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BRIEF COMMUNICATION

Oxidative stress and taxol production induced by fungal elicitor in cell suspension cultures of *Taxus chinensis*

L.J. YU*, W.Z. LAN, W.M. QIN, W.W. JIN and H.B. XU

School of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430074, China

Abstract

Treatment of *Taxus chinensis* cell suspension cultures with fungal elicitor resulted in an oxidative stress characterized by H_2O_2 production, malondiadehyde (MDA) accumulation and cell death. This oxidative stress was dependent on the concentration of elicitor. Cells exposed to elicitor accumulated taxol, however, not proportional to elicitor concentration. High production of taxol occurred in cells treated with the suitable elicitor concentration. We concluded that oxidative stress had the deleterious effect on taxol production. Simultaneous treatment with elicitor and ascorbic acid (ASA) changed the oxidative stress and taxol production. Production of taxol in cells treated with 200 mg dm^{-3} elicitor and ASA was enhanced compared with that in cells treated with only 200 mg dm^{-3} elicitor, while production of taxol in cells treated with 100 and 50 mg dm^{-3} elicitor and ASA was decreased compared with that in cells treated with 100 and 50 mg dm^{-3} elicitor.

Additional key words: cell death, hydrogen peroxide, lipid peroxidation.

Microbial invasion on intact plants would trigger rapid and localized defence response, the so-called active oxygen species (AOS) burst (Levine *et al.* 1994, Allen and Tresiki 2000). AOS burst is characterized by the rapid production of AOS, *e.g.* H_2O_2 within minutes at the site of infection (Allen and Tresiki 2000). AOS in response to elicitors have been suspected to have roles not only in induction of local and systemic resistance (Allen and Tresiki 2000, Kuzniak and Urbanek 2000), but also in oxidative stress (Pavlovkin *et al.* 1986, Kuzniak and Urbanek 2000). They rapidly interact with membrane lipids to induce lipid peroxidation (Pavlovkin *et al.* 1986), thus disrupt the normal metabolism of the cells, and even cause cell death (Levine *et al.* 1994). Application of ascorbic acid (ASA), an antioxidant, could reduce the oxidative stress.

Elicitors were successfully used to increase the yield of secondary metabolites in plant cell cultures (Dörnenburg and Knorr 1995) including taxol, a novel anti-cancer diterpenoid secondary product in cell suspension cultures of *Taxus* (Ciddi *et al.* 1995, Ketchum *et al.* 1999). The combination of elicitors could enhance taxol production more than individual elicitor did in cell suspension culture of *Taxus chinensis* (Zhang *et al.* 2000). Few of these studies have provided detailed information concerning oxidative stress induced by elicitor and the relationship between oxidative stress and taxol biosynthesis in suspension cultures of *Taxus*. The aim of the present study was to investigate the effects of fungal elicitor on oxidative stress, as well as the possible relationship between oxidative stress and taxol biosynthesis in cell suspension cultures of *Taxus chinensis*.

Taxus chinensis cell line, initiated from zygote embryo, was maintained on Gamborg's B_5 medium supplemented with 3 % sucrose, 10 μM α-naphthalene

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Abbreviations: AOS - active oxygen species; ASA - ascorbic acid; FE - fungal elicitor; f.m. - fresh mass; MDA - malondiadehyde.
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*Corresponding author; fax: (+86) 027 87540184; e-mail: yulongjiang@mail.hust.edu.cn

acetic acid, 0.5 mg dm^{-3} 6-benzylaminopurine, 0.5 g dm^{-3} lactalbumin hydrolysate and 30 g dm^{-3} sucrose, in the dark at 25 ± 1 °C. For taxol production, the cells were transferred to B_5 liquid medium modified accordingly to Zhang *et al.* (2000).

To determine the effects of elicitors on oxidative stress and taxol production, about 50 g (f.m.) of 14-d-old cells was incubated in 800 cm^{-3} fresh medium in a 2 dm^{-3} Erlenmeyer flask. After 10 d, various concentrations of fungal elicitor alone or in combination with ASA were applied. All the flasks were kept in a gyratory shaker at 25 °C in the dark at an agitation speed of 120 ± 5 rpm. The experiments were performed in triplicate. The fungal strain was isolated from the inner bark of *Taxus chinensis*. Preparation of fungal elicitor (FE) was according to the method described by Zhang *et al.* (2000).

Contents of H_2O_2 were measured by monitoring the absorbance (A_{415}) of the titanium-peroxide complex followed the method described by Brennan and Frenkel (1977). Lipid peroxidation was determined by estimating malondiadehyde (MDA) content following the method of Heath and Packer (1968). The amount of MDA-thiobarbituric acid complex (red pigment) was calculated from A_{532}. Cell death was measured by monitoring the A_{600} of the supernatant following the method of Baker and Mock (1974).

For HLPC analysis for taxol the cells were taken 4 and 8 d after elicitation, washed with deionized water and filtrated under vacuum to remove residual medium. The cells were then freeze-dried for 30 h for dry mass determination. Dried samples (100 mg) were extracted with 4 cm^{-3} methanol:dichloromethane (1:1, v/v) with sonication for 1 h at room temperature for 3 times. The extract was evaporated to dryness with rotary evaporator equipped with a condenser for solvent recovery, then redissolved in 2 cm^{-3} methanol. The methanol extracts were centrifuged at 3 400 *g* for 5 min prior to HPLC analysis. Also samples of 5 cm^{-3} from the cell free medium were extracted with 2 cm^{-3} of dichloromethane for 3 times. The combined dichloromethane fraction was vacuum dried and redissolved in 2 cm^3 methanol for HPLC analysis after centrifugation. Taxol content was analyzed by HPLC according to Zhang *et al.* (2000). The taxol concentration (mg dm^{-3}) in the samples was the combination of taxol in cells and medium. Taxol standard was from National Cancer Institution (USA).

The fungal elicitor (FE) in three concentrations used induced the different AOS, lipid peroxidation and cell death, indicating that oxidative stress was elicited in cell suspension cultures of *Taxus chinensis*. Treatment with ascorbic acid reduced oxidative stress. Treatment of cultures with 50 mg dm^{-3} FE for 6 h enhance H_2O_2 content compared with the control (Fig. 1*A*). Especially, cultures treated with 100 mg dm^{-3} and 200 mg dm^{-3} FE enhanced significantly H_2O_2 contents, reaching 220 and 290 % of the control, respectively. Treatment with ASA obviously reduced H_2O_2 accumulation induced by FE. Elicitor treatments enhanced H_2O_2 content within short time of incubation (Fig. 1*B*).

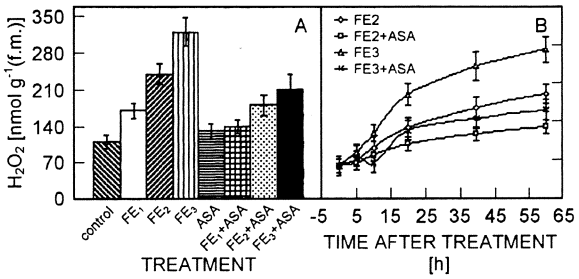

Fig. 1. The changes in H_2O_2 contents in cell suspension cultures of *Taxus chinensis* 6 h after treatment (*A*) and during the treatment (*B*). Elicitor (FE_1, FE_2 and FE_3 - 50, 100 and 200 mg dm^{-3}) and ascorbic acid (ASA, 60 mg dm^{-3}) were added into 10-d-old cultures. Means of three independent experiments. *Error bars* represent SE.

The occurrence of MDA, one of end products of the peroxidation of polyunsaturated fatty acid, was considered a useful index of lipid peroxidation. An increase in MDA content followed the enhancing concentration of FE. The contents of MDA in FE_1, FE_2 and FE_3 were 160, 230 and 360 % of the control value at the day 8, respectively. Preliminary supply of ASA attenuated the effects of FE on lipid peroxidation: the MDA content in FE_1 + ASA, FE_2 + ASA and FE_3 + ASA were reduced to 91, 86 and 76 % of its content in the respective variant FE (Fig. 2*A*).

The cell death was observed in cell suspension cultures treated with FE, particularly with FE_3 (Fig. 2*B*). Application of ASA led to a reduction of cell death to 73 % (FE_2 + ASA) and 71 % (FE_3 + ASA) of cell death in the variants FE_2 and FE_3.

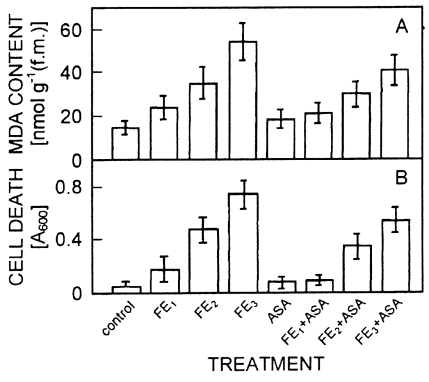

Fig. 2. The changes in MDA content (*A*) and cell death (*B*) after 8-d treatment of cell suspension cultures of *Taxus chinensis*. Elicitor and ascorbic acid were added into 10-d-old cultures. Values are means of three independent experiments. *Error bars* represent SE. Symbols as in Fig. 1.

Taxol production was enhanced by fungal elicitor treatment, and the production in FE_2 was the highest

among the treated cultures at the day 4. This was almost 1.7, 1.9 and 7.3-fold higher than that in FE_1, FE_3 and the control, respectively. Taxol production in FE_1 + ASA and FE_3 + ASA was enhanced compared with FE_1 and FE_3, respectively. However, taxol production in FE_2 + ASA was reduced to 82 % of that in FE_2 at day 4, but enhanced at day 8.

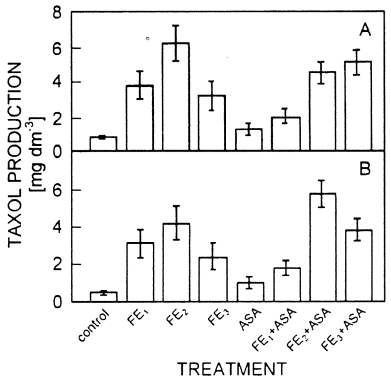

Fig. 3. The changes of taxol production at the day 4 (*A*) and day 8 (*B*) of treatment in cell suspension cultures of *Taxus chinensis*. Elicitor and ascorbic acid were added into 10-d-old cultures. Values are means of three independent experiments. *Error bars* represent SE. Symbols as in Fig. 1.

Treatment of *Taxus chinensis* cells with elicitor caused an increase in the accumulation of taxol, however, the highest production of taxol occurred in cells treated with the medium elicitor concentration. This was in accordance with Ciddi *et al.* (1995). This finding was possible due to the negative effect of oxidative stress on taxol biosynthesis. We observed that taxol production in FE_3 was significantly lower than that in FE_2, and oxidative stress in FE_3 was higher than that in FE_2. Therefore, oxidative stress must be considered when studying the enhanced production of taxol induced by elicitor.

In order to further evaluate the relationship between oxidative stress and taxol production, we applied ASA to regulate oxidative stress. Pretreatment with ASA reduced the oxidative stress induced by FE_2, but taxol production was also reduced in FE_2 + ASA compared with that in FE_2. However, treatment with ASA reduced the level of oxidative stress induced by FE_3, and taxol production was enhanced in FE_3+ASA compared with in FE_3. Furthermore, we observed that pretreatment with ASA almost eliminated oxidative stress induced by FE_1, but enhanced production of taxol still occurred in FE_1+ASA compared with that in ASA and the control (Fig. 3). Guo *et al.* (1998) reported that AOS induced phytoalexin accumulation in soybean cells, and recent data indicated that H_2O_2 might serve as intermediate signal regulating synthesis of secondary metabolites in plant-fungal interactions (Allen and Tresiki 2000). Therefore, we postulated that H_2O_2 was involved in regulation of taxol synthesis.

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