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ORIGINAL PAPER

Effects of different N fertilizers on the activity of *Glomus mosseae* and on grapevine nutrition and berry composition

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Abstract Grapevine N fertilization may affect and be affected by arbuscular mycorrhizal (AM) fungal colonization and change berry composition. We studied the effects of different *N fertilizers* on AM fungal grapevine root colonization and sporulation, and on grapevine growth, nutrition, and berry composition, by conducting a 3.5-year pot study supplying grapevine plants with either urea, calcium nitrate, ammonium sulfate, or ammonium nitrate. We measured the percentage of AM fungal root colonization, AM fungal sporulation, grapevine shoot dry weight and number of leaves, nutrient composition (macro- and micronutrients), and grapevine berry soluble solids (total sugars or °Brix*)* and total acidity. Urea suppressed AM fungal root colonization and sporulation. Mycorrhizal grapevine plants had higher shoot dry weight and number of leaves than non-mycorrhizal and with a higher growth response with calcium nitrate as the N source. For the macronutrients P and K, and for the micronutrient B, leaf concentration was higher in mycorrhizal plants. Non-mycorrhizal plants had higher concentration of microelements Zn, Mn, Fe, and Cu than mycorrhizal. There were no differences in soluble solids (°Brix) in grapevine berries among mycorrhizal and non-mycorrhizal plants. However, non-mycorrhizal grapevine berries had higher acid content with ammonium nitrate, although they did not have better N nutrition and vegetative growth.

Keywords Arbuscular mycorrhiza · Grapevine · N nutrition · *Glomus mosseae*

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Introduction

Symbiosis with the arbuscular mycorrhizal (AM) fungi is a common phenomenon observed in nearly 80% of the plant species, and it is known to improve plant nutrition (Powell and Bagyaraj 1984; Smith and Read 1997). The role of AM fungi on plant N nutrition has not been studied as extensively as that for P, but it is now receiving more attention (Hodge et al. 2000; Read and Perez-Moreno 2003). It has been shown that AM fungi directly affect N absorption and N assimilation (Barea et al. 1987), particularly in neutral to slightly alkaline soils (Azcón et al. 2001). Cruz et al. (2004) report that AM fungi may improve plant N nutrition, when grown in nutrient-poor soil, and Mäder et al. (2000) suggested that AM fungal hyphae may contribute substantially to plant N nutrition.

Nitrate ions $\left(NO_3^-\right)$ have higher mobility than ammonium ions $\left(NH_4^+\right)$, and may be delivered to the root via mass flow (Tinker and Nye 2000); therefore, the mycorrhizal system is expected to be more efficient in N uptake and mobility when the N source is NH_4^+ or when NO_3^- mobility is restricted (e.g., dry, sandy, or nutrient poor soils). However, Johansen et al. (1993) have shown that mycorrhizal hyphae have the ability to transfer inorganic N in the form of NO_3^-. In addition, in soils where ecological

conditions are not favoring nitrification, the main N source is NH_4^+. Although the effects of N on mycorrhizal fungal root colonization may vary with P levels (Sylvia and Neal 1990), the form of N may impact both root colonization and external hyphae. Hawkins and George (2001) hypothesized that AM hyphal length decreased with NH_4^+ due to a direct ion effect.

Grapevine is a mycorrhizal plant that is known to benefit from the mycorrhizal symbiosis (Karagiannidis and Nikolaou 1999) and is of high economical and agricultural interest. Grapevine N nutrition may affect grape berry composition, and consequently, must and wine quality (Bell and Henschke 2005); however, longer-term controlled studies that enable harvest of grapevine berries involving mycorrhizae have not been conducted. In addition, inorganic N sources seem to be more important for grapevine nutrition than organic N sources (Cheng and Baumgartner 2004, 2006; Christou et al. 2006).

The aim of the present work was the assessment of how different N fertilizers influence the activity of AM fungi and the investigation of how grapevine nutrition and growth is mediated by AM fungi and different N fertilizers. In addition, we assessed the influence of different N fertilizers and mycorrhiza specifically on grapevine berry composition.

Materials and methods

Plant growth and AM inoculation

The experiment took place in an open field of the Soil Science Institute of Thessaloniki in 1995 and lasted until 1998. Plastic pots of 20-l volume were used, where 1-year-old grapevine plants were transplanted, three plants per pot. The plants were developed as described in Nikolaou et al. (2003). One-node cuttings of *Vitis vinifera*, cv. Razaki, were grafted onto 250 mm cuttings of 110 Richter rootstock using a grafting machine. The cuttings had been surface sterilized with 15% household bleach (i.e., 0.78% NaOCl) and then rinsed with sterile water. The grafts were packed in wet, autoclaved sawdust, and placed in a hot room (25–28°C for 3 weeks) to promote callusing. The grafts were then planted in 100×200 mm plastic bags containing a growing medium consisting of autoclaved 1:1 peat and sand mixture and kept in a glasshouse for 1 month before being transferred outdoors.

The soil used in the experiment was poor in available P (Table 1). It was collected from the top soil (0–20 cm) of the farm of the Agricultural Research Center of Macedonia and Thrace, sieved (2 mm), stored for more than a year at room temperature, and it was not sterilized. Before using, it was examined for mycorrhizal spores with the method of wet sieving (Gerdemann and Nicolson 1963), and no live spores were found.

Table 1 Physicochemical characteristics of the soil used in this experiment

Characteristic	Value
Olsen P (mg kg–$^{-1}$)	3.5
Exchangeable K (mg kg^{-1})	235
B(mg kg^{-1})	0.76
Zn (mg kg^{-1})	1.05
Mn (mg kg^{-1})	25.9
Fe (mg kg^{-1})	6.09
Cu (mg kg^{-1})	2.7
pH (1:1)	7.15
$CaCO_3$ (%)	1.3
EC (μS cm^{-1})	0.65
Organic C (%)	1.65
Sand (%)	55
Silt (%)	27
Clay (%)	18

At the onset of the experiment, each pot received a nutrient solution containing the following nutrients and rates in mg kg^{-1} soil: 25.4 KCl, 29.7 K_2SO_4, 162 $Ca_5(PO_4)_3OH$, 27.6 $MgCl_2{\cdot}6H_2O$, which provide 90, 80, 10 kg ha^{-1}, P, K, and Mg, respectively. Nitrogen was applied in the form of either *amidic (N in urea)*, ammonium $\left(NH_4^+\right)$, nitric $\left(NO_3^-\right)$, or the combination of the last two forms $\left(NH_4^+ + NO_3^-\right)$ in the following quantities in mg kg^{-1} soil: 147.63 $CO(NH_2)_2$, 314.31 $(NH_4)_2SO_4$, 390.5 Ca $(NO_3)_2$, or 190 NH_4NO_3, which provide 200 kg N ha^{-1}. Fertilization with these nutrients and rates is common agricultural practice in the area for table grapes and has also been used by Karagiannidis and Nikolaou (2000). Application of nitrogen was repeated each year in late February to replenish N lost by leaching or otherwise. There was a slight rise in pH with $CO(NH_2)_2$ (=urea), $(NH_4)_2SO_4$, and NH_4NO_3 after their initial application; however, after about a month, the pH returned to the previous levels, and pH was no longer monitored.

Inoculum was applied by placing 30 g in the transplant hole (Hamza 1981). Non-mycorrhizal controls were also used. The inoculum consisted of *Glomus mossae* (Nicol. and Gerd) spores, hyphae and colonized maize roots, and was originally obtained from the collection of Göttingen University and was maintained at the greenhouse of the Soil Science Institute of Thessaloniki on maize roots. Plants were watered every other day at field capacity during the growing season for the duration of the experiment, and no plant protection chemicals were applied. The plants received standard vineyard practices (shoot topping, base leaf removal) and were pruned every year in mid-winter leaving only two spurs, each with two nodes. In addition,

only two flower clusters were allowed to develop per plant at the last year.

Harvest, nutrient analysis, and AM fungal colonization

At the end of the experiment (fall of 1998), the number of leaves per plant and the shoot dry weight (without leaves and berries) were recorded individually for every plant. The leaves were washed, dried at 72°C, and ground using a steel mill. Leaf nutrients were determined by ashing 1 g at 540°C, treating with concentrated HCl acid and using the Kjeldahl method (N), chromatography (P), phlogometry (K), and volumetrically using the Versanate method (Ca and Mg) (Cottenie 1980). The concentration of trace elements Mn, Zn, Fe, and Cu were determined with atomic absorption spectrophotometry (Jackson 1960).

For root AM fungal colonization part of the roots was washed and stained using trypan blue (Riedel-de-Haën, No.32700) (Phillips and Hayman 1970), and the percentage of root length colonization was estimated with microscopy (Krishna and Bagyaraj 1984). By the same way, the roots of control plants were examined for mycorrhizal colonization at the end of experiment. The number of spores per 100 g soil was assessed with the method of wet sieving described by Gerdemann and Nicolson (1963).

The berries were harvested at ripening (around 450 g per cluster), and total soluble solids (°Brix) of the berry were determined using a refractometer. Total acidity, expressed as tartaric acid, was determined by titrating 10 ml berry juice with 0.1 N NaOH from the initial pH of the juice until the final pH 8.2.

Statistical analysis

The experimental design was a completely randomized 2×4 factorial, with four forms of nitrogen, inoculated and non-inoculated plants and four replications with a total number of 32 pots, and 96 plants. The average number per pot was used for all parameters. Mycorrhizal colonization data were arcsine transformed to normalize their distributions. Data were analyzed using the statistical package MSTAT, and comparisons of means were made using the Tukey–Kramer adjustment with an experiment wise error rate at $a=0.05$.

Results

Mycorrhizal colonization

Root colonization of grapevine plants by AM fungi ranged on the average 57–77% (Fig. 1a). Control plants had no AM fungal colonization, and therefore, data are not presented. There was an interaction of nitrogen form and mycorrhizal inoculum for both AM colonization and sporulation (Table 2). Urea had reduced AM root colonization compared to the other N treatments (Fig. 1a) and the lowest sporulation (Fig. 1b), while NO_3–N favored the development of spores.

Plant growth

Mycorrhizal plants had almost twice the shoot dry weight of non-mycorrhizal plants (Fig. 2a), with an interaction of nitrogen form and mycorrhizal inoculum (Table 2), and about 50% more leaves (Fig. 2b). The form of nitrogen had no effect on non-inoculated plants, as there was no difference in dry weight or number of leaves regardless of the N source. Mycorrhizal plants had greater dry weight with NO_3–N, lower with NH_4–N and urea, and intermediate with the combination of NH_4^+ with NO_3^-; mycorrhizal plants treated with $(NH_4)_2SO_4$ had a lower number of leaves compared to other N treatments. The concentration of N was higher in mycorrhizal plants than in the non-mycorrhizal (Table 3) and tended to be higher with NH_4NO_3 or NO_3–N in both mycorrhizal and non-mycorrhizal plants. Mycorrhizal dependency or effectiveness is expressed for any plant growth parameter by the ratio of

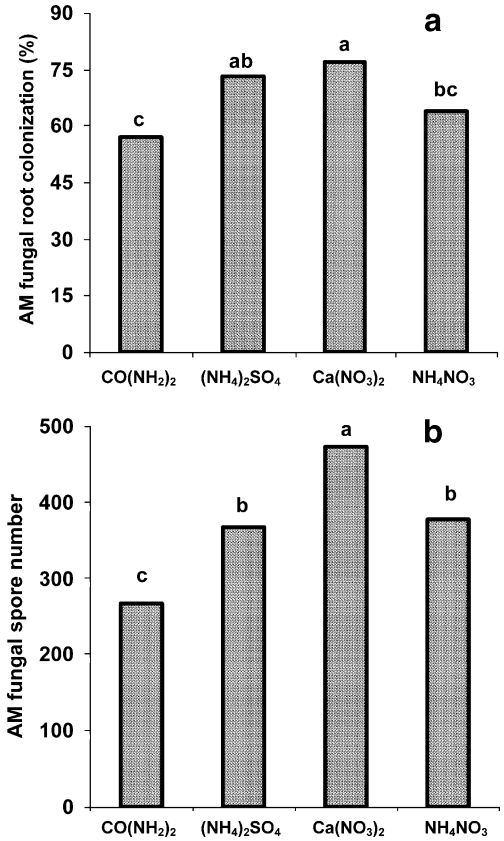

Fig. 1 Percent of arbuscular mycorrhizal fungal root colonization (**a**) and number of spores per 100 g soil (**b**) of arbuscular mycorrhizal grapevine fertilized using four different N fertilizers ($n=4$)

Table 2 ANOVA table (*P* values) for the effects of nitrogen form, AM inoculum, and their interaction on AM root colonization and sporulation, plant growth, macro- and micronutrient concentration, and berry quality parameters

Treatment	Percent RLC[a] (%)	Spore number	Shoot mass	Leaf number	N	P	K	Ca	Mg	B	Zn	Mn	Fe	Cu	Soluble solids	Total acidity
Inoculum (AM)	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0005	<.0001	<.0001	<.0001	<.0001	<.0001	0.7640	0.0111
Nitrogen (N)	0.0096	<.0001	<.0001	0.0045	0.0042	0.0005	0.0123	0.8134	0.0006	0.0002	<.0001	<.0001	0.2334	0.0003	0.0070	<.0001
AM × N	0.0096	<.0001	0.0010	0.4098	0.4474	0.0982	0.7777	0.8724	0.3098	0.0312	<.0001	<.0001	0.6151	0.0898	0.2277	0.0211

[a] Root length colonization

mycorrhizal over non-mycorrhizal plant (M/NM). These ratios, for nutrient concentrations (Table 3), and for shoot dry weight and number of leaves (not shown) reveal that the effectiveness of the AM fungi was the same for all N sources.

The other macronutrients were either higher in mycorrhizal plants (P and K), or the symbiosis did not influence their uptake (Ca, and to an extent, Mg), and were either higher with NO_3^- (as for P), or the N source had no effect (Table 3). For micronutrients, there was an interaction of nitrogen form and mycorrhizal inoculum for B, Zn, and Mn (Table 2). Mycorrhizal plants had higher B, but reduced heavy metal (Mn, Zn, Fe, and Cu) uptake, than non-mycorrhizal plants. In addition, mycorrhizal plants had higher B concentration with NO_3^-, while for Zn, NO_3^- increased concentration in non-mycorrhizal plants. For Mn, concentration decreased with urea in all plants. There was larger variation with N source for micronutrient uptake (Zn, Mn, Cu) for non-mycorrhizal than for mycorrhizal plants.

Grape berry measurements

The AM fungal colonization did not have any significant effects on total soluble solids (Fig. 3a, Table 2). On the contrary, non-mycorrhizal berries tended to have more acids than the mycorrhizal, but this was statistically true for NH_4NO_3 only, and urea had the opposite trend (Fig. 3b). In addition, there was a significant inoculum and nitrogen form interaction for total acidity. Plants were young and productivity was low and irregular. Therefore, we focused only in recording the quality of berries.

Discussion

It has been reported that the nitrogen form (particularly NH_4^+) may influence AM fungal colonization, sporulation, and external hyphae. We and others observed that NH_4–N compared to NO_3–N suppressed AM fungal sporulation and root colonization (Guo et al. 2006; Pereira et al. 1996). However, Hawkins and George (2001) found no difference in root colonization with N form, but decreased hyphal length with NH_4^+. Tu et al. (2006) reported no difference in the percentage of root colonization, but increased total root colonization with N addition. They made the point that initial soil N availability may determine the effects of N to AM fungal colonization. Jumpponen et al. (2005) showed no effect of N fertilization on root colonization by AM fungi, but more abundant coils with N, although there were changes in plant community composition with N application that could confound results. Others reported lower AM fungal diversity and lower AM fungal spores in regions

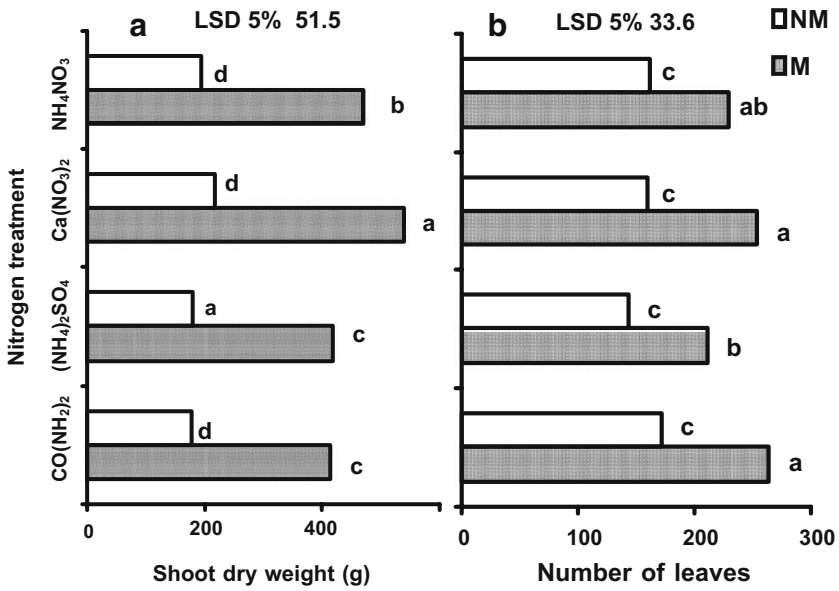

Fig. 2 Shoot dry weight (g) (**a**) and number of leaves per plant (**b**) in arbuscular mycorrhizal (M) and non-mycorrhizal (NM) grapevine fertilized using four different N fertilizers (n=4)

with high N deposition (Egerton-Warburton and Allen 2000).

There are conflicting reports over plant response to different forms of N and how this is influenced by AM fungi. Some researchers report higher mycorrhizal plant growth with NH_4–N (Ortas et al. 1996; Yoshida and Allen 2001), and others with NO_3–N, as in this study (Cuenca and Azcón 1994), or regardless of NO_3–N or NH_4–N being the N source (Guo et al. 2006; Johansen et al. 1993). There are similar conflicts for plant nitrogen uptake: increased with NH_4–N (Tanaka and Yano 2005), independent of the form supplied, or decreased with organic nitrogen (Bennett and Prescott 2004). Although mycorrhizal plants may have better growth with N supplied as NO_3^- than as NH_4^+, they may have increased N concentration with the opposite; this could be a concentration effect (Hawkins and George 2001). Mycorrhizal plants may be more efficient in N utilization than non-mycorrhizal plants (Johansen 1999). There is also evidence that the N source may influence uptake of other nutrients, as we found for most nutrients. Azcón et al. (1992) found higher P, K, and Mg uptake by AM fungal colonized *Lactuca sativa* L. plants with higher

Table 3 Macro (%), micro (mg kg^{-1}) nutrient leaf concentration, and their mycorrhizal efficiency ratio (M/NM) of mycorrhizal (AM) and non-mycorrhizal (non-AM) grapevine plants grown using four different N fertilizers (n=4)

Treatment	Macronutrients (%)					Micronutrients (mg kg^{-1})				
	N	P	K	Ca	Mg	B	Zn	Mn	Fe	Cu
AM										
$CO(NH_2)_2$	3.95abc	0.15bc	1.99abc	2.12a	0.31c	20.7bcd	22.0d	23.0e	102c	3.0cd
$(NH_4)_2SO_4$	3.89abc	0.17bc	2.15a	2.15a	0.42ab	25.4ab	23.5d	60.0d	114bc	4.0c
$Ca(NO_3)_2$	4.28ab	0.23a	2.21a	2.14a	0.43a	29.7a	18.5d	47.5d	116bc	4.5cd
NH_4NO_3	4.40a	0.18ab	2.05ab	2.09a	0.38abc	23.4bc	18.5d	47.0d	116bc	4.0c
Non-AM										
$CO(NH_2)_2$	3.46c	0.10c	1.63d	1.91a	0.29c	17.0d	38.5c	59.5d	141ab	5.0bc
$(NH_4)_2SO_4$	3.27d	0.12c	1.70d	1.88a	0.32bc	19.0cd	53.0b	91.5c	137ab	7.5ab
$Ca(NO_3)_2$	3.78bc	0.13bc	1.84bcd	1.98a	0.38abc	19.8cd	64.5a	154.0a	151a	9.3a
NH_4NO_3	3.54c	0.12c	1.72cd	1.90a	0.30c	19.4cd	48.5b	122.0b	146a	8.5a
M/NM										
$CO(NH_2)_2$	1.14	1.50	1.22	1.10	1.06	1.21	0.57	0.38	0.72	0.60
$(NH_4)_2SO_4$	1.18	1.42	1.26	1.14	1.31	1.33	0.44	0.65	0.83	0.53
$Ca(NO_3)_2$	1.13	1.76	1.20	1.08	1.13	1.50	0.28	0.30	0.76	0.48
NH_4NO_3	1.24	1.50	1.19	1.10	1.26	1.20	0.38	0.38	0.79	0.47

Means followed by the same letter are not statistically different (a=0.05)

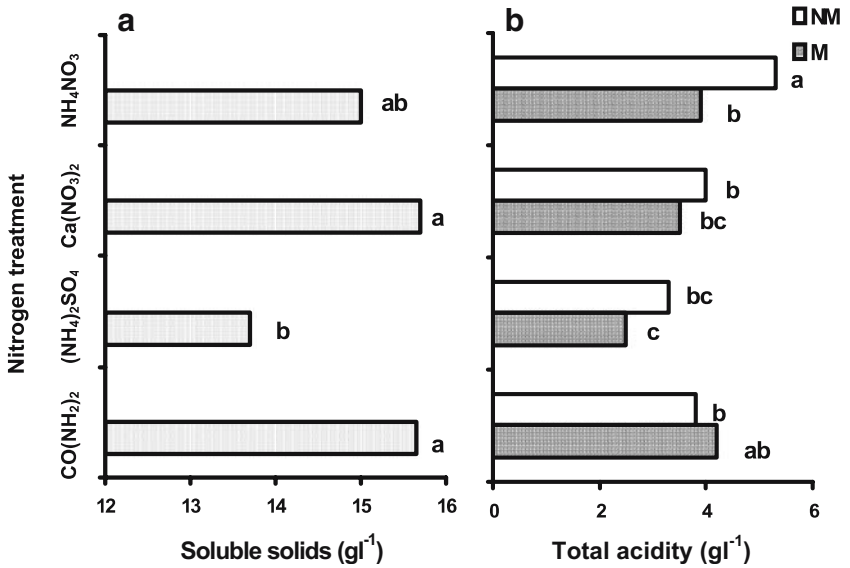

Fig. 3 Soluble solids (g l^{-1}) in grapevine berries as affected by four different N fertilizers irrespective of mycorrhizal status (**a**) (n=8) and total acidity (g l^{-1} as tartaric acid) in grapevine berries of arbuscular mycorrhizal (M) and non-mycorrhizal (NM) grapevine fertilized using four different N fertilizers (**b**) (n=4)

NO_3^- over NH_4^+ ratio. Also, Pereira et al. (1996) reported higher concentrations of K and Ca in mycorrhizal plants with NO_3^- compared to NH_4^+ as N source. Contrary to our results, Bavaresco and Fogher (1996) did not observe any increase in B by AM grapevine.

The differences in effects of various N sources may have to do with plant preferences. For instance, onion plants showed preference for NO_3^- (Guo et al. 2006). Such preference may have to do with differences in plant physiology. Fast growing species may prefer inorganic, and slow-growing species organic N forms (Weigelt et al. 2005). Co-occurring alpine plant species may have the potential to partition soil N on the basis of N form (Miller and Bowman 2002). Plants may differ in the site of nitrate reduction (root or shoot), which may be related to the associated energy cost and carbon economy (energy from photosynthesis in the shoot and oxidative pentose in the root; Andrews et al. 2004), and in the development stage and the environment (Miller and Cramer 2004). Although it has been hypothesized that slow-growing grass species perform NO_3^- reduction in the root, and fast growing grass species in the shoot, it was found that for both plant types, the main site of NO_3^- reduction was the shoot (Scheurwater et al. 2002). Tropical species have been associated with shoot nitrate assimilation, and temperate species, with root nitrate assimilation, while it has been proposed that shoot nitrate assimilation is disadvantageous at low temperature (Andrews et al. 2004). Nitrate assimilation in roots, rather than shoots of forage plants may be an adaptation to grazing (Márquez et al. 2005). Cuenca and Azcon (1994) found that *Erythrina poeppigiana* O.I., a woody tropical plant, preferred NH_4^+ as N source which was attributed to *E. poeppigiana* being a shade plant with limited photosynthesis and a higher theoretical demand in C for NO_3^- assimilation, compared to that of NH_4^+.

Grapevine is usually grafted, and therefore, there are different genotypes in root and shoot. Different rootstocks influence leaf N (Zerihun and Treeby 2002) and absorbed different amounts of P (Nikolaou et al. 2003). According to Schreiner (2003) small differences in the ability to form mycorrhizas exist among ten grapevine rootstocks examined. Mycorrhizal grapevine in this study showed better growth with NO_3^-, a form readily mobile in the xylem that may be stored in vacuoles in the root, or shoot, or storage organs (Marschner 1997). Grapevine roots have a low capacity for NO_3^- assimilation and high nitrate reductase activity (NRA) in leaves, rather than roots (Zerihun and Treeby 2002). Moreover, grapevine leaves have higher levels of Fd GOGAT protein than roots, which is a protein involved in NH_4^+ assimilation (Loulakakis et al. 2002), and in phloem companion cells of grapevine mature leaves and flowers, the enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH), that may be involved in N remobilization and trafficking, have been observed (Paczek et al. 2002).

On the other hand, the AM fungal species involved may shift plant response to the N source applied, relative to non-mycorrhizal controls, (Azcón et al. 1992; Cuenca and Azcón 1994; Guo et al. 2006). Here, we found a change in response to different N sources in micronutrient uptake among mycorrhizal and non-mycorrhizal plants. No interactions between macro- and microelements seem to exist.

More possible is that nitrogen applications and mycorrhiza change the response of plants on absorption of macro- and microelements from soil. Mycorrhizal plants had higher leaf P, K, and B concentrations. Previous works conducted by Mortimer et al. (2005) showed that grapevines inoculated with the fungus *Glomus etunicatum* were more efficient at P utilization. Similarly, Possingham and Groot Obbink (1971) reported that shoot of grapevine seedlings, grown in either unsterilized soil or in sterile soil inoculated with live mycorrhiza, had a significantly higher P content than those without mycorrhizal infection. Caglar and Bayram (2006) studied the effect of five VAM fungi (*G. etunicatum*, *Glomus caledonium*, *Glomus clarum*, *Glomus mosseae*) on four grapevine rootstocks (420 A, 41 B, 1103 P, 'Rupestris du Lot') and found that VAM fungi increased leaf P, but not N and K concentrations. In this study, non-mycorrhizal plants showed higher leaf Zn, Mn, Fe, and Cu concentrations. In contrast, Bavaresco and Fogher (1996) found that the fungus G. mosseae, inoculated on the roots of the grapevine rootstocks 3309 C and 41 B (both lime-tolerant rootstocks) increased Fe and chlorophyll concentrations in the leaves. Increased leaf Zn and Cu concentrations were found in mycorrhizal grapevine rootstocks (*Vitis* sp.) by Petgen et al. (1998). Plant response may change with different AM fungi (Azcón et al. 1992; Guo et al. 2006). Multiple AM fungal species may be more efficient in mediating N transport to the plant than a single species (Tu et al. 2006), and there may be ecotypic differences among AM fungi on their ability to take up N (Hawkins et al. 2000).

Grapevine berry ripening is determined by sugar and acid content. Although the impact of N on sugar concentration of the grapevine berries has been inconsistent, for titratable acidity, there was either no effect, or an increase in effects (Bell and Henschke 2005; Spayd et al. 1994).

According to Waschkies et al. (1994), on replant soil, inoculation of the grapevine rootstock cultivar '5C' with the fungus *G. mosseae* increased shoot length, leaf area, and shoot weight. Nitrogen addition may increase grapevine vegetative growth, creating a competing sink for carbohydrates which may delay berry maturation (Delgado et al. 2004). In addition, higher vegetative growth may increase the number of leaves, and consequently shade the berries, which may also increase titratable acidity (Bell and Henschke 2005). Zerihun and Treeby (2002) speculated that N effects on leaves may flow through to berry physiology. They found that nitrate reductase activity increased in leaves and decreased in roots with N addition, but their experiment did not reach berry harvest. In addition to N, high K levels may also reduce total acidity (Delgado et al. 2004). We found lower acidity in NH_4NO_3 fertilized mycorrhizal grapevine compared to non-mycorrhizal, which did have higher shoot growth and number of leaves, but soluble solids independent of mycorrhizal status. However, mycorrhizal plants had better K nutrition. Previously in an experiment with the same grapevine variety, rootstock, and rate of NH_4NO_3 fertilizer, we found no difference between mycorrhizal and non-mycorrhizal plants in berry total acidity and soluble solids (Karagiannidis and Nikolaou 2000); however, flower clusters allowed and time of harvest were different. Further research is needed on mycorrhizal effects on grapevine berry composition.

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