

Effect of 2,4-dichlorophenoxyacetic acid on nuclear division in stem segments of *Pisum sativum* L. cultivated *in vitro*

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Abstract

We have investigated the influence of 2,4-dichlorophenoxyacetic acid (2,4-D) on the appearance of nuclear fragments, caused by direct nuclear division, as well as on mitotic activity in cultivated internodal stem segments of *Pisum sativum* L., cv. Bördi, during 180 d of cultivation. Direct nuclear fragmentation (dNF) was indicated by the shape and structure of the nucleus as well as by the occurrence of 1C- and 3C-values of DNA, investigated cytophotometrically. The dNF occurred during the whole cultivation period in segments treated by 2,4-D in concentrations from 4 to 32 mg l⁻¹. In the presence of 2 mg l⁻¹ of 2,4-D the dNF existed in the explants only up to 90 d. Mitotic activity was not observed in the 2,4-D-free control but occurred during the whole cultivation period when 2,4-D was added in concentrations from 2 to 16 mg l⁻¹. In the presence of 32 mg l⁻¹ of 2,4-D the level of mitotic activity was very low at the beginning and ceased after 60 d in culture.

Introduction

Direct nuclear fragmentation (dNF) is a common phenomenon in plant tissue culture (D'Amato 1985). It gives rise to aneuploidy (Natali and Cavallini 1987) or to bi- and multinuclear cells (Döbel 1983) and can contribute to the variation in the culture. It can be a predominant process in plant tissue culture at the beginning of callus formation (Jelaska *et al.* 1981). In cells of *Pisum sativum*, cultivated *in vitro*, dNF has already been observed in the presence of auxin (Natali and Cavallini 1987), but the influence of components of the culture medium with mutagenic activity on the appearance of the dNF has been rarely investigated. 2,4-D is able to cause various irregularities in cells cultivated *in vitro* (Bayliss 1980, Ager *et al.* 1991, Pavlika *et al.*

Received 1 September 1993, accepted 9 November 1993.

Abbreviations: dNF - direct nuclear fragmentation; MI - mitotic index; MS-medium - medium with mineral salts according to Murashige and Skoog (1962); 2,4-D - 2,4-dichlorophenoxyacetic acid

Acknowledgements: This work was supported by DAAD scholarship given to L. Gantchev.

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1991). The mutagenic influence of 2,4-D takes place as unorganized rapid cell growth (Karp 1991) and depends on the genotype (Fransz *et al.* 1990).

Our investigations studied the influence of different concentrations of 2,4-D on the appearance of dNF on parenchyma cells of *Pisum sativum* L. under *in vitro* conditions, and its relation to the mitotic activity of these cells.

Materials and methods

Seed sterilization and culture media: Seeds of *Pisum sativum*, cv. Bördi (obtained from a local supermarket), were sterilized 3 min with 70 % ethanol, washed three times with sterilized distilled water, 4 min with sodium hypochlorite (1 : 10) and washed again three times with sterilized distilled water. The plantlets developed on MS-medium (Murashige and Skoog 1962) with ethylenediaminetetraacetate (Na-EDTA/Fe) up to 5 mg l⁻¹, thiamine up to 0.30 mg l⁻¹, sucrose 20 g l⁻¹ and agar-agar 10 g l⁻¹. The plants were grown at room temperature (25 ± 1 °C) under white fluorescent tubes (15 W m⁻²). Stem internodal segments between the first and last node from 14-d-old plantlets were used as explant sources. The explants were cut 8 - 10 mm long and cultivated in 100 ml flasks in MS-medium with 5 mg l⁻¹ Na-EDTA/Fe, 1 g l⁻¹ peptone, 30 g l⁻¹ sucrose, and 2, 4, 8, 16 or 32 mg l⁻¹ 2,4-D in darkness at 25 ± 1 °C, on a shaker (rpm = 100).

Slides for histological investigations: About 3 mm thick discs cut from the explants were fixed at 4 °C for 12 - 20 h by acrolein buffer (1.275 ml of 0.5 M NaH₂PO₄ plus 1.225 ml of 0.5 M Na₂HPO₄ filled to 30 ml of distilled water; 5 ml of concentrated acrolein were added and filled to 50 ml of distilled water). The probes were then washed twice for 2 h and once for 20 h with buffer solution (2.55 ml of 0.5 M NaH₂PO₄ + 2.45 ml of 0.5 M Na₂HPO₄ made up to 100 ml with distilled water). Dehydration took place in a refrigerator and change of solutions in an ice bath. Dehydration was as follows: ethylene-glycolmonomethylether (EGME) - 2 × 12 h, ethanol (100 %) - 2 × 12 h, *n*-propanol - 2 × 12 h and *n*-butanol - 2 × 12 h. After that the probes were placed twice for 24 - 72 h (according to the size of the probe) in solutions on the basis of *Technovit 7100* (100 ml solution + 1 g hardener I). It is important for the second treatment to use fresh solution only. Then the probes were placed in a teflon matrix which was filled with embedding medium (15 ml of the solution described above + 1 ml *Technovit 7100* hardener II). The holders of the histological blocks were fixed by special adhesive (*Technovit 3040*: one part solution plus two parts powder). All chemicals for the histology and the teflon matrix were supplied by *Kulzer & Co GmbH*, Wehrheim, Germany, except for ethanol, *n*-propanol and *n*-butanol (supplied by *Sigma*, Deisenhofen, Germany).

In the course of the histological investigations the ends of the explants were cut away in order to exclude cells of wound callus. The serial cross sections were cut on a rotating microtome (*Reichert-Jung*). The histological sections were stained with Schiff's reagent according to Yeung (1984) or with haematoxylin using *Delafield's* undiluted commercial solution (supplied by *Merck*, Darmstadt, Germany) for 1 h.

The stained specimens were embedded with *Euparal* (Carl Roth GmbH & Co., Karlsruhe, Germany).

Microscopy and cytophotometry: For light microscopy a *Olympus BH2* microscope was used. To determine the MI 150 cells were counted respectively from 10 non-successive sections for every variant. The MI is given as the relation of the mitotic cells to all cells counted. To examine the cells with nuclear fragments three times 1000 cells were counted respectively from different non-successive sections for every variant.

The slides for the cytophotometry were prepared according to Geier (1988). The DNA content of the nuclei was measured at 550 nm using an *MSP 20* cytophotometer and an *Axioplan* light microscope (Carl Zeiss, Oberkochen, Germany). The mean value of 250 prophase from specimens of root tips of one untreated plantlet ($2n = 14$) was used as a control. Three times 100 prophase were counted respectively from the specimens of explants cultured with 2 mg l^{-1} 2,4-D.

Statistics: Statistical evaluation was made by analysis of variance followed by Newman-Keuls-test at $\alpha = 0.05$.

Results

In the course of the development of the explants in medium with 2 mg l^{-1} of 2,4-D, direct nuclear divisions were already observed in parenchyma cells between the superficial layers and the vascular bundles after 3 d in culture. A typical dumbbell shaped form of the nucleus resulting from the dNF is shown in Fig. 1A. The cell in Fig. 1B contains three nuclear fragments. We are not able to decide whether this figure is the result of one or more than one direct nuclear divisions. In some cells the dNF was accompanied by other morphological aberrations; as for example increased number of nucleoli and occurrence of chromocenters (Fig. 1C). Fig. 1D shows a cell pair with one nucleus each. The two nuclei possess similar size and form and are located close by the cell wall between them. The size and form of the nuclei give rise to the assumption that the two nuclei are parts of a bigger original single nucleus.

By measurement of DNA-contents during the first 9 d in culture it was found that the number of nuclei with 1C-, 3C- and 4C-values increased significantly (Fig. 2). During the first 2 d all investigated nuclei possessed a 2C-value. In the following days there was a shift in the values to higher ploidy levels (3C- and 4C-values).

During the cultivation of explants for 365 d in auxin-free medium, which was in all other components equivalent to the medium used for our investigations dNF and mitosis did not take place in the explants. At any 2,4-D concentration there was already after 7 d in culture a relatively high number of cells with nuclear fragments. 2 mg l^{-1} of 2,4-D caused an increase in the number of cells with nuclear fragments from 14 up to 35 d in culture. After a decrease between the 60th and the 90th d of the cultivation, no cells with nuclear fragments were observed in the following period

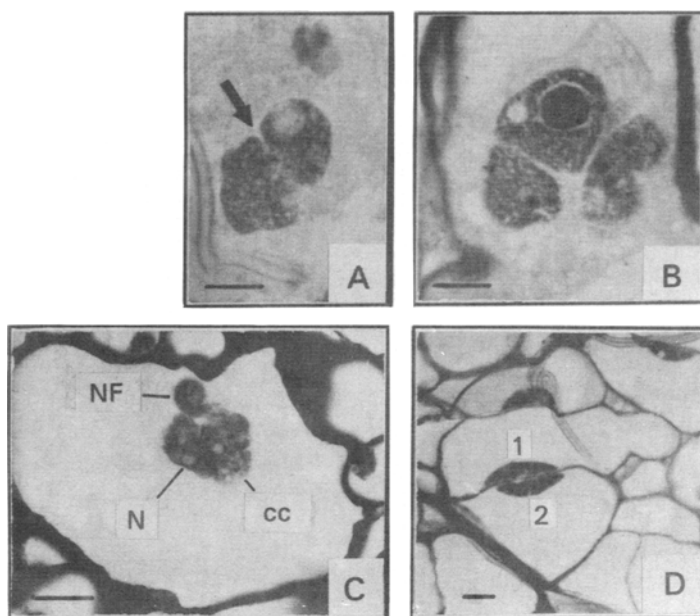


Fig. 1. Direct nuclear fragmentation in parenchyma cells of *Pisum sativum* stem segments cultivated in liquid medium with 2 mg l^{-1} of 2,4-D for different periods. *A* - typical dumbbell shaped form of the nucleus after 14 d of cultivation. *B* - tripartite nuclear fragmentation after 30 d of cultivation. The nucleolus structure shows vacuolization. *C* - separation of a small fragment of the nucleus (NF) after 7 d of cultivation. Nucleoli (N) were distinguished from the chromocenters (cc) by Feulgen staining. *D* - a cell pair separated by cytokinesis after direct nuclear fragmentation after 14 d of cultivation. The two nuclei are indicated by 1 and 2. Staining with haematoxylin according Delafield (*A*, *B*, *D*). Section thickness $20 \mu\text{m}$. Bars indicate $10 \mu\text{m}$.

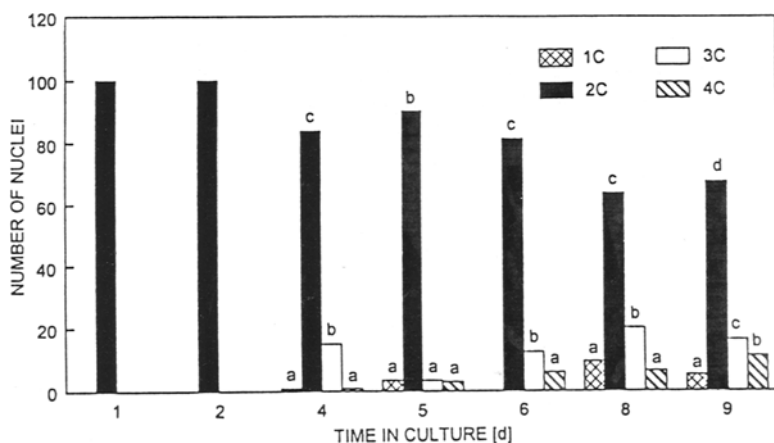


Fig. 2. Number of nuclei with 1C-, 2C-, 3C- and 4C-values in parenchyma cells of *Pisum sativum* stem segments during 9 d culture period in liquid medium containing 2 mg l^{-1} of 2,4-D. Significance is indicated by different letters.

(Fig. 3A). However, in all other concentrations nuclear fragmentation was observed during the total culture period of 180 d. In the concentration range from 4 mg l⁻¹ to 32 mg l⁻¹ of 2,4-D the number of cells with nuclear fragments increases continuously (Fig. 3, B-E). The increase in cells with nuclear fragments from the 14th to the 60th d in culture with 32 mg l⁻¹ of 2,4-D is remarkable - it was the highest range obtained in our investigations.

Mitotic activity was observed during the whole culture period by concentrations of 2,4-D from 2 to 16 mg l⁻¹. At 32 mg l⁻¹ of 2,4-D, the MI was at a very low level till the 60th d in culture and was not detectable afterwards.

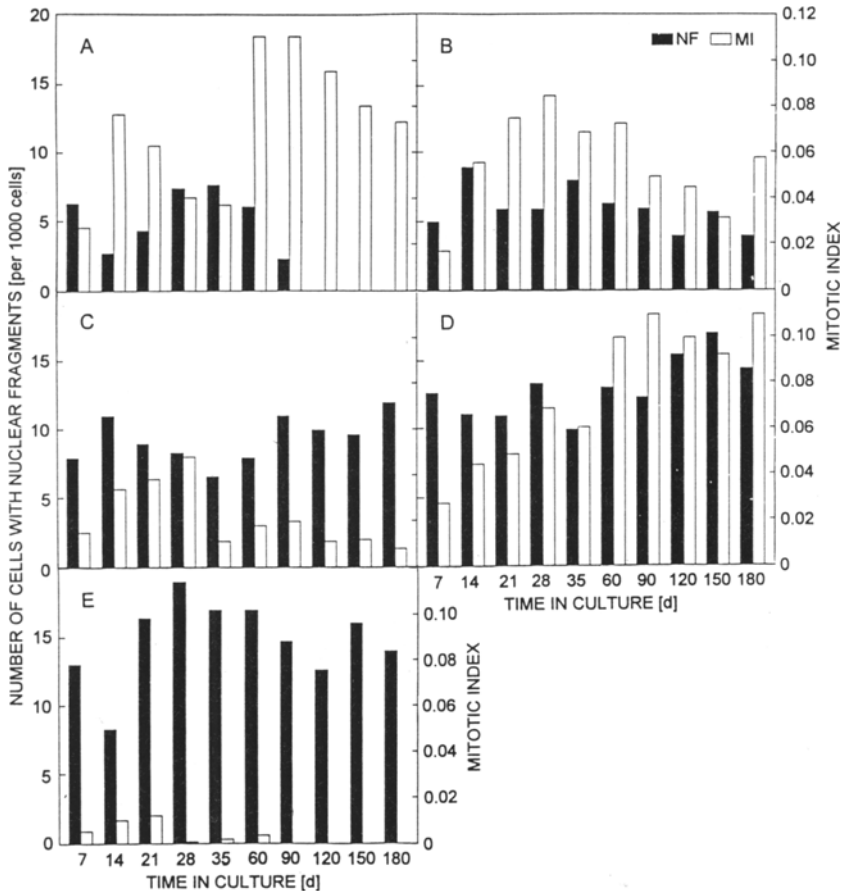


Fig. 3. Direct nuclear fragmentation indicated by the number of cells with nuclear fragments (NF) and mitotic index (MI) from parenchyma cells of *Pisum sativum* during 180 d culture period at different concentrations of 2,4-D: A - 2 mg l⁻¹; B - 4 mg l⁻¹; C - 8 mg l⁻¹; D - 16 mg l⁻¹; E - 32 mg l⁻¹ of 2,4-D.

The comparison between the MI and the number of cells with nuclear fragments during the culture period shows that a general increase in the MI was accompanied

by a decrease in the number of cells with nuclear fragments and *vice versa*. The highest MI-value was observed at 2 mg l⁻¹ of 2,4-D in the period of strongest decrease of the number of cells with nuclear fragments. No dNF was detectable after 90 d of culture at this concentration. However, the mitotic activity remains at a relatively high level until the end of the culture period. Only the data presented in Fig. 3D differ from this tendency. In spite of the high mitotic activity by this variant (16 mg l⁻¹ of 2,4-D) the number of cells with nuclear fragments was very high.

Discussion

The presence of nuclei with 1C- and 3C-values (Fig. 2) indicates a direct nuclear division in the cultures. No aberrations in the chromosome number in our specimens investigated were observed (data not shown). Similar results were obtained on *Pisum sativum* by Natali and Cavallini (1987) and interpreted as an indication of amitotic activity. In this paper, according to Nagl (1980), we prefer to use the terms "direct nuclear fragmentation" to distinguish this process from amitosis in animal cells. 1C- and 2C-values together with chromosome number variation can also indicate the entrance of cells with different chromosome numbers into mitosis (Papes *et al.* 1991).

It is usually assumed that after a dNF, cytokinesis does not take place (Nagl 1980). The mitotic apparatus is accepted to be the prerequisite for cytokinesis (Fahn 1990). But there is no definite information excluding the possibility that nuclear fragments derived from dNF cannot be divided by a newly formed cell wall. The cell pair presented in Fig. 1D give rise to the assumption that in this case cytokinesis took place after dNF as indicated by: 1) the identical shape and size of the two nuclei and 2) the location of the two nuclei close by the cell wall dividing the two cells.

There are two main conditions which make the dNF possible: volume increase of the nucleus and alteration of the metabolic relations in dependence on the cell position (Jacobj 1942). In our investigations the dNF was also observed on nuclei with increased volumes (Fig. 1A, B). It is commonly known that the volume increase of the nucleus causes a decrease in mitotic activity (Davidson *et al.* 1978). No cells with nuclear fragments were found in the 2,4-D-free control variant. This finding gives rise to the assumption that the dNF was induced by 2,4-D. Cells do not possess specific enzymes for the decomposition of 2,4-D (Nagl and Rücker 1972). Therefore, a high activity of 2,4-D in comparison with native auxins can be assumed during the whole *in vitro* culture period.

The volume increase of the nuclei results from DNA-synthesis in the nucleus, stimulated by 2,4-D, even at concentration levels suppressing the mitosis (Nagl and Rücker 1972). The general tendency for an increase in the number of cells with nuclear fragments at higher 2,4-D concentrations (Fig. 3A-E) makes it evident that the suppression of the mitosis was accompanied by an activation of the process of dNF in our cultures.

The dNF is accompanied by morphological aberrations, heterochromatin accumulation (chromocenters) and increase in the number of nucleoli per nucleus up

to 6 (Fig. 1C). The chromocenters cause position effects which exert a depressive influence upon the gene expression of close loci (Gould 1984, DeBoni 1988, Knösche 1990). The increase in the number of nucleoli indicates an activated synthesis in the nucleus and high proliferation ability of the cell.

A high MI-value from the beginning of *in vitro* cultivation is a general criterion for optimal culture development. The optimal development of our culture was established at 2 mg l⁻¹ of 2,4-D (Fig. 3A). Criteria for this are the high MI-value during the culture period and the low number of cells with nuclear fragments per 1000 cells till the 90th d with absence afterwards. The need for a definite phytohormone concentration in the medium is genotypically specific (Gould 1984).

At very high 2,4-D concentrations (32 mg l⁻¹), dNF also took place when mitotic activity had totally ceased (Fig. 3E). 16 mg l⁻¹ of 2,4-D (Fig. 3D) stimulated direct and indirect nuclear division in the cultures. Natali and Cavallini (1987) observed two mitotically active cell populations in their cultures, descended from mitotic cells and from cells that underwent direct nuclear division (amitosis by Natali and Cavallini) just before. It seems possible that in some cases cells can undergo mitosis after dNF (Bennici *et al.* 1976, Cionini *et al.* 1978, Natali and Cavallini 1987). The high MI-value in Fig. 3D was accompanied by very high number of cells with nuclear fragments. In this case it seems not to be possible that cells can enter mitosis after dNF and contribute to the increase in the MI. Therefore, it may be concluded that there are two cell populations at this 2,4-D concentration: one with preferred mitotic activity and another restricted to direct nuclear division.

Our results point to the fact that 2,4-D induces direct nuclear division in explants from internodal stem segments of *Pisum sativum*, and can stimulate this process at a range of higher concentrations. The extent of direct nuclear division and of mitosis and their relationship depend on the 2,4-D concentration in the incubation medium.

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