Vitis **56**, 147–154 (2017) DOI: 10.5073/vitis.2017.56.147-154

Arbuscular mycorrhizal contribution to nitrogen uptake of grapevines

F. Scandellari

Faculty of Science and Technology, Free University of Bolzano-Bozen, Bolzano-Bozen, Italy

Summary

The role of arbuscular mycorrhizal fungi (AMF) in supporting tree nutrition has been recognized for many species, including grapes. This study aimed at determining whether AMF contribute to nitrogen (N) uptake by grapevines using ^{15}N-enriched fertilizer to follow N transfer from the soil to the plant. Grapevines ['Nero'/'SO4'], grown in sand for 10 weeks, were divided into three fertilization treatments: (1) unlabeled NH_4NO_3; (2) $^{15}NH_4{}^{15}NO_3$ provided to root; (3) $^{15}NH_4{}^{15}NO_3$ provided to hyphae. The latter was obtained by splitting the pots in two compartments by a net impenetrable to roots, and adding the fertilizer only where AMF hyphae could develop. The vines were excavated and dry matter, total N and ^{15}N concentration of each organ determined. Root AMF colonization (RLC) was evaluated on fresh roots. The nitrogen derived from fertilizer (N_{dff}) was calculated from the excess of ^{15}N respect to its natural abundance.

Total biomass growth (~37 g/vine) and RLC (38 % on average) were not statistically different among the three treatments. ^{15}N was mostly allocated to roots, shoots and leaves, while trunks were only barely enriched. The vines receiving N directly to roots had higher N concentration and total N than vines relying on AMF, however the amount of N_{dff}, roughly 500 µg·vine^{-1}, was not different between the two treatments. These results indicate that vines growing in the compartmentalized pots might have had an initial shortage of N due to not fully developed AMF. Once the hyphal compartment was colonized, AMF contributed to N translocation to vines, as demonstrated by the same amount of N_{dff} found in the two treatments. Although preliminary, this study demonstrates the potentially important role of AMF to mineral nitrogen nutrition of grapevines and calls for further studies in pot and in the field.

K e y w o r d s : AMF; root colonization; stable isotopes; pot experiment; ^{15}N-enriched fertilizer; mineral nutrition.

Introduction

Arbuscular mycorrhizal fungi (AMF) are symbiont with most terrestrial plant species, including crops such as grapevine (Holland *et al.* 2013, Likar *et al.* 2013, Trouvelot *et al.* 2015). In adequate environmental conditions, AMF obtain carbon from the plant while the fungus transfers nutrients and water to the plant (Smith *et al.* 2010). The transfer occurs through structures created by the AMF within the root cell cytoplasm. Simultaneously, mycorrhizal extra-radical hyphae spread out of the root surface and explore the surrounding soil to penetrate sites that roots are unable to reach, greatly expanding the potential of nutrient and water uptake (Gianinazzi *et al.* 2010). AMF symbiosis has been especially studied in relation to the uptake of phosphorous; however, its role in the uptake, translocation and transfer to the plant of nitrogen is also well established (Hodge and Storer 2015).

Grapevines do not need a particularly high supply of nitrogen (N) (Marenghi 2005); however, a correct balance of N supply is necessary in grapevines to avoid disproportionate vegetative growth and the production of an excessive number of berries that generally translates into a low quality product (Metay *et al.* 2015). Especially in wine grape, nitrogen fertilization is particularly important to maintain a proper level of yeast assimilable nitrogen (YAN) to ensure a good quality of the final product (Palliotti *et al.* 2015).

It is well established that arbuscular mycorrhizal fungi absorb ammonium (Hawkins and George 2001). However, there are indications that nitrogen is not transferred from the extra-radical mycelium to the intra-radical mycelium in this form, but it is first incorporated in the amino acid arginine, one of the molecule classified as YAN (Tian *et al.* 2010). Although it was believed that arginine must be back-converted in NH_4^+ before being transferred to roots (Jin *et al.* 2005), recent evidences show that AM colonization might improve the uptake of certain amino acids, among which arginine, by colonized plants (Whiteside *et al.* 2012). The contribution of AM fungi to the nutrition of grapevines might therefore not only be related to the general nutrient status of the plant as demonstrated for other species, but may also contribute to the optimal ripening of grape berries (Karagiannidis *et al.* 2007).

One of the most interesting techniques to study nitrogen uptake and transfer, also coupled to the action of mycorrhizal fungi, is the application of ^{15}N-enriched nitrogen. Compounds that are known to be absorbed by roots and by mycorrhizal hyphae, such as ammonium nitrate, urea and amino acids, can be obtained with nitrogen containing a concentration of ^{15}N higher than at its natural abundance, which is 0.3663 atom %. This enrichment allows to easily

Correspondence to: Dr. F. Scandellari, Faculty of Science and Technology, Free University of Bolzano-Bozen, Piazza Università 5, 39100 Bolzano-Bozen, Italy. E-mail: francesca.scandellari@unibz.it

follow the fate of the nitrogen introduced into the system. Using labeled glycine, ammonium and nitrate, for example, Gallet-Budynek *et al.* (2009) demonstrated that glycine constitutes a relevant source of nitrogen for temperate ecosystem forests in the USA, especially those dominated by oak, beech and hemlock. More recently, Tomè *et al.* (2015) used double-labeled ammonium nitrate to evaluate the uptake of nitrogen by arbuscular mycorrhizal fungi and its transfer to the associated strawberry plants in a pot experiment. A similar approach had been previously used by Cheng and Baumgartner (2004, 2006) to demonstrate the transfer of nitrogen from cover crops to the associated grapevine mediated by arbuscular mycorrhizal fungi. To my knowledge, no work has been done on the use of ^{15}N-labeled ammonium nitrate to the study of nitrogen uptake by mycorrhizal fungi and its transfer to associated grapevines. This system should provide a useful tool to study mineral nutrition of grapevines and could be further implemented by using different sources of nitrogen, such as amino acids.

The aim of this work was to determine the uptake of mineral nitrogen through the external hyphae of arbuscular mycorrhizal fungi and to compare it with the direct uptake through roots. To distinguish between the nitrogen already present in the substrate-plant system, a mineral fertilizer enriched in the ^{15}N was provided, namely $^{15}NH_4{}^{15}NO_3$. To separate extra-radical mycorrhizal hyphae, pots were split in two compartments by a mesh net allowing the growth of hyphae, but not that of roots.

Material and Methods

Experimental setup: One-year-old grapevines of the cultivar 'Nero' grafted on an 'SO4' rootstock were obtained from a commercial nursery (Wineplant Ltd., Bolzano-Bozen). On August 11, 2015, the vines were transplanted into pots made as described below. Three plants were destructed the day of transplantation to evaluate the time 0 status of the vines. Vines were separated in three treatments, described below, named "^{15}N-to-hyphae", "^{15}N-to-roots" and "control" and excavated on October 21. Vines were randomly assigned to the treatments. In total, there were 7 vines in the ^{15}N-to-hyphae treatment; 3 in the ^{15}N-to-roots treatment and 4 in the control pots. The different number of vines per treatment was due to a technical problem in the climatic chamber, which caused the death of some plants just after the transplanting.

The pots were plastic boxes 266 mm large, 368 mm long and 264 mm deep (suppl. Fig. 1). In the pots allocated to the treatment ^{15}N-to-hyphae, a mesh net was installed so to divide the pot in two compartments, one double as large as the other. In the bigger compartment, a vine was transplanted. The mesh had openings of 50 µm, which allowed the growth of mycorrhizal hyphae into the smaller compartment, but blocked the growth of vine roots. The pots allocated to the treatment ^{15}N-to-roots and those of the control were not provided with the mesh. The pots were filled with sand that was pre-washed with hydrochloric acid (9 M HCl) until a pH of roughly 6.5, the optimal conditions for plant growth. Sand was then thoroughly washed with tap water to eliminate HCl excess.

The vines were left during the experimental period in a climatic chamber. The day/night regime was adjusted to 14/10 h and directed by twelve OSRAM Fluora (58W/77) fluorescent lamps, which were set parallel at 1.18 m distance to the pots. Within the day period, the temperature was kept constant at 24 °C and the relative humidity at 70 %, while during the night period temperature decreased to 19 °C whereas the relative humidity remained at 70 % via a centrifugal humidifier (Carel, HumiDisk65).

The vines were fertilized with the Hoagland solution whose general 1x composition is 2.3 $mmol \cdot L^{-1}$ $(CaNO_3)_2$, 1.7 $mmol \cdot L^{-1}$ K_2SO_4, 0.5 $mmol \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.1 $mmol \cdot L^{-1}$ KH_2PO_4, 20 $\mu mol \cdot L^{-1}$ Fe-EDTA, 1 $mmol \cdot L^{-1}$ NH_4NO_3, 1.75 $\mu mol \cdot L^{-1}$ $CuSO_4$, 12 $\mu mol \cdot L^{-1}$ H_3BO_3, 0.75 $\mu mol \cdot L^{-1}$ $H_3MoO_4 \cdot H2O$, 20 $\mu mol \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 8 $\mu mol \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$; pH is adjusted to 5.5 with HCl. The pots allocated to the treatment ^{15}N-to-roots and those of the control received a 2x Hoagland solution daily for the first 3 weeks and a 0.25x solution for the next 5.5 weeks. The pots allocated to the treatment ^{15}N-to-hyphae received the 2x Hoagland solution daily for the first 3 weeks in the big compartment, then the 0.25x Hoagland solution was delivered daily to both compartments, then for 10 days the 0.25x Hoagland solution was delivered daily only to the small compartment.

On October 10, 2015, a solution of either double ^{15}N labeled (final label was 0.802 ± 0.112 atom %) or unlabeled ammonium nitrate (NH_4NO_3) was prepared to provide each plant with 30 mg of labeled N. The pots allocated to the treatment ^{15}N-to-roots received the ^{15}N labeled solution homogeneously distributed over the sand surface. The pots allocated to the treatment ^{15}N-to-hyphae received the ^{15}N labeled solution in the small compartment, where roots were not allowed to grow. The pots allocated to the control received the unlabeled solution homogeneously distributed over the sand surface.

No other fertilizer was added to plants before the final harvest. During the experimental period, each vine received a total of 850 mg of N, 110 mg of P and 230 mg of S. Soil humidity was checked weekly with a Theta probe (AT Delta-T Devices, Cambridge, UK) at a depth of 10 cm and kept constant throughout the experimental period by adding an appropriate amount of water.

Sample collection and biomass analysis: Plants sampled at transplanting and at the end of the experiment were divided in leaves, shoots, trunk and roots (suppl. Fig. 2). No flowers or fruits developed during the experimental period. The sand from the two compartments was also collected. At the final harvest, only roots grown outside the block of nursery substrate were cleaned and measured, assuming that no new growth occurred within the old substrate. Since both at transplanting and at the final harvest, all roots had a diameter below 2 mm, no further subdivision of root type was made. The whole root biomass was washed under tap water, mixed, and a subsample of roughly a forth was transferred to plastic tubes containing distilled water to evaluate the mycorrhization degree. All

organs were weighted fresh and transferred to a ventilated oven kept at 65 °C until constant weight was reached. Total root dry weight was estimated from the fresh and dry weight of the subsample put in the oven.

Mycorrhization index: Mycorrhizal colonization of roots was evaluated by using a modified staining methodology (Phillips and Hayman 1970) consisting in softening them in 2.5 % (w/v) potassium hydroxide (KOH) in a water bath at 95 °C for 30 min. Roots were then cleared with 3 % hydrogen peroxide for 20 min, stained for 3 min at 80 °C with a solution of 5 % blue ink (Pelikan, 4001) and 5 % acetic acid, flushed with 5 % acetic acid for 20 min at room temperature and fixed in 85 % lactic acid. Once stained, 50 root pieces of 1 cm each were laid on a microscope slide and the presence of mycorrhizal structures (either vesicles or arbuscules) was counted under an optical microscope at a 40x magnification (Leica DMLS, Leica Microsystems Wetzlar GmbH). The mycorrhization index is given as percentage of the total counted root pieces (Trouvelot *et al*. 1986).

Total and labeled nitrogen analysis and calculations: All dried samples, including sand samples, were ground to a fine powder with a ball mill (Retsch), weighted in tin cups and analyzed for total N and ^{15}N using an Elemental Analyzer (EA, Flash 2000, Thermo Scientific) coupled with a Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS, Delta V Advantage, Thermo Scientific). The isotopic values of ^{15}N abundance are expressed as atom % relative to Air (Hayes 2004).

The total N (N_{tot} in mg) contained in an organ was calculated multiplying the concentration of N (N%) of that organ by its dry weight. The total N contained in the vines was calculated as the sum of the N contained in each organ.

The ^{15}N excess (atom %) in the plants was calculated subtracting the natural ^{15}N abundance of vine organs grown in the control pots from the ^{15}N abundance of the respective organs from the vines fertilized with the ^{15}N-enriched fertilizer.

The nitrogen derived from fertilizer (N_{dff}) in tree organs was calculated as follows:

$$N_{dff}(\mu g) = \frac{N_{tot}(mg) \cdot {}^{15}N_{excess}(\text{atom } \%)}{100} \cdot 1000$$

The total amount of N_{dff} found in vines was calculated as the sum of the N_{dff} in the individual organs of each vine. Because of its low and extremely variable values, the trunk was excluded from the calculation of the total.

The total nitrogen absorbed during the growing season was estimated using two methods:

Method 1: the total dry weight of the transplanted plant was estimated from their diameter using a relationship built on the three plants that were harvested on August 17, 2015; the total N of the transplanted plants was estimated by multiplying the estimated dry weight by the weighted average concentration of the three harvested plants; the absorbed nitrogen was calculated as the difference between the total N contained in the plant at harvest on October 21 and the estimated total N contained in transplanted plants on August 17.

Method 2: the absorbed nitrogen was calculated as the difference between the N contained in the individual organs of the harvested plants on October 21 and the average of N contained in the individual organs of harvested plants on August 17.

The advantage of the first method is that it minimizes the error by avoiding calculations using mean values; the advantage of the second method is that it provides the amount of N allocated to each individual organ. The results were comparable, although Method 1 results were systematically higher by 25 % on average than the results given by method 2. The values reported in the Result section are the average between the two methods.

Statistical analysis: Statistical analysis was performed using StatGraphics Centurion XV and the R software (R Core Team 2014). Normality of data was tested with Shapiro-Wilk test and homoscedasticy of variance with Levene's test. Biomass, total N and ^{15}N in sand data were analyzed using the analysis of variance (ANOVA) with the Bonferroni procedure as *post-hoc* test to discriminate among different means. Mycorrhization index, ^{15}N excess in the vines and N_{dff} data were analysed using the Kruskal-Wallis test followed by the Dunn test. Data are reported in tables as average ± standard error.

Results

Biomass: At transplanting, the fresh weight of vines ranged between 450 and 870 g including the nursery substrate. The three excavated vines had a total dry weight of 26.5 ± 1.3 g of which 21 % were of leaves including petiole, 17 % shoots, 53 % above- and below-ground woody parts and 9 % roots (Tab. 1). At harvest, the total dry weight was on average 63.4 ± 2.1 g of which 21 % were leaves including petiole, 22 % shoots, 38 % above- and below-ground woody parts and 19 % roots. No statistical differences between vines allocated to the different treatments were found. The mycorrhization index was relatively variable (Tab. 1) and no statistical difference was detected between vines in the three fertilization treatments.

Total and ^{15}N labeled nitrogen: The concentration of N in vine organs was not different from the beginning to the end of the experiment. Only roots of the ^{15}N-to-roots treatment had a significantly higher N concentration than the roots in the other vines. The weighted average N concentration for the whole harvested vines at transplanting was 1.48 ± 0.10 % (Tab. 2). The total nitrogen content of whole vines was higher at harvest than at transplanting (Tab. 3). Shoots and roots contained a higher amount of N at harvest than at transplanting, while the N content of leaves was statistically higher only in the ^{15}N-to-roots vines. Only a small amount of N was allocated to the trunk so that no significant difference was detected between the end and the beginning of the experiment. No differences in the amount of N contained in vine organs was detected among plants belonging to different treatments at the end of the experiment (Tab. 3). The weighted average of ^{15}N excess in vines was higher in the vines treated with ^{15}N than those

Table 1

Biomass of vines (g·vine^{-1}) at transplanting and at the final harvest and mycorrhization index at transplanting. Data are reported as average ± standard error

		Leaves	Shoots	Trunk	Roots	Total	Mycorhization index (%)
At transplanting		5.6 ± 0.8b	4.6 ± 0.2b	14.0 ± 0.5	2.3 ± 0.2b	26.5 ± 1.3b	N.A.
At harvest	control	12.7 ± 1.6a	13.8 ± 0.7a	24.3 ± 4.0	11.1 ± 1.5a	61.8 ± 5.4a	32 ± 9
	^{15}N-to-roots	13.4 ± 1.0a	14.0 ± 1.4a	25.0 ± 2.8	12.2 ± 2.0a	64.6 ± 5.2a	51 ± 12
	^{15}N-to-hyphae	13.2 ± 0.5a	14.1 ± 0.7a	23.9 ± 2.1	12.5 ± 1.2a	63.7 ± 1.6a	36 ± 3
p-value		0.0015	0.0001	n.s.	0.004	0.0001	n.s.

N.A. = not analyzed; n.s. = not significant for $p < 0.05$.

Table 2

Nitrogen concentration (%) in vine organs at transplanting and at the final harvest. Data are reported as average ± standard error

		Leaves	Shoots	Trunk	Roots
At transplanting		1.98 ± 0.38	1.34 ± 0.06	1.07 ± 0.21	1.45 ± 0.08b
At harvest	control	2.39 ± 0.33	1.09 ± 0.08	0.95 ± 0.17	1.45 ± 0.08b
	^{15}N-to-roots	2.72 ± 0.35	1.67 ± 0.54	1.29 ± 0.45	2.03 ± 0.43a
	^{15}N-to-hyphae	2.02 ± 0.20	1.01 ± 0.08	0.95 ± 0.09	1.26 ± 0.06b
p-value		n.s.	n.s.	n.s.	0.04

n.s. = not significant for $p < 0.05$.

Table 3

Total nitrogen in vine organs (mg·vine^{-1}) at transplanting and at the final harvest. Data are reported as average ± standard error

		Leaves	Shoots	Trunk	Roots	Total
At transplanting		113.1 ± 29.6b	61.0 ± 3.1b	183.6 ± 2.3	33.6 ± 4.2b	391.4 ± 30.9b
At harvest	control	312.4 ± 78.5ab	150.5 ± 14.8a	221.8 ± 39.3	159.4 ± 23.5a	844.2 ± 72.5a
	^{15}N-to-roots	364.0 ± 46.7a	227.5 ± 78.5a	292.8 ± 65.4	230.5 ± 42.8a	1114.9 ± 194.6a
	^{15}N-to-hyphae	265.4 ± 25.0ab	141.8 ± 13.7a	224.7 ± 26.4	155.6 ± 13.5a	787.5 ± 23.2a
p-value		0.0338	0.0009	n.s.	0.0009	0.0007

n.s. = not significant for $p < 0.05$.

of control, but there was no difference between the two groups treated with labeled fertilizer (Fig. 1). In particular, leaves and shoots appeared to be particularly enriched in ^{15}N, while in roots the standard error was very high and likely hindered the differences between treatments (Tab. 4). The ^{15}N excess of the trunk was not statistically different irrespective to treatment.

The total amount of N derived from the fertilizer (N_{dff}) found in the vines treated with the labeled N was the same whether the fertilizer was provided to roots (treatment ^{15}N-to-roots) or to hyphae (treatment ^{15}N-to-hyphae) (Fig. 2). As expected, leaves and shoots clearly allocated the labeled N, while in roots the wide experimental error hindered possible differences (Tab. 5). Trunks clearly did not contain any N_{dff}. The concentration of N in the sand was very low (0.034 ± 0.004 % on average), as it solely derived from the fertilizer (data not reported). In the treatment ^{15}N-to-hyphae, the abundance of ^{15}N of sand was significantly higher in the hyphal compartment than in the root compartment, and this one was not statistical different from the ^{15}N abundance detected in the sand of treatment ^{15}N-to-roots and of the control (Fig. 3).

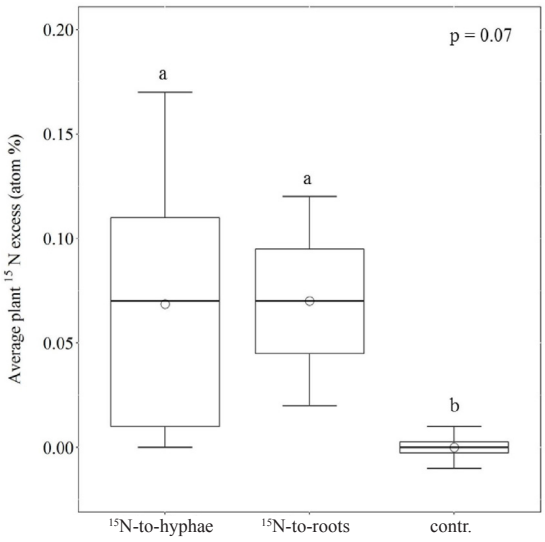

Fig. 1: Weighted average of ^{15}N excess (atom %) of the whole vines. The thick horizontal bars indicate the median while the empty points indicate the average; the box goes from the 25 % to the 75 % percentile and the hinges of the whiskers indicate 1.5 times the inter-quartile range.

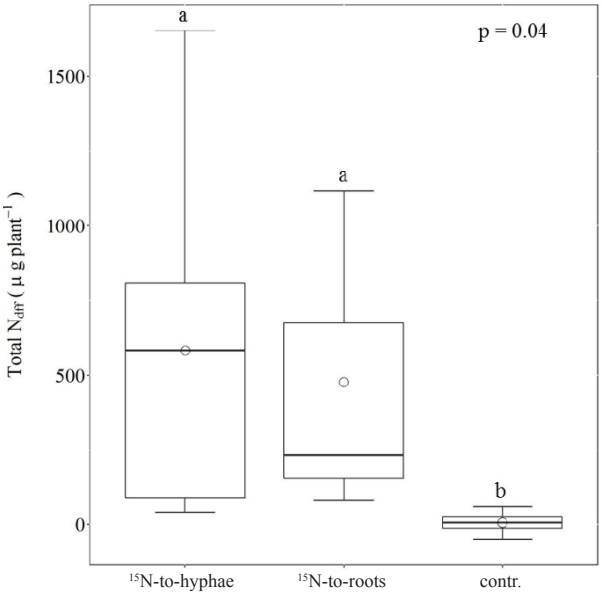

Fig. 2: Total N derived from the labeled fertilizer (N_{dff}). The thick horizontal bars indicate the median while the empty points indicate the average; the box goes from the 25 % to the 75 % percentile and the hinges of the whiskers indicate 1.5 times the inter-quartile range.

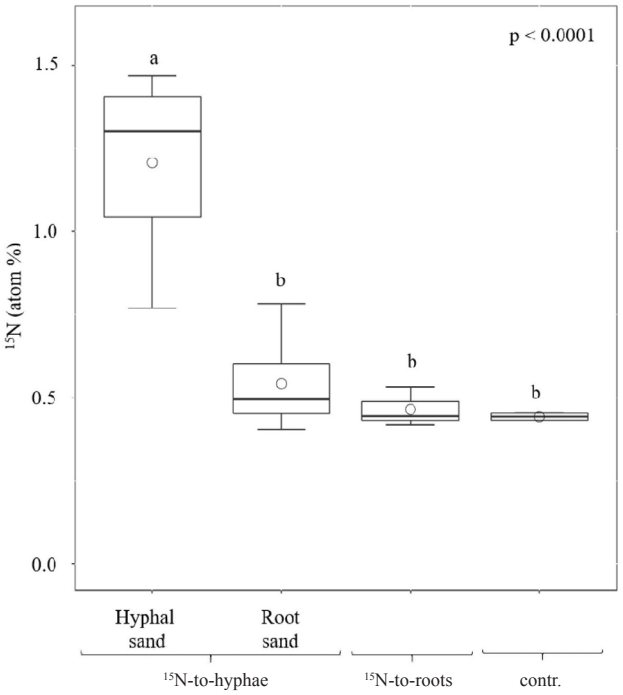

Fig. 3: ^{15}N abundance (atom %) in the sand contained in the different pot compartments. The thick horizontal bars indicate the median while the empty points indicate the average; the box goes from the 25 % to the 75 % percentile and the hinges of the whiskers indicate 1.5 times the inter-quartile.

Discussion

This paper reports a preliminary study aiming at understanding whether the experimental setting would be proper (aim 1) to quantify the contribution of arbuscular mycorrhizal hyphae to the uptake of N and its transfer to grapevines (aim 2). In fact, although similar techniques have been previously used with other plant species (Tomè *et al.* 2015), it has been rarely used for grapevines (Cheng and Baumgartner 2004, 2006).

Appropriateness of the experimental set up: There are essentially two main concerns related to this experimental setting. The first is the efficacy of the mesh net to stop the growth of grape roots and to allow that of mycorrhizal hyphae. In this experiment, AMF were indeed present in relatively high, though highly variable, amounts, as demonstrated by the index of mycorrhization. It is true, though, that the amount of mycorrhizal fungi found in roots does not necessarily relate to the extent of the extra-radical mycelia (Jakobsen 1995, van Aarle and Olsson 2003). This is probably true even in this experiment, as no direct

Table 4

^{15}N excess (atom %) in vine organs at the final harvest.
Data are reported as average ± standard error

		Leaves	Shoots	Trunk	Roots
At harvest	control	0.00 ± 0.00 b	0.00 ± 0.00 b	-0.21 ± 0.11	0.01 ± 0.01
	^{15}N-to-roots	0.07 ± 0.03 a	0.06 ± 0.06 a	0.01 ± 0.03	0.08 ± 0.07
	^{15}N-to-hyphae	0.07 ± 0.02 a	0.09 ± 0.03 a	-0.07 ± 0.06	0.14 ± 0.06
p-value		0.07	0.03	n.s.	n.s.

n.s. = not significant for $p < 0.05$.

Table 5

Nitrogen derived from fertilizer (N_{dff}, µg·vine^{-1}) in vine organs at the final harvest.
Data are reported as average ± standard error

		Leaves	Shoots	Trunk	Roots
At harvest	control	-5.6 ± 12 b	-3.6 ± 3.5 b	-546.4 ± 309.8	14.8 ± 12.0
	^{15}N-to-roots	248.8 ± 124.3 a	68.2 ± 70.2 a	-7.2 ± 74.2	159.9 ± 132.5
	^{15}N-to-hyphae	182.7 ± 63.2 a	147.0 ± 53.1 a	-187.3 ± 159.1	252.7 ± 119.8
p-value		0.03	0.04	n.s.	n.s.

n.s. = not significant for $p < 0.05$.

relationship was found between the mycorrhization index and the amount of N_{dff} found in the vines (data not shown). However, the labeled N was effectively transferred from the hyphal compartment to the vine. This transfer can be due to vine roots growing in the other side of the net, to cross-contamination among pots of different treatments, to abiotic transfer or to mycorrhizal hyphae grown through the mesh net. The growth of vine roots through the mesh net was visually excluded at harvest when no roots were found in the hyphal compartment. The relatively big diameter of grape roots (Anderson *et al.* 2003) and the fact that mesh nets with the same diameter have already been used (Tomè *et al.* 2015, Tomè *et al.* 2016) support the reliability of this observation. Cross-contamination was excluded because the weighted average of ^{15}N in vines in the control plots was not statistically different from the weighted average of ^{15}N vines excavated at transplanting, *i.e.* before any plant was in contact with labeled fertilizer (data not shown).

The abiotic transfer of mineral nitrogen, especially nitrate (NO_3^-), through the net to the root compartment is the second main concern of this technique. To minimize this effect, several actions were undertaken. The pots were slightly slanting so that the hyphal compartment receiving the labeled N was always lower than the root compartment. In this way, possible leaching would move away from the root compartment instead of towards it. In addition, the risk of leaching was kept as low as possible by adding a minimum amount of irrigation water. Finally, the labeled fertilizer was added only at the end of the experiment and the vines were exposed to it for a mere 10 days, enough to be absorbed by vines, but short enough to minimize undesired side effects. In addition, no further fertilizer was added after the addition of the label N to stimulate its uptake. The efficacy of these measures is supported by the fact that no ^{15}N in excess above the natural abundance was found in the sand of the root compartment in the 15N-to-hyphae treatment pots.

Contribution of arbuscular mycorrhizal hyphae to the uptake of N and to its transfer to grapevines: According to these premises, the ^{15}N excess found in vines of the 15N-to-hyphae treatment was transferred through the mycorrhizal network from the sand in the hyphal compartment to the plant. Therefore, in this work arbuscular mycorrhizal hyphae appear to substantially contribute to N uptake, with the same magnitude of roots. This result contrasts with that reported by Cheng and Baumgartner (2006) who reported a low contribution of extra-radical hyphae to N uptake in a field experiment. The authors suggest that their result could reflect the fact that their vines were regularly fertilized. On the contrary, the level of fertilization in this experiment was kept as low as possible precisely to stimulate mycorrhizal development (Cheng *et al.* 2008).

A surprising result was that the amount of N absorbed and transferred to the vine was not statistically different whether supplied to mycorrhizal hyphae or to roots, which necessarily bear mycorrhizal structures inside the root (arbuscules and vesicles) and extruding from the root (extra-radical hyphae). In fact, the expected result would have been a higher uptake from roots and their associated mycorrhizal structures than from extra-radical mycorrhizal hyphae alone. This result might be explained by a root system not particularly healthy and therefore not prone to absorb nutrients, as suggested by Cheng and Baumgartner (2006), due to an inappropriate substrate (pure sand). This explanation, however, is not supported by the visual verification at harvest of the good health of the whole plant and of the root system in particular. Alternatively, roots might have been perfectly functioning and might have absorbed the amount of N necessary to the plant without any contribution from mycorrhizae when the nutrient was supplied directly to roots. A third alternative might be a downregulation of N uptake from roots in presence of active mycorrhizal symbiosis, as it has been demonstrated for phosphate uptake, although there are no direct proofs that this applies also to N (Bücking and Kafle 2015). The data obtained from this experiment were not sufficient to distinguish among these hypotheses; to this aim, further experiments should be performed.

On average, the vines absorbed a good 50 % of the N given as fertilizer. Similar values are reported by Williams (2015), up to 40 %, but with much higher available N (25 g N per vine respect to 0.85 g N per vine in this experiment). In this case, since the substrate was washed sand, the fertilizer was the only source of nitrogen, fact that might justify the relatively high fertilizer use efficiency. Nonetheless, the amount of N_{dff} represented a very low portion of the total N contained in the vines' biomass, 0.1 % on average, with no statistical difference between ^{15}N-to-roots and ^{15}N-to-hyphae plants. However, there is indication of a different allocation of the N_{dff} whether absorbed from roots or from hyphae. In particular, root-absorbed N was all allocated to leaves and scarcely to shoots. On the contrary, the hyphal-absorbed N was equally allocated to shoots and leaves. In both treatments, between 30 and 40 % of N_{dff} remained in the roots, while no N_{dff} was allocated to trunk.

Final remarks: This work was meant to be a preliminary experiment to test the opportunity to use vines grown in pots to study the role of arbuscular mycorrhizal fungi on grapevines mineral nutrition. The experiment highlighted some criticism that must be taken into account in future experiments. First, this experiment lacked of any control of AMF. The substrate was pure sand, likely free of AMF propagules also because of the initial acid wash. However, the roots of the vines were already colonized from the nursery and it was not possible to know if they were naturally colonized or if this was the result of an artificial inoculum. This meant to work with highly variable colonization, which most likely determined the high variability of ^{15}N in vine organs. In addition, there was no knowledge on the species present in the pots, which might have also contributed to the high variability of the results. The absence of a negative control, on the contrary, is an educated choice, due to the knowledge that no vine growing in soils will ever experience a lack of colonization (Smith *et al.* 2011). Experiments with completely non-colonized roots, therefore, produce data that are important to understand the basic physiological mechanisms, but are not transferable to field conditions.

Another limit of this experiment is the use of only young plants, whose results might not be applicable to older

vines. There is no real contraindication to use old plants with this experimental setting, except the dimension of the pots and of the site to keep them, as it occurs for any pot experiment. This last aspect also limited the time allowed for this experiment, since roots rapidly colonized the whole volume allowed. For bigger trees and longer experiments, it is therefore necessary to consider bigger pots to leave enough space for root growth.

Despite these critical points that must be carefully considered when planning similar experiments, the technique allowed to detect the uptake of ^{15}N labeled ammonium nitrate by vines through roots and through mycorrhizal hyphae even though it was given in little amounts and for a short time span. Plans for the future include the use of different nitrogen forms and of artificial mycorrhizal inocula with known composition, in addition to field experiments allowing to verify the results of this experiment in productive vineyards.

Conclusions

The results presented in this paper confirm the role of arbuscular mycorrhizal fungi in nitrogen uptake – specifically NH_4NO_3 – and transfer to grapevine. This knowledge is already established for many species, but the data related to *Vitis vinifera* are scarce. In addition, the technique applied here proved to be applicable also to a woody plant, although young, since most of the reports published up to now consider herbaceous species. This paper therefore provides agronomic and technical support to further studies aiming at understanding the strict and pivotal relationship between grapevine roots and arbuscular mycorrhizal fungi.

Acknowledgements

The author acknowledges Dr. M. Boschiero for helping in vine transplant; Dr. C. Ceccon for isotopic analysis; Dr. E. di Marino for supporting in evaluating mycorrhizal colonization; Dr. L. Cavani and an anonymous reviewer for their valuable suggestions to improve the manuscript. Grapevines were provided by the nursery Wineplant Ltd., Bolzano-Bozen. This research is part of the projects Micovite (project n. I52I13000250005) and Myvine (project n. I52F15000130005), funded by the Free University of Bolzano-Bozen.

References

Anderson, L. J.; Comas, L. H.; Lakso, A. N.; Eissenstat, D. M.; 2003: Multiple risk factors in root survivorship: a 4-year study in Concord grape. New Phytol. **158**, 489-501.

Bücking, H.; Kafle, A.; 2015: Role of Arbuscular Mycorrhizal Fungi in the Nitrogen Uptake of Plants: Current Knowledge and Research Gaps. Agronomy **5**, 587-612.

Cheng, X.; Baumgartner, K.; 2004: Arbuscular mycorrhizal fungi-mediated nitrogen transfer from vineyard cover crops to grapevines. Biol. Fertil. Soils **40**, 406-412.

Cheng, X.; Baumgartner, K.; 2006: Effects of mycorrhizal roots and extraradical hyphae on 15N uptake from vineyard cover crop litter and the soil microbial community. Soil Biol. Biochem. **38**, 2665-2675.

Cheng, X.; Euliss, A.; Baumgartner, K.; 2008: Nitrogen capture by grapevine roots and arbuscular mycorrhizal fungi from legume cover-crop residues under low rates of mineral fertilization. Biol. Fertil. Soils **44**, 965-973.

Gallet-Budynek, A.; Brzostek, E.; Rodgers, V. L.; Talbot, J. M.; Hyzy, S.; Finzi, A. C.; 2009: Intact amino acid uptake by northern hardwood and conifer trees. Oecologia **160**, 129-138.

Gianinazzi, S.; Gollotte, A.; Binet, M. N.; Tuinen, D.; Redecker, D.; Wipf, D.; 2010: Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza **20**, 519-530.

Hawkins, H.; George, E.; 2001: Reduced ^{15}N-nitrogen transport through arbuscular mycorrhizal hyphae to *Triticum aestivum* L. supplied with ammonium vs. nitrate nutrition. Ann. Bot. **87**, 303-311.

Hayes, J. M.; 2004: An Introduction to Isotopic Calculations. Woods Hole Oceanogr. Inst. Woods Hole MA.

Hodge, A.; Storer, K.; 2015: Arbuscular mycorrhiza and nitrogen: implications for individual plants through to ecosystems. Plant Soil **386**, 1-19.

Holland, T. C.; Bowen, P.; Bogdanoff, C.; Hart, M. M.; 2014: How distinct are arbuscular mycorrhizal fungal communities associating with grapevines? Biol. Fertil. Soils **50**, 667-674.

Jakobsen, I.; 1995: Transport of phosphorus and carbon in VA mycorrhizas. In: A. Varma, B. Hock (Eds): Mycorrhiza: structure, function, molecular biology and biotechnology, 297-324. Springer Berlin Heidelberg, Berlin, Heidelberg.

Jin, H.; Pfeffer, P. E.; Douds, D. D.; Piotrowski, E.; Lammers, P. J.; Shachar-Hill, Y.; 2005: The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol. **168**, 687-696.

Karagiannidis, N.; Nikolaou, N.; Ipsilantis, I.; Zioziou, E.; 2007: Effects of different N fertilizers on the activity of Glomus mosseae and on grapevine nutrition and berry composition. Mycorrhiza **18**, 43-50.

Likar, M.; Hančević, K.; Radić, T.; Regvar, M.; 2013: Distribution and diversity of arbuscular mycorrhizal fungi in grapevines from production vineyards along the eastern Adriatic coast. Mycorrhiza **23**, 209-219.

Marenghi, M.; 2005: Manuale di viticoltura: impianto, gestione e difesa del vigneto Edagricole, Bologna.

Metay, A.; Magnier, J.; Guilpart, N.; Christophe, A.; 2015: Nitrogen supply controls vegetative growth, biomass and nitrogen allocation for grapevine (cv. Shiraz) grown in pots. Funct. Plant Biol. **42**, 105.

Palliotti, A.; Poni, S.; Silvestroni, O.; 2015: La Nuova Viticoltura: Innovazioni Tecniche per Modelli Produttivi Efficienti e Sostenibili Edagricole, Milano.

Phillips, J. M.; Hayman, D. S.; 1970: Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. **55**, 158-IN18.

R Core Team; 2014: R: A Language and Environment for Statistical Computing R Foundation for Statistical Computing, Vienna, Austria.

Smith, S. E.; Facelli, E.; Pope, S.; Andrew Smith, F.; 2010: Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. Plant Soil **326**, 3-20.

Smith, S. E.; Jakobsen, I.; Gronlund, M.; Smith, F. A.; 2011: Roles of Arbuscular Mycorrhizas in Plant Phosphorus Nutrition: Interactions between Pathways of Phosphorus Uptake in Arbuscular Mycorrhizal Roots Have Important Implications for Understanding and Manipulating Plant Phosphorus Acquisition. PLANT Physiol. **156**, 1050-1057.

Tian, C.; Kasiborski, B.; Koul, R.; Lammers, P. J.; Bucking, H.; Shachar-Hill, Y.; 2010: Regulation of the Nitrogen Transfer Pathway in the Arbuscular Mycorrhizal Symbiosis: Gene Characterization and the Coordination of Expression with Nitrogen Flux. Plant Physiol. **153**, 1175-1187.

Tomè, E.; Tagliavini, M.; Scandellari, F.; 2015: Recently fixed carbon allocation in strawberry plants and concurrent inorganic nitrogen uptake through arbuscular mycorrhizal fungi. J. Plant Physiol. **179**, 83-89.

Tomè, E.; Ventura, M.; Folegot, S.; Zanotelli, D.; Montagnani, L.; Mimmo, T.; Tonon, G.; Tagliavini, M.; Scandellari, F.; 2016: Mycorrhizal contribution to soil respiration in an apple orchard. Appl. Soil Ecol. **101**, 165-173.

Trouvelot, A.; Kough, J. L.; Gianinazzi-Pearson, V.; 1986: Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de methods d'estimation ayant une signification fonctionnelle. In: V. Gianinazzi-Pearson, S. Gianinazzi (Eds): Physiological and genetical aspects of mycorrhizae, 217-221. INRA Presse, Paris, France.

Trouvelot, S.; Bonneau, L.; Redecker, D.; van Tuinen, D.; Adrian, M.; Wipf, D.; 2015: Arbuscular mycorrhiza symbiosis in viticulture: a review. Agron. Sustain. Dev. **35**, 1449-1467.

Van Aarle, I. M.; Olsson, P. A.; 2003: Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Appl. Environ. Microbiol. **69**, 6762-6767.

Whiteside, M. D.; Garcia, M. O.; Treseder, K. K.; 2012: Amino acid uptake in arbuscular mycorrhizal plants. PLoS One **7**, e47643.

Williams, L. E.; 2015: Recovery of 15N-labeled Fertilizer by Thompson Seedless Grapevines: Effects of N Fertilizer Type and Irrigation Method. Am. J. Enol. Vitic. **66**, 509-517.

Received March 17, 2017
Accepted July 12, 2017