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Nuclear architecture and DNA location in two VAM fungi

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Summary. The ultrastructural organization of nuclei in *Glomus versiforme* and *G. mosseae* hyphae was examined by sampling at different times during the fungus life cycle. In young and active hyphae, the nucleus is chromocentric with dispersed chromatin and a well-developed nucleolus. On aging, and particularly during arbuscule collapse, the chromatin condenses to give a pyknotic nucleus with blebs in the envelope and conspicuous spaces between this and the nucleoplasm. DNA location by immunogold or enzyme-gold techniques resulted in uniform labelling over the chromatin. Nuclear degeneration is mirrored by decreased gold labelling and may thus be regarded as an important parameter for evaluation of fungus viability and the ephemeral life of the arbuscule.

Key words: Vesicular-arbuscular mycorrhizal fungi – Nucleus – Chromatin – DNA – Immunogold location

Introduction

Although vesicular arbuscular mycorrhizal (VAM) fungi are among the most widespread soil fungi, existing as symbiotic root colonizers of about 80% of land plants (Harley 1989), their cell biology (i.e. morphogenesis, cell cycle, ultrastructural organization of the protoplasm) has been largely neglected. This is primarily because VAM fungi readily germinate in vitro but are then reluctant to grow on a pure culture medium in the laboratory (Hepper 1984); thus we know little about their presymbiotic phase. The infection process has been described in many host plants, but the regulation mechanisms (production of appressoria as well as the formation and death of arbuscules) are poorly known.

This paper forms part of a study of the role of the nucleus in the fungal cell cycle and morphogenesis and describes the nuclear organization of two VAM fungi (*Glomus versiforme* and *G. mosseae*) during the symbiotic phase, from the early production of extraradical-presymbiotic hyphae to arbuscule collapse. Morphological observations coupled with gold location of DNA molecules have shown that nuclear degeneration proceeds from the extraradical hyphae to the inner arbuscular tips.

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Materials and methods

Mycorrhizal plants

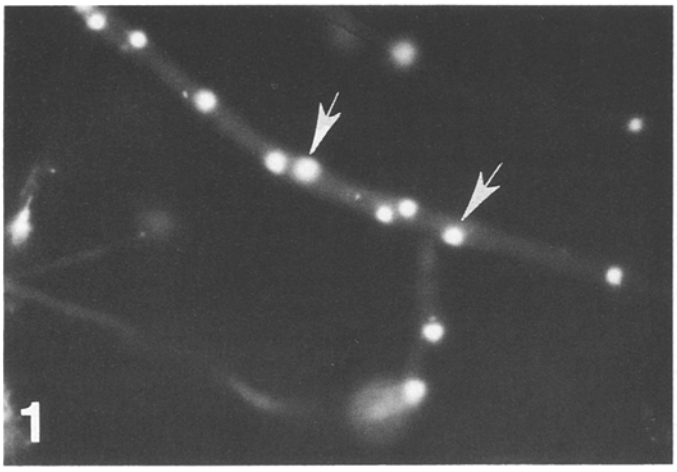
Seeds of *Allium porrum* L. cv Monstruoso di Caretan and *Ginkgo biloba* L. were sown in sterilized quartz sand and the germinated seedlings were watered three times per week with low-phosphorus Long Ashton solution. Mycorrhizal plants were obtained by injection around the seeds of a spore suspension (100 mg fresh wt./ml) obtained from *G. versiforme* (Karst) Berch fruit bodies. Mycorrhizal roots were sampled 1 and 2 months after sowing.

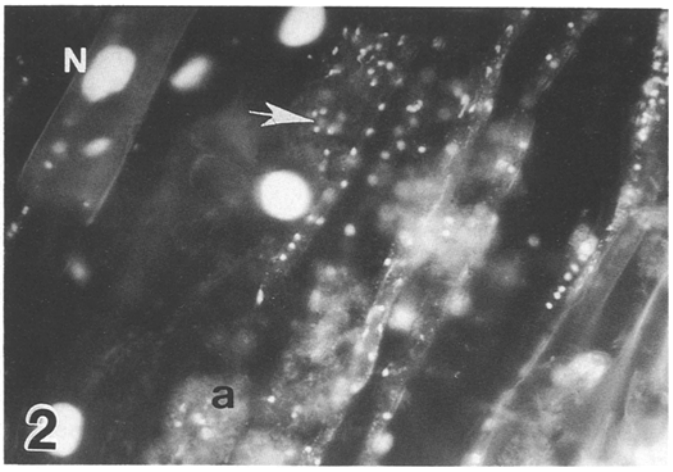
Presymbiotic mycelium

Mycelium of *G. mosseae* (Nicol & Gerd.) Gerdemann & Trappe developed from a mycorrhizal clover plant towards two lateral PVC containers through an intervening nylon net (60 µm mesh). According to Wyss et al. (1991), only the growing mycelium passes through the nylon net. The mycelium developing in the two containers was collected 7–10 days after setting up the experiment.

Fluorescence microscopy

Mycorrhizal roots from the two host plants were fixed in 4% paraformaldehyde in MTSB (50 mM PIPES, 5 mM $MgSO_4$, 5 mM EGTA pH 6.9) overnight at 4° C. Then the samples were infiltrated with 1.5 M sucrose and 0.5 M paraformaldehyde in MTSB for at least 3 days at 4° C. After the samples had been cut with a cryostat (MICROM, Heidelberg, FRG), the frozen sections (20 µm thick) together with the extraradical mycelium were stained with a saturated solution of DAPI (4′,6-diamidino-2-phenylindole) 2 µg/ml. Extraradical and intraradical mycelia were ob-





Figs. 1, 2. Nuclear distribution in *Glomus versiforme* after DAPI staining. **Fig. 1.** Round-shaped nuclei (*arrows*) are regularly distributed along the extraradical hyphae. ×643. **Fig. 2.** Frozen section of a leek root colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus. The large fluorescent bodies are the host nuclei (*N*), while the small ones (*arrow*) are those of the fungi. They are organized in regular rows inside the intercellular hyphae or in an irregular manner inside the arbuscules (*a*). ×314

served under a fluorescence microscope (Zeiss) equipped with an excitation filter G 365 and a barrier filter 420.

Electron microscopy

Extraradical mycelium and mycorrhizal roots were fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 3 h at room temperature, rinsed, postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, washed and dehydrated in an ethanol series. The samples were then embedded in Durcupan ACM resin (Fluka, Buchs, Switzerland) at 60° C and thin sections were cut and stained with uranyl acetate and lead citrate. To increase the contrast, other sections were stained with the periodic acid-thiocarbohydrazide-silver proteinate (PATAg test), according to Roland and Vian (1991). Parallel samples were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature for 3 h, washed in the same buffer, dehydrated in an ethanol series, and embedded in LR White resin (Polysciences, Warrington, Pa., USA) at 60° C.

DNA labelling

Enzyme gold-affinity labelling. DNase I (EC 3.1.21.1) purified from calf thymus (Sigma) was tagged with colloidal gold particles to form a DNase-gold complex according to Bendayan (1984). Briefly, 50 ml of a 15 nm colloidal gold solution was adjusted to pH 6.0. A 200-µl aliquot of a stock solution of DNase I (10 mg/ml) was added whilst stirring. After 5 min, 2.5 ml of 1% polyethyleneglycol (PEG) was added and the enzyme-gold mixture was centrifuged at 14000 rpm in a Beckman rotor Ti for 1 h at 4° C. The mobile pellet was collected in 4 ml of 0.02 M saline phosphate buffer (PBS), pH 6.0, to which 0.5 mg/ml PEG was added. The complex was used immediately. Thin sections from the LR White embedded samples were handled as floating sections. After rinsing in the same PBS buffer, they were exposed to the DNase-gold complex for 1 h at 30° C, floated on the incubation buffer alone for 10 min, and rinsed twice in distilled water, before staining with uranyl and lead salts. Control sections were treated with inactivated heated enzyme and with colloidal gold alone, according to Bendayan (1984).

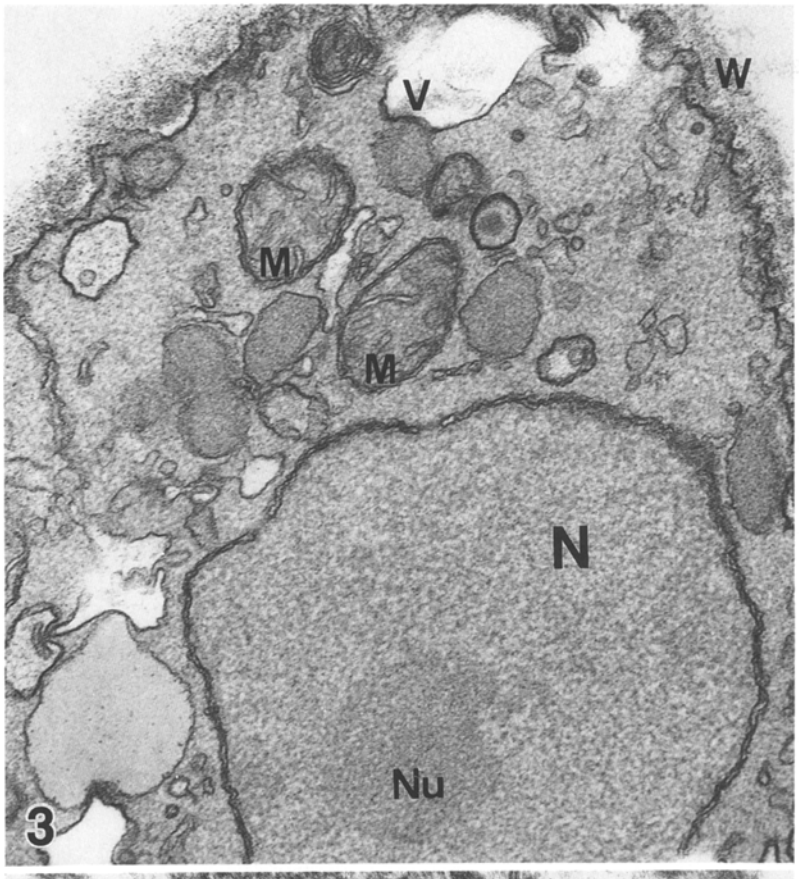
DNA immunolocation. Thin sections from the samples embedded in LR White resin and handled as described were treated with a monoclonal antibody (mAb) that binds to single- and double-stranded DNA molecules (Scheer et al. 1987), incubated with normal goat serum for 15 min, and then exposed to the undiluted mAb overnight at 4° C. After washing with 0.05 M TRIS saline buffer (TBS) pH 7.6 for 20 min and TBS containing 0.1% bovine serum albumin (BSA) for 10 min, they were incubated with 15 nm colloidal gold-goat anti-mouse immunoglobulin complex (Janssen, Beerse, Belgium) for 1 h. The second antibody was diluted 1:20 in 0.02 M TBS containing 1% BSA at pH 8.2. The sections were then washed with TBS, rinsed twice in distilled water and poststained with uranyl and lead salts. The controls were a parallel set of sections either treated with antibody preabsorbed with DNA (10 µg/ml in TBS buffer), or lacking the primary antibody treatment (Scheer et al. 1987).

Results

Nuclear distribution

All *G. versiforme* and *G. mosseae* hyphae are multinucleate. According to previous observations (Bonfante-Fasolo et al. 1987; Cooke et al. 1987), nuclei are roundish to oval and are located either in the middle of the hypha or close to its wall (Fig. 1). In the intercellular hyphae they form regular rows (Fig. 2).

Figs. 3–6. Ultrastructural features of nuclei in presymbiotic hyphae of *G. mosseae*. **Fig. 3.** The hypha possesses a nucleus (*N*) with diffuse chromatin and an electron-dense nucleolus (*Nu*). *W*, Wall, *V*, vacuole, *M*, mitochondria. ×19000. **Fig. 4.** Detail of a nucleus with a well-stained envelope after the PATAg reaction. ×41300. **Fig. 5.** The nucleus shows peripheral patches of chromatin (*arrows*) and a nucleolus with an evident nucleolar organizer (*Nor*). ×54000. **Fig. 6.** Magnification of a nucleolus showing fibrillar (*F*) and granular portions (*G*). ×74000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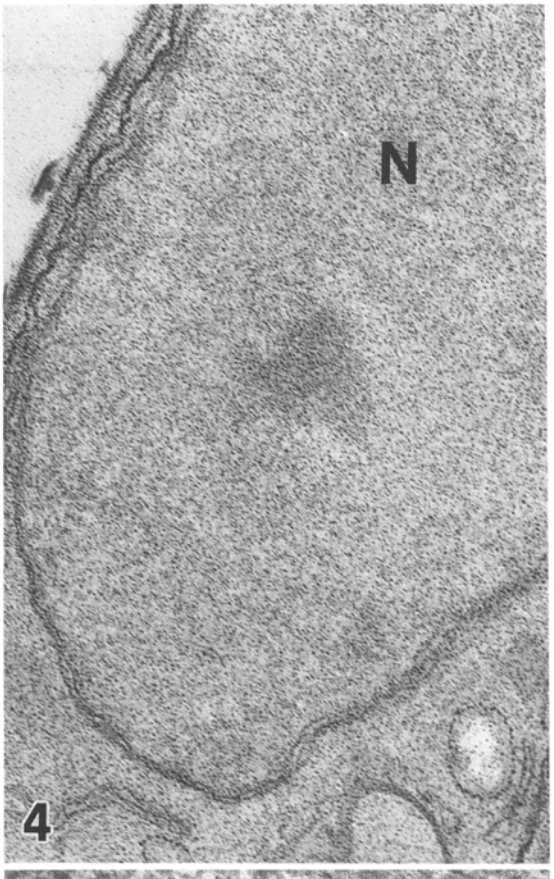
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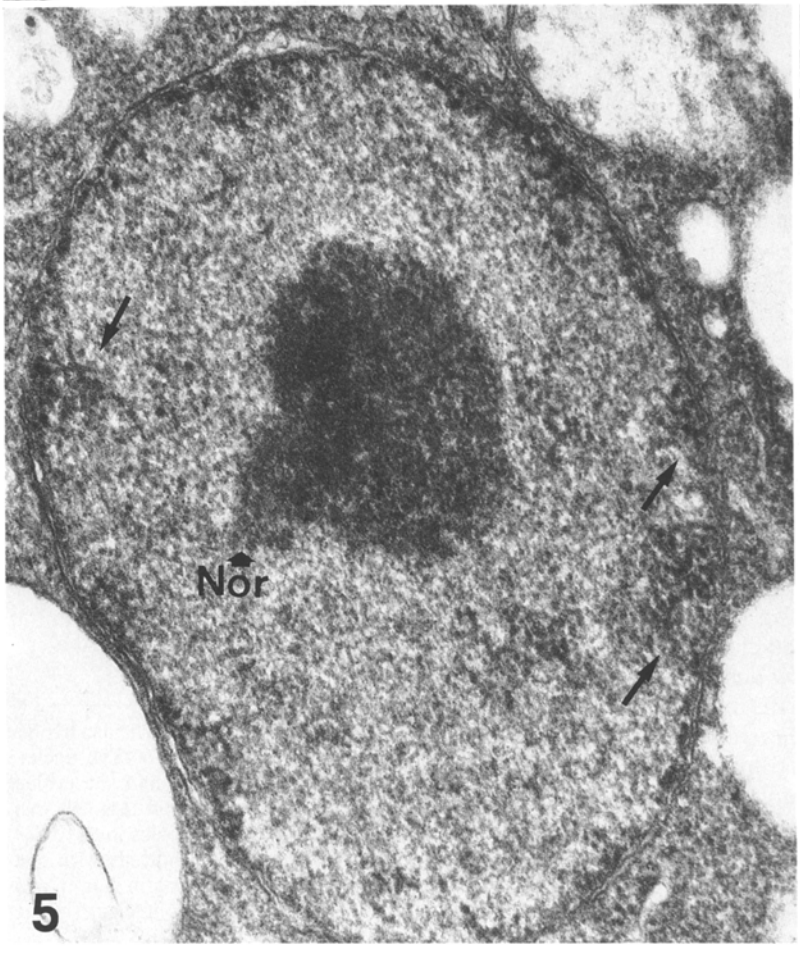
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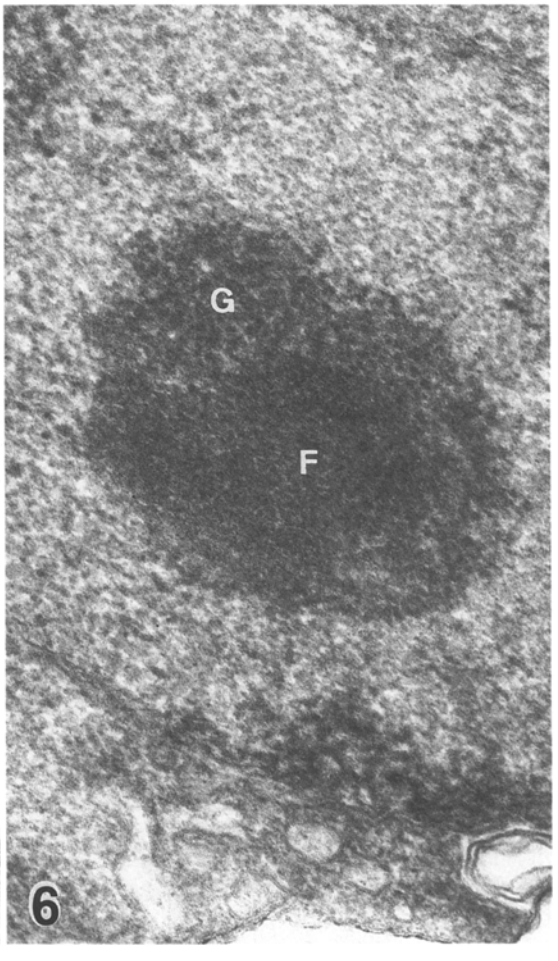
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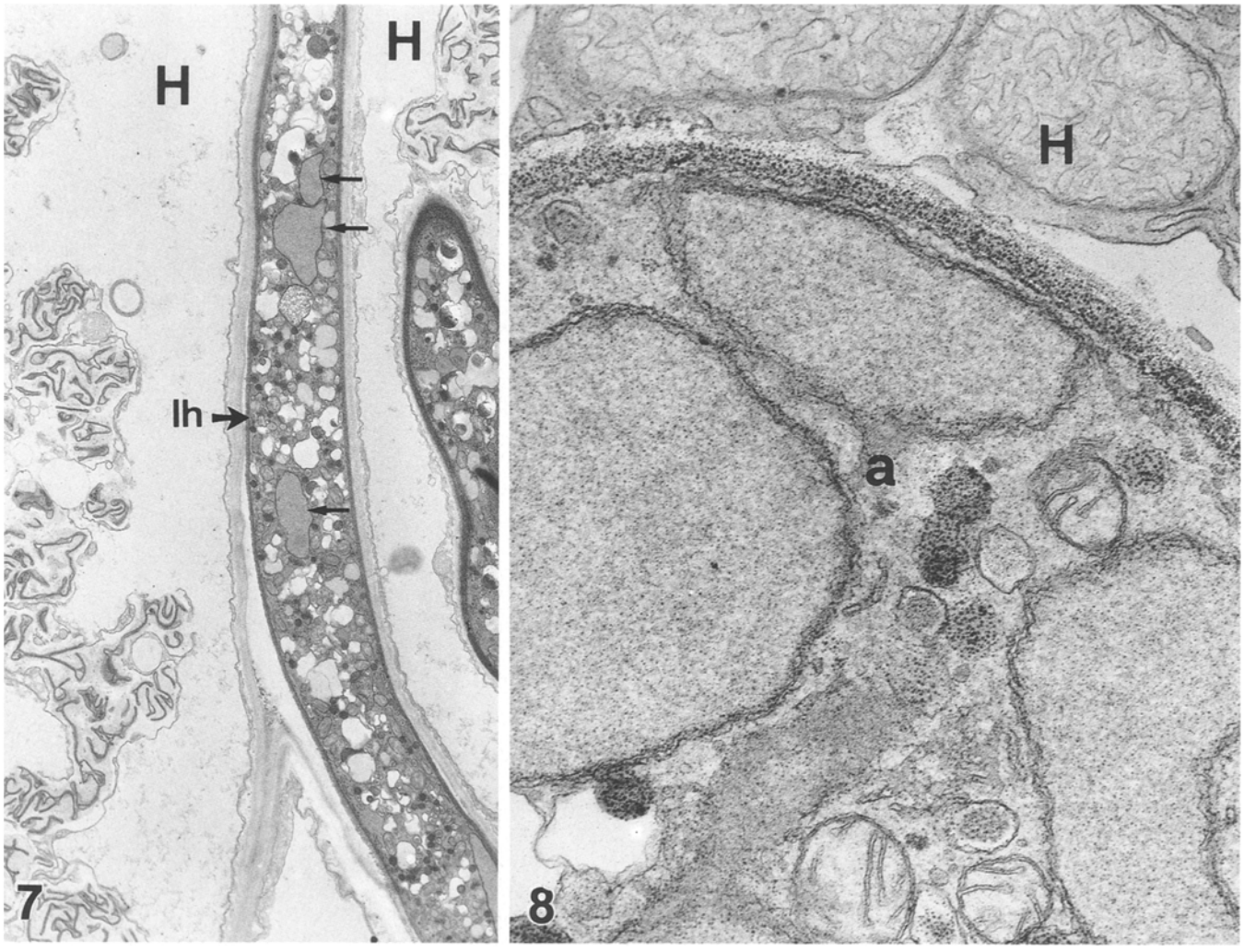


Figs. 7, 8. Ultrastructural features of nuclei in intraradical hyphae of *G. versiforme* colonizing *Ginkgo biloba*. **Fig. 7.** A running intercellular hyphae (*Ih*) shows numerous nuclei with electron-transparent chromatin (*arrows*). *H*, Host cells. ×4100. **Fig. 8.** Magnification of an arbuscular hypha (*a*) showing three nuclei with diffuse chromatin. PATAg reaction. *H*, Host. ×44000

Ultrastructural nuclear morphology

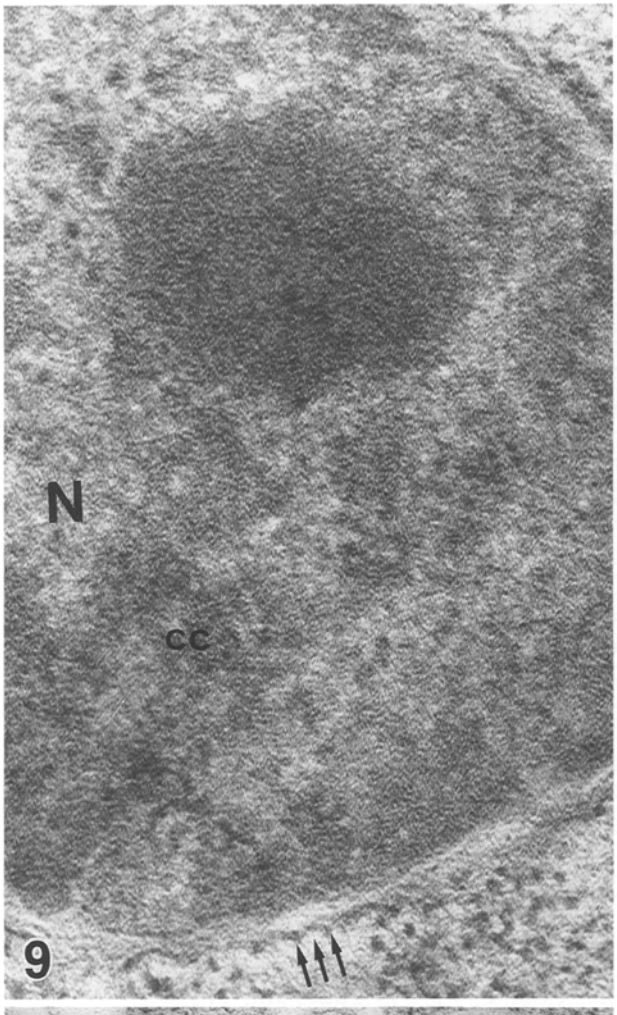
The nuclei of the two VAM fungi are morphologically very similar: the results are thus presented independent of the fungus species and the host plant.

The preinfective mycelium displays a thin wall with fine radiating fibrils, a cytoplasm rich in mitochondria, well-developed membrane systems and few small vacuoles (Fig. 3). The nucleus is bounded by a regular double envelope, which is strongly stained by the PATAg test; its loose electron-transparent chromatin (dispersed chromatin) has a granular organization (Fig. 4). A few dense chromatin patches are sometimes present near the nuclear envelope (Fig. 5). The nucleolus is a more electron-dense area, in which the granular and fibrillar components and the nucleolar organizer are recognizable (Fig. 6).

These features are also found in the intraradical phase of the young infection units. The numerous nuclei display a dispersed chromatin in both intercellular and arbuscular hyphae (Figs. 7, 8). In the glutaraldehyde-fixed samples, the nuclei of young arbuscules show a nucleoplasm with a weft of loose filaments (not illustrated).

In hyphae with increasing vacuolation, the nucleus is substantially different: patches of condensed chromatin increase in number and size, and an electron-transparent space appears between the envelope and the nucleoplasm (Fig. 9). Chromatin condensation becomes more marked, as does blebbing of the envelope (Figs. 10, 11).

Figs. 9–12. Ultrastructural features of nuclei in arbuscular hyphae of *G. versiforme* colonizing *Allium porrum*. **Fig. 9.** The nucleus possesses electron-dense chromatin masses (*cc*) and an evident space between the envelope membranes. ×115000. **Fig. 10.** Nucleus with condensed chromatin masses (*cc*) and blebs in the envelope (*B*). ×14000. **Fig. 11.** Magnification of a nucleus with conspicuous blebs in the envelope (*B*). The chromatin has a condensed structure (*cc*). ×80000. **Fig. 12.** Pyknotic nucleus (*PN*) in a vacuolated arbuscular hypha. ×63000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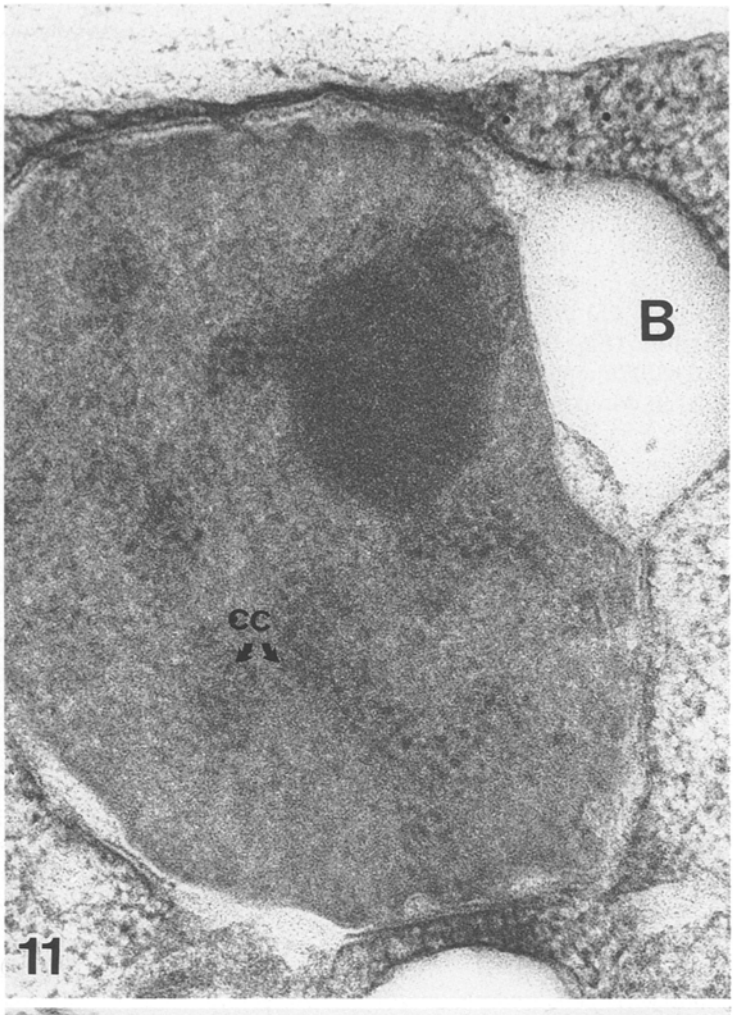
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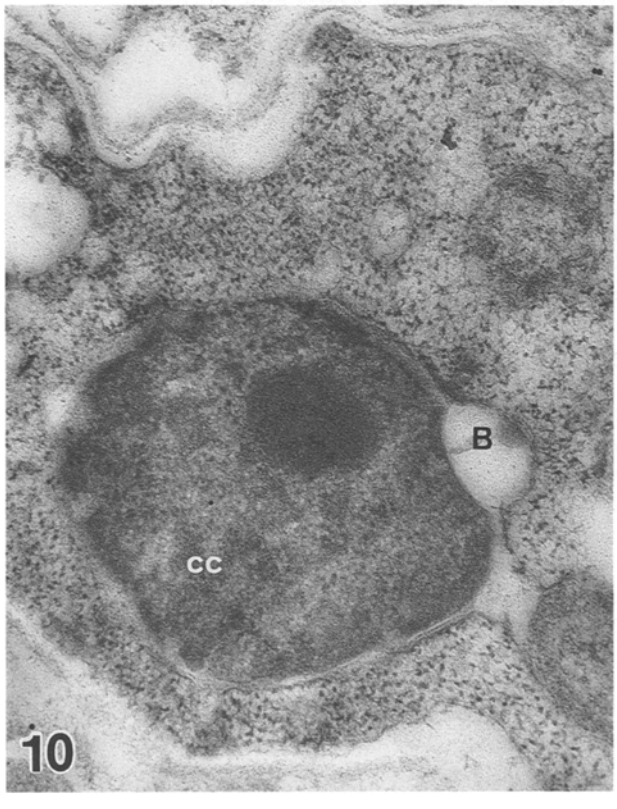
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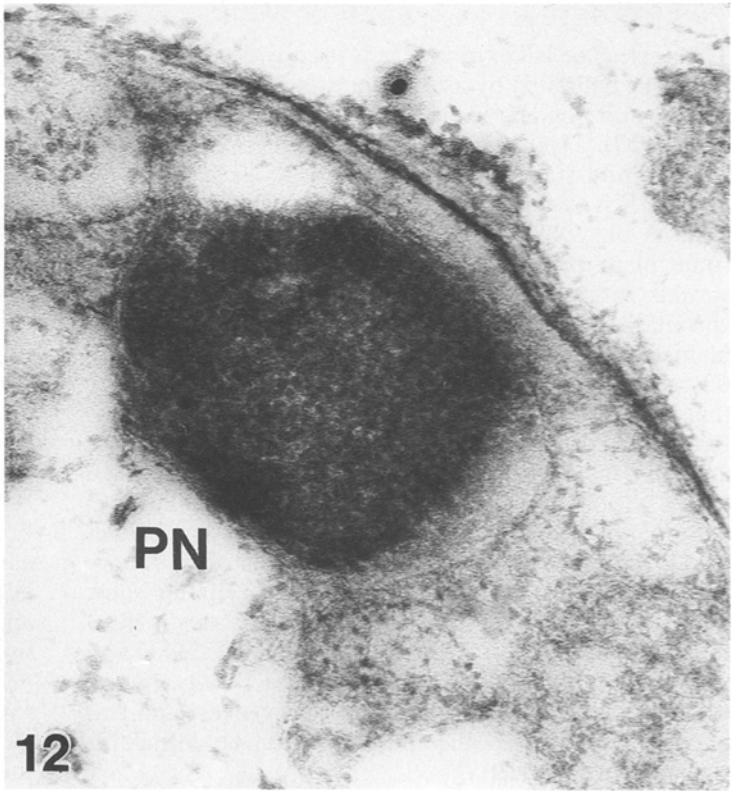
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Finally, in the collapsing arbuscules, the nucleus is fully pyknotic with highly condensed chromatin (Fig. 12).

The mAb against DNA results in regular distribution of gold granules over the nucleoplasm (Fig. 13), but no granules over the nucleolus and the cytoplasm (Fig. 14). Only the small bacterial-like organisms regularly found in the fungal cytoplasm (Scannerini and Bonfante-Fasolo 1991) show labelling over their nucleoid area (not illustrated). In the nuclei with condensed chromatin, labelling is present over transparent and electron-dense areas (Fig. 15) but strongly decreases when the nucleus is pyknotic (Fig. 16). No labelling was observed in the control experiments (Fig. 17). The DNase-gold complex results in comparable labelling (data not shown).

Discussion

Ultrastructural observations of the nuclei of *G. versiforme* and *G. mosseae* before and during colonization of their host plants demonstrate that: (1) the fungal chromatin is usually in a dispersed state; (2) this organization is progressively lost during aging of the hyphae, mostly during arbuscule collapse, resulting in condensed chromatin and a smaller pyknotic nucleus; (3) the morphological nuclear modifications are mirrored by changes in the labelling density of DNA molecules.

These results lead to questions about: (1) the ultrastructural organization of the VAM fungal chromatin, and (2) nuclear degeneration during the arbuscule life cycle.

Eukaryotic cells and chromatin ultrastructure

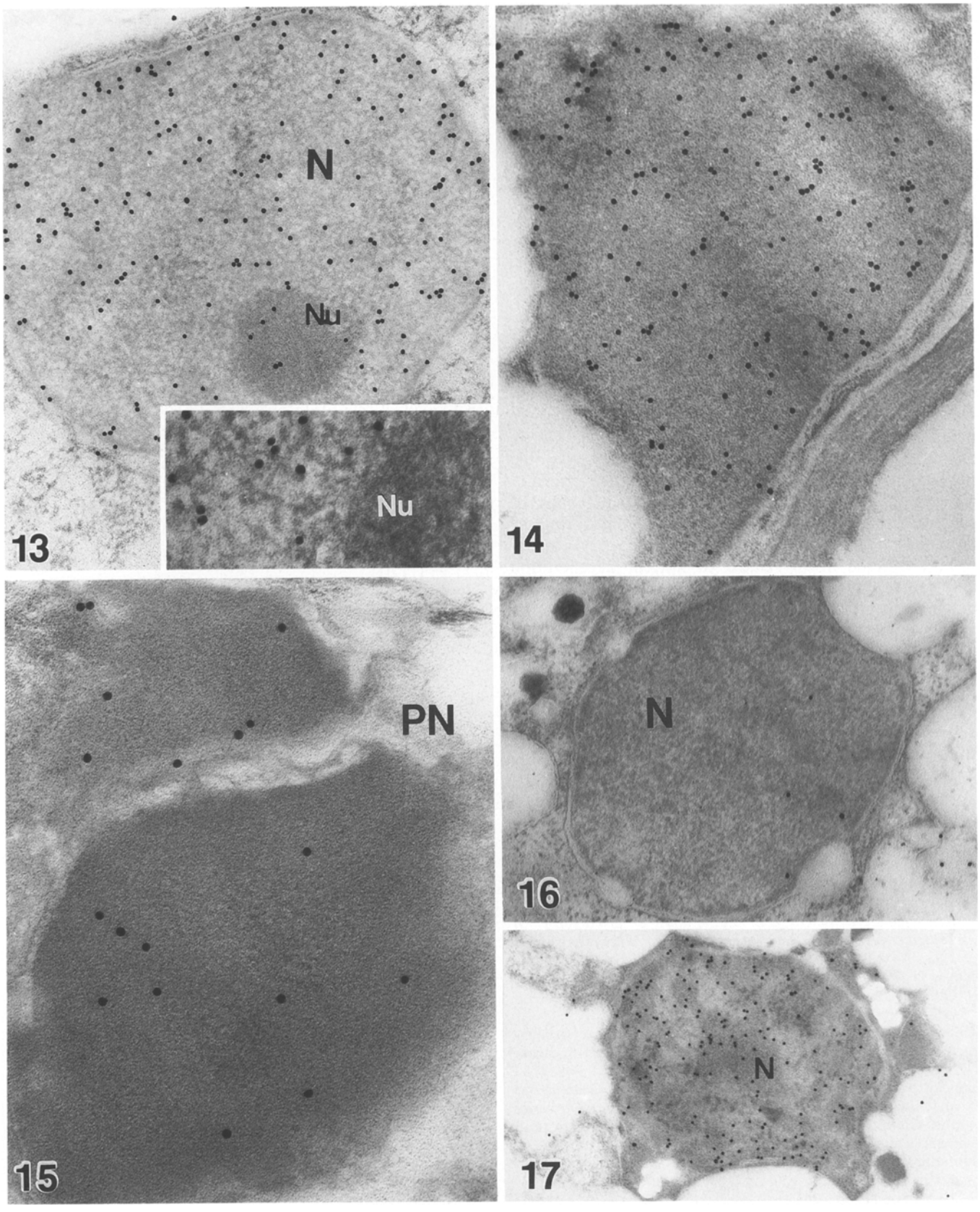
The nucleus of eukaryotic cells is usually described as an organelle delimited by an envelope and containing a nucleolus and condensed or dispersed chromatin (Alberts et al. 1989). The condensed form is also called heterochromatin and stains unusually darkly in electron micrographs. It is thought to be transcriptionally inactive (Alberts et al. 1989). According to Jordan et al. (1980), some plant nuclei possess numerous dense chromatin strands interspersed with regions of finely dispersed chromatin (reticulate nuclei), while those from other plants display only occasional peripheral, dense chromatin patches (chromocentric nuclei). It has been suggested that these gross morphological differences represent differences in the quantity of both DNA per chromosome and/or per cell, and of repetitive DNA (Jordan et al. 1980). Chromatin organization in fungal nuclei has previously not been discussed in detail (Gull and Oliver 1981), though ultrastructural observations of both conventionally fixed and freeze-substituted samples show that dispersed chromatin is the usual state; this is illustrated, for example, by the results of Beakes (1981) and Cho and Fuller (1989). The living and active hyphae of the two VAM fungi in the present study clearly possess nuclei with dispersed chromatin and can be defined as chromocentric nuclei.

Cytofluorimetry throughout the life cycle of *G. versiforme* has shown that each nucleus contains about 0.25 pg DNA (Bianciotto et al. 1991). This is comparable with the quantities reported for other fungi (Cavalier-Smith 1985), but is very low compared to other eukaryotes (Alberts et al. 1989). The dispersed chromatin observed in *Glomus* may be related to a reduced genome size. The nuclei of VAM fungi, however, are minute (about 2 μm in diameter) and, in contrast to other fungi (Rodriguez and Yoder 1991), the content of repetitive DNA, if any, is not known.

Changes in nuclear organization during infection

Formation of VAM infection units is a continuous process: in laboratory experiments the symbiotic fungus usually grows for 2–3 months after inoculation. The age of the symbiotic hyphae is therefore uncertain, despite the demonstration by Alexander et al. (1988) that the arbuscule cycle can span 7 days in some crops, and that the phase of arbuscule development is about 2.5 days, compared to a degeneration phase of about 4.5 days. However, many ultrastructural features can be regarded as markers of viability: organization of the cytoplasm, membrane preservation, degree of vacuolization, wall thickness, the lipid store and the presence of clumps. In our two VAM fungi, the nucleus of hyphae considered young and active always has dispersed chromatin. In contrast, aging of the hyphae, particularly the arbuscular hyphae, is accompanied by the appearance of condensed chromatin patches as well as blebs on the envelope. The nucleus becomes smaller and eventually pyknotic. Modifications of chromatin ultrastructure have been reported in some pathogenic fungi, such as *Uromyces phaseoli* (Heath and Heath 1978), but no assessment was made of the physiological meaning. Damage to the envelope, e.g. increasing of the perinuclear space or conspicuous blebbing, is observed in some pathogenic fungi treated with fungicides, such as the 2-aminopyrimidines, which disturb nucleic acid synthesis (Hippe 1991).

The changes in nuclear organization shown by our two VAM fungi during their life cycle are such that they can be considered as viability markers. In addition, the greater number of pyknotic nuclei in arbuscular compared to intercellular hyphae in the same infection unit may be a clue to arbuscule significance. Arbuscules are thought to be the structures in which fungus-host transfer is more efficient (Smith and Smith 1990; Gianinazzi-Pearson et al. 1991). They are nonetheless a threat to the life of the fungus. When a VAM fungus penetrates a cortical cell, the fate of its intracellular branch is sealed: in a few days it collapses, following a time-course which appears to be under the control of the fungus, irrespective of the host plant (Alexander et al. 1987). Many experiments have suggested that increased branching is a stress symptom for some filamentous fungi (Robson et al. 1991 and references therein). The ephemeral life of the arbuscule may thus be the outcome of a stress situation created by the intracellular environment; hyphal



Figs. 13–17. DNA localization in nuclei of intraradical hyphae of *G. versiforme* colonizing *Allium porrum*. **Fig. 13.** A regular distribution of gold granules occurs over the nucleus with diffuse chromatin after the use of the mAb against DNA. ×63000. *Insert*: Magnification of a nucleolus showing a weak, or negligible labelling. ×110000. **Fig. 14.** Nucleus with peripheral patches of electron-dense chromatin. Labelling is present in both forms of chromatin. ×70000. **Fig. 15.** Pyknotic nuclei showing very weak labelling after immunogold reaction. ×115000. **Fig. 16.** Control reaction on a section treated with the secondary antibody alone. ×44000. **Fig. 17.** Nucleus treated with DNase-gold complex showing gold granule labelling over the chromatin. ×38000

branching and nuclear degeneration may represent a response to this situation.

Nuclear morphology and DNA location

In situ techniques have provided important information on the biology of both plant and animal nuclei. The location of the ribosomal genes and the role of the fibrillar centres in onion or Ehrlich tumour cells have recently been demonstrated (Thiry et al. 1988; Martin et al. 1989) by using antibodies against DNA or nuclease-colloidal gold complexes. To our knowledge, this approach has never before been applied to VAM fungi.

Our results show that both dispersed and condensed chromatin is well labelled, whereas the RNA-rich nucleolus is usually not labelled. They thus extend the observations that anti-DNA mostly binds to the heterochromatin in plant and animal cells but binds uniformly to prokaryotic DNA molecules (Scheer et al. 1987; Martin et al. 1989). However, when the nucleus is fully pyknotic, the labelling density is negligible. Quantitative cytofluorimetric data are not yet available; even so, it may be suggested that the nuclear degeneration seen during arbuscule collapse is mirrored by DNA loss. In conclusion, arbuscule death related to nuclear degeneration and (possibly) to DNA loss may represent a second block point in the cell cycle of VAM fungi, in addition to that reported in axenic culture and probably due to a lack of DNA reduplication (Burggraaf and Beringer 1989).

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