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Belowground microbes mitigate plant-plant competition

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ABSTRACT

Dimorphandra wilsonii, a Cerrado endemic Fabaceae tree, is threatened by land-use changes. The few remaining individuals occur in areas dominated by alien grasses like *Urochloa decumbens*. We tested the impact of nitrogen (N) availability and symbionts' presence on mitigating the effects of competition from *U. decumbens*.

Dimorphandra wilsonii seedlings were 50-week pot-cultivated under limiting (3 mM) or non-limiting (10 mM) N, with or without *U. decumbens*, and inoculated or not with a N-fixer (*Bradyrhizobium* sp.) and an arbuscular mycorrhizal fungus (AMF – *Glomus etunicatum*), both forming symbioses in the field.

Since *D. wilsonii* seedlings grew more and 'lost' fewer nutrients under the symbionts' presence, symbionts mitigated plant-plant competition. Under limiting N, inoculated *D. wilsonii* seedlings grew more (despite no nodulation), but N fixation was only suggested when inoculated *D. wilsonii* seedlings competed with *U. decumbens*. *D. wilsonii* ^{13}C, and substrate's carbon and respiration suggest that only the microbes performing key functions received plant carbon. Under non-limiting N, inoculated *D. wilsonii* seedlings became enriched in ^{13}C, substrate accumulated carbon and microbial respiration increased, suggesting a more generalist microbial community. Data suggest inoculating *D. wilsonii* seeds/seedlings with AMF and N-fixers as a conservation measure. However, long-term field-studies need to confirm these conclusions.

1. Introduction

Cerrado, also known as the Brazilian savannah, is a biodiversity hotspot of global conservation importance [1]. It is the second largest biome in Brazil (~200 Mha – http://www.projetobiomas.com.br/bioma/cerrado), which develops on weathered and oligotrophic soils, frequently presenting aluminum toxicity [2]. Since the second half of the twentieth-century, the Brazilian savannah vegetation has been removed from ~100 Mha, and the soil used for agriculture or pasture (http://www.projetobiomas.com.br/bioma/cerrado). Consequently, full communities were removed and some species became extinct [3]. *Dimorphandra wilsonii*, an endemic tree occurring in the transition between the savannah and semi-deciduous forest [4], has been critically threatened by extinction (http://www.iucnredlist.org) since 1986 [5]. Now, the remaining *D. wilsonii* individuals are located in small, isolated populations in pasture areas dominated by species of the genus *Urochloa* (Poaceae) [6]. The conversion of forests into agricultural land is usually associated with drastic changes in the bioavailability of nutrients, mainly of nitrogen (N): N mineralization and nitrificationin agrosystems occur at higher rates than in forests [7]. Therefore, conversion of the Cerrado to agrosystems is changing the biotic and abiotic characteristics of the habitat where *D. wilsonii* and other native and endemic species have evolved.

Not much is known about *D. wilsonii*, but it is recognized that it can grow in oligotrophic soils when it establishes symbiotic associations with N-fixing bacteria, arbuscular mycorrhizal fungi (AMF) and ectomycorrhiza, which provide greater supplies of nutrients, especially N and phosphorus (P) [8], highlighting that symbioses are a key factor for success [9], being the rule rather than the exception. Colonization of *D. wilsonii* roots by N-fixing bacteria occurs not only via root hairs, but also through relatively large and slightly disorganized infection chains at the level of epidermal and adjacent cortical cells [10]. The bacteroids formed in the permanent infection chains are capable of expressing nitrogenase, fixing N_2 and transferring the fixed N to the plant. It is also known that *U. decumbens* can inhibit the positive effect of the symbiosis between *D. wilsonii* and N-fixing bacteria [10].

With the objective of determining whether the 'new' biotic and abiotic conditions constrain the establishment of *D. wilsonii* seedlings, we tested the impacts of belowground interactions with N-fixing bacteria and AMF (which are both very abundant in the agrosystems where *D. wilsonii* exists and have been shown to colonize *D. wilsonii* [8]) on mitigating the effects of competition from the alien grass *Urochloa*

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decumbens. Since N-fixing bacteria and AMF would improve the nutrition of *D. wilsonii* seedlings [11], and thus provide an advantage in reducing competition for nutrients (mostly N and P), we hypothesize that the presence of these symbiotic microbes could mitigate the effects of competition from *U. decumbens* on *D. wilsonii*. However, symbioses have costs: between 20 and 50% of the C newly fixed by photosynthesis is translocated to the roots and used to support the rhizospheric microbial community [12–14]. If organic C is produced in excess by a plant (adequate photosynthetic conditions but growth is limited by water, low nutrients, etc., or *vice versa*), many interactions can be established with microbial components of the rhizosphere that are recruited almost stochastically [15]. By contrast, if organic C is not produced in excess by a plant (sub-optimal photosynthetic conditions, and/or growth are limited by water, low nutrients, etc.), root exudates decrease and the rhizospheric community is assembled on the basis of C economy. Under this latter scenario, only the microbes performing key functions will be rewarded with C [16]. The natural abundance of carbon ($\partial^{13}C$) may provide clues on the fate of the rhizodeposited C: when a plant exudes a big fraction of C from its roots, as the lighter carbon isotope (^{12}C) moves faster, a higher proportion of the exudates will be impoverished in the heavier C isotope (^{13}C), which will be left behind in the plant root. As a result, plant roots will present higher values of $\partial^{13}C$ [17] than plants exudating less and/or providing carbon to microbes which inhabit its roots.

Since the interactions between woody seedlings and herbaceous vegetation [18] impose light limitation, we evaluated the growth of *D. wilsonii* seedlings and *U. decumbens* in a carbon limitation situation (low radiation) under two levels of availability of N in the form of ammonium: one limiting plant growth; and the other non-limiting, which in many species triggers symptoms of ammonium toxicity [19]. Because direct N absorption is energetically more efficient [20], high N availability is known to inhibit nodulation and mycorrhization [20,21]. Therefore, we hypothesized that the presence of the symbiotic microbes could have a beneficial effect on *D. wilsonii* growth, especially under limiting N availability, characteristic of the Cerrado [22].

2. Materials and methods

2.1. Experimental design and biological material

We grew the plants under two concentrations of N in the form of ammonium (NH_4^+): 3 mM and 10 mM as ammonium sulfate. For each N dose, we applied the following treatments:

i) *D. wilsonii* alone, designated **Control**;
ii) *D. wilsonii* grown in the presence of the N-fixing bacterial strain BHCB8.5 (*Bradyrhizobium* sp.) isolated from *D. wilsonii* individuals in the field, and the AMF *Glomus etunicatum* that also colonizes *D. wilsonii* field plants, designated **Symb**;
iii) *D. wilsonii* and the alien grass *U. decumbens* grown in the presence of *Bradyrhizobium* sp. and *G. etunicatum*, designated **Symb + *Ud***; and
iv) *D. wilsonii* and *U. decumbens* without the inoculation of *Bradyrhizobium* sp. or *G. etunicatum*, designated as ***Ud***.

The experimental design was completely randomized in a factorial scheme, totaling eight treatments with five replicates each.

Seeds of *Dimorphandra wilsonii* Rizz. were collected from 13 individuals forming three populations, scarified to break dormancy, and sterilized by being placed in ethanol 70% (v/v) for one minute, then in sodium hypochlorite 2.5% (v/v) for 10 min, and then washed in sterilized distilled water. We planted three seeds per 2.5 L pot, which had been pre-filled with a mixture of sand and vermiculite (2 kg pot^{-1}) in the proportion 1:1 (v/v), sterilized and autoclaved at 121 °C for 60 min. After germination, we kept only one seedling of approximately 10 cm height per pot. The 40 *D. wilsonii* plants were randomly distributed to the eight treatments, each containing five pots/replicates.

Seeds of *Urochloa decumbens*, purchased from PróSementes – Sementes para Pastagem (http://www.prosementes.com.br), were scarified in sulfuric acid (96%, 36 N) for 15 min to break dormancy, washed in sterilized distilled water and planted in pots containing the same substrate as the *D. wilsonii* plants. After 10 days, four seedlings of 5 cm height were transplanted to the pots containing *D. wilsonii* according to the treatments (Symb + *Ud* and *Ud*).

The bacterial strain BHCB8.5 (*Bradyrhizobium* sp.), previously isolated from root nodules collected from *D. wilsonii* plants, was grown in YMB culture medium [23] at 28 °C, constantly shaken, for 48 h. Microbial cells were washed, centrifuged and then resuspended in NaCl 0.9% sterilized solution. The root zones of the respective treatments (Symb and Sym + *Ud*) were inoculated with 1 mL (108 cfu mL^{-1}) of bacterial suspension together with 200 spores of AMF *Glomus etunicatum* (acquired from Simbyom, Czech Republic).

Plants were grown for fifty weeks, in a greenhouse with natural light, maximum photosynthetic active radiation between 650 and 800 μmol m^{-2} s^{-1}, and ambient temperature between 16 and 32 °C. Twice a week we supplied 50 mL of modified Hoagland nutrient solution: 3 or 10 mM $(NH_4)_2SO_4$; 1.5 mM K_2HPO_4; 1 mM $CaCl_2{\cdot}2H_2O$; 0.25 mM $MgSO_4{\cdot}H_2O$; 50 μM KCl; 25 μM H_3BO_3; 2 μM $MnSO_4{\cdot}H_2O$; 2 μM $ZnSO_4{\cdot}7H_2O$; 0.5 μM $CuSO_4{\cdot}5H_2O$; 0.5 μM $(NH_4)_6Mo_7O_{24}$; 20 μM FeNaEDTA. We used plant drip trays to prevent nutrient leaching. Throughout the experiment, *U. decumbens* shoots were cut to 5 cm above the substrate every fifteen days, to simulate herbivory. *U. decumbens* shoots were dried to constant mass at 60 °C, then stored.

At harvest, we collected three samples of ± 5 g of substrate from each pot, at 3 cm from the *D. wilsonii* plant stem base. Each sample was divided into two sub-samples: i) one for determining the inorganic N pools and substrate respiration rates, which was kept at 4 °C until analysis; and ii) the other for determining the C concentration, which was dried at 60 °C until constant weight. *D. wilsonii* plants were separated into roots and shoots, and dried at 60 °C until constant mass. *U. decumbens* were also harvested and dried at 60 °C until constant mass. Total *U. decumbens* biomass was calculated as the sum of the biomass at harvest (root and shoot) plus the shoot biomass that was cut along the experiment.

We evaluated *D. wilsonii* roots for the presence/absence of nodules and for mycorrhization on segments of 1 cm length cut 1–2 cm above the root apices. These root segments were stained [24], and mycorrhizal colonization was evaluated on quadrilateral plaques in accordance with Giovannetti and Mosse [25].

2.2. Chemical analyses

Water extracts of the substrate samples were prepared in the proportion of 1:10 m/v, agitated for one hour at room temperature, centrifuged (Centrifuge Eppendorf 5403) at 5000 rpm for 20 min at 4 °C, and the supernatant collected and analyzed colorimetrically (Spectrophotometer Tecan Spectra Rainbow A-5082) for NH_4^+ and NO_3^-. Nitrate was determined using a modified Cataldo method [26], and ammonium was determined using a modified Berthelot reaction [27]. We calculated inorganic N (inorg-N) as the sum of the two N forms. The forms of N were expressed in mg N pot^{-1} considering the dry substrate (Table S1).

We analyzed *D. wilsonii* plants for carbon (C), nitrogen (N) and phosphorus (P) while *U. decumbens* plants were only analyzed for C and N. The dried plant material was ground into powder using a ball mill (Retsch MM 2000). N and C concentrations in the plant material, and C in the substrate were determined using an elemental analyzer (EuroVector) by combustion – DCT [28] while the P concentration was determined by sulfuric digestion and colorimetry [29]. We calculated the C, N and P contents of plants by combining biomass and concentrations.

The natural abundance of ^{13}C in the roots of *D. wilsonii* was determined using mass spectrometry (IRMS, Micromass-GV Instruments,