Effects of Phosphorus Supply Levels on NO Uptake and Utilization Characteristics in Malus hupehensis (Pingyi Tiancha) Seedlings (Postprint)

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Abstract

Using 15N tracing and non-invasive micro-measurement techniques, we investigated the effects of different phosphorus supply levels (0 mmol L 1, 1.0 mmol L 1, 2.0 mmol L 1, 3.0 mmol L 1, 4.0 mmol L 1, 6.0 mmol L 1, 8.0 mmol L 1, 12.0 mmol L 1, and 16.0 mmol L 1 H2PO4) on NO3 -N absorption and utilization characteristics in Malus hupehensis (Pingyi Tiancha) seedlings, to provide a theoretical basis for improving nitrogen fertilizer use efficiency in orchards. The results showed that at low phosphorus levels (0–1.0 mmol L 1), seedlings exhibited shorter root length, smaller total root surface area, and fewer root tips. With increasing phosphorus supply, biomass, root length, total root surface area, and root tip number were significantly higher at phosphorus concentrations of 2.0–4.0 mmol L 1 compared to other treatments. However, at 6.0–16.0 mmol L 1, excessive phosphorus supply inhibited root growth, causing substantial reductions in root length and surface area, and a sharp decline in root tip number. Non-invasive scanning ion-selective electrode tests indicated that Malus hupehensis absorbed NO3 when the phosphorus concentration in the growth medium was 3.0–6.0 mmol L 1, with the highest absorption rate occurring at 3.0 mmol L 1. Under phosphorus concentrations of 0–2 mmol L 1 and 8.0–16.0 mmol L 1, seedlings exhibited NO3 efflux. With increasing phosphorus supply, the contribution rate of 15N absorbed and allocated from fertilizer to the total nitrogen content of each organ (Ndff) and plant nitrogen utilization efficiency showed a trend of initially increasing then decreasing. Nitrogen utilization efficiency peaked at 4.0 mmol L 1 phosphorus concentration, reaching 42.24%, and decreased significantly when exceeding 4.0 mmol L 1. Adequate phosphorus supply stimulated seedling root growth, thereby promoting nitrogen acquisition in Malus hupehensis. Excessive NO3 inhibited root growth and simultaneously suppressed leaf nitrate reductase activity, resulting in lower nitrogen absorption and utilization efficiency. Therefore, a phosphorus concentration of 3.0–4.0

mmol L 1 was most conducive to the growth of Malus hupehensis seedlings and their nitrogen absorption and utilization.

Full Text

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Characteristics of NO Absorption and Utilization in Malus hupehensis Rehd. Seedlings under Different Phosphorus Levels

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Abstract: In recent years, excessive application of nitrogen and phosphorus fertilizers has not only wasted resources but also created high potential risks for environmental pollution. Long-term unreasonable fertilization has damaged soil physical and chemical properties, including soil porosity and nutrient content. Therefore, promoting scientific nutrient utilization, increasing fertilizer use efficiency, and reducing nitrogen leaching, volatilization, and loss are crucial for sustainable fruit tree production. To determine the key factors influencing nitrogen utilization under different phosphorus levels, we used [1] N-labeled tracer and non-invasive micro-test techniques to investigate NO absorption and utilization characteristics in Malus hupehensis Rehd. seedlings under nine phosphorus levels (0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, and 16.0 mmol · L [1] H PO). The study aimed to improve nitrogen fertilizer utilization efficiency and provide a theoretical basis for scientific and efficient phosphate fertilizer application in apple orchards.

The results showed that under phosphorus deficiency (0–1.0 mmol · L [1]), seedlings had lower root length, root surface area, and fewer root tips. With phosphorus addition of 2.0–4.0 mmol · L [1], plant biomass, root length, root surface area, and root tip number increased significantly compared to other treatments. However, excess phosphorus (6.0–16.0 mmol · L [1]) inhibited root growth, substantially reducing root length, surface area, and root tip number. Non-invasive micro-test technique revealed significant NO absorption by M. hupehensis seedlings at 3.0–6.0 mmol · L [1] phosphorus, with the highest absorption rate at 3.0 mmol · L [1]. In contrast, phosphorus treatments of 0–2 mmol · L [1] and 8.0–16.0 mmol · L [1] showed NO efflux.

With increasing phosphorus levels, Ndff (percent of nitrogen derived from fertilizer) and nitrogen utilization efficiency initially increased then decreased. The

maximum nitrogen use efficiency (42.24%) occurred at 4.0 mmol · L ¹ phosphorus, with significant reductions above this concentration. Appropriate phosphorus supply stimulated root growth, promoting nitrogen acquisition, while excess NO inhibited root growth and suppressed leaf nitrate reductase activity, resulting in lower nitrogen absorption and utilization efficiency. Therefore, phosphorus concentrations of 3.0–4.0 mmol · L ¹ were most favorable for seedling growth and nitrogen absorption/utilization in M. hupehensis.

Keywords: Apple rootstock; Malus hupehensis Rehd.; Phosphorus level; NO absorption; Nitrogen utilization efficiency

Introduction

In recent years, rapid increases in fertilizer application by farmers have promoted soil organic matter growth and fertility improvement, but excessive fertilization has caused increasingly severe problems such as nitrate contamination of groundwater and eutrophication of lakes [1]. Overuse of nitrogen and phosphorus fertilizers not only threatens the environment but also significantly affects soil quality, leading to mineral nutrient imbalance, accelerated decomposition of soil organic matter, and serious problems like soil compaction and declining fertility [2]. Therefore, scientific and rational nutrient utilization to improve fertilizer absorption and use efficiency is key to sustainable development of modern fruit industries.

Phosphorus can promote nitrogen absorption by roots. Ding et al. [3] showed that appropriate phosphorus application enhances crop nitrogen absorption from soil, while phosphorus deficiency significantly reduces nitrogen accumulation [4]. Yuan et al. [5] demonstrated that phosphorus fertilization stimulated wheat (Triticum aestivum L.) root development, thereby promoting nitrogen absorption. Additionally, the yield-increasing effect of phosphorus raised crop nitrogen demand, increasing nitrogen uptake and ultimately reducing soil nitrate accumulation. Graciano et al. [6] found that phosphorus fertilization promoted dry matter accumulation and nitrogen and sulfur absorption in Eucalyptus grandis, with enhanced nitrogen uptake and significantly higher organ nitrogen content compared to nitrogen-only treatments.

Phosphorus plays an important role in plant nitrogen metabolism. Research indicates that nitrate reductase requires NADP /NADPH as electron acceptors to catalyze the reduction of NO to NO [7–8], which is the rate-limiting step in NO metabolism. Non-invasive micro-test technique (NMT), as a novel electrophysiological technology, can measure molecular and ion concentrations, flux rates, and three-dimensional movement information in real physiological environments, and has been widely applied in plant physiology and development studies [9–10]. Luo et al. [11] used NMT to study nitrogen absorption characteristics in different root zones of Tamarix chinensis L., finding significant NO

influx in root tips, meristematic zones, and elongation zones, while NH showed efflux trends.

Current research on phosphorus level effects on plant nitrogen absorption and utilization has mainly focused on nitrogen uptake and distribution to organs [4,12–13], emphasizing result description with limited investigation into the mechanisms of plant nitrogen absorption responses to different phosphorus levels. Moreover, few studies have examined apple (Malus pumila Mill.) rootstocks. Malus hupehensis Rehd., a unique Chinese plant resource with apomixis characteristics, produces uniform seedlings with high nutrient absorption efficiency, good resistance, and strong adaptability, making it widely used as an apple rootstock in the Bohai Bay apple production region. Using non-invasive scanning ion-selective electrodes, this study investigated the effects of different phosphorus levels on NO -N absorption and utilization characteristics in M. hupehensis seedlings to elucidate the mechanisms by which phosphorus levels affect nitrogen absorption and utilization, thereby improving apple nitrogen fertilizer use efficiency and providing a theoretical basis for rational phosphate fertilizer application in orchards.

Materials and Methods

1.1 Experimental Materials and Design

The experiment was conducted from March to August 2014 at the Horticultural Experimental Station of Shandong Agricultural University and the National Apple Engineering Technology Research Center Laboratory. One-year-old M. hupehensis seedlings were used as experimental material. In early March, stratified seeds were sown in plug trays under normal temperature and humidity management. When seedlings developed 4–5 true leaves, they were transplanted into pots filled with quartz sand (washed and dried before use). After one week of recovery with deionized water, seedlings were irrigated twice with half-strength Hoagland nutrient solution (once every 3 days), followed by full-strength Hoagland nutrient solution every 3 days with daily watering. When seedlings reached approximately 10 true leaves, uniform seedlings were selected for different phosphorus concentration treatments.

Nine H PO concentrations were established: 0, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, and 16.0 mmol·L ^{1}, with 15 replicates per treatment and 4 plants per pot. Other macronutrients [Ca(NO) 5 mmol·L ^{1}, KNO 5 mmol·L ^{1}, KCl 5 mmol·L ^{1}, MgSO 2.5 mmol·L ^{1}, EDTA-Fe 0.2 mmol·L ^{1}] and micronutrients (MnSO , CuSO , ZnSO , H BO , H MoO) were maintained at normal Hoagland solution concentrations.

When seedlings developed 7–8 true leaves (approximately 90 days), 0.1 g Ca(^{1} NO) was added during each nutrient solution irrigation (totaling 1 g, applied in 10 portions) for ^{1} N labeling. After 102 days of treatment, samples were collected on August 15 during the slow growth period of new shoots.

1.2.1 Measurement of NO Absorption Rate

On August 15, six seedlings were randomly selected from each treatment' s 15 replicates to measure NO absorption/efflux rates using non-invasive scanning ion-selective electrodes. First, approximately 10 mm of electrolyte solution was injected into the microelectrode tip, and NO -selective ion exchanger was perfused at the electrode tip (15–20 m). The prepared electrode was then connected to the preamplifier via an Ag/AgCl electrode holder with a solid reference electrode. Before measurement, electrodes were calibrated to obtain the Nernst slope and intercept; electrodes with calibration slopes within 29±3 were considered qualified.

The test solution contained 0.1 mmol · L ^{1} KNO , 0.1 mmol · L ^{1} CaCl , and 0.3 mmol · L ^{1} MES (pH 6.0), with NO concentrations of 0.05, 0.5, and 0.1 mmol · L ^{1} for calibration. For M. hupehensis seedlings under different phosphorus treatments, measurements were taken approximately 2 mm from lateral root tips. The electrode was positioned 3–5 m from the root surface as the starting point, performing reciprocating measurements perpendicular to the root surface with 30 m intervals between movements. Voltage differences measured between two points were converted to concentration differences using the Nernst slope, and flux rates were calculated using MageFlux software.

1.2.2 Determination of Leaf Enzyme Activity and Root Morphology Indices

After treatment, six uniform seedlings per treatment were selected. Nitrate reductase (NR) activity in newly fully expanded leaves was measured using the method of Li [14]. Root systems were washed and scanned using an Epson Perfection V750 transmission scanner to obtain whole-plant root images, which were then analyzed using WinRHIZO software (Regent Instruments Inc., Canada) to determine root length, total root surface area, and root tip number.

1.2.3 Determination of Plant Dry Weight and Nitrogen Content

After treatment, plants were separated into roots, stems, and leaves, killed at 105°C for 30 minutes, dried at 80°C to constant weight, and weighed for organ dry matter content. Samples were then ground using a stainless-steel electric mill, passed through a 0.25 mm sieve, and analyzed for ^{1} N abundance and total nitrogen content. Total nitrogen was determined by Kjeldahl method, and ^{1} N abundance was measured using a MAT-251 mass spectrometer.

Ndff = (^{1} N abundance in plant sample% -natural abundance%) / (^{1} N abundance in fertilizer% -natural abundance%) × 100% (1)

Nitrogen fertilizer utilization efficiency = [Ndff × organ total nitrogen content (g)] / fertilizer application rate (g) × 100% (2)

1.3 Data Processing

Data were processed using Microsoft Excel 2007. Significance and correlation analyses were performed using single-factor experimental statistical analysis in the DPS data processing system, with multiple comparisons using LSD method. Figures were prepared using Microsoft Excel 2003 and GraphPad Prism 5.

Results

2.1 Biomass of M. hupehensis under Different Phosphorus Levels

As shown in Table 1 , organ biomass under different phosphorus treatments showed a consistent trend of leaf > root > stem. The 0 mmol · L ^{1} phosphorus treatment produced the lowest biomass, with per-plant root, stem, and leaf biomass of 0.17 g, 0.12 g, and 0.28 g, respectively. Root, stem, leaf, and total biomass increased significantly with phosphorus concentration, reaching maximum values at 3 mmol · L ^{1} (root: 0.93 g, stem: 0.60 g, leaf: 1.47 g). Beyond this concentration, biomass showed a slow declining trend, remaining relatively stable under 6–12 mmol · L ^{1} phosphorus treatments.

2.2 Root Morphology Indices of M. hupehensis under Different Phosphorus Levels

Table 2 shows that root total length, surface area, and tip number were significantly affected by phosphorus supply. Under low phosphorus (0–1 mmol · L ^{1}), root length, surface area, and tip number were relatively small. At 2–4 mmol · L ^{1} phosphorus, root growth accelerated significantly, with root length, surface area, and tip number reaching maximum values. However, under 6–16 mmol · L ^{1} phosphorus, excess supply inhibited root growth, substantially reducing root length, surface area, and causing a sharp decline in root tip number.

2.3 NO Absorption Rate of M. hupehensis under Different Phosphorus Levels

Root NO absorption capacity differed significantly among phosphorus levels (Figure 1 [Figure 1: see original paper]). Non-invasive scanning ion-selective electrode tests showed NO absorption at 3–6 mmol · L ^{1} phosphorus, with strong absorption at 3 mmol · L ^{1} (average rate 39.66 pmol · cm ^{2} · s ^{1}). At 0–2 mmol · L ^{1} and 8–16 mmol · L ^{1} phosphorus, seedlings exhibited NO efflux, with obvious efflux trends at 16 mmol · L ^{1} (absorption rate: -91.01 pmol · cm ^{2} · s ^{1}).

2.4 Leaf Nitrate Reductase (NR) Activity under Different Phosphorus Levels

As shown in Table 3 , leaf NR activity was low at 0 mmol · L ^{1} phosphorus. Activity increased significantly at 1–3 mmol · L ^{1} phosphorus, maintaining levels above 30 g · g ^{1} · h ^{1}, allowing rapid reduction of NO transported from roots to

NO for participation in leaf nitrogen metabolism. At 4–16 mmol · L ^{1} phosphorus, NR activity decreased, indicating weaker nitrogen metabolism and lower nitrogen utilization.

2.5.1 Ndff Values of Different Organs

Table 4 shows that at 0–1 mmol · L ^{1} phosphorus, Ndff (percent of nitrogen derived from fertilizer) [15] was low in all organs, following the pattern stem > root > leaf. Weak root growth limited absorption capacity, with excess nitrogen translocated to shoots, resulting in higher stem Ndff than root Ndff. At 2–4 mmol · L ^{1} phosphorus, Ndff increased significantly in all organs, following root > stem > leaf, indicating enhanced root nitrogen absorption capacity. Roots required more nitrogen to maintain greater growth, thus translocating less nitrogen to shoots. At 6–16 mmol · L ^{1} phosphorus, Ndff values did not continue increasing but declined slowly as the promotive effect of phosphorus on root growth diminished and root growth became inhibited, weakening nitrogen absorption capacity.

2.5.2 Nitrogen Utilization Efficiency

Figure 2 [Figure 2: see original paper] shows extremely low nitrogen utilization efficiency (7.22%) at 0 mmol · L ^{1} phosphorus. Efficiency increased significantly with phosphorus supply, with 3–4 mmol · L ^{1} phosphorus treatments showing significantly higher efficiency than others. However, continuous excess phosphorus caused a sharp decline, with efficiency maintaining 20–30% under 6–16 mmol · L ^{1} phosphorus treatments.

Discussion and Conclusion

Root tips are the most active sites for nitrogen absorption and secretion in roots. Tissues farther from root tips are older and develop a fibrous layer that hinders nitrogen absorption [6]. Additionally, root tips in the maturation zone produce numerous root hairs that greatly increase absorption surface area and play important roles in nitrogen uptake [16]. Under low phosphorus levels, M. hupehensis seedlings had smaller root biomass, surface area, length, and fewer root tips, resulting in relatively low nitrogen absorption capacity.

Correlation analysis showed that nitrogen mobilization capacity (Ndff) was significantly positively correlated with root surface area, root length, and root tip number ($R^2 = 0.86$, $\mathbf{R^2 = 0.82}$, $R^2 = 0.91^{**}$). With increasing phosphorus supply, adequate nutrients met root growth requirements, resulting in rapid root growth with significantly increased root length, surface area, and tip number, which significantly increased Ndff values in all organs and improved plant nitrogen uptake.

NO flux rates directly reflect plant nitrogen absorption status. Using NMT, this study measured NO absorption rates in M. hupehensis roots under differ-

ent phosphorus levels. Results showed strong NO efflux under low phosphorus, while at 3.0 mmol · L ^{1} H PO , seedlings showed strong NO absorption, indicating high nitrogen mobilization capacity. At 8.0–16.0 mmol · L ^{1} H PO , high phosphorus treatments resulted in intracellular NO concentrations exceeding test solution concentrations. Combined with nitrogen accumulation, high leaf NO content can regulate shoot-root allocation [17–19], inhibiting root growth and causing sharp declines in root tip number and nitrogen absorption capacity, thus significantly reducing nitrogen mobilization and showing strong NO efflux. This indicates that both phosphorus deficiency and excess are detrimental to nitrogen absorption, while appropriate phosphorus supply (3–4 mmol · L ^{1}) promotes root nitrogen absorption, providing reliable evidence for investigating nitrogen uptake mechanisms.

After nitrate enters plant cells, it can act as a signaling molecule to induce nitrate reductase production [20]. Nitrate reductase is the rate-limiting enzyme in nitrate assimilation, directly regulating NO reduction and thus nitrogen metabolism [8]. Phosphorus is a component of NADPH, and NADP /NADPH serves as an electron carrier for nitrate reductase, transferring electrons from FAD to NO [7]. Phosphorus deficiency may inhibit leaf nitrate reductase activity due to insufficient electron carriers and energy supply, preventing rapid assimilation of root-absorbed nitrogen and resulting in NO accumulation in leaves and low nitrogen use efficiency. Adequate phosphorus supply ensures sufficient electron carriers and ATP activity, with leaf nitrate reductase activity significantly higher than under phosphorus deficiency, accelerating NO assimilation and maintaining high nitrogen use efficiency. Excess phosphorus inhibits nitrogen and phosphorus absorption and significantly reduces leaf nitrate reductase activity, suppressing both nitrogen absorption and metabolism, resulting in low nitrogen use efficiency in M. hupehensis seedlings.

In conclusion, different phosphorus levels affect nitrogen absorption and utilization by influencing root growth and ATP/electron carrier supply. Under phosphorus deficiency, insufficient nutrient and energy supply and low leaf nitrate reductase activity limit nitrogen absorption and NO metabolism. Appropriate phosphorus supply stimulates root growth, promoting nitrogen acquisition, while adequate ATP/electron carriers and high nitrate reductase activity ensure normal NO reduction. However, excess phosphorus increases leaf NO accumulation, inhibits root growth, and suppresses leaf nitrate reductase activity, resulting in low nitrogen absorption and utilization efficiency. Therefore, phosphorus concentrations of 3.0–4.0 mmol · L ^{1} are optimal for growth and nitrogen absorption/utilization in M. hupehensis seedlings.

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Note: Figure translations are in progress. See original paper for figures.