Available online at www.sciencedirect.com
SCIENCE DIRECT®
Nitric Oxide 11 (2004) 298–306
NITRIC OXIDE Biology and Chemistry
www.elsevier.com/locate/yniox

Involvement of nitric oxide in elicitor-induced defense responses and secondary metabolism of *Taxus chinensis* cells

Jian Wen Wang, Jian Yong Wu*

Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

Received 21 June 2004; revised 23 September 2004
Available online 19 November 2004

Abstract

This work was to characterize the generation of nitric oxide (NO) in *Taxus chinensis* cells induced by a fungal elicitor extracted from *Fusarium oxysporum* mycelium and the signal role of NO in the elicitation of plant defense responses and secondary metabolite accumulation. The fungal elicitor at 10–100 μg/ml (carbohydrate equivalent) induced a rapid and dose-dependent NO production in the *Taxus* cell culture, which exhibited a biphasic time course, reaching the first plateau within 1 h and the second within 12 h of elicitor treatment. The NO donor sodium nitroprusside potentiated elicitor-induced H_2O_2 production and cell death but had little influence on elicitor-induced membrane K^+ efflux and H^+ influx (medium alkalinization). NO inhibitors N^{ω}-nitro-L-arginine and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide partially blocked the elicitor-induced H_2O_2 production and membrane ion fluxes. Moreover, the NO inhibitors suppressed elicitor-induced activation of phenylalanine ammonium-lyase and accumulation of diterpenoid taxanes (paclitaxel and baccatin III). These results suggest that NO plays a signal role in the elicitor-induced responses and secondary metabolism activities in the *Taxus* cells.

Keywords: Nitric oxide; Fungal elicitor; *Taxus* cells; Oxidative burst; K^+/H^+ fluxes; Paclitaxel

Nitrogen monoxide or nitric oxide (NO) is a free radical gas formed endogenously in many biological systems and performs a wide range of biological activities. The physiological role of NO has been extensively characterized in mammalian systems where NO plays crucial roles in many cellular and organ functions in the nervous, cardiac, vascular, and immune systems, and acts as an intracellular and intercellular signal molecule in several pivotal regulatory pathways [1]. Although the formation and functions of NO in plants have been less widely characterized so far, several recent studies have suggested its involvement in the regulation of plant growth, development, and defense responses [2]. NO has been shown to affect photomorphogenesis, leaf expansion, root growth, senescence, and phytoalexin production [3,4]. As in the mammalian systems, NO in plants can have contrasting or even opposite effects, both promotional and inhibitory on growth and development, depending on the dosage, and the cellular and environmental conditions.

Because of its chemical identity as a reactive chemical species, most previous studies on NO action in plant cells have been directed at the defense responses mediated or caused by reactive oxygen species (ROS), such as the hypersensitive response (HR) or programmed cell death and phytoalexin biosynthesis. NO has been shown to induce or potentiate some of the responses which are mediated by ROS signal, such as defense-gene activation in tobacco cells [5] and the HR in *Arabidopsis* leaves [6], and phytoalexin biosynthesis in potato cells [7] and soybean cells [8]. According to Delledonne et al. [6], ROS alone is not always sufficient to mediate a strong disease-resistance response in

* Corresponding author. Fax: +852 23649932.
E-mail address: bcjywu@polyu.edu.hk (J.Y. Wu).

1089-8603/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.niox.2004.10.003

plants, and its combination with NO can act synergistically to activate a stronger response. These findings suggest the existence of NO-mediated signaling pathway in the elicitation of plant defense responses and secondary metabolite biosynthesis.

The increases in membrane ion fluxes, Ca^{2+} influx, K^+ efflux, and H^+ influx, represent another class of early events during the elicitation of plant defense responses. The Ca^{2+} influx has been found essential for the activation of the K^+ efflux/H^+ influx and many later responses, HR, defense-gene expression and phytoalexin production in plant cells [9–11]. In addition, both ROS and NO production in plant tissue in response to elicitors and microbial pathogens have been shown to depend on membrane Ca^{2+} flux [6,12]. However, little is known about the involvement of NO in the induction of K^+ efflux/H^+ influx during elicitation of plant cells.

Plant secondary metabolites are the main defense elements of plants against pathogen and herbivore attacks, and physical stress such as UV radiation. Phytoalexins, for example, are antimicrobial secondary metabolites which accumulate in plants in response to microbial infection. On the other hand, plant secondary metabolites constitute the most important class of natural products with diverse and valuable chemical properties and biological activities. Paclitaxel (Taxol, Bristol-Myers Squibb, New Brunswick, NJ), a diterpenoid secondary metabolite in various *Taxus* (yew) species, is an excellent anticancer drug which has been widely used in the treatment of breast, ovarian, and lung cancers as well as AIDS-related Kaposi's sarcoma [13]. Because of the shortage and slow-growth of yew trees in nature, Taxol supply has to resort to alternative sources rather than the natural plants. Plant cell culture of *Taxus* spp. has become a major source of Taxol and related taxanes [14]. As a secondary metabolite, Taxol production in plant tissue and cell cultures can be stimulated by biotic and abiotic elicitors such as fungus fragments and methyl jasmonate.

ROS production has been identified as an early and signal event in the elicitation of Taxol production in *Taxus* cell cultures by biotic and abiotic means [15,16]. In addition, NO has been found to induce irreversible DNA fragmentation and cell death in *Taxus brevifolia* haploid cultures [17]. However, there is still no published work on the relationship between NO and the oxidative burst, the membrane ion fluxes and the secondary metabolite biosynthesis in the *Taxus* species. This work was carried out to examine the elicitor-induced NO production and its relationship with the other elicitor responses, H_2O_2 production, membrane ion fluxes (K^+ efflux/H^+ influx) and the activation of phenylalanine ammonium-lyase (PAL) and Taxol production in *Taxus chinensis* cell cultures. The elicitor was extracted from the mycelium of a *Fusarium oxysporum* fungus, and the effect and involvement of NO in the elicitation process were detected with the aid of an exogenous NO donor and specific NO inhibitors.

Experimental procedures

Plant cell culture

A *T. chinensis* cell line induced from the young stems of a *T. chinensis* tree was used in this study. The cell line was routinely maintained in callus culture on MS medium and subcultured once every month. Cell suspension culture was maintained on liquid MS medium in 125-ml Erlenmeyer flasks on an orbital shaker at 110–120 rpm and 25 ± 1 °C in dark (referred to as shake-flask culture). Each of the culture flasks was filled with 25 ml medium and inoculated with 3.0 g fresh weight of cells from the callus culture. More details of the culture medium and conditions have been given elsewhere [16]. The experiments for studying the elicitor responses of *T. chinensis* cells were all conducted in, or with cells collected from the shake-flask culture. The fresh weight (FW) of cells in the suspension culture was obtained by filtration through a Whatman filter paper under vacuum, and the dry weight (DW) by drying the fresh cell mass at 50 °C in an oven until constant weight.

Elicitor preparation

The fungal elicitor was extracted from the mycelium of a root pathogenic fungus, *Fusarium oxysporum* f. sp. *niveum* as described by Yu et al. [18]. The mycelium was harvested from liquid culture in potato dextrose broth at 37 °C and first extracted with ethyl acetate at room temperature for 24 h to remove lipids. After the removal of liquid by filtration, the remaining solid was resuspended in deionized water, which was adjusted to pH 2 with 1 M HCl, and then autoclaved at 121 °C for 2 h. The suspension was filtered and the filtrate collected, and adjusted to pH 5.8 with 0.5 M NaOH, yielding the fungal elicitor solution. The total carbohydrate content of the elicitor solution was determined by the phenol–sulfuric acid method using glucose as a standard [19].

Elicitation experiments

Fungal elicitor was applied to the shake-flask culture on day 12 post inoculation at selected doses (carbohydrate concentrations). Sodium nitroprusside (SNP, Cat. 30190, BDH Chemicals, Poole, England) was used as an NO donor, N^{ω}-nitro-L-arginine (L-NNA, NO synthase inhibitor, Cat. N-5501, Sigma, St. Louis, MO) as an NO inhibitor, and 2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO, Cat. P-5084, Sigma) as an NO scavenger in the experiments. These NO reagents

and their dosage used in the experiments were chosen based on previous studies [6,17]. They were all predissolved in distilled water at 50–100 times of the final concentrations in the culture and sterilized by filtration. Both L-NNA (100 μM) and PTIO (100 μM) were added to the culture at 30 min prior to, and SNP (10 μM) simultaneously with the fungal elicitor.

All the following elicitor responses were measured in the shake-flask cultures except the NO production described below. All treatments were performed in triplicate and the results represented by their mean ± standard error (SE).

Measurement of elicitor-induced NO production

NO concentration was quantified by binding DAF-2 DA in a fluorometric assay [20,21]. Each 100 mg FW of cells from the shake-flask culture was transferred into 0.95 ml culture medium containing 10 μM DAF-2 DA (4,5-diaminofluorescein diacetate, Cat. D-225, Sigma) in a 2-ml microcentrifuge tube and incubated for 2 h before the elicitor and NO treatments as described above. Following the treatments, the tubes were harvested at selected time intervals and the culture supernatant was collected by filtration. In each case, 20 μl of the supernatant was diluted with distilled water at 1:50 (v/v) and then measured of the fluorescence intensity using a luminescence spectrophotometer (LS50B, Perkin–Elmer, Shelton, CT) at 495 nm excitation and 515 nm emission. The relative fluorescence value shown in the results is the ratio of fluorescence intensity at a given time to that at 0 min.

NO production in the cells was also observed by fluorescence microscopy on a Leica DMBR fluorescence microscope mounted with an I_3 filter, at 470 nm excitation and 515 nm emission.

Quantification of H_2O_2

H_2O_2 released into the culture medium was quantified by luminol chemiluminescence [22]. Briefly, 50 μl of the sample medium was added to 750 μl phosphate buffer (50 mM potassium phosphate, pH 7.9) prior to automated injection of 200 μl luminol (0.3 mM in phosphate buffer) and 100 μl $K_3[Fe(CN)_6]$ (14 mM in H_2O) by a TD-20/20 luminometer (Turner Designs, Sunny Vale, CA). The luminescence was recorded after the last injection at an integration time of 5 s and the intensity was converted to H_2O_2 concentration by calibration with pure H_2O_2 liquid.

Determination of cell viability

Cell viability was determined by Evans blue assay as described by Levine et al. [23]. In brief, the cells to be assayed were incubated in liquid MS medium containing 0.25% Evans blue for 5 min and then washed extensively with distilled water to remove excessive and unbound dye. The dye bound to dead cells was solubilized with 0.5% sodium dodecyl sulfate for 3 min in a boiling water bath and quantified by measuring the absorbance at 600 nm. The percent viability of a sample was determined based on the absorbance of intact healthy cells (100%) and that of dead cells prepared by freezing and thawing the cells (0).

Measurement of membrane K^+ and H^+ ion fluxes

Cells collected from the shake-flask culture were rinsed with an assay buffer (0.5 mM Mes adjusted to pH 6.0 with Tris, 0.175 M mannitol, 0.5 mM K_2SO_4, and 0.5 mM $CaCl_2$), and resuspended in the assay buffer (0.3 g FW/3 ml). The cells were incubated for 2 h in the shake-flask culture before the treatment by elicitor and NO reagents. H^+ flux in the cells was detected by measuring the change in the extracellular medium pH with an Orion 720 pH meter (Orion Research, Boston, MA), and K^+ flux by measuring the change in K^+ concentration in the medium with a Perkin–Elmer Analyst 100 atomic absorption spectrometer (Shelton, CT) using KCl as a standard.

Intracellular PAL assay

Phenylalanine ammonium-lyase (PAL) was extracted from fresh cell mass (300 mg FW) with 6.5 ml of 50 mM pH 8.8 Tris–HCl buffer containing 15 mM of β-mercaptoethanol in an ice-cooled mortar, ground with a pestle for about 5 min. The homogenate was centrifuged at 50,000*g* for 30 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on cinnamic acid production according to Ochoa-Alejo and Gòmez-Peralta [24]. Briefly, 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.4 ml of double distilled water, and 0.1 ml of enzyme extract were incubated at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 ml of 6 M HCl, and the product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the cinnamic acid concentration wherein was quantified with the absorbance measured at 290 nm. The protein content in the extract was determined by the Bradford method using bovine serum albumin as a standard [25].

Analysis of Taxol content

The extraction and analysis of Taxol from cells and the culture medium was based on the procedure as described by Wu and Lin [16]. In brief, intracellular Taxol was extracted from dry cell mass with methanol, and Taxol in the medium extracted by methylene chloride.

The extraction solvent was removed by evaporation under vacuum and the remaining solid extract was redissolved in acetonitrile. Taxol content in the extract solution was analyzed by reverse-phase HPLC with UV detection at 227 nm, using a 250 mm × 4.6 mm Altech Econosphere 5 μm C18 HPLC column (Altech, Deerfield, IL, USA), and a mobile phase consisting of methanol:acetonitrile:water at 25:35:45 by volume. Taxol and baccatin III, another taxane compound and a precursor for Taxol semisynthesis, were quantified with genuine standards (Cat. T-7402 and Cat. B-8154, Sigma).

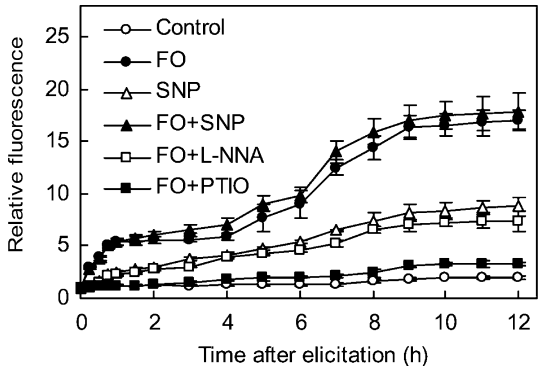

Fig. 2. Time courses of NO production (proportional to the relative fluorescence) in *T. chinensis* cell cultures subject to various treatments. FO, *Fusarium oxysporum* elicitor at 50 μg/ml; L-NNA (100 μM) and PTIO (100 μM) were added to culture 30 min prior to, and SNP (10 μM) at the same time as FO; error bars = SE, $n = 3$.

Results

Elicitor-induced NO burst in T. chinensis cell culture

The elicitor-induced NO production in the *T. chinensis* cell culture was directly observed by fluorescence microscopy, showing the green fluorescence of DAF-2 DA-stained cells (Fig. 1). Fig. 2 shows the time courses of fluorescence intensity in the DAF-2 DA-stained cultures after various treatments by the fungal elicitor and NO-related reagents. In the culture treated by the fungal elicitor only (FO), a higher fluorescence intensity over that of the control was detected within 15 min after elicitor addition, indicating the elicitor-induced NO burst. The elicitor-induced NO production exhibited a biphasic time course, reaching the first plateau in 1.5–2 h, and starting the second phase from 4 h and reaching the second and much higher plateau in 8–12 h, after the elicitor treatment. The fluorescence intensity of elicitor-treated culture was 3.5-fold higher than that of the control in the first phase (2–4 h), and 7.5-fold higher in the second phase (12 h). The culture treated with the NO donor SNP at 10 μM also exhibited a gradual increase in the fluorescence intensity in the culture, to a lower level than that induced by the fungal elicitor. This confirmed that the fluorescence detected in the experiments was arising from NO production by the DAF-2 DA-stained cells. The combination of SNP with the fungal elicitor (FO + SNP in Fig. 2) had almost a negligible effect on the fluorescence intensity of the elicitor-treated culture. The elicitor-induced fluorescence increase was effectively blocked by the NO scavenger, PTIO (100 μM), proving that the fluorescence increase was mainly a result of NO production by the elicitor-treated cells. Moreover, the elicitor-induced NO production was significantly suppressed by the NOS inhibitor,

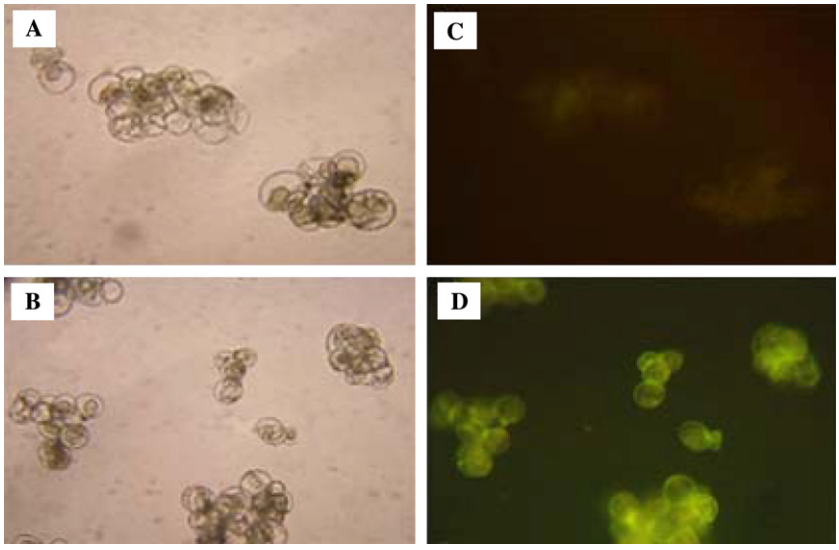

Fig. 1. Microscopy images of *T. chinensis* cells. (A) Bright-field image of normal and (B) elicitor-treated cells without DAF-2 DA staining; (C) fluorescence microscopy (470 nm excitation and 515 nm emission) of DAF-2 DA-stained cells before, and (D) after 30-min elicitor treatment (at 50 mg/l). All cells were from 12-day-old shake-flask cultures.

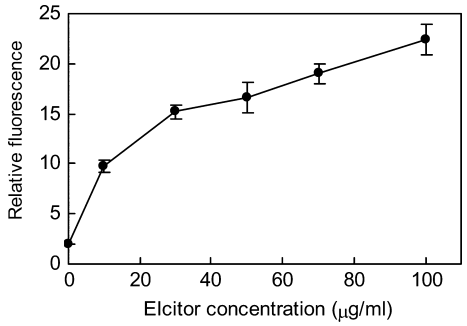

Fig. 3. NO production in *T. chinensis* cells after treatment by various doses of fungal elicitor (9 h treatment).

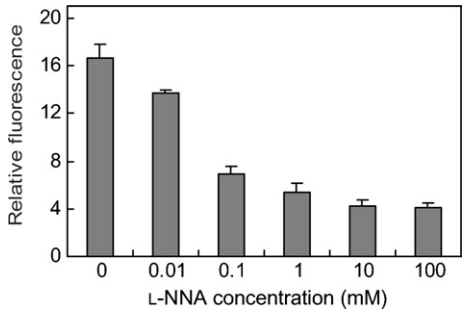

Fig. 4. NO production in *T. chinensis* cells after treatment with fungal elicitor at 50 μg/ml plus various doses of L-NNA (9 h treatment).

L-NNA (100 μM, Fig. 2), suggesting that NO synthase was a source of the elicitor-induced NO production in the *T. chinensis* cells.

The elicitor-induced NO production increased with the elicitor-concentration from 10 to 100 μg/ml, indicative of a dose-dependent effect (Fig. 3). The elicitor dose of 50 μg/ml was chosen for all subsequent experiments because of its significant stimulation of NO and the secondary metabolite production [15]. In addition, the inhibitory effect of L-NNA on the elicitor-induced NO production also was found to be dose-dependent between 0.01 and 100 mM (Fig. 4).

Elicitor-induced H_2O_2 production and cell death

The fungal elicitor-induced rapid production of H_2O_2 in the *T. chinensis* cell culture, reaching a sharp peak of 13.2 μM around 5 h, and another lower and broad peak (3.1 μM) around 6 h, post treatment (Fig. 5A). The elicitor-induced H_2O_2 production was depressed by both NO inhibitors, L-NNA and PTIO. The NO donor SNP at 10 μM in the absence of the fungal elicitor did not induce any H_2O_2, while its combination with the fungal elicitor resulted in a slight but significant increase in the H_2O_2 production (FO + SNP versus FO). The results are indicative of the relationship between NO and the elicitor-induced oxidative burst.

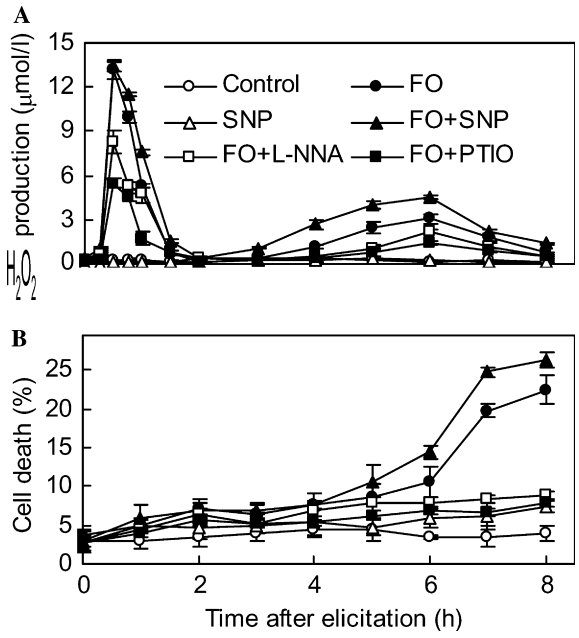

Fig. 5. Elicitor-induced H_2O_2 generation and cell death in *T. chinensis* cell cultures and the influence of NO donor and inhibitors (same procedure and dosage as specified in Fig. 2).

The treatment of *T. chinensis* cells with the fungal elicitor (50 μg/ml) resulted in notable cell death or decrease in cell viability, about 19.4% in 8 h post treatment (Fig. 5B). Compared to the oxidative burst, the elicitor-induced cell death was much slower. The NO donor SNP at 10 μM alone caused only 7.3% cell death, while its combination with the fungal elicitor resulted in 26.3% cell death, which was even higher than that caused by elicitor alone. Conversely, both NO inhibitors L-NNA and PTIO decreased the elicitor-induced cell death to 8.8 and 7.8%, respectively. Similar to the case of oxidative burst, this set of results is indicative of the relationship between NO and elicitor-induced cell death in the *T. chinensis* cell culture.

Elicitor-induced membrane ion fluxes

The elicitor treatment induced a rapid increase in K^+ concentration in the extracellular medium (Fig. 6A), indicating the efflux of K^+ through the plasma membrane, and a rapid and transient increase in medium pH (Fig. 6B), indicating the influx of H^+ through the membrane of *T. chinensis* cells. Both the K^+ and H^+ fluxes reached the peak levels in 1–2 h after elicitor addition to the culture, with a maximum K^+ efflux value of 82.6 μmol K^+/g FW cells at 2 h, and a maximum pH increase of 0.59 at 1.5 h. The NO donor SNP itself caused a slight increase of K^+ efflux, 13.6 μmol K^+/g FW cells at 2 h after treatment, but no pH change compared to that of control. Its combination with the elicitor had no significant and consistent effect on the elicitor-in-

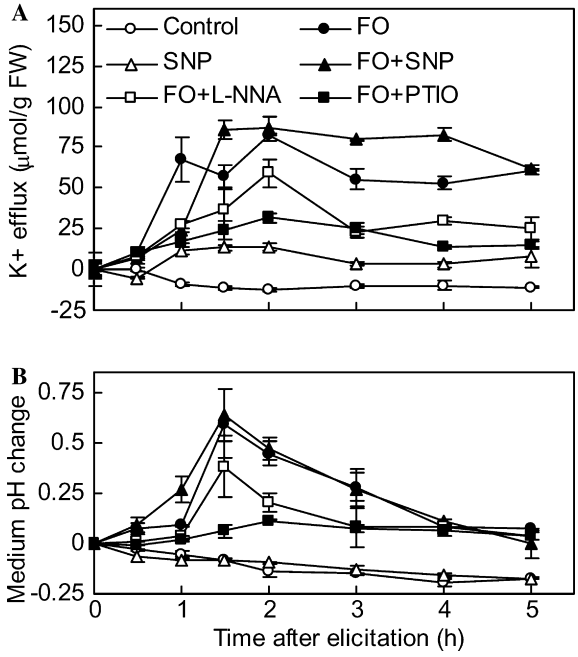

Fig. 6. Elicitor-induced membrane K^+ efflux and medium pH change (membrane H^+ influx) in *T. chinensis* cell cultures and the influence of NO donor and inhibitors (same procedure and dosage as specified in Fig. 2).

duced K^+ concentration or medium pH change (FO + SNP versus FO). In contrast, both NO inhibitors, L-NNA and PTIO, significantly suppressed the elicitor-induced K^+ and H^+ fluxes. Therefore, most of the results here are indicative of the influence of NO on elicitor-induced membrane ion-fluxes.

Elicitor-induced PAL activity and taxane biosynthesis

As shown in Fig. 7, the PAL activity in the elicitor-treated *T. chinensis* cell culture (FO) increased rapidly after the elicitor treatment, reaching a maximum in 4 h, which was more than 2-fold of the control level.

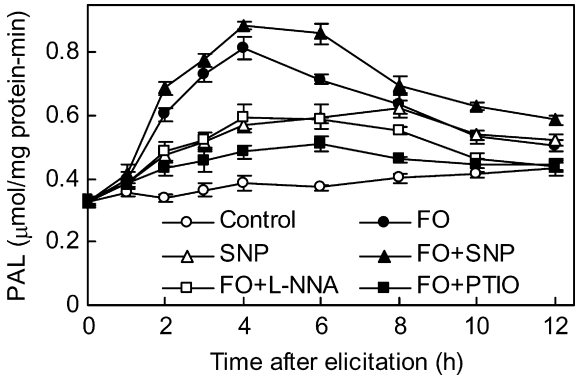

Fig. 7. Elicitor-induced activation of PAL activity in *T. chinensis* cells and the influence of NO donor and inhibitors (same procedure and dosage as specified in Fig. 2).

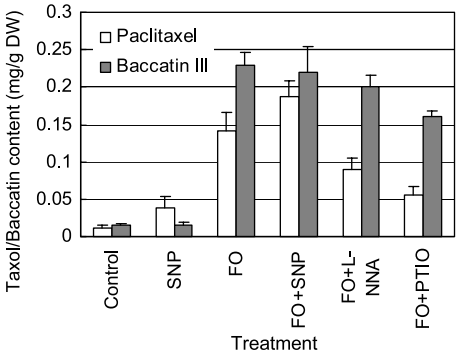

Fig. 8. Effect of NO donor and inhibitors (with the same procedure and dosage as specified in Fig. 2) on elicitor-induced paclitaxel and baccatin III production in *T. chinensis* cell cultures (6-day treatment).

The NO donor SNP significantly stimulated the PAL activity by itself, and also slightly enhanced the elicitor-induced PAL activity (FO + SNP versus FO). In contrast, the elicitor-induced PAL activity was depressed by both NO inhibitors, L-NNA and PTIO.

The elicitor treatment stimulated Taxol and baccatin III production (Fig. 8), increasing the Taxol content of cell by about 12-fold (0.14 mg/g DW for FO versus 0.011 mg/g DW for the control) and the baccatin III content by more than 13-fold (0.23 mg/g DW with FO versus 0.016 mg/g DW for the control) after 6 days of treatment. The NO donor SNP slightly but significantly stimulated the Taxol production by itself (SNP) and in combination with the fungal elicitor (FO + SNP). The elicitor-induced Taxol production was significantly depressed by both the NOS inhibitor L-NNA and the NO scavenger PTIO. However, the elicitor-induced baccatin III production was only slightly depressed by L-NNA but not significantly influenced by SNP and PTIO.

The amount of Taxol released from cells to medium was no more than 15% and that of baccatin III was less than 10% at all conditions, and the related data were not shown as they would have negligible influence on the trend of treatment effects.

Discussion

Elicitors derived from mycelium extracts of *Fusarium oxysporum* fungi have been reported previously to induce multiple plant defense responses in *Taxus* cell cultures, including the oxidative burst, lipid peroxidation, PAL activity and secondary metabolite accumulation, and cell apoptosis [15,18,26]. Most of these responses also have been realized in our present study in *T. chinensis* cell cultures. More importantly, our study has shown the elicitor-induced NO burst and its quantitative and temporal relationships with the other responses in the *Taxus* cell cultures. The elicitor-induced NO production

exhibited a clear dose-dependency and tended to become saturated at relatively larger doses. The similar saturation kinetics has been reported for the H_2O_2 accumulation in *Larix decidua* cells [27] and the PAL activity in date palm, induced by the hyphal wall fragment of *F. oxysporum* [28]. The dose-dependent and saturation effects may suggest the presence of specific receptors for elicitor perception and signal transduction leading to the subsequent defense responses including NO release. On the other hand, the elicitor-induced NO production was enhanced by the NO generator (SNP) and inhibited by the NO-synthase inhibitor (L-NNA) and NO scavenger (PTIO). These results were consistent with those found in other plant species in response to challenge by microbial pathogens [6] and mechanical stress [21]. In our study, however, the elicitor-induced NO production was not completely arrested by L-NNA even at much larger doses (up to 100 mM, Fig. 4) than those used in previous studies (0.1–5 mM). This may be due to the fact that the *Taxus* cells contain a very high level of endogenous L-arginine [17], as L-NNA is a competitive inhibitor of NOS with arginine in plant cells. Another explanation is that NO can be derived from alternative pathways, e.g., from an NO_2^- dependent side reaction of nitrate reductases, and non-enzymatically from NO_2^- accumulating in chloroplasts and the cytosol [29,30].

The biphasic time course of H_2O_2 accumulation after elicitor induction in the *Taxus* cell cultures shown in our study (FO in Fig. 5A) is a common feature of the ROS production during the elicitation of plant cells by microbial pathogens or their constituents [11,31,32]. Similarly, the elicitor-induced NO accumulation also exhibited a biphasic trend (Fig. 2), although the phase periods for the two reactive species differed. This is in agreement with the finding from soybean tissues after challenge by a microbial pathogen that the NO production kinetics closely matched that of ROS production [6]. The suppression of elicitor-induced H_2O_2 production by NO inhibitors observed in our study suggests that intracellular NO plays a regulatory role in activating the oxidative burst. In addition, exogenous NO supplied by SNP-induced cell death, and also potentiated the elicitor-induced cell death of *T. chinensis* cells. In cell culture of another *Taxus* species, *T. brevifolia*, NO also has been shown to physically induce programmed cell death [17]. Furthermore, the elicited *Taxus* cells in the presence of a NOS inhibitor, L-NNA, PTIO or their relatives, significantly decreased the NO production and cell death. This suggests that NO is implicated in mediating the elicitor-induced cell death of *Taxus* cells.

Elicitor-induced membrane $H^+$and K^+ fluxes in plant cell cultures have been widely reported as a transient decrease in the external acidification (pH drop) or even alkalinization (pH rise), followed by acidification recovery, and the reversion of K^+ influx into efflux [10,33,34]. In our present study, the cross-membrane K^+ efflux/H^+ influx induced by the *Fusarium* elicitor was partially blocked by NO inhibitors, suggestive of NO involvement in the induction of K^+/H^+ flux changes during elicitation. In animal systems, the cross talk between Ca^{2+} and NO in a crucial signaling network is implicated in the regulation of numerous cellular processes [35,36], and NO appears to be involved in the regulation of cytosolic Ca^{2+} levels as well as Ca^{2+}-permeable channels in animal cells [37,38]. Although the linkage between NO and cytosolic Ca^{2+} is still not clear in plants, a study has suggested that cytosolic free Ca^{2+} is implicated in NO-induced defense responses in tobacco [5]. Further exploration on the regulation of membrane ion channels and ion fluxes by NO should provide valuable insights into the mechanisms of NO signaling in plant cells.

Phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid pathway, is typically sensitive to the physiological state of plants and activated in the plants under biotic or abiotic stress [39]. In cell cultures of various *Taxus* species, increased PAL activity has been induced by various biotic and abiotic factors, such as heavy metal ions, fungal elicitors, methyljasmonate, and mechanical stress [15,16,40]. In our present study, the fungal elicitor-induced PAL activity was potentiated by the NO donor but inhibited by the NO inhibitors, suggesting that the activation of PAL may be regulated by the NO produced endogenously during fungal elicitation. In a previous study [6], L-NNA prevented the accumulation of transcripts encoding *PAL* and chalcone synthase (*CHS*), the first enzyme in a branch of the phenylpropanoid pathway, while the NO donor SNP induced the expression of *PAL* and *CHS* genes, in soybean cell cultures inoculated with an avirulent bacterium. NO induction of the gene encoding PAL was also observed in tobacco plants and cell cultures [5].

Finally, our present study has shown that the elicitation of paclitaxel and other taxane biosynthesis in *Taxus* cells by the fungal elicitor is strongly dependent on the elicitor-induced NO production. To the best of our knowledge, this is the first report of the relationship between NO and taxane biosynthesis in *Taxus* plants. This finding, together with the effect of NO on PAL activity mentioned afore, indicates a role for NO as a signal molecule to activate the fungal elicitor-induced secondary metabolism responses. However, the blockage of NO accumulation by putative NO inhibitors did not completely inhibit the taxane accumulation, due perhaps to the existence of alternative signal pathways, such as those mediated by ROS, jasmonates, ethylene, and SA. Since elicitation has been a successful strategy for enhancing the production of many bioactive secondary metabolites in plant tissue cultures, the elucidation of elicitor-induced NO production and its relationship with other elicitor responses will not only bring about new in-

sights into the physiological mechanisms of elicitation but also new and effective strategies for the production of desired metabolites.

Acknowledgments

This work was supported by grants from the Hong Kong Polytechnic University (PD93 and ASD fund) and the Areas of Excellence Scheme established under the University Grants Committee of the Hong Kong SAR, China (AoE/P-10/2001).

References

[1] R.F. Furchgott, Special topic: nitric oxide, Annu. Rev. Physiol. 57 (1995) 659–682.
[2] L. del Río, F.J. Corpas, J.B. Barroso, Nitric oxide and nitric oxide synthase activity in plants, Phytochemistry 65 (2004) 783–792.
[3] Y.Y. Leshem, Nitric Oxide in Plants: Occurrence, Function and Use, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000.
[4] P. Wojtaszek, Nitric oxide in plants: To NO or not to NO, Phytochemistry 54 (2000) 1–4.
[5] J. Durner, D. Wendehenne, D.F. Klessig, Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose, Proc. Natl. Acad. Sci. USA 95 (1998) 10328–10333.
[6] M. Delledonne, Y. Xia, R.A. Dixon, C. Lamb, Nitric oxide functions as a signal in plant disease resistance, Nature 394 (1998) 585–588.
[7] T. Noritake, K. Kawakita, N. Doke, Nitric oxide induces phytoalexin accumulation in potato tuber tissues, Plant Cell Physiol. 37 (1996) 113–116.
[8] L.V. Modolo, F.Q. Cunha, M.R. Braga, I. Salgado, Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp. *meridionalis* elicitor, Plant Physiol. 130 (2002) 1288–1297.
[9] M.M. Atkinson, L.D. Keppler, E.W. Orlandi, C.J. Baker, C.F. Mischke, Involvement of plasma membrane calcium influx in bacterial induction of the K^+/H^+ and hypersensitive responses in tobacco, Plant Physiol. 92 (1990) 215–221.
[10] M.M. Atkinson, S.L. Midland, J.J. Sims, N.T. Keen, Syringolide 1 triggers Ca^{2+} influx, K^+ efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene Rpg4, Plant Physiol. 112 (1996) 297–302.
[11] T. Jabs, M. Tschöpe, C. Colling, K. Hahlbrock, D. Scheel, Elicitor-stimulated ion fluxes and $O_2^{\bullet-}$ from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley, Proc. Natl. Acad. Sci. USA 94 (1997) 4800–4805.
[12] R. Schwacke, A. Hager, Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein kinase activity, Planta 187 (1992) 136–141.
[13] R.N. Patel, Tour de paclitaxel: biocatalysis for semisynthesis, Annu. Rev. Microbiol. 52 (1998) 361–395.
[14] M. McCoy, Lining up to make a cancer drug, Chem. Eng. News 82 (2004) 12–14.
[15] Y.J. Yuan, C. Li, Z.D. Hu, J.C. Wu, Signal transduction pathway for oxidative burst and taxol production in suspension cultures of *Taxus chinensis* var. *mairei* induced by oligosaccharide from *Fusarium oxysporum*, Enzyme Microb. Technol. 29 (2001) 372–379.
[16] J. Wu, L. Lin, Enhancement of taxol production and release in *Taxus chinensis* cell cultures by ultrasound, methyl jasmonate and in situ solvent extraction, Appl. Microbiol. Biotechnol. 62 (2003) 151–155.
[17] M.C. Pedroso, J.R. Magalhaes, D. Durzan, Nitric oxide induces cell death in *Taxus* cells, Plant Sci. 157 (2000) 173–180.
[18] L.J. Yu, W.Z. Lan, W.M. Qin, H.B. Xu, Effects of salicylic acid on fungal elicitor-induced membrane-lipid peroxidation and taxol production in cell suspension cultures of *Taxus chinensis*, Process Biochem. 37 (2001) 477–482.
[19] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[20] N.N. Tun, A. Holk, G.F.E. Scherer, Rapid increase of NO release in plant cell cultures induced by cytokinin, FEBS Lett. 509 (2001) 174–176.
[21] H. Garcês, D. Durzan, M.C. Pedroso, Mechanical stress elicits nitric oxide formation and DNA fragmentation in *Arabidopsis thaliana*, Ann. Bot. 87 (2001) 567–574.
[22] J.A. Glazener, E.W. Orlandi, G.L. Harmon, J.C. Baker, An improved method for monitoring active oxygen in bacteria treated suspension cells using luminol-dependent chemiluminescence, Physiol. Mol. Plant Pathol. 39 (1991) 123–133.
[23] A. Levine, R. Tenhaken, R. Dixon, C. Lamb, H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response, Cell 79 (1994) 583–593.
[24] N. Ochoa-Alejo, J.E. Gòmez-Peralta, Activity of enzymes involved in capsaicin biosynthesis in callus tissue and fruits of chili pepper (*Capsicum annuum* L.), J. Plant Physiol. 141 (1993) 147–152.
[25] M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Chem. 72 (1976) 248–254.
[26] Y.J. Yuan, C. Li, Z.D. Hu, J.C. Wu, A.P. Zeng, Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var *mairei* for taxol production, Process Biochem. 38 (2002) 193–198.
[27] M. Bach, H.U. Seitz, Elicitor-induced defence responses of a suspension-cultured woody plant (*Larix decidua*) and possible mechanisms of signal transduction, Can. J. Bot. 75 (1997) 1243–1251.
[28] C. El Modafar, A. Tantaoui, E.S. El Boustani, Differential induction of phenylalanine ammonia-lyase activity in date palm roots in response to inoculation with *Fusarium oxysporum* f. sp. *albedinis* and to elicitation with fungal wall elicitor, J. Plant Physiol. 158 (2001) 715–722.
[29] H. Yamasaki, Y. Sakihama, Stimultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species, FEBS Lett. 468 (2000) 89–92.
[30] D.J. Derzan, M.C. Pedroso, Nitric oxide and reactive nitrogen oxide species in plants, Biotechnol. Genet. Eng. Rev. 19 (2002) 293–337.
[31] C.J. Baker, E.W. Orlandi, N.M. Mock, Harpin, an elicitor of the hypersensitive response in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells, Plant Physiol. 102 (1993) 1341–1344.
[32] P.S. Low, J.R. Merida, The oxidative burst in plant defense: function and signal transduction, Physiol. Plant 96 (1996) 533–542.
[33] G.P. Bolwell, Role of active oxygen species and NO in plant defence responses, Curr. Opin. Plant Biol. 2 (1999) 287–294.
[34] A. Schaller, C. Oecking, Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defenses in tomato plants, Plant Cell 11 (1999) 263–272.
[35] M.J. Berridge, P. Lipp, M.D. Bootman, The versatility and universality of calcium signaling, Nat. Rev. Mol. Cell Biol. 1 (2000) 11–21.
[36] A. Charles, Nitric oxide pumps up calcium signaling, Nat. Cell Biol. 1 (1999) E193–E195.

[37] K. Hirooka, D.E. Kourennyi, S. Barnes, Calcium channel activation facilitated by nitric oxide in retinal ganglion cells, J. Neurophysiol. 83 (2000) 198–206.
[38] A. Zsombok, S. Schrofner, A. Hermann, H.H. Kerschbaum, Nitric oxide increases excitability by depressing a calcium activated potassium current in snail neurons, Neuosci. Lett. 295 (2000) 85–88.
[39] R.A. Dixon, N.L. Paiva, Stress-induced phenylpropanoid metabolism, Plant Cell 7 (1995) 1085–1097.
[40] R.M. Cusidó, J. Palazón, A. Navia-Osorio, A. Mallol, M. Bonfill, C. Morales, M.T. Piñol, Production of Taxol and baccatin III by a selected *Taxus baccata* callus line and its derived cell suspension culture, Plant Sci. 146 (1999) 101–107.