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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,

UK) and the expression:

$\delta^{13}C = (R\ sample/R\ standard - 1) \times 1000$

where R is the ratio $^{13}C/^{12}C$, in the sample and in the standard.

2.3. Respiration rates of the substrate

We used the MicroResp® system [30] to determine substrate respiration. This consists of two microplates (96 wells) placed face to face. Each well of the deep-well microplate (1.2 mL capacity, 96-deep-well microplate, NUNC) contained the substrate sample (± 500 µL substrate) with the carbon source (30 µL per well, 6.2 mg of C per well as glucose). The second microplate contained the detection gel (cresol red dye, 12.5 ppm), potassium chloride (150 mM) and sodium bicarbonate (2.5 mM) set in a 1% gel of noble agar (final volume 150 µL per well). The two microplates were assembled together with a silicone seal (allowing airflow between the interconnecting wells), clamped together, then incubated in the dark for 6 h at room temperature. CO2-trap absorbance was measured at 590 nm (Spectrophotometer Tecan Spectra Rainbow A-5082). CO2 production was calculated based on a calibration curve (MTI 200 micro-catharometer) and the dry weight of the substrate samples, expressed as µg CO_2 mg^{-1} of substrate h^{-1}. The calibration curve was as follows:

percentage of $CO_2 = A + B\,x\,e^{-kx}$

where k is $-\ln(R)$, R is 0.106, B is 0.384, A is 0.222, and x is A_{590}.

2.4. Calculations and statistics

The impact of the symbiotic microbes on modulating the effects of competition from *U. decumbens* on *D. wilsonii* was quantified in the relation to the respective control using the following equation:

Δ of parameter due to the presence of *U. decumbens* (%) = $(m_n - M_{cont})/M_{cont} \times 100$

where 'm' corresponds to the individual values (biomass, N and P contents), 'M_{cont}' corresponds to the mean value for each parameter for the control, and 'n' to the replicates for each treatment. For the treatments with symbiotic microbes (+Symb), the 'control' was the treatment Symb, while for the treatments without symbiotic microbes (-Symb), the 'control' was the control consisting solely of *D. wilsonii* plants (Fig. 1).

We calculated the nitrogen use efficiency (NUE) of *D. wilsonii* and of *D. wilsonii* together with *U. decumbens* plants as the% of N in plant biomass in relation to the total N added in the nutrient solution throughout the 50 weeks of the experiment:

NUE (%) = N plant(s)/N total × 100

'N total' was calculated considering the addition of 50 mL of nutrient solution two times a week, during 50 weeks. The 100 mL added weekly were equivalent to 4.2 and 14 mg N, which over the 50 weeks resulted in a total of 210 and 700 mg N, respectively. In the case of the Symb + *Ud* and *Ud* treatments, the N stored in *U. decumbens* biomass was added to that of *D. wilsonii* (Fig. S2).

The effect of the presence/absence of the symbiotic microbes on *D. wilsonii* biomass and N and P contents was tested separately using a two-way ANOVA, with the presence/absence of the symbiotic microbes and N dose as fixed factors. The effect of the treatments on plant and soil parameters was tested separately using a two-way ANOVA, with treatment and N dose as fixed factors. Scheffé post hoc multiple comparisons tested for differences ($p < 0.05$) between treatments. Differences between the N contents of plant(s) and the total N added were analyzed by Student's *t*-test ($p < 0.05$). In all cases, preliminary analyses were performed to ensure that there was no violation of the assumptions regarding the tests' application. SPSS software, version 23.0, was used for all tests.

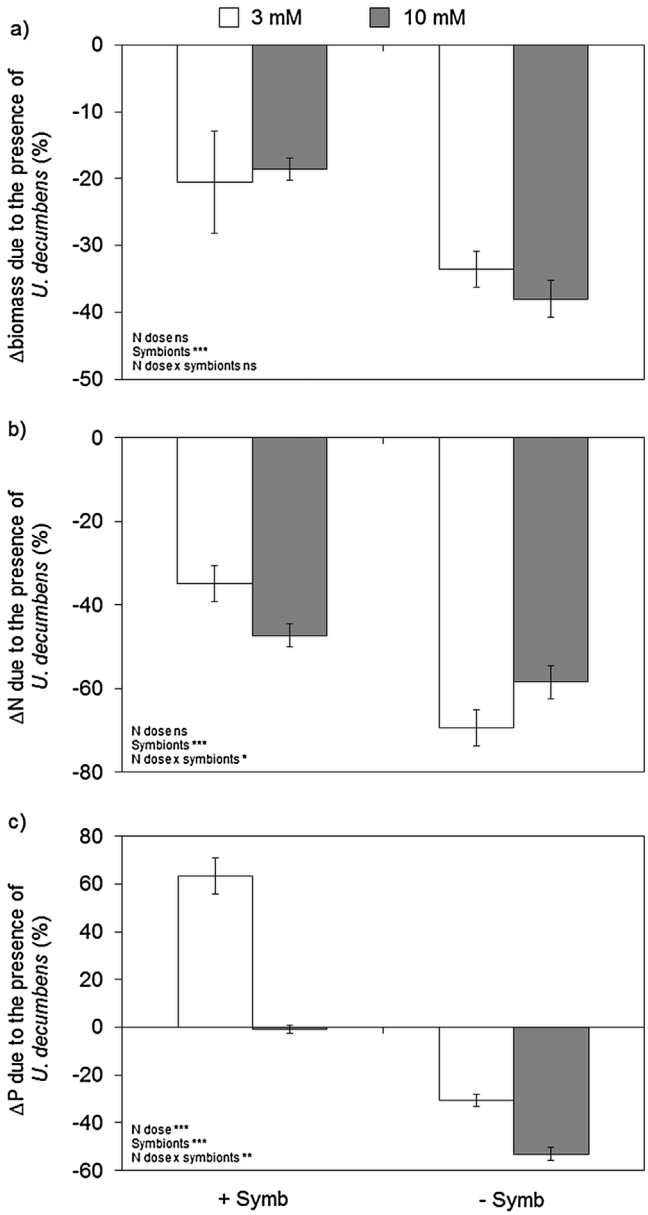

Fig. 1. Modulation of the beneficial impact of the symbiotic microbes by N availability on *D. wilsonii's* biomass (Δbiomass – a), N (ΔN – b) and P contents (ΔP – c). Significant effects are shown: * 5% level; ** 1% level; *** 0% level; and ns non-significant. Bars are the mean ± 1SE (n = 5).

3. Results

3.1. The symbiotic microbes impact the effects of competition from U. decumbens on D. wilsonii

When the role of the symbiotic microbes in modulating the effects of competition between the two plant species was isolated, it was shown that *D. wilsonii* plants grew more and 'lost' fewer nutrients to *U. decumbens* when the symbiotic microbes were present (Fig. 1). Furthermore, *D. wilsonii* seedlings grown in the presence of the symbiotic microbes actually 'gained' P but only under 3 mM N. Therefore, the presence of the symbiotic microbes under limiting N availability had a significant beneficial effect on P acquisition by *D. wilsonii* seedlings.

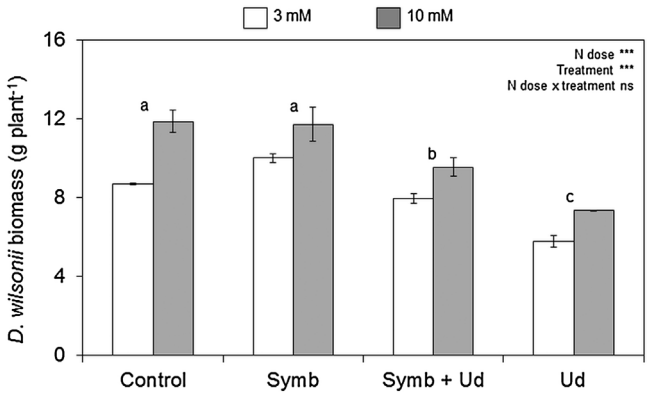

Fig. 2. Impact of the N dose and treatments on biomass accumulation of *D. wilsonii* seedlings. Significant effects are shown: * 5% level; ** 1% level; and *** 0% level; and ns non-significant. Different letters refer to significant differences between treatments at the 5% level. Bars are the mean ± 1SE (n = 5).

3.2. Impact of (a)biotic factors on the establishment of D. wilsonii seedlings

After fifty weeks of plant growth, the inorganic N pools (NH_4^+, NO_3^- and inorganic N) of the substrates were similar under both applied N doses (Table S1). By contrast, the N dose had an impact on plant biomass: *D. wilsonii* seedlings and *U. decumbens* plants accumulated more biomass when grown under 10 mM N than under 3 mM (Figs. 2 and S1). Given that *U. decumbens* grew much faster than *D. wilsonii*, the greatest total plant biomasses were attained in the treatments where *D wilsonii* and *U. decumbens* were grown together (Symb + *Ud* and *Ud*), being 4–6-fold higher than in treatments without *U. decumbens* (Fig. S1). The *D. wilsonii* plants grown under 3 mM which accumulated the most biomass were those inoculated with the symbionts and without *U. decumbens* (Symb), which also had the highest N content (Fig. S2) even though no nodules were observed (Table S2). Indeed, no nodules could be observed in the roots of any of the *D. wilsonii* seedlings, subject to any N dose or treatment, while mycorrhization was only detected in *D. wilsonii* roots (and *U. decumbens* roots) of the treatment Symb + *Ud* grown under 3 mM N.

The N contents of *D. wilsonii* plants grown under 3 mM N differed between treatments (Fig. S2). Furthermore, considering the total N stored in plant biomass (also accounting for *U. decumbens* plants of the Symb + *Ud* and *Ud* treatments – Fig. S2), N use efficiency (NUE) was generally c.a. 100% (Fig. 3), meaning that the N in the plant biomass

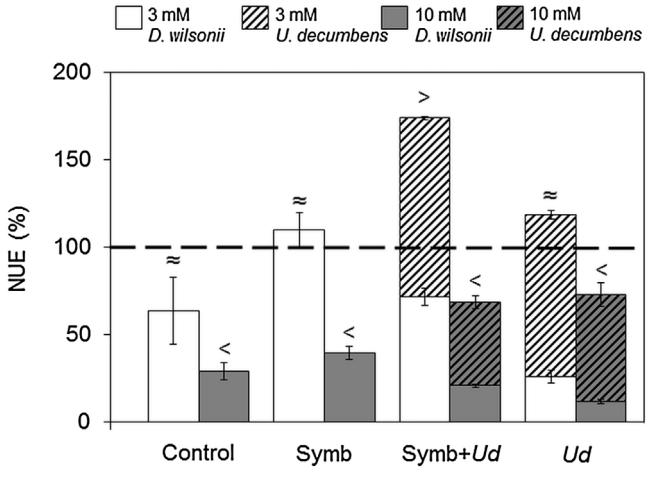

Fig. 3. Impact of the N dose and treatments on plant(s) N use efficiency (NUE). 100% NUE means that all the N that was added as nutrient solution throughout the experiment was retrieved in the plant material. Therefore, the N stored in plant biomass was lower (<), similar (≈) or higher (>) than that provided by fertilization (t-test $p < 0.05$). Bars are the mean ± 1SE (n = 5).

was similar to the total N applied throughout the 50 weeks of the experiment, thus ruling out the possibility that N_2 fixation had occurred. In contrast, and despite the absence of nodules (Table S2), the N stored in the plant biomass in the Symb + *Ud* treatment grown under 3 mM N was higher than 210 mg (Fig. S2), resulting in a NUE higher than 100% (Fig. 3) and suggesting that an additional N input had occurred: biological N_2 fixation. Furthermore, *D. wilsonii* roots from this treatment presented a mycorrhization rate of 34% (Table S2) and the highest phosphorus content (Table S3).

The *D. wilsonii* seedlings grown under 10 mM N that accumulated the most biomass were those grown without *U. decumbens* (Control and Symb), while those grown together with *U. decumbens*, with or without the symbionts (Symb + *Ud* and *Ud*), accumulated even less biomass than the control (Fig. 2). Comparing the N stored in the plant biomass (*D. wilsonii* together with *U. decumbens* if applicable – Fig. S2) with the N that was applied throughout the 50 weeks of the experiment shows that when plants were grown under 10 mM N, NUE was always below 100% (Fig. 3).

3.3. How N availability modulated the impact of the symbiotic microbes?

Under 3 mM N, the treatments had no impact on the isotopic C signature ($\partial^{13}C$) of *D. wislonii* plants (Fig. 4a), but when grown under 10 mM N, the inoculated *D. wilsonii* plants (Symb and Symb + *Ud*) changed their root's ^{13}C signature, becoming enriched in the heavier isotope (^{13}C). In agreement, the C contribution to the maintenance of the microbial community in the root zone (i.e., rhizodeposition) was highest in the substrates of those treatments (Symb and Symb + *Ud* grown under 10 mM – Fig. 4b): the C content of the substrate of these treatments was c.a. 4–5-fold greater than that of the substrate from the Symb and Symb + *Ud* treatments under 3 mM. Furthermore, the C in the substrate was most likely being used by the microbial community in the root zone since the respiration rates in the substrates of the Symb and Symb + *Ud* treatments grown under 10 mM were c.a. 4-fold higher than those from the equivalent treatments grown under 3 mM (Fig. 4c).

4. Discussion

4.1. Impacts of the symbiotic microbes on mitigating the effects of competition from U. decumbens on D. wilsonii

We tested if the presence of symbiotic microbes could mitigate the effects of competition from *U. decumbens*, alien herbaceous vegetation, on *D. wilsonii*, native woody seedlings. As hypothesized, the impact of *U. decumbens'* presence on *D. wilsonii* growth and establishment was less negative in the presence of the symbiotic microbes (Fig. 1): even though the presence of *U. decumbens* increases competition for nutrients, the presence of the symbiotic microbes may attenuate this competition. The modulation of the beneficial impact of the symbiotic microbes on P acquisition by N availability may be related to the latter's influence on plant-symbionts dynamics (). Our data suggest that, even though the habitat of *D. wilsonii* has been unbalanced by deforestation and introduction of exotic plant species (such as *U. decumbens*), the promotion of the symbiotic interactions at the rhizosphere under limiting N availabilities, may represent an advantage to *D. wilsonii*, when co-existing with *U. decumbens*. However, long-term studies and field observations need to confirm these conclusions.

4.2. Impact of (a)biotic factors on the establishment of D. wilsonii seedlings

The presence of the symbiotic microbes (*Bradyrhizobium* sp., a N-fixing bacteria, and *G. etunicatum*, an AMF) had a positive impact on biomass accumulation by *D. wilsonii* plants. However, given that sustaining microbes constitute a cost to the plant (20–50% of the C newly fixed by photosynthesis is translocated to the roots and used to support the rhizospheric microbial community [12–14,31]) under limiting N

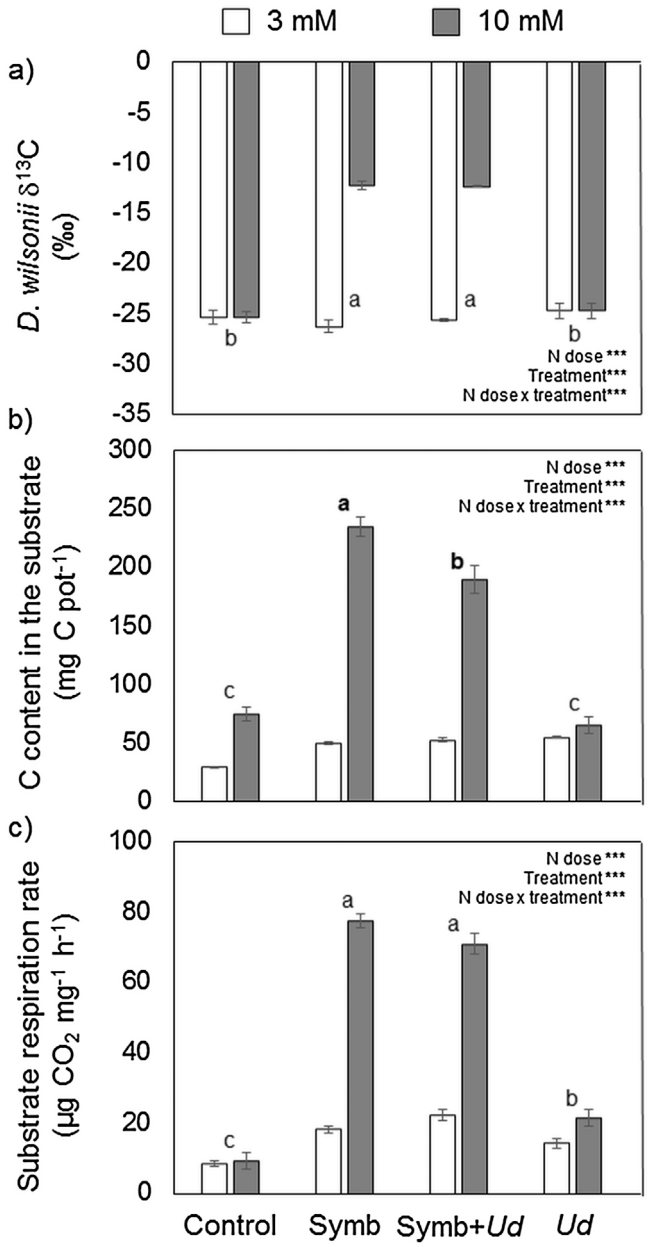

Fig. 4. Impact of the N dose and treatments on the $\delta^{13}C$ of *D. wilsonii* seedlings (a), on the C content in the substrate (b), and on the substrate respiration rate (c). Significant effects are shown: * 5% level; ** 1% level; and *** 0% level. Different letters refer to significant differences between treatments at the 5% level. Bars are the mean ± 1SE (n = 5 plants).

(3 mM) and C (low radiation) the rhizospheric community was most likely assembled on the basis of C economy so that only the microbes performing key functions were being rewarded with C [16]. This N and C limitation may explain why *D. wilsonii* seedlings: i) were not nodulated, contrary to previous observations [32]; and ii) only formed mycorrhiza when N was very limiting to plant growth, supplied as 3 mM and under competition from *U. decumbens* (Table S2). Low viability of the inoculants cannot explain the results, since although no nodules were observed and not all plants were mycorrhized, several treatments and parameters were affected by the inoculation ().

Rhizobium and *Bradyrhizobium* species are rhizobacteria known to promote plant growth through their capacity to produce phytohormones, Nod factors and vitamins, and solubilize phosphates [33]. Furthermore, *Rhizobium* and *Bradyrhizobium* species are also able to fix N at a root surface without nodule formation [34], and/or form large infection chains invading epidermal and adjacent cortical cells with bacteroids that are efficient in fixing N and transferring it to the plant [32]. These hypotheses may explain why the total amount of N stored in plant biomass was higher than the amount of N provided to the plants in the pots where inoculated *D. wilsonii* were grown together with *U. decumbens* under 3 mM N (Fig. 3). It is interesting that this effect was only significant at limiting N (3 mM ammonium), when both plant species (*D. wilsonii* and *U. decumbens)* were mycorrhized (Table S2) and *D. wilsonii* plants had higher P content (Table S3). It is known that for a certain N availability, AMF favor P absorption [35,36], and such higher P concentrations in biomass also favors N fixation [21]. Which may suggest a synergistic effect between mycorrhization and nodulation, as has been described for the tripartite interactions [32–35].

4.3. Mechanisms which may explain how N availability modulated the impact of the symbiotic microbes

In *D. wilsonii* seedlings of treatments Symb and Symb + *Ud* receiving 10 mM N, the non-occurrence of nodulation or mycorrhization (Table S2), and consequently the non-promotion of growth by inoculated symbiotic microbes (Fig. 2), was expected since high N availability inhibits nodulation and mycorrhization [20,21]. However, it had not been expected that the isotopic C signature ($\partial^{13}C$) of the plants from those same treatments (Symb and Symb + *Ud* grown under 10 mM N) would reveal an enrichment in the heavier isotope (^{13}C) relative to the equivalent treatments under lower N availability (Fig. 4a). When grown individually, C3 plants (*D. wilsonii*) typically present $\partial^{13}C$ between −20‰ and −35‰, while in C4 plants (*U. decumbens*) it is between −11‰ and −15‰ (Fig. S3) [37]. Although symbiosis with N fixing bacteria and AMF may influence stomatal conductance and thus increase plants' water use efficiency [38,39], this hypothesis was ruled out by the photosynthetic indicator we measured (photochemical reflectance index – [40] – data not shown). Alternatively, we suggest that the *D. wilsonii* seedlings from Symb and Symb + *Ud* treatments under 10 mM N were exuding more C from their roots: as the lighter isotope moves faster, a higher proportion of the exudates will be impoverished in the heavier C isotope, which was left behind in the plant root, resulting in higher values of root $\partial^{13}C$. This is in agreement with the higher concentration of organic C detected in the root medium (substrate) of Symb and Symb + *Ud* plants grown under 10 mM N, relative to those grown at 3 mM N (Fig. 4b). Liljeroth et al. [17] obtained similar results with wheat and barley grown under high N availabilities. Furthermore, microbial respiration of the root medium showed 3–4 fold higher rates under the influence of the plants inoculated with the symbiotic microbes (Symb and Symb + *Ud*) grown at 10 mM N (Fig. 4c). Altogether, these results (Fig. 4) highlight the capacity of microbes to qualitatively and quantitatively modify plant rhizodeposition, and the influence of N availability on microbial rhizosphere assembling [16,41,42]. Thus, the hypothesis that the change of $\partial^{13}C$ in *D. wilsonii* plants from Symb and Symb + *Ud* (10 mM N) treatments was caused by increased rhizodeposition cannot be dismissed (Fig. 5). Somehow, the $\partial^{13}C$ of *U. decumbens* (C4 plant species – Fig. S3) also corroborates the hypothesis that rhizospheric symbiosis changes plant $\partial^{13}C$ at high N availabilities (10 mM N), but not at low (3 mM N).

The effects of N availability on root exudates, organic C in the root medium and microbial respiration suggest that under limiting N availability (3 mM N), the C compounds exuded by the plant are mainly directed to the N fixers and arbuscular mycorrhizal fungi, which in symbiosis with the plant ensure that the *D. wilsonii,* (the host plant) benefits directly from the activities of this specialized microbial community. In agreement, since AMF are obligate biotrophs, this could explain why inoculated *D. wilsonii* seedlings actually 'gained' P when grown under limiting N (Fig. 1c). Under non-limiting N (10 mM), a greater proportion of the root exudates is probably being used to maintain a more generalist microbial community (Fig. 4), and therefore

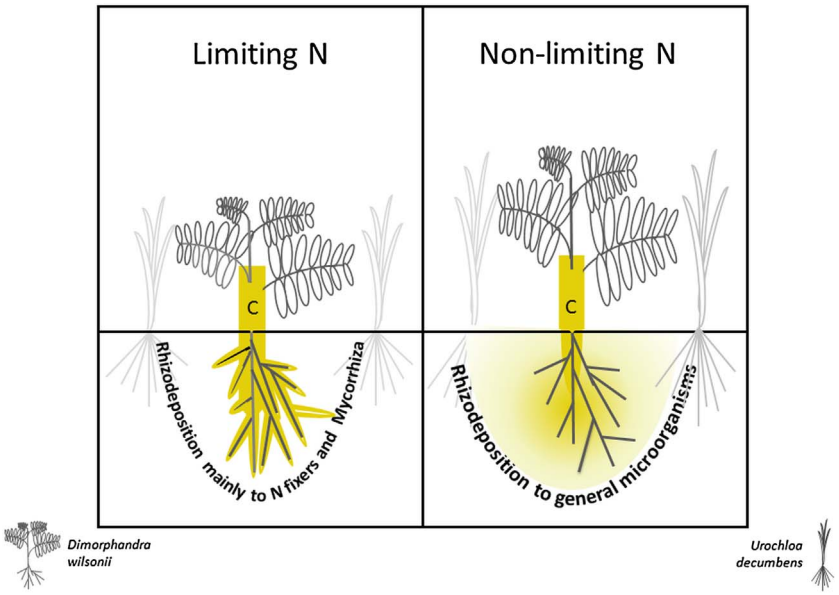

Fig. 5. Conceptual representation of how N availability may condition the fate of plant rhizodeposition. The role of N availability is represented by plant size. Flux of C newly fixed through photosynthesis is represented in yellow (aboveground thickness is considered to be equal). The fate of plant rhizodeposition (yellow density) will depend on whether the microbial community is mostly inhabiting *D. wilsonii*'s roots (limiting N) or not (non-limiting N – see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the microbe-derived benefits become available to both plant species (Fig. 1).

In conclusion, as *D. wilsonii* seedlings grew more and 'lost' fewer nutrients to *U. decumbens* in the presence of the symbiotic microbes, belowground microbes mitigated plant-plant competition. *D. wilsonii* ^{13}C, and substrate's carbon and respiration suggest that under limiting N, only the microbes performing key functions received plant carbon, while under non-limiting N, the microbial community was more generalist. 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.

Author contributions

MBF, CC, TD and MGCF conceived and designed the experiment. MBF and MMC conducted the experiment and analysis. CC and MGCF contributed with reagents and analytical tools. MBF and TD analyzed the data. MBF, TD and CC wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.06.006.

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