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RESEARCH ARTICLE

Nitrogen allocation to biochemical fractions shapes functional traits and N use efficiency in tobacco cultivars

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SUPPLEMENTARY MATERIALS

S1 Morphological and functional partitioning of nitrogen

S1.1 Conceptual framework for nitrogen partitioning

Nitrogen (N) within plant tissues exists in diverse biochemical forms that fulfill distinct physiological roles. To facilitate interpretation of nitrogen use efficiency (NUE), plant nitrogen was categorized from two complementary perspectives: morphological nitrogen fractions (defined by solubility and molecular association) and functional nitrogen pools (defined by physiological roles in photosynthesis, respiration, structure, or storage)[1].

Morphological nitrogen fractions included SDS-insoluble nitrogen ($N_{in\text{-}SDS}$), SDS-soluble nitrogen (N_s), water-soluble nitrogen (N_w), and non-protein nitrogen (N_{np}). Functional nitrogen pools were defined following the LUNA framework[1], including photosynthetic nitrogen (N_{psn}), respiratory nitrogen (N_{resp}), structural nitrogen (N_{str}), and storage nitrogen (N_{store}).

S1.2 Determination of morphological nitrogen fractions

Morphological nitrogen fractions were determined in frozen root, stem, and leaf tissues following a modified protocol of Takashima et al.[2]. Approximately 1.0 g of fresh tissue was flash-frozen in liquid nitrogen and homogenized in 1.0 mL of 100 mmol·L^{-1} sodium phosphate buffer (pH 7.5) containing 2 mmol·L^{-1} $MgCl_2$, 0.4 mol·L^{-1} D-sorbitol, 5 mmol·L^{-1} dithiothreitol, 5 mmol·L^{-1} iodoacetate, 10 mmol·L^{-1} NaCl, and 5 mmol·L^{-1} phenylmethylsulfonyl fluoride.

The homogenate was centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was collected as water-soluble nitrogen (N_w).

The remaining tissue was resuspended in phosphate buffer containing 3% sodium dodecyl sulfate (SDS), incubated at 90 °C for 5 min, and centrifuged at 5500 g for 8 min. The resulting supernatant was collected as SDS-soluble nitrogen (N_s), mainly composed of soluble functional proteins.

The remaining insoluble fraction was rinsed with anhydrous ethanol, filtered through quantitative filter paper, treated with 20% trichloroacetic acid, washed again with ethanol, air-dried, and digested using H_2SO_4–H_2O_2. Nitrogen in this fraction was designated as SDS-insoluble nitrogen ($N_{in\text{-}SDS}$), corresponding primarily to structural nitrogen.

Non-protein nitrogen (N_{np}) was calculated as the difference between total nitrogen and the sum of protein-associated nitrogen fractions ($N_w + N_s + N_{in\text{-}SDS}$), and includes nitrate, ammonium, free amino acids, and other low-molecular-weight nitrogenous compounds. Nitrogen concentrations were determined using continuous flow analysis (AA3, Seal Analytical Inc., Southampton, UK).

S1.3 Determination of Functional Nitrogen Fractions in Leaves

Functional nitrogen fractions were quantified only in leaves. All functional nitrogen components were initially calculated on a fresh weight basis. To ensure consistency with total nitrogen and morphological nitrogen fractions (measured on a dry weight basis), values were converted using sample-specific fresh-to-dry weight ratios.

Gas exchange measurements were conducted one day before harvest using a portable photosynthesis system (LI-6400XT; LI-COR Biosciences, Lincoln, NE, USA). Net photosynthetic rate (*An*) and *A/Ci* response curves were recorded to derive the maximum carboxylation rate (*Vc,max*) and maximum electron transport rate (*Jmax*)[3].

The photosynthesis model equations were:

$$A_n = \frac{V_{c,max}(C_i - \Gamma^*)}{C_i + K_c(1 + O/K_o)} - R_d \quad (1)$$

$$A_n = \frac{J_{max}(C_i - \Gamma^*)}{4C_i + 8\Gamma^*} - R_d \quad (2)$$

where C_i is intercellular CO_2 concentration (μmol·mol^{-1}), Γ^* is the CO_2 compensation point (μmol·mol^{-1}), K_c and K_o are Michaelis constants for carboxylation and oxygenation, respectively, O is the O_2 concentration (210,000 μmol·mol^{-1}), and R_d is mitochondrial respiration in the light (μmol·m^{-2}·s^{-1} CO_2).

Chlorophyll a and b were extracted from frozen leaf disks using 80% acetone for 48 h and quantified spectrophotometrically (UV2102 PCS, Unico, China)[4].

Calculation of Functional Nitrogen Pools

Photosynthetic nitrogen (N_{psn}) was calculated as the sum of nitrogen allocated to the carboxylation system (N_{cb}), electron transport system (N_{et}), and light-harvesting system (N_{lc}):

$$N_{psn} = N_{cb} + N_{et} + N_{lc} \quad (3)$$

N_{cb} was estimated as:

$$N_{cb}=\frac{V_{c,max}}{6.25\times V_{cr}\times f_{Vc,max}} \quad (4)$$

where 6.25 is the Rubisco-to-nitrogen conversion factor, V_{cr} is the Rubisco carboxylation rate (20.78 μmol CO_2 g^{-1} Rubisco s^{-1} at 25 °C), and $f_{Vc,\max}$ is the temperature correction factor (0.359 at 15 °C).

N_{et} was calculated as:

$$N_{et}=\frac{J_{max}}{8.06\times J_{mc}\times f_{J_{max}}} \quad (5)$$

where 8.06 is the cytochrome f nitrogen coefficient, J_{mc} is the electron transport capacity of cytochrome f (155.65 μmol·s^{-1} at 25 °C), and $f_{J\max}$ is the temperature correction factor (0.714 at 15 °C).

N_{lc} was calculated from chlorophyll content:

$$N_{lc}=\frac{C_c}{C_B} \quad (6)$$

where C_c is chlorophyll concentration (mmol $\cdot m^{-2}$) and C_B is the chlorophyll-to-nitrogen ratio (2.15 mmol·g^{-1}).

Respiratory, Structural, and Storage Nitrogen

Respiratory nitrogen (N_{resp}) was estimated as:

$$N_{resp}=\frac{R_t}{33.69\times f_r} \quad (7)$$

where $R_t = 0.015 \times V_{c,\max}$, 33.69 represents respiratory nitrogen use efficiency at 25 °C, and f_r is the temperature correction factor (0.522 at 15 °C).

Total leaf nitrogen (N_a) was partitioned as:

$$N_a=N_{psn}+N_{resp}+N_{str}+N_{store} \quad (8)$$

Structural nitrogen (N_{str}) was defined as $N_{in\text{-}SDS}$.

Storage nitrogen (N_{store}) comprised three components:

- Water-soluble storage nitrogen (N_{ow}):

$$N_{ow}=N_w-N_{cb}-N_{resp} \quad (9)$$

- SDS-soluble storage nitrogen (N_{os}):

$$N_{os}=N_s-N_{lc}-N_{et} \quad (10)$$

- Non-protein storage nitrogen (N_{np}), representing residual nitrogen not included in protein-associated fractions.

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