Dual Inoculation of *Sorghum bicolor* (L.) Moench ssp. *bicolor* with Vesicular Arbuscular Mycorrhizas and *Acetobacter diazotrophicus*

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Abstract

Four different Vesicular Arbuscular Mycorrhiza (VAM) fungal strains (*Glomus mosseae, G. occultum, G. constrictum* and *Scutellospora persica*), originally isolated from sand dunes, were tested for efficiency and compatibility in promoting growth of *Sorghum bicolor* cv. Keller, singularly or in association with *Acetobacter diazotrophicus*. Shoot dry weight was significantly higher in mycorrhizal plants (P<0.05); the highest values were recorded with *Glomus* strains. Fungal infection was however significantly lower (P<0.05) in *Acetobacter diazotrophicus* inoculated plants. Mycorrhizal inoculation increased the number of *A. diazotrophicus* cells in roots, stems and leaves. *A. diazotrophicus* was even detected at the spike level as well as in newly formed VAM fungal spores. Nitrogen concentration was significantly increased by *A. diazotrophicus,* even more in associations with the efficient fungal strains. Mycorrhizal plants showed a significant (P<0.05) increase in specific root length and root branching; the highest values were observed in *A. diazotrophicus* treatments in combination with *G. mosseae* and *G. constrictum.* Such morphological modifications may enhance water and nutrient uptake. Our results confirm the importance of

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studying the plant-microbial interrelationships to provide useful information for agricultural system management.

1. Introduction

Sweet sorghum (*Sorghum bicolor* (L.) Moench ssp. *bicolor*) is largely used as a forage or an intercalary cereal crop in rotation with winter cereals. In central and southern Italy sorghum is cultivated on arid and marginal agricultural areas as substitute of maize (Grimaldi et al., 1990). At present, the optimization of biomass yields is obtained by chemical fertilization (Bonciarelli, 1989). To decrease production costs and pollution risks, the inoculation of plants with useful microorganisms is attempted.

Among rhizosphere microorganisms, diazotrophic bacteria play a major role in the nitrogen cycle. Most reports consider N_2-fixing bacterial symbioses with leguminous plants; several studies, however, considered also the influence of diazotrophs on non-leguminous field crops, and the relationships between biological N_2-fixation and sustainable agriculture (Kennedy and Tchan, 1992). Recently, an acid-tolerant diazotrophic bacterium, *Acetobacter diazotrophicus*, was isolated from sugar cane stems in Brazil (Gillis et al., 1989). These bacteria have previously been observed inside roots, stems and leaves of sugar cane, in numbers as high as 10^7 cells g^{-1} fresh tissue (Stephan et al., 1988). *A. diazotrophicus* may fix atmospheric N_2 also in the presence of nitrate (Teixera et al., 1987). Such incomplete inhibition of N_2-fixation is of ecological and agronomic relevance, since it may allow the complementation of biological nitrogen-fixation in the presence of other N sources. This aspect may favour the re-cultivation of agronomic lands, following a prolonged period of chemical fertilization, alleviating immobilization of N by soil micro-organisms.

Positive effects of dual colonization of non-leguminous plant roots by vesicular arbuscular-mycorrhizal (VAM) fungi and diazotrophic bacteria were investigated on sugar cane (Boddey et al., 1991) and rice (Dhillion, 1992). Dual inoculation could be specially advantageous in the case of *A. diazotrophicus*, since this bacterium has not been isolated from soil, and the bacteria are mainly transmitted from plant to plant through vegetative propagation by stem pieces (Cavalcante and Döbereiner, 1988). Recent investigations have shown that *A. diazotrophicus* may also be introduced into sterile micropropagated sugarcane, sweet potato and sweet sorghum seedlings via vesicular arbuscular mycorrhizae (Paula et al., 1991, 1992). Inoculation of *A.

diazotrophicus with, or inside, VAM spores allowed this bacterium to penetrate and to colonize the roots of these plants, passing to the aerial tissues. Inoculation of VAM fungi seems therefore to be an essential condition for colonization of whole plants by N_2-fixing bacteria.

Selection of VAM-fungal strains for the improvement of crop yields and diazotrophs efficiency should therefore consider intersymbiont compatibility besides host-plant compatibility, in order to avoid unsuccessful field inoculations. In the present work four different AM and VAM fungi, isolated from Italian sand dunes, were inoculated singularly or in combination with *A. diazotrophicus* on sweet sorghum seedlings, to evaluate the effect of each type of symbiosis, individually or in combination, on growth, nutrition and microbial infection of the host plant.

2. Materials and Methods

Fungal inoculum

Fungal strains were originally isolated from coastal sand dunes in central Italy, and maintained on sand pot cultures of *Trifolium repens* cv. Huja. The inoculum consisted of mixed sand, roots, and spores. The spore numbers for 100 g of inoculum were respectively: 189 spores for *Glomus mosseae*, 1276 spores for *Glomus occultum*, 993 spores for *Glomus constrictum*, and 156 spores for *Scutellospora persica*.

Bacterial inoculum

Acetobacter diazotrophicus, strain PAL5 (ATCC 49037), originally isolated from sugarcane, was kindly provided by J. Döbereiner (Embrapa-CNPBS, Seropedica-Rio de Janeiro) and kept on modified agarized LGI-P medium (Cavalcante and Döbereiner, 1988). For the inoculation, *A. diazotrophicus* was grown at 28°C, for 48h at 120 rpm, to the end of the log phase, in liquid LGI-P supplied with $1g\ l^{-1}$ NH_4Cl. The inoculum was prepared by diluting the culture with 34 mM phosphate buffer, pH 6, to obtain 1×10^7 CFU/ml.

Experimental design

Sweet sorghum (*Sorghum bicolor* cv. Keller) seeds were sterilized with 10% H_2O_2 for one hour, and germinated in aseptic conditions in steam-sterilized sand (twice at 120°C for 30 min, with an interval of 24h between both autoclavings). Ten days after germination, seedlings were transplanted in 4 l

pots (four seedlings/pot) into sterilized sand. Each seedling received 50 g of mycorrhizal inoculum and 5 ml of bacterial suspension. Ten treatments (three pots each) resulting from different combinations of *A. diazotrophicus* with *Glomus mossae*, *G. occultum*, *G. constrictum* and *Scutellospora persica*, were established as follows: i) not inoculated control; ii) *A. diazotrophicus* , ii) *G. mossae*; iv) *G. constrictum*; v) *G. occultum*; vi) *S. persica*; vii) *G. mossae + A. diazotrophicus*, viii) *G. constrictum + A. diazotrophicus* ix) *G. occultum + A. diazotrophicus*, x) *S. persica. + A. diazotrophicus*.

Treatments were arranged in a completely randomized design.

After inoculation (1 June) plants were grown for one month under controlled conditions (26–20°C and 50–100% RH day/night, 16 h day-length, 215 mE sec^{-1} m^{-1}), and then outdoors until the harvest (1 October). Control and VAM inoculated plants received once a week Hoagland nutrient solution (Hoagland and Arnon, 1950). Treatments inoculated with *A. diazotrophicus*, singularly or in combination with VAM fungi, received once a week a modified Hoagland solution (without NH_4NO_3 and with 1 g l^{-1} $CaCO_3$ added to stabilise the pH). Deionized water was added when needed.

Plant measurements and analyses

At harvest, plants were cut at ground level. The following parameters were measured: shoot height, shoot diameter, shoot dry weight, root dry weight, ears dry weight (dry weights were estimated after oven-drying at 70°C for 72h). From these data, total dry weight, shoot/root dry weight ratio (S/R), and ear dry weight/total dry weight ratio were also calculated.

Roots of VAM inoculated plants were stained according to Koske and Gemma (1989) and root length, linear frequency of root tips and percent mycorrhizal infection were estimated by the gridline intersect method (Giovannetti and Mosse, 1980). From these data, specific root length (SRL, as root length per unit weight) and a root branching index were also calculated.

Total nitrogen was measured on plant aereal parts by macro-Kjeldahl method (Bergersen, 1980).

Evaluation of plant microbial colonization

Bacterial colonization was estimated in different plant sections as follows: healthy roots from each plant were chosen and gently rinsed in tap water to remove soil particles; 1 g (fresh weight) of each was crushed with a sterilized mortar and pestle, and serially diluted (10^{-2} to 10^{-8}) in sugar solution (5% cane sugar in deionized water). Washed sections (1 g fresh weight) of basal (10 centimeters from collar) and apical (4 cm below ear) stems, leaves, and ears of

each plant were homogenized and diluted as above. From each dilution 1 ml replicate samples were inoculated into 3 10-ml serum vials containing 5 ml of a semisolid nitrogen-free medium (pH 5.5) according to Cavalcante and Döbereiner (1988), LGI-P, with 10% cane-sugar. *A. diazotrophicus* was identified by thick yellow-orange pellicles on the surface of the medium and clearing of the medium below. Most Probable Number was calculated by Mc Grady tables (Postgate, 1969). Identity of the bacterium was confirmed by inoculating a loopfull of the pellicles on acetic semisolid LGI medium acidified with acetic acid to pH 4.5 (Micales et al., 1985) and agar concentration increased to 2.2 grams per liter, and on potato agar plates (Döbereiner, 1980), modified by omitting malate and increasing the sugar concentration to 10%.

Bacterial colonization of VAM spores

Colonization of newly formed fungal spores by *A. diazotrophicus* was evaluated on spores collected at harvest from pot soil with a standard wet sieving and decanting technique. Spores were surface sterilized according to Watrud (1982). Twenty intact or crushed spores of each fungal species were separately placed in vials containing N-free LGI-P semi-solid medium and incubated at 30°C for eight days. The presence of *A. diazotrophicus* was detected by a thick yellow-orange pellicle.

Statistical analyses

Data were statistically analysed using the GLM-ANOVA subroutine of NCSS statistical package (Hintze, 1992). LSD was evaluated with the same package using Fisher's test (P = 0.05).

3. Results and Discussion

Mycorrhizal infection and Acetobacter effect on fungal colonization

All fungal strains colonized plant roots (Fig. 1); *Glomus* strains produced significantly (P<0.05) higher infection rates than *Scutellospora*. VAM infection was also significantly lower (P = 0.001) in dual inoculated plants. The reduction of VAM infection observed when VAM were coinoculated with *A. diazotrophicus*, varied for each of the tested fungus. This was possibly due to a lowering of pH caused by bacterial metabolism: all the mycorrhizal fungi utilised in the present experiment were isolated and occur in alkaline soils (Mosse and Hepper, 1975; Puppi and Riess, 1987). On the contrary,

enhancement of mycorrhizal root colonization in dual inoculated sweet potato, sugarcane and sweet sorghum plants was reported (Paula et al., 1991; 1992) when *A. diazotrophicus* was coinoculated with the acid-tolerant AM fungus *Glomus clarum* (Giovannetti, 1981).
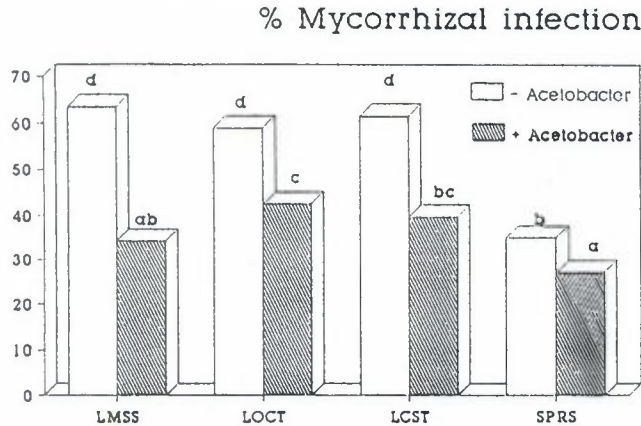
% Mycorrhizal infection

Figure 1.   Percent mycorrhizal infection of inoculated sorghum plants at harvest in the presence and in the absence of *Acetobacter diazotrophicus*. LMSS = *Glomus mosseae*, LOCT = *G. occultum*, LCST = *G. constrictum*, SPRS = *Scutellospora persica*. Different letters indicate statistically significant differences (p<0.05; n = 12).

Effect of VAM fungi on A. diazotrophicus colonization

A. diazotrophicus, when coinoculated with VAM fungi, successfully colonized different sections of sweet sorghum plants, at different extents, however, according to the coinoculated mycorrhizal strain (Fig. 2).

Differences in bacterial counts of *Glomus* inoculated plants with respect to plants inoculated with *A. diazotrophicus* alone or in combination with *Scutellospora persica* became more evident with increases in plant height. In dual inoculated plants, bacterial cells were detected even in the ears, showing the highest values in combination with *G. mossae* and *G. constrictum*. Similar results were reported for vegetatively propagated sugarcane and sweet potato (Paula et al., 1991; 1992), as well as on sweet sorghum (Paula et al., 1991). Bacteria, however, were not translocated to the plant tops. Our results point out that a large amount of bacterial cells not only colonizes plant roots, but also stems, leaves and ears. These findings suggest that mycorrhizal infection may have promoted penetration and spread of the diazotroph not only in cortical root regions, but also in root structures that allow translocation to the stems and
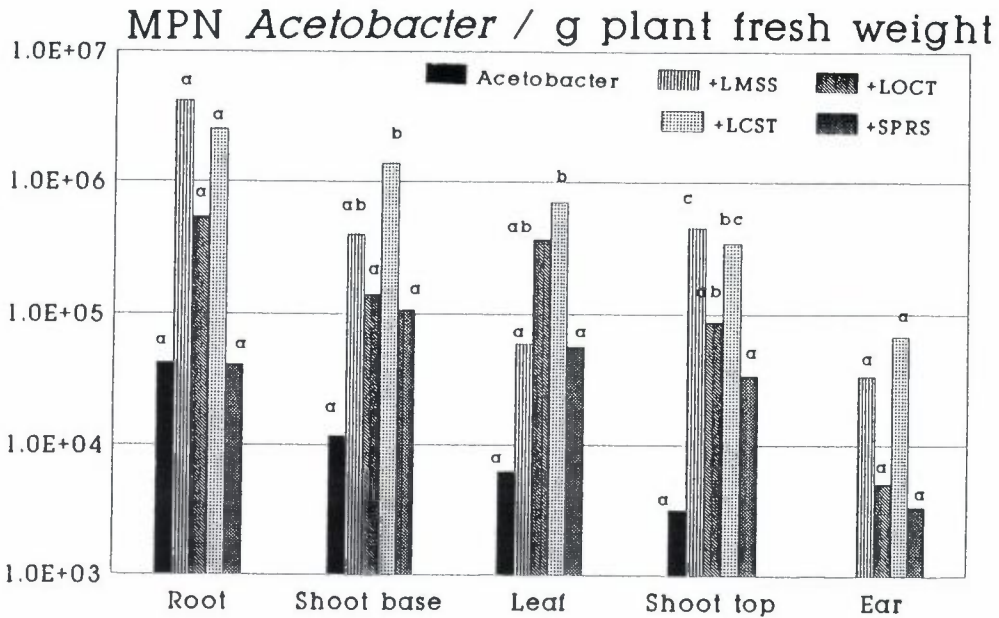
MPN *Acetobacter* / g plant fresh weight

Figure 2. Most Probable Number of *Acetobacter diazotrophicus* cells g^{-1} plant fresh weight when inoculated alone or with different VAM species. LMSS = *Glomus mosseae*, LOCT = *G. occultum*, LCST = *G. constrictum*, SPRS = *Scutellospora persica*. Different letters indicate statistically significant differences for each plant section (p<0.05; n = 12).

ears. Whether the two micro-organisms interact directly via some physiological change, mediated by the production of growth regulators, secondary metabolites, or other chemical signals in the host plant, is not yet determined. Our results suggest also some degree of different compatibilities between VAM fungi and *Acetobacter diazotrophicus*.

Acetobacter propagules in newly formed VAM spores

All vials containing crushed or intact VA fungal spores formed the typical yellow-orange pellicle on the surface of the medium.

The successful spreading and maintenance of infection might therefore be ensured by the fact that newly formed mycorrhizal fungal spores bear *Acetobacter* propagules. The presence of hyphosphere bacteria on the surface and within spores of VAM fungi was reported by many authors (Mosse, 1962; Varma et al., 1981; Tilak et al., 1989; Vancura et al., 1989; Klyuchnikov and Kozhevin, 1990) and the ability of *A. diazotrophicus* to successfully colonize sorghum plants using VAM spores as infection carriers was already observed

(Paula et al., 1991). Notwithstanding such reports, the association of VAM fungal spores with plant growth promoting rhizobacteria is a topic still much overlooked and in need of study.

Inoculation effects on sorghum

Inoculation of seedlings with VAM fungi alone caused a general increase of shoot dry weight and height (P<0.05), while *A. diazotrophicus* inoculum did not significantly affect these parameters. *G. occultum* and *G. mossae* inocula appeared to have the most remarkable effect on shoot dry weight, while plant height was increased mainly by *G. occultum* inoculum (Fig. 3a and Table 1). AM fungi, especially by *G. constrictum* and *G. occultum* inoculum, significantly increased shoot diameter, which in turn was lowered (P<0.05) by the presence of *A. diazotrophicus* (Table 1). The growth promotion effect *G. mossae*, *G. constrictum* and *G. occultum* inocula were more efficient than the *S. persica* inoculum. Root colonization, which was higher in *Glomus* spp. inoculated plants, in some cases was significantly correlated to shoot dry weight (P = 0.02, r = 0.43 in LCST treatment; P = 0.01, r = 0.68 in LOCT treatment).

Table 1.   Effect of VAM and *Acetobacter diazotrophicus* inoculation on sorghum growth at harvest. LMSS = *Glomus mosseae*, LOCT = *G. occultum*, LCST = *G. constrictum*, SPRS = *Scutellospora persica*. Means followed by different letters in a column are significantly different (p<0.05) (n = 12).

| Treatment	Shoot height (cm)	Shoot diameter (mm)	Ear d.w. (g)	SRL (cm/mg)	Root branching /g	Linear frequency of root tips /cm
Control	37.1 a	4.1 ab	0.03	nd	nd	nd
Acetobacter	41.5 ab	4.0 ab	0.12	nd	nd	nd
LMSS	45.7 ab	4.3 ab	0.06	2.75 abc	447 a	0.17 a
LOCT	55.7 b	6.3 d	0.12	1.73 a	328 a	0.20 a
LCST	46.2 ab	5.5 cd	0.05	4.19 c	744 bc	0.18 a
SPRS	48.0 ab	4.9 abc	0.03	1.69 a	500 ab	0.30 b
LMSS + *Acetobacter*	45.1 ab	4.9 bc	0.09	3.90 c	794 c	0.19 a
LOCT + *Acetobacter*	54.2 b	4.2 ab	0.08	2.31 ab	329 a	0.17 a
LCST + *Acetobacter*	54.2 b	3.8 a	0.16	4.21 c	756 bc	0.18 a
SPRS + *Acetobacter*	39.8 ab	4.5 ab	0.04	3.47 bc	630 abc	0.18 a
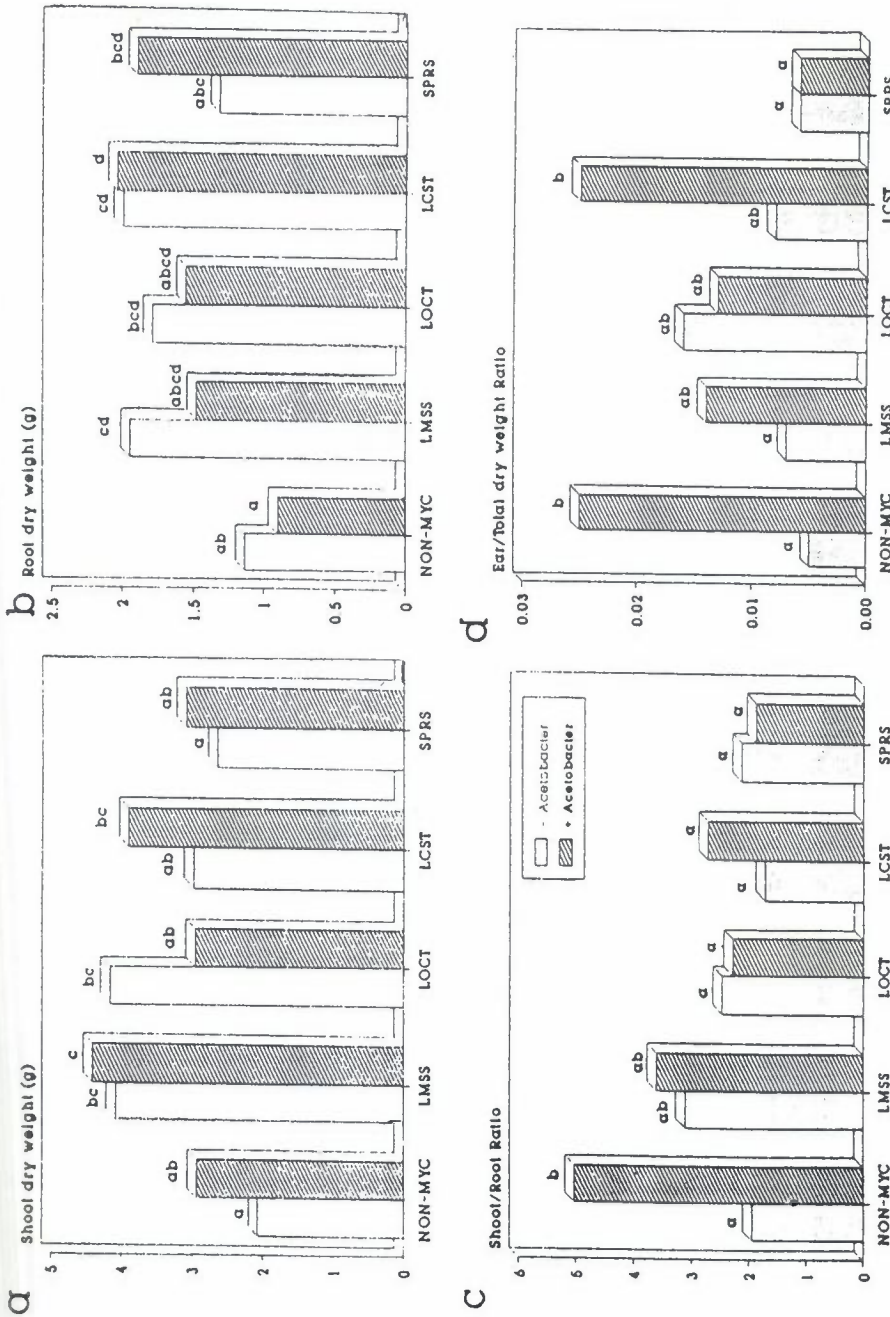
Figure 3. Shoot dry weight (a), Root dry weight (b), Shoot/root ratio (c), and Ear dry weight/Total dry weight ratio (d) of *Sorghum bicolor* plants, at harvest, inoculated with *Acetobacter diazotrophicus* alone or in combination with different mycorrhizal strains. LMSS = *Glomus mosseae*, LOCT = *G. occultum*, LCST = *G. constrictum*, SPRS = *Scutellospora persica*. Different letters indicate statistically significant differences (p<0.05; n = 12).

G. mossae alone and *G. constrictum* both alone and in combination with *Acetobacter* increased root dry weight, while *Acetobacter* did not affect this parameter. *Acetobacter* alone, however, increased S/R ratio; such effect was no longer observed in mycorrhizal plants (Fig. 3b and 3c).

Acetobacter inoculation seemed also to increase, although not significantly, ear dry weight; the biomass allocation to ear, as ear weight/total dry weight ratio, was significantly increased in *Acetobacter* and *Acetobacter + G. constrictum* treatments (Fig. 3d). These findings agree with those of Pereira et al. (1988), who reported yield increase of sorghum plants inoculated with N_2-fixing bacteria only for grain sorghum varieties, while growth performance of sweet sorghum varieties was not improved.   The enhancement of ear dry weight in *A. diazotrophicus* inoculated plants, possibly due to a greater allocation of nitrogen to this part of the plant, may lead to an efficient partitioning, but, also, less efficiency in water acquisition and tendency to lodging.

Acetobacter tended also to increase specific root length and root branching in mycorrhizal plants. Fungal effects on these parameters varied with the strain, tending to be higher in *G. constrictum* inoculated plants, singularly and in combination, and in *G. mossae + Acetobacter* treatments (Table 1). According to Fitter (1987), a highly branched, absorbing root system limits the volume of soil explored, but optimizes nutrient transport and cost efficiency. The enhancement of SRL detected in *A. diazotrophicus* inoculated mycorrhizal plants leads to a thinner and more elongated root pattern (exploratory root tipology) (Hetrick, 1991), more adaptable to intermittent stress, such as water deficiency.

Plant nitrogen content

An increase in plant nitrogen concentration was observed in all mycorrhizal plants; dual inoculation of *A. diazotrophicus* with the three *Glomus* strains increased further the N content (Fig. 4). *Acetobacter* inoculation increased plant nitrogen percent – even if the plant were not supplied with any mineral nitrogen – much more than the application of single VAM. Moreover, plant inoculated with VAM fungi alone, received a weekly application of mineral nitrogen. Nitrogen concentrations appear related to the number of bacterial cells detected in plants.

The possibility of introducing diazotrophic microorganisms into sweet sorghum plants by VAM fungal infection may allow a large utilization of this cereal as annual crop minimizing N fertilizers addition. In this experiment positive results could be observed in terms of bacterial colonization, nitrogen content and plant growth. Different fungal strains differed however in their
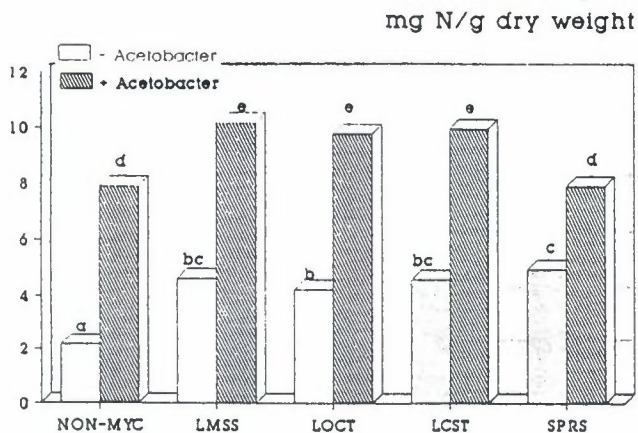
Figure 4.   Nitrogen content of *Sorghum bicolor* shoots, at harvest, inoculated with *Acetobacter diazotrophicus* alone or in combination with different mycorrhizal strains. LMSS = *Glomus mosseae*, LOCT = *G. occultum*, LCST = *G. constrictum*, SPRS = *Scutellospora persica*. Different letters indicate statistically significant differences (p<0.05; n = 3).

efficiency in promoting sorghum growth and *Acetobacter* colonization. Our results support therefore the possibility of screening microbial compatibility and efficiency previous to field applications.

Acknowledgements

REFERENCES

Bergersen, F.J. 1980. Measurement of nitrogen fixation by direct means. In: *Methods for Evaluating Biological Nitrogen Fixation*. F.J. Bergersen, ed. Wiley, Chichester, New York, pp. 65–110.

Boddey, R.M., Urquiaga, S., Reis, V., and Döbereiner, J. 1991. Biological nitrogen fixation associated with sucar cane. *Plant and Soil* **137**: 111–117.

Bonciarelli, F. 1989. *Fondamenti di Agronomia Generale*. Edagricole, Bologna.

Cavalcante, V.A., and Döbereiner, J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant and Soil* **108**: 23–31.

Dhillion, S. 1992. Dual inoculation of pretransplant stage *Oryza sativa* L. plants with indigenous vesicular-arbuscular mycorrhizal fungi and fluorescent *Pseudomonas* spp. *Biology and Fertility of Soils* **13**: 147–151.

Döbereiner, J. 1980. Forage grasses and grain crops. In: *Methods for Evaluating Biological Nitrogen Fixation*. F.J. Bergersen ed. Wiley, Chichester, New York, pp. 535–555.

Fitter, A.H. 1987. An architectural approach to the comparative ecology of plant root systems. *New Phytologist* **106** Suppl: 61–77.

Gillis, M., Kerters, K., Host, B., Janssens, D., Kroppenstedt, R.M., Stephan, M.P., Teixeira, K.R.S., Döbereiner, J., and De Ley, J. 1989. *Acetobacter diazotrophicus* sp. nov., a nitrogen fixing acetic acid bacterium associated with sugar cane. *International Journal of Systematic Bacteriology* **39**: 361–364.

Giovannetti, M. 1981. Some anatomic features and spore germination of the vesicular-arbuscular endophyte *Glomus clarus* Nicolson and Schenck. *Annali di Microbiologia* **31**: 103–107.

Giovannetti, M., and Mosse, B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* **84**: 489–500.

Grimaldi, A., Bonciarelli, F., and Lorenzetti, F. 1990. Sorgo e cereali estivi minori. In: *Coltivazioni erbacee*, Edagricole. Bologna, pp. 56–64.

Hetrick, B.A.D. 1991. Mycorrhizas and root architecture. *Experientia* **47**: 355–361.

Hintze, J.L. 1992. *Number Cruncher Statistical System*. Kaysville, Utah.

Hoagland, D.R., and Arnon, D.I. 1950. *The water-culture method for growing plants without soil*. California Agricultural Experimental Station Circular 347, University of California.

Kennedy, I.R., and Tchan, Y.T. 1992. Biological nitrogen fixation in non-leguminous field crops: Recent advances. *Plant and Soil* **141**: 93– 118.

Klyuchnikov, A.A., and Kozhevin, P.A. 1990. Dynamics of *Pseudomonas fluorescens* and *Azospirillum brasiliense* populations during the formation of the vesicular-arbuscular mycorrhiza VAM fungi. *Plant Physiology* **79**: 562–563.

Koske, R.E., and Gemma, J.N. 1989. A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research* **92**: 488–505.

Micales, B.K., Jonson, J.L., and Claus, G.W. 1985. Deoxyribonucleic acid homologues among organisms in the genus *Gluconobacter*. *International Journal of Systematic Bacteriology* **35**: 79–85.

Mosse, B. 1962. The establishment of vesicular-arbuscular mycorrhizae under aseptic conditions. *Journal of General Microbiology* **27**: 509–520.

Mosse, B., and Hepper, C.M. 1975. Vesicular-arbuscular mycorrhizal infections in root organ cultures. *Physiological Plant Pathology* **5**: 215.

Paula, M.A., Reis, V.M., and Döbereiner, J. 1991. Interaction of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato *Ipomea batatas*, sugarcane *Saccharum* spp., and sweet sorghum *Sorghum vulgare*. *Biology and Fertility of Soils* **11**: 111–115.

Paula, M.A., Urquiaga, S., Siqueira, J.O., and Döbereiner, J. 1992. Synergistic effects of vesicular-arbuscular mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato *Ipomea batatas*. *Biology and Fertility of Soils* **14**: 61–66.

Pereira, J.A.R., Cavalcante, V.A., Baldani, J.I., and Döbereiner, J. 1988. Field inoculation of sorghum and rice with *Azospirillum* spp. and *Herbaspirillum seropedicae*. *Plant and Soil* **110**: 269–274.

Postgate, J.R. 1969. Viable counts and viability. In: *Methods in Microbiology* Vol. 1. J.R. Norris and D.W. Ribbons, eds, Academic Press, New York, pp. 611–628.

Puppi, G., and Riess, S. 1987. Role and ecology of VA mycorrhizae in sand dunes. *Angewandte Botanik* **61**: 115–126.

Stephan, M.P., Teixeira, K.R.S., and Döbereiner, J. 1988. Nitrogen fixation physiology of *Acetobacter nitrocaptans*: Effect of oxygen, pH and carbon source on respiration and nitrogenase activity. In: *Nitrogen Fixation: Hundred Years After*. H. Bothe., F.J. de Bruyn, and W.E. Newton, eds. Gustav Fischer, Stuttgart, p. 207.

Teixeira, K.R.S., Stephan, M.P., and Döbereiner, J. 1987. Physiological studies of *Saccharobacter nitrocaptans* a new acid tolerant N2-fixing bacterium. *4th International Symposium on Nitrogen Fixation with Non-legumes*, Rio de Janeiro, Final Program Abstracts, p. 149.

Tilak, K.V.B.R., Li, C.Y., and Ho, I. 1989. Occurence of nitrogen-fixing *Azospirillum* in vesicular-arbuscular mycorrhizal fungi. *Plant and Soil* **116**: 286–288.

Vancura, V., Orozco, M.O., Gravová, O., and Prikryl, Z. 1989. Properties of bacteria in the hyphosphere of a vesicular-arbuscular mycorrhizal fungus. *Agriculture, Ecosystem and Environment* **29**: 421–427.

Varma, A.K., Singh, K., Lall, V.K. 1981. Lumen bacteria from endomycorrhizal spores. *Current Microbiology* **6**: 207–211.

Watrud, L.S., 1982. Spore germination and axenic culture of endomycorrhizae. A. Spore germination of vesicular-arbuscular mycorrhizal fungi. In: *Methods and Principles of Mycorrhizal Research*. N.C. Schenck ed. APS, St. Paul, pp. 81–83.