ORIGINAL PAPER

Nuclear changes in pathogen-infected tomato roots

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SUMMARY

We have investigated nuclear changes induced in tomato (*Lycopersicon esculentum*) root plants by two different pathogens, a phytoplasma of the stolbur group and the soil-borne fungus *Phytophthora nicotianae* var. *parasitica*, using light and electron microscopy as well as flow cytometry. Our results show that both pathogens strongly influenced nuclear structure, causing pyknosis and chromatolysis, and induced variations in proportion of nuclear populations with different DNA content, increasing the 2C and decreasing the 4C nuclear populations. These latter results suggest a block of the endoreduplication process in the presynthetic phase of the host cell cycle. Moreover, in pathogen-infected roots, nuclei of the same DNA content were less fluorescent, and therefore had a lower DNA content, compared to those of controls, as confirmed by morphological analyses. Our findings suggest that different stimuli may evoke similar nuclear changes, and confirm the usefulness of flow cytometry as a tool to recognize the stress conditions related to the presence of a pathogen.

Abbreviations: DAPI - 4',6-diamidino-2-phenylindole

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INTRODUCTION

Nuclear changes induced in plant host cells by pathogens (fungi, viruses, bacteria and nematodes) have generally been well described, but little information is available about the quantitative variations of DNA induced by the infection (Baluska *et al.* 1995). High percentages of polyploid cells and high levels of ploidy are common among plants with a small DNA content per genome (Grif, 2000). Polyploidy has been thoroughly studied in understanding the significance of DNA amount per genome in plant biosystematics (Grif, 2000), and during differentiation (D'Amato, 1998; Traas *et al*, 1998); less information exists on the effects of environmental factors on ploidy levels, although some factors (among which light) are known to influence them (Gendreau *et al*., 1998).

Phytoplasmas are prokaryotes, obligate parasites, belonging to the class Mollicutes; their presence has been well documented in the shoot phloem tissue of many plants of economic interest including tomato (Lisa *et al*., 1982). Their presence and effects on roots have been shown by a relatively small number of authors (Sinclair and Griffiths 1992; Cousin 1995, Lherminier and Boudon-Padieu 1996).

In this paper we have analysed morphological and quantitative nuclear changes induced in the roots of tomato (*Lycopersicon esculentum*) by a phytoplasma of the stolbur group (Lee *et al.,* 1998; Seemüller *et al.,* 1998) and by a soil-borne fungus, *Phytophthora nicotianae* var. *parasitica,* using light and electron microscopy and flow cytometry. Tomato nuclei have a low DNA amount (2C=2pg), and they should therefore be very plastic (D'Amato, 1998); moreover, in the roots only the meristematic apical cells are consistently diploid, while in the differentiated portion of the roots there are three levels of DNA content (2C, 4C, 8C) (Fusconi *et al*., 1997; Lingua *et al.*, 2001; Berta *et al*., 2001) that could be influenced by the presence of a pathogen.

MATERIAL AND METHODS

Plant growth and infection

Seeds of *Lycopersicon esculentum* cv Early Mech were germinated in Petri dishes on moist filter paper for 3-4 days. The seedlings were then transferred to quartz sand (0.5 mm coarse grade) in 1.5 l polyethylene pots. The pots were placed in growth chambers, 16/8 hours light/dark photoperiod, 24/22°C light/dark thermoperiod, 130mmol. m^{-2} s^{-1} light irradiance at pot height and watered 3 days a week (alternate days) each with 100 ml of half strength Long Ashton nutrient solution, according to Trotta *et al*., 1996.

Three weeks after sowing, ten plants were grafted with scions of tomato infected with STOL-S1 (Minucci and Boccardo 1997), a phytoplasma of the stolbur group; ten were grown ungrafted and ten grafted with healthy scions, as controls. All the plants were harvested 60 days after pathogen infection, when disease symptoms were clear, and their root systems were processed for light and electron microscopy and flow cytometry.

In another experiment, three weeks after sowing nine plants were inoculated with 100,000 zoospores of *Phytophthora nicotianae* var. *parasitica* Breda De Haar (isolate 201, subsequently referred to as *P. parasitica*); other plants of the same batches remained uninoculated. Plants were harvested 10 days post inoculation (again when disease symptoms were evident) and processed as above.

Light and electron microscopy

Small segments were sampled from the adventitious roots of 3 plants per treatment. The segments were cut at about 6 cm from the apex, then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for 3 h at room temperature, post-fixed in 1% OsO_4 in the same buffer for 2 h at room temperature, dehydrated and embedded in LR White resin at 60°C. Semithin and thin sections were cut with a Reichert Ultracut E ultramicrotome. The former were stained with 1% toluidine blue in 1% sodium tetraborate and examined under a light microscope. The latter were contrast-stained with 0.5% uranyl acetate and lead citrate (Reynolds, 1963), and examined under a Philips CM 10 electron microscope.

Evaluation of phytoplasma and *P. parasitica* infections

To confirm the presence of phytoplasmas in the roots, semithin and thin sections were observed by light and electron microscopy, respectively.

P. parasitica infection was checked microscopically on semithin and hand cut sections. An immunocytological analysis was performed using polyclonal antibodies against *P. megasperma* (kindly provided by E. Schmelzer, MPI, Cologne), according to Cordier *et al.* (1996). Indirect immunolabelling with alkaline phosphatase conjugated secondary antibody was used to visualize *P. parasitica* hyphae in 4% paraphormaldehyde pre-fixed whole root fragments.

Flow cytometry

Three two-months old tomato plants infected and uninfected by phytoplasmas, and six one month old tomato plants infected and uninfected by *P. parasitica* were processed for flow cytometry. Nuclei were extracted from whole root systems, after the removal of their apices. The roots where chopped with a razor blade in a few drops of extraction buffer (0.1 M citric acid, 0.5% Tween 20). Nuclei were extracted after 30 min incubation in this buffer by filtering through a 20 μm nylon mesh. All the steps were carried out on ice. The samples were fixed by adding two volumes of ice-cold ethanol-acetic acid (3:1) to one volume of nuclei suspension and stored at –20°C.

Two ml of the fixed samples were centrifuged at 500g on a 1 M sucrose cushion in order to elimi-

nate the fixative and reduce the debris. The pellet was resuspended in 500 ml PBS, the nuclei were counted and diluted with PBS buffer (phosphate-buffered saline, prepared according to Sambrook *et al*. 1989) to adjust their concentration to 5 x 10^5 ml^{-1}. Due to the severe damage inflicted by *P. parasitica* to the roots, the number of nuclei was lower than from phytoplasma-infected roots; therefore the six nuclear suspensions were pooled to form just one sample. They were stained with 4', 6-diamidino–2-phenylindole (DAPI, Sigma) at saturating concentration (5.6 μM).

Polyscience Fluorsbryte beads (4.2 μm) were used as a standard to calibrate the instrument and to check the instrument stability. Therefore, the beads were run before and after each sample analysis in order to compare the intensities of fluorescence of the various samples.

A mercury arc lamp was used in conjunction with BG1 and UG1 filters and a TK420 dichroic mirror to select the exciting wavelength (UV) of a Partec PAS-IIIi flow cytometer (Partec GmbH, Munster, Germany). DAPI fluorescence was detected using a GG415 barrier filter. All the filters and dichroic mirrors were provided by the flow cytometer producer. Data were acquired and analyzed with Parteclist, the software included in the instrument package. All the data on phytoplasmas were compared with a Student t-test, using a t value with a 95% confidence level (t=2,776, d.f.=4). A total of almost 28,000 nuclei from the phytoplasma-infected roots and of over 24,000 from control roots were analyzed.

The differences between *P. parasitica* infected and uninfected roots were evaluated by calculating standard errors and limits of confidence at 99% (t=2.576; d.f.= ∞) for each fluorescence value. In addition, the data regarding the same ploidy levels from the two different treatments were compared with a "u test" (a.k.a. "z test") for binomial distributions. In this way we tested the differences between the percentages of 2C, then of 4C and finally of 8C. A total of about 3,800 nuclei from *P. parasitica*-infected roots and of more than 6,200 from control roots were analyzed.

Flow cytometry results were displayed on histograms. On the y-axis was displayed the number of nuclei, on the x-axis the intensity of fluorescence. Hence, the nearer a peak was to the origin, the lower the fluorescence, and viceversa.

RESULTS

Phytoplasma infected roots

The phytoplasmas were observed in tomato roots, which as a consequence showed extensive necrosis. They were present in every root zone checked, inducing senescence in the phloem elements (Figs. 1a, b, c), in the companion cells and in the surrounding tissues, where cell walls were thin and distorted (Figs. 1a, 2b).

The nuclei of the control (uninfected) roots were elongated and showed the usual chromocentric features (Fig. 2a), i.e. decondensed, diffuse chromatin and only small patches of condensed chromatin (chromocentres).

The nuclei of infected companion cells were elongated, and in some cases, partly fragmented. Some showed lysed chromatin and very large, irregularly arranged chromocentres at their periphery (Fig. 2b). Even further degraded nuclei were also present, with very loosely packed chromatin, in senescing tissues other than the phloem, such as cortex (Fig. 2c). These forms of nuclear degeneration can, according to Woolhouse (1984), be termed "chromatolysis".

P. parasitica -infected roots

P. parasitica induced massive necrosis in the roots.

Immunocytological analyses showed the presence of *P. parasitica* in the apex (Fig. 3a) and in the differentiated zone, where hyphae frequently appeared to penetrate through root hairs (Fig. 3b), but also through epidermal cells (Fig. 3c). Hyphae grew between and into the cortex cells (Fig 3c), to reach the central cylinder.

The presence of *P. parasitica* in root tissues resulted in death and necrosis, not only of invaded cells, but also of adjacent uninfected cortical cells, that were plasmolytic, with distorted and thin walls, autophagic vacuoles, and senescent nuclei. The latter were in lateral position, chromatolytic or pyknotic, generally rounded, sometimes lobed (Fig. 3c). The most affected nuclei had clear rings around the nucleolus, caused by degradation and loss of chromatin (Fig. 2d).

Flow cytometry analyses

Both pathogens influenced distribution of the nuclei in the three populations, significantly increasing that with the lowest DNA content, i.e. 2C nuclear population, and decreasing the 4C nuclear
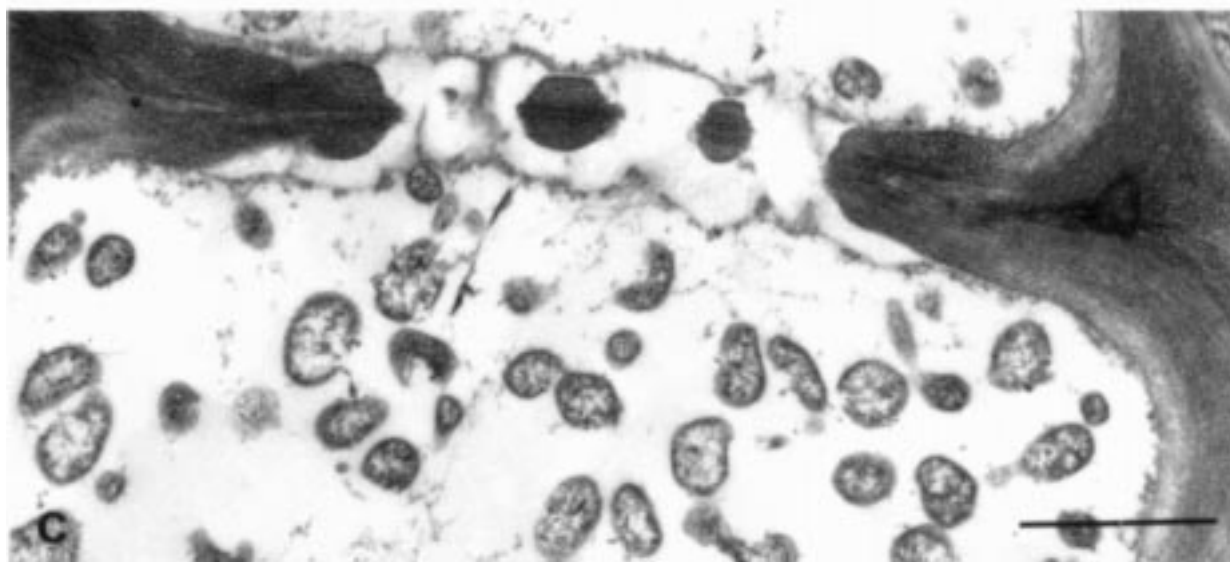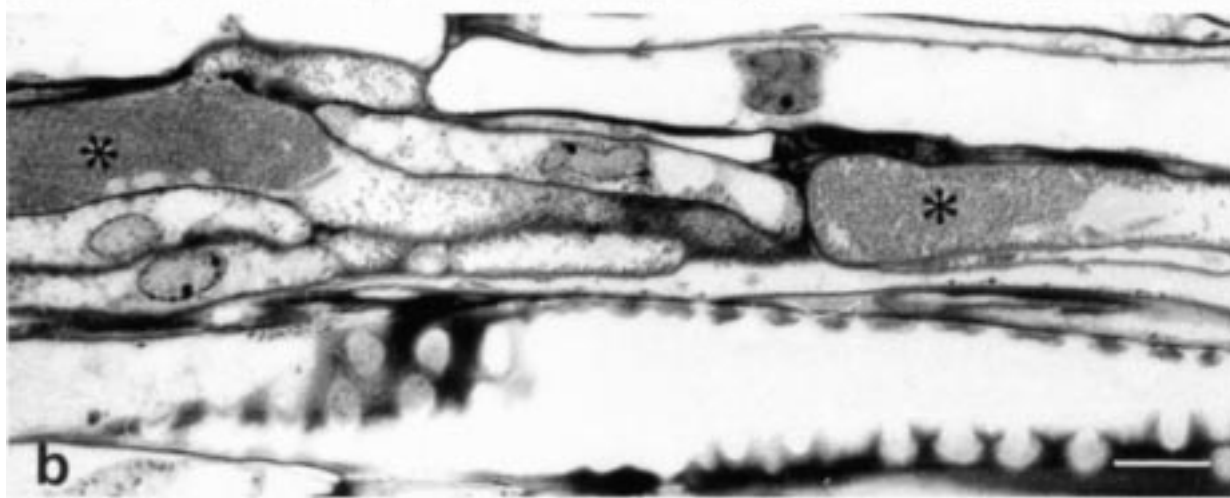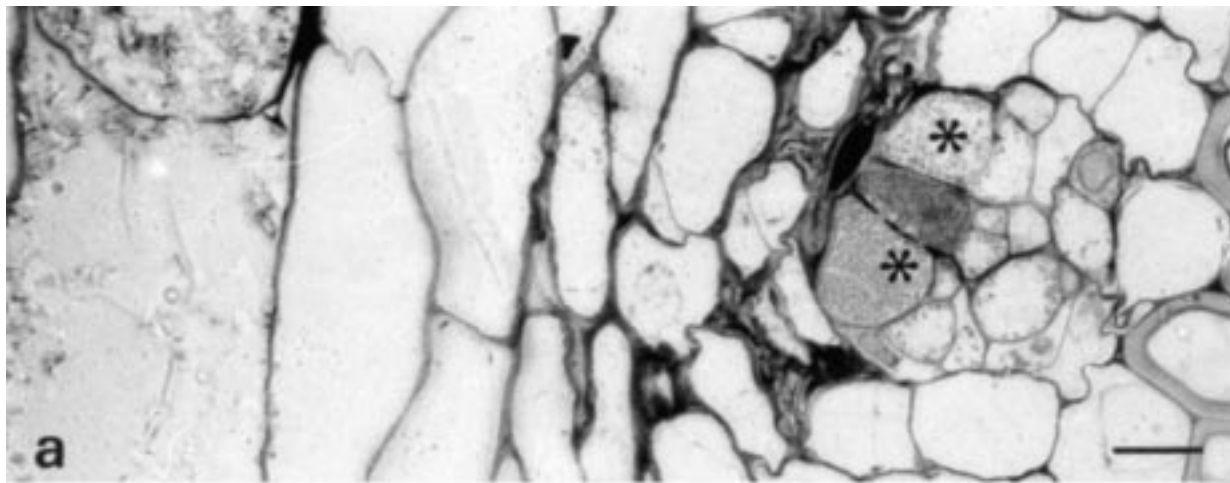
Table I
Percentage of nucleus populations with different DNA content (2C, 4C, 8C) in tomato roots ± standard errors. PTP = phytoplasma; asterisk (*) indicates significant differences according to the Student t-test, with 95% confidence limits

DNA content	control plants	PTP infected plants	t
2C	21.53 ± 1.72	35.58 ± 4.12	-3.145
4C	72.06 ± 2.30	60.78 ± 3.82	2.528
8C	6.41 ± 1.49	3.63 ± 0.33	1.823

Table II
Percentage of nucleus populations with different DNA content (2C, 4C, 8C) in tomato roots ± standard errors. Pp = *P. parasitica*; asterisk (*) indicates significant differences according to the Student t-test, with 95% confidence limits

DNA content	control plants	Pp infected plants	u
2C	16.723 ± 0.005	20.453 ± 0.006	4.619*
4C	80.324 ± 0.005	76.410 ± 0.007	4.585*
8C	2.953 ± 0.002	3.137 ± 0.003	0.517

population. No difference was found between 8C nuclei in any of the two comparisons between pathogen infected roots and controls (Fig. 4; Tables I and II).

Saturating DAPI staining caused lower fluorescence in pathogen infected root nuclei of the same ploidy levels, compared to those extracted from control roots, i.e. 2C and 8C nuclei extracted from phytoplasma-infected roots were significantly less florescent than 2C and 8C nuclei of controls, respectively (Fig. 4; Table III). Comparing *P. parasitica*-infected plants and their controls, all the differences were significant (Table IV).

DISCUSSION

We have investigated nuclear changes in roots of tomato plants infected with two different pathogens by light and transmission electron microscopy and by flow cytometry. For both pathogens we observed nuclear chromatolysis and pyknosis, variations in the proportions of nuclear populations, and a lower intensity of fluorescence of pathogen-infected plant nuclei at some levels of DNA content. These results have been obtained in spite of the different ages at which the plants from the two different experimental systems were analyzed, due to the different times required for the development of the two pathogenic infections.

Light and electron microscopy results on pathogen-infected plant nuclei were not different from others in the literature on senescing nuclei (Woolhouse, 1984; Fusconi *et al.*, 1999) and showed highly degraded chromatin, resulting in chromatolysis and pyknosis. These features are probably caused by a loss of DNA or the integrity of DNA, as confirmed by the lower fluorescence observed. We have previously shown that the nuclei of tomato plants infected with *P. parasitica*

Fig. 1 - Sections of tomato adventitious roots infected by stolbur phytoplasma. (**a-b**) Light micrographs showing sieve elements filled with dense particles (*) identified by electron microscopy as phytoplasmas. (**a**) In cross section, in which thin and distorted walls are visible (**b**) in longitudinal section; note the presence of nuclei with different degree of chromatolysis (arrows). Bars represent 10 μm. (**c**) Electron micrograph showing many phytoplasmas, the amoeboid-shaped bodies, in a sieve element. Bars represent 1 μm.
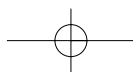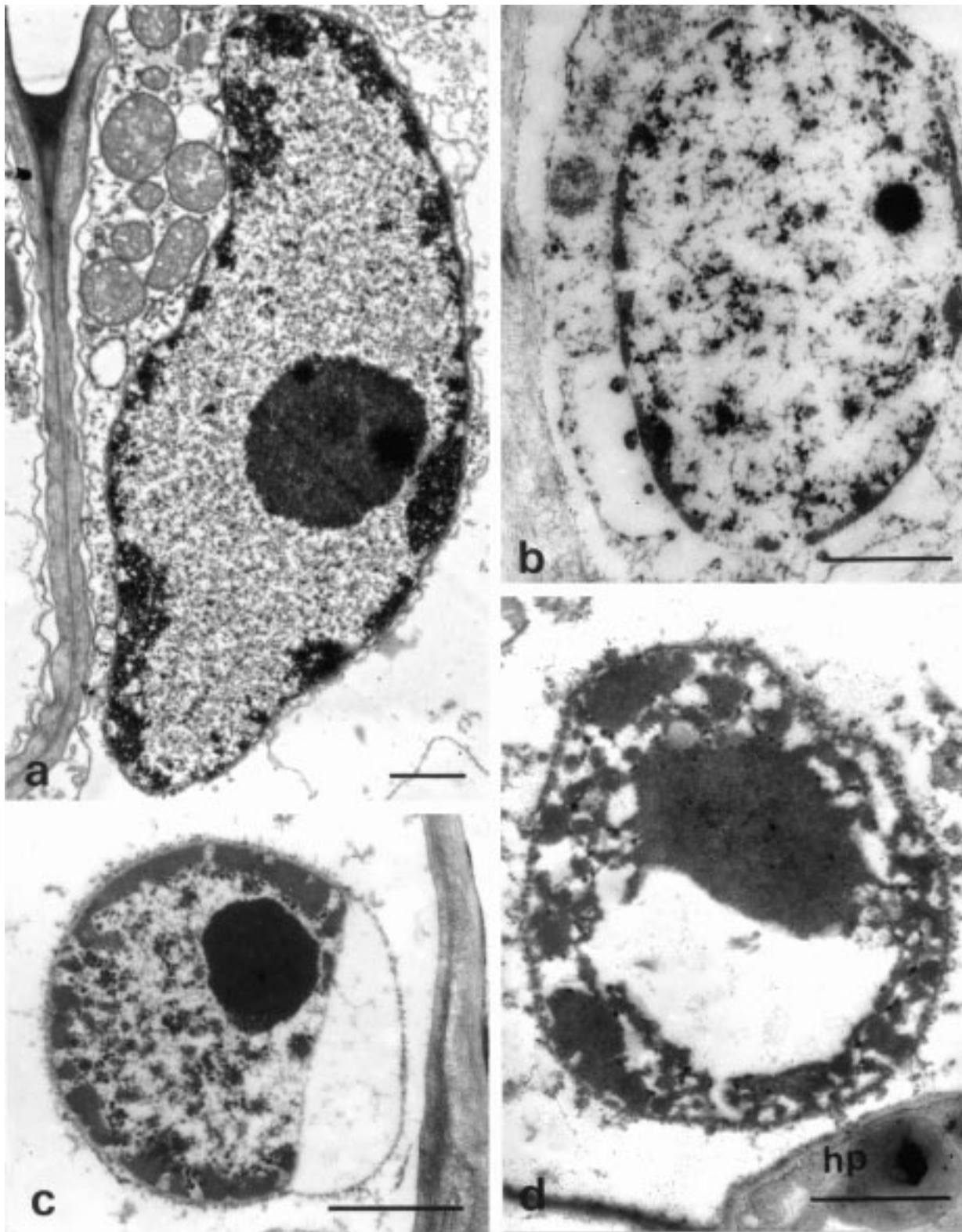
nuclei in senescing plant cells; explained as a possible apoptosis-like pathway, as has already been shown in pathogen infected plants (reviewed by Pennell and Lamb, 1997).

The lower percentages of 4C and 8C nuclear populations and significantly higher 2C nuclear populations observed in pathogen-infected roots point out the plasticity of the genome of tomato as has been shown in plants infected by the arbuscular mycorrhizal fungus *Glomus mosseae*. In *Glomus mosseae*, however, a higher percentage of 8C nuclei has been observed, and explained by polyploidization (Berta *et al.,* in press). The effects induced by pathogens may therefore be different from those induced by symbionts: infact, an increase of DNA quantity has already been reported not only in mycorrhizae, but also in the host nuclei of legumes when infected by rhizobia, though the results need further investigation (Brewin, 1991).

The increases in 2C nuclear populations should not be ascribed to larger cribro-vascular cambium tissues or to other root morphogenetic modifications: tomato plants infected with *P. parasitica* had thinner roots than controls (Fusconi *et al.*, 1999), as did phytoplasma-infected roots, whose mean diameter was significantly lower than that of controls (phytoplasmas: mm 0.77 ± 0.04; controls: mm $1.12 \pm 0,07$). In addition, no difference was found between the percentages of nuclear populations extracted from lateral or from adventitious roots (Berta, unpublished results).

The present findings suggest a block of endoreduplication in the presynthetic phase of the cell cycle, leading to an increase in 2C nuclear populations in pathogen-infected roots, in agreement with previous results obtained by static cytometry (Fusconi *et al*. 1997). Biotic stresses, such as those induced by pathogens, could possibly control the step at which cells exit the mitotic cycle and become committed to rounds of endoreduplication, as has been shown for at least one abiotic stress, water deficiency (Artlip *et al*. 1995). In this case, carbohydrate metabolism and

Fig. 3 - Light micrographs of *Lycopersicon esculentum* adventitious roots infected with *Phytophthora parasitica* after immunolocalization (**a, b**) or toluidine blue staining (**c**). (**a**) A root apex observed *in toto* ; the cap is deeply stained for the presence of the fungus. (**b**) A fungal hypha (*) penetrating the root through a root hair. (**c**) Semi-thin section of a differentiated zone; fungal hyphae are clearly visible penetrating the root through epidermal cells and spreading between cortical cells. Bars represent 10 µm.

are less accessible to propidium iodide than those of uninfected plants (Lingua *et al.,* 1996). These results, together with the present ones, are in agreement with the findings of O'Brien *et al.* (1998), who have shown a large decrease in fluorescence intensity of propidium iodide-stained

Fig. 2 - Electron micrographs of adventitious root nuclei in *Lycopersicon esculentum*. (**a**) A control cortical cell nucleus, in which small patches of condensed chromatin (chromocentres) are visible at the periphery. (**b, c**) nuclei from phytoplasma-infected roots. (**b**) Strongly chromatolytic nucleus in a companion cell. (**c**) A cortical cell nucleus in which both chromatolysis (on the right) and pyknosis are evident. (**d**) A nucleus with dramatic chromatolysis in a cortical cell in which a *Phytophthora parasitica* hypha is visible (hy). Bars represent 1 µm.
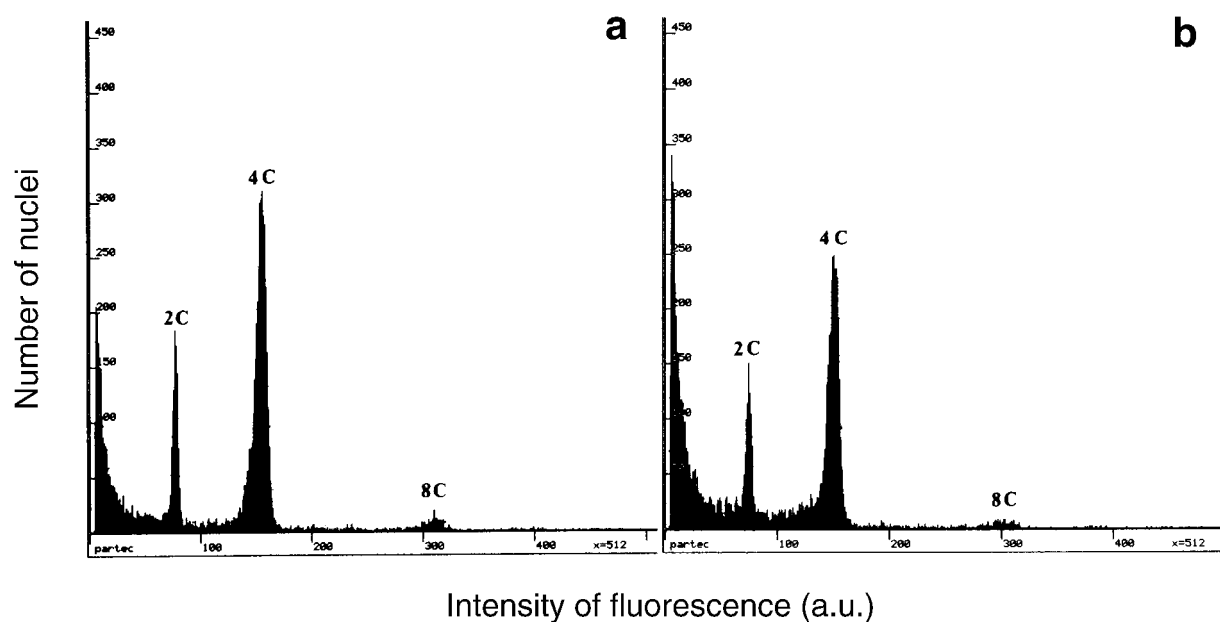
Fig. 4 - Flow cytometry histograms of DAPI-stained nuclei extracted from tomato roots. (**a**) Control plants, (**b**) phytoplasma-infected plants. On the x-axis the intensity of fluorescence, measured in arbitrary units; on the y-axis the number of nuclei. In each graph three peaks, corresponding to 2C, 4C and 8C nuclear populations, are visible. The mean fluorescence intensity of the peaks of the phytoplasma-infected plants were always lower than those of the control plants (i.e., they were nearer to the origin on the x-axis).

translocation could be involved. *P. parasitica*, as other fungal pathogenic invaders, should tend to increase the amount of carbohydrate transported to infected root zones (Isaac, 1992). After phytoplasma infection, the presence of the microorganisms in the phloem tubes impairs the transport pathway, and, as a result, the supply of photosynthate to root tissues is reduced (Catlin, 1975; Braun and Sinclair, 1978; Lepka *et al*., 1999).

Our results indicate that different stimuli may evoke a similar senescence pattern and allowed us to consider flow cytometry as a new tool in investigating the physiological condition of a root system. In fact, it allows detection in the host nucleus not only of DNA loss (Lingua *et al.* 1996), but also of DNA quantity variations, which are strongly influenced by the presence of pathogens.

ACKNOWLEDGEMENTS

Table III

Mean fluorescence intensity of DAPI stained nuclei in tomato roots ± standard errors. PTP = phytoplasma; asterisk (*) indicates significant differences according to the Student t-test, with 95% confidence limits

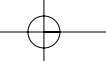| DNA content	control plants	Pp infected plants	t
2C	75.67 ± 1.48	66.67 ± 1.59	4.142*
4C	148.33 ± 4.00	133.33 ± 4.23	2.577
8C	298.00 ± 8.98	264.67 ± 7.73	2.815*

Table IV
Mean fluorescence intensity of DAPI stained nuclei in tomato roots ± the limits of confidence calculated at 99% (t 2.576; d.f. = ∞). Standard errors are in breakets. Pp = *P. parasitica*: All differences were significant, according to the calculated confidence limits

DNA content	control plants	Pp infected plants
2C	77.50 ± 0.34 (0.13)	74.00 ± 0.14 (0.06)
4C	153.00 ± 0.24 (0.09)	149.50 ± 0.15 (0.06)
8C	309.00 ± 0.43 (0.17)	301.00 ± 0.65 (0.25)

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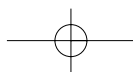
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