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Age-related responses of suspension cultured *Taxus cuspidata* to hydrodynamic shear stress

Yan-Wen Gong, Shu-Ying Li, Rong-Bin Han, Ying-Jin Yuan *

Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, P.O. Box 6888, Tianjin 300072, PR China

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Abstract

Hydrodynamic shear sensitivity is considered one of the obstacles for large-scale plant cell culture. Understanding the mechanism of shear sensitivity might assist the industrial application of plant cell culture. Here we investigated the responses of suspension cultured *Taxus cuspidata* in different culture phases under shear stress using a Couette reactor. It was found that the pH in medium and peroxide hydrogen (H_2O_2) in cells increased more rapidly in the exponential phase than that in lag phase under shear stress. The pH and H_2O_2 concentration in the exponential phase were also higher than those in the lag phase. Inhibition studies showed that there existed a 30–45 min delay in the action time of G-protein, Ca^{2+} channel and phospholipase C of *T. cuspidata* cells in the lag phase than that in the exponential phase. Age-related different membrane fluidity and H^+-ATPase activity may partially contribute to the observed responses. These early responses might be indicators for selecting shear-resistant cell lines and for cell damage caused by shear stress.

Keywords: Hydrodynamic stress; Plant cell culture; Bioreactors; Large-scale cultivation; Physiology; Membrane fluidity

1. Introduction

Plant cell biotechnology focusing on the production of plant secondary metabolites has evolved as a promising new area within the field of biotechnology in the past two decades [1,2]. One obstacle of large-scale cell culture is the cell damage caused by hydrodynamic shear under aeration and agitation [3]. The negative effects of hydrodynamic shear on cell growth and production of secondary metabolites have been widely investigated [3,4]. It was shown that various plant cell lines exhibited significant different biological responses to shear stress and the responses varied with cultivation age [5–7]. Age-related different responses to shear stress are commonly attributed to cell/aggregate size. Meijer et al. suggested that shear susceptibility was also related to history, maintenance conditions, and cultures may become "adapted" to growth under high shear conditions [7]. However, little is known about the mechanism involved in the different biological responses of plant cells in different cultivation age to hydrodynamic shear stress.

Recent work focusing on defense mechanisms in plant cells provides a new insight to understand the age-related biological responses to shear stress [8]. Experiments have demonstrated that generation of reactive oxygen species (ROS) were the early response while plant cells were exposed to mechanical stress and other stresses [9–11]. Our previous studies have shown that shear stress was directly related to ROS generation and cell death in suspension cultures of *Taxus cuspidata* (*T. cuspidata*) [10]. In general, shear stress induces the activation of G-protein, which may stimulate Ca^{2+} ion channels and phospholipase. These signal molecules then enhance NADPH oxidase activity and cause ROS generation [9,10]. Besides the role of molecule to activate defense responses, ROS also caused cell death. Stavreva and Gichner found DNA damage induced by hydrogen peroxide (H_2O_2) was dependent on the cell growth stage in tobacco cells [12]. Changes of pH and membrane fluidity were also early defense responses to shear stress and cause metabolite variation [13,14]. Therefore, we presume that the responses in the early stage may account for the age-related responses. These early responses may be a criterion for cell shear sensitivity. To date, there has been no systematic comparison of the defense responses between different cultivation ages while plant cells are exposed to hydrodynamic shear stress.

* Corresponding author. Fax: +86 22 27403888.
E-mail addresses: yjyuan@public.tpt.tj.cn, yjyuan@tju.edu.cn (Y.-J. Yuan).

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In this work, we presented the early responses of suspension cultured *T. cuspidata* in the lag and exponential stages at shear rate with 0.141 Pa. The studies also provide a new insight for shear sensitivity studies and provide a criterion for cell susceptibility under shear stress.

2. Materials and methods

2.1. Cell line and culture conditions

T. cuspidata cells were maintained on solid B_5 medium at 25 °C in the dark. The suspension cultures of *T. cuspidata* were grown in a modified B_5 medium with shaking at 110 rpm. The pH of the medium was adjusted to 5.8 before sterilization. Suspension cultures were sub-cultured every 10 days for five generations before the experiments. The *T. cuspidata* cells underwent a lag phase growth within the first 4 days of sub-culture and an exponential growth phase between 5 and 15 days.

2.2. Apparatus and experimental procedures

The Couette-type shear reactor was composed of two coaxial cylinders with the height of 300 mm. The radii of the cylinders were 25 and 26.5 mm, respectively. The sample port was set at 10 mm above the bottom of the outer cylinder. The outer cylinder was kept static and the inner cylinder was rotated at 50 rpm during experiments. Laminar shear rate τ was calculated according to the equation [15]:

$$\tau = \frac{2\pi\mu RN}{\delta}$$

where N is the rotating rate, R the radius of inner cylinder, δ the space between inner and outer cylinders, μ is the viscosity of culture. The value of μ is 1.62 cP (cell concentration is 0.08 g FW/mL). The shear rate was 0.141 Pa according to the above equation. Our previous studies have shown cells are less damage and have phenomenal effects at this shear rate [10].

The shear reactor was sterilized before inoculation, and the temperature was maintained at 25 °C. A 70 mL suspension sample of *T. cuspidata* sub-cultured for 2 or 10 days was directly loaded into the shearing reactor. The concentration of cells was adjusted to 0.08 g FW/mL in experiments.

2.3. Measurement of H_2O_2 and pH

H_2O_2 concentration was measured by the Ti-NH_3 method. The details of the method were described by Mukherjee and Choudhuri [16]. Extracellular pH was measured at 2 or 10 days after sub-culture. Continuous measurement of pH was performed after addition benzyl alcohol (BA) or activator fusicoccin of H^+-ATPase. Preliminary experiments demonstrated that 5 μM fusicoccin or 18 μM BA was little influence on cell viability within 6 h.

2.4. Measurement of plasma membrane H^+-ATPase

A membrane fraction enriched in plasma membrane was prepared as described by Allesteros et al. [17]. ATP hydrolysis activity was calculated from the amount of inorganic phosphate released at 37 °C for 30 min with a protein concentration 10–15 μg [17]. H^+-ATPase activity was expressed as nmol Pi mg^{-1} protein min^{-1}.

2.5. Measurement of membrane fluidity

Membrane fluidity was determined by FTIR. The details of the method were described by Szalontai et al. [18]. For FTIR measurement, membrane was resuspended in D_2O-based buffer. FTIR measurements were carried out on FTIR spectrometer at 1 cm^{-1} spectral resolution. At near 2850 cm^{-1}, the frequency indicated $\nu_{sym}CH_2$ symmetric stretching vibration. This frequency has been shown as an indicator of membrane fluidity. The increase of $\nu_{sym}CH_2$ frequency indicated the increase of membrane fluidity. To improve the precision, the spectra were fitted with Gaussian components using peak fitting module (Origin 7.0 software).

2.6. Statistical analysis

The results presented were the means of three independent experiments. Sample variability was given as the standard error of the mean.

3. Results and discussion

3.1. Age-related pH increase under shear stress

pH increase in culture medium was observed in both lag and exponential phases after shear stress (Fig. 1). Cell cultures in the exponential phase exhibited a rapid increase of pH within 20 min after shear stress and then became less changed. By contrast, pH in the lag phase started to increase 20 min after shear stress and less changed after 1 h. The changes of pH in lag phase were 50% lower than that in exponential phase.

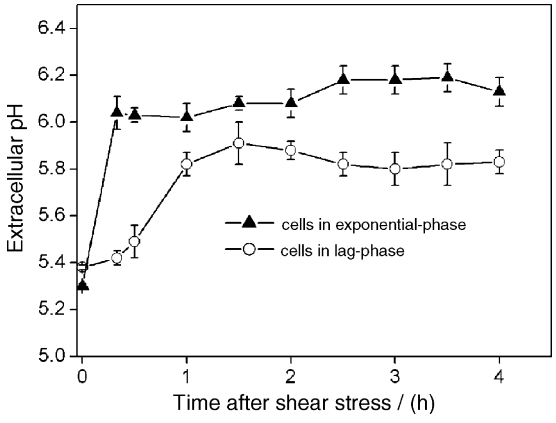

Fig. 1. pH chances of suspension cultured *Taxus cuspidata* in the exponential (10 days, ▲) and lag (2 days, ◯) phases at the shear rate of 0.141 Pa.