Investigations on the ultrastructure of the callus tissue of *Liriodendron tulipifera* L. developed *in vitro*

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(Received: February 22, 1982)

Abstract

Fragments of internodes, petioles and fragments of leaves of *Liriodendron tulipifera* L. were cultured *in vitro* in order to induce them to regeneration. Abundant callus tissue was only produced by unligified internodes and petioles. Application of various combinations of media was not successful in inducing callus organogenesis. The callus did not survive long and cytological analysis of its cells revealed the presence of severe abnormalities.

INTRODUCTION

Liriodendron tulipifera L. is an exotic ornamental plant and in Poland it grows in some parks and Botanical Gardens. An increased interest in the propagation and cultivation of this tree is noted, mainly because it contains various chemical substances of practical importance (Rzędowski 1981). The classical methods of vegetative propagation of *Liriodendron tulipifera* L. are but little efficient (Seneta 1981). Therefore, various efforts are undertaken to propagate it through the *in vitro* culture method. However, as it ensue from the literature the potentials of regeneration are very meagre. Usually the cultured *in vitro* explants produce small amounts of callus which only rarely is able to form organs (Hu 1978 quoted after Rzędowski 1981). Fully plants were obtained only from callus originated from apical buds (Furmanowa and Rzędowski 1979). Unfortunately they soon died after transferring to soil (Rzędowski 1981).

Abbreviations: IAA — indolyl-3-acetic acid, NAA — naphthyl-1-acetic acid, 2,4-D — 2,4-dichlorophenoxyacetic acid, KIN — 6-furfurylaminopurine (kinetin), BAP — 6-benzylaminopurine.

The present study concerns the culture of various explants of *Liriodendron tulipifera* L. and the ultrastructural analysis of the developing callus.

MATERIAL AND METHODS

The following experimental material as internodes, leaf petioles and blades of *Liriodendron tulipifera* L. were collected in the Institute of Dendrology of the Polish Academy of Sciences in Kórnik and Botanical Garden in Poznań. In the experiments carried out in the summer months of 1981 only annual new shoots were used. Material was sterilised in 3 per cent sublimate for 10 min and washed thoroughly with sterile distilled water. Then the internodes, petioles of about 0.5 cm in length and fragments of leaves with the vascular bundle were cut out (a rectangle of 0.8×0.2 cm). The explants were transferred under sterile conditions to test tubes. Fragments of the internodes were placed in the medium in the vertical position (reversed 180° from their natural position), on the contrary fragments of petioles were laid horizontally. The following agar media, each containing 3 per cent of sucrose, were used: 1) Murashige and Skoog (1962) — MS — and its modifications containing two- or ten-times reduced concentration of macro and micro-elements (1:2 and 1:10);

2) Nitsch and Nitsch (1969).

All the combinations of media were enriched with auxins and cytokinins in various concentrations and combinations (Table 1). As control were used the above mentioned media but without growth substances. The cultures were kept under photoperiod conditions L:D-8:16 and in darkness at $25^\circ \pm 2^\circ\text{C}$ and relative humidity about 60 per cent. In experiments where the photoperiod was applied white light from fluorescent tubes was used of 860 lux intensity.

Table 1

Various doses of exogenous growth substances added to the Murashige and Skoog and Nitsch and Nitsch media (concentration in mg/dm^3)

Cytokinin	+	Auxin
KIN—0.5	+	2,4-D—0.5 or 1
KIN—0.5	+	NAA—0.5 or 1
KIN—0.5	+	IAA —0.5 or 1
BAP—1	+	2,4-D—0.5 or 1
BAP—1	+	NAA—0.5 or 1
BAP—1	+	IAA —0.5 or 1
BAP—2	+	0

For cytological observations the callus tissue from the first and second passage was first fixed in the Karnovsky solution (1965) in cacodylate buffer, pH 6.8 and then in OsO_4 in the same buffer. Later it was dehydrated in an increasing acetone gradient and with propylene oxide and embedded in Epon 812. Material was sectioned (semithin 1-3 μm and ultrathin) on a Tesla BS 490 or LKB-Ultratome III ultramicrotome. For observation in a light microscope the semithin sections were stained with toluidine blue after Kay (1965). The size of the cells was determined by measuring them randomly in a micrometric ocular and the calculations were performed according to the commonly used statistical methods (Okta 1976). Photographs were taken in the light microscope „Amplival” with an attachment for automatic determination of the time of irradiation “mf-matic” (Zeiss). Ultrathin sections after contrasting with uranyl acetate and lead citrate were observed and photographed in a transmission electron microscope JEM 7A.

RESULTS AND DISCUSSION

The regeneration ability of three types of explantates of *Liriodendron tulipifera* L. were analysed. Callus tissue developed only in one half of the explanted internodes and petioles. Fragments of leaves did not produce calluses and shortly after the culture they degenerated (Table 2). Callus was formed only in the presence of exogenous growth substances, among which most effective proved to be the combination of 2,4-D with kinetin (KIN). Callus was most abundant on fragments of internodes and petioles in the third week after transferring onto the Nitsch and Nitsch medium with addition of 2,4-D — 0.5 mg/dm^3 + KIN — 1 mg/dm^3 or MS (1:2) containing only BAP — 2 mg/dm^3 . In both cases the callus was very soft and watery and it grew very slowly.

Table 2
Callus formation from various explants

Explant	Leaf	Petiole	Internode
Total number of inoculated explants	438	483	416
No. of explants per one variant of medium	8.4	9.2	8.0
Explants forming callus, %	a) 0 b) 0	52 48	49 45

a) Nitsch and Nitsch medium: 2,4-D — 0.5 mg/dm^3 ;

b) MS medium (1:2): BAP — 2 mg/dm^3

Light had no effect on the development of callus or on its rate of growth. In darkness callus was whitish-yellow, whereas under photo-periodic conditions it was light green. The colour of calluses did not change during the culture, only those areas which start to degenerate turned brown (28th day since inoculation). In spite of transferring calluses onto fresh medium with various concentrations of auxins and cytokinins the degeneration of calluses proceeded further.

As it ensue from the literature the apical buds exhibit a high morphogenetic potency since from callus regenerate fully developed plants (Furmanowa and Rzędowski 1979, Rzędowski 1981). Fragments of stems produce callus tissue capable of forming roots (Rzędowski 1981) and the unligified internodes and leaf petioles tested for the first time in the present study, only undifferentiating callus, whereas fragments of leaves when explanted on various media show no potency of regeneration.

This ordering of the morphogenetic potency may be helpful when undertaking further investigations since, a suitable selection of the explant plays an important role for getting positive results.

As already mentioned, callus tissue degenerated very soon, therefore it was worth while to examine it cytologically. It was hoped that due to a detailed analysis of this tissue it would be possible to find a cause or causes responsible for degeneration process. Therefore, 4-day-old light green callus obtained from internodes and the same type showing the first symptoms of degeneration (after 27 days of culture) were analysed.

The observations made in a light microscope indicated that 4-day-old callus consisted of light green minute different-shaped closely adhering cells (Fig. 1) of $39.23 \mu\text{m}$ mean size.

Analysis of the electronograms from this variant showed the presence of two cell types: with a large central vacuole (these prevailed — Fig. 2) and cells resembling "meristematic nests" (Fig. 5). Cells of the former type had an electron-dense cytoplasm (Figs. 2, 3). Like in other normal callus cells it formed a thin layer along the wall, becoming wider only near the nuclei (Fig. 2). In these cells nucleoli were mostly highly "vacuolised" (Fig. 4). The structure of plastids was alike that of chloroplasts at their very early stage of development. It was possible to distinguish inside them some structures resembling the development of grana (Fig. 3a). Plastids were seen only sporadically, their "steplike" grana included 6-12 thylakoids (Fig. 3b). The presence of relatively numerous mitochondria with a well developed system of cristae may be indicative of a high metabolic activity of these cells. The second type of cells consisted of much smaller cells filled almost completely

Figs. 1-3. Light green (4-day) callus tissue from internodes of *Liriodendron tulipifera* L. on Nitsch and Nitsch medium (1969) with 0.5 mg/dm^3 2,4-D + 1 mg/dm^3 KIN added under conditions of L:D — 8:16 photoperiod, toluidin blue staining. Cw — cell wall, M — mitochondrion, N — nucleus, P — plastid, T — thylakoid, V — vacuole. Fig. 1. Part of callus tissue in light microscope. $\times 650$. Fig. 2. Fragments of the same tissue in electron microscope. $\times 2500$. Fig. 3a. Plastids and mitochondria in callus tissue. $\times 30\,000$. Fig. 3b. Plastid with higher degree of organisation of thylakoid system. $\times 30\,000$

Figs. 4-6. Light green (4-day) callus tissue from internodes of *Liriodendron tulipifera* L. on Nitsch and Nitsch medium (1969) with 0.5 mg/dm^3 2,4-D + 1 mg/dm^3 KIN added under conditions of L:D-8:16 photoperiod, toluidin blue staining. Cw — cell wall, ER — endoplasmic reticulum, G — Golgi apparatus, M — mitochondrion, N — nucleus, Nu — nucleolus, NV — nucleolar vacuole, P — plastid, V — vacuole. Fig. 4. Cell fragment with nucleus containing vacuolised nucleolus. $\times 15\,000$. Fig. 5. Cell from the area of the "meristematic nests". $\times 4000$. Fig. 6. Enlarged fragment of "meristematic" cell with typical plastid. $\times 20\,000$.

Figs. 7-9. Degenerating (28-day-old) callus tissue from internodes of *Liriodendron tulipifera* L. on Nitsch and Nitsch medium (1969) containing 0.5 mg/dm^3 2,4-D + 1 mg/dm^3 KIN under conditions of L:D-8:16 photoperiod, toluidin blue staining. Cw — cell wall, Gl — globule, V — vacuole. Fig. 7. Loosely arranged callus cells visible in light microscope. $\times 400$. Fig. 8. Enlarged tissue fragment. $\times 2000$. Fig. 9. Cell with large unidentified globule situated centrally in vacuole. $\times 2000$

Figs. 10, 11. Degenerating (28-day-old) callus tissue from internodes of *Liriodendron tulipifera* L. on Nitsch and Nitsch (1969) medium containing 0.5 mg/dm^3 2,4-D + 1 mg/dm^3 KIN under conditions of L:D-8:16 photoperiod. Staining with toluidin blue. Cw — cell wall, ER — endoplasmic reticulum, L — lipid globule, M — mitochondrion, P — plastid, St — starch, V — vacuole. Fig. 10. Cell fragment with amyloplasts. $\times 7500$. Fig. 11. Electron micrograph of part of cell in final stage of lysis. $\times 4000$

with cytoplasm of lower electron density (Fig. 5). They resembled in their appearance the meristematic cells. Their vacuoles were minute and the plastids corresponded morphologically to proplastids (Fig. 6). Numerous polyribosomes were present in the cytoplasm, both free ones and others bound with the endoplasmic reticulum. There were also relatively numerous active mitochondria and Golgi structures. On the other hand, degenerating callus (brown) was built of larger loosely lying cells of mean size $46.68 \mu\text{m}$. Some of them contained vacuoles staining with toluidin blue to bluish with dark blue globules of various dimensions inside (Fig. 7).

Observations in the electron microscope revealed that only a small proportion of the cells had preserved the normal appearance of viable callus cells (Figs. 8, 9, 10), whereas the contents of the remaining ones underwent various degrees of lysis (Fig. 11). Within the vacuoles of these still living cells of brown callus electron-dense globules were noted (Fig. 9). The cytoplasm and the intraplasmic structures were normal. Only the plastids which in light green 4-day-old callus resembled chloroplasts or proplastids, in the degenerating callus (28-day) resembled typical amyloplasts, almost completely filled with numerous large starch grains (Fig. 10). Beside these cells of more or less normal appearance others which usually showed various degrees of degeneration (Fig. 11).

The cytological observations do not enable to elucidate what responsible such considerable deformations of the calluses cells, however, in combination with the results of the other authors they may serve as basis for postulating the following hypothesis. It is known that liriodenin present in this species, possesses strong mitostatic properties (Rzędowski 1981). The investigations of this author (1981) indicate that the amount of liriodenin in callus developed from fragments of stems or buds was two or even three time higher than that in the explantates. It is, thus, possible that the high increase in the concentration of liriodenin in the callus inhibited the division of its cells. It should be added that also the cell size may favour this hypothesis. Therefore, the mean size of the 4-day-old callus cells (viable, light green) was compared with that of 28-day callus (brown, degenerating). To ensure objective numerical data, Student's "t" test was applied. The differences in the mean size of the cells between the two types of callus were found to be statistically significant at the $\alpha=0.05$ level. This seems to indicate that divisions are less frequent in the brown callus than in the light green one.

Further investigations are necessary to clarify, why regenerating processes in *Liriodendron tulipifera* L. are so meagre and wheat factor

(factors) are inhibiting organogenesis in calluses. Particularly it should be elucidated what role plays the high concentration of lirioidenin present in those mother cells which produce the callus tissue.

Acknowledgements

We wish to thank Professor Maciej Zenkteler for critical reading and help in preparing the manuscript.

This research was partially financed from problem MR.II/16.

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Badania ultrastrukturalne tkanki kalusowej otrzymanej metodą mikrorozmnażania Liriodendron tulipifera L.

Streszczenie

Stosując nie badane do tej pory eksplantaty *Liriodendron tulipifera* L. prowadzono próby mikrorozmnażania tej rośliny. Obfitą tkankę kalusową wytwarzały jedynie niezdrewniałe międzywęzła i ogonki liściowe. Mimo stosowania różnych kombinacji pożywek nie udało się doprowadzić do zainicjowania procesów organogenezy kalusa, który po pewnym czasie zamierał. Prowadzone równoległe obserwacje cytologiczne ujawniły w nim znaczne zmiany degeneracyjne komórek.