Osmotic adjustment is a significant mechanism of drought resistance in *Pinus pinaster* and *Pinus canariensis*

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Abstract

Mechanisms of drought resistance were studied in two xeric populations of *Pinus canariensis* and *Pinus pinaster*. Seedlings were grown in a hydroponic culture for four months. Gradual controlled drought was imposed during two weeks by adding Polyethylene glycol (PEG-6000) to the growing medium. Two levels of water deficit (Ψ = -1MPa, Ψ = -2 MPa) and a control treatment (Ψ = -0.03 MPa) were tested. Relative water content (RWC) was markedly low at the end of the experiment. Both populations showed a high capacity for osmotic adjustment in needles as shown by the osmotic index, 0.63 (1.33 MPa at 80% RWC) for *P. canariensis* and 0.54 (1.25 MPa at 80% RWC) for *P. pinaster*. Root growth and root:shoot (stem + needles) ratio were increased by water deficit. Opposite patterns of relative biomass allocation were assessed between stressed and control plants. While stressed seedlings assigned more dry matter to roots, non-stressed plants showed a higher relative needle weight. The growing media caused additional stress to the plants, thus comparisons with other drought protocols should be made carefully.

Key words: hydroponic culture, Polyethylene glycol, biomass partitioning, Maritime pine, Canary Island pine.

Resumen

El ajuste osmótico como mecanismo de resistencia a la sequía en poblaciones áridas de *P. pinaster* y *P. canariensis*

Se estudiaron mecanismos de resistencia a la sequía en poblaciones áridas de *P. canariensis* y *P. pinaster*. Tras cuatro meses en cultivo hidropónico, se sometió a las plantas a un tratamiento de sequía durante dos semanas añadiendo polietilenglicol (PEG-6000) a la solución. Se evaluaron dos niveles de déficit hídrico (Ψ = -1MPa, Ψ = -2 MPa) y un tratamiento control (Ψ = -0.03 MPa). El contenido hídrico relativo (RWC) de las plántulas fue muy bajo durante todo el ensayo. Las dos poblaciones mostraron una gran capacidad de ajuste osmótico con índices de ajuste osmótico de 0.63 (ajuste osmótico de 1.33 MPa a un 80% de RWC) en *P. canariensis* y 0.54 (ajuste osmótico de 1.25 MPa a un 80% de RWC) en *P. pinaster*. El crecimiento radical y la relación raíz:parte aérea fueron estimulados por la sequía. Se encontraron patrones de reparto de biomasa opuestos entre las plantas estresadas y las control. Mientras que las primeras destinaron más materia seca a las raíces, las no estresadas invirtieron más en biomasa foliar. El medio de cultivo causó un estrés adicional a la planta por lo que las comparaciones de ensayos de sequía utilizando PEG con otros protocolos deben de tenerlo en cuenta.

Palabras clave: cultivo hidropónico, polietilenglicol, reparto de biomasa, pino marítimo, pino canario.

Introduction

The first years are the most critical for the establishment of forest species because mortality occurs mostly in the first stages of development, and consequently, they play a crucial role in reforestation programmes. Under Mediterranean conditions the success of regeneration is hampered not only due to the length of the annual drought period (sometimes longer than seven months), but also because of erratic rainfall. According

to the accepted models of climate change, the severity of the climate will be increased in similar latitudes to those of the Iberian Peninsula and the Canary Islands (Lal *et al.*, 2002; Castro *et al.*, 2005). Therefore, drought tolerance during seedling establishment will be more critical for the success of plantations and natural regeneration of forest tree species in the future. While testing plants in large-scale trials under different conditions can take several years (Danjon, 1994; O'Brien *et al.*, 2007), various field and laboratory screening methods have been used successfully to control water input, including line-source sprinkler irrigation, rainout shelters, and the use of polymers with a high molecular weight such as polyethylene glycol. Polyethylene glycol facilitates a fine control of water availability of seedlings in the growing media by their osmotic effect (Kato *et al.,* 2008). In addition, water deficit is applied in a more homogeneous manner than that attained by controlling the supply of water through different watering regimes. Nevertheless, PEG toxicity has been reported in *Pinus banksiana* and *Eucalyptus grandis* after a short exposure to this polymer, since it was absorbed by roots, lowering relative water content and damaging leaf tissues (Fan y Blake, 1997), but not in *P. halepensis* (Calamassi *et al.*, 2001) and *P. pinaster* (Harfouche, 2003). Therefore its suitability for the early screening of drought resistance in woody species must be tested.

Among the general mechanisms of tolerance to water stress, osmotic adjustment is important in maintaining cell turgor under drought (Hsiao *et al.*, 1976; Jones y Turner, 1980). Osmotic adjustment is defined as the net accumulation of solutes in cells that experience water deficit. It entails lowering of osmotic potential and maintenance of turgor pressure, cell expansion and stomatal conductance. Biomass partitioning is also a good predictor of drought tolerance since commonly plants from drier environments increase relative allocation to roots, consistently with the optimal partitioning theory (Bloom *et al.*, 1985).

Pines are the most frequent species used in reforestation programmes in the Mediterranean Basin because of their ability to survive harsh environmental conditions and their rapid growth when established. Particularly in Spain, *Pinus pinaster* has been intensively used for production and protection purposes between 1940 and 1982 (Alía *et al.*, 1996). Striking differences among provenances in growth, survival and drought resistance have been previously assessed (Alía *et al.*, 1995, 1997; Fernández *et al.*, 1999, 2000; Nguyen-Queyrens y Bouchet-Lannat, 2003; Chambel *et al.*, 2007) and rainfall in the place of origin was the factor that best explained these differences in common garden provenance trials (Danjon, 1994; Alía *et al.*, 1997).

Pinus canariensis is an endemic pine of the Canary Islands. Despite its narrow distribution area it is adapted to live in a wide range of ecological conditions: from desert-like environments to mesic forests. The species' resistance to drought has been tested in field trials located in the Canary Islands and Israel. Plants from mesic environments exhibited lower survival in dry conditions than plants from xeric locations (López *et al.*, 2007).

In the present study we test the prospect of using PEG-6000 in drought simulation experiments with two marginal populations of *P. pinaster* and *P. canariensis* grown in hydroponic media. We compare the growth, biomass partitioning, relative water content and osmotic adjustment after 14 days of water deficit treatment. Both populations inhabit poor soils and very dry environments, and may exhibit unusual adaptation to drought. We aim to address the influence of two drought treatments on the survival of seedlings, analysing the physiological mechanisms of drought resistance involved in plant adaptations to specific environments. The knowledge of these mechanisms can help us to predict species responses to climate aridification.

Material and methods

One provenance of *P. canariensis*, Arguineguín (Gran Canaria), and one provenance of *P. pinaster*, Oria (Almería) (Table 1) were chosen based on the dryness at their sites of origin. Arguineguín is located in southern Gran Canaria and only around one hundred over-aged trees constitute the forest. Mean annual rainfall is less than 200 mm with an annual drought period over 9.5 months. These extreme conditions produce decay in many trees and lack of natural regeneration. Oria is a natural forest in the Penibetica Range on calcareous soils and under Mediterranean climate, annual rainfall of 357 mm and 6 months of drought period. Natural regeneration is scarce because of grazing and the high density of some stands.

Two weeks after germination in perlite, fifty seedlings per provenance were transferred to an aerated hydroponic culture with Hoagland nutrient solution, which was renewed once a week, for four months. Nutrient concentrations were gradually increased, 1/16, 1/8, 1/4, 1/2 every two weeks until the full strength Hoagland nutrient solution. The solution was oxygenated with air diffusers to prevent root asphyxia. Plants were maintained during the whole experiment in a growth chamber with a pho-

Table 1. Location and ecological features of the two populations used in the study

Species	Population	Latitude (N)	Longitude (W)	Altitude (m)	Mean Annual Temperature (ºC)	Annual Rainfall (mm)	Drought Period (months)
P. canariensis	Arguineguín	27º49'	15º41'	300	20.5	144	9.5
P. pinaster	Oria	37º30'	2º20'	1150	15.8	357	6

toperiod of 16 h, day/night temperatures of 25/20 ºC and 60/80% relative humidity. At the beginning of the fifth month, Polyethyleneglycol (PEG 6000) was progressively added to the nutrient solution. PEG-6000 is a non-ionic water polymer and is widely used to mimic drought since PEG-induced osmotic stress produces a decline in water potential that is similar to soil drying.

Every two days the solution water potential (Ψ) was lowered 0.5 MPa until reaching the target water potential of the different treatments: control plants, without addition of PEG (t_0, Ψ = -0.03 MPa), moderately water-stressed (t_1, Ψ = -1 MPa) and severe water-stress (t_2, Ψ = -2 MPa). In t_1 Ψ was reached the 5th day after beginning the water deficit treatment and in t_2 the 9th day. At the beginning of the experiment, Ψ was established with a psychrometer-hygrometer (HR-33T, Wescor, Logan, UT) from the relationship between PEG 6000 concentration and nutrient solution water potential.

Water deficit period endured 14 days. During this period, culture solutions were replaced twice a week maintaining PEG 6000 concentration. Relative water content (RWC) and osmotic potential (Ψ_Π) were measured six times during the treatment period (3, 5, 7, 9, 11 and 14 days after the start of addition of PEG) in six plants per provenance and treatment. On each date, six juvenile developed needles of the same seedlings were collected at dawn, three for RWC and three for Ψ_Π measurements. Plants had enough leaf production to maintain good performance despite the needle harvest thus this did not affect seedling vigour. The RWC was obtained by weighing the needles immediately after harvest (FW = fresh weight), then they were placed in distilled water at 4 ºC for 24 hours to determine fresh weight at full hydration (HW). Finally, needles were oven-dried for 48 h at 70 ºC to determine dry weight (DW). RWC was calculated as RWC = (FW – DW) / (HW – DW). To determine Ψ_Π, needles were cut into small pieces and frozen in liquid nitrogen. After thawing, Ψ_Π was measured with a psychrometer-hygrometer.

We followed the method proposed by Morgan (1980) to calculate osmotic adjustment due to the positive evaluation of this method in forest tree species (Nguyen-Queyrens y Bouchet-Lannat, 2003; Warwick y Thukten, 2006). Logarithmic plots of relative water content against osmotic potential: $\ln RWC = k (-\ln(-\Psi_\Pi))$, where k is a constant for each species, were elaborated to determine the osmotic adjustment (OA) (Morgan 1992). A RWC of 80% was considered to compare active osmotic adjustment between provenances since this value is close to the turgor lost point in *P. pinaster* (Fernández *et al.*, 1999). The index of osmotic adjustment proposed by Turner (2006) was also estimated to compare measurements carried out using different protocols and across species. This index applies to all the patterns of $\ln(RWC)$ *vs.* $\ln(\Psi_\Pi)$ observed in experiments and is independent of the amount of drying achieved. The index is related to the slope of the relationship between RWC and Ψ_Π in logarithmic plots. In case of the lack of osmotic adjustment, the slope is 1.0 and the index will be 0, but for tissues that adjust osmotically, the slope will be less than 1.0 and the osmotic index will be 1.0 – slope.

The last sampling day, 10 plants per species and treatment were harvested, divided into roots, stems and needles, dried at 70 ºC for one week and weighed to calculate biomass partitioning. Besides absolute weights, relative weights were calculated, i.e. the relative contribution of roots, stem and needles to the total dry weight.

Repeated measures analyses of variance (ANOVA) were conducted with relative water content and osmotic potential as dependent variables and with treatment and species as independent factors. Both factors were considered fixed. Comparisons of slopes for the regression lines between RWC and Ψ_Π and in the allometric analysis were made with analysis of covariance (ANCOVA). All statistical analyses used software of the SAS Institute (v.9.1, Cary, NC).

Results

Water status and osmotic potential

Predawn relative water content decreased with time. On the last day of the experiment, it ranged between

mean values of 78% in control plants and 44% in t_2 plants (Fig. 1a). Water deficit induced decreases in RWC of similar magnitude in both species. Moreover, treatments t_1 and t_2 did not differ significantly in RWC during the whole test but they were different from the control after day 5 (Fig. 1a).

Needle Ψ_Π declined with time and it was lower in Canary Island pine (*P. canariensis*) than in maritime pine (*P. pinaster*) during the whole experiment (Fig. 1b). Both treatment and species effects were significant (Table 2). The drop of Ψ_Π was substantial from day 7. The most pronounced fall occurred between day 11 and day 14, particularly in t_2 (variation of 2 MPa between the two measurements). All treatments differed significantly from day 7 onwards when solution of t_1 maintained $\Psi = -1$MPa and t_2 decreased to $\Psi = -1.5$ MPa in day 7 and reached $\Psi = -2$MPa in days 9, 11 and 14.
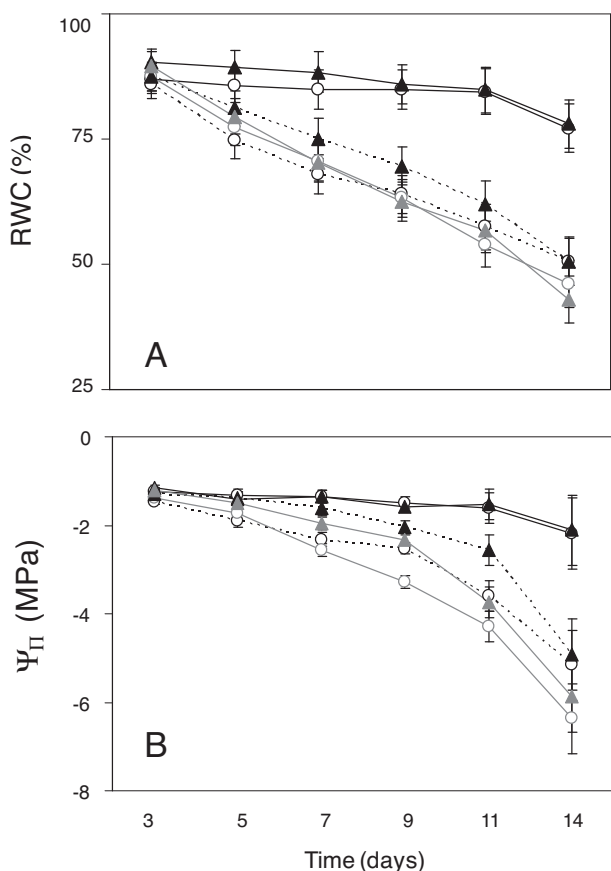
Osmotic adjustment ($\Delta\pi_a$)

The lnRWC / -ln(-Ψ_Π) plots (Fig. 2) showed a marked deviation from a passive osmotic adjustment since both slopes differed significantly from 1. A slope of 1 indicates that the tissue is not carrying out active osmoregulation. Regression lines fitted to the data intersected the *y*-axis at RWC of 96% and 97% for *P. canariensis* and *P. pinaster* respectively. The regression slope of *P. canariensis* was significantly lower than the slope of *P. pinaster*, indicating a higher capacity of osmotic adjustment of the former species, with a slope of 0.37 ± 0.02 compared to 0.46 ± 0.02 (Table 3). The osmotic index (1.0 – slope) was 0.63 for *P. canariensis* and 0.54 for *P. pinaster*. When net solute accumulation was determined at RWC 80%, the ranking of the species did not vary and it was 6% higher in *P. canariensis* ($\Delta\pi_a = 1.33$) than in *P. pinaster* ($\Delta\pi_a = 1.25$).

Biomass allocation

In all treatments *P. pinaster* presented higher dry mass than *P. canariensis* (Fig. 3). Imposed water deficit affected only root growth and root:shoot ratio (Table 4). Under stress, root dry mass was 2.5 times higher in *P. canariensis* and 3.3 times in *P. pinaster* than in the control treatment. Root:shoot ratio also increased with both t_1 and t_2 and it was not significantly different between species in any treatment. The allometric analysis showed changes in allocation patterns in response to water deficit. The slope and the intercept of the regression lines fitted for t_0 and t_2 were significantly different. Therefore, when comparisons are made at the same sample size, differences between control and high stressed plants existed.

When relative dry mass was considered no differences were found between species (Table 4) but relative root and needle mass diverged between t_0 and the stress treatments. The two traits followed opposite patterns and while relative root weight increased from 0.32 in t_0 to 0.50 and 0.51 in t_1 and t_2, needle relative weight decreased from 0.50 in t0 to 0.38 and 0.37 in t_1 and t_2.

A high proportion of seedlings of *P. pinaster* displayed axillary dwarf shoots at the end of the experiment. All plants of *P. canariensis* in the t_2 absorbed their stem pith, showing empty stems in the final harvest.

Figure 1. A) Evolution of relative water content, RWC, and B) osmotic potential, Ψ_Π, during the stress period. Vertical bars correspond to the standard errors of the mean values of seedlings for each species, ○ *Pinus canariensis*, ▲ *Pinus pinaster*, in every treatment, __t_0, ...t_1, ...t_2..

Table 2. F-values for repeated measures analysis of variance of relative water content (RWC) and osmotic potential (Ψ_Π). ns, not significant, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RWC		Ψ_Π	
Between-subjects effects		Between-subjects effects	
Species	1.11 ns	Species	4.97 *
Treatment	24.59 ***	Treatment	22.85 ***
Species x Treatment	0.01 ns	Species x Treatment	0.48 ns
Within-subjects effects		Within-subjects effects	
Time	126.73 ***	Time	79. 56 ***
Time x Sp	0.85 ns	Time x Sp	0.40 ns
Time x Treat	17.12 ***	Time x Treat	11.45 ***
Time x Sp x Treat	0.46 ns	Time x Sp x Treat	0.27 ns
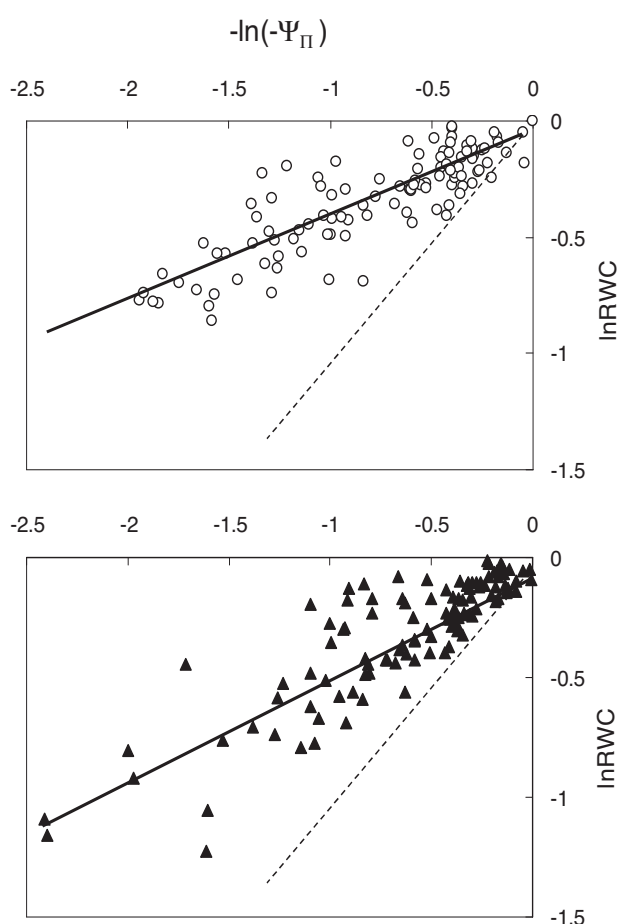
Figure 2. Logarithmic plots of osmotic potential (Ψ_Π, MPa) and relative water content (RWC) of needles of *Pinus canariensis* (○) and *Pinus pinaster* (▲). A regression line is fitted to the response of each species to the stress treatment (continuous lines). Regression parameters are provided in Table 3. The 1 : 1 line (dotted line) is the increase in solute concentration caused only for the loss of water without osmotic adjustment.

Discussion

Pinus canariensis and *Pinus pinaster* can grow in xeric environments, thus they must show drought resistance adaptations. The relative water content decreased progressively as the hydroponic solution culture water potential decreased. However, it did not differ either between the two levels of water potential or the two species. We suggest that the water deficit imposition was too fast to produce differences due to the inability of the plants to trigger all their drought resistance mechanisms. Rapid rates of water deficit imposition exceed the capacity for acclimation via osmotic adjustment (López *et al.*, 2008). Furthermore, at comparable water potentials, RWC of osmotically stressed seedlings of woody species are lower than in soil dried seedlings due to the inhibition of root water transport caused by PEG viscosity (Fan y Blake, 1997). These two hypothesis would explain the extremely low values of RWC on the last date of the experiment, very close to the permanent wilting point found in other pine species adapted to long periods of water deficit (Parker, 1952; Fan *et al.*, 1994; Lee *et al.*, 2004) or in olive trees, where RWC reached 40% under drought conditions, allowing release of about 60% of the water stored in their tissues to transpiration (Dichio *et al.*, 2006).

The initial Ψ_Π in the control treatment was low in both species, between -1.2 and -1.4 MPa, and the RWC was never 100%. As the water potential of the growing medium decreased, seedlings reduced their Ψ_Π. This decline was more remarkable in *P. canariensis*, although *P. pinaster* recorded values which also demonstrated a clear response to water stress. Divergences among treatments were patent. Osmotic potential of plants under t_1 dropped to -5.16 MPa in *P. canariensis* and -4.92 MPa

Table 3. Regression parameters of lnRWC against $-\ln(-\Psi_\Pi)$. Osmotic adjustment at 80% RWC (OA_{80}) and Index of osmotic adjustment (OA Index, Turner 2006). Slopes with different letters are significantly different at P = 0.05

| Species	r^2	Slope (MPa^{-1}) Regression line	OA_{80} (MPa)	OA Index
Pinus canariensis	0.73	0.37 a y = 0.37 x – 0.04	1.33	0.63
Pinus pinaster	0.75	0.46 b y = 0.46 x – 0.03	1.25	0.54

in *P. pinaster*, values much lower than those found for *P. pinaster* under moderate water deficit, even in provenances remarkably adapted to drought like Tamjout from Morocco (Nguyen y Lamant, 1989; Nguyen-Queyrens y Bouchet-Lannat, 2003) but they were in accordance with values obtained in *Pinus canariensis* grown in hydroponic medium under a fast imposed water deficit treatment (López *et al.*, 2008).

We found a linear response between RWC and Ψ_Π, contrasting with previous results with both species which observed a biphasic response (Nguyen-Queyrens y Bouchet-Lannat, 2003; López *et al.*, 2008). In our experiment, the initial phase of absolute osmotic adjustment without changes in RWC was absent. We think that this was not because of the seed origin but because of the water stress imposition rate and the growing medium. These seem to be essential factors when comparing osmotic adjustment besides the different measurements methods. The indexes of osmotic adjustment, 0.63 for *P.*

Figure 3. Biomass allocation of *Pinus canariensis* and *Pinus pinaster* seedlings under the three stress levels (t_0, t_1, t_2) 14 days after the beginning of PEG addition. Vertical bars correspond to the standard errors of the mean values of seedlings of each species. ■ root dry weight, ▨ stem dry weight, □ needle dry weight.

canariensis and 0.54 for *P. pinaster*, are in accordance with values found in *P. pinaster* for the Tamjout provenance (Nguyen-Queyrens y Bouche-Lannat, 2003) but higher than indexes calculated for five provenances of Canary Island pine from wetter origins (López *et al.*, 2009).

P. pinaster gained more dry mass than *P. canariensis* in all treatments, and a high proportion of seedlings displayed axillary dwarf shoots at the end of the experiment. Seedlings of Canary Island pine show the slowest ontogenetic development among the Mediterranean pines even with maturity hastening protocols (Chambel *et al.*, 2007). Stem and needle dry masses were not affected by the level of water deficit applied. Nevertheless, root dry mass, relative root dry mass, and root:shoot ratio increased with water deficit. This tendency seems to be common to plants growing under arid conditions (Nguyen y Lamant, 1989). They invest more resources in root development at the expense of shoot growth (Bongarten y Teskey, 1987). This is consistent with the optimal partitioning theory; plants shift carbon allocation to the organs collecting the most limiting resource, roots in the case of water deficit (Bloom *et al.*, 1985). Although root dry weight was significantly different between species, root:shoot ratio did not differ between them, thus the two species followed a similar allocation pattern.

The absorption of the stem pith in seedlings of *P. canariensis* under the most stressful treatment could be interpreted as an extreme response of the species since this pine stores reserve substances in the stem which allow the reconstruction of the crown after fire (Climent *et al.*, 2004). If the conditions are exceptionally hard, seedlings would mobilize all their reserves in order to survive, even emptying the stem. Reductions of the reserve storage under prolonged drought have been detected in roots of *Pinus palustris* (Sayer y Haywood, 2006). Furthermore, heat and water stress induced stem reserve mobilization in wheat as an extra source of carbon for grain filling when photosynthesis was inhibited
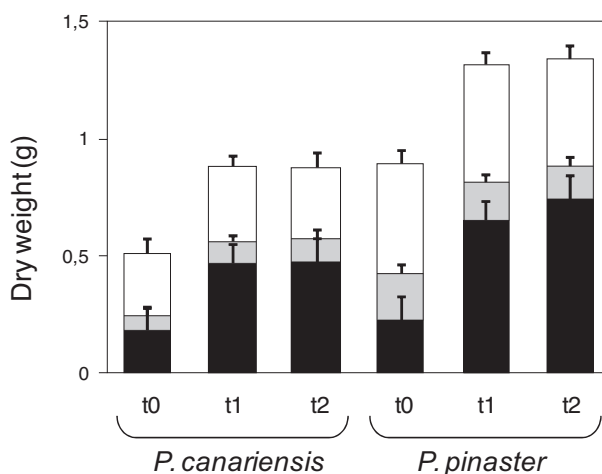
Table 4. Percentage of the explained variation due to species, treatment and the interaction species by treatment and significance values from the General Linear Models for biomass components. ns, not significant, *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Source	RDW	SDW	NDW	TDW	RDW/SDW	rRDW	rSDW	rNDW
Species	12.43 *	25.17 **	33.27 **	29.39 ***	ns	ns	ns	ns
Treatment	28.94 ***	ns	ns	17.72 **	30.31 ***	44.97 ***	ns	33.62 ***
Species x Treatment	ns	ns	ns	ns	ns	ns	ns	ns

by drought or high temperatures (Blum *et al.*, 1994; Blum, 1998).

The water shortage responses of both species, osmotic adjustment and increase of root biomass, showed regulations to extract water from dry media and they could be related to high yields and survival under stress.

Since water deficit imposition rate was too fast and the growing media caused additional stress to the plants, comparisons with other drought protocols should be made carefully.

Populations included in this work are marginal populations with regeneration problems, conservation practices designed to preserve and enlarge them must be adopted. An increase of effective population numbers is advisable to avoid inbreeding depression (González-Martínez *et al.*, 2004; Vaxevanidou *et al.*, 2006).

Acknowledgements

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