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RESEARCH ARTICLE

Investigating applied drought in *Miscanthus sinensis*; sensitivity, response mechanisms, and subsequent recovery

Mohamad Al Hassan | **Kasper van der Cruijsen** | **Dianka Dees** | **Oene Dolstra** | **Luisa M. Trindade**

Laboratory of Plant Breeding, Wageningen University & Research (WUR), Wageningen, The Netherlands

Correspondence
Luisa M. Trindade, Laboratory of Plant Breeding, Wageningen University & Research (WUR), Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands.
Email: luisa.trindade@wur.nl

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Abstract

Miscanthus is renowned for its excellent water-use efficiency and good adaptability to a wide range of environmental conditions, making it suitable for cultivation on marginal soils. Drought is a major cause of this marginality, and its occurrence is becoming more frequent and prolonged due to climatic change. Developing drought tolerant genotypes of miscanthus would ensure the maintenance of economically viable yields on lands prone to periodic water-deficiency. To better understand the underlying response and tolerance mechanisms, pre-screen for better survivability at plot setup on marginal lands, and identifying early biomarkers of stress, we explored the genetic diversity present in *Miscanthus sinensis* under applied drought. Young plants of 23 genotypes underwent 3 weeks of water-deprivation in glasshouse-controlled conditions, followed by an equal period of recovery. Leaves harvested at the end of both experimental phases were the focus of extensive biochemical analyses. Coupled with monitoring several growth and yield parameters, this was instrumental in evaluating stress impact and responses. The most productive genotypes suffered the most in terms of yield reduction and chlorophyll degradation when stress was applied. In parallel, proline and simple soluble sugars accumulated to readjust the osmotic potential in the cytosol and vacuoles, respectively. The necessary carbon skeletons for this buildup were partially acquired from resources diverted away from cell wall synthesis and maintenance, whose content dropped under stress in parallel to increasing drought-sensitivity. Correspondingly, expressional and biochemical analyses revealed a dynamic turnover of starch and soluble sugars in stressed leaves. Meanwhile, better avoidance of stress enabled a more efficient post-drought recovery, which was characterized by restoring pre-stress hydraulic status and unplugging stress response mechanisms.

KEYWORDS
biomarkers, drought, marginal land, *Miscanthus sinensis*, recovery, stress response

1 | INTRODUCTION

Lignocellulosic crops have the potential to serve as a promising source of bioenergy, especially high-yielding C4 perennial species, such as miscanthus and switchgrass (van der Weijde et al., 2013). Miscanthus, is renowned for its high productivity (Anderson et al., 2011), good water-use efficiency (Cosentino et al., 2007; Ghannoum et al., 2010), low nutrient requirement (Davis et al., 2014; van der Weijde et al., 2013), and its adaptability to adverse environmental conditions (Clifton-Brown et al., 2017; Fonteyne et al., 2016). This makes it suitable for marginal lands exploitation, to avoid the need to use farmland for energy production (Carlsson et al., 2017). However, the genus' most commonly grown species, *Miscanthus* x *giganteus* (MxG), is sterile due to its triploid nature. This presents a major obstacle for its adoption on a wider commercial scale given its costly plot setup via rhizome propagation (Xue et al., 2015), in addition to hindering its optimization via breeding (Jakob et al., 2009). Alternatively, the seed-based *M. sinensis* is gaining interest as a suitable replacement, owing to its genetic diversity (Clifton-Brown et al., 2017; Sacks et al., 2013), better tolerance to abiotic stresses (Clifton-Brown & Lewandowski, 2000a; Weng et al., 2022), and generally lower cell wall lignin contents (Qin et al., 2012; van der Weijde et al., 2017a). The latter is strongly associated with a higher saccharification efficiency (Van der Weijde et al., 2017c; Zhao et al., 2012).

Using marginal lands for cultivating biofuel crops has become an inevitable option to meet set carbon and clean-energy goals (Schueler et al., 2016) without competing for land use with food crops. In most cases however, the cause of marginality is abiotic (Blanco-Canqui, 2016), ranging from floods, alkalinity, salinity, to most importantly, drought (Gopalakrishnan et al., 2011; Von Cossel et al., 2019). The latter is forecasted by climate models to increase in frequency of occurrence, duration, and geographic spread due to global warming (Salinger et al., 2005). In fact, water deficiency reduces crop yields more than any other environmental stress (Cattivelli et al., 2008), negatively impacting every developmental stage in a plant's life. As such, promoting tolerance in biofuel crops is vital, to ensure better survival on marginal lands, specifically during field establishment when mortality in miscanthus is highest (Clifton-Brown & Lewandowski, 2000b; Jørgensen et al., 2003). Furthermore, resistance to mild and moderate droughts warrant consistent and substantial yields under unfavorable conditions. This is crucial for the success of cellulosic biorefineries (van der Weijde et al., 2013), since the criterion for success is efficient and stable crop production rather than mere plant survival.

Developing a blueprint toward optimized water-deficiency endurance requires a better understanding of the response and tolerance mechanisms to drought. Equally important is identifying biomarkers of stress, ensuring an early detection of vulnerability; crucial for pre-screening in breeding programs and reducing mortality rates in plot setups on marginal lands. This pressing need, manifested in the scientific community's growing interest in examining the impact of drought on second generation biofuel crops (Chen et al., 2020; Lovell et al., 2016; Taylor et al., 2016; Weng et al., 2022). Some studies, however, especially those on miscanthus, focused on the stress-imparted changes to cell wall quality and saccharification efficiency (Da Costa et al., 2019; Van Der Weijde et al., 2017b). Moreover, those that did assess the latter, like Ings et al. (2013) and Stavridou et al. (2019) among others, included but a handful of investigated taxa, thus overlooking the extensive genetic diversity offered by the miscanthus genus in general, and *M. sinensis* in particular (Clifton-Brown et al., 2008).

Drought triggers a series of basic stress response mechanisms that include among others, curtailing gas exchange to reduce water loss, the accumulation of compatible solutes (osmolytes), and the activation of antioxidant systems (Ashraf et al., 2011; Yang et al., 2021). These responses are conserved in most plants and do not necessarily confer tolerance. Some genotypes however retain an edge over their more sensitive counterparts due to a multitude of reasons, for example, more efficient activation of these pathways, earlier detection of stress, better avoidance, etc. (Yang et al., 2021). Accordingly, exploiting *M. sinensis* ample genetic diversity via comparative studies, is pivotal in screening for better performing genotypes and the consequent breeding efforts toward improved tolerance in this crop (Weng et al., 2022). This was evident in the dissimilar impact of water-deficiency (Van Der Weijde et al., 2017b) and salt (Chen et al., 2017) on diverse populations of *M. sinensis* genotypes, and likewise the differential expression of common regulatory pathways after flooding and drought treatments (De Vega et al., 2021).

A comparative study of genetically related taxa with different tolerance potentials undergoing applied stress would pinpoint tolerance-conveying mechanisms, and identify early stress biomarkers (Al Hassan, 2016). In this work, we tried to address the lack of such in-depth comparative study in *M. sinensis*, as well as investigating the uncharted post-drought recovery in this species. The latter would prove especially important for exploiting marginal lands prone to occasional droughts. Our working hypothesis was that those mechanisms contributing to drought tolerance would be more efficiently activated in the more tolerant genotypes, whilst earlier and more pronounced

detection of stress biomarkers would be distinctive of the more susceptible ones.

A two-step experiment was carried out under controlled glasshouse conditions on potted plantlets of 23 *M. sinensis* genotypes. Studied plants underwent 3 weeks of drought followed by 3 weeks of recovery (reinstating irrigation to stressed plants). Several growth parameters were monitored during the full duration of the experiment, with a harvest taking place following each experimental phase (stress and recovery). Leaves then underwent extensive biochemical and expressional analyses to investigate the stress response mechanisms. The recorded growth and yield data were used in setting up an index of sensitivity to stress (similar to the approach described by Malinowska et al., 2020), which allowed for the clustering of investigated genotypes into contrasting groups with respect to their vulnerability to drought. This facilitated pursuing our research goals: identifying early stress biomarkers of drought, elucidating possible mechanisms of tolerance and early post-drought recovery in *M. sinensis*, and a better understanding of stress-imparted changes on its leaf carbon allocation.

2 | MATERIALS AND METHODS

2.1 | Plant material

The experimental setup involved 24 *M. sinensis* genotypes developed by Wageningen University and Research (WUR), 15 of which were used during the EU-funded project OPTIMSC (Lewandowski et al., 2016). Each was given a code starting with G, to ease its terminology throughout this manuscript (Table S1). Van Der Weijde et al. (2017b), applied water-deficiency stress on nine of those genotypes, that were included in this work to serve as a benchmark for tolerance identification. One genotype (G6) was excluded from analyses, as its feedstock was considered unsuitable (lack of homogeneity in the starting material). Its pots, however, were kept in the experimental setup as gap fillers (labelled as pot x).

Rhizomes served as the starting material, whereby new buds were split-off and collected for the initiation of individual plants. These explants were placed each in plug-sized seed trays for 6 weeks on a commercial potting mix and irrigated with half-strength Hoagland nutritive solution. Then tiller-forming plugs were transplanted to 5 L pots filled with the same potting mix supplemented with slow-release Osmocote Exact Standard 5–6 M, to prevent any nutritive deficiency in the water-stressed plants during treatments. Plants were then allowed to acclimatize and grow for 10 weeks before initiating treatments. Flowering shoots were "pruned" to keep the plants in a vegetative growing state and normalize the starting material within each genotype.

2.2 | Experimental design

Plant material preparation and the subsequent trials were carried out in a controlled-environment chamber, at the greenhouse facilities of WUR, The Netherlands. The following conditions were used: long-day photoperiod (16 h of light) with irradiance kept at a minimum of 200 Wm^{-2}, temperature averaging around 20°C, and air humidity set to a minimum of 80%.

An experiment with two successive phases was carried out, each for 3 weeks; applied drought followed by reinstated irrigation (recovery). All 23 genotypes were studied for the first phase, whereas only 16 were included in the latter. Prior to stress application, plants (4 months old) within every genotype were screened phenotypically based on their developmental stage and growth, to remove any outliers. Sick or damaged plants were discarded outright, alongside those that were outliers within each genotype's population of clonal replicas. This was essential to keep good homogeneity and minimize any non-stress caused differences. Potted plants were placed in a randomized block design (Figure S1), distributed across four tables (blocks), each divided with a waterproof barrier into two compartments. One compartment in each block was designated for control pots (regular irrigation) and the other for drought/recovery, alternating in position between different blocks. Every compartment was further subdivided into three sections, each with 24 pots (one of every genotype) randomly shuffled within every section. Genotypes that did not have at least 20 plants in the design at the start of the treatments, were only studied in the applied drought phase (five plants were harvested from each genotype per treatment after each, the drought and recovery periods). Any remaining pots of the excluded genotypes (seven in addition to G6, Table S1) were kept as gap fillers (pot X) during the recovery study.

One week before starting treatments, all pots were flooded and drenched to ensure the activation of the Osmocote supplement. Applied drought was implemented by withholding irrigation completely for 3 weeks in the designated drought/recovery compartments, while their control counterparts were watered thrice a week via flooding. Soil moisture contents of several pots in every compartment were regularly checked with a soil moisture meter (MO750, EXTECH Instruments, USA), to ensure the uniformity of applied treatments. At the end of the drought treatments, the aerial parts of five randomly selected plants per treatment for every genotype were harvested (rhizomes and roots were discarded). Fresh stem

and leaf tissue were separated and weighed prior to further processing. The recovery study commenced afterward on the remaining pots, as regular irrigation (similar to controls) was reinstated to previously stressed plants for 3 weeks. A second similar harvest took place at the end of the recovery trial.

2.3 | Monitored growth parameters and feedstock processing

Several growth and physiological parameters were checked during the trials on a weekly basis. These included the following: stem length (measured at the node associated with the highest fully expanded leaf, on the tallest none-flowering stem), flowering occurrence, chlorophyll content using a portable "chlorophyll meter" SPAD-502Plus (Konica Minolta, Japan), photochemical efficiency of photosystem II after dark adaptation (Fv/Fm) using an OS/30-P portable fluorometer (Optics-Science Inc., USA), and the number of stems/tillers (counted only at harvests).

In both harvests, a part of the raked feedstock (leaves and stems) was snap-frozen with liquid nitrogen and stored at −80°C. The rest was separately weighed and placed in an oven at 50°C, for 4 days until stable weight was reached. Dried material was weighed again to determine the dry weight (DW) and by extension, water moisture content at harvest. Dried leaf samples were then ground using a hammer mill with a 1-mm screen. Throughout the manuscript, dry and fresh yield per plant would refer to aboveground biomass exclusively.

2.4 | Developing a sensitivity index

A multi-trait approach instead of single trait plasticity was thought to be more representative of stress impact. As such, four parameters (fresh and dry yields per plant, in addition to stem length and count), highly correlated with plant's performance under applied drought, were considered to set up a vulnerability range. The inclusion of both fresh weight (FW) and DW, aimed at putting emphasis on DW yield stability by overlapping it, while taking moisture content under stress into account. Stem length on the other hand is among the earliest physiological markers of water stress (Ings et al., 2013), and alongside stem count, was deemed influential in explaining miscanthus yield response in both control and stress conditions (Malinowska et al., 2020). Index of plasticity (IP) for each trait was calculated according to equation (1) similar to the method described by Malinowska et al. (2020). The average per genotype for every trait at favorable conditions was reported as $\text{trait}_{\text{control}}$ and that under unfavorable ones was $\text{trait}_{\text{drought}}$.

$$\text{IP} = \left(\text{trait}_{\text{control}} - \text{trait}_{\text{drought}}\right) * \text{trait}_{\text{control}}^{-1} \quad (1)$$

The 23 genotypes were then ranked according to their cumulative IPs, before arranging them into contrasting groups of sensitivity via k-means cluster analysis.

2.5 | Biochemical assays

Harvested leaves were the subject of several biochemical analyses to investigate the impact of the applied treatments and the responses they instigate. The concentrations of renowned osmolytes such as free amino acids (notably proline) and simple soluble sugars were quantified, besides evaluating the changes in ionic and starch contents, and assessing the stress-inflicted damage on the photosynthetic machinery.

2.5.1 | Photosynthetic pigments

Chlorophyll contents of studied *M. sinensis* plants were checked weekly during treatments with a portable "chlorophyll meter" SPAD-502Plus. However, stress induced rolling and curling of the leaves rendered some unsuitable for SPAD measurements. Consequently, a biochemical acetone-assisted quantification of the photosynthetic pigments was used, to assess the stress-induced damage to the photosynthetic machinery. Chlorophyll a (Chl a) and b (Chl b), and total carotenoids (Ca) were quantified, according to the method described by Straumite et al. (2015). Fresh deveined leaf tissue (the midrib was excluded, ≃50 mg) was pulverized and extracted twice using 5 mL acetone, by shaking at room temperature for 30 mins. The supernatants of both successive extractions were combined before measuring their optical density at 470, 645, and 662 nm. Further dilutions were made whenever the optical density was over one. The following equations (2–4) were then used to calculate the contents of the three pigments:

$$C_{\text{Chl a}} = 12.25A_{662} - 2.79A_{645} \quad (2)$$

$$C_{\text{Chl b}} = 21.5A_{645} - 5.1A_{662} \quad (3)$$

$$C_{\text{Ca}} = \left(1000A_{470} - 1.82C_{\text{Chl a}} - 85.02C_{\text{Chl b}}\right)/198 \quad (4)$$

2.5.2 | Simple soluble sugars

Water-soluble sugars were extracted using 80% ethanol from ≃50 mg of fresh leaf material. Samples were

extracted thrice at 80°C before drying the pooled liquid via a SpeedVac vacuum concentrator. The dried extracts were then redissolved with milliQ water and diluted to fall within a linear dynamic range of the used standards for every measured sugar. The remaining solid pellet was used for measuring starch. Simple soluble sugars were quantified via a HPAEC-PAD Dionex ICS5000+ DC equipped with a Dionex CarboPac PA1 Column (2 x 250 mm), using the programmed setup described by Dinh et al. (2019). The eluent was monitored by a thermostatic Thermo Scientific ICS5000 pulsed electrochemical detector, and the output was processed with the software ChromeleonTM Chromatography Data System version 7 (Thermo Scientific, USA). Standards of known concentrations for every detected sugar were run in parallel and used for calculations.

2.5.3 | Starch

Leaf starch contents were determined using an assay kit (no. 0207748, Boehringer, Mannheim, Germany). The solid pellet from the extraction of simple soluble sugars was solubilized in a solution of 8 M HCl and DMSO before being incubated at 60°C for 1 h. The mixture was then neutralized with NaOH and citrate buffer (pH 4.6), according to the protocol described by the kit providers. The clear supernatant was then used to determine starch contents via a multi-well plate reader at a wavelength of 340 nm.

2.5.4 | Free amino acids

An EZ:faast™ (Phenomenex Inc.) kit was used to quantify free amino acids in the leaves of *M. sinensis*. About 50 mg of fresh leaf tissue was extracted twice using 1 mL 80% ethanol by sonicating for 30 min at 60°C. Cleaning and derivatization of the extracts was performed according to the manufacturer's instructions (Phenomenex, 2003). A GC/FID (Agilent Technologies, Santa Clara, USA) was used to identify and quantify the free amino acids.

2.5.5 | Ionic content

Leaf ion concentrations were measured using an ion chromatography system 850 Professional (Metrohm, Switzerland), following the programmed setup described by Jaramillo Roman et al. (2020). Dried leaf samples were ashed in a furnace at 550°C for 5 h. Out of which ≃10 mg was dissolved in 1 mL of formic acid (3 M) and heated at 102°C for 15 min with shaking at 600 rpm. The extracts were then diluted to 10 mL using milliQ water. This was followed by further dilutions to ensure that the readings were made within the linear range of the used calibration curves for every detected ion. Data was expressed in mg ion/g DM.

2.5.6 | Cell wall analyses

Neutral detergent fiber, acid detergent fiber, and acid detergent lignin were determined in the leaves of some genotypes (one per each of the formulated sensitivity clusters). This was done according to the protocol described by van Soest (1967) and developed by ANKOM technology, with the inclusion of modifications relayed by Van Der Weijde et al. (2017b). All analyses were carried out in duplicates on dried and processed leaf material (milled to 1-mm particles), and were used to estimate leaves cell wall, hemicellulose, cellulose, and lignin contents.

2.6 | Expressional studies

Several key genes involved in starch turnover and its direct offshoots (e.g., sucrose synthesis) were targeted for expressional analyses under stress and after recovery via qPCR. These genes were selected and studied based on their importance and contribution to starch granules synthesis and degradation, in agreement with Zeeman et al. (2010), and Stitt and Zeeman (2012). Gene mining and primer design were performed based on *M. sinensis* v7.1 pre-released genome on the Phytozome database (https://phytozome.jgi.doe.gov/). Genes of interest, their abbreviations, and respective loci, as well as the sequences of used primers are presented in Table S2.

Snap-frozen leaf tissue was used for total RNA extraction and subsequent DNAse treatment, with Qiagen RNeasy mini-kit. Quantity and quality of isolated RNA were assessed using Qubit 2.0 fluorometer (Thermo Fisher Scientific) and agarose gel electrophoresis. This preceded cDNA synthesis via iScript™ cDNA Synthesis Kit, and qPCR runs with Biorad iQ™ SYBR® Green Supermix. The eukaryotic initiation factor 4A (EIF4) renowned for its stability under abiotic stress (Sudhakar et al., 2016) was used as a housekeeping gene. Relative expression ratio in between treatments within the same sensitivity subgroup was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.7 | Statistical analyses

General analyses of variance (ANOVA) were performed on the yield and growth parameters, to determine the

significance ($p < 0.05$) of sources of variance for: genotype, treatment, block, and interaction of genotype and treatment. The replicas of every genotype undergoing a single treatment per block were used as a fixed block effect with a nested plot design on which treatment was applied (treatment within block). The analyses were performed following a fixed effect model; where Y_{ijk} is the response variable, μ is the grand mean, G_i is the genotype effect, T_j is the treatment effect, GT_{ij} is the interaction term between genotype and treatment, $B(T_j)_k$ is the treatment (nested) within block effect, and e_{ijk} is the residual error.

$$Y_{ijk} = \mu + G_i + T_j + GT_{ij} + B(T_j)_k + e_{ijk} \quad (5)$$

Significant genotypic differences ($p < 0.05$) within each treatment, and in between sensitivity clusters were carried out via multiple comparative analyses (one-way ANOVA) with post-hoc comparisons using Fisher's least significant difference (LSD). Meanwhile, significant differences between treatment groups (in case of pairs), were assessed by unpaired two-sample *t*-tests. Before the analysis of any variance however, the Shapiro–Wilk test was used to check for validity of normality assumption and the Levene test for the homogeneity of variance. Whenever the latter was violated, Welch's ANOVA was used (or Welch's *t*-test in case pairs are compared). Sensitivity groups were created from the investigated 23 genotypes, using k-means cluster analysis (squared-Euclidean, with three clusters) of the calculated cumulative IPs (section 2.4). This was preceded by determining the optimal number of clusters via the Elbow method. Correlation analyses in between traits to identify strength and direction of their interrelationships were carried out using Pearson correlation coefficients. All statistical analyses were performed with Statgraphics Centurion XVIII and IBM SPSS Statistics 25.

3 | RESULTS

3.1 | Drought's impact on growth

Applied drought had a significant effect on the measured growth parameters (Table 1), although its impact varied markedly in between the 23 studied genotypes (Table 2). On the other hand, the uniformity of applied stress across the experimental setup was confirmed (both block effect and residual errors were not significant) (Table S3).

Water deprivation for 3 weeks caused an overall reduction in vegetative growth among studied genotypes by nearly 61% in fresh yield (FW), 19% in dry yield (DW), and 22% in stem count, compared to controls (Table 1). Both leaves and stems were strongly affected by drought, suffering from a loss of 37 and 28% of their control moisture contents, respectively. Other growth parameters were also adversely affected by the applied stress, for example, stem length was 20% lower on average among stressed plants than in controls, while stem-to-leaf ratio dropped. Reproductive success was similarly impaired, as the number of flowering plants decreased from 25 reported cases

TABLE 1 Overall impact of 3 weeks of drought on the measured growth parameters in 23 studied genotypes of *Miscanthus sinensis*. CV (%) = coefficient of variation (standard deviation/mean ×100%). Fresh and dry yield data are presented in g per plant

Trait	Treatment	Average	Min	Max	Range	CV(%)	Reduction (%)
Fresh yield (g)	Control	98.5	28.8	245.5	216.7	39.9	60.8
	Drought	38.6	22.5	52.6	30.1	18.4	
Dry yield (g)	Control	23.3	5.6	51.3	45.7	39.5	19.3
	Drought	18.8	6.9	30.1	23.2	22.2	
Stem-to-leaf ratio	Control	0.5	0.1	1.0	0.9	37.4	29.9
	Drought	0.4	0.1	1.2	1.2	50.1	
Leaf moisture	Control	73.5	66.1	78.9	12.8	3.8	36.9
	Drought	46.4	10.3	74.9	64.6	37.4	
Stem moisture	Control	80.3	68.3	89.5	21.2	4.8	28.1
	Drought	57.7	35.5	84.8	49.3	17.8	
Stem count	Control	10.6	4.0	28.0	24.0	50.9	22.3
	Drought	8.2	3.0	16.0	13.0	37.0	
Stem length	Control	88.4	52.1	143.5	91.4	20.3	20.1
	Drought	70.6	36.0	105.8	69.8	20.7	
Fv/Fm	Control	0.8	0.7	0.8	0.1	1.6	9.6
	Drought	0.7	0.5	0.8	0.3	11.1	

TABLE 2 Impact of 3 weeks of drought on the growth of 23 *Miscanthus sinensis* genotypes. The genotypic means (average of five replicas per treatments) are presented followed by ± their standard error (SE)

	Fresh yield (g)		Dry yield (g)		Stem count		Stem length (cm)	
Genotype	Control	Drought	Control	Drought	Control	Drought	Control	Drought
G1	83.3 ± 9.6[a]	46.4 ± 2.3[a]	21.7 ± 2.8	19.3 ± 1.3	10.2 ± 2.9	7.8 ± 1.1	91.9 ± 6.8[a]	69.9 ± 4.3[a]
G2	64.5 ± 18.5	41.2 ± 2.9	16.6 ± 5.2	17.7 ± 1.8	9.0 ± 2.7	8.2 ± 1.8	86.9 ± 9.4	79.0 ± 6.4
G3	132.6 ± 12.1[a]	43.6 ± 3.4[a]	29.8 ± 3.1[a]	20.4 ± 1.3[a]	11.3 ± 0.9[a]	6.4 ± 0.8[a]	99.1 ± 3.8[a]	66.0 ± 3.2[a]
G4	81.5 ± 16.1[a]	38.2 ± 2.6[a]	18.7 ± 4.0	17.2 ± 1.7	6.6 ± 1.5	6.0 ± 0.9	80.3 ± 7.0	60.6 ± 3.3
G5	118.4 ± 10.4[a]	35.1 ± 1.7[a]	27.0 ± 3.0	21.1 ± 1.7	10.0 ± 1.2	8.2 ± 0.7	79.5 ± 1.7[a]	61.5 ± 2.3[a]
G7	77.4 ± 8.2[a]	39.6 ± 4.5[a]	18.5 ± 2.4	23.4 ± 0.9	7.5 ± 0.9	9.3 ± 1.3	85.9 ± 2.0[a]	77.0 ± 2.1[a]
G8	123.6 ± 10.5[a]	42.0 ± 3.2[a]	30.1 ± 1.9[a]	17.5 ± 1.5[a]	14.0 ± 1.1[a]	9.4 ± 1.8[a]	98.7 ± 8.9[a]	60.4 ± 6.2[a]
G9	177.4 ± 22.6[a]	33.9 ± 2.9[a]	38.3 ± 4.5[a]	22.1 ± 2.2[a]	21.0 ± 3.3[a]	9.8 ± 0.7[a]	83.2 ± 1.3[a]	61.2 ± 4.9[a]
G10	115.6 ± 14.6[a]	29.3 ± 1.6[a]	29.3 ± 4.3[a]	18.5 ± 1.3[a]	9.4 ± 1.1	7.8 ± 0.7	108.5 ± 2.0[a]	78.6 ± 3.8[a]
G11	95.7 ± 3.9[a]	43.1 ± 2.6[a]	24.9 ± 1.3	23.6 ± 2.1	8.3 ± 1.2	8.3 ± 1.4	87.7 ± 5.9[a]	65.7 ± 4.6[a]
G12	77.8 ± 8.7[a]	35.4 ± 3.2[a]	18.1 ± 2.1	18.9 ± 1.8	7.0 ± 1.6	8.4 ± 0.5	81.9 ± 4.4	69.8 ± 2.5
G13	99.6 ± 16.9[a]	33.4 ± 5.1[a]	20.1 ± 3.8	19.2 ± 1.2	11.3 ± 2.5	11.2 ± 2.0	79.4 ± 2.2	67.7 ± 6.8
G14	161.5 ± 19.8[a]	37.0 ± 1.2[a]	36.9 ± 4.0[a]	26.1 ± 1.5[a]	16.3 ± 1.4	12.8 ± 0.9	107.3 ± 4.9[a]	71.8 ± 5.1[a]
G15	119.6 ± 11.9[a]	39.7 ± 1.2[a]	30.9 ± 4.6[a]	17.9 ± 1.4[a]	17.8 ± 2.8[a]	8.2 ± 0.9[a]	99.2 ± 6.2	85.3 ± 3.3
G16	89.1 ± 10.2[a]	34.3 ± 4.0[a]	21.7 ± 2.1[a]	14.1 ± 0.7[a]	8.6 ± 1.2	7.3 ± 2.2	90.9 ± 10.3	72.0 ± 6.3
G17	124.9 ± 2.8[a]	40.3 ± 3.1[a]	27.6 ± 2.1[a]	18.5 ± 1.9[a]	10.8 ± 1.5	7.6 ± 1.7	88.3 ± 5.6	83.0 ± 3.5
G18	75.5 ± 6.0[a]	34.5 ± 1.6[a]	16.6 ± 1.7	17.4 ± 0.9	12.2 ± 0.7[a]	7.4 ± 0.4[a]	61.7 ± 3.9	50.8 ± 6.0
G19	75.7 ± 5.5[a]	47.5 ± 1.7[a]	18.6 ± 1.1	18.5 ± 1.4	7.6 ± 0.7	7.0 ± 1.4	76.3 ± 7.7[a]	49.4 ± 2.1[a]
G20	52.8 ± 7.0	43.7 ± 1.6	13.2 ± 1.6	17.0 ± 1.6	7.8 ± 1.3	10.0 ± 1.6	64.1 ± 4.1	55.4 ± 2.1
G21	76.5 ± 7.3[a]	44.2 ± 1.7[a]	20.4 ± 2.0	17.6 ± 0.8	6.3 ± 0.8	5.0 ± 0.7	85.4 ± 5.8	84.9 ± 5.7
G22	62.5 ± 6.3[a]	35.4 ± 1.9[a]	13.4 ± 1.6	12.7 ± 2.0	4.6 ± 0.4	4.6 ± 0.4	81.7 ± 7.2	86.0 ± 5.4
G23	123.8 ± 9.5[a]	33.0 ± 1.9[a]	29.9 ± 2.2[a]	18.2 ± 0.8[a]	18.5 ± 2.1[a]	11.0 ± 1.0[a]	107.7 ± 4.2[a]	80.3 ± 3.2[a]
G24	82.2 ± 12.0[a]	39.2 ± 3.2[a]	19.7 ± 3.5	16.6 ± 2.5	8.2 ± 0.9	7.3 ± 1.1	113.4 ± 9.9	87.4 ± 10.6

[a]Statistically significant difference (Welch's *t*-test, $p < 0.05$) per trait, between plants undergoing different treatments for each genotype.

among 115 harvested control plants, to a mere 12 in their stressed counterparts, during 3 weeks of applied drought.

In between the investigated *M. sinensis* genotypes, FW stability under stress, ranged from 19.1% in G9 to 82.3% in G20 (Table 2). Our findings showed a strong negative correlation ($r = -0.88$) between FW stability under stress and plant fresh yield in favorable conditions (bigger plants being more susceptible to drought). This held true concerning dry yield stability as well with a correlation of −0.82, where again G9 and G20 were at opposing extremities. The number of stems was generally lower in plants experiencing drought, although not in all studied genotypes; for instance, G20, G7, and G12 had 28, 23, and 20% more tillers under stress in comparison to their controls, respectively. On the other hand, G15 and G9 were the most affected with a stress-induced decrease of 54% in their stem count. Expectedly, those two genotypes had the highest number of stems in favorable conditions. Differing from the three aforementioned growth parameters, stem length stability under stress did not show a strong negative correlation with higher growth in controls ($r = -0.36$).

3.2 | Formulating contrasting clusters of sensitivity

The plasticity indices of the four growth parameters (DW, FW, number of stems, and stem length) were calculated for every studied genotype (genotypic data for these traits are presented in Table 2). The indices were then tallied per genotype, to set up an index of sensitivity. As expected G20 and G9 were on the opposing ends of the cumulative index of plasticity (Figure 1), ranging from −0.26 for G20 (performing better under stress for those traits than in control conditions) to 2.03 for G9. Contrast in stress impact and response within the studied panel of 23 *M. sinensis* genotypes, was improved by formulating 3 groups of divergent sensitivity via k-means clustering (mildly, moderately, and severely

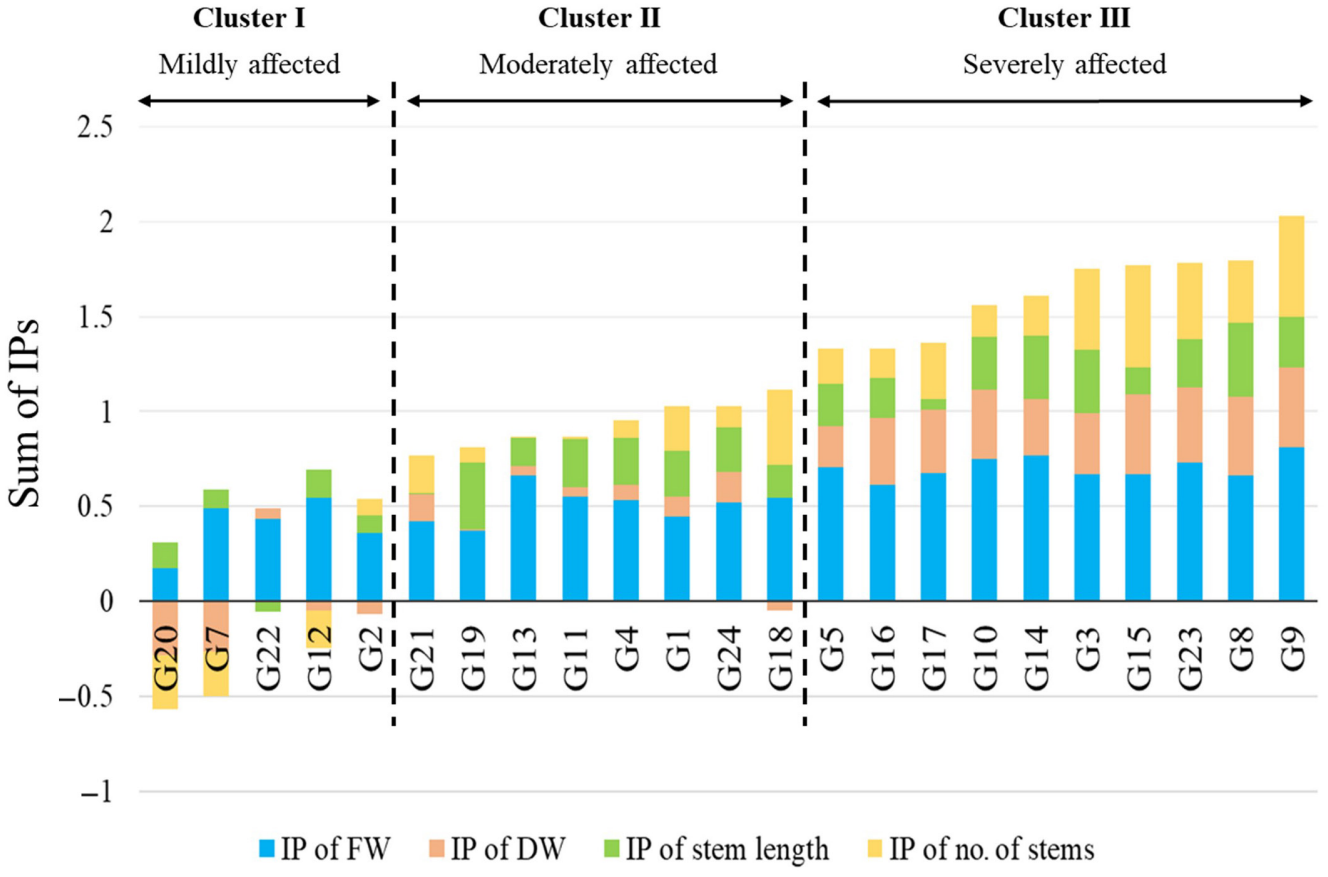

FIGURE 1 The sum of indices of plasticity (IP) of four growth parameters: fresh yield per plant (FW), dry yield per plant (DW), stem length, and stem count used to rank the studied genotypes according to their sensitivity to applied drought. A k-means cluster analyses grouped the studied genotypes into mildly affected (cluster I), moderately affected (cluster II), and severely affected genotypes (cluster III)

affected; labelled as clusters I, II, and III, respectively). Genotypes of cluster I were the smallest in size under favorable conditions (Table S4), contrary to those of cluster III.

3.3 | Degradation of photosynthetic pigments

On average, leaves of stressed plants had 53.4, 41.2, and 36.7% less chlorophyll a (Chl a), chlorophyll a (Chl b), and total carotenoids (Caro), respectively, than their control counterparts (Table 3). However, this drought-imparted drop in photosynthetic pigments was differential among the investigated genotypes, ranging from less than 30% in G22, G24, G2, and G20 to over 66% in G5, G13, G14, and G9. The decrease in pigment concentrations was mirrored for the three measured compounds across all three formulated clusters, although the severity increased in parallel to the presumed vulnerability. On this subject, the photochemical efficiency of photosystem II (Fv/Fm) was monitored as well, indicating a stress-induced decrease (Table 1).

3.4 | Osmotic adjustment

Although no ions were supplemented as part of the applied drought treatments, ionic homeostasis notably that of potassium is important, due to its role in stomatal closure/opening. To this end the contents of various leaf ions were quantified. The applied stress did not cause a significant change in the averaged detected ions (Table 4). Nevertheless, the compositional ratio was somewhat altered as potassium reported an increase contrary to phosphate, but it did not discriminate the clusters. The differences in total measured ions in between the investigated genotypes were not significant either, nor show a correlation with the established sensitivity index (data not shown). However, the increase in potassium contents and its share of the quantified ionic total was slightly stronger in genotypes of cluster III (the stress-caused increase was statistically significant for all three clusters).

Accumulation of neutral water-soluble molecules or osmolytes is a well-known plant response mechanism to maintain cell turgor pressure under abiotic stress. Hence, it was of interest to quantify the leaf contents of some notable osmolytes, such as free amino acids and simple soluble

TABLE 3 Concentration of photosynthetic pigments in leaves studied *Miscanthus sinensis* genotypes after 3 weeks of water deprivation. The genotypic means (average of five replicas per treatment) are presented followed by ±their standard error (SE). The reported means of the three sensitivity groups are averaged from $n = 5$, 8, and 10 genotypes for clusters I, II, and III (Figure 1), respectively

	Chl a (mg. g^{-1} DW)		Chl b (mg. g^{-1} DW)		Caro (mg. g^{-1} DW)	
Genotype	Control	Drought	Control	Drought	Control	Drought
G1	10.3 ± 0.8	4.1 ± 0.8	3.1 ± 0.2	1.3 ± 0.2	1.9 ± 0.2	1.2 ± 0.2
G2	14.4 ± 1.5	9.8 ± 1.1	3.7 ± 0.3	3.6 ± 0.5	2.7 ± 0.3	2.1 ± 0.3
G3	13.2 ± 1.5	6.6 ± 1.3	3.2 ± 0.4	2.5 ± 0.3	2.5 ± 0.2	2.0 ± 0.7
G4	14.9 ± 0.6	6.2 ± 1.8	3.6 ± 0.1	1.6 ± 0.5	1.9 ± 0.1	1.7 ± 0.6
G5	14.6 ± 0.7	3.1 ± 0.9	3.6 ± 0.2	1.2 ± 0.3	1.8 ± 0.2	0.6 ± 0.2
G7	10.2 ± 0.8	4.1 ± 0.3	2.6 ± 0.2	1.6 ± 0.2	1.8 ± 0.1	1.2 ± 0.1
G8	9.5 ± 1.1	5.9 ± 0.8	2.8 ± 0.4	1.8 ± 0.1	2.7 ± 0.2	1.6 ± 0.2
G9	11.4 ± 1.8	3.3 ± 0.7	3.0 ± 0.7	1.4 ± 0.2	3.1 ± 0.4	0.7 ± 0.2
G10	12.1 ± 0.6	4.4 ± 1.0	3.2 ± 0.4	1.7 ± 0.5	1.1 ± 0.1	1.4 ± 0.3
G11	11.3 ± 0.6	9.0 ± 3.7	2.8 ± 0.3	2.3 ± 0.7	1.2 ± 0.1	2.4 ± 0.7
G12	12.2 ± 1.0	4.4 ± 0.6	3.1 ± 0.3	1.4 ± 0.2	2.3 ± 0.2	1.2 ± 0.1
G13	14.1 ± 1.5	4.2 ± 1.1	3.8 ± 0.4	1.2 ± 0.3	2.6 ± 0.3	1.1 ± 0.3
G14	13.2 ± 1.6	3.6 ± 0.2	3.4 ± 0.5	2.1 ± 0.1	4.5 ± 0.4	0.5 ± 0.1
G15	12.6 ± 0.7	6.4 ± 0.8	3.1 ± 0.2	1.7 ± 0.2	0.4 ± 0.1	1.8 ± 0.2
G16	8.3 ± 1.3	5.1 ± 1.3	2.4 ± 0.3	1.7 ± 0.4	2.6 ± 0.2	1.3 ± 0.2
G17	11.4 ± 1.0	4.5 ± 0.9	3.1 ± 0.3	1.4 ± 0.2	2.9 ± 0.2	1.3 ± 0.4
G18	14.2 ± 1.3	5.1 ± 0.3	3.4 ± 0.3	1.6 ± 0.1	2.6 ± 0.2	1.7 ± 0.3
G19	11.8 ± 1.5	6.8 ± 0.8	3.2 ± 0.4	2.0 ± 0.2	2.1 ± 0.3	1.6 ± 0.2
G20	11.7 ± 1.0	8.3 ± 1.2	3.5 ± 0.3	2.5 ± 0.4	2.3 ± 0.2	1.9 ± 0.2
G21	11.3 ± 0.8	6.4 ± 1.1	3.2 ± 0.2	2.1 ± 0.3	1.2 ± 0.2	1.5 ± 0.3
G22	11.4 ± 1.1	8.5 ± 1.5	3.2 ± 0.5	2.3 ± 0.3	1.0 ± 0.2	2.0 ± 0.2
G23	13.7 ± 2.9	5.7 ± 0.8	4.0 ± 1.0	2.6 ± 0.4	4.5 ± 0.4	1.5 ± 0.3
G24	13.3 ± 1.6	10.6 ± 2.0	3.7 ± 0.4	3.5 ± 0.4	2.8 ± 0.3	2.3 ± 0.5
Cluster I	12.1 ± 0.6	7.0 ± 1.2	3.2 ± 0.2	2.3 ± 0.4	2.2 ± 0.1	1.7 ± 0.2
Cluster II	12.6 ± 0.6	5.9 ± 0.7	3.4 ± 0.1	1.8 ± 0.2	2.4 ± 0.1	1.5 ± 0.1
Cluster III	12.0 ± 0.6	4.8 ± 0.4	3.1 ± 0.2	1.8 ± 0.2	2.2 ± 0.1	1.3 ± 0.2
Overall	12.2 ± 0.4	5.9 ± 0.4	3.2 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	1.5 ± 0.1

sugars. Quantitatively, simple sugars showed an increase of nearly 2.9-fold in the leaves of stressed *M. sinensis* plants, compared to their control counterparts (Figure 2a). This was equivalent to an increment from 1.9 to 5.6 in DM%. The compositional profile of measured sugars was similar under both treatments (control and drought), with an overall dominance of sucrose and a noticeable increase of glucose's share of the detected total, under stress. On a genotype level, changes in the concentrations of measured sugars under stress varied markedly (Table S5). For instance, the increase in glucose and fructose ranged from 1.4- and 1.5-fold, respectively, in G12, to 7.7- and 8.2-fold in G9. Likewise, sucrose reported a surge in drought affected leaves, ranging from a 1.7-fold increase in G19 and G7, to 5.6- and 6.5-fold in G9 and G18, respectively. This indicated a positive correlation between the drought-induced accumulation of soluble sugars and the postulated vulnerability index ($r = +0.35$). Correspondingly, stressed leaves of cluster III genotypes retained the highest concentration of simple soluble sugars (statistically insignificant difference to that accumulated in the stressed leaves of clusters I and II) (Figure 2b).

Free amino acids are multifunctional, notably in their stress-related roles, for example as osmolytes, signal molecules, etc. Contents of 15 different amino acids in the leaves of control and stressed plants were quantified and reported in Figure 3a. Some, like proline (Pro) and serine (Ser) recorded a substantial overall increase under stress, amounting to a 27.7- and 7.2-fold (equivalent to 1.44 and 0.86 in log change), respectively. Conversely, a decrease by 53% was reported for threonine (Thr) and leucine (Leu). Under applied stress, Pro leaf contents increased in all 23 studied genotypes (Figure S2). Among the three sensitivity clusters, Pro content was slightly though not significantly higher in the stressed leaves of cluster III genotypes (Figure 3c).

TABLE 4 Average ionic content and composition in the leaves of control and stressed plants of the 23 investigated *Miscanthus sinensis* genotypes. Means are presented followed by ±their standard error (SE). Reported means of the three sensitivity clusters are averaged from n= 5, 8, and 10 genotypes for clusters I, II, and III (Figure 1), respectively

			% Of total measured ions						
Group	Treatment	Total measured ions (mg. g^{-1} DW)	Chloride (Cl^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})	Sodium (Na^+)	Potassium (K^+)	Magnesium (Mg^{2+})	Calcium (Ca^{2+})
Overall	Control	48.8 ± 1.0	6.3	28.1	8.8	0.8	40.0	6.4	9.6
	Drought	48.2 ± 1.0	6.4	24.4	8.1	0.8	45.3	6.3	8.7
Cluster I	Control	49.2 ± 1.9	7.1	27.8	8.9	0.9	38.9	6.5	10.1
	Drought	46.7 ± 2.1	6.4	24.7	8.1	0.8	43.1	6.8	10.1
Cluster II	Control	47.5 ± 1.9	6.8	28.2	9.0	0.8	40.2	5.9	9.1
	Drought	46.1 ± 1.6	6.6	24.3	8.2	0.7	45.9	5.9	8.3
Cluster III	Control	49.4 ± 1.5	5.2	28.2	8.7	0.8	40.7	6.8	9.7
	Drought	50.5 ± 1.3	6.0	24.0	8.1	0.8	46.5	6.3	8.4

3.5 | Post-drought recovery

Regular irrigation was restored to water-deprived plants after 3 weeks of applied stress. A subgroup of genotypes from clusters I and III (mildly and severely affected, respectively) were selected for further in-depth analyses. Those were G2, G7, and G20 from cluster I and G9, G14, and G15 from cluster III.

Study of plant biomass harvested after 3 weeks of applied drought showed that the controls of cluster III genotypes (three aforesaid genotypes only) had 2.4-folds higher FW, than those of the aforesaid cluster I genotypes. Their water content, however, was similar, accounting to about 75% of fresh yield (Figure 4a). This similarity in controls was not seen in stressed plants, as moisture's share in FW dropped to 54 and 40% in the studied cluster I and cluster III genotypes, respectively. This discrepancy was similar in terms of dry matter under stress, whereby a slight increase was reported in the mildly affected subgroup, compared with their controls, contrary to a 37.8% drop in the severely affected ones relative to their respective controls. After the recovery period, both subgroups of recovering plants regained substantial amounts of their water content. Scoring a 1.85- and 1.90-fold increase in FW, compared to their first harvest values, for the mildly and severely affected subsets, respectively (Figure 4b). Weight gain garnered by stressed plants during recovery, however, was mostly in moisture with minimal increase in DW matter. For instance, in the post-drought recovering plants, only 17.9 and 11.7% of the gained growth was in dry matter, for the mildly and severely affected subgroups, respectively (Figure 4c).

3.6 | Leaf carbon allocation under drought and its subsequent recovery

Carbon partitioning is a deciding factor in stress response and the subsequent recovery once optimal water supply is restored. To better understand this, expressional analyses of the starch turnover pathways were performed in leaf tissue from both harvests. For each sensitivity subgroup (mildly and severely affected), equal amounts of total RNA from one plant per genotype (listed in section 3.5), were pooled prior to cDNA synthesis. This was done in duplicates for every treatment per harvest (control and drought, and then control and recovery). Expression in stressed leaves for every gene was presented as a log-fold difference relative to their respective controls per time-point in Figure 5a. For ease of jargon, M3 and S3 represented the stressed leaves of the mildly and severely affected subsets, respectively, after 3 weeks of water stress. Similarly, M6 and S6 represented the recovering leaves of

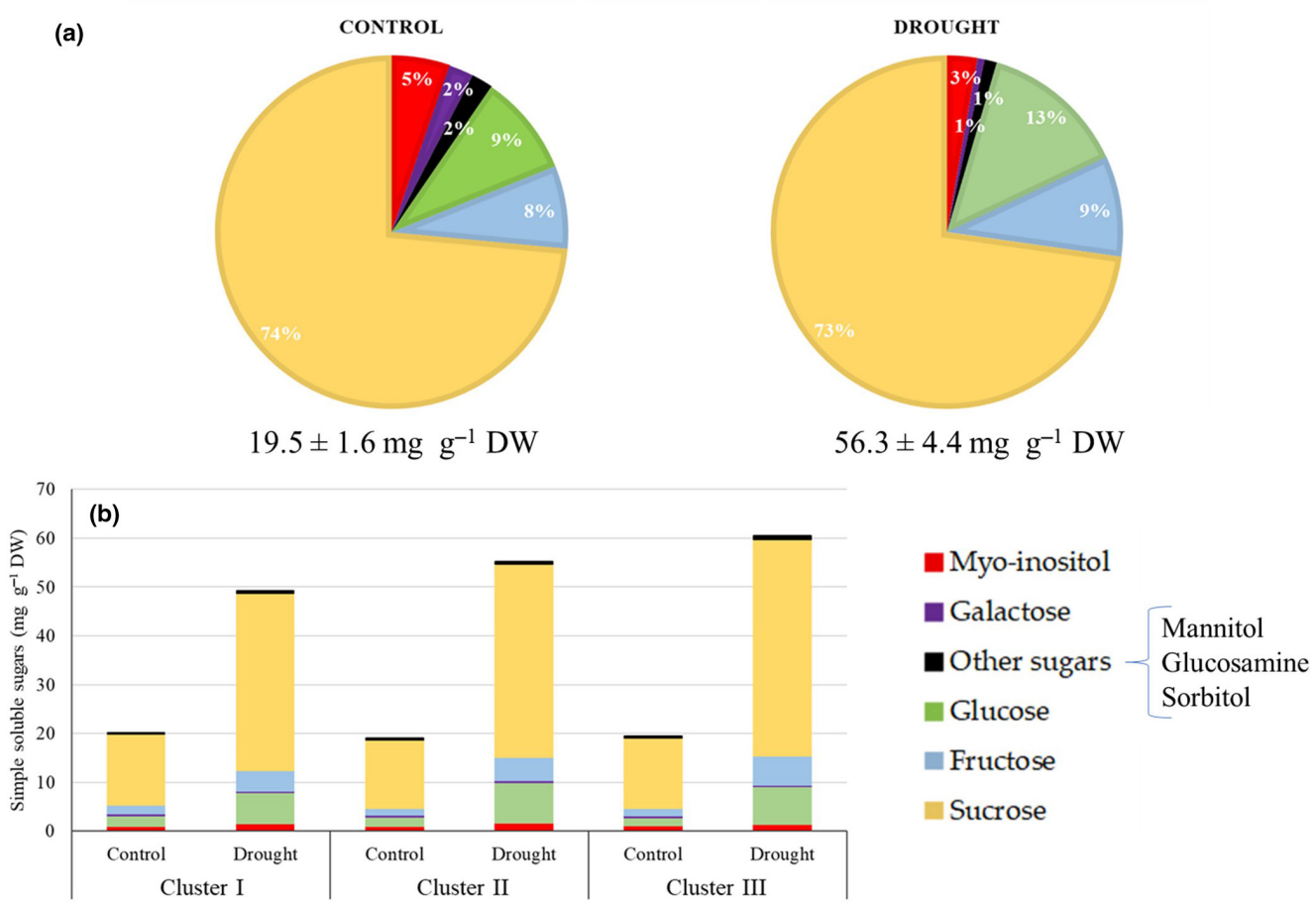

FIGURE 2 Averaged composition and concentration of measured simple sugars in the leaves of (a) control and stressed plants of all studied *Miscanthus sinensis* genotypes, and (b) in the three sensitivity clusters

those subgroups, following the subsequent 3 weeks of recovery. In parallel, simple soluble sugars and starch contents were quantified in the leaves of those genotypes after both harvests.

Several key genes in the starch degradation pathway, namely BAM3, AMY3, and ISA3, were overexpressed in S3 leaves (Figure 5a). In M3, however, the latter two were downregulated, while BAM3 reported a milder overexpression. Moreover, a spike in BAM1 expression after 3 weeks of stress (both M3 and S3) was observed. On the biochemical level, M3 and S3 both accumulated higher contents of simple soluble sugars (Figure 5c) than their controls, with an overwhelming sucrose dominance. This was likely due to the reported increased expression of sucrose synthesizing genes Susy and SPS. Meanwhile, the higher quantified concentrations of glucose and fructose under stress paralleled the overexpression of cytoplasmic invertases CINV1 and CINV2 for both S3 and M3.

Leaf starch contents of both mildly and severely affected subgroups, increased after 3 weeks of applied stress (Figure 5b), from 0.6 and 0.8% of DM in control plants, respectively, to 1.1 and 1.3% in their stressed equivalents. This was coupled with a higher gene expression in both S3 and M3, of key genes in the starch biosynthesis pathway, such as GBSS, SBE, SS1, and SS3 (Figure 5a). In addition, an explorative study of leaf cell wall composition using one genotype per sensitivity cluster was performed to understand the implications of this buildup in soluble sugars under stress for other carbon-demanding pathways. Cell wall content (NDF%) was lower in stressed leaves by 6 to 8% DM compared with their controls, in the studied mildly (G20) and severely affected genotypes (G9), respectively. The compositional makeup of the cell wall revealed that the difference was mainly in structural carbohydrates (cellulose and hemicellulose) in DM%, although cellulose's share of the cell wall increased under stress for G9 and G11. Moreover, leaf lignin levels remained unchanged after 3 weeks of drought (Table 5).

After 3 weeks of post-drought recovery the expression of genes encoding major starch degrading enzymes, such as AMY3 and BAM3, was still elevated in S6, though not as high as in S3. In parallel, higher contents

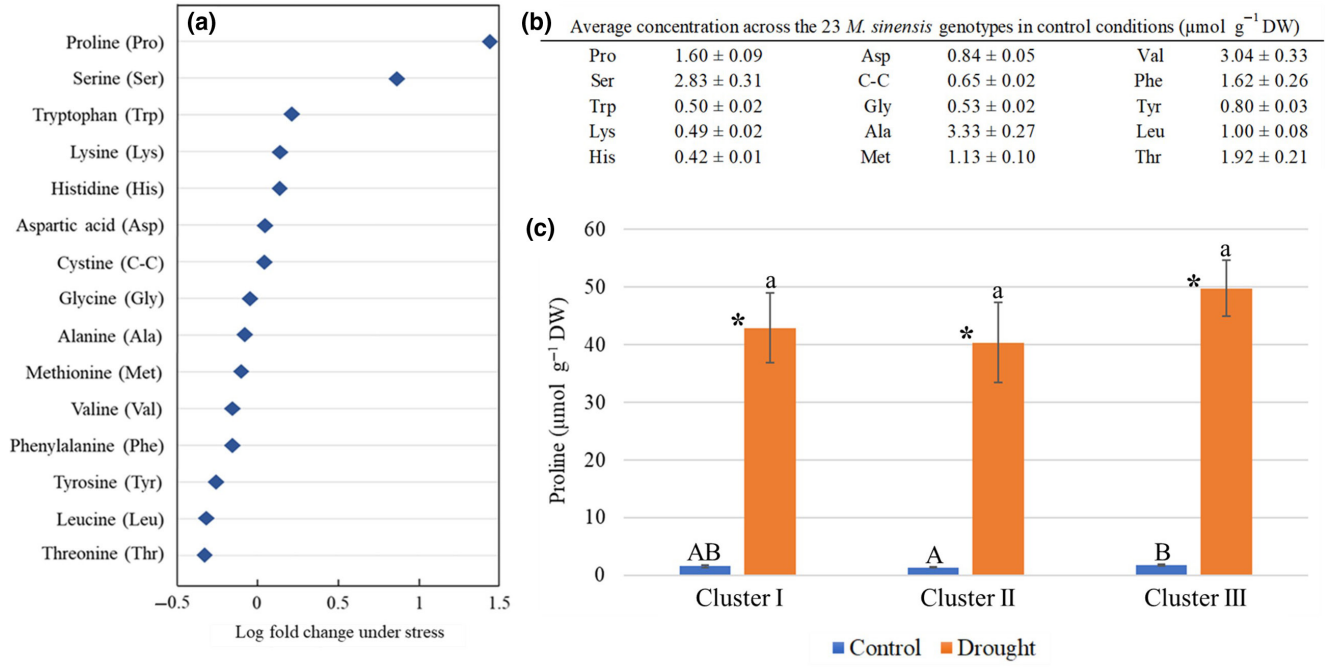

FIGURE 3 Averaged free amino acids concentrations in the leaves of 23 *Miscanthus sinensis* genotypes after 3 weeks of drought. (a) log-fold change under stress of 15 detected free amino acids and (b) the mean concentration of each of the measured amino acids under control conditions expressed in µmol. g^{-1} DW followed with ±its standard error. (c) The impact of drought on proline content across the three sensitivity clusters ($n = 5$, 8, and 10 genotypes for clusters I, II, and III, respectively). Error bars represent the standard error of the average proline content. An asterisk (*) is added whenever there is significant difference between the two treatments of the same cluster, according to Student *t*-test at $p < 0.05$. Different letters (uppercase for controls and lowercase for stressed plants) indicate significant differences between the different clusters undergoing the same treatment, according to LSD test ($\alpha = 0.05$)

of simple soluble sugars were still found in the leaves of recovering plants relative to their controls, but lower than their own 3 weeks earlier (leaves of stressed plants) at the end of drought treatments (Figure 5c). The reported higher levels of sucrose, glucose, and fructose in S6 (compared to controls) was mirrored with a continual overexpression of SuSy, SPS, CINV1, and CINV2 genes (bottom of Figure 5a). This, however, was not paralleled neither in gene expression or in quantified sugars for M6. Moreover, starch content increased in the leaves of control and stressed plants of both subgroups after recovery (compared to their counterparts, 3 weeks earlier), and was higher in recovery leaves than their controls (Figure 5b). Correspondingly, most of the studied genes in the starch synthesis pathway were still overexpressed after recovery in both M6 and S6.

4 | DISCUSSION

Second generation biofuel crops are promising candidates for sustainable cultivation on marginal lands (Pancaldi & Trindade, 2020), especially C4 perennials such as miscanthus and switchgrass. Nonetheless, several studies on both crops reported a substantial decrease in yield under drought (Barney et al., 2009; Berdahl et al., 2005; Weng et al., 2022). Enhancing tolerance, however, ensures yield stability; a prerequisite for economically viable exploitation of drought-affected lands for bioenergy production (Ings et al., 2013).

The genetic diversity offered by *M. sinensis* provides ample genetic resources for tolerance improvement in this crop (Clifton-Brown et al., 2008). We explored this in a controlled glasshouse experiment, via an in-depth comparative analysis of the underlying response mechanisms to drought and subsequent recovery using young plantlets of 23 *M. sinensis* genotypes. Drought's impact on growth and yield was assessed, showing a strong association of stress development with the size of studied plants in favorable conditions. Stressed leaves experienced a degradation of its chlorophyll contents, alongside an accumulation of osmolytes such as proline and simple soluble sugars; both findings could be considered as good biomarkers of drought in miscanthus. Interestingly, this was correlated with a decrease in cell wall synthesis, highlighting a change in carbon allocation under stress. Finally, early post-drought recovery showed the importance of better drought avoidance in maintenance of cell functions, and consequent more efficient regaining of normal homeostasis once stress subsides.

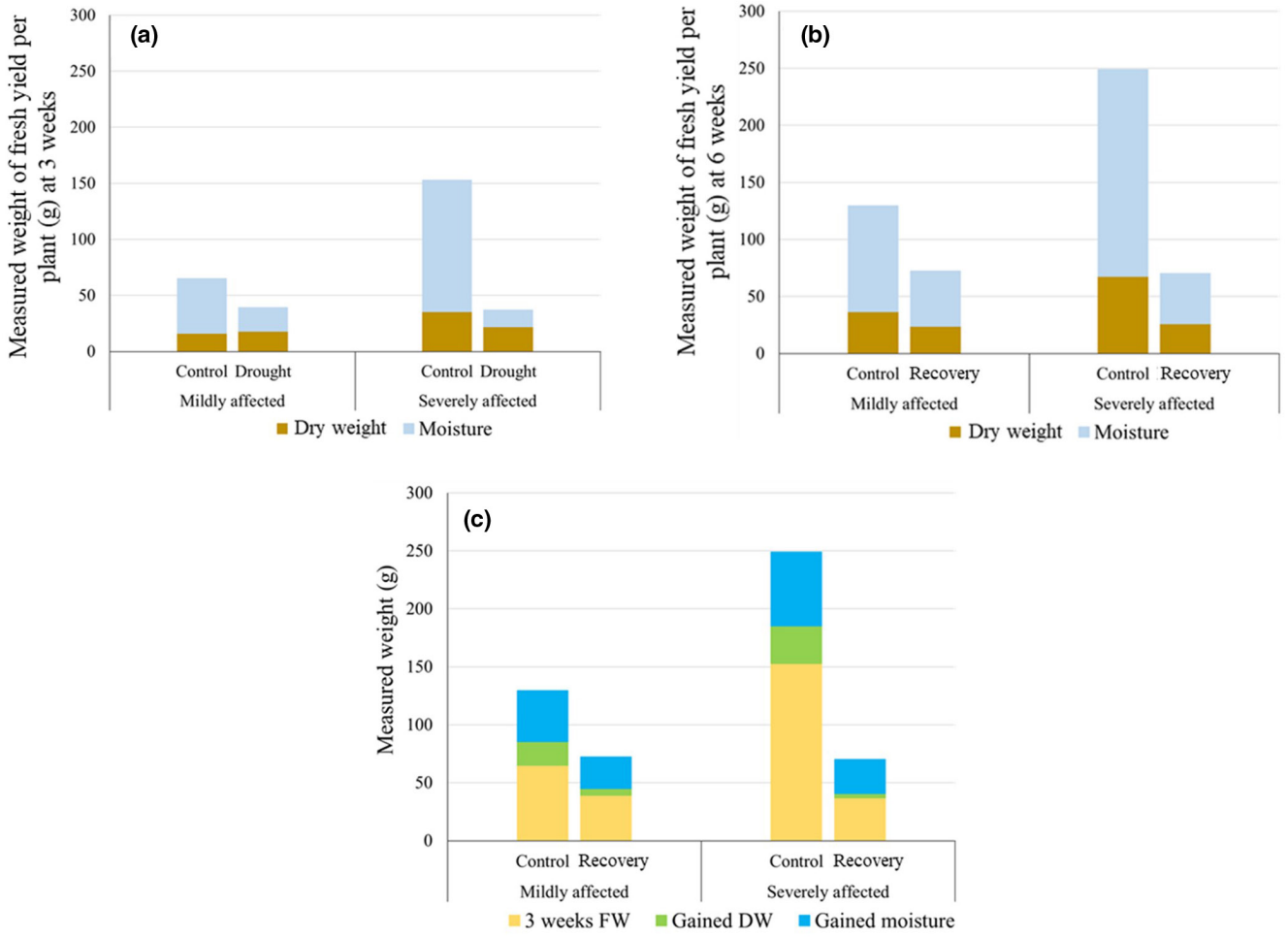

FIGURE 4 Compositional makeup of harvested feedstock of a subgroup of mildly (cluster I) and severely affected (cluster III) genotypes after 3 weeks of applied water stress and following the subsequent 3 weeks of recovery ($n = 3$ genotypes for each). (a) the weight of moisture and dry matter is presented for both subgroup's superterranean plant material after 3 weeks of stress, (b) the following 3 weeks of recovery, and (c) estimated gained masses in terms dry weight and moisture during 3 weeks of recovery

4.1 | Growth vigor in optimal conditions is offset by higher sensitivity to drought

Variability in yield and tolerance potentials offered by wide-ranging genetic diversity is crucial to get meaningful contrasts in stress response comparative studies. Notably when considering the trade-off between a plant's ability to grow under favorable conditions and its capacity to perform in suboptimal ones (Bazzaz, 1996). This was evident in this work with a noticeable variability in growth vigor among the studied genotypes (Tables 1 and 2). For instance, FW per plant among controls ranged between 28.8 and 245.5 g per plant. This was mirrored across all monitored growth parameters.

Water availability is critical for the survival, reproduction, and yield in all crops (Ordoñez et al., 2009), and *M. sinensis* is no exception. Vegetative growth was curtailed in most *M. sinensis* genotypes undergoing applied drought both in our experiment and that of Van Der Weijde et al. (2017b). In fact, the eight analyzed genotypes commonly used in both studies had a similar order of sensitivity to drought. As an average across all involved genotypes, 3 weeks of water deprivation caused a 60.8, 19.3, and 22.3% reduction compared to the control values for FW, DW, and number of stems, respectively (Table 1). Genotypes with smaller plants, however, recorded only a modest reduction in growth under stress (Table 2), with some even reporting an increase in DW compared with their controls. This dissimilarity in stress impact can be credited to reduced water use/loss in smaller plants especially ones with smaller leaf surface area (Blum, 2005); warranting a prolonged availability of soil moisture, and as a result a more moderate water deficit over the same period of treatments (average soil moisture content after 3 weeks of applied stress was 18.3 ± 1.4, 7.1 ± 1.0, and 1.9 ± 0.4%, in the pots of clusters I, II, and III plants, respectively). Correspondingly, leaf rolling, a known water stress avoidance response (Kadioglu & Terzi, 2007;

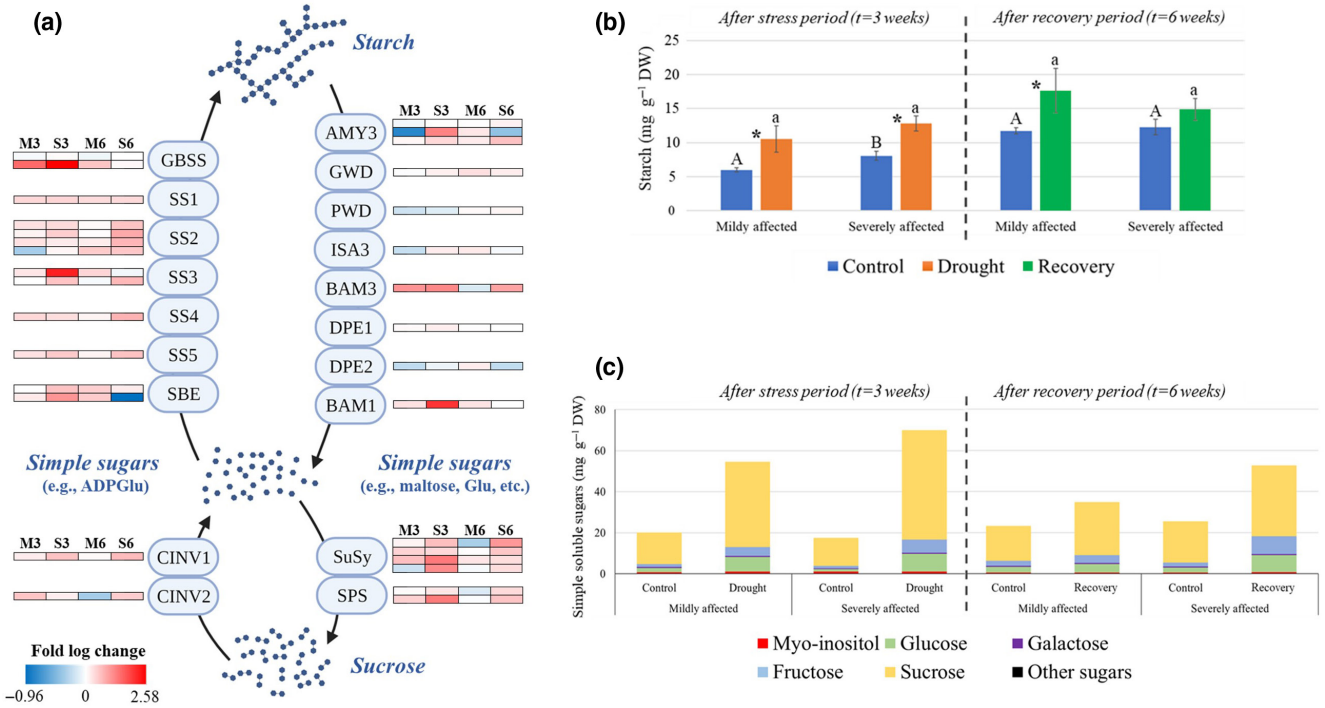

FIGURE 5 Expressional analyses of carbon economy pathways in the leaves of *Miscanthus sinensis* experiencing applied drought for 3 weeks, followed by an equivalent period of recovery. (a) log-fold change of genes involved in starch and sucrose turnover pathways, in the leaves of treated plants relative to their respective controls, in a number of mildly and severely affected genotypes after 3 weeks of applied drought (M3 and S3, respectively), and 3 weeks of subsequent recovery (M6 and S6, respectively). Genes with several entries highlight data of paralogs, using the same order displayed in Table S2. (b) Average starch leaf content of the aforementioned subgroups of genotypes after stress and the following recovery period. Error bars on top of each column represent the standard error of each mean. An asterisk (*) is added whenever there is a significant difference between the two treatments for the same measured group per time point, according to Student *t*-test at $p < 0.05$. Different letters (uppercase for controls and lowercase for stressed plants) indicate significant differences between the different groups undergoing the same treatment within a time point, according to LSD test ($\alpha = 0.05$). (C) averaged composition and concentration of measured simple sugars in the leaves of control and treated plants in the aforesaid two sensitivity subgroups after applied stress and recovery periods

TABLE 5 Cell wall quantification and composition in the leaves of three *Miscanthus sinensis* genotypes (one from each sensitivity cluster) after 3 weeks of applied drought. The average cell wall percentage (NDF) is presented in DM%. Genotypic means (average of clonal replicates per treatment run in technical duplicates) are reported followed by ± their standard error

				% of NDF		
Group	**Genotype**	**Treatment**	**NDF in DM%**	**Hemicellulose**	**Cellulose**	**Lignin**
Cluster I	G20	Control	73.9 ± 0.5[a]	46.5 ± 1.8	49.1 ± 1.6	4.4 ± 0.8
		Drought	68.3 ± 0.7[a]	46.0 ± 0.6	49.3 ± 0.7	4.8 ± 0.3
Cluster II	G11	Control	74.2 ± 0.9[a]	48.7 ± 0.4[a]	47.4 ± 1.0	3.9 ± 0.2
		Drought	66.8 ± 1.0[a]	46.6 ± 0.5[a]	49.6 ± 0.8	3.8 ± 0.6
Cluster III	G9	Control	73.6 ± 0.4[a]	49.7 ± 0.8[a]	46.3 ± 0.2[a]	3.9 ± 0.1
		Drought	65.2 ± 0.3[a]	47.5 ± 0.4[a]	49.1 ± 0.4[a]	3.5 ± 0.3

[a]Statistically significant difference (Student *t*-test, $p < 0.05$, adjusted with Bonferroni correction) between treatments of one genotype per reported trait.

Touchette et al., 2009), was noticed in the most productive genotypes (G9, G14, and G23), only 10 days after stress initiation. Meanwhile, stressed leaves of those with smaller plants (G20 and G22) did not register such a response, not even after 3 weeks of applied drought (average leaf moisture content after 3 weeks of drought was 50.2 ± 5.3, 47.3 ± 2.3, and 38.1 ± 3.8%, for stressed plants of clusters I, II, and III, respectively, compared with ≈73% in the leaves of control plants—similar for all three clusters). It can, thus, be assumed that the noticeable divergent response to

stress within the studied set of genotypes is due to relative vulnerability governed by leaf surface area, whereby stress is occurring in a temporal gradient among simultaneously stressed plants.

Reproductive success was also adversely affected by applied drought. A reduction in flowering occurrences was observed in stressed plants (by about 50%), especially among the severely affected genotypes. This finding likely is a result of flowering delay or perhaps premature shoot death. An assumption supported by similarly reported delays of heading in MxG (Cosentino et al., 2007), and several *M. sinensis* and *M. sacchariflorus* accessions (Jensen et al., 2011), experiencing periods of water shortage.

4.2 | Drought-induced chlorophyll degradation: an indicator of oxidative stress

Among the many injurious effects of drought, impairing the photosynthetic capacity is one of the earliest detected symptoms in stressed plants alongside reduced stem growth (Ings et al., 2013). The latter is a result of diminishing turgor pressure during water deficiency, which restricts further cellular division and expansion (Farooq et al., 2009). This was evident in the stress-caused decrease of stem length, and the gradual drop in its elongation rate (Figure S3), in parallel to increasing sensitivity and duration of applied drought.

Photosynthesis and vegetative growth are locked in a positive feedback loop, explaining their mutual disruption under stress. Reduced turgor pressure induces stomatal closure in stressed plants, to lessen water loss via transpiration (Chaves et al., 2009), promoting the accumulation of reactive oxygen species (ROS) (Mittler, 2002). The deleterious effects of ROS buildup include among others, lipid peroxidation and the resulting chlorophyll degradation (Foyer et al., 1994; Sharma et al., 2012). Correspondingly, all the studied genotypes reported a decrease in leaf photosynthetic pigments (chlorophylls a and b, and carotenoids) under stress. These findings are in agreement with earlier studies on several water-stressed miscanthus species and hybrids, suffering from an oxidative stress-induced decrease in chlorophyll content and fluorescence (Ings et al., 2013; Stavridou et al., 2019). The strongest recorded decrement in chlorophyll content was among genotypes of the severely affected cluster III (Table 3), whereas the opposite was reported for the mildly affected one (cluster I). This highlights a strong correlation between stress vulnerability and the degree of chlorophyll degradation, confirming the latter's status as a good indicator of oxidative stress in miscanthus.

4.3 | Vacuolar osmotic adjustment

Drought induces a loss of cell turgor, curtailing the ability of stressed plants to take up water and nutrients (Ashraf et al., 2011). A common response mechanism is the re-adjustment of the osmotic potential to re-enable water absorption (Blum, 2005). This is achieved either via biosynthesizing and accumulating organic solutes (osmolytes), or by uptaking and compartmentalizing inorganic ions (Turner, 2018). Both are fueled by redirected resources from vegetative growth to upregulate stress defense mechanisms, indirectly exacerbating yield reduction under stress (Munns & Tester, 2008).

Leaf ionic contents did not change significantly under stress (Table 4). An increase in potassium (K^+) was noticeable however, being slightly higher in the stressed leaves of cluster III genotypes. This increment in leaf K^+ concentration, could be accredited to its restricted diffusion toward other tissues (i.e., rhizomes and roots) during stress (Wang et al., 2013). Potassium is renowned for its role in guard cell regulation and thereby stomatal closure, crucial for reducing water loss (Marschner, 2012). Other K^+ drought-mitigating functions include vacuolar osmotic adjustment, increasing aquaporin activity, and ROS detoxification (Wang et al., 2013). However, potassium can hardly be considered as an indicator of vulnerability or a key contributor to drought tolerance in our study, given its minor stress-induced increment and the lack of its correlation with perceived sensitivity.

Osmolytes are very diverse, including among others, simple sugars, sugar alcohols, some amino acids, and quaternary ammonium compounds (Gil et al., 2013). Likewise, their functions are manifold, including but not limited to osmotic adjustment, counteracting photoinhibition, ROS scavenging (Hare et al., 1998). Overall, drought induced a 2.88-fold increment of leaf simple soluble sugars (sugar alcohols were included within, but they made up a small fraction of the detected total). This buildup was observed in all 23 studied genotypes (Table S5), likely to alleviate the presumed drought-induced imbalance in vacuolar osmotic potential (Gil et al., 2013; Sanchez et al., 1998). Similarly, starch contents were higher in leaves of stressed plants than in their control, in both mildly and severely affected genotypes (Figure 5). This increase of leaf transitory starch under stress could be attributed to plant's spatial readiness to counteract drought by ensuring local energy reserves in sinks (Muller et al., 2011; Thalmann & Santelia, 2017). Accumulation of simple sugars was seemingly governed by the degree of perceived stress, as the severely affected genotypes (cluster III) showed the highest averaged concentration in its leaves under drought.

On the other hand, the compositional ratio of detected simple sugars was similar for both treatments (control and

drought) across all genotypes, although under stress a noticeable increase in glucose and fructose share was evident (Figure 2). This could be attributed to the overexpression of invertases, cleaving sucrose into glucose and fructose, to feed the hexose phosphate pools and depending downstream processes (Fàbregas & Fernie, 2019). Those include free energy-producing pathways (e.g., pentose phosphate pathway and by extension, glycolysis), glutamate-mediated proline biosynthesis, and serine synthesis (Fàbregas & Fernie, 2019; Zanella et al., 2016). Sucrose on the other hand, being the main form of energy transport in between plant cells and tissues (Zimmermann & Ziegler, 1975), was expectedly the overwhelming form of measured simple sugars (≃75%). Especially since the activation of stress response mechanisms in affected tissues (leaves being the most vulnerable to desiccation) warrants a higher energy demand (Hare et al., 1998). This, however, comes at a cost, as the stress-induced accumulation of soluble sugars is enabled by redirecting resources from other carbon-demanding pathways (such as cell wall synthesis). This assumption was confirmed by our findings (Table 5), after probing leaf cell wall contents and composition in the different postulated sensitivity clusters. In stressed leaves, a decrease in the cell wall content (NDF) was recorded, almost reverse-complementary to the reported increment of leaf sugar levels (simple and starch), in each of the three clusters. These stress-induced changes, along with the overexpression of starch and sucrose metabolism pathways (both breakdown and synthesis), were all more pronounced in the severely affected genotypes (Figure 5a). Thus, highlighting the drought intensity-dependent adjustment of carbon allocation in miscanthus, specifically in terms of energy siphoned away from cell wall synthesis and maintenance to vacuolar sugars accumulation.

4.4 | Free amino acids: proline, a reliable early stress biomarker

Amino acids are central for plant metabolism, serving as building blocks of proteins, important regulatory and signal molecules, and precursors of nucleic acids (Galili et al., 2016). Some amino acids contribute as well to abiotic stress response and tolerance mechanisms (Rai, 2002), notably proline (Pro), whose buildup under stress is renowned and well documented (Szabados & Savouré, 2010). Proline accumulation, however, does not necessarily confer drought tolerance in many species -including miscanthus- (Ings et al., 2013), rather it is considered a good biomarker of stress. In agreement, Pro accumulated in the stressed leaves of all 23 investigated genotypes (Figure S2), regardless of the presumed degree of vulnerability. This confirmed Pro as a reliable early water stress biomarker in miscanthus even in mildly affected genotypes. Nonetheless, Pro peaking at 0.81% DM -in the stressed leaves of genotype G9-, likely contributes to the readjustment of the cytosolic osmotic balance, and as an osmo-protectant through its ROS scavenging capacity (Bartels & Sunkar, 2005). The necessary carbon skeletons for this buildup under stress are procured from the degradation of the previously discussed leaf transitory starch, mediated notably by the activity of BAM1 in mesophilic cells (Zanella et al., 2016). Correspondingly, the latter's encoding gene was sharply overexpressed under stress in our study (Figure 5a).

Other quantified free amino acids revealed substantial stress-induced changes in their contents. Among those reporting a large increment, serine (Ser) was second only to Pro in its increase (Figure 3a), contrary to the reported downregulation of Ser metabolism in miscanthus leaves under drought, by De Vega et al. (2021) transcriptomics analyses. This could be a form of a negative feedback control to reverse its stress-induced accumulation to almost toxic levels. Nonetheless, Ser catabolism and accumulation have both been implicated with increased tolerance in various species (Kishor et al., 2020), a discrepancy attributed to its complex functionality and homeostasis. Tryptophan (Trp) and lysine (Lys) levels also increased during stress. The former is a known precursor of auxin and the stress-alleviating melatonin (Zhang et al., 2015), in addition to acting as an osmolyte and ion transport regulator in its free form (Rai, 2002). Lysine on the other hand, is converted under stress to glutamate, the main precursor of proline, thus contributing to the latter's synthesis and accumulation (Galili et al., 2001). Conversely, threonine (Thr) and leucine (Leu) contents decreased, possibly due to their conversion to branched-chain amino acids during stress (Fàbregas & Fernie, 2019; Joshi et al., 2010).

4.5 | Early post-drought recovery: restoring moisture and unplugging response mechanisms

Post-drought recovery in biofuel crops like miscanthus remains virtually undocumented irrespective of its importance for exploiting marginal lands, particularly those in arid and semi-arid areas. We attempted to bridge this scientific gap, by reinstating regular irrigation to water-stressed plantlets of *M. sinensis* and analyzing some indicators of its recovery (water content, osmotic adjustment, and yield). Compositional analyses of the harvested feedstock showed that recovery commenced by restoring moisture content to its pre-drought status. This phenomenon was universal among the studied genotypes regardless of their sensitivity to stress, albeit being

slightly stronger in the severely affected ones (Figure 4b,c). A similar discrepancy in leaf simple soluble sugars contents was noticeable after recovery (Figure 5c), where the diminution of accumulated osmolytes is a sign of restored water potential, and subsequent downregulation of stress response mechanisms (Abid et al., 2018; Dien et al., 2019). For instance, the mildly affected cluster reported 50% less simple soluble sugars in its recovering leaves than its post-drought contents, versus a mere 20% reduction recorded in their severely affected equals. Likewise, a stronger reduction in the expression of the starch degradation pathway was noticeable in the mildly affected (Figure 5a), accompanied with a higher accumulation of starch (Figure 5b). This confirms a faster and more efficient recovery among mildly affected genotypes (Chai et al., 2010), enabled by stress avoidance, resulting in better maintenance of cellular functions during applied stress (Abid et al., 2018).

Moisture content in the recovering plants at the second harvest was slightly lower than that of their control counterparts (by about 5% in the mildly affected subgroup and 10% in the severely stressed one). This highlights the incomplete restoration of the pre-stress hydraulic potential within the short timeframe of our experiment, preventing us from documenting a possible post-drought overcompensation in vegetative growth. A longer period of recovery could confirm this postulation, though it is common for plants to fall short of complete or immediate recovery after severe droughts (Yin & Bauerle, 2017). Our findings underline the path of stressed plants to recovery and renormalization of their metabolism, by regaining turgor and downregulating their now redundant energy-draining stress response mechanisms.

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

Drought avoidance via minimized water loss was found pivotal for delaying stress-induced injuries in *M. sinensis*. However, this was achieved with a substantial yield penalty especially in the more vigorous genotypes, given the trade-off between growth vigor in favorable conditions and performance under stress. In addition, the comparative analyses employed in this study reaffirmed chlorophyll degradation and proline accumulation as good early stress biomarkers. It also drew attention to the modifications in carbon allocation under drought, where leaves acted as strong carbon sinks with a higher accumulation of soluble sugars and amino acids, as stress intensifies. The latter came at the expense of downregulating cell wall biosynthesis, namely its structural polysaccharides. Moreover, regaining moisture content and unplugging energy-draining stress response mechanisms were trademarks of early post-drought recovery.

An economically viable exploitation of marginal lands for bioenergy production calls for a shift toward achieving stress-endurance in biofuel crops. This, however, calls for developing drought tolerant varieties of miscanthus and other biofuel crops, warranting further similar in-depth research of stress effects and its responses. In parallel, further studies of recovery are necessary to investigate possible drought legacy effects on yield and feedstock quality.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, M.A.H. and L.M.T.; experimental analyses, M.A.H. and D.D.; data curation, M.A.H., D.D., and O.D.; graphics and visualization, M.A.H.; original draft preparation, MA.H., K.v.d.C., O.D., and L.M.T.; reviewing and editing, M.A.H., K.v.d.C., O.D., and L.M.T.; supervision, L.M.T.; funding acquisition, L.M.T. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in "figshare" at https://doi.org/10.6084/m9.figshare.19397528.v1.

ORCID

Mohamad Al Hassan https://orcid.org/0000-0002-2297-3394
Dianka Dees https://orcid.org/0000-0002-5319-7527
Oene Dolstra https://orcid.org/0000-0003-0305-0938
Luisa M. Trindade https://orcid.org/0000-0003-1541-2094

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