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## Mutual positive effects between shrubs in an arid ecosystem

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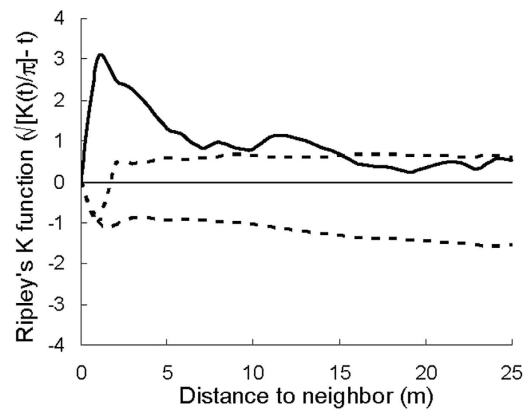
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One-way facilitation in plants has been found in many harsh environments and their role as structural forces governing species composition in plant communities is now well established. However, reciprocal positive effects benefiting two interacting species have seldom been reported and, in recent reviews, conceptually considered merely as facilitation when in fact there is room for adaptive strategies and evolutionary responses. We tested the existence of such reciprocal positive effects in an arid environment in SE Spain using spatial pattern analysis, a species removal experiment, and a natural experiment. We found that the spatial association between *Maytenus senegalensis* and *Whitania frutescens*, two shrub species of roughly similar size intimately interacting in our community, resulted in mutual benefit for both species. Benefits included improved water relations and nutritional status and protection against browsing, and did occur despite simultaneous competition for resources. Our data suggest two-way facilitation or, rather, a facultative mutualism among higher plant species, a process often overlooked which could be a main driver of plant community dynamics allowing for evolutionary processes.

Positive interactions among plants have frequently been reported in the last 20 years<sup>1,2</sup> and many examples are now documented in very different communities and climates, from deserts to arctic environments often involving partners of different size, growth form, or functional type<sup>3–5</sup>. Plants are able to alter environmental conditions and their interactions usually encompass a variety of effects<sup>6,7</sup>. For instance, plants buffer climate extremes by shading<sup>4</sup>, can increase soil water available to neighbors through hydraulic lift<sup>8,9</sup>, increase soil nutrients beneath the canopy<sup>3,10</sup>, or protect from herbivory<sup>11–13</sup>. Such positive effects occur despite competition for resources<sup>14</sup> and are context-dependent<sup>15–17</sup>. The same pair of species can be mutualists, commensals, or even parasites under different conditions<sup>18</sup>, the “positive” term in the interaction being just one end of a continuum of possible outcomes<sup>6</sup> that could stretch to mutualism. Although interspecific mutualism represents one of the most important and widely addressed interactions in ecology<sup>18–21</sup>, plant-plant mutualistic interactions have been guessed<sup>18</sup> but seldom reported. There is, however, some evidence pointing to such interactions. Pugnaire *et al.*<sup>10</sup> evidenced a mutualistic interaction between *Retama sphaerocarpa* shrubs and *Marrubium vulgare*, a smaller shrub growing in its understory. When growing together, both shrubs had higher productivity, more leaf N, and overall better physiological status than growing isolated. Holzapfel and Mahal<sup>22</sup> also found in the Mojave Desert that the community of annual species growing under *Ambrosia dumosa* shrubs had positive effects on the shrub water status, growth, and reproductive output while annual species simultaneously benefited from shrub presence improving survival, biomass production, and seed production. However, despite such empirical evidence, mutually positive interactions between higher plant species have not attracted much attention. Several reasons explain why they may be underreported. One is that few mutualisms involve species occupying the same trophic level<sup>23</sup>, a point particularly hard to assume in sessile organisms such as plants sharing similar resources. Mutualism is generally found between different trophic levels (plant-insects, animal-bacteria, algae-fungi) and even at the intraspecific level (e.g., social organisms)

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**Figure 1.** Second-order spatial analysis of the distribution pattern of *Maytenus senegalensis* and *Whitania frutescens* in an ‘artinera’ shrubland in El Ejido (Almería, Spain). The plot of the derived statistics of Ripley’s K function ( $\sqrt{K(t)/\pi} - t$ ) versus neighbor distance ( $t$ ) reveals spatial patterns at various values of the neighborhood distance  $t$ . Positive values indicate aggregation, while negative ones signify regularity. Dotted lines give 95% confidence intervals for complete spatial randomness (resulted from 1000 randomizations of actual data). Data represent means calculated from three plots.

	<i>Maytenus</i>			<i>Whitania</i>		
	Winter	Spring		Winter	Spring	
$\Psi_{pd}$ (MPa)	$-0.63 \pm 0.08$	$-1.11 \pm 0.10$	**	$-0.66 \pm 0.05$	$-1.92 \pm 0.33$	***
$\Psi_{md}$ (MPa)	$-1.77 \pm 0.12$	$-1.60 \pm 0.26$	n.s.	$-1.36 \pm 0.72$	$-3.20 \pm 0.44$	***

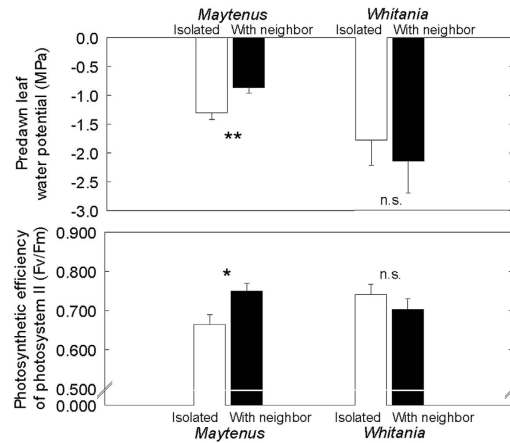
**Table 1.** Pre-dawn ( $\Psi_{pd}$ ) and mid-day ( $\Psi_{md}$ ) leaf water potential of *Maytenus* and *Whitania* shrubs growing alone in winter and spring ( $n = 8-12$ ). Data are mean  $\pm$  1 SE; statistically significant differences (U Mann-Whitney test) noted by \*\*\*( $P < 0.001$ ) and \*\*( $P < 0.01$ ).

but there are very few examples of mutualism within the same trophic level. Another reason is that the concept of mutualism is often restricted to interactions showing specificity and long evolutionary history. Finally, the unobtrusive nature of such interactions among plants could contribute to this lack of attention. Let us remind that facilitation, now widely acknowledged as a main driver of community dynamics, took decades to become recognized<sup>7,24</sup>.

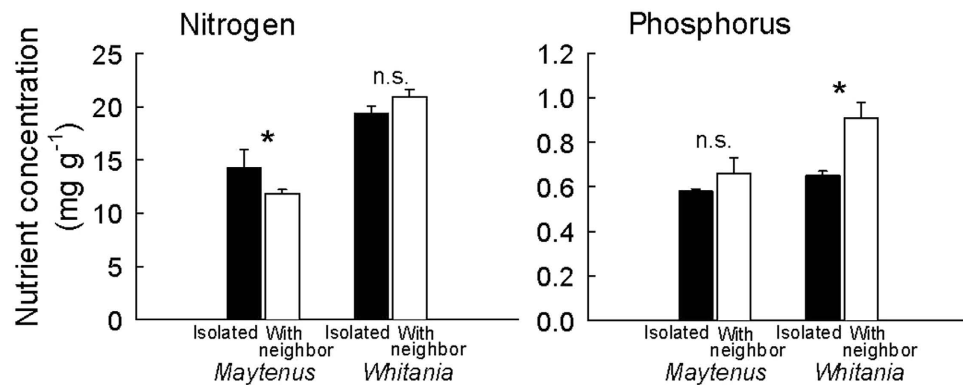
Aware of the possibility that two plants growing together may have effects benefitting both of them –leading to bi-directional facilitation or mutualism–, we addressed the interaction between two shrub species of similar size that intimately interact in semiarid environments in southeast Spain. *Maytenus senegalensis* (Lam.) Exell. subsp. *europaeus* (Celastraceae) is a tall, thorny, evergreen shrub found in coastal zones in SE Spain where it forms characteristic communities within its canopy, locally known as ‘artineras’. *Whitania frutescens* Pau (Solanaceae) is a tall, drought-deciduous shrub of African origin found also in more mesic sites in SE Spain. Individuals of *W. frutescens* (*Whitania* hereafter) in these patchy communities can grow isolated or within the canopy of the dominant species, *M. senegalensis* (*Maytenus* hereafter). We tested whether the observed spatial association between the two species was statistically significant and explored the nature of the interaction between both species through observational and manipulative experiments. We hypothesized that 1) each species will be able to modify the growing conditions for the other; 2) there is competition for resources but, overall 3) both species benefit from growing together.

## Results

Second order spatial analysis evidenced a strong association between *Maytenus* and *Whitania* shrubs for distances between 1 and 15 meters (Fig. 1) showing the two species are associated with one another. Plant physiological status depended on neighbour removal, species, and season. Overall predawn water potentials ( $\Psi_{pd}$ ) considerably decreased from winter to spring in both species but the decrease was more pronounced in *Whitania* (Table 1). In winter, *Maytenus* and *Whitania* –either isolated or with neighbors– did not differ in  $\Psi$  or photosynthetic efficiency of photosystem II ( $F_v/F_m$ ). In spring, water status of *Whitania* was unaffected by the presence of *Maytenus*, but  $\Psi$  of *Maytenus* was higher in plants living with *Whitania* than in isolation, both predawn (Fig. 2) and midday ( $\Psi_{isolated} = -2.14 \pm 0.22$  vs.  $\Psi_{+Whitania} = -1.09 \pm 0.30$ ,  $P = 0.033$ ). In spring, predawn  $F_v/F_m$  was also higher in *Maytenus* living with *Whitania* (Fig. 2) but similar at midday (*Maytenus*:  $F_v/F_m_{isolated} = 0.646 \pm 0.022$  vs.  $F_v/F_m_{+Whitania} = 0.673 \pm 0.035$ ,  $P = 0.62$ );



**Figure 2. Predawn leaf water potentials and predawn photosynthetic efficiency of photosystem II ( $F_v/F_m$ ) of *Maytenus* and *Whitania* shrubs in spring.** Clear bars represent isolated individuals, and solid bars plants with neighbors.  $N = 4-6$  shrubs. Data are mean  $\pm$  1 SE; statistically significant differences (U Mann-Whitney test) noted by \*\* ( $P < 0.01$ ) and \* ( $P < 0.05$ ).



**Figure 3. Nitrogen and phosphorus leaf concentration in *Whitania* and *Maytenus* shrubs growing isolated (clear bars) or with neighbors (solid bars).**  $n = 3$ . Data are mean  $\pm$  1 SE; statistically significant differences at  $P < 0.05$  (U Mann-Whitney test) noted by \*.

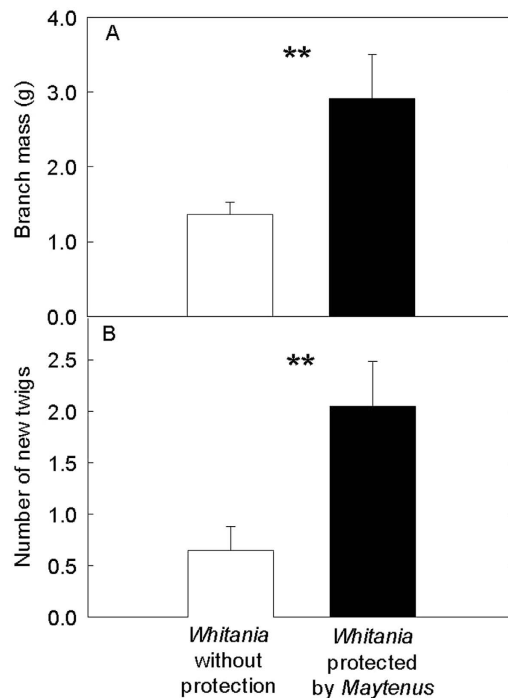
*Whitania* shrubs living with and without *Maytenus* did not differ in  $F_v/F_m$  (Fig. 2). Overall, our data show that there was no detectable influence between neighbors in winter regarding water status and fluorescence emission (an indication of stress), while in spring *Maytenus*' physiological status benefited from growing with *Whitania*, as shown by its improved water potential and fluorescence.

*Whitania* shrubs living isolated did not differ from plants living with *Maytenus* regarding leaf N content, but leaf P was higher in plants living with *Maytenus* (Fig. 3). Conversely, N content in *Maytenus* leaves was highest in plants living with *Maytenus*, while P did not differ between treatments (Fig. 3).

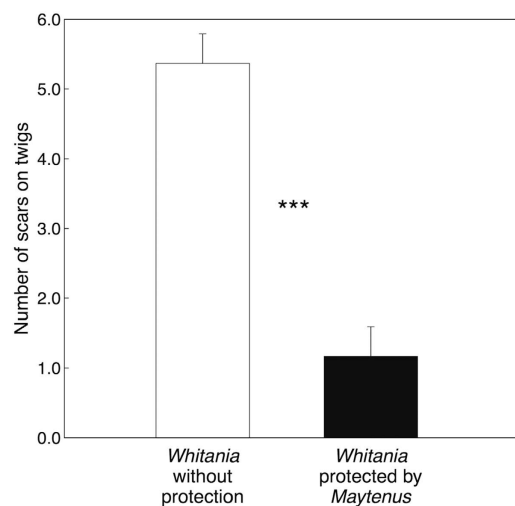
*Whitania* shrubs living isolated in gaps were strongly affected by browsing. Most sampled branches in isolated shrubs had been eaten and lost nearly 50% of their mass (Fig. 4A), being shorter ( $16.6 \pm 1.1$  cm vs  $24.7 \pm 2.0$  cm,  $P < 0.01$ ) and with less twigs than protected branches (Fig. 4B). Differences in branch width at the base were not statistically significant ( $5.34 \pm 0.38$  mm protected vs.  $6.20 \pm 0.35$  mm unprotected;  $P = 0.19$ ) but differences in leaf mass were ( $0.68 \pm 0.10$  g vs.  $0.18 \pm 0.03$  g;  $P < 0.0001$ ) while woody stem mass did not differ between protected and unprotected branches ( $2.24 \pm 0.51$  g vs.  $1.57 \pm 0.42$  g;  $P = 0.27$ ). *Whitania* shrubs living isolated in gaps were strongly affected by browsing in comparison to shrubs protected from *Maytenus*, with number of scars on twigs being five times higher (Fig. 5).

## Discussion

The association of different species in patches is a notable feature of arid and semiarid environments often considered indicative of positive interactions<sup>25-27</sup> but seldom tested<sup>28,29</sup>. Our data show that, indeed, there was a significant spatial aggregation between *Maytenus* and *Whitania* and an improved performance of individuals living in patches compared to individuals living isolated, suggesting a mutually beneficial interaction. This two-way facilitation was consequence of the interaction between plants rather than effect of environmental factors, as the presence of microsites was not evident in this system. We do not



**Figure 4.** Dry mass of branches (A) and number of new twigs per branch (B) of *Whitania* plants without protection and protected by thorny shrubs.  $N = 20$  *Whitania* plants. Data are mean  $\pm 1$  SE; statistically significant differences (U Mann-Whitney test) noted by \*\* ( $P < 0.001$ ).



**Figure 5.** Number of scars on twigs of *Whitania* individual plants (mean  $\pm 1$  SE) without protection and protected by thorny *Maytenus* shrubs.  $N = 20$  plants. Data are model estimates from linear mixed effect model; statistically significant differences are noted by \*\*\* ( $p < 0.0001$ ).

have fitness data and are therefore unable to test the full consequences of this interaction. But we did find that *Maytenus* benefited from the presence of *Whitania* by showing values of water potential and photochemical efficiency of photosystem II higher than isolated plants. Simultaneously, *Whitania* benefited from grazing protection provided by the intricate and thorny *Maytenus* canopy.

Water potential of both species declined substantially from winter to spring, evidencing a shortage in water availability. In winter, water was abundant enough to keep  $\Psi$  close to zero but in spring, the time of highest growth rate, *Maytenus*  $\Psi_{pd}$  and  $F_v/F_m$  improved with the presence of *Whitania* (Fig. 2) despite the higher transpiring leaf surface in patches and the likelihood of increased water limitation derived from competition for water. *Whitania* water status, however, was not affected by the presence of *Maytenus* and, if anything, tended to be worse in patches. Water status and photochemical efficiency

have a strong impact on overall plant performance<sup>30</sup> so that the improved physiological status of plants living in patches can likely lead to higher fitness, as is often the case in arid zones<sup>27</sup>.

Patterns of  $\Psi$  in *Whitania* are consistent with a superficial root system, while *Maytenus*  $\Psi$  data suggest it has access to deeper water resources<sup>31–33</sup>. Our data suggest that *Whitania* helps improve *Maytenus* water relations most likely through soil shading or hydraulic lift. Shade reduces thermal amplitudes and decreases soil water evaporation under the canopy<sup>34</sup>, which promotes an increase in soil moisture compared to bare ground and overall, a better water balance for plants<sup>2,4,35</sup>. Hydraulic lift is common whenever species have a dual root system accessing soil layers differing in water potential<sup>9,36,37</sup>. Increases in soil moisture linked to reduced evapotranspiration have been identified as a key mechanism driving positive interactions in dry ecosystems<sup>4,35,38</sup>. In addition, by growing within the *Maytenus* canopy, *Whitania* may increase the boundary layer conductance of the whole canopy, lessening transpirational demand<sup>39</sup> hence improving overall plant water status.

The contrasting effects on the neighbors' leaf nutrient content show that close interacting plants may compete for some resources while simultaneously improving availability for others. In our field site *Maytenus* growing with *Whitania* had lower leaf N than isolated individuals, suggesting competition for N (but simultaneously displayed higher  $\Psi_{pd}$  and  $F_v/F_m$ ). Conversely, *Whitania* growing with *Maytenus* had higher leaf P than isolated individuals, pointing to higher P availability under *Maytenus* canopies most likely linked to higher micorrhizal inocula in the understorey. This is often the case in arid environments where micorrhizas play a crucial role<sup>40</sup> and where vegetation patches act as micorrhiza reservoirs<sup>41</sup>.

Thorny *Maytenus* shrubs provided an effective protection to *Whitania*, a palatable species highly browsed on when growing isolated. Protection from grazing appears as a major benefit for *Whitania* shrubs growing with *Maytenus*, as in other systems under high herbivory pressure<sup>1,12</sup>. Examples of associational defences have been widely reported<sup>42,43</sup>. In SE Spain, unpalatable *Artemisia barrelieri* shrubs facilitate seed germination and seedling establishment of more palatable *Anthyllis cytisoides* shrubs, providing shelter from herbivory during early growth stages<sup>44</sup>. *Stipa tenacissima* tussocks have also been found to reduce browsing by rabbits<sup>38,45</sup>. Alados *et al.*<sup>46</sup> observed a positive association among palatable and unpalatable species which was more evident for the most palatable species, *Ballota hirsuta*, whose association with unpalatable species increased with grazing pressure. In most cases, benefactor species appear to physically shelter or hide beneficiary species from herbivores<sup>42,47</sup>.

Our results suggest that plants interact in a multidimensional space, with synergic or antagonistic effects depending on the factor considered<sup>42,35</sup>. Such effects depend on plant traits which have rarely been assessed in the framework of the interaction between beneficiary and benefactor species (but see refs. 48–50). There are well-known traits that characterize facilitator species<sup>49</sup>, like the ability to fix N, casting a shade<sup>1,51</sup> or buffer temperature extremes<sup>14,51</sup>. Thus complementary suites of traits in different species can lead to mutually beneficial interactions, and selection should strongly favour traits promoting benefits from neighbours<sup>20</sup> particularly in harsh environments<sup>49,50</sup>.

Our two species improve conditions regarding specific resources while competed for others with an overall positive balance for both and should have consequences for fitness –which we did not measure. Despite previous evidence on reciprocal beneficial effects between different plant species, such results still challenge our current understanding of plant interactions. We suggest that the interaction between *Maytenus* and *Whitania* may be a facultative mutualism, a term which is currently considered to subsume even transient interactions of small effect as long as both partners experience a net positive effect<sup>20,23,52</sup>. This mutual interaction in our field site is based on herbivory. In a grazed landscape facilitation is likely to promote fitness of palatable species that otherwise would tend to disappear, being able to recruit and maintain a viable population. In other words, facilitation occurred when the species were deviated from their optimum niche<sup>15–17</sup>.

Therefore, complex interactions –which may have been overlooked to date– result on reciprocal positive interactions which influence community structure. This adds to the better-known, one-way positive plant-plant interactions which have proved to be main drivers of plant community dynamics<sup>24</sup> and evolution<sup>53</sup>.

## Methods

**Field site and species.** In winter 1998 a research site was established in a *Maytenus* shrubland in El Ejido (36° 47' N, 2° 46' W, 80 m elevation), Almería the best preserved population of this species in Spain. The climate is Mediterranean semi-arid, with a pronounced dry season from June to September with almost no rain in most years. Mean annual precipitation is 280 mm and mean annual temperature 18.5°C, with January (mean temperature 12.5°C) and August (mean temperature 28.0°C) as the coldest and warmest months, respectively. Soils are calcic regosols and cambisols, with a mixed clayey substrate with a calcic hardpan close to the soil surface.

The dominant shrubs *Maytenus* and *Whitania* appear frequently aggregated in clumps where branches are intimately associated but many other species are found in these patches, from shrubs (up to 13 different species in a patch<sup>54</sup>) to forbs and grasses while the space in between is only scantily covered by small shrubs and geophytes. *Maytenus* is a thorny shrub up to 3 m tall, with coriaceous perennial leaves and an intricate spherical canopy. *Whitania* grows up to 2.5 m and has thin leaves that are shed before the dry season. Browsing by sheep and goat is frequent in the area and exposed *Whitania* individuals are preferred fodder, showing smaller and altered canopy shapes.

Both species have fleshy fruits and the spatial association of the two species may be just a consequence of dispersal patterns (the main dispersers are frugivorous birds). However, isolated individuals are frequently observed in more mesic habitats (such as shady canyons or higher elevation sites) suggesting that dispersal is rather random and the co-occurrence of both species has further implications.

**Spatial analysis.** Three 50 m × 50 m plots were established in the study site in October 1999. The coordinates of the estimated center of every *Maytenus* and *Whitania* individual taller than 10 cm were recorded to the nearest 5 cm. Spatial distribution patterns were analyzed by using Ripley's *K* function<sup>55</sup>. The null hypothesis of complete spatial randomness between the two species was tested with a modified procedure for analysis of bivariate distribution patterns, and statistical analysis of the data was performed using the SPPA software<sup>56</sup>. Ripley's *K* function is a recommended technique for bivariate point pattern analysis<sup>57</sup>. It considers each plant as the central point of a circle of radius *t*, counting the number of points found within the circle. We used the weighting approach to correct for edge effects<sup>56</sup>. If the distribution of the points is Poisson random, the expected value of the cumulative function *K*(*t*) equals  $\pi t^2$ , i.e. the area of a circle of radius *t*. For an easier interpretation, the derived sample statistic  $\sqrt{K[(t)/\pi]} - t$  is plotted, as this expression has a zero expectation for any value of *t* when the pattern is Poisson random, being positive when it is aggregated and negative when regular (uniform).

**Specific neighbor effect.** To investigate the specific effect of *Maytenus* and *Whitania* on each other, in February 1998 a removal experiment was set up. 18 patches similar in physiognomy and size (about 3 m tall, 2 m in diameter) were selected within a 2-ha plot in which spatial analyses were conducted and individuals of either *Maytenus* or *Whitania* were removed in a subset of six randomly selected patches, while another six patches were kept as control. Stems of non-target species were clipped to the base at the onset of the experiment and sprouts were removed frequently.

Two years after removal we measured physiological variables in late winter (February 2000) and at the time of maximum growth in spring (late March 2000) but not in summer because *Whitania* is a summer-deciduous species. Physiological status was assessed by measuring predawn and midday water potential ( $\Psi_{pd}$ ) (*n* = 4–6 per patch) in branch tips, randomly selected at ~1.5 m height in the canopy facing east, with a pressure chamber (Skye Instruments, Powys, UK) and predawn and midday photosynthetic efficiency of PS II ( $F_v/F_m$ ) in 30' dark-adapted leaves (*n* = 4–6) with a chlorophyll induction fluorimeter (PEA; Hansatech, Kings Lynn, UK).

**Nutrient analysis.** We determined nutrient content of *Maytenus* and *Whitania* leaves of individuals living isolated and in manipulated patches. The first mature, fully developed leaf in several branches per individual, always with similar orientation and position in the canopy, were collected, combined to make a composite sample, dried at 70 °C, and analyzed for N and P (*n* = 3 per treatment and species) by Kjeldahl and colorimetric techniques, respectively.

**Browsing assessment.** Livestock browsing by goats and sheep is frequent in the area and its effect on plants is evident at our field site. We assessed browsing by comparing *Whitania* with and without protection from *Maytenus*. We selected the tallest 1 year-old branch and recorded branch diameter at the base with a digital caliper in 20 *Whitania* shrubs growing under thorny *Maytenus* branches and paired them with another 20 *Whitania* individuals living without protection. Paired individuals were between 1 and 5 m from each other. All individuals were similar in size (canopy diameter between 0.90 and 1.4 m). We sampled plants in May 2001, at the end of the growing season, in an attempt to capture browsing effects after potential growth compensation. In each plant we harvested the selected branch and recorded branch diameter, total length to the closest millimeter, and the number of new twigs. In the laboratory we separated leaves from stems, and plant material was dried at 70 °C for 72 hours and weighed. In addition, we used scars from browsing events on *Whitania* twigs as indicator of browsing intensity, as they were clearly visible. We recorded scars in 20 pairs of *Whitania* shrubs, each pair consisting of a *Whitania* individual growing under thorny *Maytenus* branches and another *Whitania* individual living in a gap. For each *Whitania* shrub we recorded the number of clearly visible scars in three 0.1 × 0.1 m quadrats at 1 m height distributed at angles of 0°, 120° and 240° towards the shrub basal centre. For each shrub the 0° was always south. If a framed area did not contain *Whitania* twigs, the frame was moved to the closest area of the shrub with *Whitania* twigs. Visible scars deeper than 0.2 m from the shrub canopy were not recorded, as such scars were older and less visible.

**Data analysis.** Data (but for estimates of browsing intensity) were analyzed with Data Desk 6 (Data Description, Ithaca, New York, USA). Due to heterocedasticity of some variables, data were analyzed by non-parametric U Mann-Whitney tests. Water potential and chlorophyll fluorescence data were tested at the species level for differences between dates (winter vs. spring) and between treatments. Values in text and figures represent mean ± 1 standard error.

To analyse estimates of browsing intensity we used a linear mixed effects model<sup>58</sup> in the R environment, version 3.0.2. Number of scars was used as response variable and *Whitania* with or without protection from *Maytenus* as fixed factor. Random effects were modelled as angle of estimate nested in shrub pair. The model was found satisfactory from standard diagnostics on variance of residuals and outliers.

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## Author Contributions

F.I.P. and R.T. designed the experiment, R.T. and K.A.B. performed the experiments and analyzed the data, and F.I.P., R.T. and K.A.B. wrote the paper.

## Additional Information

**Competing financial interests:** The authors declare no competing financial interests.

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