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Symbiotic nitrogen contribution and biodiversity of root-nodule bacteria nodulating *Psoralea* species in the Cape Fynbos, South Africa

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ABSTRACT

The genus *Psoralea* (tribe Psoraleae, family Leguminosae) is indigenous to the Cape fynbos of South Africa and little is known about its symbiosis and/or adaptation. The aim of this study was to assess root nodulation and N_2 fixation in eight of the 50 *Psoralea* species, as well as the biodiversity of their associated nodulating microsymbionts. The eight species studied (namely, *Psoralea pinnata*, *Psoralea aphylla*, *Psoralea aculeata*, *Psoralea monophylla*, *Psoralea repens*, *Psoralea laxa*, *Psoralea asarina* and *Psoralea restioides*) all had round-shaped, determinate type (desmodioid) nodules, and data from ^{15}N natural abundance showed that they obtained 60–88% of their N nutrition from symbiotic fixation. These *Psoralea* species also transported their fixed-N as ureides (allantoin and allantoic acid) in the xylem stream, a symbiotic trait that links them very closely to the tribe Phaseoleae. Bacteria isolated from root nodules of the eight *Psoralea* species varied in phenotypic characteristics, nodulation promiscuity, and N_2-fixing efficacy. Furthermore, 16S rDNA gene sequence data showed that *Psoralea* species can form root nodules with different soil bacteria, including *Rhizobium*, *Mesorhizobium* and *Burkholderia* strains. This is not only evidence of nodulation promiscuity, but also an indication of the species' adaptation to the nutrient-poor, low-N, sandy acidic soils of the Cape fynbos.

1. Introduction

The Cape Fynbos of South Africa is rich in indigenous legumes, which belong to many genera, including *Amphithalea*, *Aspalathus*, *Bolusafra*, *Cyclopia*, *Dipogon*, *Hypocalyptus*, *Liparia*, *Podalyria*, *Polhillia*, *Psoralea*, *Stirtonanthus*, *Virgilia*, *Wiborgia*, and *Xiphotheca* (Schutte, 2000). Some genera such as *Bolusafra* have only one species (*Bolusafra bituminosa* L. Kuntse), while others like *Aspalathus* has as many as 281 species (Cupido, 2007; Boatwright and Cupido, 2011; Stirton and Muasya, 2011). Although some work has been done on the symbioses of some Fynbos legumes (Marumo, 1996; Muofhe, 1997; Muofhe and Dakora, 1999; Spriggs, 2004; Spriggs et al., 2003; Elliott et al., 2007; Spriggs and Dakora, 2007, 2008, 2009a,b), little is known about the N contribution of these species/genera and the bacterial symbionts nodulating them. The nodulation status of some of these legumes also remains unknown.

Measuring N_2 fixation in wild perennial legumes such as those endemic in Cape Fynbos can pose a challenge due to many inherent limitations, including choice of reference plants (Unkovich et al., 2008). Despite these problems, the ^{15}N natural abundance technique has been found to provide reliable data on N_2 fixation by tree and shrub legumes in natural ecosystems similar to the Cape Fynbos. In quantifying N_2 fixation in wild *Cyclopia* species using the natural abundance method, Spriggs et al. (2003) found that many non-legume reference plants in the Fynbos had very negative $\delta^{15}N$ values, possibly due to $^{15}N/^{14}N$ fractionation during mycorrhizal infection. Measuring N_2 fixation therefore proved difficult due to the sometimes very close, or more negative, $\delta^{15}N$ values of the reference plants relative to that of legume. However, this was overcome by using reference plants with the same mycorrhizal status as the test legume to quantify N-fixed. Where legumes transport ureides (allantoin and allantoic acid) as the product of N_2 fixation, the ureide assay can also be used to assess the symbiotic status of a legume. Because very little is known about members of the tribe Psoraleae, the aim of this study was to i) assess nodulation status and nodule morphology of *Psoralea* species, ii) measure species' dependency on N_2 fixation for their N nutrition, iii) bioassay for ureides in aqueous plant extracts of *Psoralea* species, and iv) isolate/characterize the bacterial symbionts nodulating *Psoralea* species.

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2. Materials and methods

2.1. Field sites and plant sampling

Field trips were undertaken to six study sites [namely, Betty's Bay (BB), Kleinmond (KM), Arabella Country Estate (ACE), Rockview Dam Valley (RDV), Kogelberg Nature Reserve (KNR), and Rooiles Nature Reserve (RNR)] in the Western Cape Province of South Africa. To assess species' dependence on N_2 fixation for their N nutrition, eight *Psoralea* species (namely, *Psoralea aculeata*, *Psoralea aphylla*, *Psoralea asarina*, *Psoralea laxa*, *Psoralea monophylla*, *Psoralea pinnata*, *Psoralea repens* and *Psoralea restioides*, see Table 3) were sampled at six study sites. At each site, young branches (plus leaves) were removed from four different replicate plants, placed in paper bags, and transported to the laboratory for processing. For each *Psoralea* species sampled per site, branches (plus leaves) of non-fixing reference plants (see Table 2) with similar phenology were also harvested for processing in the laboratory.

To ascertain nodulation status, assay for ureides, and isolate bacteria from root nodules of *Psoralea* species, about eight young plants per species were dug up with intact roots at different sites, placed in large plastic buckets containing ice, and taken to the laboratory for nodulation studies, solute extraction for ureide assay, and bacterial isolation from root nodules. Four replicate plants per species were used for solute extraction and ureide assay, and the other four replicate plants per species used for nodulation studies and bacterial isolation.

2.2. Processing plant parts for ^{15}N analysis

Each sampled branch plus leaves was oven-dried (60 °C) for 72 h, weighed, ground into fine powder (0.85 mm), and stored in tightly capped vials prior to ^{15}N analysis. The reference plants obtained from each site were similarly processed and stored for ^{15}N analysis.

2.3. ^{15}N isotope analysis and percent N derived from fixation

The ratio of $^{15}N/^{14}N$ and N concentration (%N) in plant sample were determined using a Carlo Erba NA 1500 Elemental Analyzer coupled to a Finnigan MAT 252 isotope ratio mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) via Conflo II Open-Split Device at the University of Cape Town. A 2.0 mg mass of each plant sample was weighed into an aluminium capsule, loaded onto the Carlo Erba system, combusted in evacuated quartz tubes in the presence of cupric oxide and metallic copper. The resultant gases were cleaned online before being introduced into the mass spectrometer. A similar amount of ground material from *Nasturtium* was included as internal N-containing standard to run concurrently with every five runs of sample to correct for machine errors (Belane and Dakora, 2011).

The $^{15}N/^{14}N$ isotopic composition ($\delta^{15}N$) was measured as the difference in the number of atoms of ^{15}N to ^{14}N in atmospheric (atm) N_2, using the following relationship (Mariotti et al., 1981; Unkovich et al., 1994):

$$\delta^{15}N\ (‰) = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{atm}}{(^{15}N/^{14}N)_{atm}} \times 1000$$

where the $\delta^{15}N$ value is the ^{15}N natural abundance of plant sample.

The $\delta^{15}N$ values obtained from the $^{15}N/^{14}N$ ratios were used to calculate the percent N derived from fixation of atmospheric N_2 (%Ndfa). The %Ndfa was calculated from the ^{15}N natural abundance of the legume species and non-N_2-fixing reference plants as described by Shearer and Kohl (1986) and Unkovich et al. (2008):

$$\%Ndfa = \frac{\delta^{15}N_{ref} - \delta^{15}N_{leg}}{\delta^{15}N_{ref} - B} \times 100$$

where $\delta^{15}N_{ref}$ is the mean ^{15}N natural abundance of reference plants, $\delta^{15}N_{leg}$ is the ^{15}N natural abundance of legume, and B is the ^{15}N natural abundance of *P. pinnata* that was solely dependent on N_2 fixation for its N nutrition.

2.4. Determination of B value for P. pinnata

The B value is the ^{15}N natural abundance of an inoculated test legume raised on an N-free growth medium to ensure complete dependence of N nutrition on N_2 fixation. Because of difficulty in collecting seed material of wild *Psoralea* species, the B value was determined for only *P. pinnata* (whose seed was easier to obtain), and then used to estimate %Ndfa of all the other *Psoralea* species (for clover, see Bolger et al., 1995). Seedlings of *P. pinnata* were raised from surfaced-sterilized seed in sterile Leonard jars (Vincent, 1970) and inoculated with broth culture of root-nodule bacterial strain isolated from *P. pinnata*. Three replicate jars were used. The seedlings were grown with modified ¼-strength Hoagland's N-free solution as a source of nutrients. Three uninoculated jars containing *P. pinnata* seedlings were included as a check against contamination. After 14 months, branches plus leaves were harvested from the *P. pinnata* plants, oven-dried (60 °C) for 72 h, weighed, and ground into fine powder (0.85 mm) for ^{15}N analysis in order to determine the B value.

2.5. Solute extraction from plant parts for ureide assay

Four replicate plants per species were dug up with intact roots and nodules at some field sites and placed in ice. In the laboratory, the soil was washed off roots, and each plant separated into leaves, stems, nodules and roots for solute extraction for ureide assay (Unkovich et al., 2008). For leaves, stems and roots, 0.5 g organ was removed, chopped into small pieces, and soaked in 10 mL of hot water for 10 min. After removing the plant pieces, the aqueous extract was frozen prior to ureide analysis. With nodules, however, 0.5 g fresh weight was ground in 10 mL hot water, centrifuged, and the supernatant similarly frozen prior to ureide assay. The aqueous extracts from plant organs of different *Psoralea* species were assayed for ureides as described by Dakora et al. (1992), and the absorbance of ureides measured spectrophotometically at 525 nm as the phenylhydrazone of glyoxylate (Trijbels and Vogels, 1966).

2.6. Nodulation assessment and nodule morphology in Psoralea species

Young plants of *P. aculeata*, *P. aphylla*, *P. asarina*, *P. laxa*, *P. monophylla*, *P. pinnata*, *P. repens* and *P. restioides* dug up with intact roots, were washed with water to remove any associated soil, and observed for nodulation. To determine nodule morphology in the genus *Psoralea*, fully developed mature nodules were selected from test species, blotted dry, and photographed.

2.7. Isolation and phenotypic characterization of root-nodule bacteria

Harvested nodules were thoroughly washed in sterile distilled water, surfaced sterilized by immersion in 95% ethanol for 10 s and submerged in bleach for 3 min (Vincent, 1970). They were then rinsed in 6 changes of sterile distilled water, after which each nodule was aseptically handled. Four mature nodules per plant were