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Seasonal changes in the physiological activity of regeneration under a natural light gradient in a *Pinus pinea* regular stand

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Abstract

Seasonal courses of chlorophyll *a* fluorescence, gas exchange parameters and water status, joined to environmental variables, were measured in four age classes of stone pine (*Pinus pinea* L.) natural regeneration, selected within a natural light gradient in order to cover the whole range of incident light detected within a stone pine regular stand throughout 2007 and 2008. Selection of light conditions was based a priori on the distance of seedlings to the parent trees, expressed by crown radius distances. One-year old seedlings were worse acclimated to the extreme drought conditions recorded in summer (especially in 2008), showing lower predawn and midday Fv/Fm values, slightly lower photosynthetic rates at midday, lower ΦPSII values, lower water potentials (Ψ_{pd} and Ψ_{min}), and high mortality (90%). The oldest seedlings (class 4, 51-200 cm high), although they do not experience the lowest Ψ_{min} in July 2008, were not able to maintain a positive carbon gain at midday, even showing the lowest photosynthetic rates. However, they rapidly recovered once the climatic conditions were softened in early autumn. During late autumn low Fv/Fm values were found especially in one-year old seedlings, indicating that maximal photochemical efficiency of PSII is sensitive to the autumn temperatures below cero recorded during both years. Thus, stone pine regeneration acclimates its physiology to the seasonal course of temperature and water availability, and this acclimation depended on age class. However, seedling physiology is only slightly affected by differences in light environment, probably related to the low variability recorded in GSF values (0.55 ± 0.01), due to the low density of the stand. We suggest that such low stand densities cannot assure one-year old seedlings' survival, but are sufficient for older seedlings.

Key words: stone pine; natural regeneration; drought; cold; gas exchange; photochemical efficiency; water status.

Resumen

Cambios estacionales en la actividad fisiológica del regenerado bajo un gradiente natural de luz en una masa natural de *Pinus pinea*

Durante 2007 y 2008 se midió la evolución estacional de la fluorescencia a la clorofila, de los parámetros de intercambio gaseoso y del estado hídrico, junto con variables ambientales, en el regenerado de cuatro clases de edad de pino piñonero (*Pinus pinea* L.) en una masa regular de esta especie. Las plantas se eligieron dentro de un gradiente natural de luz, con el objeto de cubrir todo el rango de luz incidente en este tipo de masas. La selección de las condiciones de luz se realizó a priori basándose en la distancia de las plantas a los árboles adultos, expresada en radios de copa. Las plantas de un año se aclimataron peor a las condiciones de sequía extrema del verano (principalmente en 2008), mostrando valores menores de Fv/Fm al mediodía y al amanecer, de tasa fotosintética al mediodía, de ΦPSII, de potenciales hídricos (Ψ_{pd} y Ψ_{min}) y una mayor mortalidad (90%). Las plantas de mayor edad (clase 4, 51-200 cm de altura), aunque no experimentaron los valores de Ψ_{min} menores en julio 2008, no fueron capaces de mantener una fotosíntesis positiva al mediodía, incluso mostrando los valores menores. Sin embargo, se recuperaron rápidamente al suavizarse las condiciones climáticas en otoño. A finales de otoño se obtuvieron valores bajos de Fv/Fm, especialmente en las plantas de un año, indicando que la máxima eficiencia fotoquímica del PSII fue sensible a las temperaturas bajo cero registradas en ambos otoños. Por tanto, la regeneración del pino piñonero aclimata su fisiología a los cambios estacionales de temperatura y disponibilidad hídrica, estando esta aclimatación influida por la clase de edad. Sin embargo, la fisiología de las plantas no se vió afectada por el gradiente natural de luz encontrado en la masa estudiada, probablemente debido a la baja variabilidad registrada en GSF y a la baja densidad de la masa. Se sugiere que esa densidad es insuficiente para asegurar la supervivencia de las plantas de un año, pero suficiente para el regenerado de más edad.

Palabras clave: pino piñonero; regeneración natural; sequía; frío; intercambio gaseoso; eficiencia fotoquímica; estado hídrico.

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Introduction

Natural regeneration of disturbed Mediterranean forest ecosystems is very uncertain and fraught with serious obstacles (Calama and Montero, 2007; Montero *et al.,* 2008). Stone pine (*Pinus pinea* L.) in the Northern Plateau of Spain shares some common problems with other Mediterranean species: (1) the masting habit, which requires synchrony between good crop years and favourable climatic conditions to attain regeneration success; (2) the low density of Mediterranean stands, favouring the presence of large gaps in the forest where big gravity-disseminated seeds (*e.g.* stone pine nut) are poorly dispersed and regeneration seldom appears; and (3) summer dryness, which represents the main constraint for seedling establishment (Castro *et al.,* 2005; Mendoza *et al.,* 2009). Other problems are more specific to stone pine: (1) the marked continental climate, thus seedlings suffered from severe winter freezing and high summer temperatures, joined to the irregular spring weather that can produce plant dieback (Resco de Dios *et al.,* 2007); (2) the commercial use of the edible pine nuts which conditions the sylvicultural practices applied, that maximize pine nut yield, but reduces the amount of seed reaching the floor necessary to assure regeneration success; (3) the presence of sandy soils that overheat and drain easily in the summer aggravating the problems of water availability. Under this scenario of severe limitations, natural regeneration of stone pine stands is doomed to failure. Despite major interest of forest managers in resolving this problem (Masetti and Mencuccini, 1991; Calama and Montero, 2007; Barbeito *et al.,* 2008), few studies have focused on the physiological aspects of this topic.

Mediterranean ecosystems usually have to face a double stress: summer drought and winter cold (Ogaya and Peñuelas, 2003). The response of natural regeneration to summer drought —characterized by low precipitation, high irradiance, high temperatures and high water vapour pressure deficit— includes reduced water potential and low photosynthetic rates due to a great stomatal control of transpirational water loss (Zweifel *et al.,* 2005), reduced CO_2 uptake (*e.g.* Chaves *et al.,* 2002), reduced nutrient uptake and reduction in photochemical efficiency (Rodríguez Calcerrada *et al.,* 2007); as a consequence, growth is reduced and survival may become problematic in cases of extreme soil water depletion (Hernández-Santana *et al.,* 2009; Prieto *et al.,* 2009). In addition, there is substantial evidence that photosynthetic capacity is directly affected by soil moisture (Keenan *et al.,* 2009). Several studies have described restricted plant activity in the Mediterranean area during the summer months due to heat and drought stress (Tenhunen *et al.,* 1990; Peñuelas *et al.,* 2007; Ogaya and Peñuelas, 2003). Thus, under drought summer conditions seedlings' physiological responses will be conditioned (Rodríguez-Calcerrada *et al.,* 2007).On the other hand, low photosynthetic rates during early winter (García-Plazaola *et al.,* 1997) and partial photo-inhibition of PSII under low temperatures have also been described for woody species (Oliveira and Peñuelas, 2000, 2001).

Plant performance depends on the underlying patterns of resource availabilities (Latham, 1992). Thus, variability among overstory conditions is correlated with seedling growth; however, regeneration responses to different overstory environments may be complex and nonlinear in nature (Powers *et al.,* 2009). Moreover, changes in physiological traits during the aging of woody plants can provide insight into factors that influence the recruitment and survival of a species (Donovan and Ehleringer, 1991; Kolb and Stone, 2000). It is important to note that the response of physiological traits through the year and mainly during drought can be different in seedlings and trees (Pardos *et al.,* 2005). Generally, seedlings showed more negative predawn water potentials than trees, and probably is a function of the rooting depth and the pattern of soil water recharge and depletion from surface to deep layers (Matzner, 2003; Cordeiro *et al.,* 2009). Thus, both the extent and susceptibility to drought varies with seedling age. During summer, seedlings growing in open areas are exposed to higher air and soil temperatures, higher vapour pressure deficit and lower soil water availability than those under canopy protection (Aussenac, 2000). In addition, published studies from a variety of experimental situations generally indicate that both photosynthesis and stomatal conductance are reduced with the age of trees, although there are exceptions to this general trend (Bond, 2000), For instance, higher rates of transpiration and photosynthesis in larger trees can be related to a greater water availability as their roots can tap deeper water resources (*e.g.* Dawson and Ehleringer, 2003; Dawson, 1996). In seedlings, the strategy of higher rates of A and gs under more severe drought conditions might provide the growth potential they need for establishment, but with a greater risk of mortality (Bond, 2000). Conifers appear to diverge from angiosperms in terms of age-related physiological changes, showing a stronger pattern of age-related declines in photosynthetic capacity (Thomas, 2010).

Pinus species is considered a drought-avoiding species that only encounters a mild drought stress, and whose carbon assimilation under drought conditions is suppressed because of stomatal closure (Martínez-Ferri *et al.,* 2000). The physiological response of stone pine natural regeneration to environmental factors through the year (but mainly under the severe summer drought and late autumn climatic conditions) will determine its survival and performance over time and will help to understand the physiological background of regeneration failure. Thus, the knowledge of the effect of these factors and their interactions on the physiology of seedlings is essential for the management of natural stands. Several studies have examined stone pine seedling's physiological performance under controlled growing conditions, but few have been done in the field (Petropoulou *et al.,* 1995; Manetas *et al.,* 1997). Our aim is to study the effect of seedling age on different physiological traits (water status, leaf gas exchange and photoinhibition) of stone pine natural regeneration under a natural light gradient in a 2 years field study, including inter-annual and inter-seasonal variability in the plants' response to environmental conditions. We expected drought (in the summer) and low temperature effects (in late autumn) to affect the physiological activity of natural regeneration throughout the different seasons, but we also expected a different response according to seedling age. The following questions are addressed: (i) What is the dependence of photosynthetic capacity (and related parameters) in seedling age of stone pine natural regeneration?; (ii) Does low late autumn temperatures condition stone pine regeneration differently than summer drought?; (iii) If the droughty summer conditions followed by a dry autumn recorded in 2008 become a standard, will stone pine natural regeneration be seriously endangered?

Materials and Methods

Study site

The study site was located in an even aged 100-120 years old stone pine stand located in the Northern Plateau in Spain (41° 28' 23" N, 4° 43' 26" W). The management of the stand is focused in nut production. Thus, basal area is largely reduced by applying 3 to 4 early and severe low thinnings, so that the final density (150-200 trees per ha) is achieved at age 60. In this way, the development of big crowns that maximize nut yield is favoured. A 0.48-ha (80 m × 60 m) permanent plot was set up in a flat area located at an altitude of 700 m, with more than 12,000 ha of stone pine. This area is characterized by sedimentary sandy soils with a large layer of quartzitic sand deeping up to 2 m. The sand content is over 90%, with soil water retention capacities between 80 and 100 mm. Soils also show very low organic matter levels ($<0.25\%$) and practical absence of carbonates. Average annual rainfall is 400 mm. Climate is extreme continental with maximum temperatures of 45°C in summer and a minimum of –15°C in winter (Calama and Montero, 2007). Regeneration cuttings by shelterwood system were done in 2002-2003, reducing basal area from 18.5 m^2/ha (200 trees/ha) to 9.70 m^2/ha (80 trees/ha).

Experimental design

Inside the 0.48 ha permanent plot, seedlings from four age-height classes [C1: one-year old seedlings; C2: two-year old seedlings; C3: seedlings between 21 and 50 cm high (four to eight years old seedlings, most needles are juvenile, with isolated presence of secondary needles); C4: saplings between 51 and 200 cm high (older than 10 years, with primary and secondary needles)] were selected along a natural gradient and tagged for subsequent physiological measurements. Forty-eight seedlings were measured during 2007 and thirty-two during 2008. In order to control the light environment, seedlings within each class were chosen along five 4 m × 80 m transects, based on the distance of seedlings to the nearest stem (d), expressed by crown radii. In this way, three distances were defined ($0 < d < 0.5R$, $0.75R < d < 1.25R$ and $d > 1.5R$, where R is the crown radius). By this method we achieved a balanced representation of all the possible range of natural light enviroments. Available light on each measured seedling was afterwards estimated from hemispherical photographs, which were taken during 2007 with a Nikon FM camera and a Sigma 8-mm fish-eye lens. From the images, the Hemiview software (Hemiview 2.1, Canopy Analysis Software, Delta-T Devices Ltd., Burwell, Cambridge, UK) calculated the global site factor (GSF). Since no major canopy disturbances occurred between 2007 and 2008, the level of light recorded in 2007 was taken to be representative of the average light environment over the 2-years study period.Temperature and air humidity were monitored at 10 minute intervals by an automatic HOBO data logger (Pro v2, ONSET, Massachusetts, USA) installed in a gap in the centre of the plot. Precipitation was recorded from a nearby meteorogical station. Soil moisture measurements in the first 18 cm (by time domain reflectometry, TRIME-FM2 TDR, IMKO Micromodultechnik GmbH, Ettlingen, Germany) matched up with physiological measurements, which were made in one day of mid-June (d179), mid-July (d206), mid-September (d254) and early December (d343) of 2007 and in one day every end of month between May and September 2008 (d149, d175, d204, d231, d272) and in November 2008 (d317).

Physiological measurements

Chlorophyll *a* fluorescence, gas exchange parameters and water status were measured in one 5 to 7 cm long terminal twig bearing sunlit needles, in twelve seedlings per age class in 2007 and eight seedlings per age class in 2008, under natural light conditions. While chlorophyll *a* fluorescence and gas exchange parameters were always measured in the same shoot, water potential measurements in C1 and C2 seedlings were made in a shoot of a nearby seedling (within a radius of 20 cm from the tagged plant). A portable infrared gas analyser (IRGA LCA 4 Analytical Development Corporation, UK) was used to measure photosynthesis (A, $\mu mol\ CO_2\ m^{-2}\ s^{-1}$) and stomatal conductance (g_s, $mmol\ H_2O\ m^{-2}\ s^{-1}$) at midday. A and g_s were expressed on a projected leaf area basis. Intrinsic efficiency of water use was estimated as $IWUE = A/g_s$ ($\mu mol\ CO_2 \cdot mmol\ H_2O^{-1}$) and vapour pressure deficit (VPD, KPa) was calculated from air temperature and relative humidity. Chlorophyll *a* fluorescence was measured in the same twig, at midday with a portable pulse-modulated fluorometer (FMS 2, Hansatech Instruments Ltd., UK). Maximum photochemical efficiency of PSII (Fv/Fm) was measured at predawn and midday after a 30 minutes dark adaptation period of the leaves. Apparent photosynthetic electron transport rate (ETR) at midday was estimated as $ETR = \Delta F/F'm \times PPFD \times 0.84 \times 0.5$, and the yield of photochemistry in PSII (Φ_{PSII}) was calculated as $\Phi_{PSII} = (F'm\text{-}Fs)/F'm$. Predawn ($\Psi_{pd}$, MPa) and midday water potential (Ψ_{min}, MPa) were measured with a pressure chamber (PMS Instrument Co. 7000, Corvallis, Oregon).

Statistical analysis

Since physiological traits were measured on several occasions every year on the same plant, the joint effect of age-height (categorical factor) and incident light (continuous variable defined by GSF) on physiological status were evaluated following a repeated measurements analysis of covariance (RMANCOVA) using the SAS procedure GLM (Delaney and Maxwell, 1981). Analyses were carried independently for each year, and including 4 measurements in 2007 and 6 measurements in 2008. Evaluated response *Y* variables at plant level were Fv/Fm, ETR, Ψ_{min}, Ψ_{pd}, A, g_s, PSII. Explanatory attributes were size class *C* (categorical), day of measurement *d* (categorical) and light, which enters the model as a continuous covariate defined by the global site factor (*GSF*) measured at each plant. General expression for the proposed RMANCOVA is:

$$Y_{ijk} = \mu + C_j + \beta\, GSF_i + \beta_j \left[C_j \times GSF_i\right] +$$

$$+ d_k + C_j \times d_k + \beta_k \left[d_k \times GSF_i\right] + \beta_{jk}\left[C_j \times d_k \times GSF_i\right] + \varepsilon_{ijk}$$

where subscripts *i*,*j* and *k* refers to the i_{th} sample plant of size class *j* measured during the k_{th} day. The main interest in the analysis was to evaluate the level of significance for both *between subject* effects (those whose levels remain constants within each plant, *i.e.,* C_j, GSF_i and $C_j \times GSF_i$) and *within-subject* effects (those that change within subjects, *i.e.,* d_k, $C_j \times d_k$, $d_k \times GSF_i$, $C_j \times d_k \times GSF_i$). However, considering the impact of the covariate on the significance of main effect of the repeated measure variable (d_k) in RMANCOVA (Delaney and Maxwell, 1981), we carried a two-step fit, assessing first the main effect of d_k and $C_j \times d_k$ in a separate repeated measurements ANOVA, and then the main effect of the rest of factors (C_j, GSF_i, $C_j \times GSF_i$, $d_k \times GSF_i$ and $C_j \times d_k \times GSF_i$) with a RMANCOVA including GSF (Annaz *et al.,* 2009; Thomas *et al.,* 2009). Standard F-test for between subjects effects have been used, while to assess significance of within subject effect either the univariate Huynn-Feldt test or the multivariate ANOVA test based on Roy's Greatest Root statistic were selected according to sphericity hypothesis (Moser *et al.,* 1990; SAS, 1988). The existence of significant differences between size classes within the same day of measurement were also evaluated following a univariate ANOVA and a multiple means comparison test. Normality and heterocedasticity were evaluated, and when severe departures from standard assumptions were detected, logarithm transformation was proposed.

Relationships between the physiological traits A, g_s and *ETR* and between these traits and other leaf-level variables (Ψ_{pd} *and* Ψ_{min}) were evaluated by graphical analysis of the analysed variables and age classes along the different days of measurements and subsequent fit of linear regression models. Both seasonal and within-season age-class differences in the pattern of the relationships were checked by testing homogeneity of slopes.

Results

Enviromental conditions

The climate of the studied area is continental Mediterranean type. Mean temperature was very similar in 2007 and 2008 (ca. 11°C), while total rainfall was almost half in 2008 (402 mm) than in 2007 (730 mm) (Fig. 1), mainly due to the rain fallen between August and November 2007. Mean temperature in July and August was also similar (21.2-21.6°C), but again rainfall was much lower in summer 2008 (11.6 mm) than in summer 2007 (79.8 mm). Maximum mean temperatures during these months reached 40°C at midday; while minimum mean temperatures were under 9°C, thus thermal oscillation during the day was very high. The lowest absolute minimum mean temperatures were shown in November 2007 (–4.6°C) and December 2008 (–3.3°C). Soil moisture showed variations during the study period; the lowest values were reached in both summers, the highest values were shown in spring and autumn-winter, following the rainfall distribution (Fig. 1). Evaporative demands (VPD) were maximum coinciding with highest mean and maximum temperatures and lowest precipitation, *i.e.,* July 2007 and August 2008. Light availability in seedlings (GSF) ranged from 0.32 to 0.68 for all four age classes. Although seedlings were chosen along the widest range in light gradient detected within the stand, differences in GSF were low (mean ± SE: 0.55 ± 0.01; CV = 0.168).

Seasonal differences in physiological status

Results of RMANCOVA (Tables 1 and 2) showed large significant within-year seasonal differences in the analysed physiological traits, while no global significant differences were shown between age classes.

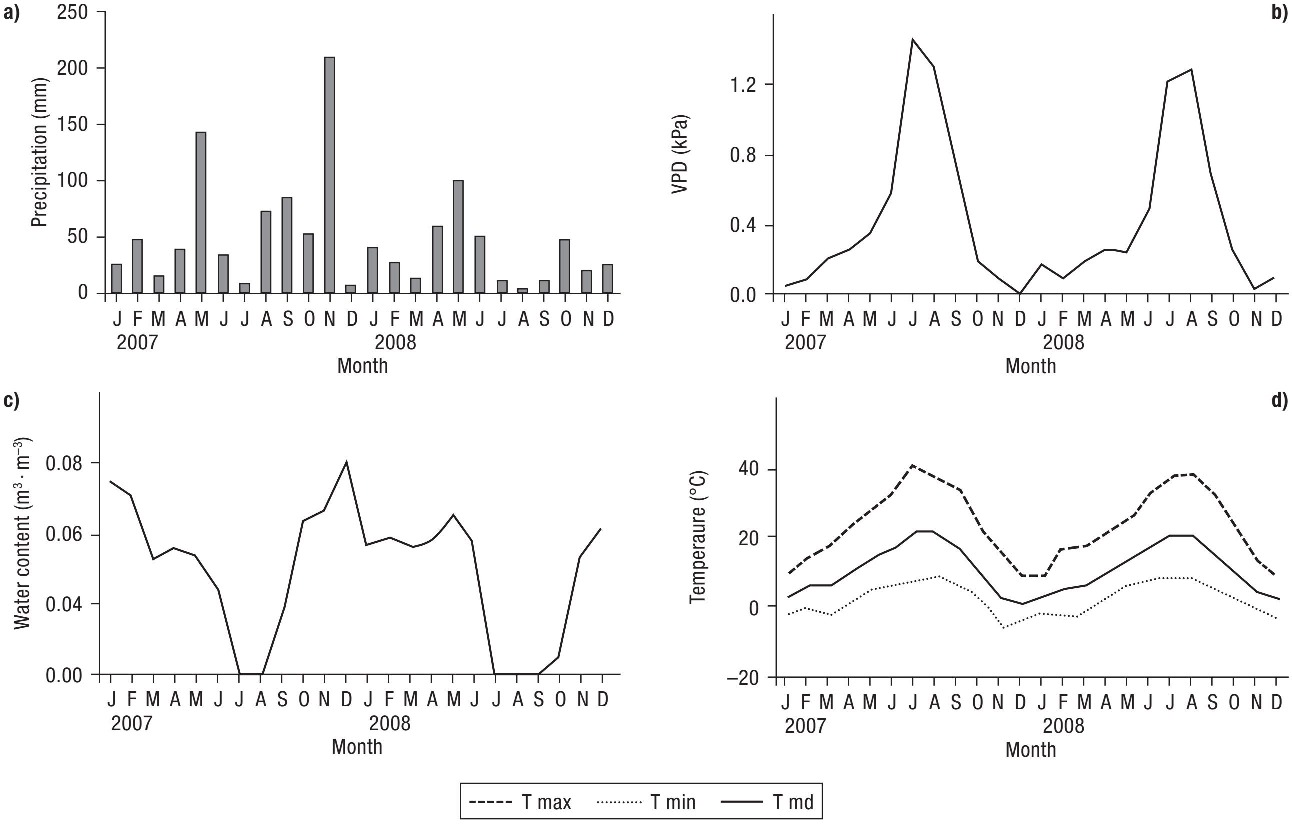

Figure 1. Seasonal course of precipitation, mm (a); mean water vapour deficit, KPa (b); soil moisture at 20 cm depth (c); and mean monthly temperature, °C (d) at the study site during 2007 and 2008.

Table 1. Results of the RMANCOVA analysis in 2007, for different physiological traits

	Ψ_{pd}	Ψ_{min}	**Midday** A	**Midday** g_s	**Midday** Φ_{PSII}	**Midday** ***ETR***	**Predawn** ***Fv/Fm***	**Midday** ***Fv/Fm***
Between Subjects								
GSF	ns	ns	ns	ns	ns	ns	ns	0.0668
Class	ns	ns	ns	ns	ns	ns	ns	ns
GSF × Class	ns	ns	ns	ns	ns	ns	ns	ns
Within Subjects								
Time	< 0.0001	< 0.0001	0.0001	< 0.0001	ns	0.0094	< 0.0001	0.0036
Time × GSF	ns	ns	ns	ns	ns	ns	ns	0.0056
Time × Class	< 0.0001	0.0047	0.0088	0.0117	ns	0.0387	0.0064	ns
Time × GSF × Class	ns	ns	ns	ns	ns	ns	0.0287	0.0425

Significant time × age class effects were detected for most of the physiological attributes, but not in midday Φ_{PSII}. These results showed a clear seasonal pattern in the physiological status of the seedlings, and no constant age-class but seasonal age-class differences in the physiological status of the plants, mainly concentrated during the summer period and affecting one-year seedlings. Finally, no common, seasonal or age-class specific responses in physiological traits due to light irradiance (GSF) were detected.

Predawn (Ψ_{pd}) and midday (Ψ_{min}) leaf water potentials were relatively high in late spring, suffered a steep decline during summer drought (more steeper in 2008) and recovered rapidly in September, after the first late-season rainfalls (Fig. 2). Data for minimum leaf water potential were closely related with minimum values of rainfall, soil water content and moisture, and maximum values of vapour pressure deficit, occurring during July 2007 and August-September 2008. Concerning age-class seasonal specific response, one-year old seedlings showed the lowest ($p\text{-value} < 0.05$) midday water potential during summer drought (June-July and August) for both years, and remained the lowest during 2008 fall.

Maximum photochemical efficiency of PSII at predawn ($Fv/Fm_{\_pd}$) was maintained between 0.76 and 0.84 during spring until mid-summer, suffering a significant decrease in autumn (seasonal differences $p\text{-value} < 0.0001$) (Fig. 3). One-year old seedlings showed significant ($p\text{-value} < 0.05$) lowest values during summer. Down-regulation of PSII at midday brought about a decline in *Fv/Fm*. Mean midday *Fv/Fm* ($Fv/Fm_{\_md}$) showed seasonal variability within each year ($p\text{-value} < 0.01$) and annual variability comparing both years of study. $Fv/Fm_{\_md}$ minimum values in 2007 were reached in late autumn (0.72 ± 0.02), while in 2008 the maximum drop occurred in July 2008 (0.74 ± 0.04). Again, one-year old seedlings were more sensitive and showed the highest decrease in $Fv/Fm_{\_md}$ ($p\text{-value} < 0.05$) along the year (Figure 3). The yield of

Table 2. Results of the RMANCOVA analysis in 2008, for different physiological traits

	Ψ_{min}	**Midday** A	**Midday** g_s	**Midday** Φ_{PSII}	**Midday** ***ETR***	**Predawn** ***Fv/Fm***	**Midday** ***Fv/Fm***
Between Subjects							
GSF	ns	ns	ns	ns	ns	ns	ns
Class	ns	ns	ns	ns	ns	ns	ns
GSF × Class	ns	ns	ns	ns	ns	ns	ns
Within Subjects							
Time	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0020	< 0.0001	< 0.0001
Time × GSF	0.0205	ns	ns	ns	ns	ns	ns
Time × Class	0.0168	0.0040	0.0489	ns	ns	< 0.0001	< 0.0001
Time × GSF × Class	0.0075	ns	ns	ns	ns	0.0826	ns

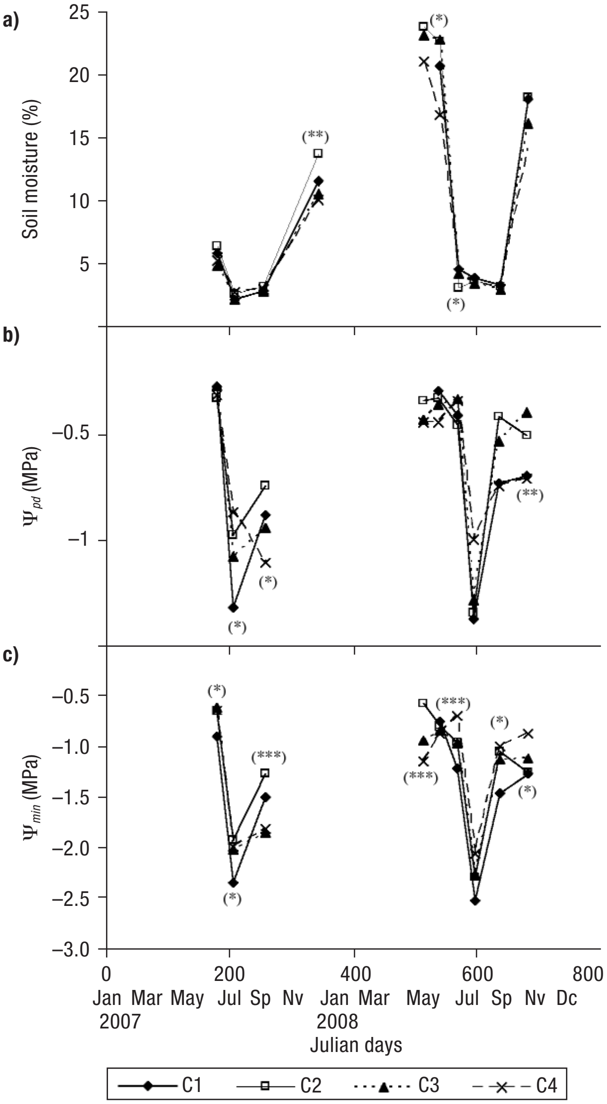

Figure 2. Seasonal course of soil moisture in the first 18 cm (a); predawn leaf water potential, MPa (b); and midday leaf water potential, MPa (c) in stone pine seedlings for the different age classes. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ statistical significance for the differences between age classes for each measuring date (seedlings measured per age class: $n = 12$ in 2007 and $n = 8$ in 2008).

photochemistry in PSII (Φ_{PSII}) of one-year old seedlings also showed minimum values under drought summer conditions (July 2007 and July-August 2008, $p\text{-value} < 0.01$), though seasonal differences in this parameter are less accurate than in others.

All seedlings showed the highest net CO_2 uptake rates (*A)* in spring (seasonal differences p-value < 0.0001), while the highest transpirational rates (*E* were shown in late autumn ($p\text{-value} < 0.0001$), specially during 2007 (an unusual humid and warm season). *A* and g_s declined steeply during summer drought, when leaves showed very low *A* and g_s values, even negative *A* values in July 2008 (Fig. 3). *A* values were higher during summer 2007 than in summer 2008. Due to low precipitations in September 2008, the recovery in g_s did not occur until late autumn. This delay in rainfall occurrence affected again more negatively to *A* and g_s of one-year old seedlings.

Relationship between physiological traits and environmental conditions

A, g_s and $Fv/Fm_{\_md}$ were affected differently by water stress during the year. Midday *A* and g_s decreased steeply from values of Ψ_{pd} lower than –0.5 MPa, while $Fv/Fm_{\_md}$ remained highly stable throughout the range of Ψ_{pd} values (Fig. 4). As no differences were found between measuring dates and age classes, pooled data were used to show the above-mentioned relationships.

Midday *A* and apparent photosynthetic electron transport rate (*ETR*) showed a linear relationship in 2007 ($p\text{-value} < 0.0001$, $R^2 = 0.44$) and 2008 ($p\text{-value} < 0.0001$, $R^2 = 0.60$). To evaluate seasonal differences in the relationship *A vs ETR* tests of hypothesis of homogeneity of slopes between seasons were done, showing slight significant differences in 2007 ($p\text{-value} = 0.0521$), in particular between spring and summer ($p\text{-value} = 0.0421$) (Fig. 5). Within-seasonal differences among age-classes in *A vs ETR* relationship were shown in spring 2007 ($p\text{-value} = 0.0053$), summer 2007 ($p\text{-value} = 0.0578$) and summer 2008 ($p\text{-value} = 0.0008$) (see Table 3 for details of among-age classes within-seasonal contrasts). The relationship between *A* and g_s also showed a clear linear fit for both

Table 3. Contrasts for the test of hypothesis of homogeneity of slopes between age classes for each season in the relationship between net CO_2 uptake (*A*) and apparent photosynthetic electron transport rate (*ETR*). Contrasts only shown when significant

Between age-class contrasts	Spring 2007	Summer 2008	Summer 2008
1-2	0.0063	0.0818	0.0072
1-3	n.s.	n.s.	0.0099
1-4	0.0043	n.s.	n.s.
2-3	0.0327	n.s.	n.s.
2-4	n.s.	0.0122	0.0016
3-4	0.0912	0.0778	0.0017

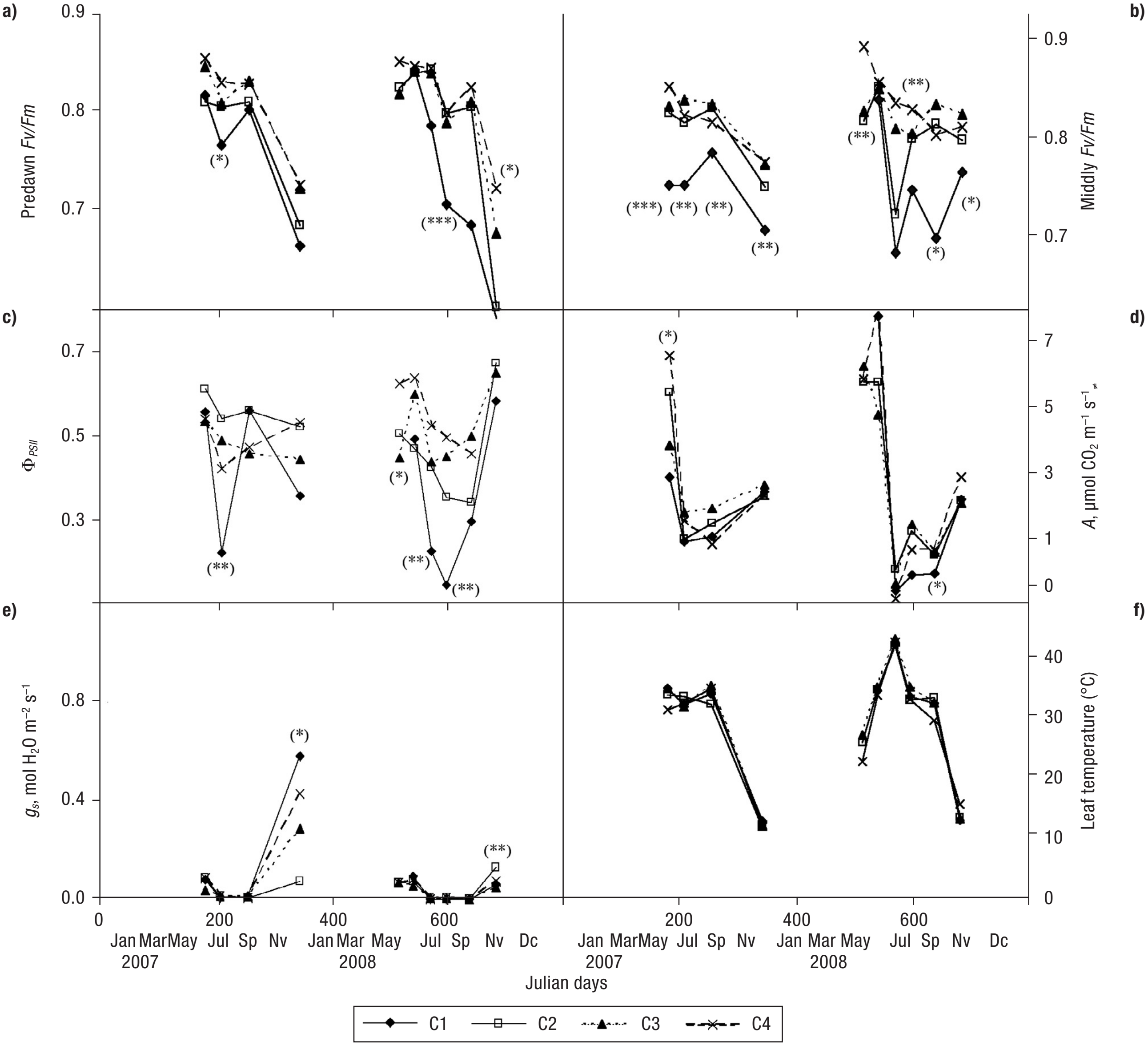

Figure 3. Seasonal course of *Fv/Fm* at predawn (a); *Fv/Fm* at midday (b); yield of photochemistry in PSII, Φ_{PSII} (c); photosynthesis rate, *A* (d); stomatal conductance, g_s (e); and leaf temperature (f), in stone pine seedlings for the different age classes. * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$ statistical significance for the differences between age classes for each measuring date (seedlings measured per age class and measuring date: n = 12 in 2007 and n = 8 in 2008).

years (p-value < 0.0001, $R^2 = 0.50$ in 2007; p-value < 0.0001, $R^2 = 0.68$ in 2008). The test of hypothesis of homogeneity of slopes between seasons showed significant differences between spring and summer (p-value < 0.0001 in 2007 and 2008), spring and autumn (p-value < 0.0001 in 2007, p-value = 0.0486 in 2008) and summer and autumn (p-value = 0.0566 in 2007, p-value = 0.0338 in 2008), detecting higher slopes in spring and summer than in autumn (Fig. 6). Concerning with seasonal age-class differences in the relation, significant contrasts were only detected in spring 2007 (p-value = 0.0062), with class 1 showing significant differences with classes 2 (p-value = 0.0944), 3 (p-value = 0.0020) and 4 (p-value = 0.0028).

Discussion

Variation in seedling water status is highly related to slight changes in soil moisture (Kozlowski and

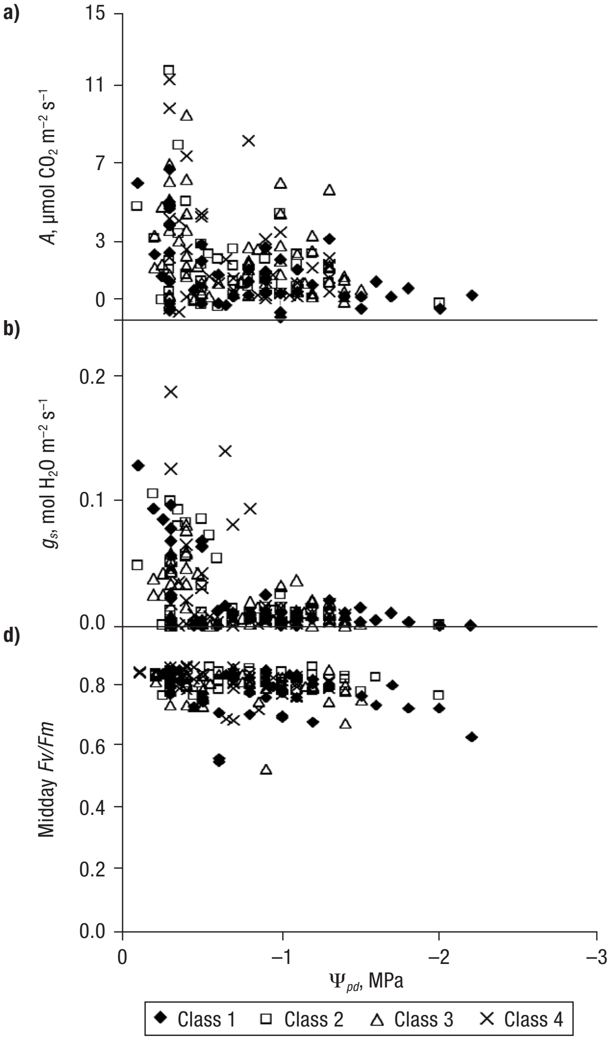

Figure 4. Midday response of net assimilation rate, *A* (a), stomatal conductance, g_s (b) and maximum photochemical efficiency, *Fv/Fm* (c) to decreasing predawn leaf water potencial in the four age classes for stone pine seedlings during 2007 and 2008 (pooled data for 2007 and 2008 are used. Seedlings measured per age class and measuring date: n = 12 in 2007 and n = 8 in 2008).

Pallardy, 2007; Rodríguez-Calcerrada *et al.,* 2007) and, thus, to weather conditions. One-year old seedlings were more affected than older seedlings by prolonged low soil moisture (rainfall lower than 12 mm), joined to high temperatures (maximum mean temperatures were over 30°C) and high VPD demands (over 2.2 KPa) during summer. Thus, during mid-summer in 2007 (July) and summer (July-August) and early autumn (September) in 2008, one-year old seedlings suffered the highest water stress. Such conditions were translated in higher mortality in these younger seedlings (90% in class 1, compared to 23% in class 2, 5% in class 3 and 0% in class 4). It is also interesting to note that while the oldest seedlings (class C4, seedlings between 51 and 200 cm) suffered less from the summer droughty conditions in terms of midday water potential, the recovery of their water status after the late summer-autumn precipitations recorded was slow in 2007.

During these months, mean predawn *Fv/Fm* ($Fv/Fm_{\_pd}$) in one-year old seedlings was less than 0.78 and even reached 0.68 in the droughty September of 2008. All four age classes showed a $Fv/Fm_{\_pd}$ value below the optimal in August 2008. Low water availability, high irradiances and high temperatures during summer also reduced midday *Fv/Fm* in all seedlings, but more steeply in one-year old seedlings, indicating a higher degree of photoinhibition in one-year old seedlings that is associated with a more severe photoinactivation of PSII (Martínez-Ferri *et al.,* 2000). Such susceptibility to photoinhibition decreased once seedlings were two-years old. The stability of predawn and midday *Fv/Fm* along the gradient of water potential showed the high stability of the photosynthetic apparatus of Mediterranean species to drought (Flexas and Medrano, 2002; Valladares *et al.,* 2005, Rodríguez-Calcerrada *et al.,* 2007). A decrease in Ψ has been related to a drop in the photochemical efficiency of PSII (Demmig-Adams *et al.,* 1989; Epron *et al.,* 1992). In addition, in the hottest days, one-year old seedlings showed the lowest Φ_{PSII} values, related to the low *Fv/Fm* and as an indirect inference of its increased capacity to dissipate excess excitation energy (Chaves et al 2008); while the oldest seedlings (class 4) showed the highest Φ_{PSII} values.

The difference between Ψ_{pd} and Ψ_{md} ($|\Psi_{md} - \Psi_{pd}|$) increased with decreasing Ψ_{pd}, therefore allowing a greater increase in the driving force for transpiration as edaphic drought developed (Poyatos *et al.,* 2008). A decrease of seedling water potential affected negatively to stomatal conductance and net assimilation rate during summer drought, irrespective of age class. Such limitations to stomatal conductance were relieved once water was not a limiting factor (*i.e.* spring and late autumn), joined to a decrease in VPD (Panek and Goldstein, 2001). The relationships between *A* and g_s *versus* Ψ_{pd} showed that the reduction in g_s caused by summer water stress was higher than the reduction in *A*, indicating that stomatal closure was the main constraint to net assimilation rate during summer drought, and a mechanism to avoid excessive water loss during central hours (*i.e.* Tenhunen *et al.,* 1981; Llusia and Peñuelas, 2000; Martínez-Ferri *et al.,* 2000). Such control of stomatal aperture can be explained in terms

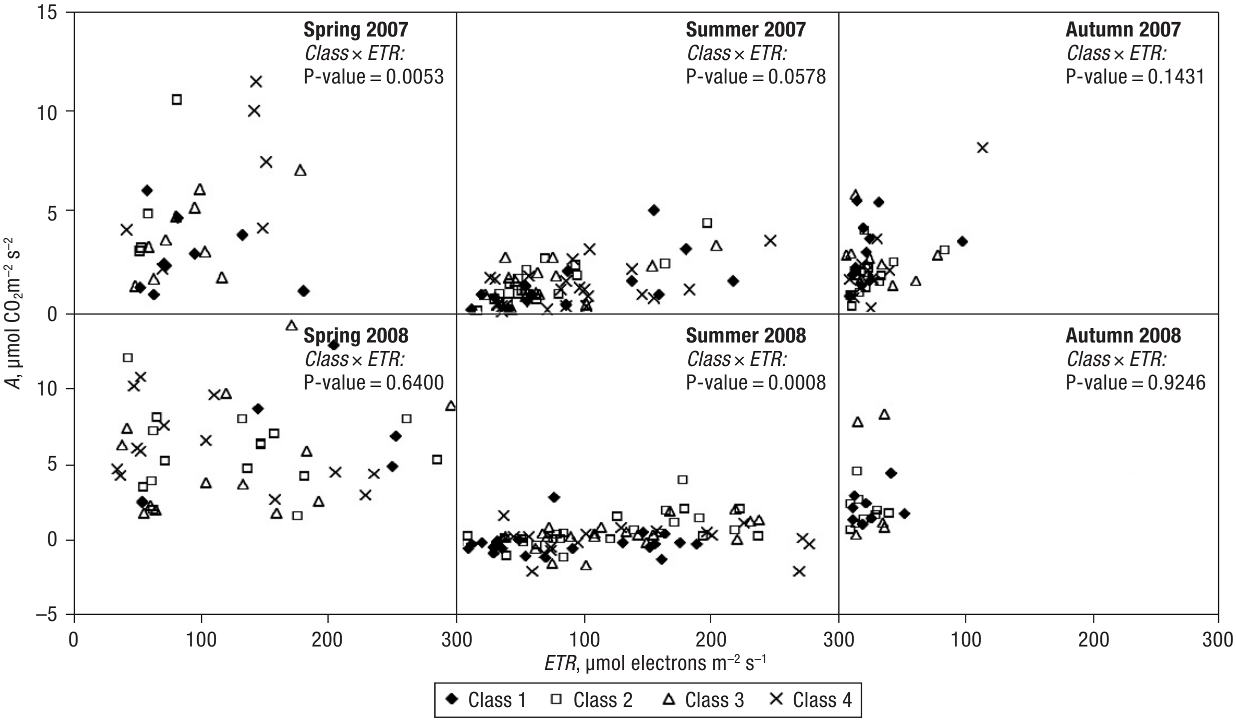

Figure 5. Relationship between net CO_2 uptake (A) and apparent photosynthetic electron transport rate (ETR) at midday in the four age classes for stone pine seedlings during spring, summer and late autumn of 2007 and 2008 (seedlings measured per age class and measuring date: n = 12 in 2007 and n = 8 in 2008). *Class* × *ETR* refers to the test of hypothesis of homogeneity of slopes between age classes for each season.

of sensitivity of stomata to increasing VPD (Irvine *et al.,* 1998; Kolb and Stone, 2000; Cordeiro *et al.,* 2009). The Ψ_{pd} values that caused stomatal closure in summer were relatively high (–0.8 to –1.2 MPa in 2007 and –0.5 to –1.3 MPa in 2008), showing a drought-avoiding behaviour of stone pine, similarly to the Californian Mediterranean *Pinus ponderosa* (Panek and Goldstein, 2001), but in contrast to the drought-tolerance behaviour of other Mediterranean evergreen oak species (Fotelli *et al.,* 2000).

Under the severe drought conditions experienced in July 2008 all seedlings showed negative or almost null photosynthesis rates at midday. Such negative trend was maintained for one-year old seedlings in August and September 2008. Higher photorespiration rates as a consequence of drought and high temperatures might have contributed to such negative photosynthetic rates (Ogaya and Peñuelas, 2003b). Linear regressions between net assimilation rate and g_s measured at midday showed good fit for most measuring dates. The proportionality between A and g_s during summer drought indicates again that photosynthesis is mainly controlled by stomatal aperture. Lower A and g_s values at midday in summer in one-year old seedlings, despite similar IWUE for all age classes showed that one-year old seedlings are able to cope worse with summer heat and drought.

Periods without water and cold stresses are widely described as the most favourable seasons for photosynthetic activity of Mediterranean species (Savé *et al.,* 1999; Ogaya and Peñuelas, 2003; Filella *et al.,* 1998), with a strong season-dependent effect (Prieto *et al.,* 2009). In our study, the highest net CO_2 assimilation rates values were reached in spring (May-June), followed by late autumn (November-December), when A rates in the morning were higher than at midday, similarly to results obtained with Mediterranean *Pinus halepensis* (Maseyk *et al.,* 2008a; Maseyk *et al.,* 2008b). In these two periods, the oldest seedlings (class 4, between 51 and 200 cm high) showed higher photosynthetic rates, related to higher leaf temperatures. Stomatal conductance was highest in late autumn, despite the low minimum mean temperatures (–4°C in 2007, –2°C in 2008), but joined to a rainy October-November period (260 mm in 2007 and 65 mm in 2008). Under these environmental conditions, one-year old seedlings showed the highest stomatal conductances in late autumn. During spring, when no cold or drought stress occurred, *Fv/Fm* values remained high all day, but usually lower

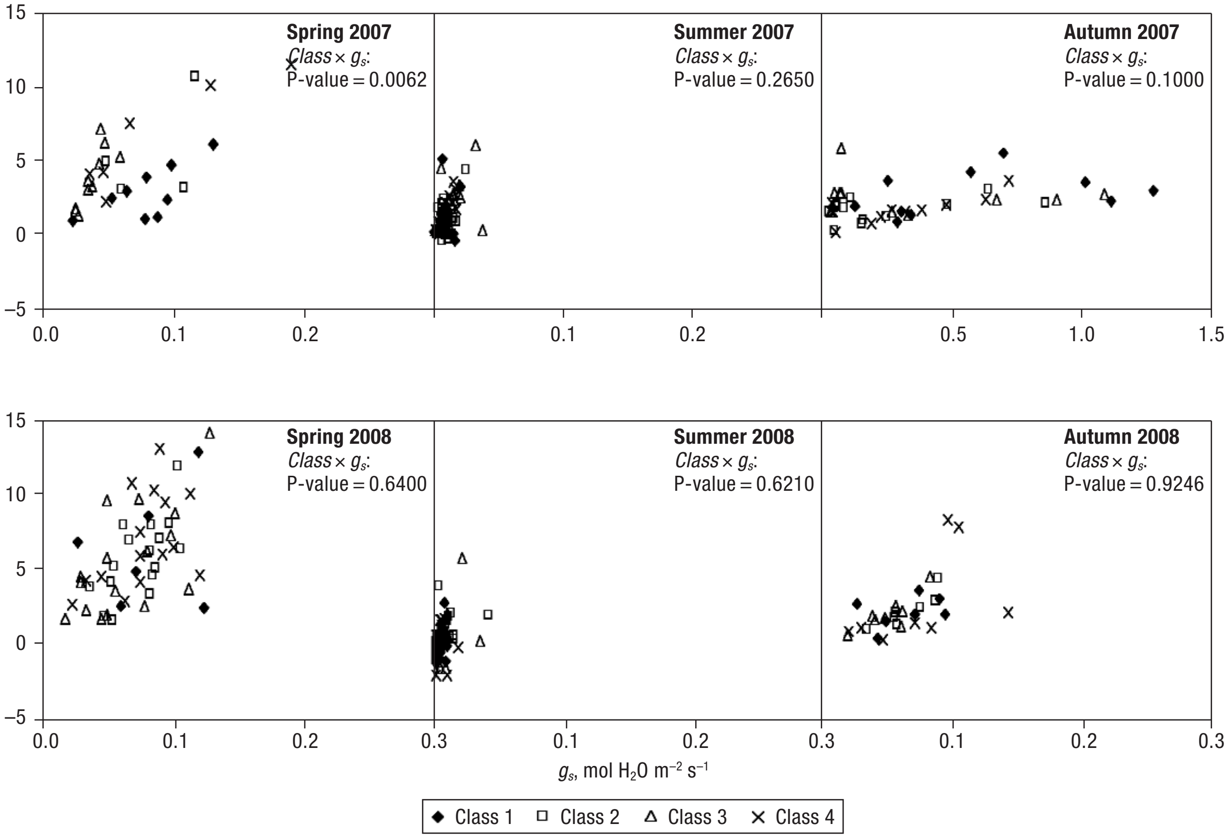

Figure 6. Relationship between net CO_2 uptake (*A*) and stomatal conductance (g_s) at midday in the four age classes for stone pine seedlings during spring, summer and late autumn of 2007 and 2008 (seedlings measured per age class and measuring date: n = 12 in 2007 and n = 8 in 2008). *Class* × g_s refers to the test of hypothesis of homogeneity of slopes between age classes for each season.

in one-year old seedlings. The yield of photochemistry in PSII (Φ_{PSII}) was high in spring and late autumn and low under drought summer conditions.

Predawn *Fv/Fm* ($Fv/Fm_{\_pd}$) was very sensitive to low temperatures (Oliveira and Peñuelas, 2000, 2001; Ogaya and Peñuelas, 2003b) and, in second term, to summer drought. Low temperatures in late autumn reduced predawn and midday Fv/Fm in all seedlings; but midday *Fv/Fm* suffered a stronger decrease in one-year old seedlings due to the combined effect of cold and seedling size. In spring and mainly in summer a marked and reversible decrease in *Fv/Fm* occurred at midday. Such depression seemed to be related to the combination of high irradiance and water limitations (Zunzunegui *et al.,* 1999) and was in concordance with a strong stomatal limitation to photosynthesis.

During late autumn, when minimum temperatures in the measuring dates were –6.8°C in 2007 and –2°C in 2008, and mean temperatures were under 4°C, a chronic down-regulation of PSII occurred, but only determined a potential decrease in *A* (Oliveira and Peñuelas, 2004). This demonstrated that stomatal regulation impeded gas exchange far more than a reduction at the PSII activity alone (Valladares and Pearcy, 1997). The decrease of F_v/F_m in autumn can be related to plant hardening, as a photoprotective mechanism against freezing temperatures (Öquist *et al.,* 2001). However, this hardening process did not suppress photosynthesis, so the differences found between age classes in F_v/F_m had no influence on carbon gain and only reflected possible different mechanisms of cold acclimation between age classes or between juvenile and adult needles (Climent *et al.,* 2009).

Higher photorespiration rates in one-year old seedlings and the oldest seedlings (class 4) during maximum summer drought (July 2007 and August 2008) is suggested as a possible mechanism of dissipating excess of photochemical energy in water-stressed and heat-stressed plants (Peñuelas and Llusiá, 2002). In late autumn one-year old seedlings showed higher photoinhibition rates (lower ETR values) than other age classes. The lower slope in the net assimilation rate

(*A*) *vs ETR* relationship in mid-summer compared to late autumn may be explained by higher photorespiration rates during summer drought (Filella *et al.,* 1998).

As mentioned before, air temperature was different between seasons, and so was leaf temperature. In all seedlings it seems that the optimum temperature for net CO_2 uptake and *gs* is seasonally adjusted depending on the range of temperatures recorded in each season (Ogaya and Peñuelas, 2003). The oldest seedlings (class 4) showed higher tolerance to high temperatures than one-year old seedlings, at least until leaf temperatures reached ca. 43°C (July 2008), when *A* showed null or negative values in all seedlings; and a trend to lower tolerance to winter cold.

Conclusions

Stone pine regeneration adapts its physiology to the seasonal course of temperature and water availability, showing a different photosynthetic performance according to age class, mainly one-year old seedlings versus the oldest seedlings (class 4, 51 to 200 cm high). Thus, stone pine regeneration develops different physiological protective mechanisms to combat the stresses of the Mediterranean climate (excessive irradiance and high summer-low winter temperatures, irregular water availability), including photoprotection, stomatal closure and water potential control within safety margins for functionality of the hydraulic system. However, seedling physiology is not affected by the natural light gradient found in the studied stand, probably related to the low variability recorded in GSF values and the low density of the stand. Interestingly, during late autumn, when mean temperatures were under 4°C, a chronic down-regulation of PSII occurred, but only determined a potential decrease in *A*. This result points out to a certain degree of non-stomatal inhibition of photosynthesis during late autumn that will inhibit shoot growth even when water is available in the soil.

Our results suggest that the low stand densities of *Pinus pinea* devoted to nut production cannot assure one-year old seedlings' survival, but are sufficient for older seedlings, once they are able to survive the first droughty summer, but only if fall rainfalls are assured. Considering the well known strong climatic control exerted over nut production and germination of stone pine (Calama and Montero *et al.,* 2007), and the now found climatic control over seedling survival and photosynthetic performance of this species, we propose more graduated and less intense regeneration fellings that will guarantee sufficient amount of seed reaching the floor necessary to assure regeneration, even when extreme climatic episodes occur.

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