

Two-way nitrogen transfer between *Dalbergia odorifera* and its hemiparasite *Santalum album* is enhanced when the N₂-fixing host effectively fixes nitrogen

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Abstract

Understanding plant-parasite interactions between root hemiparasite *Santalum album* and its host trees has theoretical and practical significance in plantations of precious sandalwood as well as tree nutrition or fertilization management. Nutrient translocation from a host plant is vital to the growth and survival of its root parasitic plant, but few studies have investigated whether a parasitic plant is also able to transfer nutrients to its host. The role of N₂-fixation in nitrogen (N) transfer between 7-month-old *Dalbergia odorifera* T. Chen nodulated with *Bradyrhizobium elkanii* DG and its hemiparasite *Santalum album* Linn. was examined by external ¹⁵N labelling in a pot study. Four paired treatments were used, with ¹⁵N given to either host or hemiparasite and the host either nodulated or grown on combined N. N₂-fixation supplied 41–44% of total N in *D. odorifera*. Biomass, N and ¹⁵N contents were significantly greater in both nodulated *D. odorifera* and *S. album* grown with paired nodulated *D. odorifera*. Significantly higher total plant ¹⁵N recovery was in N-donor *D. odorifera* (68–72%) than in N-donor *S. album* (42–44%), regardless of the nodulation status in *D. odorifera*. Nitrogen transfer to *S. album* was significantly greater (27.8–67.8 mg plant⁻¹) than to *D. odorifera* (2.0–8.9 mg plant⁻¹) and 2.4–4.5 times greater in the nodulated pair than in the non-nodulated pair. Irrespective of the nodulation status, *S. album* was always the N-sink plant. The amount of two-way N transfer was increased by the presence of effective nodules, resulting in a greater net N transfer (22.6 mg plant⁻¹) from host *D. odorifera* to hemiparasite *S. album*. Our results may provide better N management strategies for successfully mixed field plantation of *S. album* with *D. odorifera*, both are in great market demanding as precious fragrant timbers, but have been globally over-exploited in the field.

Key Words: δ¹⁵N, ¹⁵N-recovery, *Bradyrhizobium*, haustorium, N₂-fixation, nodulation

Introduction

Nitrogen (N) transfer between plants is of fundamental importance in agricultural and natural ecosystems (Richards et al. 2010). Numerous studies over the past two decades have demonstrated that N transfer between N₂-fixing and non-N₂-fixing plants is through both soil mass flow and diffusion (Høgh-Jensen 2006, Nygren and Leblanc 2015) and common mycorrhizal network (CMN) linkages (He et al. 2009, Teste et al. 2015). Parasitic plants (~19 families, 227 genera and 4,100 species) are mainly classified into hemiparasites (containing chlorophyll), holoparasites (lacking chlorophyll), xylem and phloem feeders or stem and root parasites (Bell and Adams 2011). Root parasitic plants are linked to their hosts by haustoria for taking up water, mineral nutrients or organic compounds (Pate 2001). For instance, ¹⁵N labelling and ¹⁵N natural abundance methods have demonstrated that up to 70% of N in the parasites is from the hosts (Pate 2001, Cameron and Seel 2007), indicating that such N translocations may be vital for the growth and survival of the parasitic plants. However, no studies have investigated whether a parasitic plant is also able to transfer N to its host. Indian sandalwood (*S. album* L.) has been over-exploited for its aromatic heartwood and root, which have cosmetic, religious and medicinal significance. Over the last two decades, several large-scale plantations of *S. album* have been established to meet future market requirements in Australia, China, India and Indonesia. Both pot and field plantations have shown that the growth performance of *S. album* is greatly enhanced by its successful attachment to suitable hosts, particularly to N₂-fixing species (e.g., *Acacia*, *Casuarina* and *Sesbania*) (Li 2003). Compared to a single-species plantation, a mixed plantation of suitable high-value non-N₂-fixing or N₂-fixing host trees with *S. album* is important to the socio-economy of local growers. *Dalbergia odorifera* T. Chen, one of most precious rosewoods in the world with diverse medicinal and commercial values, has been successfully planted with *S. album* in Hainan Island, China (18°42'N, 108°49'E) since 1989. Unfortunately, to date, an appropriate understanding of the N₂-fixation capacity, parasitic relationship and either one-way or two-way N transfer has not been studied between these two precious tropical forest trees.

In a pot study, using $(^{15}\text{NH}_4)_2\text{SO}_4$ as ^{15}N tracer to address the possible interplant N movement and role of N_2 -fixation in N transfer between 7-month-old hemiparasite *S. album* and its N_2 -fixing host *D. odorifera*, we asked: (1) Does N transfer occur between hemiparasitic *S. album* and host *D. odorifera*? (2) If N transfer occurs, how much is transferred? (3) Is N transfer affected by N_2 -fixation? (4) In which direction does net N transfer occur? Answers to these questions could reveal N movement between the hemiparasite *S. album* and its N_2 -fixing host *D. odorifera* and provide N management strategies for these two high-value tropical trees.

Methods

Plant growth

Seeds of *S. album* and *D. odorifera* were surface-sterilized with 3% NaOCl for 5 min, rinsed with distilled water, and germinated on sterilized sand at 28–30 °C for 4 weeks. Two emerging seedlings with 1 cm radicals, either in a single species or with *S. album* as a pair, were transplanted into one 15 × 11 × 14 cm plastic pot with 250 g potting mix (vermiculite : perlite = 2 : 1, v/v, pH 6.8 ± 0.2), grown under 33/25 °C 65/75 relative humidity (day/night) for 7-months. *Bradyrhizobium elkanii* DG, an effective N_2 -fixing strain was inoculated to germinated N_2 -fixing *D. odorifera* seedlings whereas autoclaved *B. elkanii* inoculants to *S. album*. Seedlings were weekly watered twice with distilled water, and with 5 ml Jensen's N-free nutrient solution and 2.5 ml 0.5% $(^{14}\text{NH}_4)_2\text{SO}_4$ (2.65 mg N pot⁻¹ week⁻¹) for the first 6 months (127.14 mg N pot⁻¹ year⁻¹). The ^{15}N signature of $(^{14}\text{NH}_4)_2\text{SO}_4$ was 0.366783 ± 0.000032 (^{15}N , atom %) or 1.32 ± 0.10 ($\delta^{15}\text{N}$, ‰).

Experimental design and ^{15}N labelling

Either *D. odorifera* or *S. album* served as the ^{15}N donor, and thus net N-transfer could be calculated from the difference of ^{15}N translocation from *D. odorifera* to *S. album* and from *S. album* to *D. odorifera*. Four pairs or treatments with four replicates of each were examined: (1) non-nodulated *D. odorifera*/*S. album* ($\text{Dnod}^- \rightarrow \text{S}^-$), (2) *S. album*/non-nodulated *D. odorifera* ($\text{S}^- \rightarrow \text{Dnod}^-$), (3) nodulated *D. odorifera*/*S. album* ($\text{Dnod}^+ \rightarrow \text{S}^+$) and (4) *S. album*/nodulated *D. odorifera* ($\text{S}^+ \rightarrow \text{Dnod}^+$) (see Lu et al. 2013). The two non-nodulated pairs were used to measure N transfer through the growth medium (soil pathway), while the two nodulated pairs were to study the effect of N_2 fixation on N transfer. One leaf from an N donor seedling was inserted into a 1.5 ml sterilized centrifuge tube containing 1.0 ml 0.15% $(^{15}\text{NH}_4)_2\text{SO}_4$ (N dissolved in sterilized Milli-Q water, 99.36 at % ^{15}N) solution. For each N donor seedling, a total of 3.0 ml $(^{15}\text{NH}_4)_2\text{SO}_4$ (1.006 mg N) was given as 1.0 ml each to the third, fourth and fifth fully expanded leaves. The centrifuge tubes were vertically attached to the iron wire and immediately sealed using Parafilm (Menasha, WI, USA) after the branch wrapping to avoid spillage and evaporation (Figure 1, see Lu et al. 2013). All ^{15}N solution had been imbibed after 4 weeks and no ^{14}N was supplied to the donor and receiver plants during this 4 week period.

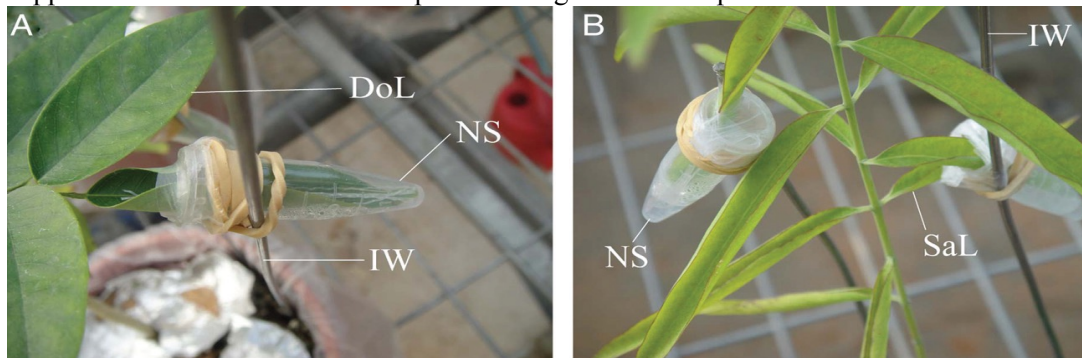


Figure 1. Setup of 99.36 at % ^{15}N labeling with plant leaves in a greenhouse. (A) *Dalbergia odorifera* and (B) *S. album*. Abbreviations: DoL, *D. odorifera* leaf; SaL, *S. album* leaf; IW, iron wire; NS, 0.15% N solution.

Plant harvest and analysis

Fresh leaves of *D. odorifera* before ^{15}N labelling were collected to estimate N_2 -fixation using the ^{15}N natural abundance method. Seedlings of *D. odorifera* and *S. album* were separately harvested after 4-weeks of ^{15}N addition to the N-donor. Oven-dried (70 °C for 72 h) tissues were ground into fine powder for the analysis of total N and ^{15}N (Isotope Ratio Mass Spectrometer, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Nitrogen transfer was expressed in following three parameters:

$$\% \text{N}_{\text{transfer}} = (^{15}\text{N}_{\text{content}}_{\text{receiver}} \times 100) / (^{15}\text{N}_{\text{content}}_{\text{receiver}} + ^{15}\text{N}_{\text{content}}_{\text{donor}}) \quad (1)$$

$$\text{N}_{\text{transfer}} (\text{mg plant}^{-1}) = (\% \text{N}_{\text{transfer}} \times \text{total N donor}) / (100 - \% \text{N}_{\text{transfer}}) \quad (2)$$

$$\% \text{NDFT} (\% \text{ of N in the receiver derived from transfer}) = (\text{N}_{\text{transfer}} \times 100) / \text{Total N}_{\text{transfer}} \quad (3)$$

Results

Formation of haustoria and nodules and N₂-fixation capacity of D. odorifera

Bell-shaped *S. album* haustoria attached to *D. odorifera* roots (Figure 2A, see Lu et al. 2013) and penetrated into *D. odorifera* nodules (Figure 2B–D, see Lu et al. 2013). The numbers of haustoria averaged 7.5 ± 3 per pair and were similar between all four paired treatments. Nodules from both the nodulated *D. odorifera* donor and receiver were reddish-brown with pink internal tissues, indicating that they were active in N₂-fixation. N₂-fixation supplied $\sim 43.00\%$ of the total N requirement, and the specific nodule activity was $\sim 45.0 \mu\text{g N mg}^{-1} \text{d}^{-1}$ wt nodule. In addition, after 4-weeks ¹⁵N labelling, foliar $\delta^{15}\text{N}$ was significantly highest in nodulated *D. odorifera* donors ($672.42 \pm 38.89\text{‰}$), less in the three non-N₂-fixing plants ($3.20\text{--}3.87\text{‰}$) and the least in the nodulated receivers ($2.61 \pm 0.45\text{‰}$).

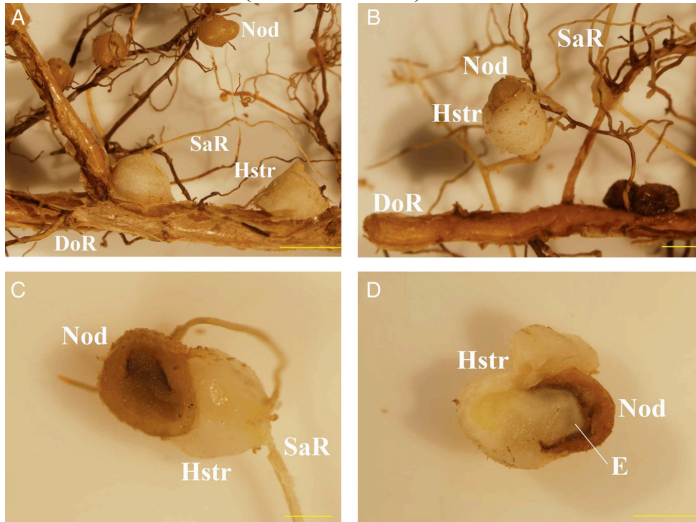


Figure 2. Haustoria of *S. album* attaching on *D. odorifera* roots (A) and nodules (B, C, D) in 7-month-old plants grown in pots. An immature haustorium (C) and a mature haustorium (D) attaching on functional nodule. Scale bars, 2 mm (A), 1 mm (B, D) and 0.5 mm (C). DoR, *D. odorifera* root; E, endophyte of haustorium; Hstr, haustorium; Nod, nodule; SaR, *S. album* root.

Plant biomass production, nitrogen concentration and content

Plant biomass production, % N or N content were similar whether *D. odorifera* and *S. album* served as the N donor or N receiver. Significantly more (1.4 – 1.5 times) biomass was produced for both *D. odorifera* and *S. album* in the nodulated pair than in the non-nodulated pair. *D. odorifera* produced about 2.5 times as much biomass as *S. album* irrespective of whether it was nodulated or not. Both *D. odorifera* and *S. album* had significantly higher % N in the nodulated pair than in the non-nodulated pair. In contrast, plant % N was similar between *D. odorifera* and *S. album* regardless of the nodulation status of the former. Plant N content in both *D. odorifera* and *S. album* was 1.9 – 3.2 times greater in the nodulated pair than in the non-nodulated pair, and *D. odorifera* had significantly more N than *S. album* whether *D. odorifera* was nodulated or not. Both greater biomass and N content were found in the nodulated pair. In addition, plant biomass production positively correlated with plant N content in both species ($r^2 = 0.94 - 0.99$).

Nitrogen transfer between D. odorifera and S. album seedlings

Two-way N transfer occurred in all four *D. odorifera*/*S. album* pairs, regardless of the nodulation status. In both nodulated and non-nodulated treatments, % N transfer, % NDFT and the amount of N transferred were significantly higher from *D. odorifera* to *S. album* (37.5 and 43.8 %, 60.8 and 81.6 %, and 27.8 and 67.8 mg plant⁻¹, respectively) than from *S. album* to *D. odorifera* (10.2 and 16.6 %, 7.4 and 12.8 %, and 2.0 and 8.9 mg plant⁻¹, respectively) (Figure 3A–C, see Lu et al. 2013). Our study was able to calculate net N-transfer in paired *D. odorifera*/*S. album* seedlings (Figure 3D, see Lu et al. 2013). The amounts of net N transfer were significantly higher in the nodulated ($22.6 \pm 4.7 \text{ mg plant}^{-1}$) than in the non-nodulated treatment ($16.9 \pm 0.8 \text{ mg plant}^{-1}$). Regardless of the nodulation status, *S. album* was the sink plant for N acquisition from *D. odorifera*.

Conclusion

Our results showed that almost half of N in *D. odorifera* was from N₂-fixation, and N₂-fixation enhanced N accumulation, biomass production and N transfer between host *D. odorifera* and hemiparasite *S. album*. When nodulated *D. odorifera* as the N donor, and when *S. album* as the N donor and partnered with nodulated *D. odorifera*, plant biomass production and N content were maximized in both plant species. Our results support

previous reports that N_2 -fixation plays an important role in N translocation to non- N_2 -fixation partner. This would have practical consequences and may provide better N management strategies for concurrently mixed plantations of precious fragrant timbers of both *D. odorifera* and *S. album* or other tree species in the field.

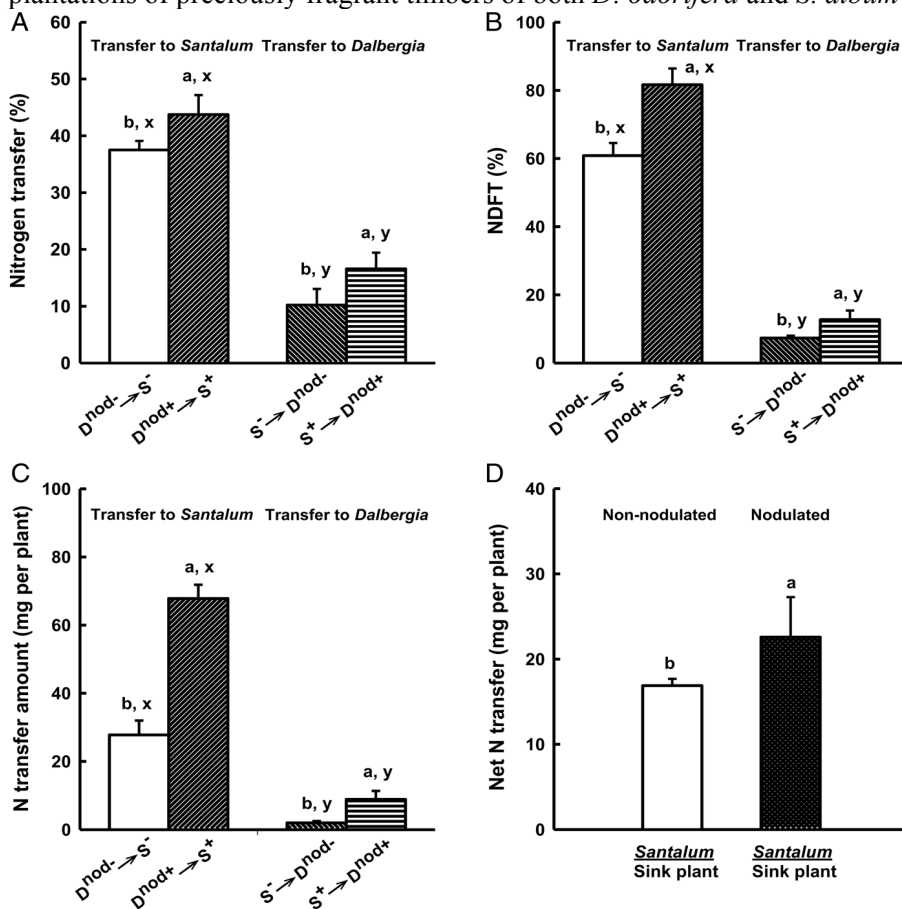


Figure 3. Nitrogen transfer (%), N in the receiver derived from transfer (NDFT), amount of N transfer (C) and net N transfer (D) in 7-month-old *D. odorifera*/*S. album* pairs as affected by nodulation (nod) and species of N donor or N receiver. Data (means \pm SE, $n = 4$) followed by different letters designate significant differences ($P = 0.05$) between treatments for a given transfer direction (a, b) and between transfer directions in pairs for a given treatment (x, y). D, *D. odorifera*; S, *S. album*; nod⁺ or nod⁻, inoculated or non-inoculated with a *Bradyrhizobium* strain; S⁺ or S⁻, *S. album* paired with inoculated or non-inoculated *D. odorifera*.

References

- Bell TL and Adams MA (2011). Attack on all fronts: functional relationships between aerial and root parasitic plants and their woody hosts and consequences for ecosystems. *Tree Physiology* 31, 3–15.
- Cameron DD and Seel WE (2007). Functional anatomy of haustoria formed by *Rhinanthus minor*: linking evidence from histology and isotope tracing. *New Phytologist* 174, 412–419.
- He XH, Xu M, Qiu GY and Zhou J (2009). Use of ^{15}N stable isotope to quantify nitrogen transfer between mycorrhizal plants. *Journal of Plant Ecology* 2, 107–118.
- Høgh-Jensen H. (2006). The nitrogen transfer between plants: an important but difficult flux to quantify. *Plant and Soil* 282, 1–5.
- Li YL. (2003). Sandalwood Introduction and Research (in Chinese). Science Press, Beijing, China.
- Lu JK, Kang LH, Sprent JI, Xu DP and He XH (2013). Two-way transfer of nitrogen between *Dalbergia odorifera* and its hemiparasite *Santalum album* is enhanced when the host is effectively nodulated and fixing nitrogen. *Tree Physiology* 33, 464–474.
- Nygren P and Leblanc HA (2015). Dinitrogen fixation by legume shade trees and direct transfer of fixed N to associated cacao in a tropical agroforestry system. *Tree Physiology* 35, 134–147.
- Pate JS (2001). Haustoria in action: case studies of nitrogen acquisition by woody xylem-tapping hemiparasites from their hosts. *Protoplasma* 215, 204–217.
- Richards AE, Forrester DI, Bauhus J and Scherer-Lorenzen M (2010). The influence of mixed tree plantations on the nutrition of individual species: a review. *Tree Physiology* 30, 1192–1208.
- Teste FP, Veneklaas EJ, Dixon KW and Lambers H (2015). Is nitrogen transfer among plants enhanced by contrasting nutrient-acquisition strategies? *Plant, Cell, and Environment* 38, 50–60.