Effect of morphactin (chlorfluorenol IT 3456) on the mitotic activity and cell growth in roots of *Pisum sativum* L.

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Abstract

Morphactin in a concentration of 100 ppm does not retard pea root growth, however, it reduced the frequency of cell division and shifted the main wave of mitoses in the 24 h period from 1 to 2 mm of root segment. The mean cell cycle duration is prolonged from 14 h in the control to 22 h in morphactin-treated roots. In the presence of morphactin the decrease of ^3H -thymidine incorporation and diminution of labelling index is accompanied by reduction of the nuclear surface area. The described changes are not accompanied by shortening of cell length. The results obtained suggest that morphactin disturbs the mechanisms regulating the initiation of S phase and its regular course. Moreover, it inhibits the endomitotic replication of DNA.

INTRODUCTION

Morphactins (fluorene-9-carboxylic acid-derivatives) are synthetic growth regulators affecting various physiological and morphological processes in plants (Schneider 1964, 1970, Ziegler 1970, Smoliński et al. 1972). These substances act on: germination, seedling growth, stem elongation growth, formation of shoot organs, root growth and root branching (ref. Schneider 1970).

Morphactins inhibit mitosis in the root meristem and disturb the polarity of cell division (Ringe and Denffer 1967, cited by Ziegler 1970, Denffer et al. 1969, cited by Schneider 1970). Moreover they inhibit cell division in the prothallia of *Anemia phyllitidis* (Schraudolf 1967) or in the apical cell of *Stypocaulon scoparium* (Denffer et al. 1969, cited by Schneider 1970). However, stimulation of cell division has been observed. Roots of *Pisum sativum* treated with morphactin show an increased parenchyma cell division and proliferation of new tissues (Saniewski et al. 1968).

Although morphactins reduce greatly the rate of cell division in meristems, they can inhibit (Ziegler et al. 1966, Sankhla and Sankhla 1967, Gaither 1975, Parups 1980) or stimulate (Linke and Marinos 1970, Pilet 1970) elongation of seedlings.

Preliminary observations showed *Pisum sativum* roots to grow well in water or morphactin solution and to have a similar length in the successive days of growth. In view of this two questions arose, firstly, whether morphactin inhibits cell division in pea roots, what could be accompanied by an increment of cell length and, secondly, whether mitosis initiation in control and morphactin-treated roots occurs at different times in the 24-h period. The present investigations were, therefore, undertaken to study morphactin action on the mitotic activity, cell cycle duration, DNA synthesis and cell growth in pea roots.

MATERIAL AND METHODS

Investigations were made on the cortex cells of primary roots of *Pisum sativum* L. var. "Cukrowy Hlowiecki" seedlings.

After washing with soap, seeds were soaked in distilled water (control) or aqueous solution of morphactin (100 ppm) for 24 h. Seeds sown on morphactin or water moistened cotton on trays were kept in darkness at room temperature. For mitotic counts and determination of diurnal mitotic rhythm, 10 mm long roots were collected over 24 h at 2-h intervals and fixed for 2 h in a mixture of ethanol/acetic acid (v:v, 3:1) and Haematein stained after 1 h hydrolysis in 4N HCl at 20°C. Squashes were prepared from 1, 2, 3, 4, 5, 6 and 7 mm of the root after exclusion of the root cap. For the mitotic index a random count of approximately one-thousand cells per 1 mm segment was made.

The cell cycle duration of meristematic cells was determined by the colchicine method (Van't Hof and Sparrow 1963). Roots of seedlings grown in morphactin or water were immersed for 2 h in 0.025% aqueous solution of colchicine. After 8-h postincubation in distilled water the roots were fixed at 2-h intervals during 16 h. The time elapsing between colchicine treatment and the appearance of the highest number of tetraploid cells defined the mean time of the cell cycle.

To determine morphactin action on DNA synthesis, 10 mm roots were incubated with 50 μ Ci/ml 6-³H-thymidine (21.6 Ci/mM) for 2 h. After fixation in FAA (formalin/ethanol/acetic acid, v:v:v, 1:18:1), the roots were cut into 1-mm segments (as above), macerated in 5% aqueous solution of pectinase and after squashing covered with L4 Ilford liquid emulsion. The autoradiograms developed after 14 days of exposure were stained with Haematein. The labelling index was estimated in an average of 2000 cells per 1 mm segment, in five roots per case.

The length of cells and nuclear diameter were estimated with a Zeiss ocular planimeter. The average cellular length and nuclear surface area were calculated from 150 measurements of cells from seven root segments, fixed in FAA and prepared as described above.

RESULTS

In roots growing in water and fixed at the time chosen randomly 10.78% mitotic activity was observed in the first and 0.14% in the 2-mm segment. Mitoses were not observed in the higher segments of roots.

Morphactins distinctly reduce the number of mitoses up to 0.08% in the 1-mm segment of the root. Mitotic activity was not visible in the next and in the further segments of root. These observations confirm the data reported earlier, that morphactin inhibits cell division in meristems (Ringe and Denffer 1967, cited by Ziegler 1970, Denffer et al. 1969, cited by Schneider 1970, Linke and Marinos 1970). Quite different results have been obtained, however, when the roots were collected at 2-h intervals over 24 h and mitotic figures were counted. Mitotic activity in roots in water and in morphactin solution is observed in three segments calculated from root cap. Most of the dividing cells are within the 1st and 2nd mm segment (Fig. 1). The highest frequency of mitoses in control roots appears in the 1-mm segment, reaching 40.15% at 11⁰⁰ p.m. Besides this main sharp peak of mitotic activity, two smaller peaks are visible: one at 9⁰⁰ a.m. and the other at 3⁰⁰ p.m.

In morphactin-treated roots the number of mitotic figures in the 1-mm segment diminishes and the peaks are shifted in time: the highest number of mitoses appears at 9⁰⁰ p.m., it is lower at 3⁰⁰ a.m., and the lowest at 7⁰⁰ a.m.

In the 2nd segment a number of mitoses in the control material diminishes and five small peaks are visible, among them two more distinct appear: at 1⁰⁰ and 9⁰⁰ a.m. It is noteworthy that in the 2-mm segment of morphactin-treated roots a large number of mitoses appears. Such a high mitotic index is seen at 7⁰⁰ a.m. and 9-11⁰⁰ p.m.

In the 3rd segment the frequency of mitotic activity diminishes markedly. In control roots a small number of mitotic figures appears at 1⁰⁰ a.m., but in roots growing in morphactin solution cell divisions are visible twice during the 24 h period at 7⁰⁰ a.m. and 9⁰⁰ p.m.

The number of mitotic figures, although the highest in the 1-mm segment of the control root at 11⁰⁰ p.m., in morphactin-treated roots is more distinct in the 2nd segment at 9-11⁰⁰ p.m. and in a little lower degree at 7⁰⁰ a.m. (Fig. 1). Moreover, in the three successive segments

Fig. 1. Changes in mitotic activity in three subsequent segments of control (—) and morphactin-treated (---) root cells of *Pisum sativum*.

of morphactin-treated roots (unlike in control material) the highest number of mitoses is observed at 7⁰⁰ a.m. and 9⁰⁰ p.m. i.e. at the same time.

We may assume that morphactin not only reduced the number of mitotic figures in the first segment of the root, but also shifted the main wave of cell division to the 2nd and in some degree to the 3rd segment of pea root. In addition, morphactin caused pronounced changes in the time of mitosis initiation during the 24-h period.

The mean duration of the cell cycle (i.e. the time between colchicine treatment and the appearance of the highest number of tetraploid metaphases) undergoes changes in the presence of morphactin (Fig. 2). While the mean duration of cell cycle in control material was 14 h, in morphactin-treated cells it becomes prolonged to 22 h. Simultaneously divergences between the mean and the minimum duration (time of first tetraploid metaphases appearance) of the cell cycle are observed. This seems to indicate a heterogeneity in the population of pea root cells.

Incorporation of ³H-thymidine into interphase nuclei has shown that DNA synthesis occurs in all root segments (Fig. 3). Because the mitoses

Fig. 2. Duration of cell cycle in control (○—○) and morphactin-treated (○—○) pea roots, estimated on the basis of the colchicine method

were observed only in the three segments of morphactin-treated and control roots, ^3H -thymidine incorporation into other root parts indicates endomitotic replication of DNA. The number of nuclei incorporating ^3H -thymidine is the highest in the 1-mm segment of control roots and gradually diminishes with the distance from the root tip, reaching the minimum in the 7-mm segment. In morphactin-treated roots, the labelling index, although much lower than in control material, becomes similar in the 1- and 2-mm segment, and beginning with the 3rd mm a number of nuclei incorporating ^3H -thymidine undergoes marked reduction.

The radioactivity of labelled nuclei calculated per $100\ \mu\text{m}^2$ increases significantly in the 2 mm segment of control pea roots, then gradually diminishes reaching the lowest level in the 6 mm segment (Fig. 4). In

Fig. 3. Labelling index in control (●—●) and morphactin-treated (○—○) root cells of *Pisum sativum*

treated material the number of silver grains per 100 μm^2 of nucleus decreases linearly and beginning with the 5-mm segment radioactivity disappears almost completely.

Fig. 4. Intensity of DNA synthesis in control (●—●) and morphactin-treated (○—○) root cells of *Pisum sativum*

In spite of pronounced differences in DNA synthesis in the 1-2 mm segment of control and morphactin-treated material the surface area of nuclei in three root segments becomes almost the same (Fig. 5). Beginning from the 3-mm segment morphactin caused a marked decrease of nuclei.

The surface area of nuclei in control material increases with cell length, however, these processes are not strictly connected in particular

Fig. 5. Effect of morphactin (○—○) on nucleus surface area in root cells of *Pisum sativum*, control (●—●)

root segments (Fig. 6). Measurements in the series of segments showed that the highest elongation of cells in control roots occurs between 6-7 mm and 2-3 mm, when the size of nuclei does not change.

Fig. 6. Changes in cell length of control (●—●) and morphactin-treated (○—○) roots of *Pisum sativum*

In morphactin-treated cells there is no correlation between nuclear surface area and cell length. The increase in cell length is not accompanied with the enlargement of nuclear dimensions, since the nuclear surface area is alike in all root segments. Cell growth in morphactin-treated roots undergoes a slight reduction in regions from 2 to 4 mm, however, between 5 and 7 mm the cell length increases again (Fig. 6). In spite of variations in the cell length in particular root segments, the length of cells in control and in morphactin-treated roots is alike.

DISCUSSION

Most authors agree that morphactin inhibits cell division in meristems. However, the nature of morphactin involvement in affecting cell division is not yet clear. Generally, two hypotheses of morphactin action on cell division are acceptable. The first, that the specific effect of morphactin activity may be dependent on the cell position within the plant (Linke and Marinos 1970) and the second, that the mechanism of their action is mainly correlated with the inhibition of IAA transport (Pilet 1970).

It seems likely, that morphactin derivatives exert their regulatory effect on auxin transport through their attachment to a site on the plasmalemma (Thomson and Leopold 1974), although such a site is separate from that to which auxins become bound (Hertel et al. 1972). According to Bridges and Wilkins (1973) morphactins acting at the plasmalemma might inhibit the process responsible for the maintenance of the polarized transport system.

In 1980 Parups demonstrated that morphactin causes a decrease in the levels of IAA, ABA, GA₁ and GA₉, but ILA (indol-3-yl-lactic acid) content increases slightly in bean roots with increasing dosage of morphactin.

According to Ziegler (1970), morphactins probably exert their effect on roots through reduction of the IAA level, possibly by inducing higher IAA-oxidase activity. Therefore, they seem to interfere reversibly with morphogenesis at a post-transcriptional level. However, when Raste and Johri (1979) used morphactin together with chlorogenic acid, an inhibitor of IAA-oxidase activity, they obtained only a marginal increase in the fresh weight of crown gall tissue of *Datura innoxia* as compared with the effect of morphactin alone.

If morphactin inhibits IAA transport or synthesis of IAA-oxidase, cell division will be reduced. An analysis of mitotic rates in root tips from both treated and control pea seedlings revealed a pronounced effect of morphactin on cell division. Generally, the total amount of mitotic indices in all root segments of water- and morphactin-treated pea roots was high over the 24 h period and reached 138.7% and 123.1% respectively. Thus about 15.5% of cells during the 24-h period do not divide in the presence of morphactin.

These data support earlier reports of the inhibitory effects in roots (Ringe and Denffer 1967, cited by Ziegler 1970). In the experiments with onion 5 per cent of nuclei divided in the control while only 0.5 per cent in morphactin-treated roots (Denffer et al. 1969, cited by Schneider 1970).

Quite opposite results were obtained by Linke and Marinos (1970). After 24 h of pregermination application of morphactin to seeds of *Pisum sativum* they demonstrated no effect on cell division in the primary root meristem, although a complete mitotic block in shoots was induced within 10 days by morphactin.

Morphactin shifted the wave of mitoses from the 1- to the 2-mm segment in pea root, it changed the diurnal mitotic rhythm and prolonged the cell cycle time. The roots in dormant embryos of *Pisum sativum* contained mixed G1 and G2 populations (Van't Hof 1974). Thus, it may be expected that morphactin inhibits one of the premitotic phases and only those cells which are in G2 phase would enter mitosis. In

cells which are in G1 the DNA should be synthesised before they start mitosis. The lowering of the labelling index in segments characterized by a high mitotic activity seems to suggest that morphactin acts on the level of DNA replication. Thus the S phase would undergo prolongation after morphactin treatment. Although such data are as yet not available, the longer mean duration of cell cycle in morphactin-treated cells seems to support this suggestion.

On the other hand, Barlow and Macdonald (1973) indicate that in corn meristem the durations of S and G2 are relatively constant. The one exception is the quiescent centre, but the cells of this region are not the subject of the present paper. Moreover, the main difference between the subsequent regions of corn roots is in the mean duration of G1. The G1 seems to be the most variable phase of the cell cycle (Mitchison 1971, Taylor and Clowes 1978).

Electron microscopic observations of morphactin-treated pea roots revealed numerous "vacuoles" in the nucleoli (Gabara unpublished). Such "vacuolated nucleoli" according to Kuroiwa and Tanaka (1971) appear at the end of the G1 period. Since only 8.8% of "vacuoles" were observed in control nucleoli and 35.4% in morphactin-treated ones, it seems possible that the G1 is prolonged by morphactin. Without determination of the duration of particular phases of the cell cycle, however, it is impossible to establish distinctly which phase of the cycle is sensitive to morphactin.

Morphactin caused a marked reduction in the intensity of DNA synthesis. Activity of DNA polymerase is the highest in root regions with the highest rates of DNA synthesis and lower where there is a low rate of DNA replication (Olszewska and Kononowicz 1979, Bryant et al. 1981). Thus the activity of DNA polymerase shows a general correlation with the ability of cells to replicate DNA. It cannot be excluded therefore, that morphactin may act on DNA synthesis through the enhancement of the level of this enzyme. In view of the decrease in the labelling index accompanied by a lower number of silver grains per nuclear surface area, one may suppose that morphactin impairs the mechanisms which initiate and regulate the S phase. Apart from any specific effect on the S phase, the diminution in the amount of incorporated ^3H -thymidine might be also explained by the reduced total speed of the cycle (Giménez-Martin et al. 1971).

Incorporation of ^3H -thymidine into root segments which do not show mitotic activity indicates endomitotic replication (Olszewska 1976). It is known that the size of nuclei increases with the differentiation of root cells. This fact is usually correlated with endomitotic polyploidization, although Bregnard and Ruch (1974) found no correlation between the level of ploidy and nuclear volume. In Kuran's opinion (1979) the enlargement of nuclear dimensions during cell growth

and differentiation occurs as the consequence of their karyoplasm hydration and not as an effect of the increase in dry mass of nuclei. The marked diminution of radioactivity over nuclei and the decrease of size of nuclei in morphactin-treated roots as compared with control material indicate that morphactin would inhibit the endomitotic replication of DNA in root cells of *Pisum sativum*.

As indicated by the present paper morphactin does not affect cell length in pea roots. On the contrary, Linke and Marinos (1970) have reported that morphactin stimulates cell elongation in pea root and has an inhibitory effect on shoot cell length in this plant. An enhancement of cell elongation was observed also in wheat coleoptile (Krelle and Libert 1968) and in epicotyl segments of *Lens culinaris* (Pilet 1970). In Schneider's opinion (1970) an inhibition of cell division by morphactin occurs along with continuing cell differentiation. The lack of morphactin action on the cell length of pea roots supports Schneider's suggestion. Normal cell growth in morphactin-treated roots accompanied by inhibition of endoreplication process negates the conception of the role of endoreplication in the elongation of cells.

Morphactins are composed mainly of three different components (Schneider 1970). In addition, these substances seem to be quickly converted into glycosides (ref. Ziegler 1970), or they disintegrate to 2-phenylbenzoic acid within the plants (Katehar and Geissler 1977). Therefore, it seems reasonable to suppose that the different action of morphactin is rather due to the appearance and accumulation of these breakdown products than to morphactin alone and the direction of their effectivity would depend upon the level of these products in the cell.

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Wpływ morfaktyny (chlorfluorenołu IT 3456) na aktywność mitotyczną i wzrost komórek w korzeniu Pisum sativum L.

Streszczenie

Morfaktyna w stężeniu 100 ppm nie hamuje wzrostu korzenia *Pisum sativum*, jednakże redukuje liczbę podziałów komórkowych i przesuwą fałę mitoz w ciągu doby z 1 do 2 mm korzenia. Średni czas trwania cyklu komórkowego ulega wydłużeniu z 14 godz. w kontroli do 22 godz. w komórkach roślin traktowanych morfaktyną. Morfaktyna powoduje spadek włączania radioaktywnej tymidyny oraz obniżenie indeksu znakowania, czemu towarzyszy zmniejszenie powierzchni jąder. Opisanym zmianom nie towarzyszy skrócenie komórek. Uzyskane dane sugerują, że morfaktyna zakłóca mechanizmy regulujące inicjację i prawidłowy przebieg fazy S, oraz hamuje endomitotyczną replikację DNA.