

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

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Abstract – One-year-old *Pinus halepensis* seedlings were subjected to four water stress conditioning treatments (control, mild = –1.2 MPa, moderate = –1.8 MPa and strong = –2.2 MPa) for 2 months. After conditioning, several parameters related to the water economy of seedlings, the root growth capacity, and the shoot and root nitrogen and non-structural carbohydrate concentration were analysed. Moderate and strongly conditioned seedlings showed a significantly lower minimum transpiration rate than the control and mildly conditioned seedlings. In a subsequent drought cycle after conditioning, these latter treatments exhibited a lower predawn water potential than the moderate and strong conditioning treatments. Drought did not induce any osmotic adjustment or changes in the cell wall elasticity of shoots. Similarly, treatments did not differ in their dehydration tolerance as determined by the percentage of electrolyte leakage. Mildly and moderately conditioned plants concentrated more nitrogen in shoots and roots, respectively. Shoot starch was concentrated more in the moderate and strong conditioning treatments while no differences were observed in roots. Soluble sugars showed the reverse trend, the moderately and strongly conditioned plants exhibiting a higher concentration than control plants in roots but not in shoots. Root growth capacity was significantly reduced in the strongly conditioned plants. (© Inra/Elsevier, Paris.)

drought resistance / electrolyte leakage / Mediterranean / minimum transpiration / plant quality

Résumé – Effet d'un préconditionnement par la sécheresse sur les relations hydriques, la capacité de croissance des racines et les concentrations en azote et hydrates de carbone non structuraux de jeunes plants de *Pinus halepensis* Mill. Des plants de *Pinus halepensis* âgés de 1 an ont été conditionnés par application de quatre niveaux de stress hydrique (Témoin, Faible = –1.2 MPa, Modéré = –1.8 MPa et Élevé = –2.2 MPa) pendant deux mois. Après le préconditionnement, certains paramètres hydriques des plants, la capacité de formation de nouvelles racines et les concentrations en azote, amidon et sucres solubles des parties aériennes et racinaires ont été mesurés. Comparativement aux plants soumis aux conditionnements Témoin et stress hydrique Faible, ceux conditionnés par des niveaux de stress hydrique plus forts (traitements Modéré et Élevé) ont présenté i) des taux de transpiration minimale plus faibles (*table I*), ii) des concentrations en amidon dans les parties aériennes et des sucres solubles dans les racines plus élevées (*table II*) iii), des potentiels hydriques de base supérieurs lors d'un cycle de dessèchement ultérieur lent (*figure 1*). En revanche, la capacité de croissance de nouvelles racines a été réduite chez les pins préconditionnés par un stress hydrique élevé (Élevé) (*table I*). Le stress hydrique n'a induit ni ajustement osmotique ni modification de l'élasticité des parois cellulaires. Également, on n'a pas observé de différences parmi les traitements par rapport à la tolérance à la déshydratation, déterminée par le pourcentage de libération d'électrolytes (*table I*). (© Inra/Elsevier, Paris.)

électrolytes / méditerranéen / qualité des plants / résistance à la sécheresse / transpiration minimale

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1. Introduction

Water stress is the main limiting factor for plant life in the Mediterranean region. The almost complete absence of rainfall during the hottest months and its irregular distribution in the cold season can impair performance of forest plantations [4]. This situation can be further complicated if winters are cold, as occurs in many areas of the interior of the Iberian Peninsula, a fact which, in many cases, forces planting to be delayed until spring. In this context, utilisation of species and stock-types resistant to drought seems to be a basic requirement.

Resistance to water stress in plants can be achieved by a series of morphological and physiological features and responses which can, to a great extent, be conditioned in the nursery by certain cultural practices [10, 34]. Among these, application of restricted watering has been proved to promote osmotic adjustments and changes in cell wall elasticity [9, 14] and to increase root growth capacity [2, 22]. It can also induce a reduction of the transpiration rate after drought recovery [7, 30, 37] and improve dehydration tolerance [25]. All these responses have been considered as mechanisms that may improve resistance of plants to water stress. However, drought may inhibit nutrient acquisition [5] and photosynthesis and, in this way, induce an undesired effect on the performance of plantations, which has been positively related to plant nitrogen [15, 33] and non-structural carbohydrates concentration [19].

This study aims to analyse the suitability of restricted watering in the last stages of plant growth in the nursery as a practice to improve the drought resistance of *Pinus halepensis* (Aleppo pine) seedlings. This pine is a native of the Mediterranean basin and is widely utilised in reforestation on limestone soils owing to its ability to thrive under dry conditions and on poor and shallow soils. The specific objectives of this study were to scrutinise the 1) the water relations, 2) the root growth capacity and 3) the nitrogen and non-structural carbohydrate concentration of seedlings subjected to different water stress conditioning treatments.

2. Materials and Methods

2.1. Plant material

Seeds from an inland Levante provenance were sown at the end of March 1995 in Forest Pot[®] containers (cavity volume 300 mL) containing an 80:20 peat/vermiculite mixture. Plants were grown in the nursery of Tragsa-El Palomar, in San Fernando de Henares (Madrid). From June to mid-September each plant received a total of

27.3 mg N, 50.9 mg P and 63.5 mg K. Seedlings were watered every day; the mean predawn water potential, determined over 3 days of August, was -0.3 MPa. Mean seedling height and collar diameter measured in mid-September were 16.6 and 0.25 cm, respectively.

2.2. Experimental design

Application of conditioning treatments started on 14 September 1995 and lasted 2 months. Thirty-six containers (1 800 plants) were randomly assigned to four groups, each group corresponding to a water stress conditioning treatment. All containers were randomly arranged in the available space. Water stress was imposed through drought cycles which consisted in restricting watering until the mean predawn xylem water potential (Ψ_{pd}) of seedlings reached a pre-established value. Once the target drought level was reached, plants were watered until saturation. Conditioning treatments were:

mild conditioning – irrigation took place when Ψ_{pd} was -1.2 MPa;

moderate conditioning – irrigation took place when Ψ_{pd} was -1.8 MPa;

strong conditioning – irrigation took place when Ψ_{pd} was -2.2 MPa;

control – irrigation once a week.

Control treatment consisted of the typical irrigation schedule applied in several Spanish nurseries during the hardening phase in which plants are watered once weekly, this imposing a very slight water stress. Ψ_{pd} of control seedlings was measured every morning before the plants were irrigated, the mean Ψ_{pd} being -0.77 ± 0.08 MPa (mean \pm SE; $n = 5$). The Ψ_{pd} limit of the strong conditioning treatment coincided approximately with the osmotic potential at turgor loss point of the plants at the beginning of the conditioning experiment ($\Psi\pi_{tlp} = -2.1 \pm 0.05$), as determined by pressure–volume curves on four seedlings [21].

Seedling cultivation and conditioning experimentation was carried out in the open-air, except on rainy days when plants were covered with a transparent plastic sheet to avoid wetting. Fertilisation during conditioning was restricted to a single application at the end of the first drought cycle, each plant receiving 0.42 mg N, 2.64 mg P and 3.7 mg K.

At the end of the preconditioning period in mid-November all treatment plants were watered and allowed to recover from drought for 3 days before analysing differences in water relations and root growth capacity. At this date, the moderate and strong conditioning treat-

ments had experienced two complete drought cycles (two cycles + 20 and 22 days of drought, respectively), whereas the mild conditioning treatment had completed four drought cycles (four cycles + 8 days).

2.3. Pressure–volume curves

One to eight days after the recovery period, ten seedlings per treatment were subjected to pressure–volume curves according to the method described by Robichaux [21]. Plants were saturated by watering them the previous afternoon and were maintained in the dark until shoot sampling the following morning. From each curve, the osmotic potential at the turgor loss point ($\Psi_{\pi_{tlp}}$), the osmotic potential at full turgor (Ψ_{π_s}) and the water saturation deficit at turgor loss point (WSD_{tlp}) were calculated as described by Tyree and Hammel [31]. The modulus of elasticity (ϵ) of cell walls was determined as the change in turgor pressure divided by the change in WSD from full turgor to the turgor pressure at a 3 % WSD.

2.4. Minimum transpiration

Nine days after the recovery period, ten seedlings per treatment were watered and enclosed in an opaque plastic bag to ensure saturation overnight. In the morning shoots were excised and left to dry in a room in which mean temperature and water vapour pressure deficit were maintained at 16 °C and 0.9 kPa, respectively. Shoot fresh mass was measured gravimetrically to the nearest 1 mg at intervals of 0.5–1 h. Plotting shoot fresh mass versus time, a curvilinear relationship is obtained in which the linear portion represents water loss from plant surfaces after stomatal closure. Minimum transpiration rate of each shoot was calculated on a mass basis as the ratio of the slope of the linear portion (calculated by linear regression, $r^2 = 0.99$) and the shoot dry mass measured after drying at 80 °C for 48 h. Minimum transpiration is an estimate of cuticular transpiration.

2.5. Predawn xylem water potential evolution along a drought cycle and electrolyte leakage

After recovering from drought for 3 days at the end of the conditioning period, 70 seedlings per treatment with similar shoot heights were selected. Plants were irrigated and placed in an unheated greenhouse and subjected to a new drought cycle by withholding water from containers. Every 4–10 days, lateral twigs from ten plants per

treatment were sampled for predawn water potential (Ψ_{pd}), water content (WC), and electrolyte leakage (EL) measurements. On the first four sampling dates (days 0, 9, 13 and 21), all treatments were sampled simultaneously and plants in each treatment were randomly selected. Afterwards, and due to the different desiccation rates exhibited by the four treatments, subsequent sampling was directed to obtain an ample range of Ψ_{pd} , WC, and EL values in each treatment. Ψ_{pd} was measured with a pressure chamber. Electrolyte leakage was expressed as a percentage of total tissue electrolyte content and was calculated as the ratio

$$EL = Ci/Cf \times 100$$

where Ci and Cf are the electric conductivity of the tissue effusate before (Ci) and after (Cf) autoclaving the twigs. Laboratory details of EL determination are explained in Villar-Salvador et al. [35]. Twig water content was calculated as:

$$(\text{fresh mass-dry mass})/\text{dry mass} \times 100$$

2.6. Root growth capacity (RGC)

Fifteen seedlings from each treatment were planted in 3-L pots (one plant per pot) containing perlite. Pots were placed in a completely randomised design in a greenhouse where the mean maximum and minimum temperatures were 26.5 °C and 6.5 °C, respectively. Plants were irrigated every other day and fertilised with slow release fertiliser. After 40 days, seedlings were cleaned from the potting medium and the number of new roots longer than 1 cm protruding out of the plug was counted and measured to the nearest millimetre.

2.7. Nitrogen and non-structural carbohydrates determination

Nitrogen and carbohydrates were analysed from three independent samples, each one of seven plants. Peat was gently washed from the roots and the entire root system and shoots were oven-dried at 60 °C for 72 h and ground. Nitrogen was assessed by the standard Kjeldahl procedure. Starch and soluble sugars were extracted according to Spiro [27]. Soluble sugar and starch concentrations were determined by the anthrone and the perchloric acid methods, respectively [23, 27].

2.8. Data analysis

The effect of water stress conditioning treatments on plant parameters was analysed by one-way ANOVA followed by a least significant difference (LSD) test to separate means [36]. Most variables were normally distributed and had homogeneous variances. Only Ψ_{pd} had to be transformed (logarithm) to ensure homoscedasticity. Differences in dehydration tolerance among treatments were assessed by comparing the electrolyte leakage at a specific water content value. For each treatment, a quadratic predictive model relating EL (dependent variable) and water content (independent variable) was built. Water content was used instead of Ψ_{pd} because a reliable fitting of the Ψ_{pd} – EL relationship was not possible. Determination coefficients and predictive equations for each treatment were: Control ($r^2 = 0.89$; $EL = 24E - 4WC^2 - 1.56WC + 262.6$), mild conditioning ($r^2 = 0.93$; $EL = 15E - 4WC^2 - 1.06WC + 197.1$), moderate conditioning ($r^2 = 0.94$; $EL = 23E - 4WC^2 - 1.49WC + 247.1$) and strong conditioning ($r^2 = 0.90$; $EL = 15E - 4WC^2 - 1.09WC + 202$). A predicted EL value and its confidence interval were estimated at a 100 % water content, which is the WC limit when seedlings started to die (data not shown). Confidence intervals were utilised to calculate the standard error of each EL prediction and thus assess, by Student's *t*-tests, if EL differences among treatments were statistically significant.

3. Results

After 3 days of recovery from the conditioning period, the four treatments showed the same Ψ_{pd} (day 0 in *figure 1*). However, when subjected to a subsequent drought cycle they showed distinct desiccation rates. Thus, 2 weeks after the beginning of a new drought cycle, both control and mildly conditioned plants presented a lower Ψ_{pd} than the other treatments (*figure 1*). The differences were maintained after 21 days, the Ψ_{pd} of the moderate and the strong conditioning treatments being 0.82 and 0.55 MPa higher than the mildly conditioned treatment (*figure 1*).

Average $\Psi_{\pi lp}$ and $\Psi_{\pi s}$ of the four treatments was -2.22 and -1.75 Mpa, respectively, whereas mean WSD_{tp} and ϵ were 16.5 % and 12.8 MPa, respectively. None of these parameters nor the EL values calculated at a 100 % twig water content showed statistically significant differences among conditioning treatments (*table I*).

The moderately and strongly conditioned plants showed a significantly lower (25–28 %) minimum tran-

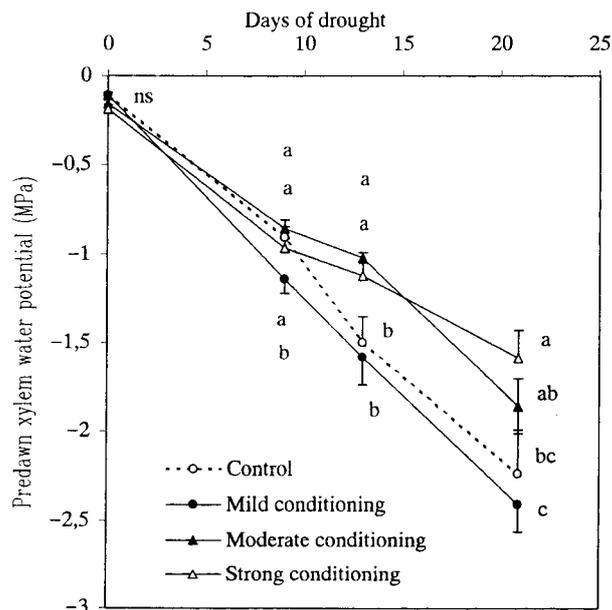


Figure 1. Predawn xylem water potential evolution along a subsequent drought cycle in *Pinus halepensis* seedlings ($n = 10$) which have been subjected previously to different water stress conditioning treatments. Means with the same letter indicate no statistically significant difference ($P \geq 0.05$, ANOVA and LSD separation test).

spiration rate than the control and the mildly conditioned ones which did not differ among them (*table I*).

After 40 days all plants produced new roots. The average number of new roots per plant that were longer than 1 cm ranged from 30 to 43. Strongly conditioned seedlings produced a statistically significant lower number of roots than the other treatments, which in turn did not differ among them (*table I*).

Nitrogen and soluble sugars accumulated more in shoots than in roots, which in turn concentrated more starch (*table II*). Differences among treatments in N concentration were small but shoot N concentration was significantly higher in the mild conditioning treatment than in the other treatments. Control plants had a significantly lower root N concentration than the other treatments, whereas the moderately conditioned ones presented the highest concentration (*table II*).

Shoot starch concentration increased with conditioning severity. Moderate and strong conditioning treatments exhibited the highest concentration, accumulating 55 % more starch than control plants (*table II*). Root starch did not show statistically significant differences among treatments. Shoot soluble sugar concentration did

Table I. Shoot water relation parameters ($n = 10$) and the root growth capacity ($n = 15$) as determined as the number of new white roots longer than 1 cm (means \pm 1 SE) of *Pinus halepensis* seedlings following different water stress conditioning treatments. Means with the same letter indicate no significant difference ($P \geq 0.05$, ANOVA and LSD separation test). $\Psi_{\pi\text{tlp}}$, osmotic potential at the turgor loss point; $\Psi_{\pi\text{fs}}$, osmotic potential at full turgor; WSD_{tlp} , water saturation deficit at the turgor loss point; ϵ , modulus of elasticity; EL, electrolyte leakage determined at a 100 % WC.

Treatment	$\Psi_{\pi\text{tlp}}$ (MPa)	$\Psi_{\pi\text{fs}}$ (MPa)	WSD_{tlp} (%)	ϵ (MPa)	Minimum transpiration (mmol g ⁻¹ h ⁻¹)	EL (%)	RGC (number of roots)
Control	-2.22 \pm 0.07 ^a	-1.73 \pm 0.02 ^a	17.1 \pm 0.99 ^a	13.3 \pm 0.70 ^a	1.13 \pm 0.071 ^a	45.6 \pm 1.85 ^a	41.9 \pm 4.09 ^a
Mild stress	-2.16 \pm 0.08 ^a	-1.75 \pm 0.09 ^a	16.0 \pm 1.17 ^a	12.4 \pm 0.83 ^a	1.11 \pm 0.117 ^a	42.9 \pm 1.90 ^a	43.2 \pm 4.52 ^a
Moderate stress	-2.26 \pm 0.03 ^a	-1.76 \pm 0.02 ^a	16.6 \pm 0.72 ^a	12.5 \pm 0.93 ^a	0.85 \pm 0.096 ^b	40.6 \pm 1.35 ^a	42.1 \pm 2.12 ^a
Strong stress	-2.24 \pm 0.08 ^a	-1.76 \pm 0.08 ^a	16.5 \pm 1.06 ^a	13.2 \pm 1.36 ^a	0.81 \pm 0.037 ^b	44.3 \pm 2.04 ^a	30.4 \pm 3.0 ^b

Table II. Shoot and root nitrogen, starch and soluble sugars concentration (mg g⁻¹) in *Pinus halepensis* seedlings (mean \pm 1 SE; $n = 3$) subjected to different water stress conditioning treatments. Means with the same letter indicate no statistically significant difference ($P \geq 0.05$, ANOVA and LSD separation test).

Conditioning Treatment	Nitrogen		Starch		Soluble sugars	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
Control	11.0 \pm 0.06 ^a	9.4 \pm 0.07 ^a	22.4 \pm 2.78 ^a	34.0 \pm 10.8 ^a	34.9 \pm 1.06 ^a	17.3 \pm 1.08 ^b
Mild	11.7 \pm 0.09 ^c	9.8 \pm 0.08 ^b	28.1 \pm 4.12 ^{ab}	33.6 \pm 7.78 ^a	30.3 \pm 2.87 ^a	20.9 \pm 0.40 ^b
Moderate	11.3 \pm 0.04 ^b	10.1 \pm 0.10 ^c	34.6 \pm 1.35 ^b	37.7 \pm 6.91 ^a	34.8 \pm 1.43 ^a	23.7 \pm 0.98 ^a
Strong	11.1 \pm 0.02 ^a	9.8 \pm 0.05 ^b	34.9 \pm 2.79 ^b	46.0 \pm 1.60 ^a	36.8 \pm 3.08 ^a	25.4 \pm 1.74 ^a

not differ among treatments but, in roots, the moderate and strong conditioning treatments accumulated significantly more soluble sugars (table II).

4. Discussion

Water stress conditioning in the nursery and applied in the autumn did not induce osmotic adjustments or changes in the cell wall elastic properties in *P. halepensis* seedlings. Several reasons can be given to explain such lack of response. First, plants might have dried out too fast inhibiting osmotic adjustments [1]. Seedlings in this study desiccated at a rate that varied from 0.08 to 0.1 MPa/d. Collet and Guehl [6] observed a higher osmotic adjustment in *Quercus petraea* when dried at a rate of 0.013 MPa/d than at 0.05 MPa/d. Second, many plants experience osmotic adjustments induced by low temperatures and short days [32]. This seems to have occurred in our experiment as mean $\Psi_{\pi\text{fs}}$ of control plants experienced a statistically significant decrease (data not shown) from -1.51 MPa in late July to -1.73 MPa in mid November. Thus, $\Psi_{\pi\text{fs}}$ in November may be a limit which drought conditioning could not reduce. Third, as in other woody species [10], *P. halepensis* might not be able to experience osmotic or cell wall elasticity adjustments in response to drought conditioning. This is supported by

the results of this study and those reported by Tognetti et al. [30], who observed neither significant osmotic adjustments nor ϵ variations in several provenances of *P. halepensis* subjected to recurrent droughts.

Electrolyte leakage, as determined in this study, has been considered as an indicator of plant dehydration tolerance [13, 25]. Water stress conditioning did not induce significant differences in twig EL measured at a 100 % WC among conditioning treatments, which indicates that water stress does not enhance dehydration tolerance of Aleppo pine seedlings. This lack of response coincides with that observed in *Juglans nigra* [13] but contrasts with that found in *Populus deltoides* clones [8] and other woody species [13]. Dehydration tolerance improvement has been linked with the capacity for osmotic adjustment [3, 9]. Therefore, the inability of *P. halepensis* seedlings to increase their dehydration tolerance seems to be in accordance with the absence of an osmotic adjustment.

In comparison with other Mediterranean pine species, Aleppo pine has a lower minimum transpiration rate [16]. In this study we have demonstrated that minimum transpiration in *P. halepensis* seedlings can be reduced by a moderate and strong water stress conditioning treatment. Similarly, Rook [22] reported the same response in drought-conditioned *P. radiata* seedlings, suggesting that this was related to a cuticle thickening.

When seedlings were subjected again to a drought cycle after 3 days of recovery from the conditioning period, the moderate and especially the strong conditioning treatments maintained a higher predawn water potential than the control and mild treatment. This suggests that the two most strongly conditioned treatments transpired less water. As seedlings from the different treatments had similar shoot sizes it is improbable that a distinct amount of foliage surface could explain the observed results. Many conifer species, including *P. halepensis*, diminish their transpiration rate by reducing stomatal conductance in response to water stress conditioning [7, 22, 30, 37]. Although in this study gas exchange measurements were not made, the lower desiccation rate exhibited by the moderately and strongly conditioned plants was probably the consequence of a reduction in stomatal conductance.

RGC has been considered as an indicator of plant vigour and in some studies it has been positively correlated with plant performance in the field (see [26]). Contrary to previous studies [2, 22, 34], RGC in *P. halepensis* was not improved by drought conditioning. Rather, the strong conditioning treatment produced significantly fewer roots than the other treatments. In agreement with our results, Tinus [29] found a significant reduction in RGC in *Pseudotsuga menziesii* seedlings when subjected to a water stress of -2.2 MPa. However, 2 years after planting we have found no significant differences in survival and growth among treatments (P. Villar-Salvador, unpublished data), which indicates that the RGC reduction was of little significance. A similar response was reported by Tinus [29], suggesting that the strongly conditioned seedlings experienced a small but reversible loss of vigour.

Water stress conditioning did not reduce either nitrogen or non-structural carbohydrate concentration, in fact it even increased it slightly. From a plant quality point of view, these results are relevant because field performance of conifer species has been positively related to shoot nitrogen [15, 33] and non-structural carbohydrate concentration [19]. Soluble sugars play an important role in osmotic adjustment [9, 18] and in dehydration tolerance [24] processes. Thus, the absence of differences among treatments in the shoot soluble sugar concentration is in accordance with the lack of osmotic adjustments and dehydration tolerance differences observed in this study. However, the distinct concentrations found in roots suggest that osmotic adjustments may have occurred in roots but not in shoots [12].

Several previous studies have also reported a positive effect of water stress on nutrient and starch concentration [20, 28]. This response has been explained because growth is depressed earlier by drought than are photo-

synthesis or nutrient absorption [11, 17]. In our case, the distinct concentrations are difficult to explain, as no differences in shoot mass have been observed (P. Villar Salvador, unpublished data), and root mass was not determined. The lower N concentration in control plants might be due to nutrient lixiviation from plugs caused by heavier irrigation.

In conclusion, the results of this study demonstrate that water stress conditioning of *P. halepensis* seedlings induced modifications which reduce desiccation rate and minimum transpiration but do not cause osmotic and cell wall elasticity adjustments nor improved dehydration tolerance. Drought conditioning did not improve RGC, strong conditioning depressing formation of new roots. Neither nitrogen nor non-structural carbohydrate concentration were diminished with respect to the control, and were even increased. Considering all the results together, recurrent droughts up to -1.8 MPa would produce the potentially best plants to thrive under water stress conditions. They would consume the soil water reserves more slowly, have a high RGC and concentrate more nitrogen and non-structural carbohydrates than non-conditioned plants.

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