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Superoxide anion burst and taxol production induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*

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Abstract

Generation of reactive oxygen species (ROS) induced by Ce^{4+} in suspension cultures of *Taxus cuspidata* was investigated. The burst of superoxide anions ($O_2^{\bullet -}$) occurred rapidly after the addition of Ce^{4+} and reached maximum at 4.3 h, while the total level of the cellular reactive oxygen species maintained unchanged. The intracellular superoxide dismutase (SOD) and catalase (CAT) were activated while the intra/extracellular peroxidases (PODs) were inhibited accompanying the $O_2^{\bullet -}$ burst. The pretreatment of the suspension cultures with diphenylene iodonium (DPI), a suicide inhibitor of the NADPH oxidase, blocked the $O_2^{\bullet -}$ burst, inhibiting the cell apoptosis and taxol production induced by Ce^{4+}. These results show that NADPH oxidase played a key role in $O_2^{\bullet -}$ burst and $O_2^{\bullet -}$ served as a mediator of Ce^{4+} for cell apoptosis and taxol production. The pretreatments of the suspension cultures with anthracene-9-carboxylate, an ion-channel blocker, nifedipine, a Ca^{2+}-channel blocker, neomycin, a phospholipase C (PLC) inhibitor, or suramin, a G-protein inhibitor, decreased $O_2^{\bullet -}$ burst induced by Ce^{4+}. It is thus inferred that Ce^{4+}-induced $O_2^{\bullet -}$ burst, which mediated cell apoptosis and taxol production by activating the ion-channels, PLC, G-proteins and NADPH oxidase.

Keywords: Ce^{4+}; *Taxus cuspidata*; Superoxide anion; NADPH oxidase; Taxol; Apoptosis

1. Introduction

The suspension cultures of *Taxus* spp. have been regarded as a very promising alternative for production of taxol, an anti-neoplastic agent [1]. However, the yield of taxol is very low because of the inherent characteristics of plant cell cultures. Therefore, various elicitors have been introduced aiming at a high taxol production. Our previous studies showed that the rare earths [2] and the oligosaccharide from *Fusarium oxysprum* [3] were effective abiotic and biotic elicitors, respectively. There existed a close relationship between the oxidative burst and taxol production in suspension cultures of *Taxus chineses* var. *mairei* induced by oligosaccharide from *F. oxysprum* [3]. Many studies showed that oxidative burst could activate some defense genes, regulate the biosyntheses of some secondary metabolites and induce apoptosis in various suspension cultures of plant cells [4,5].

The transient generation of reactive oxygen species (ROS), known as oxidative burst, is a hallmark of plant defense responses to a broad range of biological and physical stimuli such as pathogen infections, osmotic shock and wounding [4–6]. The release of ROS including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) not only impacts on the attacking pathogen and host cells at the infection site to limit the spread of the pathogen, but also

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acts as a signal molecule in both animals and plants, triggering a series of cellular responses such as related gene expression and specific enzyme activation [7]. A number of studies indicated that H_2O_2 might serve as a signal molecule for activating defense genes and enhancing the biosynthesis of secondary metabolites [8]. However, there are increasing evidence that $O_2^{\bullet -}$ from oxidative burst might be another potential inducer for defense responses of plant cells [4,9,10]. For example, phytoalexin accumulation in parsley cell suspensions and lesion formation in lesion-stimulated disease resistance in *Arabidopsis* mutants were specifically induced by superoxide rather than by H_2O_2 [10].

Recently, the NADPH-dependent oxidase in the plasma membrane, which is similar to that in mammalian neutrophils, receives much attention as the main enzyme for ROS generation in plants [11]. The complex in neutrophil cells is composed of an unusual b-type cytochrome with two subunits, $p22^{phox}$ and $gp91^{phox}$ [12], utilizes NADPH as its substrate and catalyzes the one-electron reduction of oxygen to superoxide [13]. In studies of plant defense responses, diphenylene iodonium (DPI), a mammalian NADPH oxidase inhibitor, usually is used to estimate whether the NADPH oxidase is involved in the ROS generation [14]. In addition, a cell wall peroxidase [15], apoplastic amine, diamine and polyamine oxidase-type enzymes were also proposed as potential mechanisms of ROS generation [16]. ROS, however, is a double-edged sword for plant cells. To protect from the cytotoxicity of excessive ROS, cells possess an effective ROS-scavenging system, in which superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) are considered as the key enzymes. Superoxide anions ($O_2^{\bullet -}$) are readily dismuted into H_2O_2 and O_2 either spontaneously or by SOD, followed by reduction into H_2O by several cellular enzymes such as CAT and POD [17]. Nevertheless, such a mechanism alone is not adequate to explain the rapid and transient production of large amounts of ROS that takes place under the stress conditions.

Increasing evidence proved that some signal molecules mediate the oxidative burst, ensuring an elicitor-induced response that is quantitatively appropriate, accurately timed and highly coordinated with other activities of the host plant cells. These signal molecules usually include G-protein [18], Ca^{2+} [4], protein kinases/phosphatases [14] and phospholipase [19].

Ce^{4+}, which could induce an apoptosis in suspension cultures of *Taxus cuspidata* [20], has been proved to be of a higher ability in terms of enhancing taxol production in suspension cultures of *Taxus* spp. than other rare earth ions (La^{3+} and Nd^{3+}) [2]. However, there is little understanding of how the *T. cuspidata* cells responded to Ce^{4+}. As oxidative burst serves as a mediator of elicitor-induced phytoalexin production and cell apoptosis in many cell cultures [4,10,14], a new question arises of whether Ce^{4+} could induce taxol production and cell apoptosis as a result of oxidative burst in *T. cuspidata*.

In this work, the burst of superoxide anion induced by Ce^{4+} in suspension cultures of *T. cuspidata* were investigated with respect to the related enzymes and signal transduction pathway so as to provide a better understanding of the apoptosis and taxol production induced by Ce^{4+}.

2. Materials and methods

2.1. Chemicals

Diphenylene iodonium, neomycin, suramin, anthracene-9-carboxylate, nifedipine and $Ce(NH_4)_2(NO_3)_6$ were purchased from Sigma. Neomycin and $Ce(NH_4)_2(NO_3)_6$ were dissolved in sterilized distilled water and others were in DMSO before use. Control cultures were treated with equal amounts of sterilized distilled water or DMSO.

2.2. Cell line and culture conditions

The cell line from young stems of *T. cuspidata* was sub-cultured on solid B_5 medium at 25 °C in dark [21]. Cell suspensions were cultured every 8–10 days for totally five generations in 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 with 0.1 M NaOH or 0.1 M HCl. The cell cultures (100 ml) were maintained at 25 °C in dark with shaking at 110 rpm in 500 ml flasks. Fresh cells (2.5 g) from the suspension cultures of the fifth generation were inoculated into 50 ml 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 (at day 18), and the cell samples were collected at predetermined time intervals for analyses. Besides control 1 (without any additives), control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)) was set to exclude the effects of NH_4^+ and NO_3^- on culture system. All the data were the average of triplicate samples and the errors were within ±10%.

2.3. Electron spin resonance

The accumulation of free radicals including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxy ($OH^{\bullet}$) in cells was measured using an ESR spectrometer [3,22]. Cells were frozen in liquid nitrogen and lyophilized for 18 h. The dried cells were transferred to a quartz sample tube and their radicals detected. The instrument was calibrated with MnO_2. Relative signal amplitudes were calculated according to the difference between peak and trough values at $g = 2.00$.

2.4. Measurement of $O_2^{\bullet -}$

Superoxide anions were assayed following the method of Wang and Lou [23] with a slight modification. Extracellular media (cell-free) (0.5 ml) or suspension cultures (with cells) (0.5 ml) were mixed with 1 ml of 1 mM hydroxylammonium chloride and 0.5 ml of 50 mM phosphate buffer (pH 7.8) and the mixture was incubated at 25 °C for 60 min. Then 1 ml sulphanilamide (17 mM) dissolved in 30% (w/w) acetic acid and 1 ml naphthalene diamine dihydrochloride (7 mM) were added and the mixture was incubated at 25 °C for 20 min. The absorbance was measured at 530 nm using a spectrophotometer (UV-9100).

Calibration curve of OD_{530} against NO_2^- concentration was established ($r^2 = 0.9947$). The concentration of $O_2^{\bullet -}$ was calculated as twice of that of NO_2^- based on the following reaction:

$$2O_2^{\bullet -} + H^+ + NH_2OH \rightarrow H_2O_2 + H_2O + NO_2^-.$$

2.5. Extraction of related enzymes and activity assay

For enzyme extracts, 250 mg (DW) of cells was ground with 2 ml pre-cooled 0.1 M phosphate buffer (pH 6.0) containing 0.1 mM EDTA, 0.3% (w/v) Triton X-100, 4% (w/v) polyvinylpolypyrrolidone for SOD assay or containing 2 mM EDTA, 4 mM dithiothreitol and 2% (w/v) polyvinylpyrrolidone for assay of others. The mixture was homogenized at 4 °C and centrifuged at 10,000 × g for 25 min. The supernatant was collected for enzyme assay.

Peroxidase was assayed using guaiacol as a substrate at 470 nm, and catalase was assayed using H_2O_2 as a substrate at 240 nm [24]. Superoxide dismutase was assayed following the method of Giammoplitis and Ries [25]. Proteins were determined according to the Bradford method with bovine serum albumin as a standard [26].

2.6. Hoechst 33342–propidium iodide co-staining

One milliliter of cell suspensions was centrifuged at 1100 × 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 aggregation. The cells were re-suspended in 0.2 ml of 0.1 M phosphate buffer of pH 5.8 for staining with fluorescence dyes, Hoechst 33342 and propidium iodide following the method of Ormerod et al. [27]. For comparison, part of the cells were killed by heating to 60 °C for 15 min and stained according to the same procedures. The stained cells were collected by centrifugation, washed with 10 ml of 0.1 M phosphate buffer of pH 5.8 and re-suspended 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.

2.7. Extraction of taxol and analysis by HPLC

Taxol extraction and analysis was performed as previously described by Wu et al. [21].

3. Results

3.1. Burst of $O_2^{\bullet -}$ induced by Ce^{4+}

Fig. 1A is the time courses of the amount of $O_2^{\bullet -}$ in the suspension cultures (with cells) with and without the addition of $Ce(NH_4)_2(NO_3)_6$. In order to exclude

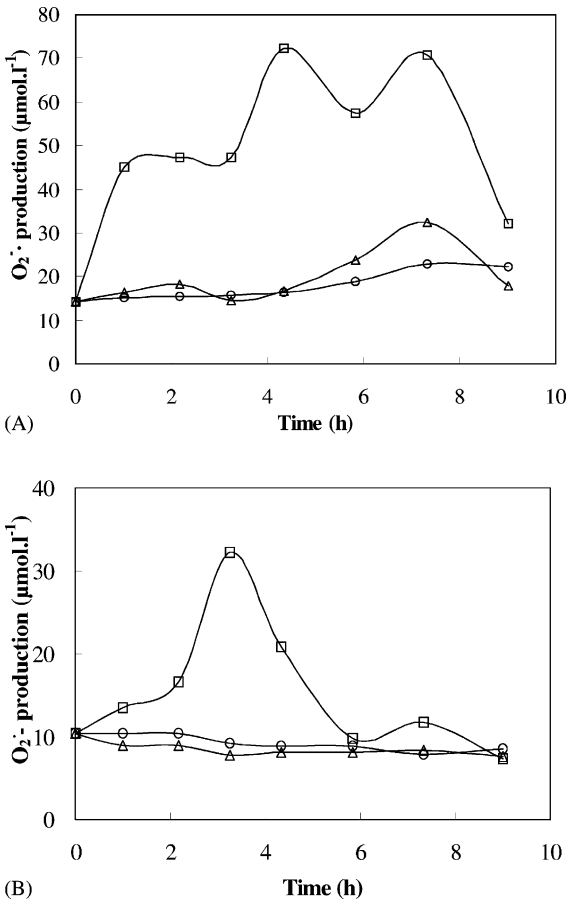

Fig. 1. Time course of $O_2^{\bullet-}$ burst in suspension cultures (A) and extracellular medium (B) induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*. $Ce(NH_4)_2(NO_3)_6$ (1 mmol/l) was added at day 18 of the cultivation: (○) control 1 (without any additives); (△) control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)); (□) Ce^{4+}-treated cultures.

the effects of NH_4^+ and NO_3^-, the cultures with equal amounts of NH_4^+ and NO_3^- but without Ce^{4+} were also investigated. From Fig. 1A it is seen that the $O_2^{\bullet-}$ levels in the two cultures without Ce^{4+} were almost the same and independent of time, indicating that NH_4^+ and NO_3^- did not affect the accumulation of $O_2^{\bullet-}$. However, in the culture system containing Ce^{4+}, the amount of $O_2^{\bullet-}$ continuously increased with time and reached a maximum after 4.3 h, which was 4.2-fold higher than that of the control cultures, then returned to the same levels with those in the two control cultures at ca. 9 h.

Fig. 1B is the time courses of the amount of $O_2^{\bullet-}$ from extracellular medium (cell-free). It is seen that the accumulation of $O_2^{\bullet-}$ in the extracellular medium began to increase after the addition of $Ce(NH_4)_2(NO_3)_6$ and reached a maximum at about 3 h, then decreased to control levels at approximately 4 h. However, the amounts of $O_2^{\bullet-}$ in the two control cultures almost maintained unchanged with time.

The total amounts of cellular ROS including $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ were monitored by ESR. Fig. 2 shows that no significant difference in signal intensities between Ce^{4+}-treated and control cells was observed within 10 h (Fig. 2A), indicating that the total amount of cellular ROS was not affected by the presence of Ce^{4+}.

3.2. Effects of Ce^{4+} on activities of SOD, POD and CAT

The activities of the protective enzymes including superoxide dismutase, catalase and peroxidases changed on addition of $Ce(NH_4)_2(NO_3)_6$ (Figs. 3–5). Intracellular CAT was activated immediately after addition of Ce^{4+} and its activity reached a maximum at about 3 h. Intracellular SOD was activated at 1 h but maintained at the higher activity only for a short time then returned to the control level. Both intra- and extracellular POD were strongly inhibited by the addition of Ce^{4+}. However, the activities of intracellular SOD, CAT and intra/extracellular POD between the two control cultures had no obvious differences, which is in consistence with the less change in $O_2^{\bullet-}$. This result further confirms the presumption that the effect of $Ce(NH_4)_2(NO_3)_6$ on the redox system of cells was resulted from Ce^{4+}, not from NH_4^+ and NO_3^-. It thus seems that the changes in activities of scavenging enzymes of ROS were partially responsible for the $O_2^{\bullet-}$ burst and maintenance of the total ROS in Ce^{4+}-treated system.

3.3. Effect of NADPH oxidase blocker on $O_2^{\bullet-}$ burst induced by Ce^{4+}

It has been reported that NADPH oxidase is the main enzyme responsible for ROS generation in plants [11]. To assess the action of NADPH oxidase in our

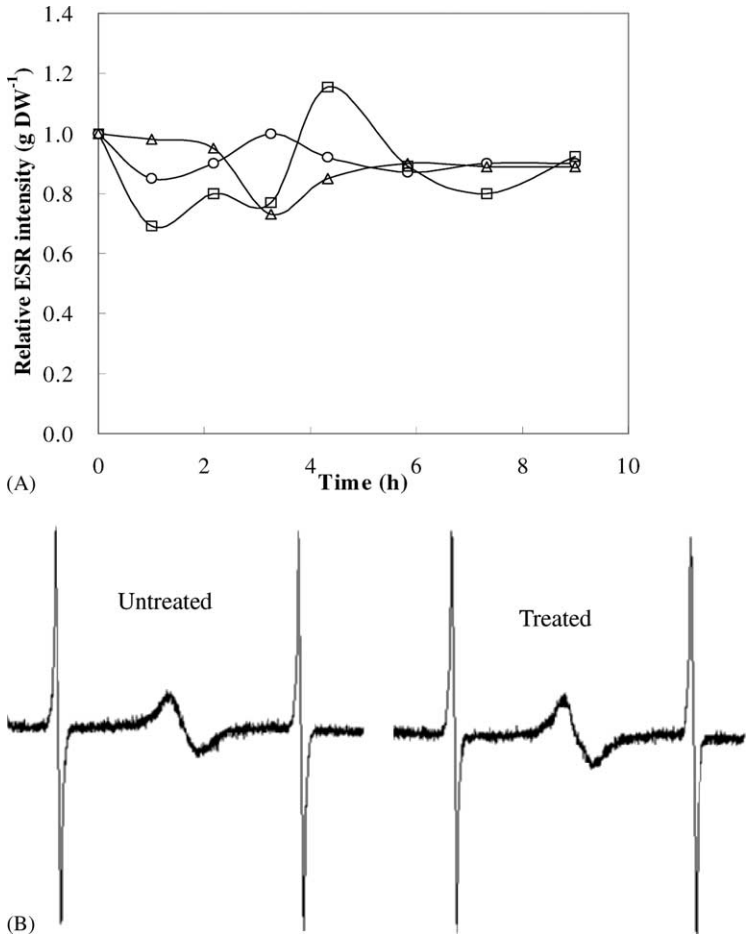

Fig. 2. Time course of ROS in suspension cultures of *Taxus cuspidata*. (A) *Taxus* suspension cultures were incubated in presence or absence of Ce^{4+} for 9 h: (○) control 1 (without any additives); (△) control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)); (□) Ce^{4+}-treated cultures. (B) ESR signal intensity from *Taxus* suspension cells induced by Ce^{4+} at 4 h was between two MnO_2 signals as standard ROS as measured by ESR.

system, DPI, a suicide inhibitor of NADPH oxidase, was introduced in the suspension cultures at 30 min prior to the addition of Ce^{4+} and the amount of $O_2^{\bullet -}$ in suspension cultures was measured at 3 and 6 h after addition of Ce^{4+}. Fig. 6 shows that the $O_2^{\bullet -}$ burst in the suspension cultures induced by Ce^{4+} was almost completely blocked by 5 μM DPI, while DPI alone (without Ce^{4+}) did not affect the $O_2^{\bullet -}$ level. These results indicate that Ce^{4+} activated NADPH oxidase, which was mainly responsible for the generation of $O_2^{\bullet -}$ in Ce^{4+}-treated system.

3.4. Effects of the inhibitors of ion-channels, phospholipase C and G-proteins on $O_2^{\bullet -}$ burst

The signal transduction pathway of $O_2^{\bullet -}$ burst induced by Ce^{4+} was investigated by introducing various pharmacological inhibitors into the culture system respectively at 30 min before the addition of Ce^{4+}. The $O_2^{\bullet -}$ in suspension cultures was detected at 3 h after the addition of Ce^{4+}.

As shown in Fig. 7, the $O_2^{\bullet -}$ burst was partially inhibited by the addition of nifedipine, a

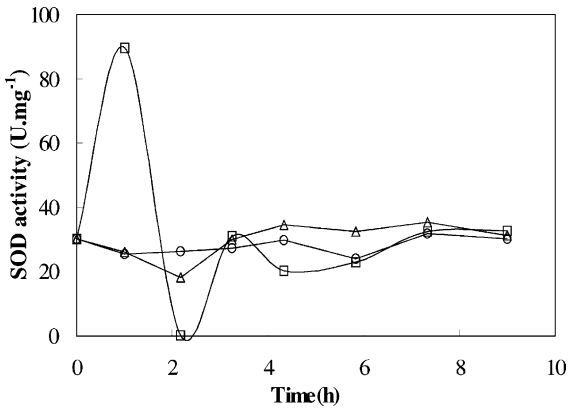

Fig. 3. Time course of intracellular superoxide dismutase (SOD) activity induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*. One enzyme unit (U) is defined as 50% inhibition of the colorimetric reaction. The SOD activity is expressed as the enzyme units contained per milligram of intracellular proteins: (○) control 1 (without any additives); (△) control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)); (□) Ce^{4+}-treated cultures.

calcium-channel blocker, neomycin, a phospholipase C (PLC) inhibitor, or suramin, a G-protein inhibitor. However, no $O_2^{\bullet -}$ burst was observed in the systems after the introduction of anthracene-9-carboxylate, an ion-channel blocker. In order to test whether the

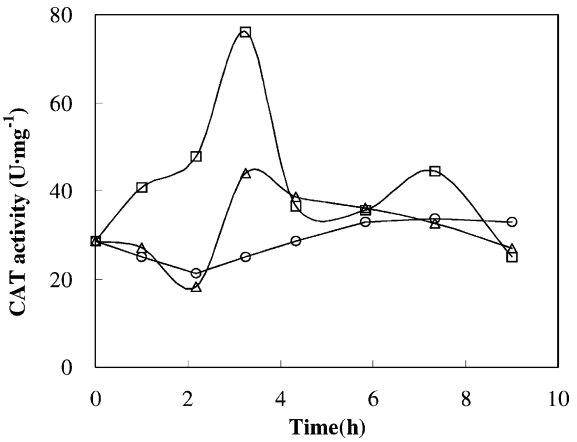

Fig. 4. Time course of intracellular catalase (CAT) activity induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*. One enzyme unit (U) is defined as the variation of absorbance of 0.01 min^{-1}. CAT activity was expressed as the enzyme units contained per milligram of intracellular proteins: (○) control 1 (without any additives); (△) control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)); (□) Ce^{4+}-treated cultures.

$O_2^{\bullet -}$ burst was blocked due to the inhibition of the additives to the normal cells, we measured the $O_2^{\bullet -}$ burst in the presence of inhibitors but without Ce^{4+} (Fig. 7). It is seen that the contents of $O_2^{\bullet -}$ in the inhibitors-containing systems maintained at the same levels with those in the two control cultures. Therefore, the $O_2^{\bullet -}$ burst was generated by the activation of NADPH oxidase that was mediated by ion fluxes, G-proteins and PLC.

3.5. Effects of DPI on cell apoptosis and taxol production

DPI was introduced into the culture systems at 30 min before the addition of Ce^{4+} and the cells at day 9 after addition of Ce^{4+} were stained and observed under a fluorescence microscopy. It is seen that ca. 20% of the total cells were induced by Ce^{4+} to undergo an apoptosis, which was hardly observed in the two control cultures. However, the pretreatment of the culture system with DPI effectively prevented the cells from apoptosis (data not shown). The addition of DPI also inhibited the taxol production (Fig. 8). It thus seems that NADPH oxidase participated in the apoptosis and taxol production by generation of $O_2^{\bullet -}$.

4. Discussion

4.1. Burst of $O_2^{\bullet -}$ induced by Ce^{4+}

The generation of $O_2^{\bullet -}$ has been identified in a diverse array of plant–pathogen interactions [5], even in heavy metal ions and wounding-induced systems [28]. Generally, there is a biphasic oxidative burst in response to an avirulent microbial pathogen. The massive burst of $O_2^{\bullet -}$ in phase II, usually appearing at 2–6 h after challenge, only occurs as an response to avirulent pathogen, while the weak burst of $O_2^{\bullet -}$ in phase I, occurring within 1 h, is a non-specific response to both avirulent and virulent pathogen [14]. In our case, the burst of $O_2^{\bullet -}$ began rapidly after the addition of Ce^{4+} into the culture systems and lasted for ca. 9 h (Fig. 1A). It is likely that Ce^{4+} induced the $O_2^{\bullet -}$ burst in a manner similar to an avirulent pathogen. Recent evidences have shown that the NADPH oxidase is one of the most important enzymes for $O_2^{\bullet -}$ generation in addition to the cell wall

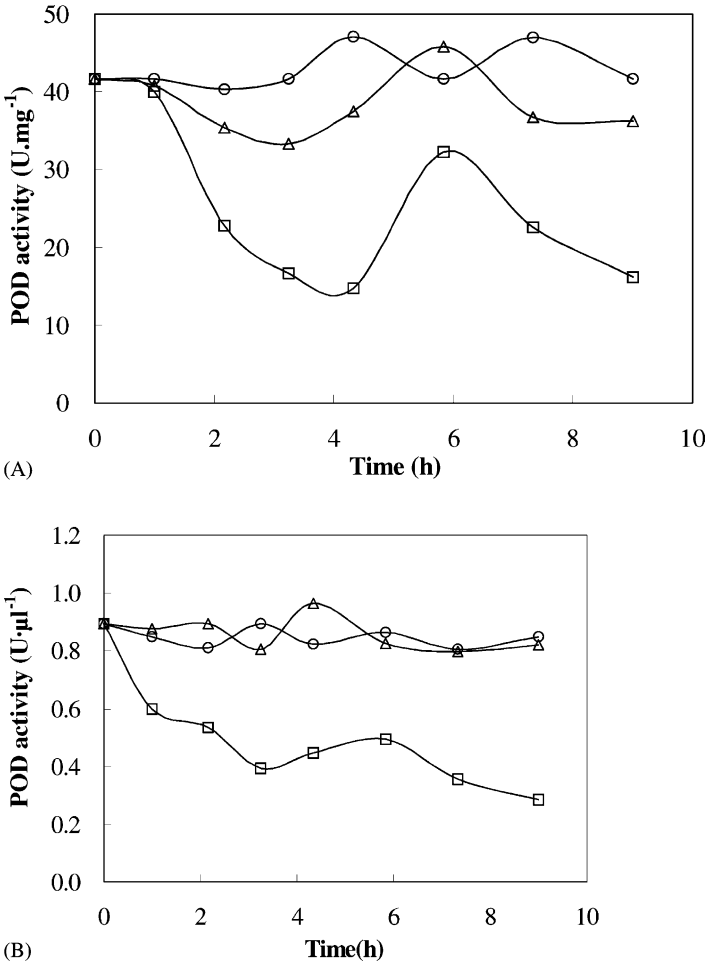

Fig. 5. Time course of intracellular (A) and extracellular (B) peroxidase (POD) activity induced by Ce^{4+} in suspension cultures of *Taxus cuspidata*. One enzyme unit (U) is defined as the variation of absorbance of $0.01\,s^{-1}$. Intra/extracellular POD activity was expressed per milligram of intracellular proteins or as the enzyme units contained per microliter of extracellular medium: (○) control 1 (without any additives); (△) control 2 (with addition of NH_4NO_3 (2 mmol/l) and $NaNO_3$ (4 mmol/l)); (□) Ce^{4+}-treated cultures.

peroxidase and the oxidases of apoplastic amine, diamine and polyamine [11]. DPI, a potent inhibitor of NADPH oxidase, is often used as a pharmacological agent to judge whether NADPH oxidase is responsible for the oxidative burst [14]. DPI ($IC_{50} = 2\,\mu M$) in the concentration range of 1–100 μM has been shown to block the elicitor-induced oxidative burst in many plant cell cultures [5,14]. Our experiments showed that DPI (5 μM) blocked the $O_2^{\bullet -}$ burst (Fig. 6A), suggesting that Ce^{4+} induce $O_2^{\bullet -}$ burst through a NADPH oxidase-dependent pathway.

It has been known that NADPH oxidase transfers electrons from intracellular NADPH to extracellular molecular oxygen [13]. The generated $O_2^{\bullet -}$ is unable to penetrate through the biological membrane due to its negative charge. Thus, it is presumed that the $O_2^{\bullet -}$ produced from $O_2^{\bullet -}$ burst mainly existed outside protoplasts and a small part of $O_2^{\bullet -}$ released into the extracellular medium. Therefore, the $O_2^{\bullet -}$ burst was also observed in the extracellular medium (Fig. 1B). The $O_2^{\bullet -}$ burst in extracellular medium lasted a shorter time compared with that in the suspension

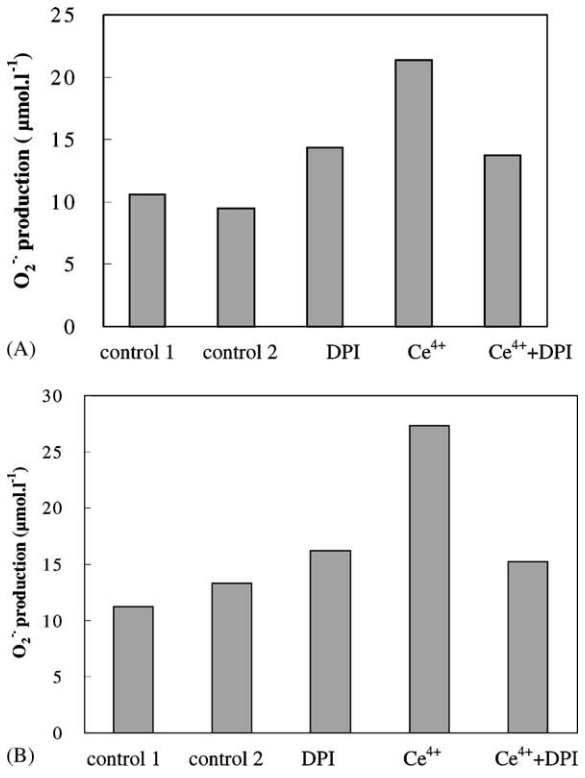

Fig. 6. Inhibition of Ce^{4+}-induced $O_2^{\bullet-}$ burst by DPI (5 μM). $O_2^{\bullet-}$ in suspension cultures was detected at 3 h (A), and 6 h (B) after the addition of Ce^{4+} (1 mmol/l). DPI was added to the culture system at 30 min before the addition of Ce^{4+}.

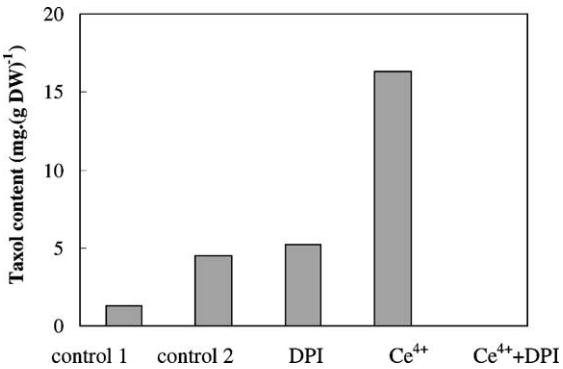

Fig. 8. Inhibition of Ce^{4+}-induced taxol production by DPI (5 μM). The taxol production was determined at 48 h after addition of Ce^{4+}, respectively. DPI was added at 30 min prior to the addition of Ce^{4+}.

cultures (Fig. 1A). This phenomenon might be explained on account of the activation of the intracellular mechanisms of ROS generation such as mitochondria, chloroplasts and peroxisome, leading to the increase of intracellular $O_2^{\bullet-}$ and the subsequent increase of $O_2^{\bullet-}$ in suspension cultures. However, it seems that the activation of the intracellular mechanisms of ROS generation was dependent on $O_2^{\bullet-}$ originated from NADPH oxidase, as the pretreatment with DPI could completely inhibit the $O_2^{\bullet-}$ burst occurring at 6 h (Fig. 6B).

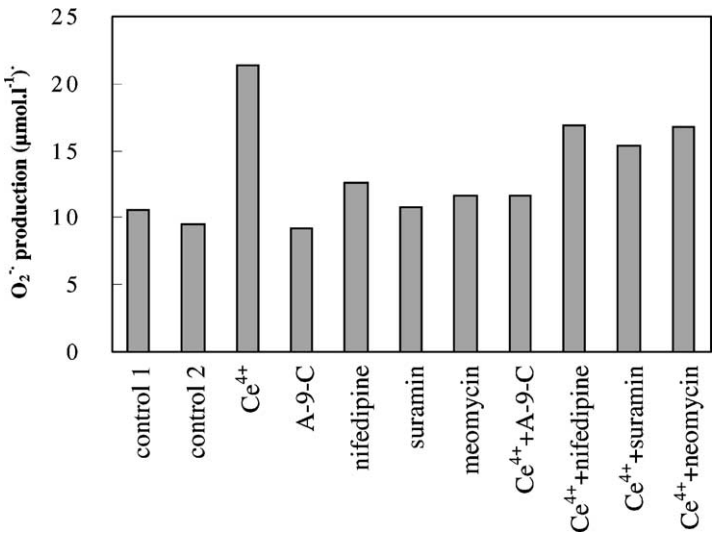

Fig. 7. Effects of anthracene-9-carboxylate (300 μM), nifedipine (100 μM), neomycin (100 μM) or suramin (50 μM) on $O_2^{\bullet-}$ burst. The additives were introduced at 30 min prior to the addition of Ce^{4+}. The $O_2^{\bullet-}$ in suspension cultures was detected at 3 h after addition of Ce^{4+}.

It is interesting to note that in the presence of Ce^{4+} the total cellular ROS ($O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$) monitored by ESR was almost unchanged during the $O_2^{\bullet-}$ burst (Fig. 2A and B). So the amounts of H_2O_2 or $OH^{\bullet}$ should decrease. This might be ascribed to the influence of Ce^{4+} on the ROS-scavenging system. ROS damages biological macromolecules and intracellular structures at high concentrations, although it plays a key role in signal transduction by regulating some of the most important biological processes at relatively low concentrations, so cells possess an efficient ROS-scavenging system [17]. SOD, an enzyme responsible for scavenging excess $O_2^{\bullet-}$, was activated only at 1 h and thus unable to cope with the rapid $O_2^{\bullet-}$ generation, leading to $O_2^{\bullet-}$ burst in suspension cultures. On the other hand, although the intra/extracellular POD were strongly inhibited, the CAT with the same function as POD was activated once the Ce^{4+} was introduced and the activation lasted for ca. 5 h, implying that H_2O_2 did not accumulate in cells and the total cellular ROS maintained unchanged.

4.2. Signal transduction pathway involved in $O_2^{\bullet-}$ burst induced by Ce^{4+}

As NADPH oxidase was proved to be the main enzyme responsible for $O_2^{\bullet-}$ generation [11], the signal molecules relevant to NADPH oxidase should be considered in investigating the signal transduction pathway of $O_2^{\bullet-}$ generation induced by Ce^{4+}. Many studies over the past years have shown that the pharmacological agents known to affect signal transduction in mammalian cells influences the defense responses of cultured plant cells [14]. Ion fluxes, calcium uptake [4], G-proteins [18], kinase cascades [14] and polyphosphoinositides [19] have been shown to participate in signal transduction inducing phytoalexins and oxidative burst.

The activation of ion-channels is regarded as one of the earliest events of cells in response to elicitors [18]. The alkalinization of extracellular medium (data not shown) and the experiment with nifedipine, respectively, showed the existences of H^+ influx and Ca^{2+} influx across plasma membrane. The complete block of anthrecene-9-carboxylate, an ion-channel blocker, to the burst of $O_2^{\bullet-}$ indicates that the opening of ion-channels played an important role in $O_2^{\bullet-}$ generation. Nifedipine, a blocker of Ca^{2+}-channel, however, only partially inhibited the $O_2^{\bullet-}$ burst, suggesting that the Ca^{2+} influx be essential but insufficient for the activation of NADPH oxidase. The increase of cytosolic Ca^{2+}, a critical factor for initiating downstream signals, originates not only from the influx of extracellular medium, but also from the release from vacuoles [29]. It is presumed that the release of Ca^{2+} from intracellular calcium storage was regulated by other ions entering into cells through ion-channels as is supported by the complete inhibition of $O_2^{\bullet-}$ burst by anthrecene-9-carboxylate. The addition of the inhibitors of PLC and G-proteins and even Ca^{2+}-channel blocker did not inhibit the pH increase in the culture systems induced by Ce^{4+} (data not shown), demonstrating that the H^+ influx occurred prior to the activation of Ca^{2+}-channel, PLC and G-proteins. Thus, H^+ influx led to the acidification of cytoplasm, which might be beneficial to the opening of ion-channels in membrane of vacuolus, endoplasmic reticulum and mitochondria, causing the release of Ca^{2+} from the calcium storage to cytoplasm.

It has been reported that heterotrimeric G-proteins function downstream of elicitor reception in the induction of the oxygen burst [18]. Suramin, which interferes with the GTP binding site of the alpha submit of G-proteins [29], completely inhibited the $O_2^{\bullet-}$ burst (Fig. 7), indicating the importance of G-proteins for Ce^{4+}-induced $O_2^{\bullet-}$ burst. Neomycin, a PLC inhibitor, also inhibited the $O_2^{\bullet-}$ burst, showing that PLC served as a mediator of $O_2^{\bullet-}$ burst. The activation of PLC would lead to the release of inositol phosphates and diacylglycerol as the second messengers [30], inducing different biochemical pathways in the elicitor responses. A role of inositol 1,4,5-triphosphate produced by phospholipase C in engagement of plant oxidative burst has been proposed by Legendre et al. [19].

These results are in line with those obtained by Rajasekhar et al. in soybean (*Glycine Max*) cv Williams 82 suspension cultures induced by *Pseudomonas Syrinage* pv *glucinea* (*psg*), where ion-channels, PLC and G-proteins all mediated the signal transduction of oxidative burst and the activation of ion-channels preceded the other signal events [18].

In our case, it is inferred that the activation of H^+-channel was followed by an upstream Ca^{2+}-channel, G-proteins and PLC before the NADPH oxidase-dependent step in the signal transduction pathway leading to the $O_2^{\bullet-}$ burst.

4.3. The key role of $O_2^{\bullet -}$ burst in taxol production and cell apoptosis

The cellular-signaling process regulates the cellular redox state, but the latter in turn affects the former, leading to pathogen accumulation and cell apoptosis. It has been shown that Ce^{4+} could induce taxol production [2] and cell apoptosis [20] in suspension cultures of *T. cuspidata*. The complete block of taxol production (Fig. 8) and cell apoptosis in the system pretreated with DPI might indicate that $O_2^{\bullet -}$ took part in mediation of the previous two processes. Many studies showed the key role of H_2O_2 from oxidative burst in orchestration of apoptosis and secondary metabolisms [8]. Recently, it was reported that phytoalexin accumulation in parsley cell suspensions [4], the lesion formation in lesion-stimulated disease resistance in *Arabidopsis* mutants [10] and the initiation of the death-signaling pathway of *Arabidopsis* mutants [9] were specifically induced by superoxide not by H_2O_2, indicating that $O_2^{\bullet -}$ from oxidative burst could induce apoptosis and second metabolism. In our case, the level of total intracellular ROS almost maintained unchanged, while the amounts of both total and extracellular $O_2^{\bullet -}$ increased in the presence of Ce^{4+}. Therefore, it is thought that H_2O_2 might not accumulate in our system, and the Ce^{4+}-induced apoptosis and taxol synthesis was mediated by $O_2^{\bullet -}$ rather than by H_2O_2.

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