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Biological Responses of Suspension Cultures of *Taxus chinensis* var. *mairei* to Shear Stresses in the Short Term

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Abstract

The biological responses of *Taxus chinensis* var. *mairei* suspension cultures to a range of shear rates were investigated in a Couette-type shear reactor. It was found that the shear rate below 458 s^{-1} enhanced the primary metabolism, increasing mitochondrial activity and protein expression and inhibiting the activity of phenylalanine ammonia lyase (PAL), an enzyme relevant to the secondary metabolism. However, the shear rate over 719 s^{-1} damaged taxus cells to some extent, decreasing the mitochondrial activity, increasing the membrane permeability, and even causing cell hypersensitive responses. As a result, PAL and intracellular peroxidase, an enzyme in cells relevant to reactive oxygen species scavenging, were activated and extracellular phenolics were accumulated. Additionally, shear-induced accumulation of free radicals and alkalization of the culture medium were observed. The results might shed light on a better understanding of the mechanism of plant cells responses to shear stress through the signal transduction pathway.

Index Entries: Suspension culture; *Taxus chinensis* var. *mairei*; shear; biological activity; secondary metabolism; hypersensitive response.

Introduction

Plant cell cultures have been regarded as a promising alternative to traditional cultivation methods or chemical synthesis routes for the production of many pharmaceuticals, fragrances, flavors, and dyes *(1,2)*. However, plant cells are sensitive to fluid shear stress, which affects cell

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growth and metabolism and, thus, the production of the desired secondary metabolites. The sensitivity of plant cell suspension cultures to shear stress has been attributed to the large size, rigid wall, and large vacuole as well as the aggregation of cells *(3,4)*. This is one of the main factors limiting the scale-up of plant cell cultures.

The methods for study of the effects of shear stresses on plant cells can be roughly divided into two kinds: (1) The cells are exposed to shear forces under growth conditions during the whole cultivation; (2) the cells are exposed to well-defined shear forces (e.g., laminar or turbulent flow in Couette, capillary, or submerged jet devices) for a short period of time, generally under nongrowth conditions *(1,5)*. The effects of shear stresses on plant cell cultures include the reduction in cell viability *(6–8)*, release of intracellular components *(9–11)*, changes in metabolism *(9,12,13)*, cell morphology, and aggregation patterns *(14–16)*.

It is well known that under some environmental stresses (pathogenic invasion, mechanical stimulation, etc.), plant cells can activate defense genes through the signal transduction pathway. Via enhancing a series of metabolic responses that produce some secondary metabolites such as phytoalexin, lignin and so on, plant cells can alleviate the external injury through these hypersensitive responses (HRs) *(17,18)*.

Phenylalanine ammonia lyase (PAL), one of the key enzymes in phenylpropanoid metabolism pathway, takes on an important role in regulating the syntheses of secondary metabolites such as lignin and phytoalexin. Additionally, it is relevant to plant resistance. Phenolics, a kind of phytoalexin against external stimulation, are synthesized at higher rates in hypersensitive reacting cells. They can strengthen the cell wall and kill pathogens, such as a toxin. However, when phenolics accumulate over a certain level, they might be unfavorable to cell growth and metabolism.

Some biotic and abiotic stresses can induce H^+ and Ca^{2+} influxes, alkalization, and oxidative burst (OXB) in plant cell suspension cultures *(19–21)*. H^+, Ca^{2+}, and reactive oxygen species (superoxide radical [$^{\bullet}O_2^-$], hydrogen peroxide [H_2O_2], and the hydroxyl radical [$OH^{\bullet}$]) can transduce signals and cause a series of metabolic responses in cells as secondary messengers. The mechanical stimulation can induce OXB *(22)*, leading to a large accumulation of reactive oxygen species (ROS) in cells. Suitable levels of ROS are favorable to strengthening cell wall, enhancing secondary metabolism, and inducing expression of defense genes. However, cells will suffer from oxidative stress when the accumulation of ROS reaches a higher level. Among various enzymes involved in the scavenging of ROS, peroxidase (POD) is one of the key enzymes, as it participates in the breakdown of H_2O_2 *(23)*. Plant cells can increase POD activity to protect themselves against oxidative stresses *(24)*.

It is thus presumed that the fluid shear stress stimulates the surface of cells as do the other environmental stresses, transducing the signals into protoplast and inducing various cell defense responses. However, most studies of the effects of shear stresses on plant cells focused on the investi-

gation of the extent of physical damages of cells and the productivity of the objective products *(25)*; little attention is paid to considering the shear stress as a stimulating signal of metabolic control and the pathway of the shear signal transduction.

In this work, the biological responses of *Taxus chinensis* var. *mairei* suspension cultures to a range of shear rates were investigated in a Couette-type shear reactor by measuring the biological activities and metabolic effects so as to have a better understanding of the mechanism of the signal responses of plant cells to shear stress.

Materials and Methods

Cell Line and Culture Conditions

The cell line *Taxus chinensis* var. *mairei* (Y416-Shi-T) was provided by the Institute of Botany, Chinese Academy of Sciences (Beijing, China). All of the 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 fresh modified B5 medium (350 mL) 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.

Apparatus and Experimental Procedures

The Couette-type shear reactor consisted of two concentrically arranged cylinders with radii of 55 and 60 mm, respectively. The height of the reactor was 160 mm. In order to minimize the end effects, the bottoms of two cylinders were set as close as possible and the sample port was set at 10 mm above the bottom of the outer cylinder. The outer cylinder was static and the inner cylinder was operated at speeds of 150–750 rpm. Although the Reynolds number *(26)* and Taylor number *(27)* indicated that the flow fields in our experiments varied from the laminar region to the transition region, the laminar shear rate was calculated as a guideline for comparison. The shear rate γ was estimated approximately according to the simplified equation *(28)*

$$\gamma = 2\Omega/(1 - K^2)$$

where Ω is the angular velocity and K is the ratio of the inner to outer cylinder radius. Using this calculation method, rotational speeds of 150, 350, 550, and 750 rpm corresponded shear rates of 196, 458, 719, and 981 s^{-1}, respectively.

The shear reactor was placed at in as sterile an environment as possible, and the temperature was maintained at 25°C by recirculating water. The 150-mL suspension sample of *Taxus chinensis* var. *mairei* (cell density: 150 g fresh weight [FW]/L) subcultured for 5 d was directly loaded into the shearing reactor in the exponential growth phase. Then, the reactor was rotated at the predetermined speed and 15-mL samples were withdrawn

hourly for various analyses. All data were the average of triplicate experiments and the errors were within ±10%.

Measurement of Cell Viability

The samples from the reactor were filtered through a funnel at a reduced pressure and the fresh cells were collected and weighed. Part of the fresh cells was freeze-dried at –50°C and the dry weight was measured.

The mitochondrial activity was evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC) assay *(29)*. The relative mitochondrial activity was expressed as the ratio of absorbance of the cell sample from the shearing reactor to that from the shake flask.

The permeability (or nonintegrity) of the cell membrane was analyzed using Evans blue dye following the procedure of Suzuki et al. *(30)*.

Analysis of Phenolics

A 5-mL filtrate of suspension sample was mixed with 10 mL ethyl acetate, and then 5 mL supernatant (rich in ethyl acetate) was collected and left at 25°C for evaporation. The residual was dissolved in 3 mL of 75% ethanol, and the extracellular phenolics were analyzed spectrophotometrically at 280 nm *(31)*.

Extraction of Related Enzymes and Activity Assay

Fresh cells (250 mg) were frozen in liquid nitrogen first. After 2 mL of 0.1 *M* phosphate buffer, pH 6.0, containing EDTA (2 mmol/L), dithiothreitol (4 mmol/L), and polyvinyl pyrrolidone (2%, w/w) were added, the frozen cells were ground with a mortar and pestle. The mixture was homogenized at 4°C and then centrifuged at 10,000*g* for 25 min. The supernatant was collected for enzyme assay.

The activity of PAL was evaluated at 290 nm according to the procedure described by Yuan et al. *(21)*. POD activity was assayed at 470 nm using guaiacol as a substrate *(32)*. Soluble proteins were determined according to the Bradford method with bovine serum albumin as a standard *(33)*.

ESR Spectroscopy

Accumulation of free radicals in cells was measured by an electron spin resonance (ESR) spectrometer *(21,34)*. 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. Relative signal amplitudes were calculated from the peak-to-trough values of the $g = 2.00$ feature.

Results

Effect of Shear on Cell Biological Activities

Figure 1 is the time-course of the mitochondrial activity at different shear rates. At a shear rate of 196 s^{-1}, the mitochondrial activity was stimulated to a level of approx 120% higher than that of the control cells. Similar findings have been reported by Dunlop et al. *(6)* for the suspension of

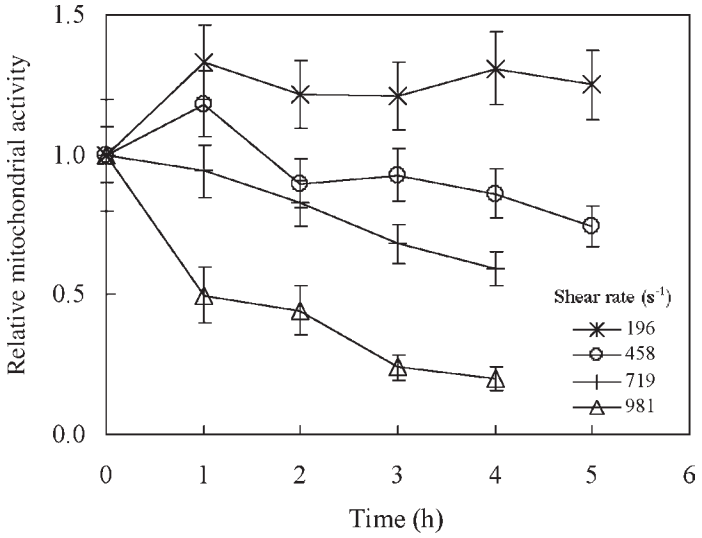

Fig. 1. Time-course of mitochondrial activity at different shear rates.

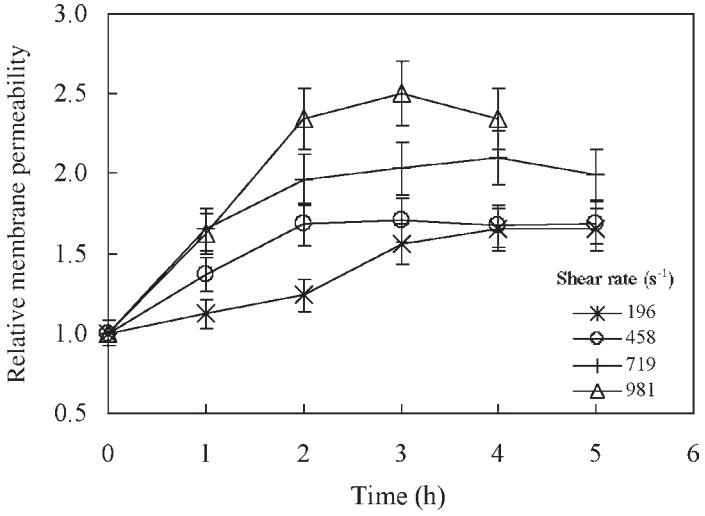

Fig. 2. Time-course of membrane permeability at different shear rates.

Daucus carota and by Scragg et al. *(35)* for the suspension of *Catharanthus roseus*. These phenomena show that the shear rate at an appropriate level is favorable to cell growth and primary metabolism, possibly by creating a suitable size distribution of cell aggregates favoring mass transfer. At a shear rate of 458 s^{-1}, the mitochondrial activity less changed within 5 h compared to that of control cells. However, the mitochondrial activity decreased with time when the shear rate was further increased (over 458 s^{-1}).

Figure 2 is the time-course of the permeability (or nonintegrity) of the cell membrane at different shear rates. It is evident that the permeability of cell membrane increased almost linearly with time within the first 2 h, and,

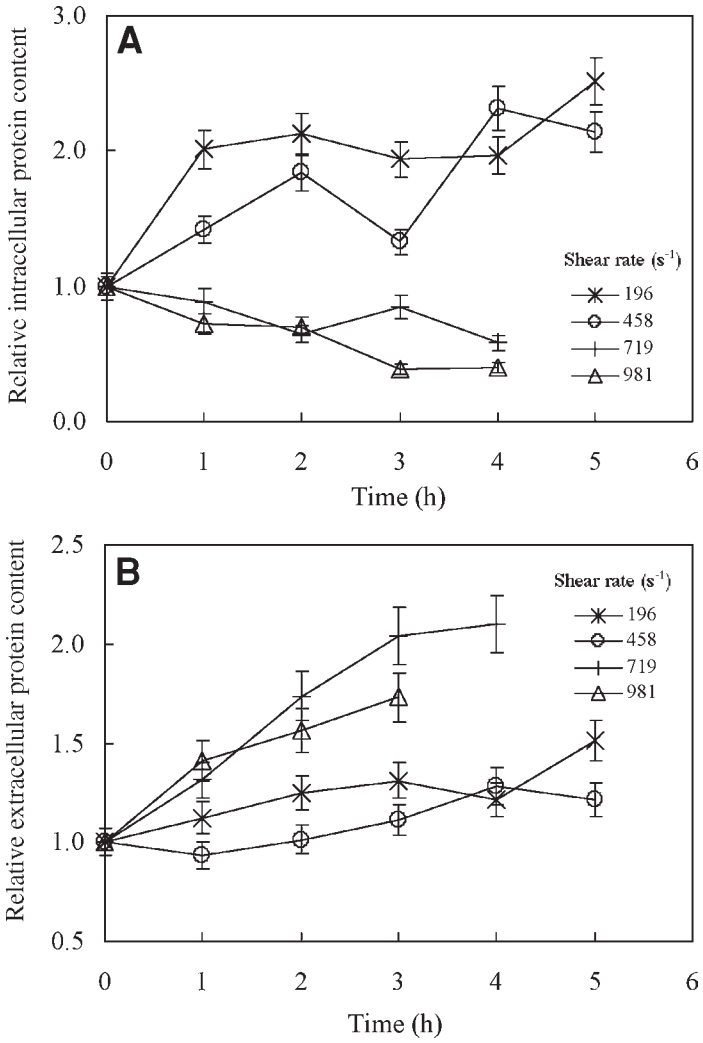

Fig. 3. Time-course of intracellular **(A)** and extracellular **(B)** soluble protein contents at different shear rates.

afterward, it became less changed. In the beginning of exposure to shear, the cell membranes most sensitive to the shear were first damaged, so the permeability of the cell membranes increased rapidly with time. Afterward, the remaining cells could withstand the shear to some extent and thus the changes of membrane permeability became less remarkable. Markx et al. *(7)* reported a similar observation by measuring the dielectric permittivity of plant cell suspensions undergoing a high shear stress.

Effect of Shear on Cell Metabolism

Extracellular/Intracellular Soluble Proteins

Figure 3 is the time course of the contents of intracellular and extracellular soluble proteins at different shear rates. Figure 3A indicates that the

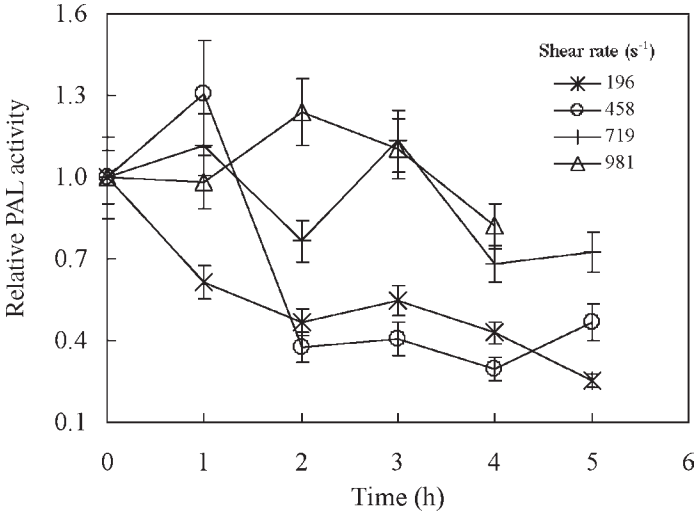

Fig. 4. Time-course of PAL activity in cells at different shear rates.

content of intracellular soluble proteins tended to increase with exposure time when the shear rate was below 458 s^{-1}, whereas it tended to decrease with time when the shear rate was over 719 s^{-1}. It is worth mentioning that the contents of intracellular proteins at the two higher shear rates (719 and 981 s^{-1}) were very close, and a similar phenomenon was observed for those at the two lower shear rates (196 and 458 s^{-1}). The content of intracellular proteins was higher than that of control culture at lower shear rate and the result was reverse at higher rate.

Figure 3B shows that the content of the extracellular soluble proteins tended to increase with exposure time. At a lower shear rate, the variation in the content of extracellular proteins was not very significant compared to that at higher shear rate. Similarly, the contents of the extracellular proteins at the two higher shear rates were very close and a similar phenomenon occurred in the case of the two lower rates. It should be noted that the content of extracellular proteins was larger than that of control culture regardless of the shear rate.

Thus, at a lower shear rate, both the contents of intracellular and extracellular proteins increased, indicating that a lower shear rate was favorable to cell growth and primary metabolism, resulting in the syntheses of proteins, possibly including expressions of some enzymes and syntheses of some new proteins such as the stress proteins. Namdev and Dunlop *(2)* and Prokop and Bajpai *(36)* reported the syntheses of proteins in yeast and in *Nicotinana tabacum* cultures as a consequence of cell responses to the fluid shear stress, respectively.

PAL Activity

Figure 4 shows that at a lower shear rate (below 458 s^{-1}), the PAL activity was inhibited rapidly within the first 1–2 h, then became less

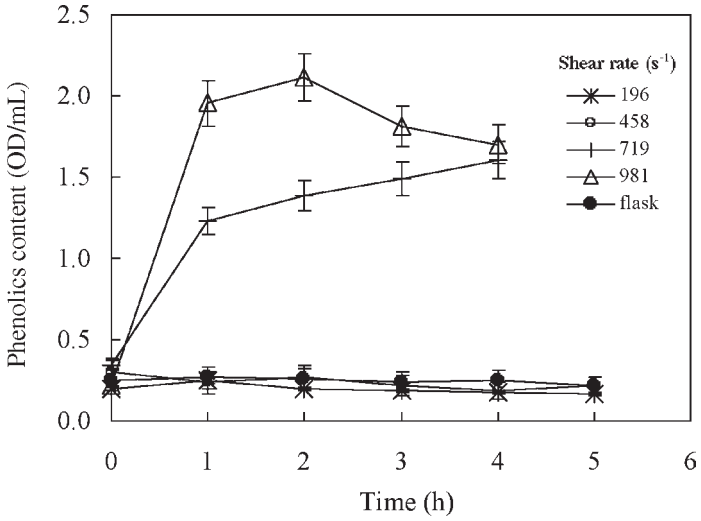

Fig. 5. Time-course of extracellular phenolics concentration at different shear rates.

changed. In contrast, at a higher shear rate (above 719 s^{-1}), the PAL activity tended to be promoted, resulting in the occurrence of the peak of PAL activity.

Extracellular Phenolics Accumulation

Figure 5 shows that at a lower shear rate, the concentration of extracellular phenolics was almost maintained at a constant level, less affected by the shear rate. Contrarily, at a higher shear rate, the phenolics concentration significantly increased with the exposure time, especially within the first 1 h. Moreover, when the shear rate was as high as 981 s^{-1}, the phenolics concentration began to decrease with exposure time after 2 h.

Extracellular/Intracellular POD Activity

Figure 6 shows the time-courses of extracellular and intracellular POD activities. From Fig. 6A, it is seen that at a lower shear rate (below 458 s^{-1}), the extracellular POD activity changed less with the exposure time. However, a higher shear rate (above 719 s^{-1}) led to a marked decrease in extracellular POD activity within the first 1–2 h; then, the activity maintained a relative low level. Furthermore, the higher the shear rate, the more the decrease. Inhibition in extracellular POD detectable in the culture medium of shear-treated cells was apparent at the shear rates higher than 458 s^{-1}.

Figure 6B indicates that at a lower shear rate (below 458 s^{-1}), the activity of intracellular POD in cells was less affected by the exposure time. In contrast, at higher shear rates (above 719 s^{-1}), the intracellular POD activity was significantly promoted with the exposure time. There occurred a peak of the activity of intracellular POD at 2 h after the shear treatment

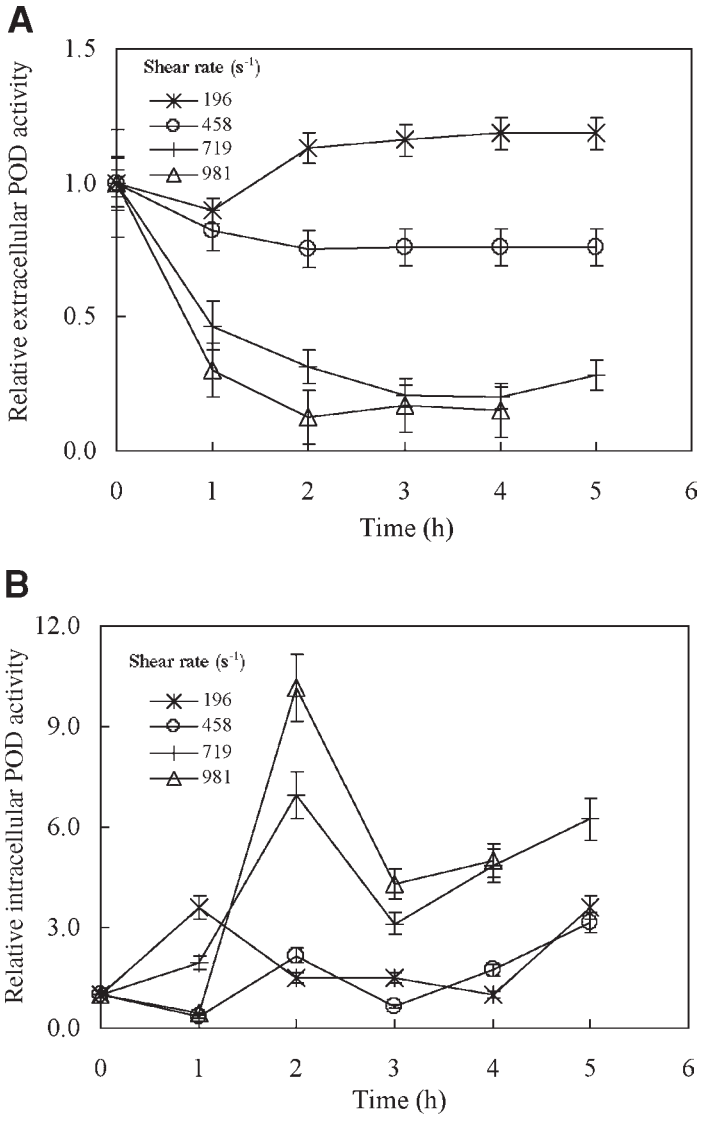

Fig. 6. Time-course of extracellular **(A)** and intracellular **(B)** POD activities at different shear rates.

(above 719 s^{-1}), and then following a little inhibition, the intracellular POD activity was slightly promoted again.

From the above results, it seems that there existed a critical shear rate, beyond which some deleterious effects on the primary metabolism of cells such as loss in mitochondrial activity, increase in membrane permeability, promotion in PAL activity, inhibition in extracellular POD, activation in intracellular POD activity, and release of intracellular proteins and phenolics occurred. On the basis of the experimental data, the critical shear rate should be around 458 s^{-1}.

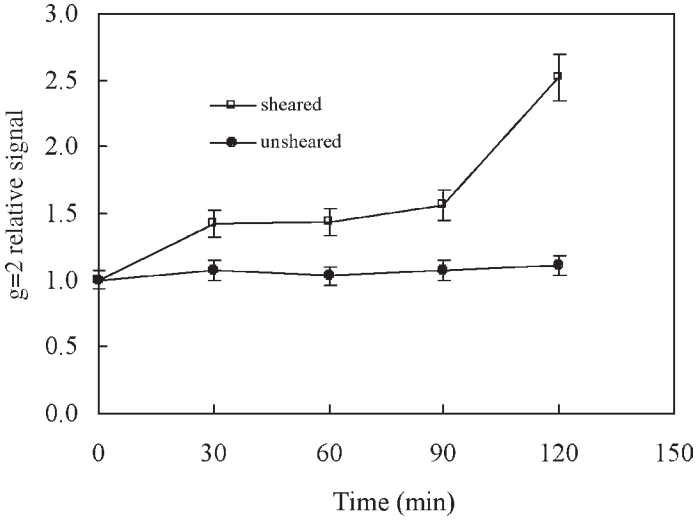

Fig. 7. Shear-induced generation of a free-radical signal in suspension cultures of *Taxus chinensis* var. *mairei* in the exponential growth phase (shear rate: 589 s^{-1}). The accumulation of free radicals in cells was measured using an ESR spectrometer described in the Materials and Methods section.

Free-Radical Accumulation

Figure 7 shows that the shear rate of 589 s^{-1} induced a distinct accumulation of free radicals in taxus cells during the 120-min treatment. The ESR signal intensity in shear-treated cells was about 2.5-fold higher than that of control cells at 120 min.

Effect of Shear on Extracellular pH of Suspension Cultures

Taxus cell suspension culture medium at all the shear rates were alkalized during 1 h of the shear treatments (*see* Fig. 8). Moreover, the higher the shear rate was applied, the more rapidly the pH of the suspension culture medium increased. After that, extracellular pH decreased slowly, except for that under the 981-s^{-1} shear rate, which led to a fast decrease of pH.

Discussion

Effect of Shear on Cell Membrane Integrity and Permeability

A lower shear rate (below 458 s^{-1}) hardly affected cell membrane integrity and permeability, thus less influenced the content of extracellular proteins (*see* Fig. 3B). In contrast, a higher shear rate (above 719 s^{-1}) caused an increase in cell membrane permeability and even the breakup of cells, resulting in the release of some intracellular substances such as phenolics and proteins *(37)*. The membrane function might change. Therefore, the content of extracellular proteins and phenolics rapidly increased and large amounts of cell debris were observed. Necrosis of many cells occurred. One possibility is altered plasmalemma function during the hypersensitive

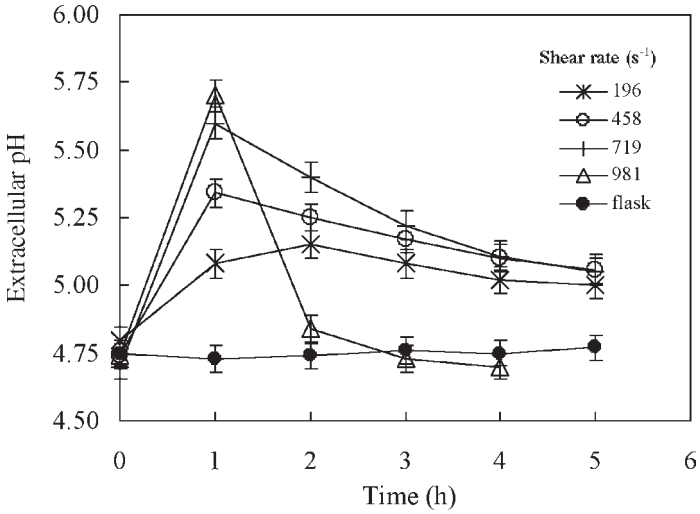

Fig. 8. Effects of the different shear rates on extracellular pH in the *Taxus chinensis* var. *mairei* suspension cultures.

response *(34)*. At a shear rate of 981 s^{-1}, about 70% of cells were completely damaged after 3 h (data not shown).

It was observed that at a lower shear rate or a shorter exposure time, the cell aggregates were of smaller size and dispersed more homogeneously compared to the control system in shake flasks. In contrast, at a higher shear rate (above 981 s^{-1}), the cell aggregates were hardly observed after 3 h. Instead, large quantities of single cells and cell debris occurred. In addition, the plasmolysis was also found by microscopic observation of the suspension samples dyed with Evans blue. The occurrence of cell debris reduced the number of cells available for dying, leading to the decrease in absorbance of the dyed cell sample (*see* Fig. 2). As the broken and dead cells lost the ability of producing phenolics, the amount of phenolics began to decrease after 2 h at a shear rate of 981 s^{-1} (*see* Fig. 5).

Because of the increase in cell membrane permeability and even the breakup of cell membrane and vacuole, the H^+ and other acid substances in cells were released into suspension culture medium, which resulted in the decrease of pH. Furthermore, at the shear rate of 981 s^{-1}, a large amount of cells were broken up quickly, thus the extracellular pH of the suspension cultures declined rapidly (*see* Fig. 8).

Effect of Shear on Cell Growth and Primary Metabolism

Proteins are the main composition of plant cells. They have many special biological functions and play a key role in the life action of plant cells. The content of proteins in plant cells, the activity of mitochondria, and the extent of membrane integrity are closely related to the growth and primary metabolism of plant cells. Under the action of a high shear rate, the mitochondrial activity of taxus cells was inhibited, the cell membrane was

damaged, and the intracellular proteins content was decreased, which might lead to unfavorable effects on the cell growth and primary metabolism. The intensity of shear at an appropriate level enhanced the cell growth and primary metabolism, increasing mitochondrial activity and protein expression and inhibiting the activity of PAL and the syntheses of phenolics.

Effect of Shear on Secondary Metabolism and Defense System

At a higher shear rate (above 719 s^{-1}), the taxus cells were adversely affected, leading to the increase in PAL activity and, subsequently, the enhancement of the secondary metabolism. As a result, the ability of resistance against environmental stresses improved. As the products of phenylpropanoid metabolism, therefore, phenolics synthesized and released rapidly (*see* Fig. 5). However, when the accumulation of phenolics reaches a certain level, they might be unfavorable to taxus cell growth and metabolism. At a higher shear rate, the taxus cells also established a defense system, possibly by promoting the activity of polyphenoloxidase (PPO) that oxidized phenolics. Chen and Huang *(38)* reported that the PPO activity was promoted by high shear stress in the suspensions of *Stizolobium hassjoo*.

The shear stimulation signals might be recognized by receptors or mechanosensors on the cell surface and cause a hypersensitive response, followed by OXB. Generally, $^{\bullet}O_2^-$ can be converted into H_2O_2 by dismutation with or without superoxide dismutase (SOD), and H_2O_2 can be broken down by POD. The inhibition in extracellular POD activity (*see* Fig. 6A) might be beneficial to the formation and accumulation of H_2O_2 outside of the plasma membrane from dismutation of $^{\bullet}O_2^-$, and then H_2O_2 would enter into cells through plasma membrane as a second messenger to transduce signals. However, the increase in the activity of intracellular POD (*see* Fig. 6B) might indicate that the accumulation of H_2O_2 in cells reached a higher level so that H_2O_2 itself would hurt cells; thus, taxus cells would decompose excessive H_2O_2 and eliminate oxidative stress by promoting the activity of intracellular POD. The sustaining high activity of intracellular POD in the experiments might imply the possible accumulation of H_2O_2 in cells. Figure 7 shows that shear can induce a distinct accumulation of free radicals in taxus cells during the treatment. Additionally, the time-course of POD activity (*see* Fig. 6) further confirms the occurrence of the oxidative burst in cells. These findings suggest that the responses involve production of H_2O_2 and perhaps other ROS, which could spawn free-radical events. It is thus presumed that H_2O_2 is one important component of shear-induced ROS.

Additionally, Anderson et al. also observed a distinct inhibition in the POD activity in the culture medium and a rapid generation of free radicals in the bean suspension cells during 12 h of elicitor treatment *(34)*. This phenomenon is very similar to our experiments and might indicate that there is a similar effect to some extent on ROS in plant cells between mechanical stimulation and biotic elicitation.

The taxus cells suspension culture medium became alkalized under the action of shear (*see* Fig. 8). This might indicate that the induction of shear opened the H^+ channels, leading to the influx of H^+, which will transduce signals to protoplast and cause a series of signal responses as a second messenger *(2,38)*. Additionally, these data indicate the changes in plasmalemma functions and lipid peroxidation and might also suggest that cellular necrosis may result from altered membrane functions that are the consequence of increasing levels of free-radical accumulation *(34)*.

General Remarks

The shear stress could not only directly damage the taxus cells resulting in a viability loss but also cause defense responses leading to the changes of secondary metabolism. Thus, the taxus cells might respond to the shear rate in a way similar to some extent to the mechanism that plant cells respond to other environmental stimuli such as biotic elicitation. H_2O_2 and H^+ might be involved in the pathway of shear signal transduction into the physiological responses of cells. Takeda et al. *(39)* reported that cytosolic calcium mediates the signal of the hydrodynamic stress into the metabolism as a second messenger. Recent research indicates that the second messengers (H^+, Ca^{2+}, H_2O_2, etc.), cell cytoskeleton proteins *(40)*, and wall–plasmalemma–cytoskeleton continuum *(41)* play very important roles in the perception and transduction of signals induced by environmental stresses. Additionally, the mechanical stimulation can be recognized by mechanosensors on the surface of plant cells. Bogre et al. *(42)* reported that the mechanosensor is a mitogen-activated protein kinase (MAPK) and can be activated after suffering a mechanical stimulation of 1 min.

Therefore, in order to have a better understanding of the effects of shear stress on plant cells, further research should be centered on the examination of the mechanism by which the fluid mechanical signals are converted into a biochemical response and of the messengers involved in the transduction of the external stimulus to the plant cells. Among them, the questions concerning signal messengers and pathways as well as the roles they may play should be answered first.

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