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Preferential Sporulation of *Glomus fasciculatum* in the Root Nodules of Herbaceous Legumes

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Abstract

Massive spore formation by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus fasciculatum* in root nodules of alfalfa (*Medicago sativa* L.) and clover (*Trifolium repens* L.) is described. Sporulation was higher in alfalfa than in clover nodules. Although most of the root system was colonized by the VAM fungus, sporulation preferentially occurred in the nodules. Colonization of the distal parts of the nodules was more intense. In clover, external hyphae attached to the nodule surface and penetrated the intercellular spaces between the cortical cells. Occasionally, non-mycorrhizal roots had nodules colonized by the fungus, suggesting that colonization occurred directly from the external rather than from the internal root mycelium.

Keywords: root nodules, *Glomus fasciculatum*, sporulation, vesicular arbuscular mycorrhizas, *Medicago sativa*, *Trifolium repens*

1. Introduction

The coexistence of two types of symbiotic associations in the root system of most legumes is well-known: nitrogen-fixing nodules, induced by *Rhizobium* (or *Bradyrhizobium*), and vesicular-arbuscular mycorrhizas (Barea and Azcón-Aguilar, 1983; Hayman, 1986; Azcón-Aguilar and Barea, 1992). The endophytes improve the supply of the two major plant nutrients, N and P, and may also cause some physiological changes, such as modifying root respiration

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rates and photosynthetic activity (Brown and Bethlenfalvay, 1988; Pacovsky and Fuller, 1988).

It has been shown that vesicular-arbuscular mycorrhizal (VAM) fungi and *Rhizobium* do not compete for infection sites. However, VAM fungi can change the pattern of distribution of nodules along the root system (Patterson et al., 1990). Both endophytes colonize the root system simultaneously (Smith and Bowen, 1979), but prior inoculation with one endophyte has been found to impede the development of the other (Bethlenfalvay et al., 1985), suggesting competition for carbohydrates.

The two microsymbionts do not normally share the same host-plant organs: *Rhizobium* bacteroids colonize the cells of the central zone of the root nodules, while hyphae of VAM have been found only on the surface but never within the nodules (Crush, 1974; Smith et al., 1979). The mechanism that excludes VAM fungi from legume root nodules is not known.

On the other hand, hyphae of VAM have been found in cortical tissues of young and mature actinorrhizal (*Frankia*) root nodules. However, the fungal endophyte did not enter the zone of the nodule containing the actinomicete endophytes (Rose and Yougberg, 1981; Daft, 1983; Gardner, 1986).

Thus, the two types of root nodules show some similarity in excluding VAM, but the degree of their tolerance of the presence of VAM is different. The purpose of this paper is to report, for the first time, the occurrence of significant invasion of legume root nodules by VAM, and to describe the circumstances of this phenomenon.

2. Materials and Methods

Alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens* L.) seeds were surface-sterilized with 2.5% $HgCl_2$ for 12 min, rinsed ten times with sterile distilled water, kept for 2 hr in the last rinse for imbibition, and germinated in the dark on moist filter paper. Five two-day-old seedlings, showing a radicle of about 5 mm long, were transferred to 500 mL plastic pots containing a steam-sterilized (1 hr during three consecutive days) soil: sand (5:2; v/v) mix.

The inoculum of the VAM endophyte, *Glomus fasciculatum* (Thaxter sensu Gerd.) Gerd and Trappe, consisted of washed mycorrhizal roots with the external mycelium and spores attached but free from soil particles. One g (fresh weight) of the inoculum, cut into 1-cm fragments, was placed as a layer at half height of the pot to allow contact with the roots as they grew.

Alfalfa and clover plants were inoculated with local isolates of *Rhizobium meliloti* and *Rhizobium trifolii*, respectively. The bacteria were grown in Allen

medium (Allen, 1951). Two mL (10^8 cells/mL) were applied to the radicles at transplanting.

Plants were grown in a greenhouse for 16 weeks (during October–February in Granada, Spain). Daylength was extended to 16 hr by fluorescent lamps providing a photosynthetic photon flux density of 650 μmol s^{-1} m^{-2}. Day and night temperatures ranged from 25 to 19° C. Plants were watered daily and fed every week with 10 mL per pot of a modified Long Ashton nutrient solution (Hewitt, 1952) lacking N and P.

At harvest, the root systems were detached from the shoot and carefully washed. Nodulation was observed under the stereomicroscope, and the percentage of mycorrhizal colonization assessed by microscopic observation of stained (Phillips and Hayman, 1970) root samples by using the gridline intersect technique (Giovanetti and Mosse, 1980).

3. Results

Alfalfa plants nodulated abundantly and most of the nodules contained many *G. fasciculatum* propagules (Plate 1a,b,c,d). These large, spore-like structures within the nodules, unlike vesicles, do not take up stain, as routinely observed with external spores, hence they appeared to be spores rather than vesicles.

Although most of the root system (90%) was colonized by the VAM fungus, sporulation preferentially occurred in the nodules (Plate 1a,b). In most cases, roots bearing the spore-containing nodules had very few spores, if any (Plate 1a,b,c,d). The distal parts of the nodules usually contained many more spores than the basal ones, which were often not colonized, or sparsely so (Plate 1c).

In the case of clover, about 80% of the root system was mycorrhizal, but only 40% of the nodules contained spores. External hyphae, attached to the distal surfaces of the nodules, sometimes penetrated the intercellular spaces between the cortical cells, without penetrating the cells themselves (Plate 2a,b). Occasionally, roots bearing colonized nodules were not mycorrhizal (Plate 2c,d), suggesting that nodule colonization originated from the external mycelium rather than from the subtending root. In fact, most of the nodules became colonized through the nodule meristem, leaving the tissues near the base uncolonized, while the younger tissue near the tip contained the VAM spores or hyphae.

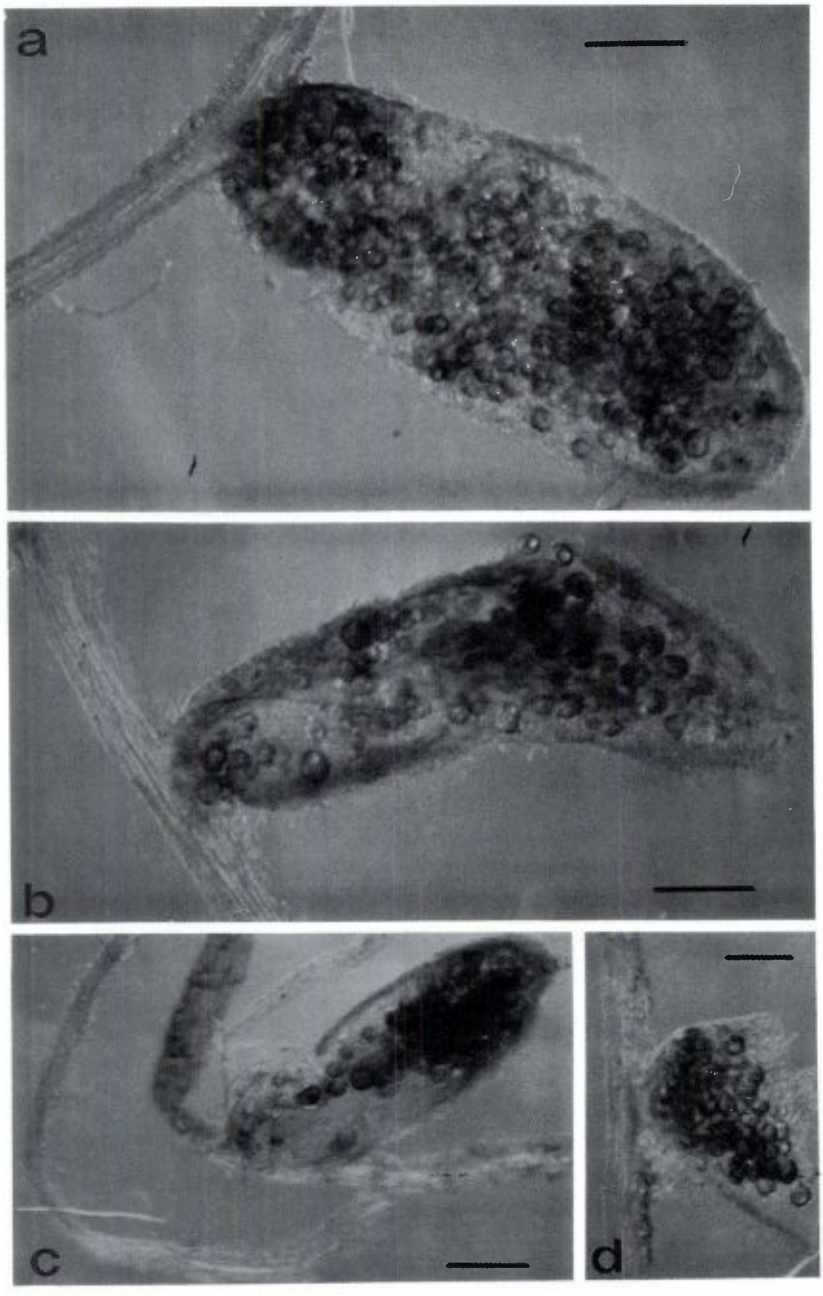

Plate 1. Alfalfa nodules containing *Glomus fasciculatum* spores: (a) and (b) massive sporulation of the fungus in the nodules; (c) initiation of colonization in the distal part of the nodule; and (d) broken nodule showing preferential sporulation in the nodule rather than in the roots. Bars = 240 μm.

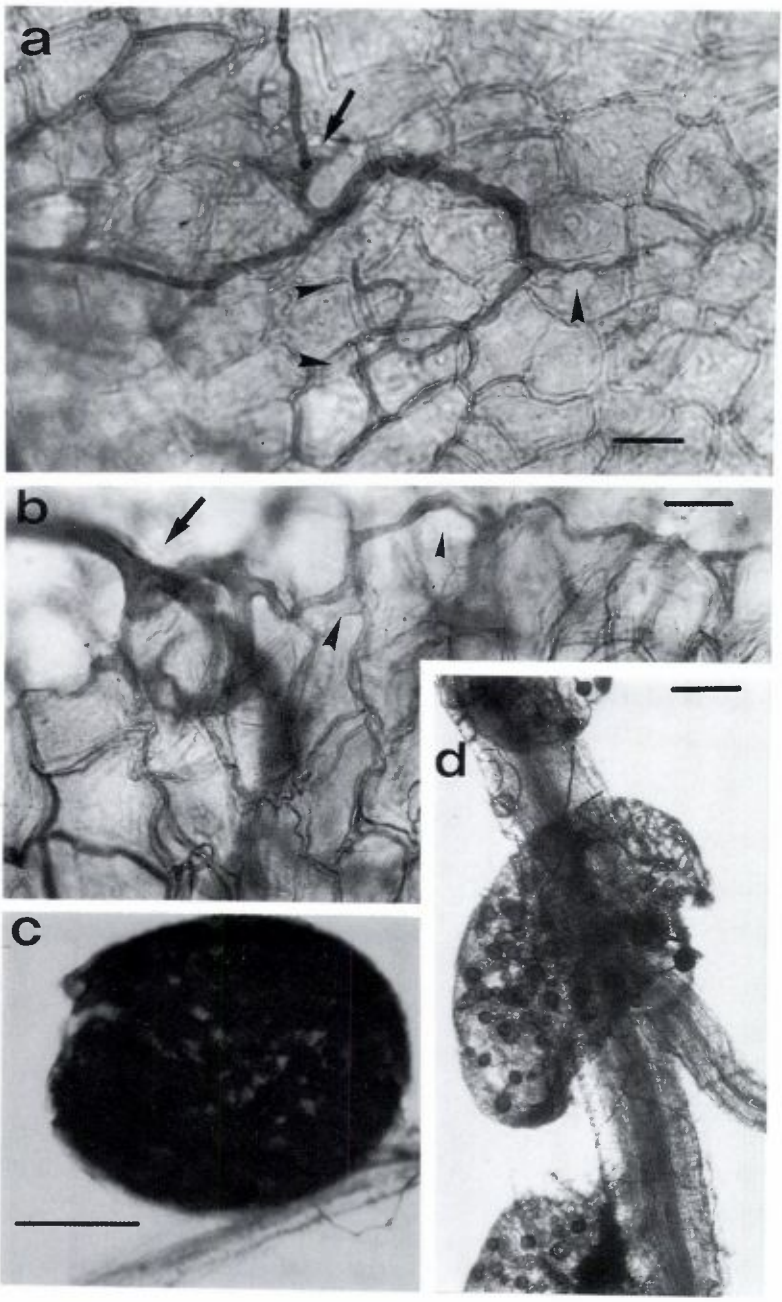

Plate 2. Nodules in mycorrhizal clover roots: (a) and (b) external hyphae attached to the nodule surface (arrows) and penetrating through the cortical cells (arrowheads) (bar = 22 μm); (c) and (d) nodules containing *G. fasciculatum* spores and showing preferential colonization of the root nodules by *G. fasciculatum*. Bar = 220 μm.

4. Discussion

Our observations showed preferential sporulation of *G. fasciculatum* in *Rhizobium*-induced nodules, but did not establish whether fungal colonization was initiated in active or senescent nodules.

It appears that VAM hyphae were attracted by the nodule tips. Since alfalfa and clover nodules belong to the indeterminate type with persistent apical meristems, such preferential colonization could be similar to that described for the apical root meristem (Mosse and Hepper, 1975; Harley and Smith, 1983; Glenn et al., 1988). Although penetration of external hyphae between the cells of the nodule cortex was also observed, formation of a functional mycorrhiza, with intracellular colonization and arbuscule development was not found.

The preferential sporulation of *G. fasciculatum* in the nodules might be explained on the basis of more suitable physico-chemical conditions inside the nodule. In fact, these are different from those of the root system due to the sensitivity of nitrogenase to the presence of O_2. Because of this, a physical barrier to gas diffusion develops in *Rhizobium* nodules. This barrier greatly reduces O_2 flux to the central part of the nodule (Witty et al., 1986). It can also limit the movement of other gases involved in nodule metabolism, such as CO_2, although under normal conditions, CO_2 concentration within the nodule seems to be controlled. VAM fungi are aerobic organisms, but their growth is stimulated by increased levels of CO_2 (Bécard and Piché, 1989). These microaerophilic conditions, or increased CO_2 levels under certain conditions, presumably stimulate VAM sporulation.

Alternatively, the chemical characteristics of the nodule could stimulate sporulation. VAM fungi have a high N requirement to synthesize chitin, the main constituent of their walls. In fact, a correlation between the N:P ratio in root tissues and sporulation of several VAM fungi had been described (Douds and Schenck, 1990a,b). The high N content of the nodules (Virtanen and Miettinen, 1963) might, at least partially, account for the massive sporulation in the nodules.

Nevertheless, the massive sporulation in the nodules is more likely to be due to the presence or absence of certain secondary metabolites. Compounds such as flavonoids and isoflavonoids, known as important transcriptional signals in *Rhizobium*, have been shown to stimulate VAM spore germination and growth *in vitro* (Gianinazzi-Pearson et al., 1989; Nair et al., 1991; Tsai and Phillips, 1991) and to increase root colonization of the host plant (Nair et al., 1991; Siqueira et al., 1991). There is general agreement that certain flavonoids play an important role in the growth and probably in the differentiation of VAM. In fact, certain flavonoids have been described to stimulate the production

of auxiliary cells without having any effect on hyphal growth (Bécard et al., 1992). These compounds, naturally present in roots and nodules, can in some cases accumulate preferentially in rhizobial nodules (Morandi and Le Quere, 1991). Alternatively, changes in hormonal balances known to occur in nodules could underlie the reported facts.

Work is in progress to determine the stages of nodule colonization by VAM and its implications with the functioning of the components of the tripartite legume symbiosis.

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