Aggregate characteristics of callus derived from woody plant *Eucommia ulmoides*

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**Abstract**

*Eucommia ulmoides* was cultivated in suspended culture and formed aggregates with broad size spectrum. According to the morphological features, density and size, the aggregates were classified into daughter aggregate (DA), meristemoid-like aggregate (MA) and old aggregate (OA). DA can grow into MA, which proliferates DAs again at some vigorously growing points on their surface. Coexistence of DAs and MAs implies better conditions for callus growth. It was revealed that mechanical disintegration of callus into smaller size in the subculture has a negative effect on callus growth due to the damage of active cells located at the aggregate surface and/or the characteristics of original big aggregate which belongs to OA before fragmentation. The growth rates of spontaneous DAs and MAs were about two times higher than that of the disintegrated callus. As an approach for scaling-up process, spontaneously proliferated aggregates can provide the rapidly growing callus of *E. ulmoides* culture compared with the mechanically disintegrated callus.

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**Keywords:** Woody plant cell; *Eucommia ulmoides*; Daughter aggregate; Meristemoid-like aggregate; Old aggregate

1. Introduction

*Eucommia ulmoides* (Tochu in Japanese and Du-Zhong in Chinese), which belongs to Eucommiaceae family, is one of the most famous traditional woody trees growing in Japan and China for nutrition and folk medicine. *E. ulmoides* was confirmed to produce more than 30 active compounds from bark and leaves [1] but considered as one of the least used woody plants for in vitro studies. The leaves contain some factors enhancing collagen synthesis of mice [2], and the raw meat texture of cultured eels was improved by feeding of its leaf powder [3]. A rapid-growing suspension culture of *E. ulmoides* is necessary for its practical application, however, it has not been established. One method to enhance the growth rate of the culture was to provide the callus with large surface area by disintegrating the big aggregates mechanically into smaller ones [4]. The growth rate was still insufficient due to the wound stress to cells, although the mechanical treatment was partially successful. In the present paper, a possible alternative cultivation strategy for *E. ulmoides* cells is discussed based on the observation of the morphological, physical and growth characteristics of the cultured callus.

2. Materials and methods

2.1. Culture conditions

Callus was induced from infant leaves of aseptically grown seedlings of *E. ulmoides*. Suspension cultures of *E. ulmoides* cells were conducted in Gamborg’s B5 medium [5] supplemented with 40 g sucrose and 0.1 mg 2,4-dichlorophenoxyacetic acid (2,4-D) per liter of medium. The medium pH was adjusted to 5.7 before autoclaving. The cells were subcultured every 4 weeks by inoculating 100 ml of fresh medium with 2 g-fresh weight (FW) of inoculum cells to 500 ml Erlenmeyer flasks and kept under about 8000 lx light irradiation. The flasks were maintained at 27°C on a rotary shaker at 100 rpm.

2.2. Cell preparation

Small aggregates of 0.4 g-FW, whose sizes were less than 5 mm in diameter, were inoculated into 100 ml Erlenmeyer flasks.
flask with 20 ml medium. Aggregates of 2 g-FW, whose sizes were between about 10 and 15 mm in diameter, were transferred to 500 ml Erlenmeyer flask with 100 ml medium. The above two size categories are identified later as DA (daughter aggregate) and MA (meristemoid-like aggregate). The control culture was prepared by mechanically disintegrating big aggregates by scalpel and inoculated into 100 ml Erlenmeyer flask with 20 ml medium with same composition mentioned above. The mean initial diameter of disintegrated aggregates was about 1 mm. Three replicate flasks were set up for each treatment and all initial cell densities were adjusted at 20 g-FW per liter.

2.3. Analytical method

Aggregate volumes were measured by measuring the increased amount of water in the 5 and 50 ml glass cylinder, respectively. In the measurement of DA volume, five aggregates with similar size were measured. The FW was measured by weighing the sampled aggregate after draining the medium by filter paper.

2.4. Histological observation

The aggregates were fixed in solution containing 40%(v/v) ethanol, 4%(v/v) formaldehyde, 3%(v/v) propionic acid and 3%(v/v) acetic acid for 48 h. Afterwards, they were dehydrated by using 2-methoxyethanol, absolute alcohol, n-propanol and n-butanol. Each step was carried out for 12 h at 4 °C. Finally, they were embedded into histoparaffin (Wako Pure Chem. Ind. Ltd., Osaka, Japan) according to the previous method [6]. Thin serial sections were cut using an ultramicrotome (Yamato Kohki Ind. Co., Saitama, Japan). The samples were stained using toluidine blue (0.05%)

Fig. 1. Morphological characteristics of *E. ulmoides* aggregates: (A) in flask culture; (B) the different categories of aggregates: upper line—daughter aggregate (DA); middle line—meristemoid-like aggregate (MA); lower line—old aggregate (OA); (C) outer view of MA; (D) cross-sectional view of MA with inner cavity; (E) protruded region on MA surface; and (F) MA surface without the protruded region, respectively. Scale bars indicate (A), (B), 20 mm, (C), (D), 10 mm and (E), (F), 0.1 mm.
in citrate buffer at pH 4.1 and observed under an optical microscope (BH-2, Olympus Co., Tokyo, Japan).

2.5. Image analysis

The aggregates were transferred into a sterile Petri dish in the clean bench. Projected images of the aggregates in Petri dish placed on the grid sheet were obtained by using a digital camera (Olympus Co., Japan). The image analysis software was Scion Image for Windows (Scion Co., MD, USA) and the cross-sectional areas of all aggregates were determined after setting the scale and digitalizing the image. The aggregate volume was obtained by Eq. (1):

\[ V = \frac{4}{3}\pi \left(\frac{A}{\pi}\right)^{\frac{3}{2}} \]

where \( V \) and \( A \) are the mean aggregate volume and the cross-sectional area, respectively.

3. Results and discussions

3.1. Morphological aspects

During cultivation, the callus grew in aggregates of different sizes as shown in Fig. 1A and B. Morphologically, it was observed that some of the callus aggregates (meristemoid-like aggregate: MA) produced small aggregates (daughter aggregate: DA) that had appeared on MA surface and sloughed off into the medium. These newly formed small aggregates grew in size. MAs kept their stuffed structure till a late stationary phase of the culture. The callus that ceased to produce DAs grew in size continuously but finally went into the old callus (old aggregate: OA) that became soft and friable with a cavity at the center as the nutrient exhaustion in the inner part took place. The MA had an irregular shape with cavity in the middle of aggregates at the end of cultivation (Fig. 1C and D), while DA was spherical or elliptical shape with a smooth surface as shown in Fig. 1B. These observations point out that the cells at the surface of MA were heterogeneous in its growth activity.

To obtain more knowledge about the callus features, histological observation was conducted. Obviously small and densely packed cells located orderly at the superficial layer (ca. 400 \( \mu \)m) of MA consisting of meristemoid-like cells (Fig. 1E). In more internal part from the aggregate surface, parenchyma-like cells existed disorderly. In addition, the meristemoid-like cells were abundant at the protruded region and resulted in pushing away the thin epidermal layer (Fig. 1F). Moreover, in the examination of DA, no cavity was observed at the inner part of aggregate and the cells were completely condensed (data not shown). A practical culture undergoes the coexistence of DAs and MAs which implies the higher activity in the growth cycle of \( E. ulmoides \) aggregate.

3.2. Aggregate density

The relationship between aggregate volume (\( V \)) and fresh weight (FW) are shown in Fig. 2. At aggregate volume less than about 1.2 ml, the slope was about 1.03, which was similar to an individual cell density. This result indicates that most aggregates of this region are thickly packed. In the volume region between 1.2 and 1.5 ml, that FW/V was approximately 1.00. The decline of the slope was due to the presence of cavity in the aggregate. The cells at the edge and in the center of a large aggregates experience different environments owing to diffusion limitations of nutrient uptake such as carbon source and/or oxygen. Consequently, the inner part of aggregates was decayed [7]. Assuming that the cavity formation is caused by material diffusion limitation, the diffusion coefficients of sucrose and dissolved oxygen were evaluated to be \( 2.45 \times 10^{-2} \) and \( 7.56 \times 10^{-2} \) cm\(^2\) h\(^{-1}\), respectively (Appendix A). These values are comparable to those reported so far in similar situations [8–10].

3.3. Aggregate volume

In suspension culture, there were various sizes of aggregates as shown in Fig. 1A. The aggregates were classified into four categories as described in the morphological aspects. Comparing with the density data, the category can be quantitatively related to the aggregate volume. The volumes of DA, MA\(_1\), MA\(_2\), and OA were less than 0.5 ml (ca. 1.03 of density), between 0.5 and 1.5 ml (1.00 ≤ density ≤ 1.03), between 1.5 and 4.0 ml (density < 1.00), and larger than 4.0 ml (density < 1.00), respectively. MA\(_2\) and MA\(_3\) are the subcategory of MA, which was defined by the morphological observation. DA grew up to MA\(_1\) then to MA\(_2\), which contains cavity. The images of MA\(_2\) are shown in

Fig. 2. Volume and fresh weight correlation in various types of \( E. ulmoides \) aggregates. DA, MA and OA indicate daughter, meristemoid-like and old aggregates, respectively. A broken line shows the line with slope 1. Abbreviations above the graph are same as those in Fig. 1.
Fig. 3. Aggregate size distribution during the cultivation. Z-axis indicates the frequency of aggregate number. DA and MA indicate initial aggregate types, (A) daughter aggregate and (B) meristemoid-like aggregate, respectively.

Fig. 4. Time course of aggregate volume. Initial aggregate type was meristemoid-like aggregate. Each symbol indicates each aggregate. Abbreviations beside the graph are same as those in Fig. 1.

3.4. Aggregate growth

The aggregate growth curves were shown in Fig. 5. The growth behaviors for both kinds of aggregates, DA and MA, are almost similar to each other. The growth rates of DA and MA were about two times higher than the maximum growth rate of mechanically disintegrated callus [4], even though the initial concentrations were same. It is clear that the disintegration process disturbs callus growth probably due to the damage of active cells located at the aggregate surface or the characteristics of big original aggregate which has al-
ready become OA before fragmentation. On the other hand, spontaneous proliferation of aggregates keeps the structure integrity of callus tissue, which plays a vital role in the growth improvement. Considering the rapidly growing callus, selection of DAs and/or MAs can provide an alternative preparation for \textit{E. ulmoides} culture rather than the mechanically disintegrated callus.

4. Conclusions

Callus of woody plant, \textit{E. ulmoides}, was cultivated in suspension culture, where it grew up consisting of three types of aggregates, i.e., daughter aggregate (DA), meristemoid-like aggregate (MA) and old aggregate (OA), which were defined based on the morphological and physical characteristics. The growth rates of DA and MA were about two times higher than those of mechanically disintegrated callus. Using DA and MA will be a promising strategy for the culture seed to overcome the slow cell growth rate of \textit{E. ulmoides}.

Appendix A

Assuming the aggregate shape is sphere and the substrate mass transfer in the aggregate to follow the diffusion mechanism, the mass balance for respective substrates can be described as follows.

\[ D_i \frac{d}{dr} \left( r^2 \frac{dC_i(r)}{dr} \right) = r_i \rho \]  
(A.1)

where \( D_i \), \( C_i(r) \), \( r_i \), and \( \rho \) are intracellular diffusion coefficient of substrate, substrate concentration, distance from the aggregate center, consumption rate of substrate and aggregate density, respectively.

The boundary conditions are written as follows:

\[ C_i(r) \bigg|_{r=R} = C_{ib} \]  
(A.2)

\[ \frac{dC_i(r)}{dr} \bigg|_{r=0} = 0 \]  
(A.3)

where \( C_{ib} \), and \( R \) are the substrate concentration in the bulk and the aggregate radius, respectively.

Assuming that the consumption rate of substrate is constant, the substrate concentration, \( C_i(r) \), can be expressed as Eq. (A.4) from Eq. (A.1) under Eqs. (A.2) and (A.3).

\[ C_i(r) = C_{ib} + \frac{r_i \rho}{6D_i} (r^2 - R^2) \]  
(A.4)

From Eq. (A.4), the effective diffusivity, \( D \) is evaluated by Eq. (A.5) by assuming the substrate concentration being zero inside the cavity.

\[ D = \frac{r_i \rho}{6C_{ib}} (R^2 - R_c^2) \]  
(A.5)

where \( R_c \) is the cavity radius.

References


