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Making the best of the worst of times: traits underlying combined shade and drought tolerance of *Ruscus aculeatus* and *Ruscus microglossum* (Asparagaceae)

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Abstract. The genus *Ruscus* (Asparagaceae) consists of evergreen, woody monocot shrubs with modified photosynthetic stems (phylloclades) that occur in dry, shaded woodland areas of the Mediterranean Basin and southern Europe. The combined drought and shade tolerance of *Ruscus* species challenges the 'trade-off model', which suggests that plants can be either drought or shade adapted, but not both. To clarify the potential mechanisms that enable *Ruscus* species to survive in shaded environments prone to pronounced soil drought, we studied form–function relations based on a detailed trait survey for *Ruscus aculeatus* L. and *Ruscus microglossum* Bertol., focusing on gas exchange, hydraulics, morphology, anatomy, and nutrient and isotope composition. We then compared these trait values with published data for other species. *R. aculeatus* and *R. microglossum* exhibited numerous traits conferring drought and shade tolerance via reduced demand for resources in general and an ability to survive on stored water. Specific traits include thick phylloclades with low rates of maximum photosynthetic CO_2 assimilation, low stomatal conductance to water vapour (g_s), low respiration rate, low light compensation point, low shoot hydraulic conductance, low cuticular conductance, and substantial water storage tissue. *Ruscus* carbon isotope composition values of –33 ‰ were typical of an understory plant, but given the low g_s could be associated with internal CO_2 recycling. *Ruscus* appears to be a model for extreme dual adaptation, both physiologically and morphologically, enabling its occupation of shaded sites within drought prone regions across a wide geographical range, including extremely low resource understory sites.

Additional keywords: carbon isotopes, functional morphology, gas exchange, hydraulic conductance, Mediterranean climate, phylloclades, understory.

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Introduction

Plant ecological distributions are constrained by several factors including tolerance of environmental conditions such as light and water availability (Sack 2004; Niinemets and Valladares 2006; Hallik *et al*. 2009; Sterck *et al*. 2011). According to the 'trade-off model' hypothesised by Smith and Huston (1989), a plant's adaptations can either allow it to tolerate low light or low water availability. However, many plant species have been reported to tolerate sites prone to strong combinations of drought and shade, including *Ruscus aculeatus* L. (Sack *et al*. 2003*b*), which occurs in dry, shaded understory habitats subjected to annual seasonal drought.

Previous studies have shown several species can tolerate combined shade and drought in experiments (Sack 2004; Martínez-Tillería *et al*. 2012) and in the field (Caspersen 2001; Engelbrecht and Kursar 2003; Niinemets and Valladares 2006), but the physiological mechanisms contributing to this ability have not received detailed study. The ability of *Ruscus* species to survive very strong combinations of shade and drought in the field and in experiments (Sack *et al*. 2003*b*; Sack 2004) makes it a model for such dual adaptation. However, the species have received little detailed study, and previous work has emphasised its adaptation via phenology (de Lillis and Fontanella 1992; Martínez-Pallé and Aronne 1999), high biomass allocation to roots, and its apparent conservative resource use (Sack *et al*. 2003*b*).

The objective of this research was to clarify the wide range of potential adaptations of *Ruscus* that contribute to its remarkable ability to survive and regenerate in shaded sites prone to occasional or seasonal soil drought. To achieve this objective, we studied 57 traits relating to gas exchange, hydraulics, morphology, anatomy, and nutrient and carbon isotope composition in two *Ruscus* species, *R. aculeatus* and *Ruscus microglossum* Bertol. (Fig. 1). We then compared trait values with those hypothesised to confer shade tolerance, drought avoidance or both. Overall, for 21 traits we had *a priori* hypotheses of a benefit for shade tolerance and for 24 traits we had *a priori* hypotheses of a benefit for drought avoidance. We then compared the traits for *Ruscus* with values compiled from the literature for: (1) temperate and tropical broadleaf evergreen species; (2) Mediterranean species; and (3) woody angiosperms in general; in order to put *Ruscus* trait values in a global context. This approach involved measuring a large number of key aspects of structure and function and, when possible, compiling specific hypotheses for traits potentially involved in the shade and drought tolerance *Ruscus* species relative to comparator species (Tables 1–5). According to the previous literature on shade and drought tolerance (for example, Givnish 1988; Jones 1992) these suites of traits in *Ruscus* are expected to directly or indirectly contribute to mechanisms operating across cell types and levels of leaf organisation conferring combined shade and drought adaptation.

Thus, on the general understanding of shade and drought tolerance traits, we hypothesised that *Ruscus* species would have mechanisms of drought adaptation including traits enabling the delay of tissue dehydration, and traits enabling maintained function even as tissue dehydrates. Such traits include a high water-use efficiency (Wright and Westoby 2003), as well as water storage tissue with high water storage capacitance associated with low bulk leaf modulus of elasticity, high relative water content at turgor loss point, and low cuticular conductance (Sack *et al.* 2003*a*; Pasquet-Kok *et al.* 2010; Ogburn and Edwards 2012). Traits potentially contributing to shade tolerance include low rates of maximum photosynthetic CO$_2$ assimilation per leaf area and per leaf mass, low light compensation point, low maximum rate of carboxylation, low maximum rate of electron transport, and more negative carbon isotope ratios (Walters and Reich 1999). Traits that potentially confer a combined drought and shade tolerance through a general conservative and cost-efficient resource use include thick lamina with thick epidermis, low respiration rates per area and mass, low stomatal conductance, and low shoot hydraulic conductance (Sack *et al.* 2003*b*). Given the exceptional biology of these species – their extreme tolerance and their possession of phylloclades – we also qualified additional traits, in particular the detailed anatomical traits such as cell sizes, for which we could not compile hypotheses due to the paucity of comparative data in the published literature. However, such anatomical traits have been argued to be strongly associated with environmental adaptation in principle (Haberlandt 1914), and thus these data for *Ruscus* are likely to be important as future studies provide comparative data for many species.

Materials and methods

Study species and site

Ruscus (Asparagaceae) is a genus of six species of evergreen sclerophyllous woody shrubs native to western and southern Europe (including north to southern England), Macaronesia, north-west Africa, and south-western Asia ranging east to the Caucasus (de Lillis and Fontanella 1992; Martínez-Pallé and Aronne 1999). *Ruscus* is thus found in a wide range of temperate forests as well as in Mediterranean-type climates characterised by wet, cool winters and dry, warm summers that result in an annual seasonal period of low water availability, or drought (Matalas 1963; Dracup 1991; Cowling *et al.* 1996). *Ruscus* exhibits phylloclades, which are flattened photosynthetic stems that resemble leaves, and are considered intermediate organs that combine stem and leaf features (Fig. 1; Cooney-Sovetts and Sattler 1987). *Ruscus aculeatus* L. is a

Table 1. Mean values for morphological traits ± s.e. for *Ruscus aculeatus* and *R. microglossum*, with units and replication

For given traits, expectations are given for whether *Ruscus* should have a higher or lower value relative to comparator species according to the hypotheses of shade or drought adaptation. Comparator species data were taken according to availability in the previously published literature and number of species, and minimum, mean, and maximum trait values are provided, or mean ± s.e. if only these were available. Sources of comparative data: leaf area (Sack *et al.* 2012); LMA (Wright *et al.* 2004); density (Niinemets 1999); LDMC (Vile 2005); and SWC (Vendramini *et al.* 2002; Ogburn and Edwards 2012)

Morphological traits	Units	*R. aculeatus* Mean ± s.e.	N	*R. microglossum* Mean ± s.e.	N	Hypotheses Shade adapted	Drought adapted	Comparator species (N)	(min, mean, max)
Leaf area	cm^2	1.80 ± 0.06	92	19.3 ± 0.34	84		Lower[A]	Dicots (485)	0.10, 17.8, 280
Leaf mass per area (LMA)	g m^{-2}	122.8 ± 8.9	92	91.0 ± 0.9	84	Higher	Lower[A,B,C]	Temperate broadleaf evergreen (132)	58.0, 153, 429
								Tropical broadleaf evergreen (72)	40.6, 145, 370
Density	g cm^{-3}	0.39 ± 0.03	10	0.27 ± 0.01	10	Higher	Lower[A,B,C]	Woody trees and shrubs (38)	0.09, 0.41, 1.33
Leaf dry matter content (LDMC)	g g^{-1}	0.389 ± 0.016	6	0.310 ± 0.005	6	Higher[A]	Lower[B,C]	Shrubs (>100)	0.384 ± 0.0084
Saturated water content (SWC)	g g^{-1}	1.59 ± 0.12	6	2.23 ± 0.05	6		Higher[B,C]	Evergreen trees and shrubs (6)	0.67, 1.82, 6.0
								Succulents (45)	1.70, 11.7, 52.0

[A] An expectation according to a hypothesis was confirmed for *R. aculeatus*.

[B] An expectation according to a hypothesis was confirmed for *R. microglossum*.

[C] Expectations for these traits are based on drought tolerance conferred by water storage tissue (‘succulence’); the opposite expectations would arise for drought tolerance conferred by the ability to maintain turgor with dehydration.

Table 2. Mean values for anatomical traits ± s.e. for *Ruscus aculeatus* and *R. microglossum*, with units and replication

For given traits, expectations are given for whether *Ruscus* should have a higher or lower value relative to comparator species according to the hypotheses of shade or drought adaptation. Comparator species data were taken according to availability in the previously published literature and number of species, and minimum, mean, and maximum trait values are provided. Sources of comparative data: tissue thickness and airspace (Sack and Frole 2006); D_m (Brodribb *et al.* 2007). NS, not statistically significant at $P < 0.05$

Anatomical traits	Units	*R. aculeatus* Mean ± s.e.	*R. aculeatus* N	*R. microglossum* Mean ± s.e.	*R. microglossum* N	Hypotheses Shade adapted	Hypotheses Drought adapted	Comparator species (N)	Comparator species (min, mean, max)
Tissue thicknesses and airspace									
Lamina	µm	278 ± 3.34	5	296 ± 8.57	5	Higher^{A,B}	Higher^{A,B}	Tropical evergreen trees (10)	156, 267, 512
Cuticle	µm	3.52 ± 0.22	5	3.29 ± 0.23	5	Higher	Higher	Tropical evergreen trees (10)	1.25, 4.60, 10.5
Epidermis	µm	20.2 ± 0.513	5	23 ± 0.657	5	Higher^{A,B}	Higher^{A,B}	Tropical evergreen trees (10)	9.75, 14.0, 17.3
Epidermis cell wall	µm	3.41 ± 0.11	5	3.69 ± 0.25	5				
Mesophyll	µm	72.6 ± 1.79	5	83.0 ± 2.72	5				
Water storage	µm	88.7 ± 7.05	5	86.2 ± 5.87	5		Present^{A,B}		
% total leaf air space	%	10.5 ± 1.38	5	20.8 ± 1.73	5				
Cell dimensions									
Epidermis cell area	µm^2	447 ± 27.0	5	599 ± 35.3	5				
Epidermis cell perimeter	µm	78.9 ± 2.61	5	94.1 ± 2.78	5				
Mesophyll cell area	µm^2	657 ± 21.5	5	846 ± 42.6	5				
Mesophyll cell perimeter	µm	94.5 ± 1.72	5	110 ± 2.41	5				
% chloroplast area in mesophyll cell	%	21.2 ± 1.49	5	21.1 ± 3.07	5				
Mesophyll area/ total leaf area (A_{mes}/A)		24.7 ± 1.3	5	20.5 ± 0.45	5				
Water storage cell area	µm^2	3414 ± 196	5	5972 ± 847	5				
Water storage cell perimeter	µm	221 ± 5.72	5	291 ± 19.1	5				
Vascular traits									
Minimum distance from vein to epidermis (D_m)	µm	203 ± 18.0	5	370 ± 49.3	5	Higher^{B}	Lower^{A}	Dicotyledons (11)	129, 233, 428
Inter-veinal distance (*IVD*)	µm	272 ± 27.5	5	509 ± 71.5	5				
Fibrous bundle sheath cell height	µm	18.4 ± 1.27	5	17.5 ± 1.49	5				
Fibrous bundle sheath cell width	µm	17.4 ± 0.66	5	16.3 ± 0.92	5				
Fibrous bundle sheath cell wall thickness	µm	4.57 ± 0.41	5	2.48 ± 0.23	5				
Average theoretical xylem conduit conductivity in the midrib	× 10^{-5} mmol m^{-2} s^{-1} MPa^{-1}	1.24 ± 0.25	5	1.98 ± 0.55	5				
Average theoretical xylem conduit conductivity in an intermediary vein	× 10^{-5} mmol m^{-2} s^{-1} MPa^{-1}	0.63 ± 0.14	5	1.19 ± 0.38	5				
Average theoretical xylem conduit conductivity in a minor vein	× 10^{-5} mmol m^{-2} s^{-1} MPa^{-1}	0.37 ± 0.12	5	0.28 ± 0.06	5				

(*continued next page*)

Table 2. (*continued*)

Anatomical traits	Units	*R. aculeatus* Mean ± s.e.	N	*R. microglossum* Mean ± s.e.	N	Hypotheses Shade adapted	Hypotheses Drought adapted	Comparator species (N)	Comparator species (min, mean, max)
Mean maximum xylem conduit diameter in the midrib	µm	9.66 ± 0.44	5	11.0 ± 0.78	5				
Mean maximum xylem conduit diameter in an intermediary vein	µm	8.31 ± 0.55	5	9.49 ± 0.72	5				
Mean maximum xylem conduit diameter in a minor vein	µm	7.15 ± 0.72	5	7.19 ± 0.48	5				

[A] An expectation according to a hypothesis was confirmed for *R. aculeatus*.

[B] An expectation according to a hypothesis was confirmed for *R. microglossum*.

typical understory species in forests in the Mediterranean basin (Fig. 1; de Lillis and Fontanella 1992; Martínez-Pallé and Aronne 1999; Sack *et al*. 2003*b*). *Ruscus microglossum* Bertol. is a hybrid produced by crossing species *Ruscus hypoglossum* L., from the Black Sea region, and *Ruscus hypophyllum* L., from North Africa (Fig. 1; Thomas 1992; USDA, ARS, National Genetic Resources Program 2009).

Experiments were conducted on *R. aculeatus* and *R. microglossum* plants in the Mildred E Mathias Botanical Garden at the University California, Los Angeles, from June to August 2009. Measurements were made in shaded understory sites on at least three individuals of each species. Diurnal light measurements were made on sunny days above the *Ruscus* plant canopies with a quantum sensor (Li-190S, Li-Cor Biosciences, Lincoln, NE, USA). Individuals received full sunlight averaging ~1500 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) for ~1 h each day, and otherwise experienced PAR of ~50 µmol m^{-2} s^{-1} interspersed with sunflecks averaging ~190 µmol m^{-2} s^{-1}. Plants were irrigated as needed, and all *Ruscus* individuals were watered before the start of this study to reduce any differences in water availability between individuals.

Phylloclade morphology

We determined leaf morphological traits on recently formed mature phylloclades. Although methods were applied to phylloclades, for convenience we retained the names of methods and traits as applied to leaves (e.g. leaf mass per area). Phylloclade area was measured on excised samples with an area meter (Li-3100, Li-Cor Biosciences). Samples were dried in an oven at >70°C for more than 48 h to determine dry mass and calculate leaf mass per area (LMA) as dry mass divided by area. Phylloclade thickness was measured with electronic digital calipers (Fisher Scientific, Pittsburgh, PA, USA), and density was calculated as mass per area divided by thickness (Witkowski and Lamont 1991).

Phylloclade anatomy

We sampled phylloclades of each species and prepared cross-sections for anatomical measurements. Phylloclades were preserved in formalin acetic acid (37% formaldehyde, glacial acidic acid, 95% ethanol, and deionised water in a 10 : 5 : 50 : 35 mixture). We measured the transverse cross-sectional anatomy using sections cut halfway along the phylloclade length, embedded in LR White (London Resin Co., London, UK), cut 0.5 µm thick using a microtome (Ultracut E, Reichert-Jung Ultracut E, Leica Microsystems, Arcadia, CA, USA), stained with 0.01% toluidine blue in 1% sodium borate, and viewed under the light microscope using a 20–40 × objective (DMRB; Leica Microsystems, Wetzlar, Germany).

We measured tissue thicknesses and cell dimensions in the lamina, and dimensions of vascular bundles and of xylem conduits using Image J software (ver. 1.42 q; National Institutes of Health, Bethesda, MD, USA) on microscope images. We measured thickness for the lamina, cuticle, epidermis, epidermis cell wall, mesophyll, and water storage compartment. We averaged three measurements of each type for each cross-section. The phylloclade tissues were arranged

Table 3. Mean values for gas exchange traits ± s.e. for *Ruscus aculeatus* and *R. microglossum*, with units and replication

For given traits, expectations are given for whether *Ruscus* should have a higher or lower value relative to comparator species according to the hypotheses of shade or drought adaptation. Comparator species data were taken according to availability in the previously published literature and number of species, and minimum, mean, and maximum trait values are provided. Sources of comparative data: A_{area}, A_{mass}, R_{area}, R_{mass}, g_s (Wright *et al*. 2004); LCP (Walters and Reich 1999); A/g_s (Gulias *et al*. 2003); $V_{c,max}$, J_{max} (Wullschleger 1993); g_{min} (Kerstiens 1996). NS, not statistically significant at $P < 0.05$

Gas-exchange traits	Abbreviation	Units	*R. aculeatus* Mean ± s.e.	N	*R. microglossum* Mean ± s.e.	N	Hypotheses Shade adapted	Hypotheses Drought adapted	Comparator species (N)	(min, mean, max)
Light-saturated rate of photosynthesis per area	A_{area}	µmol m^{-2} s^{-1}	5.22 ± 1.33		4.51 ± 0.47	6	Lower^{A,B}		Temperate broadleaf evergreens (78)	2.62, 9.42, 22.65
Light-saturated rate of photosynthesis per mass	A_{mass}	nmol CO$_2$ g^{-1} s^{-1}	0.043 ± 0.011	4	0.050 ± 0.005	6	Lower^{A,B}		Temperate broadleaf evergreens (78)	22.8, 74.6, 209
Respiration rate per area	R_{area}	µmol m^{-2} s^{-1}	0.044 ± 0.009	8	0.156 ± 0.015	10	Lower^{A,B}	Lower^{A,B}	Temperate broadleaf evergreens (50)	0.32, 0.93, 1.70
Respiration per mass	R_{mass}	nmol CO$_2$ g^{-1} s^{-1}	3.56E-04 ± 7.02E-05	8	1.71E-03 ± 1.66E-04	10	Lower^{A,B}	Lower^{A,B}	Temperate broadleaf evergreens (50)	3.33, 7.06, 15.7
Maximum stomatal conductance per area	g_s	mmol m^{-2} s^{-1}	33 ± 0.007	4	35 ± 0.006	6	Lower^{A,B}	Lower^{A,B}	Temperate broadleaf evergreens (48)	49, 142, 309
Light compensation point	LCP	µmol photons m^{-2} s^{-1}	4.85 ± 0	4	3.61 ± 0	6	Lower^{A,B}		Tropical evergreen shade-tolerators (15)	1, 6.58, 24
Intrinsic water use efficiency	A/g_s	µmol mol^{-1}	154 ± 8	4	142 ± 19.0	6		Higher^{A,B}	Mediterranean species (78)	23.3, 59.4, 142
Ratio of intercellular to ambient [CO$_2$]	C_i/C_a		0.327 ± 0.106	4	0.431 ± 0.064	6				
Maximum rate of carboxylation	$V_{c,max}$	µmol m^{-2} s^{-1}	26.6 ± 3.18	4	24.7 ± 5.42	5	Lower^{A,B}		Temperate hardwood species (19)	11, 47, 119
Maximum rate of electron transport	J_{max}		60.1 ± 6.35	5	50.6 ± 7.86	5	Lower^{A,B}		Temperate hardwood species (19)	29, 104, 237
Leaf cuticular conductance	g_{min}	mmol m^{-2} s^{-1}	0.379 ± 0.082	10	0.295 ± 0.082	12		Lower^{A,B}	Vascular plants (201)	0.1, 13.2, 107
Stem cuticular conductance	g_{min}	mmol m^{-2} s^{-1}	0.095 ± 0.025	6	0.030 ± 0.003	4				

[A] An expectation according to a hypothesis was confirmed for *R. aculeatus*.

[B] An expectation according to a hypothesis was confirmed for *R. microglossum*.

Table 4. Mean values for hydraulics traits ± s.e. for *Ruscus aculeatus* and *R. microglossum*, with units and replication

For given traits, expectations are given for whether *Ruscus* should have a higher or lower value relative to comparator species according to the hypotheses of shade or drought adaptation. Comparator species data were taken according to availability in the previously published literature and number of species, and minimum, mean, and maximum trait values are provided, or mean ± standard error, if only these were available. Sources of comparative data: K_{shoot} (Sack and Holbrook 2006); Ψ_{tlp}, RWC_{tlp}, Π_o, ε (Bartlett *et al.* 2012); C_{ft} (Scoffoni *et al.* 2008);. NS: not statistically significant at $P < 0.05$

Hydraulics traits	Abbreviation	Units	*R. aculeatus*		*R. microglossum*		Hypotheses		Comparator species	
			Mean ± s.e.	N	Mean ± s.e.	N	Shade adapted	Drought adapted	(N)	(min, mean, max)
Shoot hydraulic conductance	K_{shoot}	mmol m^{-2} s^{-1} MPa^{-1}	2.16 ± 0.10	10	2.69 ± 0.13	11	Lower^{A,B}	Lower^{A,B}	Temperate woody angiosperms (38)	8 ± 1
									Tropical woody angiosperms (49)	13 ± 1.5
Osmotic potential at full turgor	π_o	MPa	–1.28 ± 0.10	6	–0.65 ± 0.03	6	Higher^{A,B}	Higher^{A,B,C}	Evergreen woody species (182)	-3.4, –1.83, –0.49
Turgor loss point	Ψ_{tlp}	MPa	–1.84 ± 0.10	6	–1.13 ± 0.07	6	Higher^{A,B}	Higher^{A,B,C}	Evergreen woody species (158)	-4.25, –2.20, –0.54
Modulus of elasticity	ε	MPa	11.0 ± 1.41	6	5.88 ± 0.53	6		Lower^{A,B,C}	Evergreen woody species (139)	3.56, 17.1, 73.4
Relative water content at turgor loss point	RWC_{tlp}	%	88.8 ± 1.09	6	89.3 ± 0.85	6		Higher^{A,B,C}	Evergreen woody species (19)	71, 81.2, 91.1
Relative capacitance at full turgor	C_{ft}	MPa^{-1}	0.067 ± 0.008	6	0.102 ± 0.010	6		Higher^{A,B,C}	Evergreen species (6)	0.040, 0.066, 0.113
Relative capacitance at turgor loss	C_{tlp}	MPa^{-1}	0.104 ± 0.015	6	0.089 ± 0.003	6				
Predawn water potential	Ψ_{pre}	MPa	–0.45 ± 0.08	4	–0.88 ± 0.08	4				
Midday water potential	Ψ_{mid}	MPa	–1.41 ± 0.08	3	–1.29 ± 0.28	4				

[A] An expectation according to a hypothesis was confirmed for *R. aculeatus*.

[B] An expectation according to a hypothesis was confirmed for *R. microglossum*.

[C] Expectations for these traits are based on drought tolerance conferred by water storage tissue ('succulence'); the opposite expectations would arise for drought tolerance conferred by the ability to maintain turgor with dehydration.

Table 5. Mean values for tissue composition traits ± s.e. for *Ruscus aculeatus* and *R. microglossum*, with units and replication, and significance of differences between species (*t*-tests)

For given traits, expectations are given for whether *R. aculeatus* should have a higher or lower value relative to comparator species according to the hypotheses of shade or drought adaptation. Comparator species data were taken according to availability in the previously published literature and number of species, and minimum, mean, and maximum trait values are provided. Sources of comparative data: $\delta^{13}C$ (Körner *et al.* 1991); N_{mass}, N_{area} (Wright *et al.* 2004). NS, not statistically significant at $P < 0.05$

Tissue composition traits	Abbreviation	Units	*R. aculeatus* Mean ± s.e.	N	*R. microglossum* Mean ± s.e.	N	Hypotheses Shade adapted	Drought adapted	Comparator species (N)	(min, mean, max)
N per mass	N_{mass}	%	1.94 ± 0.06	9	1.92 ± 0.07	9	Lower	Higher^{A,B}	Temperate broadleaf evergreen (129)	0.58, 1.19, 2.31
N per area	N_{area}		2.38 ± 0.07	9	1.75 ± 0.06	9	Lower	Higher^{A,B}	Temperate broadleaf evergreen (129)	0.727, 1.73, 3.63
C per mass	C_{mass}	%	44.4 ± 0.48	9	43.6 ± 0.52	9				
Carbon to nitrogen ratio	C:N		23.1 ± 0.68	9	22.9 ± 0.58	9				
Carbon isotope ratio	$\delta^{13}C$	‰	–33.32 ± 0.29	9	–33.05 ± 0.20	9	Lower^{A,B}	Higher	Temperate species (35)	–27.64, –26.03, –24.09

[A] An expectation according to a hypothesis was confirmed for *R. aculeatus*.

[B] An expectation according to a hypothesis was confirmed for *R. microglossum*.

symmetrically, with adaxial and abaxial layers of mesophyll, epidermis and cuticle above and below a central, achlorophyllous water storage tissue (Fig. 2). The per cent air space in each of the adaxial and abaxial mesophyll, and in the water storage compartment was estimated to the nearest 5%, and the total phylloclade airspace was calculated:

$$\frac{\sum \%\text{Air space in each tissue} \times \underline{\text{fraction of leaf cross section occupied by that tissue}}}{100}. \quad (1)$$

As indices of cell size, the cross-sectional areas and perimeters were measured for three cells in the epidermis (adaxial and abaxial), the mesophyll (adaxial and abaxial, i.e. above and below the water storage tissue), and the water storage tissue. The area occupied by chloroplasts within a mesophyll cell was measured for three cells (adaxial and abaxial) and the percent cross-sectional chloroplast area was calculated as the ratio of chlorophyll area divided by mesophyll cell area.

We calculated the surface area of mesophyll cells per leaf area (A_{mes}/A) as described by Sack *et al.* (2013*a*), a measure of the area available for CO_2 uptake for mesophyll cell layers. Given the lack of palisade-form cells, we modelled all mesophyll cells as spheres for these calculations.

We also measured vascular anatomy to quantify traits related to the efficiency of water transport within and outside the xylem. We measured the inter-veinal distance (*IVD*) and also the minimum distance from edge of bundle sheath to epidermis (D_m) as the hypotenuse between the distance between veins and the distance to the epidermis (Brodribb Feild and Jordan 2007):

$$D_m = \sqrt{\frac{IVD^2}{2} + (\text{distance from bundle sheath edge to epidermis})^2}. \quad (2)$$

We averaged *IVD* and D_m from three values for each cross-section.

For all anatomical traits, except those relating to the central water storage tissue, measurements were made both adaxial and abaxial halves, and values for the two halves were averaged when not significantly different (at $P < 0.05$ in paired *t*-tests), except they were summed for total A_{mes}/A.

To characterise the midrib, for three typical fibrous bundle sheath cells we measured the cross-sectional heights, widths and cell wall thicknesses, calculating mean values for each trait from three measurements per cross-section. To characterise the xylem anatomy and theoretical conductivity of xylem conduits, for a typical conduit within the midrib, an intermediary vein, and a minor vein of each sampled phylloclade, we treated the conduit as an ellipse and determined the major and minor axis diameters. We calculated the theoretical hydraulic conductivity of the xylem conduit using Poiseuille's equation for ellipses based on conduit dimensions (Lewis and Boose 1995; Cochard Nardini and Coll 2004):

Fig. 1. (*a*) *Ruscus aculeatus* and (*b*) *R. microglossum* growing at the Mildred E Mathias Botanical Garden. Note that what at first glance appear to be leaves are in fact phylloclades.

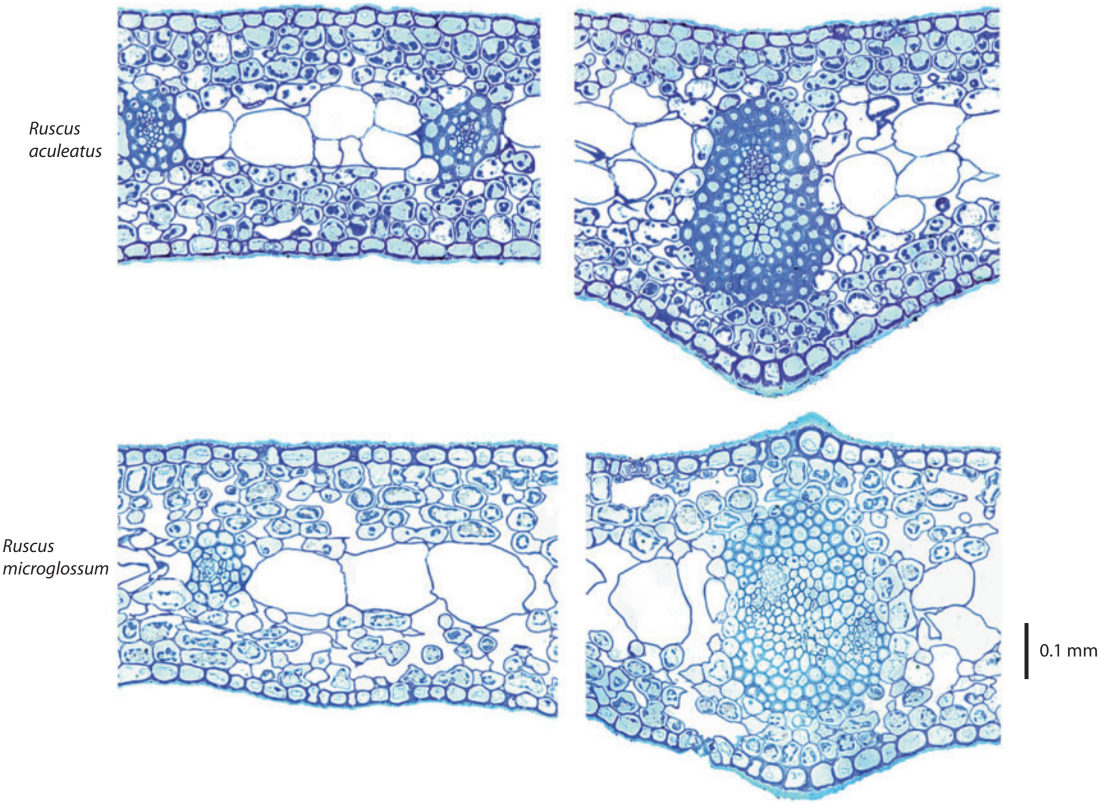

Fig. 2. Lamina and midrib cross-sections of *Ruscus aculeatus* and *R. microglossum* phylloclades (0.5 μm thick). Note that both species exhibit shade tolerance features such as absence of palisade tissue, as well as drought tolerance features such as large water storage compartment and thick-walled epidermis and fibrous bundle sheath cells surrounding the xylem and phloem for both major and minor veins, especially prominent in *R. aculeatus*, which is native to drier habitats.

$$K_t = \frac{\pi a^3 b^3}{64\eta(a^2 + b^2)}, \tag{3}$$

where a and b are the major and minor axes of the ellipse and η is water viscosity at 25°C.

Gas-exchange measurements, responses to light and CO_2, and cuticular conductance

In July and August 2009, photosynthetic light response curves and CO_2 response curves were measured using a Li-6400 portable photosynthesis system (Li-Cor Biosciences) with light provided by a red-blue light source (6400–02B no. SI-710; Li-Cor Biosciences). Gas-exchange measurements were made on at least 1–2 phylloclades per individual.

For light-response curves, phylloclades were acclimated for at least 5 min at 1500 μmol m^{-2} s^{-1} PAR, at temperatures of 25–27°C, with RH maintained at ~50%, and CO$_2$ concentration of 400 μmol mol^{-1}. Then, phylloclades were measured for net CO$_2$ assimilation per leaf area at PAR steps of 1600, 1400, 1200, 1000, 800, 600, 500, 400, 300, 200, 100, 50, and 0 μmol m^{-2} s^{-1}, with 180–240 s stabilisation at each irradiance step. We determined light-saturated photosynthetic rate per area and mass (A_{area} and A_{mass}); dark respiration rate per area and mass (R_{area} and R_{mass}), i.e. the negative A at zero PAR; maximum stomatal conductance per area (g_{s}); light compensation point (LCP) as the x-intercept; intrinsic water use efficiency (WUE, $A_{\mathrm{area}}/g_{\mathrm{s}}$); and the ratio of intercellular to ambient CO$_2$ concentration ($C_{\mathrm{i}}/C_{\mathrm{a}}$) at 100 μmol m^{-2} s^{-1} PAR. We then harvested the phylloclades measured for gas exchange to determine the nitrogen concentration and carbon isotope ratio (see below).

For CO$_2$-response curves, phylloclades were allowed to equilibrate at 400 ppm to induce stomatal opening, and the net CO$_2$ assimilation per leaf area was determined at C_{i} steps of 400, 300, 200, 100, 50, 400, 400, 500, 600, 800, 1200, 1400, 1600 ppm, with 3–4 min equilibration time at each step. We determined maximum rate of carboxylation and maximum rate of electron transport per leaf area ($V_{\mathrm{c,max}}$ and J_{max}) from plots of C_{i} vs A, corrected to 25°C (Farquhar and von Caemmerer 1980).

Cuticular conductance (i.e. minimum epidermal conductance; g_{min} *sensu* Kerstiens 1996) was determined for 3–4 mature phylloclades and 10 cm lengths of stems from each of three individuals of each species. Phylloclade and stem samples were hydrated, and then the cut ends were sealed with wax. Samples were dried for at least 30 min on a laboratory bench, at PAR of <10 μmol photons m^{-2} s^{-1} to induce stomatal closure, then samples were weighed for at least eight intervals of 30 min, during which the slope of water loss versus time was highly linear ($R^2 > 0.995$) and therefore taken to represent transpiration after stomata had closed fully. The g_{min} was calculated as the transpiration rate divided by the mole fraction vapour pressure deficit (VPD, determined from a weather station; HOBO Micro Station with Smart Sensors, Onset, Bourne, MA, USA).

Pressure–volume curve parameters

Pressure–volume curve parameters were determined for mature shoots using the bench-drying method (Koide *et al*. 2000; Sack *et al*. 2003*a*). Shoots were ~10–15 cm long, with about four phylloclades per shoot for *R. microglossum* and 15–20 phylloclades for *R. aculeatus*. Shoots were progressively dried on a laboratory bench, and measured at intervals by equilibrating for 10 min in a Whirlpak bag (Whirl-Pak, Nasco, Fort Atkinson, WI, USA) before weighing and measuring for leaf water potential (Ψ_{leaf}) with a pressure chamber (Model 1000, Plant Moisture Stress Instruments, Albany, OR, USA). Subsequently, dry mass was determined after more than 48 h in an oven at 70°C. We determined the leaf dry matter content (LDMC), saturated water content (SWC), turgor loss point (Ψ_{tlp}), relative water content at turgor loss point (RWC_{tlp}), and osmotic potential at full turgor (π_{o}), relative capacitance at full turgor (C_{ft}; $\Delta RWC/\Delta\Psi_{\mathrm{leaf}}$), and relative capacitance at turgor loss point (C_{tlp}; $\Delta RWC/\Delta\Psi_{\mathrm{leaf}}$). We determined the modulus of elasticity (ε) as the linear slope of the line fitted for pressure potential versus relative water content above and including turgor loss point (Sack *et al*. 2013*b*).

Shoot hydraulic conductance

We measured the hydraulic conductance of mature shoots (K_{shoot}) ~10–15 cm long, bearing about four phylloclades per shoot for *R. microglossum* and 15–20 phylloclades for *R. aculeatus*, using the evaporative flux method (Sack *et al*. 2002). Samples were harvested and the ends were re-cut under distilled, degassed water with a fresh razor blade, then hydrated overnight. Shoots were connected to tubing containing distilled, degassed water, running to a graduated cylinder on a balance interfaced to a computer logging data every minute to calculate the flow rate of water from the balance into the sample. Samples were held in place above a fan on wood frames strung with fishing line. Lights were arranged above a plexiglass container of water that acted as a heat trap, producing PAR of >1200 μmol m^{-2} s^{-1} at shoot level. When steady-state transpiration was achieved, samples were covered with a plastic bag and removed from the tubing, and the leaf water potential was determined with a pressure chamber (Model 1000, Plant Moisture Stress Instruments). K_{shoot} was calculated as the steady-state transpirational flow rate (E, mmol m^{-2} s^{-1}) divided by the water potential driving force ($\Delta\Psi_{\mathrm{lea}F} = -\Psi_{\mathrm{leaf}}$; MPa), further normalised by total phylloclade area. K_{shoot} values were standardised to 25°C to correct for the temperature dependence of the viscosity of water (Sack *et al*. 2003*a*).

Foliar nitrogen concentration and carbon isotope ratio

To analyse total tissue nitrogen concentration and carbon isotope composition (δ^{13}C), for three replicates from each of three individuals for each species, phylloclade samples were oven-dried at >70°C for more than 48 h, and ground by mortar and pestle. The nitrogen concentration and δ^{13}C were determined at the UC Davis Stable Isotope Facility using an elemental analyser (PDZ Europa ANCA-GSL, Sercon Ltd, Cheshire, UK) interfaced to an isotope ratio mass spectrometer (PDZ Europa 20–20, Sercon Ltd) at the UC Davis Stable Isotope Facility. Final δ^{13}C content values are expressed relative to international standard Vienna Pee Dee belemnite (V-PDB) for carbon:

$$\delta^{13}\mathrm{C}(‰) = ((^{13}\mathrm{C}/^{12}\mathrm{C}\ \text{of sample})/ (^{13}\mathrm{C}/^{12}\mathrm{C}\ \text{of standard}) - 1) \times 1000 \qquad (4).$$

δ^{13}C of plant tissues can provide a measure of intrinsic WUE (A/g_{s}) at the time that carbon was assimilated, giving long-term water-use efficiency.

Comparative data compilation and trait comparison

To consider *Ruscus* trait values in a global context, we compiled data from the literature for: (1) temperate and tropical broadleaf evergreen species; (2) Mediterranean species; and (3) woody angiosperms in general. When available, we considered data for plants grown in the shade and for species that are shade tolerant;

however, eco-physiological trait data is generally collected for sun leaves or plants, making a comparison solely for shade grown plants not possible. For more commonly studied traits (e.g. $A_{\max}$), comparative data was taken from studies with large databases and multiple traits (e.g. GLOPNET; Wright *et al.* 2004). We determined minimum, mean, and maximum values for traits from comparative studies, or the mean and standard error if raw species values were not available. Studies that were included for comparative data are referenced in the captions for Table 1–5 in association with the specific traits we compared. Differences between *Ruscus* trait values and comparative data from the literature were determined for both *R. aculeatus* and *R. microglossum*. Hypotheses were deemed supported for a *Ruscus* species if the trait value was higher or lower than the mean comparator value in the way predicted.

Results

Both *Ruscus* species studied had leaf morphology consistent with adaptation to shade and drought, relative to comparator species (Tables 1–5). We constructed a radar graph to encapsulate the key traits that would confer adaptation to shade, drought, and the combination, for *R. aculeatus*, which had the more extreme adaptation of the two *Ruscus* species considered (Fig. 3). This figure summarises the major results of our study, with values for comparator species appearing as the inner circle and *R. aculeatus* trait values displayed as the bold, outer line.

Gross morphology of phylloclades

Both *R. aculeatus* and *R. microglossum* had LMA values lower than comparator species, and *R. microglossum* had phylloclades larger than the mean leaf area for comparator species. Both *Ruscus* species had lower bulk tissue density than comparator species, consistent with the water storage in the *Ruscus* phylloclades. Notably, despite its water storage tissue, the LDMC and SWC values of *R. aculeatus* were typical of those for comparator species, whereas *R. microglossum* had a lower LDMC and a higher SWC than for comparator species sets (Table 1).

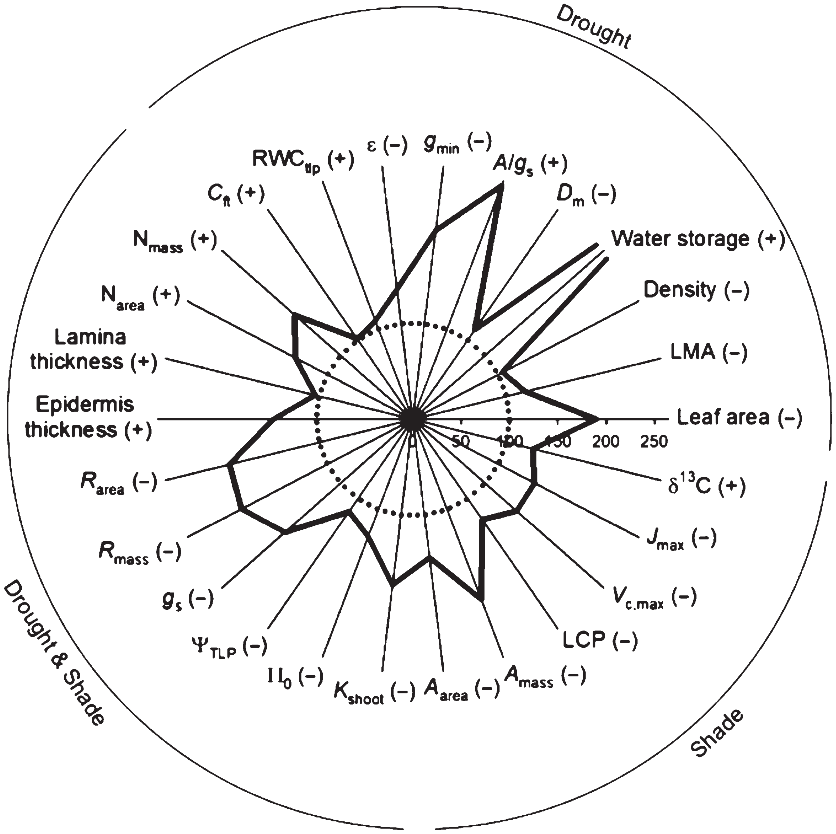

Fig. 3. Radar graph illustrating percent difference between selected traits of *Ruscus aculeatus* with comparative data (see Tables 1–5 for symbols and sources of comparative data). Values for *R. aculeatus* outside the circle indicate shade and/or drought tolerance. Traits are arranged according to whether they would contribute shade tolerance, drought tolerance or both. The inner disc represents the mean for comparative species for given traits, and the values for *R. aculeatus* is scaled relative to the magnitude of that value (%), with a value outside the disc representing greater shade and/or drought tolerance; (+) and (–) indicate if the axis is scaled such that a value outside the circle represents percent higher or lower than comparative values respectively. For traits expressed as negative values, (+) and (–) indicate more negative or less negative respectively.

Anatomy of phylloclades

The *Ruscus* species possessed numerous anatomical traits consistent with benefits for both shade and drought tolerance (Table 2). The phylloclade cross-sections were symmetrical (Fig. 2), and thus the cross-sectional anatomy of the adaxial and abaxial halves were not different for all traits (paired *t*-test; $P > 0.05$) and values were averaged within species. The two species were similar in their substantial leaf thickness and in the thickness of cuticle, epidermis cell walls and water storage compartment (Table 2). The fractions of the lamina occupied by the epidermis, mesophyll and water storage tissues were 15, 52–56, and 29–32% respectively.

Both species had parallel longitudinal veins of three sizes (midrib, intermediate, and small veins). Consistent with drought adaptation, the two species had low D_m, *R. aculeatus* having the lower value, and *R. microglossum* had a higher *IVD* (Table 2). The two species had, on average, the same maximum conduit diameters in their midribs, intermediate veins, and small veins, and the maximum conduit diameter decreased ~35% from the midrib to the small veins. The average theoretical hydraulic conductivity of xylem conduits did not differ between the species, and decreased by up to 86% from the midrib to the minor vein. *R. aculeatus* had, on average, walls that were 85% thicker in the fibrous bundle sheath (Table 2).

Gas-exchange measurements

Consistent with adaptation to simultaneous shade and drought, the *Ruscus* species had very low values for R_{area}, R_{mass}, and g_s, (Table 3) relative to comparator temperate broadleaf evergreen species (Fig. 3). The A_{area}, A_{mass}, LCP, $V_{c,\max}$ and $J_{\max}$ were also very low relative to comparator species (Fig. 3; Table 3), consistent with shade adaptation. The two *Ruscus* species had very high values for A/g_s (Table 3), consistent with excellent WUE. Consistent with strong drought tolerance via retention of stored water, both species had very low values for leaf and stem $g_{\min}$, especially relative to comparator vascular plant species (Fig. 3; Table 3).

Hydraulic conductance, pressure volume curve parameters and leaf water storage

Consistent with expectations for combined drought and shade tolerance, both *Ruscus* species had low K_{shoot} relative to comparator tropical and temperate woody angiosperms (Fig. 3; Table 4). The pressure–volume curve parameters of *Ruscus* were consistent with achieving drought tolerance through tissue water storage. Both species had less negative π_o and ψ_{tlp} than mean values for comparative evergreen woody species (Fig. 3; Table 4). Notably, both species had lower ε values than comparative evergreen woody species, and higher RWC_{tlp} and C_{ft} values (Fig. 3; Table 4), consistent with drought tolerance.

Nitrogen concentration and carbon isotope composition

Both species had high values for phyllyclade N_{area} and N_{mass} relative to comparative temperate broadleaf evergreen species, consistent with drought adaptation (Fig. 3). The $\delta^{13}C$ values were very negative and typical of values often observed for understory plants (da Silveira *et al.* 1989), consistent with shade tolerance. Indeed, the $\delta^{13}C$ values were notably strongly negative given the high WUE found for these species.

Testing hypotheses for shade and drought tolerance with trait survey data

Overall, we quantified 57 traits for the two *Ruscus* species, and for 21 traits we had *a priori* hypotheses for a benefit for shade tolerance and for 24 traits we had *a priori* hypotheses for a benefit for drought tolerance. Using comparative data, we found that 16 of 21 hypotheses were supported for shade tolerance, and 22 of 24 were supported for drought tolerance. Of the nine hypotheses for traits that would contribute to both shade and drought tolerance simultaneously (i.e. expectations were both for higher or lower values than comparative species), eight were supported by trait data. All these proportions were significantly higher than the 50% support that would have been expected to arise only from chance ($P = 0.001$–0.058; proportion tests). Notably, in the seven cases when shade tolerance traits would conflict with drought tolerance traits, four indicated a benefit for drought tolerance rather than shade tolerance for both species (Tables 1–5).

Discussion

Both *R. aculeatus* (Fig. 3) and *R. microglossum* showed trait values consistent with combined shade and drought tolerance. Numerous traits were consistent with a combined shade and drought tolerance through improving carbon balance, enabling a conservative resource use, i.e. via slow respiration and long-lived parts. Other traits would contribute specifically to drought tolerance via reduced demand for water during active photosynthesis and the ability to survive strong drought after stomatal closure. Notably, many such traits were related to water storage, providing new insights into effective forms of succulence in shaded habitats. This suite of traits would contribute importantly to the ability of *Ruscus* to occupy shaded sites prone to strong drought across a wide geographical range.

Traits contributing to simultaneous drought and shade tolerance

Ruscus species showed specialisation associated with conservative resource use consistent with tolerance of shade and drought. These specialised traits included thick lamina and component tissues that contribute to long tissue life-spans (shoots last >5 years; Sack *et al.* 2003*b*; Wright *et al.* 2004). Additionally, *Ruscus* species had very low gas-exchange rates, including low g_s, and low K_{shoot}, which would correspond to a low investment in vascular tissue (Tyree and Zimmermann 1983; Sack *et al.* 2003*b*), and low R_{area}, R_{mass}, A_{area} and A_{mass}, all representing an ability to maintain photosynthesis and growth with low requirements for light and water.

Traits contributing to drought tolerance

According to Jones *et al.* (1992), drought tolerance can be achieved through avoidance of plant water deficits, tolerance of plant water deficits or efficiency mechanisms. *Ruscus* species showed traits associated with drought tolerance either by providing the ability to maintain photosynthesis and growth in drying soil and/or the ability to survive chronic drought, as previously shown experimentally for *R. aculeatus* (Sack 2004). Traits that would contribute to the ability to maintain gas exchange in drying soil include small leaf size, high WUE and low *IVD*. High WUE, achieved in part with high N_{area}, means these *Ruscus* species can attain positive carbon balance even with extremely low g_s. Low *IVD* and water storage capacitance allow the phylloclades to maintain water supply to the mesophyll and tolerate transiently high evaporation rates, for example, due to sunflecks, without desiccating the leaf (Sack *et al*. 2003*a*). Traits contributing to the ability of the phylloclades to survive extended drought included those enabling a low evaporation rate per leaf area once stomata have shut, such as low g_{min} in leaf and stem, and those related to specialised water storage tissue, linked with low leaf density, low ε, and π_o values that were low in magnitude.

Ruscus *water storage*

The water storage tissue of *Ruscus* that occupied a third of the leaf thickness, although contributing most directly to drought tolerance, is also consistent with shade tolerance, given its contribution to reduced tissue costs for the phylloclade as a whole. The water storage tissue had thin cell walls, reflected in the low bulk ε, and low solute concentration, contributing to bulk π_o values that were low in magnitude.

This water storage tissue would also contribute to both types of drought tolerance – the ability to maintain photosynthesis in drying soil and to survive after stomata have shut during extended drought. The ‘succulence’ of *Ruscus* phylloclades is distinctive relative to more typical succulent-leafed and succulent-stemmed species, which tend to have high leaf water content and capacitance values (Vendramini *et al*. 2002; Ogburn and Edwards 2012). In contrast, in *Ruscus* species, the SWC was low relative to typical leaf succulent species, and for *R. aculeatus*, C_{ft} fell within the range of typical evergreen leaves. We note that the strong tissue differentiation in *Ruscus* (i.e. separation of mesophyll cell and water storage in space and their distinctions in anatomy) would contribute to high effectiveness of water storage, even if the bulk tissue overall had low SWC and capacitance. Indeed, across species there tends to be no relationship between the magnitude of SWC or capacitance and the degree of within-leaf tissue differentiation (Ogburn and Edwards 2012). Notably, such differentiation would contribute special advantages for supply of water, whether stomata are open or closed, as the large, thin-walled water storage cells with low solute concentration can yield their water to supply the evaporative load, while the photosynthetic tissues can maintain their volume according to their thicker walls and stronger solute concentration.

Although the capacitance and SWC values were low for *Ruscus* species, these values would be more substantial if considered relative to water demand. Across several species, C_{ft} has been found to correlate with K_{shoot} and with g_s (Sack *et al*. 2003*a*; Blackman *et al*. 2010), indicating that leaves tend to be built with capacitance to match their maximum flux rates, and thus to buffer the leaf water potential against surges in transpiration. Thus, because *Ruscus* has low g_s when stomata are open and low K_{shoot}, the capacitance would be expected to supply transpiration transiently during sunflecks or high VPD. Likewise, when stomata close, the capacitance supplies ongoing water loss via cuticular conductance. Given the extremely low g_{min} of *Ruscus*, even its moderate C_{tlp} can enable survival for weeks (Sack *et al*. 2003*b*; Sack 2004). Further, at turgor loss, the water content would equal SWC × RWC_{tlp}, and the relatively high RWC_{tlp} would contribute to the high water content once turgor is lost. Thus, the ‘succulence’ of *Ruscus* is moderate in absolute terms, but combined with its other very strong mechanisms to reduce transpiration when stomata are open or closed, i.e. low g_s and g_{min}, even this moderate capacitance would provide strong functionality.

Water-use efficiency and carbon isotope composition

It was noteworthy that despite extremely high WUE values, phylloclade $\delta^{13}C$ values of the *Ruscus* species were very negative. This presents a strong anomaly worthy of further investigation, as species with high WUE typically have higher $\delta^{13}C$ (less negative, i.e. closer to zero). The $\delta^{13}C$ can be influenced by a host of processes including source CO_2, stored plant carbon, and time-integrated CO_2 concentration at the site of carboxylation (Farquhar *et al*. 1989). For *Ruscus*, although $\delta^{13}C$ values were typical of an understory plant, they did not appear to be driven by internal CO_2 concentration because the leaf isotopic values were depleted in ^{13}C, whereas the high WUE determined by gas exchange would likely promote enriched isotopic values. It is more likely that $\delta^{13}C$ in this species was determined by source CO_2 or stored carbon, or recycling of respired CO_2 (da Silveira *et al*. 1989). Our study individuals of *Ruscus* were cultivated in a shaded understory, similar to their natural habitat. Previous studies have shown that there can be higher concentrations of respired CO_2 in the forest understory than in the canopy, resulting in more negative carbon isotope ratios in understory plant tissue than canopy plant tissue (da Silveira *et al*. 1989). This is a function of decomposing leaves and litter cover, slow air mixing, as well as plant environmental responses.

A second possible explanation for the $\delta^{13}C$ values of *Ruscus* is related to its growth form and phenology, as *Ruscus* has extensive rhizomes (Sack *et al*. 2003*b*), which act as a carbon store for the plant. Using this recycled carbon during the growth season, when the plant may not be able to meet its carbon requirement by photosynthesis alone because of low maximum rates under light limitation, may also contribute to more negative $\delta^{13}C$ values (Vizzini 2003). Notably, *Ruscus* stems are hollow and thus can also store relatively large amounts of CO_2, for use during a growth period when carbon is otherwise limiting, especially given very low g_s. Some of this stored carbon might be photosynthetically fixed by the stem (Nilsen and Sharifi 1997). In each of these cases, respired, stored, or recycled CO_2 would supply carbon that was previously fixed by Rubisco with more negative $\delta^{13}C$ values (–27‰) than air (–8‰).

There was also a dissonance between the $\delta^{13}C$ value and C_i/C_a. The fully mature phylloclades which were selected and used for gas-exchange and $\delta^{13}C$ measurements were produced during late winter and early spring with mild temperatures (average at midday 19.0–22.0°C) and low atmospheric VPD (average at midday 0.6–1.5 kPa). However, the gas-exchange measurements were taken during summer with high temperature (average at midday 30.0–35.5°C) and high VPD (average at midday 2.5–3.5 kPa). We harvested the phylloclades that we used for gas exchange to determine the carbon isotope ratio. Although the low value of instantaneous C_i/C_a may be caused by high temperature and high atmospheric VPD, the very low value of $\delta^{13}C$ may represent the time when the phylloclade's carbon was assimilated (during mild temperature and low VPD).

Implications for drought and shade tolerance: Ruscus *as a model*

We found strong support for a large number of hypotheses for trait-based shade and drought tolerance, providing a strong trait basis for combined tolerance. Although the detailed functional trait survey conducted here is relatively novel in its breadth (see also Pasquet-Kok *et al*. 2010), this is a logical extension of the traditional approach for understanding the basis for plant adaptation to environment, i.e. testing expectations for individual traits established by previous studies of the functional significance of these traits in other species. We acknowledge there is some degree of uncertainty in interpreting a large number of traits simultaneously based on studies of other species. First, the interpretation of the value of traits based on other species may not be in all cases equally valid for *Ruscus*. Some trait variation may relate to other functions. Further studies, for example, using mutants, would be necessary as conclusive evidence of the value of specific traits in a given species. However, one advantage of testing numerous expectations for each hypothesis is that the key finding will be robust to the removal of some traits from the analysis if those are later found to be inappropriate. Ideally, when a model for estimating plant performance from leaf traits becomes available, one could determine how the specific quantitative combinations of traits presented here scales up to plant shade and drought tolerance.

The shade and drought tolerance of *Ruscus*, consistent with the suite of traits examined here, is one case demonstrating how plants can avoid a general trade-off between shade and drought tolerance. Further, *Ruscus* is noteworthy as one of only a few stem photosynthetic plants that occupy a shaded habitat. Historically, it is likely that shade tolerance preceded drought tolerance given this species' ancestors were species of moist tropical forests (Kim *et al*. 2010), and thus *Ruscus* or its ancestor apparently evolved drought tolerance while expanding its range into drier habitat or during past climate change. Considering its unique adaptations and trait values, *Ruscus* can serve as an excellent model for the basis of combined shade and drought tolerance.

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References

Bartlett MK, Scoffoni C, Sack L (2012) The determinants of leaf turgor loss point and prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecology Letters* **15**, 393–405. doi:10.1111/j.1461-0248.2012.01751.x

Blackman CJ, Brodribb TJ, Jordan GJ (2010) Leaf hydraulic vulnerability is related to conduit dimensions and drought resistance across a diverse range of woody angiosperms. *New Phytologist* **188**, 1113–1123. doi:10.1111/j.1469-8137.2010.03439.x

Brodribb TJ, Feild TS, Jordan GJ (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology* **144**, 1890–1898. doi:10.1104/pp.107.101352

Caspersen JP (2001) Interspecific variation in sapling mortality in relation to growth and soil moisture. *Oikos* **92**, 160–168. doi:10.1034/j.1600-0706.2001.920119.x

Cochard H, Nardini A, Coll L (2004) Hydraulic architecture of leaf blades: where is the main resistance? *Plant, Cell & Environment* **27**, 1257–1267. doi:10.1111/j.1365-3040.2004.01233.x

Cooney-Sovetts C, Sattler R (1987) Phylloclade development in the Asparagaceae: an example of homoeosis. *Botanical Journal of the Linnean Society* **94**, 327–371. doi:10.1111/j.1095-8339.1986.tb01053.x

Cowling R, Rundel P, Lamont BB, Arroyo MK, Arianoutsou M (1996) Plant diversity in Mediterranean-climate regions. *Trends in Ecology & Evolution* **11**, 362–366. doi:10.1016/0169-5347(96)10044-6

da Silveira L, Sternberg L, Mulkey SS, Wright SJ (1989) Ecological interpretation of leaf carbon isotope ratios: influence of respired carbon dioxide. *Ecology* **70**, 1317–1324. doi:10.2307/1938191

de Lillis M, Fontanella A (1992) Comparative phenology and growth in different species of the Mediterranean maquis of central Italy. *Plant Ecology* **99-100**, 83–96. doi:10.1007/BF00118213

Dracup JA (1991) Drought monitoring. *Stochastic Hydrology and Hydraulics* **5**, 261–266. doi:10.1007/BF01543134

Engelbrecht BMJ, Kursar TA (2003) Comparative drought-resistance of seedlings of 28 species of co-occurring tropical woody plants. *Oecologia* **136**, 383–393. doi:10.1007/s00442-003-1290-8

Farquhar GD, von Caemmerer S (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149**, 78–90. doi:10.1007/BF00386231

Farquhar GD, Ehleringer JR, Hubick K (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 503–537. doi:10.1146/annurev.pp.40.060189.002443

Givnish TJ (1988) Adaptation to sun and shade: a whole-plant perspective. *Australian Journal of Plant Physiology* **15**, 63–92. doi:10.1071/PP9880063

Gulias J, Flexas J, Mus M, Cifre J, Lefi E, Medrano H (2003) Relationship between maximum leaf photosynthesis, nitrogen content and specific leaf area in Balearic endemic and non-endemic Mediterranean species. *Annals of Botany* **92**, 215–222. doi:10.1093/aob/mcg123

Haberlandt G (1914) 'Physiological plant anatomy.' (MacMillan: London)

Hallik L, Niinemets U, Wright J (2009) Are species shade and drought tolerance reflected in leaf-level structural and functional differentiation in Northern Hemisphere temperate woody flora? *New Phytologist* **184**, 257–274. doi:10.1111/j.1469-8137.2009.02918.x

Jones HG (1992) 'Plants and microclimate: a quantitative approach to environmental plant physiology.' (Cambridge University Press: Cambridge)

Kerstiens G (1996) Cuticular water permeability and its physiological significance. *Journal of Experimental Botany* **47**, 1813–1832. doi:10.1093/jxb/47.12.1813

Kim JH, Kim DK, Forest F, Fay MF, Chase MW (2010) Molecular phylogenetics of Ruscaceae *sensu lato* and related families (Asparagales) based on plastid and nuclear DNA sequences. *Annals of Botany* **106**, 775–790. doi:10.1093/aob/mcq167

Koide RT, Robichaux RH, Morse SR, Smith CM (2000) Plant water status, hydraulic resistance and capacitance. In 'Plant physiological ecology: field methods and instrumentation'. (Eds R Pearcy, JR Ehleringer, H Mooney, P Rundel) pp. 161–183. (Kluwer: Dordrecht, The Netherlands)

Körner C, Farquhar GD, Wong SC (1991) Carbon isotope discrimination by plants follows latitudinal and altitudinal trends. *Oecologia* **88**, 30–40. doi:10.1007/BF00328400

Lewis AM, Boose ER (1995) Estimating volume flow rates through xylem conduits. *American Journal of Botany* **82**, 1112–1116. doi:10.2307/2446063

Martínez-Pallé E, Aronne G (1999) Flower development and reproductive continuity in Mediterranean *Ruscus aculeatus* L. (Liliaceae). *Protoplasma* **208**, 58–64. doi:10.1007/BF01279075

Martínez-Tillería K, Loayza AP, Sandquist DR, Squeo FA (2012) No evidence of a trade-off between drought and shade tolerance in seedlings of six coastal desert shrub species in north-central Chile. *Journal of Vegetation Science* **23**, 1051–1061. doi:10.1111/j.1654-1103.2012.01427.x

Matalas NC (1963) Probability distribution of low flows. USGS Professional Papers 434-A, US Government Printing Office, Washington DC.

Niinemets U (1999) Research review: components of leaf dry mass per area – thickness and density – alter leaf photosynthetic capacity in reverse directions in woody plants. *New Phytologist* **144**, 35–47. doi:10.1046/j.1469-8137.1999.00466.x

Niinemets U, Valladares F (2006) Tolerance to shade, drought, and waterlogging of temperate northern hemisphere trees and shrubs. *Ecological Monographs* **76**, 521–547. doi:10.1890/0012-9615(2006)076[0521:TTSDAW]2.0.CO;2

Nilsen ET, Sharifi MR (1997) Carbon isotope composition of legumes with photosynthetic stems from Mediterranean and desert habitats. *American Journal of Botany* **84**, 1707–1713. doi:10.2307/2446469

Ogburn RM, Edwards EJ (2012) Quantifying succulence: a rapid, physiologically meaningful metric of plant water storage. *Plant, Cell & Environment* **35**, 1533–1542. doi:10.1111/j.1365-3040.2012.02503.x

Pasquet-Kok J, Creese C, Sack L (2010) Turning over a new 'leaf': multiple functional significances of leaves versus phyllodes in Hawaiian *Acacia koa*. *Plant, Cell & Environment* **33**, 2084–2100. doi:10.1111/j.1365-3040.2010.02207.x

Sack L (2004) Responses of temperate woody seedlings to shade and drought: do trade-offs limit potential niche differentiation? *Oikos* **107**, 110–127. doi:10.1111/j.0030-1299.2004.13184.x

Sack L, Frole K (2006) Leaf structural diversity is related to hydraulic capacity in tropical rain forest trees. *Ecology* **87**, 483–491. doi:10.1890/05-0710

Sack L, Holbrook NM (2006) Leaf hydraulics. *Annual Review of Plant Biology* **57**, 361–381. doi:10.1146/annurev.arplant.56.032604.144141

Sack L, Melcher PJ, Zwieniecki MA, Holbrook NM (2002) The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of Experimental Botany* **53**, 2177–2184. doi:10.1093/jxb/erf069

Sack L, Cowan P, Jaikumar N, Holbrook NM (2003*a*) The "hydrology" of leaves: co-ordination of structure and function in temperate woody species. *Plant, Cell & Environment* **26**, 1343–1356. doi:10.1046/j.0016-8025.2003.01058.x

Sack L, Grubb P, Maronon T (2003*b*) The functional morphology of juvenile plants tolerant of strong summer drought in shaded forest understories in southern Spain. *Plant Ecology* **168**, 139–163. doi:10.1023/A:1024423820136

Sack L, Scoffoni C, McKown AD, Frole K, Rawls M, Havran JC, Tran H, Tran T (2012) Developmentally based scaling of leaf venation architecture explains global ecological patterns. *Nature Communications* **3**, 837. doi:10.1038/ncomms1835

Sack L, Chatelet D, Scoffoni C, *PrometheusWiki* contributors (2013*a*) "Estimating the mesophyll surface area per leaf area from leaf cell and tissue dimensions measured from transverse cross-sections," *PrometheusWiki*. Available at http://www.publish.csiro.au/prometheuswiki/tiki-pagehistory.php?page=Estimating the mesophyll surface area per leaf area from leaf cell and tissue dimensions measured from transverse cross-sections&preview=16 [accessed June 18, 2013]

Sack L, Pasquet-Kok J, *PrometheusWiki* contributors (2013*b*) "Leaf pressure-volume curve parameters," *PrometheusWiki*. Available at http://prometheuswiki.publish.csiro.au/tiki-index.php?page=Leaf+pressure-volume+curve+parameters.

Scoffoni C, Pou A, Aasamaa K, Sack L (2008) The rapid light response of leaf hydraulic conductance: new evidence from two experimental methods. *Plant, Cell & Environment* **31**, 1803–1812. doi:10.1111/j.1365-3040.2008.01884.x

Smith T, Huston M (1989) A theory of the spatial and temporal dynamics of plant communities. *Vegetatio* **83**, 49–69. doi:10.1007/BF00031680

Sterck F, Markesteijn L, Schieving F, Poorter L (2011) Functional traits determine trade-offs and niches in a tropical forest community. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20 627–20 632. doi:10.1073/pnas.1106950108

Thomas GS (1992) 'Ornamental shrubs: climbers and bamboos.' (John Murray Publishers: Great Britain)

Tyree MT, Zimmermann MH (1983) 'Xylem structure and the ascent of sap.' (Springer-Verlag: Berlin)

USDA, ARS, National Genetic Resources Program (2009) Germplasm Resources Information Network (GRIN) online database. National Germplasm Resources Laboratory, Beltsville, Maryland. Available athttp://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?410577 [Accessed 20 July 2009]

Vendramini F, Díaz S, Gurvich DE, Wilson PJ, Thompson K, Hodgson JG (2002) Leaf traits as indicators of resource-use strategy in floras with succulent species. *New Phytologist* **154**, 147–157. doi:10.1046/j.1469-8137.2002.00357.x

Vile D (2005) Specific leaf area and dry matter content estimate thickness in laminar leaves. *Annals of Botany* **96**, 1129–1136. doi:10.1093/aob/mci264

Vizzini S (2003) δ^{13}C and δ^{15}N variability in *Posidonia oceanica* associated with seasonality and plant fraction. *Aquatic Botany* **76**, 195–202. doi:10.1016/S0304-3770(03)00052-4

Walters M, Reich PB (1999) Research review: low-light carbon balance and shade tolerance in the seedlings of woody plants: do winter deciduous and broad-leaved evergreen species differ? *New Phytologist* **143**, 143–154. doi:10.1046/j.1469-8137.1999.00425.x

Witkowski E, Lamont BB (1991) Leaf specific mass confounds leaf density and thickness. *Oecologia* **88**, 486–493.

Wright IJ, Westoby M (2003) Nutrient concentration, resorption and lifespan: leaf traits of Australian sclerophyll species. *Functional Ecology* **17**, 10–19. doi:10.1046/j.1365-2435.2003.00694.x

Wright IJ, Reich PB, Westoby M, Ackerly DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, Flexas J, Garnier E, Groom PK, Gulias J, Hikosaka K, Lamont BB, Lee T, Lee W, Lusk C, Midgley JJ, Navas M-L, Niinemets U, Oleksyn J, Osada N, Poorter H, Poot P, Prior L, Pyankov VI, Roumet C, Thomas SC, Tjoelker MG, Veneklaas EJ, Villar R (2004) The worldwide leaf economics spectrum. *Nature* **428**, 821–827. doi:10.1038/nature02403

Wullschleger SD (1993) Biochemical limitations to carbon assimilation in C_3 plants – a retrospective analysis of the A/C_i curves from 109 species. *Journal of Experimental Botany* **44**, 907–920. doi:10.1093/jxb/44.5.907