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Arbuscular Mycorrhizas formed by *Asphodelus fistulosus* and *Glomus coronatum*: Three-Dimensional Analysis of Plant Nuclear Shift Using Laser Scanning Confocal Microscopy

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Abstract

Two main morphological types of arbuscular mycorrhizas (AM) are recognised, the *Arum*-type and the *Paris*-type. Most research has focused on the *Arum*-type, including studies of the influence of colonisation on the plant at the cellular level. Modifications in the size, position and shape of plant nuclei in colonised and uncolonised cells of the *Paris*-type AM formed by *Asphodelus fistulosus* L. inoculated with *Glomus coronatum* Giovannetti were investigated in this study. The plants were grown in nurse pots to achieve near synchronous colonisation. Laser

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scanning confocal microscopy (LSCM) was used to visualise root material and to quantify plant nuclear shift in three dimensions, which has not been achieved in the past. In each colonised cell containing arbusculate coils there was a quantifiable increase in the distance from the centre of the nucleus to the closest cell wall and in the volume of the plant nucleus. Similar effects occurred in cells with hyphal coils but were not quantifiable because of small numbers. The position and size of the nucleus did not change in uncolonised cells of either inoculated or uninoculated plants. The significance of the results is discussed with respect to the development of *Paris*-type AM.

Keywords: *Asphodelus fistulosus*, *Paris*-type, arbuscular mycorrhizas, laser scanning confocal microscopy, nuclei

1. Introduction

Arbuscular mycorrhizas (AM) are formed by most terrestrial plant species in association with some species of Glomalean fungi (Morton and Benny, 1990). These associations usually result in the transfer of nutrients between the two partners (Smith and Read, 1997). The relationships between the plant and the fungi are complex. The establishment and functionality of AM differs in associations between different plant and fungal species and is influenced by environmental conditions.

There are two main morphological types of AM, the *Arum*-type and the *Paris*-type (Gallaud, 1905). The morphology of the two types is distinct. In the *Arum*-type intercellular hyphae are produced, which give rise to arbuscules (highly branched, intracellular, terminal structures). In the *Paris*-type intracellular hyphal coils and arbusculate coils are produced, and there is no intercellular phase of colonisation. However, some AM are an intermediate between the two types. The time-course of development of the *Arum*- and *Paris*-types is also different (Rosewarne et al., 1997; Cavagnaro et al., 2001).

The majority of detailed research on AM has involved cultivated plants (Cooke et al., 1993), which tend to form the *Arum*-type (Smith and Smith, 1997). The *Paris*-type has received much less attention and little is known about its development and function. This is a large gap in knowledge because the *Paris*-type is reported to occur in more plant families than the *Arum*-type (Smith and Smith, 1997). Furthermore, it is estimated that 80% of terrestrial plant species form AM associations (Bonfante-Fasolo, 1984). Given the widespread nature of these associations (Schwab et al., 1991) and our limited knowledge about what is reportedly the more abundant morphological type, there is a need for research into *Paris*-type AM.

There have been a number of studies of plant and fungal nuclei in AM systems. It has been established that in cortical cells colonised by arbuscules there is an increase in the size of the plant nucleus (hypertrophy) (Blair et al., 1988; Berta et al., 1990); it becomes lobed, the chromatin is decondensed (Balestrini et al., 1992), and there is a delay in nuclear senescence in colonised cells (Lingua et al., 1999). There is also a positional change of the plant nuclei from the periphery towards the centre of cells containing arbuscules (Balestrini et al., 1992). Despite these studies, and a number of others, there have been no studies of the influence of *Paris*-type structures (only *Arum*-type structures, Balestrini et al., 1992) on the size and position of plant nuclei. The aim of the present study was to determine whether the size or position of the plant nucleus in cells of *Asphodelus fistulosus* L. is altered by the formation of *Paris*-type AM with *Glomus coronatum* Giovannetti, using laser scanning confocal microscopy (LSCM) in a time course experiment.

2. Materials and Methods

Plant growth

Establishment of nurse pots and transplanting methods are the same as in Cavagnaro et al. (2001), briefly: Three pre-germinated *Allium porrum* L. cv Vertina (Leek) seeds were planted in a triangular arrangement into 70 mm diameter, plastic, non-draining pots (Polar Cup, Australia) containing 400 g of a 1:9 (w/w) mixture of inoculum and soil/sand mix. The inoculum was a mixture of soil and roots derived from a *G. coronatum* pot culture raised on *Allium* in the same soil/sand mix as that used for the experiment. The soil/sand mix was composed of a 1:9 (w/w) mixture of washed river sand and soil collected from Mallala, South Australia. The soil contained 16 mg bicarbonate extractable phosphate (P) kg^{-1} soil (Colwell, 1963). The soil/sand mix had a final bicarbonate extractable P concentration of 6.63 mg kg^{-1}. Uninoculated controls were prepared by replacing the inoculum with soil and roots derived from non-inoculated pot cultures. These nurse pots were grown for 42 days and were watered three times a week to 10% of soil dry weight (w/w). Once a week, starting two weeks after planting, the pots were fertilised using a modified Long Ashton solution minus P (Cavagnaro et al., 2001).

Uninoculated *Asphodelus* seedlings for transplantation into nurse pots were produced as follows: Seeds collected from Ardrossan, South Australia were surface sterilised, imbibed and allowed to germinate. After five days the germinated seeds were planted into 200 mm diameter, plastic, free draining pots, containing a sterile 1:9 (w/w) mixture of Mallala soil and sand (as

described above). A total of 37 seeds were planted in an evenly spaced pattern in each of 2 pots. The plants were watered three times each week. Forty two days after planting the *Allium* nurse plants, soil was removed from the centre of each nurse pot using an 8 mm diameter cork borer. One carefully washed, 14 day old *Asphodelus* seedling was placed into the hole and the soil was backfilled. Seedlings of a uniform size were selected for transplanting in order to minimise variation between plants. A total of 20 pots were established.

Two *Asphodelus* plants were harvested at 4, 8, 12, 20 and 28 days after transplanting (DAT) from both inoculated and uninoculated treatments by carefully washing them free of soil with reverse osmosis (RO) water. The plants were blotted dry and the roots and shoots separated. The roots were kept for sectioning and staining. Root samples were embedded in gelatin and freeze-sectioned using a modification of the method of Smith and Dickson (1991) to give 120 µm thick, longitudinal sections. Sections were stained overnight with acid fuchsin (1 mg ml^{-1}) at room temperature to stain plant and fungal cell walls. The sections were then stained with DAPI (4',6-diamindo-2-phenylindole) (1 µg ml^{-1}) for ten minutes at room temperature, to stain nucleic acid. Finally, the sections were mounted on microscope slides in glycerol. A sub-sample of the roots was stained using Trypan Blue (Phillips and Hayman, 1970) and percent colonisation was determined using the grid line intersect method (Giovannetti and Mosse, 1980).

Microscopy (LSCM)

A MRC-1000UV Laser Scanning Confocal Microscope System (Bio-Rad) in combination with Ar laser and a Nikon Diaphot 300 inverted microscope in fluorescence mode were used to visualise acid fuchsin staining (excitation at 488/10 nm and emission at 522/32 nm) and DAPI staining (351/8 nm and 455/30 nm). Samples were observed with a × 40 water-immersion objective lens with a numerical aperture of 1.15 and working distance of 210 µm. A series of optical xy-sections, each with a 2 µm interval on the z-axis, was collected for both inoculated and uninoculated plants. Each image was averaged over 4 scans using a Kalman filtering process and saved as a digital file with the size of 768 × 512 pixels and the intensity of staining expressed in 256 levels of grey (0 = black, 255 = white).

Distortion of images due to differences in the refractive index of the immersion medium (water) of the objective lens and the combination of the embedding medium (glycerol) and the fungal and plant tissue was accounted for by using the correction factor calculated by Dickson and Kolesik (1999). The axial distortion reduced the nominal z-interval between the xy-slices from 2 µm to 1.42 µm.

Measurements

The volume of each nucleus was determined stereologically by summing the product of the cross-sectional area of each optical slice of the nucleus by the z-step. Measurements were only made in cells where this could be done without interference from fungal structures. Six nuclei were measured for each treatment from each of two replicate plants 28 DAT. The distance from the centre of the plant nucleus to the closest cell wall was measured using Comos image analysis software (Bio-Rad, UK). The distance in the z-axis was measured by counting the number of optical slices from the centre of the nucleus to the top and bottom of the cell. The number of optical slices was then multiplied by the z-step (1.42 μm). The distance from the centre of the nucleus to the closest cell wall in the x- and y-axes was measured using a line draw function of the Comos software. The distance was measured in ten cells containing arbusculate coils (AC) in sections collected from two plants for each treatment at each harvest. The volume of colonised and uncolonised cells was measured at 8 DAT, to ensure that there was no change in the volume due to colonisation.

3. Results

Development of the symbiosis

Asphodelus colonised by *G. coronatum* formed a *Paris*-type mycorrhizal morphology. The LSCM provided clear images of the hyphal coils (HC) and AC. Examples of uncolonised cells, HC and AC are shown in Figs. 1a, b and c respectively. The AC and HC were observed in the inner and outer cortex of the roots respectively. The surface of the HC was smooth (Fig. 1b). The AC had looped hyphae similar to those of the HC, with small arbuscule-like protrusions on their surfaces (Fig. 1c). At no stage of the experiment were intercellular hyphae observed. The roots were 6.0 (±1.2), 13.6 (±3.0), 14.6 (±2.1), 30.7 (±2.7) and 45 (±5)% colonised 4, 8, 12, 20 and 28 DAT, respectively.

Fungal nuclei

Fungal nuclei were observed in the thick looped hyphae of the HC (Fig. 1b). Nuclei were also observed in the looped hyphae and thicker arbuscular branches of the AC (Fig. 1c). The fungal nuclei were spherical in shape. They were small; approximately 2 μm in diameter; therefore, the volume could not be accurately estimated from images collected using a z-step of 1.42 μm. There were up to six fungal nuclei per 30 μm of hyphae in the HC. The distribution of nuclei was irregular, although they were often paired.

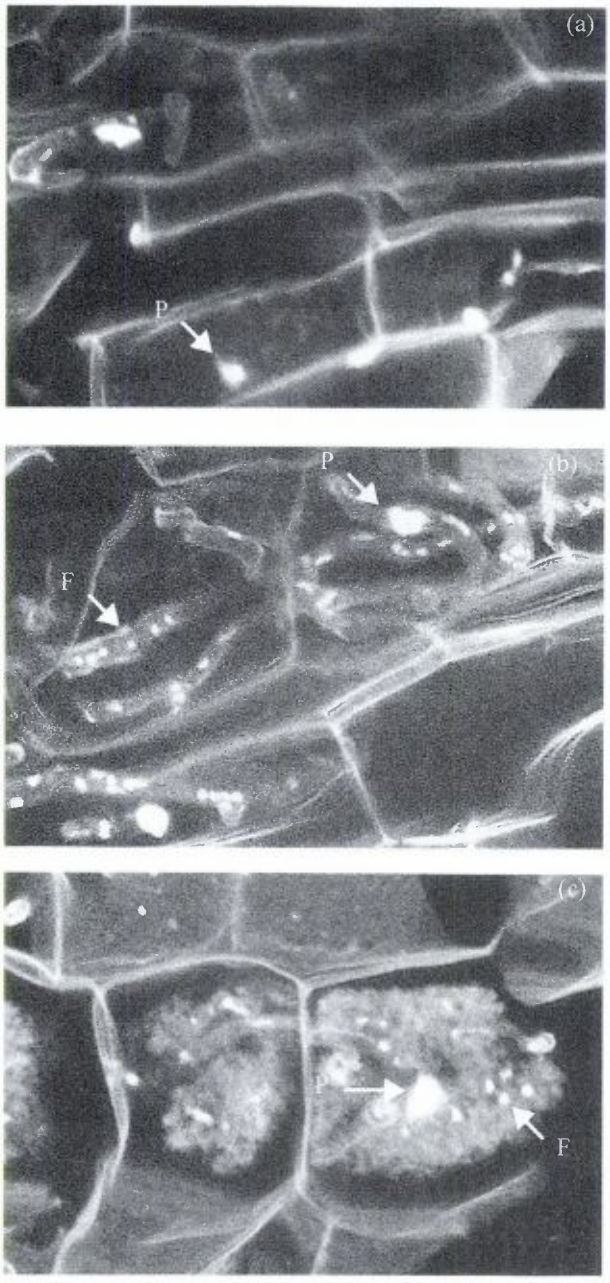

Figure 1. LSCM extended focus images of (a) uncolonised cells, (b) hyphal coils, and (c) arbusculate coils. Plant nuclei and fungal nuclei are labeled P and F, respectively. Note: Compare position of plant nuclei in (a) and (b). The horizontal dimension of the images is 144 μm.

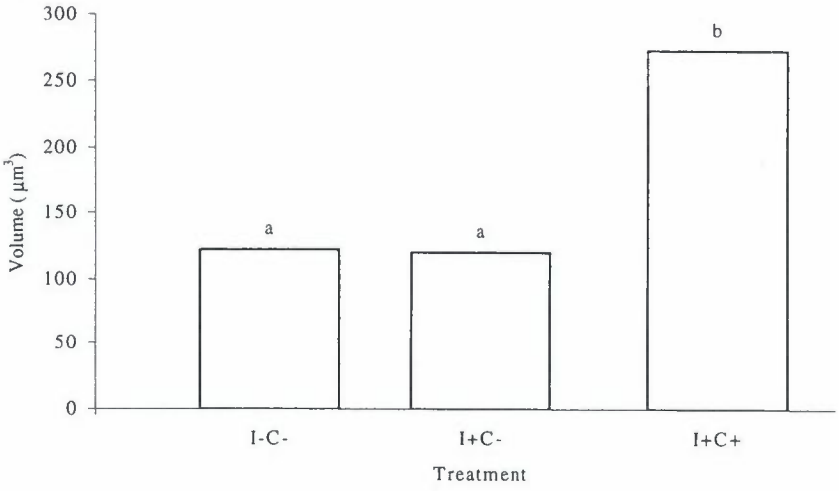

Figure 2. Volume of plant nuclei in of uncolonised cells of uninoculated plants (I–C–), uncolonised cells of inoculated plants (I+C–), and colonised cells (AC) of inoculated plants (I+C+). Means with the same letter when the least significant differences (LSDs) are compared ($P<0.05$). n=6.

Volume of the plant nuclei

In colonised cells the plant nuclei were generally found to be lobed, although this was not the case of all nuclei. The volumes of the plant nuclei at 28 DAT in the uncolonised cells of inoculated and uninoculated plants and colonised cells of inoculated plants are given in Fig. 2. In cells colonised by AC the nuclei were larger (hypertrophied) compared to uncolonised cells of inoculated and uninoculated plants. This was also apparent in cells containing HC, however, the sample size was insufficient to quantify this change.

Position of the plant nuclei

The first HC and AC were noted 8 DAT, so measurements of nuclear size and position for 4 DAT were not made. Colonisation of cells by hyphae did not result in a change in the cell volume (data not shown $P = 0.9$) compared to uncolonised cells of either inoculated or uninoculated plants, thus allowing for a direct comparison of nuclear position measurements. The distance from the centre of the plant nucleus to the nearest plant cell wall at 8, 12, 20 and 28 DAT in colonised cells and uncolonised cells of inoculated plants and uncolonised cells

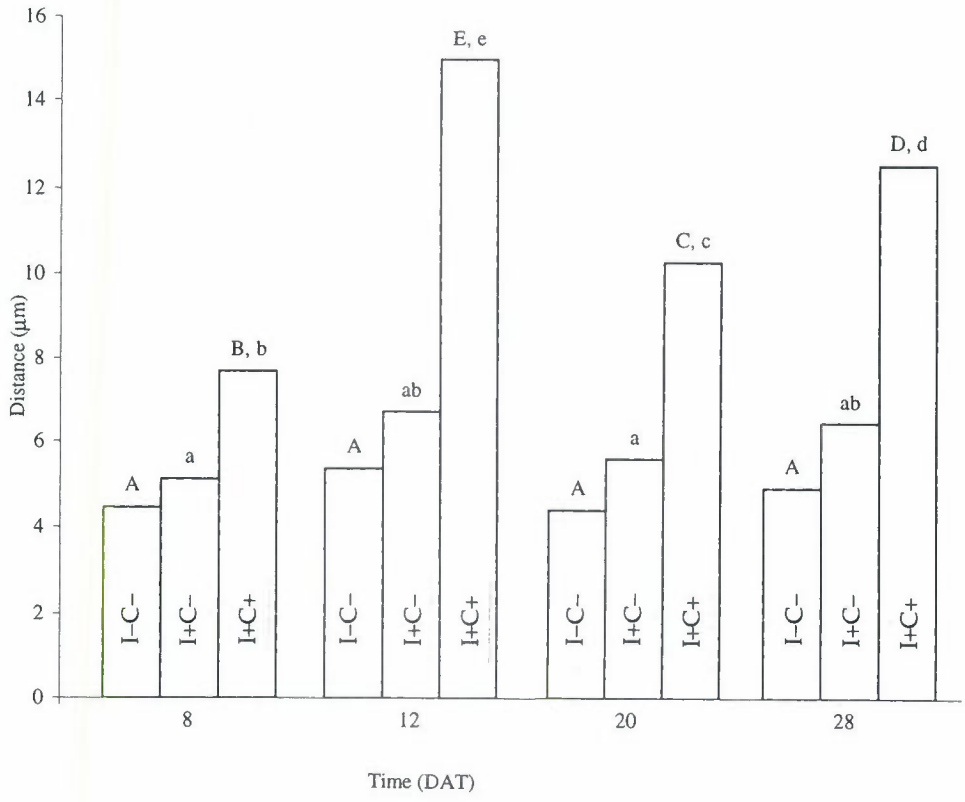

Figure 3. Mean distance from the centre of the plant nuclei to the nearest plant cell walls of uncolonised cells of uninoculated plants (I–C–), uncolonised cells of inoculated plants (I+C–), and colonised cells (AC) of inoculated plants (I+C+). Because of the different levels of interaction (see Table 1) valid comparisons can only be made between means with uppercase and lowercase labels. Means with the same letter (uppercase or lowercase) are not significantly different when the least significant differences (LSDs) are compared ($P<0.05$).

of uninoculated plants is given in Fig. 3. The distance in uncolonised cells of either inoculated or uninoculated plants is considerably less than that in colonised cells. This demonstrates that there is a major change in the position of the nucleus in cells colonised by AC. The distance in uncolonised cells of both inoculated and uninoculated plants is given in Table 1. There was no significant two-way interaction between harvest time and inoculation treatment, therefore, the one-way interactions are presented (Table 1). The position of the nucleus in cells containing HC also changed (see Fig. 1b).

Table 1. Mean distance from the centre of the plant nuclei to the nearest plant cell walls in uncolonised cells of inoculated and uninoculated plants and for each harvest time.

Treatment	Distance (μm) 8 DAT	12 DAT	20 DAT	28 DAT
Both inoculated and uninoculated plants	4.80a	6.05c	4.99ab	5.69bc

	Distance (μm) Inoculated	Uninoculated
All harvest times	4.78a	5.98b

Values followed by the same letter are not significantly different when the least significant differences (LSDs) are compared ($P<0.05$). Comparisons between means can only be made for either inoculation treatment or harvest time. n=10.

4. Discussion

Development of the symbiosis

Asphodelus colonised by *G. coronatum* formed a *Paris*-type morphology as reported previously (Cavagnaro et al., 2001). The HC and AC were similar to those described for *Acer* by Yawney and Schultz (1990) and Cooke et al. (1993). In *Asphodelus* the smooth looped hyphae of the HC (Fig. 1b) were similar to those observed using LSCM in *Panax* (Melville et al., 1998) and *Lillium* colonised by *Scutellospora calospora* (Dickson and Kolesik, 1999). The small arbuscule-like branches on the surface of the thick looped hyphae of the AC are similar to the smaller branches of arbuscules. The percent colonisation and rate of colonisation of the roots was similar to that observed in previous studies of the same plant and fungal species (Cavagnaro et al., 2001).

Fungal nuclei

Bago et al. (1998, 1999) studied nuclei in the external hyphae of AM fungi in great detail. However, the nuclei of AM fungi when forming HC and AC have not been studied. From our observations, it is apparent that the nuclei in the HC are similar in shape, size and irregularity of position to those observed in external hyphae and intercellular hyphae of AM. This is not unexpected given the similar diameter of hyphal coils, external hyphae and intercellular

hyphae. The nuclei in the thicker branches of the AC were distorted in a similar manner to those observed in arbuscules formed by *Gigaspora margarita* in *Allium porrum* (Bianciotto and Bonfante, 1992).

Volume of the plant nuclei

Lobed plant nuclei have previously been observed in colonised cells of *Allium* (*Arum*-type) (Balestrini et al., 1992). Given the varied and irregular shape of the plant nuclei observed, LSCM can provide a more accurate measure of change in size using stereology, rather than simply measuring diameter. This, in addition to the ability to make measurements in three-dimensions, highlights the advantage of using the LSCM for such studies. In cells containing AC the nuclei were hypertrophied (Fig. 2), which is a common reaction of plants to microbial infection due to unfolding of chromatin (Berta et al., 1990). In AM systems there is no evidence that this heterotropy is due to an increase in ploidy. Unfolding of the chromatin implies an increase in transcriptional activity within the cell. The increase in the volume of the nucleus in cells colonised by HC and AC is important because it implies fungal/plant interactions similar to those established by arbuscules.

Position of the plant nuclei

In cells containing AC, the position of the plant nuclei changed compared to uncolonised cells of both inoculated and uninoculated plants (Fig. 3). At 8 DAT the distance from the centre of the plant nucleus to the closest cell wall was greater in colonised cells. This distance increased between 8 and 12 DAT, but remained the same at later times. The fact that there was no further increase in distance after 12 DAT in colonised cells suggests that the change in the nuclear position must be a rapid process. Given the rate of morphological development of AM in nurse pots (Brundrett et al., 1985; Rosewarne et al., 1997; Cavagnaro et al., 2001) this is not unexpected.

There was no significant two-way interaction between inoculation treatment and harvest time affecting the distance from the centre of the plant nuclei to the closest plant cell wall in uncolonised cells of both inoculated and uninoculated plants. The difference in the distance from the centre of the plant nuclei to the closest plant cell wall in colonised cells and uncolonised cells was in the range of approximately 2.5 to 10 μm (Fig. 3). When comparing the inoculation treatments (Table 1), the distance differed by considerably less, approximately 1.2 μm. This implies that although there may be an effect of colonisation of adjacent cells on the position of the nuclei, it is not nearly as marked as that seen in colonised cells (Fig. 3).

In cells colonised by HC there was also a movement of the plant nucleus to the centre of the plant cell (Fig. 1b). The small number of sections containing HC meant that it was not possible to quantify changes in position. Balestrini et al. (1992) observed no change in the position of the nuclei of *Allium* in cells containing entry coils in the hypodermis. This difference between entry coils and HC with respect to the position of plant nuclei probably reflects differences in the effect on the plant and possibly the function of the two types of coils.

The significance of the movement of the plant nuclei in cells colonised by arbuscules, AC and HC is not well understood. The position of plant nuclei is closely linked to the spatial organisation of the plant cytoskeleton (Balestrini et al., 1992). Bonfante and Perotto (1995) speculated that nuclear movement probably results from modifications in the organisation of the plant cytoskeleton as in pathogenic systems (see Koboyashi et al., 1992). However, Genre and Bonfante (1998) found that the actin filaments reassemble in relation to the different spatial organisation of arbuscule-containing cells (particularly in relation to organelle movement), rather than nuclear movement as a result of modifications in the organisation of the plant cytoskeleton. The same presumably occurs in cells containing AC or HC. However, this is yet to be determined.

We believe that it is unlikely that the repositioning of the nucleus in colonised cells is due to mechanical movement of the nuclei by the fungi alone. When a hypha colonising a cell invaginates the membrane of the cell, it may simply force the nucleus away from the periphery of the cell. However, if a hypha were to enter the cell from a wall opposite the nucleus, we would expect the nucleus to be forced towards the opposite cell wall. Given that the nucleus could be adjacent to any of the six walls, it is more likely that the hypha would enter the cell through a wall from which the nucleus was distant.

5. Conclusions

LSCM has been used in the past to investigate the morphology, cytoskeleton and physiology of AM (Ayling et al., 1997; Genre and Bonfante, 1998; Melville et al., 1998; Dickson and Kolesik, 1999). In this experiment LSCM allowed us to make more detailed and accurate measurements of positional and volume changes than would have been possible using conventional light microscopy. This study quantitatively demonstrates that the AC formed by *G. coronatum* have similar effects on their host plant (*Asphodelus*) with respect to the position and size of the plant nuclei as cells of *Allium* containing arbuscules. Similar but not quantified effects occur in cells containing HC. At present, there

is insufficient evidence to determine what factors initiate, control or facilitate nuclear shift in colonised cells.

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