Mycorrhiza (1993) 4:59–62

Mycorrhiza

Original papers

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Succinate dehydrogenase activity of external and internal hyphae of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae* (Nicol. & Gerd.) Gerdmann and Trappe, during mycorrhizal colonization of roots of leek (*Allium porrum* L.), as revealed by in situ histochemical staining

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Abstract. The succinate dehydrogenase (SDH) activity of hyphae of the vesicular-arbuscular (VA) mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerdmann and Trappe, in symbiotic association with leek (*Allium porrum* L.) roots, was investigated by histochemical staining in situ. Leek seedlings were transplanted to sand culture and inoculated with spores of *G. mosseae* placed just below the base of the stem. At intervals (14, 25, 35 and 60 days) after transplanting, the growth medium of seedlings was flooded with nitro blue tetrazolium chloride solution, thereby displacing the nutrient solution. This allowed sites of SDH activity of external and internal fungal structures of the mycorrhizas to be stained without physically disturbing the symbiotic system. After counterstaining harvested roots and mycelium with acid fuchsin, it was possible to differentiate clearly metabolically active and inactive regions of the fungus. The lengths of external hyphae and infected root both increased nearly exponentially, and were in constant proportion (1.4 m hyphae per cm of infected root) for up to 60 days. The percentage length of external hyphae with SDH activity remained almost constant (80%). In each infected length of root there was a gradation of SDH activity from inactive distal (older) hyphae to uniformly active proximal (younger) hyphae. These findings are discussed in relation to the symbiotic activity of the mycobiont.

Key words: VAM fungi – Infection – Vital staining – Hyphae – Colonization

Introduction

It is widely accepted that external hyphae of vesicular-arbuscular (VA) mycorrhiza take up phosphorus (P) from the soil solution and translocate it to internal hyphae and thence to root cells, probably via arbuscules. For a full understanding of the functioning of VA mycorrhiza it is necessary to measure the lengths and metabolic activity of external and internal hyphae in relation to development of the host and colonization of the root system of the host by the mycobiont.

The viability of external hyphae extracted from soil has been studied with fluorescein diacetate (FDA) (Schubert et al. 1987). However, the fluorescence of this fluorochrome fades rapidly and the study of internal structures of roots is confused by autofluorescence. Histochemical staining for dehydrogenase activity has been used on external (Sylvia 1988; Hamel et al. 1990) and internal (Macdonald and Lewis 1978; Carr 1981; Kough and Gianinazzi-Pearson 1986) hyphae. This technique has been used to evaluate the effect of agrochemicals and low photon irradiance on viability of internal VA mycorrhizal hyphae (Ocampo and Barea 1985; Kough et al. 1987; Smith and Gianinazzi-Pearson 1990; Pearson et al. 1991).

External hyphae have been isolated from soil by wet sieving (Sylvia 1988) or on a sucrose gradient (Schubert et al. 1987). These procedures involve breaking hyphae and might directly affect their viability (Söderström 1979). Moreover, Jasper et al. (1989) found that soil disturbance severely reduced the infectivity of external hyphae of VA mycorrhizal fungi. Reliable measurement of the viability of external mycelium clearly requires minimal disturbance.

This paper describes a method of assessing viability of internal and external hyphae by in situ staining for succinate dehydrogenase (SDH) activity. We also examine change in SDH activity of internal hyphae as a function of distance from the point of inoculation.

Materials and methods

Growth conditions

Seeds of leek (*Allium porrum* L. Musselburgh) were surface sterilized for 3 min in saturated $Ca(OCl)_2$ solution and then germi-

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** Deceased

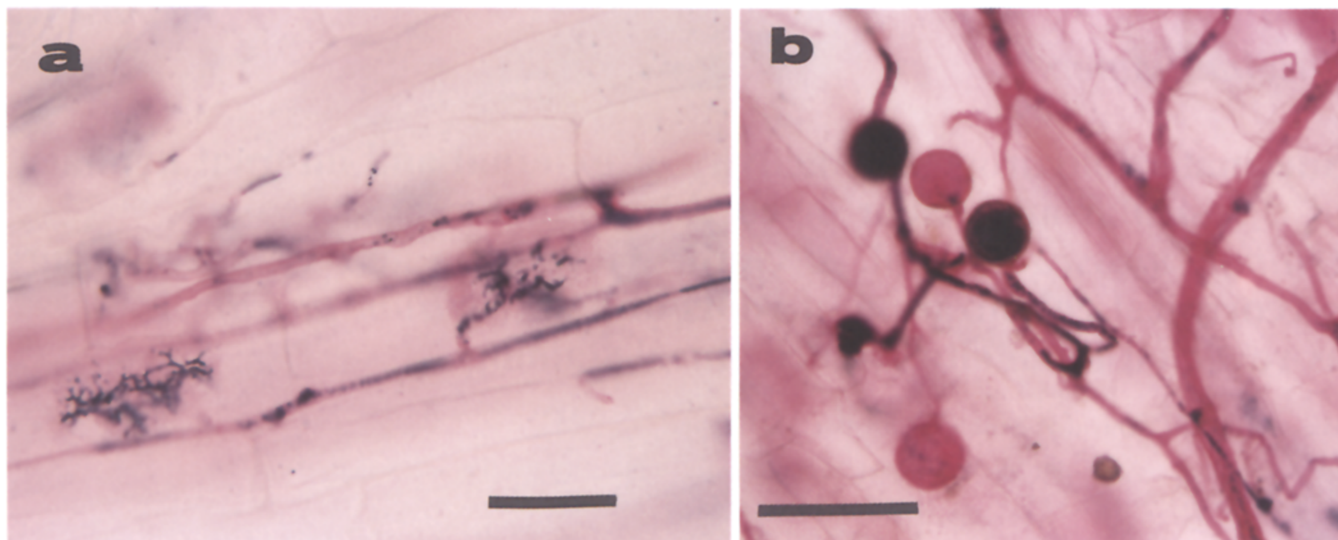

Fig. 1a, b. Hyphae of *Glomus mosseae* stained with nitro blue tetrazolium chloride and counterstained with acid fuchsin. Succinate dehydrogenase (SDH)-active hyphae were stained blue-black and inactive pink. **a** Young arbuscules in a root of leek (*Allium porrum*). Note the intense staining in arbuscular branches. *Bar* = 50 μm. **b** External hyphae and spores on the surface of a root of leek. *Bar* = 100 μm

nated on moist paper tissue. Seven-day-old seedlings were transplanted singly into tubes (50 ml disposable polypropylene syringes without a plunger) filled with autoclaved sand wetted to field capacity with the following solution ($mg\ l^{-1}$): $Mg(NO_3)_2 \cdot 6H_2O$, 183; K_2SO_4, 87.0; $MgSO_4 \cdot 7H_2O$, 92.0; $CaCl_2 \cdot 6H_2O$, 221.0; FeNa-EDTA, 9.2; $MnSO_4 \cdot 7H_2O$, 0.07; $CuSO_4 \cdot 5H_2O$, 0.07; H_3BO_3, 0.47; $NaH_2PO_4 \cdot 2H_2O$, 2.5; the pH was adjusted to 7.0. Approximately 20 spores of *Glomus mosseae* (Nicol. & Gerd.) Gerdmann and Trappe were placed below each seedling root at the time of transplanting. The tubes were covered in black plastic and placed in a growth cabinet (21/16°C, 14-h photoperiod, photon flux densitiy between 500 and 750 $\mu mol\ m^{-2}\ s^{-1}$ at 400–700 nm). Aliquots (15 ml) of nutrient solution were added three times per week. At 14, 25, 35 and 60 days after transplanting, three plants were harvested to measure development of colonization, lengths of internal and external hyphae and SDH activity of hyphae.

In situ staining method

A solution of nitro blue tetrazolium chloride (NBT) ($1\ mg\ ml^{-1}$, Sigma) was prepared in 50 mM Tris/HCl buffer (pH 7.4) containing 0.1 M sodium succinate and 0.5 mM $MgCl_2$ (Macdonald and Lewis 1978). NBT solution (80 ml) was added gradually to each tube, thereby displacing the nutrient solution. The base of the tube was sealed with Parafilm and the tube incubated in daylight at 25°C for 5 h.

After incubation, the contents of the tube were transferred to a 50-cm Petri dish containing water. External hyphae were carefully excised under a dissecting microscope. Remaining hyphae were collected from a 100-μm mesh sieve by wet sieving. Hyphae were counterstained by immersion in 0.01% acid fuchsin in lactic acid for 5 min. Hyphal lengths and SDH-active lengths were measured by grid intersection on a 2-cm^2 grid of 1-mm squares by the intersection method (Newman 1966). Subsamples were taken when the mass of hyphae was too large to spread on to one slide. The hyphae were classified into three categories of SDH activity: very active, active and inactive, corresponding to dense staining, normal staining and no staining, respectively.

Roots from which hyphae had been removed were boiled in chloral hydrate (100% w/v) solution for 1 h, rinsed with water, and then stained with acid fuchsin/lactic acid for 2 days to allow complete penetration by the counterstain. The lengths of total root and infected root were measured by grid intersection (Newman 1966).

The primary adventitious root showing the longest spread of infection in a tube was selected for detailed examination of the hyphae. The root was placed on a slide glass and squashed with a cover slip. At 5-mm intervals from the base of the stem, the intersected hyphae were classified according to the three categories of SDH activity and the number of vesicles.

Results

In situ staining

SDH-active sites in external and internal mycelium were stained dark blue in situ with NBT and could easily be distinguished from the pink-stained (acid fuchsin) inactive sites (Fig. 1). Roots stained with NBT under vacuum showed staining of internal hyphae similar to roots stained by incubation in situ, suggesting that the penetration of dye in the latter method was uniform and reliable.

Growth and viability of external hyphae

Figure 2 shows changes with time in shoot dry mass, total and infected root length. An approximately exponential increase in the length of infected root began two weeks after initiation of infection. The length of external hyphae was proportional to that of infected root, remaining at an almost constant ratio of 1.4 m hyphae per cm of infected root (Figs. 2b, 3b). Figure 3a shows that the percentage of viable hyphae was always more than 75%, and remained almost constant during the experiment.

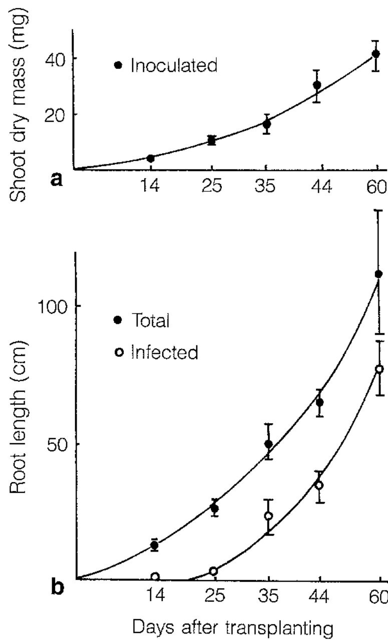

Fig. 2. Change with time in **a** shoot dry mass of leek, and **b** total and infected *Glomus mosseae* root length. *Vertical bars* indicate standard errors of means

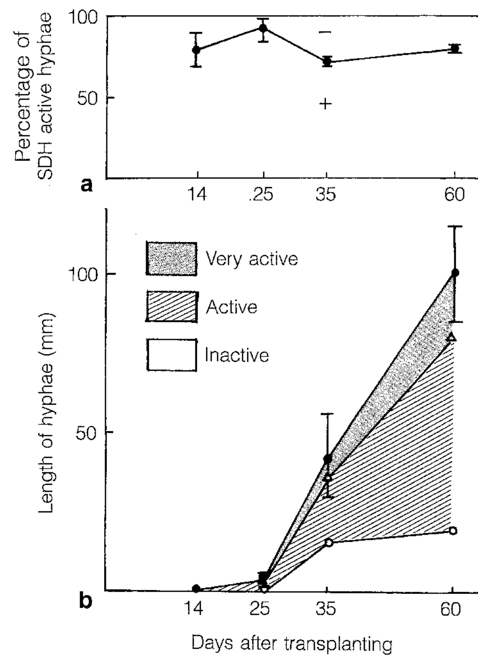

Fig. 3. **a** Change with time in percentage of external hyphae of the *Allium porrum/Glomus mosseae* mycorrhizal association active in SDH. The active hyphal fraction indicated as (+) is the sum of those very active and active in SDH. **b** Change with time in length of external hyphae very active, active or inactive in SDH

Viability of internal hyphae

Figure 4 shows the distribution of internal hyphae and their SDH activity as a function of distance along the primary adventitious root at 25 and 35 days. Although there was some variability in the distribution between replicates, the trend was consistent. Infection from the inoculum spread both proximally and distally but the SDH activity of the older part of the infection 'segment' (as defined by Amijee et al. 1989), i.e., at the point of inoculation, was much less than of the younger, advancing 'front' (Amijee et al. 1989), with a continuous variation in activity between these points. Vesicles were also most common in the older part of the segment (Fig. 4).

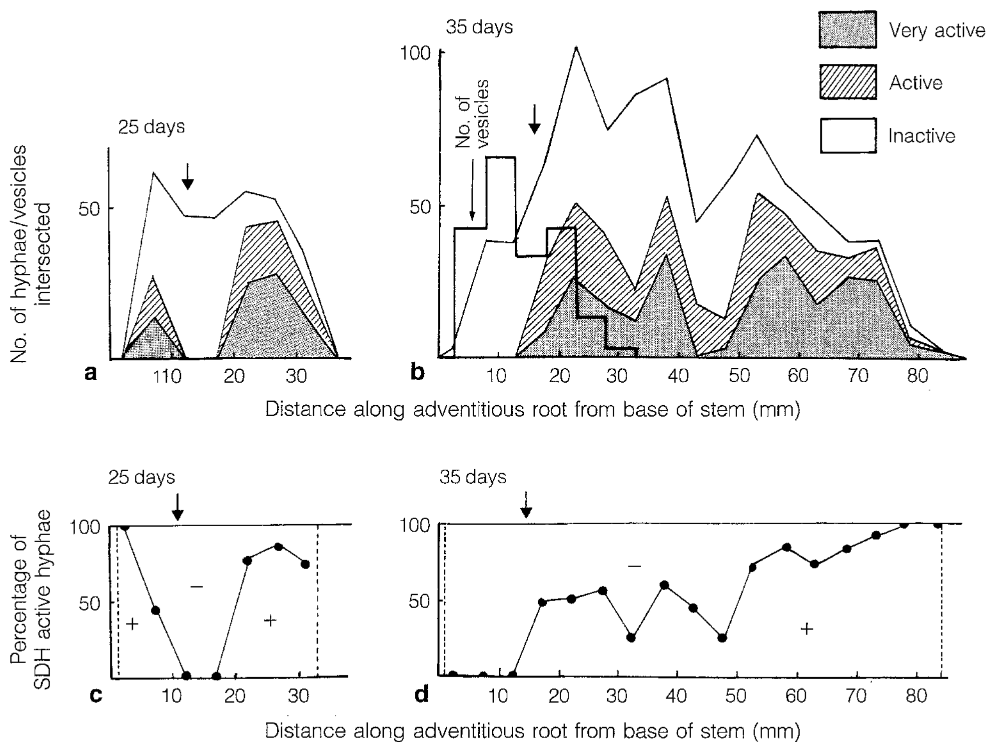

Fig. 4a–d. Distribution of internal hyphae of *Glomus mosseae* along adventitious roots of leek 25 and 35 days after transplanting. *Black arrows* indicate the position of the inoculum. **a, b** Distribution of hyphae very active, active or inactive in SDH and of vesicles. **c, d** Percentage of internal hyphae active in SDH. The active hyphal fraction indicated by + is the sum of those very active and active in SDH

Discussion

Our finding of a constant proportion of viable external hyphae contrasts with previous reports. Schubert et al. (1987), working with *Trifolium repens* (clover) inoculated with *Glomus clarum*, found that the length of hyphae stained with FDA decreased from 100% to less than 15% after 5 to 8 weeks. In contrast, Sylvia (1988) found in a *Pasulum notatum/Glomus* spp. association that the percentage length of external hyphae staining with indonitro tetrazolium increased from zero to more than 30% in 9 weeks. Such inconsistencies might arise from the extraction procedures used to isolate hyphae from soil. Söderström (1979) showed that fungal hyphae rapidly lost the ability to stain with FDA when mechanically disrupted and concluded that no useful inferences about in situ viability could be drawn from staining such damaged hyphae. In the present study, hyphae were stained in situ with no mechanical disturbance. The reduced viability in older parts of infection segments suggests that VA mycorrhizal hyphae do lose viability with age: the constant proportion of external hyphae observed here almost certainly occurred because their total length increased nearly exponentially. This finding has important implications for a young, rapidly growing plant as it suggests that the functioning of the external hyphal network remains unaffected during ontogeny. Further work is needed to test this assumption.

This paper has shown that the infection segment is not internally homogeneous, as the older internal hyphae are much less physiologically active than the younger hyphae at the advancing fronts. Since translocation of P is only by physiologically active hyphae (Cooper and Tinker 1981), our data imply that only the younger parts of a segment are active in P uptake. This in turn might mean that a pattern of VA mycorrhizal infection with many small units of varying length of infection (Smith and Walker 1981) is more efficient in taking up soil P than infection with long continuous segments in a banded inoculum (Buwalda et al. 1984).

Acknowledgements. M. S. is grateful to the Science and Technology Agency, Japan for financial support that enabled him to work in the UK.

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