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Flow cytometry as a tool to investigate nuclear senescence in symbiotic and pathogenic systems

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Summary — Arbuscular mycorrhizae have been reported to moderate negative effects (growth inhibition, necrosis, death) caused by soil-borne pathogenic fungi. In this paper, we assessed root viability with two DNA fluorescent flow cytometry stains that can be usefully combined to evaluate chromatin changes. Nuclei were extracted from arbuscular mycorrhizal (*Glomus mosseae*) and non-mycorrhizal tomato roots, infected or uninfected with *Phytophthora nicotianae* var *parasitica* and watered with a low phosphate nutrient solution. Nuclei extracted from non-mycorrhizal roots infected with the pathogenic fungus showed lower fluorescence values after staining, compared to arbuscular mycorrhizal roots, suggesting that the presence of the pathogen may result in DNA loss and condensation. Infection by either fungus (symbiotic and pathogen) reduced the ratio of 4c to 2c nuclei in the differentiated root.

arbuscular mycorrhiza / chromatin / root pathogen / flow cytometry / senescence

Résumé — La cytométrie de flux comme moyen pour étudier la sénescence nucléaire dans des systèmes symbiotiques et pathogènes. Les mycorhizes arbusculaires sont connues pour réduire les effets négatifs (croissance, nécrose, mort) causés par les pathogènes telluriques. Dans ce travail, nous avons évalué par cytométrie de flux, la viabilité des racines, en utilisant deux colorants d'ADN qui, en combinaison, permettent d'estimer les changements au niveau de la chromatine. Les noyaux ont été extraits des racines de tomate mycorhizées ou non avec *Glomus mosseae* ou infectées ou non avec *Phytophthora nicotiana* var *parasitica* et ayant reçu une solution nutritive pauvre en phosphore. Les noyaux extraits des racines non mycorhizées, infectées avec le champignon pathogène, ont donné des valeurs plus faibles de florescence par rapport à ceux obtenus avec des noyaux extraits des racines mycorhizées. Cela suggère que la présence de champignons pathogènes se traduit au niveau de l'ADN par une perte de condensation. L'infection par les deux champignons (symbiotique et pathogène) réduit le rapport du nombre de noyaux 4c par rapport aux 2c dans les racines différenciées.

mycorhize arbusculaire / chromatine / cytométrie de flux / sénescence

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INTRODUCTION

Soil-borne root pathogenic fungi generally produce extensive root necrosis. However, this may be moderated by the arbuscular mycorrhizal (AM) symbiosis (Lindermann, 1992; Hooker et al, 1994; Liu, 1995). In tomato plants, for example, arbuscular mycorrhizae significantly reduce root necrosis, decrease root infection by pathogens and reduce inhibition of plant growth (Caron et al, 1985, 1986; Cordier et al, 1996; Trotta et al, 1996). However, mechanisms of biocontrol are not well understood.

The analysis of events occurring at the cellular and nuclear level offer the opportunity to improve understanding of the mechanisms underlying biocontrol. The nuclei of senescing root cortical cells may be either strongly condensed or chromatolytic due to DNA loss. Evaluation of chromatin structure and DNA content is therefore a useful indicator of root viability. One method available to analyse these parameters is flow cytometry of nuclei extracted from tissues or organs, coupled to staining with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) DNA-specific dyes. This is a powerful technique and the use of these stains in combination has previously given information about both quantitative and structural chromatin variations in a variety of systems (Cowden and Curtis, 1981; Mazzini et al, 1983; Darzynkiewicz et al, 1984; Evenson et al, 1986; Sgorbati et al, 1986, 1993; Bonaly et al, 1987; Berta et al, 1990, 1996).

This paper describes the use of these techniques in the investigation of nuclear senescence in roots of non-AM and AM tomato plants, infected or uninfected with the pathogenic fungus *Phytophthora nicotianae* var *parasitica*.

MATERIALS AND METHODS

Tomato plants (*Lycopersicon esculentum* Mill cv Early Mech) were grown in quartz sand under controlled conditions (16/8 h light/dark photoperiod, 24/20 °C light/dark thermoperiod, 130 µmol m^{-2} s^{-1} light irradiance at pot height, fluorescent lamps Sylvania F36W/133ST, cool white) and fed on alternate days with a Long Ashton nutrient solution containing 32 µM phosphate. Mycorrhizal plants were inoculated at sowing with the AM fungus *Glomus mosseae* (Nicol and Gerd) Gerdemann and Trappe (BEG12) by mixing colonized roots of leek (*Allium porrum* L) into the growth substrate. After 1 month, some plants were infected with *Phytophthora nicotianae* var *parasitica* Breda De Haar (isolate 201, subsequently referred to as *P parasitica*). These four groups of plants were identified as: C = uninoculated and uninfected controls; C + P = *P parasitica*-infected plants; M = AM plants; M + P = *P parasitica*-infected AM plants. Plants were harvested when they were 2 months old (1 month after *P parasitica* infection) and the intensity of mycorrhizal infection (M%) was calculated according to Trouvelot et al (1986). Only plants with M% values higher than 40% were used for investigations.

For flow cytometry, fresh roots deprived of their apices were chopped with a razor blade in a petri dish, in the presence of a few drops of extraction buffer (0.1% citrate, 0.5 M Tween 20). Nuclei were isolated after 30 min incubation in this medium by filtering through a 20 µm nylon mesh.

Propidium iodide staining

Nuclei were diluted in 0.1% citrate, 0.1% triton X-100 to obtain a concentration of 10^5 nuclei mL^{-1}, and then stained with 50 µg/mL PI. Polyscience beads (4.2 µm) were used as a standard. This procedure provides qualitative information because of the way that PI binds to DNA; as PI is intercalating, highly decondensed chromatin is easier to stain (Biradar and Rayburn, 1994).

4',6-diamidino-2-phenylindole staining

The four samples were diluted with 0.4 M dibasic phosphate to adjust the concentration of nuclei to 10^5 mL^{-1}. They were then stained with 5.6 µM DAPI. Human lymphocytes (HL), previously fixed in 70% ethanol and stained with DAPI, were used as the internal standard. Nuclear fluorescence intensity was evaluated as a percentage of the intensity of HL fluorescence.

Flow cytometry

A Partec PAS IIIi instrument was used. For measurement of DAPI staining, a mercury arc lamp was used in conjunction with BG1, UG1 filters and a TK420 dichroic mirror to select the exciting wavelength (UV). DAPI fluorescence was detected using a GG415 barrier filter. For PI staining, an argon laser (wavelength = 488 nm) was used as the exciting light source and a GG610 barrier filter was used to detect the fluorescence emission. Mean, integral (number of particles) and variation coefficients (CV%) were calculated using 'Parteclist' (Partec GmbH, Münster, Germany). The ratio between the number of nuclei with 4c DNA and those with 2c DNA content was calculated and the data compared by analysis of variance (ANOVA).

Light microscopy

Roots inoculated or not with *G mosseae* were fixed for 2 h in 4% formaldehyde in 0.1 M phosphate buffer, pH 7, postfixed in OsO_4 1%, dehydrated and embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Median longitudinal sections were cut on a Reichert ultramicrotome, and nucleus morphology was examined using light microscopy (Zeiss Axiolab, Carl Zeiss Jena GmbH, Jena, Germany), after staining with 1% toluidine blue in 1% sodium tetraborate.

RESULTS

Propidium iodide staining

Two peaks, belonging to two nucleus populations with 2c and 4c DNA content, were recognized after PI staining. Nuclei from M + P roots showed the highest fluorescence values, while those extracted from C + P roots showed the lowest ones (table I and fig 1). CVs ranged from 6.38 to 11.06%.

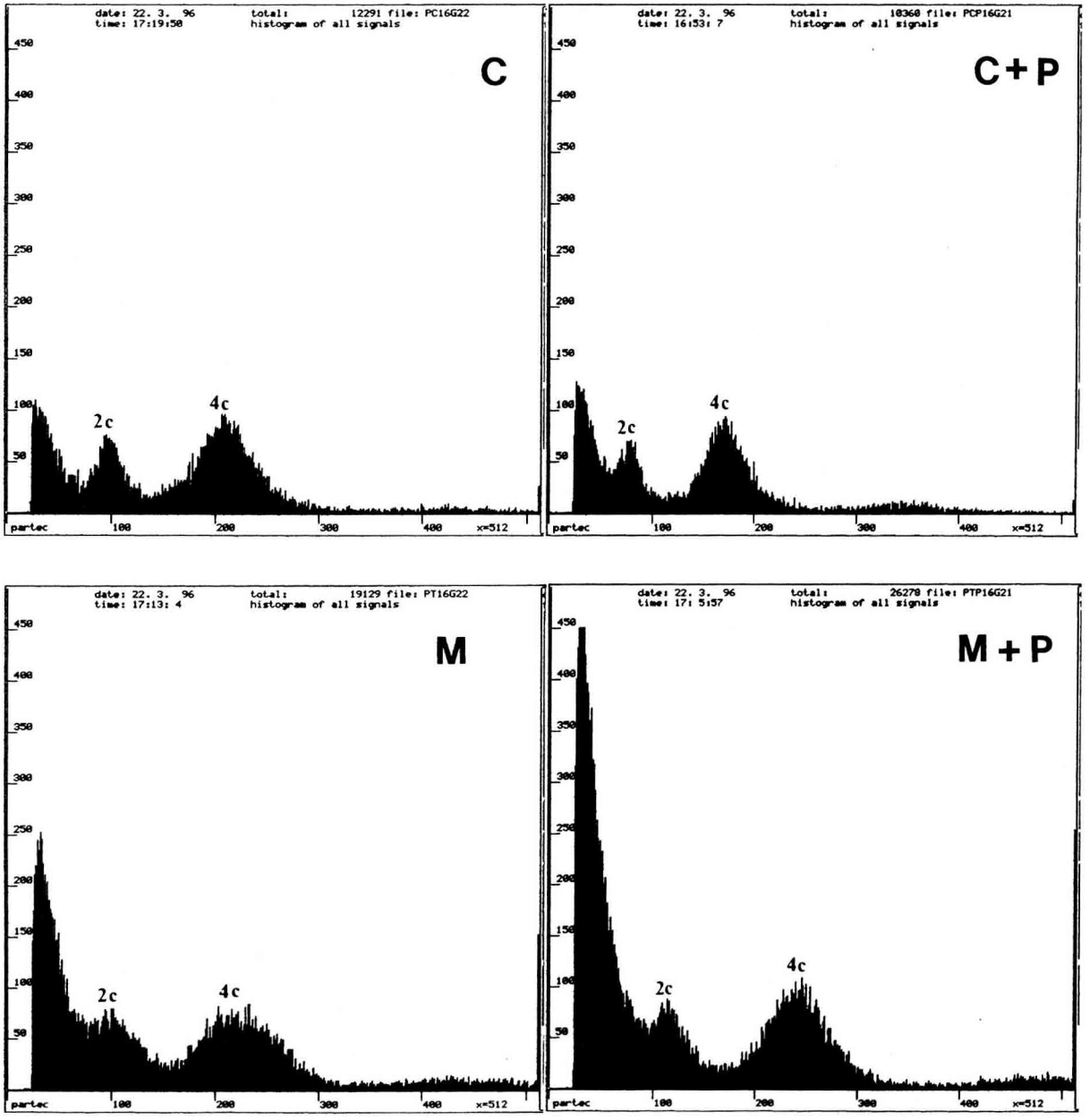

Fig 1. Flow cytometry histograms of propidium iodide (PI)-stained nuclei extracted from tomato roots. C: control plants; C + P: *Phytophthora nicotianae* var *parasitica*-infected plants; M: arbuscular mycorrhizal plants; M + P: *Phytophthora nicotianae* var *parasitica*-infected arbuscular mycorrhizal plants. In each histogram, the left peak represents the 2c nuclei population, the right one the 4c nuclei population.

4',6-diamidino-2-phenylindole staining

Nuclei of M plants exhibited the highest fluorescence after DAPI staining, followed by those from the M + P treatment (table II and fig 2). Those from C and C + P plants were very similar, but the 4c peak of C roots had a higher fluorescence, while 2c nuclei from C + P roots showed higher fluorescence. CVs ranged from 1.29 to 3.86%, with the C + P treatment having the highest values (table II).

In C plants, the mean ratio of 4c to 2c was higher than 3, but below 4 (table III), and significantly different from values of both C + P and M + P ($P < 0.01$) and M ($P < 0.05$). These data were confirmed by PI staining (data not shown).

Nuclear morphology – light microscopy analysis

Nuclei in the differentiated part of uninfected roots were in a peripheral position in cells and were mostly lens-shaped (fig 3a). In M and M + P roots, nuclei were often round or lobe-shaped and in a central position where the AM infection was well developed and especially in arbuscule-containing cells. Decondensed chromatin was clearly visible (fig 3b).

In contrast, nuclei were in a peripheral position, lens-shaped and pyknotic or weakly stained in C + P roots (fig 3c).

DISCUSSION

In various AM systems, the presence of the symbiotic fungus has been reported to result in significant hypertrophy of the host cell nucleus (Protsenko and Shemankova, 1971; Holley and Peterson, 1979; Toth and Miller, 1984; Sgorbati et al, 1993). Moreover, data obtained with a variety of techniques (Berta et al, 1986, 1990; Blair et al, 1988; Berta and Squadrone, 1993; Sgorbati et al, 1993) have shown that this nuclear hypertrophy is not due to polyploidization, but to an increase in chromatin decondensation.

Table I. Intensity of fluorescence of propidium iodide (PI)-stained nuclei for each of the four treatments.

	Mean fluorescence intensity of nuclei with 2c DNA content	*Variation coefficients (2c nuclei) (%)*	*Mean fluorescence intensity of nuclei with 4c DNA content*	*Variation coefficients (4c nuclei) (%)*
C	86.5	6.38	181	6.56
C + P	78	11.06	171.5	6.44
M	100	10.95	222.5	10.65
M + P	115	8.36	242	8.67

C: control plants; C + P: *Phytophthora nicotianae* var *parasitica*-infected plants; M: arbuscular mycorrhizal plants; M + P: *Phytophthora nicotianae* var *parasitica*-infected arbuscular mycorrhizal plants.

Table II. Intensity of fluorescence of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei for each of the four treatments.

	Mean fluorescence intensity of nuclei with 2c DNA content	*Variation coefficients (2c nuclei) (%)*	*Mean fluorescence intensity of nuclei with 4c DNA content*	*Variation coefficients (4c nuclei) (%)*
C	34.0	1.29	67.9	3.46
C + P	34.6	3.18	67.2	3.86
M	36.6	2.45	73.4	1.72
M + P	35.9	2.84	72.4	2.54

Abbreviations as in table I.

Chromatin decondensation is known to be related to transcription in both animal and plant cells (Nagl, 1985), and the shape and position of a nucleus is also thought to depend on its functional activity. In the *A porrum + G mosseae* system, round, centrally positioned nuclei, which are more common in the arbuscule-containing cortical cells, show higher labelling after ^{3}H-uridine uptake than peripheral ones, indicating that they are more active (Berta et al, 1996).

The differences observed in the 4c to 2c ratio for tomato roots + *G mosseae* in this study also point to the absence of polyploidization, and suggest that the presence of either a mycorrhizal and/or a pathogenic fungus in root tissues affects the host cell cycle, as in has been observed for mycorrhiza in *A porrum + G mosseae* (Berta et al, 1991).

Fluorescence of PI stained nuclei was lower in C and C + P than in M and M + P roots. The lower fluorescence values of the C nuclei can be partly explained by chromatin condensation, indicating a lower transcriptional activity and sug-

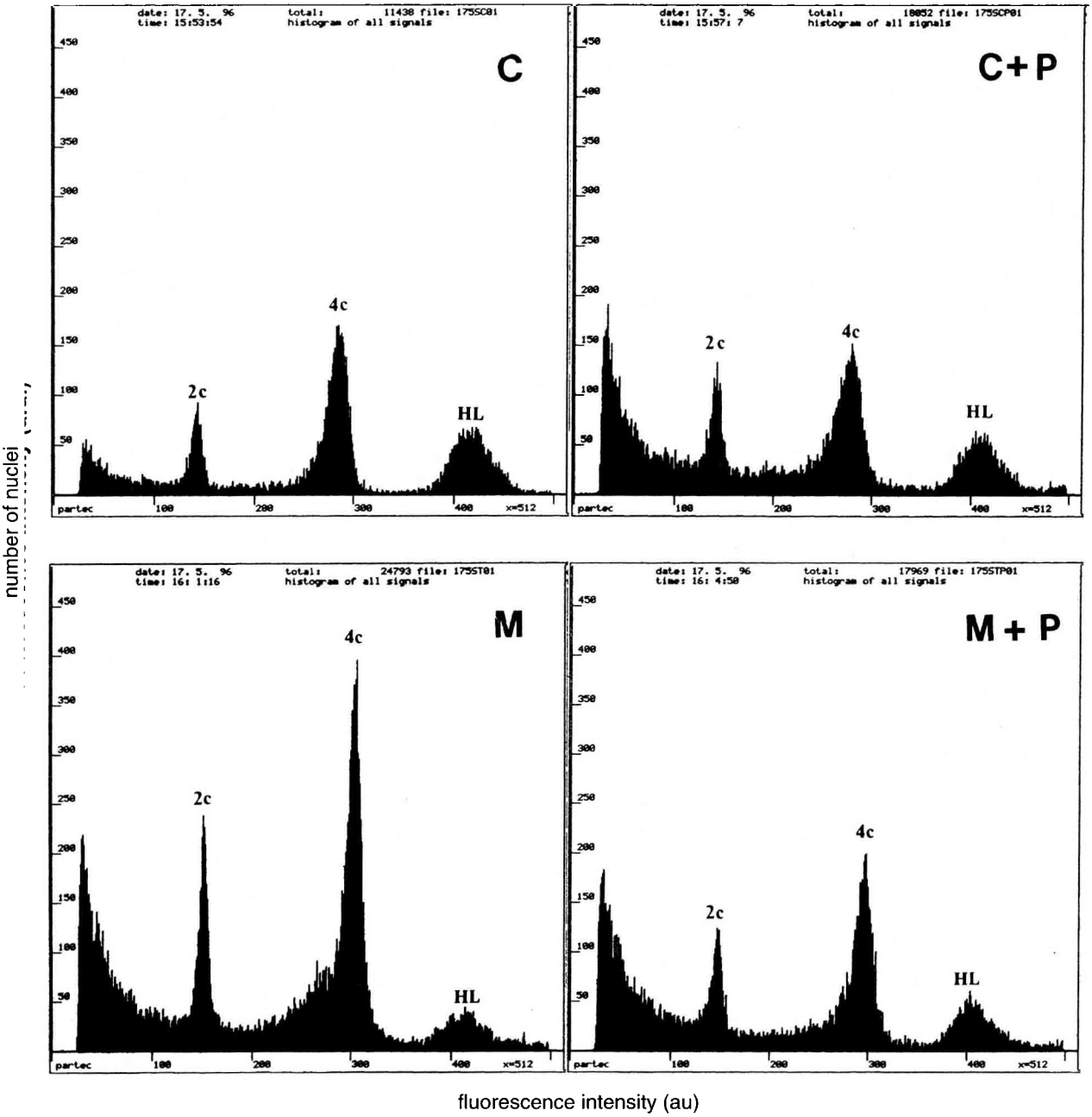

Fig 2. Flow cytometry histograms of 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei extracted from tomato roots. C: control plants; C + P: *Phytophthora nicotianae* var *parasitica*-infected plants; M: arbuscular mycorrhizal plants; M + P: *Phytophthora nicotianae* var *parasitica*-infected arbuscular mycorrhizal plants. In each histogram, the left peak represents the 2c nuclei population, the central one the 4c nuclei population, and the right peak is given by the human lymphocytes (HL) (standard).

Table III. Ratio between the number of nuclei with 4c DNA content and number of nuclei with 2c DNA content in five repetitions.

	I	*II*	*III*	*IV*	*V*	*Mean*
C	3.90	3.05	4.29	3.98	3.83	3.81 ± 0.21
C + P	2.95	3.24	2.99	2.50	2.42	2.82 ± 0.16
M	2.65	2.59	2.68	4.07	2.65	2.93 ± 0.29
M + P	2.89	2.87	2.09	2.36	2.34	2.51 ± 0.16

Abbreviations as in table I.

gesting that the control roots are mostly ageing or senescing.

Nuclei of AM tomato plants were always most fluorescent after DAPI staining, as previously reported for other AM systems (Berta et al, 1986; Sgorbati et al, 1993). This may be attributable to replication of part of the DNA in nuclei from AM roots. The lower fluorescence of C + P nuclei might be due to their partial fragmentation, as observed by light microscopy, or to direct DNA loss, through the stress imposed by the pathogenic fungus.

Both chromatin condensation (revealed by PI) and DNA loss (evaluated by DAPI), which are clear signals of senescence whatever the reason (root age or pathogen infection), were reduced with AM colonization of tomato roots.

Useful information was also provided by comparisons of CVs of data obtained with the two stains. Data from PI staining generally exhibited high CVs: these data were less precise than those obtained with DAPI. It is of interest that the CV of C + P were usually the highest, with both stains, suggesting that the C + P population of

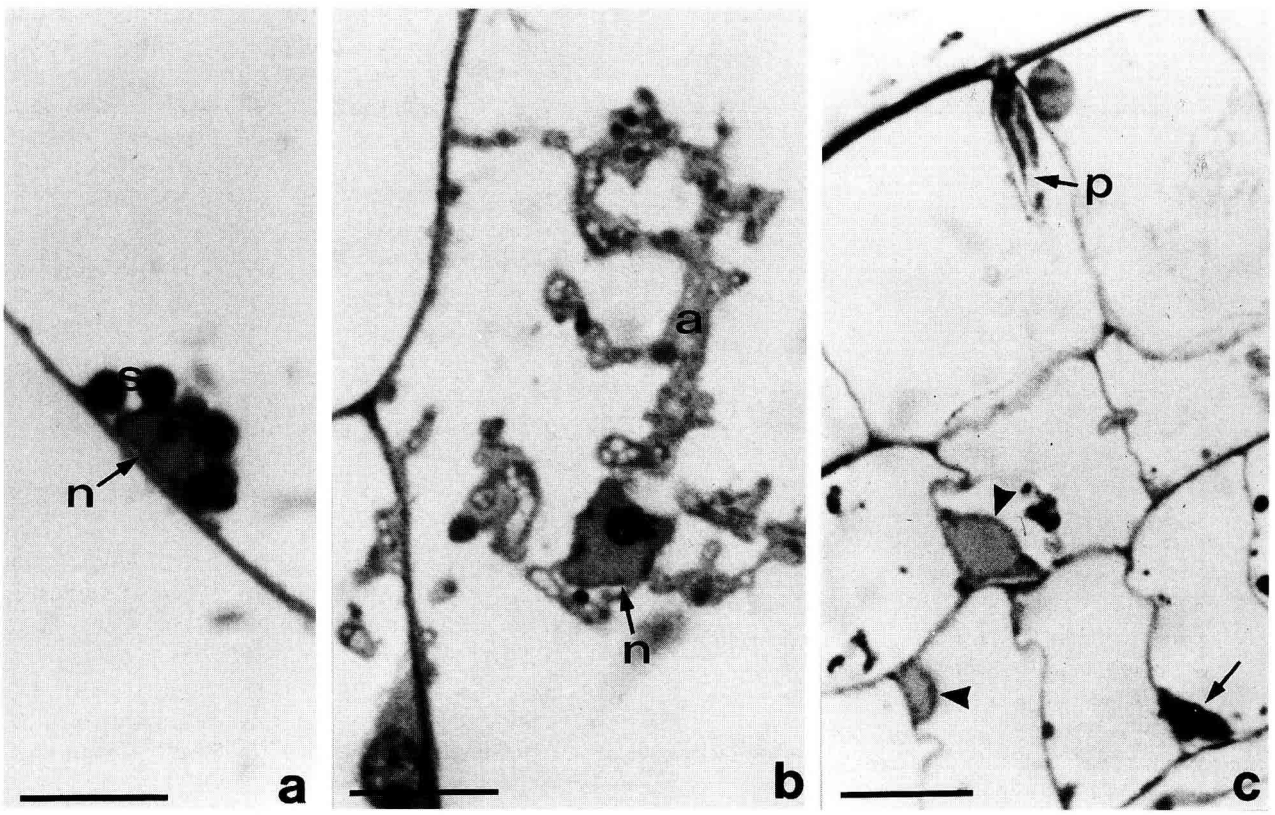

Fig 3. Nuclei in tomato root cortex tissue. **a**) Uninfected root, the nucleus (n) with an evident nucleolus is adjacent to the cell wall, surrounded by starch grain (s); **b**) arbuscular mycorrhizal root, the nucleus (n), in central position, shows decondensed chromatin, peripheral chromocentres and a large, round, nucleolus, a: arbuscule; **c**) *Phytophthora nicotianae* var *parasitica*-infected root, the nuclei are in lateral position, pyknotic (arrow) or weakly stained (arrowheads); p: pathogenic hypha. Horizontal bars represents 10 μm.

nuclei was the least homogeneous of the four analyzed.

In conclusion, our results show that flow cytometry gives valuable information about root senescence and suggests that it could be especially useful in investigating the effects of AM and pathogenic fungi on plant cell nuclei.

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