HOKKAIDO UNIVERSITY

Title	Autoradiographic Observation on Growth and Differentiation of Cultured Potato Tissues
Author(s)	OKAZAWA, Yozo
Citation	Journal of the Faculty of Agriculture, Hokkaido University, 57(4), 431-442
Issue Date	1974-09
Doc URL	https://hdl.handle.net/2115/12888
Type	departmental bulletin paper
File Information	57(4)_p431-442.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

AUTORADIOGRAPHIC OBSERVATION ON GROWTH AND DIFFERENTIATION OF CULTURED POTATO TISSUES

Yozo OKAZAWA

(Department of Botany, Faculty of Agriculture,
Hokkaido University Sapporo)

Received Dec. 25, 1973

INTRODUCTION

As pointed out in the previous investigation (OKAZAWA et al 1967), parenchymatous tissue excised from potato tuber induces callus formation on the medium with an adequate supply of auxin alone, and produces adventitious roots instead of callus formation when auxin in combination with a relative higher concentration of kinetin is incorporated into the medium. We may reasonably conjecture that an induction of cell multiplication proceeds by inciting DNA duplication in the quiescent cells of the tuber tissue due to the application of the growth substances. Accordingly it is worthwhile to evaluate variation with time in effectiveness of the growth substances on DNA synthesis occurring in the nuclei of cells in culture during the progress of their growth.

In a pioneer study (REICHARD and ESTBORN 1951), it has already shown the fact that thymidine was a precursor of DNA and was not diverted to the synthesis of RNA. In the experiments described below, therefore, an attempt was made to study this problem by following the incorporation of radioactive thymidine to the cell nuclei of tissues cultured under several conditions, by means of histological and microautoradiographic techniques.

MATERIALS AND METHODS

The experimental materials used throughout the investigation were isolated from the mature tubers of *Solanum tuberosum* L., commercial variety "Irish Gobbler" which were stored in cellar. The preparation of explant has been described in detail by OKAZAWA et al (1967). The explants were cultured at 25°C in darkness on the solid nutrient medium, which was the modified White's mineral salts mixture in 2 per cent sucrose as described in the earlier publication (OKAZAWA et al 1967). Cultures were

selected at random on 0, 1, 3, 6, 9, 12, 15 and 27 day after inoculation, harvested and studied histologically. Light microscope autoradiography was carried out as follows. The experimentally obtained explants were cut medianly into slices about 2 mm in thickness, perpendicular to the surface which was previously in contact with the medium. The slices were incubated by immersing in small volume of solution on a slowly-shaking machine to aid in aeration. The isotope used was thymidine (methyl-T, 23.5 c/mM) obtained from the Radiochemical Center, Amershan UK. Incubation solution contained 20 μc/ml of isotope, α-naphthaleneacetic acid (NAA) and kinetin (KIN) where applicable. After incubation the slices were chased for 2 hr in non-radioactive solution containing 17.0 μg/ml thymidine, plus the appropriate concentration of the hormones. Each of the tissues immediately after chasing was fixed in FAA, dehydrated by passage through an ethanol-tertiary butyl alcohol series and embedded in pareffin as outlined by JENSEN (1962). The paraffin embedded materials were cut at 8 μ with a rotary microtome, and affixed to glass slides by chrome-alum adhesive, followed to hydrate in the usual manner in ethanol-water series. The slides were then coated by dipping in Sakura NRM-2 nuclear emulsion diluted 1:1 with water, and exposed for 14 days at 2°C. The slides were then developed for 4 minutes at 20°C in Conidol-X (Sakura Co.), rinsed in a one per cent acetic acid stop bath and fixed in Conifix (Sakura Co.) for 15 minutes. The developed sections were stained with safranin and fast green.

RESULTS

Histologically. the explant tissues were composed of uniform parenchymatous cells, in which stored a large amount of starch grains. Within a four week culture period, the explants soon turned dark brown with neither formation of callus nor initiation of adventitious root on the medium devoid of auxin. At the end of culture, cell wall of large vacuolated cells was stained prominently red with safranin, which cells were localized along the outer periphery of explant tissue. When NAA was fed at 0.3 mg/l into the basal medium, the mitotic figures became to be evident in the preparation of the cultured tissues within two days after inoculation (Plate I-2). At this stage all of these mitotic figures were observed in the cells which are close behind the outermost layer ones of tissues. An outgrowth of callus proceeded by taking place a fairly high rate of cell division from all side of explants 5-7 days after inoculation (Plate I-3). Finally the appearance of proliferated callus was buff to light brown at the end of the fourth week (Plate I-8).

In the following experiment, when the cultures were received 0.05 mg/l of NAA in combination with kinetin at 1.0 mg/l, similar appearance of mitotic figures on the periphery layer of explant was also resulted within a few days. However the suberous wall of outermost layer cells became to be stained deeply with safranin, and active dividing cell clumps scattered randomly among the large vacuolated quiescent cells (Plate I–2, 3 and 4). The outgrowth of the cell clumps originated deep within explant and the interior cell groups of the clumps exhabited tracheidal thickening that were not in an organized pattern (Plate I–5, 6 and 7). These tissues differentiated into nodule-like nests of cells, which tissues differed from the mother cells in the tissue by being smaller, thinner walled, lacking apparent vacuoles and having densely stained nuclei and cytoplasm (Plate I–9, and 10). About two weeks later, the meristematic nodules changed into apical meristems of root and succeeded to grow aerial root or some of which penetrated deep into the medium (Plate I–11 and 12). Finally, in this case of culture, it became to be a dark brown decicated tissue with numerous adventitious roots and their germs.

To obtain information as to the distribution of the cells with active nuclei in DNA synthesis, following microautoradiographic techniques were employed. Within a day after inoculation, the tritiated thymidine was incorporated rapidly into the nuclei of several quiescent cells in the explants, which were cultured on the medium supplemented with or without growth substances (Plate II–1 and 2). These cells with active nuclei were randomly scattered throughout the tissues (Plate II–3 and 4). However these inocula failed to proceed proliferation of callus cells when cultured in the absence of exogenous auxin, and the labelling activity of these cells decreased steeply to be almost nil within additional 5 days (Figure 1 and 2). On the other hand, the addition of NAA without kinetin to the medium elicited an outgrowth of the callus, and the quiescent cells in the interior of explants were remained as core (Plate I–8). While labelling activity of nucleus in the auxin culture showed a decreasing trend similar to that of control for the first 6 days wtih advancing of culture (Figure 1 and 2), a stimulating incorporation of the tritiated thymidine at the meristematic region preceded a vigorous outgrowth of callus from the flanks and upper peripheral layer of inocula due to the application of NAA. These marked incorporation passed through a maximum on the 10th or 12th day, and then decreased constantly as the stationary phase of callus growth approached (Figure 1, 2 and 3). This is virtually compatible with the fact that the cell dividing zone accompanying with callus proliferation was coincided with

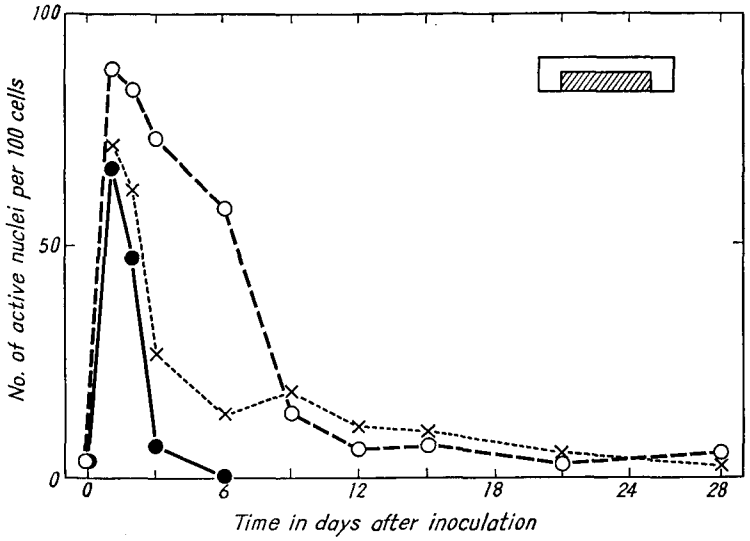


Figure 1. Incorporation of tritiated thymidine into the nuclei of potato tissues cultured on the several media. Counting cells were distributed in the interior core of the explants, which are showed as hatching part in the figure. Control (—●—), NAA 0.3 mg/l (···×···) and NAA 0.3 mg/l plus KIN 1.0 mg/l (- -○- -).

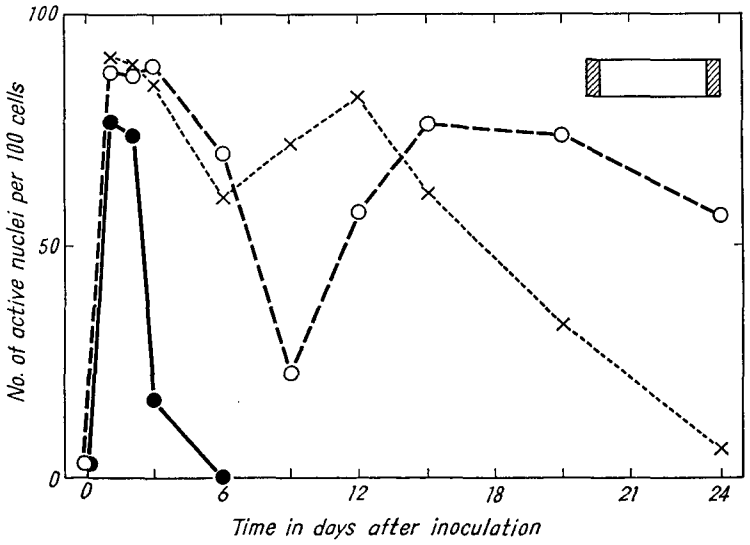


Figure 2. Incorporation of tritiated thymidine into nuclei of potato tissues cultured on the several media. Counting cells were distributed in the marginal part of the explants, which are showed as hatching part in the figure. Control (—●—), NAA 0.3 mg/l (···×···) and NAA 0.3 mg/l plus KIN 1.0 mg/l (- -○- -).

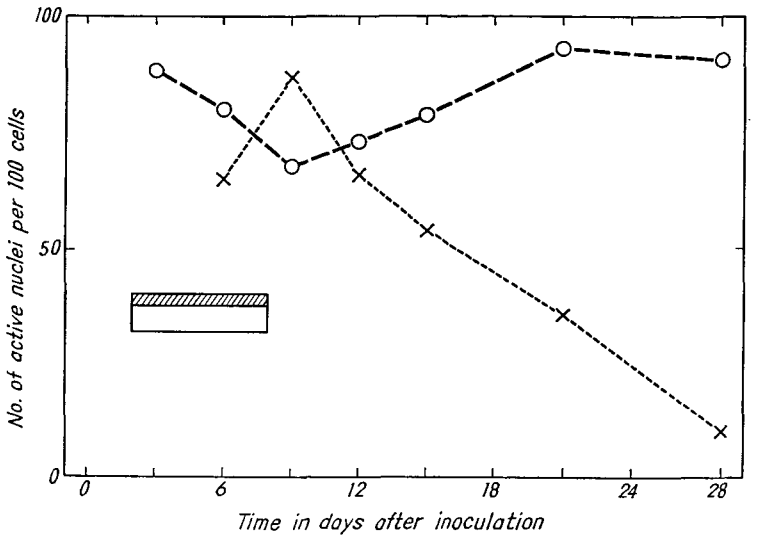


Figure 3. Incorporation of tritiated thymidine into nuclei of potato tissues cultured on the several media. Counting cells were distributed in the upper peripheral layer of the explants, which are showed as hatching part in the figure. NAA 0.3 mg/l (···×···) and NAA 0.3 mg/l plus KIN 1.0 mg/l (--○--).

the regions where cells with labelled nuclei were apparently detected.

In the experiment with potato tissue cultured on the medium applied with NAA and KIN, it was shown the most considerable increase in activity of labelling which was about 25 per cent over the control within 24 hr, as illustrated in Figure 1 and 2, then change in number of active nucleus was followed to display a similar tendency to be progressively decrease with time. However the labelling activity of the cells in this case was still quite significant even on the 6th day when the activity of control cultures became to be almost inert, and succeeded to decrease gradually until it became equal level to the core cells of the tissues. The changes in the labelled nuclei distributed in the upper peripheral layers and flanks of tissues with auxin and KIN showed also fundamentally the same pattern as the cultures with auxin alone. However, the type of growth induced by this medium was qualitatively different in spite of the similarity in the pattern of distribution of active nuclei during the first stage of culture. At the advanced stage of culture, the nuclei densely stained with silber grain were confined to the aggregated small cell clamps being somewhat apart from the margin of inoculum tissues when cultured with auxin alone.

On the other hand, small active dividing cells of the meristematic

nodules which were densely stained with fast green were predominantly localized along the outer periphery of inoculum cultured with auxin plus KIN, and the pattern of labelling distribution persisted until the end of culture (Plate II–5 and 6). At the harvesting time, the heavy labelling exclusively resulted in the apical region of newly formed root tips which emerged from the meristematic nodules. To obtained further information as to the effect of KIN on the labelling of nuclei, these cultures were carried out supplying with auxin at a high or low concentrarion with or

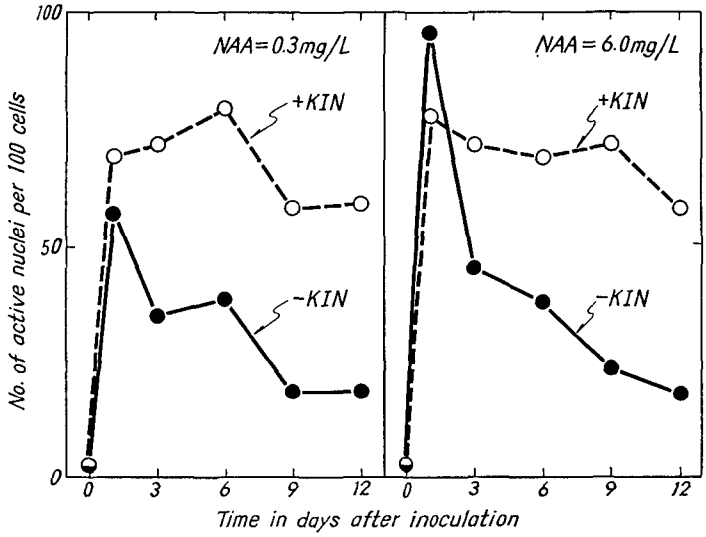


Figure 4. Effect of aging treatment on the incorporation of tritiated thymidine into nuclei of potato tissues cultured on the several media. Explants were subjected to the aging treatment on the plain agar with or without kinetin (1.0 mg/l) for two weeks, afterwards they transferred to culture on the nutrient media with NAA at 0.3 mg/l and 6.0 mg/l for 12 days. Figure showed changing in number of active nuclei scattered in the tissues during the course of culture.

without KIN as illustrated in Figure 4. At both the high and low concentration of NAA, maintenance of labelling activity appears to be caused by enrichment of KIN in the medium. The proposal that changing levels of endogenous cytokinin may participate in callus proliferation of potato tissues cultured with auxin alone has been documented by the finding that an aging treatment of freshly excised inocula led to cause a shortage of cytokinin in them (OKAZAWA 1970). To further substantiate this finding, DNA replication was examined with the aged explants which were trans-

ferred to culture on the nutrient medium for six days. The aging treatment was performed by allowing the initial explants to stand on the plain agar at 25°C in darkness for 2 weeks prior to culturing on the nutrient medium. As shown in Figure 4, in the subsequent culture on the KIN-free medium a marked decline of labelling activity occurred in the aged explants on the first day of culture, even when applied an adequate concentration of NAA to callus proliferation of non-aged explants. The inhibiting effect on labelling due to aging treatment was remarkably alleviated by the simulatneous application of sufficient amount of KIN in the medium.

DISCUSSION

From the experimental results as stated above, it was manifested that the DNA synthesis was an essential prerequisite for initiation of cell division in the cultured explants, even though it was not always led starting of the cell division. The DNA replication in the cell nuclei of the explants was rapidly stimulated throughout the tissue within 24 hr after inoculation, irrespective of being present auxin in the medium or not. However the presence of auxin in the medium is certainly indispensable for the initiation of succeeding cell division, which fact added confirmatory support to the previous conclusion (OKAZAWA 1967). With regard to this point, YEOMAN and MITCHELL (1970) have already advanced the view that in the absence of auxin the cell division was prevented, although DNA synthesis and its accumulation occurred in the cultured tissues of Jerusalem artichoke tubers. DAS et al (1958) have reported that DNA synthesis without following cell division was detected during incubation of excised tobacco pith tissue in a nutrient medium without auxin. FOSKET (1968) has also found a similar fact using cultured stem segments of *Coleus*, and suggested that some of cells may have attained a higher level of ploidy without dividing. In the case of aging experiment using potato tuber tissues, the synthesis of nuclear DNA without following cell division was observed when the tissues were incubated for a while in the absence of any growth regulators (WATANABE and IMASEKI 1973). One possible interpretation of such a behavior may be to attribute the preliminary DNA replication in the explant cells to a wound response caused by excision of the tubers. Regard to the wound response of potato tuber, RAPPAPORT and WOLF (1967) have revealed a stimulation of DNA synthesis occurred soon after excision of potato tuber, suggested that there is a nucleic acid synthesis in response to wounding. According to WATANABE and IMASEKI (1973), this is accounted for by assuming that the wounding of potato tuber by itself must trigger

the induction of DNA synthesis. Subsequently commencement of cell division ensuing from DNA synthesis was confined to the cells which are distributed in the peripheral layer of the explants, while in the remaining cells localized in the inner part of explant neither DNA replication nor cell division succeeded. This interior inactive core persisted until the end of culture. There are several reasons why the appearance of the core does occur in the cultured tissues. As a point in reference to this problem, YEOMAN et al (1968) have suggested that localization of the cell division in the explants is a concequence of a local provision of a products of autolysis. At the surface of explant, autolysis occurred as a result of injury imposed by excision. They concluded that the surface autolysis led to an enhancement of surface meristematic activity. However, in the present experiment, the initial explants were thoroughly washed by sterile water just before inoculation in order to inhibit to stimulate autolysis. Accordingly a beneficial effect of the autolysate on the promotion of cell division may be nearly out of the bound of possibility. Taking the multiple physiological factors on callus growth into consideration, it would be incapable of excluding the possibilities that there might be less availability of oxygen or nutrient and more accumulation of carbon dioxide and inhibitors in the interior core of tissues compared to the peripheral layer of them. Therefore, a completely convincing documentation of this problem does not seem to exist, since sufficient informations are not yet available. On the other hand, although it cannot be overlooked an important role of endogenous cytokinins in the explant, a possibility of shortage of cytokinins in the core of explant looks exceedingly remote because of a evidence as to the behavior of cytokinin (OKAZAWA 1968, 1969 and 1970).

An initial lag phase of these cultures is conventionally regarded as a preparing period of callus growth. This lag phase extending over the first several days appeared in accordance with the time of the initial DNA replication and thereafter cell division. Therefore the DNA synthesis during the lag phase period is definitely implicated in following outgrowth of callus.

The fact that a rhizoid-like outgrowth occurred when a higher level of KIN in combination with NAA at 0.05 mg/l was supplied to the medium, has already reported in the previous paper (KATSURA et al 1970). In the present paper, it appeared that the cells of nodule-like meristem originated from deep within the explant tissues and finally differentiated into roots. This is in line with results obtained other investigation (GAUTHERET 1966; NAKAJIMA and YAMAGUCHI 1968). Therefore it is not surprising fact that

a localization of cells with active nuclei was limited to the meristematic nodule.

In the previous paper on the aging treatment of initial explant (OKAZAWA 1970), an assumption that a disappearance of endogenous cytokinin in the explants due to the aging treatment resulted in a failure of callus proliferation of them cultured on the medium with auxin alone, is much strengthened by the present evidence that there was no DNA replication of the aged explants unless kinetin is supplied exogenously. Accordingly this made it possible to conclude that the presence of cytokinin in the explants is essential to incite the DNA synthesis of explant cells.

SUMMARY

Histological and autoradiographic observations were made on characteristics of development of tissue cultures under the several hormonal conditions. The results reported here concern the influences of auxin and cytokinin on differentiation of cells, preceded by the incorporation of tritiated thymidene into the nuclear DNA. Within a few days after inoculation, incorporation of tritiated thymidine took place rapidly into the nuclei of cells which were randomly scattered throughout the explant tissue, irrespective of the absence of auxin in the medium. The labelling of nucleus might be due to an induction of DNA synthesis in response to wounding by excision. However, no further cell division was succeeded in the tissue cultured on the medium devoid of auxin. As the development of callus or meristematic nodule proceeded, localization of cells with active nucleus was limited to the meristematic region. Unlike the freshly cut explants, the aged explants were incapable of initiation of DNA replication without cytokinin, even if appropriate concentration of auxin was supplied. Therefor it can be concluded that the presence of cytokinin as well as auxin is necessary for the induction of nuclear DNA synthesis followed by the cell multiplication of potato tuber tissues.

LITERATURE CITED

1) DAS, N. K., K. PATAU, and F. SKOOG, (1958) Autoradiographic and microspectrophotometric studies on DNA synthesis in excised tobacco pith tissue. Chromosoma 9: 606-617,

2) FOSKET, D. E. (1968) Cell division and the differentiation of wound-vessel members in cultured stem segments of Coleus. Proc. Acad. Sci. US 56: 1089-1096.

3) GAUTHERET, P. J. (1966) Factors affecting differentiation of plant tissues grown *in vitro*, in "Cell differentiation and morphogenesis" North Holland Pub. Co., (Amsterdam) 55-95.

4) JENSEN, W. A., (1962) Botanical histochemistry, W. H. Freeman Co., 55-99.
5) KATSURA, N., Y. OKAZAWA and T. TAGAWA, (1970, a) Physiological studies on the potato tissue cultured *in vitro*. Part I. Effect of α-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid on the callus and root formation. (in Japanese) Memo. Facl. Agric. Hokkaido Univ., 7: 301-306.
6) KATSURA, N., Y. OKAZAWA and T. TAGAWA, (1970, b) Physiological studies on the potato tissue cultured *in vitro*. Part II. Significance of α-naphthaleneacetic acid and kinetin in rooting process. (in Japanese) Memo. Facl. Agric. Hokkaido Univ., 7: 416-422.
7) MITCHELL, J. P., (1969) RNA accumulation in relation to DNA and protein accumulation in Jerusalem artichoke callus cultures. Ann. Bot., 33: 25-34.
8) NAKAJIMA, T. and T. YAMAGUCHI, (1968) Histological observation on callus tissue derived from root-tuber of sweet potato (in Japanese) Proc. Crop Sci. Soc. Japan, 37: 619-623.
9) OKAZAWA, Y., N. KATSURA, and T. TAGAWA, (1967) Effect of auxin and kinetin on the development and differentiation of potato tissue cultured *in vitro*. Physiol. Plant., 20: 862-869.
10) OKAZAWA, Y., (1968) Significance of native cytokinin in callus growth of potato tissue cultures. Proc. Crop Sci. Soc. Japan, 37: 522-527.
11) OKAZAWA, Y., (1969) Occurrence of natural cytokinin in potato tuber. (in Japanese) Proc. Crop Sci. Soc. Japan, 38: 25-30.
12) OKAZAWA, Y., (1970) Physiological significance of endogenous cytokinin occurred in potato tubers during their developmental period. Proc. Crop Sci. Soc. Japan, 39: 171-176.
13) RAPPAPORT, L. and N. WOLF, (1967) Regulation of bud rest in tubers of potato, *Solanum tuberosum* L. IV Gibberellins and nucleic acid synthesis in excised buds, in "Biochemical regulation in diseased plant or injury." Phytopathol. Soc., Japan.
14) REICHARD, P. and B. ESTBORN, (1951) Utilization of deoxyribosides in the synthesis of polynucleotides. J. Biol. Chem., 188: 839-846.
15) WATANABE, A. and H. IMASEKI, (1973) Induction of deoxyribonucleic acid synthesis in potato tuber tissue by cutting. Plant Physiol., 51: 772-776.
16) YEOMAN, M. M., A. F. DYER and A. I. ROBERTSON, (1965) Growth and differentiation of plant tissue cultures, I Changes accompanying the growth of explants from *Helianthus*. Ann. Bot. N. S., 29: 265-276.
17) YEOMAN, M. M., P. K. EVANS and G. G. NAIK, (1966) Changes in mitotic activity during early callus development. Nature 209: 1115-1116.
18) YEOMAN, M. M. and P. K. EVANS, (1967) Growth and differentiation of plant tissue cultures, II Synchronous cell divisions in developing callus cultures. Ann. Bot. N. S., 31: 323-332.
19) YEOMAN, M. M., G. G. NAIK and A. I. ROBERTSON, (1968) Growth and differentiation of plant tissue cultures, III The initiation and pattern of cell division in developing callus cultures. Ann. Bot., 32: 301-313.
20) YEOMAN, M. M. and J. P. MITCHELL, (1970) Changes accompanying the addition of 2,4-D to excised Jerusalem artichoke tuber tissue. Ann. Bot., 34: 799-810.

EXPLANATION OF PLATES

Plate I. Photographs of histological observation of the cultured tissues.

1) Vertical section of the initial explant showing vacuolated parenchyma cells. × 112.

2) Vertical section of the developing callus (day 3) showing an anticlinal division in a cell of the peripheral layer. × 400.

3) Vertical section of the developing callus (day 6) showing several rows of new cells immediately beneath the upper surface of the explant. The layer of outermost large cells started to degenerate to the safranin positive layer. × 40.

4) Vertical section of developing callus (day 6). The arrow indicates a region of meristematic nodule being stained with fast green. × 40.

5), 6), 7) Enlarged meristematic nodules including tracheides. × 112.

8) Vertical section of proliferating callus cultured with auxin alone (day 21). Friable callus cells grew out along the flank of explants. The arrow indicates a core in which remained a considerable amount of starch grains in the cells even at this stage. × 40.

9) Median section of developing meristematic nodule (day 14). The arrow indicates small active dividing cells of meristematic nodule densely stained with fast green. × 40.

10) Median section of nodule-like cell clump (day 14). This cell clump soon originated meristematic tissue as showing in plate I–9. × 112.

11), 12) Median sections of adventitious root originated from the meristematic nodule. × 40.

Plate II, Microautoradiographic photographs of cells of cultured potato tissues.

1) Inactive nucleus for the incorporation of the tritiated thymidine. × 960.

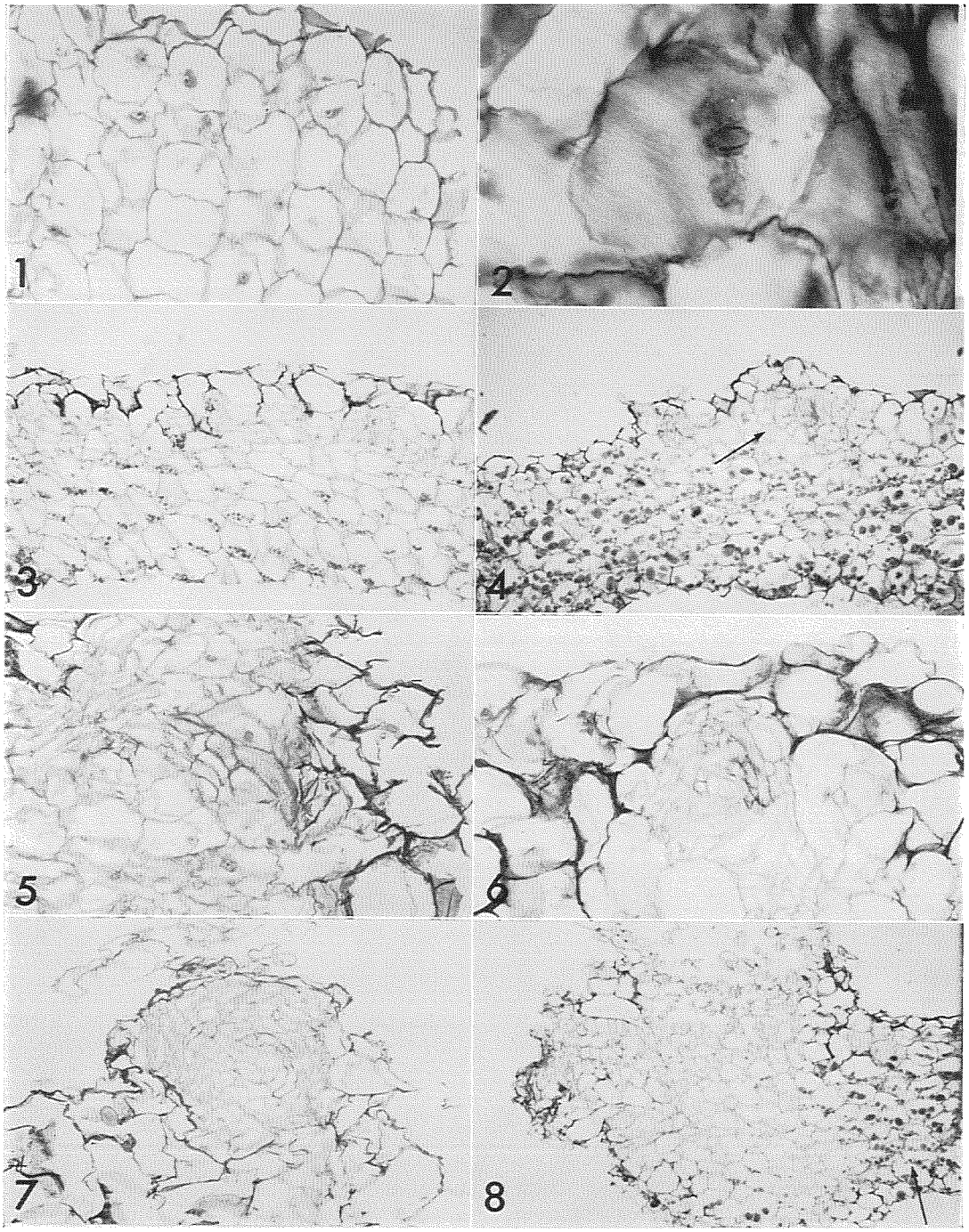
2) Active nucleus for the incorporation of the tritiated thymidine. Both of 1) and 2) are cell nucleus in the explants cultured on the medium (day 1). × 960.

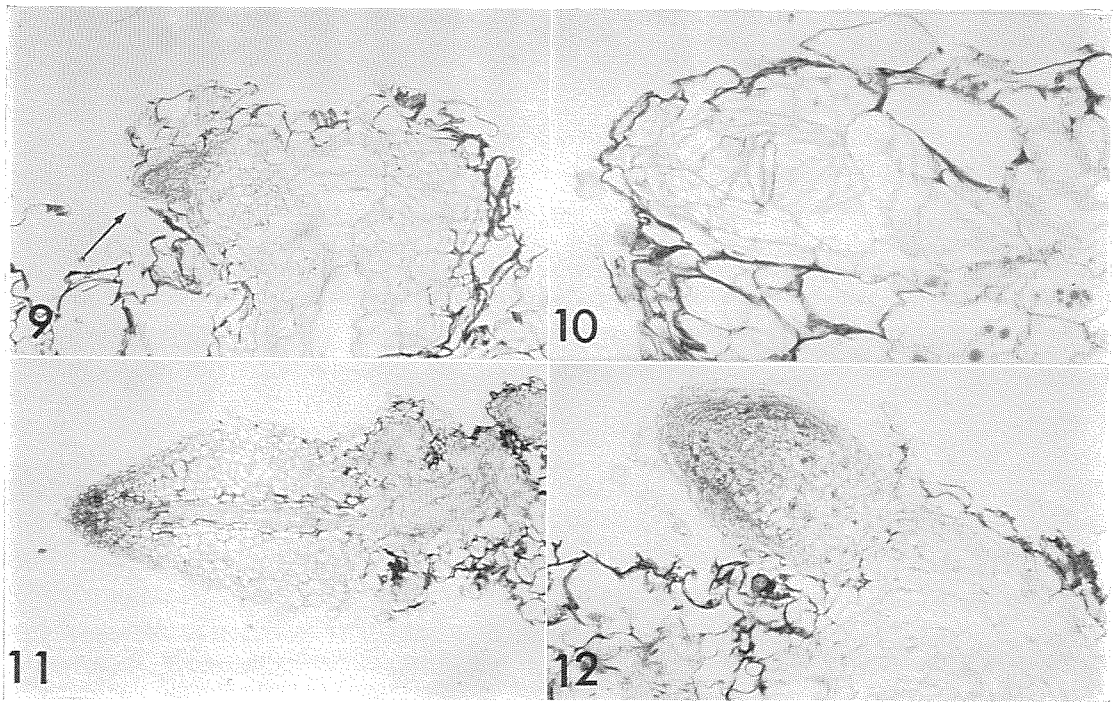
3) Vertical sections of explant (day 1). The arrows indicate active nuclei scattered randomly throughout the tissues. × 112.

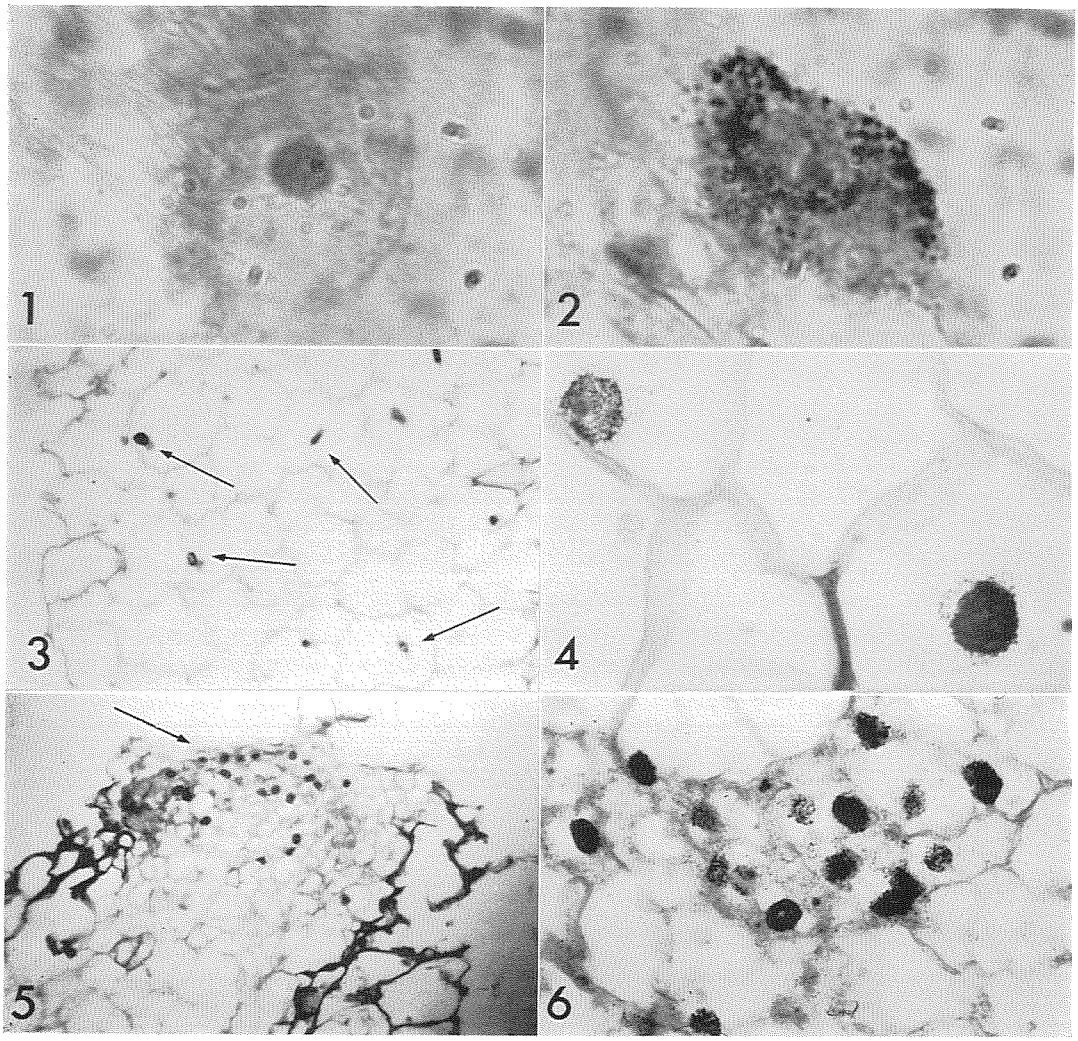
4) An enlarged picture of active cells. × 400.

5) Autoradiographic feature of meristematic cells with active nuclei lacated at the pheriphery of meristematic dome, which cells are small and densely stained with fast green, where is indicated by arrow. × 112.

6) An enlarged photograph of meristematic cells with active nucleus which showing by densely stained with silber grains. × 400.


1
2
3
4
5
6
7
8


9
10
11
12


1
2
3
4
5
6