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Original article

Nursery fertilisation affects the frost-tolerance and plant quality of *Eucalyptus globulus* Labill. cuttings

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Abstract – *Eucalyptus globulus* is widely used in productive exotic plantations but the expansion of these plantations is limited by low temperatures, as its cold hardening capacity is limited (0.5 to 3.0 °C). It is not well understood how nursery fertilisation affects the field performance of plants. This led us to study the effect of three mineral nutrients (N, P and K) on both plant quality and frost tolerance. The experiment comprised eight growth treatments in which a high dose (H-) or a low dose (L-) of each nutrient was applied. Nitrogen was the nutrient that determined shoot growth, new root growth after transplanting (root egress), frost tolerance and field performance. Performance was better with treatment H-N than with treatment L-N, leaf nitrogen contents being 1.53 and 0.89% respectively. The effects of phosphorus and potassium were not significant between treatments for any parameter. The exception was P which, when interacting with N, favoured root egress for the H-N treatment. It was concluded that nursery fertilisation offers a management tool for eucalyptus growers concerned with plant stock quality.

field performance / frost tolerance / mineral nutrients / non-structural carbohydrates / root egress

Résumé – La fertilisation en pépinière affecte la tolérance au froid et la qualité des plants bouturés d'*Eucalyptus globulus* Labill. *Eucalyptus globulus* est largement utilisé dans des plantations exotiques productives, mais l'expansion de ces plantations est limitée par les basses températures, étant donné que l'endurcissement potentiel au froid de cette espèce est limité (0,5 à 3,0 °C). On ne comprenait pas bien comment la fertilisation en pépinière pouvait affecter la performance en plantation des plants. Ceci nous a amené à étudier l'effet de trois nutriments minéraux (N, P et K) sur la qualité des plants et la résistance au froid. L'expérimentation a comporté huit traitements pour l'étude de la croissance pour lesquels une forte dose (H-) ou une faible dose (L-) de chaque nutriment a été apportée. L'azote a été le nutriment qui a déterminé la croissance de la pousse, la croissance de nouvelles racines après transplantation (émission de racines), la résistance au froid et la performance en plantation. Les performances étaient meilleures avec le traitement H-N que avec le traitement L-N, la teneur en azote des feuilles atteignant respectivement 1,53 et 0,89 %. Les effets du phosphore et du potassium n'ont été significatifs pour aucun des paramètres. L'exception a concerné le phosphore qui lorsqu'il était en interaction avec l'azote a favorisé l'émission de racines dans le traitement H-N. On conclut de cette étude que la fertilisation en pépinière offre un outil de gestion pour les producteurs d'eucalyptus confrontés au problème de la qualité des plants.

performance en plantation / tolérance au froid / nutriments minéraux / hydrates de carbones non structuraux / émission de racines

1. INTRODUCTION

Plant quality is a term used to describe the extent to which a nursery plant may be expected to successfully survive and grow after outplanting, this being heavily dependent on factors such as species and genetics, nursery culture and site conditions. Temperature is one of these factors because the cold-hardening process during the cold season induces morphological and physiological changes that influence both frost tolerance and plant quality [4, 37]. These changes allow plants to respond better to transplantation and coping with new environmental conditions. For *Eucalyptus* spp., the most widely grown commercial hardwood in the world (planted on about 15 Mha of land [2]), various studies have been carried out on the effects of cold conditions on the hardening process [1, 9, 10, 22, 24, 33, 55]. In particular, *Eucalyptus globulus* Labill. (planted on more than 1 Mha in the Iberian Peninsula [52]) revealed low frost tolerance. This trait, along with water stress resistance, are the main factors that restricting crop areas because their plantations are limited to sites where minimum temperatures usually do not fall below –5 °C [40, 45, 47, 59].

In addition, nursery fertilisation plus the plants' mineral nutrients' content affect their quality [16,30,57]. It is also common to apply fertilisers during the plantation stage to encourage plant vigour, varying the dosage according to soil conditions [19]. Among the fertilisation regimes used in nurseries, nutrient-deprivation practices have been even proposed as a useful tool for strengthening plants against photodamage [10].

It has been proven that eucalyptus responds to water stress, by modifying dry matter partitioning, leaf morphology and cell osmotic potential [49]. The resulting concentration effect on cell electrolytes of the last factor (osmotic adjustment)

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Table I. Maximum and minimum average temperatures (TMmax, TMmin), maximum and minimum absolute temperatures (Tmax, Tmin) and accumulated chill hours ≤ 8 °C (h_8), from the start to the end of the nursery assay.

Dates (month-day)	Day	TMmax (°C)	TMmin (°C)	Tmax (°C)	Tmin (°C)	h_8
11-25 to 12-08	1st to 14th	21.9	11.9	23.5	7.0	6
12-09 to 12-22	15th to 28th	20.6	10.9	22.5	8.5	6
12-23 to 01-05	29th to 42th	18.9	7.9	20.5	5.0	55
01-06 to 01-19	43th to 56th	19.0	6.5	20.0	5.0	164
01-20 to 02-03	57th to 71th	18.4	4.4	25.5	–1.0	302
02-04 to 02-17	72th to 85th	22.0	6.2	25.5	3.5	402

is thought to aid survival by reducing the risk of intracellular freezing, owing to the lowering of the cytoplasm freezing point, and by maintaining cell turgor [11]. These strategies contribute to increasing the resistance in later unfavourable situations such as water stress or frost. The plant nutritional state also contributes in part to this capacity [21] due to its effect on plant growth, root growth capacity, biomass partitioning, leaf area, photosynthesis and/or nutrient mobilization from old to current growth after outplanting [13, 42, 58]. This depends, however, on species, nutrient concentrations and contents [8, 57, 58]. Nevertheless, it still remains uncertain how fertilisation in nurseries influences plant quality and post-transplantation responses to low temperatures for most species [3], including *E. globulus* [16]. Changes to fertilisation schedules in late summer and autumn can be a part of this hardening process [15, 28, 32], by modifying quantities and proportions of the nutrients, particularly N, P and K. Therefore, it was hypothesised that N, P and K, supplied during the hardening stage in the nursery, affect plant quality and frost-tolerance of *Eucalyptus globulus* Labill. To verify this hypothesis, morpho-physiological and growth parameters have been taken into account, as well as the field performance of plants one year after transplantation.

2. MATERIALS AND METHODS

2.1. Plant material

Seven month-old *Eucalyptus globulus* Labill. rooted cuttings were grown at the nursery of Grupo Empresarial ENCE, S.A. (San Juan del Puerto, Huelva, Spain), in SLC containers (Super-leach®, 125 cm^3, 3.5 cm diameter at the top, 22 cm long, 400 plants m^{-2}) filled with a peat-bark pine mix (1:1 v/v). Four-leaf cuttings, 10 cm long, were placed in a greenhouse at 20–25 °C, high relative humidity (> 80%). There was a natural photoperiod and the cuttings were well-watered. When they had formed roots, after two months approximately, they were taken to the nursery under a 60% shaded mesh, and three months later were exposed to full sun light. Fertiliser was applied once a week (10-2.5-5 + micronutrients). The cuttings were randomly collected from a group of 70 plus-trees from the breeding program at ENCE, S.A. In the second week of October 2004, they were taken to the nursery at the University of Huelva (La Rábida, Huelva) and maintained under a 50% shaded mesh. They were watered daily and fertilised once a week (Compo® Universal liquid fertiliser, 7-5-6 + micronutrients) until the beginning of the experiment (nursery assay + field trial), which started in the last week of November. At the beginning of the nursery assay, the plants measured 26.06 ± 0.57 cm in height and 2.83 ± 0.04 mm in stem diameter, the leaf nutrients' contents were 1.12% N, 0.08% P and 0.68% K. Freezing tests, as described below (Sect. 2.3), were carried out at –2, –4, –5 and –6 °C, and the results revealed that temperatures below –5 °C were lethal, with 100% of damage to the plant leaves. The maximum and minimum temperatures in the nursery 14 days before the start of the nursery assay averaged 22.2 °C and 12.7 °C respectively. Table I shows the temperature evolution during the 12-weeks nursery assay. The chill hours accumulated at the end of the nursery assay were 242 h (≤ 7 °C), 402 h (≤ 8 °C) or 670 h (≤ 10 °C).

2.2. Experimental design

The plants were randomly distributed among 24 trays (12 plants/tray). The three abovementioned nutrients (N, P, K) were considered as fixed factors. Each nutrient was applied in two different dosages, High (H-) and Low (L-), where L = 1/10 H. It allowed eight possible combinations of nutrient and dosage (Tab. II) in a balanced three-way treatment structure:

- 2 N treatments × 2 P treatments × 2 K treatments = 8 nutrient solutions,
- 8 nutrient solutions × 3 trays = 24 trays.

The trays were randomly distributed at the nursery and rotated weekly to avoid microclimatic differences. Treatment No. 1 (126 ppm N, applied in the form of NO_3^-; 71.2 ppm P and 89.9 ppm K) was in the usual range used in the nurseries during the growing phase of the plants [28]. It was taken as a reference for calculating the dosage of nutrients applied in the other treatments. The remaining macro- and micro-nutrients were also added to the different nutrient solutions in similar quantities to avoid possible deficiencies. Fertilisation was carried out once a week from the beginning to the end of the nursery assay. Each tray was fertilised with its respective nutrient solution, whose dose was 804 cm^3 per tray (67 cm^3 per plant). Distilled water (8 μS cm^{-1}) was added after every second fertilisation to recover any losses caused by evapotranspiration. At the end of each week, before continuing with the next fertilisation stage, the remaining nutrient solution in the root ball was removed by watering with distilled water, allowing it to leak through the hole in the bottom of the tray. The abovementioned process was then repeated. A check was made to ensure that the balls were permanently moistened to field capacity by the watering solutions.

Table II. Mineral nutrition treatments applied to the plants. The quantity of nutrients (N, P and K) contained in the watering solution, as well as the relative proportions of the nutrients, is shown in each case.

Watering solutions (H = High, L = Low)			Watering solution (mg/L)			Relative proportions	
			N	P	K	P/N – K/N	P_2O_5/N – K_2O/N
H-N	H-P	H-K	126.0*	71.23*	89.93*	0.57 – 0.71	1.30 – 0.86
		L-K	126.0	71.23	8.99	0.57 – 0.07	1.30 – 0.09
	L-P	H-K	126.0	7.12	89.93	0.06 – 0.71	0.13 – 0.86
		L-K	126.0	7.12	8.99	0.06 – 0.07	0.13 – 0.09
L-N	H-P	H-K	12.6	71.23	89.93	5.65 – 7.14	12.95 – 8.59
		L-K	12.6	71.23	8.99	5.65 – 0.71	12.95 – 0.86
	L-P	H-K	12.6	7.12	89.93	0.57 – 7.14	1.30 – 8.59
		L-K	12.6	7.12	8.99	0.57 – 0.71	1.30 – 0.86

* Reference treatment.

2.3. Measurements

As well as frost tolerance, other parameters such as morphology, plant growth, nutrients content and root egress were assessed. Additionally, the nursery assay was followed by a field trial to evaluate plant growth and survival after 12 months in the field. The parameters measured during the experiment were the following:

2.3.1. Morphology and growth

At the beginning of the test, ten plants were selected at random for each treatment (permanent sample). Their height (H) and diameter (D) were measured four times during the nursery assay (days 1, 28, 56 and 84), which made it possible to calculate the plants' absolute growth rate with regard to these two parameters (AGR_H and AGR_Drespectively). Since we are dealing with very young nursery plants, which were measured over a short period of time (12 weeks), we believe that these two parameters are useful for assessing plant growth.

$$\mathrm{AGR} = (x_2 - x_1)/(t_2 - t_1)$$

x_1 and x_2 are the parameter studied (i.e. H, D) between dates t_1 and t_2 respectively. At the end of the assay, six plants per treatment were selected at random from among the ten permanent plants and oven-dried at 70 °C to constant weight. This was followed by measurements of the shoot and root dry weights (SDW, RDW respectively). Using these parameters, it was possible to determine the total dry weight (TDW = SDW + RDW), the shoot to root dry weight ratio (SDW/RDW), and slenderness (H/D).

2.3.2. Root egress from the root ball

This was carried out at the end of the nursery assay and involved five plants per treatment. The plants were taken out of their containers, making every effort to keep their root balls intact. White pre-existing roots (usually less than 5 and shorter than 2 cm long) grown out from each ball were cut [23, 56]. It was previously ascertained, using the plants harvested for the dry weight measurements, that there were not white roots inside the root ball at this time. After this, the plants were put in 2.5 L containers, filled with moistened perlite before being taken to a greenhouse with favourable temperature (25/18 °C, day/night) and humidity (40/80% relative humidity) conditions. The plants were watered daily and fertilised once a week with the solution from treatment No. 1. After three weeks in the greenhouse, they were removed from their containers. After careful cleaning, the length of the new roots from the root ball (root egress) was measured and classified under seven categories: 0 = no regenerated roots; 1 = several roots < 1 cm long; 2 = 1 to 3 roots > 1 cm; 3 = 4 to 10 roots > 1 cm; 4 = 11 to 30 roots > 1 cm; 5 = 31 to 100 roots > 1 cm; 6 = more than 100 roots > 1 cm. To determine their dry weight, the roots were then oven-dried at 70 °C.

2.3.3. Frost tolerance

This was determined through freezing tests at the end of the nursery assay, using excised leaves from five plants per treatment [3]. Full-expanded leaves were taken from the third or fourth whorl, below the terminal bud, and then exposed to the minimum temperature used in the test. Tests were carried out at temperatures of –6 and –7 °C. These minimum temperatures were set after carry out freezing tests at an earlier stage of the experiment at –2, –4, –6, –7 and –8 °C. The leaves (two leaves per plant) were placed in glass tubes measuring 2.2 cm in diameter and 15 cm in length. These were put into a standard freezer, which had been fitted with a temperature programmer (West® 4400, ISE Inc., Cleveland, OH, USA) and two ventilators that allowed the air to pass around inside. The temperature was slowly (3 °C h^{-1}) and progressively decreased from 15 °C until the predetermined minimum value for each test was reached. Once this value had been reached, it was then maintained for three hours and slowly (4 °C h^{-1}) increased until reaching room temperature (the complete cycle lasted for a minimum of 16 h). The test tubes were then taken out of the freezer and distilled water was added (≤ 2 cm^3) until the cut sections of the leaf petioles were covered. The leaves were kept for one day in a growth chamber at temperatures of 25/17 °C (day/night), high relative humidity conditions (> 60%), and a photoperiod of 14 h. The resulting frost damage on the surface of the leaves could be visually observed the following day. Leaf damage (LD) was assessed by rating the leaves into 25% leaf damage classes [3]. This test was designed and put into practice following comparisons with other freezing tests (whole plant freezing test, electric conductivity of leaf discs and stem segments). The results of this test were previously calibrated to the whole plant freezing test for these kind of leaves, and proved

Table III. Mean values (±SE) at the end of the nursery assay, for high and low nitrogen treatments (H-N and L-N respectively) of: absolute growth rate of height (AGR_H) and diameter (AGR_D), height (H), slenderness (H/D), total dry weight (TDW) and the shoot/root dry weight ratio (SDW/RDW). AGR represents the average value from the start to the end of the nursery assay. p = level of significance for N factor.

Treatment	AGR_H (mm/day)	AGR_D (μm/day)	H (cm)	H/D (cm/mm)	TDW (g)	SDW/RDW
H-N	1.13 ± 0.09	6.30 ± 0.33	34.2 ± 0.9	10.6 ± 0.3	3.64 ± 0.20	2.69 ± 0.15
L-N	0.24 ± 0.02	2.75 ± 0.27	27.8 ± 0.8	8.9 ± 0.3	2.81 ± 0.18	1.95 ± 0.09
p	< 0.001	< 0.001	< 0.001	< 0.001	0.003	< 0.001

to be faster and more precise and allowed us to test the same plant several times. The visual evaluation was clear: the leaves that had not been affected by low temperatures kept their green colour (5GY 6/10 to 7,5GY 5/6, Munsell®) and those that had been affected turned to a brownish colour (5YR 3/4 to 7,5YR 4/4, Munsell®).

2.3.4. Mineral nutrients (N, P, K) and non-structural carbohydrates (soluble sugars -SS-, starch -St-)

Once weighed, the six harvested plants were used for the purposes of analysing the macronutrients (N, P, K), soluble sugars (SS) and starch (St) content in both the shoot and root portions. The following analytical procedures were applied: elemental analysis for N (EA FLASH 1112 CHNS, CE Instruments Ltd., UK), blue-molybdate colorimetry for P, flame photometry for K (Flame photometer 410, Corning Ltd, Essex, England), hydro-alcoholic extraction and colorimetric titration with anthrone for soluble sugars [50], and acidic hydrolysis, followed by titration with anthrone for starch [44]. A spectrophotometer (UVmini-1240, Shimadzu, Tokyo, Japan) was used for P, SS and St analysis.

2.3.5. Survival and growth in the field

In late February, once the testing period was over, ten plants per treatment were taken and planted 0.8 × 1.0 m^2 apart, in the field belonging to the Polytechnic School of the University of Huelva (Huelva, Spain 37° 12' N, 6° 54' W). Although the plot of land was flat, homogenous and small, it was divided into 5 sections or 'blocks' containing 2 plants per treatment each, to avoid possible microenvironmental effects. After 12 months, the height, stem diameter and survival of the plants were measured.

2.3.6. Data analysis

The effects of treatments (N, P and K) on the abovementioned parameters were assessed using the general linear model procedure (multifactorial ANOVA, SPSS® 12.0, SPSS Inc.). The factors were considered to be fixed, with the model used being: $y_{ijk} = \mu + N_i + P_j + K_k + NP_{ij} + NK_{ik} + PK_{jk} + NPK_{ijk} + \varepsilon$. Terms i, j, and k varied between 1 and 2. Data were checked for normality and homocedasticity. A repeated-measures ANOVA was used for H and D of the permanent sample. The block effect in the field assay was not statistically significant and was therefore eliminated from the model. The Tukey HSD test (Tukey Honest Significant Difference) was used as a means of comparison when differences were significant ($p \leq 0.05$). Correlation analysis was used to examine the relationship between parameters.

3. RESULTS

3.1. Morphology, growth and nutrient content

Nitrogen was the more discriminating factor for most of the morphological and growth parameters ($p \leq 0.003$), with the exception of RDW ($p = 0.586$). The highest values were for those treatments with more N (Tab. III). The effects of two other factors (P and K) and their interaction with one another were not significant ($p \geq 0.350$). Although Table III only shows the average values for AGR for the entire nursery assay, differences among treatments were noticed from the start and became greater as the assay developed. On the other hand, correlation analysis revealed that dry weights (i.e. SDW, RDW, TDW) were strongly correlated to one another ($r > 0.830$, $p < 0.001$, $n = 48$), so we are going to focus only on one of them (TDW) henceforth. Moreover, these three parameters were correlated positively to AGR_H ($r \geq 0.770$, $p < 0.001$, $n = 48$) and to AGR_D ($r \geq 0.610$, $p \leq 0.068$, $n = 48$).

The leaf's nitrogen concentration was highly influenced by the quantities of N in the watering solutions. At the end of the nursery assay, the average values of N in the leaves were 1.53 ± 0.20% and 0.89 ± 0.15% for H-N and L-N respectively ($p < 0.001$). However, the different amounts of P and K supplied with the watering solutions did not result in significant differences in the leaf concentration of these two elements (0.09 ± 0.02% P, $p = 0.833$, and 0.74 ± 0.18% K, $p = 0.235$, for all of the treatments as a whole). In respect of non-structural carbohydrates (SS, St), there were not significant differences for any of the factors (N, P, K) or their interactions ($p \geq 0.230$). The average values in the leaves for all of the treatments were 4.89 ± 0.50% SS and 8.00 ± 0.65% St. Nutrient and carbohydrate concentrations in the roots revealed a similar pattern to the leaves. The average values in the roots for all of the treatments were: 0.12% P, 0.84% K, 3.62% SS, 8.16% St, and 1.03% N (1.19% N for H-N, and 0.87% N for L-N, $p = 0.002$).

3.2. Performance attributes

In terms of root egress, only factors N ($p = 0.001$) and P ($p = 0.007$) had a significant effect on new root growth (dry weight). The more of these nutrients there were in the watering solution, the more new roots were generated. Interactions N × P ($p = 0.020$, Fig. 1) and N × P × K ($p = 0.015$) were also significant. It is worth highlighting here that plants suffered

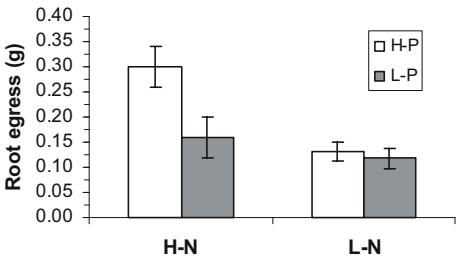

Figure 1. Mean values (±SE) of root regeneration after transplanting (root egress, $g_{dry\ weight}$) for each treatment at the end of the nursery assay, resulting from high (H-) and low (L-) supplies of nitrogen and phosphorus.

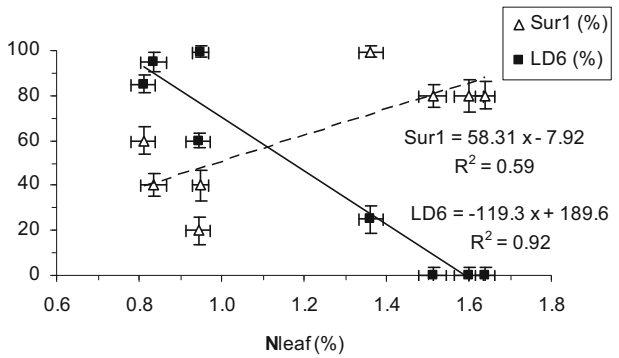

Figure 2. Leaf nitrogen concentration at the end of the nursery assay (N_{leaf}) versus leaf damage in the freezing test at –6 °C (LD_6) and survival after one year in the field (Sur_1). Each point is the mean value (± SE) per treatment.

Table IV. Mean values (±SE) of survival (Sur_1) and growth (ΔH_1, ΔD_1) in the field one year after plantation. ΔH_1 and ΔD_1 are height and diameter increments.

Treatment	Sur_1 (%)	ΔH_1 (cm)	ΔD_1 (mm)
H-N	85.0 ± 5.7	75.4 ± 3.1	9.7 ± 0.6
L-N	40.0 ± 7.8	49.8 ± 5.1	5.3 ± 0.6
p	< 0.001	< 0.001	< 0.001

more from a reduction of N than of P, because the positive effects of P were only noticeable when there was a high supply of N (Fig. 1). An evaluation of root egress growth using the established numerical code (categories) did not reveal significant differences between treatments for any factor (N, P or K). Nor were there significant differences in the interactions between them ($p \geq 0.200$). The average value for all of the treatments as a whole for the numerical code was 4.88 ± 0.09.

The freezing test at –7 °C caused a lot of damage in all of the treatments ($LD_7 > 75\%$) and there were not significant differences between them ($p = 0.827$). At –6 °C, however, test results did differ significantly among treatments in relation to factor N ($p < 0.001$). Factors P and K and the interactions either with each other, or with nitrogen, were not significant ($p \geq 0.120$). Plants that received a larger quantity of nitrogen (H-N) responded better to frost ($LD_6 = 6.3 \pm 6.3\%$) than those that received less (L-N, $LD_6 = 85.0 \pm 6.5\%$), regardless of the quantities of phosphorus and potassium provided.

3.3. Field response and correlation analysis

After one year in the field, those plants that had received more N in the nursery during the hardening phase revealed greater growth and survival rates (Tab. IV). While, for the factors P and K, there was a tendency towards lower values for treatments L- but the differences were not significant ($p \geq 0.127$); nor were the interactions between the factors.

Plant survival (Sur_1) and stem diameter growth (D_1) in the field were significantly and positively correlated to one another. In addition, both positively correlated to the leaf nitrogen concentration at the end of the nursery period (N_{leaf}). The correlation of these three parameters to the leaf damage (LD_6) was negative (Tab. V). Plants containing less than 1.0% N in their leaves were more susceptible to frosts (≥ 60% LD6) and to field mortality (≤ 60% Sur_1) than plants containing more than 1.3% N, which showed more than 75% Sur_1 and a lesser damage by frost (≤ 25% LD6). The larger concentration of N in these tissues corresponded to those plants that had developed for longer in the nursery and had a greater proportion of biomass in their shoots (SDW/RDW). However, the concentration of P, K, SS and St did not correlate significantly to any other parameter. In terms of root egress, there were not significant correlations to other parameters, except total dry weight in the nursery. Larger plants revealed a greater capacity for root regeneration.

4. DISCUSSION

The fact that growth was significantly smaller in L-N treatments confirms once more the relevant role of this mineral on plant growth [35]. The concentrations of this element in leaf tissues have been taken into account when elaborating models of growth for *Eucalyptus grandis* [26]. In our experiment, leaf nitrogen values below 1.0% significantly prevented plants from growing. Adding more N to the watering solutions also influenced biomass distribution between shoot and root, typically favouring the former over the latter [39]. None of this excessively affected or damaged the plants [51] because SDW/RDW were positively correlated to growth and survival in the field. On the other hand, the lack of significant differences between treatments as a result of the effects of P and K led us to believe that the applied doses, even in treatments with lower quantities of these elements, did not affect growth or biomass distribution. Consequently, they did not behave as limiting nutrients in this experiment, as was obtained, at least for the P application, by Pinkard et al. [38].

The average concentration of leaf nutrients in eucalyptus in Australian forests presents a traditional range of 0.80–1.50% N, 0.05–0.10% P and 0.50–0.80% K whereas, in plantations, it is usually 1.00–2.30% N, 0.05–0.15% P and 0.40–1.40% K [20, 36, 41]. In Spain, clones of *E. globulus* during the

Table V. Correlation matrix for some parameters measured in the experiment. Above: Pearson correlation coefficient. Below: level of significance. The correlations among parameters were made by taking into account average values for each of the eight treatments (n = 8).

	Sur_1	D_1	LD_6	RE	N_{leaf}	P_{leaf}	K_{leaf}	SS_{leaf}	TDW	SDW/RDW
Sur_1	1.000									
D_1	0.719	1.000								
	0.044									
LD_6	–0.736	–0.805	1.000							
	0.037	0.016								
RE	0.590	0.375	–0.540	1.000						
	0.124	0.360	0.167							
N_{leaf}	0.768	0.815	–0.961	0.525	1.000					
	0.026	0.014	0.000	0.182						
P_{leaf}	0.177	0.027	–0.428	–0.075	0.497	1.000				
	0.676	0.949	0.290	0.860	0.210					
K_{leaf}	0.315	–0.233	–0.203	0.184	0.199	0.408	1.000			
	0.447	0.578	0.630	0.663	0.636	0.316				
SS_{leaf}	–0.171	–0.151	–0.121	0.088	0.123	0.618	–0.190	1.000		
	0.686	0.720	0.775	0.836	0.771	0.102	0.653			
TDW	0.827	0.599	–0.699	0.831	0.662	0.097	0.143	0.176	1.000	
	0.011	0.117	0.054	0.011	0.074	0.820	0.736	0.676		
SDW/RDW	0.867	0.873	–0.918	0.576	0.869	0.179	0.152	–0.064	0.830	1.000
	0.005	0.005	0.001	0.135	0.005	0.672	0.720	0.881	0.011	

Survival (Sur_1) and stem diameter (D_1) one year after field plantation. Leaf damage at –6 °C (LD_6). Root egress (RE). Leaf concentration of nutrients (N_{leaf}, P_{leaf}, K_{leaf}) and soluble sugras (SS_{leaf}). Total dry weight (TDW) and shoot to root dry weight ratio (SDW/RDW).

hardening stage in the nursery have revealed leaf nutrient levels of 1.2% N, 0.08% P and 1.04% K [16], and 0.10% P and 0.79% K [7]. Besides, one year-old seedlings of *E. globulus* grown in a pot-culture experiment with an abundant or deficient supply of nutrients, had 2.10% N, 0.27% P, 1.05% K and 0.98% N, 0.14% P, 0.47% K, respectively [25]. Consequently, our values are within the normal range for cultivated plants and at the upper limit for values normally observed in natural forests.

In the case of potassium, a monovalent cation, with numerous important physiological functions, it is worth mentioning that it does not form part of the structure of the plant. Therefore, its effect on growth is not as noticeable as the effect of nitrogen and phosphorus, unless its content was reduced to below a critical value (i.e. to around 0.4–0.5% K in leaves) [12, 29]. It is possible that the quantity of K in this assay (0.74% in leaves) was higher than that critical value. This is just speculative, however; and it would therefore be advisable to determine standards of nutrient concentrations for the species under different growth conditions [41] and its relationships to cold hardening [3]. The results of our experiment could be considered a step forward to tackling the Diagnostic and Recommendation Integrated System (DRIS) for *Eucalyptus globulus*. This technique has been successfully used for forest species [43], and it is very useful for identifying which nutrient or nutrients are more limiting, as well as illustrating nutritional imbalances.

If we consider that the optimal values of total non-structural carbohydrates (SS+St) are between 10–15% [34], then the total average of SS and St found in this study was somewhere in between. The concentration of SS in leaves obtained in this test (4.9%) was similar other studies [6, 40, 46] but far from the 8.0% that this species is able to store when it carries out osmotic adjustment induced by water stress [14]. If this had taken place up to the latter level, frost tolerance would probably have been much greater due to the lowering of the freezing point [11].

The new roots protruding from the root ball (root egress) are an indicator of plant vigour [5] and could be linked to the content of P, due to the effect of this element on metabolic processes and growth [31]. Using this parameter to predict the post-transplantation response of plants can have its limitations, however [48]. In our experiment, plants fertilised with greater quantities of P and N were more vigorous and had a better root egress. It is possible that the noticeably pronounced effect of N application on plants, to certain extent disguised the effect of P. It is worth mentioning that root egress revealed a certain level of correlation to Sur_1, LD_6 and N_{leaf} ($r^2 > 0.27$). Although this was not significant, it demonstrated that this parameter was an indicator of the physiological state of plants even though its usefulness for predicting post-transplantation responses was limited.

The enhanced nutritive state of the plants fertilised with a greater quantity of nitrogen made them more frost tolerant

at the end of the hardening period. A high N level, however, has been reported as reducing hardiness [54], or has been shown either to decrease frost hardiness in the fall or to accelerate cold dehardening in the spring [57]. Nevertheless, it is species dependent and, although a over-consumption of macro-elements is generally considered to reduce hardiness [3], it is advisable to differentiate between optimal, sub-optimal and luxuriant levels of N content and their relationships to cold hardening [3, 13]. For instance, *Picea mariana* needed at least 1.28% N in shoot tissues to ensure hardening, while, with 0.64% N, it did not harden and seedlings with 0.87% N showed a lesser degree of hardiness than those with 1.28% [3]. Besides, other factors such as K/N ratio [15] and P/N ratio [58] could be important in cold hardening and growth. If we examine the values of leaf N concentration in both ours and other related experiments [16], we can observe that plants with more than 1.25% N responded to the freezing test significantly better than those that had less than 1.0%. It is possible that with N concentrations below 1.0%, this nutrient acts as limiting element and disguises the effect of other factors. Consequently, in order to determine the optimal concentration of these three nutrients in the hardening phase of the plants, it will be necessary to carry out further studies since it is known that sub-optimal levels of N, P and/or K can interfere with the process of hardening against frost [11, 13, 57].

As our results did not show significant differences between N treatments in K and SS contents, the positive effect of N-fertilisation on frost tolerance would be explained with difficulty. When exposed to low temperatures, plant cells encounter three main problems; alterations in the availability and status of water (dehydration), changes in the spatial organization of biological membranes, and a retardation of biochemical and chemical reactions [27]. Freezing injury is regarded to be a consequence of membrane lesions that are caused by the dehydration that occurs during freezing [11], although other factors may also contribute to the cellular damage induced by freezing. In general, the function of the solutes (e.g. SS) is to maintain turgor in dehydrating cells, but they may also have protective effects on macromolecules. Soluble sugars content is known to vary according to the hardening status of a tissue. Nevertheless, in addition to SS, woody plants could accumulate other solutes, such as amino acids, organic acids, glycosides and inorganic salts, when exposed to low temperatures [27], that could explain the positive effect of N on cold hardiness. Besides, because the plasma membrane is thought to be the primary site of injury during freezing [11, 27], most of the alterations are aimed at preserving the integrity of membranes. The lipid-protein ratio of cell membranes and the soluble protein concentration change during cold acclimation in a way that the threshold temperature of cell damage is lowered compared to non-acclimated plants [27, 53]. These proteins include apoplastic proteins having antifreeze activity, cryoprotective proteins, dehydrins, storage proteins, etc., and their concentrations may be increased by nitrogen fertilisation [27]. However, the function of many cold-induced proteins in eucalyptus species is not yet known.

Eucalyptus globulus reveals a certain capacity for increasing its frost tolerance, usually between 0.5 and 3.0 °C [1, 17, 22, 47]. Although the absolute value is not large (≤ 3 °C), it is enough to differentiate between genotypes with more or less frost tolerance [1, 17]. Low temperatures can be a major factor affecting the level of frost tolerance but, as we have seen, mineral nutrition also exerts a strong influence. Consequently, in the case of nursery plants, a balanced fertilisation regime that maintains the levels of leaf nitrogen concentration well above 1.25% will improve their rooting, survival and growth responses in the field, at least for the first season after fertilisation and for this type of plant material [18, 58]. As leaf N concentrations did not exceeded 1.65% in our experiment, thus no recommendations can be made on the upper limit of N concentration in relation to cold hardening and plant quality.

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REFERENCES

[1] Almeida M.H., Chaves M.M., Silva J.C., Cold acclimation in eucalypt hybrids, Tree Physiol. 14 (1994) 921–932.

[2] Beadle C., Sands P., Synthesis of the physiological, environmental, genetic and silvicultural determinants of the growth and productivity of eucalypts in plantations, For. Ecol. Manage. 193 (2004) 1–3.

[3] Bigras F.J., Gonzalez A., D'Aoust A.L., Hébert C., Frost hardiness, bud phenology and growth of containerized *Picea mariana* seedlings grown at three nitrogen levels and three temperature regimes, New For. 12 (1996) 243–259.

[4] Boorse G.C., Ewers F.W., Davis S.D., Response of chaparral shrubs to below freezing temperatures: acclimation, ecotypes, seedlings vs. adults, Am. J. Bot. 85 (1998) 1224–1230.

[5] Burdett A.N., Understanding root growth capacity: Theoretical considerations in assessing planning stock quality by means of root growth test, Can. J. For. Res. 17 (1987) 768–775.

[6] Caldeira M.C., Fernandez V., Tomé J., Pereira J.S., Positive effect of drought on longicorn borer larval survival and growth on eucalyptus trunks, Ann. For. Sci. 59 (2002) 99–106.

[7] Carvajal F.L., Caracterización morfo-fisiológica de plantas forestales cultivadas en dos viveros de diferentes localidades, Final-year Dissertation, Escuela Politécnica Superior, Huelva University, Spain, 2004.

[8] Cheaïb A., Mollier A., Thunot S., Lambrot C., Pellerin S., Loustau D., Interactive effects of phosphorus and light availability on early growth of maritime pine seedlings, Ann. For. Sci. (2005) 575–583.

[9] Close D.C., Beadle C.L., Brown P.H., Holz G.K., Cold-induced photoinhibition affects establishment of *Eucalyptus nitens* (Deane and Maiden) Maiden and *Eucalyptus globulus* Labill., Trees 15 (2000) 32–41.

[10] Close D.C., Brown P.H., Hovenden M.J., Beadle C.L., Eucalypt seedling hardiness to low temperature: a synthesis, Combined Proceedings International Plant Propagators Society 53 (2003) 116–119.

[11] Colombo S.J., Glerum C., Webb D.P., Day length, temperature and fertilisation effects on desiccation resistance, cold hardiness and root growth potential of *Picea mariana* seedlings, Ann. For. Sci. 60 (2003) 307–317.

[12] Dalla-Tea F., Marcó M.A., Fertilisers and eucalypt plantations in Argentina, in: Attiwill P.M., Adams M.A. (Eds.), Nutrition of eucalypts, CSIRO, Australia, 1996, pp. 327–333.

[13] Dumroese R.K., Hardening fertilization and nutrient loading of conifer seedlings, in: Riley L.E., Dumroese R.K., Landis T.D., USDA Forest Service, Proceedings RMRS-P-28, 2003, pp. 31–36.

[14] Fernández M., Arias M.T., Pardos J.A., Ruiz F., Soria F., The *Eucalyptus globulus* clone resistance to *Phoracantha semipunctata* depends on its response to water stress, Proceedings of the 10th MEDECOS CONFERENCE: Ecology, Conservation and Management of Mediterranean Climate Ecosystems, 2004 Rhodes (Greece), April 5 to May 1.

[15] Fernández M., Royo A., Gil L., Pardos J.A., Effects of temperature on growth and stress hardening development of phytotron-grown seedlings of Aleppo pine (*Pinus halepensis* Mill.), Ann. For. Sci. 60 (2003) 277–284.

[16] Fernández M., Ruiz S., Tapias R., Soria F., Mineral nutrition affects cold hardening development of rooted cuttings *Eucalyptus globulus* Labill. clones, in: Borralho N.M.G., Pereira J.S., Marques C., Coutinho J., Madeira M., Tomé M. (Eds.), *Eucalyptus* in a changing world, Proc. IUFRO Conf., Aveiro (Portugal), 2004, 11–15 October, pp. 509–512.

[17] Gallino J.P., Fernández M., Tapias R., Alcuña M.M., Cañas I., Aclimatación al frío en diferentes clones de *Eucalyptus globulus* Labill. durante el régimen natural de endurecimiento, Proceedings of the 2° Simposio Iberoamericano de *Eucalyptus globulus*, Vigo University, Pontevedra (Spain), 2006, October, 17–21.

[18] Gaspar M.J., Borralho N., Lopes Gomes A., Comparison between field performance of cuttings and seedlings of *Eucalyptus globulus*, Ann. For. Sci. 62 (2005) 837–841.

[19] Graciano C., Guiamet J.J., Goya J.F., Impact of nitrogen and phosphorus fertilisation on drought responses in *Eucalyptus grandis* seedlings, For. Ecol. Manage. 212 (2005) 40–49.

[20] Judd T.S., Attiwill P.M., Adams M.A., Nutrient concentrations in eucalypts: a synthesis in relation to differences between taxa, sites and components, in: Attiwill P.M., Adams M.A. (Eds.), Nutrition of eucalypts, CSIRO, Australia, 1996, pp. 123–153.

[21] Harvey H.P., van den Driessche R., Nutrition, xylem cavitation and drought resistance in hybrid poplar, Tree Physiol. 17 (1997) 647–654.

[22] Harwood C.E., Frost resistance of subalpine *Eucalyptus* species. I. Experiments using a radiation frost room, Aust. J. Bot. 28 (1980) 587–599.

[23] Helenius P., Louranen J., Rikala R., Physiological and morphological responses of dormant and growing Norway spruce container seedlings to drought after planting, Ann. For. Sci. 62 (2005) 201–207.

[24] Hovenden M.J., Warren C.R., Photochemistry, energy dissipation and cold-hardening in *Eucalyptus nitens* and *E. pauciflor*, Aust. J. Plant Physiol. 25 (1998) 581–589.

[25] Kaul O.N., Gupta A.C., Tandon V.N., Nutrition studies on *Eucalyptus* IV: Diagnosis of mineral deficiencies in *Eucalyptus globulus* seedlings, Indian Forester 94 (1980) 453–456.

[26] Kriedemann P.E., Cromer R.N., The nutritional physiology of the eucalypts nutrition and growth, in: Attiwill P.M., Adams M.A. (Eds.), Nutrition of eucalypts, CSIRO, Australia, 1996, pp. 109–121.

[27] Kontunen-Soppela S., Dehydrins in Scots pine tissues: responses to annual rhythm, low temperature and nitrogen, Oulu University Press, Oulu, Finland, 2001, 44 p., URL: http://herkules.oulu.fi/isbn9514259114/.

[28] Landis T.D., Mineral nutrients and fertilization, in: Landis T.D., Tinus R.W., McDonald S.E., Barnett J.P. (Eds.), The container tree nursery manual, Vol. 4, Agriculture Handbook 674, USDA, Forest Service, 1989, pp. 1–70.

[29] Landis T.D., Macronutrients – Potassium, Forest Nursery Notes, USDA, Forest Service, R6-CP-TP-11-04, 2005.

[30] Landis T.D., van Steenis E., Macronutrients – Nitrogen: part 2, Forest Nursery Notes, USDA, Forest Service, R6-CP-TP-01-04, 2004.

[31] Landis T.D., van Steenis E., Macronutrients – Phosphorus, Forest Nursery Notes, USDA, Forest Service, R6-CP-TP-07-04, 2004.

[32] Lantz C.W., Biesterfeldt R.C., Moorheal D.J., Aycock O.E., Barham R.O., Brissette J.C., Dierauf T.A., Dougherty P.M., Fryar R.D., Ross O.E., Schroeder R.A., Stauder A.F., Care and planting of Southern pine seedlings, USDA, Forest Service, Southern Region, Management Bulletin, RB-ME39, 1996.

[33] Leborgne N., Teulieres C., Cauvin B., Travert S., Boudet A.M., Carbohydrate content of *Eucalyptus gunnii* leaves along a annual cycle in the field and during induced frost-hardening in controlled conditions, Trees 10 (1995) 86–93.

[34] Marshall J.D., Carbohydrates status as a measure of seedling quality, in: Duryea M.L. (Ed.), Evaluating seedling quality: principles, procedures, and predictive abilities of major tests, Oregon State University, Corvallis, OR (USA), 1985, pp. 49–58.

[35] Marschner H., Mineral nutrition of higher plants, Academic Press limited, London, UK, 1995.

[36] Negi J.D.S., Sharma S.C., Mineral nutrition and resource conservation in Eucalyptus plantations and other forest covers in India, in: Attiwill P.M., Adams M.A. (Eds.), Nutrition of eucalypts, CSIRO, Australia, 1996, pp. 399–416.

[37] Pardos M., Royo A., Gil L., Pardos J.A., Effect of nursery location and outplanting date on field performance of *Pinus halepensis* and *Quercus ilex* seedlings, Forestry 76 (2003) 67–81.

[38] Pinkard E.A., Baillie C.C., Patel V., Mohammed C.L., Effects of fertilising with nitrogen and phosphorus on growth and crown conditions of *Eucalyptus globulus* Labill. experiencing insect defoliation, For. Ecol. Manage. 231 (2006) 131–137.

[39] Pinkard E.A., Baillie C.C., Patel V., Paterson S., Battaglia M., Smethurst P.J., Mohammed C.L., Wardlaw T., Stone C., Growth responses of *Eucalyptus globulus* Labill. to nitrogen application and severity, pattern and frequency of artificial defoliation, For. Ecol. Manage. 229 (2006) 378–387.

[40] Pita P., Pardos J.A., Growth, leaf morphology, water use and tissue water relations of *Eucalyptus globulus* clones in response to water deficit, Tree Physiol. 21 (2001) 599–607.

[41] Prado J.A., Toro J.A., Silviculture of Eucalypt plantations in Chile. in: Attiwill P.M., Adams M.A. (Eds.), Nutrition of eucalypts, CSIRO, Australia, 1996, pp. 357–369.

[42] Rikala R., Heiskanen J., Lahti M., Autumn fertilization in the nursery affects growth of *Picea abies* container seedlings after transplanting, Scand. J. For. Res. 19 (2004) 409–414.

[43] Romanyà J., Vallejo V.R., Nutritional status and deficiency diagnosis of *Pinus taeda* plantations in Spain, For. Sci. 42 (1996) 192–197.

[44] Rose R.W., Rose C.L., Omi S.K., Forry K.R., Durall D.M., Bigg W.L., Starch determination by perchloric acid vs. enzymes: evaluating the accuracy and precision of six colorimetric methods, J. Agric. Food. Chem. 39 (1991) 2–11.

[45] Ruiz de la Torre J., Flora Mayor, Organismo Autónomo Parques Naturales, Ministerio de Medio Ambiente, Madrid, Spain, 2006.

[46] Sánchez-Olate M., Zapata J., Ríos D., Pereira G., Escobar R., Efecto del fotoperíodo en el desarrollo de plantas de *Eucalytus globulus* Labill. ssp. *globulus* cultivadas en vivero, Bosque 24 (2003) 23–28.

[47] Scarascia-Mugnozza G., Valentiny E., Kuzminski E., Giordano E., Freezing mechanisms, acclimation processes and cold injury in *Eucalyptus* species planted in the Mediterranean region, For. Ecol. Manage. 29 (1989) 81–94.

[48] Simpson D.G., Ritchie G.A., Does RGP predict field performance? A debate, New For. 13 (1997) 253–277.

[49] Snowdon P., Nutritional disorders and other abiotic stresses of *Eucalyptus*, in: Keane P.J., Kile G.A., Podger F.D., Brown B.N. (Eds.), Diseases and pathogens of *Eucalyptus*, CSIRO Publishing, Collingwood, Australia, 2000, pp. 385–410.

[50] Spiro R.G., Analysis of sugars found in glycoproteins, in: Neufeld E.F., Ginsburg V. (Eds.), Methods in ezymology, Vol. VIII, Complex carbohydrates, Academic Press, New York, 1966, pp. 3–26.

[51] Thompson B.E., Seedling morphology: what you can tell by looking, in: Duryea M.L. (Ed.), Evaluating seedling quality: principles, procedures, and predictive abilities of major tests, Oregon State University, Corvallis, OR (USA), 1985, pp. 59–71.

[52] Toval G., The *Eucalytus globulus* clonal silviculture in Mediterranean climate, in: Borralho N.M.G., Pereira J.S., Marques C., Coutinho J., Madeira M., Tomé M. (Eds.), *Eucalyptus* in a changing world, Proc. Int. IUFRO Conf., 2004, Aveiro (Portugal) 11–15 October, pp. 70–78.

[53] Ukaji N., Kuwabara C., Takezawa D., Arakawa K., Fujikawa S., Accumulation of pathogenesis-related (PR) 10/Bet v 1 protein homologues in mulberry (*Morus bombycis* Koidz.) tree during winter, Plant Cell Environ. 27 (2004) 112–1121.

[54] Van den Driessche R., Effects of nutrients on stock performance in the forest, in: Van den Driessche R. (Ed.), Mineral nutrition of conifer seedlings, CRC Press, Boca Raton, Florida, 1991, pp. 229–260.

[55] Valentini R., Scarascia-Mugnozza G., Giordano E., Kuzminsky E., Influence of cold hardening on water relations of three *Eucalyptus* species, Tree Physiol. 6 (1990) 1–10.

[56] Villar-Salvador P., Planelles R., Oliet J., Peñuelas-Rubira J.L., Jacobs D.J., González M., Drought tolerance and transplanting performance of holm oak (*Quercus ilex*) seedlings after drought hardening in the nursery, Tree Physiol. 24 (2004) 1147–1155.

[57] Villar-Salvador P., Puértolas J., Peñuelas J.L., Planelles R., Effect of nitrogen fertilization in the nursery on the drought and frost resistance of Mediterranean forest species, Invest. Agrar.: Sist. Recur. For. 14 (2005) 408–418.

[58] Warren C.R., McGrath J.F., Adams M.A., Differential effects of N, P and K on photosynthesis and partitioning of N in *Pinus pinaster* needles, Ann. For. Sci. 62 (2005) 1–8.

[59] Whitehead D., Beadle C.L., Physiological regulation of productivity and water use in *Eucalyptus*: a review, For. Ecol. Manage. 193 (2004) 113–140.