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Reactive Oxygen Species, Cell Growth, and Taxol Production of *Taxus cuspidata* Cells Immobilized on Polyurethane Foam

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Abstract

Dynamic changes in reactive oxygen species (ROS) of *Taxus cuspidata* cells immobilized on polyurethane foam were investigated and the relation between ROS content and taxol production was discussed. Immobilization shortened the lag period of cell growth and moderately increased H_2O_2 and $O_2^{-\bullet}$ contents inside the microenvironment within the first 15 d. After 20 d, excessive production of H_2O_2 and $O_2^{-\bullet}$ was observed accompanied by marked increases in membrane lipid peroxidation and cell membrane permeability. The taxol content of immobilized cells was fourfold that of suspended cells at d 35. The addition of exogenous H_2O_2 barely affected malondialdehyde content and cell membrane permeability but led to an obvious accumulation of taxol. It is inferred that the intracellular and extracellular H_2O_2 inside the microenvironment might be one factor promoting taxol biosynthesis under the immobilization stress.

Index Entries: Immobilization; polyurethane foam; reactive oxygen species; taxol; *Taxus cuspidata*.

Introduction

Taxol, extracted from the bark of *Taxus* trees, is viewed as a promising anticancer drug for the treatment of ovarian and breast cancers, owing to its unique mode of preventing microtubule from disintegration *(1)*. The global scarcity of *Taxus* trees severely limits the therapeutic applications

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of taxol. *Taxus* spp. cell cultures have been considered a potential alternative for large-scale taxol production. Many strategies have been proposed to improve taxol production of *Taxus* spp. cells *(2–4)*, and immobilization is considered a very useful method of improving taxol production *(5,6)*, because it can prevent cells from shear stress, improve cell density, promote cell organization and differentiation, make the cells reusable, and so on *(7)*. Immobilization of cells on polyurethane foam provided cells with a peculiar spatial microenvironment where cells were regulated by reticulated foam and nutriment transportation, obviously affecting cell growth and production *(8,9)*. Over the past 15 years, much attention has been paid to the effect of oxygen supply and transportation on the production of immobilized cells *(10,11)*. Reactive oxygen species (ROS) play many important roles in the control of biologic processes such as cell growth and death and biotic and abiotic stress responses *(12)*. Our studies have shown that ROS burst induced by biotic and abiotic elicitors is closely related to taxol production *(3,4)*. Angelova et al. *(13)* reported that antioxidant enzymes of superoxide dismutase (SOD) and catalase (CAT) of immobilized *Aspergillus niger* cells changed obviously compared with those of suspended cells. Less attention, however, is paid to the changes of ROS content in both the immobilized cells and microenvironment, especially to the physiologic behavior and role of ROS in the microenvironment.

In the present work, *Taxus cuspidata* cells were immobilized on polyurethane foam, the changes in ROS content in both the immobilized cells and microenvironment were investigated, and the relation between ROS content and taxol production was discussed, in order to better understand the physiologic mechanisms of the responses of the cells to the immobilization microenvironment.

Materials and Methods

Cell Line and Culture Conditions

The cell line *T. cuspidata* was provided by the Institute of Botany, Chinese Academy of Sciences, Beijing, China. All chemicals used were of analytical grade and obtained commercially.

Suspensions of the cell line were subcultured every 10 d for a total of five generations in 50 mL of fresh modified B_5 medium containing sucrose (25 g/L), naphthylacetic acid (2 g/L), and 6-benzyl aminopurine (0.15 mg/L) in 1000-mL shake flasks at 25°C in the dark with shaking at 110 rpm. The pH was adjusted to 5.8. Fresh cells (2.5 g) from the suspension cultures of the fifth generation were inoculated into 55 mL of fresh modified B_5 medium in 250-mL Erlenmeyer flasks. Cells in flasks were harvested at predetermined times for the subsequent experiments. All data were the average of triplicate independent experiments. Statistical analysis was performed using a Student's *t*-test.

Immobilization and Release of Cells

Cells were immobilized on polyurethane foam following the method of Ishida *(9)* with a slight modification. Foam cubes (1 × 1 × 1 cm) were soaked for 2 to 3 d in 80% (v/v) ethanol and rinsed three times with deionized water. Four foam cubes were threaded onto a stainless steel wire, which was bent to hold the cubes flat against the bottom of a 250-mL wide-mouthed Erlenmeyer flask. To each flask was added 55 mL of culture, followed by autoclaving.

The release of immobilized cells was conducted following the method of Kannaiyan et al. *(14)* with a minor modification. Cells were released from the immobilized state by gently shaking polyurethane foam with sterile nippers in a 50-mL beaker loaded with phosphate buffer (0.1 *M*, pH 5.8). Then the cell suspensions were centrifuged at 3000*g* for 5 min.

Measurement of Cell Biomass

Foam cubes were removed from the stainless steel wires and filtered under vacuum through a filter paper, rinsed with deionized water, and dried at 80°C in an oven until no weight loss was detected. The cell weight was calculated by subtracting the weight of the dry foam cubes. Suspended cells were dried in the same way as that used for immobilized cells.

Determination of Malondialdehyde Content

Malondialdehyde (MDA) content was determined by thiobarbituric acid reaction following the method of Sofo et al. *(15)*.

Analysis of Cell Membrane Permeability (or Nonintegrity)

Cell membrane permeability (nonintegrity) was analyzed using Evans blue dye following the procedure of Suzuki et al. *(16)*.

Detection of $O_2^{-\bullet}$ Content

The content of $O_2^{-\bullet}$ in extracellular medium was detected using the nitroblue tetrazolium method *(17)*. Briefly, 1 mL of cell culture medium was harvested by centrifugation (10,000*g*, 20 s, 25°C), and the $O_2^{-\bullet}$ concentration in the supernatant was measured by monitoring the reduction of nitroblue tetrazolium (100 µ*M*) at 530 nm. SOD (100 U) was added in a duplicate cuvet to access the specificity of the changes of absorbance with $O_2^{-\bullet}$ concentration. The amount of $O_2^{-\bullet}$ was calculated based on ε_{550} nm = 12.8 mM^{-1}cm^{-1} *(17)*.

Detection of H_2O_2 Content

Intracellular H_2O_2 content was measured using Ti-NH_3 following the method of Mukherjee et al. *(18)*.

H_2O_2 concentration in the extracelluar medium was tested following the method of Bellincampi et al. *(19)*. The incubation medium (500 µL) was

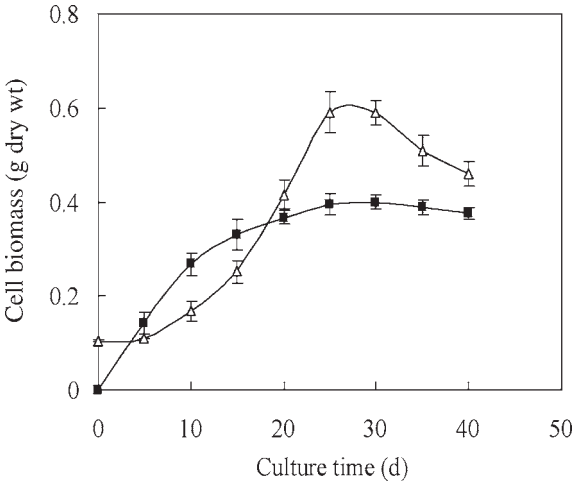

Fig. 1. Change in cell growth as function of cultivation time: (■) immobilized cells; (△) suspended cells.

added to 500 µL of the assay reagent (500 µ*M* ferrous ammonium sulfate, 50 m*M* H_2SO_4, 200 µ*M* xylenol orange, and 200 mm of sorbitol). The absorbance of the Fe^{3+}–xylenol orange complex (A_{560}) was detected after 45 min. The specificity for H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture with CAT. Standard curves of H_2O_2 were obtained for each independent experiment by adding an appropriate amount of H_2O_2 to 500 µL of basal medium mixed with 500 µL of assay reagent.

Assay of Phenylalanine Ammonialyase Activity and Detection of Taxol Content

Phenylalanine ammonialyase (PAL) activity and taxol content were analyzed as described previously *(20)*.

Results and Discussion

Effect of Immobilization on Cell Growth

Figures 1 and 2 show the cell growth and cross-dissection photographs of immobilized cells, respectively. The immobilized and suspended cells had different growth models. The biomass of immobilized cells was obviously higher than that of the suspended cells in the first 15 d of cultivation ($n = 3$, $p < 0.05$) and much lower after 20 d ($n = 3$, $p < 0.01$).

Immobilization increases cell density and protects cells from the hydrodynamic fluid shear stress. Cells tune their redox state to respond to change in the microenvironment *(21)*. Direct cell-to-cell interactions alter the cellular redox state, and the redox alterations in turn modulate signal pathways related to cell growth *(22)*. There were enough nutriments in the microenvironment in the early time of cultivation, favoring cell growth.

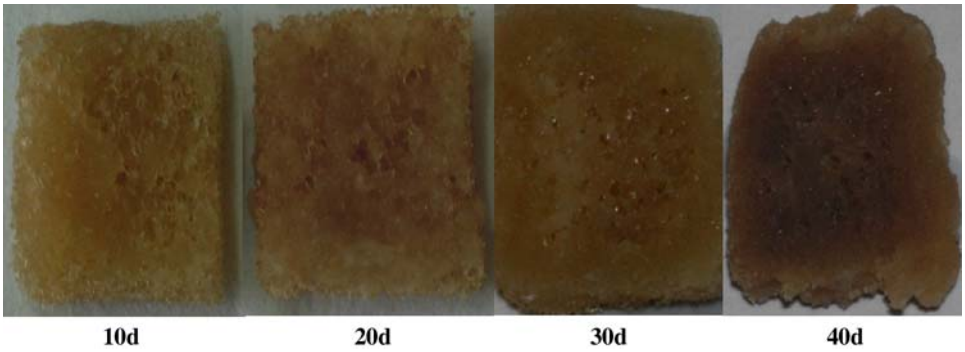

Fig. 2. Cross-dissection photographs of immobilized cells.

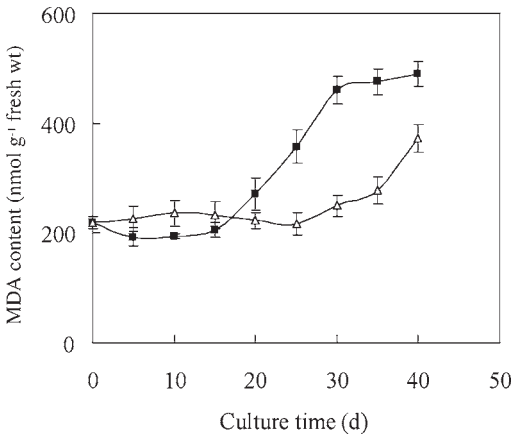

Fig. 3. Change in MDA content with cultivation time: (■) immobilized cells; (△) suspended cells.

Therefore, the lag period of the immobilized cells was shortened *(8,9)*. After 20 d, some cells grew out of foam and formed a layer of tight tissue at the foam surface, which was obviously visible at d 30 and 40 (Fig. 2). Thus, the cell organization was intensified, making the mass transfer difficult within the microenvironment. The biomass of immobilized cells was therefore much lower than that of the suspended cells after 20 d of cultivation, and the stationary period of the immobilized cells was also prolonged. The decreased cell growth rate and the intensified cell organization contributed to the secondary metabolisms *(23)*.

Effect of Immobilization on MDA Content

Figure 3 indicates that the membrane lipid peroxidation (MDA) content of immobilized cells did not differ obviously from that of the suspended cells in the first 15 d but was much higher than that of the suspended cells after 20 d ($n = 3$, $p < 0.01$).

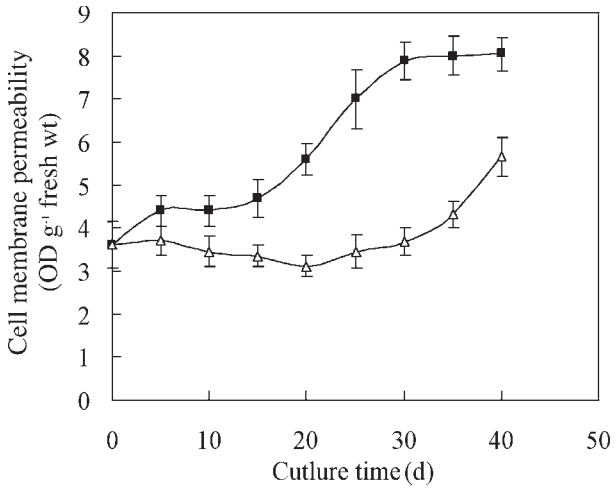

Fig. 4. Change in cell membrane permeability with cultivation time: (■) immobilized cells; (△) suspended cells. OD = optical density at 600 nm.

Immobilization increases the activities of antioxidant enzymes such as SOD and CAT *(13)*, keeping ROS content below the threshold of damaging the cell membrane. Therefore, MDA content did not change obviously within the first 15 d of cultivation. After 20 d, the mass-transfer limitation within the microenvironment became obvious with the intensification of cell organization, leading to the decrease in cell viability. Moreover, the nutriment limitation would promote the production of ROS *(24)*, resulting in oxidative stress and membrane lipid peroxidation. At d 30, the MDA content of immobilized cells was 184% that of the suspended cells. Similarly, a notable increase in MDA content was caused by fungal elicitor–induced ROS burst *(25)*. The accumulation in MDA would result in a loss of cell membrane functions and an increase in cell membrane permeability.

Effect of Immobilization on Cell Membrane Permeability (Nonintegrity)

Figure 4 shows that the membrane permeability of the immobilized cells gradually increased within 20 d, differed obviously with that of the suspended cells ($n = 3$, $p < 0.01$) afterward, and was twofold that of the suspended cells at d 30.

Immobilization activates the cell-specific metabolism pathway relevant to cell membrane permeability, increasing the cell membrane permeability *(26)*, which, to some extent, favors substance transmembrane transportation, thus promoting cell growth *(27)*. After 20 d, the MDA content increased markedly (Fig. 3), harming cell membrane selective permeability. The significantly increased cell membrane permeability would lead to the loss of cell membrane integrity and cause the death of cells. An obvious brown color in the immobilized cells was observed at d 40 (Fig. 2), indicating the dying of some cells in the core foam.

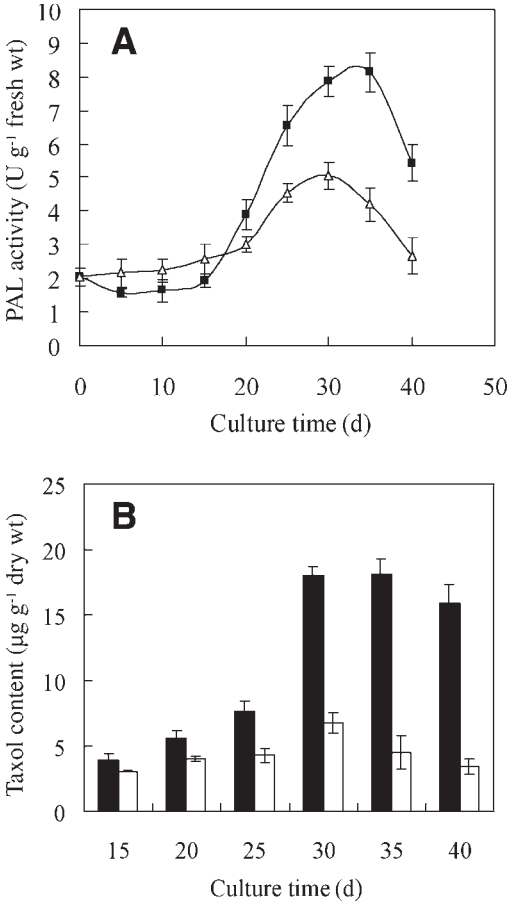

Fig. 5. Change in **(A)** PAL activity and **(B)** taxol content as function of time: (■) immobilized cells; (△,□) suspended cells. One PAL unit was defined as the change in absorbency of 0.1/min at 290 nm, and the enzyme activity was expressed as units per gram fresh weight.

Effects of Immobilization on PAL Activity and Taxol Content

Figure 5 shows the changes in PAL activity and taxol content. The PAL activities (Fig. 5A) of immobilized and suspended cells were almost the same within the first 20 d of cultivation. Correspondingly, the taxol content (Fig. 5B) of the immobilized and suspended cells also had no obvious difference. Both PAL activity and taxol content differed significantly between the immobilized and suspended cells after 25 d ($n = 3$; $p = 0.01$), and the taxol content of the immobilized cells was fourfold that of the suspended cells at d 35.

The microenvironment favored cell growth within the first 15 d (Fig. 1). The PAL activity of rapidly growing cells is generally low *(23)*. With the intensification of cell organization, the microenvironment became unfavorable for cell growth (Fig. 1). Thus, the activity of PAL, an induced

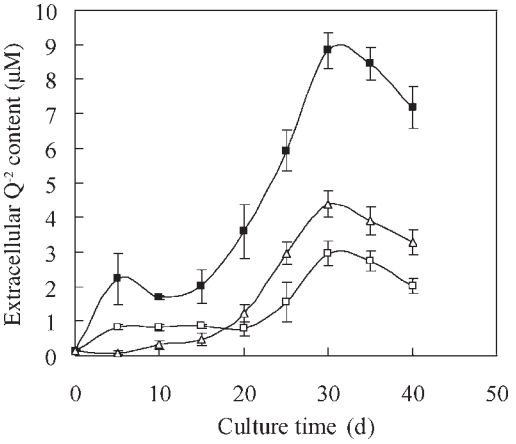

Fig. 6. Change in $O_2^{-\bullet}$ content in extracelluar medium as function of time: (□) outside microenvironment; (■) inside microenvironment; (△) suspension medium.

enzyme, started to increase to defend the stress of the deteriorated microenvironment after 20 d. Furthermore, because PAL is also the key enzyme in the general phenylpropanoid pathway forming the side chains during taxol biosynthesis, the increased PAL activity promoted taxol production.

Effect of Immobilization on Superoxide Anion ($O_2^{-\bullet}$) in Extracellular Medium

Figure 6 shows that the $O_2^{-\bullet}$ content inside the microenvironment was higher than that outside the microenvironment and in the suspension until d 40 ($n = 3$, $p < 0.01$). The $O_2^{-\bullet}$ concentration gradient between the inside and outside microenvironment was more significant after 25 d than before.

$O_2^{-\bullet}$ is mainly generated by a plasma membrane–located NAD(P)H oxidase transferring electrons from intracellular NADPH to extracellular molecular oxygen and is unable to penetrate through the biologic membrane, owing to its negative charge *(28)*. Therefore, $O_2^{-\bullet}$ mostly exists outside cells *(28)* and probably confers its effect on the cell membrane *(29)*. Direct cell interaction at high density alters the cell redox state *(22)*. Immobilization might activate membrane NAD(P)H oxidase to produce a moderate amount of $O_2^{-\bullet}$ in the microenvironment before 15 d. After 20 d, the $O_2^{-\bullet}$ had a sharp accumulation induced by the microenvironmental stress. The excess amount of $O_2^{-\bullet}$ would attack the cell membrane lipid, causing lipid peroxidation (Fig. 3) and increasing cell membrane permeability (nonintegrity) (Fig. 4).

Effect of Immobilization on Intracellular H_2O_2 Content

Figure 7 shows that the H_2O_2 content of immobilized cells slightly increased compared with that of the suspended cells in the first 20 d ($n = 3$; $p < 0.05$) and was much higher than that of the suspended cells afterward until 40 d ($n = 3$; $p < 0.01$).

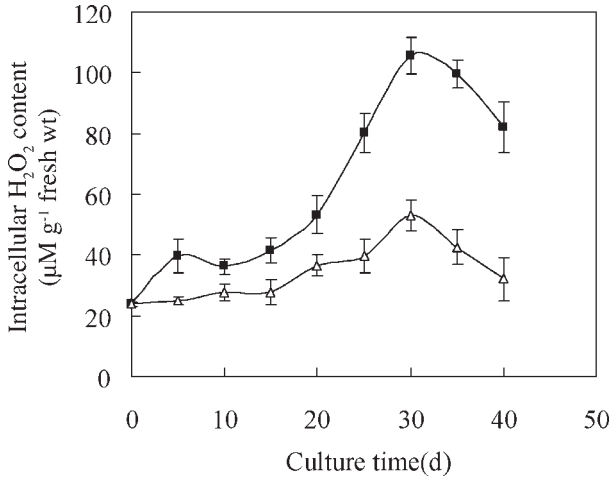

Fig. 7. Change in intracellular H_2O_2 content with cultivation time: (■) immobilized cells; (△) suspended cells.

Immobilization increased cell density and the cells grew as aggregates in the reticulated foam. Direct cell-cell interaction at high density changed the redox state of the cells *(22)*, so the immobilized cells increased H_2O_2 content to challenge the change in the redox state. The increase in H_2O_2 content, to some extent, could trigger the gene expression related to cell proliferation and growth *(30,31)*. When a low content of ROS acted on proliferating cells for a long time, the sustained signal pathway also lasted for a prolonged time *(32)*. The immobilization gave cells with a continuous stimulus, so the moderate increase in H_2O_2 promoted cell growth within the first 15 d (Fig. 1). With the intensification of cell organization, the nutriment limitation became significant in the microenvironment. The H_2O_2 content notably increased when cells grew under nutriment-limiting conditions *(33)*. At d 30, the H_2O_2 content of the immobilized cells was twice that of the suspended cells. The marked increase in H_2O_2 content promoted the expression of defense genes and secondary metabolisms *(34)*, favoring taxol production.

Effect of Immobilization on H_2O_2 Content in Extracelluar Medium

Figure 8 shows that H_2O_2 content inside the microenvironment was obviously higher than that outside the microenvironment and in the suspension before 40 d. The H_2O_2 content gradient between the inside and outside microenvironment was larger after 20 d than before.

The H_2O_2 content in the microenvironment moderately increased owing to the change in the redox state of the immobilized cells in the first 15 d. With the worsening of nutriment limitation, significant H_2O_2 accumulation occurred in the microenvironment after 20 d. At d 30, the H_2O_2 content in the microenvironment was 230 and 169% that outside the microenvironment and in the suspension, respectively. The significantly increased H_2O_2

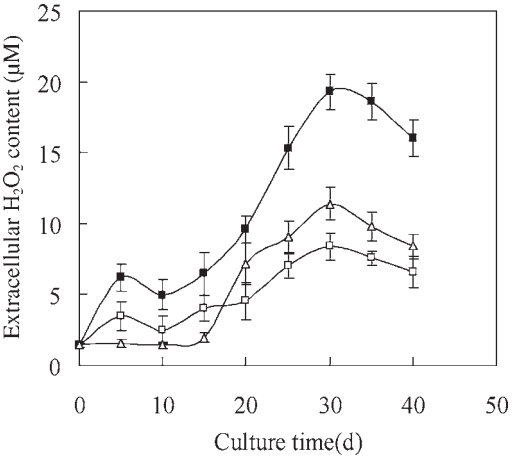

Fig. 8. Change in H_2O_2 content in extracellular medium with time: (□) outside microenvironment; (■) inside microenvironment; (△) suspension medium.

content in the microenvironment adapted to defend the stress of the adverse microenvironment.

The immobilization provided cells with a peculiar microenvironment, regulating the cell growth (Fig. 1) and secondary metabolisms (Fig. 5A,B). However, the extracelluar H_2O_2 in the microenvironment may potentially affect the immobilized cells, which needs to be further studied by investigating the effect of exogenous H_2O_2 on the liberated cells to understand better the physiologic mechanisms of H_2O_2 in the microenvironment.

Change in Intracellular H_2O_2 Content in Liberated Cells

Figure 9 shows that the H_2O_2 content in liberated cells tended to decrease with increasing time. At 2 h, the H_2O_2 content of the liberated cells was near that of the suspended cells. This further demonstrates that the immobilization conferred cells on the persistent stimuli and did promote the increase in intracellular H_2O_2 content. Therefore, the subsequent experiments were conducted by adding exogenous H_2O_2 to the medium every 2 h for six times to investigate the effect of exogenous H_2O_2 on cell membrane permeability and secondary metabolisms.

Effect of Exogenous H_2O_2 on Cells

Figure 10 shows the effects of exogenous H_2O_2 on MDA content, cell membrane permeability, PAL activity, and taxol content.

H_2O_2 at 15 and 20 μ*M* barely affected MDA content and cell membrane permeability (Fig. 10A,B), which is similar to the observation of Han and Yuan *(29)* in their inhibition study.

By contrast, the exogenous H_2O_2 obviously affected PAL activity and taxol production (Fig. 10C,D). The taxol contents of the suspended cells treated with 15 and 20 μ*M* H_2O_2 were, respectively, 115 and 164% that of the

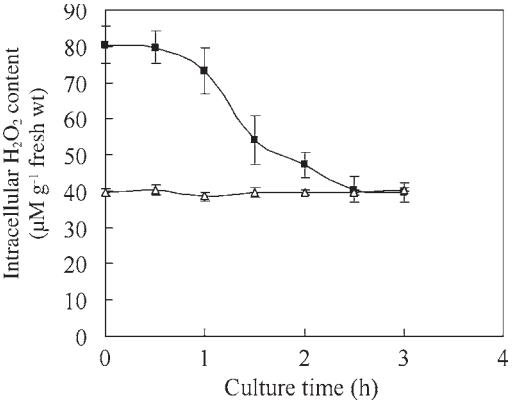

Fig. 9. Change in intracellular H_2O_2 content of liberated and suspended cells with cultivation time: (■) liberated cells; (△) suspended cells.

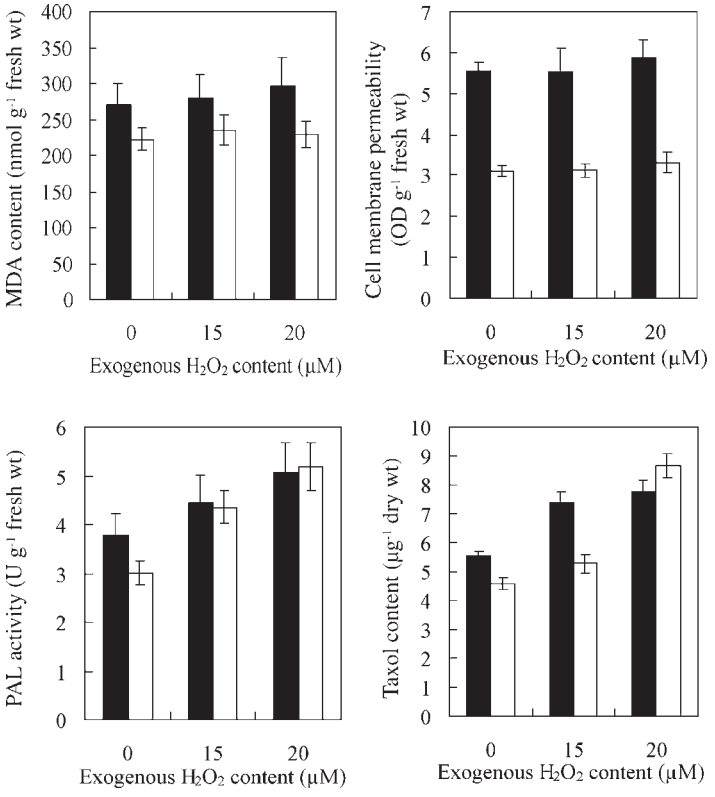

Fig. 10. Effects of exogenous H_2O_2 on cell membrane permeability, MDA content, PAL activity, and taxol production. Exogenous H_2O_2 was added to the medium every 2 h for six times to obtain the final concentrations at 15 and 20 μ*M*, respectively. Both the immobilized and suspended cells were cultured for 20 d. **(A)** MDA content was tested at 2 h after the sixth addition of H_2O_2: (■) liberated cells; (□) suspended cells. **(B)** Cell membrane permeability was tested at 2 h after the sixth addition of H_2O_2: (■) liberated cells; (□) suspended cells. **(C)** PAL activity was tested at 2 h after the sixth addition of H_2O_2: (■) liberated cells; (□) suspended cells. One PAL unit was defined as the change in absorbency of 0.1/min at 290 nm, and the enzyme activity was expressed as units per gram fresh weight. **(D)** Taxol content was analyzed at 24 h after the sixth addition of H_2O_2: (■) liberated cells; (□) suspended cells. OD = optical density at 600 nm.

cells without H_2O_2 treatment. Similarly, the taxol contents of the liberated cells treated with 15 and 20 μ*M* H_2O_2 were, respectively, 131 and 138% that of the cells without H_2O_2 treatment. The exogenous H_2O_2 may function as an extracelluar elicitor to activate the secondary metabolisms, increasing taxol production. Zhao et al. *(25)* reported that the sole addition of exogenous H_2O_2 increased indole alkaloid production of *Catharanthus roseus* cells. Han and Yuan *(29)* showed that exogenous H_2O_2 promoted PAL activity of secondary metabolisms. It can be concluded that the addition of exogenous H_2O_2 did promote taxol production regardless of the suspended or liberated cells.

Relation Between ROS and Taxol Production

The ROS burst induced by biotic and abiotic elicitors could activate cell defense genes and secondary metabolisms *(3,25,29)*, promoting taxol production *(3)*. The significantly increased intracellular H_2O_2 content in the immobilized cells was relevant to the defense metabolisms.

The exogenous H_2O_2 obviously promoted PAL activity and taxol production (Fig. 10C,D). Different from the conventional culture systems of free cells *(19,35)*, immobilization provided cells with a peculiar spatial microenvironment, where cells were confined within the reticulated foam, suffering from the sustainable stimuli and nutriment limitation. Moreover, H_2O_2 was the most stable product that freely penetrated through the cell membrane and entered into cells *(36)*, so the released H_2O_2 in the microenvironment may serve as an elicitor promoting secondary metabolisms and taxol biosynthesis of the adjacent cells after 20 d. The signal transudation pathway of activating the secondary metabolisms by the released H_2O_2 may differ from that mediated by the intracellular H_2O_2 *(35)* and deserves further study. Therefore, the release of H_2O_2 in cells and inside the microenvironment may be one factor in the promotion of taxol production of the immobilized *T. cuspidata* cells.

Conclusion

Immobilization moderately increased the intracellular H_2O_2 content of *T. cuspidata* cells in the first 15 d, favoring cell growth. Afterward, both H_2O_2 and $O_2^{-\bullet}$ were accumulated excessively, and the MDA content and cell membrane permeability increased significantly. The taxol content of the immobilized cells was three times higher than that of the suspended cells at d 35. The addition of exogenous H_2O_2 obviously promoted taxol biosynthesis. It is thus inferred that the intracellular and extracellular H_2O_2 in the microenvironment might be one factor leading to the increase in taxol production under the immobilization stress.

Acknowledgments

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