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REGULAR ARTICLE

The dual symbiosis between arbuscular mycorrhiza and nitrogen fixing bacteria benefits the growth and nutrition of the woody invasive legume *Acacia cyclops* under nutrient limiting conditions

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Abstract
Background and aims *Acacia cyclops* is an invasive species within Mediterranean ecosystems, characteristically low in soil nutrients. Thus associations with nitrogen-fixing bacteria (NFB) and arbuscular mycorrhiza (AM) may provide an advantage to these legumes. This study investigated the role of AM and NFB in the growth and nutritional physiology of *A. cyclops*.

Methods Seedlings were inoculated with naturally occurring NFB, *Glomus mosseae* or both, and grown under glasshouse conditions for 5 months. Plants were cultivated in sand and supplied with a 20 % strength nutrient solution. Xylem sap nutrients, photosynthetic rates, biomass and chemical compositions, were recorded.

Results The dual inoculation decreased the colonization of both symbionts, compared to a single symbiosis with

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either symbiont. Despite low colonization levels, the dual symbiosis increased host biomass and relative growth rates. This was associated with increased photosynthetic rates and enhanced nutrition. Additionally, dual symbiotic plants had enhanced N and P acquisition and utilization rates. Xylem sap analysis showed higher levels of NH_4^+ being exported from the roots to the shoots in the dual symbiotic plants compared with other treatments.

Conclusions These findings suggest the dual symbiosis is an important factor in the growth and development of *A. cyclops* under nutrient limiting conditions.

Keywords Arbuscular mycorrhiza · Nitrogen fixing bacteria · Dual symbiosis · P and N nutrition · Xylem sap · Invasive species · *Acacia cyclops*

Introduction

Many of the Australian *Acacia* species are known to be invasive species within Mediterranean ecosystems, resulting in major losses of biodiversity from these sensitive ecosystems (Stock et al. 1995; Richardson et al. 1996; Marchante et al. 2003; Carvalho et al. 2010). *Acacia cyclops* A.Cunn. ex G.Don is one such species, occurring as a shrub or small tree in dense thickets, especially along coastal areas where it was introduced to stabilize sand dunes (Richardson et al. 1996).

Soils within these ecosystems are generally nutrient poor, characteristically low in N and P. These two nutrients are of specific importance to legumes, as nodule formation and the increased photosynthetic rates characteristic of symbiotic plants require increased levels of N and P. Thus, invasive legume species with the ability to fix nitrogen, such as *A. Cyclops*, exploit these nutrient-poor conditions and accrue an aboveground biomass rich in N. Consequently, these plants transform local growing conditions by converting prevailing soil conditions from low to high N cycling regimes (Richardson and Van Wilgen 2004; Stock et al. 1995; Vitousek et al. 1987). The enriched soils resultant of invasive stands are therefore to the detriment of the local plants, which are not well adapted to these levels of high soil nutrients, often resulting in growth depressions of the indigenous plants (Witowski 1989, 1991; Marchante et al. 2009). In contrast, the indigenous nitrogen-fixing plants do not have this negative effect on the soils as they do not dominate specific ecosystems or have the same aboveground biomass as the invasive species do.

The role of mutualisms is now widely appreciated as being an important factor that facilitates invasions of alien species in many habitats (Stock et al. 1995; Reinhart and Callaway 2006). Arbuscular mycorrhizal (AM) fungi and nitrogen fixing bacteria (NFB), such as *Rhizobia*, are largely responsible for the supply of P and N to legumes in soils lacking adequate concentrations of these minerals, thus providing benefits to invasive species and allowing for increased growth under these nutrient-limiting conditions (Stock et al. 1995; Rodriguez-Echeverria et al. 2008). The improved aboveground nutrition of these invasive plants (Richardson and Van Wilgen 2004; Stock et al. 1995; Vitousek et al. 1987) can be mediated via the export of organic and inorganic N compounds in the xylem sap. It is known that legumes can alter the composition of N containing solutes in xylem sap (Winter et al. 1981; Peoples et al. 1986). In addition, mycorrhizal colonization can also affect the xylem sap fractions of N compounds, as well as the concentrations of xylem sap P (Bell and Pate 1995). These nutritional benefits provided by the respective root symbioses, impose a C cost on the host plants, which are often reflected in the increased photosynthetic and respiratory rates of the hosts (Kaschuk et al. 2009; Mortimer et al. 2008, 2009).

However, despite the additional costs of supporting two root symbionts, the associated C demands generally do not result in a growth depression of the host. In fact, as a result of dual colonization of the roots, there is usually an increase in host growth over-and-above that of singular inoculation (Chalk et al. 2006). One of the reasons accounting for this is that the improved nutritional status of the host allows for the maintenance of a higher photosynthetic rate, which in turn offsets the carbon losses incurred by the large sink demand of the two symbionts (Fitter 1991; Mortimer et al. 2008; Wright et al. 1998). Furthermore, the dual symbiotic plants have also been shown to be more efficient at using the available nutrients, this can be seen in a number of studies showing the improved assimilation and incorporation of both N and P in plants colonized by both AM fungi and *Rhizobia* (Brown and Bethlenfalvay 1988; Kaschuk et al. 2009; Mortimer et al. 2008).

Despite numerous studies showing the synergistic benefits of the dual inoculation of legumes and a

number of these pertaining to the reliance of invasive species on root symbioses, the contribution of a double symbiosis with AM fungi and NFB to the growth and nutrition of *A. cyclops*, has not yet been investigated. This study will provide a fundamental understanding of how these invasive species are able to become rapidly established in nutrient poor soils and outcompete native plants, to the detriment of the indigenous flora. The primary aim of the study is to determine the role of the dual symbiosis in enabling *A. cyclops* to grow in nutrient-poor soils. This was investigated on the basis of biomass allocation, macronutrient acquisition, the xylem export of assimilated nutrients and photosynthetic C costs in various combinations of symbiotic states.

Materials and methods

Plant growth conditions

Surface-sterilized seeds of *A. cyclops* were scarified by soaking the seeds in concentrated H_2SO_4 for 3 hours and then rinsed 10 times in distilled water. Seeds were then soaked overnight in distilled water and planted in vermiculite the following day. After 3 weeks the seedlings were transferred to 20-liter pots containing acid-washed river sand. The plants were grown for 5 months between September and January, in a north-facing glasshouse at the University of Stellenbosch, Stellenbosch, South Africa. The average maximum daily photosynthetically active irradiance was between 800 and 1000 $\mu mol.m^{-2}.s^{-1}$ and the average day/night temperatures and humidities were 26/17 °C and 35/75 % respectively. Plants were watered twice weekly, at 100 % water holding capacity of the pots (pre-determined), with a 20 % strength Long Ashton nutrient solution (Hewitt 1966), modified to contain 100 μMN and 20 μM P for five months.

Plants were either colonized (+M) or uncolonized (−M) by the mycorrhizal fungus *Glomus mossae* and grown with (+R) or without (−R) NFB. Mycorrhizal treatments were inoculated with 10 g (per pot) of *Glomus mosseae* (strain YV) inoculum (MicroBio Ltd, Cambridge, UK), by placing a thin layer of mycorrhizal inoculum below the surface of the sand when seedlings were planted. *A. cyclops* seedlings not inoculated with AM received a filtered inoculum solution, which was prepared by filtering the inoculum through a 37 μm mesh, which removed all fungal material. Indigenous NFB were isolated by collecting nodules from the roots of *A. cyclops* growing at the Coetzenberg Mountain area of the University of Stellenbosch. The nodules were first surface-sterilized with 95 % ethanol and then soaked in 3.5 % hypochlorite solution. The nodules were crushed, using a sterile mortar and pestle in 1 ml of deionised distilled water. Bacteria were streaked on Yeast Extract-Mannitol Agar plates. Single colonies were selected and re-streaked for purity. For inoculation with the cultured NFB, *A. cyclops* seeds were soaked in a turbid suspension (100 ml) of indigenous cell cultures. The seedlings were re-inoculated with 10 ml of the same cell suspension upon planting into pots.

Each treatment consisted 10 replicates and at the time of transplanting the seedlings to pots, seedlings were harvested (t_1) and initial growth and nutrition parameters were determined. After 5 months the potted plants were harvested (t_2) and growth and nutrition parameters were again measured.

Photosynthesis

Before harvesting, at 5 months, the youngest fully expanded phyllode of each plant was used for the photosynthetic determinations. Saturation light levels (1100 $\mu mol.m^{-2}.s^{-1}$) were determined using Light-response curves (see addendum for more information) and then used to measure maximum photosynthetic rates. Light levels of 1100 $\mu mol.m^{-2}.s^{-1}$ are appropriate and in line with other studies on *Acacia* seedlings, often *Acacia* seedlings grow in the shade of densely spaced mother plants and are thus shade adapted (Yu and Ong 2003; Possel and Hewitt 2009). Readings were taken at midday, using a portable infrared gas analyzer (PP-Systems CIRAS, Hitchen, UK). The photosynthetic measurements were taken with the following conditions in the environmentally controlled leaf chamber:

a) Photosynthetic photon flux density=1100 μmol quanta $m^{-2}.s^{-1}$
b) Relative humidity=44 %
c) Leaf vapor pressure deficit=1.83 kPa
d) Flow rate=400 $\mu mol.s^{-1}$
e) Reference CO_2=400 ppm
f) Leaf temperature=25 °C

Percentage mycorrhizal colonization

At the time of harvesting roots were cut into 1 cm segments and rinsed and cleared with 20 % KOH for 3 days at room temperature. The KOH was rinsed off and the segments acidified with 1 % HCL overnight. Thereafter the roots were stained with 0.05 % (w/v) aniline blue and left overnight. The roots were then destained in a 1 % HCL/glycerol mix. Root segments were placed on slides and the colonization components were determined according to the line-intersect method (Brundrett et al. 1994).

Chemical analyses of plant tissues and xylem sap

Total N and P in shoots and roots (root and nodule segments used) were determined colorimetrically after Kjeldahl digestion of ground and dried samples. Xylem exudates were collected from root stumps in the early morning with a micropipette, according to the methods described by Cramer and Richards (1999). The stem was cut with a scalpel just above the root system, and root pressure was allowed to exude the xylem sap at the cute-surface. The initial few drops of sap, collected during the first 5–10 min, were discarded and the rest of the xylem exudation was siphoned with a 20 micro-liter Gilson micropipette over a period of 60 min and stored in Eppindorf tubes on ice. Thereafter the tubes were kept at −20 degrees Celsius, until further analyses were performed. Ureide concentrations (allantoin) were determined following Vogels and Van der Drift (1970), amino acid concentrations were determined according to the ninhydrin method by Rosen (1957), NH_4^+ according to Solorzano (1969) and phosphate, using the method of Murphy and Riley (1962). Spectrophotometry was performed using a Varian UV-VIS spectrometer (Varian Analytical Instruments, California, USA)

N and P uptake and utilization

Specific P absorption rate (SPAR) ($mgP.g^{-1}$ root $dm.d^{-1}$) is the calculation of the net P absorption rate per unit root dry mass (dm) (Nielson et al. 2001):

$$SPAR = (M_2 - M_1)/(t_2 - t_1) \times (\log_e R_2 - \log_e R_1)/(R_2 - R_1)$$

Where M is the P content per plant (mg), t is the time (days) and R is the root dm (g). The subscript "$_1$" represents the P content, time and dm of the former harvest and the subscript "$_2$" represents the P content, time and dm of the latter harvest.

Specific P utilization rate (SPUR) ($gdm.mg^{-1}P.d^{-1}$) is a measure of the amount of dm gained for the P taken up by the plant (Nielsen et al. 2001):

$$SPUR = (W_2 - W_1)/(t_2 - t_1) \times (\log_e M_2 - \log_e M_1)/(M_2 - M_1)$$

Where W is the plant dm (g), t is the time (days) and M is the P content (mg) of the plant. The subscript "$_1$" represents the P content, time and dm of the former harvest and the subscript "$_2$" represents the P content, time and dm of the latter harvest.

The specific nitrogen absorption rate (SNAR) ($mgN.g^{-1}$ root $dm.d^{-1}$) and the specific nitrogen utilization rate (SNUR) ($gdm.mg^{-1}N.d^{-1}$) was adapted from the above equations to include N instead of P, according to Mortimer et al. (2008).

Statistical analysis

The mycorrhizal percentage data (% colonization) were arcsine transformed (Zar 1999). The effects of the factors and their interactions were tested with an analysis of variance (ANOVA) (SuperAnova, Statsgraphics Version 7, 1993, Statsgraphics Corporation, USA). Where the ANOVA revealed significant differences between treatments the means ($n=10$) were separated using a post hoc Student Newman Kuehls (SNK), multiple range test ($P \leq 0.05$).

Results

Symbiont biomass

Uninoculated plants remained free of root symbionts for the duration of the experiment (Fig. 1b,c). Nodule growth was reduced when combined with AM colonization (Fig. 1b) and similarly, the percentage AM colonization was higher in the single symbiosis than in combination with the (Fig. 1c).

Plant biomass

Compared to uninoculated control plants, the plant dry mass increased with dual inoculation and with single

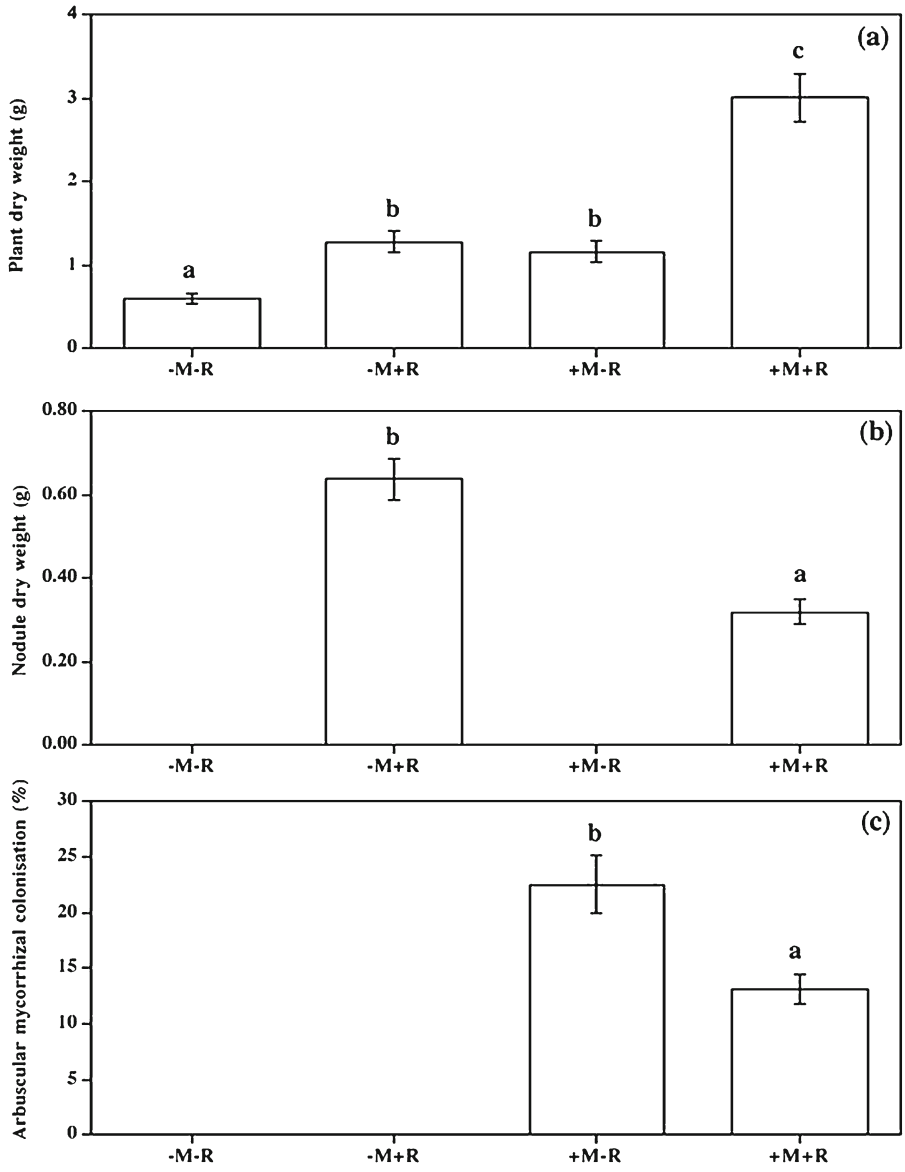

Fig. 1 Plant dry mass (**a**), nodule dry mass (**b**) and mycorrhizal infection (**c**) of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** nitrogen fixing bacteria and were either colonized **(+M)** or remained uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means ($n=10$). Different letters indicate significant differences between each treatment ($P\leq 0.05$)

inoculation of either AM fungi or NFB as symbiotic partners (Fig. 1a). The increase in plant biomass for the double symbiosis was not related to different allocations to root or shoot dry matter per plant, but to the enhanced relative growth rates and the growth of both root and shoot components, as evidenced by the lack of differences in the root:shoot, root:plant and shoot:plant ratios (Table 1). Dual symbiotic plants were approximately 2-fold larger than non-symbiotic plants (Fig. 1a) and the shoot growth of the dual symbiotic plants was increased approximately 2.5 and 5-fold compared to single inoculated or uninoculated plants respectively (Table 1). Although slightly less pronounced, a similar pattern was observed for root growth with the same treatments (Table 1).

Xylem sap nutrients

The xylem export of nutrients from the symbiotic roots was greatly affected by the symbiotic status of the plants (Fig. 2a–d). The xylem sap phosphate concentration was the highest in the host plants with AM colonization only, this was reduced by approximately 50 % when the host plant was colonized by both AM and NFB (Fig. 2a). In the absence of AM colonization, the plants with nodules had lower P export than the

Table 1 Plant biomass of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** Rhizobial inoculation and were either colonized **(+M)** or uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means (n=10). Different letters along each row, indicate significant differences between each treatment ($P \leq 0.05$), with the standard error of the means

Plant growth parameters	−M ; −R	−M ; +R	+M ; −R	+M ; +R
Relative growth rate ($mg.g^{-1}.day^{-1}$)				
Total plant	68.81±9.24a	105.29±8.40b	118.52±14.8b	184.82±19.38c
Dry mass (g)				
Shoot	0.46±0.04a	0.95±0.13b	0.86±0.12b	2.36±0.22c
Root	0.15±0.03a	0.33±0.07b	0.31±0.03b	0.66±0.11c
Organ dry mass ratios				
Root:Shoot	0.32±0.05a	0.38±0.09a	0.38±0.03a	0.28±0.03a
Root:Plant	0.25±0.03a	0.27±0.04a	0.27±0.02a	0.22±0.02a
Shoot:Plant	0.79±0.03a	0.77±0.04a	0.73±0.02a	0.79±0.02a

uninoculated control plants (Fig. 2a). Furthermore, the ureide concentration in xylem sap was lowest in the uninoculated plants (Fig. 2b). Although AM plants had greater ureide levels than the uninoculated plants, the presence of NFB had the greatest influence the ureide concentrations in the xylem sap, irrespective of the presence or absence of mycorrhiza (Fig. 2b).

Compared to plants with double root symbionts or the uninoculated controls, the host plants with a single root symbiont, generally had lower NH_4^+concentrations in xylem sap. Furthermore, the lowest NH_4^+concentrations in xylem sap were found in host plans with only NFB as the symbiont (Fig. 2c). The amino acid concentrations in the xylem sap were the lowest in the host plants with AM colonization, either as single or double symbiotic plants, whereas the host plants with nodules only, had the highest concentration of amino acids (Fig. 2d).

N and P nutrition

Plant P and N were highest in the dual symbiotic plants, as was shoot N (Table 2). Shoot P was highest in AM plants only. The roots colonized by NFB maintained the highest levels of P, with the dual symbiotic roots having the highest overall. No differences were found in the root N concentrations across treatments (Table 2). Furthermore, the AM plants had the greatest specific P absorption rates (Fig. 3a) and the dual symbiotic plants had about a 5-fold higher rate than any of the other treatments, similarly, the specific nitrogen absorption rates were approximately 5-fold higher for the dual symbiotic plants (Fig. 3b). An almost identical pattern was found in the specific phosphate utilization rates across the treatments (Fig. 4a). The roots colonized by NFB maintained the highest specific nitrogen utilization rates, yet once again, the dual symbiotic plants had higher rates than any of the other treatments (Fig. 4b).

Photosynthesis

The maximum photosynthetic rate was recorded in the dual symbiotic plants (Fig. 5). Although there was no difference in the photosynthetic rates between the plants with either the single AM colonization or with NFB, the uninoculated plants displayed the lowest photosynthetic rates (Fig. 5).

Discussion

The synergistic benefits of the dual inoculation of *A. cyclops* were apparent in the improved relative growth rates, biomass and nutrition of the host plants. These are likely to be attributing factors to this plant's ability to develop and rapidly colonize nutrient poor soils.

The physiological advantages associated with the dual inoculation of *A. cyclops* occurred in spite of the reduction in growth of both symbiotic partners. The decline in the degree of colonization by the respective symbionts concurs with the work of Catford et al. (2003) who proposed that the symbiotic partners in tripartite relationships can autoregulate, and thus limit

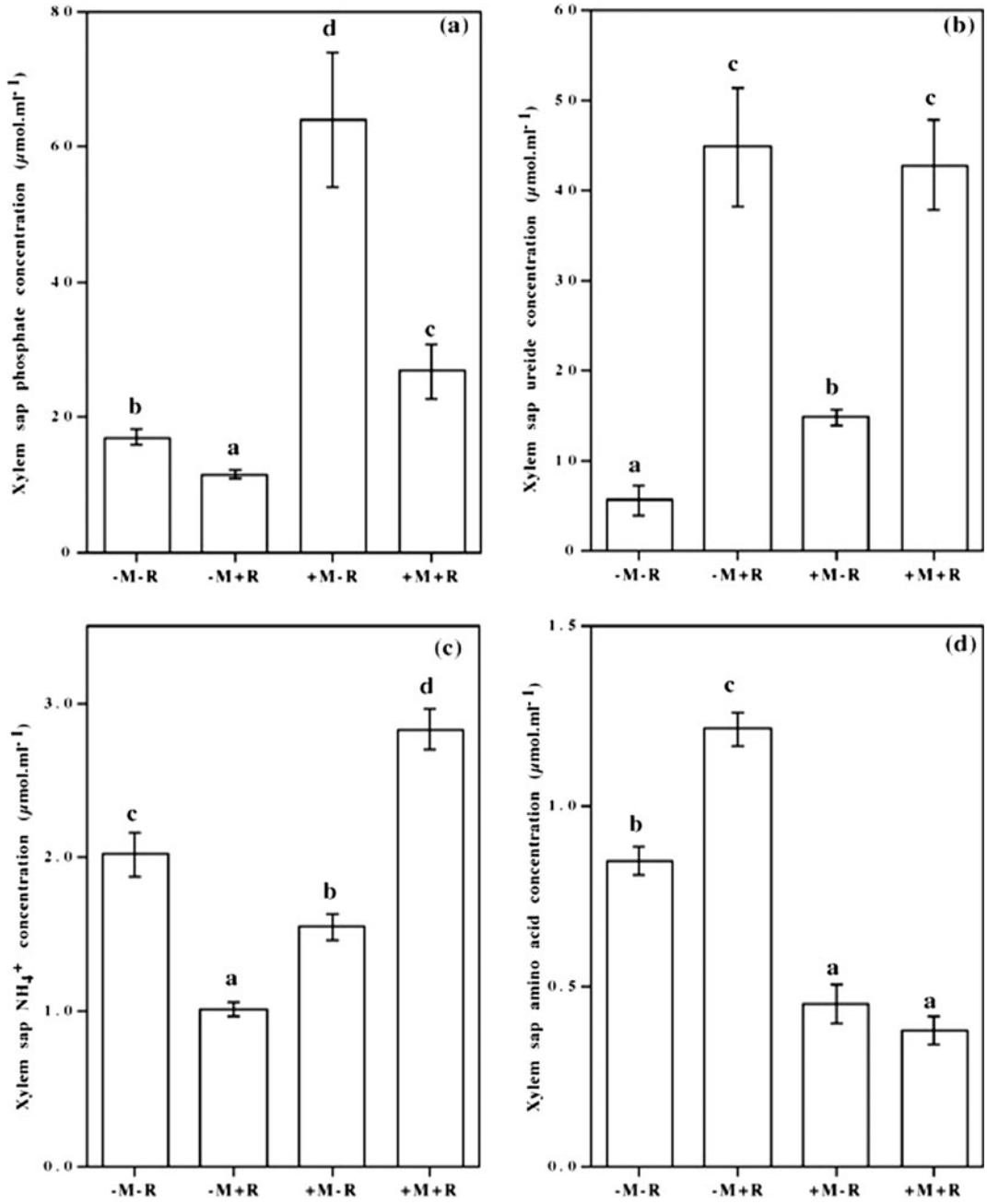

Fig. 2 Phosphate (**a**), ureide (**b**), NH_4^+ (**c**) and amino acid (**d**) concentrations in the xylem sap of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** nitrogen fixing bacteria and were either colonized **(+M)** or remained uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means (n=10). Different letters indicate significant differences between each treatment ($P \leq 0.05$)

the development of the other symbionts. The need for such autoregulation may be ascribed to the fact that a double symbiosis would involve a larger C sink burden than either symbiont on its own. Therefore, by limiting symbiont development of both mycorrhiza and nodules, the amount of C used by the respective symbionts can be regulated. In support of this, Pearson et al. (1993) found that competition for root C could

Table 2 Tissue N and P concentrations of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** Rhizobial inoculation and were either colonized **(+M)** or uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means (n=10). Different letters along each row, indicate significant differences between each treatment ($P \leq 0.05$)

	−M ; −R	−M ; +R	+M ; −R	+M ; +R
Tissue P (mmol. g^{-1} dw)				
Plant P	0.04±0.006a	0.08±0.012a	0.29±0.079b	1.48±0.205c
Shoot P	0.1±0.007a	0.09±0.005a	1.02±0.193c	0.62±0.056b
Root P	2.99±0.093ab	3.13±0.167ab	2.81±0.185a	3.44±0.095bc
Tissue N (mmol. g^{-1} dw)				
Plant N	1.09±0.140a	2.99±0.386b	1.18±0.127a	7.23±0.682c
Shoot N	0.12±0.020a	0.15±0.013a	0.64±0.081b	0.94±0.041c
Root N	3.07±0.122a	2.86±0.033a	2.86±0.090a	3.14±0.058a

limit the extent of AM colonization. However, these findings are contrary to other studies showing improved growth and development of both symbionts under tripartite conditions (Chaturvedi and Singh 1989; Kaschuk et al. 2009; Mortimer et al. 2008; Pacovsky et al. 1986). The improved growth of the dual symbiotic plants, in spite of the decline in symbiont growth, is in agreement with the study carried out by Rodriguez-Echeverria et al. (2008) who found a positive correlation between dual colonization of *Rhizobia* and AM and the improved growth of *Acacia longifolia*, also an invasive species in Mediterranean regions. This improved growth is most likely a result of improved nutrition, leading to higher photosynthetic rates.

The increased photosynthetic rates of the dual symbiotic plants resulted in improved plant biomass, which has also been shown to be true in a number of past studies (Jia et al. 2004; Kaschuk et al. 2009; Mortimer et al. 2008). The ability of the host plants to achieve and maintain higher rates of photosynthesis is likely to have been driven by two factors. Firstly a greater below ground C sink due to the two symbionts and secondly by the improved nutrition of the host plant. Interestingly, despite the below ground C sink, more C was invested into the above ground tissue of the dual symbiotic plants in comparison to the other treatments. This was likely the result of a cost-benefit effect between the C and nutrient sinks. To begin with, the higher demand for belowground C resulting from having to support both symbionts, may have caused an increase in the photosynthetic rates of these plants, as was found for this study and shown in previous works as well (Jia et al. 2004; Mortimer et al. 2008; Kaschuk et al. 2009). Additionally, the increased N concentrations found in the roots, would have required further C-skeletons for N assimilation. Thus, due to the competition for C-skeletons in the belowground tissues, the high NH_4^+ concentration in the xylem sap may indicate that inorganic N was being translocated to the shoots for assimilation. This is in accordance with the work of Valentine and Kleinert (2006) who suggested that AM competed with NH_4^+ assimilation for host C in the roots. Further evidence for the competition of C driving the N to the shoots lies in the high concentration of ureides (high N:C) in comparison to the low amino acid concentration (low N:C) in the xylem sap of the dual symbiotic plants. Consequently, high shoot N concentrations would allow for increased shoot growth and photosynthetic rates. The occurrence of increased above-ground growth was also reported by Rodriguez-Echeverria et al. (2008), who found that *A. longifolia* plants colonized by both AM and nodule bacteria, had greater above-ground biomass. Rodriguez-Echeverria et al. (2008) attributed this to the increased N levels, resulting from improved biological N fixation (BNF). Additional evidence in favor of the argument regarding the C and nutrient sinks resulting in higher above ground C investment, is that the dual symbiotic plants were dramatically more efficient in terms of N and P capture and utilization than any of the other treatments. Thus allowing for more C to be available for growth in place of nutrient assimilation. The concept of improved shoot N resulting in higher photosynthetic rates and improved shoot growth is not new, past studies have shown that

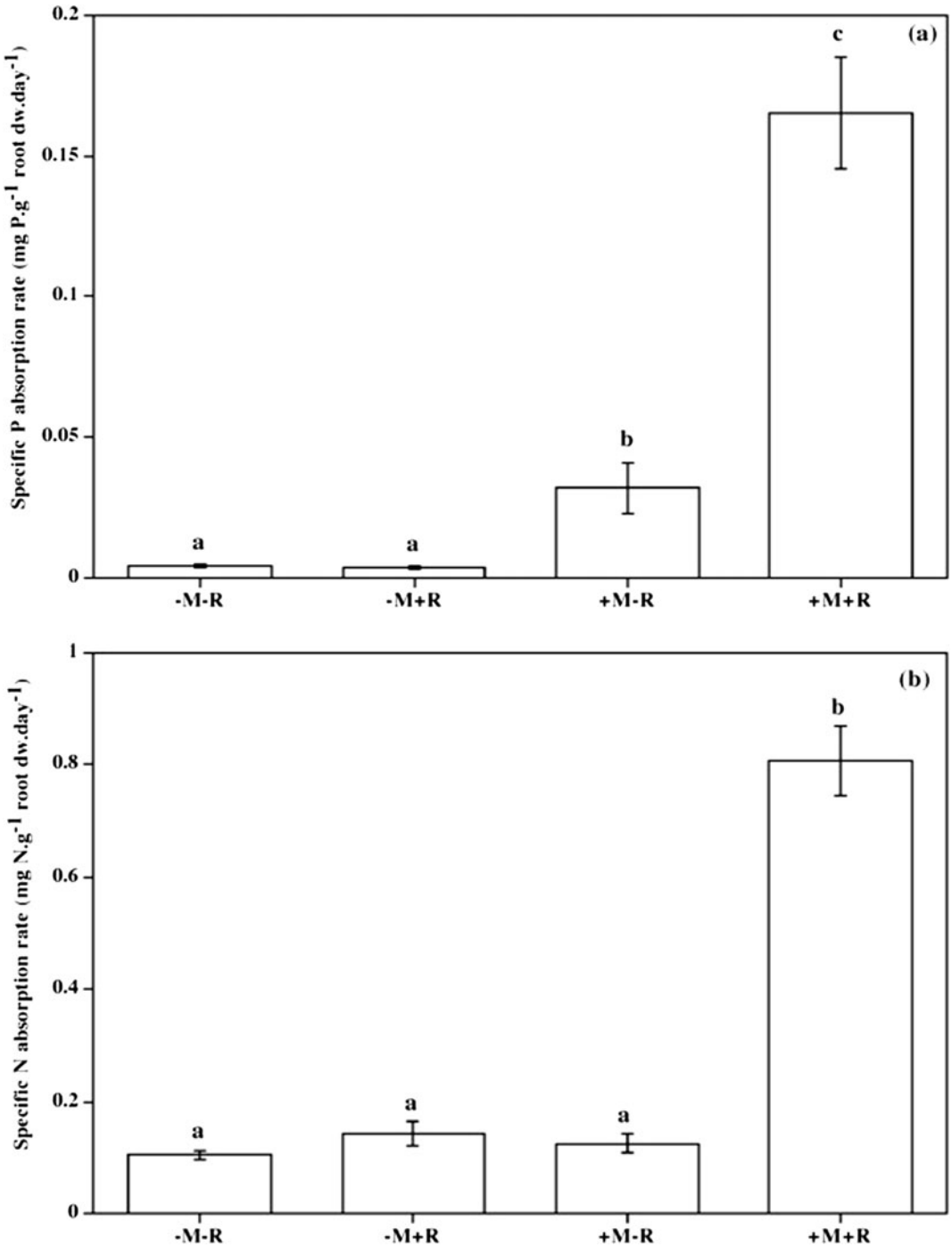

Fig. 3 Specific phosphate absorption rate (**a**) and specific nitrogen absorption rate (**b**) of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** nitrogen fixing bacteria and were either colonized **(+M)** or remained uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means (n=10). Different letters indicate significant differences between each treatment ($P \leq 0.05$)

improved N nutrition and N-use efficiency of shoots led to improved photosynthetic rates and growth, thus confirming our findings (Epo 1991; Lima et al. 2008; Mortimer et al. 2012).

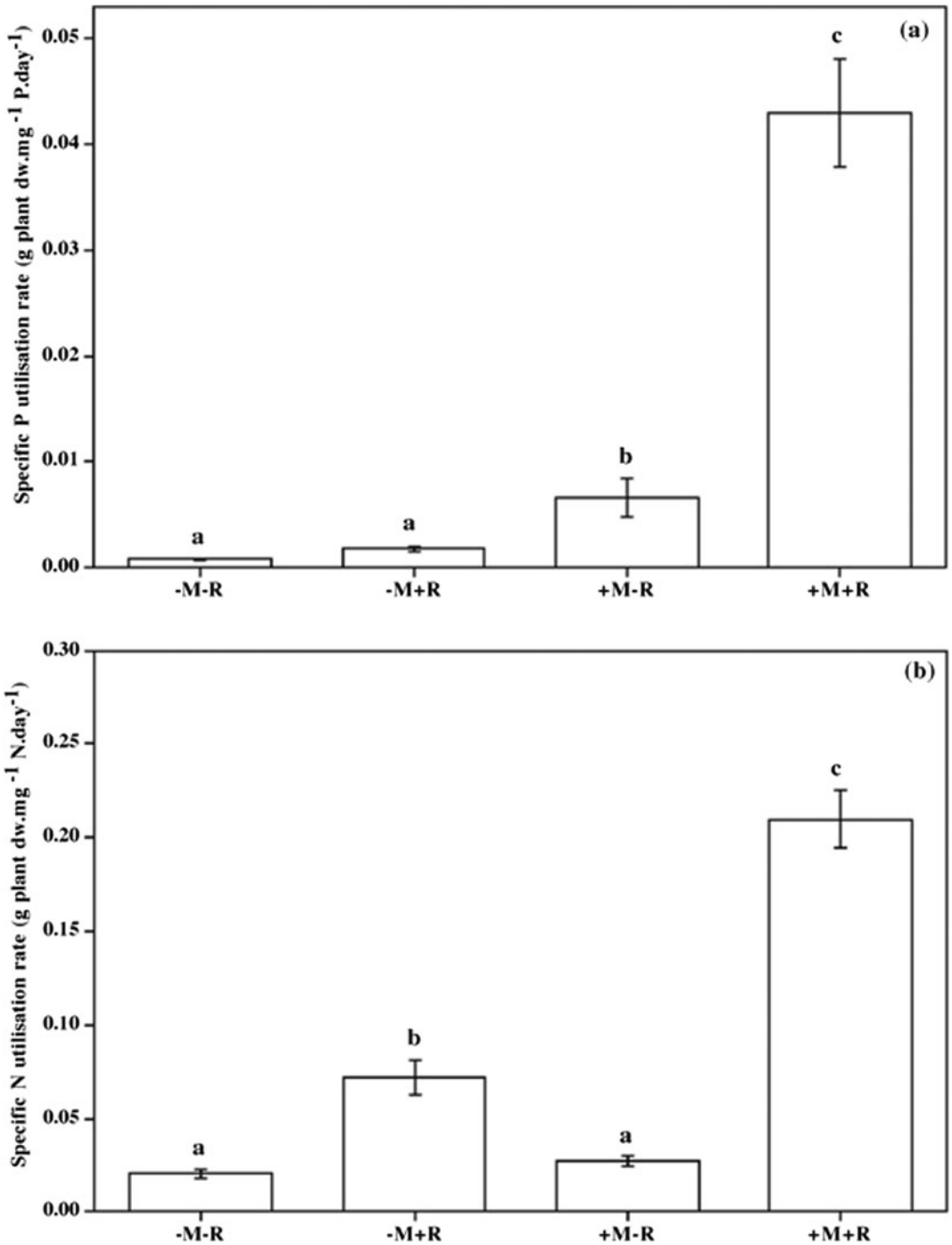

Fig. 4 Specific phosphate utilisation rate (**a**) and specific nitrogen utilisation rate (**b**) of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** nitrogen fixing bacteria and were either colonized **(+M)** or remained uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means ($n=10$). Different letters indicate significant differences between each treatment ($P\leq 0.05$)

Another physiological benefit of inoculation of nodulated *A. cyclops* with AM was the increased N and P nutrition of the host plants, despite the decline in the growth of both symbionts. Thus it is apparent that

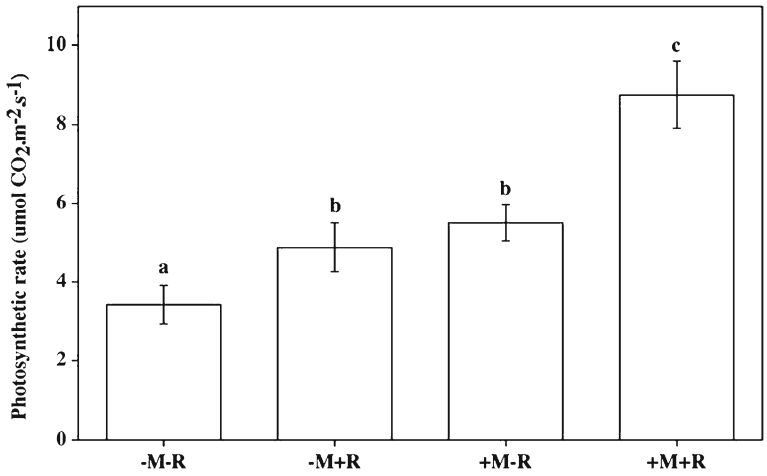

Fig. 5 Leaf photosynthetic rate of *Acacia cyclops*, grown for 5 months in P and N deficient sand culture, under glasshouse conditions. Host plants were grown with **(+R)** or without **(−R)** nitrogen fixing bacteria and were either colonized **(+M)** or remained uncolonized **(−M)** by the arbuscular mycorrhizal fungus, *Glomus mossae*. Values are presented as means (n=10). Different letters indicate significant differences between each treatment ($P \leq 0.05$)

the symbionts present on the roots remained effective in terms of nutrient capture. The AM plants had dramatically increased levels of plant P, especially in comparison with the non-AM plants. However, a large portion of the P remained below ground, as evidenced by the lower P export from the roots and the low shoot P levels of the dual symbiotic plants, though the shoot P levels still remained higher than those of the non-symbiotic plants. This is likely the result of the high P demand of the nodules, which are known to be strong sinks for P and may accumulate several fold higher P concentrations than other plant organs (Høgh-Jensen et al. 2002; Tang et al. 2001; Vadez et al. 1999). The increased levels of root P would ensure continued nodule function, thus providing additional N to be used in the photosynthetic process of the host plant. This is evident from the higher transport of N to the shoots and subsequent increase in above ground biomass, as discussed earlier.

It is apparent that the AM play both an indirect and a direct role in the N nutrition of the host. The high N concentrations found in the nodulated AM plants (2.4-fold higher than the nodulated non-AM plants) indicate the indirect the role that AM play in the N nutrition of the host, by enhancing BNF. Other studies evaluating the effect of a tripartite symbiosis between legumes, AM fungi and nodule bacteria have found that the presence of AM led to increased rates of biological N fixation and increased plant N concentrations (Toro et al. 1998, Kaschuk et al. 2009; Mortimer et al. 2008, 2009). The high levels of plant N found in the non-nodulated AM plants, compared to the uninoculated controls, indicate the direct influence that AM can have on the N levels of the host pant. This is in line with previous studies showing that AM are able to directly assimilate and provide N to the host plant (Constable et al. 2001; Govindarajulu et al. 2005; Marschner and Dell 1994; Mortimer et al. 2009; Toussaint et al. 2004).

These findings suggest that AM plays an important role, both directly through nutrition, and indirectly through the enhanced synergistic benefits of the dual colonization, in the ability of *A. cyclops* to become established in soils low in N and P. Although the current experiment was carried out in a glasshouse, factors such as the higher relative growth rates, increased biomass, greater photosynthetic rates, improved nutrition and more efficient use of nutrients would allow for the rapid establishment of *A. cyclops* colonies. Rodriguez-Echeverria et al. (2008) attributed the ability of *A. longifolia* to rapidly form nodules, thus establishing high rates of BNF at an early stage, as an integral process allowing for the growth of invasive species in low nutrient soils. It was further proposed that these traits would provide a competitive advantage over other flora trying to grow in the same soils.

In summary, the dual symbiotic plants experienced improved N and P nutrition and enhanced growth

when compared with non-symbiotic plants or plants with only one of the root symbionts. This was achieved in two ways; firstly, these plants were vastly more efficient at the assimilation and use of acquired nutrients, thereby allowing for more C to be available for growth. Secondly, by the translocation of N to above ground tissues for assimilation, thus reducing the below ground C sink for photosynthates.

It is evident that the dual inoculation of *A. Cyclops* resulted in the enhanced performance of these plants in nutrient poor soils, relative to single symbiont inoculation or uninoculated plants. These physiological advantages may remain prevalent under the field conditions, where these plants occur in competition for resources in low nutrient soils. Although this was a glasshouse-based study, it is tempting to suggest that this symbiotic partnership may contribute to the invasive success of the host species.

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