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## HORMONE-INDUCED ENDOREDUPPLICATION PRIOR TO MITOSIS IN CULTURED PEA ROOT CORTEX CELLS<sup>1</sup>

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### A B S T R A C T

One-mm-thick cortical explants excised aseptically from 10–11 mm behind the tip of 3-day-old roots of the garden pea, *Pisum sativum*, cv. 'Little Marvel' were cultured on a synthetic nutrient medium supplemented with auxin or auxin and cytokinin. Nuclear DNA contents were measured in cells of the explants at the outset and at specified times during culture up to seven days. Fixed and sectioned preparations were stained with the Feulgen method using the DNA-specific dye auramin-O. Fluorescent microspectro-photometric measurements of individual nuclei were made from each cortical population. At day zero all cortical nuclei measured were either 2c or 4c with respect to their DNA content. In the presence of the auxins, indoleacetic acid and 2,4-dichlorophenoxyacetic acid, and the cytokinin, kinetin, DNA values increased to multiples of the 2c level with populations at the 8c and 16c level predominating after three days of culture as well as at seven days. In the presence of auxins alone no change in DNA values was observed during three days. Kinetin concentrations as low as 0.01 ppm were already effective. The data are interpreted to show that cytokinin, in the presence of auxin, induces two rounds of DNA synthesis prior to the first mitoses, the first round being connected with chromosome doubling by endoreduplication and the second one with normal mitosis. From this we inferred that tetraploid cells in leguminous root nodules might have arisen in the same way, i.e., by endoreduplication prior to the first mitoses induced by the rhizobial division stimulus, unless the chromosome number of root cortical cells had already been doubled by endoreduplication in the normally differentiating root systems.

IN MOST LEGUMINOUS SPECIES root nodules arise in the mature root cortex (Bond, 1948). Two explanations are given for the occurrence of tetraploid cells in root nodules. Either the tetraploid cells stem from a preexisting tetraploid cortical cell, which is selectively triggered by the infecting microorganism to divide (Wipf and Cooper, 1940; Nutman, 1965, many others), or the tetraploid cells stem from diploid cortical cells in which the chromosome complement was doubled by endoreduplication brought about by the infecting microorganism (Bhaskaran and Swaminathan, 1958; Kodama, 1968, 1970). Torrey (1961) found that in pea root segments cultured on a synthetic nutrient medium auxins trigger divisions in diploid cells and auxins plus cytokinins in tetraploid cells. Since in this system the dividing diploid and tetraploid cells belong to two different tissue types, the

pericycle and the cortex, respectively, the question remained whether or not auxins plus cytokinins act specifically on preexisting endotetraploid (cortical) cells. In order to study this problem we selected the pea cultivar 'Little Marvel' since preliminary experiments proved that most mature root cortical cells of this cultivar are diploid. The experiments reported here involve the determination of the relative DNA content of individual nuclei of cortical cells before and after culture in a synthetic nutrient medium containing auxins with or without added kinetin.

**MATERIALS AND METHODS—Culture Methods—**The materials and methods used by Torrey and Fosket (1970) were modified for the experiments described here. After surface sterilization, seeds of *Pisum sativum*, cv. 'Little Marvel' were imbibed in sterile distilled water for 15 hr and germinated in the dark at 25 C in 10 cm petri plates containing a thin layer of 0.7% water agar. Roots longer than 1.5 cm were selected 72 hr after sterilizing the seeds. Segments 1 mm long were excised at 10–11 mm behind the root tip. The central cylinders of the segments were sufficiently uniform in diameter so that they could be removed with a standard tissue punch (Fig. 1B). Holders for segments of diam 1.4 and 1.5 mm were used. The steel punches were sterilized in an oven at 150 C

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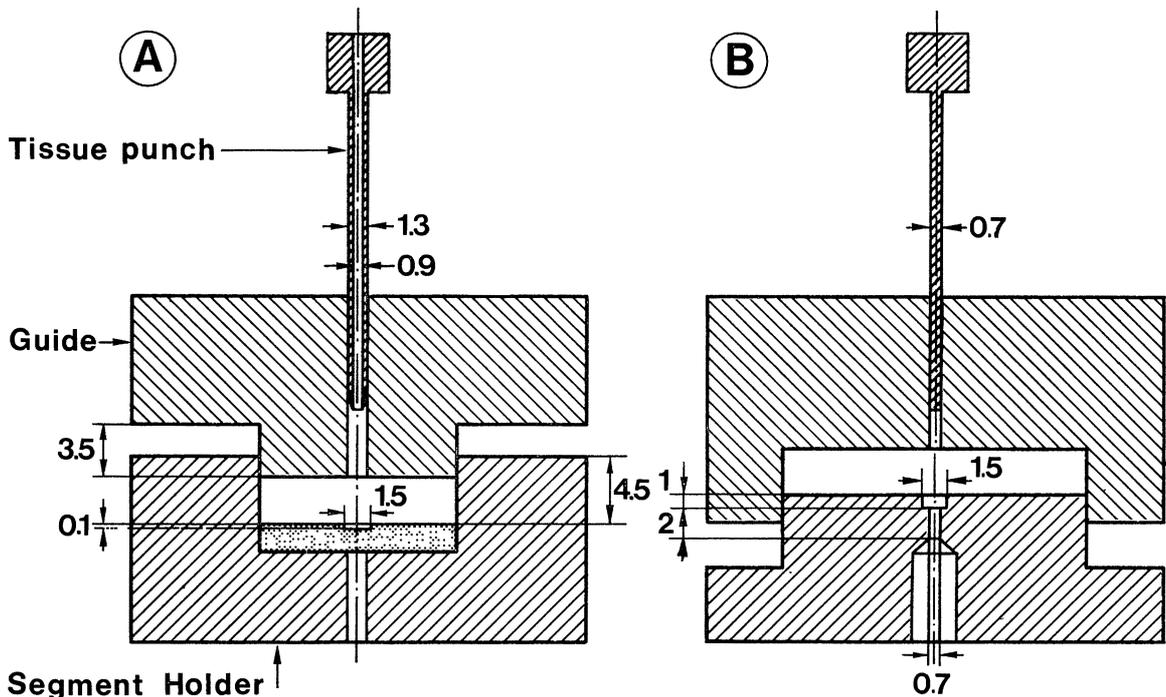


Fig. 1. Tissue punches for removal of the stele (B) or stele plus inner cortex (A) from pea root segments. For further explanation see text. Dimensions are given in mm.

for several hr; the guides and segment holders, which were made of plexiglass, were surface sterilized by soaking in 70% ethanol for 20 min and dried under an ultraviolet sterilamp for 1 hr. In experiments using cortical explants from other parts of the root, tissue punches with adapted dimensions were used. In a few experiments the central cylinder plus 3–5 cell layers of the inner cortex were removed with the tissue punch in Fig. 1A. The punch was made from a #18 hypodermic needle. Explants were checked for accurate centering of the punch before culture. No attempt was made to remove the epidermis.

Cortical explants were transferred to the S2M agar medium used previously (Torrey and Fosket, 1970), which includes the auxins indoleacetic acid at 0.175 mg/liter ( $10^{-6}$ M) and 2,4 dichlorophenoxyacetic acid at 1.1 mg/liter ( $5 \times 10^{-6}$ M). Explants were cultured for periods up to 7 days on S2M medium or S2M supplemented with kinetin at different concentrations, ranging from 0.01 mg/liter ( $4.6 \times 10^{-8}$ M) to 1.0 mg/liter ( $4.6 \times 10^{-6}$ M).

**Microtechnique and staining**—Cortical explants were fixed in FAPA (37% formaldehyde 50 ml, 95% ethanol 250 ml, glacial acetic acid 15 ml, propionic acid 15 ml, distilled water 170 ml) under reduced pressure, carried through an alcohol series and xylene, and embedded in paraplast. They were cut at  $30 \mu$  and mounted in 4% form-

aldehyde on glass slides with Haupt's adhesive and dried overnight in formaldehyde vapor at 40 C.

Dried sections were placed overnight in xylene, then carried through an alcohol series to distilled water. The Feulgen hydrolysis was in 6N HCl at 20 C for 8 min, followed by a distilled-water rinse. Sections were stained with a freshly prepared 0.5% aqueous solution of auramin-O (Chroma, Stuttgart). Immediately prior to use,  $\text{SO}_2$  was bubbled through the staining solution, and then the stain was placed under a coverslip supported above the sections. Sections were stained 30 min, rinsed with two changes of fresh  $\text{SO}_2$  water and with distilled water, and mounted in glycerine.

**Cytological procedures**—Cytological procedures were modified from Torrey and Barrios (1969). Cortical explants were fixed in Carnoy's solution for 12–24 hr, rinsed in water, treated with a freshly prepared saturated solution of Pectinol R-10 (Rohm and Haas, Philadelphia) in 0.2M acetate buffer of pH 4.5 for 1.5 hr at room temperature, hydrolyzed in 1N HCl at 60 C for 15 min, stained with Schiff's reagent for 2 hr, rinsed with  $\text{SO}_2$  water and tap water and squashed on gelatin-coated slides. Coverslips were removed with the dry-ice method. The squashes were then air dried and mounted with permount. Chromosomes in metaphase plates were counted.

**DNA fluorescence measurements**—The method was modified from Bosshard (1964). Measurements of nuclear DNA fluorescence were made with a Leitz Ortholux fluorescent microscope equipped with the MPV microscope photometer with circular measuring stops. Illumination was with an Osram HBO 200 W high pressure mercury lamp with excitation filters UG1 and BG 12 in combination with red suppression filter BG 38 and with blocking filter K 530. Auramin-O-SO<sub>2</sub> stained sections were scanned for nuclei using incandescent light. For random sampling of nuclei the sections were moved in parallel lines along the intersection of a crossline eyepiece micrometer. Every whole nucleus which was "hit" by the intersection was centered in the appropriate measuring stop. Then the illumination was switched to UV for the photometer reading. In every section observation under ultraviolet illumination was minimized to avoid bleaching. Local background fluorescence was also determined for each nuclear measurement and subtracted from each reading. DNA values per nucleus were recorded on an arbitrary photometric scale. DNA values could be calibrated on mitotic cells, where each half of diploid anaphase figures equalled 2c, metaphase diploids equalled 4c, and metaphase tetraploids equalled 8c.

**RESULTS AND THEIR INTERPRETATION**—*Relative DNA content of individual nuclei of cortical cells before and after culture in a synthetic nutrient medium*—Figure 2 represents the DNA values (readings on the photometric scale corrected for background) of a sample of individual interphase nuclei from cortical explants at day zero (the time of explanting) (A), and at day 3 on S2M (B), on S2M + 0.01 ppm kinetin (C), and on S2M + 0.05 ppm kinetin (D). The DNA values of individual 4c and 8c mitotic (metaphase) figures are shown in the graphs as clear values. Figure 2A shows two populations of nuclei with respect to DNA content at day zero: (1) a population comprising values from 150 to about 300 representing 65% of the nuclei with an average DNA content per nucleus of 221 arbitrary units; (2) a population comprising values from about 350–600 representing 35% of the nuclei with an average DNA content per nucleus of 445 arbitrary units. For the purpose of calibrating these values we plotted in this same graph the DNA values of 4c metaphase figures taken from segments with dividing cells. Such cells had an average DNA content per mitosis of 463, which falls within the second population of interphase nuclei in Graph A.

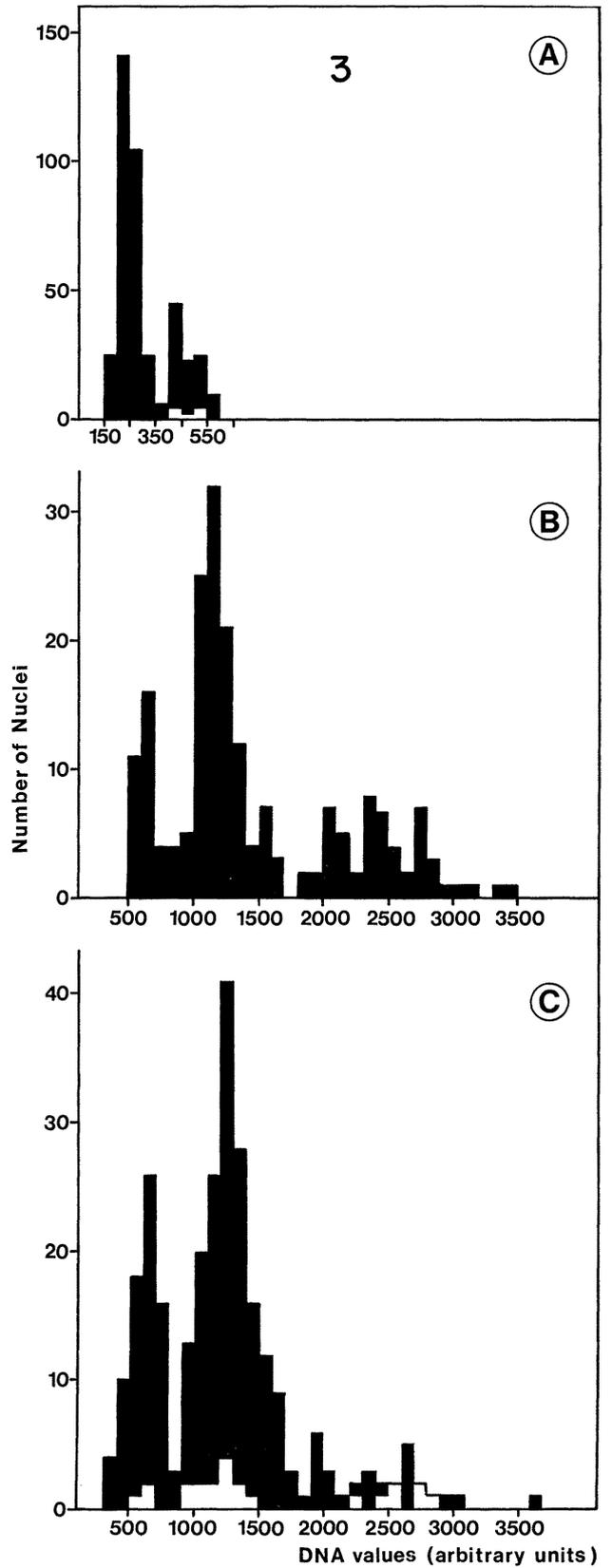
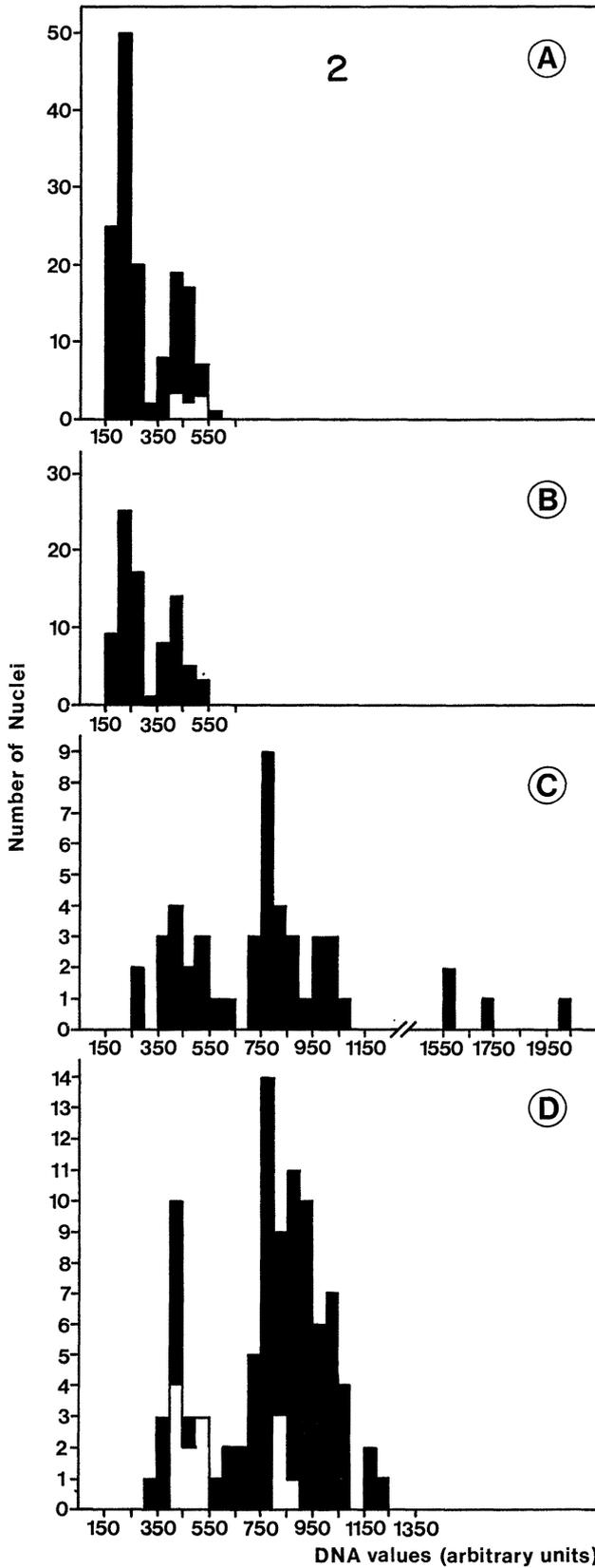
We interpret these data as representing two populations of nuclei, one at the 2c level and the other at the 4c level of DNA. Thus, cortical nuclei at the beginning of the experiment may be of three types. The predominant population is composed

of diploid nuclei arrested at G<sub>1</sub> at the 2c level of DNA. The 4c nuclei may be either nuclei at G<sub>2</sub> of a diploid population or nuclei at G<sub>1</sub> of a tetraploid population. Nuclei of the latter population would already possess twice the number of chromosomes (not replicated) as compared with nuclei at the 2c level of DNA. From observation of interphase nuclei in this material, it is not possible to distinguish between these latter two possibilities.

In Fig. 2B are plotted DNA values of interphase nuclei from cortical explants cultured on S2M medium for 3 days. The two frequency distributions of DNA values shown in Fig. 2A and Fig. 2B are very much alike, the 2c population in graph B representing 64% of the nuclei with an average DNA content per nucleus of 230 arbitrary units and the 4c population representing 36% of the nuclei with an average DNA content per nucleus of 423 arbitrary units. Hence from these data we find no evidence for DNA synthesis when explants were cultured for three days on S2M. No mitoses were observed in these explants.

A series of experiments was run in which cortical explants were cultured on S2M medium with different concentrations of kinetin ranging from 0.01–1 ppm. In earlier experiments it was shown (Matthyse and Torrey, 1967; Torrey and Fosket, 1970) that 1 mg/liter kinetin was about optimal for induction of polyploid mitoses with most cells entering division at about 72 hr in culture. In other experiments (Torrey, 1961; Phillips and Torrey, 1970) it was shown that in cultured pea root segments the threshold for stimulating polyploid mitosis was around 0.01 mg/liter kinetin. From Fig. 2C it is evident that even this threshold concentration of kinetin (0.01 ppm is about  $4.6 \times 10^{-8}$ M) brought about a drastic shift from lower to higher values in the frequency distribution of DNA content per individual nucleus by 72 hr of culture; the 2c population of nuclei disappeared and a major new population appeared at the 8c level of DNA. A few nuclei were at the 16c level of DNA. Fig. 2D shows approximately the same shift in DNA values in explants cultured for three days of S2M + 0.05 ppm kinetin.

Mitotic figures were observed in both the 0.01 ppm and 0.05 ppm kinetin explants. DNA values of metaphase figures are plotted in Fig. 2D (clear bars) and fall at the center of the peaks for 4c and 8c nuclei. In Feulgen-stained squash preparations of these segments metaphase plates clearly showing 4n (28) chromosomes were recorded. At day 3 in culture most cells were about to enter their first division. Therefore the marked 8c peak in Fig. 2C and D most likely represents tetraploid nuclei at G<sub>2</sub>, the 4c peak diploid nuclei at G<sub>2</sub>. However, we may not exclude the possibility that some tetraploid cells are again (or still) at G<sub>1</sub> (4c) or in S (4c–8c). Also some octaploid cells may be at G<sub>1</sub> (8c) or S (8c–16c). In any case it is



clear that the cell population has changed from predominantly diploid at the beginning of the experiment to predominantly polyploid by the time most cells are about to enter their first mitosis on S2M + 0.01 or 0.05 ppm kinetin.

Earlier experiments showed that the proliferation of cells in pea root cortical explants was limited by (an) unknown endogenous substance(s) (Libbenga, 1970) and that the explants showed local differences in proliferation: i.e., the proliferation of the inner cortex was relatively high and that of the middle and outer cortex relatively low. Although excised from younger root material the explants used in the present study also showed this response. Therefore, in order to ensure a more homogeneous system, the following experiments were performed with explants from which the inner cortex was removed along with the central cylinder and the endodermis. Figure 3A shows DNA values in a sample of cortical nuclei from these explants at day zero, the time of explanting. Again we found two populations of nuclei with respect to DNA content: a marked peak representing nuclei at the 2c level of DNA (about 74% of the nuclei measured, average DNA content per nucleus 241 arbitrary units) and a second peak at the 4c level of DNA (about 26%, average DNA content per nucleus 471 arbitrary units). In Fig. 3B and C are plotted data from samples of nuclei of explants cultured on S2M + 1 ppm kinetin for three and seven days, respectively. The 4c, 8c, and 16c mitotic figures recorded in these explants are given as clear values. In Fig. 3C the average DNA values per mitotic figure were: 4c: 580, 8c: 1211, and 16c: 2602. Note that in this series the DNA values for mitoses and interphase nuclei were systematically higher than in the previous series (Fig. 2A to D and Fig. 3A). The reason for this is not clear.

Figure 3B shows the frequency distribution of DNA values in a sample of nuclei from explants harvested at day 3. In this sample relatively few nuclei were found at the 4c level of DNA. A much larger population varying around the 8c level was found. The third broad peak in the graph represents nuclei with DNA values roughly centering at the 16c level. As in explants with the inner cortex intact, which were cultured in lower concentrations of kinetin (Fig. 2C and D), the data of Fig. 3A and B point to a change from a predominantly diploid cell population at the be-

ginning of the experiment into a predominantly polyploid population (most likely comprising a major tetraploid and a minor octaploid population) present at day 3 of culture.

Distributions of DNA values of individual interphase nuclei in asynchronously dividing cell populations such as diploid apical root meristems show two marked peaks: one comprising values roughly centered at the 2c level and one at the 4c level of DNA (McLeish and Sunderland, 1961; Deeley, Davies, and Chayen, 1957 and others). From the data discussed previously we concluded that within the first three days of culture of S2M plus kinetin the cell population of cortical explants changes into a major population of tetraploid cells and minor populations of diploid and octaploid cells. Once this composite population of cells is dividing asynchronously on S2M + kinetin we may expect a distribution of DNA values comprising two major peaks roughly centered at the 4c and 8c levels and two minor peaks at the 2c and 16c levels. The frequency distribution of DNA values which were recorded in explants at day 7 in culture on S2M + 1 ppm kinetin (Fig. 3C) satisfies our expectation, except the 2c peak, which is missing. However, the diploid mitotic figures we found occasionally in sections of these explants prove the presence of a diploid dividing population. The absence of DNA values at the 2c level in the sample of Fig. 3C may be due to sampling error, i.e., they were present in very small numbers but were not included in the population of nuclei measured.

*Ratio of diploid and polyploid mitoses in cortical explants excised at increasing distances from the root tip*—A series of experiments was carried out to determine at which level of differentiation of the root cortex auxins plus cytokinins induce polyploid division in vitro. Torrey (1961) found only diploid mitoses in callus of 1 mm pea root tips cultured for seven days on S2 medium containing auxin plus 1 ppm kinetin.

Cortical explants with the inner cortex intact were excised at one-mm intervals along the root tip of 3-day-old pea seedlings. These 1 mm long explants were cultured on S2M medium supplemented with 1 ppm kinetin. After seven days of culture the percentages of diploid and polyploid mitoses were determined. The results are shown in Fig. 4. In cortical explants excised at 1–2 mm behind the tip 75% of the mitoses had the diploid

←  
 Fig. 2, 3. 2. Histograms relating nuclear number (ordinates) and DNA content per nucleus in arbitrary units (abscissae) in pea root cortical explants with the inner cortex intact. A: Day 0, the time of explanting; clear area represents 4c mitotic figures; B: Day 3, on S2M; C: Day 3 on S2M + 0.01 ppm kinetin; D: Day 3 on S2M + 0.05 ppm kinetin; the clear areas represent 4c and 8c mitotic figures. 3. Histograms relating nuclear number (ordinates) and DNA content per nucleus in arbitrary units (abscissae) in pea root cortical explants with the inner cortex removed. A: Day 0, the time of explanting; clear area represents 4c mitotic figures; B: Day 3 on S2M + 1 ppm kinetin; C: Day 7 on S2M + 1 ppm kinetin; clear areas represent 4c, 8c and 16c values from mitotic figures.

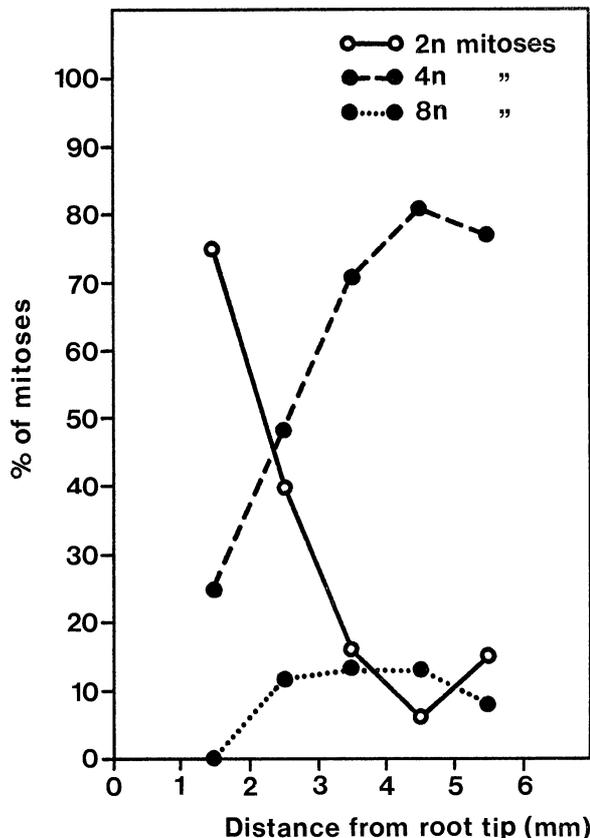


Fig. 4. Graph representing percentages of diploid and polyploid mitoses in cortical explants excised at increasing distances from the root tip. The explants were cultured for seven days on S2M + 1 ppm kinetin. Values are plotted midway in area from which explants were taken. The 0–1 mm section was not examined in this experiment.

number and 25 % the tetraploid number of chromosomes. No octaploid mitoses were observed. This ratio progressively changed with explants excised at increasing distances behind the root tip: at 5–6 mm 85 % of the mitoses were polyploid (77 % 4n plus 8 % 8n) and 15 % diploid.

**DISCUSSION AND CONCLUSIONS**—Endoreduplication is a natural process occurring in all sorts of differentiating plant tissues (D'Amato, 1964a, b). The data from the literature on endoreduplication in root cortical tissues in leguminous species show differences according to species and even varieties. In the mature root cortex of *Trifolium alexandrinum* Bhaskaran and Swaminathan (1958) reported nuclei to be only on the 2c level of DNA. From counts of chromocenters, Kodama (1968) claimed the mature root cortex of *Astralagus sinicus* also consists of only diploid cells. Deeley, Davies, and Chayen (1957) reported a shift from lower to higher values in the frequency distribu-

tion of DNA content per individual nucleus in the root cortex of *Vicia faba* at increasing distances from the root tip, with a population roughly consisting of 70 % 4c and 30 % 8c cells at 12–20 mm behind the tip. From data published by Mitchell (1965) one can estimate that the mature root cortex of *Pisum sativum*, cv. 'Meteor' consisted approximately of 80 % 2c and 20 % 4c cells, although Libbenga (1970) reported 6 % 2c, 80 % 4c and 14 % 8c, for the mature root cortex of *Pisum sativum*, cv. 'Rondo.' This latter distribution is similar to that found by McLeish and Sunderland (1961) in a sample of nuclei from whole roots of *Pisum sativum*, cv. 'Sutton's Meteor' at 6–8 mm behind the tip. In the present study we found that in the mature root cortex of 'Little Marvel' peas approximately two-thirds of the cells are at the 2c level of DNA.

The predominant chromosome number of meristematic cells in root nodules of most leguminous species investigated is tetraploid (Wipf, 1939; Kodama, 1970), even if the root nodules originate in a root cortex of which most cells are diploid (Bhaskaran and Swaminathan, 1958; Mitchell, 1965; Kodama, 1968). According to Kodama (1968, 1970), the chromosome number of interphase nuclei in the root cortex of *Astralagus sinicus* doubles by endoreduplication prior to the first mitoses induced at early nodulation. We came to the same conclusion for polyploid mitoses induced by auxin-cytokinin treatment in root cortical explants of 'Little Marvel' peas.

Our present ideas based on our data and that from the literature can be summarized in the following way. In species which form tetraploid root nodules most root cortical cells are able to double their chromosome number by endoreduplication. However, the fraction of these cells actually doubling their chromosome number during differentiation varies according to species and variety. If the mature cortical cells are exposed to a division stimulus (e.g., root nodule bacteria in infection threads or auxins plus cytokinins provided to root cortical explants in tissue culture), then the diploid cells double their chromosome number by endoreduplication and, after a new round of DNA synthesis, enter mitosis as tetraploid cells. Those cells which had already doubled their chromosome number prior to exposure to a division stimulus may enter mitosis after one round of DNA synthesis but without preceding endoreduplication. Thus, the two explanations given in the literature for the origin of tetraploid root nodules (summarized in the introduction to the present paper) are not mutually exclusive, provided we drop the presumption that the division stimulus acts *specifically* on preexisting endotetraploid cells. Actually there is no longer a need to maintain that presumption, as was already pointed out by Libbenga (1970).

Recently Kodama (1970) reported that in some species root nodules are diploid. He assumed that the root nodules in these species originated in the pericycle. As far as we know, this has only been proven for *Arachis hypogea*. One cannot exclude the possibility that in some species most root cortical cells maintain the diploid chromosome number even when they are stimulated to divide by the infecting nodule bacteria. Clearly, this possibility should be carefully explored.

From our data and conclusions it is reasonable to suggest that both auxin and cytokinin are involved in root nodule initiation. Recently, this possibility has been presented seriously by Libbenga (1970), Phillips and Torrey (1970), and Phillips (1971).

Virtually nothing is known about the control mechanisms of endoreduplication in differentiating plant tissues. Perhaps in the *in vitro* system used in our investigation, it is possible to control both the occurrence and the degree of endoreduplication in the cell populations by manipulation of the auxin and cytokinin levels provided exogenously. This would raise the important question whether the occurrence of endopolyploidy observed in normally differentiating root systems (cf. Torrey, 1965) and elsewhere in the plant (D'Amato, 1964a) is controlled by such factors as the supply of endogenous auxins and cytokinins within the plant. Our results are consistent with those of Das, Patau, and Skoog (1958) and Patau, Das, and Skoog (1957) but show even more clearly the specific dependence of cells on cytokinin for DNA synthesis. These conclusions are in contrast to those of Simard (1971) with tobacco pith tissue who claimed no effect of kinetin of DNA synthesis.

Data from Torrey (1961) and our present data on the percentages of diploid and polyploid mitoses in cortical explants at increasing distances from the root tip suggest that meristematic cells are somehow resistant to the auxin-cytokinin endoreduplication stimulus. The mechanism of this resistance is worthy of exploration since it is part of the answer to the question of what is a root meristem.

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