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ORIGINAL PAPER

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# Positive correlation between VAM-induced changes in root exudation and mycorrhizosphere mycoflora

**Abstract** The influence of vesicular-arbuscular mycorrhizal (VAM) fungi on rhizosphere mycoflora was studied together with the possible mechanism involved in this process. Six combinations of VAM fungi and phosphorus fertilizer treatments were applied to *Leucaena leucocephala* roots and quantitative and qualitative observations were made periodically of the rhizosphere mycoflora and constituents of root exudates. The results obtained indicate that the presence of specific mycoflora in the rhizosphere of mycrorrhizal roots is mediated through root exudates rather than being an outcome of improved *P* nutrition.

**Key words** Vesicular-arbuscular mycorrhizal (VAM) fungi · *Leucaena leucocephala* · Mycorrhizosphere Root exudation · Phosphorus nutrition

## Introduction

The term "mycorrhizosphere" describes the rhizosphere of a root colonized with a mycorrhizal fungus. When a vesicular-arbuscular mycorrhizal (VAM) fungus forms a symbiotic association with a plant root, the physiology of the plant is altered. As a consequence, the microbial community in a mycorrhizosphere differs from that in a nonmycorrhizal rhizosphere soil (Garbaye 1991; Jalali and Jalali 1991; Paulitz and Linderman 1991; Sharma and Mukerji 1992). The mechanism by which the symbiotic association with a mycorrhizal fungi alters the existing rhizosphere mycoflora is of fundamental interest to mycorrhizal research.

The influence of root exudates on microbial populations in the vicinity of roots is well known (Curl and Truelove 1986; Hawes 1990; Rovira 1985; Bansal and Mukerji 1994). A VAM fungus, after it has established itself inside a fine root, changes the exudation pattern both quantitatively and qualitatively (Graham et al. 1981). Whether the VAM fungi-induced changes in rhizosphere microflora are mediated by root exudates needs to be explored in detail.

The usually accepted explanation the effects of VAM fungi in an ecosystem is nutritional, especially involving phosphorus (Paulitz and Linderman 1991). Whether the observed changes in the rhizosphere mycoflora, including suppression of soil-borne plant pathogens, are confounded by nutritional or phosphorus effects or whether VAM fungi directly interact with other microbes is not known. In most of the earlier experiments, no attempt was made to compensate for a phosphorus effect by supplying nonmycorrhizal plants with appropriate amounts of phosphorus. Such experiments would clarify the role of *P* nutrition in the suppression of disease by VAM fungi. The present investigation posed the following questions:

1. What are the quantitative and qualitative effects of *Glomus macrocarpum* on sugars and amino acids in root exudates?

2. Is there a correlation between changes in rhizosphere mycoflora and root exudation?

3. Can VAM-induced changes in rhizosphere mycoflora be mimicked by supplying soluble sources of phosphorus exogenously?

## Materials and methods

### Field setup

The experimental setup consisted of six experimental plots (8×20 m) located in the Botanical Garden, University of Delhi. The plots were deep ploughed and the soil was sterilized thoroughly with 0.1% formalin.

Seeds of *Leucaena leucocephala* (Lam.) de Wit Var. K-8 were procured from the National Bureau of Plant Genetic Resources, New Delhi. The VAM inoculum of *G. macrocarpum* Tul. & Tul.

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(105 spores/g soil) was prepared from rhizosphere soil of *Prosopis juliflora* located in the Ridge area of the University of Delhi.

Seeds were sown in six combinations of VAM incoulum and two phosporus fertilizers as given below:

1. Control without VAM and phosphorus
2. Superphosphate at a concentration of 60 kg/ha
3. $KH_2PO_4$ at a concentration of 60 kg/ha
4. VAM with a spore density of 105/g soil
5. Superphosphate and VAM at the same concentrations as in 2 and 4
6. $KH_2PO_4$ and VAM at the same concentrations as in 3 and 4

Collection of samples

Samples where collected from the experimental site at monthly intervals starting 15 days after seedling emergence. Roots were carefully dug out to avoid damaging fine roots.

Root exudate collection and analysis

The collected roots were washed thoroughly, kept in sterilized distilled water for 24 h at room temperature and then filtered through predried, preweighed filter paper (Whatman no. 1). The filtrate was lyophilized and stored at −20° C until use. Fine roots remaining on the filter paper were dried in oven at 72° C and the dry weight measured. Colorimetric estimation of amino acids was performed with the method of Spies (1957), and the anthrone method (Yemm and Willis 1954) was used for total sugar estimation. Student's *t*-test was applied to determine the significance of differences between means of treated and control plants.

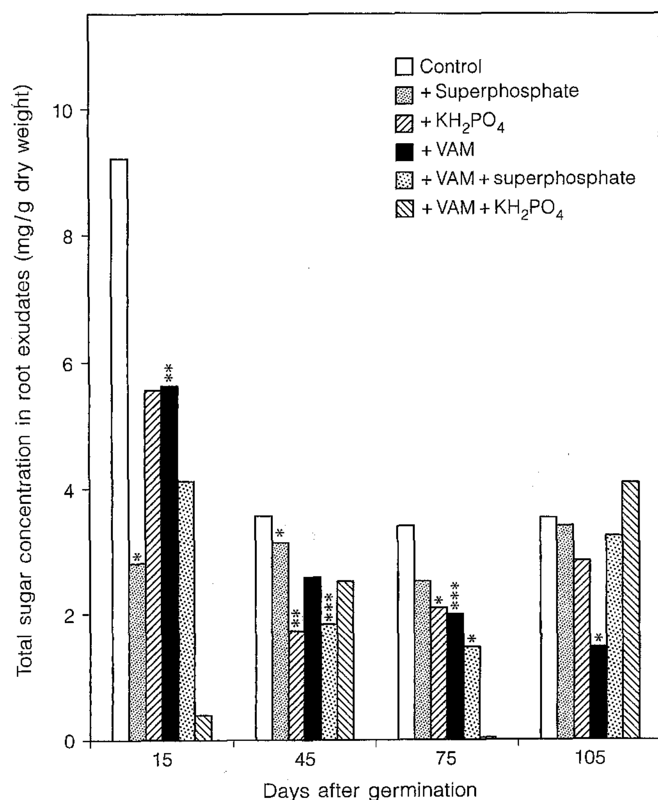


**Fig. 1** Variation in sugar concentration in root exudates in different treatments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

Rhizosphere and rhizoplane mycoflora

The dilution plate technique (Dickinson 1971) was used for isolation of rhizosphere and rhizoplane fungi, employing Czapex's Dox yeast extract agar medium (Thom and Raper 1945).

The growth and occurrence of soil fungi isolated was expressed as:

1. Average no. of colonies at a dilution of 1:1000 per gram of rhizosphere soil or per cm of root segement

$$= \frac{\text{Average no. of colonies per Petri plate}}{\text{Volume of diluted suspension plated per Petri plate or no. of segments per Petri plate}}$$

2. Percent frequency of occurrence

$$= \frac{\text{No. of samples in which fungus occurs}}{\text{Total no. of samples}} \times 100$$

Percent mycorrhiza formation

Mycorrhizal colonization of roots was measured according to Phillips and Hayman (1970). Root pieces were thoroughly washed in water and kept in 10% potassium hydroxide for 3–4 days depending upon the thickness of the roots. The roots were then thoroughly washed tap water and stained with 0.1% trypan blue (CDH, Delhi, India) in lactophenol (phenol:lactic acid:glycerine:water, 1:1:2:1). Stained roots were cut into 1-cm-long sections and mounted in lactophenol. Fifty segments were mounted and examined and the results expressed as the mean of three readings. Percent mycorrhiza formation was calculated as follows:

$$\text{Percent mycorrhiza formation} = \frac{\text{Total no. of secondary lateral root sections colonized}}{\text{Total no. of lateral roots sections}} \times 100$$

## Results and discussion

The VAM fungus, *G. macrocarpum* reduced the exudation of both sugars and amino acids from the fine roots of *L. leucocephala* (Figs. 1, 2). A similar decrease was brought about by externally supplied phosphorus fertilizers and was statistically significant ($P < 0.05$–$0.001$). Colonization by VAM fungi and an increase in soil phosphorus is also known to reduce the leakage of metabolites from roots of *Pinus radiata, Zea mays, Citrus aurantium* and *Sorghum* sp. (Bowen 1969; Graham et al. 1981; Laheurte and Berthelin 1988; Ratnayake et al. 1978; Schwab et al. 1984).

The endomycorrhizal fungus also caused a decrease in the total number of mycoflora colonies per gram of soil in the rhizosphere and rhizoplane regions (Figs. 3, 4). This correlated well with decreases in the leakage of sugars and amino acids. In contrast, increase in soil phosphorus led to an increase in mycoflora around the fine roots. Thus the suppression by VAM fungi of rhizosphere mycoflora is not an outcome of improved *P* nutrition but is a direct consequence of variation in exudation. Besides alterations of nutrients in root exudates, the release of mycotoxic inhibitors (Schwab et al. 1984) may also be involved. This is supported by the fact that phosphorus caused an equivalent decrease in

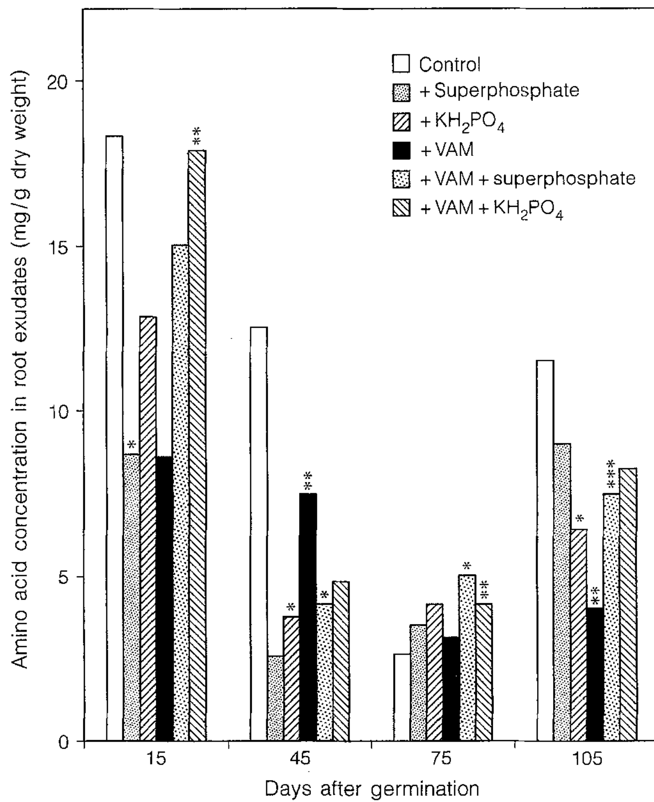


**Fig. 2** Exudation of amino acids in different treatments. *P* values as in Fig. 1

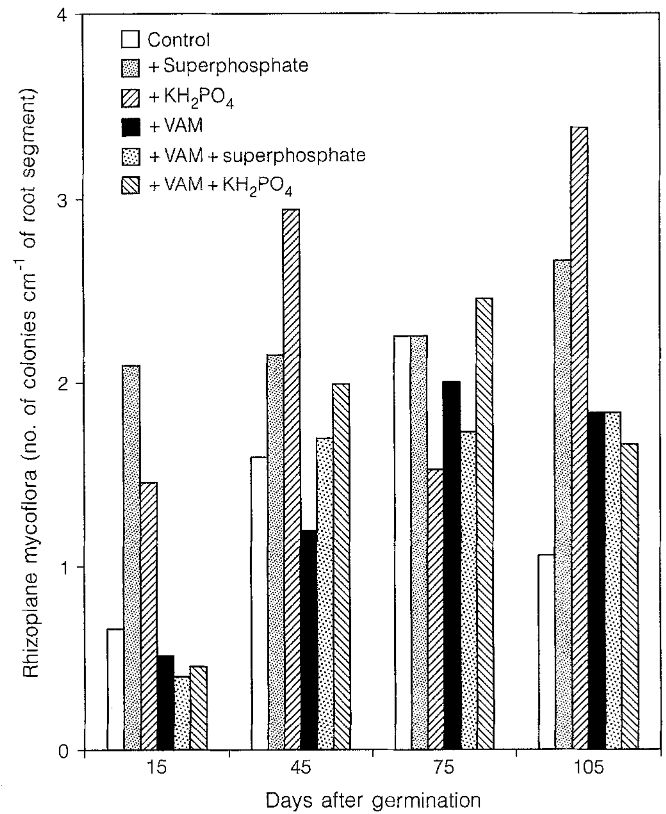


**Fig. 4** Effects of different treatments with VAM inocula and phosphorus fertilizers on rhizoplane mycoflora

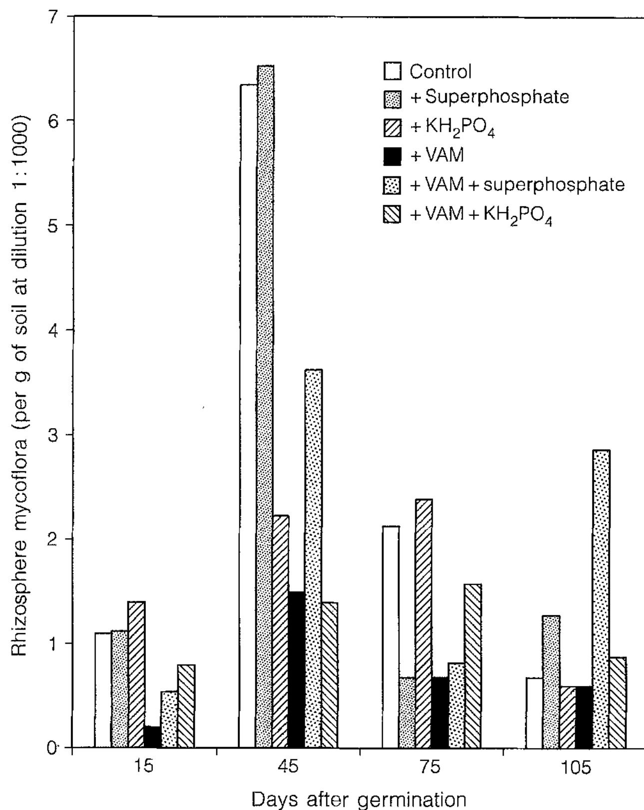


**Fig. 3** Effects of different treatments with VAM inocula and phosphorus fertilizers on rhizosphere mycoflora

leakage of metabolites as well as an increase in the number of colonies in the rhizosphere soils.

The category of specialized mycorrhizosphere organisms (Garbaye 1991) includes those organisms that depend completely on simple organic molecules (sugars, amino, acids, organic acids, etc.) released by plant roots and fungal symbionts. In the present study, the fungi included in this category are *Aspergillus clavatus, A. luchuensis, A. fischeri, A. humicola, Cladosporium cladosporioides, Humicola grisea* and *Neurospora crassa,* as they were absent in the respective nonmycorrhizal controls (Tables 1–4). The alterations in mycorrhizosphere mycoflora caused by *G. macrocarpum* show a tendency towards the suppression of pathogenic fungi, e.g. *Fusarium* sp., and the stimulation of saprophytes, e.g. *Cladosporium* sp. The present results support the earlier reports of Jalali and Jalali (1991) and Sharma (1990), who observed increased resistance to many soilborne plant pathogens.

How VAM fungi change the spectrum of microflora in the mycorrhizosphere is still a matter of conjecture. Several hypotheses have been put forth to explain the mechanisms involved (Table 5), but most await experimental investigation. The present investigation took into account two important mechanisms, viz root exudation (physiological) and improved *P* nutrition (indirect). Our results indicate that of the four proposed mechanisms (Table 5), physiological effects are most

**Table 1** Percent frequency of occurrence of rhizosphere (R) and rhizoplane (Rp) mycoflora of *Leucaena leucocephala* 15 days after germination

| Fungus | Control | | +Superphosphate | | $+KH_2PO_4$ | | +VAM | | +VAM +superphosphate | | +VAM $+KH_2PO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp |
| *Alternaria* sp. | 66.6 | 33.3 | — | — | — | — | — | 33.3 | — | — | — | — |
| *Aspergillus clavatus* | — | — | — | — | — | — | — | — | — | — | 16.6 | — |
| *Aspergillus flavus* | 100 | 83.3 | 100 | 100 | 50.0 | 33.3 | 33.3 | 83.3 | 33.3 | 33.3 | 33.3 | 100 |
| *Aspergillus nidulans* | — | — | — | 16.6 | 66.6 | — | — | — | — | — | — | — |
| *Aspergillus niger* | 33.3 | — | 100 | 100 | — | 16.6 | 33.3 | — | 33.3 | 33.3 | — | — |
| *Aspergillus sulphureus* | 16.6 | 16.6 | — | — | — | — | — | — | — | — | — | — |
| *Aspergillus terreus* | — | — | — | — | 16.6 | — | — | — | — | — | — | — |
| *Aspergillus wentii* | — | — | — | — | 16.6 | — | — | — | — | — | — | — |
| *Cladosporium cladosporioides* | — | — | — | — | — | — | 83.3 | 66.6 | — | — | — | — |
| *Fusarium moniliforme* | 50.0 | 16.6 | — | — | — | — | — | — | — | — | — | — |
| *Fusarium oxysporum* | 83.3 | 100 | 33.3 | 33.3 | 100 | 83.3 | — | — | — | — | 66.6 | — |
| *Humicola griesea* | — | — | — | — | — | — | 66.6 | — | 16.6 | — | — | — |
| *Phoma hybemica* | — | — | — | — | — | — | — | — | 16.6 | 16.6 | — | — |

**Table 2** Percent frequency of occurrence of rhizosphere (R) and rhizoplane (Rp) mycoflora of *L. leucocephala* 45 days after germination

| Fungus | Control | | +Superphosphate | | $+KH_2PO_4$ | | +VAM | | +VAM +superphosphate | | +VAM $+KH_2PO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp |
| *Alternaria* sp. | 33.3 | 16.6 | — | 16.6 | — | — | — | — | — | — | — | — |
| *Aspergillus flavus* | 83.3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 83.3 | 83.3 | 83.3 |
| *Aspergillus fumigatus* | 33.3 | — | — | — | — | — | — | — | 16.6 | — | 33.3 | 83.3 |
| *Aspergillus nidulans* | 83.3 | 100 | 100 | 100 | 100 | 33.3 | — | — | 100 | 33.3 | 33.3 | — |
| *Aspergillus niger* | 83.3 | — | 66.6 | 100 | 100 | 100 | 66.6 | 33.3 | — | — | 33.3 | 83.3 |
| *Aspergillus terreus* | 33.3 | — | 83.3 | 66.0 | 100 | 50.0 | 100 | 33.3 | — | — | — | — |
| *Aureobasidium pullulans* | 33.3 | — | — | — | — | — | — | — | — | — | — | — |
| *Cladosporium cladosporioides* | — | — | — | — | — | — | 33.3 | 66.6 | — | — | — | — |
| *Fusarium moniliforme* | 16.6 | — | — | — | — | — | — | — | — | — | — | — |
| *Fusarium oxysporum* | — | — | — | 16.6 | — | — | — | — | 16.6 | — | — | — |

**Table 3** Percent frequency of occurrence of rhizosphere (R) and rhizoplane (Rp) mycoflora of *L. leucocephala* 75 days after germination

| Fungus | Control | | +Superphosphate | | $+KH_2PO_4$ | | +VAM | | +VAM +superphosphate | | +VAM $+KH_2PO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp |
| *Alternaria* sp. | 33.3 | — | — | — | — | — | — | — | — | — | — | — |
| *Aspergillus flavus* | 100 | 100 | 100 | 100 | 100 | 100 | 66.6 | 100 | 100 | 100 | 66.6 | 100 |
| *Aspergillus humicola* | — | — | 33.3 | — | — | — | — | — | — | — | — | — |
| *Aspergillus nidulans* | 66.6 | — | 100 | — | 100 | 16.6 | 66.6 | 33.3 | — | — | 66.6 | — |
| *Aspergillus niger* | 50.0 | 100 | 100 | 83.3 | 16.6 | 16.6 | 66.6 | 16.6 | 66.6 | 100 | 66.6 | 66.6 |
| *Aspergillus terreus* | — | — | — | — | — | — | — | 50.0 | — | — | — | — |
| *Cladosporium cladosporioides* | — | — | — | — | — | — | 16.6 | — | — | — | — | — |
| *Colletorichum falcatum* | — | — | — | — | — | — | 16.6 | — | — | — | — | — |
| *Fusarium oxysporum* | — | — | — | — | — | — | — | — | — | — | 33.3 | 16.6 |
| *Mucor heimalis* | — | — | — | — | — | — | — | — | 16.6 | — | — | — |
| *Neurospora crassa* | — | — | — | — | — | — | 16.6 | — | 16.6 | — | — | — |
| *Rhizopus nigricans* | — | — | — | — | — | — | — | — | 16.6 | — | — | — |

**Table 4** Percent frequency of occurrence of rhizosphere (R) and rhizoplane (Rp) mycoflora of *L. leucocephala* 105 days after germination

| Fungus | Control | | +Superphosphate | | $+KH_2PO_4$ | | +VAM | | +VAM +superphosphate | | +VAM $+KH_2PO_4$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp | R | Rp |
| *Alternaria* sp. | 16.6 | — | — | — | — | — | — | — | — | — | — | — |
| *Aspergillus flavus* | 66.6 | 66.6 | 50.0 | 50.0 | 16.6 | 100 | 66.6 | 83.3 | 16.6 | 83.3 | 16.6 | 16.6 |
| *Aspergillus fumigatus* | — | — | — | — | — | — | — | — | 33.3 | 33.3 | — | — |
| *Aspergillus humicola* | — | — | — | — | — | — | — | 50.0 | — | — | — | — |
| *Aspergillus nidulans* | 33.3 | — | 16.6 | 66.6 | 50.0 | 16.6 | 66.6 | — | — | — | 50.0 | 50.0 |
| *Aspergillus niger* | 33.3 | 16.6 | 33.3 | — | 50.0 | 100 | 16.6 | 50.0 | 16.6 | — | 50.0 | 50.0 |
| *Aspergillus terreus* | — | — | 16.6 | — | — | — | — | — | — | — | — | — |
| *Aureobasidium pullulans* | — | — | 16.6 | — | — | — | — | — | — | — | — | — |
| *Cephalosporium roseum* | 16.6 | 50.0 | 100 | 100 | — | — | — | — | — | — | 100 | 100 |
| *Cladosporium cladosporioides* | — | — | — | — | 66.6 | — | 83.3 | — | 100 | — | — | 16.6 |
| *Colletorichum falcatum* | — | — | — | — | 16.6 | — | — | — | 16.6 | — | — | — |
| *Mucor heimalis* | — | — | — | — | — | — | — | — | 16.6 | — | — | — |
| *Phoma hybernica* | — | — | — | — | — | — | — | — | 16.6 | — | — | — |
| *Rhizopus nigricans* | — | — | — | — | — | — | — | — | 16.6 | — | — | — |

**Table 5** A summary of mechanisms proposed for the interaction of mycorrhiza and root-borne pathogens

| Type | Principle | References |
|---|---|---|
| 1. Physical | 1. Include formation of Hartig's net and mantle which provides a physical barrier to the pathogen | Garbaye (1991), Perrin and Garbaye (1983) |
| | 2. Is supported by absence of systemic effects | Davis and Menge (1980), Rosendahl (1985) |
| 2. Physiological | 1. Alteration of pattern of root exudation both quantitatively and qualitatively | Graham and Menge (1982), Paulitz and Linderman (1991), Schwab et al. (1984) |
| | 2. Influencing phenol metabolism and lignification | Dahne and Schonbeck (1979) |
| | 3. Chitinase activity increased | Dahne et al. (1978) |
| | 4. Ultrastructural changes | Marx et al. (1982), Gianinazzi-Pearson et al. (1981), Dexhemier and Pargney (1991) |
| | 5. Antibiotics | Duchense et al. (1988), Sylvia and Sinclair (1983) |
| 3. Microbial | Alteration of microbial population and enhancing the population of antagonistic organisms to plant pathogens | Garbaye (1991), Paulitz and Linderman (1991) |
| 4. Indirect effect | Is an outcome of increased up take of *P* and other nutrients | Graham and Menge (1982), Paulitz and Linderman (1991) |

important in the alteration of rhizosphere mycoflora. Contrary to suggestions by Paulitz and Linderman (1991), we believe that VAM-induced changes in mycorrhizosphere mycoflora are not an outcome of improved *P* nutrition but a result of physiological interaction between plant roots and the fungal symbiont.

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