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Status of nuclear division in arbuscular mycorrhizal fungi during in vitro development

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Summary. The number of nuclei in spores and along hyphae of an arbuscular mycorrhizal fungi *Gigaspora margarita* was measured in digital images of fluorescence arising from mithramycin stained cultures. Typical dormant spores (250 µm diameter) contained 2000 nuclei. Eight hundred nuclei were mobilized during the first 3 days of germination. The number of nuclei in the spores nearly returned to the initial number after 22 days of hyphal growth. The average relative DNA content in the nuclei of dormant spores and in the nuclei of spores incubated for 22 days was comparable, as judged from fluorescence intensity. Hyphal elongation occurred with 460 nuclei per cm under a special set of in vitro conditions that promote extensive hyphal growth of arbuscular mycorrhizal fungi. We found an average total of 26 000 hyphal nuclei per germinating spore after 22 days. The specific DNA polymerase α inhibitor aphidicolin did not inhibit spore germination but it rapidly reduced the rate of hyphal growth and arrested growth after 4 days. No nuclei were produced de novo during this time. These results demonstrate that *G. margarita* replicates nuclear DNA and undergoes nuclear division when grown in vitro even in the absence of a plant host.

Keywords: Charge-coupled device; DNA fluorescence; *Gigaspora margarita*; Image processing; In vitro culture; Nuclear division.

Introduction

Vesicular-arbuscular (VA) mycorrhizal fungi are obligate biotrophs. Attempts to grow them in pure culture have been unsuccessful for more than thirty years (Williams 1991). One of the key steps in the life cycle of these microorganisms is the acquisition of their symbiotic mode of growth (Bécard and Piché 1989 a). This begins after spore germination and terminates when the first root colonization and arbuscules are formed.

Early hyphal growth depends on spore reserves and requires some stimulatory root factors (M1 mechanism) whereas symbiotic growth is exclusively root dependent (M2 mechanism) (Bécard and Piché 1989 a). It has been proposed that both modes of growth may not be fundamentally different if one considers that only the carbon source changes and that the stimulatory root factors first required are also necessary throughout the symbiotic growth period (Bécard and Piché 1989 a). Carbon dioxide in addition to root exudates were found to be the critical factors for hyphal growth stimulation of germinated spores of *Gigaspora margarita* Becker and Hall (Bécard and Piché 1989 b). An appropriate CO₂ concentration (2%), and the flavonol quercetin (10 µM) provided abiotic conditions suitable to reproducibly sustain extensive in vitro growth of *G. margarita* (Bécard et al. 1992). If the hypothesis proposed above is correct, we would expect the fungus at this stage not to lack any fundamental biological functions like DNA replication and nuclear division. This is different from what has been recently proposed, i.e., that VA mycorrhizal fungi lack the ability to synthesize nuclear DNA in the absence of their host (Burggraaf and Beringer 1988, 1989; Viera and Glenn 1990).

The objective of the present study is to examine the nuclear status of *G. margarita* at various physiological stages of development. For this purpose, we combined epifluorescence microscopy and digital image analysis to accurately resolve and count for the first time the nuclei of this fungus and to compare their brightness as an indication of their DNA content.

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- Chen W-T (1981) Mechanism of retraction of the trailing edge during fibroblast movement. *J Cell Biol* 90: 187–200
- Farsi JA, Aubin JE (1984) Microfilament rearrangements during fibroblast-induced contraction of three-dimensional hydrated collagen gels. *Cell Motil* 4: 29–40
- Fleischer M, Wohlfarth-Bottermann KE (1975) Correlation between tension force generation, fibrillogenesis and ultrastructure of cytoplasmic actomyosin during isometric and isotonic contractions of protoplasmic strands. *Cytobiologie* 10: 339–365
- Geeves MA (1991) The influence of pressure on actin and myosin interactions in solution and in single muscle fibres. *J Cell Sci [Suppl]* 14: 31–35
- Gutfreund H (1982) The use of pressure perturbations to investigate the interaction of rabbit muscle myosin subfragment 1 with actin in the presence of ADP. *FEBS Lett* 140: 11–15
- Grolig F, Williamson RE, Parke J, Miller C, Anderton BH (1988) Myosin and Ca^{2+} -sensitive streaming in the alga *Chara*: detection of two polypeptides reacting with a monoclonal anti-myosin and their localization in the streaming endoplasm. *Eur J Cell Biol* 47: 22–31
- Hejnowicz Z, Sievers A (1981) Regulation of the position of statoliths in *Chara* rhizoids. *Protoplasma* 108: 117–137
- Heslop-Harrison J, Heslop-Harrison Y (1989) Myosin associated with the surface of organelles, vegetative nuclei and generative cells in angiosperm pollen grains and tubes. *J Cell Sci* 94: 319–325
- Höner B, Jockusch BM (1988) Stress fiber dynamics as probed by antibodies against myosin. *Eur J Cell Biol* 47: 14–21
- Kadota A, Wada M (1992 a) Reorganization of the cortical cytoskeleton in tip-growing fern protonemal cells during phytochrome-mediated phototropism and blue light-induced apical swelling. *Protoplasma* 166: 35–41
- (1992 b) Photoinduction of formation of circular structures by microfilaments on chloroplasts during intracellular orientation in protonemal cells of the fern *Adiantum capillus-veneris*. *Protoplasma* 167: 97–107
- Kolega J (1986) Effects of mechanical tension on protrusive activity and microfilament and intermediate filament organization in an epidermal epithelium moving in culture. *J Cell Biol* 102: 1400–1411
- Kuroda K (1990) Cytoplasmic streaming in plant cells. *Int Rev Cytol* 121: 267–307
- Marston SB (1982) Formation and dissociation of actomyosin complexes. *Biochem J* 203: 453–460
- McCurdy DW, Williamson RE (1991) Actin and actin-associated proteins. In: Lloyd CW (ed) *The cytoskeletal basis of plant growth and form*. London, Academic Press, pp 3–14
- Ohmori H, Toyama S, Toyama S (1992) Direct proof that the primary site of action of cytochalasin on cell motility processes is actin. *J Cell Biol* 116: 933–941
- Picton JM, Steer MW (1981) Determination of secretory vesicle production rates by dictyosomes in pollen tubes of *Tradescantia* using cytochalasin D. *J Cell Sci* 49: 261–272
- Pope DG, Thorpe JR, Al-Azzawi MJ, Hall JL (1979) The effect of cytochalasin B on the rate of growth and ultrastructure of wheat coleoptiles and maize roots. *Planta* 144: 373–383
- Putnam-Evans C, Harmon AC, Palevitz BA, Fehcheimer M, Cormier MJ (1989) Calcium-dependent protein kinase is localized with F-actin in plant cells. *Cell Motil Cytoskeleton* 12: 12–22
- Sievers A (1990) Transduction of the gravity signal in plants. In: Ranjeva R, Boudet AM (eds) *Signal perception and transduction in higher plants*. Springer, Berlin Heidelberg New York Tokyo, pp 297–306 (NATO ASI Series, series H, vol 47)
- Schnepf E (1981) Morphogenesis and polarity of tubular cells with tip growth. In: Kiermayer O (ed) *Cytomorphogenesis in plants*. Springer, Wien New York, pp 265–299 (Cell biology monographs, vol 8)
- Schröter K (1971) Versuch einer Kausalanalyse der geotropischen Reaktionskette im *Chara*-Rhizoid. *Planta* 96: 339–353
- Kruse S, Kuo-Huang L-L, Wendt M (1989) Statoliths and microfilaments in plant cells. *Planta* 179: 275–278
- Kramer-Fischer M, Braun M, Buchen B (1991 a) The polar organization of the growing *Chara* rhizoid and the transport of statoliths are actin-dependent. *Bot Acta* 104: 103–109
- Buchen B, Volkmann D, Hejnowicz Z (1991 b) Role of the cytoskeleton in gravity perception. In: Lloyd CW (ed) *The cytoskeletal basis of plant growth and form*. Academic Press, London, pp 169–182
- Staiger CJ, Schliwa M (1987) Actin localization and function in higher plants. *Protoplasma* 141: 1–12
- Tang X, Hepler PK, Scordilis SP (1989) Immunochemical and immunocytochemical identification of a myosin heavy chain polypeptide in *Nicotiana* pollen tubes. *J Cell Sci* 92: 569–574
- Tazawa M, Shimmen T (1987) Cell motility and ionic relations in characean cells as revealed by internal perfusion and cell models. *Int Rev Cytol* 109: 259–312
- Tewinkel M, Kruse S, Quader H, Volkmann D, Sievers A (1989) Visualization of actin filament pattern in plant cells without pre-fixation. A comparison of differently modified phallotoxins. *Protoplasma* 149: 178–182
- Volkmann D, Czaja AWP (1981) Reversible inhibition of secretion in root cap cells of cress after treatment with cytochalasin B. *Exp Cell Res* 135: 229–236
- Czaja I, Sievers A (1988) Stability of cell polarity under various gravitational forces. *Physiologist* 31 [Suppl]: 40–43
- Buchen B, Hejnowicz Z, Tewinkel M, Sievers A (1991) Oriented movement of statoliths studied in a reduced gravitational field during parabolic flights of rockets. *Planta* 185: 153–161
- Wang YL (1984) Reorganization of actin filament bundles in living fibroblasts. *J Cell Biol* 99: 1478–1485
- White RG, Sack FD (1990) Actin microfilaments in presumptive statocytes of root caps and coleoptiles. *Amer J Bot* 77: 17–26

Materials and methods

Fungal material

Azygospores of *G. margarita* Becker and Hall (DAOM 194757) were produced in greenhouse pot culture, collected, purified, and surface sterilized as already described (Bécard et al. 1992). In one experiment, spores produced in vitro using the root organ culture system (Bécard and Piché 1991) were used. Two month-old spores were removed from 6 month-old petri dish cultures. Except when specified, spores with a diameter between 230 and 270 μm were studied. Six to eight spores were used per treatment.

Fungal growth conditions

Conditions for spore germination and growth in 2% CO_2 incubators (32 $^\circ\text{C}$) were as described by Bécard et al. (1992). The spores were germinated in dishes incubated in inverted position, and were transferred, the day of germination (2nd day), to square petri dishes (one spore per dish) incubated vertically. Culture media were M medium (Bécard and Fortin 1988) supplemented or not with 10 μM quercetin (Bécard et al. 1992) and aphidicolin (Sigma Chemical Co., St. Louis, Mo.). Aphidicolin inhibits specifically DNA polymerase α , the enzyme responsible for nuclear DNA replication. Aphidicolin (150 μM) was added axenically to the culture medium after it had been autoclaved and allowed to cool to 45 $^\circ\text{C}$. The stock solution (74 mM)

was prepared with DMSO. Control plates were supplied with an equivalent volume of DMSO. All media were gelled with 0.3% (wt/vol) gellan gum (Gel-Gro; ICN Biochemicals, Cleveland, Ohio).

Assessment of fungal growth

Before measurement, hyphal elongation of *G. margarita*, observed through a dissecting microscope, was marked on the bottom half of the petri dish. The hyphal length (in centimeters) was then measured by using a 2 mm grid. The number of clusters of auxiliary cells was also determined.

Nuclear staining

Nuclei were stained with the DNA specific fluorescent stain mithramycin. The stain was prepared by dissolving 100 $\mu\text{g}/\text{ml}$ mithramycin A (Sigma Chemical Co.) in 15 mM MgSO_4 (Heath 1987). For staining spore nuclei, spores were severed from the experimental dishes with a sharp blade scalpel and directly transferred into 20 μl of mithramycin solution laid on a microscope slide. The cover glass (No. 1 22 \times 22 mm) was used to gently crush the spore and expel its contents. Microscopic observations were made immediately. Images of spore nuclear content were digitalized and stored for further analysis. The exact same procedure was followed for each spore to minimize variations between the readings.

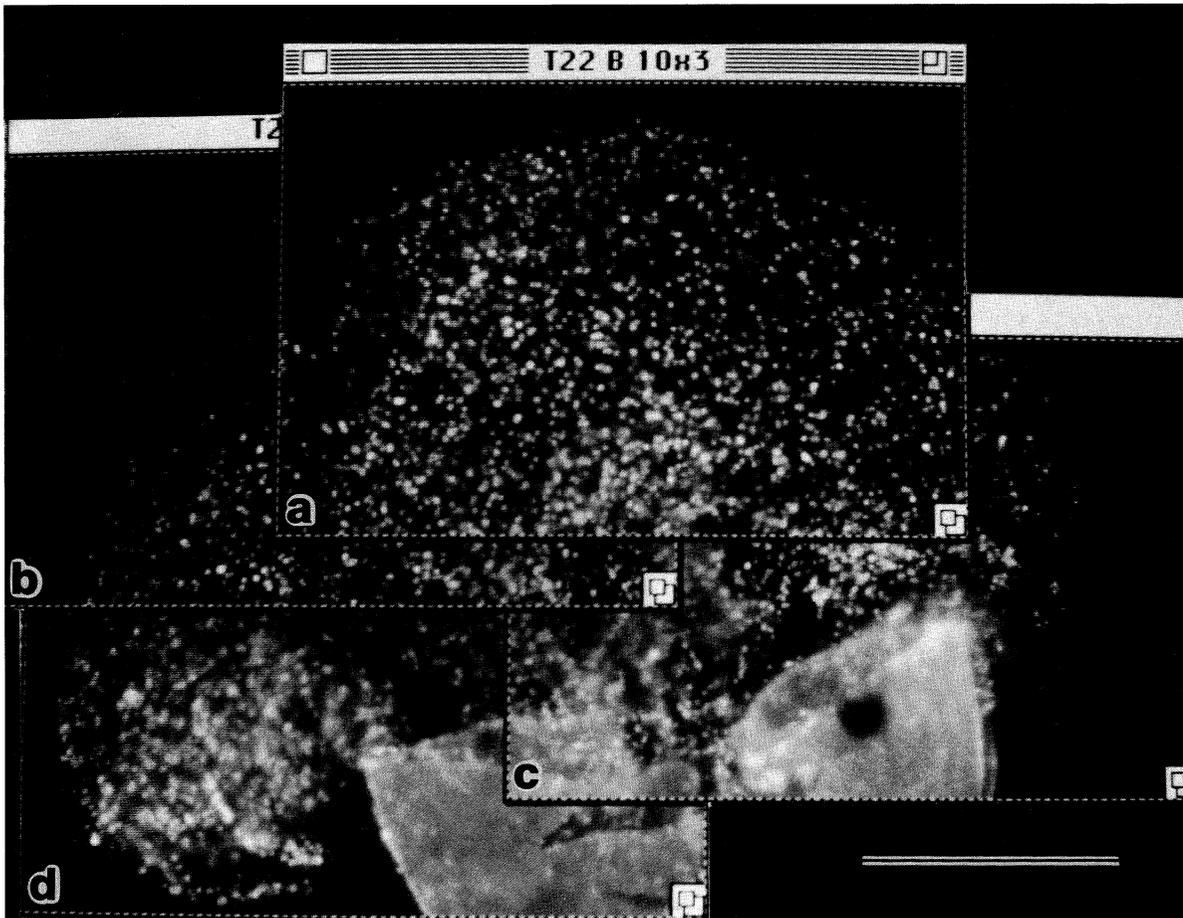


Fig. 1. Crushed spore of *Gigaspora margarita* showing mithramycin stained nuclei. The picture was restored with four (1/2 \times) digitalized images (a–d) previously acquired. This montage allows to select the regions to be further analyzed. Bar: 200 μm

For staining hyphal nuclei, the growing hyphae left after severing the spores were fixed overnight in the culture dishes with 2% glutaraldehyde (in phosphate buffered saline; ICN Biomedicals Inc., Costa Mesa, Calif.) at 4°C. They were then rinsed several times with deionized water for 24 h. Prior to hyphal nuclei staining, the dishes were dried in an oven at 75°C for 3 h so that hyphae were spread flat on the bottom of the dishes. Ten to twenty segments of hyphae intersecting a pattern of parallel (15 mm apart) lines were stained *in situ* with a drop (10 µl) of mithramycin. When hyphal growth was limited (aphidicolin experiment), only the germ tube, 1 cm away from the spore, was examined. To optimize the penetration of the stain, the selected segments were gently incised with a razor and put in contact with mithramycin for 24 h. Prior to observations hyphae were rinsed once with deionized water.

Optical and image processing devices

An inverted Nikon Diaphot microscope equipped with an epifluorescence illumination (100 W mercury lamp) and a B-2 A filter cube (dichroic mirror 510 nm, excitation 450–490 nm, barrier 520 nm) was used for all nuclear observations. Images were acquired (0.2 s exposure) with a charge-coupled device (CCD) Imaging System Star I (Photometrics, Tucson, Ariz.), transferred to a Macintosh II (Apple Computer Inc., Cupertino, Calif.), stored in a high-capacity storage subsystem (MacinStor, Storage Dimensions, San Jose, Calif.) and analyzed with image processing software from IPLab/Spectrum (Sig-

nal Analytics Corporation, Vienna, Virg.). Under the conditions used, almost no fading occurred, as a decrease of only 1.5% and 2% of fluorescence emission was measured within 15 s and 120 s, respectively.

Counting nuclei

Counts of spore nuclei were made by analyzing digitalized images collected at 250 × magnification. A montage of 4 images per spore was made to obtain the full picture of the contents of one spore at this magnification (Fig. 1). Subdivisions of the images were carefully made to avoid analyzing overlapped regions more than once. For subsequent processing, nuclear brightness was extracted from the images (Fig. 2 A) after two consecutive segmentations (Gonzales and Wintz 1987). The first one, which uses Sobel operators and partitions the image based on abrupt changes in gray-level, was used to isolate the nuclei as objects (Fig. 2 B). The second one, which is based on histogram thresholding of gray-level, was used to discriminate the nuclei from the background. The result of these two processing steps was then binarized (Fig. 2 C) to sum the nuclear regions. This integrated brightness related to nuclear area was finally divided by the average value previously calculated for a single nucleus. The value for a single nucleus was determined by averaging an integrated region where the nuclei had been previously counted manually. The regions used contained thirty to sixty nuclei, depending on the size of the image.

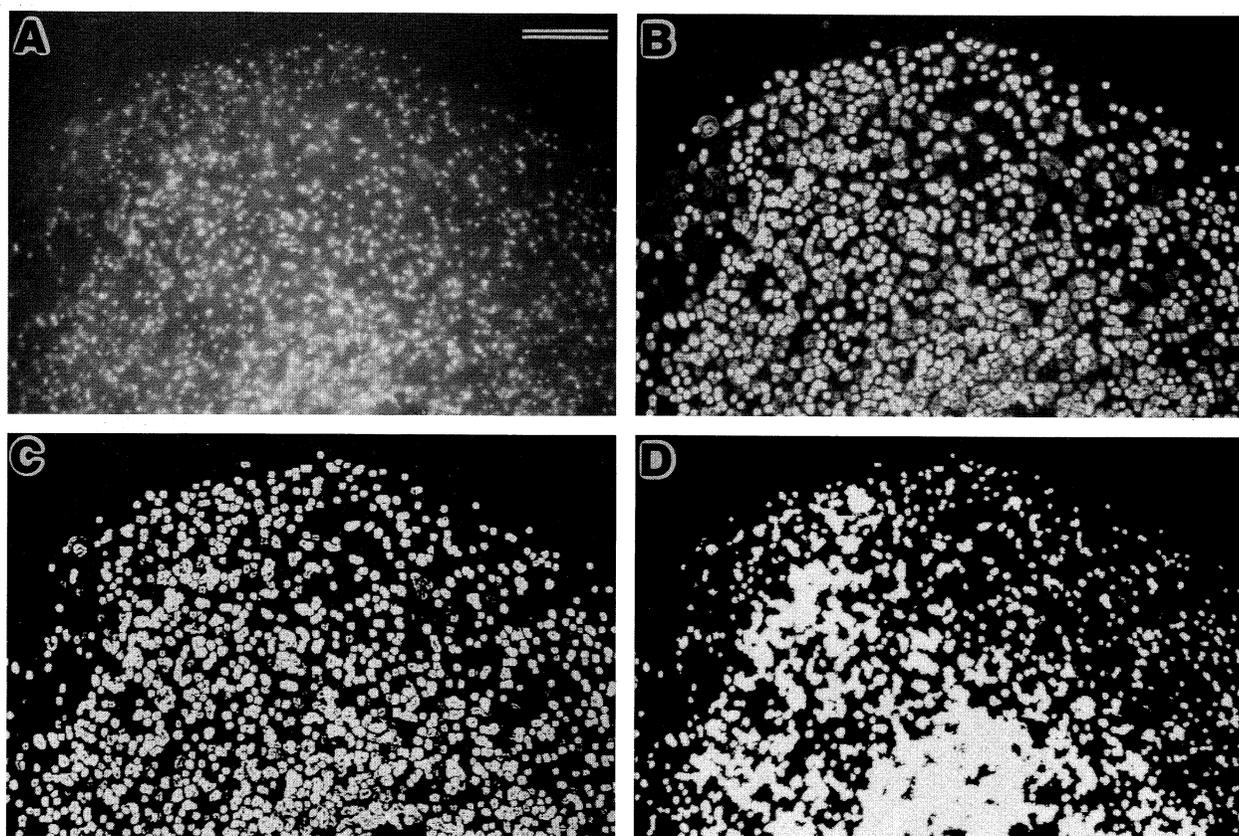


Fig. 2. Image processing of the nuclear region shown on Fig. 1 a. Bar: 75 µm. **A** First acquired original image. **B** Segmented image (A) using Sobel operators (based on abrupt changes in gray-level) to better extract nuclei from the background. **C** Binarized image (B) after a gray-level thresholding segmentation. This image is the one used to integrate the nuclear area. **D** Binarized image (A) after only one segmentation (gray-level thresholding). The nuclei are poorly resolved

Nuclei in hyphae were counted manually with the microscope at a magnification of $500\times$. Nuclei were counted for each selected hyphal segment along 2 to 3 optical fields ($680\ \mu\text{m}$ in diameter). The average number of nuclei per cm of hypha was expressed for each individual germinating spore.

Determination of DNA nuclear content

Assuming that emission of fluorescence from mithramycin in the nucleus was proportional to its DNA content (Coleman et al. 1981) and exploiting the fact that the CCD has a high sensitivity, photometric accuracy and linear response (Hiraoka et al. 1987), we used relative measurements of brightness of the digitalized images to compare the average DNA contents of nuclei in spores of different physiological stages. After subtracting the average background brightness from regions where the number of nuclei was known (> 1000), average nuclear values of brightness were determined and compared for the spores at 0 and 22 days of germination.

Results

An example of the routine image processing steps used for counting the nuclei is shown in Fig. 2A–C (see Materials and methods). Figure 2D shows the binarized result of only one segmentation (gray-level thresholding) step. If the first segmentation (edge Sobel) is not made, good discrimination of the nuclei from the background is impossible because the background is too variable.

The number of nuclei in normal size ($250\ \mu\text{m}$ in diameter) resting spores of *G. margarita* was close to two thousand and variable (ranging from 1700 to 3100, coefficient of variation (CV) 26.3% (Table 1). Less than half this number of nuclei was found in the small ($167\ \mu\text{m}$ in diameter) spores (ranging from 490 to 1530, CV 45%). Since they were only a third the size of the larger one, they contained proportionally more nuclei. Newly produced in vitro spores, originating from the same mycelium network grown in a very homogenous environment, also showed a high variability in their nuclear content (ranging from 1420 to 2740, CV 30%). One day after germination (day 3), the number of nuclei in spores declined (-800) (Fig. 3). This number increased slowly later to almost reach the original value after 3 weeks of hyphal growth.

Table 1. Mean number of nuclei in spores of *Gigaspora margarita* before germination

	Spore diameter (μm)	No. of nuclei
Normal size spores	250 (11.5) ^a	2030 (534)
Small spores	167 (17.6)	831 (374)
In vitro spores	258 (12.0)	1830 (550)

^aNumbers in brackets are standard deviations of the means

As hyphal elongation progressed, the number of nuclei per cm of hyphae remained constant (≈ 460) (Table 2). Auxiliary cell clusters had an average of 8 cells. Electron microscopy of thin sections showed an average of 15 nuclei per cell (Montpetit et al., unpubl. results). This allowed the calculation of the total number of nuclei produced during in vitro growth of *G. margarita* (Fig. 4).

The average nuclear brightness in spores before germination and after 22 days of growth gave equivalent average values, 4970 (CV 15.0%) and 5251 (17.8%), respectively.

Spore germination was not affected by the presence of aphidicolin. However, hyphal growth stopped after 4 days with a final elongation of 5.1 cm (Table 3). The 7th day, many of the hyphal tips exhibited retracted cytoplasm and became empty and septate. The numbers of nuclei in germ tubes and in spores were one third and one half of the control values, respectively

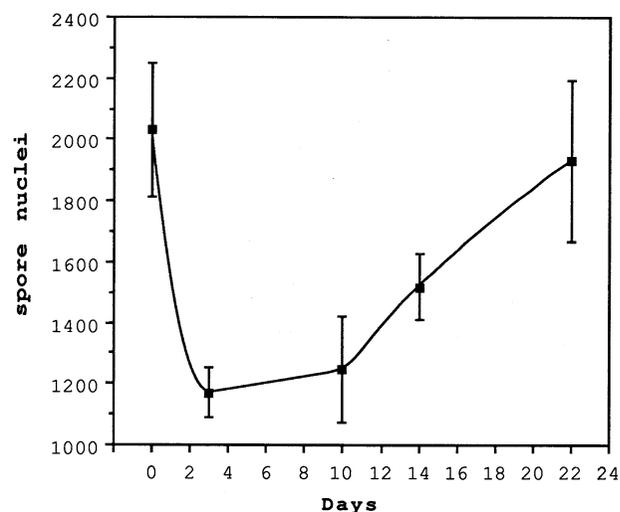


Fig. 3. Number of nuclei in germinating spores of *Gigaspora margarita* after 0, 3, 10, 14, and 22 days of culture. Vertical bars correspond to the standard errors of the means

Table 2. Hyphal growth and nuclei production from germinating spores of *Gigaspora margarita* after 3, 10, 14, and 22 days of culture

Days	Hyphal length (cm)	No. of clusters of auxiliary cells	Nuclei density (cm^{-1} of hypha)
3	1.9 (0.4) ^a	1.3 (0.8)	473 (14.0)
10	27.8 (8.9)	6.5 (2.7)	448 (20.6)
14	39.1 (4.8)	7.7 (1.9)	466 (20.6)
22	53.7 (14.8)	13.2 (5.3)	456 (30.9)

^aNumber in brackets are standard deviations of the means

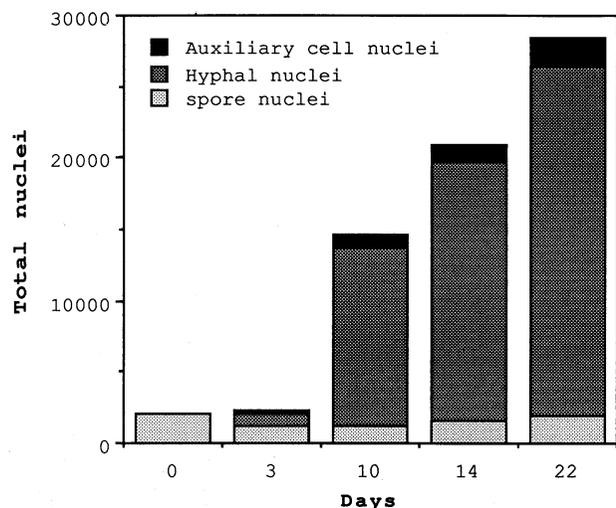


Fig. 4. Total number of nuclei produced by germinating spores of *Gigaspora margarita* after 0, 3, 10, 14, and 22 days of culture

(Table 3 and Fig. 5). Long segments of branched hyphae had no nuclei. We conclude from these observations that no nuclear division occurred in the presence of aphidicolin.

Aphidicolin also inhibited growth (50%) of *G. margarita* in a culture medium without quercetin.

Discussion

Quantification of nuclei in spores of arbuscular mycorrhizal fungi has been attempted several times. Estimates of the number of nuclei have been made either

by direct observation of the stained nuclei (2600 to 3850 for *Gigaspora gigantea* Nicolson and Gerdemann and *Scutellospora erythropha* Koske and Walker, Cook et al. 1987; over 1000 for *Glomus caledonium* Nicolson and Gerdemann, Burggraaf and Beringer 1988), or by using a model based on several assumptions regarding the spatial organization of nuclei in the spores (over 20 000 for *G. caledonium* and *G. margarita*, Burggraaf and Beringer 1989). The method we used is also based on the direct observation of all stained nuclei. The high sensitivity of the camera system, combined with image processing, obviated the need to estimate the number of nuclei present in the spores. By using a systematic procedure, means and standard deviations of actual numbers of nuclei were determined and comparisons between spores in different physiological stages were made. We used mithramycin rather than DAPI because the former specifically stains DNA, while the latter can react with polyphosphate and RNA (Allan and Miller 1980, Coleman et al. 1981). Moreover, the lower sensitivity of mithramycin insured that only nuclei would be detectable and not mitochondria. A total number of 2000 observed nuclei per spore of *G. margarita* is compatible with the range given by Cook et al. (1987) for the larger spores of *G. gigantea*. We believe that the second estimation for the number of nuclei (20 000) given by Burggraaf and Beringer (1989) was incorrect as were the assumptions regarding the nuclear organization that were used to arrive at this value.

It is not known whether nuclear division occurs inside the forming spore or whether preexisting hyphal ma-

Table 3. Effect of aphidicolin on hyphal growth and nuclei production of germinating spores of *Gigaspora margarita* after 7 days of culture

	Hyphal length (cm)	No. of clusters of auxiliary cells	Nuclei density (cm ⁻¹ of germ tube)	Spore nuclei
Control	12.4	3.4	822	1690
Aphidicolin	5.1*	2.4	275*	860*

*Values between the two treatments are significantly different (Student's *t* test, $P < 0.001$)

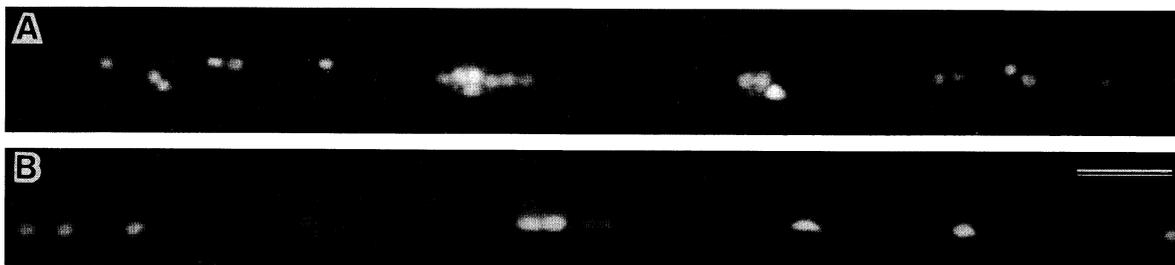


Fig. 5. Mithramycin stained nuclei in hyphae of *Gigaspora margarita* grown in the absence (A) or the presence (B) of aphidicolin. Bar: 20 μ m

terial including nuclei is used exclusively during spore formation. If the second hypothesis is true, one 250 µm diameter spore must be filled by cytoplasmic material of approximately 29 cm of a 6 µm diameter hypha. If so, a number greater than 2000 nuclei per spore should be expected unless either the sporulating hyphae have a lower nuclear density than germinating hyphae (69 nuclei/cm instead of 460) or, as the spore is forming and mainly filled by lipidic material, there is an exclusion of cytoplasm which includes the nuclei. In either event, the result gives rise to a great variability in the number of nuclei in spores, even when they are produced in vitro.

Our results strongly suggest that nuclear division was not required for spore germination. The day following germ tube emergence (day 3), the total number of nuclei in the hyphae in addition to those remaining in the spores did not exceed the initial number (see Fig. 4). Moreover, aphidicolin did not inhibit spore germination.

In the presence of aphidicolin, however, hyphal growth stopped on day 4. Considering the high demand for nuclei (460/cm) during hyphal elongation, this result is not surprising and indicates that nuclear division took place soon after spore germination. The aphidicolin experiment also showed that, without nuclear DNA replication, the 800 spore nuclei required for spore germination could only sustain a maximum of 4 days of growth and 5.1 cm of hyphal elongation.

From spores originally containing 2000 nuclei, 26 000 hyphal nuclei were produced in 22 days. Interestingly, if germination required approximately 800 nuclei, spores almost recovered their original number of nuclei by the end of the 22-day-period. We hypothesize that there is a continuous exchange of material between spores and hyphae throughout the growing period. If the nuclei present in one spore form a population of different genotypes rather than a clone, this exchange process insures that daughter spores have the same variability among their nuclear genotypes.

The fact that no noticeable difference of fluorescence emission per average nucleus was found in day 0 and day 22 spores, indicates that there is no difference in ploidy between the two population of nuclei. The proposal that nuclear division in mycorrhizal fungi during germination may take place in the absence of DNA synthesis (Viera and Glenn 1990) does not apply here, where an average of 13 times the initial number of nuclei were produced (26 000/2000) over 22 days of culture.

From our results, we conclude that arbuscular mycorrhizal fungi, following spore germination, divide their

nuclei and replicate nuclear DNA in the absence of their host. This finding contradicts what was recently proposed by Burggraaf and Beringer (1988, 1989) and supported by Viera and Glenn (1990). A possible explanation for the discrepancy is that some experiments (Burggraaf and Beringer 1989) showing negative results were misinterpreted because the in vitro conditions used for fungal growth were not optimized. For example, the experiment showing no incorporation of [³H]adenine into DNA was made with 50 spores incubated in water for 4–5 days. We now know that during this short period which includes germination time, very little hyphal tissue is produced, almost no nuclear division has taken place, and as a result, very few DNA molecules have been synthesized. Consequently, when the germinated spores were cracked open in a drop of the reagent mixture, most of the DNA to be analyzed originated from the spores. Therefore, DNA was not labelled since it was present before exposure to the isotope.

Our study shows that like the synthesis of proteins, RNA and mitochondrial DNA (Hepper 1979, Beilby and Kidby 1982), nuclear DNA replication is another fundamental function that is intrinsically performed by arbuscular mycorrhizal fungi. This knowledge should stimulate efforts to grow these microorganism in pure culture.

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References

- Allan RA, Miller JJ (1980) Influence of S-adenosylmethionine on DAPI-induced fluorescence of polyphosphate in the yeast vacuole. *Can J Microbiol* 26: 912–920
- Bécard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytol* 108: 211–218
- Piché Y (1989 a) New aspects on the acquisition of biotrophic status by a VAM fungus, *Gigaspora margarita*. *New Phytol* 112: 77–83
- (1989 b) Fungal growth stimulation by CO₂ and root exudates in vesicular-arbuscular mycorrhizal symbiosis. *Appl Environ Microbiol* 55: 2320–2325
- (1992) Establishment of VA mycorrhizae in root organ culture: review and proposed methodology. In: Norris JR, Read DJ, Varma AK (eds) *Methods in microbiology*, vol 24. Academic Press, London, pp 89–108
- Douds DD, Pfeffer PE (1992) Extensive hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO₂ and flavonols. *Appl Environ Microbiol* 58: 821–825

- Beilby JP, Kidby DK (1982) The early synthesis of RNA, protein, and some associated metabolic events on germinating vesicular-arbuscular mycorrhizal fungal spores of *Glomus caledonius*. *Can J Microbiol* 28: 623-628
- Burggraaf AJP, Beringer JE (1988) Nuclear division and VA-mycorrhizal in vitro culture. In: Sylvia DM, Hung LL, Graham JH (eds) *Mycorrhizae in the next decade*. University of Florida, Gainesville, p 190
- — (1989) Absence of nuclear DNA synthesis in vesicular-arbuscular mycorrhizal fungi during in vitro development. *New Phytol* 111: 25-33
- Coleman AW, Maguire MJ, Coleman JR (1981) Mithramycin- and 4'-6-diamidino-2-phenylindole (DAPI)-DNA staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids, and virus particles. *J Histochem Cytochem* 29: 959-968
- Cook JC, Gemma JN, Koske RE (1987) Observations of nuclei in vesicular-arbuscular mycorrhizal fungi. *Mycologia* 79: 331-333
- Gonzales RC, Wintz P (1987) *Digital image processing*. Addison-Wesley, Reading, MA
- Heath B (1987) Fluorescent staining of fungal nuclei. In: Fuller MS, Jaworski A (eds) *Zoospore fungi in teaching and research*. Southeastern Publishing, Greenville, NC, pp 169-171
- Hepper CM (1979) Germination and growth of *Glomus caledonius* spores: the effects of inhibitors and nutrients. *Soil Biol Biochem* 11: 269-277
- Hiraoka Y, Sedat JW, Agard DA (1987) The use of charge-coupled device for quantitative optical microscopy of biological structures. *Science* 238: 36-41
- Viera A, Glenn MG (1990) DNA content of vesicular-arbuscular mycorrhizal fungal spores. *Mycologia* 82: 263-267
- Williams PG (1991) Axenic culture of arbuscular mycorrhizal fungi. In: Norris JR, Read DJ, Varma AK (eds) *Methods of microbiology*, vol 24. Academic Press, London, pp 203-220