Trade-offs between seedling growth, plant respiration and water-use efficiency in two Mediterranean shrubs *Rhamnus alaternus* and *Rhamnus ludovici-salvatoris*

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Abstract

Seedling recruitment is a critical developmental stage in regeneration of plant populations under Mediterranean conditions that strongly depends on water availability. Seed mass and relative growth rate (RGR) may affect the early establishment of seedlings through different physiological processes. Here, we examined the effects of the seed mass and carbon balance on seedling growth under two water regimes in *Rhamnus alaternus* L. and *Rhamnus ludovici-salvatoris,* two Mediterranean shrubs, showing a different ability to recruit seedlings. Plant water consumption and biomass accumulation (ΔB) were measured during three periods of the growth in order to estimate water use efficiency (WUE), RGR, and its components. Additionally, net photosynthesis and leaf, stem, and root respiration were measured in plants grown in pots well watered and under progressive drought. *Rhamnus alaternus* showed the higher seed mass, ΔB, and plant WUE than that of *R. ludovici-salvatoris* in all periods and water regimes. The higher RGR of *R. alaternus* was observed during the first and the second period, but the reverse trend was registered during the third period as a consequence of the higher initial biomass of *R. alaternus.* Also, *R. alaternus* showed a higher specific leaf area and estimated carbon balance than that of *R. ludovici-salvatoris*. The observed differences in ΔB, estimated carbon balance, seed mass, and WUE between both species could explain their different distribution and ability to recruit seedlings under natural conditions.

Additional key words: chilling temperature; leaf area ratio; seedling survival; specific leaf area.

Introduction

Summer drought and precipitation in short periods, when soil water availability and temperature are suitable for plant growth, are the major climatic factors responsible for restriction of growth, productivity, and survival of evergreen woody plants in Mediterranean-type regions (Larcher 2000). In these areas, summer drought severely influences the distribution and composition of vegetation due to water deficit in the leaf tissue and hydraulic failure, which affect many physiological processes and can have ultimate consequences for plant growth and survival (Lambers 1998, McDowell *et al.* 2008, Duncan and Sperry 2014), especially in early phases of development (Boyer *et al.* 2003).

In widely distributed species, different populations are usually subjected to different biotic and abiotic conditions, and therefore to environments with contrasting limitations on seedling establishment. It is well known that, under Mediterranean conditions, seedling recruitment is a critical developmental stage that may strongly depend on water availability (Moles and Westoby 2004) and on species ability to establish quickly before the onset of the drought period. In fact, seedling survival during the first growth period has been described as one of the main bottleneck processes in plant regeneration of several Mediterranean species, such as *Olea europaea* (Rey and Alcantara 2000),

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Abbreviations: DM – dry mass; *E* – transpiration rate; g_s – stomatal conductance; LA – leaf area; LAR – leaf area ratio; LMR – leaf mass ratio; P_N – net photosynthetic rate; P_{Nm}/R_{Dm} – net carbon assimilation to dark respiration ratio on a mass basis; R_D – respiration rate; RGR – relative growth rate; R/S – root:shoot ratio; SLA – specific leaf area; SWC – soil water content; VPD – vapor pressure deficit; WUE_i – intrinsic water-use efficiency (= P_N/g_s); WUE_{WP} – plant water-use efficiency.

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Phillyrea latifolia (Herrera *et al.* 1994), *Rhamnus alaternus* (Gulías *et al.* 2004), and *Rhamnus ludovici-salvatoris* (Traveset *et al.* 2003).

Species ability to accumulate biomass in short periods, when water availability and temperature are favorable, is expected to be a key factor determining its ability to establish and, thus, to successfully regenerate its population under Mediterranean conditions. Relative growth rate (RGR) estimates biomass accumulation per unit of time and can be divided into morphological components, specific leaf area (SLA) and leaf area ratio (LAR), which indicate plant´s investment into leaves, and physiological components, such as net assimilation rate (NAR) and plant respiration that determine the plant's carbon economy (Lambers *et al.* 1989, Pérez-Ramos *et al.* 2010).

Plant ability to produce biomass under water deficit conditions is related to its water use efficiency (WUE). Moreover, species plasticity to regulate stomatal conductance (g_s) and WUE under water limiting conditions is an important trait to determine its specific ability for growth and survival under limiting available water, and it appears to be a key trait in plant adaptation to both different environments and climate change scenario (Lázaro-Nogal *et al.* 2013). WUE can be defined as the balance between biomass gain (kg of biomass produced or mol of CO_2 assimilated) and losses of water (m^3 of water used or mol of water transpired). This term can be measured at different levels (plant community or leaf level) and at different time scales, from months to minutes (Morison *et al.* 2008). At the leaf level, it is common to use single-leaf gas exchange measurements, relating net CO_2 assimilation rate (P_N) either to g_s, designated intrinsic WUE_i (P_N/g_s), or P_N to leaf transpiration rate (E), defined as instantaneous WUE_i (P_N/E). Whole-plant WUE (WUE_{WP}) reflects the actual WUE, at a larger spatial (whole plant) and temporal scale (whole growth period) than at that of leaf-level estimates. The interspecific variability in WUE at the leaf level has been studied in many species (Anyia *et al.* 2007, Medrano *et al.* 2009, Barbour *et al.* 2010, Galmés *et al.* 2011, Lelièvre *et al.* 2011, Moreno-Gutiérrez *et al.* 2012, Gratani *et al.* 2013). However, WUE_{WP} has been reported in fewer studies, and mainly in cultivated plants, such as sunflower (Virgona and Farquhar 1996), cowpea (Ismail and Hall 1992), *Malus* rootstocks (Xiaowei *et al.* 2010), coffee (Meinzer *et al.* 1992), or rice (Impa *et al.* 2005). Nevertheless, several articles have attempted to measure or estimate WUE_{WP} of noncultivated species (Wildy *et al.* 2004, Smith and Sperry 2014).

On the other hand, seedling growth can vary according to other life-habit traits of plant species, such as seed mass, specific leaf area, and leaf lifespan. The seed mass represents the amount of reserve provided for the young plant during its early life, being one of the main characteristics influencing seedling early growth (Hou and Romo 1998, Pérez-Ramos *et al.* 2010), although other traits linked to life form are also related to differences in early seedling growth (Houghton *et al.* 2013).

Rhamnus alaternus L. is a perennial dioecious shrub distributed throughout the Mediterranean Basin, flowering during the late winter and early spring, with a peak in mid-February. It produces fleshy fruits, which ripen in the late spring and early summer, and thus represent important water and nutrient source for birds and small mammals (Gulías *et al.* 2004). *Rhamnus ludovici-salvatoris* Chodat is an endemic species of the Balearic Islands, taxonomically related to *Rhamnus alaternus* L. Both are sclerophyllous shrubs and represent a typical case of contrast between phylogenetically related species, with similar morphological traits, but showing a different distribution. *R. ludovici-salvatoris* is able to survive in a wide range of habitats (from 1,000 m a.s.l. and precipitation of 1,200 mm year^{-1} to semiarid areas at sea level and less than 300 mm year^{-1}) probably due to a relatively high plasticity in its water strategy (Lázaro-Nogal *et al.* 2013). Nevertheless, its area of distribution has declined in the last decades, probably due to land use changes and its scarce seedling recruitment ability (Traveset *et al.* 2003) and its low seedling ability to emerge from deep burial (El Aou-ouad *et al.* 2014), which hinders species regeneration after ecosystem disturbance.

The main objective of this study was to compare biomass accumulation, relative growth rate, and water-use efficiency of *R. ludovici-salvatoris* and *R. alaternus* seedlings under well-watered and drought conditions in order to determine their relative contribution to seedling recruitment ability under Mediterranean conditions. Here, we addressed the following specific objectives: (*1*) What is the effect of the seed mass on early seedling biomass accumulation and RGR? (*2*) What is the relative contribution of whole plant photosynthesis and respiration to explain different ability of each species to recruit seedlings under field conditions? (*3*) To what extent water-use efficiency at plant level can explain differences in species seedling recruitment?

Materials and methods

Plant material and experimental design: The experiment was carried out at the University of the Balearic Islands experimental field from September 2009 to July 2010. Since there are no known community, where populations of both species can be found in Mallorca, seeds were collected at two different sites in June and July when fruits were ripened. Seeds of *R. alaternus* were collected at Esporles, a site located at 200 m a. s. l. on a north slope of a hill in the west of the Mallorca Island, with a mean annual precipitation of 629 mm, an average of the daily maximum temperatures of the warmest month of 30.2°C, and an average of the minimum daily temperature of the coldest month of 7.4°C (Guijarro 1986). Seeds of *R. ludovici-salvatoris* were collected at Binifaldo (the site is located at 600 m a.s.l., near the Lluc Monastery; 39°49'N, 2°534'E). Mean annual precipitation is 1,265 mm; the average of the daily maximum and minimum temperatures of the warmest and the coldest months are 27.5°C and 2.5°C, respectively. The seeds were collected in plastic bags from ten individuals per species.

The seeds of both species were germinated in seed benches under glasshouse conditions in September. Seedlings were transplanted to 2-L pots and placed outdoors until mid-January, under a rain shelter in order to prevent rain interferences. The seedlings were grown in a mixture of 40% horticultural substrate, 40% clay calcareous soil, and 20% sand. Pots were covered with a 2-cm layer of Perlite to avoid evaporation from the soil surface.

Soil water regime was controlled by weighing each pot on alternate days and restoring the soil water content specific to each treatment. Water consumption was calculated as the difference of pot mass between days. Three growth periods were considered: the early-spring (from 5 to 24 April); mid-spring (from 25 April to 24 May), and late-spring (from 24 May to 11 July). All plants were maintained under well watered (WW) conditions at a field capacity until late May (24 May) at the end of the second growth period. Thereafter, both species were divided into two groups: (*1*) plants that were maintained under WW conditions, at the field capacity and (*2*) plants that were subjected to a progressive drought (D) and were kept at 30% of the field capacity. Environmental conditions during the experiment were those typically registered under Mediterranean climate (14.0ºC < early spring < 14.7°C; 17.6°C < mid-spring < 18.4°C; 17.7ºC < late spring < 30.5°C; 86% < RH < 51%).

Biomass production, relative growth rate and water-use efficiency: The seed mass was determined on 100 seeds per species before sowing. Plant biomass was measured on six seedlings per species and treatment at the following stages: after emergence (before the expansion of the first leaf) (H0); at the beginning of the 1st growing period (H1), at the end of the 2nd growing period (H2); and at the end of the 3rd growing period (H3). In order to determine dry biomass, leaves, stems, and roots were separated and oven-dried for 48 h at 70ºC. Specific leaf area (SLA, m^2 kg^{-1}) was measured in a sub-sample by measuring leaf dry mass (DM) and leaf area by using a scan (*HP Scanjet G3010*, *HP*®, USA) and a computer program (*Image J 1.42*). Total plant leaf area was then calculated as SLA × total leaf biomass.

Relative growth rate (RGR) of each growing period was calculated as follows:

$$\mathrm{RGR} = [\mathrm{Ln}(\mathrm{B}_{(n+1)}) - \mathrm{Ln}(\mathrm{B}_n)]/\mathrm{n^o days},$$

where B$_n$ is the average plant biomass (n = 6) at the beginning of the growing period, and B$_{(n+1)}$ is plant biomass of each seedling at the end of the growing period. Since biomass determination is a destructive measurement, initial biomass (B$_n$) was assumed the same for each seedling at a given harvest, species, and treatment. The nº of days is the time elapsed between n and n+1.

Leaf area ratio (LAR) was calculated as plant leaf area/plant biomass, leaf mass ratio (LMR) as leaf biomass/plant biomass, and root to shoot ratio (R/S) as root biomass/shoot biomass (Lambers *et al.* 1998).

Plant water-use efficiency (WUE$_{WP}$) was estimated at three growth periods (H1, H2, and H3) as follows:

$$\mathrm{WUE_{WP}}\,(\mathrm{g/\,L}) = (\mathrm{B}_{(n+1)} - \mathrm{B}_n)/\text{total water consumed.}$$

Plant respiration: All tissue samples (leaf discs, stem, and root segments) were harvested and chosen to represent the entire canopy, entire stem, and roots. Each replicate (tissue from individual plant) consisted of four leaf discs or several segments from distal to proximal ends of stem or roots. Tissues were incubated during 30 min in 30 mM MES-CaCl$_2$ buffer (pH = 6.2). Oxygen uptake rates in the dark were measured in 5 ml of the above buffer at 25ºC using a Clark-type of oxygen electrode (*Dual Digital Mod 20; Rank Brothers Ltd*, UK). Buffer was well stirred in a closed system. Respiration measurements were performed with the oxygen electrode technique to avoid the gasket-related leak with the CO$_2$ gas-exchange measurements (Long and Bernacchi 2003, Hurry *et al.* 2005).

Leaf gas exchange: Net CO$_2$ assimilation rate per unit of leaf area (P_N), g_s, and leaf transpiration (E) were measured at between 10:00 and 12:00 h on fully expanded and sun-exposed leaves of six different healthy plants per treatment and species using an infra-red gas analyser *Li-6400* (*Li-cor Inc*., Lincoln, NE, USA). The cuvette conditions were fixed at 1,500 μmol(photon) m^{-2} s^{-1}, to ensure light-saturated photosynthesis, CO$_2$ partial pressure was similar to that of the atmosphere (400 ppm). Intrinsic and instantaneous water use efficiencies were calculated as P_N/g_s and P_N/E, respectively.

Data analysis: All statistical analyses were performed using the *SPSS* package (*SPSS, v. 17.0*). One-way analysis of variance (*ANOVA*) was used to assess the differences between means and to calculate the correlation coefficients between both species and in each harvest and treatment. Variables analyzed [dry mass in each growth period, biomass variation (ΔB), and RGR] were log-transformed in order to normalize their distribution.

Results

Soil water content (SWC) was above 90% in the WW plants (both *R. alaternus* and *R. ludovici-salvatoris*) (Fig. 1). There were no significant differences in SWC between both species. Under water deficit, SWC was reduced progressively until 30% of the field capacity after four and five weeks in *R. alaternus* and *R. ludovici-salvatoris*, respectively. *Rhamnus alaternus* showed a fast drop of SWC in the first week of treatment (from 90 to 70%), and then it fell progressively to 30% by the end of June. *Rhamnus ludovici-salvatoris* showed a similar decrease of SWC down to 30%, although this decrease was more gradual than that showed by *R. alaternus* during the first week.

Biomass production, relative growth rate and water use efficiency: Growth pattern from seeds to up to 10-month-old plants was exponential in both species, although biomass was always significantly higher in *R. alaternus* than that of *R. ludovici-salvatoris* (Fig. 2). The seed mass of *R. alaternus* was three times higher than that of *R. ludovici-salvatoris*, which was maintained in seedlings with two cotyledons (Fig. 2). Thereafter, the difference in biomass between species slightly increased (Fig. 2). Biomass accumulation (ΔB) was always significantly higher in *R. alaternus* than that in *R. ludovici-salvatoris* (Fig. 3*A*). Both species showed a different pattern of RGR (Fig. 3*B*) along harvest periods and treatments. There were no differences in RGR between species during H0-H1 and H2-H3 stages in the D plants, while *R. alaternus* showed significantly higher RGR than that of *R. ludovici-salvatoris* at H1-H2 and H2-H3 under WW.

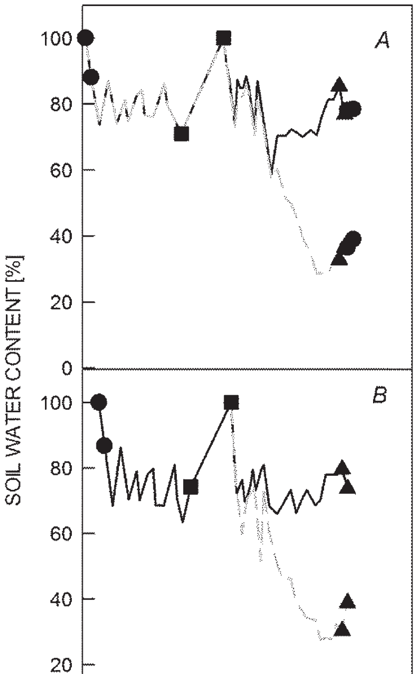

Fig. 1. Soil water content of *Rhamnus ludovici-salvatoris* (*A*) and *R. alaternus* (*B*) under well-watered (*black line*) and progressive drought (*gray line*) conditions during the course of experiment. Harvest symbols: *circle* – first harvest, *square* – second harvest, *triangle* – third harvest under well-watered and water deficit conditions.

Both species showed higher WUE_{WP} during the second period than that of the first one as a consequence of a great biomass accumulation regardless of the very high amount of water lost during the second period (Fig. 4). On the other hand, differences between the WW and D plants in WUE_{WP} were significant only in *R. alaternus*.

Additionally, *R. alaternus* presented as much as three times higher WUE_{WP} than that of *R. ludovci-salvatoris* in both periods and under both soil water conditions.

Mean values of plant growth, total leaf area (LA), specific leaf area (SLA), leaf area ratio (LAR), leaf mass ratio (LMR), and root:shoot ratio (R/S) were determined and are presented in Table 1. Both species increased LA and reduced SLA during the harvest periods, regardless of watering conditions. SLA was significantly higher in *R. alaternus* than that in *R. ludovici-salvatoris* at H3, regardless the water treatment.

R. ludovici-salvatoris showed a similar or slightly higher LAR and LMR than that of *R. alaternus*. The seedling R/S ratio was significanly higher in *R. ludovici-salvatoris* than that of *R. alaternus*, what implies a higher resource allocation to aerial biomass in this species.

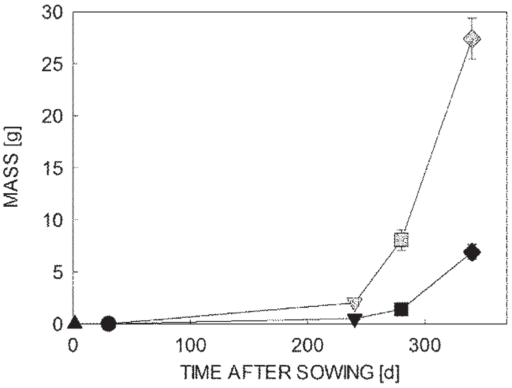

Fig. 2. Evolution of dry mass (from the seed mass up to ten months-old plants) of *Rhamnus alaternus* (*gray symbols*) and *R. ludovici-salvatoris* (*black symbols*). Day 0 correspond to the seed mass, day 30 correspond to seedling after emergence (before the first true leaf appeared); days (240, 280, and 340) correspond to biomass at H1, H2, and H3, respectively, under well-watered treatment. Values represent means of six replicates ± standard error.

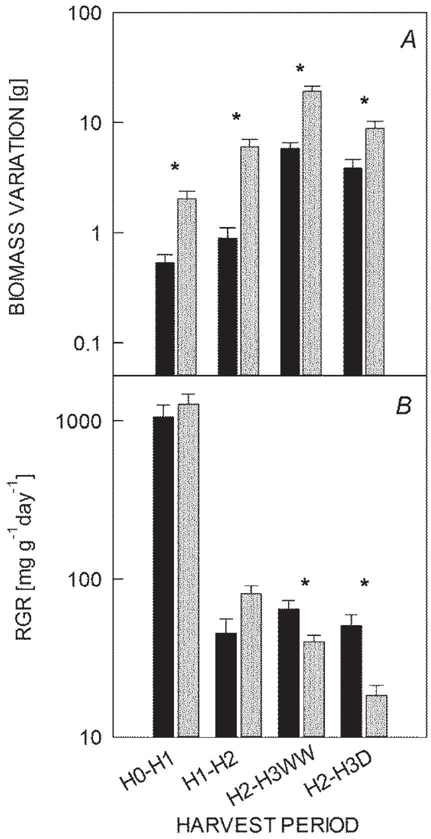

Fig. 3. Different pattern of biomass variation (ΔB) (*A*) and relative growth rate (RGR) (*B*) during harvest periods and treatment conditions of *Rhamnus alaternus* (*gray bars*) and *R. ludovici-salvatoris* (*black bars*). Harvest period symbols: H0-H1, H1-H2, H2-H3 under WW and H2-H3 under D (*i.e.* H0 – harvest after emergence; H1 – harvest 1 (7 months); H2 – harvest 2 (8 months); H3 – harvest 3 (10 months); WW – well-watered; D – drought. Columns represent mean values of six replicates ± standard error. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

Gas-exchange parameters: Mean values of P_N, g_s, E, and leaf intrinsic water use efficiency (P_N/g_s) during the harvest periods and under both treatments were determined (Table 2). *R. alaternus* showed significantly higher P_N than that of *R. ludovici-salvatoris* at H1 and H3 under WW conditions. By contrast, g_s was only significantly higher in *R. alaternus* at H1. As a consequence, this species showed a significantly higher P_N/g_s at all harvests and treatments. Water deficit lowered P_N and g_s in both species, although such a decrease was larger in *R. alaternus* than that in *R. ludovici-salvatoris*. Both species presented higher WUE$_i$ under drought.

Respiration of different tissues of both species decreased significatly at H3. Leaf and stem respiration were slightly higher in *R. ludovici-salvatoris* than that in *R. alaternus* at four harvest periods, although these differences were not significant in most cases (Fig. 5). By contrast, root respiration was slightly higher in *R. alaternus* than that in *R. ludovici-salvatoris* at three harvest periods under WW conditions, but *R.ludovici-salvatoris* presented slightly higher root respiration than that of *R. alaternus* under D (Fig. 5). The only significant

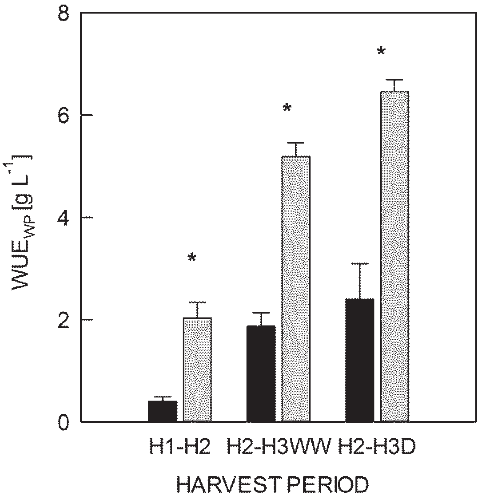

Fig. 4. Whole-plant water-use efficiency [WUE$_{WP}$; total dry matter production/consumed water] under well-watered (H1-H2 and H2-H3 in WW; *see* Materials and methods for further explanations) and drought (H2-H3 under D) conditions of *Rhamnus alaternus* (*grey bars*) and *R. ludovici-salvatoris* (*black bars*). Values are means of six replicates ± standard error. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

Table 1. Morphological components of plants growth, total leaf area (LA), specific leaf area (SLA), leaf area ratio (LAR), leaf mass ratio (LMR), root:shoot ratio (R/S) of *Rhamnus alaternus* and *R. ludovici-salvatoris* under well-watered (WW) and drought (D) treatments. Values are means ± standard error. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

Parameter	*R. alaternus*				*R. ludovici-salvatoris*			
	H1	H2	H3WW	H3D	H1	H2	H3WW	H3D
LA [cm^2]	119.7 ± 16.5*	371.6 ± 47.3*	787.2 ± 85.5*	622.6 ± 118.4*	25.4 ± 4.8	75.2 ± 9.5	211.5 ± 17.3	161.8 ± 28.7
SLA [cm^2 g^{-1}]	108.8 ± 4.9	91.0 ± 1.7	75.3 ± 4.5*	89.5 ± 13.2*	84.8 ± 10.9	91.7 ± 6.2	62.3 ± 6.1	55.9 ± 3.0
LAR [cm^2 g^{-1}]	58.7 ± 3.2	46.2 ± 1.5	28.7 ± 2.7*	37.1 ± 5.3	48.7 ± 5.9	52.6 ± 3.2	32.0 ± 3.2	30.1 ± 2.3
LMR [g g^{-1}]	0.55 ± 0.01*	0.50 ± 0.01*	0.37 ± 0.02*	0.41 ± 0.06*	0.57 ± 0.02	0.57 ± 0.01	0.51 ± 0.01	0.53 ± 0.03
R/S	0.31 ± 0.01	0.26 ± 0.02*	0.31 ± 0.02*	0.24 ± 0.02*	0.38 ± 0.03	0.37 ± 0.02	0.36 ± 0.02	0.35 ± 0.03

Table 2. Leaf net photosynthesis (P_N), stomatal conductance (g_s), transpiration (E), and intrinsic water-use efficiency (WUE$_i$) of *Rhamnus alaternus* and *R. ludovici-salvatoris* under well-watered (WW) and drought (D) treatmens. Values are means ± standard error of six replicates. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

Parameter	*R. alaternus*				*R. ludovici-salvatoris*			
	H1	H2	H3WW	H3D	H1	H2	H3WW	H3D
P_N [µmol(CO$_2$) m^{-2} s^{-1}]	12.07 ± 0.79*	11.10 ± 1.33	9.58 ± 0.88*	4.10 ± 0.55	6.74 ± 0.4	9.67 ± 0.81	7.33 ± 0.76	5.82 ± 1.12
g_s [mol(H$_2$O) m^{-2} s^{-1}]	0.20 ± 0.01*	0.12 ± 0.02*	0.09 ± 0.01*	0.02 ± 0.00*	0.09 ± 0.01	0.20 ± 0.03	0.14 ± 0.02	0.07 ± 0.01
E [mmol(H$_2$O) m^{-2} s^{-1}]	1.89 ± 0.17	2.64 ± 0.32*	1.75 ± 0.16*	0.66 ± 0.10*	1.74 ± 0.16	3.90 ± 0.43	3.01 ± 0.45	1.78 ± 0.29
WUE$_i$ [µmol(CO$_2$) mol^{-1}(H$_2$O)]	106.3 ± 8.7*	92.6 ± 3.4*	113.5 ± 6.4*	198.4 ± 35.3*	76.6 ± 9.7	54.3 ± 5.5	59.5 ± 11.3	75.5 ± 10.6

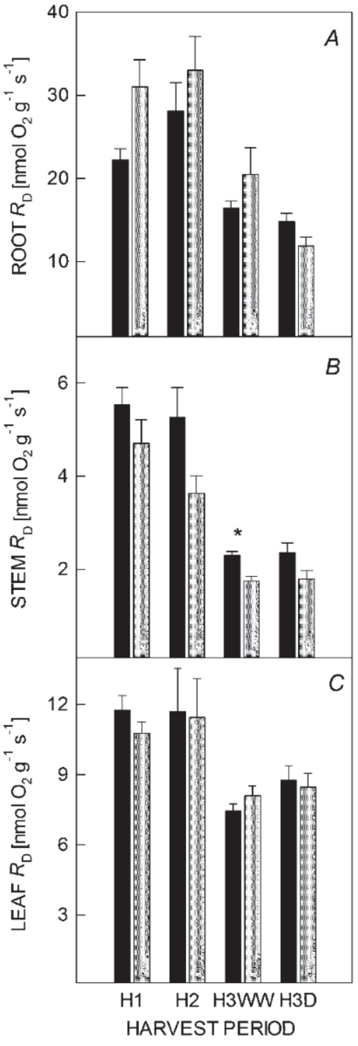

Fig. 5. (*A*) Root, (*B*) stem, and (*C*) leaf respiration (R_D) during four sampling periods and under well-watered (WW) and water-deficit (D) conditions of *Rhamnus alaternus* (*gray bars*) and *Rhamnus ludovici-salvatoris* (*black bars*). Harvest periods and treatments are the same as in Fig. 3. Each value is the mean of six replicates ± standard error. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

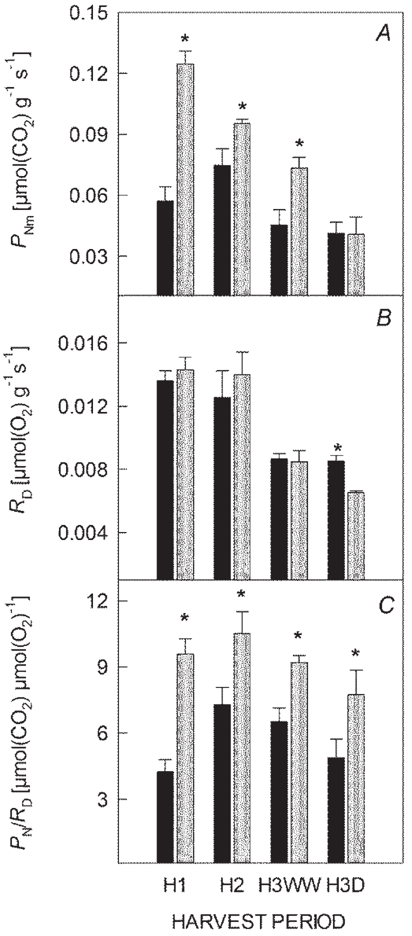

Fig 6. (*A*) Net CO$_2$ assimilation rate on a mass basis (P_{Nm}), (*B*) dark respiration rate on a mass basis (R_D), and (*C*) the ratio of net CO$_2$ assimilation to dark respiration rates on a mass basis (P_N/R_D) at different harvests and treatments (harvest periods and treatments are the same as in Fig. 3). Values are means of six replicates ± standard error. * – significant differences ($P<0.05$) between both species at each harvest and treatment.

difference between both species was found in stems of H3 period under WW conditions (Fig. 5). Similarly, the calculated whole plant respiration was significantly higher in *R. ludovici-salvatoris* than that in *R. alaternus* at H3 under D conditions (Fig. 6*B*). By contrast, photosynthesis on a mass basis was significantly higher in *R. alaternus* than that in *R. ludovici-salvatoris* at all harvests except at H3 under D (Fig. 6*A*).

We used the ratio of net carbon assimilation to dark respiration rates on a mass basis ($P_{\mathrm{Nm}}/R_{\mathrm{Dm}}$), *i.e.* moles of CO_2 incorporated per moles of O_2 consumed, as a simple approach to leaf carbon balance which may allow a qualitative comparison between species (Pattison *et al.* 1998). Estimated carbon balance was significantly higher in *R. alaternus* than that in *R. ludovici-salvatoris* at all harvests and treatments (Fig. 6*C*). Differences in carbon balance between species and along harvest periods followed a very similar pattern to biomass accumulation, with *R. alaternus* showing significantly higher values of both parameters than those in *R. ludovici-salvatoris* (Figs. 3*A*, 6*C*). Moreover, both parameters increased along the harvest periods, although the slight and insignificant decrease in estimated carbon balance was observed in both species at the last harvest period.

Discussion

Biomass accummulation and relative growth rate: *Rhamnus alaternus* showed a significantly higher biomass accummulation than that of *R. ludovici-salvatoris* at all harvests and treatments, in accordance with the different seedling recruitment ability reported for these species under field conditions (Traveset *et al.* 2003, Gulías *et al.* 2004).

The observed RGR values of both *Rhamnus* species (Fig. 3) were similar to those reported by Castro-Diez *et al.* (2003) for *Rhamnus alaternus* and *Rhamnus lycioides.* Although species were at the same phenological stage at the beginning of the experiment, they presented different patterns of RGR variation along both time and water treatments. RGR was significantly higher in *R. alaternus* than in *R. ludovici-salvatoris* during the H1-H2 and H2-H3 periods under WW, but not at H0-H1 and H2-H3 under D. The reported differences between the biomass accumulation and the RGR patterns were due to the larger increase of the initial biomass in *R. alaternus* than that in *R. ludovici-salvatoris* (Fig. 3). This resulted in a low relative increase of biomass in this species despite its higher biomass accumulation (Myers and Kitajima 2007; Mediavilla and Escudero 2010).

On this regard, the low biomass accumulation of *R. ludovici-salvatoris* plants in the early spring (H1) compared to *R. alaternus* could partially explain the low ability of that species to accumulate biomass at the early stages of seedling recruitment. This can limit seedling recruitment to a great extent under Mediterranean conditions, where the optimum period for plant growth is shortened by both winter chilling temperatures and summer drought (Chaves *et al.* 2002, Valladares *et al.* 2008, Gulías *et al.* 2009). This agrees with the low seedling survival of *R. ludovici-salvatoris* observed under field conditions (Traveset *et al.* 2003).

Seedling growth was also conditioned by the seed mass differences between both species, since those differences were similar to that observed along the experiment (Fig. 2). Early seedling mass appears to be a trait determined largely by the seed mass (Hou and Romo 1998). Similar results were reported by Pérez-Ramos *et al.* (2010), showing that during early growth, younger seedlings were more dependent on their seed mass. Thus, the direct link between the seed size and plant growth suggests a translocation of seed carbohydrates to plant organs, in support of previous works that found that early seedling growth was strongly determined by seed cotyledonary reserves (Kennedy *et al.* 2004, Urbieta *et al.* 2008). In addition, the significant differences between both species might be due to a direct causal relationship. For instance, Castro *et al.* (2008) found that plants grown from small seeds have a higher RGR than plants grown from large seeds. In another study, Villar *et al.* (2010) showed that the seed mass was negatively correlated with morphological variables (LAR and SLA).

Drought stress caused a larger RGR decrease in *R. alaternus* than in *R. ludovici-salvatoris* (Fig. 3*B*). Similar patterns of RGR were observed in other Mediterranean shrubs under both well-watered and water deficit conditions (Galmés *et al.* 2005). Water deficit decreased the overall growth of both species included in this study (Table 1, Fig. 3*A*). In both species, biomass allocation to leaves (LMR) and SLA slightly decreased under D conditions compared with H1 and H2. Consequently, LAR also decreased. Similar trends in morphological component variations were observed in *Pistacia lentiscus* L. and *Hypericum balearicum* L. (Galmés *et al.* 2005). *Rhamnus alaternus* showed the higher SLA than *R. ludovici-salvatoris*, which is in accordance with its higher biomass accumulation capacity since high SLA has been described as a trait of fast-growing species (Poorter and Remkes 1990; Garnier 1992; Marañon and Grub 1993; Galmés *et al.* 2005), because a low leaf area to biomass ratio decreases the efficiency of biomass investment by reducing the amount of leaf area available for light interception and hence photosynthetic carbon gain (Lambers 1989, Reich *et al.* 1998). *Rhamnus ludovici-salvatoris* showed the higher R/S than that of *R. alaternus*, what implies a higher resource allocation to aerial biomass of this species, mainly due to a higher stem biomass. This higher allocation to stem biomass in *R. alaternus* would allow a more efficient light interception in this species than in *R. ludovici-salvatoris*, what shows another important difference between both species, *i.e.* plant architecture.

Net photosynthesis was higher in *R. alaternus* than that in *R. ludovici-salvatoris* in all harvest periods under WW conditions, while both species presented similar dark respiration rates in most analysed tissues. Root respiration was somewhat higher in *R. alaternus* than in *R. ludovici-salvatoris* as expected in fast-growing species, due to their higher rates of growth and anion uptake (Van der Werf *et al.* 1988, Poorter *et al.* 1991), although these differences were not significant. Estimated whole plant photosynthesis and respiration was three to five times higher in *R. alaternus* than in *R. ludovici-salvatoris* along the different harvest periods. Photosynthesis and respiration are generally closely linked (Dutilleul *et al.* 2003; Atkin *et al.* 2005), therefore, the estimated carbon balance resulted in higher values in *R. alaternus* than that in *R. ludovici-salvatoris* (–60% of total carbon balance of *R. alaternus*). The balance between photosynthesis and respiration determines the whole-plant carbon balance, and hence the capacity of plants to produce new biomass for growing and developing reproductive structures (Poorter *et al.* 1992). As reported by Poorter *et al.* (1992), and despite the limitations of the scaling up from punctual leaf measurements to whole plant photosynthesis, our results clearly showed that plant carbon balance reflects biomass accumulation (compare Figs. 3*A*, 6*C*). Usually, more than half of the total carbon assimilated in photosynthesis is lost in the respiratory processes necessary for growth and maintenance, nonetheless this balance may change under drought. For instance, although photosynthesis may decrease up to 100%, thus becoming totally impaired under severe water-stress, the respiration rate may either increase or decrease under stress, but may never become totally impaired (Flexas *et al.* 2005). In the present study, the level of drought applied was moderate which significantly decreased photosynthesis, but had almost no effect on respiration, which is consequently reflected on biomass accumulation (Galmés *et al.* 2007). Moreover, *R. ludovici-salvatoris* showed higher respiration rates than *R. alaternus* in all tissues under D conditions, which is in accordance with its low biomass accumulation observed under these conditions. This fact impairs ability of *R. ludovici-salvatoris'* to attain a positive carbon balance and its seedling survival capacity during the first summer drought period. This is consistent with other results reported by Gratani and Varone (2004) and Gratani *et al.* (2008).

Water-use efficiency differences between species: The present results showed that both species followed the same trend in WUE_{WP} under WW and D conditions when calculated by the gravimetric method. Under WW conditions, WUE_{WP} increased from the first to the last period as a consequence of the increasing biomass accumulation, probably because of photosynthesis and biomass accumulation were partially limited by air temperature in the early spring. Also, the decrease in g_s and E in both species, as a consequence of the VPD increment during the spring, probably contributed to the observed increase in WUE. However, this was not the case of *R. ludovici-salvatoris* from H1 to H2, which presented increasing g_s and E. Nevertheless, and as previously pointed out, this species showed a poor abilty to accummulate biomass during the early spring.

In general, pattern of intrinsic WUE_i (*i.e.* P_N/g_s) was not similar to WUE_{WP} (Fig. 4 and Table 2). On the other hand, both intrinsic and WUE_{WP} were higher in *R. alaternus* than that in *R. ludovici-salvatoris* although the differences were greater for WUE_{WP}. WUE_i takes into account P_N but plant growth depends also on respiration and therefore on the carbon balance. On this regard, respiration can indirectly affect WUE_{WP} by modifiying the carbon balance of the plant, especially under drought. According to this, different studies have shown that leaf level estimates of water use efficiency, either instantaneous (P_N/E; P_N/g_s) or integrative (Δ^{13}C in leaf dry matter) do not always reflect the actual WUE_{WP} (Martim *et al.* 2009, Gulías *et al.* 2012, Tomàs *et al.* 2012).

As reported by several studies, adaptation to environmental conditions under the Mediterranean-type climate implies that plants must use water in an efficient way. On this regard, the low water use efficiency has been related to a decline in performance of seedlings and saplings after a drought event in some Mediterranean species (Gulías *et al.* 2002, Lloret *et al.* 2004; Ponton *et al.* 2002). Similarly, the low WUE registered in this study in *R. ludovici-salvatoris*, when compared to *R. alaternus* at all harvest periods and treatments, would play a key role in the ability of these species to recruit seedlings under field conditions.

Conclusion: In conclusion, the higher growth and biomass accumulation of *R. alaternus* seedlings in comparison to *R. ludovici-salvatoris* can be explained by its higher carbon balance as a consequence of its higher photosynthetic rates on the mass basis and similar respiration rates at the plant level. Moreover, *R. ludovici-salvatoris* carbon balance was specially impaired under both early spring (probably as a consequence of chilling temperatures) and drought conditions. In addition, *R. ludovici-salvatoris* showed the low water use efficiency at the plant level during all periods and treatments. These traits could partially explain the low ability of this species to recruit seedlings under Mediterranean conditions and thus its limited distribution area.

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