Nitrogen isotope discrimination in open-pollinated and hybrid canola suggests indirect selection for enhanced ammonium utilization

Yi Hu1,2, Robert D. Guy2* and Raju Y. Soolanayakanahally3*

1Key Laboratory of Mountain Surface Processes and Ecological Regulation, Institute of Mountain Hazards and Environment, Chinese Academy of Sciences, Chengdu, China, 2Department of Forest and Conservation Sciences, Faculty of Forestry, University of British Columbia, Vancouver, BC, Canada, 3Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

Nitrogen isotope discrimination ($\Delta^{15}$N) may have utility as an indicator of nitrogen use in plants. A simple $\Delta^{15}$N-based isotope mass balance (IMB) model has been proposed to provide estimates of efflux/influx ($E/I$) ratios across root plasma membranes, the proportion of inorganic nitrogen assimilation in roots ($P_{\text{root}}$) and translocation of inorganic nitrogen to shoots ($T_{\text{root}}/T_{\text{t}}$) under steady-state conditions. We used the IMB model to investigate whether direct selection for yield in canola (Brassica napus L.) has resulted in indirect selection in traits related to nitrogen use. We selected 23 canola lines developed from 1942 to 2017, including open-pollinated (OP) lines developed prior to 2005 as well as more recent commercial hybrids (CH), and in three separate experiments grew them under hydroponic conditions in a greenhouse with either 0.5 mM ammonium, 0.5 mM nitrate, or 5 mM nitrate. Across all lines, $E/I$, $P_{\text{root}}$ and $T_{\text{root}}/T_{\text{t}}$ averaged 0.09±0.03, 0.82±0.05 and 0.23±0.06 in the low nitrate experiment, and 0.31±0.06, 0.71±0.07 and 0.42±0.12 in the high nitrate experiment, respectively. In contrast, in the ammonium experiment average $E/I$ was 0.40±0.05 while $T_{\text{root}}/T_{\text{t}}$ averaged 0.07±0.04 and $P_{\text{root}}$ averaged 0.97±0.02. Although there were few consistent differences between OP and CH under nitrate nutrition, commercial hybrids were collectively better able to utilize ammonium as their sole nitrogen source, demonstrating significantly greater overall biomass and a lower $P_{\text{root}}$ and a higher $T_{\text{root}}/T_{\text{t}}$, suggesting a somewhat greater flux of ammonium to the shoot. Average root and whole-plant $\Delta^{15}$N were also slightly higher in CH lines, suggesting a small increase in $E/I$. An increased ability to tolerate and/or utilize ammonium in modern canola hybrids may have arisen under intensive mono-cropping.

KEYWORDS
nitrogen isotopes, carbon isotopes, nitrate, ammonium, model, canola
Introduction

Nitrogen (N) is the primary limiting nutrient for most plants growing in natural and agricultural ecosystems (Glass, 2003) and nitrate (NO$_3^-$) and ammonium (NH$_4^+$) are the two most important inorganic N sources for plants. Modern agricultural systems depend heavily on the use of N fertilizers for greater crop yield and more than 50% of the applied nitrogen is lost to the environment through leaching, greenhouse gas emissions (Vitousek et al., 1997; Hirel et al., 2007; Guo et al., 2010) and groundwater contamination (Guo et al., 2010). Canola is a relatively new crop, derived from oilseed rape (Brassica napus L.), and has become one of the world’s most important oil crops (Raymer, 2002). Like other non-legume crops, nitrogen is usually the limiting nutrient and N fertilizer is the biggest input needed for seed production (Wang et al., 2014; Zhang et al., 2022). Though very little is known about N uptake and assimilation in oilseed rape and canola, different N-use efficiencies among cultivars have been reported (Stahl et al., 2016; Li et al., 2020). Better understanding and characterization of whole-plant N-use and N-partitioning patterns among diverse canola lines is necessary for improving N-use efficiency (NUE), and beneficial in moving towards more sustainable agricultural production.

Both nitrate and ammonium have two major transport systems responsible for N uptake: high-affinity (HATS) and low-affinity (LATS) transport systems that are most effective at low or high N concentrations, respectively (Glass et al., 2002). Nitrate assimilation is more complex than ammonium. Once retained by the root, nitrate must first be reduced to nitrite and then ammonium before it can be assimilated further. Nitrate is converted into nitrite in the cytoplasm by nitrate reductase (NR), and nitrite is converted to ammonium by nitrite reductase (NiR). Ammonium, whether directly taken up by root cells or produced from nitrate, is assimilated into glutamine by glutamine synthetase (GS). It has been reported that under certain conditions, a substantial portion of the nitrate and/or ammonium initially taken up by plants (influx) returns to the rooting medium (efflux) before it can be assimilated (Kronzucker et al., 1997; Hawkins and Robbins, 2010). The ratio of efflux over influx (E/I) describes the bi-directional movement of inorganic N between root and rhizosphere and relates to the N-uptake efficiency; i.e., a low E/I indicates a high uptake efficiency because there is less leakage of inorganic N back to the rooting medium.

There are two stable isotopes of nitrogen (14N and 15N), with 14N being the predominant form (99.636% of global nitrogen). However, the stable isotopes of N may show differences in rates of chemical reaction, or in physical processes such as diffusion, that result in small changes in 15N/14N ratios between N pools. Changes in the relative abundance of 14N and 15N, called isotope fractionation, can provide integrated or tracer information about N fluxes within/through ecosystems (Evans, 2001; Robinson, 2001). It is well recognized that changes in plant δ15N occur during nitrate or ammonium assimilation, often causing plants to be depleted in 15N by about 2 to 3‰ compared to the soil N (Evans, 2001). This difference implies that there is discrimination against the heavier isotope, in either N transport or assimilation. It is now more than thirty years since the first reports of N isotope effects associated with NR and GS were measured in vitro using preparations from spinach leaves, yielding discrimination factors of ~15% for NR (Ledgard et al., 1985) and ~17% for GS (Yoneyama et al., 1993). Subsequent work has indicated that discrimination by either enzyme is probably somewhat higher (Needoba et al., 2004; Karsh et al., 2012; Carlisle et al., 2014; Cui et al., 2020). Nonetheless, under N-limited conditions, plant δ15N tends to be close to the δ15N value of the available soil inorganic N because local supplies of both isotopes are assimilated to near completion (Evans, 2001; Kolb and Evans, 2003). There is greater observed discrimination when substrate concentrations are high and/or there is a greater opportunity for residual heavy nitrogen to diffuse away from roots before being taken up again, but rarely (if ever) does the δ15N reflect the full discrimination factor of NR or GS (Yoneyama et al., 2001; Pritchard and Guy, 2005).

In a series of articles, Kalciscts and Guy (2013a), Kalciscts and Guy (2013b), Kalciscts and Guy (2014) presented an isotope mass balance (IMB) model that combines δ15N and tissue N content to derive (1) E/I at the root, (2) leaf vs. root partitioning of N assimilation, and (3) fluxes of inorganic and organic N to the shoot. Kalciscts and Guy (2013a), Kalciscts and Guy (2016a) and Hu and Guy (2020) took different approaches to assess the validity of the IMB model. The first approach was to test the IMB model by comparing it to a compartmental analysis of tracer efflux (CATE) using stable isotope tracing to determine root E/I in balsam poplar (Populus balsamifera L.). The highly correlated E/I from the two methods suggested the IMB model can be used for estimating N-uptake efficiency (1 – E/I), which is difficult to measure directly. In the second approach, the IMB model was tested by measuring the δ15N of inorganic and organic N forms in xylem sap in black cottonwood (Populus trichocarpa Torr. & Gray) to compare the calculated proportion of inorganic nitrogen assimilated in roots (Proot) and translocation of inorganic nitrogen to shoots (Troot/Ttop) to direct measurements. Hu and Guy (2020) validated and improved the model estimates by adjusting the discrimination factor for NR to 22‰. Significant genotypic variations in N-use traits from the IMB model were reported in balsam poplar under either NO$_3^-$ or NH$_4^+$ nutrition (Kalciscts and Guy, 2016b), and in black cottonwood (Hu and Guy, 2020) and heart-leaved willow (Salix eriocephala Michx.) under NO$_3^-$ (Hu et al., 2022).

For heart-leaved willow, Hu et al. (2022) used the IMB model and tissue C/N ratios to study variation in N-uptake efficiency and NUE, and stable carbon isotope analysis to study variation in water-use efficiency (WUE). The carbon isotopic
composition ($\delta^{13}C$) of plant tissue can provide information on intrinsic water-use efficiency of C3 plants (Sun et al., 1996; Seibt et al., 2008). Fractionation of carbon isotopes occurs principally during diffusion of $CO_2$ into the leaf and at fixation by RuBisCO, which both discriminate against $^{13}C$. Net discrimination can be simply modelled as a function of the atmosphere-to-leaf $CO_2$ diffusion gradient, which also determines the intrinsic WUE of photosynthesis (Farquhar et al., 1982).

Canola has been under strong selection in breeding programs for higher yield and better oil quality. In this study, we selected 23 Canadian canola lines developed from 1942 to 2017, including open-pollinated (OP) lines developed prior to 2005 as well as more recent commercial hybrids (CH), and in three separate experiments grew them under hydroponic conditions in a greenhouse with either 0.5 mM ammonium, 0.5 mM nitrate or 5 mM nitrate. We hypothesized that direct selection for yield in canola may have resulted in the indirect selection of N-uptake efficiency, NUE and WUE.

Material and methods

Plant material, hydroponics system and experimental design

The experiment used 23 historical canola lines developed in Canada from 1942 to 2017, including open-pollinated lines developed prior to 2005 and more recent commercial hybrids (Table 1). Three separate experiments were conducted under hydroponic conditions in a greenhouse with either 0.5 mM ammonium, 0.5 mM nitrate (low nitrate), or 5 mM nitrate (high nitrate). Because of toxicity, it was not possible to grow plants in 5 mM ammonium.

The hydroponic system (Figure 1) had four 150 L bench-mounted acrylic tubs, a 1400 L vertical ground tank (RK400; CANWEST, Surrey, Canada), two submersible water pumps (Little Giant, Fort Wayne, IN, USA) and connecting PVC piping (1-inch diameter; WaterTec, Langley, BC, Canada). One water pump sat in the bottom of the main tank and continuously circulated media to the four tubs, each holding a floating “raft” made of dense foam bolted between black (lower) and white (upper) sheets of Perspex. Each raft held 32 plants in drilled holes (2.5 cm, one plant per hole) fitted with partially slit foam plugs. For each experiment there were initially four replicate plants per line (one per raft, randomly arranged; extra holes were filled with additional canola plants that were not part of this study). The depth of water in the tubs was set by an overflow outlet draining into a receiving reservoir at ground level containing a float switch-activated pump that returned media to the main tank. The total volume of the system was maintained at 2000 L. To ensure aeration of the entire system, an air pump was connected to an air stone in the receiving reservoir. Exposed parts of the circulation system not

| Year of release | Group | Seed source |
|----------------|-------|-------------|
| 1942           | Open-pollinated | AAFC        |
| 1966           | Open-pollinated | AAFC        |
| 1968           | Open-pollinated | AAFC        |
| 1973           | Open-pollinated | AAFC        |
| 1974           | Open-pollinated | AAFC        |
| 1982           | Open-pollinated | AAFC        |
| 1989           | Open-pollinated | AAFC        |
| 1990           | Open-pollinated | AAFC        |
| 1992           | Open-pollinated | AAFC        |
| 1994           | Open-pollinated | Raymond Gadoua |
| 1996           | Open-pollinated | AAFC        |
| 1998           | Open-pollinated | University of Alberta |
| 2001           | Open-pollinated | Nutrien     |
| 2005           | Open-pollinated | AAFC        |
| 2007           | Commercial Hybrid | BASF    |
| 2011           | Commercial Hybrid | Corteva    |
| 2012           | Commercial Hybrid | Monsanto   |
| 2013           | Commercial Hybrid | BASF    |
| 2014           | Commercial Hybrid | Corteva    |
| 2014           | Commercial Hybrid | Nutrien    |
| 2015           | Commercial Hybrid | Corteva    |
| 2016           | Commercial Hybrid | BASF    |
| 2017           | Commercial Hybrid | Monsanto   |

AAFC (Agriculture and Agri-Food Canada), BASF (Baden Aniline and Soda Factory).

The hydroponics solution was a modified 1/10th strength Johnson’s solution (Johnson et al., 1957) supplemented with either 0.5mM or 5mM nitrate (as Ca(NO3)2; $\delta^{15}$N = 3.5‰) for the low nitrate or the high nitrate experiment, respectively, and 0.5mM ammonium (as (NH4)2SO4; $\delta^{15}$N = 0.2‰) for the ammonium experiment. The solution was monitored daily for oxygen levels, pH and temperature. Powdered calcium carbonate (CaCO3) was added to buffer pH in the range of 6-7.5. Media NO3 and NH4 concentrations were measured periodically using the perchloric acid method (Cawse, 1967) and phenolhypochlorite method (Solozzano, 1969). The solution was completely replaced every five days in an attempt to ensure there was no substantial decrease in NO3 or NH4 concentration over time that could result in major changes to the $\delta^{15}$N of the hydroponics solution.
However, based on the total nitrogen content and isotopic composition of the harvested plants, 10.22, 21.26 and 2.36% of the supplied N was consumed in the ammonium, low nitrate and high nitrate experiments, which would have resulted in isotopic enrichments of 0.5, 0.4 and 0.1‰, respectively. Accordingly, source $\delta^{15}$N values were adjusted by these amounts for purposes of calculating discrimination.

Isotope analysis and IMB model calculations

Canola plants were harvested into leaves, stems, and roots after 45 days of growth. Once freeze-dried, these parts were weighed and then pulverized using a Wiley mill (Fritsch Laborgeratebau, Terochem Scientific, Ottawa, ON, Canada) followed by a Geno/Grinder (SPEX SamplePrep, Metuchen, New Jersey, USA). Sub-samples (3 mg) were packed into tin capsules and analyzed for %C, %N, $\delta^{13}$C and $\delta^{15}$N using a Vario EL Cube Elemental Analyzer (Elementar, Germany) interfaced to an Isoprime Isotope Ratio Mass Spectrometer (GV Instruments, UK) in the Stable Isotope Lab, Agriculture and Agri-Food Canada Lethbridge Research and Development Centre, Lethbridge, Alberta, Canada.

Nitrogen isotopic composition ($\delta^{15}$N) and isotope discrimination ($\Delta$) were expressed as:

$$\delta^{15}N = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$  \hspace{1cm} (1)

$$\Delta^{15}N_{\text{sample}} = \frac{(\delta^{15}N_{\text{source}} - \delta^{15}N_{\text{sample}})}{(1 + \delta^{15}N_{\text{sample}}/1000)}$$  \hspace{1cm} (2)

where, $R_{\text{sample}}$ and $R_{\text{standard}}$ are the $^{15}$N/$^{14}$N ratios of the sample and the arbitrary standard (air $N_2$), respectively. Plants

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FIGURE 1
Simplified schematic of the greenhouse steady-state hydroponic system. Not shown are the aeration pump and a small receiving vessel containing a second submersible water pump returning media to the main reservoir. The artwork is drawn by Debbie Maizels, Zoobotanica - Science & Nature Illustration.
were divided into three major parts (leaf, stem, and root), and the whole-plant isotope discrimination was calculated as the weighted sum of these parts:

\[
\Delta^{15}N_{\text{whole-plant}} = (f_{\text{root}} \times \Delta^{15}N_{\text{root}}) + (f_{\text{stem}} \times \Delta^{15}N_{\text{stem}}) \\
+ (f_{\text{leaf}} \times \Delta^{15}N_{\text{leaf}})
\]

(3)

where, \(\Delta^{15}N\) is the discrimination relative to the external media N source and \(f_i\) is equal to the fraction of tissue nitrogen contributing to overall plant nitrogen content. The percent ratio of inorganic nitrogen (\(T_i\)) relative to total nitrogen (\(T_t\)) translocated to the leaves (\(T_{iTi}\)), as predicted by the IMB model (Kalcsits et al., 2014, Figure 2), was calculated as:

\[
\frac{T_i}{T_t} = \frac{(\Delta^{15}N_{\text{root}} - \Delta^{15}N_{\text{leaf}})}{\Delta_{\text{enzyme}}} \times 100
\]

(4)

where, \(\Delta_{\text{enzyme}}\) is the discrimination factor for NR (22‰) or GS (17‰), as appropriate for either nitrate or ammonium assimilation. Assuming leaves and roots are the major sites of nitrogen assimilation, the amount of stem N (relative to total plant N) that originated from the leaves (\(f_{\text{stem-leaf}}\)) was approximated by:

\[
f_{\text{stem-leaf}} \approx \frac{\Delta^{15}N_{\text{stem}} - \Delta^{15}N_{\text{root}}}{\Delta^{15}N_{\text{stem}}} \times f_{\text{stem}}
\]

(5)

and the proportion of total plant nitrogen assigned to the leaf pool was:

\[
f_{\text{leaf}} \text{pool} = f_{\text{leaf}} + f_{\text{stem-leaf}}
\]

(6)

\(\Delta^{15}N_{\text{stem}}\) frequently exceeded \(\Delta^{15}N_{\text{root}}\) in the ammonium experiment, in which case all stem N was assumed to originate from the roots and \(f_{\text{stem-leaf}}\) was assigned a value of zero. The proportion of total plant nitrogen assimilated in the roots (\(P_{\text{root}}\)) was given by:

\[
P_{\text{root}} = 1 - \left( f_{\text{leaf pool}} \times \frac{T_i}{T_t} \right)
\]

(7)

and efflux over influx (E/I) was then calculated as:

\[
\frac{E}{I} = \frac{\Delta^{15}N_{\text{plant}}}{\Delta_{\text{enzyme}} \times P_{\text{root}}}
\]

(8)

**Results**

Plants reached greater size in the nitrate experiments than in the ammonium experiment, but given that these were all separate experiments, we are unable to test for differences in performance between N sources or concentrations. There were no obvious signs of N limitation or toxicity in all three experiments. Foliar nitrogen averaged (±SD) 4.48±0.69% in the 0.5mM (low) nitrate experiment, 4.90±0.94% in the 5.64±1.27% in the 0.5 mM ammonium experiment, which is within the expected 3-6% sufficiency range for herbaceous plants (Römheld, 2012).

Plants showed considerable discrimination against \(^{15}\text{N}\) in the ammonium experiment. Across all 23 canola lines, the leaf, stem and root \(^{15}\text{N}\) averaged (±SD) 6.1±0.8‰, 8.2±0.9‰ and 7.1±1.0‰, respectively (\(P<0.001\)). Group means, ranges and statistical differences are summarized in Table 2. There were clear statistical differences between OP and CH lines for whole-plant biomass, root \(^{15}\text{N}\), whole-plant \(^{15}\text{N}\) and whole-plant \(^{13}\text{C}\). The difference in the whole-plant \(^{15}\text{N}\) can largely be ascribed to the difference in root \(^{15}\text{N}\). The CH group had 76% higher biomass than the OP group, while the root:shoot ratios was similar. The CH group also had significantly higher average root and whole-plant \(^{15}\text{N}\) than the OP group, by 0.6% and 0.3%, respectively. For whole-plant \(^{13}\text{C}\), the CH group was 0.5% higher than the OP group. Although the differences were small, the IMB model estimates suggested significantly higher \(E/I\) and \(T_i/T_t\), and lower \(P_{\text{root}}\) in the CH group as compared to the OP group. There was no statistical difference between OP and CH lines in whole-plant C/N, being 9.95±2.03 for the OP group and 10.40±2.44 for the CH group, respectively.

Unlike the ammonium experiment, there were no statistically significant differences between the OP and the CH groups in either the low nitrate experiment (Table 3) or the high nitrate experiment (Table 4). In the low nitrate experiment, the average whole-plant biomass across all lines was 9.55±7.65 g and the average root:shoot ratio was 0.14±0.05. Mean leaf, stem and root \(^{15}\text{N}\) was 0.7±0.8‰, 1.7±0.8‰ and 5.7±1.3‰, whereas whole-plant \(^{15}\text{N}\) and \(^{13}\text{C}\) were 1.7±0.6% and -30.8±0.7‰, respectively. Estimated \(E/I\), \(P_{\text{root}}\) and \(T_i/T_t\) averaged 0.09±0.03, 0.82±0.05 and 0.23±0.06, respectively.

In the high nitrate experiment, whole-plant biomass averaged 11.92±6.5 g and the mean root:shoot ratio was 0.16 ±0.13. Mean values for the leaf, stem and root \(^{15}\text{N}\) were 2.0 ±2.4‰, 6.0±1.2‰ and 11.3±0.9‰, and the whole-plant \(^{15}\text{N}\) and \(^{13}\text{C}\) were 4.8±1.3‰ and -31.5±0.6‰, respectively. The

**Statistics**

All statistical analyses used R version 3.5.1 (R Core Development Team, 2022). One-way nested ANOVA was used to test for differences in plant biomass, whole-plant and tissue C and N concentrations, whole-plant and tissue \(^{13}\text{C}\) and \(^{15}\text{N}\), and IMB model estimates with group effect (OPs and CHs) as a fixed factor and historical lines nested within the group effect. Pearson correlation coefficients (\(r\)) were used to examine relationships between all physiological variables determined among the 23 canola lines.
average $E/I$ was 0.31±0.06, while $P_{root}$ was 0.71±0.07 and $Ti/Tt$ was 0.42±0.12.

Genetic correlations between growth and isotope-based physiological traits in each experiment are shown in Figure 3. $P_{root}$ and $Ti/Tt$ were consistently and very strongly negatively related to each other in all three experiments ($P<0.001$), as would be expected based on Equation 7. Otherwise, the strength and direction of trait-to-trait correlations varied considerably between experiments.

In the ammonium experiment, whole-plant biomass was positively correlated to $Ti/Tt$ ($P<0.01$), and negatively to $P_{root}$ ($P<0.05$). Root:shoot ratio was almost but not quite significantly negatively correlated to whole-plant $\delta^{13}$C ($P = 0.058$). $P_{root}$ was negatively correlated with biomass ($P<0.05$). Whole-plant $\delta^{13}$C was significantly and positively correlated with the whole-plant C/N ratio ($P<0.05$).

In the low nitrate experiment, biomass was positively correlated to whole-plant $\delta^{13}$C ($P<0.01$) but not quite $E/I$
Table 2: Overall means and ranges of whole-plant (WP) biomass, root-to-shoot (R:S) ratio, organ and whole-plant level nitrogen isotope discrimination (Δ15N) and related isotope mass balance (IMB) model estimates, whole-plant carbon isotope composition (Δ13C) and carbon to nitrogen ratio (C/N) between open-pollinated (OP) lines and commercial hybrids (CH) in Brassica napus L. grown under 0.5 mM ammonium.

| Trait               | Open-pollinated (OP) lines | Commercial hybrids (CH) | Group difference |
|---------------------|----------------------------|-------------------------|------------------|
| WP biomass (g)      | Mean value ± SD            | Data range              | Mean value ± SD  | Data range | P = 0.001 |
| R:S ratio           | 0.24 ± 0.07                | 0.06–0.38               | 0.25 ± 0.08      | 0.05–0.39 | n.s.      |
| Leaf Δ15N (%)       | 6.0 ± 0.8                  | 3.8–7.7                 | 6.1 ± 0.9        | 3.9–7.7   | n.s.      |
| Stem Δ15N (%)       | 8.1 ± 1.0                  | 5.2–9.8                 | 8.2 ± 0.9        | 5.6–9.8   | n.s.      |
| WP Δ15N (%)         | 6.5 ± 0.8                  | 4.0–8.3                 | 6.8 ± 0.8        | 5.1–7.9   | P < 0.05  |
| Ti/Tt               | 0.05 ± 0.04                | 0.01–0.15               | 0.08 ± 0.04      | 0.01–0.16 | P < 0.001 |
| P_root              | 0.98 ± 0.02                | 0.88–0.99               | 0.96 ± 0.03      | 0.88–0.99 | P < 0.001 |
| E/I                 | 0.39 ± 0.05                | 0.23–0.51               | 0.41 ± 0.05      | 0.29–0.50 | P < 0.01  |
| WP Δ13C (%)         | -31.7 ± 1.0                | -33.5–29.3              | -31.1 ± 1.0      | -31.0–29.5| P < 0.05  |
| WP C/N              | 9.95 ± 2.03                | 6.28–17.21              | 10.4 ± 2.44      | 6.88–16.63| n.s.      |

n.s., not significant.

(P = 0.061), and negatively correlated to root:shoot ratio (P < 0.05). Similar to the ammonium experiment, E/I was not correlated with either Ti/Tt or P_root, however P_root was also not significantly correlated with biomass. In sharp contrast to the ammonium experiment, whole-plant Δ13C was positively related to E/I (P < 0.01) and negatively related to whole-plant C/N, but not quite significantly so (P = 0.063).

Discussion

There are three major competing fates for inorganic N taken up by roots, including (1) assimilation in the root cytosol, (2) loading of unassimilated N into the xylem for root-to-shoot translocation, or (3) efflux back to the rooting medium (Glass, 2003). Since the assimilating enzymes (NR and GS) prefer lighter N (14N) over

Table 3: Overall means and ranges of whole-plant (WP) biomass, root-to-shoot (R:S) ratio, organ and whole-plant level nitrogen isotope discrimination (Δ15N) and related isotope mass balance (IMB) model estimates, whole-plant carbon isotope composition (Δ13C) and carbon to nitrogen ratio (C/N) between open-pollinated (OP) lines and commercial hybrids (CH) in Brassica napus L. grown under 0.5 mM nitrate condition.

| Trait               | Open-pollinated (OP) lines | Commercial hybrids (CH) | Group difference |
|---------------------|----------------------------|-------------------------|------------------|
| WP biomass (g)      | Mean value ± SD            | Data range              | Mean value ± SD  | Data range | n.s.      |
| R:S ratio           | 0.14 ± 0.04                | 0.04–0.23               | 0.13 ± 0.05      | 0.04–0.24 | n.s.      |
| Leaf Δ15N (%)       | 0.7 ± 0.9                  | -0.7–2.5                | 0.4 ± 0.7        | -1.7–1.9  | n.s.      |
| Root Δ15N (%)       | 5.7 ± 1.0                  | 2.9–7.8                 | 5.6 ± 1.5        | 1.9–8.4   | n.s.      |
| Stem Δ15N (%)       | 1.8 ± 0.7                  | 0.6–3.7                 | 1.5 ± 1.0        | -0.2–3.6  | n.s.      |
| WP Δ15N (%)         | 1.7 ± 0.5                  | 0.9–3.1                 | 1.5 ± 0.6        | 0.5–3.2   | n.s.      |
| Ti/Tt               | 0.23 ± 0.06                | 0.10–0.38               | 0.24 ± 0.07      | 0.11–0.35 | n.s.      |
| P_root              | 0.82 ± 0.05                | 0.70–0.94               | 0.81 ± 0.07      | 0.77–0.96 | n.s.      |
| E/I                 | 0.09 ± 0.03                | 0.05–0.17               | 0.08 ± 0.03      | 0.03–0.16 | n.s.      |
| WP Δ13C (%)         | -30.8 ± 0.7                | -32.4–29.0              | -31.0 ± 0.7      | -32.9–29.8| n.s.      |
| WP C/N              | 12.64 ± 2.01               | 8.35–17.82              | 12.52 ± 2.25     | 9.99–17.93| n.s.      |

n.s., not significant.
heavier N\(^{15}\)N during assimilation, organic N in plants is generally depleted in \(^{14}\)N while remaining unassimilated inorganic N is enriched (Ledgard et al., 1985; Yoneyama et al., 1993). In this study, we found that root and whole-plant \(\Delta^{15}\)N were both positive across all three experiments, indicating that lighter inorganic N forms (\(^{14}\)NH\(_4\) or \(^{14}\)NO\(_3\)) were preferentially assimilated by plants while some fraction of the heavier unassimilated inorganic N (\(^{15}\)NH\(_4\) or \(^{15}\)NO\(_3\)) returned to the hydroponic medium. Although the three experiments were not conducted simultaneously, mean whole-plant \(\Delta^{15}\)N was highest in the ammonium experiment (6.6‰) and lowest in the low nitrate experiment (1.7‰), with the high nitrate experiment in the middle (4.8‰). The leaf \(\Delta^{15}\)N, which represented up to 70% of plant N in this study and is often used as a proxy for whole-plant \(\Delta^{15}\)N, also followed the same order.

Although discrimination against \(^{15}\)N by NR is thought to exceed discrimination by GS (Hu and Guy, 2020), we observed greater overall discrimination in the ammonium experiment than in the nitrate experiments. Higher whole-plant discrimination indicates greater efflux of unassimilated N back to the rooting medium – in other words, a greater futile cycling of inorganic nitrogen across root cell membranes. Our IMB model analysis indicates that the average root \(E/I\) was 0.09 in the low nitrate experiment, 0.31 in high nitrate experiment and 0.41 in the ammonium experiment. A much greater futile cycling for ammonium as compared to nitrate has been commonly observed in many plants (Britto and Kronzucker, 2001), and, at higher concentrations, may contribute to ammonium toxicity (Britto et al., 2001). A recent study from Hachiya et al. (2021), however, has attributed ammonium toxicity in Arabidopsis to

**TABLE 4** Overall means and ranges of whole-plant (WP) biomass, root-to-shoot (R:S) ratio, organ and whole-plant level nitrogen isotope discrimination (\(\Delta^{15}\)N) and related isotope mass balance (IMB) model estimates, whole-plant carbon isotope composition (\(d^{13}\)C) and carbon to nitrogen ratio (C/N) between open-pollinated (OP) lines and commercial hybrids (CH) in Brassica napus L. grown under 5 mM nitrate condition.

| Trait                  | Open-pollinated (OP) lines | Commercial hybrids(CH) | Group difference |
|------------------------|---------------------------|------------------------|------------------|
| WP biomass (g)         | Mean value ± SD           | Data range             | Mean value ± SD  | Data range         | n.s.         |
| R:S ratio              | 0.16 ± 0.15               | 0.02–0.96              | 0.19 ± 0.12      | 0.04–0.48          | n.s.         |
| Leaf \(\Delta^{15}\)N (%a) | 2.0 ± 2.5                 | -3.5–6.4               | 1.7 ± 2.3        | -2.3–5.7           | n.s.         |
| Stem \(\Delta^{15}\)N (%a) | 11.2 ± 0.9                | 8.5–12.2              | 11.3 ± 0.8       | 8.8–12.4           | n.s.         |
| WP \(\Delta^{15}\)N (%a) | 4.7 ± 1.4                 | 1.2–7.1               | 4.8 ± 1.3        | 3.0–8.0            | n.s.         |
| Ti/Tt                  | 0.42 ± 0.12               | 0.15–0.71             | 0.43 ± 0.11      | 0.28–0.63          | n.s.         |
| \(P_{root}\)           | 0.71 ± 0.07               | 0.55–0.82             | 0.71 ± 0.05      | 0.62–0.85          | n.s.         |
| \(E/I\)                | 0.30 ± 0.07               | 0.08–0.40             | 0.31 ± 0.06      | 0.20–0.43          | n.s.         |
| WP \(d^{13}\)C (%a)    | -31.5 ± 0.8               | -33.7–30.2            | -31.5 ± 0.6      | -32.5–30.3         | n.s.         |
| WP C/N                 | 8.04 ± 1.56               | 5.06–11.00            | 8.36 ± 1.76      | 4.59–12.63         | n.s.         |

n.s., not significant.
acidiﬁcation caused by overly high rates of NH$_4^+$ assimilation catalyzed by a plastidic GS.

Measuring E/I has been difﬁcult because of high spatial and temporal heterogeneity in actively growing plants (Hawkins and Robbins, 2010). Due to the dynamic nature of N ﬂux in time and space within the plant, an integrated approach to assess E/I would be useful. There are two available methods that can measure E/I directly, namely with microelectrodes (Hawkins and Robbins, 2010) or by the CATE method using either $^{15}$N or $^{15}$N as a tracer (Kronzucker et al., 1995; Min et al., 1999; Kalcits and Guy, 2016a). These methods provide instantaneous measures of nitrogen ﬂux, whereas the IMB model is expected to better reﬂect the time-integrated (and therefore potentially less variable) efﬁciency of nitrogen uptake. Nonetheless, Kalcits & Guy (2016) compared E/I of balsam poplar as measured by the CATE method and by the IMB model under 0.5 mM ammonium or nitrate, and in both cases found that E/I was higher when plants were provisioned with ammonium than with nitrate. E/I is also known to increase as substrate concentration increases, as suggested by our nitrate experiments. Min et al. (1999) measured E/I at 0.1 mM and 1.5 mM nitrate in trembling aspen (Populus tremuloides Michx.), lodgepole pine (Pinus contorta Dougl.) and Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco), and in every case found E/I was increased at the higher concentration.

An advantage of the IMB model over other methods to measure nitrogen ﬂux is the information it provides on relative concentrations of inorganic and organic nitrogen translocated in the xylem and the proportioning of N assimilation between roots and shoots. This information is calculated from the leaf-root differences in discrimination (Equation 4) and by combining it with the organ level data on N partitioning (Equation 7). In the nitrate experiments, leaf $\Delta^{15}$N values, on average, were both close to zero, while stem $\Delta^{15}$N and root $\Delta^{15}$N were not. We found similar patterns in nitrate-grown black cottonwood and heart-leaved willow (Hu and Guy, 2020; Hu et al., 2022). Leaf $\Delta^{15}$N in these circumstances is close to zero because the transported $^{15}$N-enriched inorganic N and $^{15}$N-depleted organic N from roots essentially balance each other out. In the ammonium experiment, however, leaf $\Delta^{15}$N was 6.0% and there was a much smaller leaf-root difference in $\Delta^{15}$N. This smaller difference is expected since, in most species, ammonium is primarily assimilated in roots and not leaves, whereas the site of nitrate assimilation is more variable (e.g., Kalcits et al., 2015). The overall mean $T_i/T_t$ in the ammonium experiment was 0.07, and $P_{root}$ was 0.97. In contrast, in the low nitrate experiment $T_i/T_t$ and $P_{root}$ averaged 0.23 and 0.82, and in the high nitrate experiment, they averaged 0.42 and 0.71, respectively.

Another profound advantage of the IMB model is its utility for screening large numbers of plants. The assessment of genotypic variation in E/I and other ﬂux parameters using the CATE or microelectrode methods has been limited to a few genotypes (e.g., Abdellauoi et al., 2001) or to species with known contrasting NUE (Britto and Kronzucker, 2001; Britto et al., 2001; Glass, 2003). In the present study, we used the IMB model to assess 23 historical canola lines. Although we were unable to detect differences between speciﬁc lines because of low statistical power (there were only 3-4 individuals tested per line), our hypothesis that direct selection for yield in canola may have resulted in the indirect selection on N-uptake efﬁciency (i.e., 1- E/I) was supported by the group differences we found in the ammonium experiment. In this experiment, the CH group had better growth than the OP group, as well as higher $\Delta^{15}$N at the root, stem, leaf and whole-plant levels. The commercial hybrids also had a greater mean leaf-root $\Delta^{15}$N difference. These differences indicate that the commercial hybrids, which are more recent than the open-pollinated lines, tend to have higher E/I and $T_i/T_t$, and a lower $P_{root}$. The higher $T_i/T_t$ and lower $P_{root}$ suggest that hybrid lines have a slightly greater capacity for ammonium translocation in the xylem and proportionally greater ammonium assimilation in the leaves. The greater relative growth of these lines when provisioned solely with ammonium also suggests increased resistance to ammonium toxicity. In contrast, the slightly higher E/I suggests more futile cycling at the soil-root interface and a modest reduction in uptake efﬁciency. This result is surprising, as we might initially expect a greater growth demand to result in relatively less N leakage, not more. Overall, the slightly higher eﬄux implies an even faster rate of ammonium uptake by the CH group. By harvest, we calculate that gross N inﬂux and eﬄux averaged 244±18 and 97±14 mg N per plant for the OP group, and 430±38 and 182±33 mg N per plant for the CH group, respectively.

The greater stem $\Delta^{15}$N relative to root $\Delta^{15}$N observed in the ammonium experiment underscores an important limitation of the current IMB model in assuming that the root inorganic N content is negligible. Kalcits et al. (2014) noted that this assumption must result in an underestimation of the isotopic difference between root- and leaf-assimilated organic N, and thus an underestimation of $T_i/T_t$ with consequent effects on the estimation of $P_{root}$ and, to a lesser extent, E/I. They also noted that the fraction of inorganic N in roots typically varies from 1 to 10% of the total N (Evans et al., 1996; Yoneyama et al., 2001; Black et al., 2002; Kolb and Evans, 2003). The root inorganic and organic N fractions are expected to differ in $\Delta^{15}$N by an amount that approximates the discrimination factor of the assimilatory enzyme. Accordingly, Hu and Guy (2020) found that the $\Delta^{15}$N of the soluble organic and inorganic N fractions in roots of nitrate-grown black cottonwood diﬀered by 18.2%. Given that the $\Delta_{assim}$ for GS is 17‰, and if as much as 1/10th of the root N content is ammonium, the whole root discrimination could be decreased by up to 1.7‰. Indeed, if N assimilation were fully
restricted to the roots, the aerial portions of the plant would show greater discrimination than the roots by an equal amount. If only stems show greater discrimination, as in our ammonium experiment (Table 2), then some N assimilation must also occur in the leaves but with little of it making its way into other tissues. We note from Table 2 that the average leaf and stem $\Delta^{15}N$ values for the OP and CH groups were very similar and did not differ, whereas the root $\Delta^{15}N$ for the CH group was significantly greater than it was for the OP group. The difference in root $\Delta^{15}N$ may therefore indicate that the CH roots have a lower load of unassimilated ammonium than the OP roots and, if accounted for, could reduce or even eliminate the differences in $E/I$, $Ti/Tt$ and $P_{root}$. Direct measurements of root ammonium contents are needed to evaluate this possibility.

Contrary to our hypothesis, there were no group differences in C/N ratio (our proxy for NUE) or in whole-plant $\delta^{13}C$ (our proxy for WUE). We estimated the genetic correlations between key physiological traits and biomass across all 23 canola lines. The trait-to-trait correlations varied considerably between experiments (Figure 3). There appeared to be a trade-off between NUE and WUE in the low nitrate experiment, but the proxies for these traits were positively correlated in the ammonium and high nitrate experiments. Consistent with the group differences noted above for the ammonium experiment, whole-plant biomass was positively correlated with $Ti/Tt$ and negatively with $P_{root}$. The rootshoot ratio was not correlated to any N-related traits in the ammonium and low nitrate experiments, but it was positively correlated with $P_{root}$ and $E/I$ in the high nitrate experiment. The correlation with $P_{root}$ might be expected mathematically (i.e., increased root assimilation simply because of root biomass), but the calculation of $E/I$ is independent of root mass. Increased root-to-shoot ratio was also correlated with increased $E/I$ in heart-leaved willow (Hu et al., 2022) and in black cottonwood (Hu, 2022). A higher root biomass might result in a higher N supply (greater uptake) relative to N demand by the shoot (e.g., for photosynthetic proteins), allowing more unassimilated nitrate to efflux from the root (Evans, 2001).

**Data availability statement**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**Author contributions**

YH performed hydroponics experiments, analyzed data, and drafted the manuscript. RDG provided scientific insight into data analysis and edited the manuscript. RYS conceived of the study, obtained funding, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Investigate nitrogen uptake and assimilation in poplar and willow. Nitrogen and nitrate in xylem sap under near steady-state hydroponics. Variability and quantitative genetics within integrated approaches.

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