^- or as a combination of both in most standard culture media for plant cell culture. Initially, the effect of increased total nitrogen content of the medium (with NH_4^+/NO_3^- ratio of 1:2) on growth and CPT production was studied. The results in Fig. 7b show that 60 mM total nitrogen was optimal for maximum CPT production by the cells, and further increase in the total nitrogen level favored only cell growth. Furthermore, the ratio of NH_4^+/NO_3^- was varied over a specific range, at 60 mM total nitrogen level, and the results are shown in Fig. 7c. Generally, increased levels of NO_3^- favored cell growth, whereas the increased levels of NH_4^+ had a marked effect on CPT biosynthesis. At an NH_4^+/NO_3 concentration balance of 5:1 with 60 mM total nitrogen, the maximum CPT content of 48.7 μg/g DW was achieved, the cell mass concentration being 8.62 g/l (Fig. 7c). This contrasts with 29.8 μg/g of CPT produced in the control standard MS medium.

The results with nitrogen treatment suggest that increased concentration balance of ammonium to nitrate source in medium had a positive effect on CPT content. On the basis of preliminary experiments with nitrogen (Fig. 7b, c) with *N. nimmoniana* cell culture day 15 was selected for nitrogen feeding. On day 15 of culture cycle, the selected ratio of ammonium to nitrate (5:1) at a total initial nitrogen of 60 mM was fed to the culture medium and checked for the biomass and CPT accumulation. Cells

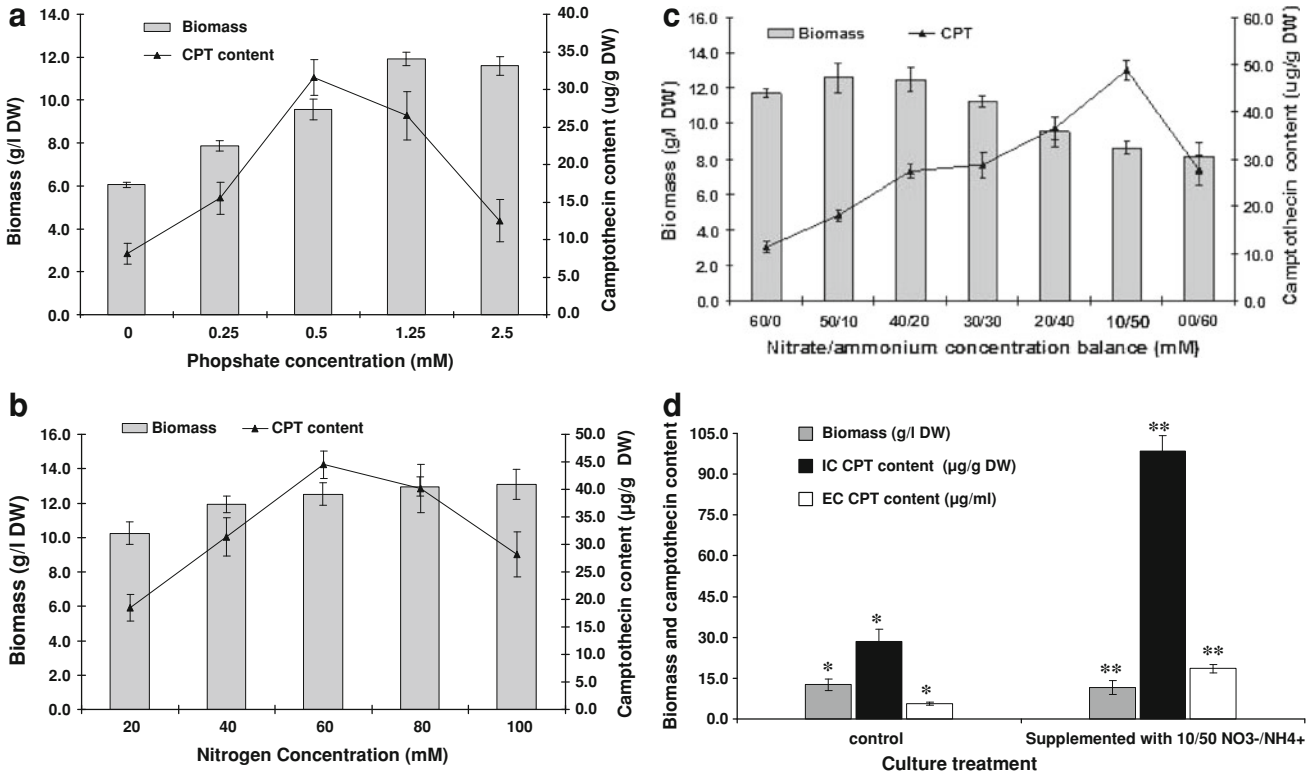

Fig. 7 Effects of different phosphate concentrations (provided as KH_2PO_4) on growth and camptothecin (CPT) production in cell suspension culture of *N. nimmoniana* (day 20) (**a**), effect of increased total nitrogen content at a constant NH_4^+/NO_3^- ratio (1/2) on growth and CPT content in *N. nimmoniana* cell cultures (day 20) (**b**), effect of ammonia to nitrate ratio (maintaining total nitrogen at 60 mM level) on growth and CPT content in *N. nimmoniana* cell cultures (day 20) (**c**), improved CPT content in suspension cells of *N. nimmoniana* by selective feeding of nitrate/ammonium (on 15th day of culture cycle) as nitrogen source (**d**). *Single asterisk* and *double asterisks* the day it reached the maximum in control (20) and nitrogen fed (24) culture, respectively. *IC* intracellular, *EC* extracellular. The data shown are mean of three replicates and ±SD values presented as *error bars*

were harvested every 3 days, after nitrogen source feeding. There was no conspicuous increase in the cell biomass over the control (12.65 g/l DW). However, the CPT content had a 2.4-fold increase, over the control culture (28.5 µg/g DW) (Fig. 7d). Surprisingly, the nitrogen feeding treatment also induced the leaching of CPT in medium in trace amounts.

The control of product formation by total nitrogen has also been shown in *L. erythrorhizon* for shikonin production (Fujita et al. 1981). The enhanced production of anthocyanin by *Vitis* sp. and diosgenin by *D. deltoidea*, with the use of optimal concentration of both NH_4^+ and NO_3^- (Yamakawa et al. 1983; Tal et al. 1982). Our results are similar to that of cell suspension cultures of *Taxus yunnanensis* to produce taxol (Chen et al. 2003) and production of tropane alkaloids from *A. belladonna* root culture (Bensaddek et al. 2001). Higher ratio of ammonium to nitrate source concentration was supportive for CPT biosynthesis. Ammonium ions would probably be rapidly assimilated into glutamate and then into glutamine which might involved in CPT biosynthesis (Yamazaki et al. 2004). However, low ammonium in medium was supported the taxoid production in cell suspension culture of *T. chinensis*, the possible reason for the same was intracellular salicylic acid which is involved in signal cascade, which regulates the taxoid biosynthesis (Zhou and Zhong 2009, 2011).

Selection of optimal nutrient combination

The present studies performed on the effect of sucrose, phosphate, and nitrogen source concentrations and feeding of nitrogen and sucrose, on CPT synthesis in *N. nimmoniana* cell cultures gave individually the optimal concentrations of these nutrients in the medium for better production of biomass and CPT. Hence, different combinations of the nutrients were used to investigate their combined effect on growth as well as alkaloid production. The cells were cultivated in MS medium containing different combinations of sucrose, nitrogen, phosphate, and growth regulators. The cultures were harvested on day 20 and analyzed for growth and CPT content (Table 1). The best combination as shown in Table 1 was as follows: a nitrogen feeding ratio of NH_4^+ to NO_3^- (5/1) at 60 mM

Table 1 Cell culture biomass and camptothecin (CPT) content in *N. nimmoniana* cell suspension cultures in MS medium containing different combinations of nutrients

Serial no.	Nutrient combination in medium				Biomass (g/l DW)	CPT content (μg/g DW)	
	Nitrogen source (60 mM) NH_4^+/NO_3^-	Phosphate source (mM)	Carbohydrate source (% sucrose)	Sucrose feeding[a] (%)		IC_CPT	EC_CPT
1[b]	1/2	1.25	3	Nil	14.1 ± 0.72	29.2 ± 1.5	8.2 ± 0.07
2	5/1	0.5	3	2	8.1 ± 0.55**	34.4 ± 2.6*	11.6 ± 0.14**
3	5/1[c]	0.5	3	2	10.6 ± 0.51**	51.7 ± 2.1**	19.1 ± 0.13**
4	1/5	0.5	3	2	14.5 ± 0.64	22.1 ± 1.4**	10.1 ± 1.7*
5	5/1	0.5	5	2	11.0 ± 0.75**	31.5 ± 2.5	12.4 ± 0.22**
6	1/5	0.5	5	2	14.2 ± 1.26	20.2 ± 0.9**	Trace

N. nimmoniana cells were cultured with 10.74 μM naphthaleneacetic acid (NAA), 0.93 μM kinetin (Kn), 2 mg/l ascorbic acid, 1 mg/l PVP and harvested on day 20 of the cell culture cycle to analyze biomass and CPT content (IC and EC corresponds to intracellular and extracellular, respectively). Values labeled * and ** were significant at $p < 0.05$ and $p < 0.01$, respectively, when compared control treatment (serial no. 1), based on a one-way ANOVA followed by Dunnett's test. The data shown are means of three replicates, and ±SD values are presented as error bars

[a] Sucrose feeding was done on day 12

[b] Control treatment

[c] Nitrogen feeding (NH_4^+/NO_3^-; 5/1) on day 15 of the cell culture cycle, initially the medium have nitrogen concentration (NH_4^+/NO_3^-) ratio of 1/5

total nitrogen concentration on day 15, phosphate at 0.5 mM, initial sucrose at 3 (% w/v), and 2 % sucrose feeding on day 12 (treatment 3 in Table 1) along with the phytohormones (as described in "Materials and methods"). Under these nutrients concentrations, 10.6 g/l culture biomass and 51.7 μg/g DW intracellular CPT were produced by *N. nimmoniana* cell culture in 20 days. On the other hand, the standard MS medium (control) with 3 % of sucrose resulted in cell mass and alkaloid concentrations of 14.6 g/l and 29.2 μg/g DW, respectively (treatment 1 in Table 1). Thus, the investigations on media optimization led to a 1.7-fold increase in CPT concentration in the modified medium compared to the standard MS medium. CPT was also leached to the extracellular medium, which reached to a maximum of 2.3-fold higher over the control treatment (treatment 3 in Table 1) on day 20. The current findings are in contrast with the Fulzele et al. (2001), who found 0.036 mg/ml of CPT in extracellular culture medium and trace amount of CPT intracellularly. The reason behind this type of culture behavior might be due to the feedback restriction posed by the leached CPT in culture medium. As CPT was found in medium as early as on day 5 compared with present case (days 10–12). Trace amount of 9-MCPT was found in suspension cells. However, it is interesting to note that, although the concentration of the intracellular CPT present in the suspension culture was lower, the overall productivity in terms of CPT content per liter culture medium per day was higher in the suspension culture (9.2 μg/l/day) compared to that obtained in the callus culture (5.1 μg/l/day).

The CPT content is variable in different morphological parts of *N. nimmoniana*. Maximum CPT content has been reported in root (1.55 %), followed by bark (0.27 %), seeds (0.10–0.054 %), stem (0.06 %), and leaves (0.058–0.081 %) (Puri et al. 1999; Fulzele and Satdive 2005; Padmanabha et al. 2006). In the present study, the *N. nimmoniana* leaf, stem, and seed explant have 0.022 ± 0.013, 0.034 ± 0.014, and 0.093 ± 0.020 % CPT content, respectively. The final optimized suspension culture derived from the *N. nimmoniana* leaf callus had 0.0051 ± 0.0003 % CPT content. However, the leaf explant has 0.022 % CPT content. CPT produced by the optimized in vitro cell culture (0.0051 %) was comparable with the leaf explant as well as leaf and stem CPT content reported by the former researchers. Due to the high amount of CPT present in the *N. nimmoniana* wood chips/bark and roots, it is harvested the most to fulfill the commercial need. Commercially, for getting 1 kg of CPT around 1,000–1,500 kg of *N. nimmoniana* wood chips is required. This makes the *N. nimmoniana* plant an endangered species. The *N. nimmoniana* bark is unfeasible for the initiation of in vitro cell line. However, the leaf, stem, and young seeds were mostly used to initiate the in vitro cell culture. The present in vitro cell culture strategy show promising results to get the CPT and it might reduce the load on the natural plant stock when properly scale up and thorough culture medium requirements were established.

Conclusions

Culture medium constituents influenced biomass production and secondary metabolite accumulation in cell

suspension cultures of *N. nimmoniana*. Sucrose was the best carbon source, while a combination of NH_4^+ and NO_3^- was favorable. Furthermore, relative high nitrate in the media supports the biomass, while high ammonium enhances the CPT content. However, the possible reason behind the nitrogen (high ammonium to nitrate ratio) induced CPT production is still to be determined. Phosphate at 0.5 mM fostered maximum CPT accumulation. Selective nutrient (sucrose and nitrogen) feeding strategies also have CPT augmenting effect. It is evident that medium manipulation is a well-established and principal way of enhancing the culture efficiency of plant cells for biomass and product accumulation. The results of the present study could be integrated with other yield enhancement strategies like precursor feeding, permeabilization, in situ product removal, elicitation for more effective improvement in CPT production, and such efforts are in progress. This further finds a way for exploring the possibility of scaling-up cell suspension cultures.

Acknowledgments V.S.K. acknowledges University Grant Commission (Basic scientific research fellowship for meritorious students, grant letter no. F.4-1/2006-BSR/7-57/2007-BSR), New Delhi for providing research fellowship. Authors also acknowledge Dr. D.P. Fulzele, Bhabha Atomic Research Centre, Mumbai, India for the kind gift of standard CPT and Prof. Suresh G. Killedar, Bharati Vidyapeeth Pharmacy College, Kolhapur, Maharashtra, India, for his kind help in collection of the *N. nimmoniana* plant specimens.

Conflict of interest The authors declare that they have no conflict of interest.

References

Akalezi CO, Liu S, Li QS, Yu JT, Zhong JJ (1999) Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*. Process Biochem 34:639–642

Anonymous (2001) Conservation assessment and management plan for medicinal plants in Maharashtra state. Foundation for Revitalisation of Local Health Traditions (FRLHT), Bangalore

Arisawa M, Gunasekera SP, Cordell GA, Farnsworth NR (1981) Plant anticancer agents XXI. Constituents of *Merriliodendron megacarpum*. Planta Med 43:404–407

Bensaddek L, Gillet F, Saucedo J, Fliniaux M (2001) The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. J Biotechnol 85:35–40

Bhambhani S, Karwasara VS, Dixit VK, Banerjee S (2011) Enhanced production of vasicine in *Adhatoda vasica* (L.) Nees. cell culture by elicitation. Acta Physiol Plant 34:1571–1578

Chen YC, Yi F, Cai M, Luo JX (2003) Effects of amino acids, nitrate, and ammonium on the growth and taxol production in cell cultures of *Taxus yunnanensis*. Plant Growth Regul 41:265–268

Ciddi V, Shuler ML (2000) Camptothecin from callus culture of *Nothapodytes foetida*. Biotechnol Lett 22:129–132

Dubois M, GuiUes KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Ferri M, Righetti L, Tassoni A (2011) Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. J Plant Physiol 168:189–195

Fujita Y, Hara Y, Ogino T, Suga C (1981) Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. Plant Cell Rep 1:59–60

Fulzele DP, Satdive RK (2003) Somatic embryogenesis, plant regeneration, and the evaluation of camptothecin content in *Nothapodytes foetida*. In Vitro Cell Dev Biol Plant 39: 212–216

Fulzele DP, Satdive RK (2005) Distribution of anticancer drug camptothecin in *Nothapodytes foetida*. Fitoterapia 76:643–648

Fulzele DP, Satdive RK, Pol BB (2001) Growth and production of camptothecin by cell suspension cultures of *Nothapodytes foetida*. Planta Med 67:150–152

Fulzele DP, Satdive RK, Pol BB (2002) Untransformed root cultures of *Nothapodytes foetida* and production of camptothecin. Plant Cell Tissue Organ Cult 69:285–288

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. Exp Cell Res 50:151–158

Gertlowski C, Petersen M (1993) Influence of the carbon source on growth and rosmarinic acid production in suspension cultures of *Coleus blumei*. Plant Cell Tissue Organ Cult 34:183–190

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr Opin Plant Biol 8:93–102

Govindachari TR, Viswanathan N (1972) Alkaloids of *Mappia foetida*. Phytochemistry 11:3529–3531

Gunasekera SP, Badawi MM, Cordell GA, Farnsworth NR, Chitnis M (1979) Plant anticancer agents X. Isolation of camptothecin and 9-methoxycamptothecin from *Ervatamia heyneana*. J Nat Prod 42:475–477

Jain R, Dixit VK (2010) Effect of miconazole and terbinafine on artemisinin content of shooty teratoma of *Artemisia annua*. Nat Prod Commun 5:1–6

Karwasara VS, Dixit VK (2011) Culture medium optimization for improved puerarin production by cell suspension cultures of *Pueraria tuberosa* (Roxb. ex Willd.) DC. In Vitro Cell Dev Biol Plant 48:189–199

Karwasara VS, Jain R, Tomar P, Dixit VK (2010) Elicitation as yield enhancement strategy for glycyrrhizin production by cell cultures of *Abrus precatorius* Linn. In Vitro Cell Dev Biol Plant 46:354–362

Karwasara VS, Tomar P, Dixit VK (2011a) Oxytocin influences the production of glycyrrhizin from cell cultures of *Abrus precatorius* Linn. Plant Growth Regul 65:401–405

Karwasara VS, Tomar P, Dixit VK (2011b) Influence of fungal elicitation on glycyrrhizin production in transformed cell cultures of *Abrus precatorius* Linn. Pharmacogn Mag 7:307–313

Komaraiah P, Kishor PB, Kavi CM, Magnusson KE, Mandenius CF (2005) Enhancement of anthraquinone accumulation in *Morinda citrifolia* suspension cultures. Plant Sci 168:1337–1344

Kulkarni AV, Patwardhan AA, Lele U, Malpathak NP (2010) Production of camptothecin in cultures of *Chonemorpha grandiflora*. Pharmacogn Res 2:296–299

Kumar R, Ved DK (2000) Illustrated field guide to 100 red listed medicinal plants of conservation concern in Southern India. Foundation for Revitalization of Local Health Traditions, Bangalore, pp 261–263

Liu S, Zhong JJ (1998) Phosphate effect of production of ginseng saponin and polysaccharide by cell suspension cultures of *Panax ginseng* and *Panax quinquefolium*. Proc Biochem 33:69–74

Lopez-Meyer M, Nessler CL, McKnight TD (1994) Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata*. Planta Med 60:558–560

Merillon JM, Rideau M, Chenieux JC (1984) Influence of sucrose on levels of ajmalicine, serpentine and tryptamine in *Catharanthus roseus* cells in vitro. Planta Med 50:561–567

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Planta 15:473–497

Namdeo AG, Sharma A, Sathiyanarayanan L, Fulzele D, Mahadik KR (2010) HPTLC densitometric evaluation of tissue culture extracts of *Nothapodytes foetida* compared to conventional extracts for camptothecin content and antimicrobial activity. Planta Med 76:474–480

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Padmanabha BV, Chandrashekar M, Ramesha BT, Hombe Gowda HC, Gunaga RP, Suhas S, Vasudeva R, Ganeshaiah KN, Uma Shaanker R (2006) Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham., in the western ghats, India: implications for identifying high-yielding sources of the alkaloid. Curr Sci 90:95–100

Pasqua G, Monacelli B, Mulinacci N, Rinaldi S, Giaccherini C, Innocenti M, Vinceri FF (2005) The effect of growth regulators and sucrose on anthocyanin production in *Camptotheca acuminata* cell cultures. Plant Physiol Biochem 43:293–298

Priel E, Showalter SD, Blair DG (1991) Inhibition of human immunodeficiency virus (HIV-1) replication in vitro by noncytotoxic doses of camptothecin, a topoisomerase I inhibitor. AIDS Res Hum Retrovir 7:65–72

Puri SC, Handa G, Gupta RK, Gupta VK, Shrivastava TN, Somal P, Sharma SN (1999) Quantitation of camptothecin in *Nothapodytes foetida*. J Indian Chem Soc 76:370–371

Ramachandra RS, Ravishankar GA (2002) Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv 20:101–153

Roja G, Heble MR (1994) The quinoline alkaloids camptothecin and 9-methoxycamptothecin from tissue cultures and mature trees of *Nothapodytes foetida*. Phytochemistry 36:65–66

Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. Plant Cell 14:185–205

Saito K, Sudo H, Yamazaki M, Koseki-Nakamura M, Kitajima M, Takayama H, Aimi N (2001) Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. Plant Cell Rep 20:267–271

Sarin R (2005) Useful metabolites from plant tissue cultures. Biotechnology 4:79–93

Satdive RK, Fulzele DP, Eapen S (2007) Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* A. Juss by elicitation and media optimization. J Biotechnol 128:281–289

Sharma T, Dixit VK (2006) Studies on Ti-mediated transformed cultures of *Artemisia annua* L. Indian J Pharm Sci 68:448–455

Shrinivasan V, Ryn DDY (1993) Improvement of shikonin productivity in *Lithospermum erythrorhizon* cell cultures by alternating carbon and nitrogen feeding strategy. Biotechnol Bioeng 42:793–799

Takimoto CH, Wright J, Arbuck SG (1998) Clinical applications of the camptothecins. Biochim Biophys Acta 1400:107–119

Tal B, Gressel J, Goldberg I (1982) The effect of medium constituent on growth and diosgenin production by *Dioscorea deltoidea* cells grown in batch culture. Planta Med 44:111–115

Thanh NT, Van KN, Paek KY (2007) Effecting of medium composition on biomass and ginsenoside production in cell suspension culture of *Panax vietnamensis* Ha et Grushv. VNU J Sci Nat Sci Technol 23:269–274

Thengane SR, Kulkarni DK, Shrikhande VA, Joshi SP, Sonawane KB, Krishnamurthy KV (2003) Influence of medium composition on callus induction and camptothecin accumulation in *Nothapodytes foetida*. Plant Cell Tissue Organ Cult 72:247–251

Trejo-Tapia G, Arias-Castro C, Rodrguez-Mendiola M (2003) Influence of the culture medium constituents and inoculum size on the accumulation of blue pigment and cell growth of *Lavandula spica*. Plant Cell Tissue Organ Cult 72:7–12

Wall ME, Wani MC, Cook CE et al (1966) Plant antitumour agents I. The isolation and structure of camptothecin, a novel alkaloidal leukaemia and tumour inhibitor from *Camptotheca acuminata*. J Am Chem Soc 88:3888–3889

Wang Y, Weathers PJ (2007) Sugars proportionately affect artemisinin production. Plant Cell Rep 26:1073–1081

Wang HQ, Yu JT, Zhong JJ (1999) Significant improvement of taxane production in suspension cultures of *Taxus chinensis* by sucrose feeding strategy. Process Biochem 35:479–483

Weathers PJ, Towler MJ, Xu J (2010) Bench to batch: advances in plant cell culture for producing useful products. Appl Microbiol Biotechnol 85:1339–1351

Weidenfeld H, Furmanowa M, Roeder E, Guzewska J, Gustowski W (1997) Camptothecin and 10-hydroxycamptothecin in callus and plantlets of *Camptotheca acuminata*. Plant Cell Tissue Organ Cult 49:213–218

White PR (1963) The cultivation of animal and plant cells, 2nd edn. Ronald, New York

Xu JF, Ying PQ, Han AM, Su ZG (1999) Enhanced salidroside production in liquid-cultivated compact callus aggregates of *Rhodiola sachalinensis*: manipulation of plant growth regulators and sucrose. Plant Cell Tissue Organ Cult 55:53–58

Yamakawa T, Kato S, Ishida K, Kodama T, Mionda Y (1983) Production of anthocyanin by *Vitis* cells in suspension culture. Agric Biol Chem 47:2185–2191

Yamazaki Y, Kitajima M, Arita M, Takayama H, Sudo H, Yamazaki M, Aimi N, Saito K (2004) Biosynthesis of camptothecin. In silico and in vivo tracer study from [1-^{13}C] glucose. Plant Physiol 134:161–170

Ya-ut P, Chareonsap P, Sukrong S (2011) Micropropagation and hairy root culture of *Ophiorrhiza alata* Craib for camptothecin production. Biotechnol Lett 33:2519–2526

Zha XQ, Luo JP, Jiang ST, Wang JH (2007) Enhancement of polysaccharide production in suspension cultures of protocorm-like bodies from *Dendrobium huoshanense* by optimization of medium compositions and feeding of sucrose. Process Biochem 42:344–351

Zhao J, Zhu WH, Hu Q, He XW (2001) Improved alkaloid production in *Catharanthus roseus* suspension cell cultures by various chemicals. Biotechnol Lett 22:1221–1226

Zhong JJ, Yoshida T (1995) High cultivation of *Perilla frutescens* cell suspensions for anthocyanin production: effects of sucrose concentration and inoculum size. Enzyme Microb Technol 7:1079–1087

Zhou X, Zhong JJ (2009) Effect of initial ammonium concentration on taxoid production and biosynthesis genes expression profile in suspension cultures of *Taxus chinensis* cells. Eng Life Sci 9:261–266

Zhou X, Zhong JJ (2011) Intracellular salicylic acid is involved in signal cascade regulating low ammonium-induced taxoid biosynthesis in suspension cultures of *Taxus chinensis*. Appl Microbiol Biotechnol 90:1027–1036