Hydrogen Peroxide from the Oxidative Burst is Not Involved in the Induction of Taxol Biosynthesis in *Taxus chinensis* Cells

Wen Zhi Lan[a*], Wen Min Qin[b], Long Jiang Yu[a], and Xi Yang[a]

[a] School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China. Fax: +86-27-87543683 3.
E-mail: lanwz73@sohu.com
[b] Department of Biology, University of Waterloo, Ontario N2L 3G1,Canada

* Author for correspondence and reprint requests

Z. Naturforsch. **58 c**, 605–608 (2003);
received January 13/March 17, 2003

In cell suspension cultures of *Taxus chinensis*, 40 mg/l fungal elicitor from *Aspergillus niger* and 20 μM $HgCl_2$ elicited 5.7 and 3.6 mg/l taxol, which was a 9-fold and 5-fold increase *vs.* compared with the control, respectively. The fungal elicitor induced hydrogen peroxide (H_2O_2) accumulation but $HgCl_2$ did not, indicating that H_2O_2 was not necessary for enhancement of taxol induced by elicitor. Compared with the treatment with fungal elicitor alone, exogenous catalase, ascorbic acid, diphenylene iodonium and superoxide dismutase induced a 0.45, 0.4, 0.7 and 1.4-fold H_2O_2, but elicited taxol production, which was 0.98, 1.2, 1.1 and 0.9-fold, respectively, *vs.* non-treated cells. Elicitor-induced taxol production was not accorded with the amount of H_2O_2 production.

Key words: Elicitor, Taxol, *Taxus chinensis*

Introduction

Oxidative burst, a transient increase in the production of reactive oxygen species (ROS), generally occurs in a number of plant pathogen interactions at the early stage. Hydrogen peroxide (H_2O_2), the most stable compound among ROS, has been implicated in plant disease resistance (Lamb and Dixon, 1997). However, reports linking H_2O_2 from the oxidative burst to biosynthesis of second metabolites, such as phytoalexin, have been contradictory, even with respect to experiments performed on the same plant species, such as soybean (Levine *et al.*, 1994; Mithöfer *et al.*, 1997; Guo *et al.*, 1998) and tobacco (Lamb and Dixon, 1997; Doreyl *et al.*, 1999).

Presently there has been increasing interest in exploiting *Taxus* spp. cell cultures to produce the anticancer drug, taxol. A wide variety of elicitors such as fungal elicitors (Zhang *et al.*, 2000; Yuan *et al.*, 2001; 2002; Yu *et al.*, 2002), methyl jasmonate (Zhang *et al.*, 2000; Spela *et al.*, 2002) and heavy metal ion (Zhang *et al.*, 2000) have been employed to induce the biosynthesis of taxol in *Taxus* cell cultures. Before the activation of *de novo* synthesis of taxol, the oxidative burst is also observed in elicitor-induced *Taxus chinensis* cultures (Yuan *et al.*, 2001, 2002; Yu *et al.*, 2002). Yuan *et al.*, (2001, 2002) concluded that the dependence of taxol production on the intensity of H_2O_2 from oxidative burst followed a modified logistic curve, and inferred that the syntheses of the side chain and nucleus of taxol were enhanced by low and high intensities of H_2O_2, respectively. However, it is not clear whether inhibition or enhancement of H_2O_2 from the oxidative burst would affect taxol biosynthesis, which is the main subject of the present paper.

Materials and Methods

Plant materials and culture conditions

Taxus chinensis cell lines, isolated from *Taxus chinensis* zygote embryos, were maintained in modified MS medium as previously described (Zhang *et al.*, 2000). With 10 g (fresh weight) of cells inoculated into a 250 ml Erlenmeyer flask containing 100 ml liquid modified MS media, 40 mg/l fungal elicitor, and 20 μM $HgCl_2$ were added to the 10-day-old cultures. The flasks were shaken at 120 ± 5 rpm at 25 ± 1 °C.

Fungal elicitor preparation

The fungal strain *Aspergillus niger* was isolated from the inner bark of *Taxus chinensis*. Preparation of fungal elicitor was according to the method described by Zhang *et al.* (2000). The elicitor dose was measured by the total carbohydrate content of the fungal homogenate, which was determined by the phenol-sulfuric acid method using glucose as standard.

H_2O_2 measurement

Contents of H_2O_2 were measured by monitoring the A_{415} of the titanium-peroxide complex according to Brennan and Frenkel (1977). Absorbance values were calibrated to the standard curve.

Taxol determination

The cells after elicitation were washed with deionized water to remove residual medium and filtrated under vacuum. The cells were then freeze-dried for 30 h for dry wt determination. Dried samples (100 mg) were mashed and extracted with 4 ml methanol/dichloromethane (1:1, v/v) with sonication for 1 h at room temperature for 3 times. The extract was evaporated to dryness with a rotary evaporator equipped with a condenser for solvent recovery, then redissolved in 2 ml methanol. The methanol extracts were centrifuged at 3400 × *g* for 5 min prior to HPLC analysis. Samples of 5 ml from the cell free medium were extracted with 2 ml of dichloromethane for 3 times. The combined dichloromethane fraction was vacuum dried and redissolved in 2 ml methanol for HPLC analysis after centrifugation. taxol was analyzed by HPLC on a reverse-phase C_{18} column at 227 nm at 25 °C using a mobile phase of methanol/acetonitrile/water (25:30:30, v/v/v). The elution rate was kept at 1 ml min^{-1}. Throughout the experiment, all injection volumes were 10 µl. Taxol concentration (mg/l) in the samples was the combination of taxol in cells and medium.

Results and Discussion

Effect of elicitor on H_2O_2 production

Several suspension cultures plant cell have been reported produce H_2O_2 during stimulation by elicitors from the fungus (Levine *et al.*, 1994; Lamb and Dixon, 1997; Mithöfer *et al.*, 1997; Guo *et al.*, 1998; Doreyl *et al.*, 1999). Fig. 1 shows the effect of the fungal elicitor from *Aspergillus niger* on H_2O_2 production in *Taxus chinensis* cell cultures. The elicitor-induced H_2O_2 production started to increase 1 to 2 h after treatment, and reached a maximum of 240 nmol/gFW at about 4 h, and decreased thereafter (Fig. 1). Treatment of *Taxus chinensis* cultures with fungal elicitor stimulated the H_2O_2 accumulation, which agrees with the observations by Yuan *et al.* (2001; 2002) and Yu *et al.* (2002). Taxus chinensis cultures without elicitor treatment accumulated little H_2O_2. 20 µM $HgCl_2$ only induced a slight increase in H_2O_2 accumulation; however, the increase *vs.* free elicitor treatment was not statistically significant ($P < 0.05$) (Fig. 1). Indeed, a low concentration of $HgCl_2$ had little effect on H_2O_2 accumulation in white clover (*Trifolium repens* L.) suspension cultures (Devlin *et al.*, 1992).

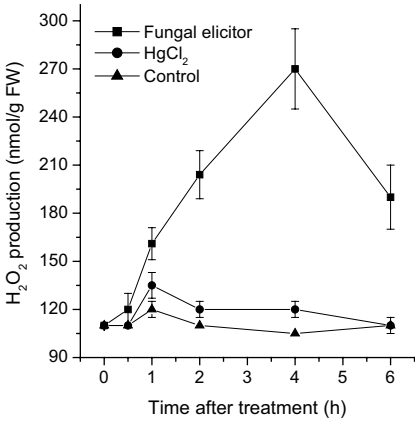

Fig. 1. H_2O_2 accumulation induced by fungal elicitor and $HgCl_2$ in *Taxus chinensis* suspension cells. The rate of H_2O_2 accumulation was determined in cultures at various times after addition of 40 mg/l fungal elicitor and 20 µM $HgCl_2$, and in the untreated control. Elicitor was added into 10-d old cultures. FW represents fresh weight. Each value is the mean ±SE from seven independent experiments.

Effect of elicitor on taxol production

The time courses of taxol accumulation in *Taxus chinensis* cultures treated with fungal elicitor and $HgCl_2$ were demonstrated in Fig. 2. Significant dif-

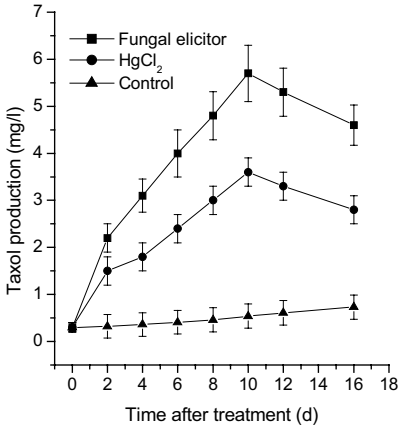

Fig. 2. Taxol accumulation induced by fungal elicitor and $HgCl_2$ in *Taxus chinensis* suspension cells. The rate of H_2O_2 accumulation was determined in cultures at various times after addition of 40 mg/l elicitor and 20 µM $HgCl_2$, and in the untreated control. Elicitor was added to 10-d old cultures. Each value is the mean ± SE from five independent experiments.

ferences in taxol concentrations between elicited cultures and the control appeared within 2 d. The highest taxol concentrations under the influence of fungal elicitor (5.7 mg/l) and $HgCl_2$ (3.6 mg/l) appeared on day 10 after treatment, which was 10-fold and 6-fold of the control, respectively.

Although H_2O_2 acts as a second messenger for the induction of some defense genes (Lamb and Dixon, 1997), Levine *et al.* (1994) and Doreyl *et al.* (1999) observed that manipulation of H_2O_2 of the oxidative burst did not modify transcripts of phenylalanine ammonia-lyase and chalcone synthase, respectively. Moreover, phytoalexin and terpenes, secondary metabolites generally present in plant-pathogen interactions, were independent on hydrogen peroxide in elicitor-treated tobacco and soybean cell cultures (Devlin *et al.*, 1992; Levine *et al.*, 1994; Mithöfer *et al.*, 1997; Guo *et al.*, 1998; Doreyl *et al.*, 1999). The present results showed that $HgCl_2$ did not induce H_2O_2 accumulation, but enhanced taxol production similarly to what the fungal elicitor did. Therefore, we postulate that H_2O_2 was not required for enhancement of taxol induced by elicitor.

Effect of scavengers and inhibitors of H_2O_2 generation on H_2O_2 accumulation and taxol production induced by fungal elicitor

To further examine the relationship between H_2O_2 accumulation and taxol biosynthesis, we analyzed these responses in the presence of some scavengers and inhibitors of ROS generation. All chemicals at the concentration used were not toxic to the cells and had little effect on taxol production. Fig. 3 shows the effect of various chemicals on H_2O_2 accumulation at 4 h and taxol production on 10 d after fungal elicitor treatment. Addition of 200 U/ml catalase (CAT) and 60 mg/l ascorbic acid (ASA), scavengers of ROS (Mithöfer *et al.*, 1997; Yu *et al.*, 2002), and diphenylene iodonium (DPI; 500 μM), an inhibitor of NAD(P)H oxidase (Levine *et al.*, 1994; Lamb and Dixon, 1997; Mithöfer *et al.*, 1997; Guo *et al.*, 1998), induced a 0.45, 0.4, and 0.7-fold H_2O_2 accumulation, and elicited a 0.98, 1.2, and 1.1-fold taxol production, higher than found with fungal elicitor alone (Fig. 3 A). Similarly, phytoalexin production was little inhibited when H_2O_2 level was decreased by DPI or ASA (Mithöfer *et al.*, 1997; Doreyl *et al.*, 1999). For reasons, yet unknown, we even found that superoxide dismutase (SOD; 300 U/ml), which alleviates the superoxide radical anion, increased the amount of H_2O_2 accumulation (1.4-fold), but decreased the taxol production (0.9-fold) compared with the treatment of fungal elicitor alone did (Fig. 3 B). These results show that elicitor-induced taxol production did not depend on the intensity of H_2O_2 from oxidative burst, which is in contrast to the observations of Yuan *et al.* (2001; 2002).

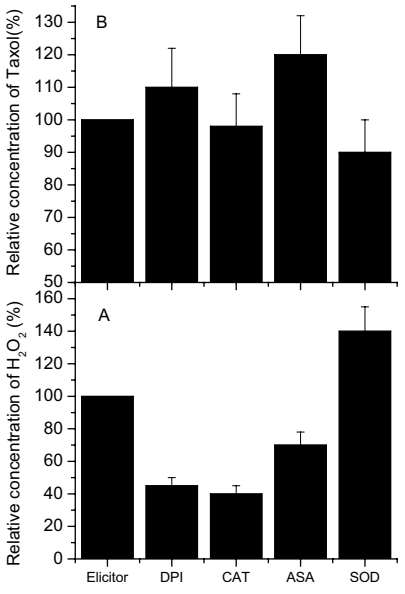

Fig. 3. Effect of inhibitors, exogenous SOD, and catalase on the production of H_2O_2 (A) taxol (B) in *Taxus chinensis* cell suspension cultures treated with fungal elicitor.
Catalase (CAT, 200 U/ml), ascorbic acid (ASA, 60 mg/l), diphenylene iodonium (DPI, 500 μM) and superoxide dismutase (SOD, 300 U/ml) were added to cultures 10 min before the addition of elicitor. Samples for H_2O_2 assay and taxol determination were taken 4 h and 10-d after treatment, respectively. FW represents fresh weight. The values of H_2O_2 and taxol production obtained from cells treated with fungal elicitor were set to 100%. Fungal elicitor was added to the 10-d old cultures. Means ± SE were calculated from six independent experiments.

Acknowledgements

This work was financially supported by the Ministry of Education (2000 year excellent youth teacher fund). We also thank National Cancer Institute for presenting the taxol standard.

Brennan T. and Frenkel C. (1977), Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol. **59**, 411–416.

Devlin W. S. and Gustine D. L. (1992), Involvement of the oxidative burst in phytoalexin accumulation and the hypersensitive reaction. Plant Physiol. **100**, 1189–1195.

Dorey1 S., Kopp M., Geoffroy P., Fritig B., and Kauffmann S. (1999), Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. Plant Physiol. **121**, 163–172.

Guo Z.-J., Lamb C., and Dixon R. A. (1998), Potentiation of the oxidative burst and isoflavonoid phytoalexin accumulation by serine protease inhibitor. Plant Physiol. **118**, 1487–1494.

Lamb C. and Dixon R. A. (1997), The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**, 251–275.

Levine A., Tenhaken R., Dixon R., and Lamb C. (1994), H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79**, 503–593.

Mithöfer A., Daxberger A., Fromhold-Treu D., and Ebel J. (1997), Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. Phytochemistry **45**, 1101–1107.

Spela B., Marjana C., Maja K., Maja R., and Jana Z. (2002), Jasmonic acid stimulates taxane production in cell suspension culture of yew (*Taxus X media*). Planta Med. **68**, 475–476.

Yu L. J., Lan W. Z., Qin W. M., Jin W. W., and Xu H. B. (2002), Oxidative stress and taxol production induced by fungal elicitor in cell suspension cultures of *Taxus chinensis.* Biol. Plant **45**, 459–461.

Yuan Y. J., Li C., Hu Z. D., and Wu J. C. (2001), 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**, 372–379.

Yuan Y. J., Li C., Hu Z. D., and Wu J. C. (2002), A double oxidative burst for taxol production in suspension cultures of *Taxus chinensis* var. *mairei* induced by oligosaccharide from *Fusarium oxysporum.* Enzyme Microb. Technol. **30**, 774–778.

Zhang C. H., Mei X. G., Liu L., and Yu L. J. (2000), Enhanced paclitaxel production induced by the combination of elicitors in cell suspension cultures of *Taxus chinensis.* Biotechnol. Lett. **22**, 1561–1564.