Journal of Experimental Botany, Vol. 23, No. 77, pp. 1099–1105, November 1972

Cytokinin Production in Relation to the Growth of Pea-root Callus Tissue

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Received 15 December 1971

ABSTRACT

The natural occurrence of free cytokinins was examined in relation to the growth of serially propagated pea-root callus tissue cultures. The relatively slow-growing pea-root callus was harvested at regular intervals throughout a 12-week period and fresh weight, dry weight, cell number, and cytokinin activity were determined. At the end of the culture period the fresh weight had increased about 34 times, the cell number about 100-fold, and the dry weight approximately 19 times over that found on inoculation. Purified ethanol extracts from pea-root callus were tested for cytokinin activity by the soybean callus bioassay. Cytokinin activity was detected in extracts made at all stages of growth tested. There was a sharp rise in cytokinin content during the early period of culture. The peak in cytokinin activity established at the beginning of the phase of growth was associated with a high frequency in mitoses. As growth proceeded there was a decline in both cytokinin content and in the mitotic index.

INTRODUCTION

Cytokinesis in tobacco pith parenchyma is dependent upon the presence and proper balance of two hormones, auxin and cytokinin (Das, Patau, and Skoog, 1956). Soybean cotyledon callus tissue has an absolute requirement for exogenous cytokinin for cell proliferation *in vitro* (Miller, 1961). The specificity of the requirement for cytokinin in soybean and tobacco callus tissues has led to the use of these tissues as bioassays for cytokinin. Many callus tissues grown in culture, however, do not require an exogenous supply of cytokinin. This fact implies that in such tissues endogenous cytokinin production is sufficient to attain the level required for cell division. Fox (1962, 1963) found that habituated tobacco callus tissue synthesized cytokinin-like compounds and Miura and Miller (1969) reported that a variant strain of soybean callus which had lost its requirement for an exogenous supply produced three cytokinins endogenously. Tegley, Witham, and Krasnuk (1971) found that tissue cultures of crown gall from *Parthenocissus tricuspidata* L. which were maintained on a medium lacking cytokinin produced a cytokinin which was similar to zeatin ribonucleoside. Extracts from crown gall tumour cells of *Vinca rosea* L., which had no cytokinin requirement in culture, were reported to contain a new class of cell division-promoting substances (Wood, 1964; Wood and Braun, 1967) which are purinone derivatives (Wood, 1970).

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In this investigation we determined whether serially propagated pea-root callus tissue cultured on a medium devoid of cytokinin was capable of producing its own free cytokinin (i.e. cytokinin not present in tRNA). In addition, changes in the cytokinin activity of pea-root callus tissue were examined during the period of growth in culture.

MATERIALS AND METHODS

Pea-root callus tissue cultures

Pea-root callus was derived from initial explants cut 10–11 mm proximal to the root tip from 48-h germinated seedling roots of peas (*Pisum sativum* L. cv. Alaska). Explants were cultured on a defined medium designated S2M medium which consisted of the following in mg/l: $CaCl_2 \cdot 2H_2O$, 440; NH_4NO_3, 1650; KNO_3, 1900; KI, 0·83; $CoCl_2 \cdot 6H_2O$, 0·025; KH_2PO_4, 170; H_3BO_3, 6·2; $Na_2MoO_4 \cdot 2H_2O$, 0·025; $MgSO_4 \cdot 7H_2O$, 370; $MnSO_4 \cdot H_2O$, 16·9; $CuSO_4 \cdot H_2O$, 0·025; $ZnSO_4 \cdot 7H_2O$, 8·6; $FeSO_4 \cdot 7H_2O$, 27·8; Na_2EDTA, 37·3; thiamine HCl, 0·1; nicotinic acid, 0·5; IAA, 0·175; 2,4-D, 1·1; *myo*-inositol, 100; sucrose, 40 000; agar, 10 000. The medium was adjusted to pH 5·5 before being autoclaved for 15 min at 15 lbf/in^2 at 121 °C. While still warm an organic nitrogen solution was added. This mixture contained the following components expressed as mg/l of S2M medium: L-glutamic acid, 147; glycine, 75; L-aspartic acid, 133; L-arginine HCl, 184; L-asparagine, 132; urea, 60. This solution was adjusted to pH 5·5 before sterilization by aseptic filtration. The callus tissue produced was relatively slow-growing and the tissue, although granular, never became friable. Routinely, three pieces of callus approximately 70 mg fresh weight per piece were inoculated on 70 ml S2M medium in 250 ml Erlenmeyer flasks at 12-week intervals. Cultures were maintained at 23 °C in the dark.

Cell number, fresh and dry weight, were measured at regular intervals throughout the experimental period. At least six flasks were harvested for each growth parameter. Cell-number determinations were made by the modification of the Brown and Rickless (1949) tissue-maceration technique employed by Fosket and Torrey (1969). Mitoses were determined by fixing callus tissue in acetic alcohol (glacial acetic acid:absolute ethanol::1:3, v/v), staining with the Feulgen technique (Matthysse and Torrey, 1967), and examining at least 1000 nuclei. Mitotic index is the percentage of nuclei in which a recognizable stage of mitosis was recorded.

Extraction of cytokinins

Harvested pea-root callus was homogenized for 3 min with sufficient ice-cold 95 per cent ethanol so that the final concentration was about 80 per cent. The homogenized tissue was extracted for 6 h in two portions of 80 per cent ethanol and centrifuged at 10 000 *g* for 20 min at 4 °C. The alcoholic extract was evaporated in a rotary film evaporator under reduced pressure at 37 °C. The resulting water phase was adjusted to pH 8·0 and extracted with three equal volumes of *n*-butanol which were then combined and evaporated to dryness. The residue was redissolved in about 100 ml of water and adjusted to pH 2·0 with conc. H_2SO_4. Fifteen ml of ice-cold saturated $AgNO_3$ solution were added and the mixture constantly stirred for 12 h at 4 °C and centrifuged at 7000 *g* for 15 min. The precipitate was washed with ice-cold 2 per cent $AgNO_3$ solution and stirred with 20 ml of 0·2 NHCl at 50 °C for 30 min before being centrifuged at 7000 *g*. This procedure was repeated twice. The combined acidic supernatants were adjusted to pH 2·5 with conc. NaOH and percolated through a Dowex Ag50W-X8, H+ (200 and 400 mesh) column, 1·0 cm × 25·0 cm, which was then washed with 100 ml of water and eluted with 300 ml 3N NH_4OH. The eluate was evaporated under reduced pressure to about 50 ml and extracted at pH 8·0 with three equal volumes of *n*-butanol which were combined and evaporated to dryness *in vacuo*. The resulting residue was dissolved in 80 per cent ethanol and applied as a streak on Whatman No. 1 filter-paper and developed with *n*-butanol:acetic acid:water::12:3:5. Each chromatogram was thoroughly air dried and cut into 10 equal Rf strips and eluted with 80 per cent ethanol. The eluates were reduced in volume and tested for cytokinin activity.

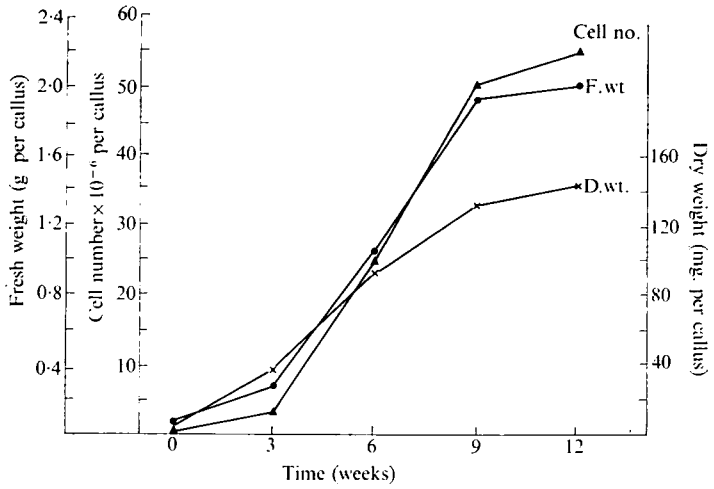

FIG. 1. Growth curves for pea-root callus tissue cultures maintained on S2M agar medium.

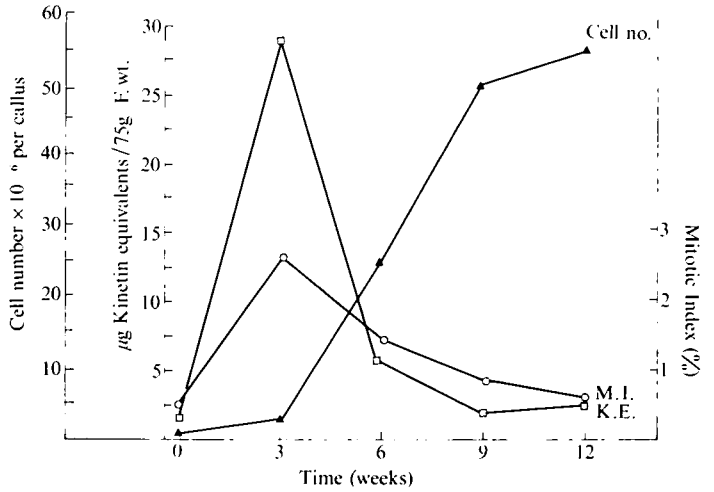

FIG. 2. Changes in cytokinin activity of pea-root callus tissue in relation to its growth in culture.

Cytokinin bioassay

The soybean (cv. Biloxi) cotyledon callus bioassay as employed by Miller (1963) was used to test for cytokinin activity. Dilutions of ethanolic eluates from Rf strips of chromatograms were incorporated before autoclaving in SCF agar medium (Fosket and Torrey, 1969). For assay of cytokinin activity four pieces of soybean callus tissue each 8 mg in fresh weight, were inoculated on 20 ml of medium in each of two replicate 125 ml Erlenmeyer flasks. In all assays, pieces were also placed on SCF medium not supplemented with extracts from pea callus to assess for possible carry-over of cytokinin. The average fresh weight of such controls ranged from 8 to 15 mg per piece at the end of the growth period. The cultures were maintained at 23 °C and received 12 h diffuse warm-white fluorescent light per day. After four weeks the average fresh weight per callus piece was determined.

RESULTS AND DISCUSSION

The growth of serially propagated pea callus tissue during a 12-week period of culture is illustrated in Fig. 1. The results show that the callus tissue was relatively

slow-growing, entering a growth phase at three weeks which continued until week 9. There was little or no growth between weeks 9 and 12 and the tissue was in a stationary phase of growth. At the final sampling the average fresh weight per callus piece had increased about 34 times over that found on inoculation. The cell number increased approximately 100 times and the dry weight about 19 times over that of the initial inoculum. Fig. 2 in which cell number and mitotic index are compared illustrates that there was a peak in mitotic index in pea-root callus tissue at three weeks which preceded a phase of rapid increase in cell number per callus piece. During the remainder of the period of growth in culture there was

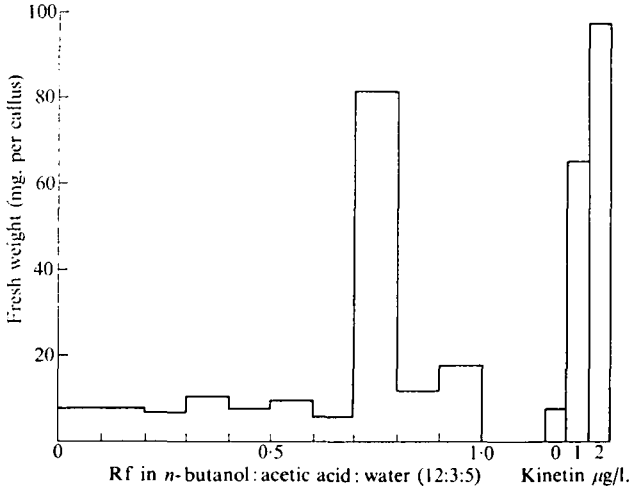

FIG. 3. Histogram of soybean callus bioassay of purified extracts of 12-week-old pea-root callus tissue after paper chromatography in a solvent system of *n*-butanol : acetic acid : water (12:3:5). Extract was tested at a concentration of 75 g fresh weight per litre of medium. The response given by kinetin standards is illustrated.

a decline in the frequency of mitoses. Essentially similar results were reported by Torrey, Reinert, and Merkel (1962) who examined changes in the mitotic index in suspension cultures of *Convolvulus* and *Haplopappus* and by Henshaw, Jha, Mehta, Shakeshaft, and Street (1966) in *Linum* and *Rubus* tissue cultures.

Preliminary experiments using crude 80 per cent ethanol extracts of 12-week old pea-root callus tissue revealed no cytokinin activity in the soybean callus bioassay. Purification of crude ethanolic extracts by precipitation with silver salts, ion-exchange chromatography, organic-solvent extraction, and paper chromatography in a solvent system of *n*-butanol : acetic acid : water : : 12 : 3 : 5 resulted in purified extracts which on assay showed a reproducible promotion of growth. The histogram in Fig. 3 represents a typical response to an extract of 12-week-old callus which was bioassayed at a concentration of 75 g fresh weight per litre of medium. Only one region of cytokinin activity was found at Rf 0·7–0·8 and the growth response was approximately equivalent to that induced by 2·3 μg/l kinetin. Bioassay of extracts from three-week-old callus produced three regions of cytokinin activity at Rf 0·1 to 0·2, Rf 0·4 to 0·5, and Rf 0·7 to 0·9 as shown in Fig. 4. The region at Rf 0·7 to 0·9 was considerably more effective in

inducing soybean tissue proliferation than the other two active zones and was approximately equivalent to the response given by 23 μg kinetin. It was only at this stage of culture that paper chromatography of extracts of pea callus exhibited three regions which were active in the soybean bioassay. Extracts from six-week-old callus revealed two regions of activity at Rf 0·5 to 0·6 and Rf 0·7 to 0·8 on paper chromatograms as described above. The more slowly migrating active band was approximately equivalent to 1·7 μg kinetin equivalents, whereas the faster-moving region was equivalent to 3·9 μg kinetin equivalents when tested at a concentration of 75 g fresh weight per litre of medium. Bioassay of extracts of pea-root

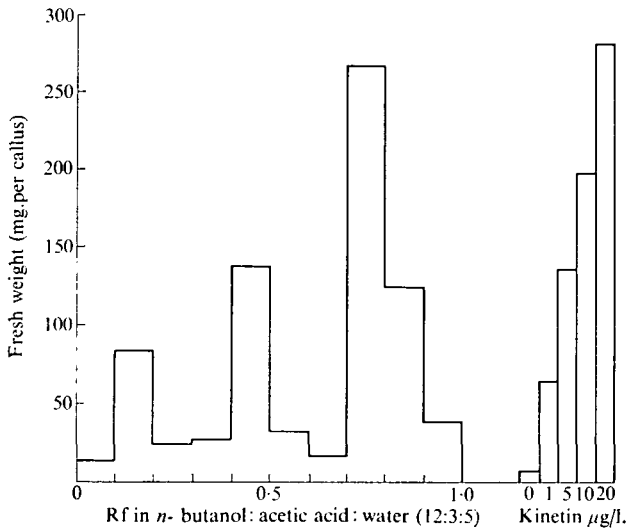

FIG. 4. Histogram of soybean callus bioassay of purified extracts of three-week-old pea-root callus tissue. Extract was tested at a concentration of 75 g fresh weight per litre of medium and the growth responses given by standard amounts of kinetin are shown.

callus tissue at other sampling times revealed a single band of cytokinin activity at Rf 0·7 to 0·8. In this investigation no attempt was made to identify chemically the biologically active compounds extracted from pea-root callus. The active factors appeared to be purine derivatives as they were precipitated by silver ions in acid solution.

Fig. 2 illustrates the changes in total cytokinin activity in pea-root callus tissue during a 12-week period in relation to the changing mitotic index and increase in cell number. In this experiment purified extracts of free cytokinins were bioassayed at a concentration of 75 g fresh weight per litre of medium. On inoculation the cytokinin content was low and was equivalent to the growth response given by 1·4 μg/l kinetin. Cytokinin activity equal to 28·9 μg kinetin equivalents was found on the third week of culture. This represented about a 20-fold increase over the value found at the outset of culture. The peak in cytokinin production in pea-root callus mirrors the rise in frequency of mitoses and corresponds with the beginning of the growth phase (Fig. 2). During the remainder of the period of growth in culture there was a decline in cytokinin production. The

value found at week 6 was 5·6 μg kinetin equivalents and at week 9 the value (1·9 μg kinetin equivalents) was approximately similar to that found on inoculation. At the final sampling the cytokinin content was slightly higher than that found at week 9. Basically similar changes in the pattern of cytokinin content to those reported here were found in serially propagated sycamore cell suspensions (Mackenzie, 1970). In pea-root callus tissue the magnitude of the increase (20-fold) in cytokinin content at the beginning of the phase of cell division over the value found at inoculation was larger than the fourfold increase reported by Mackenzie (1970) in *Acer* cells.

The origin and specific pathways of free cytokinin biosynthesis are unclear. However, from our results it can be inferred that there was an increased rate of synthesis at certain times during the growth cycle in pea-root callus. Cytokinin production was greater during the early stages of growth when there was a high frequency of mitoses than at the later stages of culture when fewer cells were undergoing cell division. Upon examining the distribution of free cytokinins in seedling pea roots, Short and Torrey (1970, 1972) found that the cytokinin content was greater in the meristematic region of the root apex than in the more mature regions of the root. Also, Letham and Williams (1969) reported that the cytokinin contents of extracts of developing seeds of apple fruitlets were much greater than the fruitlet flesh. Furthermore, extracts of the shoot apex of *Coleus* exhibited greater cytokinin activity than extracts from older portions of the shoot (Short and Fosket, unpublished). Therefore, it is proposed that the results of this investigation are not inconsistent with the hypothesis that a certain threshold level of cytokinin has to be achieved prior to the initiation of the process of cell division and that failure to attain this level may limit or prevent cell division.

ACKNOWLEDGEMENTS

We should like to acknowledge the skilled technical assistance of Miss Barbara Platz and Mrs. D. Joan Miller during this work. This work was supported by Research Grant GM–08145 from the National Institute of Health, U.S.P.H.S., to J. G. Torrey. K. C. Short gratefully acknowledges the receipt of a Maria Moors Cabot Post-doctoral Research Fellowship from Harvard University during the tenure of which this work was carried out.

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