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# Secretion of a bundle of (1→3)-β-glucan hollow fibrils from protoplasts of callus suspension under a $Ca^{2+}$-rich and acidic stressed condition

**Abstract:** A fiber bundle composed of hollow fibrils is secreted from protoplasts of white birch (*Betula phatyphylla* Sukatcher var. *japonica* Kan No. 8) leaves callus and the secretion is more efficient in an acidic medium containing high concentration of $Ca^{2+}$. The influence of the $Ca^{2+}$ concentration and pH has been investigated concerning the number of cells secreted. The secreted hollow fibers were very thin. The $^{13}C$ nuclear magnetic resonance spectra indicated that the fibers are composed of linear (1→3)-β-glucan without branches (callose). The potential utilization of such unique callose fibers and their optimized production needs more investigation.

**Keywords:** calcium ion, callose, callus, fiber spinning, hollow fibrils, protoplast

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## Introduction

Physicochemical stress alters the crystalline structure in the transition stage between primary and secondary walls in developing cells (Kataoka and Kondo 1996, 1998, 1999). The effects of stress were investigated also in leaves under various conditions of the culture medium. Interestingly, the protoplasts of higher plants could be stimulated under a certain culture condition in the presence of high concentration of $Ca^{2+}$ to produce gigantic fibers consisting of callose [(1→3)-β-glucan] (Kondo et al. 2000; Sasamoto et al. 2003; Fukumoto et al. 2005; Seyama et al. 2008; Seyama and Kondo 2012). The callose fibers consist of a bundle of hollow fibrils (Seyama et al. 2008). Callose has a linear chain composed of (1→3)-β-linked D-glucopyranosyl residues, and it is, in general, laid down at the cell plate during cytokinesis and in the cell wall during pollen development (Meikle et al. 1991). It is also synthesized in response to wounding and infection by pathogens or in the presence of high concentration of aluminum ion (Jacobs et al. 2003). In the in vitro synthesis of callose with an isolated membrane fraction including the synthase, a short fibrillar structure was formed with a few 10 nm in width (Him et al. 2001; Pelosi et al. 2003; Colombani et al. 2004). However, the unique hollow hierarchical structure in long and large fibers described by Seyama et al. (2008) was not yet observed by others.

To understand the regulatory mechanism of secretion of the callose hollow fibrils would be relevant from the scientific point of view. To this end, the present study is aiming to establish a fiber production system with higher efficiency than the previous one. Callus suspension culture, instead of *Betula* leaves, should be observed. Then, the callus fibrils should be characterized and the results will be discussed to callose hollow fibrils obtained previously from *Betula* protoplasts.

## Materials and methods

### Materials

The plantlet of *Betula platyphylla* Sukatchev var. *japonica* Kan No. 8 was employed for preparation of the callus. The plant was irradiated with visible light at 25°C (all wavelength range, 3000–4000 lux): 16 h irradiation and 8 h darkness.

### Preparation of callus and their protoplasts

*Betula* petioles were placed on agar with MS medium according to Murashige and Skoog (1962), including hormones with 2 mM α-naphthalene acetic acid (NAA) and 1 mM *N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (4PU), for the induction of the callus for 2 months at 25°C and shaken with 200 rpm. The spent medium in each flask was replaced with a fresh medium weekly. After 3 weeks, the protoplasts

were isolated by a treatment with 0.6 M mannitol solution containing the 1% of cellulase Onozuka R-S (Yakult Co., Ltd., Tokyo, Japan) and 1% Maserozyme R-10 (Yakult). After the enzymatic treatment, the suspension including the protoplasts was filtered on a sheet of nylon mesh with a pore size of 40 μm. After washing with 0.6 M mannitol, the protoplast precipitates were collected by centrifugation (800 rpm, 150 g, 3 min). Then, an appropriate amount of fresh 0.6 M mannitol solution was added again for further centrifugation, and this treatment was repeated twice.

## Induction of fiber secretion from protoplasts

The initial culture condition was selected as described earlier (Kondo et al. 2000; Sasamoto et al. 2003; Fukumoto et al. 2005; Seyama et al. 2008; Seyama and Kondo 2012); that is, the obtained protoplasts were cultured in the one-half strength of the standard MS liquid medium, including the hormones (1 mM NAA and 1 mM 4PU), and then 150 mM $CaCl_2$ was added to the culture medium with pH adjusted to 3.5. The cell densities were adjusted to approximately $5\times10^4$ cells $ml^{-1}$ in the culture well (equivalent to 2500 cells per well); incubation at 28°C in the dark. The protoplasts in the culture plate were observed with an inversed light microscope (CK40; Olympus Co., Tokyo, Japan). The parameters (pH, $CaCl_2$ concentration, enzyme solutions for protoplast isolation, and number of subcultures) were controlled.

## $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy

The fibers secreted from the protoplasts into the culture medium were collected with a micromanipulator, washed with sterilized distilled water, and placed in a small tube. $D_2O$ was added to exchange the solvent from $H_2O$ to $D_2O$. The fibers were then dissolved in 1.5% NaOD/$D_2O$ solution. The reference [curdlan powder (linier (1→3)-β-glucan) from *Alcaligenes faecalis* (Sigma-Aldrich Co. LLC, Tokyo, Japan)] was directly dissolved in 1.5% NaOD/$D_2O$. Both samples were centrifuged at the rotation rate of $1.5\times10^4$ rpm for 10 min and the supernatants were submitted to NMR analysis. Spectra were recorded with a 400 MHz NMR spectrometer (JNN-AL400; JEOL, Tokyo, Japan) using a 5 mm NMR probe. The $^{13}C$ NMR spectra were obtained from 24 k scans with a pulse delay of 2 s. All measurements were performed with $d_6$-acetone as an internal standard.

## Transmission electron microscopy (TEM) observations

The secreted fibers were collected, washed with sterilized distilled water, and dehydrated with an ethanol (EtOH) series of 30%, 50%, 70%, 90%, and 100% aqueous EtOH before embedding in an LR white resin. After the sample specimens were treated at 50°C for 24 h to allow the resin to be polymerized, the obtained embedded samples were sectioned with <100 nm in thickness with an ultramicrotome (LKB Co., Japan). As a structural characterization by immunolabeling with a monoclonal antibody, the samples on the 3.0 mm TEM grids were then subjected to immuno-gold labeling: (1) treatment with 100 ml of the blocking solution including the callose antibody (Meikle et al. 1991) as a primary antibody at 30°C for 2 h or at 4°C overnight, and (2) after washing with the serum-free blocking solution, the sections were incubated at 30°C for 2 h in 100 ml of the same blocking solution including a secondary antibody of mouse anti-rabbit immunoglobulin G that was labeled with a gold particle of 20 nm in diameter.

# Results and discussion

The secretion of callose subfibrils from protoplasts of *Betula* leaves required a long culture period of approximately 2 months (Seyama et al. 2008; Seyama and Kondo

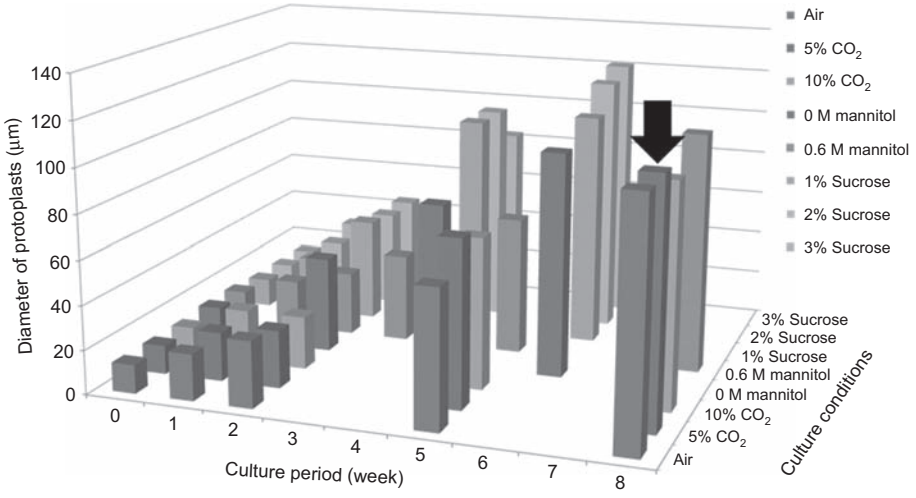


**Figure 1** Protoplast size in diameter cultured in several conditions.
The arrow indicates an accelerating condition of the fiber secretion from *Betula* protoplasts estimated by considering protoplast sizes enlarging as culture period proceeded.

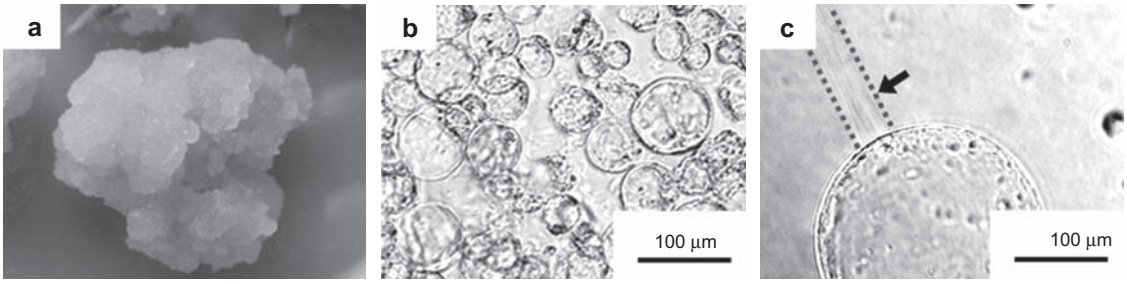


**Figure 2** Light microscopic images of callus from *Betula* (a), its protoplasts prepared from the callus (b), and an expanded protoplast secreting a fiber (c).
The arrow indicates secreting a fiber.

2012). For accelerating the process, the initial culture condition was changed in terms of sugar contents, $CO_2$ concentration, and culture periods and the protoplast diameters were measured. The diameter size in enlarged protoplasts is related to the initiation of fiber secretion, that is, as soon as protoplasts reach 100 μm in diameter, they start to secrete a fiber (Seyama and Kondo 2012). A better effect for accelerating the callose fiber production (Figure 1) was observed at 5% $CO_2$ atmosphere without mannitol (0 M) and with a low concentration of sucrose as a carbon source. However, the effect was weak so that the material produced was not enough for structural and biological analysis. Therefore, the callus of *Betula* was taken as a starting material instead of the leaves.

Within 4 weeks of inoculation, callus was proliferated from *Betula* petiole (Figure 2a). The protoplasts isolated from the callus in a mannitol solution of (0.6 M) with 1% cellulose Onozuka R-S and 1% Maserozyme R-10 are shown in Figure 2b. The callus protoplasts successfully secreted a fiber in 1 month.

The callus protoplasts secreted a fiber (Figure 2c) under the fiber secretion condition from *Betula* leaves described previously (Kondo et al. 2000; Sasamoto et al. 2003; Fukumoto et al. 2005; Seyama et al. 2008; Seyama and Kondo 2012). Afterward, the parameter pH, $CaCl_2$ concentration, and enzyme solutions were reexamined for a number of subcultures.

Figure 3 shows the number of the callus protoplast secreting a fiber as a function of culture conditions. The optimal conditions are as indicated by an arrow, that is, pH 3.5 and $CaCl_2$ concentration of 150 mM, for protoplasts isolated by the enzymatic treatment of 1% cellulose Onozuka R-S and 1% Maserozyme R-10. Under this condition, eight times more fiber was produced than from leaf protoplasts. Moreover, the induction period of spinning fibers from callus protoplasts was reduced by 50% when compared with the performance of leaf protoplasts. A fiber was spun only from one site of the surface of each protoplast (as was the case of protoplasts from leaves), and the fiber formed a gigantic callose bundle

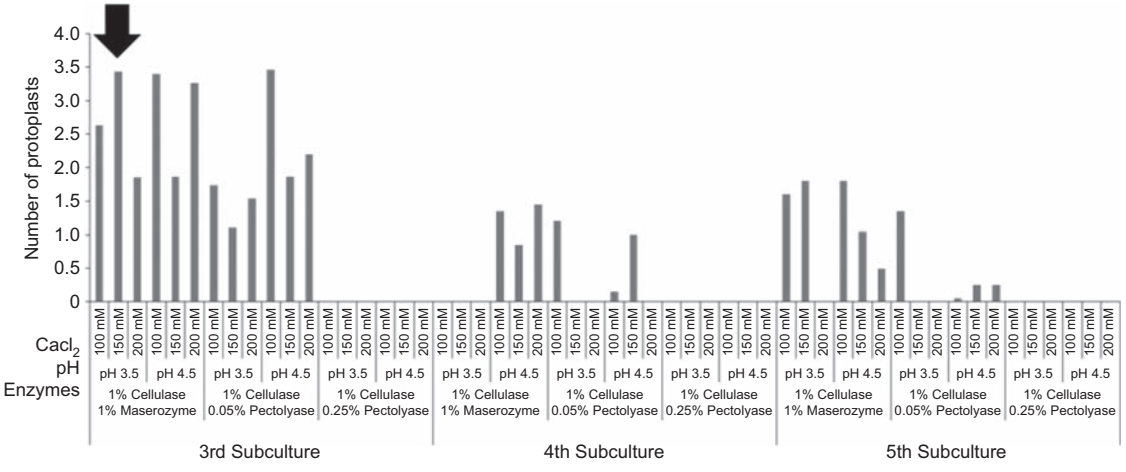


**Figure 3** Number of callus protoplasts secreting a fiber cultured in several conditions.
The arrow indicates an optimal fiber-secreting condition at pH 3.5 with the $CaCl_2$ concentration of 150 mM using protoplasts isolated by the enzymatic treatment of 1% cellulose Onozuka R-S and 1% Maserozyme R-10.

compared with other polysaccharide fibers reported by Brown et al. (1976).

As reported by Seyama et al. (2008), the $^{13}C$ NMR spectra of spun fibers and curdlan (reference) are in agreement (Figure 4). Accordingly, the main component of the fibers spun is (1→3)-β-glucan without branches (callose). It is well known that $Ca^{2+}$ induces the secretion of callose as reported by Kauss (1987, 1996). The callose is a substance produced at the interface of a plasma membrane in response to wounding, pathogen infection, and chemical and/or mechanical damages. Ohana et al. (1992) showed that β-furfuryl-β-glycoside (FG) is an endogenous activator for (1→3)-β-glucan synthase and the enzyme activation coincides with the transferring of FG from the vacuole to the cytoplasm (Ohana et al. 1993). This glycoside is proposed to be an allosteric effector, binding at a different site to that of $Ca^{2+}$ and thus inducing conformational changes in the enzyme. We have hypothesized that the pH change could be the primary factor responsible for the changes in the cytoplasmic levels of FG. A lower cytoplasmic pH would tend to suppress the activity of the antiporter responsible for the uptake of FG into the vacuole. If the rate of synthesis of FG remained constant in the cytoplasm, then the amount of FG available to activate callose synthase would increase, and as the synergism with elevated $Ca^{2+}$, callose synthesis would be initiated. In our study, the $Ca^{2+}$ and low pH seem to induce such a callose synthesis in the protoplasts.

The diameter of the fibers secreted from callus protoplasts was approximately 14 μm, whereas the fibers secreted from the leaf protoplasts were approximately 30 μm (Seyama et al. 2008). The TEM observation of the ultrathin sections of the fibers with an immune gold-labeled antibody is presented in Figure 5. The secondary antibodies with gold nanocolloids (10–20 nm in diameter) attached with the initial (1→3)-β-glucan antibodies

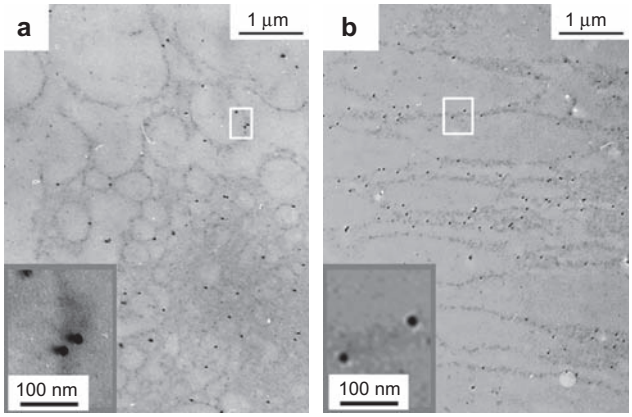


**Figure 5** TEM images of a bundle of callose hollow subfibrils with the callose immuno-gold particles labeling with monoclonal antibody to (1→3)-β-glucan.
The black dots indicate the deposition of the gold particles. Oblique sections (a) and longitudinal sections (b). The insets exhibit the enlarged images of the individual sites with white rectangles.

are visible. In the oblique section, the gold nanocolloids are concentrated on the elliptic frames having a diameter ranging from a few hundred nanometers to a few micrometers, whereas they were also concentrated on the column frames in the longitudinal section. Thus, the TEM images indicate that the gigantic callose fibers are composed of a bundle of nanoscaled and microscaled hollow fibrils. The size of the hollow fibrils is smaller than that from leaf cells. The interfacial area between the secreted fiber and the outlet on the cell is presented in Figure 6. The image may indicate either the very end of a secreted fiber comprising hollow fibers or the outlet shape of protein aggregates on the plasma membrane. The gold nanocolloids attached to callose (arrows) were not found inside of the round-shaped structure. Therefore, the hollow structure

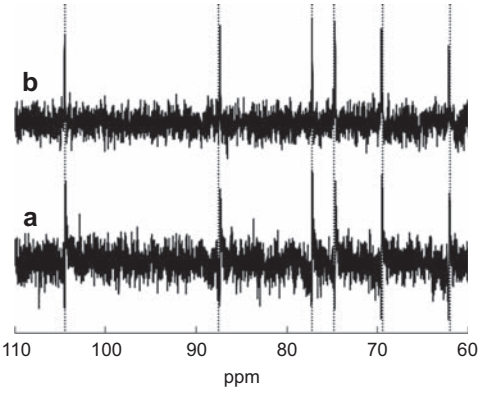


**Figure 4** $^{13}C$ NMR spectra of (a) secreted fibers and (b) curdlan.

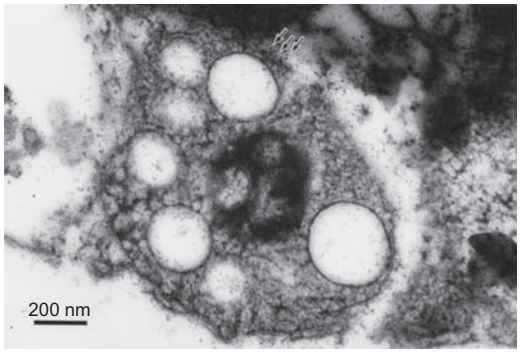


**Figure 6** TEM image of an interfacial area between a secreting fiber and the outlet on the plasma membrane of a cell.
The arrows indicate that callose that was immuno-gold labeled via monoclonal antibody to (1→3)-β-glucan similarly to the TEM image in Figure 5.

proved to be at least continuing to the very end of secreted fibers on the plasma membrane. In the case of in vitro synthesis of callose with an isolated membrane fraction including a callose synthase, a short callose fibrillar structure with a few 10 nm in width found to be formed (Him et al. 2001; Pelosi et al. 2003; Colombani et al. 2004). This indicates that the (1→3)-β-glucan synthesized in a solution was likely to be uniaxially self-assembled for some reasons to result in the formation of callose fibers. The mechanism has not been elucidated; moreover, a mechanism of formation of the hollow fibrils seen in this study has not yet been clarified at all. The present study showed the avenue to a more efficient production of bundles from hollow protoplast fibrils. The future investigation is facilitated concerning the aggregation of synthesizing enzymes on plasma membranes similar to the case of cellulose nanofiber synthesis by the Gram negative bacterium *Gluconacetobacter xylinus* (Brown et al. 1976).

## Conclusion

Callus was regenerated from white birch (*B. platyphylla*) leaves, and then protoplasts were isolated from the callus suspension and cultured under the $Ca^{2+}$-rich acidic stress condition. The fiber production efficiency of the protoplast was essentially improved as manifested in the shorter culture period and increasing number of active cells spinning a fiber. The fiber from callus protoplasts is composed of hollow fibrils of (1→3)-β-glucan without branches. It has a hollow shape even at the end on the plasma membrane throughout the whole secretion. The synthesizing enzymes for the hollow callose fibers are probably located and arranged in a round-shaped geometry on the plasma membrane.

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## References

Brown, R.M. Jr., Willison, J.H.M., Richardson, C.L. (1976) Cellulose biosynthesis in *Acetobacter xylinum*: visualization of the site of synthesis and direct measurement of the in vivo process. Proc. Natl. Acad. Sci. USA 73:4565–4569.

Colombani, A., Djerbi, S., Bessueille, L., Blomqvist, K., Ohlsson, A., Berglund, T., Teeri, T.T., Bulone, V. (2004) In vitro synthesis of (1→3)-β-D-glucan (callose) and cellulose by detergent extracts of membranes from cell suspension cultures of hybrid aspen. Cellulose 11:313–327.

Fukumoto, T., Hayashi, N., Sasamoto, H. (2005) Atomic force microscopy and laser confocal scanning microscopy analysis of callose fibers developed from protoplasts of embryogenic cells of a conifer. Planta 223:40–45.

Him, J.L.K., Pelosi, L., Chanzy, H., Putaux, J.-L., Bulone, V. (2001) Biosynthesis of (1→3)-β-D-glucan (callose) by detergent extracts of microsomal fraction from *Arabidopsis thaliana*. Eur. J. Biochem. 268:4628–4638.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P., Fincher, G.B. (2003) An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 15:2503–2513.

Kataoka, Y., Kondo, T. (1996) Changing cellulose crystalline structure in forming wood cell walls. Macromolecules 29:6356–6358.

Kataoka, Y., Kondo, T. (1998) FT-IR microscopic analysis of changing cellulose crystalline structure during wood cell wall formation. Macromolecules 31:760–764.

Kataoka, Y., Kondo, T. (1999) Quantitative analysis for the cellulose $I_a$ crystalline phase in developing wood cell walls. Int. J. Biol. Macromol. 24:37–41.

Kauss, H. (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu. Rev. Plant Physiol. 38:47–72.

Kauss, H. (1996) Callose synthesis. In: Membranes: Specialized Functions. Eds. Smallwood, M., Knox, J.P., Bowles, D.J. Bios Sci. Publ. Press, Oxford.

Kondo, T., Magoshi, J., Abe, H., Sasamoto, H. (2000) Method for producing of non cellulosic callose fibers from protoplasts and the callose fiber. Japan Patent No. 3936522.

Meikle, P.J., Bonig, I., Hoogenraad, N.J., Clarke, A.E., Stone, A.B. (1991) The location of (1→3)-β-glucans in the walls of pollen tubes of *Nicotiana alata* using a (1→3)-β-glucan-specific monoclonal antibody. Planta 185:1–8.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497.

Ohana, P., Delmer, D.P., Volman, G., Steffens, J.C., Matthews, D.E., Benziman, M. (1992) β-Furfuryl-β-glucoside: an indogenous activator of higher plant UDP-glucose: (1-3)-β-Glucan synthase. Plant Physiol. 98:708–715.

Ohana, P., Benziman, M., Volman, G., Delmer, D.P. (1993) Stimulation of callose synthase in vivo correlates with changes in intracellular distribution of the callose synthase activator β-furfuryl-β-glucoside. Plant Physiol. 101:187–191.

Pelosi, L., Imai, T., Chanzy, H., Heux, L., Buhler, E., Bulone, V. (2003) Structural and Morphological diversity of (1→3)-D-glucans synthesized in vitro by enzymes from *Saprolegnia monoica*. Comparison with a corresponding in vitro product from blackberry (*Rubus fruticosus*). Biochemistry 42: 6264–6274.

Sasamoto, H., Ogita, S., Hayashi, N., Wakita, Y., Yokota, S., Yoshizawa, N. (2003) Development of novel elongated fiber-structure in protoplast cultures of *Betula platyphylla* and *Larix leptolepis*. In Vitro Cell Dev. Biol. Plant 39: 223–228.

Seyama, T., Kondo, T. (2012) Morphological responses of *Betula* protoplasts in fiber spinning. Holzforschung 66:407–411.

Seyama, T., Kimura, S., Sasamoto, H., Abe, H., Kondo, T. (2008) Spinning of a gigantic bundle of hollow fibrils by a spirally moving higher plant protoplast. Planta 227:1187–1197.