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Tissue Culture Note

Relationship between starch accumulation and organ development at the different growth stages of callus in Kihada (*Phellodendron amurense* Rupr.)

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Abstract Microscopic observations of Kihada (*Phellodendron amurense*) callus revealed a strong correlation between starch accumulation and organ development. The starch accumulation started before any observable organ development and was prominent in cells which ultimately gave rise to organ primordia. A large amount of starch accumulation was observed in the three- or four-celled proembryo-like structure (PLS) organization of callus cultured on MS media supplemented with BAP plus 2,4-D. Two-celled PLS showed low performance for accumulation of starch. No starch accumulation was observed in globular and heart-shaped embryo-like structure ELS organization. During shoot primordium development, a decrease in the starch content of the cultured tissues was found, indicating the utilization of the glucan in the organogenic process. Starch granules disappeared with the development of the meristematic dome and leaf primordium and also started to disappear gradually with the development of cell walls and the tracheary elements.

Key words: Callus, kihada, organ development, organogenesis, starch accumulation.

Kihada (*Phellodendron amurense* Rupr.) is a medicinal woody plant belonging to the family Rutaceae and widely found in Korea, China, and Japan. This plant is well known to contain medicinal compounds like berberine, palmatine, magnoflorine etc as secondary metabolites. Most recently, research has focused mainly on berberine which is found usually in the bark, root, and fruit. This plant is used in Japan and China as crude drugs, such as an anti-stomachic for intestinal function control, an anti-inflammatory, and an anti-pyretic agents.

Plant cells and tissues have totipotency to regenerate into plants via embryogenesis and organogenesis (Mangat et al. 1990). Medium and tissue carbohydrates play important roles in this process. It is assumed that sucrose is the best carbohydrate in cell culture media because it is the main transport form of carbohydrates in most species (Strickland et al. 1987). Histological observations revealed that plant cells about to undergo *in vitro* developmental processes have elevated starch contents (Mangat et al. 1990). Ribas et al. (2000) observed starch accumulation in embryogenic cells of *Aspidosperma polyneuron*. Jensen (1963) observed an abundance of starch in the zygote during the early stages of embryo development in cotton. Using histochemical techniques, Thorpe and Murashige (1970) showed that starch was accumulated in the shoot-forming regions of the tissues just prior to the initiation of organs in tobacco callus cultures. An ultrastructural study of the shoot-initiation process also showed that starch was utilized by tobacco cells during early meristemoid formation and confirmed the mobilization of the starch from the surrounding tissue regions during the subsequent development of the organs (Ross et al. 1973). Thorpe and Meier (1974) observed evidence that continuous availability of carbohydrates is required for shoot primordium growth and/or development into leafy vegetative shoots. The pattern of starch and its use have been reported earlier in other embryogenic and organogenic systems (Blanc et al. 2002).

Cell division occurs naturally in callus. Sometimes unorganized cells become organized and developed shoots or roots under controlled conditions of plant growth regulator. To develop organ, starch accumulation is the pre-requisite and it may work as a catalyst in the

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; ELS, embryo-like structure; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; PLS, proembryo-like structure.
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cell to develop shoots or roots. The degradation products of starch are utilized for the elongation of the cells, possible in the buildup of new cell wall materials. If starch was absent in the cell, callus failed to initiate any organ.

Following this view, we attempted to examine the relationship between starch accumulation and organ development at different growth stages of callus in Kihada. We can get a lot of callus within a short time by subculture using proper plant growth regulator. Before investigating the relationships between the biosynthesis of medicinal compounds and organ development, the present study examined the relationships between starch accumulation and organ development in Kihada.

There are some reports concerning the *in vitro* culture of Kihada related to mass propagation by axillary bud/callus culture (Azad et al. 2004, 2005). To our knowledge, this is the first report on the observation of a relation between starch accumulation and organ development from hypocotyl-derived callus in Kihada.

Fruits of Kihada were obtained from a 50-year-old tree growing at the Medicinal Plant Garden of Kumamoto University, Japan. Flesh was removed from fruits, and seeds were recovered. They were washed with detergent-containing tap water for 15 min and then rinsed thoroughly with running tap water for 20 min. They were surface-sterilized with 70% ethanol for 3 min and then with 3% (v/v) sodium hypochlorite aqueous solution (Wako Pure Chem. Ind. Ltd., Japan) for 20 min. The surface-sterilized seeds were then washed with at least three changes of sterile-distilled water to remove any traces of the sterilants. Sterilized seeds were germinated on 10 ml of MS (Murashige and Skoog 1962) medium supplemented with 2.0 μM BAP in culture tubes (120×25 mm, Asahi Techno Glass, Japan). The medium contained 3% sucrose and 0.2% gellan gum, and its pH was adjusted to 5.7±0.1. The medium was autoclaved under 1.2 kg cm^{-2} pressure at 120°C for 20 min. The seeds were incubated at 25±1°C under 16-hr photoperiod with a light intensity of 50 μmol·m^{-2}·s^{-1}.

After 4 weeks, hypocotyl explants were excised from *in vitro* grown seedlings and placed in Petri dishes (9×1.5 cm, Asahi Techno Glass, Japan) containing 30 ml MS semi-solid medium supplemented with different concentrations and combinations of BAP and NAA, IBA, or 2,4-D for inducing callus. Media containing different levels (0.44, 0.89, 2.22, and 4.44 μM) of BAP in combination with NAA (1.07, 2.69, 5.37, and 10.74 μM), IBA (0.98, 2.46, 4.92, and 9.84 μM) or 2,4-D (0.90, 2.26, 4.52, and 9.05 μM) were used for induction of callus. Continuous propagation of callus was done by subculturing it on callus medium every 8 weeks. The initial callus (6–8 mm in diameter) was then subdivided into three to four small calli (3 mm in diameter), which were separately cultured on the callus media for propagation. Two types of callus were used in this experiment: calli with ELSs which were developed on MS medium supplemented with BAP plus 2,4-D and regenerative calli which were developed on MS medium containing BAP plus NAA or IBA.

Calli were sampled at 2- to 7-day intervals from callus initiation and were fixed with FAA (5 ml formalin, 130 ml ethanol, and 5 ml acetic acid) at room temperature for 2 days. They were rinsed thoroughly with running tap water for 1 day and then dehydrated with graded ethanol series (ethanol concentration from 50% to 100% (v/v), 1 day for each treatment). After ethanol was replaced with xylene, the calli were embedded in paraffin and then cut into sections 10–15 μm thick by rotary microtome (1512, Leitz, Germany). The sections were fixed with 3% gelatin (v/v) on slide glasses, deparaffined with xylene, and then stained consecutively with saffranin and fast green. Finally, stained sections were observed with normal light and a polarizing microscope (BX 51, Olympus, Japan).

Among different concentrations and combinations of the PGRs in the MS medium, 2.22 μM BAP plus 4.52 μM 2,4-D and 4.44 μM BAP plus 5.37 μM NAA showed better performance for callus initiation of Kihada.

The first cell division in vascular tissues was found after 10 days of culture, showing periclinal or anticlinal cell division. After 12 days, the second division of the two-celled proembryo-like structure (PLS) took place in one of the two cells to form a three-celled PLS or in both cells simultaneously to form a four-celled PLS. A five-celled PLS was formed after 14 days of culture. These events were reported to be derived from a single cell origin in vascular tissues (Azad et al. 2005). Among different types of PLS organization, two-celled PLSs showed relatively lower starch accumulation, where the formation of cell walls had just been initiated (Figure 1A_1, A_2). On the other hand, three- to five-celled PLSs showed greater accumulations of starch, where cell walls were observed distinctly (Figure 1B_1, B_2; C_1, C_2). Furthermore, the starch granules were fewer in the amount of them accompanied with the development of cell walls and they were randomly distributed along the cell walls. The number of starch granules was dramatically decreased during the development of ELS organization. In most cases, cell division occurred after 10 to 15 days of culture within groups of adjacent cells of vascular tissues which thereafter developed into cell masses and the globular structure of ELS. The globular ELS was formed on MS medium containing 2.22 μM BAP plus 4.52 μM 2,4-D after 7 weeks of culture. Globular ELS was composed of small cells with a dense cytoplasm. When the calli with globular ELSs were transferred to MS medium supplemented with 1.5 μM BAP plus 1.0 μM NAA or IBA, cell differentiation took

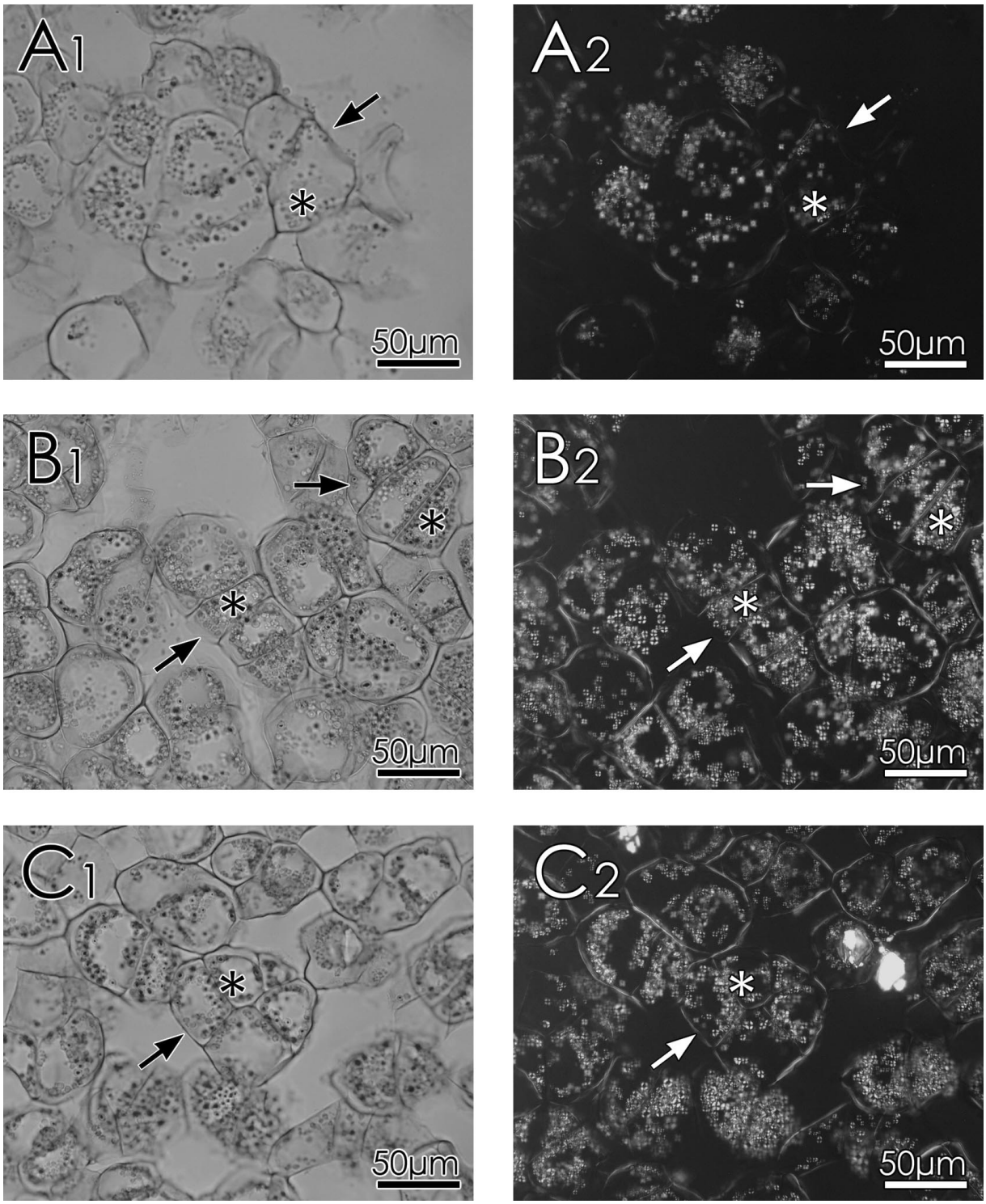

Figure 1. Transverse sections of PLS stage callus. A_1, A_2: Two-celled PLS (arrow); B_1, B_2: Three- or four-celled PLS (arrow); C_1, C_2: Five-celled PLS (arrow). Each PLS showed starch granule accumulation (asterisk) which were developed on MS medium containing 2.22 μM BAP+4.52 μM 2,4-D. A_1, B_1, C_1: Light microphotographs; A_2, B_2, C_2: Polarizing microphotographs.

place progressively. After 4 weeks of transfer, some ELSs turned to heart-shaped structures and developed a meristematic zone. No starch accumulation was observed in globular and heart-shaped ELSs (Figure 2A_1, A_2; B_1, B_2).

In the case of regenerative callus, the meristematic cells or tissues were distinguishable within 10 days of culture. The meristematic tissues formed meristematic zone and developed into a meristematic dome with procambium cells after 4 weeks of culture. It was

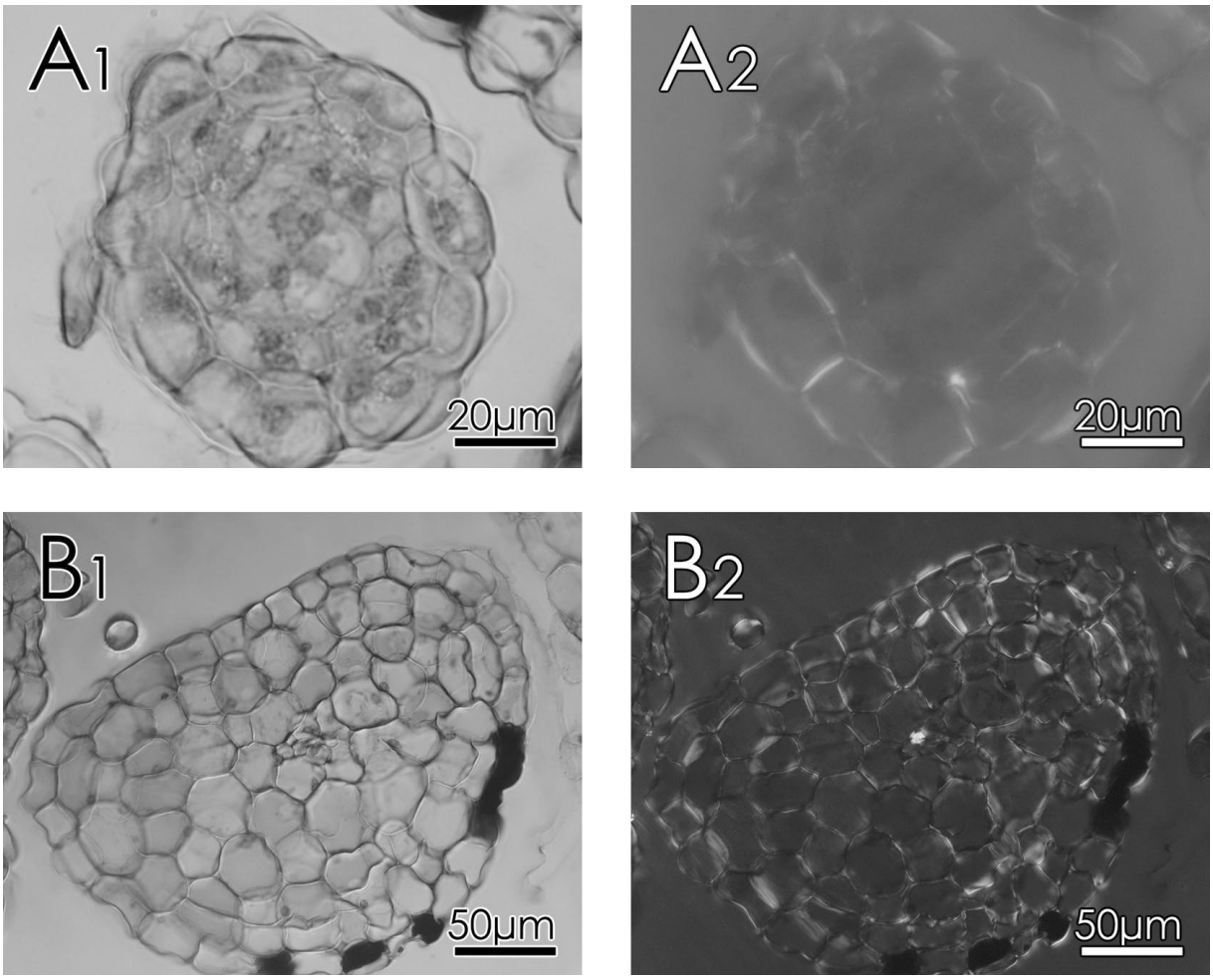

Figure 2. Transverse sections of ELS stage callus. A_1, A_2: Globular ELS; B_1, B_2: Heart-shaped ELS. These ELSs showed starch disappearance which were developed on MS medium containing 2.22 μM BAP+4.52 μM 2,4-D. A_1, B_1: Light microphotographs; A_2, B_2: Polarizing microphotographs.

recognized that the procambium cells were differentiated at the periphery of the central cylinder below the meristematic dome. After 6 weeks of culture, the active meristematic dome gave rise to a leaf initial. Each meristematic dome was surrounded by leaf initials, leaf primordia, and preliminary leaves. Starch accumulation was observed in the surrounding tissues of the meristematic zone (Figure $3A_1$, A_2), where starch granules disappeared with the development of the leaf primordium and meristematic dome (Figure $3B_1$, B_2).

Distinctive cell differentiation in cortical and medullar parenchyma, primary vascularization, and first formation of initial tracheary elements were found under the leaf primordium. These elements were found only in the BAP plus NAA- or IBA-containing MS media after 6 weeks of culture, whereas they were absent in BAP plus 2,4-D-containing medium. Starch disappearance was also observed with the development of tracheary elements (Figure $3C_1$, C_2).

This study was undertaken to gain a better understanding of starch accumulation and organ development at different growth stages of callus in Kihada. Cell division occurs naturally in callus. Unorganized cells sometimes became organized and later developed shoots or roots under controlled conditions of PGRs. It has been shown with callus cultures that the basic regulatory mechanism underlying plant organ initiation involves a balance between auxin and cytokinin (Skoog and Miller 1957). Azad et al. (2005) reported that in kihada callus proliferation was active on MS media containing BAP plus NAA, whereas the formation of ELSs was found in MS media containing BAP plus 2,4-D. In the present study, we used PLS- and ELS-stage calli produced by Azad et al. (2005).

Two- to five-celled PLSs were formed in vascular tissues within 10 to 14 days of callus culture (Figure 1). During the maturation of the proembryos, a protoderm was formed through the establishment of a peripheral zone consisting of one or several layers of cells, and vascular tissues appeared. Prior to this cell division, a significant accumulation of starch was noticed at this stage. Barciela and Vieitez (1993) have found

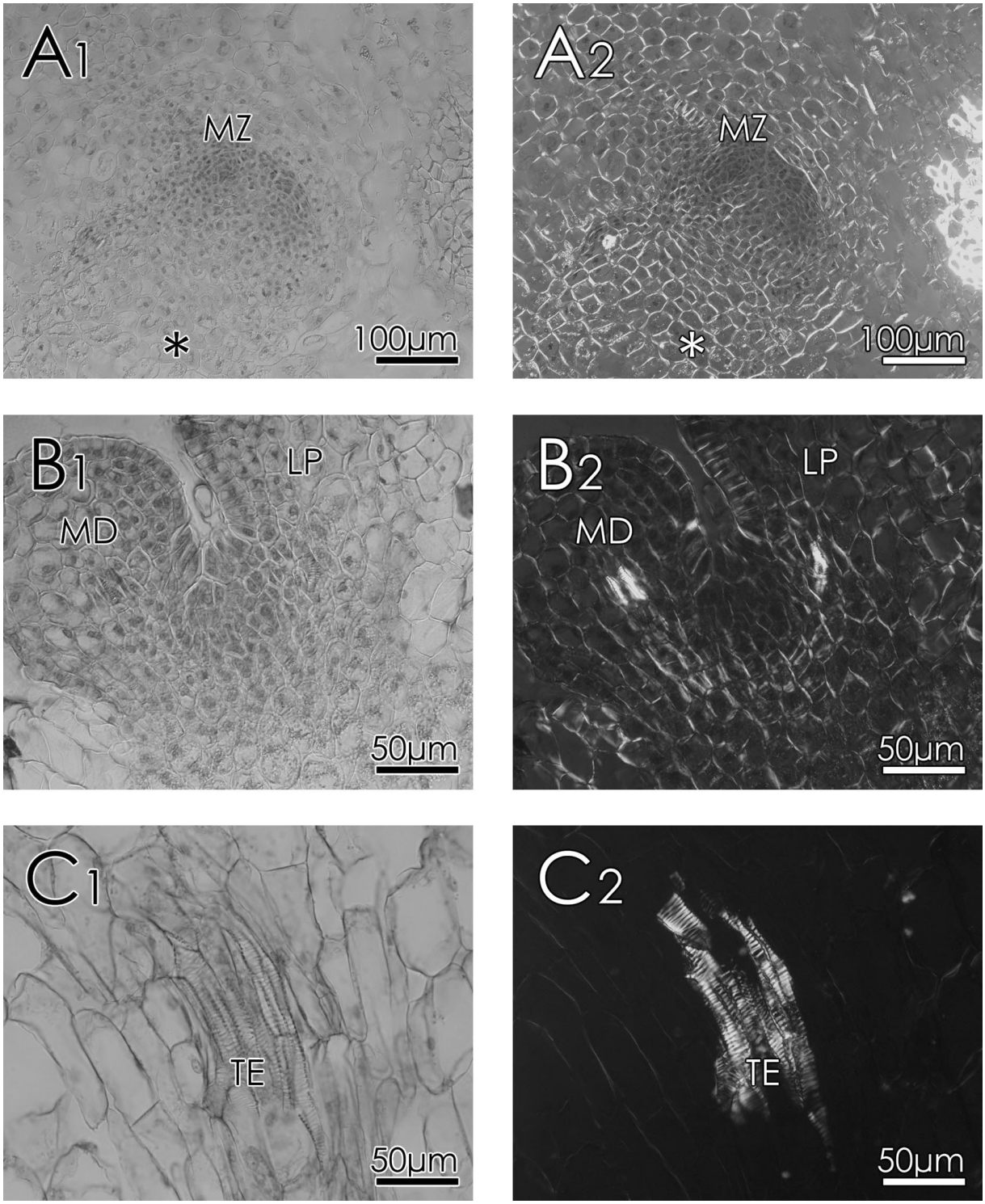

Figure 3. Starch accumulation or disappearance in callus tissues acompanied with organ development. A_1, A_2: Meristematic zone (MZ). Starch granules existed in the surrounding tissue of meristematic zone (asterisk); B_1, B_2: Leaf primordium (LP), meristematic dome (MD); C_1, C_2: Tracheary elements (TE). These structures were developed on MS medium containing 2.22 μM BAP+5.37 μM NAA. A_1, B_1, C_1: Light microphotographs; A_2, B_2, C_2: Polarizing microphotographs.

starch grain accumulation at superficial layers of proembryogenic cells of *Camellia japonica.* Maâtaoui et al. (1990) also observed starch accumulation in the proembryogenic cells of *Quercus suber*.

In the present study, we did not find starch accumulation in globular and heart-shaped ELSs (Figure 2). Starch may be used at the early growth stages of callus. Plata et al. (1991) suggested that starch should be promptly metabolized in embryogenic tissues, supplying energy for localized mitotic and metabolic activities.

During the early formation of the meristematic zone, however a significant number of starch granules were present in the surrounding tissues (Figure $3A_1$, A_2). Jordy (2004) observed an abundant amount of starch accumulation in the meristematic cells of *Pinus pinaster*. Thorpe and Murashige (1970) also observed starch accumulation in meristematic cells of tobacco callus tissues. In contrast, with the development of the meristematic zone, starch started to disappear from these cells. No starch was observed in the meristematic dome and leaf primordium (Figure $3B_1$, B_2). Starch granules also disappeared after the development of the tracheary elements (Figure $3C_1$, C_2). Ross et al. (1973) showed ultra-structrally that the disappearance of starch in tobacco callus preceded meristemoid formation in the cells directly involved in the organogenic process. Similarly, Howarth et al. (1983) also found the disappearance of starch grains during cellular differentiation in small clumps of *Lotus* callus. According to Patel and Berlyn (1983), strong amylase activity in the cells of organogenic centers of cultured explants in *Pinus coulteri* was responsible for the degradation of starch. These reports support the mobilization and utilization of starch as a prime source of energy for the differentiation and growth of shoot primordia. Sadik and Ozbun (1967) also observed a direct relationship between the presence of starch and floral induction in cauliflower. They found that floral primordia which developed into functional flowers had a very heavy accumulation of starch, while floral primordia which aborted were devoid of starch.

The accumulation of starch and its subsequent disappearance from the cells of the developing shoot primordia and the subjacent tissues suggest that starch was used both during organ initiation and its later development (Mangat et al. 1990). Although the accumulation and disappearance of starch and the need for continued free sugars in the medium have been correlated with the shoot-forming process, no information on the actual contribution of the breakdown products of starch to this process is available (Thorpe et al. 1986). The degradation products of starch are probably utilized for the elongation of growth of the cells, possibly in the buildup of new cell wall materials, and in energy production (Patel and Thorpe 1984).

The physiological significance of starch accumulation has been recognized in organ initiation. During organogenesis, energy and structural material may be supplied for the shoot apical meristem and the adjacent cells by starch hydrolysis, which may impact gene expression programs (Jordy 2004). Two major roles of starch have been proposed, namely, that of an energy reserve for the high-energy process of organogenesis and the provision of osmotic agents in the form of free soluble sugars (Thorpe et al. 1986). We suggest that the accumulation of starch functions in the initiation of organized structures in Kihada callus.

Based on the results of this investigation, it can be concluded that a close relationship exists between starch accumulation and organ development at different growth stages of callus in Kihada. The present results strongly support causative roles of starch in organogenesis.

References

Azad MAK, Yokota S, Ishiguri F, Yahara S, Yoshizawa N (2005) Histological studies of shoot regeneration system in hypocotyl-derived callus of *Phellodendron amurense* Rupr. *J For Res* 10: 377–384

Azad MAK, Yokota S, Ohkubo T, Andoh Y, Yahara S, Yoshizawa N (2004) Micropropogation of plantlets through callus in Kihada (*Phellodendron amurense* Rupr.). *J SHITA* 16: 122–130

Barciela J, Vieitez AM (1993) Anatomical sequence and morphometric analysis during somatic embryogenesis on cultured cotyledon explants of *Camellia japonica* L. *Ann Bot* 71: 395–404

Blanc G, Larder L, Martin A, Jacob JL, Carron MP (2002) Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Müll. Arg). *J Exp Bot* 53 (373): 1453–1462

Howarth MJ, Peterson RL, Tomes DT (1983) Cellular differentiation in small clumps of *Lotus corniculatus* callus. *Can J Bot* 61: 507–517

Jensen WA (1963) Cell development during plant embryogenesis. *Brookhaven Symp Biol* 16: 179–202

Jordy M-N (2004) Seasonal variation of organogenetic activity and reserves allocation in the shoot apex of *Pinus pinaster* Ait. *Ann Bot* 93: 25–37

Mangat BS, Pelekis MK, Cassells AC (1990) Changes in the starch content during organogenesis in *in vitro* cultured Begonia rex stem explants. *Physiol Plant* 79: 267–274

Maâtaoui ELM, Espagnac H, Michaux-Ferriere N (1990) Histology of callogenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) cultured *in vitro*. *Ann Bot* 66: 183–190

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

Patel KR, Berlyn GP (1983) Cytochemical investigations on multiple bud formation in tissue cultures of *Pinus coulteri. Can J Bot* 61: 575–585

Patel KR, Thorpe TA (1984) Histochemical examination of shoot initiation in cultured cotyledon explants of Radiata pine. *Bot Gaz* 145(3): 312–322

Plata E, Ballester A, Vieitez AM (1991) An anatomical study of secondary embryogenesis in *Camellia reticulata. In Vitro Cell Dev Biol* 27: 183–189

Ribas LLF, Guerra MP, Zanette F, Kulchetscki L (2000) Somatic embryogenesis in *Aspidosperma polyneuron* Mull. Arg In: Jain SM, Gupta PK, Newton RJ (eds) *Somatic Embryogenesis in Woody Plants vol 6.* Kluwer Academic Publishers, Dordrecht, Boston, London, pp 509–537

Ross MK, Thorpe TA, Costerton JF (1973) Ultrastructural aspects of shoot initiation in tobacco callus cultures. *Am J Bot* 60: 788–795

Sadik S, Ozbun JL (1967) Histochemical changes in the shoot tip of cauliflower during floral induction. *Can J Bot* 45: 955–959

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol* 11: 118–131

Strickland SG, Nichol JW, McCall CM, Stuart DA (1987) Effect of carbohydrate source on alfalia somatic embryogenesis. *Plant Sci* 48: 113–121

Thorpe TA, Meier DD (1974) Starch metabolism in shoot-forming tobacco callus. *J Exp Bot* 25: 288–294

Thorpe TA, Murashige T (1970) Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Can J Bot* 48: 277–285

Thorpe TA, Joy IV RW, Leung DWM (1986) Starch turnover in shoot-forming tobacco callus. *Physiol Plant* 66: 58–62