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Histochemical Study on the Development of Taxol-like Vesicles in Callus Cells of *Taxus cuspidata* Var. Nana

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Abstract: The formation and development of Taxol-like vesicles in callus cells of Kyaraboku (*Taxus cuspidata* var. nana.) were investigated using histochemical and quantitative methods. Many transparent vesicles revealed in the young and mature normal callus cells. The vesicles treated thoroughly with Taxol detection chemicals and immunoassay system. In case of Vanillin-sulfuric acid reagent, several big and small vesicles showed intense positive reaction and were stained brown and yellow ocher. The treatment of Dragendorff reagent showed brown color of vesicles with extreme positive reaction, whereas Anisaldehyde-sulfuric acid reagent turned vesicles purple with positive reaction. In immunoassay system, almost vesicles in callus cells had positive reaction and were revealed yellow-green. Therefore, the vesicles were determined to include Taxol-like compounds. The vesicles were originated around the nucleus of young callus cells. While vesicles were more enlarged in mature callus cells and were located around bigger vacuoles. Eventually, the vesicles started incorporating into the enlarged vacuoles of old cells. The development of vesicles exposed hypertrophy in young callus and were aggregated in mature callus.

Key words: Callus, visualization, Taxol-like vesicle, histochemical method

INTRODUCTION

Taxol was originally isolated from the bark of *Taxus brevifolia*[1] and established its chemical structure. Then the distinctive mechanism of Taxol stabilization of microtubules was discovered[2,3]. Taxol has been widely recognized as an important anticancer drug and many kinds of fruitful results were reported worldwide[4-7]. However, due to the difficulties in obtaining this compound from yew trees, the clinical use of Taxol has been limited. Among these difficulties are its low concentration and high cost of the extraction process. The limited availability of Taxol from its natural source has motivated the development of alternative production sources. Total synthesis was achieved by Nicolau *et al.*[8,9] and Holton *et al.*[10] but it is not commercially viable. An alternative approach for obtaining Taxol is the semisynthesis[11-13] application of the more abundant taxanes isolated from the needles of yew trees but this process involves a limited number of synthetic steps. Another approach for producing Taxol is by application of the endophytic fungi of *Taxus* species, which is still limited due to low production[14-16]. However, the most promising alternative for the production of Taxol is the use of plant tissue culture derived from different *Taxus* species[17-19].

Most of the previous work regarding Taxol information has been done for its extraction. On the knowledge of regarding producing plants, their structure and histochemistry is unavailable. The aim of this work was to provide more information concerning the formation, localization and development of Taxol-like vesicles in callus cells of *Taxus cuspidata* var. nana using histochemical and quantitative methods.

MATERIALS AND METHODS

The research project was carried out in the Laboratory of Chemistry and Biotechnology for Utilization of Forest Resources, Faculty of Agriculture, Ehime University during 2002 to 2003. The explants were obtained from young stem sections of Kyaraboku (*Taxus cuspidata* var. nana) in Matsuyama, Ehime, Japan.

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Explants and method of callus cultivation: The explants were sterilized by soaking in 70% ethanol for a few minutes and followed by the immersing in sodium hypochlorite (1% active chlorine) for 20 to 30 minutes by gently stirring followed by washing thricely with sterile water. After removing needle segments of the stem they were transferred into their sterilized medium. The explants were kept on solid Gamborg's B5 medium[20]. Cultivation was maintained at 25°C in the dark for 1.5, 2.5 and 3.0 months.

Visual and qualitative methods of the big and small vesicles in callus cells with chemical reagents: To clarify the presence of Taxol-like vesicles, 1.5, 2.5 and 3.0 months old callus cells were visualized by four different chemical methods. The reagents used for the methods as follows: (1) Vanillin-sulfuric acid reagent[21], (2) Dragendorff reagent[22], (3) Anisaldehyde-sulfuric acid reagent[21] and (4) immunoassay kit for Taxol (purchased from Hawaii Biotechnology Group, INC., USA)[23]. The fresh callus cells were taken out from Erlenmeyer flasks and put on the slide glass. Each three chemical reagents except for the immunoassay kit dropped on callus cells, followed by heating for 10 sec at 90°C. After 30 to 40 min, the callus cells stained with each chemical were observed under light microscope (Olympus, BH-2).

Existence of Taxol in callus cells were detected by the immunoassay method by Hawaii Biotechnology Group, INC. The fresh callus cells were put into a microtiter plate and then 50 μl of 1/1000 dilution of anti-Taxol antibody in PBS-T was added per one well. The plate was incubated for one hour at room temperature. The callus cells in the plate were washed four times with TBS-T, then 100 μl of 1/1000 dilution PBS-T containing alkaline-phosphatase labelled goat anti-mouse IgG conjugate was added per one well. After one hour incubation at room temperature, the plate was washed four times with TBS-T. And then, 200 μl per each well of 1mg ml^{-1} alkaline phosphatase substrate (pNPP) solution diluted in alkaline phosphatase substrate buffer (pH 9.5) was added per one well. The plate was incubated for one hour at room temperature. The callus cells were observed under the light microscope and photographed.

The formation and development of Taxol-like vesicles in callus cells: For observation under the light microscope, 1.5, 2.5 and 3.0 months old fresh callus samples were cut into 2 mm cubes with a feather knife. Each sample was pre-fixed immediately with the Karnovsky's solution, post-fixed with 2% osmium tetroxide solution. The specimens were dehydrated in a graded ethanol series and embedded in epoxy resin. Sections (0.5 μm tick) were cut with a Sorval UT-1000 ultramicrotome, stained with toluidine blue O and were observed under the light microscope and photograped.

Quantitative analysis of Taxol content in callus cells: Extraction of Taxol from 1.5, 2.5 and 3.0 months old callus cells of Kyaraboku was conducted using the method of Muranaka *et al.*[24]. The callus subcultured on the B5 medium were freeze-dried for five days, grounded with a pestle and extracted twice with methanol and water (1:1 v/v) for two days at room temperature. The resulting extractives were evaporated in vacuum to give crude residues. The crude residues were subsequently partitioned between dichloromethane and water (1:1 v/v) to yield, after evaporation of the solvent and drying in vacuum, dichloromethane solbles. The amount of Taxol in each sample was determined by HPLC[25].

RESULTS

External features of callus cells under the stereomicroscope: We used the stereomicroscope for clarifying the external feature of callus cells formation in explant. Fig. 1 showing the feature of pith (P), xylem (X) and cortex (Co) of the explant and the formation of callus cells. Numerous callus cells (Ca) were developed from the cortex radially. In 1.5 months old callus, the cells showed whitely yellow. They were spheroidal, oval and club shaped. The average diameter of the spheroidal and oval shaped callus cells was 51.6 μm, the club shaped callus cells was 140 vertically and 34.5 μm horizontally. Whereas 3.0 months old callus cells were yellowish brown to ocher. The average diameter of former callus cells was 84.5 μm, latter was 286 μm vertically and 73.0 μm horizontally. Fig. 2 explains the feature of non treated callus cells. Young callus cells were club and globular shaped. Numbers of transparent vesicles were observed in young and mature callus cells. Most of vesicles in mature callus cells were localized near the cell walls (CW) (Fig. 2, arrow), or were presented in cytoplasm (Fig. 2, double arrow).

Visualization and qualification of Taxol-like vesicles in callus cells with chemicals: To observe the presence of Taxol-like substance, 2.5 months old callus cells and four staining chemicals were applied on the samples. The chemicals were Vanillin-sulfuric acid reagent, Dragendorff reagent, Anisaldehyde-sulfuric acid reagent and immunoassay kit (Fig. 3-5, Table 1).

Table 1: Histochemical analysis of Taxol-like vesicles in callus cells of *T. cuspidata* var. nana

Chemicals	cell wall	cytoplasm	vesicles
Vanillin sulfuric acid reagent	-	+	+++
Dragendorff reagent	-	+	+++
Anisaldehyde sulfuric acid reagent	-	+	+++
Immunoassay kit	-	+	+++

Notes: The callus cells were incubated at 25°C in dark for 2.5 months, Abbreviations: - means negative reaction and colorless, + means weakly positive reaction, +++ means intense positive reaction and deep color

Initially the reaction with Vanillin-sulfuric acid reagent showed no effect on the cell walls (CW). While cytoplasm was stained yellow and yellow ocher weakly. Several big and small vesicles in callus cells revealed intense positive reaction and converted to brown and yellow ocher (Table 1, Fig. 3, arrow). Whereas the application of Dragendorff reagent on cell wall (CW) has given negative reaction and was colorless. Although cytoplasm stained yellow ocher (Fig. 4). While the vesicles were brown showed intense positive reaction (Table 1, Fig. 4, arrow). Third, reaction with Anisaldehyde-sulfuric acid reagent caused negative reaction on cell wall (CW) and was colorless. While cytoplasm in callus cells was stained slightly pink with a little positive reaction. On the other hand, many vesicles in callus cells showed intense positive reaction and were stained purple (Table 1, Fig. 5, arrow). In case of immunoassay method, cell wall (CW) of 2.5 months old callus cells reacted negatively showing colorless and transparent aspects. However, cytoplasm was stained buff yellow showing positive reaction. In contrast, numbers of vesicles in callus cells developed intense positive reaction with yellow-green color (Table 1, Fig. 6, arrow).

Morphological features of the formation, localization and development of Taxol-like vesicles in callus cells: Vesicles were stained throughly with all of the detective solutions for Taxol-like compounds in callus cells of Kyaraboku. Fig. 7 to 10 showed the process of formation to development of vesicles. The median longitudinal sections of 0.5μm thickness were stained with toluidine blue O and were observed under the light microscope. In 1.5 months old young callus cells, the vesicles of the sections were stained light blue. Other organs in callus cells had good stainability and were light purple, purple and deep blue. While the newly born callus cells were filled with many organs away from nucleus (N) (Fig. 7). The shape of nucleus disclosed the cell with high energies at just pre and post-phase of cell division. The small vesicles stained light blue and were formed around nucleus (N) in young callus cells (Fig. 7, arrow, Fig. 13, A). Whereas 2.5 months old mature callus cells (MC) grew up greatly and vacuoles were also enlarged. Several vesicles were formed near nucleus (N) and were segregated in cytoplasm (Fig. 8, arrow, Fig. 13, B). When the vacuoles in callus cells were enlarged more, the vesicles were localized in cytoplasm with very narrow space between cell walls to vacuoles (Fig. 8, Fig. 13, C). Then, the vesicles started incorporating into vacuoles of old callus cells (OC) (Fig. 8, arrow, Fig. 13, D). In addition, when the callus cells were aged, they were changed to yellow or light brown (Fig. 9). The cell walls and cytoplasm were stained deep blue with toluidine blue O. However, the vesicles were observed around vacuoles (Fig. 9, arrow). Contrarily, when the callus cells aged more and changed to deep brown, they occurred plasmolysis and plasma membrane (PM) was separated from the cell wall clearly but the vesicles were presented in callus cells (Fig. 10, arrow). Both Fig. 11 and 12 showed the aggregation and developmental process of vesicles in callus cells. Generally, the vesicles in callus cells were composed of single vesicles and then aggregated to form compound vesicles. There were vesicles developed by one small vesicle in young callus cell (Fig. 11, one pile arrow, Fig. 12, A). While two pieces of the vesicles were observed (Fig. 11, double arrow, Fig. 12, B). Mature callus cells revealed enlarged vacuoles. Finally, the vesicles aggregated by three pieces or four pieces were also observed (Fig. 11, white edge black arrow, Fig. 12, C).

Relationship between Taxol-like vesicles and Taxol content on the aging of callus cells: Longitudinal sections (0.5 μm tick) were observed to clarify the volume and the number of vesicles in a callus cell. In addition, we measured Taxol content in callus cells by HPLC.

The number of vesicles in each callus cell was measured by using sections after 1.5, 2.5 and 3.0 months cultivation, respectively. As a result, 4.5 vesicles were admitted in the sections after 1.5 months cultivation. Then, the vesicles increased to 9.0 pieces after 2.5 months cultivation. The vesicles decreased to 6.0 pieces after 3.0 months cultivation (Fig. 14).

Similarly, the volume of vesicles was calculated from the area they occupied in each callus cell. The area of the vesicles was about 3.4% after 1.5 months cultivation, increased rapidly to approximately 14.9% after 2.5 months cultivation and decreased suddenly to 3.3% after 3.0 months cultivation (Fig. 15).

We also measured the influence of cultivation time to increase and decrease Taxol content in callus cells by HPLC (Fig. 16). Taxol content of callus cells was about 0.013% (on the dry weight basis) after 1.5 months cultivation, increased rapidly to 0.035% at 2.5 months. Finally Taxol content decreased suddenly to 0.009% at 3.0 months (Fig. 16).

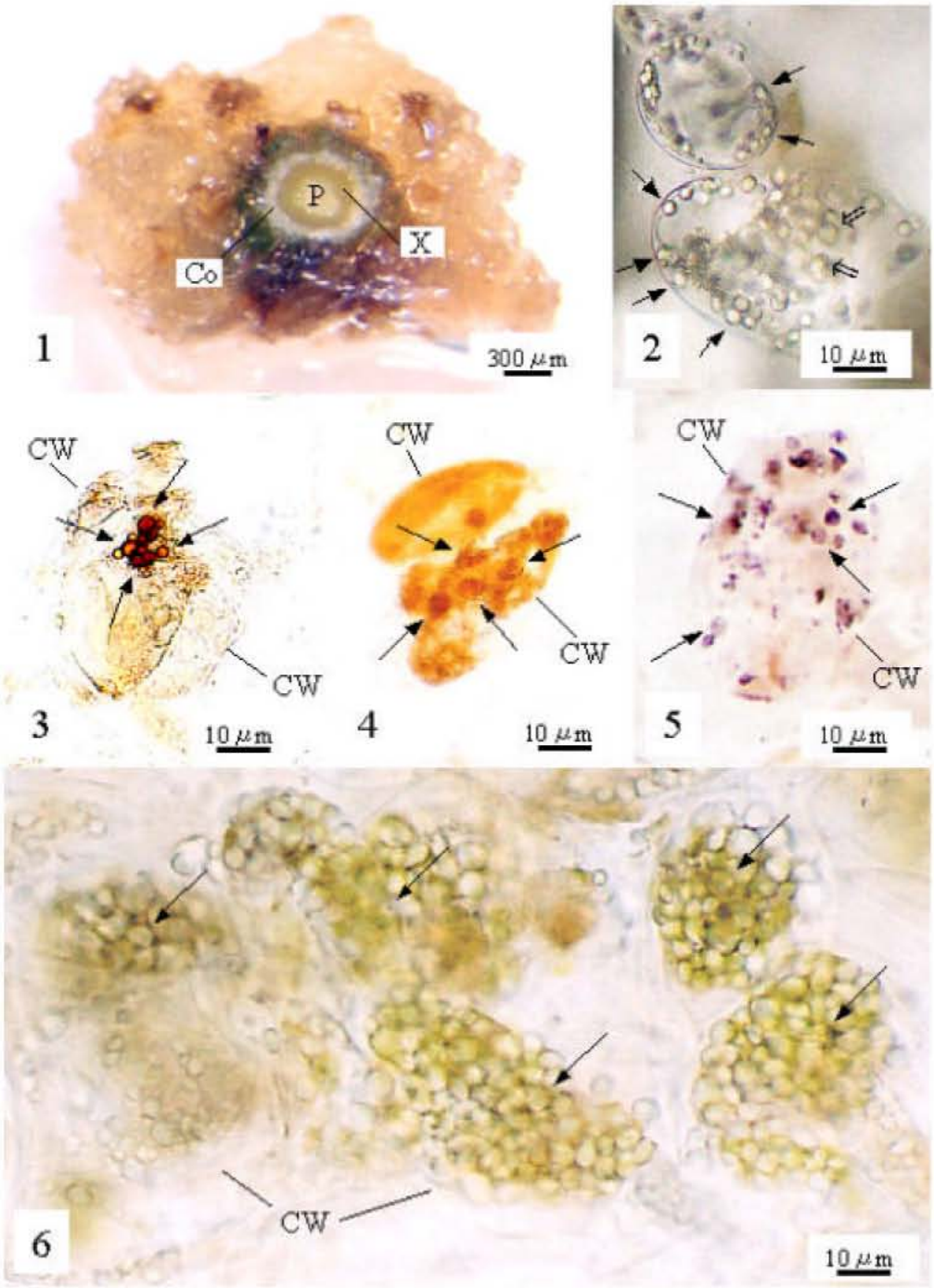

Fig. 1-6: Characteristics of callus cells in Kyaraboku (*T. cuspidata* var. nana.). 1: Callus cells formation in the young stem from the cortex (Co) at the edge of the xylem (X) and away from pith (P). 2: External features showing callus cells in spheroidal and oval shaped and several transparent vesicles (arrows) are present in callus cells. 3: Callus cells revealed many vesicles (arrows) after staining with Vanillin-sulfuric acid reagent and cell wall (CW) was colorless. 4: Callus cells showed vesicles (arrows) after staining with Dragendorff reagent and cell wall (CW) was transparent. 5: Callus cells beared vesicles (arrows) after staining with Anisaldehyde-sulfuric acid reagent and cell wall (CW) was not stained. 6: Callus cells exposed vesicles (arrows) after treatment with immunoassay system and cell wall (CW) showed negative reaction

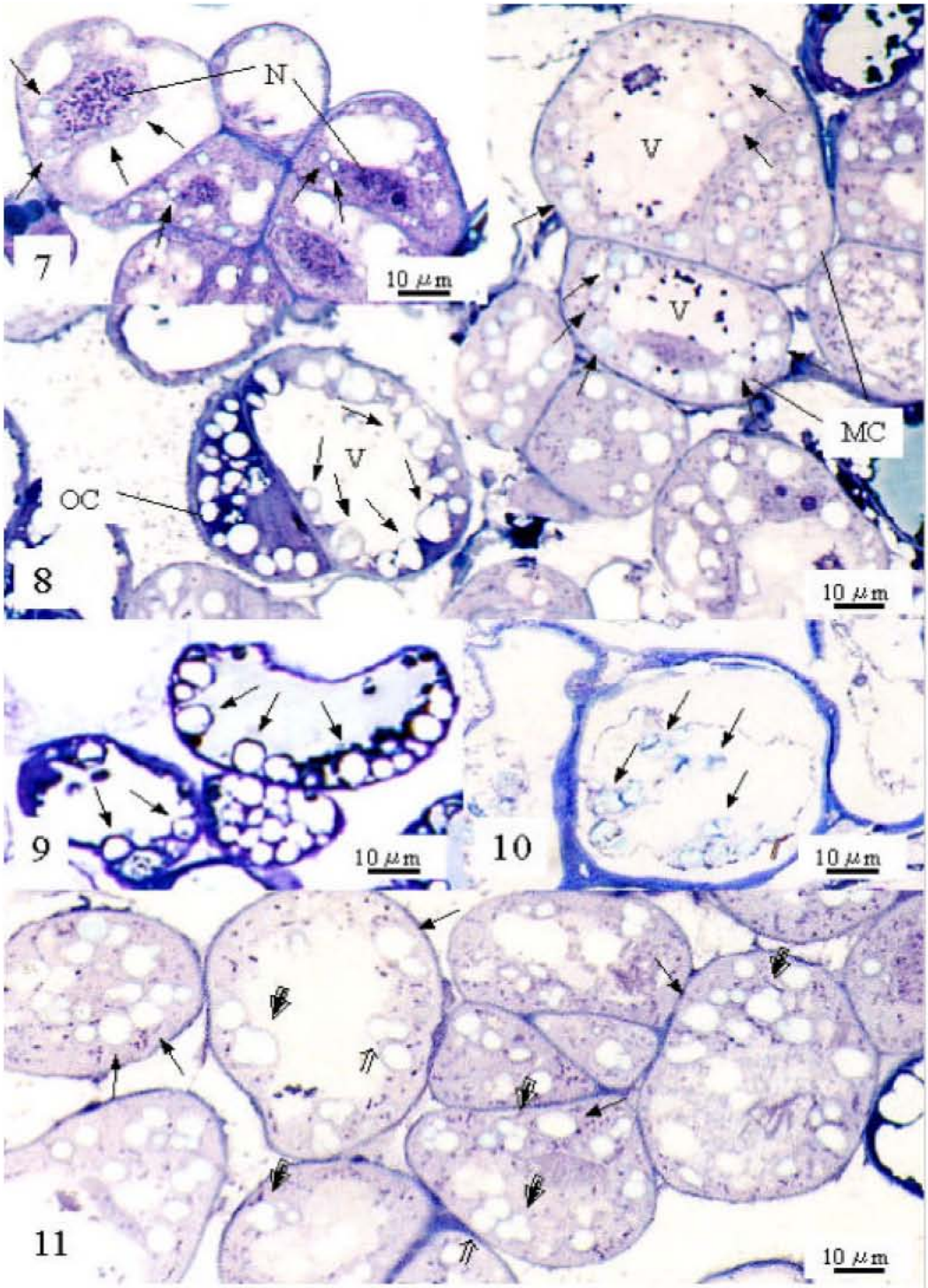

Fig. 7-11: Longitudinal features revealed formation and development of Taxol-like vesicles in callus cells of Kyaraboku (*T. cuspidata* var. nana.). 7: Taxol-like vesicles (arrows) formed around nucleus (N) in young callus. 8: Taxol-like vesicles (arrows) aggregated around enlarged vacuoles (V) in mature callus (MC) and entered into more bigger vacuoles (V) in old callus (OC). 9: Taxol-like vesicles (arrows) were incorporated into the vacuoles of necrotic callus. 10: Taxol-like vesicles (arrows) were included in vacuoles of plasmolysis callus. 11: Aggregation and developmental features of Taxol-like vesicles (arrows) in mature callus

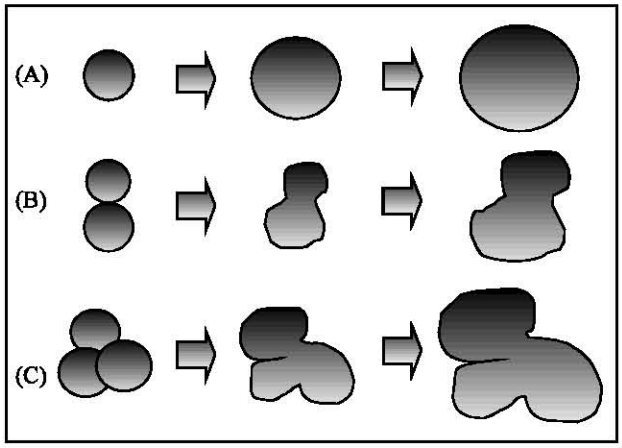

Fig. 12: Schematic features of the aggregation and development of Taxol-like vesicles in callus cells. Notes, A: The vesicles developed by one small vesicle; B: The vesicles fused by two small vesicles; C: The vesicles formed by three small vesicles

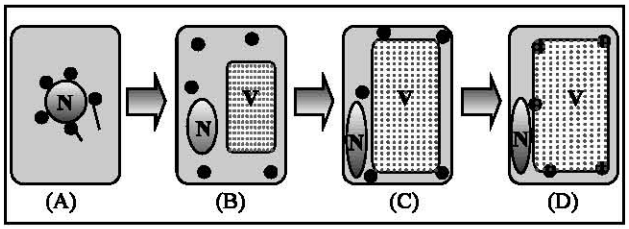

Fig. 13: Schematic features of the formation and development of Taxol-like vesicles in a callus cell. Abbreviations, N: nucleus; V: vacuole; Ve: vesicle

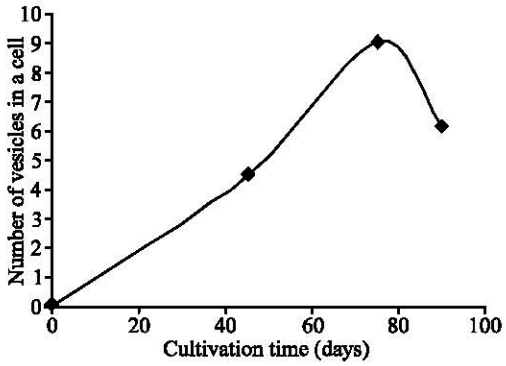

Fig. 14: Effect of days after cultivation on the number of Taxol-like vesicles in a callus cell of *T. cuspidata* var. nana. Notes: The number of vesicles in a callus cell was measured from 0.5 μm sections under the light microscope

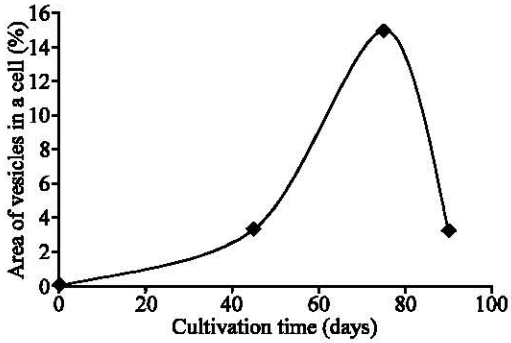

Fig. 15: Effect of days after cultivation on the development of Taxol-like vesicles in a callus cell of *T. cuspidata* var. nana. Notes: The area of vesicles in callus cell was measured from 0.5μm sections under the light microscope

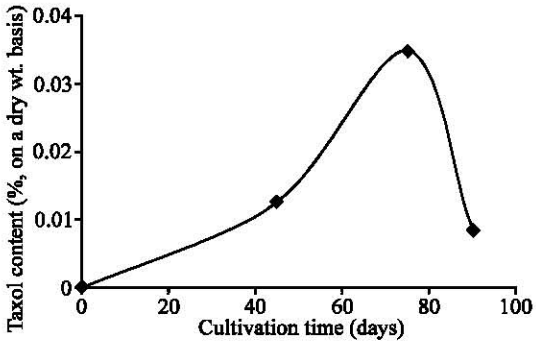

Fig. 16: Effect of days after cultivation on the content of Taxol-like vesicles in callus cells of *T. cuspidata* var. nana Notes: Taxol content in callus cells was measured by HPLC

DISCUSSION

Taxol is present virtually all part of the yew tree[26] but preferentially aggregates in the bark and needles. In contrast, the extensive work on biochemistry of Taxol, its systems of the formation and development in callus cells were not well documented.

The callus cells used in this study were generated from the cortex of Kyaraboku and were light green to whitely yellow. It is enough evidence to believe the existence of chloroplast, chromoplast or leucoplast-like organs from these colors.

Nucleus in prophase of cell division was observed in numbers of young callus cells. They were oval and spheroidal shaped. While the external zone of callus cells were tublar type. It is thought to be small oval and spheloidal cells had high activity of division and large tubuar cells started vegetative growth. Many transparent

vesicles were also existed in each cell. These vesicles were recognized close to the cell walls in the tublar shaped cells. It seemed to be usual that these vesicles were recognized of Kyaraboku as the chromoplast in the young stalks and needles.

Many researchers reported the production of Taxol with plant tissue culture of *Taxus* species[17,18,19]. Similarly we used four kinds of Taxol detection chemicals and examined the existence of Taxol in callus cells, which was cultured artificially. The vesicles in callus cells showed positive reaction with each chemical reagent. Therefore, we found many vesicles including Taxol-like compounds. However, the vesicles in callus cells did not showed the equal positive reaction with Vanillin-sulfuric acid reagent.

The staining ability was different for each vesicle. These results suggested that the vesicles may contain Taxol. In addition, the vesicles showed brown and yellow ocher with Vanillin-sulfuric acid reagent. While the vesicles revealed yellow-green with the immunoassay kit. We suggested that Taxol content may differ remarkably in each vesicle. For immunocytochemical studies, Russin *et al.*[27] described Taxol aggregate almost exclusively in cell wall rather than cytoplasm. While, Fornalè *et al.*[18] published the Taxol transport using [^{14}C]-Taxol as a tracer in *Taxus baccata* suspension culture. Consequently, they suggested that Taxol was transported from the medium to culture cells. Then they absorbed molecules from its exogenous medium, which were found to localize both in cell wall (20%) and in protoplast (80%) and was an aggregation within the vacuole[18]. While in our study, the vesicles were found more in the cytoplasm rather than cell wall.

Concomitantly, we observed the formation and development of vesicles in callus cells. It was cleared that the many small vesicles were originated around nucleus in young callus cells. This showed the transmission of signals markedly on Taxol formation. When callus cells aged, the vacuoles were more enlarged and many vesicles were departed from nucleus. In old and brown callus cells, the vesicles began to enter into vacuoles and vacuole membranes occured invagination. Generally, the formation of vesicles around nucleus in young callus cells and the incorporation of vesicles into the vacuoles were observed in the plants[28]. Our studies revealed different results where aged cell did not have vesicles accumulation.

In higher plants, Hama[29] reported that the storage starch body consist of single or compound grains. Taxol-like vesicles composed of the single small or small compound vesicles in our experiment. The callus cells just after the cell division, the vesicles was small type about 2.48 μm in the diameter. However, the vacuole in mature callus cells was bigger and observed to aggregate by two or three vesicles and these vesicles were extensively grown to 9.35 μm. Therefore, our results suggested the formation, aggregation and development of the vesicles which may promote Taxol biosynthesis in callus cells of Kyaraboku.

We examined the influence of culture times on the hypertrophy and the number of vesicles in callus cells. The number of vesicles began to increase markedly from 1.5 months cultivation and then became maximum at 2.5 months. They were decreased after 2.5 months. Further, we examined Taxol content and morphological characteristics in callus cells during 3.0 months period. Consequently, it is thought that the decrease of Taxol content was caused by the senescence of browning callus cells.

In conclusion, although Taxol biosynthesis in plant callus was known to the limited content, the histochemical and quantitative study added new knowledge in this field which will open the ways and methods for further development.

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