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Effects of Water Deficit Stress on Growth Parameters of *Robinia pseudoacacia* L. Selected Clones under In Vitro Conditions

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Abstract: Rapid screening methods for drought-resistant genotypes are urgently needed in tree improvement programs in the face of current climate change. We used a plant tissue culture technique to assess the phenotypic response of three highly productive genotypes of *Robinia pseudoacacia* to water deficit induced by mannitol and sucrose in a range of water potentials from 0 MPa to −1.5 MPa in an eight-week experiment. Our study showed genotype-specific responses to induced drought stress, indicating the potential for tree improvement in productivity and stress tolerance. Considering that all plantlets were constantly supplied with carbon, from the medium during the drought-induced experiment, our results suggest that hydraulic failure rather than carbon starvation may be the main cause of drought-induced mortality. Furthermore, our results showed different metabolic pathways of sucrose depending on the concentration of sucrose in the medium and different responses to osmoticum (mannitol vs. sucrose) and its concentration among the clones tested. We believe, that for large-scale breeding programs wanting to select for drought-tolerant genotypes, the use of culture media containing 90 gL^{-1} mannitol or 90 gL^{-1} sucrose at an early selection stage should provide satisfactory screening results. However, lab-based screening should be supported by further field trials, preferably at multiple sites, to assess the long-term impact and phenotypic stability of the early selection strategies.

Keywords: drought stress; black locust; mannitol; generalized estimating equations

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1. Introduction

Drought episodes caused by climate change have underpinned many large-scale forest dieback events in recent years, often in combination with other abiotic and biotic factors [1,2]. Today, there is undoubtedly ample evidence that forests are dying either directly or indirectly due to heat and drought [3–7]. Recent evidence suggests that rising global temperatures are already exacerbating drought-induced forest changes and affecting terrestrial net primary productivity [8]. The other consequences of prolonged droughts and higher temperatures include potential shifts in species distribution ranges and a reduction in the sustainability of forests and their benefits for ecological and social needs [2,9,10]. The understanding of drought effects on trees is thus vital for the proper management and conservation of forest ecosystems [11].

Drought stress is a multifaceted phenomenon that can be analysed at physiological, metabolomic, and genetic levels. In general, plants experience drought when water availability to the root system becomes limited or when the transpiration rate becomes so high that an imbalance between water uptake and water loss occurs [12], resulting in cavitation.

The first measurable consequence of drought stress is a reduction in growth caused by a decrease in cellular expansion [13]. The cellular expansion process and carbohydrate wall synthesis are highly dependent on water deficit [14,15], and the reduction in growth is a consequence of the shedding of these cells [16]. The duration of drought is one of the critical factors that can have a particularly negative impact on forest communities [1]. The wide range of plant adaptation mechanisms and survival strategies depends on plant species, and genotypes within species, which developed under different climatic and environmental conditions. In general, two different water management strategies have evolved in plants: an isohydric strategy and an anisohydric strategy. Previous studies have shown that differences in the behaviour of isohydric and anisohydric plants are due to differences in the sensitivity of their respective guard cells to a critical leaf threshold [17]. As a result, under both optimal conditions and mild-to-moderate drought conditions, anisohydric plants maintain higher stomatal conductance and CO_2 assimilation compared to isohydric plants and, therefore, remain more productive under drought stress conditions [17]. However, the recent advances in drought response research suggest that the traditional classification of plants as iso/anisohydric has shown large differences in their isohydrocity [18] and multi-species comparisons have shown that species are ordered on a continuum rather than a dichotomy. Very few species, if any at all, conform strictly to the definitions of isohydric or anisohydric strategies [18–20]. Nonetheless, sensitivity to drought is fundamental to the geographic distribution of individual species as well as communities [9,21] and thus there are an increasing number of studies that provide species distribution models based on climatic variables. Recent studies in Central Europe indicate that most native tree species will face a significant decrease in suitable habitat area, while future climatic conditions are likely to favour the occurrence of introduced species such as black locust (*Robinia pseudoacacia* L.) [9,22,23], which is still relatively rare in Central and Northeastern Europe.

R. pseudoacacia is a light-demanding competitive pioneer species native to North America which was introduced into Europe at the beginning of the 17th century. This non-native species has become economically important due to its multipurpose use [22–26], providing important timber and non-timber ecosystem products (e.g., honey production [27], regulating services (nitrogen fixation in wasteland and in the reclamation of surface mines, erosion prevention and control, carbon sequestration, soil formation and stabilization [24] and cultural ecosystem services (aesthetics, biotherapeutic and recreational value, especially in urban areas [26]. However, the combination of high productivity, and rapid growth with high wood density, which is rare among woody plant species [24], gave this species an advantage that has attracted worldwide interest and seems to outweigh the negative aspects of its introductions (e.g., invasiveness).

Current climate change provides an additional incentive to improve the growth rates and wood properties of black locust in sustainably managed plantations in Central Europe to limit the uncertainties of long rotation periods of native forest species and provide an additional source of woody biomass, reducing timber harvesting from natural forests. Essential for the efficient improvement of *R. pseudoacacia* is the selection of high-yielding clones, but it is evident that improved drought stress resistance is also required [28].

Drought resistance is defined as enhanced productivity under the unfavourable conditions of water deficit. *R. pseudoacacia* is a good candidate to meet these criteria as it is considered a very drought-resistant species [24]. Black locust can adapt to prolonged drought by reducing water loss through both reduced transpiration and leaf size [29] and due to an extensive root system that can access water in deeper soil layers [30]. Interestingly, *R. pseudoacacia* is also classified as an anisohydric species, characterized by the maintenance of a high transpiration rate independent of soil moisture levels until there is little water left to withdraw; however, the degree of drought tolerance within the species is genotype-specific [31,32].

To study changes in plant growth under drought, several authors have successfully used the technique of plant tissue culture as an alternative to field experiments [33,34]. This artificial setup offers advantages such as tight control of experimental conditions, and the ability to grow many plants in a limited space [35,36]. Therefore, most of the knowledge on stress physiology is based on the use of these types of artificial stress conditions [37,38].

In such studies, sucrose, mannitol, polyethylene glycol (PEG), or sorbitol have been used frequently to simulate drought stress, reducing the water potential in the plant medium to mimic soil drying [39]. These agents have no toxic effects on plants and their use is a standard procedure for screening drought-resistant clones [33,34]. The application of different concentrations of osmotic agents allows for the exploration of plant responses from mild to life-threatening stress.

The water deficit induced by mannitol or sucrose is analogous to drought conditions in the natural environment [38] and can be characterized by a decrease in soil water potential (Ψ). Values of Ψ from 0 to -0.3 MPa are typical for well-watered plants, whereas values below -0.4 MPa corresponds to moderate water stress, and values from -1.5 to -2.0 MPa represent severe stress and permanent loss of turgor in most plant species [40]. Water potentials below -2.0 MPa are likely to cause severe vascular embolism [41]. It should be noted, however, that these values vary depending on the species and drought model [42] and not only Ψ-decrease affects the plant, but also its duration affects the extent of damage [43].

In the present study, we selected three highly productive genotypes of *R. pseudoacacia* that we vegetatively propagated. We compared the effect of osmotic stress induced by mannitol and sucrose, which we used to mimic drought conditions, on two growth parameters, total shoot length and fresh weight, as well as mortality of *R. pseudoacacia* vegetative cuttings in the in vitro cultures. To evaluate the phenotypic response of the plants on stress, we exposed them to a range of water potentials (-0.0 MPa to -1.5 MPa) through the manipulation of the media. Specifically, the following factors were tested depending on the *Robinia* genotype: the dose-dependent and the time-dependent effects. In addition, we were also able to investigate the osmoticum-dependent effects on the plants. The aim of our study was to measure the variation in genotype response to a drought-induced event in order to gain a better understanding of behavioural patterns and to support the future selection of drought-resistant clones of *R. pseudoacacia*.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Three genotypes of *R. pseudoacacia* characterized by desirable traits such as stem form (straightness), high productivity (well above the average for the region), and vigour [44], originating from forest stands in the Krosno Forest District of western Poland, were selected for the drought stress experiment (Table 1). All three genotypes of *R. pseudoacacia* were selected earlier as plus trees by the National Commission and phenotyped according to the National Standards for the Selection of Forest Trees. Plus trees are selected only in selected seed stands or at least in production seed stands of selected species where the species is dominant. The stands from which these tree genotypes originated (plus trees) have been naturalized in the Polish landscape for over 200 years making them a potentially ideal source of propagation material. These stands are used commercially in various forestry practices providing seeds for reforestation; breeding activities have also been carried out for the past 20 years.

Table 1. Name and number of selected clones of black locust.

Clone Name	Clone Number
4SO	9757
6SO	9735
10PT	9755

Dormant axillary buds were collected in February from each of the three selected clones (4SO, 6SO, 10PT) and used for micropropagation. Initially, explants were cultured on Murashige and Skoog medium (MS) [45], supplemented with 2% sucrose, 0.4 mgL^{-1} of BAP (6-benzylaminopurine) and 0.1 mgL^{-1} of NAA (naphthalene-1-acetic) and mixed with 5 gL^{-1} agar, to solidify the media, the pH was adjusted to 5.8 in 8 cm jars according to Szyp-Borowska et al. [46]. Micropropagated plantlets (five in each jar) were grown at 25 °C day temperature and 20 °C night temperature under a 16-h-day and 8-h-night photoperiod. The photosynthetically active radiation (PhAR) light level was approximately 300–400 μmol $m^{-2}s^{-1}$, and the humidity was maintained at 70%.

After three subcultures, sufficient plant material (explants) was propagated from each of the three clones and the osmotic drought treatment was applied once shoots reached approximately 3 cm in height. Since there are no reports in the literature on the response of black locust to osmotic stress in vitro, we added two commonly used osmotic agents to the medium, i.e., sucrose and mannitol, to replicate the experimental conditions that are often used in studies with abiotic stress in vitro. Sucrose is accumulated in cells as an osmoprotectant during heat and drought stress [7,47]. In turn, mannitol can mimic drought stress conditions and is usually added to the media at concentrations ranging from 9 to 127 g, depending on the genotype of the plant under study [48]. To separate the osmotic effect of sugar in the medium from its role as a carbon source, we used both mannitol and sucrose in the following combinations: five explants from each clone were transferred to one of the seven treatments which induced osmotic potentials from 0.0 MPa (control) to −1.5 MPa, mimicking different intensities of drought stress conditions (Table 2). This water potential range also mirrors the viable soil water potential range for plants [36]. Each treatment (seven) included six replicate jars for a total of 210 explants per genotype and a total of 630 plants in the experiment. The cultures were maintained for eight weeks to investigate their growth potential and response to drought stress. To analyse growth parameters, total shoot length (TSL) and total fresh weight (FW) were measured for all plants in all treatments. These measurements were taken every two weeks from the beginning of the treatment. TSL (cm) was measured from the surface of the medium to the tip of the shoot using a ruler. During the same period, all plants were weighed separately to determine FW (g), before subculture to fresh medium. Mortality M (%) was estimated as the percent ratio of dead to live plants at a given time (i.e., weeks 2, 4, 6, and 8).

Table 2. Osmotic treatment number (No.), water potential (Ψ level) and the amount and type of osmotic agent used (Sucrose or Mannitol) and associated treatment abbreviations used in the eight-week experiment on black locust explants.

No.	Ψ Level (MPa)	Sucrose Concentration (gL^{-1})	Mannitol Concentration (gL^{-1})	Abbreviation *
1	0	0	0	Control (MS)
2	−0.2	30	-	S30
3	−0.3	-	25	M25
4	−0.4	60	-	S60
5	−0.6	90	-	S90
6	−1.2	-	90	M90
7	−1.5	-	120	M120

* MS—Murashige and Skoog medium, M—mannitol, S—sucrose.

2.2. Statistical Analysis

To determine the influence of osmotic agents, and time of drought stress exposure on mortality (M) of the tested clones, we used a generalized linear model (Model 1) as follows:

$$g(\mu_{ijk}) = C_i + O_j + T_k + CO_{ij} + CT_{ik} + OT_{jk} \quad (1)$$

where $g(\mu_{ijk})$ is the log link function, μ_{ijk} is the mean M for the ith clone (C) (i = 1, 2, 3) and jth osmoticum treatment (O) (j = 1, . . . , 7), ijlth jar in the kth week time point (k = 0, 2, 4, 6, 8), C_i is the main effect of the ith clone, O_j is the main effect of the jth osmoticum, T_k is the main effect of the jth time point (repeated measures effect), CO_{ij} is the clone × osmoticum interaction effect, CT_{ik} is the clone × time interaction effect and OT_{jk} is the osmoticum × time interaction effect. In Model 1 there is no third order interaction effect because the full model (with second and third-order interactions) did not converge.

In order to examine the impact of the above-mentioned effects on TSL and FW, we used a generalized linear model (Model 2) that includes a third-degree interaction effect as follows:

$$g(\mu_{ijk}) = C_i + O_j + T_k + CO_{ij} + CT_{ik} + OT_{jk} + COT_{ijk} \quad (2)$$

where $g(\mu_{ijk})$ is the identity link function, μ_{ijk} is the mean TSL or mean FW for the ith clone (i = 1, 2, 3) and jth treatment (osmoticum) (j = 1, . . . , 7) for the ijmth plant in the kth week time point (k = 0, 2, 4, 6, 8), COT_{ijk} is the clone × osmoticum × time interaction effect and other effects are the same as in Model 1.

The analysis of a generalized linear repeated measures model, as in the case of this study (five time points every two weeks: 0, 2, 4, 6, 8), should consider the possible correlation of the data at the jar or plant level—cluster (in case of mortality—105 jars—clusters; in the case of TSL and FW—519 surviving plants—clusters). It was important to identify a specified structure in the data, and thus to fit an appropriate covariance matrix. This was possible by analysing the model using generalized estimation equations GEE methodology [49]. To identify robust GEE solutions for which the covariance model provides the best approximation for the purpose of evaluating model effects, we tested the following covariance structure matrices [50]: variance components (VC), compound symmetry (CS), autoregressive of first order (AR (1)), Toeplitz (Toep), and unstructured (UN). According to the GEE fit criteria (QIC criterion) [51] the best fitting model for M was the CS matrix, for TSL it was the UN matrix, while for FW the Toep matrix was best. For significant model effects, pairwise comparisons were made between least square means with Tukey's posthoc test and the Tukey–Kramer correction for unequal sample sizes. All statistical analyses were performed using the GENMOD, GEE and MIXED procedures of SAS/STAT®® v. 14.3 (Cary, NC, US.: SAS Institute Inc., 2017).

3. Results

3.1. Effect of Water Stress on Mortality

All three *R. pseudoacacia* genotypes studied showed different behaviour in terms of mortality rate depending on the duration of drought stress, osmoticum type and its concentration (Table 3).

Table 3. ANOVA table for mortality with Wald statistics for GEE analysis (DF—degrees of freedom; chi-square—test statistics; *p*—*p*-values).

Model Effect	DF	Chi-Square	*p*
Clone	2	28.02	<0.001
Osmoticum	6	77.12	<0.001
Time	4	56.19	<0.001
Clone × Osmoticum	11	68.61	<0.001
Clone × Time	8	40.43	<0.001
Osmoticum × Time	23	59.55	<0.001

The highest mortality rate was observed for clone 4SO (Table 4), especially for the osmoticum below −0.6 MPa. After only four weeks of stress, the percentage of dead plants observed for this clone was 13%, i.e., ten times higher than for 10PT and almost three times higher than for 6SO (Table 5). After eight weeks, the mortality rate for 4SO was 37%, and 9% (10PT) and 7% (6SO) for the remaining clones. Clones 10PT and 6SO were less sensitive

to the moderate osmotic potential and the mean rate of M was at the same level for both (Table 4). The highest mannitol concentration (120 gL^{-1}) caused a plant mortality rate of 20% in 6SO and 12% in 10PT, which was also sensitive to the highest concentration of sucrose (S90). The mortality rate of these clones increased after six weeks of treatment and remained at this level until the end of the eight-week experiment (Table 5).

Table 4. Mean ± SE mortality rate (%) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) after exposure to seven osmoticum treatments. Different treatments are denoted as: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum.

Osmoticum	Clone											
	10PT			4SO			6SO			Total for Osmoticum		
control	0.0 ± 0.0	a	A	5.3 ± 1.6	b	B	2.0 ± 1.1	a	A	2.4 ± 0.7	b	
S30	0.0 ± 0.0	a	A	4.0 ± 4.0	ab	A	0.0 ± 0.0	a	A	1.3 ± 1.3	ab	
M25	2.7 ± 1.6	a	A	18 ± 4.9	b	B	0.0 ± 0.0	a	A	6.9 ± 1.9	bc	
S60	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	0.0 ± 0.0	a	
S90	10.0 ± 5.5	b	A	24.7 ± 8.7	c	B	0.0 ± 0.0	a	A	11.6 ± 3.6	cd	
M90	0.7 ± 0.7	a	A	24.7 ± 5.5	c	B	3.3 ± 1.4	a	A	9.6 ± 2.3	cd	
M120	12.0 ± 5.4	b	A	27.3 ± 4.9	c	A	20.0 ± 7.6	b	A	19.8 ± 3.6	d	
Total for clone	3.6 ± 1.2		A	14.9 ± 2.1		B	3.6 ± 1.2		A			

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

Table 5. Mean ± SE mortality rate M (%) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) after 2, 4, 6, and 8 weeks of drought stress exposure. Effect of Clone × Time.

Clone	Time (weeks)											
	2			4			6			8		
10PT	0.0 ± 0.0	a	A	1.4 ± 0.8	a	A	7.6 ± 3.9	a	B	9.1 ± 4.1	a	B
4SO	3.3 ± 1.6	a	A	13.3 ± 3.5	b	B	20.5 ± 4.2	b	C	37.2 ± 6.7	b	D
6SO	0.0 ± 0.0	a	A	4.7 ± 3.1	ab	A	6.7 ± 3.7	a	B	6.7 ± 3.7	a	B
Total for time	1.1 ± 0.6		A	6.5 ± 1.6		B	11.6 ± 2.3		C	17.6 ± 3.2		D

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

There was a significant genotype by osmoticum interaction across the seven treatments (Table 4, Figure 1). Plant mortality did not occur with the application of S60, regardless of genotype and treatment duration (Table 6). The deleterious effect of the sucrose dose was observed only for the highest concentration of this osmotic agent (S90, which corresponded to −0.6 MPa), by week four. In contrast, mannitol caused mortality in plants regardless of its concentration in the medium, or treatment duration (Table 5), with mortality increasing with increasing concentration from M25 to M120 and after the first four weeks of treatment, even at the lowest (M25, −0.3 MPa) concentration of this osmotic agent in 4SO and 10PT genotypes. The interaction effects on the mortality of the clones, relative to both the osmoticum concentration and time, are presented in Figure 1.

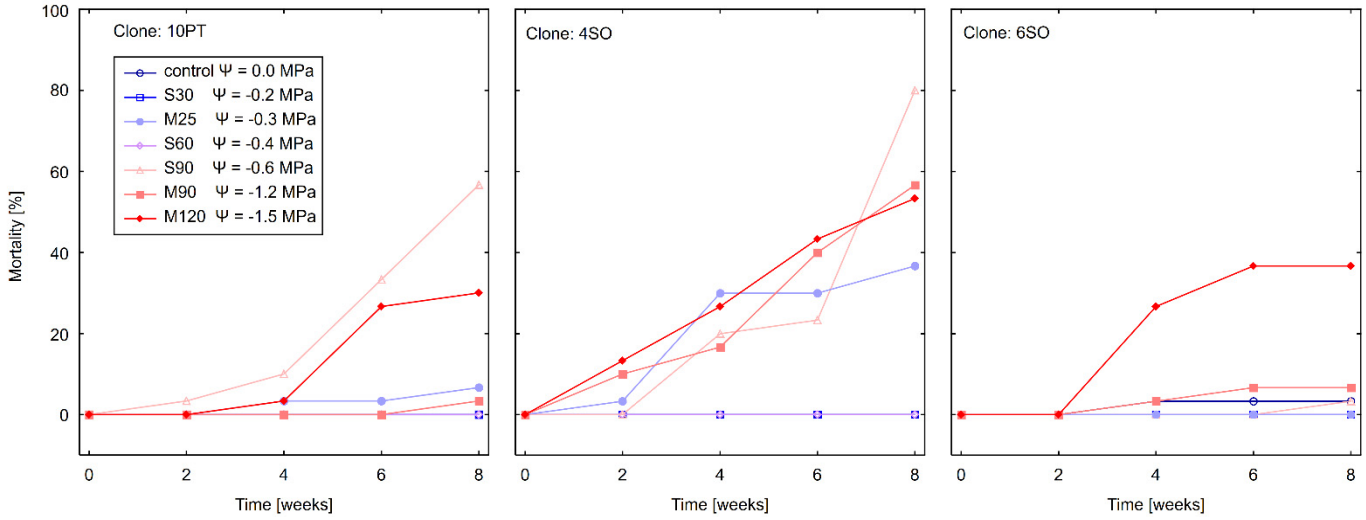

Figure 1. Effect of duration and magnitude of osmotic agents on mortality (%) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) in in vitro culture conditions over 8-weeks of exposure to seven treatments. Different treatments are denoted as: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol.

Table 6. Mean ± SE mortality rate M (%) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for different treatments measured after 2, 4, 6, and 8 weeks of drought stress exposure. Different treatments are denoted by the water potential (Ψ) and the osmotic agent and its concentration in the medium. "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Osmoticum × Time.

Osmoticum	Time (weeks)											
	2			4			6			8		
control	0.0 ± 0.0	a	A	2.2 ± 1.5	a	A	4.4 ± 1.9	ab	A	5.6 ± 2.1	a	A
S30	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	6.7 ± 6.7	ab	A
M25	0.0 ± 0.0	a	A	10.0 ± 4.2	b	B	10.0 ± 4.24	b	B	14.4 ± 7.1	a	B
S60	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A	0.0 ± 0.0	a	A
S90	0.0 ± 0.0	a	A	7.8 ± 6.7	ab	AB	15.6 ± 8.9	bc	B	34.4 ± 12.4	bc	B
M90	3.3 ± 2.4	a	A	6.7 ± 2.7	b	AB	15.6 ± 5.5	c	AB	22.2 ± 8.2	b	B
M120	4.4 ± 3.0	a	A	18.9 ± 6.9	c	B	35.6 ± 9.2	c	C	40.0 ± 10.1	c	C
Total for time	1.1 ± 0.6		A	6.5 ± 1.6		B	11.6 ± 2.3		C	17.6 ± 3.2		D

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

3.2. Effect of Drought Stress on Total Shoot Length (TSL)

After eight weeks of growth on the different media, TSL varied significantly for all terms in the model except for the clone × time interaction effect (Table 7).

The longest absolute TSL was recorded for the 10PT clone (2.66 cm), followed by 6SO (2.34 cm) and finally 4SO (2.17cm) (Table 8). However, the largest relative TSL increment under stress conditions was observed with the 6SO clone (Figure 2; Tables S1–S3 Supplementary). This clone maintained its growth even under the lowest water potentials in the medium, increasing its TSL by 13% compared with the initial TSL in M120 and M90 media, while growth was most inhibited in S90, increasing by only 8% of the initial TSL. The TSL increment in the control medium was 26% for 6SO. In contrast to 6SO, the 10PT clone was much more sensitive to drought during the eight-week stress exposure and a

significant reduction in TSL growth was observed in this clone. The TSL increment was only 3% in M120, 7% in M90 and 4% in the M25 osmoticum compared with the initial TSL. In turn, sucrose S90 in the 10PT clone did not cause such an inhibitory effect as observed in the 6SO clone. The TSL increment in the control medium for 10PT during the eight-week experiment was 27% longer compared with the initial TSL and was slightly shorter than in the S30 treatment (TSL increment by 32%). The 4SO clone was characterized by an intermediate response to the stress condition compared to 6SO and 10PT. InterestingLy, the TSL increment for the 4SO clone in S30 osmoticum was almost twice as long (41%) compared to the control (24%) during the eight-week experiment. This TSL increase is generally consistent with the overall results for the drought-induced agents (Table 8). The TSL increment of all clones was supported by the supply of sucrose in the media at a concentration of 30 gL^{-1}. The TSL of all clones in S30 was significantly greater ($p < 0.05$) than that of all other osmoticum and control treatments. In contrast, mannitol caused a significant inhibitory effect on the TSL in all clones ($p < 0.05$). The TSL was less in all clones, which became evident by week four when growth was significantly suppressed for all mannitol treatments (Table 9, Figure 2).

Table 7. ANOVA table for total shoot length TSL and total fresh weight FW for the GEE analysis of *R. pseudoacacia* (DF—degrees of freedom, F—test statistics; *p*—*p*-values).

Model Effect	DF	TSL		FW	
		F	*p*	F	*p*
Clone	2	52.05	<0.001	2.35	0.096
Osmoticum	6	22.7	<0.001	72.21	<0.001
Time	4	157.7	<0.001	257.53	<0.001
Clone × Osmoticum	12	3.87	<0.001	3.91	<0.001
Clone × Time	8	0.84	0.568	5.18	<0.001
Osmoticum × Time	24	15.29	<0.001	51.93	<0.001
Clone × Osmoticum × Time	48	2.52	<0.001	4.46	<0.001

Table 8. Mean ± SE total shoot length TSL (cm) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for different osmotic treatments. Different treatments are denoted by the water potential (Ψ) and the osmotic agent and its concentration in the medium and denoted as: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 900 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum.

Osmoticum	Clone											
	10PT			4SO			6SO			Total for Osmoticum		
control	2.94 ± 0.08	b	C	2.47 ± 0.08	bc	B	2.18 ± 0.08	a	A	2.53 ± 0.05	b	
S30	2.92 ± 0.08	b	B	2.60 ± 0.09	c	A	2.87 ± 0.08	c	B	2.79 ± 0.05	c	
M25	2.51 ± 0.08	a	B	1.96 ± 0.10	a	A	2.11 ± 0.08	a	A	2.19 ± 0.05	a	
S60	2.79 ± 0.08	b	C	2.17 ± 0.08	b	A	2.45 ± 0.08	b	B	2.47 ± 0.04	b	
S90	2.54 ± 0.09	a	B	1.97 ± 0.17	a	A	2.49 ± 0.08	b	B	2.33 ± 0.07	b	
M90	2.47 ± 0.08	a	C	1.77 ± 0.12	a	A	2.1 ± 0.08	a	B	2.12 ± 0.05	a	
M120	2.43 ± 0.09	a	B	2.26 ± 0.11	b	A	2.12 ± 0.10	a	A	2.27 ± 0.06	a	
Total for clone	2.66 ± 0.03		C	2.17 ± 0.04		A	2.33 ± 0.03		B			

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

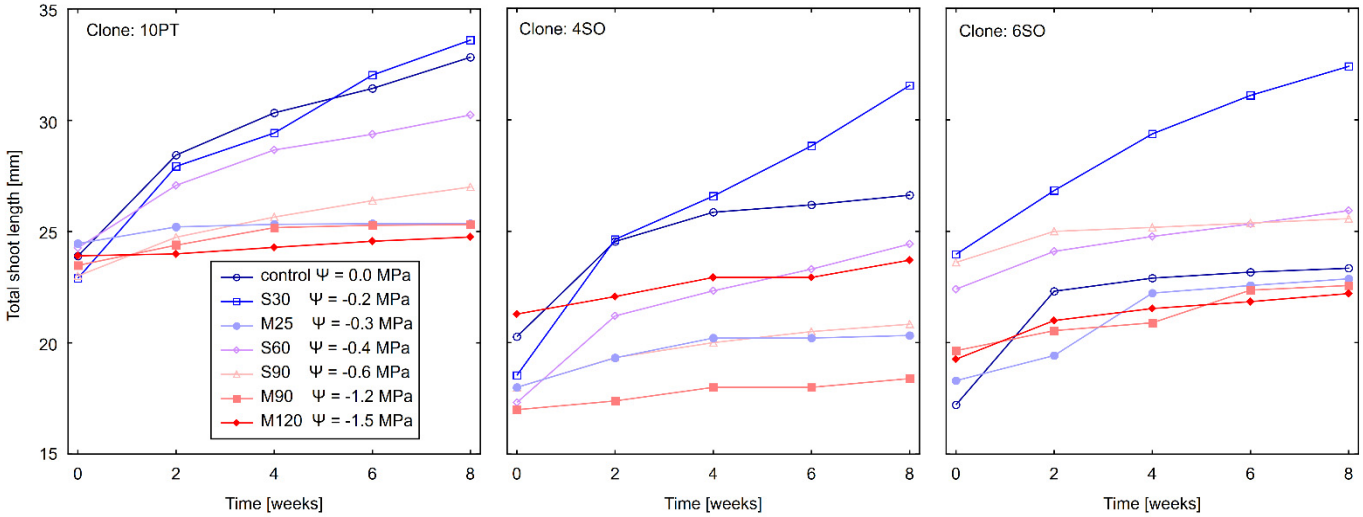

Figure 2. Effect of different osmoticum treatments on total shoot length TSL of *Robinia pseudoacacia* clones (10PT, 4SO, 6SO) during 8 weeks of treatment. Different treatments are denoted by the water potential (Ψ) and the osmotic agent and its concentration in the medium as follows: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol.

Table 9. Mean ± SE total shoot length TSL (cm) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for different treatments measured after 0, 2, 4, 6, and 8- weeks of drought stress exposure. Different treatments are denoted by the water potential (Ψ) and the osmotic agent and its concentration in the medium and denoted as "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 900 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Osmoticum × Time.

Osmoticum	**Time (weeks)**														
	0			**2**			**4**			**6**			**8**		
control	2.05 ± 0.05	a	A	2.51 ± 0.05	c	B	2.64 ± 0.05	c	C	2.69 ± 0.05	c	D	2.76 ± 0.05	c	E
S30	2.18 ± 0.05	a	A	2.65 ± 0.05	d	B	2.85 ± 0.05	d	C	3.07 ± 0.05	d	D	3.25 ± 0.05	d	E
M25	2.03 ± 0.05	a	A	2.13 ± 0.05	ab	B	2.26 ± 0.05	ab	C	2.27 ± 0.05	ab	C	2.28 ± 0.06	ab	C
S60	2.13 ± 0.05	a	A	2.41 ± 0.05	c	B	2.53 ± 0.05	c	C	2.60 ± 0.05	c	D	2.69 ± 0.05	c	E
S90	2.15 ± 0.07	a	A	2.30 ± 0.07	bc	B	2.36 ± 0.07	bc	B	2.41 ± 0.08	b	B	2.45 ± 0.08	b	B
M90	2.00 ± 0.06	a	A	2.08 ± 0.06	a	B	2.14 ± 0.06	a	C	2.19 ± 0.06	a	D	2.21 ± 0.06	a	D
M120	2.15 ± 0.06	a	A	2.24 ± 0.06	ab	B	2.29 ± 0.06	ab	C	2.31 ± 0.06	ab	C	2.36 ± 0.07	ab	C
Total for time	2.09 ± 0.02		A	2.33 ± 0.02		B	2.44 ± 0.02		C	2.50 ± 0.02		D	2.57 ± 0.02		E

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

3.3. Effect of Drought Stress on Total Fresh Weight (FW)

Based on the model used to analyse the total fresh weight, all effects were significant except for the clone (Table 7). During the eight-week experiment clones almost doubled their fresh weight but the FW increment was osmoticum dependent. The most significant increase in FW was recorded for sucrose osmoticum, in particular, the concentration of 30 gL^{-1} produced the highest results of FW, which was particularly evident for 4SO and 6SO clones (Table 10).

Table 10. Mean ± SE fresh weight FW (g) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for different osmotic treatments. Different treatments are denoted as: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum.

Osmoticum	Clone											
	10PT			4SO			6SO			Total for Sugar		
control	0.09 ± 0.02	b	A	0.089 ± 0.02	a	A	0.07 ± 0.02	a	A	0.08 ± 0.01	a
S30	0.22 ± 0.02	c	A	0.30 ± 0.02	c	B	0.37 ± 0.02	c	C	0.29 ± 0.01	c
M25	0.06 ± 0.02	ab	A	0.07 ± 0.02	a	A	0.05 ± 0.02	a	A	0.06 ± 0.01	a
S60	0.19 ± 0.02	c	A	0.19 ± 0.02	b	A	0.25 ± 0.02	b	B	0.21 ± 0.01	b
S90	0.19 ± 0.02	c	B	0.09 ± 0.04	a	A	0.20 ± 0.02	b	B	0.16 ± 0.02	b
M90	0.04 ± 0.02	a	A	0.07 ± 0.03	a	A	0.03 ± 0.02	a	A	0.05 ± 0.01	a
M120	0.06 ± 0.02	ab	A	0.08 ± 0.03	a	A	0.04 ± 0.02	a	A	0.06 ± 0.01	a
Total for clone	0.12 ± 0.01		A	0.13 ± 0.01		A	0.14 ± 0.01		A		

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

Significant differences were observed in FW between the treatments and duration of drought stress exposure (Table 11). The FW was lowest for mannitol regardless of its dose and treatment duration, while the effect of sucrose on FW changed over time, reflecting variations in the dose effect. After two weeks, there was a significant increase in FW for S30 compared to control and mannitol treatments. At four weeks, there was a significantly lower increase in FW for S90 compared to S30 and S60. After week six, the lowest FW was measured for S90, higher for S60 and the highest for S30. See Figure 3 and Tables S4–S6 in Supplementary Materials for a fuller depiction of the interaction effects of clone, time and osmoticum treatment on FW.

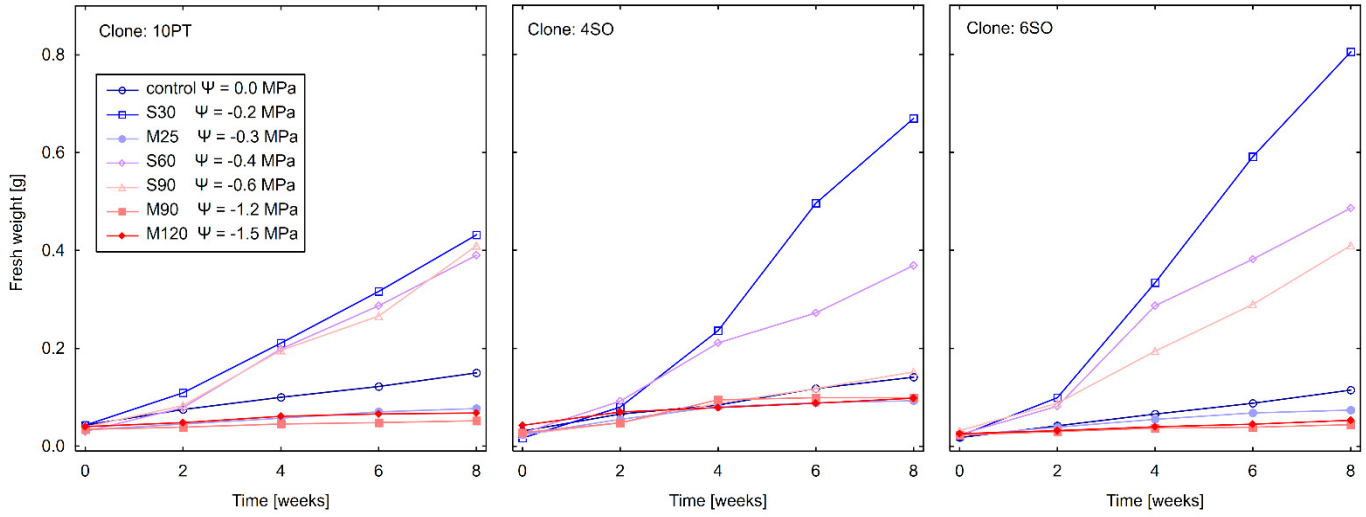

Figure 3. Effect of different osmoticum on fresh weight of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for 8 weeks treatment. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol.

Table 11. Mean ± SE fresh weight (FW) (g) of *R. pseudoacacia* clones (10PT, 4SO, 6SO) for different treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposure. Different treatments are denoted as "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Osmoticum × Time.

Osmoticum	Time (weeks)														
	0			2			4			6			8		
control	0.03 ± 0.01	a	A	0.06 ± 0.01	ab	B	0.08 ± 0.01	a	C	0.11 ± 0.01	b	D	0.13 ± 0.01	b	E
S30	0.03 ± 0.01	a	A	0.09 ± 0.01	c	B	0.26 ± 0.01	c	C	0.47 ± 0.01	e	D	0.63 ± 0.01	e	E
M25	0.03 ± 0.01	a	A	0.05 ± 0.01	a	B	0.06 ± 0.01	a	C	0.08 ± 0.01	ab	C	0.08 ± 0.01	a	C
S60	0.03 ± 0.01	a	A	0.08 ± 0.01	bc	B	0.23 ± 0.01	c	C	0.31 ± 0.01	d	D	0.41 ± 0.01	d	E
S90	0.03 ± 0.02	a	A	0.07 ± 0.02	bc	B	0.16 ± 0.02	b	C	0.22 ± 0.02	c	D	0.32 ± 0.02	c	E
M90	0.03 ± 0.01	a	A	0.04 ± 0.01	a	A	0.06 ± 0.01	a	B	0.06 ± 0.01	a	B	0.06 ± 0.01	a	B
M120	0.04 ± 0.02	a	A	0.05 ± 0.02	ab	A	0.06 ± 0.02	a	A	0.07 ± 0.02	a	A	0.07 ± 0.02	a	A
Total for time	0.03 ± 0.01		A	0.06 ± 0.01		B	0.13 ± 0.01		C	0.19 ± 0.01		D	0.25 ± 0.01		E

Means with the same lowercase letter within a column or means with the same uppercase letter within a row are not significantly different at $p \leq 0.05$; SE—standard error.

4. Discussion

Increased drought stress tolerance of forest trees will be a key feature in maintaining functional forest cover in the face of climate change. When breeding for drought resistance, morphological traits, such as growth, yield, mortality, and physiological traits, including water use efficiency (WUE), stomatal conductance, cavitation of conductive tissue, photosynthetic ability, leaf wilting, leaf water potential, and osmotic regulation, can be used as target traits for selection. However, most of these traits require considerable effort and time to measure and are thus less suitable for large-scale screening in breeding programs [52]. Although in vitro experimental setups are inherently imperfect [1,36], it allows for tight control over imposed stress levels induced by different osmotic agents, such as sucrose and mannitol, and their concentration in the media. In our preliminary study, despite all three genotypes having been selected for fast growth and straight form, the *R. pseudoacacia* explants showed clone-dependent responses to stress conditions for all traits studied. These results indicate that it is possible to both maintain tree productivity and survivability under stress conditions and that these results are clone dependent for the measured traits including mortality, TSL and FW.

As described earlier, *R. pseudoacacia* is considered in many studies to be an anisohydric (drought tolerant) species [31,32] with a more variable Ψ-leaf, maintaining open stomata and high photosynthetic rates for extended periods of time, even when leaf water potential declines [3]. This risk-taking behaviour can be beneficial when water is abundant, even under mild to moderately stressful conditions, which in our experiment corresponded to water potentials between −0.2 MPa and −0.4 MPa. However, under more intense drought conditions, of −0.4 MPa to −1.5 MPa, this behaviour can threaten the survival of *R. pseudoacacia* clones, exceeding the species' physiological range of tolerance. Under the lowest water potential (−1.5 MPa), mortality was observed in all studied clones, although tolerance to stress varied widely among the three clones. In fact, clone 6SO showed little mortality until exposed to the most severe stress conditions −1.5 MPa), whereas clone 4SO was far less tolerant to stress with mortality occurring at only −0.3 MPa.

McDowell et al. [53] showed that isohydric species are more likely to die during prolonged droughts of moderate intensity, while anisohydric trees such as *R. pseudoacacia* are more likely to die during intense, even short, droughts. In our study *R. pseudoacacia* clones showed considerable variation in their response to drought and its duration. The 6SO clone was characterized by maintaining the highest survival rate among the clones tested throughout the experimental period and the occurrence of mortality was noted only under the most severe drought stress conditions beginning in the fourth week, while the

4SO suffered drought-induced death as early as the second week under mild to moderate drought. Although we did not consider stomatal conductance and xylem cavitation in our study, the varied mortality and growth responses to drought of the clones studied in our experiment support the hypothesis that genotypes rather than species should be considered with respect to specific physiological strategies.

Further consideration should be given to the cause of the drought-induced mortality, which may have been caused by either hydraulic failure and/or carbon starvation [54]. Reduced soil water content or increased transpiration rate can impede water transport causing cavitation of the xylem vessels (filling with air), which stops water flow and leads to dehydration. Hydraulic failure is particularly likely to occur during intense drought, while carbon starvation is a relatively slow process and may occur during the later stages of prolonged drought [53]. Carbon starvation occurs when stomata close to prevent hydraulic failure. This process reduces carbon uptake through photosynthesis and starves the plant due to continuous metabolic carbohydrate demand. The results of the study by Dai et al. [27] indeed confirm the possible cause of the combination of hydraulic failure and carbon starvation for the drought-induced mortality of *R. pseudoacacia* saplings. Although we should be cautious about the results of our study in this context, considering that all plantlets had a constant supply of carbon from the medium during the drought-induced experiment, our results suggest that hydraulic failure rather than carbon starvation is the main driver for the drought-induced mortality observed.

By exposing the *R. pseudoacacia* clones in our study to a wide range of water potentials, some of which were not immediately life-threatening but negatively affected growth and productivity [4], it was possible to identify patterns in growth traits under stress conditions. Shoot growth is the most important and visible trait and is a very sensitive indicator of stress [16,39]. Many studies have attempted to determine the threshold water potential that suppresses plant growth [55–57]. For woody plants, a soil water potential threshold below −0.3 MPa can be expected to inhibit plant growth [57]. Ridolfi and Dreyer [58] found a drought threshold of −0.6 MPa for *Populus* × *canadensis* 'Robusta'. This negative plant response threshold can be used as a guide for selecting tolerant genotypes, below which fast-growing trees lose their advantage. Our study showed that intense drought indeed triggered a rapid inhibition of shoot length growth, while under mild stress (−0.2 MPa) all clones studied achieved greater TSL versus the control treatment, indicating an increase in water use efficiency under mild stress or an effect of the specific osmotic agent (sucrose) discussed below. Importantly, the responses of the three black locust clones studied were nuanced and depended on the clone (genotype), the type of osmotic agent, and its concentration, combined with the exposure time under stress conditions. The 4SO clone was the most drought sensitive among the clones studied. It was characterized by the lowest TSL increment and the highest mortality rate under moderate stress conditions (80% at week eight at a water potential of −0.6 MPa). The 10PT clone was characterized by an intermediate response to stress-induced conditions. Clone 10PT showed stable growth at a water potential of −0.6 MPa; however, mortality reached 57% after eight weeks under moderate stress conditions (at a water potential of −0.6 MPa). The highest drought resistance among the clones studied was shown by clone 6SO, which not only achieved the greatest TSL during eight weeks of drought, but also had the lowest mortality (3% at a water potential of −0.6 MPa).

The three clones also responded differently to the osmoticum and its concentration. In general, mannitol had a stronger inhibitory effect on TSL and FW increment on the black locust clones compared with sucrose. Our experiment showed that the TSL and FW increment was suppressed immediately after exposure to mannitol, regardless of its concentration in the medium (120, 90 and 25 gL^{-1}). In the case of mannitol exposure, our results are consistent with the findings of Claeys et al. [36], who indicated that plant growth is extremely sensitive to mannitol and that growth rates decreased rapidly when plants are exposed to even low concentrations.

In the present study, mannitol not only suppressed plant growth but was also responsible for the sharp increase in mortality of *R. pseudoacacia* clones, confirming that sucrose and mannitol play different roles in plant metabolism [59]. Mannitol, added to a nutrient solution, mimics a drought-stress condition and does not produce phytotoxic effects. Unlike mannitol, sucrose can be taken up and metabolized by the plant. At low concentrations (2% and 4%), sucrose is necessary for optimal growth and reproduction [60,61] and can increase dry weights [62]. All growth media used in our experiment contained 2% (*w*/*v*) sucrose. However, sucrose is known to increase stress tolerance and affect ABA signal transduction [63] and, therefore, may counteract the negative effects of oxidative stress on photosynthesis that can potentially limit growth [64]. The fact that we observed growth inhibition at low-stress levels with sucrose-containing media (S90), fits with the common view that stress-induced growth inhibition is an active process that is not dependent on carbon restriction, as was the case in our experimental conditions, where sucrose acted as a carbon source in the plant medium, significantly promoting the growth of clones in in vitro culture at a concentration of 30 gL^{-1} (S30). However, further increasing the sucrose supply reduced the water potential in the leaves in a dose-dependent manner in the leaf tissue. A concentration of 90 gL^{-1} (S90~−0.6 MPa) caused a negative effect, as evidenced by significantly lower TSL and FW growth compared to the S30 treatment, while significantly increasing tree mortality. The results obtained are consistent with the previous findings of Hoekstra et al. [65], who showed that prolonged heat and drought stress leads to a progressive accumulation of sucrose, a high concentration of which reduced the growth rate in the plants. In this context, our results showed different metabolic pathways for sucrose, depending on the concentration of sucrose in the medium, and generally different responses to the osmoticum treatments and the overall concentrations among the three clones tested, with clone 6SO being the most tolerant to drought stress, regardless of osmoticum or concentration.

As our preliminary screening showed, the relationship between productivity and drought response cannot be generalized within a species because trees of the same species can have different water conservation strategies [66]. Desirable clones for short-rotation forestry should combine increased productivity and drought stress tolerance. A full understanding of the relationship between drought response traits would be invaluable for predicting how tree species and genotypes within species will respond to future droughts. This knowledge could be used to develop tools for phenotyping in tree breeding aimed at improving drought resistance.

5. Conclusions

Our in vitro experiment, in which we used a wide range of water potentials, allowed for the rapid screening of three *R. pseudoacacia* clones under mild to life-threatening drought stress conditions. Our study revealed genotype-specific responses to induced drought stress, indicating the potential for tree improvement programs to select on both productivity and stress tolerance, which is particularly important in light of climate change with more frequent drought events. Considering that all plantlets were constantly supplied with carbon from the media, during the drought-induced experiment, our results suggest that hydraulic failure rather than carbon starvation may be the main driver for the drought-induced mortality. Furthermore, our results showed both different metabolic pathways of sucrose depending on its concentration in the medium and generally different responses to osmoticum (mannitol vs. sucrose). Nevertheless, we believe that for large-scale breeding programs designed to select for drought-tolerant genotypes, the use of culture media containing 90 gL^{-1} mannitol or 90 gL^{-1} sucrose at an early selection stage should provide early screening results. The first reliable plant responses to stress can be observed within four weeks of treatment application and exposure.

Yet, it is important to note that while in vitro screening provides precise control of stress conditions, it does not reflect the full complexity of environmental conditions affecting tree growth and survival. Therefore, we think that the potential applicability

of the in vitro rapid screening method in tree improvement programs should be further confirmed by testing under field conditions, preferably at multiple test sites, to evaluate genotype-by-environment interactions and assess the long-term impact of utilizing this early selection strategy.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f13121979/s1, **Table S1.** Total shoot length means TSL (cm) of 10PT clone of *R. pseudoacacia* in different osmotic treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 900 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time. **Table S2.** Total shoot length means TSL (cm) of 4SO clone of *R. pseudoacacia* in different osmotic treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 900 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time. **Table S3.** Total shoot length means TSL (cm) of 6SO clone of *R. pseudoacacia* in different osmotic treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 900 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time. **Table S4.** Fresh weight (FW) (g) of *R. pseudoacacia* clone (10PT) for different treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time. **Table S5.** Fresh weight (FW) (g) of *R. pseudoacacia* clone (4SO) for different treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time. **Table S6.** Fresh weight (FW) (g) of *R. pseudoacacia* clone (6SO) for different treatments measured after 0, 2, 4, 6, and 8 weeks of drought stress exposition. Different treatments are denoted: "control"—medium without osmoticum, "S30"—medium with 30 gL^{-1} of sucrose, "S60"—medium with 60 gL^{-1} of sucrose, "S90"—medium with 90 gL^{-1} of sucrose, "M25"—medium with 25 gL^{-1} of mannitol, "M90" medium with 90 gL^{-1} of mannitol, "M120" medium with 120 gL^{-1} of mannitol. Effect of Clone × Osmoticum × Time.

Author Contributions: Conceptualization, I.S.-B.; Data curation, I.S.-B. and J.U.; Formal analysis, J.U.; Funding acquisition, T.W. and M.N.; Investigation, I.S.-B.; Methodology, I.S.-B. and J.U.; Project administration, T.W. and I.S.-B.; Supervision, M.N. and J.U., Visualization, M.N., I.S.-B. and J.U.; Writing—original draft, I.S.-B., M.N. and J.U.; Writing—review and editing, all the authors (I.S.-B., J.U., M.N., T.W. and B.R.T.) All authors have read and agreed to the published version of the manuscript.

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