



Effects of the Fractionation of the Nitrogen Fertilization on Root Nitrate Uptake and Vine Agronomic Performance in Pinot Gris Grapevines in a Temperate Climate

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Abstract

The study aims to investigate at a physiological and molecular level the root absorption of nitrogen (N) during the annual cycle of grapevine. The study was performed on potted Pinot Gris plants grafted on Kober 5BB and grown for two consecutive years in a semi-controlled environment (Northeast, Italy). The study compared the response of plants N-fertilized in spring (T1), or in spring and in post-harvest (T2) or no-N fertilized (C). Results showed that under our climate conditions nitrate was taken up by the grapevines when applied both in spring or in spring and post-harvest. The nitrate acquisition in T1 roots is mediated at molecular and physiological level by a higher activation of high-affinity nitrate-transport system to take up nitrate in comparison to no-fertilized plants. Comparing the two N fertilization managements, the dynamic of nitrate uptake rates showed different patterns with an overall late response of High-Affinity Transport System when the application of part of N-fertilization was delayed to post-harvest (T2) in comparison to T1. Nevertheless, during the 2 years of the trial the fractionation of N fertilization applied in spring and post-harvest did not negatively affect the yield and quality parameters of vines. An increase of N concentration in T2 roots after two consecutive growing seasons may suggest that the fractionation can lead to beneficial effects on long period. Results of this work contribute to improve the comprehension of N acquisition in grapevine in order to optimize the use of N inputs in vineyard.

Keywords Affinity transport system · Nitrate influx · Nitrogen · NRT · Root · Transporter

1 Introduction

In agricultural aerobic soils, nitrate is one of the main nitrogen (N) forms that widely sustain N needs of crops. Physiological studies demonstrated the existence of

three mechanisms for nitrate acquisition in higher plants, which are strongly dependent on nitrate concentration in the root external solution (Glass and Siddiqi 1995; Forde and Clarkson 1999). These systems are known as Low-Affinity Transport System (LATS), constitutive High-Affinity Transport System (cHATS) and inducible High-Affinity Transport System (iHATS). When the external

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concentration of nitrate is high (at the mM range, above 1 mM nitrate) the acquisition of nitrate is mainly operated by LATs that contributes linearly to nitrate acquisition and is saturated when root external nitrate concentration is above 50 mM. When the nitrate concentration in the root external solution is very low (in the μM range and up to 0.5–1 mM nitrate) the major contribution to nitrate acquisition derives from the HATSs activity (Crawford and Glass 1998; Dechorgnat et al. 2011).

The nitrate acquisition by HATSs is tightly regulated in plants at the physiological and transcriptional level (Crawford and Glass 1998), and these systems involve one constitutive and several inducible nitrate transporters showing features comparable to the Michaelis–Menten kinetic model. The cHATS is mediated by one nitrate transporter, called NRT1.1 ($K_m = 6\text{--}20\ \mu\text{M}$; Wang et al. 2020), whereas iHATS is operated by nitrate transporters belonging to the NRT2 family ($K_m = 20\text{--}100\ \mu\text{M}$; Crawford and Glass 1998). The higher affinity of NRT1.1 allows the acquisition of nitrate even when its concentration in the external solution is at very low levels (Siddiqi et al. 1990; Aslam et al. 1992; Kronzucker et al. 1995). Under this condition, the nitrate acquisition by NRT1.1 activates the inducible components of iHATS, such as the gene expression of some nitrate transporters and nitrate associate proteins. In particular, some of NRT2 nitrate transporters require the presence of associate proteins (as NAR protein) to mediate the nitrate transport (Kotur et al. 2012; Gu et al. 2014; Liu et al. 2014).

Previous works on cultivated plants showed that the induction of nitrate transporters was responsive to sugars and light, whereas their downregulation was operated by overaccumulation of downstream metabolites (such as ammonium, glutamine and other amino acids; Quesada et al. 1997; Krapp et al. 1998; Forde 2000; Lejay et al. 2008). In maize, the root exposure to nitrate under hydroponic conditions induced the acquisition of nitrate reaching a maximum uptake rate 8 h after the beginning of the nitrate application with the activation at transcriptional level of two NRT2 transporters that mainly contribute to the iHATS (Santi et al. 2003; Liu et al. 2009; Zanin et al. 2015).

The nitrate acquisition in woody plant species has been scarcely investigated in the past years (Cerezo et al. 2000; Sorgonà et al. 2005; Nakamura et al. 2007; Pii et al. 2014; Tomasi et al. 2015; Cochetel et al. 2017; Rossdeutsch et al. 2021), whereas much more attention has been paid to herbaceous species (for a review see Fan et al. 2017; Vidal et al. 2020). The grapevine is one of the woody species most cultivated in the world. Although the effects of N fertilizer doses on the performance of grapevine have been reported (Holzapfel and Treeby 2007; Vrignon-Brenas et al. 2019), the mechanisms involved in root N acquisition and its regulation are still poorly investigated and mainly studied under hydroponic conditions (Yang et al. 2007; Pii et al. 2014;

Tomasi et al. 2015; Cochetel et al. 2017; Rossdeutsch et al. 2021). As observed in other plant species, also roots of grapevine are responsive to the external nitrate concentration through the induction of the high-affinity transport system at physiological and transcriptional level (Pii et al. 2014; Tomasi et al. 2015). The expression profile of two genes coding for high-affinity nitrate transporters (VvNRT2.4A and VvNRT2.4B) and for the accessory protein (VvNAR2.2) showed similar trend to that of the anion uptake (Pii et al. 2014). Moreover, in the grapevine the activation of nitrate uptake mechanisms seems to be strongly dependent on the rootstock and also on the characteristics of the scion (Tomasi et al. 2015).

In viticulture, N fertilization should be considered to optimize the vigor of plants and at the same time to maximize the grape quality. An excess of N fertilization may result in an excess of vegetative vigor compromising the grape quality (Verdenal et al. 2021). Other aspects that greatly influence the N availability in vineyards are linked to management practices, such as the use of cover crops (especially when non-leguminous plants are used) whose N requirements can compete with grapevines for N acquisition. When grapevine grows under N-deficiency stress, the fermentation processes and wine flavors can be also affected. It is well known, in fact, that N supply can affect the amount of Yeast Assimilable N (YAN) in the must, which is a critical parameter that controls the fermentation kinetics in wines (Bell and Henschke 2005). Moreover, the biosynthesis of numerous aroma compounds is dependent on N availability, and consequently, this latter affects the wine sensory profile (Ancín-Azpilicueta et al. 2013; Verdenal et al. 2021). Consequently, N fertilization should achieve a compromise in order to balance qualitative and quantitative aspects of grape yield (Tomasi et al. 2015).

The efficiency of N fertilization has been shown to be largely influenced by the timing of N application (Conradie 1986; Holzapfel and Treeby 2007). In general, N fertilization is recommended during periods of maximum uptake rate of N, that is reported to occur between bud break and veraison, although from harvest until leaf-fall a second peak of N acquisition has been reported (Conradie 1980). Up to date the optimal timing to perform the N fertilization for grapevine is still under debate. Some studies showed that in the early stages of the growing cycle (from bud break to bloom) the grapevine mostly relies on the N reserve rather than on N directly taken up by roots, as the N demand by the new growth could not be completely satisfied by newly absorbed N (Conradie 1986; Holzapfel and Treeby 2007). Thus, N fertilization during the post-harvest period should receive special attention to enable the plant to face the first stages of growth with optimal N reserves. Most of the studies that reported a high N uptake rate in post-harvest were performed in warm climates (Conradie 1980, 1986; Peacock

et al. 1989), whereas this phenomenon is still poorly understood in cooler regions (where the vine growing cycle is shorter). Moreover, it has been shown that under cool climates the mobilization of N into perennial plants is anticipated, starting fairly before the harvest (Schreiner 2016). In these cases, the soil N applications during the late season might be inefficient, as observed by Walker et al. (2022).

Given the lack of information regarding the optimal timing for the best management of N fertilization under Northern Italy climate conditions, we have evaluated two N application timing (spring fertilization versus spring + post-harvest fertilization) in Pinot Gris grapevines and compared the two treatments for: (I) the quality and quantity of production and (II) nitrate acquisition monitoring the uptake rates by HATS and LATS during two consecutive growing seasons. Molecular analyses on target gene expression of nitrate transporters allowed us to detail the response of plants to N treatment. Results here presented may have great relevance for developing selection programs and agronomic practices related to improve the nitrogen acquisition in the grapevine.

2 Materials and Methods

2.1 Plant Material and Growth Conditions

The study was carried out over two growing seasons (2016–2017) in the experimental site of the Research Centre for Viticulture and Oenology (CREA-VE), located in Conegliano, in the Veneto Region, Northeast of Italy (45°51' N–12°15' E). Eighteen 3-year-old Pinot Gris vines (*Vitis vinifera* L.), grafted onto Kober 5BB, were cultivated in plastic pots, volume 45 L, filled with soil (80%–15%–5% of sand–silt–clay in volume, respectively, Supplementary Table S1, Supplementary Figure S1). During winter, pruning vines were adjusted to one cane 10–12 nodes in length and positioned in rows with a spacing of 1 m between vines and 1.5 m between rows (Supplementary Figure S1). In the 2 years of the experiment, the plants were maintained under natural light and environment temperature and were well-watered throughout the vegetative seasons by replenishing the daily water usage by an automatic drip irrigation system. Daily water consumption was monitored through continuously weighing two reference fertilized vines with Laumas Elettronica ISC scales connected to a D1 Flex log 1.9 data-logger (Tecnopenta PD, Italy). The following average water volumes were supplied daily in the two study years: between budding and flowering: 0.6–0.9 L per plant; between flowering and ripe berries: 1.4–1.8 L per plant; between ripe berries and leaf fall: 0.6–0.9 L per plant. For volumes greater than 0.6 L, vines were watered twice or three times per day. All pots were equipped with a white plastic cover to prevent leaching by rainfall.

At the beginning of the trial, the vines were divided in three groups of six vines, and the following treatments were applied: C, control vines without N fertilization; T1, vines fertilized with an amount of 1.7 g N per vine per year only in spring (between budding and berry set); T2, vines fertilized with a total amount of 1.7 g N per vine per year in spring and autumn (1.1 g N per vine between budding and flowering and 0.6 g N per vine in post-harvest period, between harvest and complete fallen leaf stages). In both years, N fertilization was performed supplying 100 mL aqueous suspension containing 2.384 g $\text{Ca}(\text{NO}_3)_2$ to the soil six times between budding and flowering in treatment T1, and four times between budding and flowering plus 2 times in post-harvest in treatment T2 (Supplementary Table S2). Plants of all treatments received equal amounts of granular phosphorus (P) and potassium (K) fertilizers (1.5 g P_2O_5 per vine per year, supplied as granular PK and 1.5 g K_2O per vine per year supplied as granular K_2SO_4). No trimming was performed during the growing season, and standard viticultural practices were applied for disease control. Weather conditions at the experimental site were monitored using a local weather station coupled to a Watch Dog 1400 data logger (Spectrum Technologies, Bridgend, UK).

2.2 Agronomic and Physiologic Measurements

Leaf chlorophyll content was measured as an indicator of the vine N nutritional status. Measures were taken by using a portable Minolta SPAD-502 (Konica-Minolta, Osaka, Japan) chlorophyll meter at 4 different phenologic stages in the two study seasons: flowering, closed-bunches, veraison and harvest.

Measures were taken on 6 fully expanded leaves per vine. At flowering and closed-bunches stages (23 and 33 stages, respectively), the SPAD measure was taken on the leaf opposite to the first bunch, whereas from veraison (35 stage), SPAD measures were taken on the fourth leaf after the last bunch (Porro et al. 2001).

Grapes were harvested at the optimal technological maturity, defined as Total Soluble Solids (TSS) ≥ 19 Brix and Titratable Acidity (TA) ≤ 9 g L^{-1} of tartaric acid for Pinot Gris in the local area. Three replicates of two vines per treatment were hand-harvested, and bunches were counted and weighted electronically with a hanging scale (CH, Kern, Germany) to determine yield per vine and bunch average weight.

Technological analyses (pH, total soluble sugars and titratable acidity) were carried out in the laboratory immediately after the harvest. Thirty berries randomly selected from each replicate was crushed and the must sieved for soluble solids and titratable acidity analysis. Soluble solids were measured by refractometer (Atago PR32) at 20 °C. pH and titratable acidity (expressed as g L^{-1} of tartaric acid

equivalents) were determined using a Micro TT 2022 automatic titrator (Crisson, Barcelona, Spain) by titration with 0.1 N NaOH. Yeast Assimilable Nitrogen was quantified according to Nicolini's method (Nicolini et al. 2004).

2.3 Root Sampling Procedure

To monitor the dynamics of nitrate uptake across the growing season, root sampling was carried out at 6-time points corresponding to the following stages of the vegetative cycle (Coombe 1995): 4, budding; 15, eight leaves separated; 23, flowering; 33, closed-bunches, hard berries still green; 38, ripe berries; 45, 50% fallen leaves. Dates for root sampling and fertilizer application in the two study seasons are reported in the Supplementary Table S2. At each time point, root sampling was performed in the morning between 9:00 AM and 11:00 AM, by collecting three independent biological replicates of roots for each treatment. Each replicate consisted of root tips collected from two vines. Sampling was performed by gently digging the soil with a small scoop taking care not to damage the roots. Three random excavations for each pot were carried out in order to collect a total of 0.8 g of root tips per replicate. Roots were washed from soil residues with distilled water and placed in a solution of CaSO_4 0.5 mM for approximately 15 min. Afterward, samples were removed from the solution and quickly dried with absorbent paper. The roots of each replicate were divided into three parts: two samples of approximately 0.3–0.4 g were weighed and used for nitrate absorption analysis (one for high affinity, one for low affinity). The remaining part was frozen in liquid nitrogen and stored at -80° for the gene expression analysis.

2.4 Measurement of Nitrate Uptake

At each time point, nitrate uptake analysis was performed on three replicates per treatment, immediately after the root sample collection. A total number of 18 root samples, 9 for the determination of the nitrate high-affinity uptake rates and 9 for the determination of the nitrate low-affinity uptake rates, were placed in aerated jars containing an assay solution prepared freshly. For the measurements of the nitrate HATS, 25 mL of assay solution containing 0.5 mM CaSO_4 and 0.1 mM of $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$ was used, whereas for the measurements of the nitrate LATS, 25 mL of assay solution containing 0.5 mM CaSO_4 and 0.5 mM of $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$ was used. Nitrate uptake rates were determined by depletion method, measuring the decrease of nitrate concentration from the assay solutions as described by Rizzardo et al. (2012). Briefly, root samples were submerged in the assay solutions, and every 2 min (during 10 min of assay) each assay solution was sampled (aliquots of 200 μL) for the determination of nitrate concentration. The aliquots

were carefully mixed with 800 μL of salicylic acid (5% w/v in concentrated H_2SO_4) and incubated for 20 min at room temperature following the addition of 19 mL of 2 N NaOH. After cooling, nitrate concentration was determined by measuring the absorbance at 410 nm (Cataldo et al. 1975) with a HP 5975C mass spectrometer and 7693A automatic liquid sampler injector (Agilent Technologies). The net-nitrate uptake rate was expressed as $\mu\text{mol g FW}^{-1} \text{h}^{-1}$ (FW, Fresh Weight), and it was calculated by using a calibration line with the following concentration values of NO_3^- : 0, 0.12, 0.14, 0.16, 0.18, 0.2, 0.24 and 0.3 mM for high-affinity measurements and 0, 0.3, 0.5, 0.7, 0.8, 1.0, 1.2 and 1.5 mM for the low-affinity ones.

2.5 Root Nitrogen Concentration

A root sampling was performed during the dormant period in 2017 and 2018 to determine the stored N concentration. Sampling was performed in February by collecting three independent replicates of roots for each treatment. Each replicate consisted of approximately 10 g of secondary and primary roots collected from two vines. Samples were analyzed by an external laboratory following the Kjeldahl method (Kjeldahl 1883).

2.6 RNA Isolation and Gene Expression Analysis

For 2016 and 2017 growing seasons, expression changes of target transcripts were profiled on root samples (three independent biological replicate for each treatment) by quantitative real-time PCR (RT-qPCR). Total RNA was isolated from the root lyophilized samples; DNase treatment and cDNA synthesis were performed as previously reported (Chitarra et al. 2018). The absence of genomic DNA contamination was checked before cDNA synthesis by qPCR using ubiquitin (*VvUBI*) specific primers of grapevine. The analyzed genes encode for: three high-affinity nitrate transporters of grapevine (*VvNRT2.4A*; *VvNRT2.4B*; *VvNRT2.7*) and one accessory protein required for the NRT2 activity (*VvNAR2.2*). Their expression was previously described by Pii et al. (2014) as four of the most interesting genes involved in nitrate uptake in grapevine roots. The *VvNRT2.4A* and *VvNRT2.4B* genes are homologous to high affinity nitrate transporters *AtNRT2.1* and *AtNRT2.2*, coding for the two main HATS nitrate transporters in Arabidopsis (Pii et al. 2014).

RT-qPCR reactions were carried out in a final volume of 10 μL containing 5 μL of SYBR® Green Master Mix (Bio-Rad Laboratories, Inc.), 5 μM specific primers and 1:10 of diluted cDNA. Reactions were run in the CFX 96 apparatus (Bio-Rad Laboratories, Inc.) using the following program: 10-min preincubation at 95°C , followed by 40 cycles of 15 s at 95°C , and 30 s at 60°C . Each amplification was followed

by melting curve analysis (60–94 °C) with a heating rate of 0.5 °C every 15 s. All reactions were performed with at least two technical replicates. The relative expression of transcripts was quantified after normalization to two housekeeping genes: *VvUBI* and *VvACT*. Oligonucleotide sequences are listed in Supplementary Table S3. Gene expression data were calculated as expression ratio (relative transcript level) to C samples collected at the budding phenological stage (relative gene expression = 1).

2.7 Statistical Analysis

The experiments were performed on three biological replicates ($N=3$). SPAD and nitrate uptake datasets were analyzed by two-way repeated measures analysis of variance (ANOVA) using Newman-Keuls test ($N=3$; p value <0.05). The reports of two-way repeated measures ANOVA are provided as Supplementary Tables S4 and S5. Gene expression datasets and vine production and quality data were analyzed by one-way repeated measures ANOVA and one-way ANOVA, respectively, using Newman-Keuls test ($N=3$; p value <0.05). In both years, the datasets were normally distributed basing on the Shapiro–Wilk test. The Brown–Forsythe test showed the equality of group variances tested.

3 Results

3.1 Climate

The 2016 and 2017 seasonal temperatures were similar. Temperatures were within the average for the area, with no excessive temperature peaks (Table 1). Although there were some differences in the distribution of rainfall during the two growing seasons 2016 and 2017 (data not shown), this did not affect the fertilizer uptake as the water availability was regulated through tailored automatic irrigation.

3.2 Agronomic and Physiologic Measures

During the growing season, an overall decreased of SPAD values was observed in all vines independently to the N treatment and the year. Comparing different N treatments at the same phenological stage, the SPAD measurements

showed significant differences for the phenological stages closed bunches (33) and veraison (35) in both growing seasons (2016 and 2017). The values recorded in the treatment T1 and T2 were similar and higher than those recorded in the control C, whereas no differences were found in the other phenological stages (Table 2).

The production and quality measurements at the harvest showed statistically significant differences among treatments. In particular, the values of grape yield in 2016 and 2017 were higher for T1 (1.6 and 1.2 kg vine⁻¹ yield) and T2 (1.2 and 1.1 kg vine⁻¹ yield) treatments compared to the control (0.9 and 0.5 kg vine⁻¹ yield). Similar trend was observed for cluster weight in 2016 and 2017 with higher values for T1 (91.0 and 111.4 kg vine⁻¹ yield) and T2 (84.6 and 103.7 kg vine⁻¹ yield, Table 3).

Concerning grape quality, no differences were recorded for Total Soluble Solids (TSS), pH and Titratable Acidity (TA), while Yeast Assimilable Nitrogen (YAN) was higher in the T1 and T2 treatments than in the C control.

The vegetative growth (pruning weight) in the second year (2017) was higher in the N treatments (T1 and T2), and they were significantly different from C plants in 2017 (Table 3).

Root samples were collected during the dormant period in February 2017 and 2018 to determine the stored N concentration. In February 2017, non-significant variations in the N concentration were observed among N treatments (data not shown), whereas in February 2018 a significant increase of N concentration in T2 roots was observed in comparison to C or T1 roots (N % (dry weight): 0.88^b in C roots, 0.77^b in T1 roots, 1.2^a in T2 roots, letters refer to significant differences based on F test; $N=3$; p value <0.05).

3.3 Dynamic of Root Net-Nitrate Uptake in Grapevine

Regarding the HATS, the analyses in Fig. 1a, c and Supplementary Table S6 refer to the inducible system iHATS, as it has much greater capacity of nitrate uptake than cHATS (at the μM concentration range of nitrate; Kronzucker et al. 1995). During 2016, at eight leaves separated (15 phenological stage) and flowering (23 phenological stage), C plants showed higher nitrate uptake rates than those recorded for T1 and T2, whereas in the following sampling time a different

Table 1 Average monthly temperature (\pm standard deviations) recorded by the weather station located in the study area in the two growing seasons: from March 2016 to October 2016 and from March 2017 to October 2017

	Year	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Average
Average temperature (°C)	2016	10.0 \pm 2.4	14.4 \pm 2.5	17 \pm 3.8	21.8 \pm 2.6	25.3 \pm 2.2	23.2 \pm 2.6	21.7 \pm 2.2	13.5 \pm 2.1	19.1
	2017	11.7 \pm 3.1	13.6 \pm 3.4	18.6 \pm 3.2	23.5 \pm 2.1	24.4 \pm 1.8	25.2 \pm 3.2	17.7 \pm 2.9	13.9 \pm 2.6	19.2

Table 2 Soil Plant Analysis Development (SPAD values) measured during 2016 and 2017 at different phenological stages (23: flowering, 33: closed bunch, 35: veraison and 38: ripe berries) were investigated in C, T1 and T2 plants

Date	Stage	SPAD values			<i>p</i> value
		C	T1	T2	
26/05/2016	Flowering (23)	29.8±0.2 (A)	33.2±0.6 (A)	33.1±1.0 (A)	ns
15/06/2016	Closed bunches (33)	28.1±0.4 b (A)	33.8±0.5 a (A)	32.6±1.4 a (A)	**
25/07/2016	Veraison (35)	20.2±1.9 b (B)	26.7±1.0 a (B)	24.5±0.9 a (B)	**
25/08/2016	Ripe berries (38)	21.9±0.9 (B)	24.9±0.6 (B)	22.1±0.7 (B)	ns
	<i>p</i> value	***	***	***	
23/05/2017	Flowering (23)	23.0±1.6	26.5±0.5 (AB)	29.3±1.2 (A)	ns
15/06/2017	Closed bunches (33)	22.7±0.3 b	28.4±2.1 a (A)	28.9±1.8 a (A)	*
13/07/2017	Veraison (35)	20.6±1.1 b	25.2±0.4 a (B)	26.0±1.2 a (B)	*
09/09/2017	Ripe berries (38)	23.2±1.2	26.5±0.7 (AB)	24.6±1.0 (B)	ns
	<i>p</i> value	ns	**	**	

Different lowercase letters mean significant differences among treatments within each date (row), while different capital letters (in brackets) mean significant differences among the phenological stages within one treatment (column). Each data represents the mean of three replicates±SE (two-way repeated measures ANOVA, Newman-Keuls method; $N=3$; p value <0.05*, <0.01 **, <0.001 ***)

C, refers to no N-fertilized plants; T1, refers to plants N-fertilized only in spring; T2, refers to plants N-fertilized in spring and in post-harvest

Table 3 Production and quality records at harvest time in season 2016 and 2017. Data are means of three replicates±SE. Different letters mean significant differences among treatments

Year	2016				2017			
Parameter	C	T1	T2	<i>p</i> value	C	T1	T2	<i>p</i> value
Yield/vine (kg)	0.9±0.0 b	1.6±0.0 a	1.2±0.0 a	***	0.5±0.1 b	1.2±0.2 a	1.1±0.2 a	*
Cluster weight (g)	59.9±1 b	91.0±1 a	84.6±2 a	***	68.1±14 b	111.4±8 a	103.7±10 ab	*
Berry weight (g)	1.19±0.03	1.09±0.03	1.02±0.04	ns	0.86±0.10 b	1.23±0.06 a	1.08±0.03 ab	*
TSS (Brix)	21.4±0.1	21.5±0.2	21.9±0.4	ns	22.4±0.3	21.5±1.0	22.6±0.1	ns
pH	3.36±0.06	3.36±0.04	3.35±0.02	ns	3.55±0.02	3.53±0.03	3.56±0.01	ns
TA (g/L)	4.7±0.3	4.2±0.1	4.5±0.1	ns	4.3±0.2	3.7±0.2	4.2±0.1	ns
YAN (mg/L)	54.9±3 b	87.6±5 a	88.0±7 a	**	56.3±2 b	70.7±3 a	60.6±3 ab	**
Pruning weight (kg)	0.15±0.03	0.20±0.00	0.16±0.02	ns	0.06±0.00 b	0.19±0.00 a	0.16±0.00 a	***

The asterisks mean the significative differences between the treatments (ANOVA, Newman-Keuls method; $N=3$; p value <0.05*, <0.01 **, <0.001 ***)

C, refers to no N-fertilized plants; T1, refers to plants N-fertilized only in spring; T2, refers to plants N-fertilized in spring and in post-harvest
TSS Total Soluble Solids, TA Titratable Acidity, YAN Yeast Assimilable Nitrogen

pattern was observed as the highest value was recorded by T1 roots at the 33 phenological stage (closed bunches, hard berries still green). Roots of T2 plants showed a similar trend to those recorded by T1 roots at the beginning of the growing season; thereafter, the nitrate uptake rate reached a peak at the ripe berries phenological stage (38; Fig. 1a). In the following season (2017, Fig. 1c), high values of net-uptake rate of nitrate were again recorded by T1 and T2 plants on average: T1 plants reached the peak of uptake rate at closed bunches stage (33), whereas the T2 plants at the 50% fallen leaves stage (45; Supplementary Table S6). No-fertilized plants (C plants) reached a peak of nitrate uptake at the ripe berries phenological stage (38), although the value was not

significantly different from the rates reached by T1 and T2 at the same phenological stage (Fig. 1c).

Regarding the LATS, in the first growing season (2016, Fig. 1b, Supplementary Table S6) the highest nitrate uptake rate was reached by C plants during flowering stage (23), whereas T1 and T2 plants showed similar uptake rates values at the flowering (23), closed bunches (33) and 50% fallen leaves (45) stages. During 2017 (Fig. 1d, Supplementary Table S6), the nitrate uptake rates by LATS were almost comparable between T1 and T2 plants (with the exception for the ripe berries stage (38) where a significant difference was observed between these two treatments, as the higher value was recorded by T2 plants). Overall low nitrate uptake

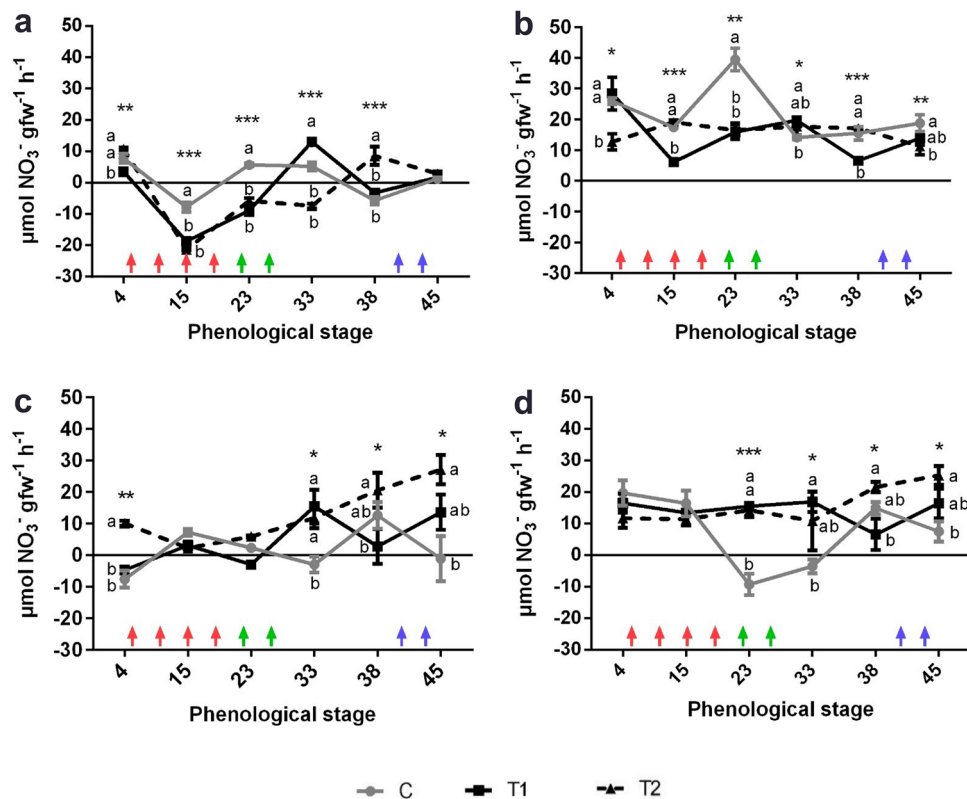


Fig. 1 Root net-nitrate uptake by High Affinity Transport System (HATS, **a** and **c**) and Low Affinity Transport System (LATS, **b** and **d**) measured during the two growing seasons, in 2016 (**a** and **b**) and 2017 (**c** and **d**). The arrows mark the fertilization timing in T1 and T2 (red arrows, fertilization events performed for both T1 and T2 plants; green arrows refer only to T1; purple arrows refer only to T2). Phenological stages of the vegetative cycle: 4, budding; 15, eight leaves separated; 23, flowering; 33, closed bunches, hard berries still green; 38, ripe berries; 45, 50% fallen leaves. Error bars indicate the mean \pm SE ($N=3$). Means followed by different letters differ significantly among treatments within each time point (two-way repeated measures ANOVA, Newman-Keuls method; $N=3$; p value $<0.05^*$, $<0.01^{**}$, $<0.001^{***}$). C, refers to no N-fertilized plants (gray circle); T1, refers to plants N-fertilized only in spring (black square); T2, refers to plants N-fertilized in spring and in post-harvest (black triangle)

rates were shown by C roots, especially at the phenological stage 23 (Fig. 1d).

3.4 Gene Expression in Root Samples

The expression of four genes (involved in nitrate acquisition: three genes coding for nitrate transporters, *VvNRT2.4A*, *VvNRT2.4B*, *VvNRT2.7*; and one gene for an accessory protein, *VvNAR2.2*) was monitored during the growing seasons in 2016 and 2017. During 2016, significant differences between C and T1 were observed at the beginning of the season (Fig. 2). At eight leaves separated stage, the expression of *VvNRT2.4A*, *VvNRT2.4B* and *VvNAR2.2* were upregulated by T1 in comparison to C (Fig. 2a, b, d). In comparison to T1, at eight leaves separated stage, T1 roots did not show significant changes in the expression values of these genes in the following stages (except for a significant reduction in the expression of *VvNRT2.4A* and *VvNAR2.2* at 50% fallen leaves, 45 stage).

ries still green; 38, ripe berries; 45, 50% fallen leaves. Error bars indicate the mean \pm SE ($N=3$). Means followed by different letters differ significantly among treatments within each time point (two-way repeated measures ANOVA, Newman-Keuls method; $N=3$; p value $<0.05^*$, $<0.01^{**}$, $<0.001^{***}$). C, refers to no N-fertilized plants (gray circle); T1, refers to plants N-fertilized only in spring (black square); T2, refers to plants N-fertilized in spring and in post-harvest (black triangle)

In 2017 (similarly to 2016), significant differences between C and T1 of the gene expression were observed mainly at the beginning of the growing season (Fig. 3). Comparing to C roots, T1 treatment induced the upregulation of *VvNAR2.2* at the budding stage (4, Fig. 3d) and of *VvNRT2.4A* at the eight leaves separated stage (15, Fig. 3a). On the other hand, C roots showed a significant higher expression of *VvNRT2.4A* at the budding stage (4) and *VvNRT2.7* at the budding (4) and eight leaves separated (15) stages than in T1 roots (Fig. 3a, c). No other significant changes were observed, except for the upregulation of *VvNRT2.4B* by T1 at the 50% fallen leaves stage (Fig. 3b).

4 Discussion

In the last decades, several works have described the processes involved in the N acquisition in different plant species, and among all N forms, nitrate has received the most attention (for review see Hachiya and Sakakibara 2017;

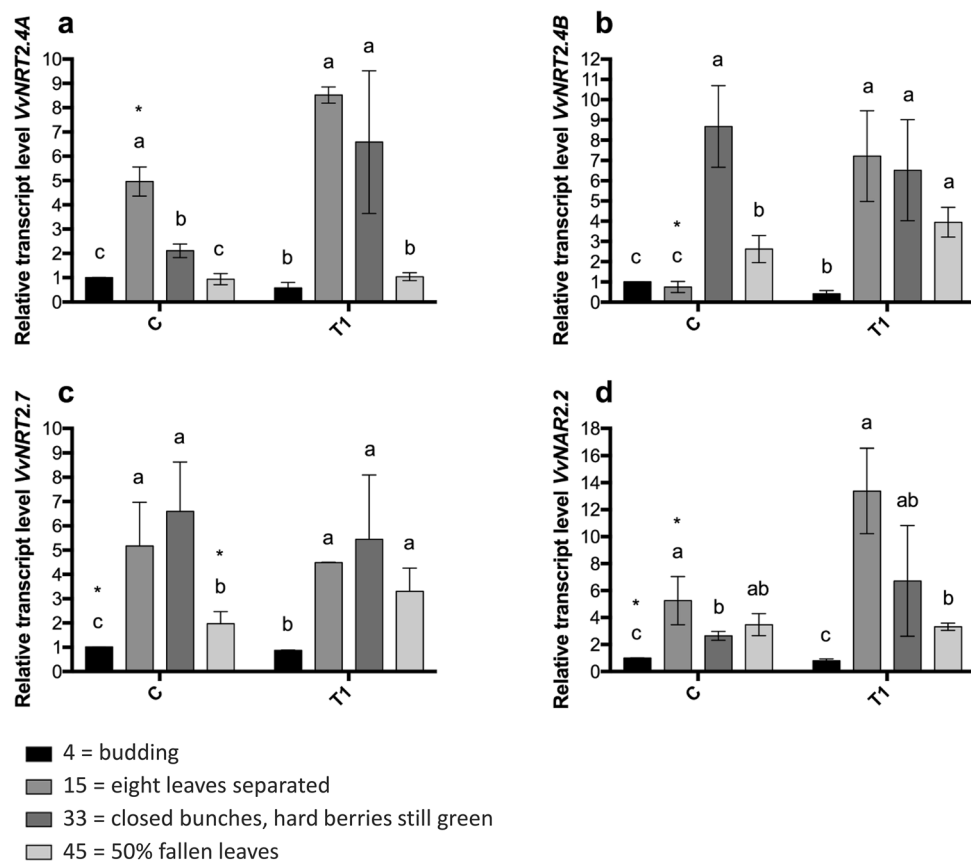


Fig. 2 Relative gene expression level of *VvNRT2.4A* (Nitrate transporter 2.4A, VIT_06s0061g00320, **a**), *VvNRT2.4B* (Nitrate transporter 2.4B, VIT_08s0040g01500, **b**), *VvNRT2.7* (Nitrate transporter 2.7, VIT_14s0066g00850, **c**) and *VvNAR2.2* (Nitrate transporter 2.2, VIT_17s0000g09470, **d**) measured by RT-qPCR in 2016. Root samples were collected during the 2016 vegetative season, and RT-qPCR signals were normalized using *VvACT* (Actin, VIT_04s0044g00580) and *VvUBI* (Ubiquitin, VIT_16s0098g00580), two housekeeping genes. Data are presented as the mean \pm standard error (SE); relative changes in gene transcript levels were calculated on the basis of the

mean transcript level of C sample at budding phenological stage (4, relative transcript level=1). Within each treatment, different letters indicate significant differences between phenological stages (one-way repeated measures ANOVA, Newman-Keuls method; $N=3$; p value < 0.05). C, refers to no N-fertilized plants; T1, refers to plants N-fertilized only in spring. Comparing two treatments (C vs T1), asterisks indicate significant differences between the same phenological stages. Phenological stages of the vegetative cycle: 4, budding; 15, eight leaves separated; 33, closed bunches, hard berries still green; 45, 50% fallen leaves

Vidal et al. 2020). To date, scarce information is available on the timing of N fertilization and on mechanisms involved in the nitrate uptake in grapevines (Yang et al. 2007; Pii et al. 2014; Tomasi et al. 2015; Rossdeutsch et al. 2021). This study was performed on Pinot Gris grapevine, a variety grown all over the world and that, in combination with Kober 5BB rootstock, shows good adaptability to clay soils (very common in the study area). Main objective of this work was the study of the plant response to different timing of N fertilization during two consecutive growing seasons. The response of no-N-fertilized (C) plants was compared with the response of plants N-fertilized only in spring (T1) or plants N-fertilized in spring and in autumn (T2). As it is known that soil moisture affects the soil availability and root acquisition of mineral N forms (Verdenal et al. 2021; Oliveira Stefanello et al. 2020), during the trial (2016 and

2017 years) plants were well-watered and subject to similar temperature conditions throughout the growing seasons. Therefore, we can exclude a strong interference of environmental conditions on plant responses.

In comparison to no-fertilized plants (C treatment), the effect of N fertilization in spring (T1) was well evident in both years, as indicated by an increase of vigor, productivity and quality of T1 grapevines. It is well known that, despite the moderate N requirement of grapevines, conditions of N restriction can reduce the annual biomass production and hence the final yield in comparison to well fertilized vines (Vrignon-Brenas et al. 2019; Zerihun and Treeby 2002). Nitrogen availability can significantly influence berry set and floral bud initiation (Spayd et al. 1993; Keller et al. 2001), which in turn can affect the final yield. This may explain the decreased bunch weight and the related lower

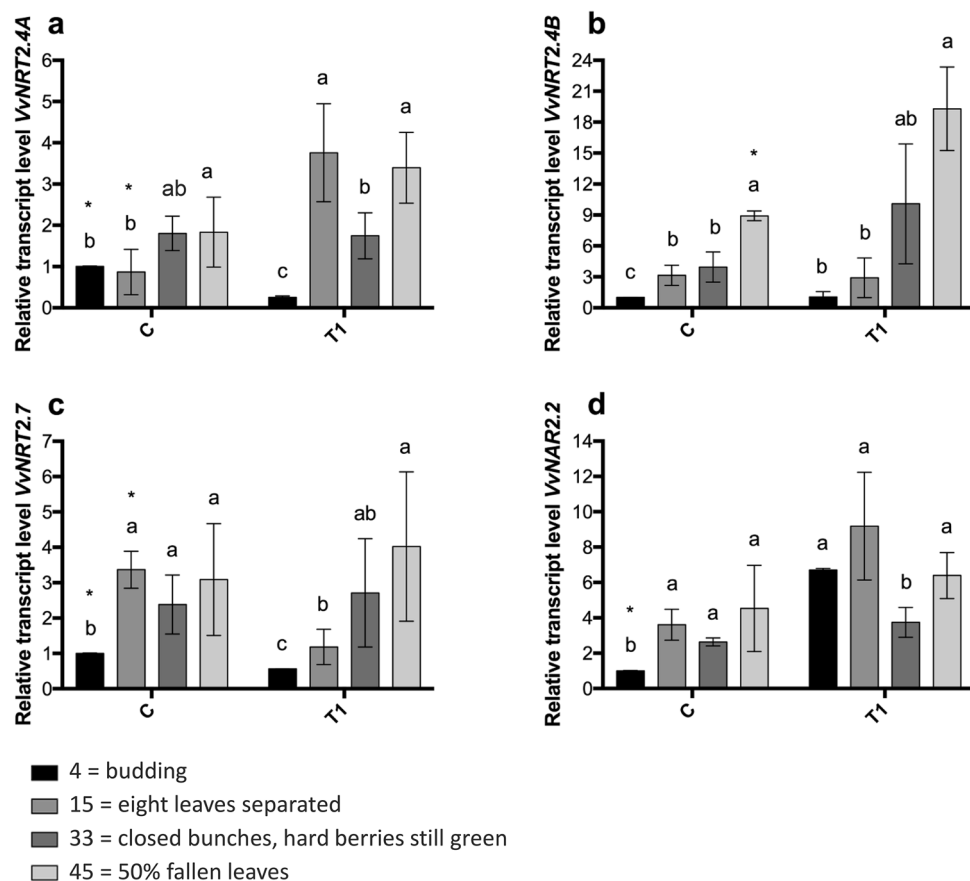


Fig. 3 Relative gene expression level of *VvNRT2.4A* (Nitrate transporter 2.4A, VIT_06s0061g00320, **a**), *VvNRT2.4B* (Nitrate transporter 2.4B, VIT_08s0040g01500, **b**), *VvNRT2.7* (Nitrate transporter 2.7, VIT_14s0066g00850, **c**) and *VvNAR2.2* (Nitrate transporter 2.2, VIT_17s0000g09470, **d**) measured by RT-qPCR in 2017. Root samples were collected during the 2017 vegetative season, and RT-qPCR signals were normalized using *VvACT* (Actin, VIT_04s0044g00580) and *VvUBI* (Ubiquitin, VIT_16s0098g00580), two housekeeping genes. Data are presented as the mean \pm standard error (SE); relative changes in gene transcript levels were calculated on the basis of the

mean transcript level of C sample at budding phenological stage (4, relative transcript level=1). Within each treatment, different letters indicate significant differences between phenological stages (one-way repeated measures ANOVA, Newman-Keuls method; $N=3$; p value < 0.05). C, refers to no N-fertilized plants; T1, refers to plants N-fertilized only in spring. Comparing two treatments (C vs T1), asterisks indicate significant differences between the same phenological stages. Phenological stages of the vegetative cycle: 4, budding; 15, eight leaves separated; 33, closed bunches, hard berries still green; 45, 50% fallen leaves

yields observed in the C vines compared to T1 in both years of the study. In C vines, the bunch weight was 51% and 63% lower than T1 vines in 2016 and 2017 respectively, while yield in C vines was reduced by 77% and 140% in 2016 and 2017, respectively, compared to T1 vines. Overall, these results show that fertilization with 1.6 g N per plant, calculated to be consistent with the conventional N fertilization practice in the area ($40 \text{ kg N ha}^{-1} \text{ year}^{-1}$), is fundamental to achieve the production standards, as yields for T1 treatment were almost double than in C in both years. These results are consistent with previous studies where similar N dosage were applied. Oliveira Stefanello et al. (2020) reported that grapevines of Alicante Bouschet cultivar subjected to $40 \text{ kg N ha}^{-1} \text{ year}^{-1}$ nitrogen supply in southern Brazil presented between two and three times higher yields than the no-fertilized control. Gaiotti et al. (2021) and Tian et al.

(2022) tested $40 \text{ kg N ha}^{-1} \text{ year}^{-1}$ N supply vs a no-fertilized control in Pinot gris and Chardonnay cultivars, respectively. In both studies, yield in control vines was 30% lower than in the N-fertilized ones due to a lower cluster weight.

In addition to the results observed on yields, the fact that in the second year of study the vine growth (pruning weight) remained steady in T1 vines while was significantly reduced in C ones (60% decreased compared to C in 2016) may suggest that further reductions of growth or yield may be expected in the mid or long term. According to this hypothesis, Vrignon-Brenas et al. (2019) demonstrated that N deficiency may influence the vine carbon balance dynamics and consequently the vine growth and production, with effects within and between seasons.

At nitrate concentrations present in soil solution, the high-affinity transport system can have great relevance for nitrate

acquisition in plants. Regarding C plants, high fluctuations in the root capacity to take up the anion were observed during the two consecutive years, and the comparison between 2016 and 2017 does not allow to outline a common trend for both high- and low-affinity transport systems. This observation can be related to multiple factors that can modulate the nitrate acquisition in plants, such as the nitrate availability in pots, the N level and N-assimilates in plants (Vidmar et al. 2000; Miller et al. 2007). Hydroponic studies indicated that in the absence of nitrate in nutrient solution, plants showed low capability to take up the anion (Zanin et al. 2015). It is plausible to suppose that, under soil conditions, the N biogeochemical processes determined fluctuations in the endogenous nitrate concentration in the C treated pots that did not allow to detect a stable trend for the nitrate uptake rate in C plants. On the other hand, the nitrate fertilization of T1 pots determined the induction of nitrate uptake operated by the high affinity transport system. Comparing 2016 and 2017, a similar trend of nitrate uptake rates was observed for T1 plants from flowering (23) to 50% fallen leaves (45), and this trend was similar to those described by Löhnertz (1988) and Verdenal et al. (2021). The highest HATS uptake rates of nitrate were reached by T1 roots in the mid-season (closed-bunches stage, 33) in both years (2016 and 2017) as at this stage, root net-nitrate uptake values by HATS were $13.0 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ in 2016 and $15.4 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ in 2017 (at the same stage, T2 recorded values equal to -7.4 and $11.8 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ in 2016 and 2017 respectively, while C values were 5.2 and $-3 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ in the 2 years). Supporting this observation, even the SPAD index values were significantly higher in the mid-season (at closed bunches, 33, and veraison, 35) for N-fertilized treatments compared to the control.

This result agrees with previous evidence in grapevine, where the acquisition of nitrogenous compounds was needed for optimum flowering and for grape development, reaching a peak of N uptake after flowering stage (23, Linsenmeier et al. 2008; Holzapfel et al. 2019). Moreover, in grapevine, it has been reported that at the end of the growing season even a second peak of N acquisition is expected to occur (from harvest until leaf-fall; Conradie 1980; Löhnertz 1988; Verdenal et al. 2021), and in fact the values of nitrate uptake rates at the end of both growing seasons (50% fallen leaves, 45) were overall higher than those detected in the previous phenological stage (ripe berries, 38). In T1 vines, HAT net-nitrate uptake increased from $-3.2 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ at ripe berries stage (38) to $2.8 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ at fallen leaves (45) in 2016, and from $1.7 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ (38) to $13.6 \mu\text{mol NO}_3^- \text{ gfw}^{-1} \text{ h}^{-1}$ (45) in 2017. A similar increasing trend was not observed in both years for C and T2 treatments. It has been observed that in warm and temperate areas, a reduction in N uptake can be observed during the ripening phase due to the hot and dry conditions that

characterize this period and that promote a decrease in the N availability in the soil (Verdenal et al. 2021). However, in our experiment soil moisture was maintained at optimum levels throughout the growing season; thus, the reduction in root N acquisition observed for T1 treatment at ripening stage (38) is more likely related to root mechanisms involved in the nitrate uptake rather than to nitrate availability in the root external solution.

The overall expression pattern of genes coding for two nitrate transporters and one accessory protein (*VvNRT2.4A*, *VvNRT2.4B* and *VvNAR2.2*) showed higher values in T1 roots than those in C in both growing seasons (2016 and 2017). Some works on different plant species have provided evidence that the presence of NAR2 accessory protein is functional for the activity of NRT2s as nitrate transporters (Zhou et al. 2000; Tong et al. 2005; Okamoto et al. 2006). Similar trend between NAR2 expression pattern and expression values of NRT2.4 s (especially NAR2 and NRT2.4A) might indicate that the translated proteins are active to mediate the nitrate acquisition in plants. As reported by Pii et al. (2014) in short-term experiments, the phenomenon of nitrate induction in grapevine was supported by a concomitant upregulation of *VvNRT2.4A*, *VvNRT2.4B* and the accessory protein *VvNAR2.2* exhibiting a similar trend to that of the anion uptake. In contrast, the expression of *VvNRT2.7* (homologous to *AtNRT2.7*) was quite different from patterns recorded for the other three genes. In agreement with Pii et al. (2014) the expression of *VvNRT2.7* did not seem to be positively modulated by the nutrient availability. Based on experimental evidence of its homologous gene in Arabidopsis, *VvNRT2.7* might play a role in the nitrate accumulation in other organs than roots (e.g. seeds) and in the nitrate storage within vacuoles (Chopin et al. 2007).

Some works have highlighted several advantages linked to post-harvest N fertilization of vineyard (Serdinescu and Suteu 1997; Suteu et al. 2000). When the full dose of nitrate is supplied to vineyard in spring, part of it can be leached in the soil with negative impacts on environment, whereas the temporal separation of N application (in spring and in post-harvest) may be a valid strategy to reduce N losses when nitrate availability exceeds plant needs limiting the pollution of ground water by nitrate. Therefore, in the present work, the effect of spring and post-harvest N fertilization (T2) was evaluated and compared with the effects of a “traditional” N fertilization when N was applied only in spring (T1).

In both years, the levels of yield per plant, yield parameters and vegetative growth (pruning weight) were comparable between the spring and spring–autumn treatments (T1 vs T2). The reduction of N supply in spring in T2 plants was supposed to influence quality parameters (Bell and Henschke 2005); however, our results show that the grape composition and in particular the YAN levels in T2 plants were similar to T1 ones. This suggests that splitting the annual amount

of 40 kg N ha⁻¹ by supplying only two-thirds in spring does not affect negatively the vine vegetative development nor those reproductive processes, such as floral bud formation or berry set, which occur in the spring period and can impact the final vine production.

Comparing the two N fertilization managements, the dynamic of nitrate uptake rates showed different patterns with an overall late response when the application of part of N-fertilization was delayed to post-harvest (T2) in comparison to T1. At the end of both growing seasons (2016 and 2017), the nitrate uptake (rates referred to HATS and LATS) in T2 roots at ripe berries stage (38) was notably higher than in T1 roots. These results indicate a significant effect of N fertilization timing on the capacity of grapevine roots to take up the anion. The measure of N concentration in roots can provide an indication of the N nutritional status of plants available to begin the vegetative season in the following year. Usually, the N refilling in roots is linked to the yield of previous years and occurs during grape maturity and continues until leaf fall (Holzapfel and Treeby 2007; Rossouw et al. 2017). In agreement with data of nitrate acquisition in T2 plants at the 50% fallen leaves (45 stage), a significant increase of N concentration in T2 roots was measured at the end of the second year (at February 2018) suggesting that the N applied in post-harvest can be efficiently stored in roots in temperate climate conditions typical of Northeast Italy. This might contribute to promote a prompt restart of vegetative growth in the following year. It must be pointed out that at this time, fertilizer application is ineffective to alleviate an existing deficiency that arises from insufficient reserve status, because roots absorb very little N before five or six leaves have unfolded on the shoots (Löhnertz 1988). Thus, an enhanced refilling of storage reserves through late season N supply may help preventing detrimental N deficiencies in the following spring.

The fact that higher N accumulation in T2 root reserves was achieved at the end of the second year suggests that benefits from fractionated N fertilization may be seen over the long term.

5 Conclusions

In the present work, application periods of N fertilizer were investigated in mature Pinot Gris grapevines grown in Northeast Italy. Under temperate climate conditions, nitrogen applied both in spring or in spring and post-harvest can be taken up by the vine with good and comparable performance in terms of plant growth and productivity. The nitrogen management determined changes in the dynamic of nitrate uptake during the two consecutive growing seasons, in particular for the nitrate uptake by high affinity transport

system. It is noteworthy that the fractionation of nitrogen fertilizer in spring and post-harvest may lead to beneficial effect on long period as demonstrated by an increase of nitrogen concentration in roots of post-harvest fertilized plants at the end of two growing seasons. As perspective of this work, it might be of interest to evaluate the plant response to the fractionation of nitrogen fertilization setting up long-term experiments to follow the vegetative growth and plant productivity. Results of this work may be useful to define new guidelines for nitrogen fertilization aiming to maximize the root capacity to take up nitrogen applied in vineyard.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing Interests The authors declare no competing interests.

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