Effects of Restricted Watering and CO₂ Enrichment in the Morphology and Performance after Transplanting of Nursery-grown *Pinus nigra* Seedlings

Carme Biel and Robert Savé

Departament de Tecnologia Hortícola, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Carretera de Cabrils s/n. 08348 Cabrils, Barcelona, Spain

Abdessamad Habrouk, Josep Maria Espelta, and Javier Retana¹

Centre de Recerca Ecològica i Aplicacions Forestals (CREAF) i Unitat d'Ecologia, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

Additional index words. reforestation, hardening, *Pinus nigra*, seedling, nursery practices

Abstract. Extensive areas occupied by *Pinus nigra* forests in Spain have burned in recent years. Recovery of these forests depends upon reforestation. In this study, we analyze the combined effects of different treatments of water restriction and air enrichment with CO₂ in the nursery as a means to increase hardening and vigor of *P. nigra* seedlings of various ages (recently germinated, 1- and 2-year-old) and their post-transplant performance in a burned area. In all cases, 2-year-old seedlings showed the highest specific leaf weight and low cuticular transpiration rates. CO₂ enrichment increased total biomass, leaf biomass and leaf area of seedlings while water restriction decreased leaf area, leaf biomass, and stem biomass, without any significant interaction between both experimental factors. Younger (recently germinated and 1-year-old) seedlings showed a larger relative response to the experimental factors than 2-year-old seedlings. After transplanting, the previous CO₂ and water treatments did not affect seedling survival, but 2-year-old seedlings showed the lowest mortality rates.

Wildfires are among the most important natural disturbances in Mediterranean ecosystems (Trabaud, 1987). However, during recent decades, the number and, especially, the surface area burned by forest fires has increased considerably (Piñol et al. 1998). These larger and more intense wildfires are now affecting areas that were not so regularly burned in the past as fire-prone Mediterranean areas (Moreno et al., 1998). These areas are occupied by species that may lack an efficient postfire regeneration mechanism. This is the case of *Pinus nigra* Arnold, a pine widespread in middle-montane areas of the Mediterranean Basin. Due to the lack of serotinous cones and the early dispersal of seeds at the beginning of spring, this species does not have a seed bank after summer wildfires (Habrouk et al., 1999), and shows very low or no postfire regeneration (Retana et al., 2002; Trabaud and Campant, 1991).

Due to this lack of successful regeneration, the only alternative to recover *P. nigra* forests is to reforest with seeds or seedlings of this species developed in nurseries. The first objective of

nursery production is to obtain plants adequate for the area where they will be planted. In the Mediterranean region, water stress is the main limiting factor for plant life (Mitrakos, 1980). The almost complete absence of rainfall during the hottest months and its irregular distribution in the cold season can impair performance of forest plantations (Baeza et al., 1991; Vilagrosa et al., 1996). For this reason, plants needed for revegetation and restoration projects in Mediterranean climates should be plants adapted to low rainfall (Araújo-Alves et al., 2000). Water requirements during establishment may be particularly high (Baeza et al., 1991), because seedlings have root systems that are not developed sufficiently to replace water lost through transpiration and because they are acclimatized to the environmental conditions of nurseries, which favor plant productivity rather than plant hardening (van der Driessche, 1991a, 1991b, 1992). The ecophysiological response of plants to harsh environmental conditions can be changed by acclimatization during nursery production or in the first stages in the field, after transplanting (Sachs, 1991). Thus, exposure of conifer seedlings to drought stress can increase their subsequent drought resistance (Kaushal and Aussenac, 1989). This resistance can be achieved by a series of morphological and physiological features that may, to a great extent, be conditioned in the nursery by certain cultural practices. Among these practices, application of restricted watering has been proved

to promote osmotic adjustment and changes in cell wall elasticity (Royo et al., 2001; Savé et al., 1993, 1995; see Villar-Salvador et al., 1999), and increase root growth capacity (Ali Abod and Sandi, 1983).

However, the most effective treatments for drought resistance in the nursery tend to produce the smallest seedlings (Royo et al., 2001, van der Driessche, 1991a). Since large seedlings usually have higher survival and growth than small seedlings under natural conditions (Egnell and Örlander, 1993; Lamhamedi et al., 1996, van der Driessche, 1991a), the second objective of nursery production should be to obtain plants of larger size than those grown with hardening treatments. To increase seedling growth and compensate for the reduced growth of hardening treatments, one possibility is to complement them with applications of high CO₂ concentrations in the atmosphere of the nursery (Savé et al., 1998), because the general conclusion of a number of studies (see reviews in Curtis and Wang, 1998; Norby et al., 1999; Tingey et al., 2000) is that tree seedlings exposed to high CO₂ generally show enhanced growth due to increased carbon assimilation. Thus, the combined effects in the nursery of drought stress and high CO₂ might improve both the hardening and productivity of tree seedlings that will be planted in the field. An enriched CO₂ atmosphere might lead to more hardened seedlings by increasing specific leaf weight, the amount of solutes (nonstructural carbohydrates), and fine roots, thus promoting better water status (Wullschlegel et al., 2002). Different studies indicate that nursery treatments can influence the performance of seedlings after planting in the field (Bayley and Kietlka, 1996; van der Driessche, 1991a, 1991b), but there is still insufficient information about the field response of seedlings receiving different treatments and the effects (synergistic or not) caused by those treatment (Arnott et al., 1993; van der Driessche, 1992). The main objective of this study was to evaluate the combined effects of different treatments of irrigation and CO₂ enriched air for improving survival and growth of *P. nigra* seedlings transplanted into burned areas in Catalonia (northeastern Spain).

Material and Methods

This work was conducted in two different stages: a nursery assay and a plantation test in the field.

Nursery assay. The nursery assay was developed in a greenhouse located in Cabrils Barcelona (2° 30' E, 41° 45' N). In September 1999, 1200 *P. nigra* seeds were sown in 300 - cm³ forest-pot containers of 17- cm² cell upper surface and 18 cm high, filled with a substrate of 1 peat : 1 perlite mixture (by volume). In October 1999, 600 one-year-old *P. nigra* seedlings (1:0) and 600 two-year-old seedlings (2:0) were obtained from a commercial nursery (Olot, northeastern Spain) and were also placed in the greenhouse. Commercial seedlings were grown in 300-cm³ forest-pot containers filled with 3 peat : 1 vermiculite (by volume). Plant density in the greenhouse was 387 plants/m. Mean, minimum and maximum temperatures

Received for publication 14 Aug. 2002. Accepted for publication 3 Sept. 2003. Thanks are due to Josep Duocastella for his permission to carry out the present experimental plantation on his property as well as for field assistance, and to Beatriz Díez, Roser Cristobal and Ramón Carrabina for field assistance. This research was funded by the PETRI project PTR1995-0511-OP and AGRIGAS.

¹Corresponding author; e-mail Retana@uab.es.

GROWTH REGULATORS

during the essay period were 17.1, 13.6, and 25.6 °C, respectively, and the average of crop daily evapotranspiration (Etc) was 2.87 mm·d⁻¹.

Seedlings of *P. nigra* were grown at three CO₂ concentrations and two irrigation treatments in a factorial experiment.

1) **CO₂ TREATMENT.** A multitunnel greenhouse (a three span tunnel type divided into three modules of 77 m² each) was used to test the effects of CO₂ on *P. nigra* seedlings. The air was enriched with a constant flow of pure industrial CO₂ (Carburros Metálicos, S.A.) through polyethylene vessels. One greenhouse module was flushed with this gas until 500 ppm CO₂ concentration was reached and the other one was flushed until a concentration of 750 ppm was reached. The third module was maintained at atmospheric CO₂ concentration (350 ppm; control treatment). The CO₂ concentration was measured by an infrared gas analyzer (IRGA) (LIRA model 3600; MSA, Spain) connected to a personal computer and controlled by a PLC OMRON C20K, an automatic device to control the opening and closure of the valves that injected the CO₂ flux. This automatic system provided a CO₂ flux that was enough to reach the concentration desired. To avoid high humidity and high temperatures effects, roof windows of all modules were opened once a day (from 10:30 AM to 15:00 PM). During this period,

CO₂ concentration was kept constant through increasing flux rates. During the experiment, ventilation rates ranged from 5.0 to 6.1 m³·s⁻¹ with a wind speed between 0 and 4 m·s⁻¹. The film cover of the three modules had 64% of transmittance, and the mean radiation received by the plants was 1772 W·m⁻²·d⁻¹.

2) **IRRIGATION TREATMENT.** Two water treatments were imposed during the essay: a control (100%), which corresponded to complete substrate saturation plus 30% of drainage (the common watering strategy in commercial nurseries in the area), and 50%, which corresponded to the half doses. The average amount of water applied daily was 9.9 L·m⁻² and 5.4 L·m⁻² in the 100% and 50% treatment, respectively. These water amounts were calculated on the basis of the average of crop daily evapotranspiration during the essay (2.87 mm·d⁻¹) plus 30% of leaching and 40% of efficiency of the irrigation system (Peñuelas and Ocaña, 1996). Water plus fertilization was daily applied with microsprinklers. Nutrient solution was 8.22 mmol·L⁻¹ NO₃⁻, 0.9 mmol·L⁻¹ PO₄H₂⁻, 1.66 mmol·L⁻¹ SO₄²⁻, 4.20 mmol·L⁻¹ K⁺, 3.92 mmol·L⁻¹ Ca²⁺, 1.50 mmol·L⁻¹ Mg²⁺, and 1.28 mmol·L⁻¹ NH₄⁺. The pH was adjusted to 6.5.

Different morphological and biomass allocation variables were measured for plants of each treatment before and five months after treatment application (in both cases, six plants per treatment were randomly selected). Biomass, expressed as a dry weight, was obtained after oven-drying to constant weight at 60° for at least 48 h. Seedling root, stem and needle biomass were measured separately. Projected leaf area

was determined for each plant with a digital analysis system (Delta-T Devices, Cambridge, U.K.). Total stem height (from ground to base of terminal bud) and basal stem diameter (with a digimatic caliper; 500 series, Mitutoyo, Japan) were measured. From these variables, different indexes were calculated: specific leaf weight (SLW, leaf weight/leaf area), leaf weight ratio (LWR, leaf weight/plant weight), leaf area ratio (LAR, total leaf area/plant weight), stem weight ratio (SWR, stem weight/plant weight), root weight ratio (RWR, root weight/plant weight), and root/shoot ratio (root weight/stem+leaf weight) (Coombs et al., 1985). Total root length and total root area were measured with a digital analysis system (Delta T Devices). Specific root length (SRL) was calculated as root length/root weight (Tagliavini et al., 1993). At the end of the experiment and just before field plantation, five samples of five leaves of seedlings per treatment were saturated overnight at 7 °C. Fresh weight was measured gravimetrically the next day at intervals of 30 and 60 min. Leaf dry weight was measured after drying at 60 °C for

48 h. Cuticular transpiration was calculated as loss of water after stomatal closure (see Villar-Salvador et al., 1999).

This nursery experiment was conducted as a factorial experiment and analyzed as a completely randomized design, with three factors tested: age [seed, 1 year old (1:0) and 2 years old (2:0)], CO₂ concentration (ambient, 350, 500, and 750 ppm), and water level (control, 100%, and 50% of water supply). There were 100 1- and 2-year-old plants, and 200 plants of seed origin per treatment. Statistical analysis was performed with SAS (SAS Institute Inc., Cary, N.C.). Analysis of variance was used to test for the effects of age, CO₂, and water treatment on all measured and calculated variables. When necessary, data were normalized by a log transformation. The sequential Bonferroni method was used to control the group-wide type I error rate (Rice, 1989). The individual values of the different levels of each variable were compared with a posthoc test (Fisher's protected least significant difference).

Field plantation. To assess the response to field conditions of the *P. nigra* seedlings grown under different greenhouse treatments, they were planted outdoors in the Castelltallat Mountains (41° 45' to 42° 6' N; 1° 38' to 2° 1' E, Catalonia, northeastern Spain). This area was affected by a wildfire in July 1998, which burned ≈14,300 ha covered extensively by *P. nigra* forests (84% of the surface). The climate of the area is dry-subhumid Mediterranean (according to the Thornwaite index), with mean annual temperature of 10 to 13° C and mean annual precipitation of 600 mm. The experimental plot (1.7 ha) was located in a burned forest of *P. nigra* with no regeneration of any tree species and barely covered by scattered shrubs and a herb layer dominated by *Brachypodium retusum*. The soil of the plot was a calcareous limestone.

Before plantation, soil ripping was carried out using a double teeth subsoiler mounted at the rear of a bulldozer, which opened parallel rips of 70 cm deep with 2.5 m between them. After site preparation, 60 seedlings of each greenhouse treatment (2 water levels × 3 CO₂ levels) and two ages [1 year old (1:0) and 2 years old (2:0) in the nursery] were randomly chosen and planted in the rips by assigning a random number before plantation to each of the 720 seedlings. No selection by size was carried out; seedlings were randomly chosen in the greenhouse. Seedlings were planted in the field at a density of 1400 seedlings/ha, a standard density value used in *P. nigra* afforestations (Espelta et al., unpublished data). We did not plant the third age treatment (seedlings arisen from seeds in the greenhouse) because they did not attain the minimum size recommended for planting. Seedlings were planted in March 2000, and their survival was monitored at the end of 2000 and 2001. Height at the end of 2001 and height growth during these 2 years in the field were measured for all surviving plants.

To study differences of survival between planted seedlings of different ages, CO₂, and irrigation treatments, models obtained from multi-way contingency tables were analyzed. When the goodness-of-fit was *p* > 0.05, data were considered to fit the model in question.

Fig. 1. Root length (A) (in cm) and specific root length (B) (in m·g⁻¹) of recently emerged, 1-year-old and 2-year-old *Pinus nigra* seedlings in the three CO₂ treatments (350, 500, and 750 ppm). Vertical bars extend over ±1 SE of the mean.

Table 1. F values from ANOVA tests of effects of age (recently-emerged, 1-year-old and 2-year-old seedlings), CO₂ treatment (350, 500, and 750 ppm) and irrigation (100% and 50% of water supply) on different morphological and biomass allocation variables of *Pinus nigra* seedlings in the greenhouse experiment. The following abbreviations have been used: root/shoot (R/S), leaf weight ratio (LWR), stem weight ratio (SWR), root weight ratio (RWR), leaf area ratio (LAR), specific leaf weight (SLW), and specific root length (SRL). Significant coefficients (at $p = 0.05$ when the sequential Bonferroni method is employed) are indicated in bold. All variables except height, LWR, SWR, RWR, and LAR were log-transformed.

Source of variation	Stem ht	Stem diam	Root length	Leaf area	Root area	Total biomass	Leaf biomass	Root biomass	R/S	LWR	SWR	RWR	LAR	SLW	SRL	Cuticular transpiration
Age (A)	113.3	599.1	280.5	342.8	381.8	781.8	544.2	509.1	11.6	82.0	23.8	9.0	211.9	142.6	82.5	59.8
CO ₂ level (C)	1.7	3.3	10.5	6.9	6.5	6.9	8.8	2.1	1.0	1.0	0.8	0.8	0.0	0.8	4.3	0.2
Irrigation (I)	22.6	9.0	0.0	21.0	2.0	4.1	25.5	1.1	31.3	32.6	5.9	30.9	22.8	1.7	2.4	5.3
A × C	0.8	2.9	7.5	2.8	3.5	4.1	4.0	2.1	1.4	2.1	0.1	1.1	1.0	1.5	11.8	1.6
A × I	2.9	2.4	15.6	9.1	4.1	1.3	6.7	0.4	6.5	11.5	0.0	5.9	16.4	1.1	35.9	2.1
C × I	3.2	3.2	0.3	3.3	0.3	0.6	3.4	0.3	2.8	5.8	0.1	2.3	3.1	0.8	2.1	1.0
A × C × I	0.6	1.8	0.9	1.7	0.3	1.8	2.4	1.1	0.9	2.3	0.9	0.7	1.7	1.0	4.3	0.6

The best model was considered to be the most parsimonious acceptable ($p > 0.05$) one. These analyses were carried out with the program STATISTICA for WINDOWS. A two-way analysis of variance was used to test for the effects of CO₂ and water treatment on final height and height growth of seedlings. To control the group-wide type I error rate, the sequential Bonferroni method was used (Rice, 1989). The individual values of the different levels of each variable were compared with a posthoc test (Fisher's protected least significant difference).

Results

Nursery assay. The effects of age, CO₂ treatment, and irrigation on morphological and biomass allocation variables of *P. nigra* seedlings in the nursery assay are summarized in Table 1. The age of seedlings influenced all variables considered (Table 2). Seedlings increased in size from recently emerged seedlings to 1- and 2-year-old seedlings for most variables considered. Seedlings of different ages also differed in the variables related to resource allocation, because LWR, LAR, and SRL decreased from recently emerged to 1- and 2-year-old seedlings, while SWR, RWR, and SLW showed the opposite pattern.

The increment of CO₂ increased total biomass, leaf biomass, and leaf area of *P. nigra* seedlings (Table 3). High CO₂ concentration also increased root length, although the interaction age × CO₂ treatment was significant for this variable and for SRL (Fig. 1). Root length increased with CO₂ in recently emerged and in 1-year-old seedlings, but not in 2-year-old seedlings.

There was no significant evidence that the decrease in water supply affected total biomass (Table 1), but it reduced stem height, leaf area, leaf biomass, and stem biomass (Table 3). As a consequence, LWR decreased, while R/S ratio and RWR increased (Table 3). However, there was a significant interaction between age × irrigation for some of the variables considered (Fig. 2). Leaf area increased with irrigation for recently emerged seedlings but not for older ones. This also led to an increment of LWR and LAR for these young seedlings but not for older ones. Root length and SRL also increased with irrigation in recently emerged and, to a lesser extent, in 2-year-old seedlings, but decreased in 1-year-old seedlings.

According to Table 1, age of plants was also the only factor significantly affecting

Table 2. Mean values of different morphological biomass allocation and physiological variables of *Pinus nigra* seedlings of the three ages considered in the greenhouse experiment. Significantly different means, based on Fisher LSD F test (at $p = 0.05$), are indicated by different letters.

Variable	Seedling		
	Recently emerged	1-year-old	2-year-old
Stem height (cm)	9.8 a	11.4 b	17.1 c
Stem diameter (cm)	1.0 a	3.3 b	4.0 c
Root length (cm)	92.8 a	768.3 b	752.0 b
Leaf area (cm ²)	14.8 a	72.1 b	78.5 b
Root area (cm ²)	7.0 a	76.6 c	45.8 b
Total biomass (g)	0.29 a	2.87 b	5.00 c
Leaf biomass (g)	0.12 a	0.90 b	1.12 c
Root biomass (g)	0.13 a	1.54 b	2.55 c
R/S (g·g ⁻¹)	0.91 a	1.16 b	1.18 b
LWR (g·g ⁻¹)	0.40 c	0.32 b	0.23 a
SWR (g·g ⁻¹)	0.15 a	0.16 a	0.23 b
RWR (g·g ⁻¹)	0.45 a	0.53 b	0.51 b
LAR (cm ² ·g ⁻¹)	50.0 c	26.1 b	16.0 a
SLW (mg·cm ⁻²)	8.1 a	12.5 b	14.3 c
SRL (m·g ⁻¹)	8.3 c	5.1 b	3.0 a
Cuticular transpiration (mg·g ⁻¹ dry weight per min)	0.90 b	0.99 b	0.36 a

Table 3. Mean values of different morphological biomass allocation and physiological variables with significant differences for *Pinus nigra* seedlings grown under three CO₂ and two irrigation treatments in the greenhouse experiment. Significantly different means based on Fisher LSD F test (at $p = 0.05$), are indicated by different letters.

Variable	Treatment		
	CO ₂		
	350 ppm	550 ppm	750 ppm
Total biomass (g)	2.5 ± 0.3 a	2.9 ± 0.4 ab	3.2 ± 0.4 b
Leaf biomass (g)	0.65 ± 0.07 a	0.79 ± 0.08 b	0.84 ± 0.09 b
Leaf area (cm ²)	50.5 ± 5.0 a	58.5 ± 6.1 ab	64.9 ± 5.8 b
	Irrigation		
	50%	100%	
Stem height (cm)	11.9 ± 0.5	14.1 ± 0.6	
Leaf area (cm ²)	53.7 ± 4.5	62.2 ± 4.7	
Leaf biomass (g)	0.68 ± 0.06	0.83 ± 0.07	
Stem biomass (g)	0.52 ± 0.06	0.63 ± 0.08	
R/S (g·g ⁻¹)	1.26 ± 0.06	0.93 ± 0.06	
LWR (g·g ⁻¹)	0.28 ± 0.01	0.34 ± 0.02	
RWR (g·g ⁻¹)	0.54 ± 0.01	0.45 ± 0.01	

cuticular transpiration of *P. nigra* seedlings. Plants of seed origin and 1-year-old showed higher values than 2-year-old plants (Table 2). The other factors and the different interactions were not significant.

Field plantation. Mean survival of *P. nigra* seedlings one season after their transplant to the field was 75%. According to the best model obtained in the analysis of the multiway contingency table of survival data, survival of *P. nigra* seedlings was not influenced by the experimental treatments previously received in the nursery, neither watering regime nor CO₂ fertilization, but did depend on seedling age (log-linear analysis, G² = 14.8, $p = 0.789$, df

= 20). Survival was higher in 2-year-old seedlings than 1-year-old seedlings (81% vs. 69% of seedlings alive). Two years after planting, seedling survival was only 51%. Again, there were no differences in survival among CO₂ or water treatments, but survival still depended on seedling age (56% and 44% for 2-year-old and 1-year-old seedlings).

Seedling height 2 years after planting was significantly affected by seedling age and the CO₂ treatment in the greenhouse but not by the irrigation level applied in the greenhouse (Table 4). Two-year-old seedlings were taller than one-year-old ones (23.2 ± 0.4 vs. 18.2 ± 0.5 cm). Seedlings receiving 550 ppm achieved higher

Fig. 2. Variations in (A) leaf area (in cm²), (B) leaf weight ratio (LWR, in g·g⁻¹), (C) leaf area ratio (LAR, in cm²·g⁻¹), (D) root length (in cm), and (E) specific root length (SRL, in m·g⁻¹) of recently emerged, 1-year-old and 2-year-old *Pinus nigra* seedlings in the two irrigation treatments (100% and 50% of water supply). Vertical bars extend over ± 1 SE of the mean.

height (22.0 ± 0.7) than seedlings receiving 750 ppm (19.4 ± 0.5 cm), while those of 350 ppm (20.5 ± 0.7 cm) did not show differences with the other two treatments. Similarly, height growth also depended on seedling age and CO₂ treatment but not on irrigation level (Table 3). The interaction between CO₂ enrichment and water regime was significant (Fig. 3a): 100%-watered seedlings were taller than 50%-watered seedlings in the 750 ppm treatment, but no differences were found in the 550-ppm treatment, and the opposite trend was observed in 350-ppm treatment. The age $\times$ CO₂ interaction was also significant (Fig. 3b). Height of 1-year-old seedlings was higher in those that had received higher CO₂ enrichment, while 2-year-old seedlings showed the highest value in the intermediate CO₂ level.

Discussion

During the nursery assay, age, water regime, and atmospheric CO₂ concentration modified the morphological traits of seedlings (Table 1). As expected, all variables related to size (height, diameter, and biomass) increased with age. Moreover, biomass allocation to roots (R/S, RWR) or stems (SWR) also increased with age, while allocation to leaves (LWR) decreased. This pattern of biomass allocation, in which

older plants allocate less to leaves and more to support tissues than younger plants, has been commonly reported in the literature (Tissue et al., 1997). Older seedlings also exhibited a higher specific leaf weight (SLW) and a lower cuticular transpiration rate. Both trends involve a progressive increase in the sclerophylly of leaves (Edwards et al., 2000), probably due to larger concentrations of secondary and structural compounds not involved in the photoassimilation process in older seedlings (Cornelissen et al., 1999). These morphological and physiological characteristics could promote some advantages, such as increased leaf longevity, increased carbon gain per unit investment, and high water use efficiency (Freitas, 1997; Turner, 1994).

Table 4. ANOVA test of effects of age (1-year-old and 2-year-old seedlings), CO₂ treatment (350, 500, and 750 ppm), and irrigation (100% and 50% of water supply) in the greenhouse on final height and height growth 2 years after planting of *Pinus nigra* seedlings. Total sample size was 252.

Source of variation	df	Final ht		Ht growth	
		F	P	F	P
Age (A)	1	66.8	<0.001	272.6	<0.001
CO ₂ level (C)	2	5.4	0.005	4.1	0.018
Irrigation (I)	1	0.2	0.875	1.0	0.323
A $\times$ C	2	1.0	0.369	5.4	0.005
A $\times$ I	1	0.9	0.346	1.5	0.222
C $\times$ I	2	1.4	0.252	5.8	0.003
A $\times$ C $\times$ I	2	1.0	0.380	1.1	0.328
Error	241				

Atmospheric CO₂ concentration significantly affected total biomass, leaf biomass, leaf area, and root length of seedlings. Total biomass was higher for 750 than for 550 ppm (e.g., 28% and 16% respectively, in comparison with seedlings grown at ambient CO₂). This enhancement of above-ground growth has been perhaps the most common manifestation of the effect of CO₂ reported on seedlings of many species (Broadmeadow and Jackson, 2000; Curtis and Wang, 1998; Norby et al., 1999), including *Pinus* species (Tissue et al., 1997), where aerial biomass may increase 40% to 60% with increasing CO₂. On the other hand, in our study, the impact of elevated CO₂ on roots was small, and the increase of root biomass found in other studies (Norby et al., 1999; Tingey et al., 2000) was not confirmed in the case of *P. nigra* seedlings. Root length and SRL increased with CO₂ in recently emerged and in 1-year-old seedlings but not in 2-year-old seedlings (Fig. 1). This pattern could be related to the fact that containers may affect root growth in the larger plants, as has been previously shown in other studies using container-grown plants (Tingey et al., 2000). The causes of this effect remain unclear, but it has been attributed to physical restrictions of growth leading to a hormonal response (Sage, 1994) or the inability of roots to absorb enough water and nutrients to fulfill requirements of the aerial part, causing water stress (Will and Teskey, 1997).

The reduced water regime applied affected stem height and biomass and leaf area, with values being 14% to 18% lower for low-watered plants. On the contrary, no effects in root biomass or area were found. Altogether, these variations lead to an increment of R/S and RWR under the reduced water supply treatment. These morphological traits, coupled with a low cuticular transpiration rate, confirm the positive effects of reduced irrigation to increase the potential hardening process in plants (Grossnickle et al. 1991; van der Driessche, 1991a, 1991b), which could confer a higher drought resistance to seedlings (Savé et al., 1993). In the case of several morphological traits (leaf area, LWR, LAR, root length, and SRL), there was an interaction between age of seedlings and irrigation. Differences between irrigation treatments were higher for recently emerged seedlings than for older ones, pointing out that the effects of hardening treatments should be applied when plants are young because they have higher levels of morphological plasticity than older ones. In spite of the separate effects reported for CO₂ and water regime, none of the

Fig. 3. Height growth (in cm) at the end of the experiment in the field of *Pinus nigra* seedlings grown in the three CO₂ treatments (350, 500, and 750 ppm). (A) Seedlings grown under the two irrigation treatments (100% and 50% of water supply). (B) Seedlings 1 and 2 years old. Vertical bars extend over ± 1 SE of the mean.

interactions between both factors was significant (Table 1), meaning that there were not combined treatments of water and CO₂ that resulted at the same time in higher drought tolerance and enhanced growth.

The final survival of seedlings (51%) 2 years after plantation in the field may be considered low in comparison to the results obtained in temperate and boreal areas (Archibold et al., 2000; Gemmel et al., 1996). However, it is similar to other plantation experiences carried out with *P. nigra* in a neighborhood area (Espelta et al., unpublished data) and fell in the range of experiences carried out in Mediterranean environments (Vallejo and Alloza, 1998), where the transplant shock caused by a climate characterized by severe water stress usually leads to high first-year mortality rates (Mesón and Montoya, 1993). Mortality could be attributed

almost entirely to water stress, as all dead seedlings presented brown-drought leaves with no signs of herbivory or pest attack (personal observation). Concerning the experimental factors assayed, age was the only significant factor affecting survival in the field, because the survival rate was 13% higher in 2- than in 1-year-old plants. As 2-year-old plants were the ones that exhibited lower cuticular transpiration (lower SLW) coupled with a lower leaf area ratio, this result confirms the paramount importance that some morphological traits may have to reduce water lost and to increase the successful establishment of seedlings (Egnell and Örlander, 1993). In contrast, neither water regime nor CO₂ enrichment modified survival of *P. nigra* seedlings after transplant in the field. Water shortage imposed in the nursery, although causing reduction in stem height and leaf area, did not modify leaf characteristics (such as sclerophilly) and did not promote enough hardening to enhance seedling survival. In spite of the lack of major effects of CO₂ enrichment or water reduction treatments on seedling survival in the field, it is important to note that some differences in height growth and final height among the different treatments applied still remained 2 years after plantation (Table 4). Interestingly, seedlings previously grown under 500 ppm of CO₂ achieved the overall better results. In view of the absence of

major differences in architecture and biomass partitioning of seedlings grown under 500 or 700 ppm of CO₂ (Table 3), better growth of the former could be due to some ecophysiological advantages (e.g., higher assimilation rates) of intermediate CO₂ enriched seedlings (Wullschlegel et al., 2002). On the other hand, considering the similar results obtained in the survival of seedlings, using larger plants in restoration projects might help to reduce soil erosion, increase water retention, and provide a better visual landscape quality (Biel, 2002).

The adoption of reduced watering regimes may be useful to reduce excessive water waste in the nurseries and to increase plant survival in the field (van der Driessche, 1991a). However, from our results we cannot conclude that the nursery treatments applied, neither water shortage nor CO₂ enrichment, lead to important variations in plant survival. Two aspects would require further analysis. On the one hand, the irrigation levels tested (100% vs. 50%) may not have been sufficiently different to change drastically the morphological and physiological traits of *P. nigra* seedlings and determine their success in field conditions. Future studies should include more severe treatments of water reduction to

force seedlings hardening. On the other hand, it remains unclear if a longer application of the experimental treatments to recently germinated seedlings (those that showed the highest response) would allow them to develop similar high specific leaf area and lower cuticular transpiration rates than those exhibited by 2-year-old seedlings. In spite of the lack of effects of CO₂ enrichment in seedling survival, it is important to stress the promising result that, for a similar survival rate, seedlings grown at 550 ppm of CO₂ were larger after transplant than control plants. This result could improve the targets of the restoration process. Since reclamation of large burned areas requires the production of a large number of vigorous seedlings in a short time (Baeza et al., 1991; Vilagrosa et al. 1996), we feel that new strategies in landscape restoration under Mediterranean environmental conditions would benefit from further research on the combined effects from hardening by water stress and CO₂ enrichment on the production of nursery seedlings.

Literature Cited

- Ali Abod, S. and S. Sandi. 1983. Effect of restricted watering and its combination with root pruning on root growth capacity, water status and food reserves of *Pinus caribaea* var *hondurensis* seedlings. *Plant Soil* 71:123–129.
- Araújo-Alves, J.P., J.M. Torres-Pereira, C. Biel, F. de Herralde, and R. Savé. 2000. Effects of minimum irrigation technique on ornamental parameters of two Mediterranean species used in xeriscaping and landscaping. *Acta Hort.* 541:353–358.
- Archibold, O.W., C. Acton, and E.A. Ripley. 2000. Effect of site preparation on soil properties and vegetation cover, and the growth and survival of white spruce (*Picea glauca*) seedlings in Saskatchewan. *For. Ecol. Mgt.* 131:127–141.
- Arnott, J.T., S.C. Grossnickle, P. Puttonen, A.K. Mitchell, and R.S. Folk. 1993. Influence of nursery culture on growth, cold hardiness, and drought resistance of yellow cypress. *Can. J. For. Res.* 23:2537–2547.
- Baeza, J.M., A. Pastor, J. Martín, and M. Ibáñez. 1991. Mortalidad postimplantación en repoblaciones de *Pinus halepensis*, *Quercus ilex*, *Ceratonia siliqua* y *Tetraclinis articulata* en la provincia de Alicante. *Studia Oecol.* 8:139–146.
- Bayley, A.D. and J.W. Kietzka. 1996. Stock quality and field performance of *Pinus patula* seedlings produced under two nursery growing regimes during seven different nursery production periods. *New For.* 13:337–352.
- Biel, C. 2002. Efectes de diferents tractaments agrònomic i de la micorizació en la bioproducció de *Rosmarinus officinalis* L. en la fase de viver i en restauracions paisatgístiques en condicions de clima mediterrani. PhD thesis. Autonomous Univ. Barcelona, Barcelona.
- Broadmeadow, M.S. and S.B. Jackson. 2000. Growth responses of *Quercus petraea*, *Fraxinus excelsior* and *Pinus sylvestris* to elevated carbon dioxide, ozone and water supply. *New Phytol.* 146:437–451.
- Coombs, J., D.O. Hall, S.P. Long, and J.M.O. Scurlock. 1985. Techniques in bioproductivity and photosynthesis. Pergamon Press, Oxford.
- Cornelissen, J.H.C., A.L. Carnelli, and T.V. Callaghan. 1999. Generalities in the growth, allocation and leaf quality responses to elevated CO₂ in eight woody species. *New Phytol.* 141:401–409.
- Curtis, P.S. and X. Wang. 1998. A meta-analysis of elevated CO₂ effects on woody plant mass, form

GROWTH REGULATORS

- and physiology. *Oecologia* 113:241–250.
- Edwards, C., J. Read, and G. Sanson. 2000. Characterising sclerophylly: Some mechanical properties of leaves from heath and forest. *Oecologia* 123:158–167.
- Egnell, G. and G. Örlander. 1993. Using infrared thermography to assess viability of *Pinus sylvestris* and *Picea abies* seedlings before planting. *Can. J. For. Res.* 23:1737–1743.
- Espelta, J.M., A. Habrouk, and J. Retana. 2003. An economic and ecological multi-criteria evaluation of reforestation methods to recover burned *Pinus nigra* forests in NE Spain. *For. Ecol. Mgt.* 180:185–198.
- Freitas, H.M.O. 1997. Drought, p.129–150. In: M.N.V. Prasad (ed.). *Plant ecophysiology*. John Wiley & Sons, London.
- Gammel, P., U. Nilsson, and T. Welander. 1996. Development of oak and beech seedlings planted under varying shelterwood densities and with different site preparation methods in Sweden. *New For.* 12:141–161.
- Grossnickle, S., J. Arnett, J. Major, and T. Tschaplinski. 1991. Influence of dormancy induction treatments on western hemlock seedlings. I. Seedling development and stock quality assessment. *Can. J. For. Res.* 21:164–174.
- Habrouk, A., J. Retana, and J.M. Espelta. 1999. Role of heat tolerance and cone protection of seeds in the response of three pine species to wildfires. *Plant Ecol* 145:91–99.
- Kaushal, P. and G. Aussenec. 1989. Transplanting shock in corsican pine and cedar of atlas seedlings: Internal water deficits, growth and root regeneration. *For. Ecol. Mgt.* 27:29–40.
- Lamhamedi, M.S., P.Y. Bernier, and C. Hébert. 1996. Effect of shoot size on the gas exchange and growth of containerized *Picea mariana* seedlings under different watering regimes. *New For.* 13:207–221.
- Meson M. and M. Montoya. 1993. *Selvicultura mediterránea*. Editorial Mundi-Prensa, Madrid.
- Mitrakos, K.A. 1980. Theory for Mediterranean plant-life. *Acta Oecol. Plantarum* 15:245–252.
- Moreno, J.M., A. Vázquez, and R. Vélez. 1998. Recent history of forest fires in Spain, p. 159–186. In: J.M. Moreno (ed.). *Large forest fires*. Backhuys Publ., Leiden.
- Norby, R.J., S.D. Wullschleger, C.A. Gunderson, D.W. Johnson, and R. Ceulemans. 1999. Tree responses to rising CO₂ in field experiments: implications for the future forest. *Plant Cell Environ.* 22:683–714.
- Peñuelas, J.L. and L. Ocaña. 1996. Cultivo de plantas forestales en contenedor. Editorial Mundi-Prensa, Madrid.
- Piñol, J., J. Terradas, and F. Lloret. 1998. Climate warming, wildfire hazard, and wildfire occurrence in coastal eastern Spain. *Climate Change* 38:345–357.
- Retana, J., J.M. Espelta, A. Habrouk, J.L. Ordóñez, and F. Solà-Morales. 2002. Regeneration patterns of three Mediterranean pines and forest changes after a large wildfire in northeastern Spain. *Ecoscience* 9:89–97.
- Rice, W.R. 1989. Analyzing tables of statistical tests. *Evolution* 43:223–225.
- Royo, A., L. Gil, and J.L. Pardos. 2001. Effect of water conditioning on morphology, physiology and field performance of *Pinus halepensis* Mill. seedlings. *New For.* 21:127–140.
- Sachs, R.M. 1991. Stress-adapted landscapes save water escape injury in drought. *Calif. Agr.* 45:19–21.
- Sage, R.F. 1994. Acclimation of photosynthesis to increasing atmospheric CO₂: The gas exchange perspective. *Photosynth. Res.* 39:351–368.
- Savé, R., J. Peñuelas, O. Marfà, and L. Serrano. 1993. Changes in tissue osmotic and elastic properties and canopy structure of strawberries under mild water stress. *HortScience* 28:925–927.
- Savé, R., C. Biel, R. Domingo, M.C. Ruiz-Sánchez, and A. Torrecillas. 1995. Some physiological characteristics of citrus plants for drought resistance. *Plant Sci.* 110:167–172.
- Savé, R., F. de Herralde, J. Retana, J.M. Espelta, and C. Biel. 1998. Effect of elevated CO₂ on plant productivity and hardening under Mediterranean conditions. In: *The earth's changing land*. GCTE-LUCC Open Sci. Conf. Global Change, Barcelona.
- Tagliavini, M., L.J. Veto, and N.E. Looney. 1993. Measuring root surface area and mean root diameter of peach seedlings by digital image analysis. *HortScience* 28:1129–1130.
- Tingey, D.T., D.L. Phillips, and M.G. Johnson. 2000. Elevated CO₂ and conifer roots: Effects on growth, life span and turnover. *New Phytol.* 147:87–103.
- Tissue, D.T., R.B. Thomas, and B.R. Strain. 1997. Atmospheric CO₂ enrichment increases growth and photosynthesis of *Pinus tadea*: A 4 year experiment in the field. *Plant Cell Environ.* 20:1123–1134.
- Trabaud, L. 1987. Dynamics after fire of sclerophyllous plant communities in the Mediterranean basin. *Ecol. Med.* 13:25–37.
- Trabaud, L. and C. Campmant. 1991. Difficulté de recolonisation naturelle du pin de Salzmann *Pinus nigra* ssp. *salzmannii* (Dunal) Franco après incendie. *Biol. Conserv.* 58:329–343.
- Turner, I.M. 1994. Sclerophylly: Primarily protective? *Funct. Ecol.* 8:669–675.
- Vallejo, V.R. and J.A. Alloza. 1998. The restoration of burned lands: the case of eastern Spain, p. 91–108. In: J.M. Moreno (ed.). *Large forest fires*. Backhuys Publ., Lieden.
- van den Driessche, R. 1991a. Influence of container nursery regimes on drought resistance of seedlings following planting. I. Survival and growth. *Can. J. For. Res.* 21:555–565.
- van den Driessche, R. 1991b. Influence of container nursery regimes on drought resistance of seedlings following planting. II. Stomatal conductance, specific leaf area and root growth capacity. *Can. J. For. Res.* 21:556–572.
- van den Driessche, R. 1992. Changes in drought resistance and root growth capacity of container seedlings in response to nursery drought, nitrogen and potassium treatments. *Can. J. For. Res.* 22:740–749.
- Vilagrosa, A., J. P. Seva, A. Valdecantos, J. Cortina, J.A. Alloza, I. Serrasolsas, M. Abril, V. Diego, J. Bellot, and R. Vallejo. 1996. Plantaciones para la restauración forestal en la Comunidad Valenciana, p. 435–546. In: *La restauración de la cubierta vegetal en la Comunidad Valenciana*. Fundación CEAM, Valencia.
- Villar-Salvador, P., L. Ocaña, J. Peñuelas, and I. Carrasco. 1999. Effect of water stress conditioning on the water relations, root growth capacity, and the nitrogen and non-structural carbohydrate concentration of *Pinus halepensis* Mill. (Aleppo pine) seedlings. *Ann. For. Sci.* 56:459–465.
- Will, R.E. and R.O. Teskey. 1997. Effect of elevated carbon dioxide concentration and root restriction on net photosynthesis, water relations and foliar carbohydrate status of loblolly pine seedlings. *Tree Physiol.* 17:655–661.
- Wullschleger, S.D., T.J. Tschaplinski, and R.J. Norby. 2002. Plant water relations at elevated CO₂—Implications for water-limited environments. *Plant Cell Environ.* 25:319–331.