Callus Formation, and Effects of Applied Pressure to the Cultured Cambial Explant of Sugi (*Cryptomeria japonica* D. Don)*

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Abstract——The callus formation was investigated with the cultured cambial explants of Sugi (*Cryptomeria japonica* D. Don). Cell proliferation was vigorous in the surface area of the explant comparing with the inner area. Phloem parenchyma cells, ray cells and a few of fusiform cells contributed to the callus formation.

In order to simulate the *in vitro* system of the cambial explant to the inner environment of the growing stem, the culture with radially applied pressure was also attempted. The applied pressure suppressed the cell proliferation in the inner area of the explant. The proliferated tissues under the pressure maintained their ordered cell arrangement. Therefore, the applied pressure was effective to the maintenance of the tissue structure in culture.

Introduction

When a piece of a plant tissue was cultured *in vitro* in a nutrient medium, living cells such as parenchyma cells are generally proliferated and turned to callus tissue with a disturbed cell arrangement. For the maintenance of the structure and function of cultured tissue depressing such callus formation, the *in vitro* system is required to be simulated to the internal environment of the plant body. Many studies[1~3] on the simulation of the *in vitro* system to the internal environment have been mainly focused on chemical factors such as nutrients, hormones, vitamins, etc., while some workers[4~6] suggested that certain mechanical factors, such as pressure, were important as well. The latter suggestion is of interest because, for instance, cambial cells in a growing tree are apparently subjected to compression stress to the radial direction, because these cells are being divided by the tangential plane and expanding radially between the central core of rigid mature xylem and peripheral phloem.

In the present paper, cambial explants of Sugi (*Cryptomeria japonica* D.Don) were cultured *in vitro* and the formation of the callus tissue was microscopically followed in order to investigate the origin of the callus. Moreover, the same cambial explants were also cultured under applied radial pressure for examining the effect of this physical factor on the maintenance of cambial structure and function.

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Materials and Methods

Cambial blocks of ca. 10 mm(L) × 50 mm(T) with mature phloem and xylem, both of which thickness were a few millimeters, were sampled from twelve-year-old stems of Sugi (*Cryptomeria japonica* D. Don) in June and July when the cambium was highly active. The cambial blocks, after soaked in 70% EtOH (30–60 sec), were sterilized in 10% NaOCl solution (5 min) and then rinsed with sterile 0.01 N HCl solution (10 min) followed by sterile H_2O (3 times).

After both radial surfaces of the sterilized cambial blocks were trimmed off to expose the new radial surfaces, the blocks were further divided along radial planes into 10 small blocks (ca. 10 mm × 5 mm) which were designated as cambial explants. The cambial explant thus obtained had the exposed new surfaces not attacked by the sterilizer only on the radial faces.

Slant agar medium for the organ culture in the test tube (25 mmϕ) was consisted of Wolter and Skoog basal medium[7] with hormones and amino acids (Table 1). In the culture without pressure, the cambial explants were planted horizontally,

Table 1. Constituents of medium

Inorganic		Organic	
Compound	mg/l	Compound	mg/l
Na_2SO_4	425	*myo*-Inositol	100
KNO_3	170	Thiamine	0.4
KCl	140	Pyridoxine·HCl	0.4
NaH_2PO_4	35	Nicotinic acid	0.5
$MnSO_4 \cdot 4\text{-}6H_2O$	14.4		
$ZnSO_4$	5.7	L-Arginine	3.5
H_3BO_3	3.2	L-Asparagine	1.0
KI	1.6	L-Aspartic acid	1.0
$MgSO_4 \cdot 7H_2O$	1565	L-Lysine	0.1
$Ca(NO_3)_2$	612	L-Phenylalanine	0.1
EDTA-Na-Fe·H_2O	5.8		
NH_4NO_3	50	2,4-D*	0.1
		NAA**	0.5
		Kinetin	0.1
		Gibberellin	0.5
		Saccharose	30 (g/l)
		Agar	8.6(g/l)

Final pH was adjusted to 5.6–6.0 with 1N NaOH or 1N HCl.

* 2, 4-Dichlorophenoxy acetic acid

** Naphthyl acetic acid

the basal end of which being submerged into the slant medium with the phloem side up and xylem side down (Fig. 1-a). In the culture under applied pressure, the cambial explant was planted similarly to the case of without pressure except followings: a rubber stopper was put on the bottom of a test tube and a stainless steel weight wrapped with Saran Wrap® was put on the phloem face of the explant in order to apply pressure (about 0.05 atm) to cambial zone (Fig. 1-b).

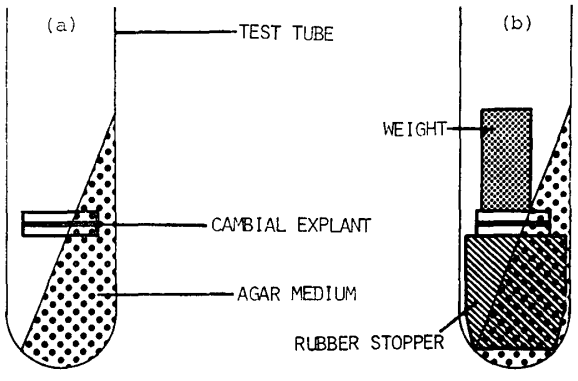

Fig. 1. *In vitro* system of the explant without (a) and under (b) applied pressure to the cambial explant.

Three series of experiments without pressure, and two series with applied pressure were examined. In each experiment, every two explants were harvested at a time after 0, 2, 5, 7, 10, 14, 21 and 30 days in culture, and fixed in FAA. They were dehydrated through a conventional EtOH-H_2O dehydration series and then embedded in celloidin. On every harvest, cross sections (30 μm thickness) were obtained from the middle area of the length of one explant, and radial sections were obtained from both outer (just beneath the radial surface) and inner areas of another one. These sections were stained with safranin-fast green for histological observation.

Results and Discussion

1. Cambial Explant without Pressure

Although expanding cells were microscopically observed on the radial surfaces of the cambial explant after only 2 days in culture, masses of callus visible to the naked eye were first observed after 7–10 days. The cross section in the vicinity of these callus (Fig. 2) shows that callus-like cells were derived from ray- and phloem parenchyma cells with abnormal enlargement and proliferation, and finally covered radial surfaces of the explant. The radial section of this area (Fig. 3) shows that a few of fusiform cells were septated to form strands (Fig. 3, arrow heads). These stranded cells were abnormally proliferated later, but most of the fusiform cells were

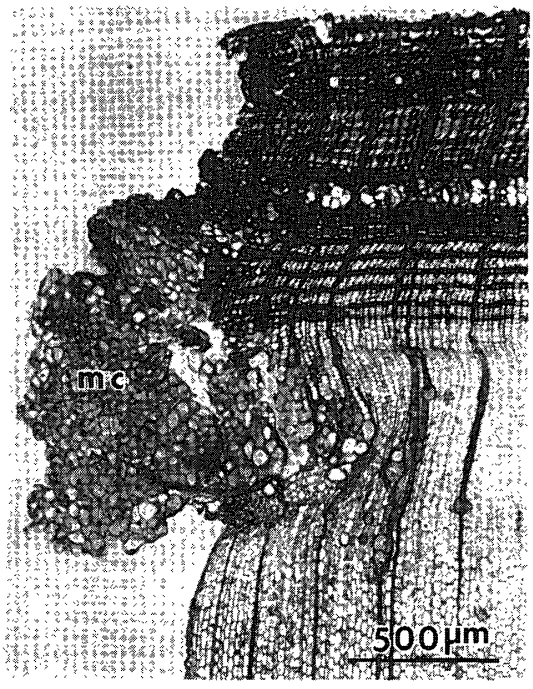

Fig. 2. The cross section of the explant after 7 days in culture without pressure. mc: a mass of callus on the radial surface of the explant.

finally crushed by other proliferated cells (Fig. 3, white arrows), without showing any divisional activity.

Cell proliferation was also observed in the inner area of the explant after about 10 days (Fig. 4, short arrows). However, the extent of the proliferation is not so great as compared with that observed in the surface area showen with longer arrow in Figure 4. Ray parenchyma cells within the mitotic zone began to enlarge after 5–7 days, and to divide after 7–10 days in this area. Phloem parenchyma cells, which had differentiated into strands, also enlarged and divided remarkably within the phloem differentiating zone after 10–14 days. In the earlier stage of cell proliferation observed in the cross sectional view of the inner area after 14 days (Fig. 5), ray cell proliferation (Fig. 5, R), which had originated within the mitotic zone, extended fairly deep into xylem differentiating zone, but not so much into phloem differentiating zone where phloem parenchyma cells were vigorously proliferating (Fig. 5, P).

Figure 6 shows a cross sectional view of the much advanced stage of proliferation observed in the cambial zone after 14 days in culture. The proliferated cells on each of the phloem and the xylem side were originated from phloem parenchyma cells (Fig. 6, P) and ray cells (Fig. 6, R), respectively (c.f. Fig. 5). Undifferentiated or immature fusiform cells were crushed and observed in "Y"-shape between the

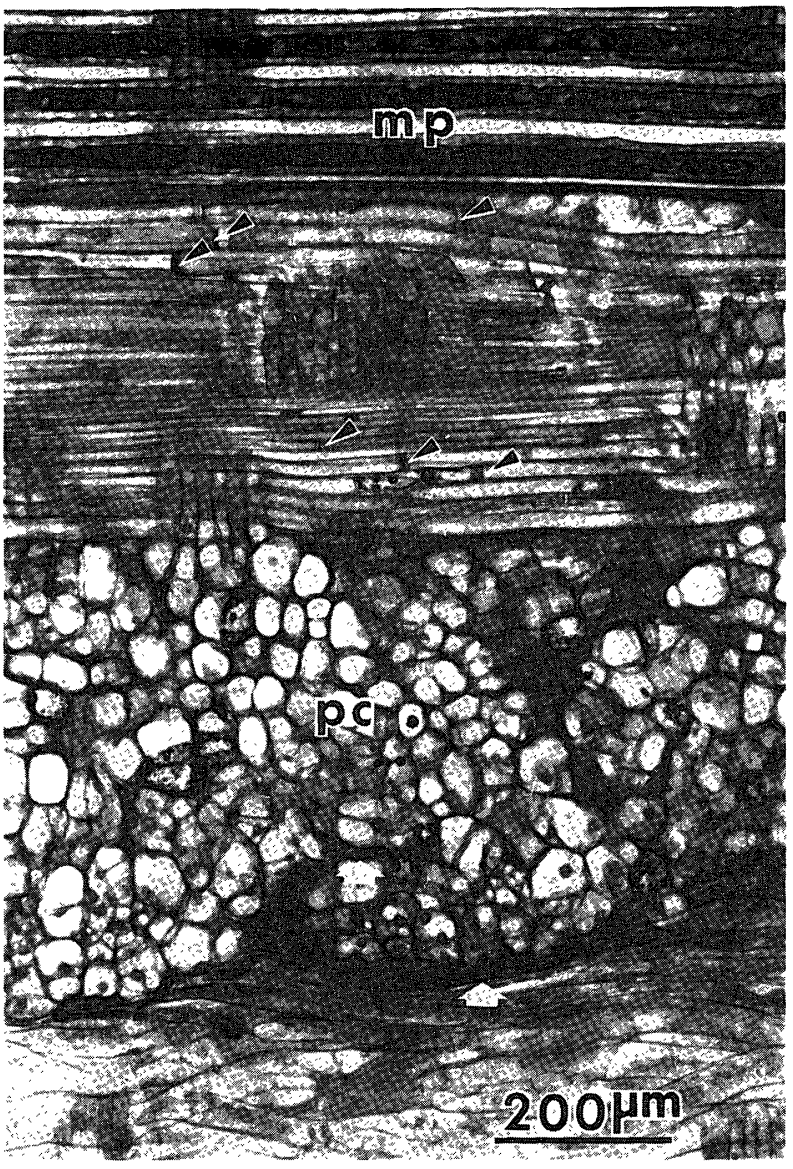

Fig. 3. The radial section just beneath the mass of callus on the radial surface of the explant after 14 days in culture without pressure, showing septations of fusiform cells (arrow heads) and crushed fusiform cells (white arrows). mp: mature phloem, pc: proliferated cells.

proliferated cells. The cells between the callus-like tissues and rigid mature xylem or mature phloem were also crushed after prolonged culture. In such a case, the callus-like tissues were frequently separated from the mature tissue by a crack.

Within the mature phloem, proliferated cell groups originated from phloem parenchyma cells were also observed between the tangential row of thick walled fibers (Fig. 4, arrow heads). Proliferation of ray parenchyma cells in the mature phloem was not so conspicuous as the one in the cambial zone.

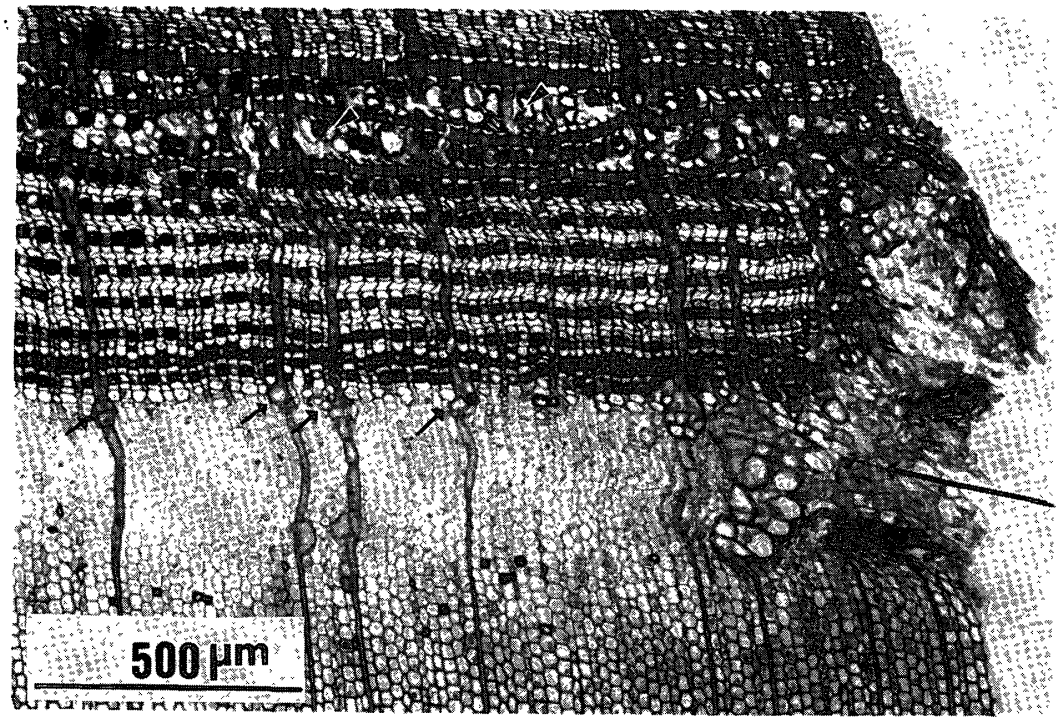

Table 4. The cross section of the explant after 12 days in culture without pressure, showing cell proliferations in the inner area (short arrows) and the surface area (long arrow) of the explant. Arrow heads indicate the proliferation of phloem parenchyma cells between the tangential row of mature phloem fibers.

As mentioned above, callus formation in cambial explants first appears in the surface area of the explant, and the initiation of callus formation in the inner area occurs a little later. The callus formation is ascribed to the proliferation of ray parenchyma cells, phloem parenchyma cells and a few of fusiform cells. Most of the fusiform cells, especially in the inner area, were crushed by other proliferated cells, without showing any divisional activity. These findings were summarized in Table 2.

Although these callus originating cells finally turned to be all similar callus cells, they showed different ways of division characteristic to each cell type in their early proliferating stages. Ray parenchyma cells changed their shape to spherical form during enlargement before division. Phloem parenchyma cells enlarged their radial axes and divided periclinally first, producing two rows of strands. Fusiform cells to be divided were first septated and shortened their length before their callus-like proliferation.

Vigorous cell proliferation in the surface areas of the explant might be ascribed to a wide open space which might be required for enlargement and proliferation of cells. On the other hand, the lesser proliferation in the inner areas might be ascribed to the limited open space. However, this idea does not fit to the fusiform

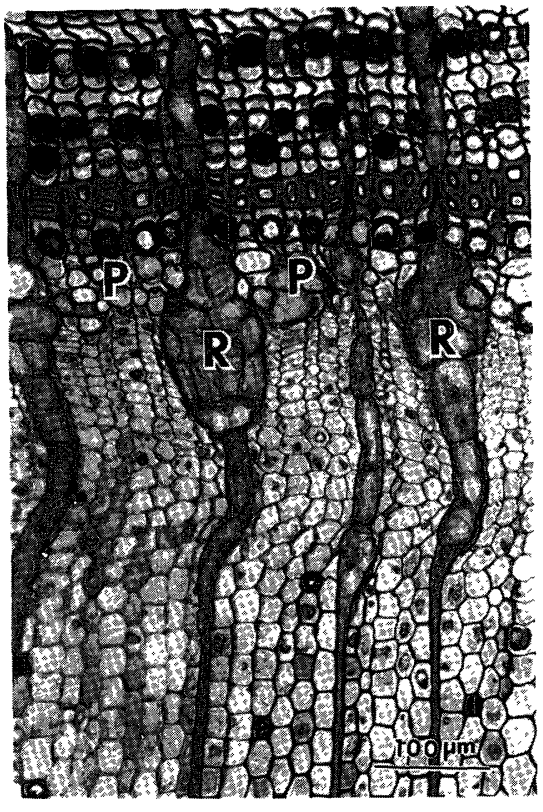

Fig. 5. The cross section of the inner area of the explant after 14 days in culture, showing an earlier stage of the proliferation of ray cells (R) and phloem parenchyma cells (P).

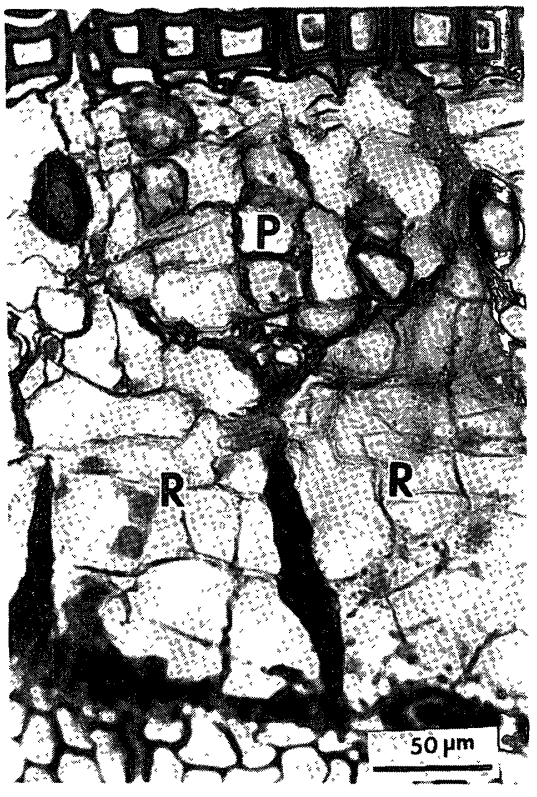

Fig. 6. The cross section of the inner area of the explant after 14 days in culture without pressure, showing the much advanced stage of proliferated phloem parenchyma cells (P) and ray cells (R). Crushed fusiform cells are observed in "Y"-shape.

Table 2. Proliferated tissue elements and degrees of the proliferation

		Surface area	Inner area
Mature phloem	P. p. c.*	+ + +	+ +
	Ray cells	+ +	+
Cambial zone	P. p. c.*	+ + +	+ +
	Ray cells	+ + +	+ +
	Fusiform cells	+	(+)

P.p.c.: Phloem parenchyma cells

cells, because their septations, which do not require open space, were observed in the surface area, rather than in the inner area. In this case, other factors affecting differently between these areas would be considered — e.g. different circumstances such as osmotic pressure, gas supply (CO_2, N_2, O_2, etc.) through the contact with air, the effect of woundings (e.g. ethylene and other wound hormones) on the surface of explant, the contact with other cells in the inner area, etc.

2. Cambial Explant under Applied Pressure

2.1 Suppression of cell enlargement and division in the inner area by applied pressure

As mentioned above, ray parenchyma cells began to enlarge and divide after 5–7 days in the inner area of the explant cultured without pressure (Fig. 7-a). Phloem parenchyma cells without pressure also enlarged and divided remarkably in the phloem differentiating zone after 10–14 days (Fig. 7-b). In the inner area of the explant cultured under applied pressure, however, ray parenchyma cells scarcely enlarged or divided after 7 days (Fig. 7-c). Even after 14 days, enlargement and division of ray cells occurred only a little, and phloem parenchyma cells did not begin to divide yet (Fig. 7-d). Such suppression of cell enlargement and division was also observed even in the mature phloem of the inner area cultured under applied pressure.

These observations suggest that the pressure applied to the explant may cause forces restricting cell enlargement and proliferation in the inner area of the explant. If, as supposed earlier in this paper, the lesser proliferation in the inner area of the explant without pressure is to be ascribed to the space-limiting factor, such a factor must be much intensified by the applied pressure in this case.

When the culture period of explants kept under applied pressure were prolonged, living cells began to proliferate well even in the inner area. This proliferation might be induced by the development of some forces (e.g. osmotic pressure) which was able to overcome the applied pressure during the prolonged culture.

Applied pressure suppressed cell proliferation only in the inner area of the explant. In the surface area of the explant, the cells had already begun to proliferate in the cambial zone and mature phloem after about 7 days in culture, in accordance with the case of pressure-free culture. However, the arrangement of the proliferated cells were different between the two culture systems. The details of the surface area under applied pressure will be mentioned below.

2.2 Arrangement of proliferated cells in the surface area under applied pressure

Callus tissues were found on the radial surfaces of the explant with applied pressure similarly to the case of the explant without pressure. The proliferated tissues just beneath these callus, however, were not similar to that of the pressure-free explant. Namely, while the proliferated cell shape was spherical in the latter case (Fig. 8-a), the proliferated tissues in the former consisted of a heap of the strand of flat cells in the radial section (Fig. 8-b). This tissue was constituted with phloem parenchyma cells and septated fusiform cells, both of which were repeatedly divided

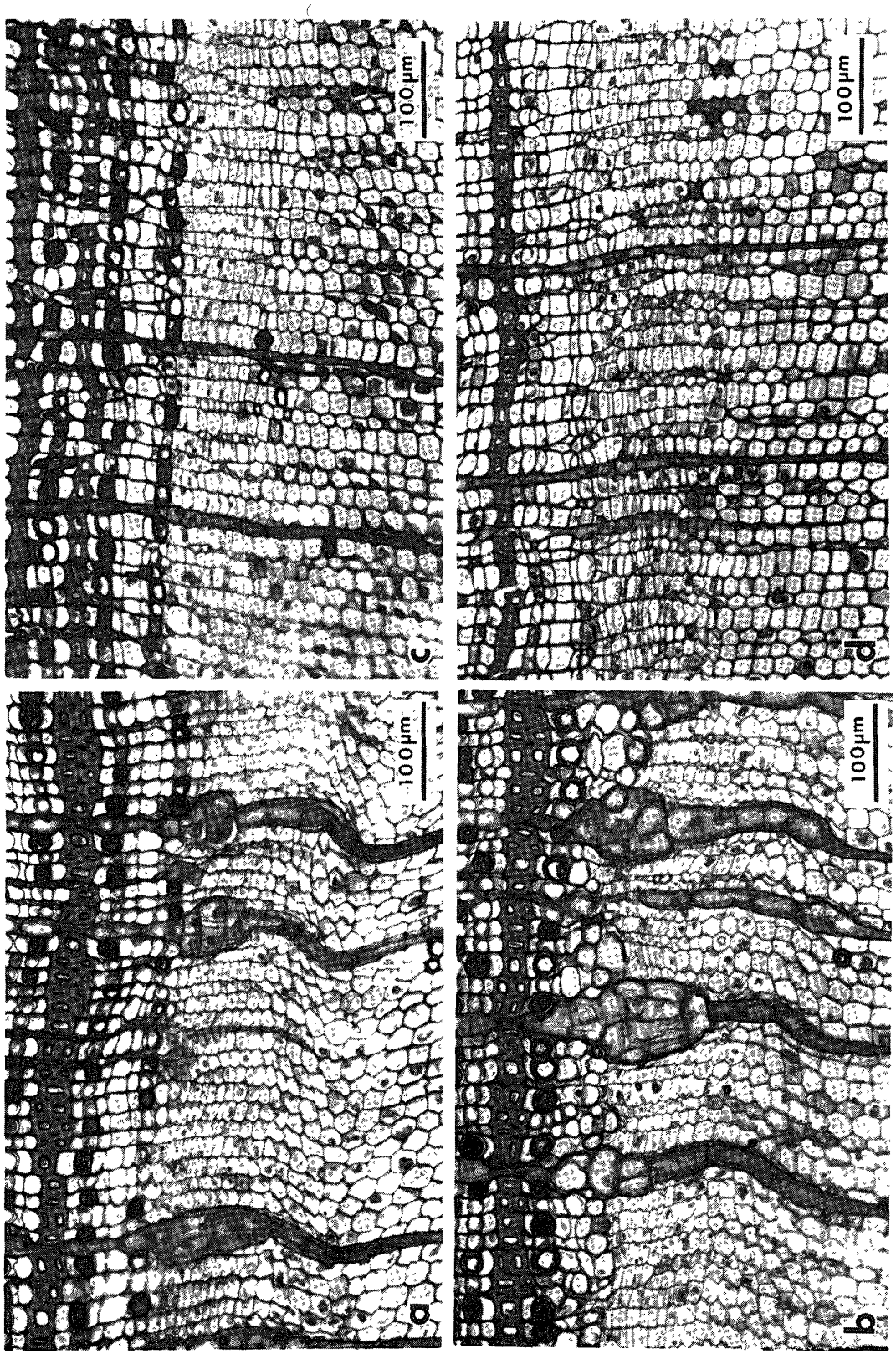

Fig. 7. The cross sections of the explants after 7 days (a, c) and 14 days (b, d) in culture without pressure (a, b) and under applied pressure (c, d).

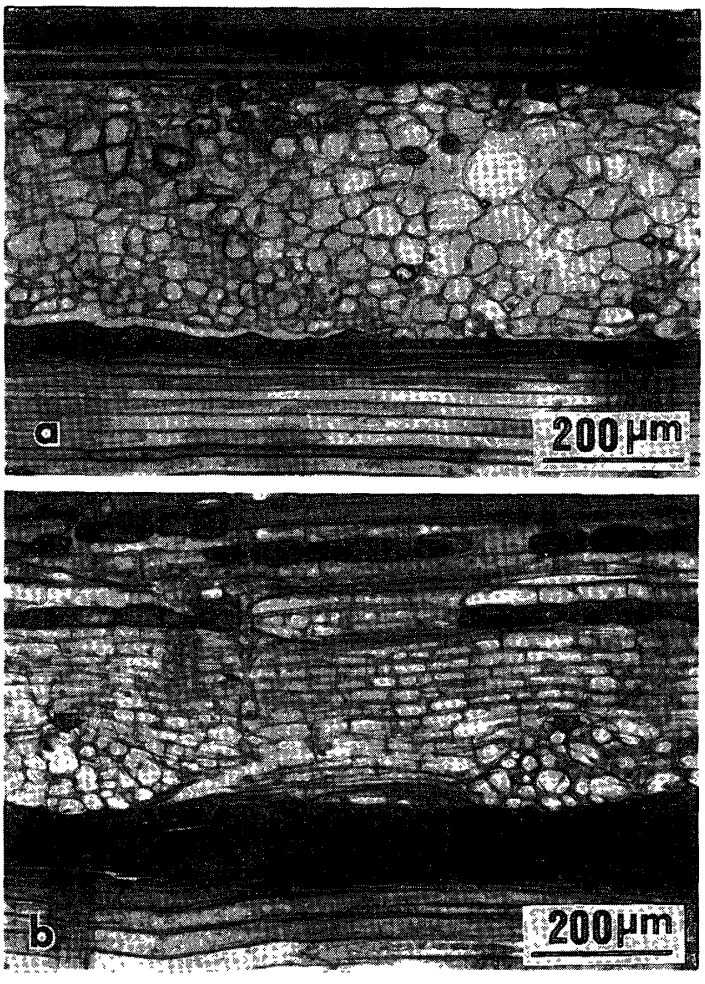

Fig. 8. The radial sections of the surface area of explants after 14 days in culture without pressure (a) and under applied pressure (b). Arrows in (b) indicate groups of spherical cells originated from ray cells.

by tangential planes maintaining these rows of strands (Fig. 8-b). Similar arrangement of proliferated cells were also observed in the inner area when the explant was cultured long enough under applied pressure.

The restricting force generated from the applied pressure seemed to maintain the cell shape by means of controlling the divisional plane[6].

Beside the above-mentioned heap of flat cells, some groups of spherical cells were observed on the xylem side of cambial zone (Fig. 8-b, arrows). These cells were apparently originated and proliferated from ray parenchyma cells in the same way as the case of pressure-free culture. Because the pressure was applied as radial compression (i.e. in parallel to the long axis of ray cells), the shape of proliferated ray cells seemed to indicate seemingly no-effect of applied pressure.

2.3 Comparison with other studies

Our experiments under applied pressure presented the two findings upon the effect of pressure: 1) suppression of cell proliferation, and 2) maintenance of the shape and arrangement of proliferated cells. These findings indicate that applied pressure effectively maintains tissue structure during *in vitro* culture. These are also consistent with the reports of Brown[5] who suggested that physical environments is an important factor as well as chemical ones for tissue differentiation.

Brown[5] who observed the differentiation of xylem elements with secondary walls in his cambial explant cultured under applied pressure, suggested that the applied pressure was closely related to secondary wall formation. Imamura[8] also reported in his study of excrescence featured Sugi wood that the wall of ray parenchyma cells were thickened when a restricting force was applied against the radial growth of the stem. In spite of the application of radial pressure on the cambial explant in the present experiments, however, wall-thickenings in the proliferated cells were not observed at all. Controlling effect of chemical factors (e.g. hormones) upon the formation of tracheary elements or wall-thickening have also been demonstrated by many workers[9~12]. Therefore, it remains to be clarified whether the fail of wall-thickenings in our explant was to be ascribed to the unfavorable magnitude of applied pressure or to the faulty constituent of medium in our *in vitro* system.

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