Vitis **29**, 5—13 (1990)

Istituto Coltivazioni Arboree dell'Università di Torino, Torino, Italia

Centro di Studio sulla Micologia del Terreno C.N.R., Torino, Italia

Effects of vesicular-arbuscular mycorrhizal fungi on micropropagated grapevines: Influence of endophyte strain, P fertilization and growth medium[1])

by

A. SCHUBERT, M. MAZZITELLI, O. ARIUSSO and I. EYNARD

Einfluß vesikulär-arbuskulärer Mykorrhizapilze auf *in vitro* vermehrte Reben: Wirkung von Pilzstamm, Phosphatdüngung und Kultursubstrat

Zusammenfassung: *In vitro* vermehrte Pflanzen der Unterlagssorte *Vitis berlandieri* × *V. rupestris* 1103 P wurden in steriles Substrat verpflanzt, das mit steigenden P-Gaben angereichert war, und während der Anpassungsphase mit verschiedenen Arten vesikulär-arbuskulärer Mykorrhizapilze (VAM) beimpft. In dem Substrat ohne P-Düngung förderten alle Pilzarten das Rebenwachstum; der positive Einfluß der Beimpfung war jedoch mit zunehmender Düngungsstufe verwischt. Zwei Pilzarten waren indessen auch noch bei der höchsten P-Konzentration in der Lage, das Pflanzenwachstum zu steigern. In einem weiteren Versuch wurde der Einfluß der VAM-Art *Glomus caledonicum* (NICOL. et GERD.) TRAPPE et GERD. in einem Torf/Sand-Gemisch geprüft, das mit 0 oder 10 % Erde versetzt war. Obwohl der VAM-Pilz die Wurzeln in beiden Fällen besiedelte, wurde eine signifikante Wachstumssteigerung nur in dem Erde enthaltenden Substrat festgestellt.

Key words: mycorrhiza, fungus, *Glomus* spp., host plant, tissue culture, micropropagation, root, growth, phosphorus, fertilization, soil, growth medium.

Introduction

Vesicular-arbuscular mycorrhizal (VAM) fungi colonize the roots of many crop plants and enhance their growth and nutrient uptake. Growth of the grapevine is strongly dependent on the presence of VAM fungi, as is shown by the large dry weight ratio between mycorrhizal and non-mycorrhizal plants, which has been observed in pot- and field-grown vines (POSSINGHAM and GROOT-OBBINK 1971; GEBBING *et al.* 1977; MENGE *et al.* 1983; SCHUBERT *et al.* 1988). Inoculation with VAM fungi can thus be beneficial for grapevine growth; however natural, non-sterilized soils contain propagula of indigenous VAM species, which are effective in enhancing plant growth (SCHUBERT and CRAVERO 1985; SCHUBERT *et al.* 1988) and thus decrease the economic interest for the artificial introduction of these fungi. On the contrary, when plants are grown in substrates lacking natural endophytes, inoculation with VAM fungi encounters no natural competition (MENGE *et al.* 1983) and may become economically interesting.

Grapevine micropropagation is a well-established technique, with potential applications for the production of virus-free plants (GALZY 1969). Micropropagated grapevines are grown in sterile media *in vitro* and transplanted thereafter in substrates which, even when not sterilized, often lack VAM propagules. At this stage, plantlets can be easily stressed by unfavourable nutritional and environmental conditions; the pres-

[1]) Paper no. 679 of the Istituto Coltivazioni Arboree.

ence of well developed mycorrhizae, absorbing nutrients and water from the substrate by the attached network of external hyphae, may be an important factor to improve plant growth.

Previous work has shown that micropropagated grapevines can be successfully colonized by VAM fungi both *in vivo* (SCHUBERT *et al.* 1987) and *in vitro* (RAVOLANIRINA *et al.* 1988) and that inoculation can enhance their growth rate. *In vitro* inoculation, however, is a lengthy and cumbersome practice, requiring isolation and sterilization of fungal spores; furthermore, after transplanting micropropagated plants replace the majority of their roots grown *in vitro* with new ones (CONNER and THOMAS 1981), and as a consequence most of the mycorrhizal roots would be lost at this stage. For such reasons, *in vivo* VAM inoculation seems more suitable for commercial application than *in vitro* inoculation. In this work we inoculated micropropagated grapevines at the beginning of the acclimatization phase and tested the effects of fungal strain, soil fertilization and growth medium composition on the growth response induced by mycorrhizal inoculation.

Materials and methods

Plants of the grapevine rootstock *Vitis berlandieri* × *V. rupestris* cv. 1103 P were micropropagated *in vitro* from axillary buds in a modified MS medium (MURASHIGE and SKOOG 1962) containing 1 mg l^{-1} benzylaminopurine and then rooted in the same medium without hormones. 4 weeks after the beginning of the rooting phase, plants were transplanted into 500 cm^3 plastic pots filled with a sterile substrate. Acclimatization was performed keeping plants at 100 % air humidity with a transparent plastic sheet cover for 1 week and then progressively opening the cover, which was definitely removed after 2 weeks from transplant.

Three experiments were carried out, the first two to study the effects of endophyte strain and of P fertilization, the third one to test the activity of VAM fungi in two different growth media:

Experiment 1: A mixture of a γ-irradiated (1 Mrad) soil and of autoclaved silica sand (1/1 by volume) was used. The characters of the soil used in the mixture are shown in Table 1. The mixture was amended with different amounts of P, i.e. 0, 20 and 40 mg kg^{-1} soil dry weight, in the form of $Ca(H_2PO_4)_2 \cdot H_2O$, weighed and mixed in each

Table 1

Chemical analysis of the potting mixtures

Chemische Analyse der Kultursubstrate

	Soil/sand 50/50	Soil/peat/sand 0/50/50	Soil/peat/sand 10/30/60
pH in water	7.5	6.3	6.7
Organic matter (%)	1.27	8.66	4.72
C/N	9.4	n.d.[3])	n.d.
C.E.C.[1]) (meq 100 g^{-1})	20.2	5.5	6.5
$NaHCO_3$ extr. P[2]) (mg kg^{-1})	12	14.2	11.5

[1]) Cation exchange capacity.
[2]) OLSEN *et al.* (1954).
[3]) Not determined.

pot. In this experiment non-mycorrhizal plants were compared with plants inoculated respectively with *Glomus caledonicum* (NICOL. and GERD.) TRAPPE and GERD., *G. constrictum* TRAPPE, *G. occultum* WALKER, and *G. versiforme* (KERSTEN) BERCH.

Experiment 2: The substrate and fertilization treatments of Exp. 1 were used in this case. Non-inoculated plants were compared with plants inoculated with *Glomus fasciculatum* (THAXTER *sensu* GERD.) GERD. and TRAPPE, *G. monosporum* GERD. and TRAPPE, and *Glomus sp.* E3.

Experiment 3: In this experiment two artificial substrates commonly employed in commercial nurseries were used, whose main characters are shown in Table 1. They were made up of a steam-sterilized (1 h at 100 °C, replicated after 5 d) sandy loam soil containing 7 mg kg^{-1} Na bicarbonate extractable P (OLSEN *et al.* 1954), peat and sand (30 min at 120 °C) at the rates 0/50/50 and 10/30/60 by volume. The VAM fungus *Glomus caledonicum*, which proved to be an efficient endophyte in Exp. 1, was used as inoculum.

In all experiments VAM fungi were obtained from 6 months old pot cultures of *Trifolium pratense* L.: 10 g soil containing spores and infected roots was used as inoculum. Plants were grown in a controlled-climate glasshouse at 22 °C ± 2 °C, 60—75 % r.h. and 8 h natural light, whose intensity ranged between 500 and 800 µmol m^{-2} s^{-1}: total photoperiod was extended to 14 h with halide vapour lamps yielding on average 250 µmol m^{-2} s^{-1} at the plants' level. Pots were hand-watered and each was given weekly 20 ml of Knop nutrient solution without P. Leaf widths were measured at regular time intervals and total plant leaf area was calculated using the equations described in a previous paper (SCHUBERT *et al.* 1986). Percent fungal root colonization was assessed at the end of each experiment on roots stained with trypan blue, using the grid intersect method (GIOVANNETTI and MOSSE 1980).

In each experiment pots were randomized and each of the treatments consisted of 5 replicate pots. Data were analyzed as two-factor analysis of variance, using Duncan's test for mean separation.

Results

Experiment 1

All inoculated plants were mycorrhizal at the end of the experiment, with relative root colonization higher than 40 % (Table 2). In non-fertilized soil, non-inoculated plants grew very little (Fig. 1) while mycorrhizal plants reached, 75 d after inoculation, a leaf surface 3 – 5 times that of non-inoculated plants. At the last harvest, leaf surfaces of all inoculated treatments were significantly larger than in uninoculated control plants, although some endophytes, e.g. *G. constrictum*, were less effective in increasing growth than the other ones.

Addition of P fertilizer to the soil increased growth of non-inoculated plants but not of the mycorrhizal ones. At the intermediate fertilization level still most inoculated plants had significantly larger leaf surfaces than uninoculated controls, while differences were no more significant at the highest fertilization level (Fig. 1).

Experiment 2

A similar pattern was observed as in Exp. 1. Roots of inoculated plants were colonized by VAM fungi (Table 2). In the absence of P fertilization non-inoculated plants showed almost no growth, while mycorrhizal plants reached, at the last measurement, leaf surfaces more than 5 times larger (Fig. 2). Addition of P enhanced growth of non-inoculated plants, although significant differences between the latter and the plants

inoculated with *G. fasciculatum* and *G. monosporum* persisted even at the highest fertilization level.

Experiment 3

Relative root colonization was 51 and 59 %, respectively, in the 0/50/50 and in the 10/30/60 mixtures; uninoculated plants were not mycorrhizal. In both substrates non-mycorrhizal plants grew at a rate comparable with that of non-inoculated plants grown in the unfertilized soil/sand mixture in Exps. 1 and 2 (Fig. 3). Inoculation with *G. caledonicum* was effective on plant growth in the mixture containing 10 % soil, while no significant differences were found in the mixture containing only peat and sand.

Table 2

Percent root colonization of grapevine roots in the soil/sand mixture · Averages of inoculated treatments followed by the same letter do not differ significantly at P = 0.05

Prozentsatz der durch Mykorrhizapilze besiedelten Wurzeln in der Boden/Sand-Mischung · Mittelwerte mit denselben Buchstaben unterscheiden sich bei P = 0,05 % nicht signifikant

	P added to the mixture (mg kg^{-1})		
	0	20	40
Experiment 1			
G. caledonium	55 b	56 b	45 b
G. constrictum	63 ab	50 b	60 ab
G. occultum	50 b	54 b	50 b
G. versiforme	85 ab	89 a	96 a
Uninoculated	0	0	0
Experiment 2			
G. fasciculatum	68 a	70 a	62 ab
G. monosporum	51 ab	53 ab	40 ab
G. sp. E3	39 ab	17 b	31 ab
Uninoculated	0	0	0

Discussion

The results of this work confirm previous observations (SCHUBERT *et al.* 1987; RAVOLANIRINA *et al.* 1988) that vesicular-arbuscular mycorrhizal fungi may enhance growth of micropropagated grapevines, as they do in the case of plants obtained as seedlings or rootlings. As expected (HAYMAN 1982), the intensity of the growth response is influenced by genetic and environmental factors, as fungal strain, soil nutrient content and soil type.

Many fungal species are known to form VA mycorrhizae on the same host plant but they vary in their efficiency in increasing plant growth (CLARKE and MOSSE 1981; PLENCHETTE *et al.* 1983). These differences may depend on genetically controlled physiological characters of the fungus, which play a role in the uptake of nutrients from the soil and in their transfer to the host root cells: such characters are e.g. the produc-

tion of extraradical mycelium (ABBOTT and ROBSON 1977) and the activity of alkaline phosphatases (GIANINAZZI-PEARSON and GIANINAZZI 1976). We had this type of results in our experiments, where *G. monosporum* and *G. occultum* significantly increased plant leaf surface at nearly all fertilization levels, while *G. versiforme* did not.

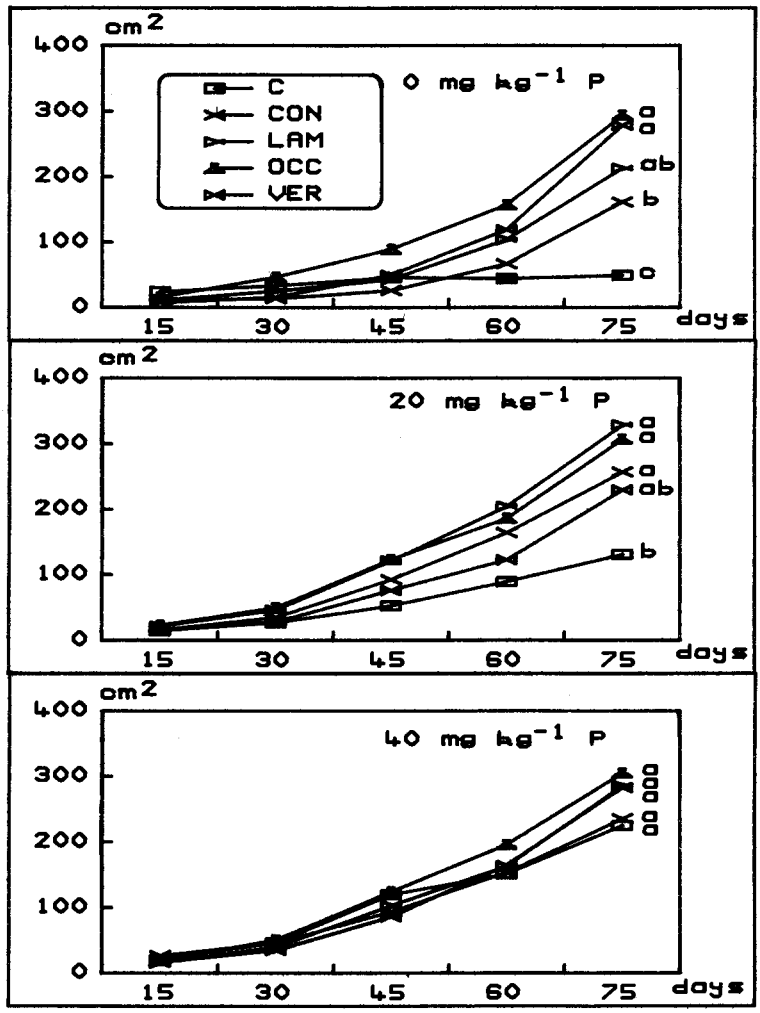

Fig. 1: Total leaf surface per plant (cm^2) at increasing time after transplanting in pots (days) of micropropagated grapevines uninoculated (C) or inoculated with *Glomus constrictum* (CON), *G. caledonicum* (LAM), *G. occultum* (OCC), or *G. versiforme* (VER), in a substrate with 0, 20, or 40 mg kg^{-1} P added (Exp. 1). For the last measurement, averages not followed by the same letter differ significantly at P = 0.05.

Veränderung der Gesamtblattfläche je Pflanze (cm^2) mit zunehmender Verweildauer der *in vitro* vermehrten Reben (d nach dem Umpflanzen) in einem Kultursubstrat, dem 0, 20 oder 40 mg kg^{-1} P zugesetzt wurden (Experiment 1). — C = nichtbeimpfte Kontrolle; CON, LAM, OCC, VER = *Glomus constrictum, G. caledonicum, G. occultum, G. versiforme*. Verschiedene Buchstaben hinter den letzten Meßwerten weisen auf signifikante Mittelwertsdifferenzen bei P = 0,05 % hin.

In this experiment *G. monosporum* and *Glomus sp.* E3 gave large growth responses in soil receiving no P amendment; they were also the most effective endophytes in a previous experiment, where grapevine seedlings were inoculated in the same soil, without P fertilization (SCHUBERT *et al.* 1988). After addition of P fertilizer to the soil, the growth response (i.e. the ratio between leaf area of mycorrhizal plants and leaf area of non-mycorrhizal plants) declined for all endophytes, but remained significant at the highest P level for *G. fasciculatum* and for *G. monosporum*. This suggests that the positive influence of these VAM endophytes on grapevine growth may not be explained by

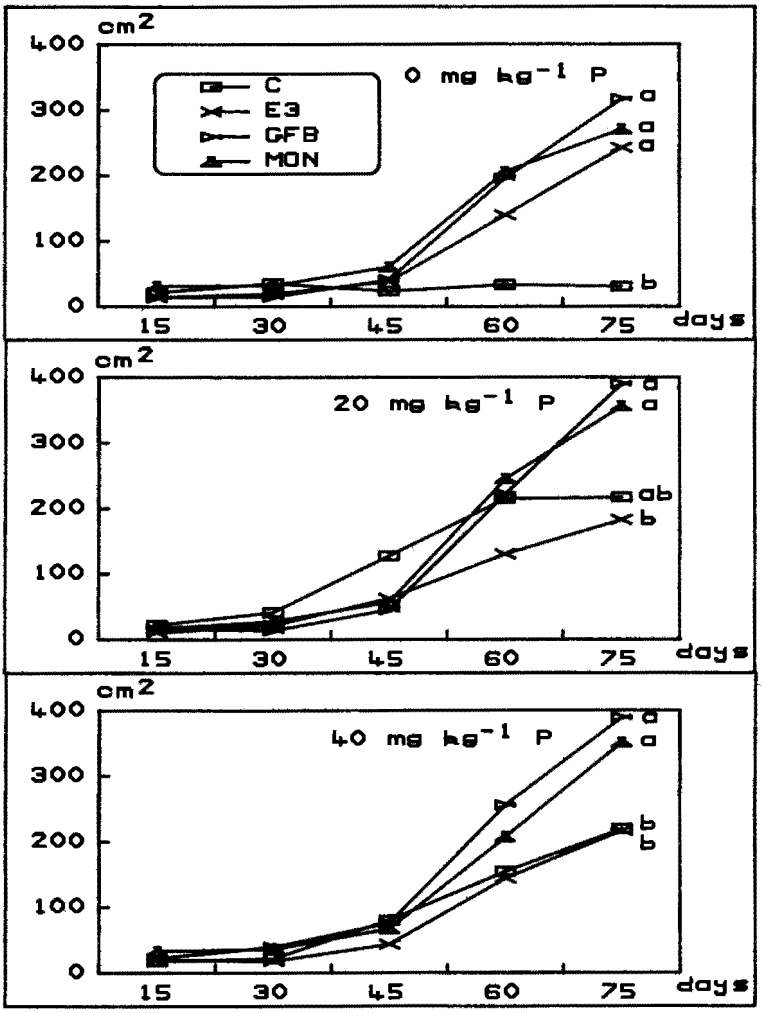

Fig. 2: Total leaf surface per plant (cm^2) at increasing time after transplanting in pots (days) of micropropagated grapevines uninoculated (C) or inoculated with *Glomus sp.* E3 (E3), *G. fasciculatum* (GFB), or *G. monosporum* (MON), in a substrate with 0, 20, or 40 mg kg^{-1} P added (Exp. 2).

Veränderung der Gesamtblattfläche je Pflanze (cm^2) mit zunehmender Verweildauer der *in vitro* vermehrten Reben (d nach dem Umpflanzen) in einem Kultursubstrat, dem 0, 20 oder 40 mg kg^{-1} P zugesetzt wurden (Experiment 2). — C = nichtbeimpfte Kontrolle; E3, GFB, MON = *Glomus* sp. E3, *G. fasciculatum*, *G. monosporum*.

improved P nutrition alone. In other plants, a positive effect of some VAM strains on plant growth when large amounts of P were present in the soil has also been reported (VERKADE and HAMILTON 1985). These findings may be partly explained by the ability of VAM fungi to affect some plant functions, as water uptake and translocation (AUGÉ *et al.* 1986), or susceptibility to root pathogens (DEHNE 1982), independently of P uptake.

The potting medium used affected growth of the mycorrhizal plants. Although the fungus colonized roots in both peat-based media, only in the medium containing soil a significant growth response to inoculation could be observed. This result is in agreement with previous findings, where addition of peat to potting media has been reported to decrease development of VAM fungi and growth response in woody and herbaceous plants (GRAHAM and TIMMER 1984; ZAIJCEK *et al.* 1987). BIERMANN and LINDERMAN (1983) tested the effects of several soil-peat substrates on growth of mycorrhizal clover and found that even low amounts of soil added to peat-based media were sufficient to induce growth responses to VAM inoculation, which were very low in the absence of soil.

Our results show that mycorrhizal inoculation of micropropagated grapevines may be useful for plant growth only if the appropriate endophytes and soil conditions are employed. The production of mycorrhizal inoculum is and will remain an expensive practice, even if new technologies will reduce its costs. Thus the knowledge of the best

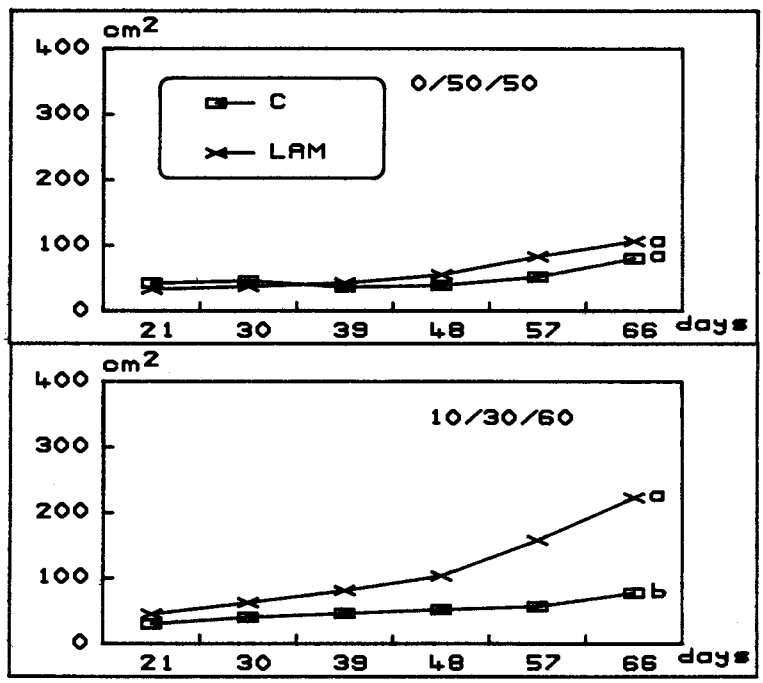

Fig. 3: Total leaf surface per plant (cm^2) at increasing time after transplanting in pots (days) of micropropagated grapevines uninoculated (C) or inoculated with *Glomus caledonicum* (LAM) in mixtures of soil, peat and sand in the ratio 0/50/50 or 10/30/60 (Exp. 3).

Veränderung der Gesamtblattfläche je Pflanze (cm^2) mit zunehmender Verweildauer der *in vitro* vermehrten Reben (d nach dem Umpflanzen) in Erde/Torf/Sand-Gemischen der Zusammensetzung 0/50/50 oder 10/30/60 (Experiment 3). — C = nichtbeimpfte Kontrolle, LAM = *Glomus caledonicum*.

conditions for growth and activity of introduced VAM fungi is of paramount importance, if these microorganisms are to be commercially exploited. If these conditions are fulfilled, inoculation with VAM fungi can be an effective biological alternative to P fertilization, and in some cases it can provide growth enhancements which cannot always be matched by P fertilization.

Summary

Micropropagated plants of *Vitis berlandieri* × *V. rupestris* 1103 P were inoculated during the acclimatization phase with several vesicular-arbuscular mycorrhizal (VAM) fungi, in a sterile substrate amended with increasing amounts of P fertilizer. All VAM fungi increased plant growth in the unamended substrate, but the positive effect of inoculation decreased with increasing fertilization rates. However, two VAM fungi were still effective in increasing plant growth at the highest fertilization level. In a further experiment the effect of inoculation with the VAM fungus *Glomus caledonicum* (NICOL. and GERD.) TRAPPE and GERD. was assessed in a peat/sand mixture amended with 0 and 10 % soil. Although the VAM fungus colonized roots in both cases, a significant growth response was observed only in the substrate containing soil.

Acknowledgement

We thank Dr. A. MARTINELLI of the Micropropagation laboratory, Zanzivivai Ferrara s.r.l., for kindly providing us with micropropagated grapevine plants.

Literature

ABBOTT, L. K.; ROBSON, A. D.; 1977: Growth stimulation of subterranean clover with vesicular-arbuscular mycorrhizas. Austral. J. Agricult. Res. **28**, 639—649.

AUGÉ, R. M.; SCHECKEL, K. A.; WAMPLE, R. L.; 1986: Greater leaf conductance of well-watered VA mycorrhizal rose plants is not related to phosphorus nutrition. New Phytol. **103**, 107—116.

BIERMANN, B.; LINDERMAN, R. G.; 1983: Effect of container plant growth medium and fertilizer phosphorus on establishment and host growth response to vesicular-arbuscular mycorrhizae. J. Amer. Soc. Hort. Sci. **108**, 962—971.

CLARKE, C.; MOSSE, B.; 1981: Plant growth responses to vesicular-arbuscular mycorrhizae. XII. Field inoculation responses of barley at two soil P levels. New Phytol. **87**, 695—703.

CONNER, A. J.; THOMAS, M. B.; 1981: Re-establishing plantlets from tissue culture: a review. Plant Propagators' Soc. Proc. **31**, 342 – 357.

DEHNE, H. W.; 1982: Interactions between vesicular-arbuscular mycorrhizal fungi and plant pathogens. Phytopathology **72**, 1115—1119.

GALZY, R.; 1969: Remarques sur la croissance de *Vitis rupestris* cultivée *in vitro* sur différents milieux nutritifs. Vitis **8**, 191—205.

GEBBING, H.; SCHWAB, A.; ALLEWELDT, G.; 1977: Mykorrhiza der Rebe. Vitis **16**, 279—285.

GIANINAZZI-PEARSON, V.; GIANINAZZI, S.; 1976: Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. I. Effect of mycorrhiza formation and phosphorus nutrition on soluble phosphatase activities in onion roots. Physiol. Vég. **14**, 833—841.

GIOVANNETTI, M.; MOSSE, B.; 1980: An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol. **84**, 489—500.

GRAHAM, J. H.; TIMMER, L. W.; 1984: Vesicular-arbuscular mycorrhizal development and growth response of rough lemon in soil and soilless media: effect of phosphorus source. J. Amer. Soc. Hort. Sci. **109**, 118—121.

HAYMAN, D. S.; 1982: Influence of soils and fertility on activity and survival of vesicular-arbuscular mycorrhizal fungi. Phytopathology **72**, 1119—1125.

KIERNAN, J. N.; HENDRIX, J. W.; STOLTZ, L. P.; MARONEK, D. M.; 1984: Characterization of strawberry plants produced by tissue culture and infected with specific mycorrhizal fungi. HortScience **19**, 883—885.

MENGE, J. A.; RASKI, D. J.; LIDER, L. A.; JOHNSON, E. L. V.; JONES, N. O.; KISSLER, J. J.; HEMSTREET, C. L.; 1983: Interactions between mycorrhizal fungi, soil fumigation and growth of grapes in California. Amer. J. Enol. Viticult. **34**, 117—121.

MURASHIGE, T.; SKOOG, F.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473—497.

OLSEN, S. R.; COLE, C. V.; WATANABE, F. S.; DEAN, L. A.; 1954: Estimation of available phosphorus in soils by extraction with soil bicarbonate. U.S. Dept. Agricult. Circ. 939.

PLENCHETTE, C.; FURLAN, V.; FORTIN, J. A.; 1983: Responses of endomycorrhizal plants grown in a calcined montmorillonite clay to different levels of soil phosphorus. I. Effect on growth and mycorrhizal development. Can. J. Bot. **61**, 1377—1383.

POSSINGHAM, J. V.; GROOT-OBBINK, J.; 1971: Endotrophic mycorrhiza and the nutrition of grape vines. Vitis **10**, 120—130.

RAVOLANIRINA, F.; GIANINAZZI, S.; TROUVELOT, A.; CARRE, M.; 1988: Production of endomycorrhizal explants of micropropagated woody fruit crops. 2nd Eur. Symp. Mycorrhizae, Prague, 14.—20.8.88, 83.

SCHUBERT, A.; CAMMARATA, S.; ARIUSSO, O.; 1986: Inoculation of grapevine plants with VA mycorrhizal fungi in unsterile soil. Proc. 3rd. Int. Symp. Grapevine Physiology, O.I.V., Paris, 303—307.

— — ; — —; EYNARD, I.; 1988: Growth and root colonization of grapevines inoculated with different mycorrhizal endophytes. HortScience **23**, 302—303.

— — ; CRAVERO, M. C.; 1985: Occurrence and infectivity of vesicular-arbuscular mycorrhizal fungi in North-Western Italy. Vitis **24**, 129—138.

— — ; MAZZITELLI, M.; GRIBAUDO, I.; 1987: Effect of inoculation with vesicular-arbuscular mycorrhizal fungi on micropropagated *Vitis berlandieri* × *Vitis riparia* 'Kober 5 BB'. Proc. Symp. Florizel 87 Arlon (Belgium), 144—153.

VERKADE, S. D.; HAMILTON, D. F.; 1985: Effect of *Glomus fasciculatum* or *Glomus mosseae* on growth of *Liriodendron tulipifera* under high fertility. J. Environ. Hort. **3**, 101—103.

ZAJICEK, J. M.; ALBRECHT, M. L.; DANIELS HETRICK, B. A.; 1987: Growth of three native prairie perennials as influenced by phosphorus fertilization, potting media, and mycorrhizae. J. Amer. Soc. Hort. Sci. **112**, 277—281.

Eingegangen am 16. 5. 1989

A. SCHUBERT
Istituto Coltivazione Arboree
dell'Università
Via P. Guria 15
10126 Torino
Italia