



Challenging the 'Immunity Hypothesis': Primary or secondary parasitism as different survival strategies for the harlequin mistletoe *Lysiana exocarpi* (Behr) Tiegh[☆]

Marina Corrêa Scalon^{a,*}, Davi Rodrigo Rossatto^b

^a Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal do Paraná, Curitiba, PR 81531-990, Brazil

^b Departamento de Biologia, Faculdade de Ciências Agrárias e Veterinárias, Univ. Estadual Paulista Campus de Jaboticabal. Via de Acesso Prof. Paulo Donato Castellane S/N, Vila Industrial 14884-900, Jaboticabal, SP, Brasil

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ABSTRACT

The relationship between host plants and parasitic plants is considered a unique and fascinating model to explore different aspects in plant physiology, especially concerning water relations and nutrient use, given that host and parasite share these resources. An even more intriguing dynamic occurs when a parasite parasitizes another parasite (epiparasitism). In theory, these epiparasites should show even more specialized physiological functions to be capable to withdraw water and nutrients from the hemiparasite, such as maintaining even lower water potential and dealing with stronger nutrient imbalance. Here we studied leaf morpho-physiological responses of the Australian harlequin mistletoe (*Lysiana exocarpi*) and box mistletoe (*Amyema miquelii*) acting as hemiparasites on pink gum (*Eucalyptus fasciculosa*) and *Lysiana exocarpi* as an epiparasite on *A. miquelii* parasitizing *E. fasciculosa*. We explored the water and nutrient use and partitioning between the different plants involved (host–mistletoe–epiparasite). We found that *L. exocarpi* showed physiological and morphological differences that represent a more acquisitive strategy of resource use with higher stomatal conductance, water use efficiency, specific leaf area and K and Mg leaf concentration when growing as an epiparasite compared to the same species growing as primary parasite. *Amyema miquelii* traits remained constantly similar irrespective whether as primary parasite or with an epiparasite sharing its resources while the effect of the epiparasite is mostly depicted in its host's host species. In conclusion, our findings suggest that by adjusting some traits, epiparasitism is an alternative option for the harlequin mistletoe. Moreover, regardless of the host–parasite association, the primary host is the most affected by the epiparasitism.

1. Introduction

Plant scientists have continually used mistletoes and other plant hemiparasites as models to investigate resource use strategies, due to their unique organs and physiological mechanisms to acquire and use water and nutrients. Specially, mistletoe–host relationships are intriguing, since there is usually only one host plant involved, and resource use and dynamics can be followed in the plant–plant system. However, much less studied, is the resource use by epiparasites (also referred to as hyperparasites; see Krasylenko et al., 2021). Epiparasites is the term usually used in relation to mistletoes (Krasylenko et al., 2021) and refers to organisms that parasitize other primary parasites, using the parasite as their host. The relationship between an epiparasite and its

primary parasite host adds layers of complexity to the already intricate dynamics of parasitic interactions. Indeed, while the concept of hyperparasitism is well-documented across various groups of organisms, its prevalence and significance within plants have often been underestimated and underexplored (Krasylenko et al., 2021; Wilson and Calvin, 2017). Facultative epiparasitism has been reported for different Santalaceae and Loranthaceae species (Clark et al., 2020; Wilson and Calvin, 2017). By targeting primary parasites, epiparasites can affect the population dynamics, distribution, and behaviour of both primary parasitic plants and their host plants, as well as influence the structure and functioning of plant communities as a whole.

There is little knowledge about the selective forces that favour epiparasitism. There may be the influence of the foraging behaviour of seed

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* Corresponding author.

E-mail address: marina_scalon@yahoo.br (M.C. Scalon).

dispersers (Heide-Jorgensen, 2008) and even mistletoe phenology (Li and Zhang, 2020). However, because of the potential extra physiological constraints in water and nutrient acquisition involved in an epiparasitism relationship, it is speculated that using another mistletoe as a host may be a last resource when there are no other hosts available (Fontúrbel et al., 2023). To establish a connection with a mistletoe and survive in a long-time span could be challenging due to physiological incompatibility. Mistletoes are known to be profligate water users, and to maintain lower water potentials than their hosts and high transpiration rates to maintain the water flowing from the host (Ehleringer et al., 1986; Scalon et al., 2021; Scalon and Wright, 2016; Schulze et al., 1984). An epiparasite would have to surpass these differences to move xylem sap from the mistletoe to its tissues, which could be critical for survival, as it may achieve even lower water potentials to divert water from the hemiparasite. Mistletoes are also known to be very efficient in withdrawing nutrients from their hosts usually showing higher leaf macronutrients concentration (Lamont and Southall, 1982; Scalon et al., 2013), which is probably progressively increased from mistletoe to the epiparasite (Ehleringer et al., 1985; Lamont and Southall, 1982; Tenakoon et al., 2011).

Parasitic plants are characterized by the specialized root, named haustorium (Kuijt, 1969). It is the haustorium that enables attachment and penetration of the parasitic plants to the susceptible host plants, forming a vascular bundle that connects the parasite and the host (Teixeira-Costa, 2021; Yoshida et al., 2016). The development of the haustorium is also known to be dependent on host-derived chemical signals (known as haustorium-inducing factors – HIFs) for some more specific parasitic plant species, but can also have very generalist behaviour (Chang and Lynn, 1986; Shen et al., 2006). Although mistletoes with epicortical roots and multiple connections are known to engage more in epiparasitic relationships (Krasnylenko et al., 2021), mistletoes with a single haustorium (Teixeira-Costa et al. 2020) could reveal more about the nutrient and water partitioning between the plants involved, since there is a single source of water and nutrient for the epiparasite. Contradicting the patterns found in African epiparasites (Midgley, 2024), in Australia facultative epiparasitic species can be found parasitizing either another mistletoe but also their host's host, which is a unique model to understand these relationships. *Lysiana exocarpi* (Behr) Tiegh. is a known generalist mistletoe occurring in over 150 host species (Clark et al., 2020) with various reports of epiparasitism in different Loranthaceae hosts, particularly in mistletoes from the *Amyema* genus (Wilson and Calvin, 2017).

Given the lack of studies on the physiological relationships of epiparasites with their hemiparasite hosts, we provide a study comparing the same mistletoe species acting as primary hemiparasite and also as an epiparasite. Our aim was to understand the water and nutrient use and partitioning between the different plants involved (host–mistletoe–epiparasite). We also explored the impact of epiparasitism on the host by comparing the same host species with the mistletoe alone and with the mistletoe attached to the epiparasite. We hypothesize that physiological constraints in water and nutrient acquisition imposed by the epiparasite will be reflected (1) in the mistletoe when associated with the epiparasite, and (2) in the host traits when associated with the combination of the mistletoe and the epiparasite together. Therefore our hypotheses are that (i) mistletoes with the epiparasite attached will show more conservative traits, with lower specific leaf area, photosynthetic rate and stomatal conductance, but higher water use efficiency compared to the same species without the epiparasite, and that (ii) the host with the mistletoe and the epiparasite will show generally lower leaf nutrients concentration and higher water use compared to the host parasitized by the mistletoe alone, since they are providing resources for two different organisms.

2. Material and methods

2.1. Study site and selected species

The study site was located in Onkaparinga River National Park borders, 40 km south of Adelaide, South Australia (35.1592° S, 138.5642° E; 139 m elevation). The vegetation is classified as a low sclerophyll woodland (Fig. 1A) dominated by pink gum (*Eucalyptus fasciculosa* F. Muell. – Myrtaceae), golden wattle (*Acacia pycnantha* Benth. – Fabaceae) and murray pine (*Callitris preissii* Miq. – Cupressaceae) (Whittington and Sinclair, 1988), characterized by moderate (5–10 m) to occasionally tall (>10 m) eucalyptus trees (the dominants) forming a non-continuous canopy, and by a dense under-growth of sclerophyllous shrubs (0.6–1.3 m) (Cochrane, 1963). The climate is Mediterranean-type with wet winters and dry summers, an annual rainfall of ca. 569 mm, mostly (≈80 %) between April and October (Department for Environment and Heritage, 2004). In September 2013, we sampled five individuals of the pink gum *Eucalyptus fasciculosa* F. Muell. with the harlequin mistletoe *Lysiana exocarpi* (Behr) Tiegh. attached as primary parasite, five individuals of *E. fasciculosa* with the box mistletoe *Amyema miquelii* (Lehm. Ex Miq.) attached as primary parasite, and five individuals of *E. fasciculosa* with *A. miquelii* as primary parasite and *L. exocarpi* attached as epiparasite. We searched for *E. fasciculosa* individuals of similar size (≈10 cm DBH – diameter at breast height, and ≈4–5 m height) with only one mistletoe attached; however, there were two *E. fasciculosa* + *L. exocarpi* pairs with two different *L. exocarpi* individuals attached. The epiparasite connection was easily distinguished because of the different branch colours between the two mistletoe species (Fig. 1C) and the evident haustorial connection formed by *L. exocarpi* (Fig. 1d)

2.2. Leaf traits

Five mature, fully-expanded sun-exposed leaves per individual were collected from the third node, immediately scanned and the area was measured using Image J software. The leaves were dried in the oven for 72 h at 65 °C and weighed using a digital scale (Sigma-Aldrich Kern EWJ 600–2 M; 0.01 ± 0.03 g precision). Specific leaf area (SLA) was calculated as the ratio of fresh leaf area to leaf dry mass. The same leaves were finely ground for nutrient analyses using a micro plant grinding machine and samples were sent to the Analytical Service Unit from the School of Agriculture and Food Science at The University of Queensland for N determination using a LECO TruSpec CHN combustion analyser. Total P, K, Ca and Mg were measured using an ICP-OES analyser (Thermo-scientific iCAP PRO XP) following nitric perchloric acid digestion.

In the field, we measured photosynthetic rate (A_{max}), stomatal conductance (g_s), and transpiration rates (E) simultaneously at saturating light intensity (2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and 400 $\mu\text{mol mol}^{-1}$ ambient CO_2 , using the portable infra-red gas analysis system Licor 6400. Ambient temperature averaged 27.7 °C ranging from 24.2 °C to 30.8 °C, VPD averaged 1.16 ± 0.17 kPa. Intrinsic water use efficiency (iWUE) was calculated as the ratio between A_{max} and g_s .

2.3. Data analysis

All data were log-transformed for the assumption of normality (Kolmogorov-Smirnov test, $P < 0.05$ for all variables). Homogeneity of variances was tested with Levene's test. To test for general differences in leaf functional traits and leaf nutrient concentration, we used multivariate analysis of variance (MANOVA) with species and combination (*E. fasciculosa* + *A. miquelii* + *L. exocarpi* – EAL; *E. fasciculosa* + *A. miquelii* – EA; and *E. fasciculosa* + *L. exocarpi* – EL) as covariates. To test for individual trait trends in relation to habit (epiparasite, parasite or host) we used two-way ANOVAs followed by Tukey tests, considering all tests to be significant when $P < 0.05$. To explore the differences between species, we performed a principal component analysis (PCA)

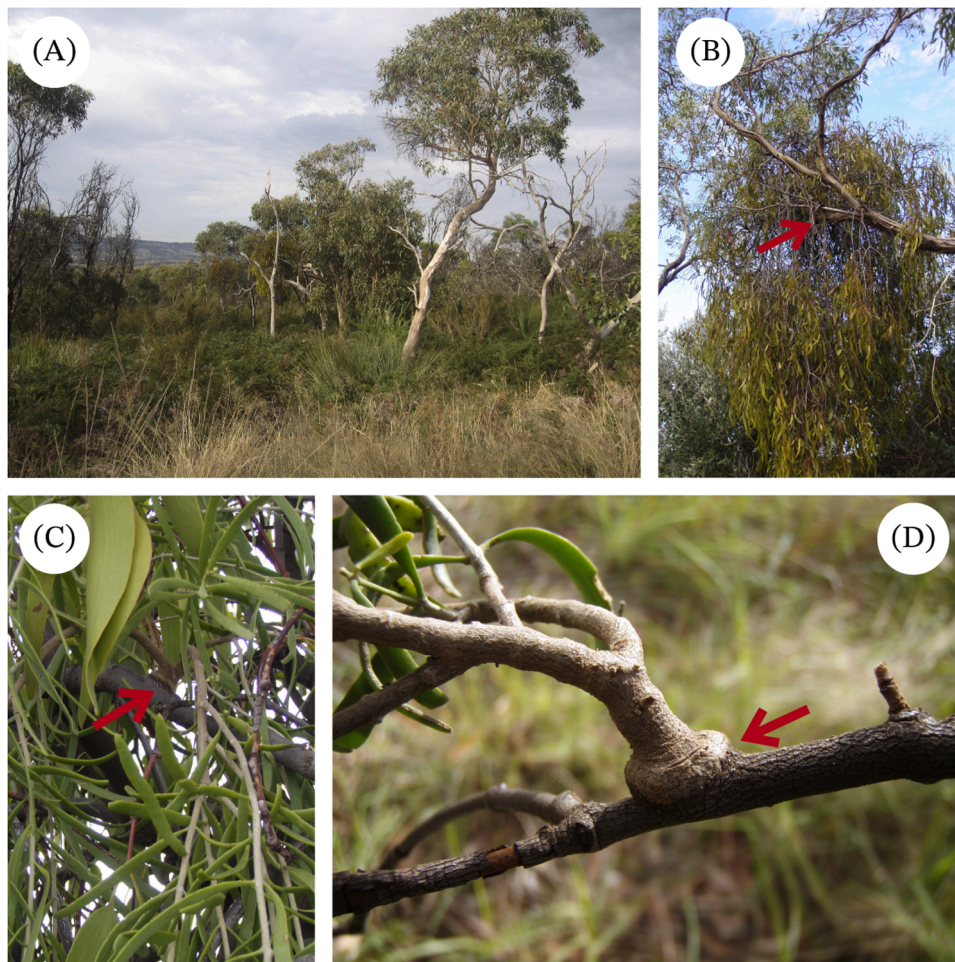


Fig. 1. (A) Onkaparinga River National Park borders showing the vegetation aspect; (B) the box mistletoe *Amyema miquelii* (Lehm. ex Miq.) Tiegh. attached to the pink gum *Eucalyptus fasciculosa* F. Muell.; (C,D) the box mistletoe *Amyema miquelii* with epiparasite, the harlequin mistletoe *Lysiana exocarpi* (Behr) Tiegh. attached showing the different branch colour (C), and the evident haustorium formed (D). Arrows in B–D indicate the haustorium. *Lysiana exocarpi* leaf length is 5.94 ± 1.08 cm for scale reference.

followed by permutational multivariate analysis of variance based on Euclidean distances, using the ‘adonis2’ function within the vegan package (Oksanen et al., 2007). All analyses were performed in R (version 4.4.0; R Core Team, 2024).

3. Results

There were differences between the three species (MANOVA: $F_{2, 27} = 20.49$, $P < 0.0001$) and an interaction between species and their different combinations (MANOVA: $F_{2, 27} = 3.50$, $P < 0.0001$), meaning that species would respond differently according to the associated host, mistletoe or epiparasite (Table 1). The host *E. fasciculosa* showed consistently higher A_{max} compared to the two Loranthaceae species (Fig. 2a). However, the host showed higher SLA (Fig. 2d) and slightly higher leaf N (Fig. 3a) when associated with a single parasite.

Lysiana exocarpi showed similar A_{max} (Fig. 2a), but higher stomatal conductance (Fig. 2b), SLA (Fig. 2d), leaf Mg (Fig. 3e), and lower iWUE (Fig. 2c) and leaf K (Fig. 3d), when growing as epiparasite compared to the same species growing as primary parasite. *Amyema miquelii* traits remained constantly similar irrespective whether as primary parasite or with an epiparasite sharing resources (Figs. 2 and 3).

Marked differences between the three species were revealed by the PCA (Adonis test, $r^2 = 0.44$, $P < 0.001$; Fig. 4). The first two principal components explained $\approx 58.8\%$ of the variation. *Eucalyptus fasciculosa* was associated with higher A_{max} , leaf N concentration and SLA, while *L.*

exocarpi generally had higher leaf nutrient concentration, except N. *Amyema miquelii* was more related to a higher stomatal conductance and leaf transpiration.

4. Discussion

From our results, there are some significant findings that improve our understanding of the ecophysiology of epiparasitism. Firstly, we partially corroborate our hypothesis since, even under very similar environmental conditions, when growing as epiparasite, *L. exocarpi* showed physiological and morphological differences that represent a more acquisitive strategy of resource use, with higher transpiration rates, higher stomatal conductance, lower water use efficiency and higher SLA and some leaf nutrients compared to *L. exocarpi* acting as primary parasite. Secondly, we found that the effect of epiparasite is mostly depicted in their host’s host species (i.e., *E. fasciculosa*), and not in the mistletoe host. The combination of mistletoe and epiparasite seems to have a stronger impact on the host *E. fasciculosa*, that showed lower SLA with the mistletoe combined with the epiparasite compared to the host with a single parasite, either *A. miquelii* or *L. exocarpi*. Thirdly, the mistletoe *A. miquelii* does not seem to be directly affected by the epiparasite, showing similar physiological traits and leaf nutrient concentrations irrespective of *L. exocarpi* presence. Finally, there were some marked differences between the two Loranthaceae species, with *L. exocarpi* showing higher leaf nutrient concentrations than *A. miquelii* in

Table 1

F-values and *P*-values for two-way ANOVA on the influence of species and species combinations (host+mistletoe+epiparasite, host+mistletoe) on maximum photosynthetic rate (A_{max}), stomatal conductance (g_s), leaf transpiration rate (E), intrinsic water use efficiency (iWUE), specific leaf area (SLA) and leaf concentrations of the macronutrients N, P, Ca, K, Mg and S.

Trait	Source of variation	DF	<i>F</i> -value	<i>P</i> -value
A_{max}	Species	2	23.65	< 0.0001
	Combination	2	2.77	0.081
	Species* Combination	2	3.87	0.033
	Residual	28		
g_s	Species	2	6.85	0.004
	Combination	2	5.88	0.007
	Species* Combination	2	2.64	0.090
	Residual	28		
E	Species	2	9.24	< 0.0001
	Combination	2	8.43	0.001
	Species* Combination	2	4.96	0.015
	Residual	28		
iWUE	Species	2	6.85	0.004
	Combination	2	5.88	0.008
	Species* Combination	2	2.64	0.089
	Residual	28		
SLA	Species	2	17.14	< 0.0001
	Combination	2	1.65	0.211
	Species* Combination	2	7.20	0.003
	Residual	28		
N	Species	2	17.16	< 0.0001
	Combination	2	3.27	0.054
	Species* Combination	2	0.25	0.779
	Residual	28		
P	Species	2	16.09	< 0.0001
	Combination	2	3.26	0.053
	Species* Combination	2	5.07	0.014
	Residual	28		
Ca	Species	2	9.88	< 0.0001
	Combination	2	0.12	0.884
	Species* Combination	2	2.95	0.069
	Residual	28		
K	Species	2	108.89	< 0.0001
	Combination	2	38.51	< 0.0001
	Species* Combination	2	20.88	< 0.0001
	Residual	28		
Mg	Species	2	7.81	0.002
	Combination	2	3.55	0.042
	Species* Combination	2	2.69	0.088
	Residual	28		
S	Species	2	17.10	< 0.0001
	Combination	2	1.67	0.207
	Species* Combination	2	2.57	0.095
	Residual	28		

general, irrespective of growing as secondary or primary parasite.

The “immunity hypothesis” posits that the inability of a mistletoe to parasitize a host is due to the host immunity (Muche et al., 2022). Midgley (2024) suggested that epiparasitism would only occur when the epiparasite is unable to primarily parasitize the host’s host, since extracting water and nutrients from the mistletoe would require even higher transpiration rates. This hypothesis was supported from surveys performed to understand host choice of mistletoes in South African thicket vegetation and also from Fontúrbel et al. (2023) reports from Chilean temperate rainforest, where facultative epiparasites were reported to not attack the host’s host locally. In our study however, we found that, even though there is the need to maintain higher transpiration rates as epiparasite, this does not prevent *L. exocarpi* from parasitizing the host’s host. Indeed, Milner et al. (2020) showed that *L. exocarpi* can be considered a generalist mistletoe over 6 bioregions across New South Wales, despite having significant preference for few host species at a local scale. The authors suggested that this species is an opportunistic parasite that can use a great number of hosts. Moreover, contrary to the reliance on a relict marsupial to disperse its fruits, which was the case for the Chilean facultative epiparasite *Tristerix corymbosus* (Fontúrbel et al., 2023), both *L. exocarpi* and *A. miquelii* are majorly

dispersed by abundant frugivorous birds (Reid, 1987; Reid et al., 1995). In addition, the flowering and fruiting periods of many mistletoes, such as *Amyema pressii*, *A. miquelii*, *A. quandong* and *L. exocarpi*, overlap (Yan, 1993), which probably favours seed germination of *L. exocarpi* on other mistletoes, given the dispersing birds foraging behaviour (Heide-Jørgensen, 2008; Kuijt, 1969). Nevertheless, the immunity hypothesis may still explain host choice in other systems and for other Loranthaceae or Viscaceae species, but it seems not to be the case for Australian harlequin mistletoe *L. exocarpi*. By adjusting some of the physiological traits to match a more resource acquisitive strategy (higher transpiration and stomatal conductance, lower water use efficiency and higher SLA) *L. exocarpi* can survive parasitising another mistletoe, but also can co-occur parasitising its host’s host.

The effect of the continuum mistletoe–epiparasite on the primary host was apparently more detrimental than one mistletoe alone, and *E. fasciculosa* showed significantly lower SLA compared to the individuals of the same species parasitized by a single mistletoe, either *A. miquelii* or *L. exocarpi*. Lower SLA implies more scleromorphic leaves, which is usually associated with limited water and nutrient availability during leaf expansion (Turner, 1994; Scalon et al., 2017). However, here we found that for all the other traits there was no difference between hosting either any of the mistletoe species or the mistletoe with the epiparasite. This suggests that shifting leaf morphology by decreasing SLA may indicate an alternative adaptation to the nutrient and water deprivation caused by the mistletoe–epiparasite infection. In studies with other tropical host species, leaves in parasitized branches also showed lower SLA compared to leaves from non-parasitized branches (Scalon et al., 2017, 2021), suggesting that nutrients were being preferentially drawn to the parasite (Schulze et al., 1984). Indeed, changes in leaf carbon economy result into distinct configuration in tissue-level anatomy, which may allow the leaf to cope with variable resource limitations (Lu et al., 2020). Therefore, the consequences for the host under compounded parasitic stress may result in lower whole plant-level carbon gain and a possible decrease in host fitness over the long term. Even though instantaneous measurements of maximum carbon assimilation remained similar for the host with a single mistletoe and with the combination of mistletoe and epiparasite, changes in SLA may imply in higher leaf construction costs, which can affect plant growth (Poorter and Villar, 1997). Given that epiparasitism is widespread and highly frequent among Australian mistletoes (Clark et al., 2020) further research is needed to explore the physiological and ecological significance of this interaction.

Interestingly, despite the effect on the main host, the primary mistletoe *A. miquelii* does not seem to be affected by the epiparasite, maintaining similar traits with or without infection by *L. exocarpi*. This finding suggests that, at least for the species investigated here, the epiparasite does not have the potential to act as a biological control over the mistletoe, as previously observed for the obligate epiparasite *Viscum loranthei* on the mistletoe *Scurrula cordifolia* in India (Pundir, 1981). However, an interesting approach would be to perform long-term studies monitoring the dynamics and duration of the relationship to verify its persistence over time. Nutrient concentrations are believed to increase from primary host to mistletoe and finally to the epiparasite, as found for the epiparasite *Viscum articulatum* growing on the mistletoe *Dendrophthoe curvata* in tropical northwestern Borneo (Tennakoon et al., 2014) and for the autoparasite *Phoradendron californicum* in Arizona (Ehleringer and Schulze, 1985). Indeed, here *L. exocarpi* was generally associated with higher nutrient concentrations (Fig. 4), specially leaf Ca, Mg and S (Fig. 3), supporting the hypothesis that epiparasites are terminal sinks for xylem transported nutrients (Glatzel and Geils, 2009). However, there were no differences in nutrient concentrations for *A. miquelii* with or without the epiparasite, and no differences for N, P, Ca and S leaf concentration for *L. exocarpi* as a primary or secondary parasite. The constant nutrient concentration in most cases irrespective of the configuration established may simply indicate differences in nutrient demands from the two Loranthaceae species, and could help

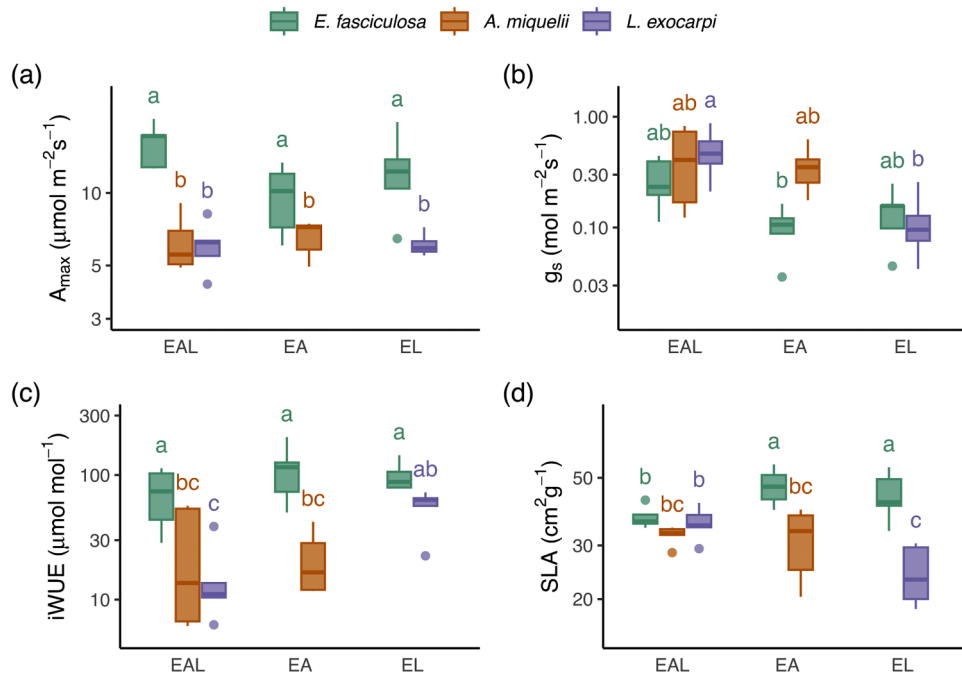


Fig. 2. Differences between species (*Eucalyptus fasciculosa* in light green, *Amyema miquelii* in light blue and *Lysiana exocarpi* in purple) in distinct combinations (*E. fasciculosa* + *A. miquelii* + *L. exocarpi* – EAL; *E. fasciculosa* + *A. miquelii* – EA; and *E. fasciculosa* + *L. exocarpi* – EL) for (a) maximum photosynthetic rate (A_{max}), (b) stomatal conductance (g_s), (c) intrinsic water use efficiency (iWUE), and (d) specific leaf area (SLA). Distinct letters show significant differences (ANOVA followed by Tukey test, $P < 0.05$; Table 1).

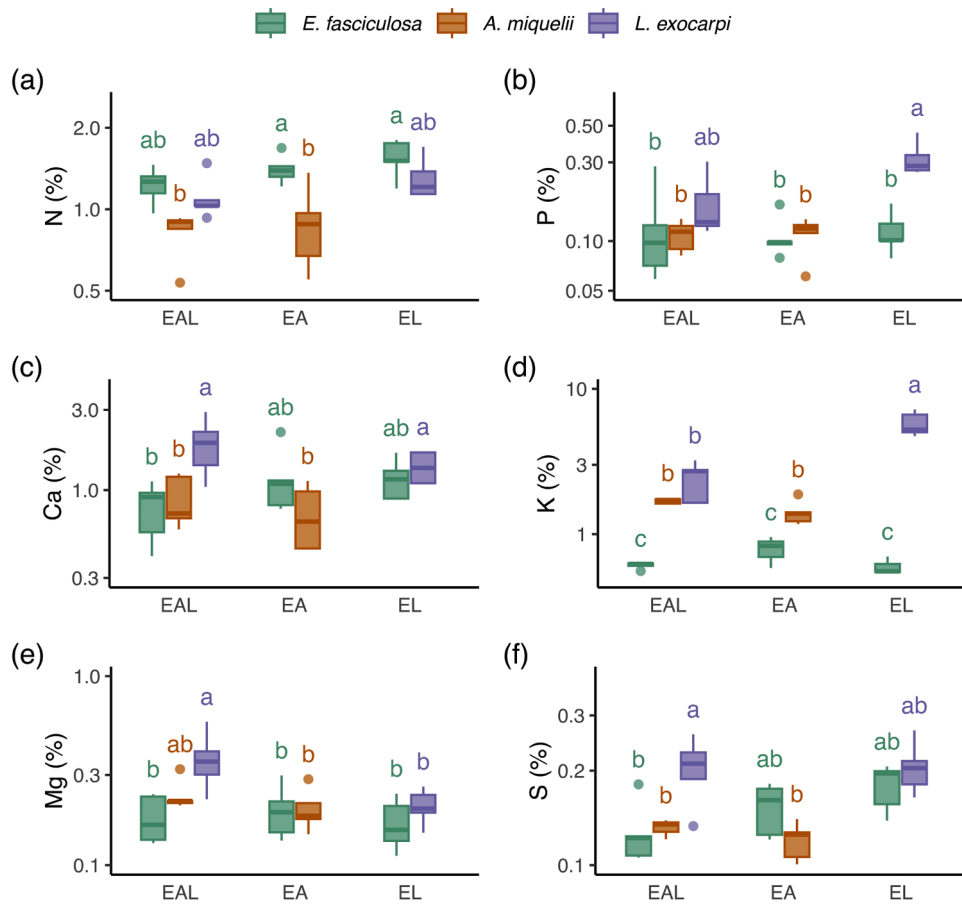


Fig. 3. Differences between species (*Eucalyptus fasciculosa* in light green, *Amyema miquelii* in light blue and *Lysiana exocarpi* in purple) in distinct combinations (*E. fasciculosa* + *A. miquelii* + *L. exocarpi* – EAL; *E. fasciculosa* + *A. miquelii* – EA; and *E. fasciculosa* + *L. exocarpi* – EL) for nitrogen (a), phosphorus (b), calcium (c), potassium (d), magnesium (e) and sulfur (f) leaf concentration. Distinct letters show significant differences (ANOVA followed by Tukey test, $P < 0.05$; Table 1).

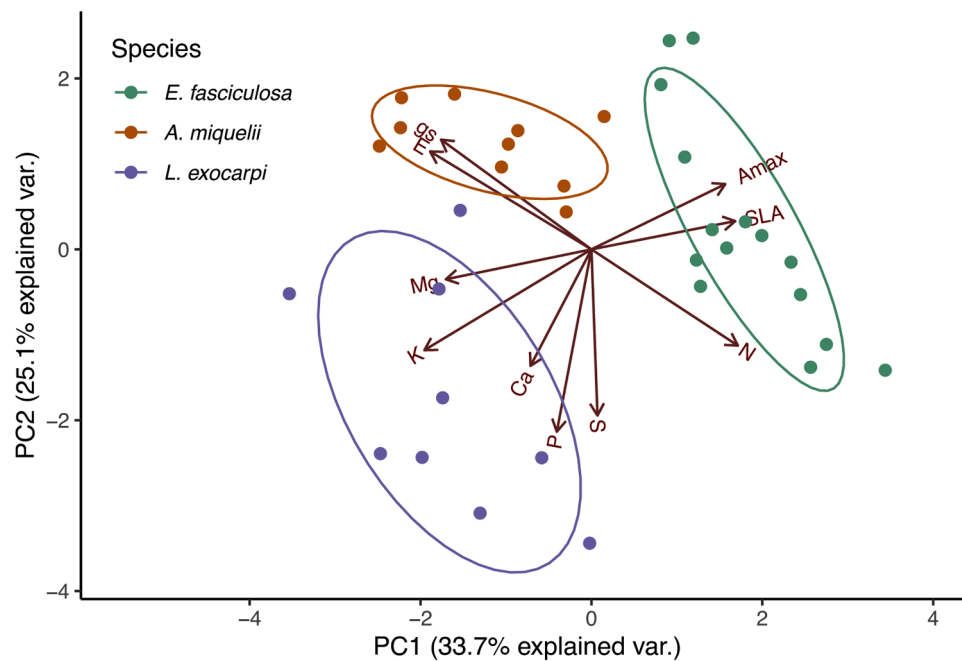


Fig. 4. Principal component analysis (PCA) showing groups formed by the three different species, the primary host *Eucalyptus fasciculosa*, the primary mistletoe *Amyema miquelii* and *Lysiana exocarpi*. Traits used were maximum photosynthetic rate (A_{max}), stomatal conductance (g_s), leaf transpiration rate (E), specific leaf area (SLA) and leaf concentrations of the macronutrients N, P, Ca, K, Mg and S.

explain why “sharing” resources might be less of a problem. Many authors have suggested that different functional groups of mistletoes may have different anatomical and physiological mechanisms to absorb and store different mineral nutrients from the host xylem (Glatzel and Geils, 2009; Mathiasen et al., 2008; Pate, 1995; Shen et al., 2006; Tennakoon et al., 2014). *Lysiana exocarpi*, for yet unknown reasons, can sustain high leaf nutrient concentration. The ecosystem consequences for *L. exocarpi* on nutrient cycling in these low-nutrient habitats also warrant further investigation.

5. Conclusions

In conclusion, our findings suggest that epiparasitism is an alternative option for the harlequin mistletoe *L. exocarpi*, which can survive in the same habitat parasitizing both the primary host, *E. fasciculosa* and the mistletoe *A. miquelii*, by increasing water use and modifying leaf specific area. While we found no evidence of a negative impact of the epiparasite on the mistletoe, there are some evidence that the primary host parasitized with both the mistletoe and the epiparasite suffers more detrimental effects. Knowing that epiparasitic relationships seems to be more common and diverse than once thought (Krasylenko et al., 2021), and some species such as *L. exocarpi* can be facultative epiparasite, the complexity of these relationships is expected to have impacts on many different levels, from the host’s and the primary mistletoe’s fitness to the cascade effect over distinct ecological scales and over different taxa involved (dispersers, herbivores, pollinators, etc.).

CRedit authorship contribution statement

Marina Corrêa Scalon: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Davi Rodrigo Rossatto:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for [Journal name] and was not involved in the editorial review or the decision to publish this article.

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Data availability

Data will be made available on request.

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