Biotechnology and Bioprocess Engineering **2005**, 10: 109-114

Ce^{4+}-Stimulated Ion Fluxes Are Responsible for Apoptosis and Taxol Biosynthesis in Suspension Cultures of Taxus Cells

Jing-Chuan Li, Zhi-Qiang Ge, and Ying-Jin Yuan*

P. O. Box 6888, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

Abstract Ion fluxes across the plasma membrane activated by 1 mM Ce^{4+}, cell apoptosis and taxol biosynthesis in suspension cultures of *Taxus cuspidata* were studied. The extracellular pH sharply decreased upon the addition of 1 mM Ce^{4+}, then increased gradually and exceeded the initial pH value over a time period of 12 h. The extracellular Ca^{2+} concentration decreased within the first 3 h after the addition of Ce^{4+}, then gradually decreased to one third of initial value in control at about 72 h and remained unchanged afterwards. Experiments with an ion channel blocker and a Ca^{2+}-channel blocker indicated that the dynamic changes in extracellular pH and the Ca^{2+} concentration resulted from the Ce^{4+}-induced activation of H^+ uptake and Ca^{2+} influx across the plasma membrane *via* ion channels. A pretreatment of the ion channel blocker initiated Ce^{4+}-treated cells to undergo necrosis, and the prior addition of the Ca^{2+}-channel blocker inhibited Ce^{4+}-induced taxol biosynthesis and apoptosis. It is thus inferred that H^+ uptake is necessary for cells to survive a Ce^{4+}-caused acidic environment and is one of the mechanisms of Ce^{4+}-induced apoptosis. Furthermore, the Ca^{2+} influx across the plasma membrane mediated both the Ce^{4+}-induced apoptosis and taxol biosynthesis.

Keywords: apoptosis, Ce^{4+}, ion fluxes, taxus cells

INTRODUCTION

Taxol is a very effective anticancer drug and has shown significant activity in clinical trials against a wide variety of tumors especially refractory ovarian, non-small cell lung cancer, AIDS-related Kaposi's sarcoma and other cancers. However, the limited supply of taxol from the bark of the *Taxus* species prompted intense efforts to develop alternative methods for taxol production. Suspension cultures of *Taxus* spp. are regarded as a very promising alternative for the production of taxol [1], but the yield of taxol is very low due to the inherent characteristics of plant cell cultures. So, various biotic and abiotic elicitors have been introduced aiming at high taxol production [2-4]. Our research showed that 1 mM Ce^{4+} is the most effective elicitor when compared with other rare earth ions including Ce^{3+}, La^{3+} and Nd^{3+} for taxol biosynthesis and cell apoptosis in suspension cultures of *Taxus cuspidata* [5,6].

Unlike other rare earth ions, Ce^{4+} creates a low pH in a solution and possesses a high oxidation-reduction potential, which stimulates an O_2^- burst in a suspension culture of *Taxus cuspidata* [7]. H^+ in plants functions as Na^+ does in animals, and the change in H^+ distribution between extracellular and intracellular spaces can produce a proton force that causes the fluxes of other ions across the plasma membrane [8]. Whether or not Ce^{4+} can cause ion fluxes across the plasma membrane is thus questioned considering its ability to acidify the environment. It has been reported that Ce^{4+} acts as an abiotic elicitor in *Taxus* spp [5-7,9]. The activation of ion fluxes is one of the early signal events in a plant-elicitor interaction. Jabs *et al.* [10] reported that the recognition of an elicitor by a plasma membrane receptor is accompanied by the activation of ion channels, resulting in transient influxes of Ca^{2+} and H^+ and effluxes of K^+ and Cl^-. Accordingly, we also questioned whether Ce^{4+} can activate ion fluxes across the plasma membrane like other elicitors do.

It is possible that Ce^{4+} acts in the same manner as other rare earth ions. The effect of rare earth ions on ion channels was reported to have two opposite modes. Ln^{3+} was shown to enhance a Ca^{2+} influx [11,12] while other rare earth ions acted as Ca^{2+}-channel blockers of the plasma membrane [13,14].

Ion fluxes over the plasma membrane are one of the early elements of these receptor-mediated responses and they play an essential role in an oxidative burst, the activation of defense-related genes and the accumulation of phytoalexin. In addition, both secondary metabolite biosynthesis and cell apoptosis have been recognized as the late elements of plant defense responses to elicitors [10]. In this work, the effects of Ce^{4+} on ion (H^+ and Ca^{2+}) fluxes across the plasma membrane were investigated in suspension cultures of *Taxus* cells by analysis of the dy-

***Corresponding author**
Tel: +86-22-2740-1288 Fax: +86-22-2740-3888
e-mail: yjyuan@public.tpt.tj.cn

namical changes of extracellular pH and Ca^{2+} concentration. Experiments with an ion channel blocker and Ca^{2+}-channel blocker were also conducted to shed light on the mechanism of action of Ce^{4+} on Taxol biosynthesis and cell apoptosis.

MATERIALS AND METHODS

Chemicals

Propidium-iodide (PI), Hoechst 33342, anthracene-9-carboxylate (A-9-C), nifedipine and $Ce(NH_4)_2(NO_3)_6$ were purchased from Sigma. $Ce(NH_4)_2(NO_3)_6$ was dissolved in sterilized distilled water. A-9-C and nifedipine was dissolved respectively in DMSO before use. Control cultures were treated with equal amounts of sterilized distilled water or DMSO.

Cell Line and Culture Conditions

The cell line from young stems of *Taxus cuspidata* was subcultured on solid B_5 medium at 25°C in the darkness. Cell suspensions were cultured every 8~10 days for a total of five generations in a fresh modified B_5 medium containing sucrose (25 g/L), naphthylacetic acid (2 mg/L) and 6-benzyl aminopurine (0.15 mg/L). The pH of the medium was adjusted to 5.8. Cell cultures (100 mL) were maintained in 500-mL flasks at 25°C in the darkness with continuous shaking at 110 rpm. Fresh cells (2.5 g) from the suspension cultures of the 5th generation were inoculated into 50 mL of fresh modified B_5 medium in a 250-mL Erlenmeyer flask. $Ce(NH_4)_2(NO_3)_6$ was added to the culture system at the late stage of cultures (day 12), and cell samples were collected at predetermined time intervals for analyses. Two control groups, Control 1 without any additive and Control 2 with NH_4NO_3 (2 mmol/L) and 4 mmol/L $NaNO_3$ (4 mmol/L), were used to test the effects of NH_4^+ and NO_3^- on the culture system. All data were the average of triplicate samples and the standard errors were within ± 8%.

Measurement of Extracellular pH and Ca^{2+} Concentration

Extracellular pH and the Ca^{2+} concentration were detected with a pH-meter and an automatic Beckman biochemical analyzer C-X7, respectively.

Hoechst 33342-PI Co-staining

One mL of cell suspension was centrifuged at 1,100 × g for 10 min and the supernatant was discarded. The remaining cells were washed three times with 10 mL of 0.1 M phosphate buffer (pH 5.8) with continuous bubbling to prevent the cells from aggregating. The cells were re-suspended in 0.2 mL of 0.1 M phosphate buffer at pH 5.8 for staining with fluorescence dyes, Hoechst 33342 and Propidium-iodide following the method of Ormerod *et al.* [15]. The stained cells were collected by centrifugation, washed with 10 mL of 0.1 M phosphate buffer at pH 5.8 and resuspended in the same phosphate buffer (0.2 mL). Cell suspensions (50 μL) were dropped on a slide and observed using a fluorescent microscope (Nikon, E-800) under UV radiation.

Extraction of Taxol and Analysis by HPLC

Taxol was extracted from the suspension cultures and analyzed by HPLC as described in a previous publication [16].

RESULTS AND DISCUSSION

Effects of Ce^{4+} on Extracellular pH

The extracellular pH of the suspension culture of *Taxus* cells in response to different Ce^{4+} concentrations was investigated (Fig. 1). The addition of 1 mM $Ce(NH_4)_2(NO_3)_6$ caused the extracellular pH to decrease immediately from 4.8 to 3.3 and then to increase to 5.5 at 24 h, while $Ce(NH_4)_2(NO_3)_6$ at a lower concentration (0.5 mM) did not cause an obvious change of the extracellular pH within 24 h. Additionally, $Ce(NH_4)_2(NO_3)_6$ at a higher concentration (5 mM) resulted in a decrease of the extracellular pH from 4.8 to 2.4, which remained unchanged with time. Fig. 2 shows the time course of the extracellular pH induced by 1 mM $Ce(NH_4)_2(NO_3)_6$. The extracellular pH sharply declined by about 3.3 pH units upon the addition of 1 mM Ce^{4+} (Stage 1). After about 1 h, the pH began to increase and reached the initial pH of 4.6 at 12 h (Stage 2). The pH then continued to increase to 6.2 at 29 h and remained at this level thereafter (Stage 3). In contrast, the extracellular pH of Control 1 (without any additive to B_5 medium) changed minimally during the whole period of time. These results indicate that the effect of $Ce(NH_4)_2(NO_3)_6$ on extracellular pH was dose-dependent, which is consistent with the dose-dependence of taxol biosynthesis. It is thus likely that only 1 mM $Ce(NH_4)_2(NO_3)_6$ activated the H^+ influx across the plasma membrane resulting in the activation of downstream signal events such as the related defense gene expression and taxol biosynthesis.

To further clarify the mechanism of 1 mM $Ce(NH_4)_2(NO_3)_6$-caused H^+ uptake, it was necessary to determine which component of $Ce(NH_4)_2(NO_3)_6$ plays the key role in this process. $Ce(NH_4)_2(NO_3)_6$ can be taken as consisting of two parts: Ce^{4+} and NH_4^+-NO_3^-. The culture system was composed of two parts: cells and the medium. To investigate whether the pH change was also caused by other components in addition to Ce^{4+}, the extracellular pH of Control 2, which contained equal amounts of NH_4^+ and NO_3^- but without Ce^{4+}, was tested (Fig. 2). The extracellular pH of Control 2 decreased slightly compared with that in Control 1 without any additives. This indicates that the change of extracellular pH caused by $Ce(NH_4)_2(NO_3)_6$ mainly resulted from Ce^{4+}. To investigate whether the pH change was caused by the effect of Ce^{4+} on the medium or cells, the Ce^{4+}-caused pH change

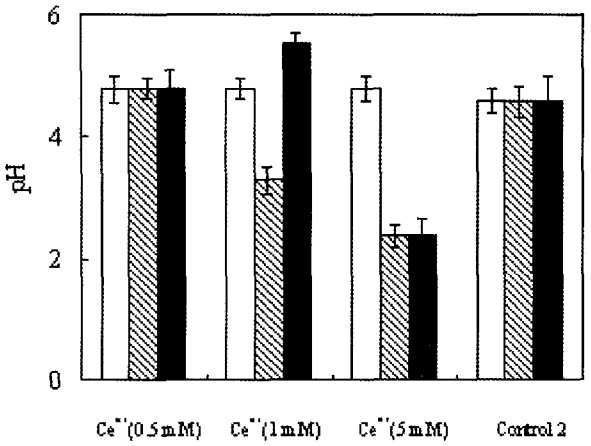

Fig. 1. Effect of Ce^{4+} concentration on extracellular pH in a suspension of *T. caspidata*. □ Before the addition of Ce^{4+}, ▨ Just after the addition of Ce^{4+}, ■ 24 h after the addition of Ce^{4+}.

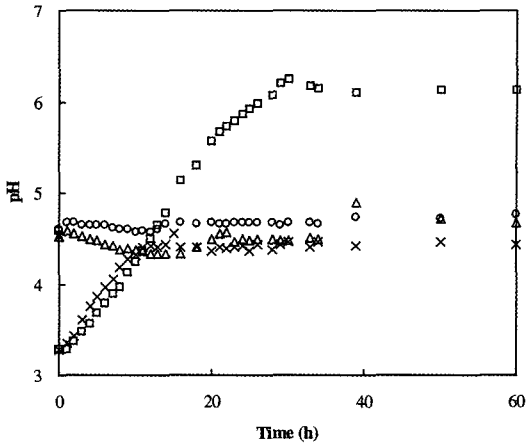

Fig. 2. Time course of extracellular pH induced by 1 mM Ce^{4+} in suspension cultures of *T. cuspidate* cells. ○ Control 1 (without any additives), △ Control 2 (with NH_4NO_3 (2 mmol/L) and $NaNO_3$ (4 mmol/L), □ Ce^{4+}-treated culture, × HCl-treated culture.

of the cell-free medium was also monitored. It is seen that the pH of the cell-free system fell sharply upon the addition of $Ce(NH_4)_2(NO_3)_6$ and remained unchanged within 24 h, while the pH of the cell-containing system decreased rapidly, and then increased immediately as shown in Fig. 2. So, the pH changes in Stages 2 and 3 resulted from the action of Ce^{4+} on cells, whereas the sharp fall of the extracellular pH in Stage 1 was a consequence of the action of Ce^{4+} on the medium.

Plant cells possess a self-regulation mechanism that responds to various environmental changes to some extent. To analyze whether the dynamic change of extracellular pH is caused by the ability of Ce^{4+} to acidify a medium, the effect of instantaneous acidification of the culture medium caused by 1 mM Ce^{4+} was modeled by regulating the medium pH with HCl. As shown in Fig. 2, the extracellular pH of the system treated with 1 M HCl also returned to its initial level (a similar trend with that of the Ce^{4+}-treated system) and subsequently remained at the initial level thereafter. It is thus inferred that the sharp decrease of pH caused by Ce^{4+} was within the endurable pH range for *Taxus* cells, and the pH could be restored to its normal level by the self-regulation of the cells. The subsequent alkalinization of the culture medium, however, is difficult to explain in terms of a cell self-regulation mechanism to the decreased pH.

Plants use protons almost exclusively as coupling ions. The efflux of H^+ generates the electrochemical gradient of protons across the plasma membrane that provides the driving force for the uptake and efflux of ions and metabolites across the plasma membrane [8]. The plasma membrane H^+-ATPase is a key component required to generate a proton motive force with a membrane potential from -120 to -160 mV (negative inside) and a pH gradient from 1.5 to 2.0 units (acid outside) by excluding H^+ from the cells [17]. Our results show that the addition of Ce^{4+} (1 mM) immediately decreased the extracellular pH by 1.3 units (Fig. 2), forming a pH gradient (acid outside) similar to that produced by PM H^+-ATPase. It is thus presumed that Ce^{4+} functioned as the plasma membrane H^+-ATPase in terms of generating proton forces that caused the fluxes of ions during Stage 2. It has been proposed that the different effects of elicitors on the plasma membrane H^+-ATPase and the resultant acidification or alkalinization of the extracellular medium is the consequence of cell responses to the difference between specific and nonspecific elicitors [18].

Effects of Ce^{4+} on Extracellular Ca^{2+} Concentration

As shown in Fig. 4, the concentration of extracellular Ca^{2+} obviously decreased within the first 3 h after the addition of Ce^{4+}, then gradually decreased to one third of initial value in control at about 72 h and remained unchanged afterwards. In contrast, the concentration of extracellular Ca^{2+} in Control 2 hardly changed with time. This result implies that Ce^{4+} caused the influx of extracellular Ca^{2+} across the plasma membrane. This is consistent with our previous presumption that the sharp increase of the cytoplasmic Ca^{2+} concentration of *Taxus cuspidata* cells (within 2~4 min after the addition of Ce^{4+}) resulted from the influx of extracellular Ca^{2+} into the cytoplasm as well as the release of Ca^{2+} from the calcium storage to the cytoplasm [19].

Effects of Ion Channel Blocker and Ca^{2+}-channel Blocker on Extracellular pH

The dynamic change of extracellular pH and Ca^{2+} suggested that the ion fluxes took place in the 1 mM Ce^{4+}-induced system. To validate this assumption, A-9-C, an ion channel blocker, and nifedipine, a Ca^{2+}-channel blocker, were added to the culture system at 30 min prior to the addition of Ce^{4+}, respectively. It was observed that A-9-C at 300 and 400 μM partially inhibited the pH increase caused by Ce^{4+} (Fig. 4), while nifedipine at 100

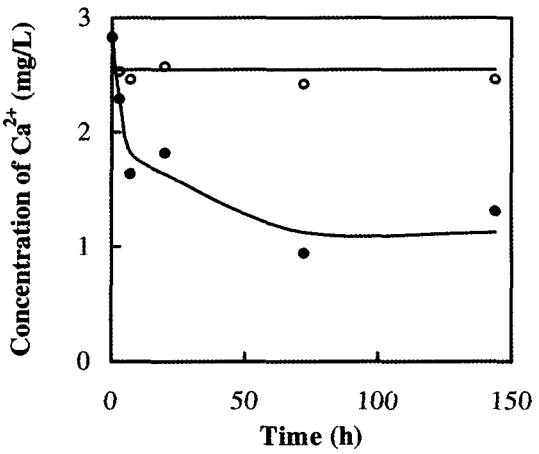

Fig. 3. Time course of the concentration of extracellular Ca^{2+} induced by 1 mM Ce^{4+} in suspension cultures of *T. cuspidate* cells. ○ Control 1 (without any additives), ● Ce^{4+}-treated culture.

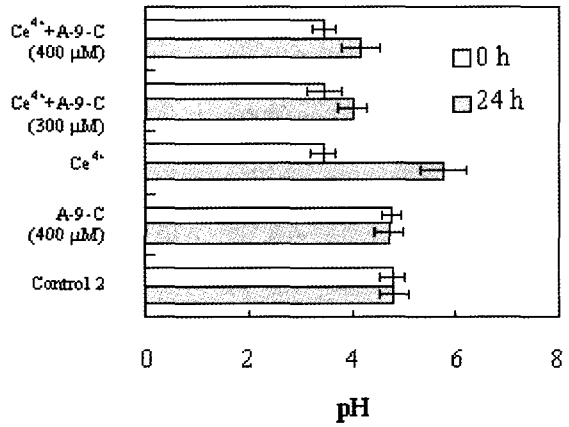

Fig. 4. Inhibition of Ce^{4+}-induced increase of extracellular pH by 300 μM Anthracene-9-carboxylate (A-9-C). Extracellular pH was detected 24 h after the addition of Ce^{4+} (1 mmol/L) in suspension cultures of *T. cuspidate* cells. A-9-C was added to the culture system 30 min prior to the addition of Ce^{4+}.

μM and 150 μM hardly affected the pH (Fig. 5). This indicates that Ce^{4+} activated the H^+ channel leading to an increase in the extracellular pH, independent of the Ca^{2+} channel activation.

It has been reported that A-9-C also inhibits elicitor-induced K^+ effluxes in parsley: *Petroselinum cripum* [10]. There also exists a plasma membrane K^+ efflux/H^+ influx exchange mechanism in tobacco [20,21]. Thus, the partial inhibition of 9-A-C to the increase of extracellular pH likely blocked the plasma membrane K^+ efflux/H^+ influx exchange.

Effects of Ion Channel Blocker on Cell Viability and Intracellular Soluble Protein Content

An intracellular pH that remains relatively constant is

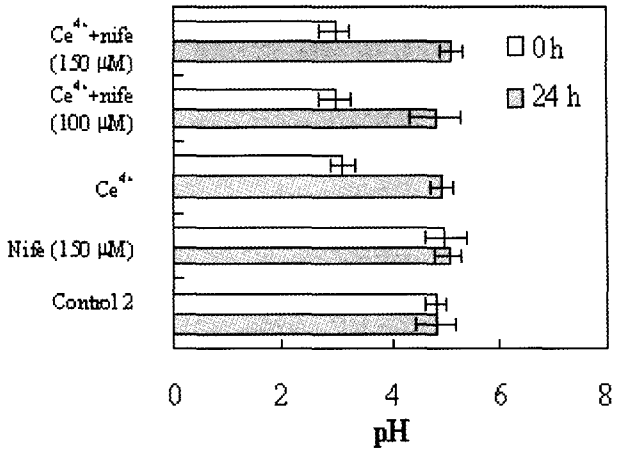

Fig. 5. Inhibition of Ce^{4+}-induced an increase of extracellular pH by nifedipine (100 μM) in suspension cultures of *T. cuspidate* cells. Extracellular pH was detected 24 h after the addition of Ce^{4+} (1 mmol/L). Nifedipine was added to the culture system 30 min before the addition of Ce^{4+}.

essential for cells to perform their physiological functions, and a slight change in pH might significantly affect cell metabolism. It was observed that the prior addition of the ion channel blocker, A-9-C, made the Ce^{4+}-containing cultures become dark-brown and the numbers of cells obviously decreased at 24 h after the addition of Ce^{4+}. In contrast, the culture system with 1 mM Ce^{4+} alone and with A-9-C alone showed a light cream color, similar to that of Control 2. The addition of A-9-C obviously decreased cell viability and the intracellular soluble protein content of the Ce^{4+}-treated cells, while the treatment with Ce^{4+} or A-9-C alone only slightly decreased cell viability and the intracellular soluble protein content (Table 1). It is thus concluded that the H^+ influx across the plasma membrane is essential for cell survival as it protects cells from the strong acidic environment caused by Ce^{4+}.

A change of pH has been regarded as a part of the biochemical response mechanism [8] and H^+ may act as a second messenger [22]. Several reports on animal cell apoptosis implicated the reduction in intracellular pH as proapoptotic signals [23-26]. As for the Ce^{4+}-induced *Taxus* suspension culture, the H^+ influx across the plasma membrane resulted in cytoplast acidification of *Taxus* cells. It has been reported that Ce^{4+} induced *Taxus* cells undergo apoptosis. It is thus presumed that the H^+ uptake induced by Ce^{4+} activated the downstream signaling events and consequently led to apoptosis.

Effects of Ca^{2+}-channel Blocker on Taxol Biosynthesis and Cell Apoptosis

Numerous reports indicated that Ca^{2+} is a critical element in elicitor-mediated signal transduction as observed in many other signaling processes [27]. The increase of cytosolic Ca^{2+} concentration can serve to transduce a particular stimulus to target proteins that guide the cellular responses. To clarify whether or not the Ca^{2+} influx across

Table 1. Effects of A-9-C on *T. cuspidate* cell viability and intracellular protein content (M ± SD, $n=3$)

	Control 2	A-9-C [d]	Ce^{4+}	Ce^{4+}+A-9-C[c]	Ce^{4+}+A-9-C[d]
Cell viability (A_{492}/ g FW)[a]	6.03 ± 0.33	5.58 ± 0.32	5.00 ± 0.26	1.61 ± 0.07	1.00 ± 0.05
Protein content (mg /g FW)[b]	9.46 ± 0.61	8.74 ± 0.52	8.25 ± 0.56	1.58 ± 0.06	0.93 ± 0.04

Anthracene-9-carboxylate (A-9-C) was introduced 30 min prior to the addition of Ce^{4+}.
[a, b] Cell viability and protein content were detected 24 h after treatment with Ce^{4+}.
[c] The concentration of A-9-C was 300 μM.
[d] The concentration of A-9-C was 400 μM.

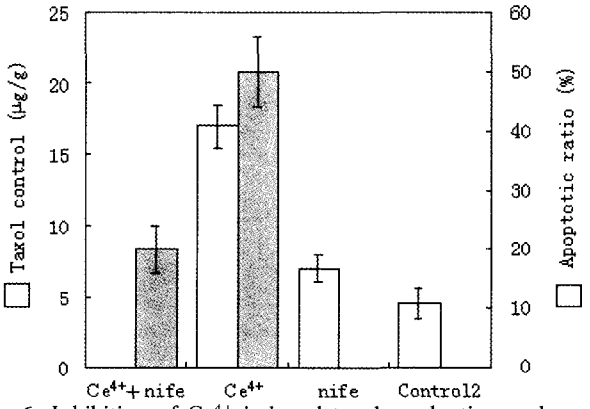

Fig. 6. Inhibition of Ce^{4+}-induced taxol production and apoptosis by nifedipine (100 μM) in suspension cultures of *T. cuspidate* cells. Nifedipine was added 30 min prior to the addition of Ce^{4+}. Taxol production was detected 48 h after the addition of Ce^{4+}.

the plasma membrane mediated 1 mM Ce^{4+}-induced taxol biosynthesis and cell apoptosis, nifedipine was introduced 30 min prior to the addition of Ce^{4+}, and the taxol contents and apoptosis ratio were detected at day 2 and day 9 after the addition of Ce^{4+}, respectively. As shown in Fig. 6, nifedipine completely blocked the taxol biosynthesis but partially inhibited the cell apoptosis induced by Ce^{4+}, indicating that the Ca^{2+} influx across the plasma membrane mediated the signal transduction pathways from Ce^{4+} to both taxol production and cell apoptosis.

CONCLUSION

The addition of 1 mM Ce^{4+} causes dynamic changes of extracellular pH and the Ca^{2+} concentration due to the activation of H^+ uptake and Ca^{2+} influx across the plasma membrane *via* ion channels. An H^+ uptake is essential for Ce^{4+}-treated cells to survive a harmful environment and is one of the mechanisms for Ce^{4+}-induced apoptosis. A Ca^{2+} influx across the plasma membrane is essential for Ce^{4+} to induce taxol biosynthesis and apoptosis.

Acknowledgement This work was supported by the National Natural Science Foundation of China (Grant No. 20176038 and 20028607).

Abbreviations A-9-C, anthracene-9-carboxylate; Nife, nifedipine; PI, propidium-iodide.

REFERENCES

[1] Hirasuna, T. J., L. J. Pestchanker, and V. Srinivasan (1996) Taxol production on suspension cultures of *Taxus baccata*. *Plant Cell Tiss. Org. Cult.* 44: 95-106.
[2] Ketchum, R. E. B., D. M. Gibson, R. B. Croteau, and M. L. Shuler (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol. Bioeng.* 62: 97-105.
[3] Yukimune, Y., H. Tabata, Y. Higashi, and Y. Hara (1996) Methyl jasmonic-induced overproduction of paclitaxel and baccatin III in *Taxus* cell suspension cultures-addition of methyl jasmonic acid as elicitor to *Taxus* baccata and *Taxus* medis suspension cell culture. *Natural Biotech.* 14: 1129-1132.
[4] Yuan, Y. J., Z. Y. Ma, Z. Q. Ge, Z. Q. Miao, and A. P. Zeng (2000) Apoptosis and taxol production in suspension cultures of *Taxus* spp. cells. *J. Chem. Ind. Eng.* 51(Suppl.): 43-46.
[5] Yuan, Y. J., G. W. Hu, and C. G. Wang (1998) Effect of La, Ce on *Taxus cuspidata* cell growth, biosynthesis and release of taxol. *J. Rare Earth* 16: 300-306.
[6] Li, J. C., Z. H. Ma, Y. J. Yuan, A. C. Sun, and C. X. Hu (2001) Dynamic effects of cerium on syntheses of soluble protein and taxol in suspension culture of *Taxus Chinensis* var. *mairei* cells. *Rare Earth* 19: 233-238.
[7] Yuan, Y. J., J. C. Li, Z. Q. Ge, and J. C. Wu (2002) Superoxide anion burst and taxol production induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*. *J. Mol. Catalysis B: Enzymatic* 18: 551-560.
[8] Serrano, R. (1989) Structure and function of plasma membrane ATPase. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 61-94.
[9] Yuan, Y. J., Z. Q. Ge, J. C. Li, J. C. Wu, and Z. D. Hu (2002) Differentiation of apoptotic and necrotic cells in suspension cultures of *Taxus cuspidata* by the combined use of fluorescent dying and histochemical staining methods. *Biotechnol. Lett.* 24: 71-76.
[10] Jabs, T., M. Tschöpe, C. Colling, K. Hahlbrock, and D.

Scheel (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexins synthesis in parsley. *Proc. Natl. Acad. Sci. USA* 94: 4800-4805.

[11] Segal, J. (1986) Lanthanum increases the rat thymocytes cytoplasmic free calcium concentration by enhancing calcium influx. *Biochim. Biophys. Acta* 886: 267-271.

[12] Powis, D. A., C. L. Clark, and K. J. O'brien (1994) Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers cathcholamine release from bovine chromaffin cells. *Cell Calcium* 16: 377-390.

[13] Gelli, A., V. J. Higgins, and E. Blumwald (1997) Activation of plasma membrane Ca^{2+}-permeable channels by race-specific fungal elicitors. *Plant Physiol.* 113: 269–279.

[14] Xu, H. and M. C. Heath (1998) Role of calcium in signal transduction during the hypersensitive response caused by basidiospore-derived infection of the *Cowpea rust* fungus. *Plant Cell* 10: 585-598.

[15] Ormerod, M. G., X. M. Sun, R. T. Snowden, and R. Davies (1993) Increased membrane permeability of apoptotic thymocytes: A flow cytometric study. *Cytometry* 14: 595-602.

[16] Wu, Z. L., Y. J. Yuan, Z. H. Ma, and Z. D. Hu (2000) Kinetics of two-liquid-phase Taxus cuspidata cell culture for production of Taxol. *Biochem. Eng. J.* 5: 137-142.

[17] Sze, H., X. Li, and M. G. Palmgren (1999) Energization of plant cell membranes by H^+-pumping ATPases: Regulation and biosynthesis. *Plant Cell* 11: 677-689.

[18] Blein, J. P., M. L. Milat, and P. Ricci (1991) Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor form *Phytophthora cryptogea*. *Plant Physiol.* 95: 486-491.

[19] Hu, G. W., J. C. Li, and Y. J. Yuan (2000) Effect of rare earth on *Taxus cuspidata* intracellular calcium concentration. *J. Tianjin Uni. (Sci. Technol.)* 33:19-20.

[20] Atkinson, M. M., L. D. Keppler, and E. W. Orlandi (1990) Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco. *Plant Physiol.* 92: 215-221.

[21] Baker, C. J., M. M. Atkinson, and A. Collmer (1987) Concurrent loss in Tn5 mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K^+/H^+ exchange in tobacco. *Phytopathology* 77: 1268-1272.

[22] Lichtenthaler, H. K. (1998) The stress concept in plants: An introduction. *Ann. N. Y. Acad. Sci.* 351: 187-198.

[23] Barry, M. A., J. E. Roynolds, and A. Eastman (1993) Etopside-induced apoptosis in human HL-60 cells is associated with intracellular acidification. *Cancer Res.* 53: 2349-2357.

[24] Chen, Q., R. S. P. Benson, A. D. Whetton, S. R. Brant, M. Donowitz, M. H. Montrose, C. Dive, and A. J. M. Watson (1997) Role of acid/base homeostasis in the suppression of apoptosis in haemopoietic cells by v-Abl protein tyrosine kinase. *J. Cell Sci.* 110: 379-387.

[25] Li, J. and A. Eastman (1995) Apoptosis in a interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. *J. Biol. Chem.* 270: 3203-3211.

[26] Rajotte, D., P. Haddad, A. Haman, E. J. J. Cragoe, and T. Hoang (1992) Role of protein kinase C and the Na^+/H^+ antiport in suppression of apoptosis by granulocyte macrophage colony-stimulating factor in interleukin-3. *J. Biol. Chem.* 267: 9980-9987.

[27] Nürnberger, T., D. Nennstiel, T. Jabs, W. R. Sacks, K. Hahlbrock, and D. Scheel (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* 78: 449-460.

[Received November 22, 2004; accepted April 13, 2005]