Molybdenum-induced effects on nitrogen absorption and utilization under different nitrogen sources in *Vitis vinifera*

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**ABSTRACT**

Nitrogen (N) in different forms has been demonstrated to play significant roles in plants. However, little is known about molybdenum (Mo) effects on N absorption and utilization in grapevine seedlings grown under different N sources. The present study used a sand culture system to analyze the impact of Mo application (0 μM; 1 μM) on N absorption and utilization in grapevine (*Vitis labrusca × V. vinifera* ‘Shine Muscat’ (rootstock 3309 m)) young potted seedlings under different N sources (NO₃⁻, NH₄NO₃ and NH₄⁺). The different N forms and Mo application significantly influenced dry matter accumulation, and root architecture and activity. The effects of Mo on total N content followed the order of (NH₄NO₃ > NO₃⁻ > NH₄⁺). Moreover, Mo and N induced VvMOT1 and VvNRT1.1 expression synergistically. Mo supply altered the utilization of NO₃⁻, NO₂⁻, and NH₄⁺ in grapevines under different N sources. NH₄NO₃ showed the highest effect while NH₄⁺ the least. Furthermore, the 15N-labeling experiment showed that the 15N content in shoot and root and the 15N-use efficiency were the highest after Mo application under NH₄NO₃ source, indicating the synergistic effects of Mo with the co-application of NO₃⁻ and NH₄⁺ sources. The study’s findings provide insights on Mo and N fertilizer utilization for cultivation and production practices in fruits.

1. Introduction

Nitrogen (N) is a primary macronutrient essential for plant function under natural ecosystems and production systems (Marschner 1995; Bell and Henschke 2005; Siddiqui et al. 2012; Rahman et al. 2021). Plants absorb two primary inorganic N forms, nitrate (NO₃⁻) and ammonium (NH₄⁺), from the soil (Li et al. 2013, 2020). NO₃⁻ needs to be converted to NH₄⁺ before it can be assimilated and used by plants (Alamri et al. 2021). NH₄⁺ is assimilated into organic compounds via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. Studies have reported differences in effects on plant physiology among the various N forms. NH₄⁺ as the sole N source reduces N-use efficiency (NUE), root activity, nitrate reductase (NR) and sucrose phosphate synthase activities, overall photosynthesis, and N metabolism (Drath et al. 2008; Zheng et al. 2012; Li et al. 2013). Meanwhile, NO₃⁻ is not only an essential source of nitrogen for plants, but also functions as a signaling molecule. NO₃⁻ stimulates whole plant nutrient transport and assimilation in response to environmental changes (Liu, Niu, et al. 2017). Zhang et al. (2012) reported lower NUE and root activity in tobacco under a floating seedling system with NH₄⁺ than those with NO₃⁻ or urea ((NH₄)₂CO·N) as the N source. Genomics and proteomics have shown that NO₃⁻ regulated the expression of genes encoding NR and a nitrate transporter (NRT1.1) and rapidly activated NO₃⁻ signaling (Wang et al. 2012; Alvarez et al. 2014; Bouguyon et al. 2016). Besides, research has demonstrated complementary effects and plant benefits with the application of a mixture of NO₃⁻ and NH₄⁺ than with either NO₃⁻ or NH₄⁺ source given alone (Britto and Kronzucker 2013; Zheng et al. 2015; Qin et al. 2017; Liu et al. 2019). A good N-absorption efficiency guarantees plant growth, development, and yield (Hirel et al. 2001; Kraiser et al. 2011; Xu et al. 2011). Several studies have positively correlated chlorophyll content, photosynthesis, and plant biomass with N availability (Liu et al. 2018). Still, less studies have reported the changes in these effects of different N sources in the presence of other important minerals (such as molybdenum) in plants.

Molybdenum (Mo) is a trace element essential for plants (Arnon and Stout 1939; Mulder 1954; Siddiqui et al. 2021; Alamria et al. 2022). It is a key catalytic component of the NR molybdenum cofactor (Moco) with a crucial role in NO₃⁻ metabolism in higher plants (Mulder 1954; Kaiser et al. 2005; Mendel and Schwarz 2011; Tejada-Jiménez et al. 2013). However, excessive amounts of N and phosphate fertilizers with inadequate trace elements and organic fertilizers have resulted in soil acidification, which tends to bring about Mo deficiency problem (von Uexküll and Mutert 1995; Wang et al. 2002; Gao et al. 2016). Mo deficiency is common in plants grown in well-drained soils, acidic soils, and iron oxide-rich soils (Kaiser et al. 2005). Almost 70% of arable land in the world is acidic, while nearly 4 million ha of cultivable land in China is deficient in Mo (Wang et al. 2002). The traditional Mo application practices are the foliar application (0.02–0.05%) during the flowering period or period of plants showing symptoms of Mo deficiency(which is often confused with N deficiency). In China, the soil available molybdenum content of the Southwest producing area and the Vitis amurensis of Northeast producing area even below 0.1 mg kg⁻¹. The Arid and Semi-arid production area in the Loess Plateau,
the Middle and Lower reaches of the Yellow River producing area and the Southern producing area were at the level of 0.10–0.15 mg kg\(^{-1}\). The threshold value of soil Mo deficiency is 0.15 mg kg\(^{-1}\) (Liu 2017). Therefore, there is an urgent need to correct Mo deficiency. Therefore, Mo deficiency is often associated with N stress (Dijkshoorn and Ismunadji 1972), increased NO\(_3\) concentration (Kaiser et al. 2005), and reduced plant biomass and yield (Kovács et al. 2015).

The deficiency of Mo mainly affects the biosynthesis of Moco, present in Mo enzymes, such as NR, sulfite oxidase (SO), aldehyde oxidase (AO), xanthine dehydrogenase (XDH), and the mitochondrial amidoxime-reducing component (mARC). In plants, the enzyme NR catalyzes the conversion of NO\(_3\) to nitrite (NO\(_2\)); it regulates molybdate transporters (MOT1, molybdate transporter type 1) and NRTs at the transcript level (Sun et al. 2015; Liu et al. 2020) and plays a vital role in N fixation and assimilation (Mendel and Schwarz 2011). Studies have shown that the deficiency of Mo causes symptoms, such as leaf yellowing (Sun et al. 2009; Gao et al. 2016), small taproots and lateral roots (Gao et al. 2016), and irregular chloroplasts and unclear membrane structures (Liu et al. 2020), which are noticeably similar to N deficiency symptoms (Mulder et al. 1959). Studies have reported impaired MOTI expression, molybdate (MoO\(_4^{2-}\)) absorption, and NR activity in MOTI-deficient mutants. The atmot1;2 Arabidopsis mutants had lower NO\(_3\) levels and NR activity than the wild-type plants (Gasber et al. 2011). These earlier findings suggested that Mo deficiency significantly affects N acquisition and assimilation. Meanwhile, a mutant deficient in NRT1.1 showed decreased NO\(_3\) absorption into guard cells (Wang et al. 2012), suggesting that regulating NRT1.1 may influence photosynthesis and N acquisition. However, no study has reported the effects of Mo application on plant Mo concentration and N absorption and its utilization in the presence of different N forms.

Understanding the physiological changes and the mechanisms improving photosynthesis through efficient N acquisition and utilization in grapevines with Mo application will contribute to less fertilizer use and better fruit quality. Therefore, the effects of Mo application with different N forms/sources on Mo absorption, chlorophyll content, root morphology and activity, and nitrate metabolism in grapevines were investigated. The study’s findings will help understand the correlation between Mo and N and provide a scientific basis for rational N and Mo fertilization programs.

2. Materials and methods

2.1. Experimental materials and treatment

The study was conducted at the Shandong Academy of Grape of the Shandong Academy of Agricultural Sciences, Jinan (Shandong, China; 36°40’N, 117°4’E). Grafted (3309 m rootstock) grapevine seedlings (Vitis labruscaxV. vinifera ‘Shine Muscat’) of uniform size obtained from Shan-Juxian Grape Research Institute were planted in plastic pots (23 cm × 21.5 cm; black) containing sand and maintained in a greenhouse at 25–28°C/5–10°C day/night temperatures, 55%–65% relative humidity (RH), and natural daylight. Seedlings of uniform size (3–4 leaves, 300 seedlings) in pots were selected after two months and cultured in a modified Hoagland solution containing dicyandiamide, a nitrification inhibitor (7.0 µM) that prevents NH\(_4\)\(^+\) oxidation. The modified Hoagland solution contained N (7.14 mmol/L total N), P, and K in a 1:0.85:1.1 ratio with other nutrients including 493 mg L\(^{-1}\) MgSO\(_4\), 2.86 mg L\(^{-1}\) H\(_2\)BO\(_3\), 30 mg L\(^{-1}\) Fe-EDTA, 2.13 mg L\(^{-1}\) MnSO\(_4\), 0.05 mg L\(^{-1}\) ZnSO\(_4\)+H\(_2\)O, and 0.05 mg L\(^{-1}\) CuSO\(_4\)+5H\(_2\)O. The seedlings were grown with different N sources: nitrate alone (NO\(_3\)), co-provision of nitrate and ammonium (NH\(_4\)NO\(_3\)), 50:50, and ammonium alone (NH\(_4\)), with (1 µmol L\(^{-1}\), +Mo) or without Mo (0 µmol L\(^{-1}\), −Mo) (Table S1). Here, Na\(_2\)MoO\(_4\)-2H\(_2\)O was used as the Mo fertilizer. Fifty replicate seedlings were grown under each condition. The nutrient solution was replaced every seven days, and the experiment was carried out for 50 days, and the grapevine seedlings were harvested with 5–8 leaves.

2.2. Measurement of growth parameters, root morphology, and activity

The whole seedling was separated into shoots and roots and dried first at 105°C for 30 min and then at 80°C for five days. The shoot and root dry weight and the total plant dry weight were recorded.

The roots were thoroughly washed, and the morphological features were determined using the WinRhizo image analysis system (V4.1 c; Regent Instruments, Quebec, Canada). Root activity was measured following the TTC (2,3,5-triphenyltetrazolium chloride) method (Liu et al. 2020).

2.3. Determination of Mo concentration, chlorophyll content of leaves, amino acid, and soluble protein content

Fresh grapevine samples (5-8 leaves) were separated into roots and shoots, dried to constant weight, and ground to a fine powder using a pulverizer. The powder was digested with 5 mL of 65% nitric acid (v/v) overnight, and the ash was dissolved in 2 mL of 30% hydrogen peroxide (H\(_2\)O\(_2\), v/v). The Mo concentration in the sample was analyzed using a mass spectrometer (NexION™ 300 ICP-MS system; PerkinElmer, Waltham, MA, USA) following the method by Filipiak-Szok et al. (2014).

Chlorophyll was extracted from leaves with 50 mL of 80% aqueous acetone, and the absorbance values at 645 and 663 nm were measured using a spectrophotometer to determine the chlorophyll a and b concentrations (Liu, Xiao, et al. 2017).

The amino acid concentrations were examined using a Ninhydrin (triketohydridine hydrate) Assay Kit (Comin, Suzhou, China) by absorption spectrometry. The sample solution (1 mL) was ground in 1 mL acetate buffer (2 mmol L\(^{-1}\), pH 5.4) and 1 mL ninhydrin, which was incubated for 15 min in a boiling water bath and then cooled in water. After 5 min, the above mixture was diluted with 3 mL 60% ethanol. The absorbance of the mixture at 570 nm was read using a spectrometer.

The soluble protein contents were analyzed using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). The sample solution (0.1 mL) was ground in 2 mL BCA detection reagent and incubation for 30 min at 37°C. After cooling to room temperature, the sample absorbance was determined at 562 nm for the measurement of the soluble protein content.
2.4. Measurement of total N content, nitrate (NO$_3^-$), and ammonium (NH$_4^+$) concentrations

The total N content was measured using the Kjeldahl method (Ding et al. 2017).

The NO$_3^-$ concentrations in the shoot and root samples were determined using the specific kits (Comin, Suzhou, China). Plant samples (1 g) were transferred to a 10 mL test tube and about 5 mL of water was added, which was then incubated for 30 min in a boiling water bath. The homogenates were centrifuged at 12,000g for 20 min after cooling and the supernatant was transferred to a tube to measure the NO$_3^-$ content using the salicylic acid method.

The NH$_4^+$ concentrations in the shoot and root samples were measured by the specific kits following the manufacturer’s guidelines (Comin, Suzhou, China). The reaction mixture contained 0.1 mL filtrate, 0.01 mL 100% K–Na tartrate, 2.4 mL redistilled water, and 0.1 mL Nessler reagent. A spectrophotometer was used to measure the absorbance at 425 nm after 5 min.

2.5. The $^{15}$N labeling method and measurement of N-use efficiency (NUE)

Grapevine seedlings were cultured with modified Hoagland solution with Ca$^{15}$NO$_3$_2 (10.20 atom% $^{15}$N, Shanghai Research Institute of Chemical Industry, SRICI), NH$_4$$^{15}$NO$_3$ (10.20 atom% $^{15}$N, SRICI), and NH$_4$Cl (10.20 atom% $^{15}$N, SRICI) for treatments with NO$_3^-$, NH$_4$NO$_3$, and NH$_4$Cl as sources, respectively, for 50 days to determine N absorption and NUE. The samples (roots and shoots) were washed by branch water, detergent, branch water, and 1% hydrochloric acid in order, and then with deionized water for 3 times. The roots and shoots were separately placed in paper envelopes and dried first at 105°C for 30 min and then at 80°C for five days. The roots and shoots were homogenized using a mortar and pestle, filtered with a fine-mesh sieve (0.25 mm). The samples were analyzed using a stable-isotope ratio mass spectrometer (MAT-251, Thermo Finnigan, San Jose, CA, USA) to determine the total N content, and $^{15}$NO$_3^-$ use efficiency at the Institute for Application of Atomic Energy (IAAE) of the Chinese Academy of Agricultural Sciences (CAAS) (Clarkson et al. 1996).

The calculation of $^{15}$N was measured according to Ding et al. (2017) as follows:

\[
Ndff(\%) = \frac{\text{abundance of } ^{15}N \text{ in plant } - \text{ natural abundance } ^{15}N}{\text{abundance of } ^{15}N \text{ in fertilizer } - \text{ natural abundance } ^{15}N} \times 100\%
\]

(1)

\[
^{15}N \text{ use efficiency (\%)} = \frac{Ndff \times \text{total } ^{15}N \text{ of organs(g)}}{^{15}N \text{ fertilization(g)}} \times 100\%
\]

(2)

2.6. Determination of N metabolic enzyme activities

The activity of N metabolic enzymes, including NR (EC 1.7.1.3), nitrite reductase (NiR, EC 1.7.2.1), glutamine synthetase (GS, EC 6.3.1.2), and NADH-glutamate synthase (NADH-GOGAT, EC 1.4.1.14), were determined following previously reported methods (Liu, Xiao, et al. 2017). Three independent biological replicates were maintained per treatment, and each measurement was repeated thrice.

NR activity assay was measured as follows: the roots and shoots (1.0 g fresh weight) were mixed with 6 mL of extraction buffer. The homogenate was then centrifuged at 8000g for 5 min at 4°C. The supernatant was mixed with 0.1 mol L$^{-1}$ HEPEs buffer (pH 7.5), 22 mmol L$^{-1}$ FAD, 15.4 mmol L$^{-1}$ KNO$_3$, and 0.227 mg L$^{-1}$ of NADH. After 30 min incubation at 28°C, the reaction was ended with the addition of 0.1 ml 1 mol L$^{-1}$ zinc acetate. Then 1% (w/v) sulfinamide mixed with 2 mol L$^{-1}$ HCl and 0.02% (w/v) 1-naphthylamine was added to the above reactant solutions. Absorbance was measured at 543 nm after 30 min incubation.

NiR activity assay was measured as follows: The enzyme extract (0.2 mL) was mixed with 1 mL 0.1 mol L$^{-1}$ potassium phosphate buffer (pH 7.5), 0.05 mL, 10 mmol L$^{-1}$ KNO$_2$, 0.05 mL, 15 mg mL$^{-1}$ methylviologen, and then 50 mg mL$^{-1}$ sodium dithionite (Na$_2$S$_2$O$_4$), dissolved in 100 mmol L$^{-1}$ NaHCO$_3$ was added to initiate the reaction. The mixture was incubated for 30 min at 25°C and was terminated by vortexing until the methylviologen color had completely disappeared. The residual NO$_2^-$ in the reaction mixture was measured by combining 0.2 mL of the mixture and 6.5 mL of water with 1.8 mL 10% (w/v) sulfinamide prepared in HCl and 1.5 mL 1% (w/v) N (1-naphtyl)-ethylene-diamine dihydrochloride. The absorbance of the mixture was measured at 540 nm.

GS activity assay followed the protocol of our previous study (Liu, Xiao, et al. 2017). Fresh roots and shoots samples were ground in an extraction buffer containing 50 mmol L$^{-1}$ Tris-HCl (pH 8.0), 2 mmol L$^{-1}$ MgSO$_4$·7H$_2$O, 2 mmol L$^{-1}$ DTT, and 0.4 mol L$^{-1}$ sucrose. The homogenate was centrifuged at 15,000g for 20 min at 4°C. Reaction mixture A comprised 0.1 mol L$^{-1}$ Tris-HCl (pH 7.4), 80 mmol L$^{-1}$ MgSO$_4$·7H$_2$O, 20 mmol L$^{-1}$ glutamic acid-Na, 20 mmol L$^{-1}$ cysteine, and 2 mmol L$^{-1}$ EGTA, and reaction mixture B contained mixture A and 80 mol L$^{-1}$ hydroxylamine hydrochloride (pH 7.4). The final reaction mixture comprised of 1.6 mL of mixture B, 0.7 mL of the crude enzyme extract, and 0.7 mL of 40 mmol L$^{-1}$ ATP, and was incubated for 30 min at 37°C. The reaction was ended with 1 mL of a color agent containing of 0.2 mol L$^{-1}$ TCA, 0.37 mol L$^{-1}$ FeCl$_3$, and 0.6 mol L$^{-1}$ HCl in 2% HCl. The absorbance of each supernatant was determined at 540 nm.

NADH-GOGAT activity was measured according to the following method: Fresh roots and shoots samples were extracted using the mixture consisting of 0.2 mol L$^{-1}$ sodium phosphate buffer (pH 7.5), 2 mmol L$^{-1}$ EDTA, 0.5% (v/v) Triton X-100, 0.1% (v/v) mercaptoethanol, and 50 mmol L$^{-1}$ KCl. The homogenates were centrifuged at 4°C at 10,000g for 20 min. NADH-GOGAT activity was examined in the supernatant by determining the decrease in absorption at 340 nm due to NADH oxidation. The reaction mixture comprised of 25 mmol L$^{-1}$ sodium phosphate buffer (pH 7.3), 0.3 mL enzyme extract, 1 mmol L$^{-1}$ EDTA, 5 mmol L$^{-1}$ 2-oxoglutarate, 100 mmol L$^{-1}$ KCl, 20 mmol L$^{-1}$ L-glutamine, and 1 mmol L$^{-1}$ NADH.

2.7. Total RNA extraction and quantitative RT–PCR

The roots and leaves were harvested and immediately frozen in liquid nitrogen. The total RNA was extracted from these samples using an RNA extraction kit (Tiangen...
Biotechnology, Beijing, China). The PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) was used to generate the complementary deoxyribonucleic acid (cDNA) for quantitative real-time polymerase chain reaction (qRT-PCR). The expression levels of Mo transporter genes, NO$_3^-$ transporter genes, and Moco biosynthetic genes were measured using the SYBR Premix Ex Taq system (Takara Biotechnology, Dalian, China). The genes and primer sequences are listed in Supplementary Table S2. The qRT-PCR was performed with an initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification. The data were analyzed following the comparative threshold cycle (CT) ($2^{-\Delta\Delta CT}$) method (Liu et al. 2020), using the $Vvactin$ gene as the reference. Three independent biological replicates and three technical replicates were used for the analysis.

2.8. Statistical analysis

Data were analyzed using SPSS Statistics (Version 19.0; IBM Corp., Armonk, NY, USA). Duncan’s multiple range test was used to measure the differences between pairs of means at $P<0.05$. GraphPad Prism (Version 6.0) was used to plot the graphs and do an ANOVA to compare the treatments.

3. Results

3.1. Mo application influences the plant parameters (growth, dry weight, root architecture, and activity) of grapevines seedlings under different N sources

Seedlings cultured with Mo grew better than those without Mo under different N supply, especially under NO$_3^-$ or NH$_4$NO$_3$ supply (Figure 1).

Among the different N forms applied, NH$_4$NO$_3$ resulted in the highest plant dry weight while NH$_4^+$ alone resulted in the lowest (Table 1). Mo application increased the shoot dry weight by 10.40%, 15.73%, and 9.94%, and the root dry weight by 16.43%, 34.84%, and 15.88% in the grapevine seedlings under NO$_3^-$, NH$_4$NO$_3$, and NH$_4^+$ sources, respectively (Table 1). Mo application significantly increased the plant dry weight compared with the −Mo treatments (Table 1).

Among the various N sources, NH$_4$NO$_3$ application led to the highest root architectural parameters and root activity, while NH$_4^+$ alone resulted in the lowest values. Mo application significantly increased root length, volume, surface area, forks per root, tips per root, and root activity of grapevine seedlings (Table 2). Meanwhile, no considerable difference was observed in average root diameter between −Mo and +Mo treatments (Table 2). The Mo treatment increased root activity by 28.97%, 32.92%, and 25.92% under NO$_3^-$, NO$_3^-$, and NH$_4^+$ sources, respectively.

![Figure 1. Effects of molybdenum (Mo) application on growth status in grapevine seedlings under different nitrogen (N) sources. A seedling without Mo under NO$_3^-$ source; B seedling with Mo under NO$_3^-$ source; C seedling without Mo under NH$_4$NO$_3$ source; D seedling with Mo under NH$_4$NO$_3$ source; E seedling without Mo under NH$_4^+$ source; F seedling with Mo under NH$_4^+$ source.](image-url)

Table 1. Root dry weight, shoot dry weight, and total dry weight of grapevine seedlings grown with or without Mo under different nitrogen (N) sources.

| N treatments | Mo treatments | Root dry weight | Shoot dry weight | Dry weight of plant |
|--------------|---------------|-----------------|------------------|---------------------|
| NO$_3^-$     | −Mo           | 7.65 ± 0.57cd   | 17.62 ± 0.60c    | 25.27 ± 0.58d       |
|              | +Mo           | 8.71 ± 0.64c    | 19.45 ± 0.83bc   | 28.16 ± 0.96c       |
| NH$_4$NO$_3$ | −Mo           | 11.48 ± 0.83b   | 21.13 ± 1.82b    | 32.61 ± 1.24b       |
|              | +Mo           | 15.49 ± 1.36a   | 24.45 ± 1.71a    | 39.94 ± 1.34a       |
| NH$_4^+$     | −Mo           | 6.31 ± 0.86d    | 15.53 ± 0.98d    | 21.84 ± 0.87e       |
|              | +Mo           | 7.31 ± 0.45cd   | 18.31 ± 1.14c    | 25.62 ± 0.36d       |

Analysis of variance

| N treatments | ****          |
| Mo treatments | ****          |

Notes: In the graph, −Mo and +Mo represent seedlings without (0 µM) and with Mo (1 µM) supply, and NO$_3^-$, NH$_4^+$, and NH$_4$NO$_3$ represent sole nitrate source, sole ammonium source, and co-application of ammonium and nitrate sources. Data are represented as mean ± SE ($n = 3$). Different lowercase letters (a, b, c, d) indicate significant differences between the treatments ($P < 0.05$; Duncan-test). Significance levels are shown as: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$, and ns = non-significant.
Table 2. Effects of molybdenum (Mo) application on the root length (A), root volume (B), tips per root (C), forks per root (D), surface area (E), average root diameter (F), and root activity (G) of grapevine seedlings under different nitrogen (N) sources.

| N treatments | Mo treatments | Root length | Average root diameter | Root volume |
|--------------|---------------|-------------|-----------------------|-------------|
| NO$_3^-$    | −Mo           | 804.61 ± 16.07e | 0.44 ± 0.025b | 0.82 ± 0.003d |
|             | +Mo           | 1030.7 ± 14.78c | 0.45 ± 0.025b | 1.02 ± 0.023c |
| NH$_4$NO$_3$| −Mo           | 1214.61 ± 17.15b | 0.49 ± 0.011a | 1.14 ± 0.026b |
|             | +Mo           | 1524.47 ± 37.84a | 0.5 ± 0.011a | 1.38 ± 0.026a |
| NH$_4^+$    | −Mo           | 724.58 ± 23.99f | 0.42 ± 0.011b | 0.68 ± 0.035e |
|             | +Mo           | 939.2 ± 17.85d | 0.43 ± 0.011b | 0.78 ± 0.06d  |

Analysis of variance

N treatments **** **** **** ****
Mo treatments **** ns ****
N×Mo ns ns ns *

| Tips per root | Forks per root | Surface area | Root activity |
|---------------|----------------|--------------|---------------|
| NO$_3^-$      | 1042.59 ± 20.92d | 5007.14 ± 3.76d | 105.96 ± 1.58d | 42.52 ± 1.95d |
| NH$_4$NO$_3$ | 1160.46 ± 23.22c | 5459.95 ± 4.10b | 123.62 ± 1.81c | 54.84 ± 1.66c |
| NH$_4^+$     | 1489.56 ± 17.98a | 5725.64 ± 1.26a | 151.32 ± 4.37a | 81.6 ± 3.96a  |

Analysis of variance

N treatments **** **** **** ****
Mo treatments **** ns **** ****
N×Mo ns ns ns *

Notes: In the table, −Mo and +Mo represent seedlings without (0 µM) and with Mo (1 µM) supply, and NO$_3^-$, NH$_4$ and NH$_4$NO$_3$ represent sole nitrate source, sole ammonium source, and co-application of ammonium and nitrate sources. Data are presented as mean ± SE (n = 3). Different lowercase letters (a, b, c, d) indicate significant differences between the treatments (P < 0.05; Duncan-test). Significance levels are shown as: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns = non-significant.

Table 3. Effects of molybdenum (Mo) application on Mo concentration in roots and shoots, chlorophyll content, amino acid content in roots and shoots, the soluble protein under different nitrogen (N) sources.

| N treatments | Mo treatments | Mo concentration (µmol g$^{-1}$ DW) | Chlorophyll a+b | Root Shoot |
|--------------|---------------|-----------------------------------|----------------|-----------|
| NO$_3^-$    | −Mo           | 0.12 ± 0.07c                      | 0.02 ± 0.01b   | 1.2 ± 0.05 |
|             | +Mo           | 0.13 ± 0.02b                      | 0.1 ± 0.009c   | 1.2 ± 0.05 |
| NH$_4$NO$_3$| −Mo           | 0.17 ± 0.05c                      | 0.25 ± 0.11b   | 3.0 ± 0.37 |
|             | +Mo           | 0.2 ± 0.09d                       | 0.32 ± 0.10a   | 3.21 ± 0.24 |
| NH$_4^+$    | −Mo           | 0.13 ± 0.039c                     | 0.17 ± 0.11b   | 3.21 ± 0.24 |
|             | +Mo           | 0.2 ± 0.087c                      | 0.2 ± 0.10a    | 3.21 ± 0.24 |

Analysis of variance

N treatments ns ns ns ****
Mo treatments **** ns ****
N×Mo ns ns ns *

Notes: Seedlings were grown in Hoagland solution containing three different N forms (NO$_3^-$, NH$_4$NO$_3$, and NH$_4^+$) with and without Mo fertilizer (Na$_2$MoO$_4$·2H$_2$O). In the table, −Mo and +Mo represent seedlings without (0 µM) and with Mo (1 µM) supply, and NO$_3^-$, NH$_4$ and NH$_4$NO$_3$ represent sole nitrate source, sole ammonium source, and co-application of ammonium and nitrate sources. Data are presented as mean ± SE (n = 3). Different lowercase letters (a, b, c, d) indicate significant differences between the treatments (P < 0.05; Duncan-test). Significance levels are shown as: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns = non-significant.

NH$_4$NO$_3$, and NH$_4^+$ sources, respectively, compared with −Mo seedlings (Table 2).

3.2. Mo application influences Mo concentration, chlorophyll content, amino acids contents and the soluble protein under different nitrogen sources in grapevine seedlings

Mo application markedly increased Mo concentrations in shoot and root of grapevine seedlings under N supply (Table 3). Mo concentration was considerably higher in roots under NH$_4$NO$_3$ than with NO$_3^-$ or NH$_4^+$ source alone (Table 3). However, no significant difference in Mo concentration in shoot and root was observed among the seedlings under different N sources grown without Mo (Table 3).

The chlorophyll (a + b) content was maximum in grapevine seedlings cultured with NH$_4$NO$_3$ in −Mo and +Mo treatments (Table 3). Compared with −Mo treatments, Mo application increased chlorophyll (a + b) content under NO$_3^-$ and NH$_4$NO$_3$ sources (Table 3). However, no differences were detected in chlorophyll b content and chlorophyll a/b ratio (data not shown).

Seedlings grown with NH$_4$NO$_3$ as the N source exhibited the highest amino acid content among the treatments (Table 3).
Mo application significantly increased GS activity in roots of grapevine seedlings under the NH₄NO₃ source (Table 6). Meanwhile, GS activity in the shoots of grapevine seedlings with Mo was markedly higher than those without Mo under all sources (Table 6). A similar trend was observed in NADH-GOGAT activity in the grapevine seedlings (Table 6).

Mo application upregulated VvMOT1 gene expression in the shoots and roots in the presence of N sources (Figure 2 (A, B)), indicating the role of Mo in activating the VvMOT1 transcript. Under Mo application, the VvMOT1 expression level reached a maximum in both roots and shoots with N source NH₄NO₃ (Figure 2(A, B)); the VvMOT1 expression level was consistent with Mo concentration. Meanwhile, Mo application and different N forms influenced the transcript levels of VvNRT1.1 (Figure 2, C and D). Mo application significantly increased VvNRT1.1 expression in both roots and shoots of grapevine seedlings under NO₃⁻ alone and NH₄NO₃ sources (Figure 2(C, D)), with no significant difference under NH₄⁺ alone (Figure 2 (C, D)).

**4. Discussion**

In terms of dry weight, plants cultured with sole NH₄⁺ or NO₃⁻ differed from co-application in NH₄⁺ and NO₃⁻ (Table 5). Mo application significantly increased GS activity in roots of grapevine seedlings under the NH₄NO₃ source (Table 6). Meanwhile, GS activity in the shoots of grapevine seedlings with Mo was markedly higher than those without Mo under all sources (Table 6). A similar trend was observed in NADH-GOGAT activity in the grapevine seedlings (Table 6).

Mo application upregulated VvMOT1 gene expression in the shoots and roots in the presence of N sources (Figure 2 (A, B)), indicating the role of Mo in activating the VvMOT1 transcript. Under Mo application, the VvMOT1 expression level reached a maximum in both roots and shoots with N source NH₄NO₃ (Figure 2(A, B)); the VvMOT1 expression level was consistent with Mo concentration. Meanwhile, Mo application and different N forms influenced the transcript levels of VvNRT1.1 (Figure 2, C and D). Mo application significantly increased VvNRT1.1 expression in both roots and shoots of grapevine seedlings under NO₃⁻ alone and NH₄NO₃ sources (Figure 2(C, D)), with no significant difference under NH₄⁺ alone (Figure 2 (C, D)).

**5. Effects of Mo on the N metabolic enzyme activities and gene expression of VvMOT1 and VvNRT1.1**

The NR activity was high in both roots and shoots of grapevine seedlings under NO₃⁻ and NH₄NO₃ sources (Table 6); however, no difference was detected in seedlings with only NH₄⁺ as the N source between +Mo and −Mo treatments (Table 6). Meanwhile, NiR activity in the leaves and roots of grapevine seedlings cultured with Mo was higher than those without Mo, especially in the presence of NH₄NO₃ (Table 6).

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**Table 4.** The total N content, nitrate (NO₃⁻) and ammonium (NH₄⁺) concentrations in roots and shoots of grapevine seedlings grown with or without molybdenum (Mo) under different nitrogen (N) sources.

| N treatments | Mo treatments | Total N content (%) | NO₃⁻ (mg.g⁻¹(DW)) | NH₄⁺ (mg.g⁻¹(DW)) |
|--------------|--------------|---------------------|--------------------|--------------------|
| NO₃⁻         | −Mo          | 1.76 ± 0.082c       | 2.54 ± 0.21d       | 702.04 ± 49.33a    | 477.05 ± 15.85a   |
|              | +Mo          | 2.0 ± 0.099b        | 3.26 ± 0.13b       | 569.48 ± 38.78b    | 364.53 ± 21.16b   |
| NH₄NO₃      | −Mo          | 1.98 ± 0.072b       | 2.91 ± 0.067c      | 500.59 ± 57.72b    | 357.35 ± 19.51b   |
|              | +Mo          | 2.52 ± 0.081a       | 3.69 ± 0.027a      | 409.12 ± 31.8c     | 229.71 ± 51.41c   |
| NH₄⁺         | −Mo          | 1.55 ± 0.16d        | 2.32 ± 0.16d       | 69.79 ± 18.32d     | 27.17 ± 23.69d    |
|              | +Mo          | 1.76 ± 0.068c       | 3.02 ± 0.14bc      | 35.44 ± 7.04d      | 24.61 ± 16.16d    |

Analysis of variance

| N treatments | Mo treatments | NO₃⁻ | NH₄⁺ |
|--------------|--------------|------|------|
|              |              | **** | *****|
| Mo treatments |              | ns   | ns   |
| N×Mo         |              | **   | ***  |

Notes: In the table, −Mo and +Mo represent seedlings without (0 µM) and with Mo (1 µM) supply, and NO₃⁺, NH₄⁺, and NH₄NO₃ represent sole nitrate source, sole ammonium source, and co-application of ammonium and nitrate sources. Data are represented as mean ± SE (n = 3). Different lowercase letters (a, b, c, d) indicate significant differences between the treatments (P < 0.05; Duncan-test). Significance levels are shown as: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, and ns = non-significant.
et al. 2010; Britto and Kronzucker 2013; Zheng et al. 2015; Qin et al. 2017; Imran et al. 2019), and a similar phenomenon has been observed in this study. The dry weight of grapevine seedlings grown under three N forms was in the order NH₄⁺ > NO₃⁻ > NH₄⁺NO₃. Additionally, NH₄NO₃ treatment resulted in the highest plant biomass with Mo application, consistent with the highest chlorophyll content and Mo content. These observations indicate an important role of Mo in promoting grapevine seedlings under different nitrogen (N) sources. Our results revealed that NH₄NO₃ can obviously increase vine Mo concentration under different N sources, consistent with the increased Mo concentration in the seedlings. Mo application increased vine Mo concentration under different N sources, indicating more nutrient absorption (Liu et al. 2020). Therefore, better root parameters indicate that Mo application might lead to efficient N absorption and assimilation. Interestingly, our results indicated that the interaction between Mo and N on root length, root volume, and root activity is synergistic, which might be beneficial to increase its absorption capacity for nutrients (N, Mo) of grapevine seedlings.

Our data showed that Mo application significantly increased Mo concentration of roots and shoots (Table 3). Similar reports were reported in winter wheat (Imran et al. 2019) and strawberry (Liu et al. 2020). However, the effect of the N treatments (different N forms) or the interaction between Mo and N on Mo concentration was not significant. The higher chlorophyll content has a positive correlation with plant growth and development (Liu et al. 2018). In terms of chlorophyll content seedlings responded with a significant difference in the N forms tested (Table 3). In addition, Mo induced significant increases in the chlorophyll content under NO₃⁻ and NH₄NO₃ treatments, which was also reported by Imran et al. (2019). The chlorophyll content was maximum in grapevine seedlings cultured with NH₄NO₃ and Mo, consistent with the highest N content and highest total biomass. Our results revealed that NH₄NO₃ can obviously enhance amino acid and soluble protein of the grapevine seedlings and Mo supply exhibited induced effects on amino acid and soluble protein, indicating more nutrient contents in seedlings.

Plant cells absorb Mo, as MoO₄²⁻, through molybdate transporters, especially those of the MOT1 family. In the present study, MoO₄²⁻ in the nutrient solution influenced VvMOT1 expression and its role in MoO₄²⁻ transport. Mo application markedly enhanced the expression of VvMOT1 in the shoots and roots under different N sources, consistent with the increased Mo concentration in the seedlings. Mo application increased vine Mo concentration under different N sources. These findings are consistent with Arabidopsis, strawberry, and rice (Tomatsu et al. 2007; Liu, Xiao et al. 2017; Huang et al. 2018; Liu et al. 2020). Yet, the

Table 6. Nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and NADH-glutamate synthase (NADH-GOGAT) activities in roots and shoots of grapevine seedlings under different nitrogen (N) sources.

| N treatments | Mo treatments | NR activity (μg NO₃⁻·g⁻¹FW h⁻¹) | NADH-GOGAT activity (umol·g⁻¹·h⁻¹) |
|--------------|---------------|-----------------------------------|-------------------------------------|
|              | Root          | Shoot                             | Root                                | Shoot                                |
| NO₃⁻         | −Mo           | 33.7 ± 1.49d                      | 27.3 ± 0.87d                        | 20.15 ± 3.44cd                      | 13.28 ± 3.44cd                      |
|              | +Mo           | 42.61 ± 4.62c                     | 34.75 ± 5.01c                      | 24.53 ± 2.47bc                      | 17.66 ± 2.47bc                      |
| NH₄NO₃       | −Mo           | 57.15 ± 4.9b                      | 43.27 ± 4.43b                      | 17.0 ± 3.42b                        | 11.32 ± 3.42b                      |
|              | +Mo           | 75.57 ± 2.82a                     | 58.58 ± 1.80a                      | 21.1 ± 3.42b                        | 27.82 ± 4.11a                      |
| NH₄⁺         | −Mo           | 8.9 ± 3.06e                       | 5.87 ± 3.47e                       | 4.03 ± 1.72d                        | 4.13 ± 1.72d                       |
|              | +Mo           | 7.9 ± 2.35e                       | 7.5 ± 2.31e                        | 11.61 ± 4.08c                       | 11.61 ± 4.08c                      |

Notes: In the table, −Mo and +Mo represent seedlings without (0 µM) and with Mo (1 µM) supply, and NO₃⁻, NH₄⁺, and NH₄NO₃ represent sole nitrate source, sole ammonium source, and co-application of ammonium and nitrate sources. Data are represented as mean ± SE (n = 3). Different lowercase letters (a, b, c, d) indicate significant differences between the treatments (P < 0.05; Duncan-test).Significance levels are shown as: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, and ns = non-significant.
characterization of VvMOT1 involved in MoO₄²⁻ absorption requires detailed analysis. In plants, the transporter NRT1.1 is responsible for NO₃⁻ absorption from soil or nutrient solution. The N treatments significantly influenced the expression of NRT1.1 by the ANOVA analysis (Figure 2). Mo application enhanced the transcript levels of VvNRT1.1, which was consistent with Liu (2017) and Imran et al. (2019). The highest expression of NRT1.1 transporter gene was found in seedlings cultured with NH₄NO₃ and Mo, indicating the combined effect of Mo and N on NRT1.1 expression is synergistic in grapevine young seedlings.

Our data showed that the N content in shoot showed no significant difference between NO₃⁻ and NH₄⁺ but for NH₄⁺-NO₃⁻ treatments. This finding was consistent with the findings of Lang et al. (2018) who argue that none of the applied N forms (NO₃⁻, NH₄⁺, urea, arginine, and glutamine) influence the N content in leaves and wood in grapevine. However, the N content in root was significantly influenced by different N forms (Table 4). The N metabolic enzymes (NR, NiR, GS, NADH-GOGAT) activities were influenced by the N forms and Mo supply, but no significant difference was observed on the interaction between Mo and N (Table 6). Mo application resulted in higher NR activity in both roots and shoots under different N sources, with a considerable effect in the presence of NH₄NO₃ and NO₃⁻ sources. This observation is consistent with Imran et al. (2019) who indicates that the maximum NR activity in plants occurs when cultured with NH₄NO₃ and Mo. As a result, Mo supply significantly decreased the NO₃⁻ content under NH₄NO₃ and NO₃⁻ sources. In addition, grapevine seedlings grown with N in three different forms exhibited higher NiR, GS, and NADH-GOGAT activities, higher NH₄⁺ content, and total N content with Mo supply, suggesting Mo fertilizer’s role in N absorption and assimilation. To verify the above results, this experiment with ¹⁵N tracer technique revealed that N forms significantly influenced the ¹⁵N absorption content and ¹⁵NUE followed the order NH₄NO₃ > NO₃⁻ > NH₄⁺, which indicated that more ¹⁵N was absorbed and utilized by seedlings grown with NH₄NO₃ source. Obviously, with increasing ¹⁵N absorption content, ¹⁵NUE increased in seedlings supplied with Mo under all N sources. The data (Table 5) provided direct evidence that Mo and N promote ¹⁵N absorption and ¹⁵NUE in grapevine seedlings synergistically.

5. Conclusion
In grapevine seedlings, the N forms and Mo application significantly influenced dry weight and root architecture and activity. Mo application improved tissue Mo levels to ensure sufficient Mo for N assimilation. Additionally, the chlorophyll content, amino acid content, and the soluble protein were affected by the N forms and Mo application. Mo application induced VvMOT1 expression and NRT1.1 expression to ensure sufficient Mo and N absorption. The reduced tissue NO₃⁻ content and the enhanced NH₄⁺ content with higher N assimilation-related enzyme (NR, NiR, GS, and NADH-GOGAT) activities indicated improvement in N absorption and assimilation with NH₄NO₃ and Mo supply. The ¹⁵N absorption and ¹⁵NUE improved after Mo application under different N sources, exhibiting a synergistic effect of Mo and N. Overall, Mo showed more complementary effects with nitrate-based sources than the sole NH₄⁺ source. In summary, Mo application together with nitrate-based nutrition may be used for grapevine in practice. Nonetheless, it is necessary to further explore the detailed interaction of Mo and N on N absorption and utilization in adult vines under field conditions.
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