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Very Early Biomarkers Screening for Water Deficit Tolerance in Commercial *Eucalyptus* Clones

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Abstract: The identification of genotypes more tolerant to water deficit is a challenge to breeding programs. In this research, our objectives were to identify and validate traits for tolerance to water deficit in eucalypts. The estimation of genotypic parameters and early selection are proposed based on mixed models, selection indexes and validation schemes. Seedlings with 110 days were grown in a greenhouse for 12 weeks, and two water deficit treatments were conducted (polyethylene glycol and water limitation). A total of 26 biomarkers were evaluated, and 15 of them were significant, exhibited adequate heritability, and used for screening: final plant height, increment in height, increment in diameter, area of mature and fully expanded leaf, nutrient contents of N, K, Ca, Mg, S, Cu, Zn, Mn and B, photosynthesis (A) and stomatal conductance (gs). Both treatments were adequate to discriminate water deficit-tolerant clones. The ranking of tolerant clones according to their phenotype in the field demonstrates the potential for early selection and is consistent with the maintenance of water-deficit-tolerance mechanisms until adulthood. There is evidence that the choice of biomarker depends on the species involved and different strategies contributing to the tolerance trait.

Keywords: drought; eucalypt breeding; phenomics; early selection

Citation: Corrêa, T.R.; Picoli, E.A.d.T.; Pereira, W.L.; Condé, S.A.; Resende, R.T.; de Resende, M.D.V.; da Costa, W.G.; Cruz, C.D.; Zauza, E.A.V. Very Early Biomarkers Screening for Water Deficit Tolerance in Commercial *Eucalyptus* Clones. *Agronomy* **2023**, *13*, 937. https://doi.org/10.3390/agronomy13030937

Academic Editors: Szilvia Veres and Tibor Janda

Received: 17 February 2023
Revised: 9 March 2023
Accepted: 18 March 2023
Published: 22 March 2023

1. Introduction

Climate forecasts for the coming decades predict water scarcity due to rising temperatures in various regions of the globe [1]. Therefore, research on how plants can maintain growth and productivity in situations of water deficit is of great relevance. Some physiological studies address the issue, such as plant adaptations to water deficit and the selection of water-stress-tolerant genotypes in order to maintain productivity [2], while others provide an overview of adaptive responses to water drought in eucalyptus [3].

For perennial species such as eucalyptus, drought-tolerant genotypes have lower productivity than expected. While genotypes that show greater growth and development are more sensitive to stress conditions [4]. Obtaining an ideal genotype, with satisfactory growth in conditions of low water availability [5], however, requires intense cycles of selection and genetic improvement. Perennial species' breeding is time-consuming, where procedures to reduce the crop cycle or contribute to the selection process can subsidize and accelerate the breeding programs. Early selection is an alternative that has been shown

to be efficient in the genetic improvement of *Eucalyptus* [6,7]. Accordingly, in the present report, "early selection" is defined as the use of traits of plant individuals at a younger stage in the evaluation process in order to be used as predictors of features in their productive age and allowing anticipating genetic gains [6,7].

The wide distribution and adaptation to the environmental range of eucalypts is emphasized by strategies that confer this type of tree options to cope with water deficit. There are growth, physiological, anatomical, morphological, nutritional, gene expression and metabolism traits, among others, that contribute to water deficit tolerance [3,8]. Growth and adaptability traits are regarded as markers for water stress tolerance and a baseline for comparison for different eucalypt species [8]. Considering the ease of access and effectiveness of traits such as petiole anatomy, allometric measures of plant height, stem and treetop diameter, and mineral content such as boron and calcium, may have diverse uses such as evaluating phenotypic plasticity and GxE interactions and uncovering biomarkers associated with growth and quality traits. Since these biomarkers can be evaluated in other plant species, and bearing in mind that phenotypic plasticity is expressed or estimated through these traits, a similar approach may also be useful for plants other than eucalypts.

In this regard, a set of restricted traits will meet the objectives of commercial plantations for sustainable productivity and resistance to stress. A derived question is "How to gather favorable traits for wood trees with long breeding cycles such as eucalyptus?" Ideally, this action is expected to be reliable, provide routine use, easy estimates, low cost and be available for a large number of samples.

Early selection for water deficit tolerance is based on the clone's precocious classification of their degree of tolerance or susceptibility. Recently, this evaluation, carried out based on specific phenotypic markers and easy to measure, allowed the discrimination of *Eucalyptus* genotypes tolerant and susceptible to dieback, a physiological disorder with complex etiology [7]. The selection of biomarkers related to water deficit tolerance and validation in the efficiency of the methodology used in this process are essential for the efficient estimation of genotypic parameters. After the identification of biomarkers, the early selection of *Eucalyptus* clones more tolerant to water deficit should support the recommendation of clones for more stable and productive plantations, even in drought conditions.

The present work aimed to identify and validate the use of morphological, nutritional and physiological traits as biomarkers for tolerance to water deficit in *Eucalyptus*. The selection of this set of biomarkers depends on the ease and its common use in the routine of commercial nurseries for the propagation of eucalypts. In addition, our goal is to provide greater confidence in clone resistance level estimates, using a broader set of biomarkers. Estimates of genotypic parameters and procedures for early selection are proposed based on selection indexes and validation schemes.

2. Materials and Methods

2.1. Plant Material, Seedling Acquisition and Management

The experiment was carried out with 19 eucalyptus genotypes, the performance data of which were made available by forestry companies. Among these, 11 genotypes were tolerant and 8 susceptible to water deficit, according to the behavior of clones at the field level in areas of commercial production with water deficit occurrence. Most of these empirical estimates were made based on observations of 10 climatic zones, according to information from Suzano S/A, which covers areas of plantations that vary from inland to coastal regions, and biomes from the Amazon to the Brazilian Savannah.

The information on the behavior of the eucalyptus genotypes (tolerance or susceptibility) and the rainfall in commercial areas, as challenged by the water deficit, was provided by the following forestry companies (Table 1). Suzano S/A (http://www.suzano.com.br/ (accessed on 18 March 2023)) and Fibria (http://www.suzano.com.br/ (accessed on 18 March 2023)) are Brazilian cellulose and paper companies that merged recently. Due to a delay in the authorization process, we were recommended to omit the third company that provided three of the eucalypt seedlings. The identification of the clones by the companies

was suppressed in accordance with a confidentiality contract. The seedlings were produced by cuttings according to the nursery protocols and supplied by the forestry companies.

Table 1. Commercial *Eucalyptus* clones and their classification as tolerant or susceptible to water deficit (empirical data, field observations provided by the companies Fibriaand Suzano S/A, based on the historical behavior of clone plantations in commercial areas with water deficit).

Clone	Genetic Background	Phenotype Associated with Water Deficit
E-01	*E. grandis* × *E. urophylla*	Susceptible
E-02	*E. grandis* × *E. pellita*	Tolerant
E-03	*E. grandis* × *E. urophylla*	Tolerant
E-04	*E. grandis* × *E. pellita*	Susceptible
E-05	*E. grandis* × *E. urophylla*	Susceptible
E-06	*E. platyphylla*	Tolerant
E-07	*E. grandis* × *E. urophylla*	Susceptible
E-08	E. grandis × *E. urophylla*	Tolerant
E-09	*E. grandis* × *E. urophylla*	Susceptible
E-10	*E. grandis*	Tolerant
E-11	*E. grandis* × *E. urophylla*	Tolerant
E-12	*E. urophylla*	Tolerant
E-13	*E. grandis*	Susceptible
E-14	*E. grandis* × *E. urophylla*	Tolerant
E-15	(GG 100)	Susceptible
E-16	Mercado I144	Tolerant
E-17	*E. grandis* × *E. urophylla*	Tolerant
E-18	*E. grandis* × *E. urophylla*	Tolerant
E-19	*E. grandis* × *E. urophylla*	Susceptible

Seedling management was carried out according to standard nursery protocols of Clonar—Resistência a Doenças Florestais Company, Cajuri (latitude: 20°47′26″ S and longitude: 42°47′48″ W), Minas Gerais, Brazil. Clonar—Resistência a Doenças Florestais is a technology-based company that has its own nursery based on the standards of the nurseries of the forestry companies.

Seedlings with 110 days were transplanted in 2 L plastic bags containing rice husk (Santa Carolina, Brazil) (Figure 1A) and fertilizer. The initial fertilization consisted of supplementing the substrate with 150 g osmocolt Plus 15-9-12 [3M] and 150 g SSP (single superphosphate) per 8 kg of rice husk. Additional fertilization was carried out for all plants with a solution of 15 g L^{-1} mono ammonium phosphate (MAP), once a month, and NPK (10–5–30) solution of 6 g L^{-1} at every 15 days. All procedures were performed in a greenhouse according to the standard protocols of Clonar—Resistência a Doenças Florestais under routine conditions (average temperature: 25 °C, natural light, and daily irrigation).

The forestry companies sent the seedlings to the facilities of Clonar Company around the end of October and the beginning of November 2015. The production and transport of the seedling were carried out in 100 mL conic and hollow plastic tubes (55 mm larger diameter) filled with vermiculite. The delivery of seedlings depended on the availability of each nursery clone from three different companies, the transport of seedlings and work routine. The acclimatization stage, experiment conduction and evaluation were carried out from November 2015 to February 2016. Some of the evaluation procedures (nutritional) lasted a little longer, depending on the laboratory's routine.

The seedlings were kept in a greenhouse with a transparent plastic cover (Figure 1A–C) for a period of 30 days for acclimatization, with an average temperature of 25 °C, average relative humidity of 70%, natural sunlight and daily irrigation. No additional or artificial light was provided. The seedlings were grown in standardized conditions, allowing the random variation expected in seedling production and meeting the internalization of the selection procedures for water deficit tolerance genotypes in the nurseries. Considering that the seedlings were approximately the same age, after the acclimatization period, seedlings

of a similar size were selected to compose the experiment. Plant height was used as the size parameter and standard for the plant chosen for the experiment.

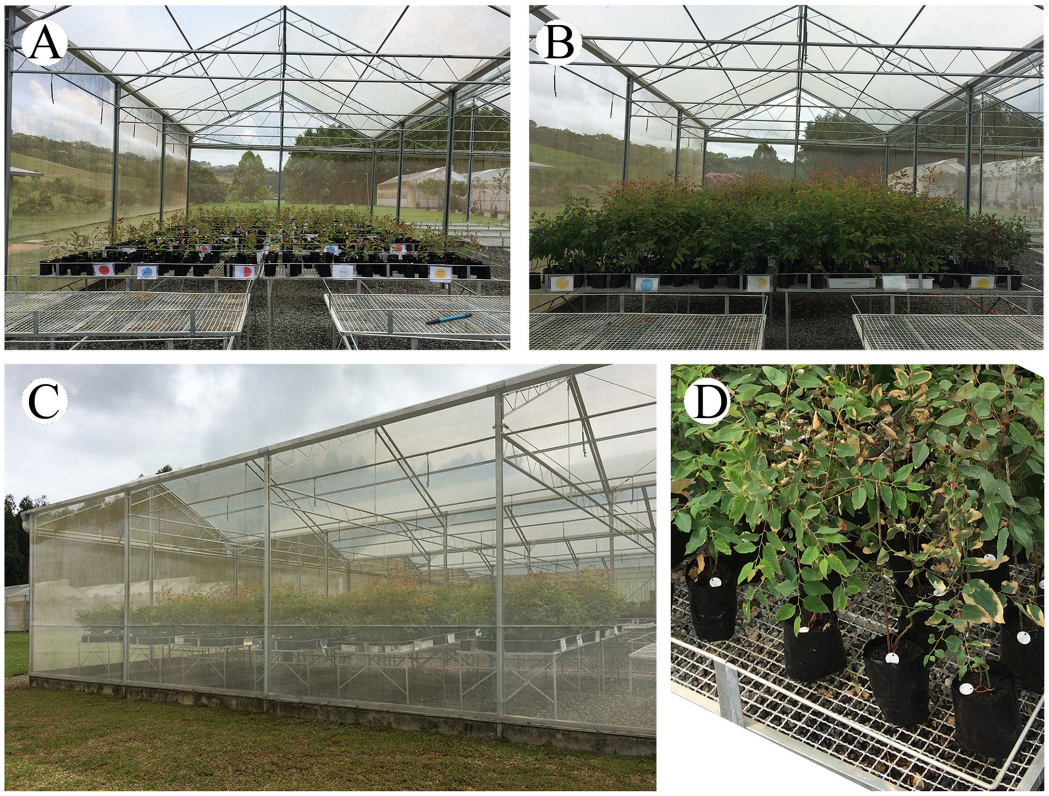

Figure 1. Greenhouse and nursery facilities to simulate water deficit under controlled conditions. (**A**) Panoramic view inside the greenhouse where the experiment was conducted. First week of acclimatization, showing the identification signs of treatments and layout of the experiment; (**B**) Panoramic view inside of the greenhouse in the eleventh week of the experiment; (**C**) External view of the greenhouse; and (**D**) detail of plants with symptoms of water deficit under treatment with 300 PEG.

Irrigation was carried out once or twice a day, automatically, using programable sprinklers set on the upper roof support of the greenhouse structure, depending on the visual assessment of the plants inside the greenhouse and daily temperature monitoring. Manual irrigation was provided, if necessary, during the acclimatization period. Seedling management practices were carried out according to the nursery procedure of Clonar—Resistência a Doenças Florestais Company, including initial fertilization, maintenance of seedlings and phytosanitary control, when and if necessary.

2.2. Control and Water Stress Treatments

The three treatments were established according to [7]. Water deficit treatment was provided by the application of 100 mL of a solution of polyethylene glycol 6000 (PEG), at 300 g L^{-1} or by restricting the amount of water that was poured into the seedling recipient (plastic bag) to 100 mL L^{-1} water per day.

The treatments are categorized as: 1—Control treatment, plants growing in a greenhouse in plastic bags containing 2 L of carbonized rice, fertilizer, an average temperature of 25 °C, natural light and daily irrigation; 2—PEG treatment (or simply 300PEG), the standard nursery procedure (control) plus water stress simulation with the application of 100 mL of a PEG solution 300 g L^{-1} of per pot, every two days; and 3—100 mL L^{-1} water treatment (or simply 100H_2O), the standard nursery procedure (control) with irrigation restricted to the application of 100 mL water per pot per day.

Water stress simulation was carried out after the acclimatization period. Subsequently, the seedlings in the plastic bags filled with rice rusk were randomly distributed on perforated metal workbenches (Figure 1C,D) in a completely randomized block design, with a split plot arrangement to accommodate all genotypes and treatments. The eucalypt clones and treatments were properly identified with plastic labels and signs (Figure 1A,B,D). Sprinkler irrigation was suspended during the experiment and irrigation was performed manually and abundantly for each plastic bag with seedlings once or twice a day, except in treatment $100H_2O$, where each plastic bag instead received 100 mL water per day.

The application of the PEG solution was carried out at two-day intervals and maintenance of irrigation according to the nursery standard was sustained concurrently with the application of PEG. Plants submitted to the water restriction treatment had their application limited to a fixed 100 mL of water per pot per day.

The level of stress supplied to the plants was defined in previous experiments [9,10], carried out under identical use of substrate and local conditions and management, where the methodology for simulating water deficit was established. The water potential of the solution of the substrate was measured for every seedling pot on the last day of the experiment and was determined using a cryoscope (ITR model MK540). For each treatment, the water potential of the substrate solution of a total of 60 plants of four different genotypes was evaluated. The freezing point of the solution sample in degrees Horvet (°H) was converted into MegaPascal (MPa) according to the previous method [11]. Higher values mean greater water availability and were observed for the control samples and $100H_2O$ treatment (~−0.08 MPa), while medium and lower values were observed for drier conditions and with less water availability, observed for moderate (100PEG; −0.35 MPa) and severe (300PEG; −0.48 MPa) water stressed samples, respectively. The information on water potential was estimated for the control and 300PEG treatments as presented, while the 100PEG treatment, as it was not used in the present report, was maintained just as a reference. The water potential of the substrate solution of treatment $100H_2O$ was considered the same as the control treatment since it consisted of the same substrate.

In the tenth week of the experiment, the leaf water potential of the plants was measured with a Scholander Chamber Model 1000 (PMS Instrument Company, Albany, OR, USA) in two periods, from 3:00 to 5:00 a.m. and from 1:00 to 3:00 p.m.

2.3. Traits

2.3.1. Morphological

The variables evaluated were the stem diameter (SD) measured 2.5 cm above the collar region; leaf area (LA) calculated from 10 fully expanded leaves of the third or fourth node, from the apex to the base of each plant; plant height (PH); increase in diameter (ID) and increase in height (IH).

The variables SD and LA were measured at the beginning and end of the experiment with calipers and a tape measure, respectively. For the LA estimate, the leaves were scanned and the individual area of each leaf was determined. The variables "ID" and "IH" consisted of the difference in diameter and height of the plant stem at the beginning and end of the 12 weeks of the experiment, respectively. SD, PH, ID and IH are expressed in cm and LA in cm^2.

2.3.2. Nutritional Traits

Twenty healthy and fully expanded leaves were collected from the middle third of each plant. The leaves were dried in an oven at 60 °C for 3 days and sent to the Forest Soils Laboratory, Department of Soil Science of the Federal University of Viçosa (UFV), where nutrient analyzes were performed according to the standard laboratory procedure. The evaluated nutrients were nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S), and the micronutrients: iron (Fe), zinc (Zn), cupper (Cu), manganese (Mn) and boron (B). N, P, K, Ca, Mg and S are expressed in dag kg^{-1}, and Zn, Fe, Mg, Cu and B in mg kg^{-1}.

Dried leaves were submitted to Nitric-Perchloric digestion as described by [12]. Phosphorus (P) levels were determined by colorimetry using the ascorbic acid method [13], those of potassium (K) by flame photometry, and those of calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn) by atomic absorption spectrophotometry. Sulfur (S) levels were determined by turbidimetry [14] and those of nitrogen (N) by the Kjeldahl method.

2.3.3. Physiological Traits

The variables transpiration (E, mmol H_2O kg^{-1} s^{-1}), stomatal conductance (gs, µmol H_2O m^{-2} s^{-1}), photosynthesis (A, µmol CO_2 m^{-2} s^{-1}), instantaneous water use efficiency (A/E), intrinsic water use efficiency (A/gs) and internal carbon/external carbon ratio (Ci/Ca) were measured using IRGA (LCpro-SD, ADC Biocientific Ltd.) 30 (first evaluation) and 60 days (second evaluation) after the installation of the experiment. The data were collected in three replications with a total of 57 plants each. In each replication, all plants representing each combination of genotype and treatment were evaluated from 9:00 to 11:30 a.m. The evaluation was carried out under constant irradiation of 1000 µmol $m^{-2}s^{-1}$, using the expanded leaves of the middle third of the plants.

2.4. Statistical Analysis

The plants were evaluated in a completely randomized block design with three replications, where one plant of each clone was conducted as an experimental unit, within the three treatments (described above) arranged in split plots. The experiment lasted 12 weeks, after which the morphological data and the leaf samples for nutritional analysis were collected. Physiological variables were collected 30 and 60 days after the beginning of the water stress simulation.

The estimates of the genotypic parameters were obtained by mixed model methodology, REML (Restricted Maximum Likelihood) procedure/BLUP (Best Linear Unbiased Prediction) and deviance analysis (Anadev) [15,16], following the model below.

$$y = Xb + Zg + Wi + e,$$

where y is the vector of observed data (biomarkers phenotypic expression), b is the vector of fixed effects (i.e., overall average, experimental blocks and treatments), g is the random effect vector of total genotypic effects, i is the interaction effect of genotypes and the three treatments (i.e., the nursery default—control, and the two induced water stress conditions) and e is the random residual, respectively. The capital letters X, Z and W are the incidence matrices for the effects b, g and i, respectively. The distributions and variance structures of the model random effects are described as: $g\left|\sigma_g^2 \sim N\left(0, I\sigma_g^2\right)\right.$, being I an identity matrix given the non-relatedness between clones; $i\left|\sigma_i^2 \sim N\left(0, I\sigma_i^2\right)\right.$ and $e\left|\sigma_e^2 \sim N\left(0, I\sigma_e^2\right)\right.$. SELEGEN-Reml/Blup software (Statistical System and Computerized Genetic Selection via Linear Models Mixed)—version 2016, model #54 [17] was used to perform the statistical analyses.

For the majority of the traits the genotype × water-stress environment interaction effects (i) were not significant in the deviance analyses (see the low values for c2int and high values for rgloc in Table 2 for the data with two treatments and in Supplementary Table S1 for the data with three treatments).

Table 2. Estimation of genotypic parameters from the seedling analysis of 19 commercial *Eucalyptus* clones submitted to water stress treatments (100H_2O and 300PEG) for a period of 12 weeks. Both treatments of water stress were considered for the estimates of morphological, nutritional and physiological traits.

	Parameters							
Traits	h_g^2	h_{mg}^2	**Acgen**	C_{int}^2	$r_{g_{loc}}$	CV_{gi}%	CV_e%	$\bar{x}$
PH	0.47	0.84	0.91	0.00	0.99	9.70	10.35	61.07
LA	0.40	0.74	0.86	0.11	0.28	20.89	23.09	177.24
ID	0.27	0.66	0.81	0.04	0.85	11.34	18.07	3.22
IH	0.31	0.73	0.85	0.00	0.99	17.13	25.67	31.70
N	0.11	0.36	0.70	0.17	0.40	6.17	15.39	1.68
K	0.29	0.58	0.76	0.28	0.51	7.48	9.01	1.08
Ca	0.19	0.54	0.73	0.32	0.37	11.66	18.72	0.79
Mg	0.54	0.84	0.91	0.08	0.86	13.38	10.86	0.42
S	0.29	0.69	0.83	0.21	0.57	17.64	23.40	0.23
Cu	0.32	0.74	0.86	0.19	0.63	16.72	20.05	5.90
Zn	0.48	0.78	0.88	0.15	0.76	16.76	14.75	29.58
Mn	0.37	0.69	0.83	0.18	0.67	14.54	16.05	678.86
B	0.34	0.77	0.88	0.10	0.76	13.13	16.78	85.92
A	0.22	0.62	0.79	0.00	0.97	20.81	39.41	6.50
gs	0.15	0.48	0.70	0.06	0.71	20.19	46.58	0.08

h_g^2: Heritability of individual plants in the broad sense; h_{mg}^2: Heritability of the genotype average; Accgen: Accuracy of genotype selection; C_{int}^2: Coefficient of determination of the effects of genotype × treatment interaction; $r_{g_{loc}}$: genotype correlation between performance in the various environments; CV_{gi}%: genotype coefficient of variation; CV_e%: coefficient of residual variation; $\bar{x}$: overall average of the experiment. Individual heritability values in accordance with [18]: low: values lower than 0.15; moderate: values from 0.15 to 0.50; and high: values greater than 0.5. PH—plant height, LA—leaf area, ID—increment in stem diameter, IH—increment in stem height, concentrations of the nutrients nitrogen—N, potassium—K, calcium—Ca, magnesium—Mg, sulfur—S, cupper—Cu, zinc—Zn, manganese—Mn, boron—B, photosynthesis—A, stomatal conductivity—gs. Averages of PH, ID and IH are expressed in cm, LA in cm^2; N, K, Ca, Mg and S concentration are expressed in dag kg^{-1}; Zn, Fe, Mg, Cu and B in mg kg^{-1}; gs is expressed in μmol H_2O m^{-2} s^{-1}; A is expressed in μmol CO_2 m^{-2} s^{-1}. A(1) and gs(1) indicate measures from the first evaluation of physiological traits conducted on the 30th day of the experiment.

The estimation of genotypic parameters based on the analysis of *Eucalyptus* clones considered the water stress treatments (100H_2O and 300PEG) together. The average, SD and CV for all clones in the control treatment is exhibited in Supplementary Table S2. The coefficient of determination of the effects of genotype × environment interaction (C_{int}^2) and genotype correlation between the performance in the different environments ($r_{g_{loc}}$) were estimated based only on the different water stress treatments. In these analyses, for purposes of estimation, water stress treatments were considered as the environmental component. The threshold values for heritability were in accordance with [18]: low, values lower than 0.15; moderate, values from 0.15 to 0.50; and high, values higher than 0.5.

2.4.1. Selection Index

The predicted genotypic values were used to calculate the selection index, based on the average of ranks [19], and the correlations of traits (possible phenotypic markers) with the tolerant and susceptible phenotype were considered.

The lowest rank value indicates a beneficial condition for the water deficit tolerance trait. Likewise, the sum of ranks of established biomarkers will provide different numbers, the smaller the combination of traits or biomarkers, the more favorable to tolerance, and the larger the combination, the more favorable to susceptibility to water deficit.

2.4.2. Selection Index Based on Weights

For the second selection index, the possible phenotypic markers found were used, which in turn were standardized, making $x \sim N(\mu = 0; \sigma 2 = 1)$. Then the model was adjusted:

$$y = b_0 + b_1X_1 + b_2X_2 + \ldots + b_{15}X_{15} + e,$$

where y is the binary variable of tolerance (1) and susceptibility (0), b_0 is the intercept value, b_1 to b_{12} are the coefficients of the multiple regression, X to X_{15} are the variables collected and selected as possible biomarkers (Table 2) and e is the residue of the model.

The stepwise backward-elimination variable selection algorithm was performed using the step function of the R software [20]. The selection index as proposed by [21] was performed by means of the equation:

$TP = b_0 + b_1X_1 + b_2X_2 + \ldots + b_{15}X_{15}$, where TP is the probability of tolerance (ranging from 0 to 1) and the bs refer to the X variables selected by the stepwise backward procedure.

To make the procedure practical and to facilitate its application directly in the field, the weights have been readjusted with the non-standard (i.e., originals) variables, so the breeder can use the variables in the magnitudes observed in the field.

2.4.3. Validation Procedures

Two validation strategies were adopted. The first consists of the "leave-one-out" method [22], of which the model is fit for 18 of the 19 clones used, and the 19th is predicted. The 19 predicted values are stored sequentially and then evaluated using two criteria: (i) RMSE (Root Mean Square Error); and (ii) accuracy "r" (correlation between observed and predicted values). This correlation between predicted and observed values refers to the predictive ability, which means whether or not the model is able to predict clone behavior (tolerant or susceptible) based on the values observed from seedlings.

The second strategy was to check the consistency of the data and modeling. The models were adjusted for both the average of all the blocks. This procedure aims to validate the predictive capacity of the models and the biological relationship between the selected variables as possible phenotypic markers of water deficit tolerance.

3. Results

Genotypic parameters for validation of phenotypic markers for water deficit tolerance.

The evaluated traits varied significantly for eucalyptus clones submitted to treatments with water deficit. Among the 26 morphological, nutritional and physiological traits evaluated, 15 showed significant heritability and high accuracy (greater than 70%) (Table 2). The variability of the estimates for these 15 traits was the criterion for classifying them as phenotypic markers for the early selection of water deficit-tolerant *Eucalyptus* clones.

The morphological variables "plant height", "increase in height", and "leaf area" and nutritional variables "Mg", "Cu", "Mn", "Zn" and "B" showed greater heritability exhibiting a high genetic control. These nine variables showed accuracy above 80%, indicating high reliability for their use in the selection process. The other selected variables, morphological "increase in diameter", physiological "A" and "gs" and nutritional "N", "K", "Ca" and "S", showed heritability between 0.11 and 0.29, classified as "medium magnitude", and were also considered suitable for analysis. These variables showed accuracy above 70% (high precision), and, together with the traits with high heritability, guarantee reliability to the selection practice.

In the analysis of the interaction G × E (Clones × Treatments), the traits LA, Zn, N, Mn, Cu, S, K, and Ca showed a complex but low interaction with a significant coefficient of determination of the effects of the genotype × treatment interaction (C2int) (11; 15, 17; 18, 19, 21, 28; and 32%, respectively). The other traits were not significant for the interaction, showing a high correlation (rgloc) between the water deficit simulation treatments, indicating greater stability between the treatments.

High heritability values reflect the relevance of the markers identified for the early selection of eucalypt clones, as well as evidence of the use of adequate water deficit

simulation conditions. The 100H_2O and 300PEG treatments were efficient in allowing the expression and estimation of significant genetic variability among the clones evaluated on the premise of early selection, where, for most of the selected variables, the heritability values differed from zero (Figure 2). Conversely, high heritability (Figure 2) points to the variability expressed by the evaluated genotypes under stress conditions, favoring the approach of their phenotypic plasticity. The average water potential of leaves of the 19 commercial eucalypt clones and control and water deficit treatments are presented in Supplementary Table S3.

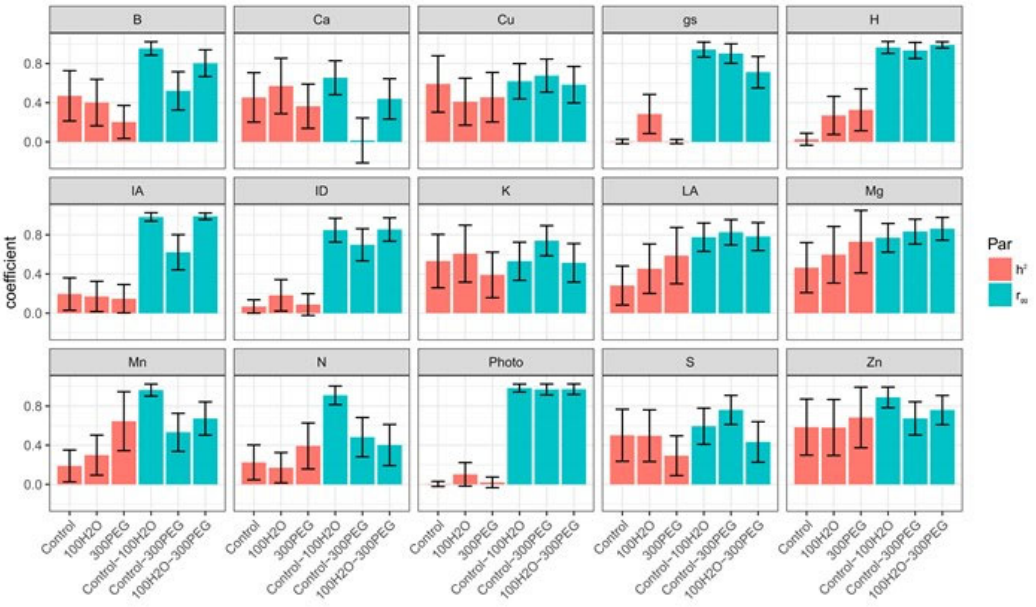

Figure 2. Heritability of individual plants in the broad sense (h^2g) of morphological, nutritional and physiological traits (orange bars) and genotypic correlation between performance in the different environments (rg) (blue bars) of *Eucalyptus* clones submitted to conditions of water deficit simulation. PH—plant height, FA—foliar area, ID—increment in stem diameter, IH—increment in stem height, concentrations of the nutrients nitrogen—N, potassium—K, calcium—Ca, magnesium—Mg, sulfur—S, cupper—Cu, zinc—Zn, manganese—Mn, boron—B, photosynthesis—A, stomatal conductivity—gs.

Selection index based on the average of ranks for water deficit tolerance.

The clones were classified based on the phenotypic traits that showed considerable variability, using the rank index of [19]. The clones that occupied the first positions in the ranking can be indicated for selection as more tolerant materials since the lower values of the ranking indicate genotypes that have a set of morphological, nutritional and physiological characteristics, which combined more favorable to tolerance to water deficit (Table 3).

The top 10 positions in the ranking were occupied by the water deficit-tolerant clones (Table 3), except clone E-01, while the last 6 positions were occupied by clones susceptible to water deficit. This classification is in accordance with the tolerance to water deficit observed at the field level (Table 1).

Validation of methodology for the selection of clones tolerant to water deficit.

The effects of phenotypic markers selected for water deficit tolerance were tested by the stepwise backward methodology. Figure 3 only includes and displays the variables that were selected by the model, out of the 15 that were evaluated. The selected variables are those that appear in the boxes. The magnitudes, as well as the direction of effects, of the variables are also presented.

Table 3. Mulamba and Mock index based on morphological, nutritional and physiological traits in seedlings of 19 commercial *Eucalyptus* clones submitted to and water deficit ($100H_2O$ and 300PEG) for 12 weeks.

Clone	Rank PH	Rank LA	Rank ID	Rank IH	Rank N	Rank K	Rank Ca	Rank Mg	Rank S	Rank Cu	Rank Zn	Rank Mn	Rank B	Rank A	Rank gs(1)	Rank Average
E-18 T	12-7	1-3	14-4	4-12	7-1	7-14	1-5	1-1	19-2	5-16	11-7	18-14	17-13	6-1	5-7	8.03
E-08 T	1-4	7-5	4-11	16-1	8-2	4-3	3-3	8-5	1-12	9-18	18-4	11-16	7-18	16-14	14-9	8.40
E-02 T	2-1	2-1	2-1	15-4	4-6	19-1	4-15	4-13	15-7	12-12	5-9	19-13	18-12	17-8	8-19	8.93
E-17 T	19-17	17-6	11-2	1-18	11-5	1-19	9-6	2-2	17-3	14-9	17-1	14-15	4-7	2-2	13-5	8.96
E-11 T	9-15	15-9	1-6	10-14	5-13	18-6	8-14	12-19	6-9	10-2	14-3	9-4	15-5	4-5	10-6	9.20
E-01 S	16-16	12-7	7-3	17-8	16-3	2-9	17-7	17-8	5-6	13-17	8-13	5-9	14-11	3-1	3-8	9.37
E-12 T	3-6	5-11	6-13	12-7	1-11	3-13	14-12	18-12	7-19	18-4	9-17	16-3	1-3	8-11	15-4	9.40
E-10 T	7-3	19-18	19-12	9-11	9-18	13-11	12-2	16-15	2-15	7-5	4-15	3-6	1-2	5-18	9-1	9.57
E-03 T	8-5	16-10	9-14	8-5	10-8	12-8	2-11	6-7	14-17	2-13	1-18	15-8	10-14	18-4	1-16	9.67
E-06 T	18-18	11-13	10-7	13-3	18-10	9-5	11-8	10-4	11-1	3-19	3-19	2-19	5-5	7-16	11-14	10.03
E-04 S	4-8	6-2	8-16	14-10	3-14	6-16	5-4	3-3	18-4	16-7	15-11	16-19	19-19	10-9	2-17	10.13
E-16 T	11-9	3-12	18-17	10-16	14-17	17-12	7-9	14-10	4-16	1-8	10-2	10-2	12-8	14-12	7-11	10.20
E-14 T	6-10	10-14	13-15	18-2	12-16	5-17	16-17	5-9	16-10	17-11	12-6	8-10	2-1	11-10	17-2	10.27
E-15 S	13-13	4-17	15-10	6-17	13-19	16-18	6-1	11-6	10-11	8-6	2-16	7-12	9-4	12-17	12-10	10.70
E-05 S	15-19	13-15	3-5	19-6	2-4	15-10	10-18	7-11	8-13	3-15	6-10	6-1	13-10	15-15	19-18	10.80
E-09 S	14-11	9-8	5-8	7-13	19-15	14-7	18-16	13-16	13-5	11-11	13-14	13-18	8-16	1-3	6-3	10.93
E-13 S	17-14	14-16	13-18	2-19	15-9	11-2	13-10	9-14	12-8	15-10	7-11	4-7	11-15	9-6	16-13	11.33
E-19 S	10-12	8-4	17-8	5-15	17-7	8-15	15-19	15-17	9-14	6-14	16-5	17-11	6-6	19-19	4-12	11.70
E-07 S	5-21	18-19	16-19	11-9	6-12	10-4	19-13	10-18	3-18	19-16	19-8	12-17	3-9	13-13	18-15	12.33

T: tolerant to water deficit; S: susceptible to water deficit; PH: plant height; LA: leaf area; ID: increment in stem diameter; IH: increase in stem height, N: concentrations of the nutrients nitrogen; K: potassium; Ca: calcium; Mg: magnesium; S: sulfur; Cu: cupper; Zn: zinc; Mn: manganese; B: boron; A: photosynthesis; gs: stomatal conductivity. Ranks for each combination of clone and variable are presented separately, the first from $100H_2O$ treatment and the second from 300PEG treatment. The average rank consists of the average of individual ranks for both water deficit treatments for each of the traits.

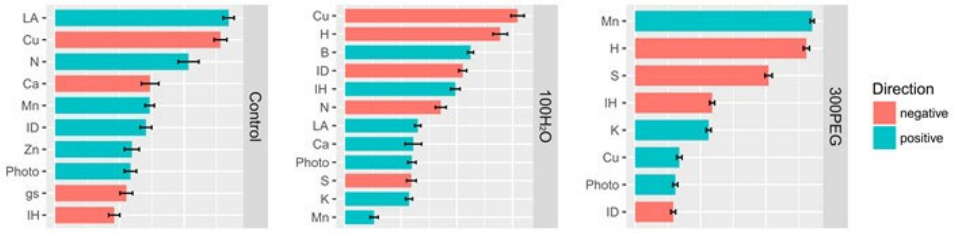

Figure 3. Comparison between effects of biomarkers using all separate blocks. Colored bars are the effect signal on the model. PH—plant height, FA—foliar area, ID—increment in stem diameter, IH—increment in stem height, concentrations of the nutrients nitrogen—N, potassium—K, calcium—Ca, magnesium—Mg, sulfur—S, cupper—Cu, zinc—Zn, manganese—Mn, boron—B, photosynthesis—A, stomatal conductivity—gs. When the bar is blue, the contribution of the variable is positive in the tolerance probability, conversely, when the bar is red, the contribution is negative in estimating the probability.

Figure 4 shows that, using the "leave-one-out" validation scheme, the selection was more reliable when the left boxplot was close to 0 (susceptible clones) and the right boxplot was close to 1 (tolerant clones) (water deficit simulation with 100H_2O and 300PEG treatments) using the selected phenotypic markers (Tables 2 and 4). In the "leave-one-out" validation scheme, tolerance (T = 1) and susceptibility (S = 0) were plotted on the *x*-axis, according to the empirical data of each clone in commercial areas (Table 1). On the *y*-axis, the probability found by the model was quantitative, according to the values obtained by the clones in each block and treatment, for all possible phenotypic biomarkers. The 300PEG treatment showed a higher percentage of probability of tolerance since the susceptibility of the clones was close to 20% and the tolerance close to 90% (Figure 4)—that is, within the criteria of values, 0 for susceptibility and 1 for tolerance, established for evaluating treatments. The 300PEG treatment was more reliable for early selection compared to 100H_2O and Control, indicating that this is an adequate methodology since the water deficit simulation provided closer results of the susceptibility and tolerance probabilities (0 and 1) to susceptible and tolerant genotypes, respectively.

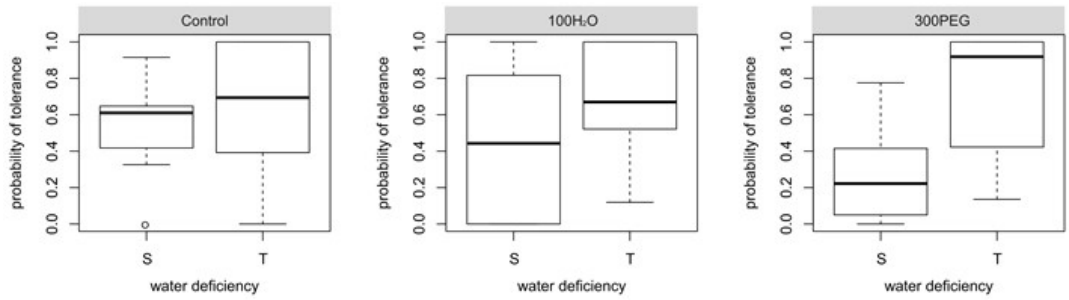

Figure 4. "leave-one-out" validation scheme for selection of clones tolerant to water deficit in the treatments: Control, 300PEG and 100H_2O, where the probability is given by S = susceptible and T = tolerant.

Table 4 shows the values of r and RMSE for all models evaluated. The 100H_2O and 300PEG treatments demonstrated the highest r and the lowest RMSE, confirming that these are the best scenarios for the early selection of water deficit tolerance. This association was made by comparing the final success rate of the prediction of tolerant/susceptible genotypes. The water stress treatments, 100H_2O and 300PEG, showed an increase in the overall prediction success rate over the control treatment. Probability tolerance values < 0.5 were taken as susceptible clones and values $\geq$ 0.5 as tolerant. In this sense, the following equations can be used to predict the potential or probability of tolerance (TP): (TP): $tp = 5.773 + 0.004\ LA + 0.028\ B - 1.339\ Ca - 0.094\ Cu + 0.048\ IH - 0.0731\ H$

$-0.819\ ID + 1.430\ K + 0.000\ Mn - 2.016\ N + 0.122\ photo - 2.828$ for the 100H_2O treatment; and $tp = 2.525 + 1.181\ Cu - 1.947\ IH - 8.242\ H - 2.120\ ID + 2.454\ K + 3.594\ Mn + 6.121$ A $- 1.379\ S$ for 300PEG treatment.

Table 4. Traits of predictive water deficit tolerance models based on seedling data from 19 commercial *Eucalyptus* clones submitted to water deficit treatments (100H_2O and 300PEG) for 12 weeks.

		Control	**100H_2O**	**300PEG**
Average of all blocks	r	0.17	0.37	0.56
	RMSE	0.55	0.49	0.42
	Observed	Success rate		
		Control	100H_2O	300PEG
Average of all blocks	Susceptible (8)	25.00%	50.00%	87.50%
	Tolerant (11)	72.73%	72.73%	63.54%
	Total (19)	52.63%	63.16%	73.68%

4. Discussion

Procedures based on water deficit simulation, treatments and biomarkers, were previously established to estimate or phenotype the level of tolerance to eucalypt dieback physiological disorder in seedlings of commercial eucalyptus clones [7]. Some of these biomarkers and treatments and the methodology established for the early selection of eucalyptus genotypes are promising and suitable for a eucalypt breeding program aimed at water deficit tolerance.

In the present work, we establish (I) establish an adequate and viable approach for a greater number of genotypes; (II) a statistical treatment confirming the accuracy of procedures for the selection of the genotype with resilience to the water deficit; reinforce (III) the feasibility of early selection of complex traits such as water deficit tolerance; (IV) the need for procedures capable of increasing resistant traits in future commercial clones; (IV) the need to conduct experiments in conditions of water deficit; and provide (V) precise identification of water deficit-tolerant genotypes provided by biomarkers of a different nature from those previously reported. Together, this information brings confidence and novelty to the identification of stress-tolerant genotypes as successful results are presented.

LA, Zn, N, Mn, Cu, S, K, and Ca have significant effects of genotype × treatment interaction (C2int) and also contributed to the discrimination of the water deficit tolerant genotypes. In this study, we opted to analyze the genotype and environment interaction across the two water deficit scenarios to approach different strategies to cope with water deficit stress. Significant interactions of environment or treatments with eucalypt genotypes are expected under water deficit conditions [8,9,23,24]. Conversely, PH, ID, IH, Ca, Mg, S, photosynthesis (A) and gs(1) represent more stable phenotypic biomarkers among water stress treatments due to the non-significant interaction between treatment genotypes. The stability of phenotypic biomarkers between treatments with water deficit may be adequate for other sets of clones or eucalypt species that may benefit from early selection.

The set of biomarkers used to select genotypes more resilient to water deficit should be sensitive enough to different environments and responses to water deficit triggers. Several biomarkers are recurrently identified as contributing to the tolerance phenotype, but they are not exactly the same as in the previous report [7]. These differences are attributed to the species that constitute the clones sampled in each report [7,25]. Despite positive and correct relationships, more accurate rankings of tolerant genotypes are expected from a set of biomarkers that encompass a greater number of strategies related to the screened germplasm.

Most, but not all, of the nutritional biomarkers reported here are in line with previous reports that also simulated water deficit and evaluated eucalypt genotypes [7,25]. Likewise, biomarkers such as stomatal conductance and photosynthesis were significant when evalu-

ated on the 30th day (first evaluation), but not on the 60th day (second evaluation), which was attributed to a possible acclimatization process. Nevertheless, this may comprise a disadvantage of the use of physiological biomarkers, since they are more susceptible to oscillations than the other biomarkers, and will need a thorough approach. In the present report, our objective was to identify and validate biomarkers that will ease and help the selection process for water deficit tolerance in eucalypts. In addition to the influence of the eucalyptus clone pedigree, significant interaction with the environment [26], severity/duration of stress [27] and nutrients [23] are reported and may contribute to these differences. Coopman et al. [28] reported an interaction between the responses of eucalyptus and the applied hardening treatments. This reinforces the influence of genotype background and interaction of the evaluated traits. Further, there seems to be no consensus on the standards for water deficit tolerance tests [8]. The present results suggest a candidate for these guidelines, at least for early selection for water deficit tolerance, as the approach used here was successful in the discrimination of more tolerant and more susceptible eucalypt clones considering a genetic base consisting of four species used commercially.

Among the 26 evaluated variables, 15 showed significant heritability and accuracy. The establishment and validation of these biomarkers is a contribution to eucalyptus breeding programs aiming at the early selection of genetic materials tolerant to water deficit.

Heritability has the function of allowing the genetic study of the character and, since its role is predictive, it will express the confidence of the phenotypic value as a guide to the genotypic value [29]. Therefore, if a variable has significant heritability and good magnitude, the selection on which it is based is reliable. The heritabilities of 15 selected traits were significant and attest to the feasibility of the early selection for water deficit tolerance based on the 100H_2O and 300PEG treatments.

Another genotypic parameter closely related to the heritability of character is selective accuracy. The accuracy shows a correlation between true and predicted genotypic values, and, the higher its value, the more confident the assessment of individuals [18]. In the present study, selective accuracy ranged from 70% to 91% for the phenotypic biomarkers associated with water deficit tolerance. According to [18,30], an accuracy greater than 70% indicates that there will be good precision in the selection of the genotypes.

Among the selected morphological biomarkers, "plant height", "increase in height", "increase in diameter" and "leaf area" showed significant heritability. Although plant height and height increase are easily measurable traits, the evaluation for early selection should be concomitant with the estimation and analysis of the other biomarkers. The "increment in diameter" was also selected for its relevant heritability and accuracy. Shao et al. [31] state that water deficit can cause a reduction in the number and size of the stem cells, leading them to become more compacted in this region, preventing water loss, but limiting the initial growth. In the present work, the stem diameter stood out as a biomarker for tolerance to water deficit, which is attributed to a similar reduction in the number and size of cells, limiting their initial growth, as described by [31].

The plasticity of the leaf area is an important trait related to the control of water use in crops [31]. The leaf area was selected as a biomarker for early selection of dieback-tolerant eucalyptus clones, a physiological disorder associated with water deficit [7]. Silva et al. [5] reported a reduction in leaf area in eucalypt clones submitted to water deficit. For these authors, the reduction in leaf area is the first line of defense against water deficit, because its reduction is associated with a reduction in water loss.

Among the evaluated nutrients, N, K, Ca, Mg, S, Cu, Zn, Mn and B are emphasized in the selection of water deficit tolerant genotypes and described as appropriate biomarkers for early selection. Waraich et al. [2] studied the role of nutrients in the response of plants to stress conditions, where, in addition to the essential character of plant growth and development, they also minimize the drastic effects of abiotic stresses, such as water deficit. According to these authors, the nutritional status of the plants plays a critical role in their resistance to low water availability. Müller et al. [25] successfully approached this issue and associated the efficiency of nutrient absorption with water deficit conditions.

Despite the lower heritability among all selected biomarkers, N had a significant genotype × treatment interaction (C2int) and relevant accuracy. This nutrient has been associated as a biomarker for early selection of tolerant eucalyptus clones with regard to tolerance to dieback, a physiological disorder potentiated by water deficit [7] and thus maintained in this analyzes. The importance of N can be directly related to A, which is also a relevant trait for the selection of water deficit tolerant eucalyptus clones.

Photosynthesis (A) was a significant biomarker (Table 2) and decreased under water stress and is also related to the leaf area, two important biomarkers for ranking water deficit tolerant eucalypt genotypes (Figures 2 and 3). Similar behavior was observed for divergent eucalypt clones under water stress [9]. The relations "leaf area" and "N" can also be linked to the capture of light, while the products of photosynthesis can act in the balance and availability of suitable carbon skeleton forms for the assimilation of nitrogen. Grassi et al. [32] state that N is one of the main determinants of the photosynthetic capacity of E. grandis since approximately 50% of photosynthetic N constitutes the enzyme RUBISCO. These reports support the interlinked plasticity and interrelation of traits expected from biomarkers that contribute to water deficit tolerance. Conversely, physiological traits such as photorespiration contribute to water deficit tolerance [33], increase with water deficit stress [34] and have a profound impact on nitrate assimilation [35]. The relevance of N in the discrimination of water-tolerant genotypes is, therefore, adequate.

Müller et al. [25] reported that drought stress significantly reduced the dry matter of leaves, roots, and the entire plant, as well as decreasing the nutritional efficiency of most clones. Under drought stress, stress-tolerant clones generally have high absorption efficiency despite low efficiency in the use of nutrients, while stress-sensitive clones have low absorption efficiency, low efficiency in root formation and high absorption efficiency in leaf formation. The contrasting behavior of tolerant and susceptible genotypes under stress [25] and the interaction with the severity/duration of stress [27] and nutrients [23] are expected to contribute even more to interlinked plasticity and interrelation of biomarkers related to water deficit tolerance. It is worth highlighting that there are trends expected for phenotypic biomarkers, whose behavior contributes to the tolerance trait [8], however, they have not been explored here, as our objective was to identify these biomarkers instead of discussing their trends to increase or decrease tolerance.

Potassium (K) stands out among nutritional biomarkers that have a positive effect on physiological and structural traits, such as osmotic adjustments and reduced damage to membranes [36], thus contributing to eucalyptus tolerance to water deficit [37,38]. Under conditions of water scarcity, K is related to reduced leaf growth and greater osmotic adjustment, the latter contributing to enhancing leaf turgor under dry periods. This short-term regulation allowed trees to react more promptly to environmental changes, in addition to increased stomatal conductivity [37]. Despite this, the increased supply of K can contribute to the increase in hydraulic dysfunction, due to greater water depletion and tree mortality [37]. Alternatively, fertilization with K resulted in increased efficiency in the use of water for stem wood biomass and canopy transpiration from the first to the third year after planting [38]. K may therefore be a crossroads that interconnects morphological, nutritional and metabolic responses to water deficit.

Conversely, calcium (Ca) has a pronounced effect on maintaining the integrity of the cell wall, participating in the signal cascade in the immediate response to stresses, which is primordial in plant metabolism [39]. The importance of Ca in the recovery of plants subjected to water stress is based on its role in maintaining cell structure mediated by the activation of the enzyme ATPase, which in turn is necessary for the recovery of nutrients lost during stress [2].

Similar to Ca, magnesium (Mg) contributes to the structuring of the cell wall [40] and was an important biomarker for tolerance to water deficit, presenting the highest heritability among the selected biomarkers. According to Epstein and Bloom [41], Mg is essential for the activation of most essential enzymes in the plant, such as ATPase, ribulose-1,5-bisphosphate (rubisco), carboxylases, RNA polymerase and protein kinase, that, among

other photosynthesis-related functions [2,35], reduces CO_2 fixation. In fact, a reduction in photosynthesis is recognized as a trend under drought stress, although it has provided different results for the evaluated eucalypt clones. This shed light on the amplitude of the responses to water deficit and their range among eucalypt genotypes, reflecting the plasticity of the phenotype and resources to respond adequately, along with other traits [42], in a combination providing less damage to growth.

Stress due to water deficit will affect processes in which copper (Cu) effectively participates [2]. Although required in small quantities, conditions under which genotypes are devoid of a suitable amount of Cu will, for example, have a potential impact on nutrition and cell wall structure, which justifies the contribution of this nutrient to water deficit tolerance.

Waraich et al. [2] mentioned the association between the lower availability of Zn and water deficit, where its deficiency reduced photosynthesis. This was attributed to the reduction in the leaf area, which reduced the interception of light and carbon uptake per unit of area, in addition to possible damage to the photosynthetic apparatus. Accordingly, an impact on the interaction among nutritional, physiological and morphological characteristics of plants under water deficit stress is expected, since leaf area, photosynthesis rates, Zn and other mineral elements were useful for the identification of eucalypt genotypes known as more tolerant to water stress.

The flag leaves of wheat under water stress that had received Zn fertilization to the soil displayed increased concentrations of phenols and flavonoids, a group of metabolites that defend cells against the attack of ROS [43]. It seems also to be true for eucalypts since tolerant clones showed higher phenolic content comparing plants subjected to the water deficit to those subjected to the control treatment [9]. In addition, there are reports of significant benefits of Zn for root growth [2] and photosynthetic improvement of the plant under water stress [43]. These results [2,10,43] suggest that greater interaction among strategies regarding water deficit tolerance in eucalypts is expected.

Manganese (Mn) was indicated for early selection of eucalypt clones, as it has a positive association with tolerance to water deficit. Mn acts as a cofactor for many antioxidant enzymes, such as ascorbate peroxidase, SOD and CAT, which are vital for the development of plants when under stress conditions as they capture free radicals [44]. Leite et al. [45] reported an accumulation of phenolic compounds and greater Mn in the tissues affected by eucalyptus blight, a physiological disorder. Although excess Mn has also resulted in increased SOD, CAT and peroxidase activity [46]. Conversely, Bloom [35] highlighted the association of Mg and Mn and the complexes that are formed with RUBISCO in favor of RuBP's oxygenation and carboxylation roles, respectively. This is relevant as RUBISCO's affinity for Mn is five times greater than for Mg [47]. Once again, it seems that the balance among different traits works together to contrast the effects of water deficit, where the ability to adequately increase or decrease processes such as photosynthesis is essential. These reports and the significant effect of mineral elements that contribute to the identification of eucalypt genotypes that are more resistant to water stress corroborate this idea.

Boron (B) is recurrently associated with physiological disorders and water deficit in *Eucalyptus* [7,26,48]. For Mattiello et al. [48], B deficiency reduces the permeability of the plasma membrane and water flow. In addition, B is fundamental in the primary structure of the cell wall [49]. It is worth emphasizing that B deficiency also affects the formation of the xylem, hypertrophy and modifications in the cellular cortex, and stoma deformation [49]. These structural effects are expected to have a direct impact, decreasing hydraulic conductivity and transport and nutrient availability, which makes plants more susceptible to water deficit. Thus, the reasoning of more efficient mechanisms in the transport or reallocation of B is understandable for water deficit tolerant Eucalyptus clones. These interpretation mechanisms are mentioned by Barros Filho [24] and endorse the expectations of B is considered a biomarker for water deficit tolerance.

Among the physiological biomarkers evaluated, A and gs were selected from this set of *Eucalyptus* clones and support the selection of tolerant genotypes (Tables 2 and 3).

A and nutritional parameters were identified as sensitive to water deficit [29,31,35,38,47]. This sensibility should be further explored as these biomarkers were significant only for the first evaluation on the 30th day of the experiment. gs is a prominent biomarker that decreased under water deficit conditions, allowing plants to bypass dehydration. By reducing stomatal conductance, plants minimize water loss and maintain cellular hydration when the water vapor pressure deficit increases under conditions of low water availability. Thus, the magnitude of gs reflects the intensity of the water deficit [50]. The authors of [50] also reported that, in the rainy seasons, the gs of *E. urophylla* plantlets were elevated, and, in the dry season, there was a drastic reduction in this trait. Although these physiological traits contribute to water deficit tolerance [2,3,7,30,31], the reason for their lack of significance, non-considered heritability and accuracy over the two evaluation periods are not fully understood.

The duration of water stress and the time plants take to adapt or respond to the stress condition is related to the availability of water at the level of cells, tissues and organisms. However, when water is scarce or any component is depleted under a stress that reflects the effects of water scarcity, this situation causes the organism to gradually collapse or die. It is reasonable to derive from these arguments that different responses to water deficit and physiological disorders reported for eucalypts are, in fact, a fraction of this gradual collapse. There is evidence that this hypothesis regarding responses to water deficit stress is true [7,51]. This is relevant as the water stress treatments, 100H_2O and 300PEG, used in the screening of eucalypts for tolerance had a positive effect on the probability of recognizing tolerance to water deficit. These treatments allowed us to capture information on the variability and phenotypic plasticity of *Eucalyptus* clones submitted to water stress.

For the confirmation of early selection based on the phenotypic biomarkers, aiming at the identification of eucalypt tolerant clones, together with the Mulamba and Mock index, the "leave-one-out" validation scheme was performed. The phenotype used as the reference in this analysis was the phenotype of the tolerant clones, based on the information we had from the commercial clone behavior in the field, and the selected biomarkers. This type of validation is useful to generate confidence in the application of the proposed methodology; therefore, if the early selection biomarkers are efficient, they can provide good results.

The effects of phenotypic markers selected for tolerance to water deficit were tested by the stepwise backward methodology. The stepwise backward methodology is performed interactively; thus, the variables are included or removed from the model until it remains the most parsimonious. We emphasize that variable selection methods such as this stepwise approach are purely statistical and can provide results that are not expected from a biological point of view. To circumvent this, it is necessary to use a larger set of clones, so that the point collinearities between variables do not exert an excluding force of elements that would be theoretically important from the biological point of view. However, the efficiency of this set of variables in predicting the probability of clonal tolerance to water deficit in *Eucalyptus* is undeniable, as indicated by higher hit rates of water stress treatments (Table 4).

There are two ways of associating biomarkers with tolerance hits, based on their physiological role as previously discussed, and based on a possible relationship with linkage disequilibrium with tolerance genes. In addition to qualitative information on nutritional, physiological, or morphological biomarkers, they can have positive or negative values that do not have a biological meaning. In this case, they would be in linkage disequilibrium with tolerance genes and, not necessarily expressing a phenotypic trait.

Regardless of the relationship between biomarkers and resistance to water deficit, both treatments, 100H_2O and 300PEG, provide conditions for satisfactory early selection, as water stress has been simulated efficiently. Although both treatments simulate the water deficit, they provide conditions for the plant to use different strategies to adapt to the water deficit condition. In the 100H_2O treatment, there is less water availability, that is, there is no water in the substrate for absorption, while in the water stress simulation with 300PEG, there is water, but it is less available. Our results indicate and assess the

existence of different strategies that contribute to the water deficit tolerance phenotype in *Eucalyptus* clones. The presence of different strategies among eucalypt commercial clones is associated with the different behaviors observed for the evaluated genotypes, because, although both treatments result in water deficit stress, the 100H_2O and 300PEG treatments provide water stress in different ways. Significant genotype and treatment interactions support the presence of distinct water deficit tolerance strategies that were not addressed in this report.

The number and nature of traits with significant effects highlight the complexity of the tolerance due to water deficit stress. The interconnected plasticity of genotypes is, therefore, a key element for tolerance to water deficit, where the establishment and interrelationships of nutritional, structural, physiological and other traits are essential for better adaptation to the stress condition. A more detailed approach is needed to attribute the direct and indirect contribution of each trait to tolerance to water stress.

In addition to previous reports based on nutritional [7,25] and morphological data [7], we validated the use of traits contributing to water deficit tolerance in *Eucalyptus* and the need for water deficit conditions to carry out the selection of tolerant genotypes. We introduced genetic information such as the inheritance and accuracy estimates that will support breeding programs and reinforce the feasibility of using phenotypic biomarkers in the early selection for water deficit tolerance.

Commercial clones divergent in tolerance to water deficit, according to empirical data in areas of commercial production, were used. Once available, the use of ideotypes identified based on more precise criteria will provide more accurate estimates to support early selection. We emphasize that the early evaluation of morphological, nutritional and physiological traits pooled from an experiment in a greenhouse allows the genotypes to be ranked according to their tolerance to water deficit, based on the evaluation of traits according to criteria of high heritability and accuracy. These procedures have been validated and effective in discriminating water deficit-tolerant and susceptible *Eucalyptus* clones, based on empirical data from commercial field conditions.

5. Conclusions

Several phenotypic traits of easy access, morphological (plant height, increase in height, increase in diameter and leaf area), nutritional (N, K, Ca, Mg, S, Cu, Zn, Mn and B) and physiological, such as photosynthesis (A) and stomatal conductance (gs), provided heritable and highly accurate biomarkers for the efficient selection of water deficit tolerant Eucalyptus genotypes.

The true identification of clones tolerant and susceptible to water deficit, according to their phenotype in the field, demonstrates the potential and importance of the biomarkers for early selection in *Eucalyptus* breeding.

Selection for water deficit tolerance should be performed under conditions of water deficit stress.

The early and correct identification of water deficit-tolerant *Eucalyptus* clones is consistent with the maintenance of water deficit tolerance mechanisms until adulthood.

The nature of the 15 selected phenotypic biomarkers supports interconnected and different strategies, contributing to water stress tolerance in eucalypts.

There is evidence that the choice of biomarkers to be used depends on the species being evaluated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030937/s1, Supplementary Table S1—Estimation of genotypic parameters from the analysis of 22* *Eucalyptus* clones submitted to water stress (100H_2O and 300PEG) and CONTROL treatments in relation to morphological, nutritional and physiological traits; Supplementary Table S2—Average, standard deviation (SD) and coefficient of variation (CV) from three replications of *Eucalyptus* clones submitted to treatments with water stress (100H_2O and 300PEG) and control treatment in relation to the morphological, nutritional and physiological traits in an experiment in a completely randomized block design and split plot arrangement; Supplementary

Table S3—Average water potential (MPa) of leaves of 19 commercial clones submitted to control treatment and water deficit simulation based on water restriction ($100H_2O$) and application of polyethylene glycol solution (300PEG). The water potential was accessed in two periods, from 3:00 a.m. to 5:00 a.m. (dawn) and from 1:00 p.m. to 3:00 p.m. (afternoon).

Author Contributions: Conceptualization, E.A.d.T.P. and T.R.C.; methodology, E.A.d.T.P., T.R.C. and R.T.R.; software, M.D.V.d.R. and C.D.C.; validation, T.R.C., R.T.R. and W.G.d.C.; formal analysis, T.R.C., R.T.R. and W.G.d.C.; investigation, E.A.d.T.P., W.L.P., S.A.C. and T.R.C.; resources, E.A.d.T.P. and E.A.V.Z.; data curation, E.A.d.T.P., T.R.C. and E.A.V.Z.; writing—original draft preparation, E.A.d.T.P. and T.R.C.; writing—review and editing, E.A.d.T.P., T.R.C., R.T.R. and W.G.d.C.; supervision, E.A.d.T.P.; project administration, E.A.d.T.P.; funding acquisition, E.A.d.T.P. and E.A.V.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This experiment was part of a collaborative research and induced demand project with the financial support of Suzano S/A (Contract number 7100515). The project also received grants and funding from the Foundation for Research Support of the State of Minas Gerais (FAPEMIG) and the National Council for Scientific and Technological Development (CNPq) (Process number 351417/2014-1 and 150596/2022/7). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001.

Data Availability Statement: The datasets for this manuscript are available upon request to Suzano S/A staff. Some of the information may not be disclosed according to a confidentiality contract. Information on climate zone x genotype distribution may also be requested and will be evaluated according to company policy. Requests to access the datasets should be directed to Edival Zauza. edivalzauza@suzano.com.br.

Acknowledgments: The company Suzano S/A, for the provision of clones to carry out experiments and permission to publish the results. Fibria (presently merged with Suzano S/A), for the provision of clones and assignment of personnel and equipment for physiological measures.

Conflicts of Interest: Author E.A.V.Z. was employed by Suzano S/A. The authors declare that this study received funding from Suzano S/A and that these resources were managed by the Sociedade de Investigações Florestais (SIF)/UFV. The funder had no role in the study design, greenhouse/nursery data collection, analysis and decision to publish. The funder had the following involvement with the study: provided part of the plant material, gathered information on the behavior of the genotypes in field conditions and (E.A.V.Z.) contributed to the review of the manuscript. This experiment was part of a collaborative research and induced demand project that was also funded by the Foundation for Research Support of the State of Minas Gerais (FAPEMIG) and the National Council for Scientific and Technological Development (CNPq). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001. Part of the information contained in this manuscript was presented in the poster section of the 3rd Agriculture and Climate Change Conference held in Budapest, Hungary, 2019. T.R.C. received a Research fellowhip Grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process number 351417/2014-1). W.G.d.C—Research fellowships granted by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (process number 150596/2022/7). This project was collaborative and induced research funded by Suzano S/A.The project resources were administrated by Sociedade de Investigações Florestais (SIF), Contract number 7100515, which E.A.d.T.P. was the project coordinator.

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