The hemiparasitic shrub *Osyris lanceolata* (Santalaceae) does not disturb the ecophysiology of its hosts

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ABSTRACT

Osyris lanceolata is a hemiparasitic dioecious shrub species growing on stabilised dunes. This study aimed to determine the spatial structure of *O. lanceolata* and its hosts, to understand the ecophysiological mechanisms involved in water and nutrient transfer, and quantify the physiological stress on its hosts. In the Asperillo stabilised dunes (Doñana, Spain) we selected one mixed plot (50 m × 50 m) with *Pinus pinea* and *Juniperus phoenicea* in which the coordinates of every individual, the hosts and the hemiparasitic species, were recorded. Additionally, we selected two study areas in which *O. lanceolata* was well represented, one dominated by *P. pinea* and the other by *J. phoenicea*. We marked 60 plants: 20 *O. lanceolata* with their respective hosts (10 *Pinus* and 10 *Juniperus*) and 20 free host plants. In all the marked plants we measured: leaf water potential, gas exchange, photochemical efficiency, morpho-anatomical leaf traits, pigments, and proline content.

Our results evidenced that *O. lanceolata* is spatially associated with its hosts. Midday leaf water potential values were always between 1.5 to 3 times more negative and transpiration rates were 6-fold higher for the hemiparasite than for the hosts. Additionally, *O. lanceolata* plants showed a high accumulation of proline in leaves and haustorium. Although the hemiparasitic species maintains an active photosynthetic canopy, its rates of CO_2 assimilation were 35–48% lower than in the hosts, which caused a 10-fold lower instantaneous water use efficiency. Through these mechanisms, the hemiparasitic plant can absorb water and nutrients from the host species. Despite this parasitic relationship, there were no significant differences in the physiological performance of the hosts in comparison to the parasite-free plants.

We conclude that *O. lanceolata* presents a specific strategy to absorb water in this dry ecosystem, without exerting negative effects on the plant community, which might indicate that there is an equilibrium in the tradeoffs between parasitism and mutualism in the interaction between species.

1. Introduction

Mediterranean landscapes are formed by patches of plant species that present different strategies to face the drought conditions of the summer season (Zunzunegui et al., 2011). Species can tolerate, avoid, or escape the absence of water associated with the elevated temperatures of summer, but there are a group of species can steal water from a host through a parasitic relationship.

Several plants can directly parasitize the vascular system of other plants via specialized transfer organs called haustorium, which allows an unidirectional connection between the vascular system of the host and the parasite. Holoparasites attach to the phloem and /or xylem while hemiparasites attach to the xylem of host plants to extract water and nutrients maintaining an autotrophic metabolism (Tĕsitel, 2016;

Svĕtlíková et al., 2018). There are two types of hemiparasitic plants, stem hemiparasites that grow as epiphytic plants and root hemiparasites which attach host roots and their above-ground canopy is usually similar to that of non-parasitic plants with which they can compete for light (Tĕsitel et al., 2015). However, there are recent pieces of evidence that hemiparasites can also acquire organic compounds from the host, partly presenting a heterotrophic metabolism (Tĕsitel et al., 2010; Mostaghimi et al., 2021). Nonetheless, hemiparasitic plants only have availability to xylem-mobile organic compounds, such as amino acids (Irving and Cameron, 2009), because they only attach the host xylem and not the phloem.

It has been proposed that root hemiparasites tend to establish and grow better in nutrient-poor, low-productivity environments because they benefit from obtaining nutrients through a passive and low-cost

system, and the competition for light with the host should be lower than in nutrient-rich environments (Dueholm et al., 2017). As a result of this interaction, hemiparasites have generally higher concentrations of nutrients in their leaves than the other species of the community, which implies high-quality litter compared with surrounding species (Quested et al., 2003), therefore they might perform a facilitation effect on the nutrient cycling in poor soils (Tĕšitel et al., 2015, 2021), increasing productivity and diversity of the community in these nutrient-poor ecosystems (Griebel et al., 2017).

Several authors have described that, in natural populations, the growth of hemiparasites may be enhanced by using multiple host species simultaneously (Marvier, 1998; Matthies, 1998). Therefore, they can function as a network connecting different individuals and species within the community (Tĕšitel et al., 2021), but it is difficult to study this underground network in natural conditions. In the field, the direct examination of host root–hemiparasite association is only possible at sites with sandy soils and low vegetation cover; in other conditions, it is necessary to employ indirect techniques.

Therefore, hemiparasite species may perform an important role in the structure of plant communities. To study this role, different authors have proposed that patterns of spatial aggregation of species can be a good indicator to infer the predominance of interspecific interactions in natural conditions (Santoro et al., 2012). For this reason, in the community assembly process of a dune community, in which abiotic and biotic filters act simultaneously, the analysis of species spatial patterns can help to understand the role of different species interactions in which hemiparasites may exert an important function, especially at a local scale.

The physiological mechanisms which control the transfer of solutes between the host and the hemiparasites might vary between annual and perennial hemiparasite species. It is accepted that in annual hemiparasites this transfer occurs through a water potential gradient between the host and the parasite, which is driven by an elevated transpiration rate and the accumulation of osmolytes (Tĕšitel et al., 2010). In perennial hemiparasites, despite presenting more conservative mechanisms, there are important differences among species. For instance, in the hemiparasite *Santalum acuminatum,* the transpiration and photosynthetic rates are lower and water use efficiencies are very similar to the values of its main host *Acacia rostellifera* (Tennakoon et al., 1997a). On the other hand, the hemiparasite species *S. album* exhibits higher transpiration rates and lower water-use efficiency than its hosts (Lu et al., 2014). The explanation of these differences might be focused on the anatomical connections between the haustorium and the host vessels; in some species, there is a vascular continuity while in others this continuity does not occur, instead, there is an interfacial parenchyma at the host–parasite interface (Tennakoon and Cameron, 2006).

Therefore, it would be important to understand the physiological mechanism underlying the transfer of water and nutrients in perennial hemiparasitic species. Particularly, when this kind of species grows on sandy soil, poor in nutrients, subjected to elevated temperatures and severe summer drought, such is the case for *Osyris lanceolata* on the stabilised dunes of Doñana. Furthermore, it might help to understand the role of this species in the complex relationships of parasitism versus facilitation that conform the coexistence of plant communities on the stabilised dunes.

Osyris lanceolata (Santalaceae) is a xylem-tapping root hemiparasitic, dioecious shrub or small tree species, which develops the haustorium in order to parasite different perennial evergreen and summer-deciduous species, such as *Juniperus phoenicea* and *Pinus pinea* (Herrera, 1984, 1988a, 1988b). We hypothesized that (1) *O. lanceolata* should be spatially associated with its hosts to facilitate root connections through the haustorium; (2) it should present a passive mechanism to control the sap extraction from its hosts through an elevated transpiration rate; (3) it should present net photosynthetic rate lower than the hosts; 4) it should produce moderate negative ecophysiological effects on the hosts, especially associated with water economy during the summer season in which water scarcity is an extremely negative factor.

In order to test these hypotheses, we studied the spatial pattern and the ecophysiology of *O. lanceolata* and two perennial host species, *J. phoenicea* and *P. pinea* (selecting infected and uninfected hosts).

2. Material and methods

2.1. Study site and species description

The present study was carried out in the stabilised sands of the coastal dune system of El Asperillo (37° 04′ N, 6° 41′ W), located at Doñana Natural Park (South-Western Spain). The climate is Mediterranean with an oceanic influence and mild temperatures (García Novo et al., 1996). The mean annual temperature is 16.8 °C, average maximum temperatures occur in July and August (33.5 °C), while January is the coldest month with an average minimum temperature of 6.9 °C. The average annual rainfall is 550 mm, although it is subject to high inter-annual variability, ranging from 170 to 1028 mm during the last 25 years (Fedriani and Delibes, 2009). Approximately 80% of the annual precipitation is concentrated from October to March (Zunzunegui et al., 2011). Annual rainfall was above the average in the two study years, 784 and 713 mm for the annual cycles 2009–2010 and 2010–2011, respectively.

The vegetation of the area is dominated by large plantations of P. pinea, several species of scrub, such as Corema album, Halimium halimifolium, H. calycinum, Stauracanthus genistoides, Cytisus grandiflorus, Osyris lanceolata and isolated patches of Juniperus phoenicea (Díaz-Barradas and Múñoz-Reinoso, 1992; Díaz-Barradas and Gallego-Fernández, 1996).

Osyris lanceolata Hochst. & Steud. (African Sandalwood) is an evergreen, dioecious hemiparasite that belongs to the family Santalaceae. Its biogeographical area extends over coastal areas in the Mediterranean basin, Macaronesia, and North African regions (Herrera, 1988c).

It is a highly branched, evergreen shrub or small tree, 2–3 m high. The leaves are alternate and coriaceous, about 13–50 mm long, sharp-pointed with a light blue-green color, and often covered with a waxy bloom. The fruits are fleshy, with an orange color when they are ripe (Valdés et al., 1987).

Pinus pinea L. and *Juniperus phoenicea* L. are two coniferous species well established in the Iberian Peninsula. They are widely distributed in the study area and, in particular, *P. pinea* exhibits an elevated cover thanks to afforestation activities during the last century, while *J. phoenicea* is restricted to small, isolated, difficult to access patches and represents the original vegetation of the area before afforestation (Díaz-Barradas and Múñoz-Reinoso, 1992). In other areas of Doñana National Park, *J. phoenicea* is the main host of *O. lanceolata* (Herrera, 1988); in the study area, based on our field survey, *P. pinea* and *J. phoenicea* appeared to be the main hosts of *O. lanceolata*.

2.2. Experimental design: spatial distribution

In the field, we marked a 50 $m \times$ 50 m sampling plot in a mixed area with *Pinus pinea* plantations and *Juniperus phoenicea*, in which *O. lanceolata* was well represented. Other species in the understory were *Cytisus grandiflorus* and *Corema album*, but they were present with low vegetation cover.

In order to facilitate the recording of plant position, we divided the plot into four 25 $m \times$ 25 m subplots and the XY coordinates of each plant were recorded with a measuring tape in relation to the sampling plot. In addition, each plant's projection area was calculated to measure the maximum and minimum orthogonal diameters of the crown projection, approaching the surface of an ellipse. With these data, we could calculate the total cover by species (Zunzunegui et al., 2010).

2.3. Experimental design: field and laboratory ecophysiological measurements

For the ecophysiological measurements, we selected two $50\,m \times 50$ m plots, one dominated by *Pinus pinea* and the other by *Juniperus phoenicea* (between 25 and 30% of the basal area dominated by these species). As the vegetation of the dunes is homogeneous, we considered that these plots were representative of each community.

In each of the study plots, we haphazardly selected 10 adult individuals of *O. lanceolata* and their respective hosts, by direct observation of the root system. We also selected 10 *P. pinea* and 10 *J. phoenicea* neighbor-free plants (no *O. lanceolata* individuals at least in a radius of 3 m) to have control of the host response. In total 60 plants were measured, 30 per study plot (in the *Pinus* plot: 10 uninfected *P. pinea*, 10 infected *P. pinea,* and 10 *O. lanceolata*; in the *Juniperus* plot: 10 uninfected *J. phoenicea;* 10 infected *J. phoenicea* and 10 *O. lanceolata*). Measurements of water potential, chlorophyll fluorescence, and morpho-anatomical leaf traits were carried out in spring and summer, the first corresponding to the optimal growing season when temperatures are mild and water is available, and the second to the stress season when temperatures are elevated, and water is scarce. Measurements of gas exchange, stomatal density, and chlorophyll and proline content were only taken in summer. In spring, temperatures oscillated between 20 and 25 °C, with relative humidity between 50 and 70% and VPD between 0.5 and 0.6 KPa, while in summer temperatures increased to 30–32 °C, relative humidity ranged between 38 and 40% and VPD between 1.8 and 2 KPa. The concentration of CO_2 ranged between 370 and 390 µl l^{-1}, independent of the season.

The leaf water potential was measured at midday between 11:30 and 13:30 h solar time in every marked plant using terminal shoots, one shoot per plant. Cut shoots were immediately inserted into a Scholander-type pressure chamber (Manofrígido, Portugal) and pressure was applied until sap was observed to flow from the cut end.

In situ, measurements of gas exchange were made in three fully-developed, sun-exposed leaves of every marked plant using a portable open-system gas-exchange analyser (LCi-portable photosynthesis, ADC, UK), which measures net photosynthetic rate (A_n, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and leaf intercellular CO_2 concentration (C_i, µmol mol^{-1}). Instantaneous water use efficiency was calculated as A_n/E (WUE, µmol $mmol^{-1}$). Measurements were made between 9:30 and 11:30 h (solar time) under saturating light conditions (PPFD between 1500 and 1800 µmol m^{-2} s^{-1}), to avoid stomatal depression.

The leaves used for gas exchange measurement were maintained in a portable fridge and digitalized in the laboratory. Leaf surface was calculated with the program Midebmdp (Ordiales, Spain, 2000). Field data of gas exchange were recalculated per leaf area.

Midday chlorophyll fluorescence kinetics were recorded in the field on healthy fully expanded leaves of all study plants using a pulse-amplitude portable fluorometer (mini-PAM, Walz, Effeltrich, Germany). For dark-adapted measurements we used dark leaf clips and for light measurements we used a leaf-clip holder 2030-B (Walz) which provides measurements of leaf temperature and photosynthetically active radiation at sample level. Three leaves were measured per plant at 9:30–11:30 h solar time (mean values per plant were used for statistical analysis).

To measure the maximum photochemical efficiency of PSII (F_v/F_m), leaves were maintained for 20 min in darkness using dark leaf clips, this period is considered appropriate to allow the total reoxidation of all the reaction centres of photosystem II. We calculated the ratio of variable fluorescence to maximum fluorescence as, $F_v/F_m = (F_m - F_0)/F_m$, where F_0 = initial and F_m = maximal fluorescence. Effective photochemical efficiency of PSII (Φ_{PSII}) was estimated on light-adapted leaves as $\Phi_{PSII} = (F'_m - F)/F'_m$, where F'_m = maximal and F steady-state fluorescence under actinic irradiance (Genty et al., 1989; Maxwell and Johnson, 2000).

Following the same authors, the electron transport rate (ETR) was calculated with the following equation:

$$ETR = \Phi_{PSII} \cdot PFDa \cdot 0.5 \cdot \left(\mu mol\,electrons\,m^{-2}\,s^{-1} \right).$$

Where PFDa is absorbed light (measured with the leaf-clip holder), and 0.5 is a factor that represents the portioning of energy between PSII and PSI.

For the analysis of morpho-anatomical leaf traits, the same leaves scanned for photosynthesis measurements were dried at 70 °C for 48 h and weighed to calculate leaf mass per area (LMA) as the ratio of dry leaf mass (M_d) to fresh leaf area (g m^{-2}). In the field, we collected a new sample of fully expanded, healthy leaves from all study plants. These leaves were used to measure relative water content (RWC) and leaf dry matter content (LDMC). They were kept in plastic bags and stored refrigerated until we arrived at the laboratory, where fresh mass (M_f) was recorded within 3 h. These leaves were then hydrated with distilled water for saturation and maintained at 5 °C. The following day, leaves were weighed to obtain saturated mass (M_s). Finally, leaf samples were dried at 70 °C for 48 h and weighed (M_d). The ratio LDMC (mg g^{-1}), known as tissue density, was calculated as M_d/M_s. We estimated RWC (%) as $(M_f - M_d) \times 100/(M_s - M_d)$.

Healthy fully-developed leaves of *O. lanceolata* ($n = 36$, 18 from plants parasitizing *P. pinea* trees + 18 from plants parasitising *J. phoenicea* trees) were haphazardly collected in the field in spring to examine stomatal density. We used transparent nail polish to obtain stomatal impressions of the adaxial and abaxial leaf sides, once dry they were observed on a glass slide by a Leica Microscope. The number of stomata was recorded in two microscopic fields on both sides of the leaf, using a 400× magnification. Stomatal density (number of stomata per mm^2) was calculated as the ratio between the number of stomata and the area of the microscopic field, which was 0.1046 mm^2.

Terminal shoots of every marked plant were frozen in liquid nitrogen in the field and transported to the laboratory, where they were kept at −22 °C until analysis of photosynthetic pigments and proline content.

Chlorophyll a (Chl a) and b (Chl b) pigments were extracted with 100% acetone and determined spectrophotometrically on a dry mass basis following Ain-Lhout et al. (2004).

Proline concentration was estimated colorimetrically using the acid-ninhydrin method (Bates et al., 1973) with samples of leaves and the haustorium of *O. lanceolata* and the host species in the two associations. We used L-proline (Pancreac) for the standard curve.

2.4. Data analysis

The spatial pattern of the trees was analysed using Ripley's K-function (Ripley, 1981; Haase, 1995). This function enables to characterize a point pattern at several r distances based on the number of neighbouring trees at distances $\leq r$ from each tree. Ripley's K-function counts the number of points of the observed plants within concentric circles of radius r and compares them with the expected number of plants within a distance r. For a given point pattern, K-function is computed as:

$$K(r) = n_r/\rho$$

Where n_r is the mean number of neighbouring trees lying within the distance r from a tree and ρ is set equal to N/A, with N being the number of trees and A the area. For a completely random process of the same value of ρ, $K(r) = \pi r^2$. Thus, for an aggregated process, where the number of neighbours tends to be greater than the random process, $K(r) > \pi r^2$. For a regular pattern, the number of neighbours tends to be lower than for a random process, and then $K(r) < \pi r^2$.

The L-function proposed by Besag (1977) is a squared transformation of the original K-function by Ripley (1976) to linearize and stabilize the variance of K. To test H_0 (random point pattern) versus H_1 (regular or aggregated point pattern), 99 simulations of a Poisson random model were used to generate a confidence interval of L-functions. Observed

values of L-function over the confidence interval of a random distribution indicate aggregation, while values under the confidence interval indicate a regular distribution. If values are inside the confidence interval, there is a random distribution. All point pattern analyses were carried out using Programita software developed by Wiegand and Moloney (2004).

The normality of all measured variables was first checked with Kolmogorov–Smirnov test. For the variables measured in spring and summer, the temporal differences of each trait for each species (*Osyris* and infected and uninfected individuals of the two host species were considered as different species for the analysis) were analysed by repeated-measures ANOVA (Potvin et al., 1990). In this analysis, the within-subject factor was season (with two categories) and the between-subject factor was species (for the *Pinus* and *Juniperus* plots). In these analyses, the replicates were the individuals. In the cases where Mauchly's sphericity was not assumed, we applied Huynh–Feldt's correction. In order to compare differences between species in one single date or temporal differences for species and variables we used one-way ANOVA. Pair-wise posterior comparison was based on the Tukey procedure. The statistical tests were considered significant at the $P < 0.05$ level. All statistical tests were made with the software package IBM SPSS Statistics for Windows, Version 24 (Armonk, NY, USA).

3. Results

3.1. Spatial distribution

The study plot was dominated by *P. pinea* (18.9% cover), followed by *O. lanceolata* (7.5%), *C. grandiflorus* (1.4%), and *C. album* (1.1%). *Juniperus phoenicea* had a small cover in the plot, only 0.5%.

The O-ring analysis confirmed that there was a significant aggregation between *P. pinea* and *O. lanceolata* only in the first 2.5 m, and a random pattern in the following meters (Fig. 1). *Juniperus phoenicea* showed a random pattern in relation to *O. lanceolata* (Fig. 1).

3.2. Water potential

The water potential of the study plants followed the seasonality of the Mediterranean climate, with minimum values during the summer season. However, the mean midday water potential of *O. lanceolata* plants was always two or three times more negative than the respective water potential of their host plant at any time of the year, being the seasonal pattern very similar in both host species (Fig. 2).

For each population, within-subject effects reveal that there were significant temporal differences, and the interaction time × species was also significant; on the other hand, between-subjects effects also present differences among species as it is shown in the repeated-measures ANOVA (Table 1).

Tukey pairwise comparisons (for each month and population) revealed that there were significant differences between the leaf water potential of *O. lanceolata* and the host species, both in the case of *P. pinea* and *J. phoenicea* ($P < 0.01$), but there were no significant differences in midday water potential of infected and uninfected plants for both host species in any study month ($P > 0.05$).

The ratio between the midday water potential of *O. lanceolata* and its hosts ranged between 2.0 to 3.2 MPa/MPa in the case of *Osyris/P. pinea*,
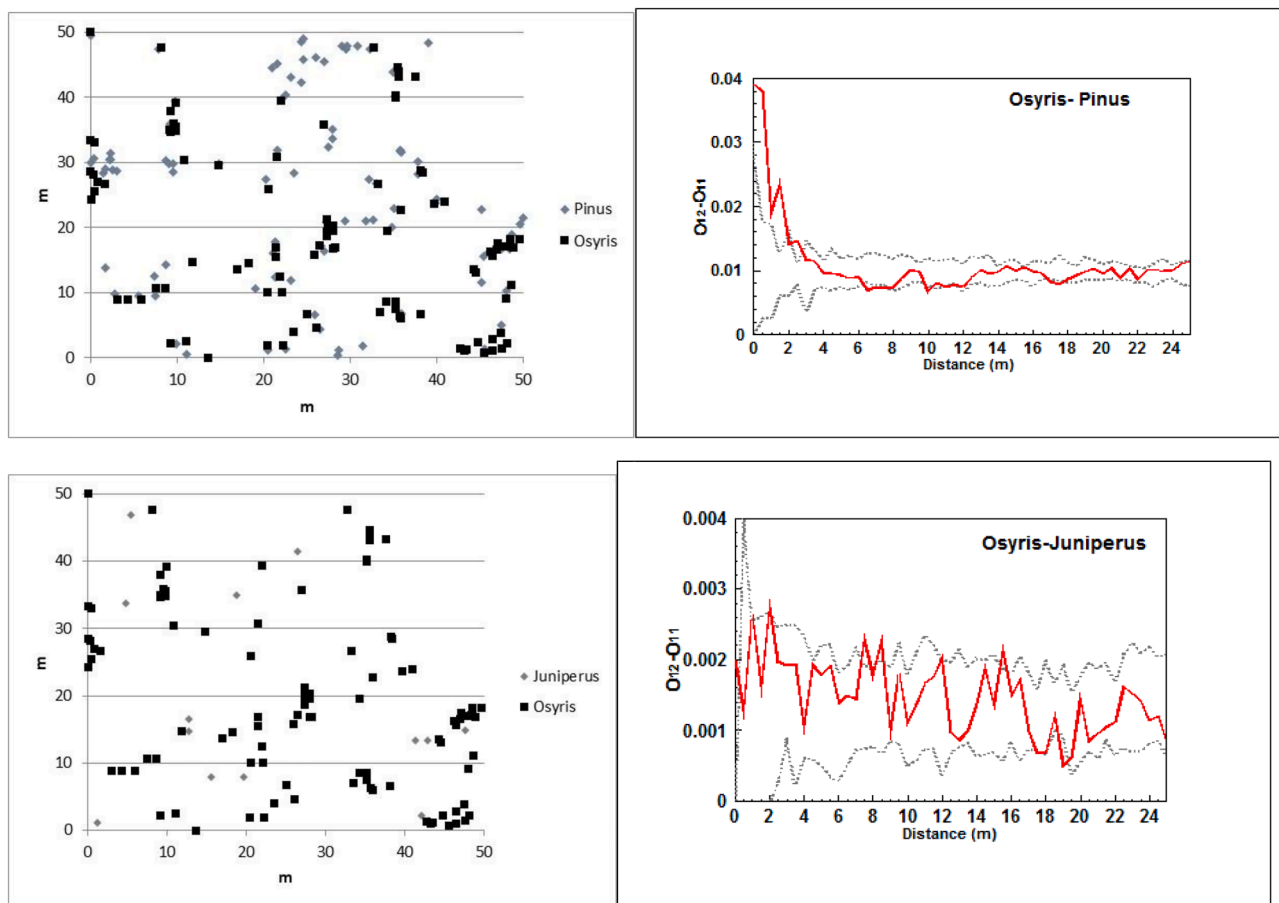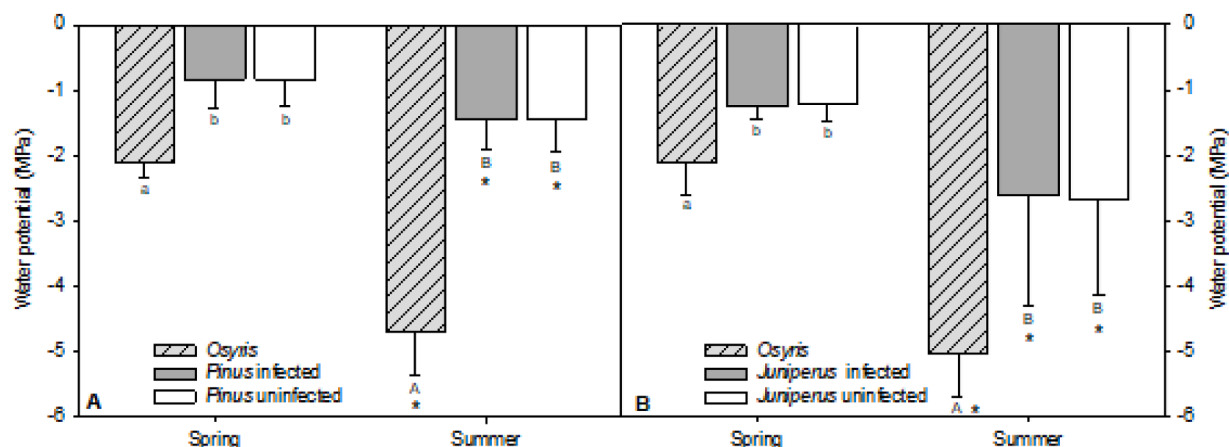

Fig. 1. Spatial distribution of *Osyris lanceolata, Pinus pinea,* and *Juniperus phoenicea* trees in the 50 *m* × 50 m study plot (left) and results of the spatial aggregation analysis (red line) using O-ring for *O. lanceolata* with *P. pinea* and *O. lanceolata* with *J. phoenicea* (right). The dotted lines indicate the confidence interval for a random distribution at any distance.

Fig. 2. Midday leaf water potential (average $\pm$ SE, $N = 10$) of *Osyris lanceolata* and the host species (infected and uninfected with the hemiparasite), measurements of spring and summer. A. Results of the *Pinus pinea* plot. B. Results of the *Juniperus phoenicea* plot. In each plot, lower-case letters show significant differences ($P < 0.05$) among groups in spring and capital letters in summer according to the Tukey test. Asterisks (at the position of summer data) show significant effects of time for each species and plot.

Table 1

Repeated-measures ANOVA on the effects of time and species on water potential in the two study plots. Significant differences at $P < 0.05$ are marked in bold in the tables.

(a) Within-subjects effects of Time $\times$ Species (error df = 27)

(b) Between-subjects effects of Species (error df = 27).

(a)	df	F (Pinus)	P (Pinus)	F (Juniperus)	P (Juniperus)
Time	1	914.56	**<0.001**	703.14	**<0.001**
Time $\times$ Species	2	250.17	**<0.001**	49.00	**<0.001**

(b)	df	F (Pinus)	P (Pinus)	F (Juniperus)	P (Juniperus)
Intersection	1	11,368.35	**<0.001**	3227.88	**<0.001**
Species	2	1785.45	**<0.001**	152.76	**<0.001**

while in the case of *Osyris*/*J. phoenicea* this ratio was always lower ranging between 1.7 to 2.2 MPa/MPa.

3.3. Gas exchange

Photosynthetic assimilation rates (A_n) were 35 to 48% lower in *O. lanceolata* than in any of the host species ($P < 0.01$; Fig. 3). On the other hand, transpiration rates (E) were six-fold higher in *O. lanceolata* than in the host species ($P < 0.001$). Transpiration rates for *O. lanceolata* ranged between 12 and 16 mmol $m^{-2} s^{-1}$ when it was infecting *P. pinea*, and between 10 and 14 mmol $m^{-2} s^{-1}$ when it was infecting *J. phoenicea*, whereas the values of the infected hosts ranged from 2 to 2.5 and from 1.8 to 2.2 mmol $m^{-2} s^{-1}$ respectively. Differences were significant in both sampling areas ($P < 0.01$). Stomatal conductance (g_s) was 12.5% lower ($P < 0.05$) in *O. lanceolata* than in *P. pinea*, but there were no significant differences in the association with *J. phoenicea* (Fig. 3).

Regarding the effect of *O. lanceolata* on the host species, no significant differences were found in gas exchange variables between infected and uninfected individuals, of any of the host species. Although uninfected plants of the host species had higher assimilation rates for a given stomatal conductance than infected plants, differences were not significant. Instantaneous water use efficiency (A_n/E) values were similar for infected and uninfected plants, but both host species have 10-fold higher WUE than the hemiparasitic species.

3.4. Chlorophyll fluorescence

According to repeated measures ANOVA, the measured variables

exhibited significant differences between spring and summer in the two study plots, nonetheless the interaction time $\times$ species was only significant for φ_{PSII} and ETR in the *Pinus* plot, and close to the limit of significance for F_v/F_m and ETR in the *Juniperus* plot. On the other hand, between-subjects effects only reveal significant differences between species for φ_{PSII} and ETR in the *Pinus* plot and F_v/F_m in the *Juniperus* plot (Table 2).

The results of the Tukey test only show a few pairwise significant differences in summer. Infected *J. phoenicea* presents lower values of F_v/F_m than those of *O. lanceolata*, while the uninfected plant exhibited intermediate values, and φ_{PSII} of *O. lanceolata* was significantly lower than infected or uninfected values of *P. pinea*. Finally, the ETR values of *O. lanceolata* were significantly lower than those of *P. pinea* (infected and uninfected) in summer (Fig. 4).

### 3.5. Leaf water content and morpho-anatomical traits

RWC, LDMC and LMA exhibited temporal differences for both plots, except for LDMC in the *Pinus* plot, which was constant throughout the seasons according to repeated measures ANOVA. The interaction time $\times$ species was significant for LDMC and LMA in the *Pinus* plot and LMA in the *Juniperus* plot. Regarding the between-subject effects, there were significant differences among species in LDMC and LMA in the *Pinus* plot and the three variables in the *Juniperus* plot (Table 3).

The values of RWC were always lower in summer than in spring. However, despite having significantly more negative leaf water potential than the host species, the leaves of *O. lanceolata* maintained similar or significantly more elevated values of RWC than infected or uninfected host species, in all the sampling dates (Fig. 5).

In the *Pinus* plot, RWC did not show significant differences among species on any sampling date. Contrarily, in the case of the *Juniperus* plot, the RWC of *O. lanceolata* leaves was higher than *J. phoenicea* leaves (infected and uninfected) in any season (Fig. 5).

The values of LDMC ranged from 300 to 500 mg g^{-1} and from 200 to 400 g m^{-2} for LMA, but we could not follow any specific pattern concerning species or months (Table 3).

Stomata were present in both leaf sides of *O. lanceolata* (ranging from 201 to 286 stomata mm^{-2} in the adaxial surface and from 298 to 326 stomata mm^{-2} in the abaxial), with stomatal density being significantly higher in the abaxial surface ($P < 0.01$). The plants infecting *J. phoenicea* presented higher stomatal density (306.7 ± 39.2 stomata mm^{-2}) than those infecting *P. pinea* (249.9 ± 69.7 stomata mm^{-2}) ($P = 0.04$).
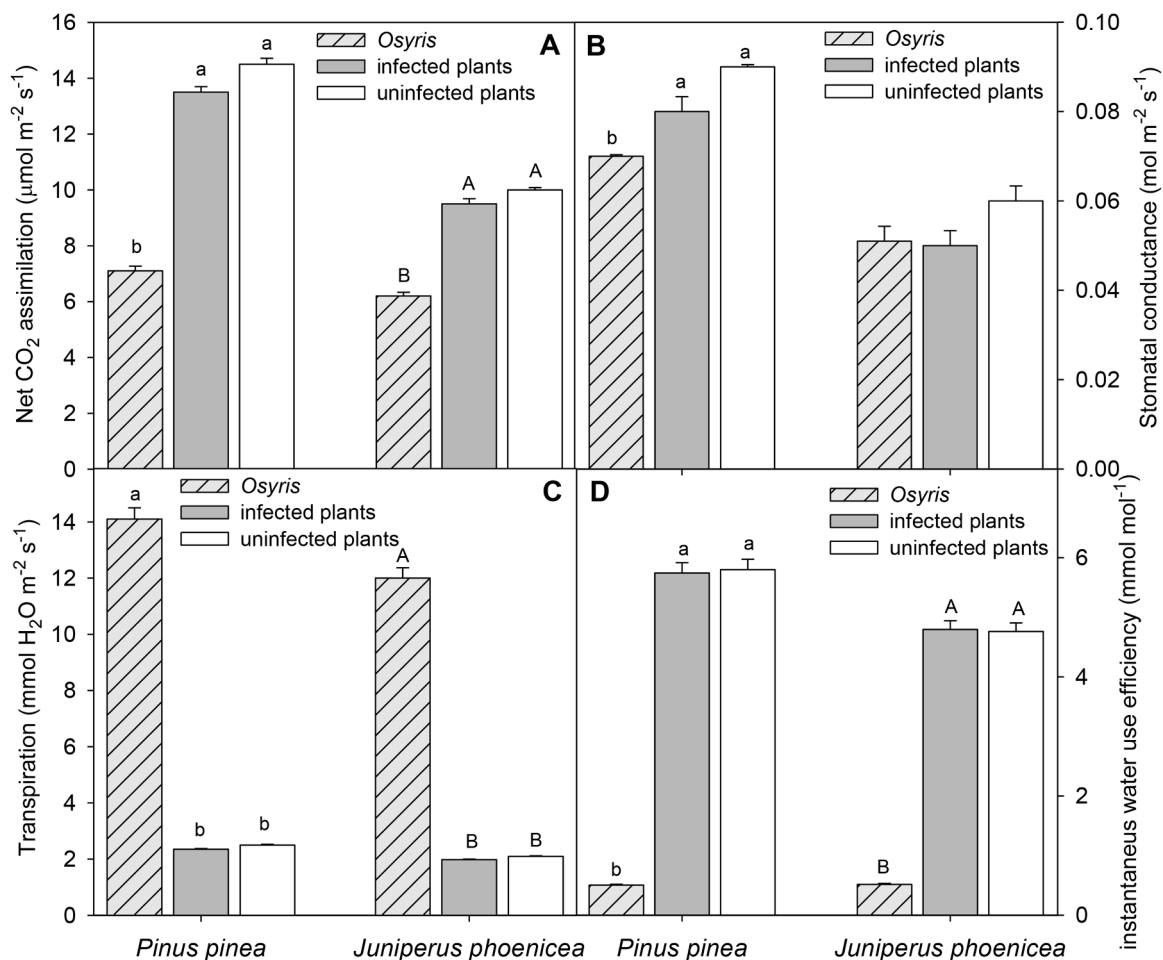
Fig. 3. Gas exchange measurements (average + SE, N = 10) of *Osyris lanceolata* and the host species (infected and uninfected with the hemiparasite), measurements of summer. A. Net CO_2 assimilation rate. B. Stomatal conductance. C. Transpiration rate. D. Instantaneous water use efficiency. Letters show significant differences among groups ($P < 0.05$) for each species association according to the Tukey test. Lowercase letters in the association between *O. lanceolata* and *Pinus pinea* and capital letters in the association between *O. lanceolata* and *Juniperus phoenicea*.

Table 2
Repeated-measures ANOVA on the effects of time and species on different chlorophyll fluorescence variables (F_v/F_m, φ_{PSII}, and ETR) in the two study plots. Significant differences at $P < 0.05$ are marked in bold in the tables.
(a) Within-subjects effects of Time × Species (error df = 27)
(b) Between-subjects effects of Species (error df = 27)

(a)				Pinus plot						Juniperus plot					
	Time				Time × Species			Time				Time × Species		
	df	F	P		df	F	P	df	F	P		df	F	P
F_v/F_m	1	20.68	**<0.001**		2	1.86	0.18	1	91.31	**<0.001**		2	3.23	0.055
φ_{PSII}	1	8.34	**0.008**		2	7.17	**0.003**	1	44.45	**<0.001**		2	2.68	0.09
ETR	1	12.33	**0.002**		2	13.80	**<0.001**	1	21.92	**<0.001**		2	3.20	0.057

(b)				Pinus plot						Juniperus plot					
	Intersection				Species			Intersection				Species		
	df	F	P		df	F	P	df	F	P		df	F	P
F_v/F_m	1	14,308.4	**<0.001**		2	0.59	0.56	1	6567.8	**<0.001**		2	6.08	**0.007**
φ_{PSII}	1	416.8	**<0.001**		2	5.31	**0.011**	1	274.16	**<0.001**		2	1.56	0.229
ETR	1	636.3	**<0.001**		2	8.03	**0.002**	1	388.25	**<0.001**		2	2.07	0.145

3.6. Leaf pigments and proline content

The amount of total chlorophyll (chlorophyll *a* + b*)* was 57 to 68% lower in *O. lanceolata* than in the host species ($P < 0.01$), but there were no significant differences between infected and uninfected plants for both host species (Fig. 6).

Our results support the existence of a 17–19 fold higher accumulation of proline in the haustoria and leaves of *O. lanceolata* compared to plant tissues of infected or uninfected plants of both host species ($P < 0.01$) (Fig. 7).
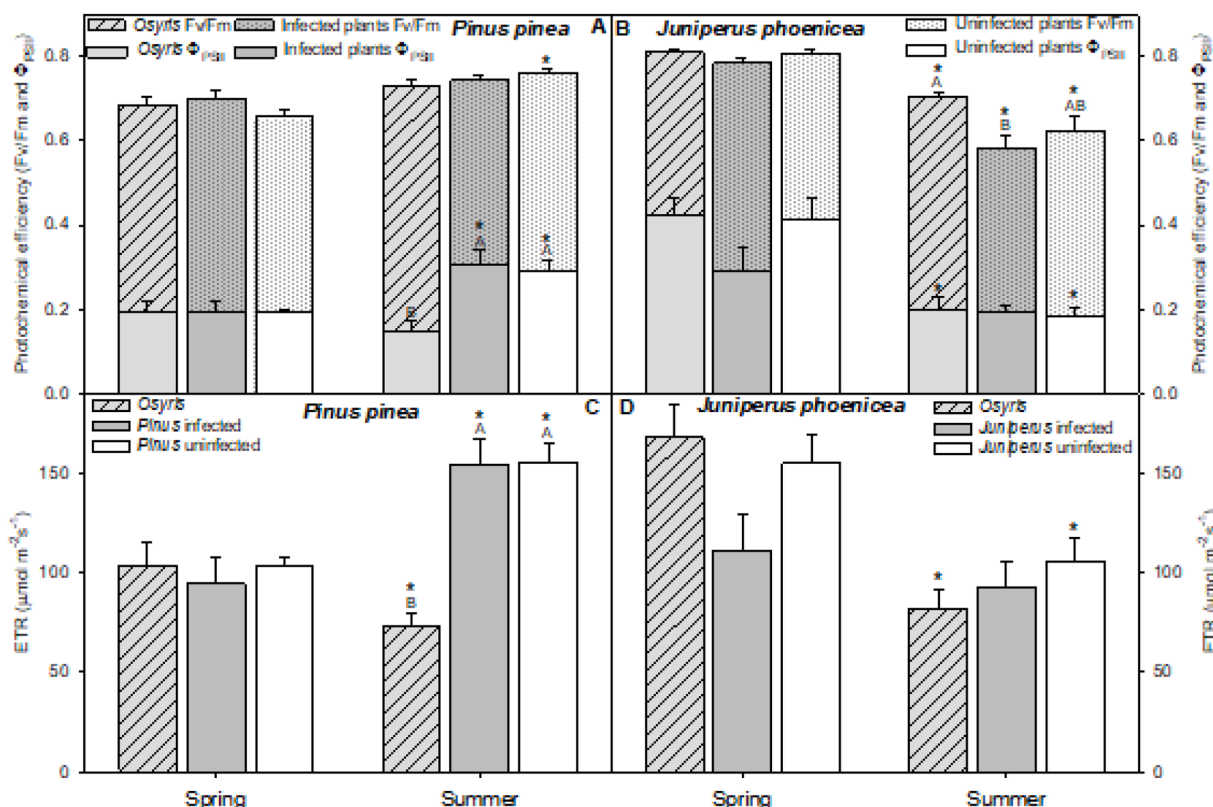
Fig. 4. Maximum quantum yield (F_v/F_m) and effective quantum yield (Φ_{PSII}) for *Osyris lanceolata* and the host species *Pinus pinea* A. and *Juniperus phoenicea* B. (infected and uninfected with the hemiparasite). ETR values for *O. lanceolata* and the host species *P. pinea* C. and *J. phoenicea* D. (infected and uninfected with the hemiparasite). Data are average + SE ($N = 10$). In each plot, capital letters show significant differences among groups in summer ($P < 0.05$) according to the Tukey test. Asterisks (at the position of summer data) show significant effects of time for each species and plot.

Table 3
Repeated-measures ANOVA on the effects of time and species on different leaf trait variables in the two study plots. Significant differences at $P < 0.05$ are marked in bold in the tables.
(a) Within-subjects effect of Time $\times$ Species (error df = 27)
(b) Between-subjects effect of species (error df = 27)

(a)		Pinus plot						Juniperus plot					
		Time			Time $\times$ Species			Time			Time $\times$ Species		
		df	F	P	df	F	P	df	F	P	df	F	P
RWC		1	51.17	**<0.001**	2	2.17	0.091	1	12.93	**<0.001**	2	0.727	0.493
LDMC		1	0.092	0.764	2	3.90	**0.036**	1	33.17	**<0.001**	2	1.06	0.360
LMA		1	250.27	**<0.001**	2	26.26	**<0.001**	1	32.70	**<0.001**	2	3.58	**0.04**

(b)	*Pinus* plot						*Juniperus* plot					
	Intersection			Species			Intersection			Species		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
RWC	1	30,354	**<0.001**	2	2.88	0.073	1	12,064	**<0.001**	2	11.1	**<0.001**
LDMC	1	11,920	**<0.001**	2	3.82	**0.035**	1	14,931	**<0.001**	2	60.6	**<0.001**
LMA	1	3705	**<0.001**	2	15.3	**<0.001**	1	625.3	**<0.001**	2	12.5	**<0.001**

4. Discussion

The results of this study highlight some of the most important ecological and ecophysiological traits in the hemiparasitic relationship between *O. lanceolata* and two of its hosts, *P. pinea* and *J. phoenicea* under field conditions.

In the plot dominated by *Pinus pinea* (18.9% plant cover), the O-ring analysis showed a significant aggregation between *P. pinea* and *O. lanceolata* in the first 2.5 m, and a random pattern in the following meters. In the same way, there was a random pattern with *J. phoenicea*

which presented low cover in this study plot. This result suggests that the *O. lanceolata* species is connected to the host xylem of *P. pinea,* which provides water and mineral nutrients to the hemiparasite. Probably, in other areas in which the cover of *J. phoenicea* is higher, a significant aggregation would appear with *O. lanceolata*. This result seems to support that root hemiparasites should have an optimal zone for growth in relation to their hosts, which means, not too close to find the maximum density of fine roots (Herrera, 1988b) or to avoid competition for light and not too far away, to provide the connection with the host especially in the early stages of the establishment (Dueholm et al., 2017).
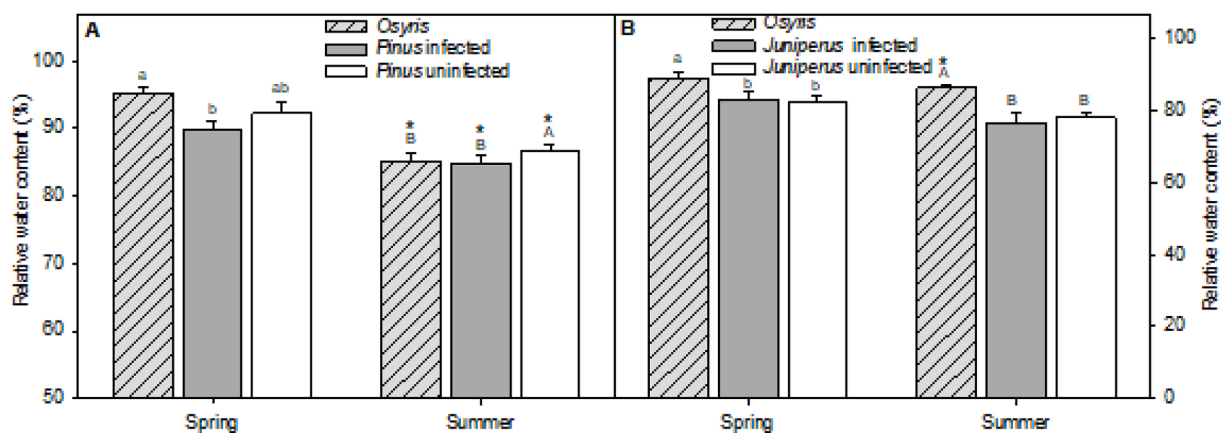
Fig. 5. RWC (%) (average + SE, $N = 10$) of leaves of *Osyris lanceolata*, and the host species (infected and uninfected with the hemiparasite) in the two study plots. In each plot, lower-case letters show significant differences among species in spring and capital letters in summer ($P < 0.05$) according to the Tukey test. Asterisks (at the position of summer data) show significant effects of time for each species and plot.
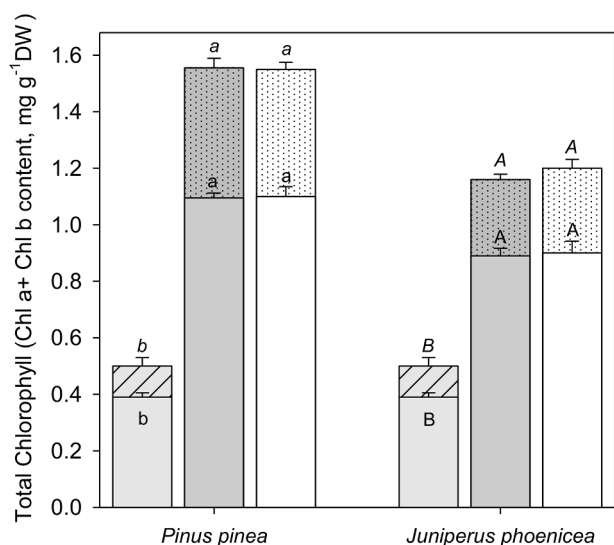

Fig. 6. Chlorophyll *a* (solid) and *b* (with pattern) contents (average + SE, $N = 10$) in leaves of *Osyris lanceolata* and its hosts (*Pinus pinea* and *Juniperus phoenicea*). The letters (in italics for Chlorophyll *b*) show significant differences among groups ($P < 0.05$), for each species association according to Tukey. Lowercase letters in the association between *O. lanceolata* and *P. pinea* and capital letters in the association between *O. lanceolata* and *J. phoenicea*. Measurements of summer.

Fig. 7. Proline content (average + SE, $N = 10$) in leaves and haustorium of *Osyris lanceolata* and leaves of its hosts (infected and uninfected *Pinus pinea* and *Juniperus phoenicea*). The letters show significant differences among groups ($P < 0.05$), for each species association according to the Tukey test. Lowercase letters in the association between *O. lanceolata* and *P. pinea* and capital letters in the association between *O. lanceolata* and *J. phoenicea*. Measurements of summer.

In this study, we have described a complex and distinctive set of physiological traits in *O. lanceolata* to obtain resources from its hosts.

In summary, the hydric extracting behavior of *O. lanceolata* presents the following characteristics, a significantly more negative water potential than its hosts, elevated transpiration rates, a low water use efficiency, and a high stomatal density on both sides of the leaves; all these mechanisms contribute to driving the parasitic resource acquisition from the host xylem. Additionally, we have measured a significant accumulation of proline in the leaves and haustoria of *O. lanceolata* which can play an important role in the regulation of enzyme activities and protection of protein folding, act as an osmoprotectant, having different protection functions against abiotic stresses (Zulfiqar et al., 2020). In the root hemiparasitic shrub *Santalum acuminatum*, proline was recorded in the haustorium and xylem of this species, but it was almost imperceptible in the xylem of most hosts (Tennakoon et al., 1997b). Several Mediterranean scrub species also accumulate proline in summer, which i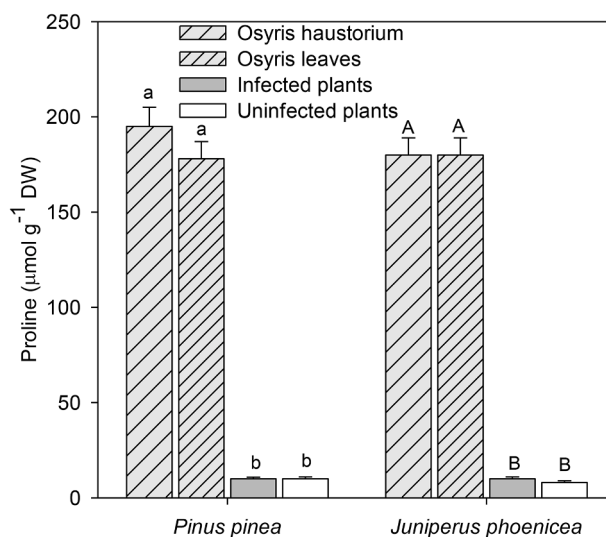s linked to the decrease in water potential and may be associated with a decrease in osmotic potential and a protective mechanism (Ain-Lhout et al., 2001; Zunzunegui et al., 2011).

Although transpiration rates of *O. lanceolata* were always larger than those of the host, these rates were lower on the hemiparasite associated with *J. phoenicea* than in the association with *P. pinea*. This result might explain the recorded differences shown in this manuscript in the water potential gradient between the parasite and its two hosts.

Despite having a direct water flow from the host to the hemiparasite, we did not find any significant differences in midday water potential between infected and uninfected trees either of *P. pinea* or *J. phoenicea*. This finding might be indicative that the effects of parasitism are not affecting the performance of the hosts, although water availability should be a primary driver of species under Mediterranean summer conditions (Zunzunegui et al., 2011). Similar results have been found on the effect of the mistletoe *Passovia ovata* on two different hosts, the evergreen *Miconia albicans* and the deciduous *Byrsonima verbascifolia* (Scalon et al., 2021). The authors have found that infected and uninfected branches exhibited similar midday water potential, but pre-dawn

leaf water potential was significantly more negative for infected branches of both hosts, which suggested limitations in the filling capacity during the night of infected branches.

Nevertheless, these exploitative hemiparasitic mechanisms evidenced in this study by *O. lanceolata* seem to agree more with the general pattern of annual than perennial hemiparasite species, or of a hemiparasitic species growing under conditions of moderate resource availability (Těšitel et al., 2015).

In annual hemiparasites such as *Striga* spp. or *Rhinanthus* spp., it is accepted that the mechanism controlling the transfer of solutes works as a passive mass flow, in which the hemiparasite has to maintain a more negative water potential than the host, through an elevated transpiration rate in the parasite's leaves, with poor stomatal regulation which determines the open stomata all day even under elevated vapor pressure deficit and water deficit stress conditions. All this is linked to the accumulation of osmoprotectants compounds (Zulfiqar et al., 2020), such as sugar alcohols, especially mannitol (Jiang et al., 2005), or free amino acids such as proline (Zunzunegui et al., 2011).

In contrast, in perennial hemiparasite species, the transfer of solutes occurs through different mechanisms. In some species, the transpiration rates of the hemiparasite species are similar to those of the hosts (Tennakoon et al., 1997a) and the transfer of solutes flows across cell walls or contact parenchyma (Těšitel et al., 2010). In these species, the gradient of water potential between the host and the hemiparasite is maintained exclusively by an increased concentration of osmotically active compounds (Těšitel et al., 2015). Nonetheless, in a study of the hemiparasitic relationships of *Santalum album* with N_2 fixing and no fixing hosts, the authors have shown that independent of the host species, *S. album* always exhibited higher transpiration and lower water-use efficiency than its host, as a mechanism to improve xylem sap extraction from the hosts, while photosynthetic activity depended on the host-type (Lu et al., 2014). All these pieces of evidence support the idea that the physiological mechanisms and anatomical connections of perennial hemiparasites and their hosts are diverse and complex, and following our results, the haustorium of *O. lanceolata* seems to have a vascular continuity with the xylem of its hosts.

Moreover, many root-hemiparasitic species display inefficient photosynthesis and high respiratory rates (Těšitel et al., 2010). The results of this study confirm this trend, *O. lanceolata* exhibited lower photosynthetic rates, and chlorophyll content than any of the host species and lower electron transport rates when parasitizing *P. pinea* in summer, despite no light limitations in most of the cases. This result is according to the literature, for example, mistletoes are able to perform photosynthesis, but the amount of carbon they obtain via an autotrophic metabolism is usually below non-parasitic plants, as they exhibit low mean CO_2 assimilation rates, low effective quantum yield, low light saturation point and low chlorophyll a/b ratio despite experiencing favourable light condition (Těšitel et al., 2011; Mostaghimi et al., 2021).

Hemiparasitic plants might compensate for their lower photosynthetic rate by acquiring some organic compounds from their hosts (Těšitel et al., 2011). The proportion of carbon heterotrophy in hemiparasitic species is variable and it has been quantified using stable isotopes (Těšitel et al., 2010; Světlíková et al., 2018; Mostaghimi et al., 2021).

Hemiparasites are considered plagues, species that usually cause negative effects on host photosynthesis and plant growth (Watling and Press, 2001). The results of Scalon et al. (2021) partly support this idea, especially during the wet season in which the mistletoe is using water and nutrients that could reach the hosts's leaves, but it is remarkable to see a different behavior between infected and uninfected branches of the same individual. However, in the present study, for the studied variables, we did not observe negative physiological effects on the host plants, neither host photosynthesis of infected plants nor maximum or effective quantum yield were significantly affected when compared with neighbouring uninfected plants.

In most of the studies, RWC is a good indicator of the hydric

conditions of the plant, and it is often correlated with leaf water potential. Our results evidence that *O. lanceolata* maintains an elevated RWC all the year, through the exploitation of the xylem of the host which implies a low leaf water potential all the year.

The benefits of this exploitative root-hemiparasitic lifestyle seem a mechanism to obtain resources in this poor and dry ecosystem of the dunes, in which plant performance is limited by low availability of water and nutrients and primary production is relatively low. However, our results point out that this hemiparasitic relationship does not significantly affect the water economy and carbon assimilation of the host species.

At the community level, many parasitic plants may parasitize multiple hosts simultaneously, creating an underground network which might have important consequences for the structure and function of the community (Phoeniix and Press, 2005; Těšitel et al., 2021). However, in our study, the results of the spatial analysis seem to evidence that *O. lanceolata* only parasites one host, or neighbouring hosts. Furthermore, previous studies have shown that the roots of *O. lanceolata* and those of its associate host had extensive areas of contact and separate shrubs must be considered independent plants (Herrera, 1988b). We suggest two non-mutual exclusive hypotheses for this spatial distribution, in the first place the germination of *O. lanceolata* is linked to dispersal by birds which feed on the fruits and perch on the trees, on the other hand, the low vegetation cover of the area, and the low water availability might render the exploitation of different hosts difficult.

The impact of hemiparasitic plants in the community is complex and they might exert even a positive effect on the community (Těšitel et al., 2021). For example, the nutrient-rich litter of hemiparasitic plants decomposes quickly (Fisher et al., 2013), which might in some cases act in a positive way enhancing nutrient cycling in communities. We have recorded that the litter of *O. lanceolata* presents higher nitrogen content than surrounding species and facilitates decomposition in multispecies litter bags (non-published results). Other studies have pointed out that hemiparasites can have a structural role on plants in local communities, acting as centres for the establishment and growth of colonizing fleshy-fruited woody species (Mellado and Zamora, 2017). In conclusion, we can assume that the dark side of a parasitic relationship might in some cases present a facilitation or mutualistic effect in the maintenance of plant communities on the dry and poor stabilised sands of Doñana.

Overall, the combined study of plant physiology and spatial distribution indicates that *O. lanceolata* presents a complex set of physiological strategies to obtain water and nutrients from its hosts without exerting negative effects on them. On the other hand, it may also present some positive relations in the community through different facilitation processes. Therefore, our results might indicate there is an equilibrium in the tradeoffs between parasitism and mutualism in the interaction between these species and *O. lanceolata* might have an important role in the biotic filters that shape plant community assembly, especially at a local scale.

Declaration of Competing Interest

None.

Data availability

The data that support the findings of this study are available from the corresponding Author MC Diaz Barradas, upon reasonable request.

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