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Dehydration response in *Stylosanthes scabra*: Transcriptional, biochemical, and physiological modulations

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Abstract

Stylosanthes scabra, popularly known as stylo, is native to the Brazilian Caatinga semi-arid region and stands out as a drought-tolerant shrub forage crop. This work provides information about the plant response during the first 48 h of water deficit, followed by a rehydration treatment. Besides root transcriptomics data, 13 physiological or biochemical parameters were scrutinized. Additionally, RNA-Seq annotated transcripts not associated with the "Viridiplantae" clade were taxonomically categorized. It was found that *S. scabra* quickly perceives and recovers from the oscillations of the imposed water regime. Physiologically, mechanisms that minimize evapotranspiration or protect the photosynthetic apparatus stood out. Biochemically, it was found that the root tissue invests in synthesizing compounds that can act as osmolytes (proline and sugars), emphasizing the importance of osmoregulation to water deficit acclimation. Consistently, transcriptome and qPCR analyses showed that a set of enriched biological processes with upregulated (UR) transcripts were involved in protective functions against reactive oxygen species or encoding enzymes of important metabolic pathways, which might contribute to *S. scabra* response to water deficit. Additionally, several UR kinases and transcription factors were identified. Finally, in an innovative approach, some naturally occurring microbial groups (such as *Schizosaccharomyces*, *Bradyrhizobium*, etc.) were identified in the *S. scabra* roots. This study reveals insights into the physiological, biochemical, and molecular mechanisms underlying the *S. scabra* response to water deficit and provides candidate genes that may be useful in developing drought-tolerant crop varieties through biotechnological applications.

Abbreviations: *A*, net CO_2 assimilation; Car, leaf carotene content; Chla, leaf chlorophyll "*a*" content; Chlb, leaf chlorophyll "*b*" content; ETR, electron transport rate; g_s, stomatal conductance; qP, photochemical quenching; RWC, leaf water content; TFAs, total free amino acids; TSCs, total soluble carbohydrates; TSPs, total soluble proteins; WUE, water use efficiency.

José Ribamar Costa Ferreira-Neto and Flávia Czekalski de Araújo contributed equally to this work.

1 | INTRODUCTION

Regarded as an exclusively Brazilian phytogeographical domain, the Caatinga is considered one of the most important natural Brazilian environments, covering most of the semiarid region of the country. This biome's flora is broad and diverse, considering the plants' morphological and molecular adaptations to the semiarid and hostile environment. Water availability is scarce and extremely oscillating, directly interfering with several organisms' distribution and survival (Bohnert et al., 1995; Castanho et al., 2020).

The Caatinga's plants include species of the genus *Stylosanthes* (Fabaceae). Factors such as rich genetic variability and the ability to survive under extreme conditions make species of this clade an excellent source of genes for breeding programs (Huang et al., 2017), especially targeting legumes. Furthermore, their ability to restore fertility and improve physical soil properties is worth mentioning due to their already reported association with rhizobia (nitrogen-fixing bacteria) and their potential to provide permanent vegetation cover (de Barcellos et al., 2008). These characteristics are of paramount importance and elevate this forage group to a level of high employability in the tropics and subtropics (Cameron & Chakraborty, 2004).

Five species of the mentioned genus—*Stylosanthes scabra*, *Stylosanthes seabrana*, *Stylosanthes hamata*, *Stylosanthes guianensis*, and *Stylosanthes viscosa*—are predominantly used as forage in humid to semiarid tropics (Chandra et al., 2006). *S. scabra* stands out from the others because of its drought tolerance (Nagaich et al., 2013) and its ability to grow in moderately acidic soils with low fertility (Edye & Topark-Ngarm, 1992). Moreover, this species is often associated with regions characterized by high soil salinity. *S. scabra* presents a substantial osmotic adjustment capacity, contributing to maintaining turgor in tissues under low water potential (Chandra et al., 2006). Its deep roots can collect water from the soil at considerably low water potentials (less than −1.5 MPa, usually associated with wilting point). These properties are essential, especially for perennial species, which need to survive the reduced water availability season in the tropics (Chandra et al., 2006; Nagaich et al., 2013).

Despite its robustness under unfavorable conditions, *S. scabra* still lacks transcriptomic studies to understand how its genetics is used under water deficit conditions. There are only four articles (Jiang et al., 2018 [*S. guianensis* cv. Reyan 2]; Jia et al., 2020 [*S. guianensis*]; Jiang et al., 2021 [*S. guianensis* cv. Reyan 2]; and Liu et al., 2022 [*S. guianensis* [Aubl.] Sw.]) addressing high-throughput gene expression studies for the *Stylosanthes* genus. None of them explores the water deficit issue, nor do they address the here studied species. Additionally, accessions from the Caatinga biome still lack physiological and biochemical data scrutinized under the above-mentioned situation.

The RNA-Seq technique is a widely used high-throughput gene expression sequencing and analysis method. Economically important legumes such as soybean (Ferreira-Neto et al., 2019) and cowpea (Ferreira-Neto et al., 2021) are being scrutinized and understood using this technology. Additionally, our research group has also been employing RNA-Seq to study regionally important species acclimated to highly hostile environments, as in *Cenostigma pyramidale* (Frosi et al., 2021), also native to the Caatinga biome.

When preparing RNA-Seq libraries, the applied protocol includes a selection step for polyadenylated RNA, which allows the detection of mRNA from eukaryotes in general. Furthermore, the RNA-Seq protocols direct the studies' focus to polyadenylated mRNA, although a reduced bacterial mRNAs quantity is a common noise (Hadfield & Eldridge, 2014; Strong et al., 2014). According to Chakraborty et al. (2016), in addition to analyzing high-throughput gene expression, such a technique represents an unbiased method to obtain insights into the presence of diverse "guests" in the analyzed target organism. Using RNA-Seq libraries, these authors identified a wide range of microbes (mostly eukaryotic; some prokaryotic) in different tissues of English walnut (*Juglans regia*). Sangiovanni et al. (2019) also highlight the importance of identifying sequences from other biological sources in works covering RNA-Seq data of a given organism. Such use would result in the "from trash to treasure" transformation of the obtained information (Sangiovanni et al., 2019). Therefore, our RNA-Seq libraries also explored this aspect, searching for the presence of endophytes in a legume, a group known to be a rich source of these microorganisms (Dudeja et al., 2012).

Given the above, this study aimed to use physiological, biochemical, and RNA-Seq data to analyze how *S. scabra* (85/UNEB, accession) responds in the first 48 h of water deficit and to the rehydration treatment. The treatment times were chosen in order to understand the initial response moments to the studied situation. Depending on the early sensing of a particular biotic or abiotic stress by rapid responses, the plants shape an appropriate action (e.g., to light, injury, heat, cold, etc.) that can lead to successful acclimation. The performed multistratified analysis enabled us to connect different layers of information, which provided a broad informative picture of the analyzed species' early response and the recovery process to the stress at issue. Furthermore, the RNA-Seq data helped identify transcripts from some endophyte clades, shedding light on the possible biotic interactions of this legume plant.

2 | MATERIALS AND METHODS

2.1 | Cultivation and plant growth conditions

Stylosanthes scabra Vogel is a species widely grown as a drought-tolerant forage crop. It belongs to the Fabaceae (Leguminosae) family, commonly found in the Caatinga biome (Brazil), and introduced in Hawaii and Australia (Forzza et al., 2016). The used accession (85/UNEB) was collected from the Caatinga and belongs to the Active Germplasm Bank of the Universidade do Estado da Bahia (UNEB)—Brazil. The plants were propagated by 10 cm long stem cuttings at EMBRAPA SEMIÁRIDO (Petrolina, Pernambuco, Brazil). They were submitted to the application of 1000 ppm of IBA (indolebutyric acid dissolved in mineral talc) and transplanted into 15 × 8 cm plastic bags (3 seedlings/pot) containing ultisol and vermiculite (3:1) as substrate. The cuttings were kept in a climate-controlled greenhouse (with 50% shade) under irrigation (twice a day).

After rooting, the plants were transferred to plastic pots (10 L)—containing substrate comprising sand-ultisol-vermiculite—and maintained

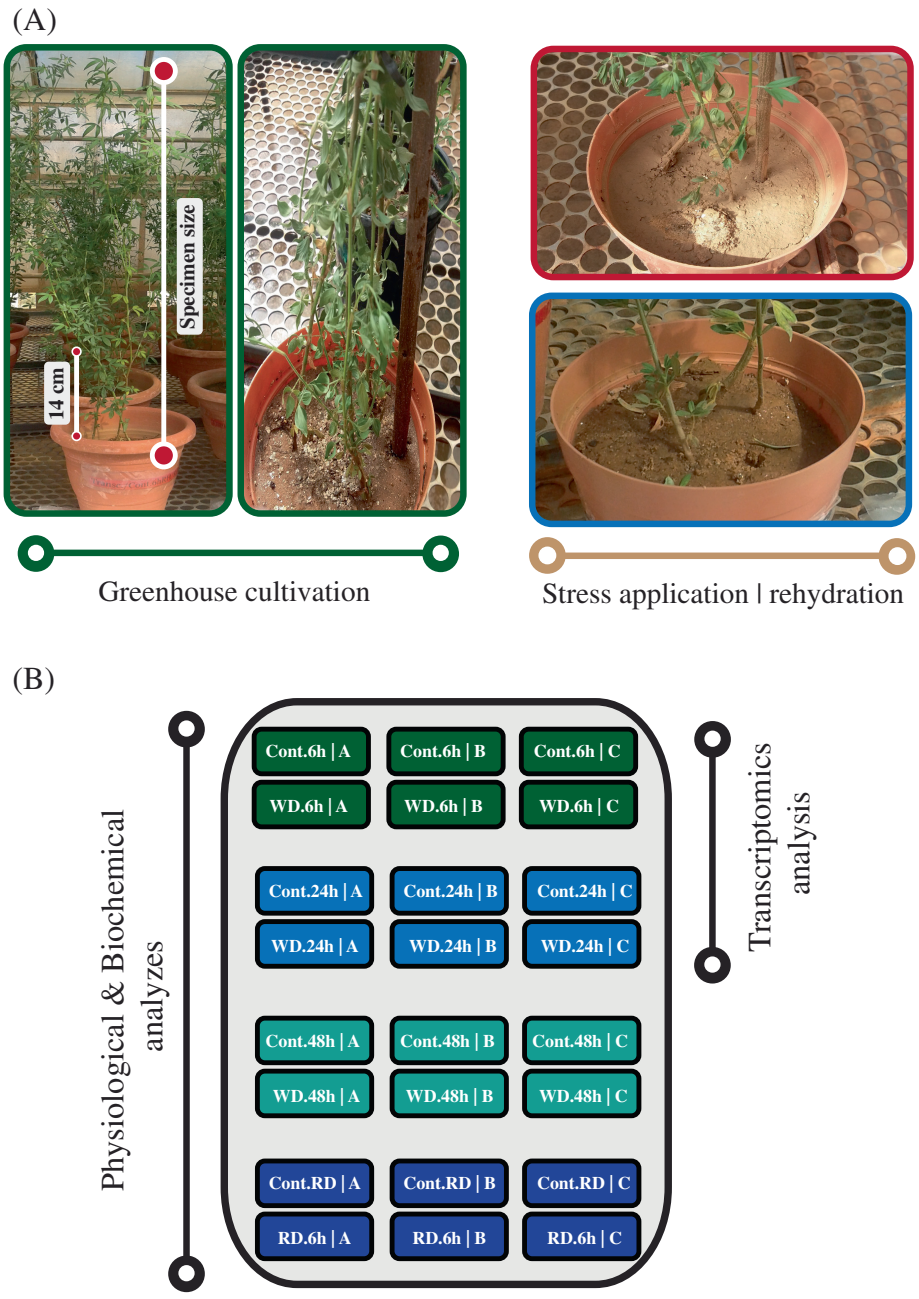

FIGURE 1 Prior actions implemented for performing the analyses in this study. (A) Growth conditions and stress application: cultivation of *Stylosanthes scabra* in a controlled environment; water deficit treatment by irrigation suspension. (B) Experimental designs implemented to perform the physiological, biochemical, and transcriptomic analyses, showing the number of biological replicates (three: A, B, C), treatment times [WD.6h (6 h under water deficit); WD.24h (24 h under water deficit); WD.48h (48 h under water deficit); RD.6h (6 h after rehydration)] and their respective controls (Cont.6h [6 h treatment under water deficit]; Cont.24h [24 h treatment under water deficit]; Cont.48h [48 h treatment under water deficit]; Cont.RD6h [rehydration treatment control]). Cont. (control); WD, water deficit

in a greenhouse under controlled conditions of temperature (25 ± 2°C), humidity (60 ± 5%), and 12 h/day natural light photoperiod (photosynthetic photon flux density [PPFD] = 1.5 × 10^3 μmol m^{-2} s^{-1} per 12 h/day) (Figure 1A, "Greenhouse cultivation" item). At this stage, the plants were watered daily, once a day at 9 a.m.

After 6 months of cultivation, the plants were subjected to water deficit stress (irrigation suspension; Figure 1A, "Stress application/rehydration" item), comprising three different treatment times (collections after 6, 24, and 48 h of irrigation suspension). To impose stress, the plants making up the treatments were not watered at 9 a.m., as usual. The plants were collected after each treatment period studied, i.e., irrigation suspension for 6 h (collection at 3 pm on the same day), 24 h (collection at 9 a.m. the next day), and 48 h (collection at 9 a.m., 2 days after the irrigation suspension started). In addition, there was the rehydration treatment (Figure 1A, "Stress application/rehydration" item). To do so, a group of plants was rehydrated (500 ml of H_2O) after 48 h of irrigation suspension. Then, these plants were collected 6 h after the rehydration process application. Each treatment had its respective control (Figure 1B). All assay elements had biological triplicates (Figure 1B), which comprised three plants per pot. After collection, the plants were immediately frozen in liquid N_2. The aerial part (leaves) and the root part were stored separately in an ultrafreezer at −80°C. The experimental design consisted of randomized blocks in a two versus four factorial schemes (two treatments vs. four time frames).

2.2 | Physiological and biochemical analyses

2.2.1 | Leaf water content (RWC_{leaf}), gas exchange, and chlorophyll fluorescence

Physiological measurements were performed at the four treatment times shown above (Figure 1B, "Physiological and Biochemical analyses" item). Trifoliate leaves were sampled for the RWC_{leaf} calculation, which was determined according to RWC (%) = [FW − DW/TW − DW] × 100 (Barrs & Weatherley, 1962), where FW is fresh leaf weight, TW is the turgid weight of the same leaves (after being submerged in deionized water for 24 h), and DW is their dry weight, obtained after 48 h in forced ventilation.

We obtained the gas exchange and chlorophyll "*a*" fluorescence values using an infrared gas analyzer (IRGA, LI-COR, model LI-6400XT) with a fluorescence chamber (6400-40), coupled with a 2 cm^2 area and a 300 mmol s^{-1} gas flow rate, with 400 ppm of CO_2 concentration. Measures were taken on mature, non-senescent leaves of the *S. scabra* trifoliolate leaves under a constant photosynthetic photon flux (PPF) of 1200 μmol m^{-2} s^{-1}.

We obtained net CO_2 assimilation (*A*) and stomatal conductance (g_s) values. Water use efficiency was calculated by the net CO_2 assimilation and transpiration rate (*E*) ratio (WUE = *A*/*E*). To evaluate chlorophyll *"a"* fluorescence, fluorescence emission at an equilibrium state (Ft) and maximum fluorescence emission (Fm′) were determined in light-adapted leaves undergoing steady photosynthesis. The photochemical *quenching* (qP) and the electron transport rate (ETR = (Δ*F* − Fm′ × PPFD × 0.5 × 0.84)) were calculated according to (Baker, 2008) using chlorophyll "*a*" fluorescence. When calculating the ETR, we used a 0.5 value as the fraction of excitation energy distributed to PSII and a 0.84 value as the fraction of incident light absorbed by the leaves.

2.2.2 | Biochemical analyses in leaf tissue

The biochemical analyses covered the four treatment times set out above (Figure 1B, item "Physiological and Biochemical analyses"). Mature leaves and roots for each treatment were collected, immediately frozen in liquid nitrogen, and then stored in an ultrafreezer at −80°C. Total soluble carbohydrates (TSCs; DuBois et al., 1956); total free amino acids (TFAs; Moore & Stein, 1948); total soluble proteins (TSPs; Bradford, 1976); the amino acid proline (Bates et al., 1973), chlorophyll *"a"* (Chla); chlorophyll *"b"* (Chlb); and carotenoids (Lichtenthaler & Buschmann, 2001) were quantified. All quantifications were determined using a dual-beam spectrophotometer (Thermo Scientific/ Model Genesys 10 S UVVIS) adjusted to the specific wavelength for each organic compound.

2.2.3 | Physiological and biochemical analyses—Statistics

The statistical analysis of the physiological and biochemical data was performed using STATISTICA 8.0 software (StatSoft. Inc.). The data were analyzed for normal distribution using the Shapiro–Wilk test of normality (*W*) and homogeneity (Levene's test). The *t*-test ($p < 0.05$) was performed for the variables that presented parametric data. For the RWC_{leaf} variable (24 h), which showed neither normal distribution nor homogeneity, the Mann–Whitney nonparametric test was used ($p < 0.05$). Prior to statistical analysis, the data regarding RWC_{leaf} (presented as percentages) were transformed using the arc sen √x/100 formula.

Principal component analysis (PCA) was made by 13 selected variables from each analyzed condition to determine whether: (1) there were trends in the grouping of treatments and (2) define the most important variables for group separation. For this multivariate analysis, the Factoextra (Kassambara & Mundt, 2020) package was used.

2.3 | Transcriptomic assay

2.3.1 | RNA-Seq libraries: Synthesis and sequencing

The total RNA was isolated using the "SV Total RNA Isolation System" kit (Promega) following the manufacturer's protocol. The concentration and quality of the total RNA extracted were evaluated using Agarose gel (1.5%) and the "Agilent 2100 Bioanalyzer" tool (Agilent Technologies). Only samples with RNA integrity number ≥8.0 were sequenced. The sequencing platform and kits used by Frosi et al. (2021) and Ferreira-Neto et al. (2021) were also used in the present work. Twelve libraries encompassing the biological replicates of the following treatments were synthesized: Cont.6h/WD.6h, and Cont.24h/WD.24h (Figure 1B, item "Transcriptomics analysis"). Thus, we aimed to study the *S. scabra* response to the first 24 h of water deficit stress at the molecular level.

2.3.2 | RNA-Seq library assembly and transcriptome annotation

We performed the de novo assembly of *S. scabra* RNA-Seq libraries. First, adapters and low-quality sequences were removed using the Trimmomatic software (Bolger et al., 2014) (adopted parameters: HEADCROP [13]; TRAILING [30]; and MINLEN [32]). The obtained reads showed a Phred value ≥30. Those not meeting the minimum quality requirements mentioned above were discarded. The quality metrics of the resulting sequences were accessed using FastQC 0.11.8 software (https://github.com/s-andrews/FastQC).

For data assembly and analysis, the "*de novo* pipeline RNA-Seq" (version 3.1.3) from the GenPipes platform (Bourgey et al., 2019) was used, which is briefly presented below. Transcriptome assembly was performed using Trinity 2.0.4 software (Haas et al., 2013). After this step, the software Transdecoder 2.0.1 (https://github.com/TransDecoder/TransDecoder/wiki) was implemented to identify candidate ORFs and translate them. Only the largest ORF of each transcript was considered. Then, we used the Trinotate 2.0.2 software (https://github.com/Trinotate/Trinotate.github.io/wiki) for the functional annotation of the identified ORFs in the resulting

transcriptomes. Trinotate makes use of a number of different methods for functional annotation, including homology search to known sequence data (BLAST+/SwissProt/Uniref90), protein domain identification (HMMER/PFAM), protein signal peptide, and transmembrane domain prediction (signalP/tmHMM), and comparison to currently curated annotation databases (EMBL Uniprot eggNOG/GO Pathways databases). In addition, we conducted the annotation of proteins originating from the Transdecoder action using similarity analysis (BLASTP; cutoff < e^{-20}) against protein sequences from the TREMBL database (https://www.uniprot.org/uniprot/?query=reviewed:no).

The completeness assessment of the transcriptome assembly was carried out using gVolante (Nishimura et al., 2017), ortholog search pipeline "BUSCO" v.5 (ortholog set: Viridiplantae). Completeness assessments performed on gVolante report scores based on not just the coverage of reference genes but also on sequence lengths (e.g., N50 scaffold length), allowing quality control in multiple aspects.

2.3.3 | Differential expression analysis of the obtained transcripts

Transcript abundance estimation for each sample was performed using the RSEM tool (RNA-Seq by Expectation–Maximization; Li & Dewey, 2011). The EdgeR software (Robinson et al., 2009) was implemented for differential expression analysis. We considered differentially expressed transcripts those that showed $-1 < Log_2FC < 1$, p-value <0.05, and FDR < 0.05.

2.3.4 | Identification of potential microbiota sequences

Sequence identification was performed using the GenPipes annotation pipeline. Thus, the annotation from the Trinotate 2.0.2 software (https://github.com/Trinotate/Trinotate.github.io/wiki) was scrutinized, starting from its output format. The example below shows the mentioned output configuration:

"ZPR1_YEAST^ZPR1_YEAST^Q:3-332,H:349-454^54,545% ID^E:4,03 e-31^RecName: Full=Zinc finger protein ZPR1;^Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Saccharomycetaceae; Saccharomyces."

Using a python script, we filtered all sequences that did not contain the term "Viridiplantae" in their annotation line. Then, the resulting group of elements had their sequences analyzed (BLASTN, cut-off <e^{-20}) against "Viridiplantae" sequences from the RefSeq database (93,436,768 sequences; August, 2021) to confirm they exhibit no significant similarity with potential plant sequences.

2.3.5 | Gene ontology enrichment analysis

For the proposed analysis, we used the NeVOmics tool (Network-based Visualization for Omics; Zuniga-Leon et al., 2018) that identifies statistically overrepresented biological processes (GO terms) within a given gene/protein set. A hypergeometric distribution (p < 0.05) and an FDR correction (FDR <0.05) were employed to identify the statistically more represented function annotations. The totality of "control versus treatments" transcriptome data was used for each treatment studied as background in the NeVOmics enrichment analyses.

The mentioned tool uses an input file in plain text with a list of genes (KEGG gene ID) or proteins (UniProt Entry ID) obtained by the omic approach. Due to the reduced number of annotated proteins for *S. scabra* in the UniProt (https://www.uniprot.org/) database, *Arachis hypogea* homologous proteins were used. This species contains a large volume of information in the mentioned database, and the genus *Arachis* is phylogenetically close to the genus *Stylosanthes* (Cardoso et al., 2013). The UniProt IDs of *A. hypogea* were obtained from the TREMBL database.

2.3.6 | Transcription factors and kinases mining

Transcription factors (TFs) and kinases mining and identification were carried out using iTAK software (Zheng et al., 2016). It bases the TFs identification and classification on the consensus rules summarized from public databases (namely: PlnTFDB 2.0 (Pérez-Rodríguez et al., 2010) and PlantTFDB 3.0 (Jin et al., 2014)), with families from PlantTFcat (Dai et al., 2013) and AtTFDB (https://agris-knowledgebase.org/AtTFDB/), using supporting evidence.

For kinase identification and classification, iTAK tool has the potential to identify up to 150 kinase families by performing two main actions: (1) identification: if sequences presented a significant hit (at least 50% compared to the Pfam domain model) to the protein kinase domains (PF00069, PF07714, or PF00481) in the Pfam database; (2) classification: after step "a," the identified VuPKs were classified into gene families by comparing sequences to a set of Hidden Markov Models (HMMs; e-value cut-off <$1.0e^{-5}$) developed by Lehti-Shiu and Shiu (2012).

2.3.7 | qPCR: Setup, cDNA synthesis, efficiency evaluation, and relative expression

These analyzes were performed according to the MIQE guidelines (Bustin et al., 2009). To evaluate the transcriptomic data's accuracy, 14 *S. scabra* transcripts (Table S1), with upregulation indicated in RNA-Seq libraries, were specifically selected for qPCR investigation. The validated transcripts were associated with enriched GO terms, TFs, and kinase proteins highlighted throughout the text. Three biological (individuals) and three technical replicates (extractions) were used per treatment, to guarantee the statistical reliability of the process. qPCR reactions were carried out in 96-well plates at LineGene 9660 (Bioer) using the SYBR Green detection method.

Aliquots of the same total RNA sample sent for sequencing of the RNA-Seq libraries were employed in this step. Possible contamination with genomic DNA was eliminated via digestion with "RNase-free

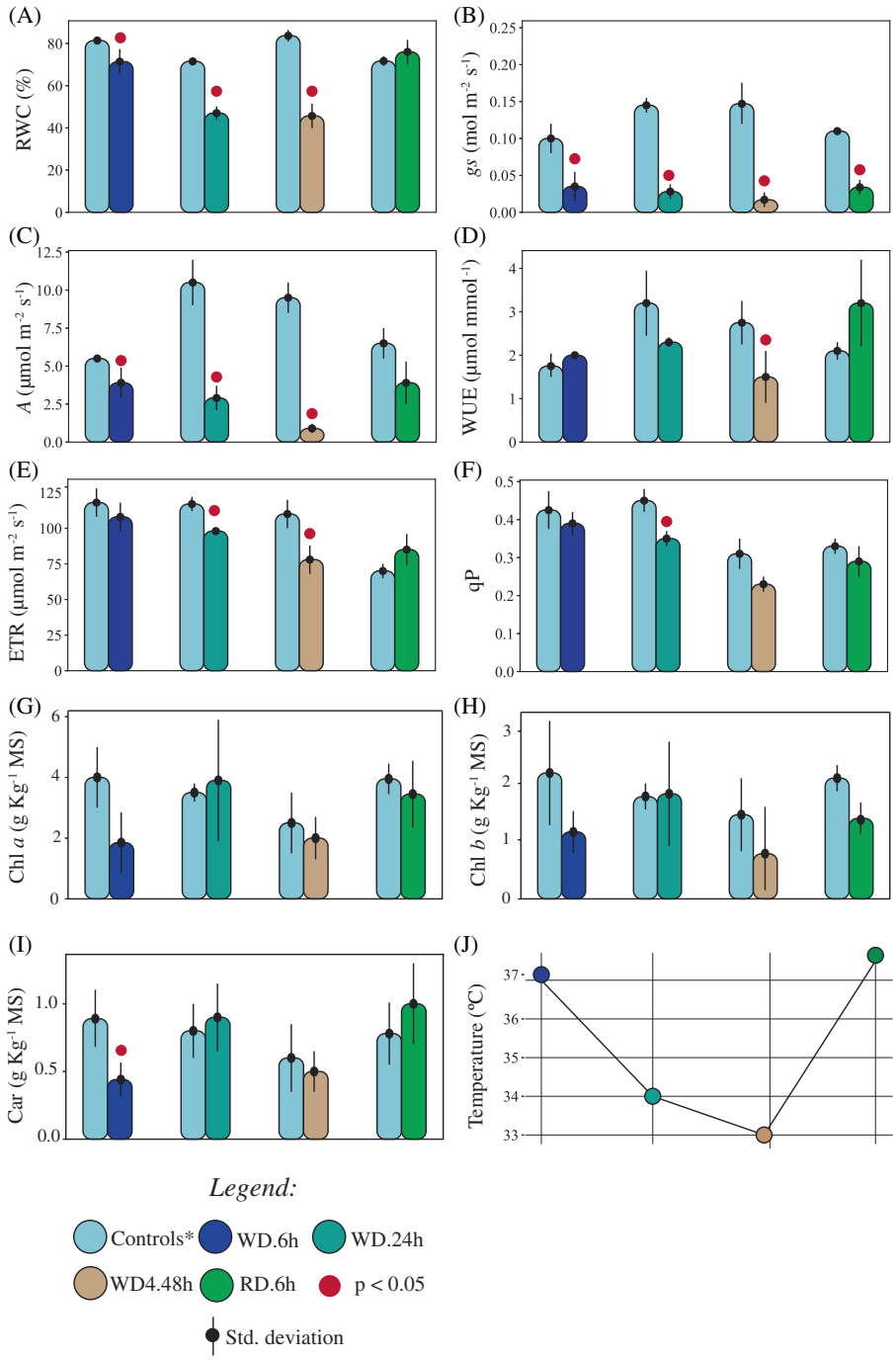

FIGURE 2 Physiological and fluorescence parameters of *Stylosanthes scabra*, and environmental temperature obtained during the studied treatments. (A) Leaf relative water content (RWC); (B) stomatal conductance (gs); (C) net CO_2 assimilation (A); (D) water use efficiency (WUE); (E) electron transport rate (ETR); (F) photochemical quenching (qP); (G) leaf chlorophyll "*a*" (Chla) content; (H) leaf chlorophyll "*b*" (Chlb) content; (I) leaf carotene (Car) content; and (J) environmental temperature recorded throughout the assay. *Each treatment analyzed presents its respective control (i.e., Cont.6h; Cont.24h; Cont.48h; Cont. RD). Bars represent mean ± SD ($n = 3$). The comparison of the means between control and treatments was determined by the Tukey test at 5% of probability ($p < 0.05$) for "B," "C," "E," "F," "G," "H," and "I"; and by the Mann–Whitney nonparametric test ($p < 0.05$) for "A". h, hours; RD, rehydration; WD, water deficit

DNase I." RNA quantity and quality were evaluated according to Ferreira-Neto et al. (2021). Total RNA (1 µg) was reverse transcribed for each sample studied to generate cDNA with the "Improm-IITM Reverse Transcriptional System" kit (Promega), using oligo primers (dT) according to the manufacturer's recommendations. qPCR setup, PCR cycling, amplification efficiency assay, primer pairs design, and melting curves analysis (Figure S1) were performed as described by Ferreira-Neto et al. (2021).

"β-tubulin" and "Ubiquitin" (Table S1) were selected as reference genes for qPCR water deficit assay data normalization. These were indicated from stability tests performed for the same cultivar, tissue, and treatment times.

The Rest2009 software (standard mode) was used for relative expression analysis of the target transcripts. Such analysis is based on paired comparisons (of target transcript and reference genes under stress conditions and controls) using randomization and bootstrapping—Pair-wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002). Hypothesis testing ($p < 0.05$) was used to determine if differences in expression of target transcripts under control and treated conditions were significant.

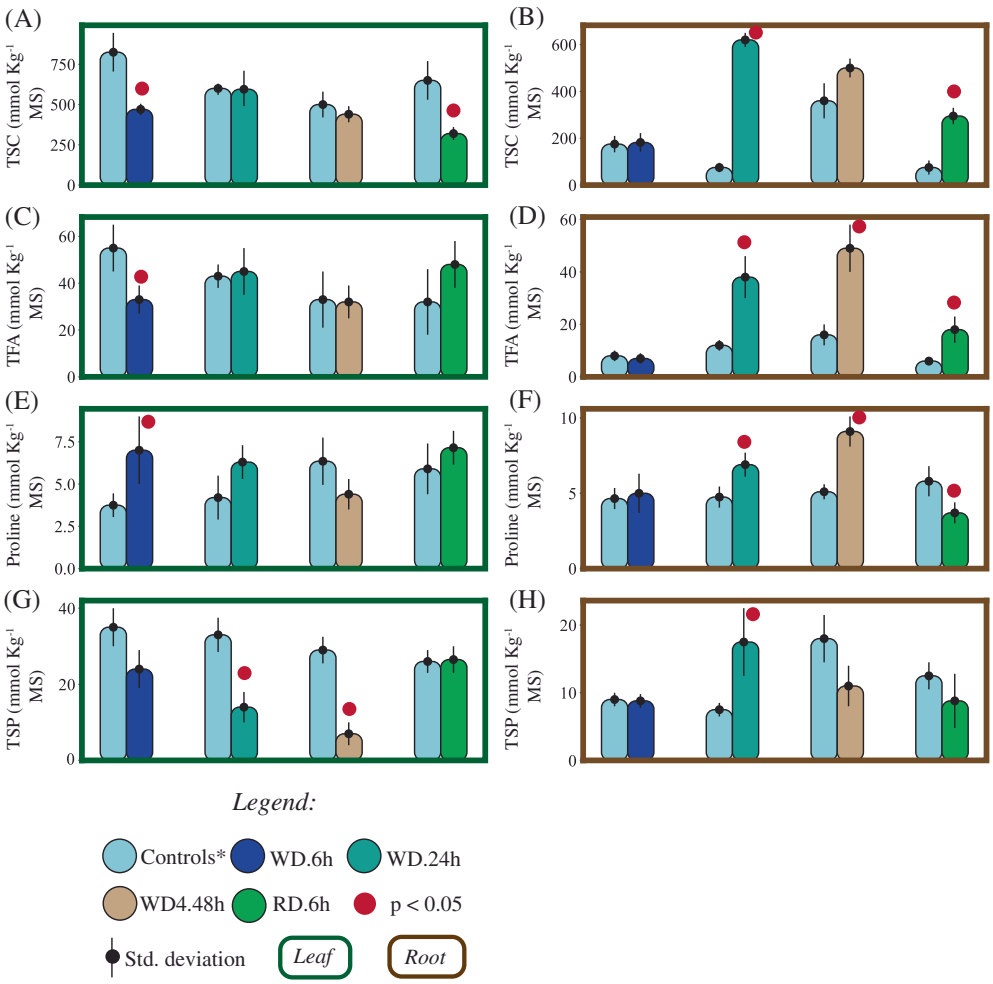

FIGURE 3 Biochemical indicators in leaf and root tissues of *Stylosanthes scabra* submitted to different water deficit and rehydration treatments. (A) Total soluble carbohydrates (TSCs) in leaves; (B) TSC in roots; (C) total free amino acids (TFAs) in leaves; (D) TFA in roots; (E) proline content in leaves; (F) proline content in roots; (G) total soluble proteins (TSPs) in leaves; and (H) TSP in roots. *Each treatment analyzed presents its respective control (i.e., Cont.6h; Cont.24h; Cont.48h; Cont.RD). Bars represent mean ± SD ($n = 3$). The comparison of the means between control and treatments was determined by the Tukey test at 5% of probability ($p < 0.05$). h, hours; RD, rehydration; WD, water deficit

3 | RESULTS

3.1 | Water deficit effects on *S. scabra* physiology

After 6 h under water withholding (WD.6h), the *S. scabra* plants already showed changes in physiological parameters. The RWC_{leaf} of the individuals under study decreased throughout the performed treatments (Figure 2A). This parameter reduction was directly proportional to the water deficit time imposed. There was a 12% decline in RWC_{leaf} for the WD.6h treatment compared to its respective control (Figure 2A). There was a 34 and 45% reduction for the WD.24h and WD.48h treatments, respectively (Figure 2A). This parameter reestablished itself to its respective control level 6 h after the rehydration period (RD6h) (Figure 2A).

Moreover, the water deficit periods imposed caused reductions in gas exchange-associated parameters. For the WD.6h treatment, we observed a 65% reduction, followed by 79 and 88% reductions in stomatal conductance (g_s) values for the WD.24h and WD48h treatments, respectively (Figure 2B). For the rehydration treatment (RD.6h), there was no full "g_s" recovery and it remained reduced. On average, rehydrated plants showed 32% of the "g_s" of their respective controls (Figure 2B) after 6 h of rehydration.

Regarding net CO_2 assimilation (A), the analyzed condition showed an important impact on this variable for all treatment times. There were 30, 73, and 91% reductions for WD.6h, WD.24h, and WD.48h, respectively, compared to their respective controls (Figure 2C). We found that at RD.6h, "A" had recovered and was restored to its control level.

For the WUE parameter, all treatments (WD.6h, WD.24h, and RD.6h), except WD.48h, remained at similar levels compared to their respective controls (Figure 2D). For the last treatment mentioned (WD.48h), however, there was a 45% reduction (Figure 2D).

Regarding photochemical parameters, the ETR showed reductions starting with the WD.24h treatment. For this treatment and the following one (WD.48h), we found a 15 and 23% decline in ETR compared to their respective controls (Figure 2E). The values observed for quenching (qP) showed differences for the respective controls only for WD.24h (reduction of 21%) (Figure 2F).

Regarding leaf pigments, we observed that the chlorophyll "*a*" (Figure 2G) and "*b*" (Figure 2H) contents in all implemented treatments remained similar to their controls. However, we found a decrease (51%) for carotenoids only for the WD.6h treatment (Figure 2I). There was no significant disturbance of this parameter in the later treatment times.

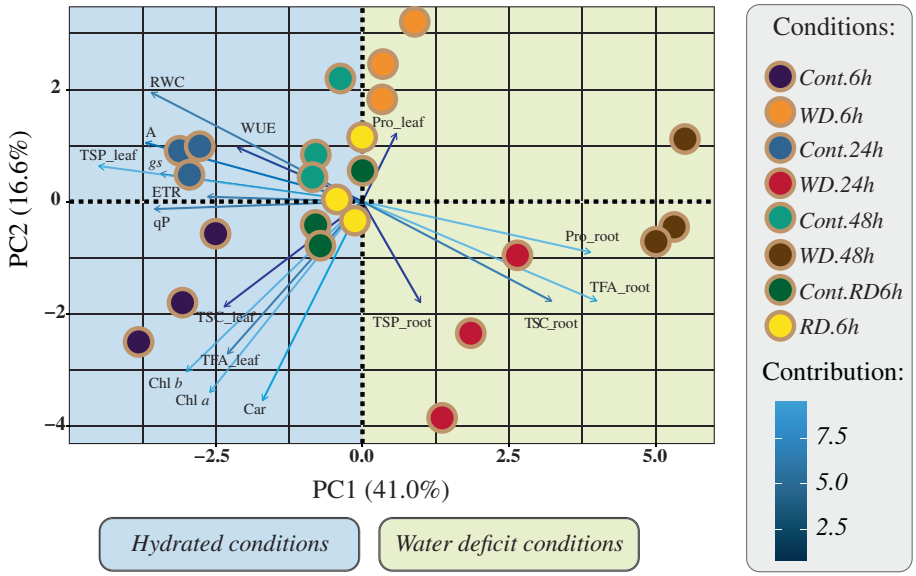

FIGURE 4 Principal component analysis (PCA)-biplot of the treatments and physiological/biochemical parameters. The treatments (colored spheres; $n = 3$) dispersed in different ordinates based on the dissimilarity among them. The length and color intensity of a vector indicate, respectively, the quality of representation and the contribution of the scrutinized parameters on the principal components. The angles between the vectors derived from the middle point of biplots exhibit positive or negative interactions of the studied parameters. Leaf relative water content (RWC); stomatal conductance (gs); net CO_2 assimilation (A); water use efficiency (WUE); electron transport rate (ETR); photochemical quenching (qP); leaf chlorophyll "*a*" (Chla) content; leaf chlorophyll "*b*" (Chlb) content; leaf carotene (Car) content; total soluble carbohydrates in leaves (TSC_leaf); total soluble carbohydrates in roots (TSC_root); total free amino acids in leaves (TFA_leaf); total free amino acids in roots (TFA_root); proline content in leaves (Pro_leaf); proline content in roots (Pro_root); total soluble proteins in leaves (TSP_leaf); total soluble proteins in roots (TSP_root); Cont., controls; PC, principal component; RD, rehydration; WD, water deficit

3.2 | Biochemical adjustments in different tissues of *S. scabra* under water deficit

The contrast in the biochemical components' content measured in the *S. scabra* root and leaf tissues showed that these organs use different strategies in an attempt to acclimatize to the water deficit condition. As for the leaf values of TSCs, this parameter was reduced (decline of 41% compared to its control) only in the WD.6h treatment (Figure 3A). In the following water deficit treatments (WD.24h and WD.48h), *S. scabra* restored leaf TSC levels to the level of their respective controls (Figure 3A). However, it is interesting to observe that there was a 51% decrease in leaf TSC levels for the RD.6h treatment compared to its respective control (Figure 3A). Regarding root tissue, we observed that the TSC tends to increase in most treatments, although only the WD.24h and RD.6h treatments, with respective increases of 735 and 205%, showed statistical significance (Figure 3B). In the remaining treatments, this parameter did not differ from their respective controls.

For leaf tissue, TFA values remained unchanged in all treatments, except for WD.6h, where a 41% reduction was observed, compared to its control (Figure 3C). TFS values in the root were higher in all treatments (Figure 3D) compared to their respective controls, except for WD.6h.

For the leaf proline content, a change in this index (in this case, 77% increase) occurred only for the initial treatment (WD.6h) (Figure 3E). For *S. scabra* root tissue, an increase in this parameter in the WD.24h (77%) and WD.48h (74%) treatments were observed (Figure 3F). However, there was an important reduction after rehydration (RD.6h treatment) (Figure 3F). The referred treatment showed 69% of the proline content of its respective control (Figure 3F).

In turn, the TSP content in the leaf tissue showed a tendency to decrease in water-deficit treatments. However, this variable was significantly reduced in WD.24h (68%) and WD.48h (76%) compared to their respective controls (Figure 3G). For root tissue, only WD.24h (157%) showed an increase for the referred parameter (Figure 3H). The rest of the treatments remained at the same level as their respective controls (Figure 3H).

3.3 | Physiological and biochemical data overview by PCA

Based on the correlation between the physiological/biochemical parameters and extracted groupings, the variation pattern in *S. scabra* treatments were also studied using PCA. This action made it possible to categorize the data points into some "information clusters." From Figure 4, the data were divided into two main quadrants, covering hydrated or water deficit samples. The WD.24h and WD.48h treatments were the most distinct from the other studied conditions. The RD.6h treatment showed similar behavior to the controls, indicating the fast *S. scabra* recovery. Interestingly, considering controls and RD.6h treatment samples, an increasing order of dissimilarity from WD.6h to WD.48h (Figure 4) was observed.

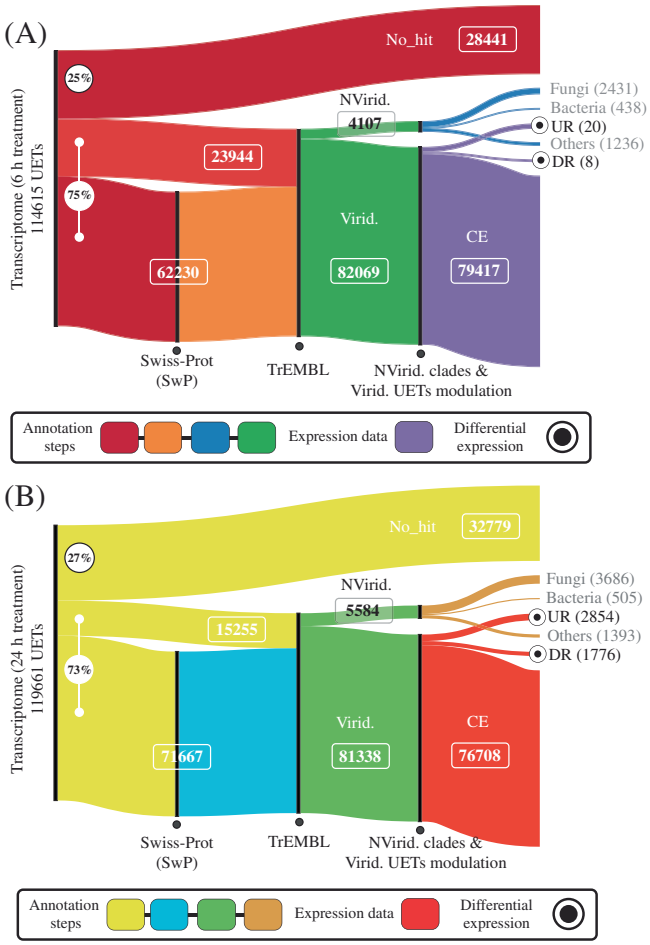

FIGURE 5 Sankey diagram showing the information processing flow—from annotation against different databases, categorization as to sequence origin, to differential expression analysis for RNA-Seq sequences associated with the Viridiplantae clade. (A) Data for RNA-Seq libraries associated with the 6 h treatment under water deficit and its respective control. (B) Data for RNA-Seq libraries associated with the 24 h treatment under water deficit and its respective control. CE, constitutive expression; DR, downregulated; NVirid, sequences annotated and not representative of the Viridiplantae clade; UETs, unique expressed transcripts; UR, upregulated; Virid, sequences annotated and representative of the Viridiplantae clade

The first two PCA components explained 57.6% of the variance in the dataset (Figure 4). The PC1 (which is associated with the water regime resolution) exhibited 41.0% of the total variability (Figure 4). The TFA and TSP root parameters were positively associated with the water deficit conditions, presenting the most substantial contributions for the formation of groupings of these treatments (Figure 4).

3.4 | Transcriptomics of *S. scabra* root tissue under water deficit

3.4.1 | Data assembly and annotation

Assembly and sequencing metrics are available in Appendix S1. After assembling the transcriptomes, we found 236,712 unique expressed transcripts (UETs; Appendix S1). For the libraries associated with the 6 h treatment (Cont.6h and WD.6h), this value was approximately 116,000 (UETs; Figure 5A). In libraries associated with the 24 h treatment (Control.24h and WD.24h), this amount was approximately 119,000 (UETs; Figure 5B).

The Swiss-Prot databases (Swp; which presents protein sequences manually annotated and reviewed) and TrEMBL (which contains protein sequences automatically annotated and not reviewed) returned the highest annotation rates. However, the largest volume of information retrieved came from the TrEMBL database (Figure 5A,B) due to its larger informational content. About 73% of the obtained transcriptomes were annotated (Figure 5A,B). In addition to the obvious presence of transcripts associated with the Viridiplantae clade ("Virid."; Figure 5A,B), we identified a smaller group of sequences similar to those of various microorganisms ("NVirid."; Figure 5A,B), mainly fungi and, in even smaller numbers, bacteria (Figure 5A,B). Approximately 27% of the transcriptomes showed no similarity with sequences from the analyzed databases (No_hit; Figure 5A,B), which may be either sequences specific to *S. scabra* and/or microorganisms with scarce sequencing data.

Regarding the differential expression analysis, we observed a great deal of variation among the treatments. The 6 h treatment under water deficit showed low amounts of modulated transcripts, with eight downregulated (DR) and 20 UR transcripts (Figure 5A). A probable cause for this may be associated with the anomalous performance of two of its biological replicates (see "RNA-Seq libraries PCA analysis," Appendix S1). Due to the low rate of molecular information (differential expression), these libraries (control and treatments) were used only as a component for mining and identifying sequences not associated with the Viridiplantae clade.

In turn, the 24 h treatment showed high transcriptional modulation. A total of 4630 unique transcripts were differentially expressed, with 1776 DR and 2854 UR (Figure 5B).

3.5 | Decoding the *S. scabra* transcriptional response to water deficit

3.5.1 | Biological processes enrichment

The analysis of enriched biological processes indicated important *S. scabra* strategies in responding to water deficit. The 2854 UR transcripts (Figure 5B) in RNA-Seq libraries from the 24 h water deficit treatment encompassed 129 distinct biological processes (Table S2), with 34 being enriched (Table S2). Nine of these were found to be biologically informative. Figure 6 shows these terms, their constituent elements, and respective modulation (in $Log_2FoldChange$ form).

The "Glutathione metabolic process" term, associated with oxidative stress metabolism, was the most enriched in the presented set. Interestingly, all of their elements (items with the type notation "A0AXXXXXXXX," in addition to B4UW81 and C7F8A3 in Figure 6) were representatives of the glutathione transferase (GST) enzyme. Most of these presented high transcriptional modulation (Figure 6).

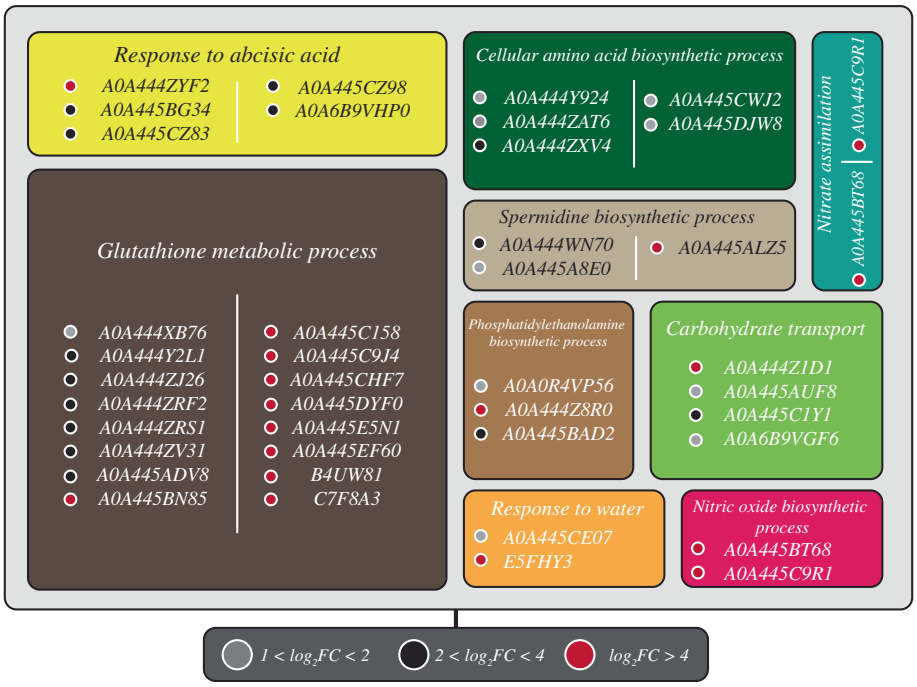

FIGURE 6 Treemap of the enriched biological processes for the 24 h treatment under water deficit, showing their respective constituent elements and transcriptional modulation (in Log2FoldChange form).

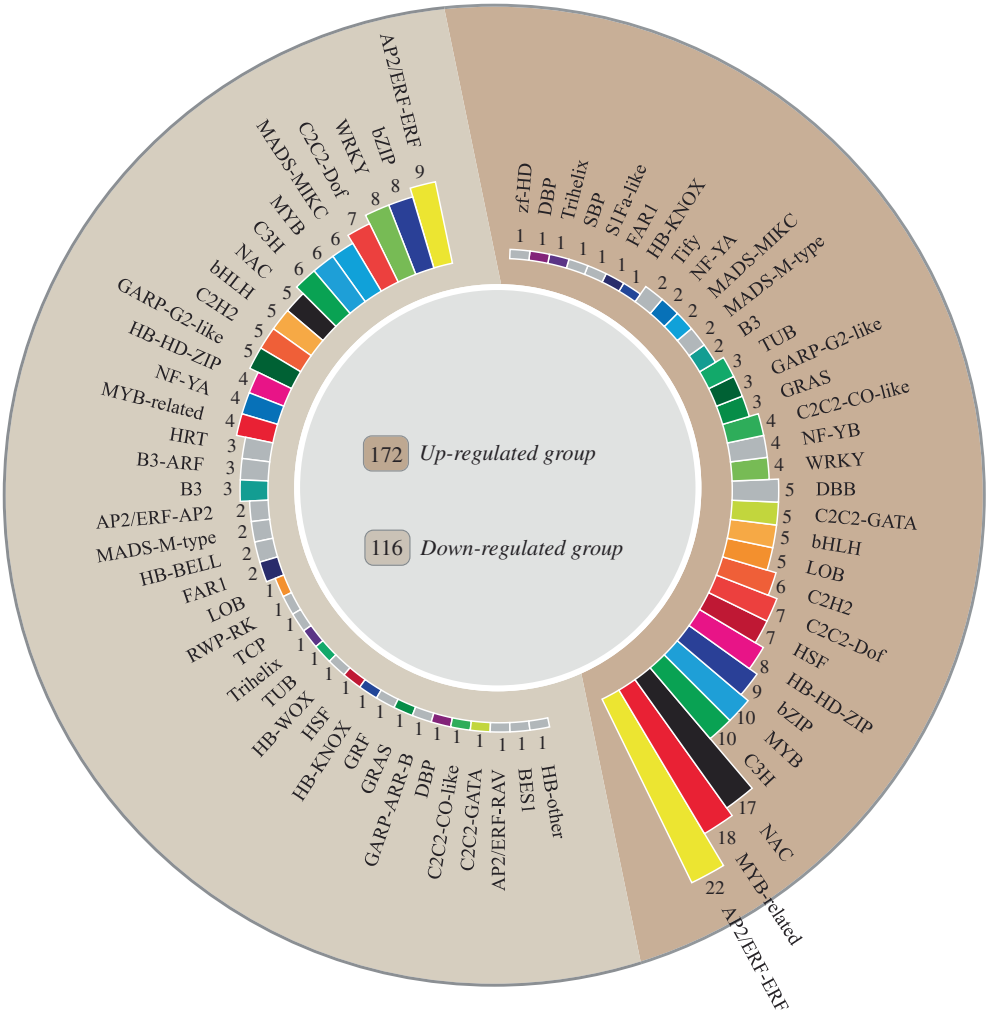

FIGURE 7 *Stylosanthes scabra* transcription factor families categorized by their differential expression (upregulated or downregulated) after 24 h of water deficit stress imposition. Gray columns represent transcription factors specific to each regulation set; similar colors in the columns of the different expression groups (upregulated or downregulated) indicate the same transcription factor family

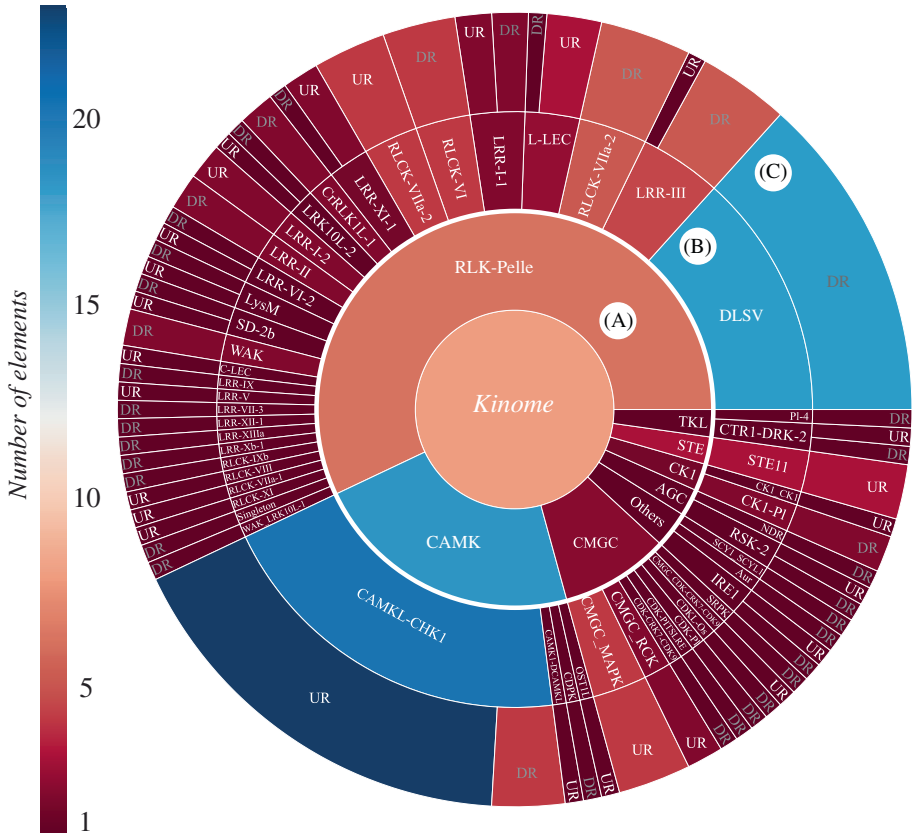

FIGURE 8 Differentially expressed *Stylosanthes scabra* kinome sequences after 24 h of water deficit, categorized into: (A) group, (B) family, and (C) transcriptional modulation. DR, downregulated; UR, upregulated

The "Response to abscisic acid" and "Cellular amino acid biosynthetic process" terms come next. Transcripts encoding HVA22-like protein (A0A444ZYF2) and Shikimate kinase (A0A444ZXV4) were the most modulated for each process mentioned above, respectively (Figure 6).

The term "Spermidine biosynthetic process," associated with this polyamine (PA) production, also stood out. This term showed the *S*-adenosylmethionine decarboxylase (SAMDC) enzyme (A0A445ALZ5) as the most modulated element (Figure 6). The "Phosphatidylethanolamine biosynthetic process," "Carbohydrate transport," and "Response to water" terms also stood out (Figure 6). The phosphatidylserine decarboxylase enzyme (A0A444Z8R0) (for the first term) and the proteins SWEET (A0A444Z1D1) (for the second) and dehydrin (E5FHY3) (for the third) were the most modulated elements (Figure 6).

Finally, we noticed the enrichment of the "Nitric oxide biosynthetic process" and "Nitrate assimilation" terms (Figure 6). Both terms showed nitrate reductase (NR) enzymes (A0A445BT68 and A0A445C9R1) as major modulating elements (Figure 6).

3.5.2 | The expressed TFome and kinome of *S. scabra* under water deficit

TFs, whose expressed set under a given condition is called the "TFome," and kinases proteins (PKs), whose set is called the "kinome," were identified. Both categories are important players in the molecular physiology of any living organism. Regarding the TFome, which is associated with the activation/deactivation of specific genes, we found a diverse pool in the 24 h treatment under water deficit. For this treatment, 288 TFs were differentially expressed, with 172 UR (associated with 32 different families), and 116 DR (associated with 38 different families) (Figure 7).

For the UR group, the following families stood out: AP2-ERF-ERF (22), MYB-related (18), and NAC (17) (Figure 7). Many families with UR transcripts also presented DR transcripts, as is the case of AP2-ERF-ERF, MYB, and NAC, among others (Figure 7). This data suggests the selection of specific isoforms for the stress response. In addition, there were set-specific TF families (columns in gray, Figure 7). Among others, DBB, NF-YB, and MAD-M-type were specific to the UR group, while HRT, B3-ARF, and AP2/ERF-AP2, for example, were specific to the DR set (Figure 7).

Regarding PKs, whose role is closely associated with cell signaling processes, mining their representatives revealed a diverse kinome for the transcriptome associated with the 24 h treatment under water deficit. This kinome consisted of eight major differentially expressed groups, presented in descending order as follows (Figure 8A): RLK-Pelle (76), CAMK (30), CMGC (12), STE, "others" (4), AGC (3), CK1 (3), STE (3), and TKL (3). They comprised 51 different families (Figure 8B).

Regarding the 26 families with at least one member induced in the 24 h treatment under water deficit (Figure 8B,C), CAMK_CAMKL-CHK1 (composed mainly of CBL-interacting serine/threonine-protein kinases; data not shown) was the one with the highest number of representatives (23) (Figure 8C); CMGC_MAPK and RLK-Pelle_RLCK-

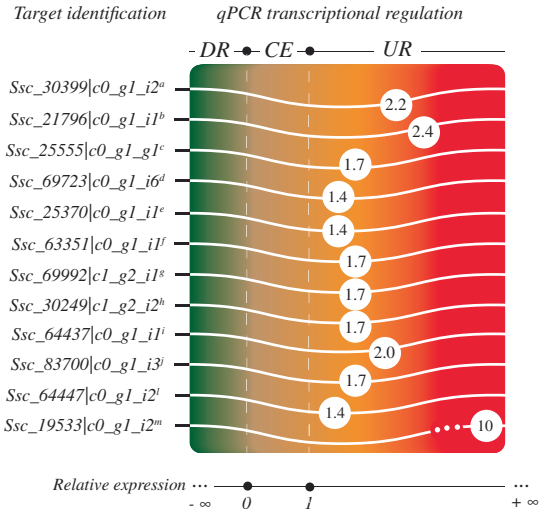

FIGURE 9 Gradient chart presenting the qPCR analyzed targets, their ID (Ssc_XXXXX_cX_gX_iX), and associated annotation (superscript letters). The color gradient is associated with the relative expression value (number inside the white circle) for the targets. Targets with a relative expression value below zero and $p < 0.05$ are considered downregulated (DR); those between zero and one, and $p \geq 0.05$ are considered constitutively expressed (CE); those greater than one and $p < 0.05$ are considered upregulated (UR). Ssc: *Stylosanthes scabra*; aMYB-related transcript factor; bNAC transcription factor; cCAMK_CAMK_CHK1 kinase; dCMCG_MAPK kinase; eRLP-Pelle_RLCK_Villa-2; fHVA22-like protein ("Response to abscisic acid" GO term); Glutathione transferase enzyme ("Glutathione metabolic process" GO term); hS-adenosylmethionine decarboxylase enzyme ("Spermidine biosynthetic process" GO term); iPhosphatidylserine decarboxylase enzyme ("Phosphatidylethanolamine biosynthetic process" GO term); jSWEET protein ("Carbohydrate transport" GO term); lNitrate reductase enzyme ("Nitric oxide biosynthetic process" and "Nitrate assimilation" GO term); mDehydrin protein ("Response to water" GO term).

VIIa-2 (composed primarily of RIPKs [receptor-interacting protein kinase] kinases and PBLs; data not shown), ranked next, both with four representatives (Figure 8C).

3.5.3 | UR transcripts' validation by qPCR

Fourteen UR transcripts for 24 h treatment under water deficit were analyzed by qPCR to confirm the reliability and robustness of the gene expression pointed out by RNA-Seq. Out of 14 tested primer pairs, 12 were functional (Figure 9) in preliminary tests (cDNA amplification and efficiency assay), being forwarded for relative gene expression analysis. The two nonfunctional primer pairs (no cDNA amplification) were associated with AP2-ERF TF (Figure 7) and Shikimate kinase (A0A444ZXV4; "Cellular amino acid biosynthetic process") (Figure 6), respectively. The transcripts encoding the following proteins were analyzed in the qPCR assay (superscript letters in Figure 9): MYB-related transcript factor; NAC TF; CAMK_CAMK_CHK1 kinase; CMCG_MAPK kinase; RLP-Pelle_RLCK_Villa-2 kinase; HVA22-like protein; GST enzyme; SAMDC enzyme; phosphatidylserine decarboxylase enzyme; SWEET protein; NR enzyme; and dehydrin protein.

The functional primer pairs (for target transcripts and reference genes) had an amplification efficiency ranging from 93.0 to 101.0% (Table S1). Their specificity was confirmed by the presence of a single peak in the melting curves (Figure S1). The gene expression of the 12 qPCR targets (Figure 9; Table S1) agreed with the RNA-Seq data. The validated transcripts were associated with enriched GO terms, TFs and kinases proteins highlighted throughout the text. This result demonstrated the quality of the RNA-Seq libraries, as well as the robustness of the gene expression statistics used in the present work.

3.5.4 | Identification of potential microorganism transcripts

About 4% of the UETs in the analyzed libraries were associated with organisms not belonging to the Viridiplantae clade ("NVirid."; Figure 5A,B). Among these, we can highlight the presence of fungi and, to a lesser extent, bacteria (Figure 5A,B). The implemented annotation strategies/tools provided secure annotation of these microorganisms at the genus level. We have mined 95 different genera of Fungi and 17 different genera of Monera (specifically, Eubacteria; data not shown). Figure 10 shows the five most abundant genera for Fungi and Eubacteria. For the first clade, this group was composed (in descending order) of: *Schizosaccharomyces*, *Saccharomyces*, *Neurospora*, *Aspergillus*, and *Filobasidiella*. For the Eubacteria, the following stood out (in descending order): *Bradyrhizobium*, *Rickettsia*, *Bacillus*, *Streptomyces*, and *Escherichia* (Figure 9).

3.5.5 | Schematic illustration of the major research findings

Major findings of the present work, regarding the four studied approaches (physiological, biochemical, and transcriptional fine-tunings, as well as identification of possible symbiotic microorganisms), are summarized in Figure 11. From this illustration, some outstanding *S. scabra* resilience characteristics are highlighted.

4 | DISCUSSION

4.1 | Physiological and biochemical dynamics of *S. scabra* under water deficit

This study conducted a robust analysis of the physiological and biochemical adaptations of *S. scabra* under water deficit and rehydration conditions. The 13 parameters analyzed in leaf or root tissues gave us a panorama of this species' ability to cope with and recover from the studied condition. Such data demonstrated that *S. scabra* was able to

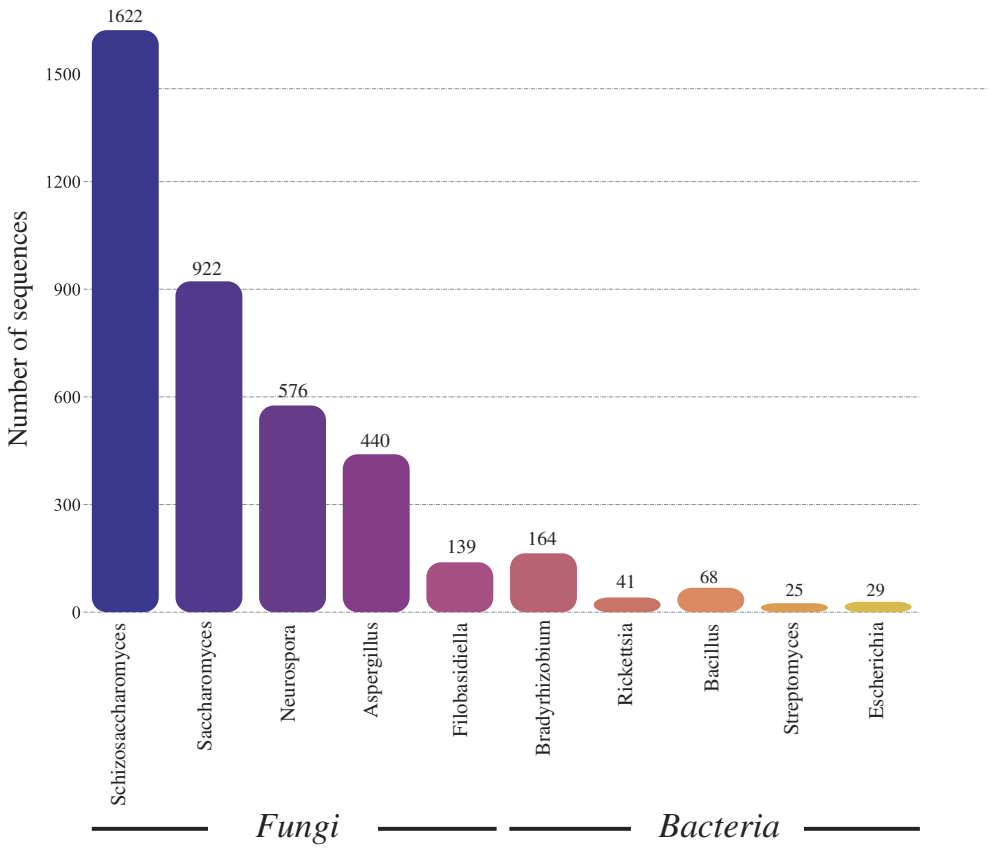

FIGURE 10 Fungal and eubacterial genera with most recurrently expressed sequences identified in the *Stylosanthes scabra* root transcriptome under analysis

quickly perceive (from 6 h water deficit treatment) changes in water availability, reorganizing its internal functions aiming at acclimatization to the new environmental condition.

According to Lawlor and Cornic (2002), the RWC is an important index for relating cellular water *status* to the body's metabolic condition due to its previously known and well-established effects. These authors mention that C3 metabolism plants (including *S. scabra*) with RWC below 50% suffer a strong negative impact on their "*fitness*." The RWC of *S. scabra* gradually decreased throughout the assay. For the WD.48h treatment, the analyzed individuals showed a 45% RWC. Nevertheless, when subjected to the rehydration condition (RD.6h), the analyzed plants recovered cell turgidity quickly, indicating high hydraulic conductivity and tolerance to low water availability, corroborating such ability for C3 species of the Brazilian Caatinga (Frosi et al., 2012; Rivas et al., 2017).

Associated with the decrease in RWC, simultaneous reductions in stomatal conductance (g_s) and net CO_2 assimilation (*A*) are commonly observed in plants in general. Stomata closure is one of the earliest responses to water deficit stress and was already observed in the WD.6h treatment in *S. scabra*. This closure aims to reduce water loss through evapotranspiration. We observed a reduction in "g_s" throughout the water deficit assay and also in the RD.6h treatment. However, the decline in "*A*" occurred only in the water deficit treatments (WD.6h, WD.24h, and WD.48h). In the RD.6h treatment, this parameter returned to a level similar to its respective control. The quick recovery of "*A*" may represent a strategy to increase WUE (whose value was significantly reduced at WD.48h), providing the possibility of plant growth with minimal water loss by evapotranspiration. According to Galle et al. (2011), this quick recovery of "*A*," coupled with still reduced "g_s" values, may occur due to mesophilic conductance. Flexas et al. (2008) point out that CO_2 conductance in the mesophyll is a dynamic process and changes in response to environmental variables, thus counterbalancing the mechanisms required to reduce stomatal conductance (aiming to decrease evapotranspiration). Regarding WUE throughout the assay, it remained at the levels of the respective controls (except for the most prolonged stress treatment, WD.48h). This parameter's maintenance in the WD.6h and WD.24h treatments—associated with the decrease of "g_s" and "*A*" in these same treatments—deserves to be highlighted. WUE refers to the biomass produced, or grain yield (or other plant parts with agronomic value), per unit of water used in crop plants (Hatfield & Dold, 2019). WUE maintenance in most of the treatments performed, added to its rapid recovery in RD.6h, reveals us an important resilience characteristic of *S. scabra* plants.

Regarding parameters associated with *S. scabra* photochemistry, although reductions in variables related to gas exchange were observed since the WD.6h treatment, qP and ETR were statistically reduced at WD.24h. In addition, ETR was statistically reduced in the WD.48h treatment. However, the quick recovery of these parameters in RD.6h to the level of the respective control is noteworthy. It is well known that water deficit can lead to changes in photosynthetic rate. These changes can be caused either by stomatal closure or damage to

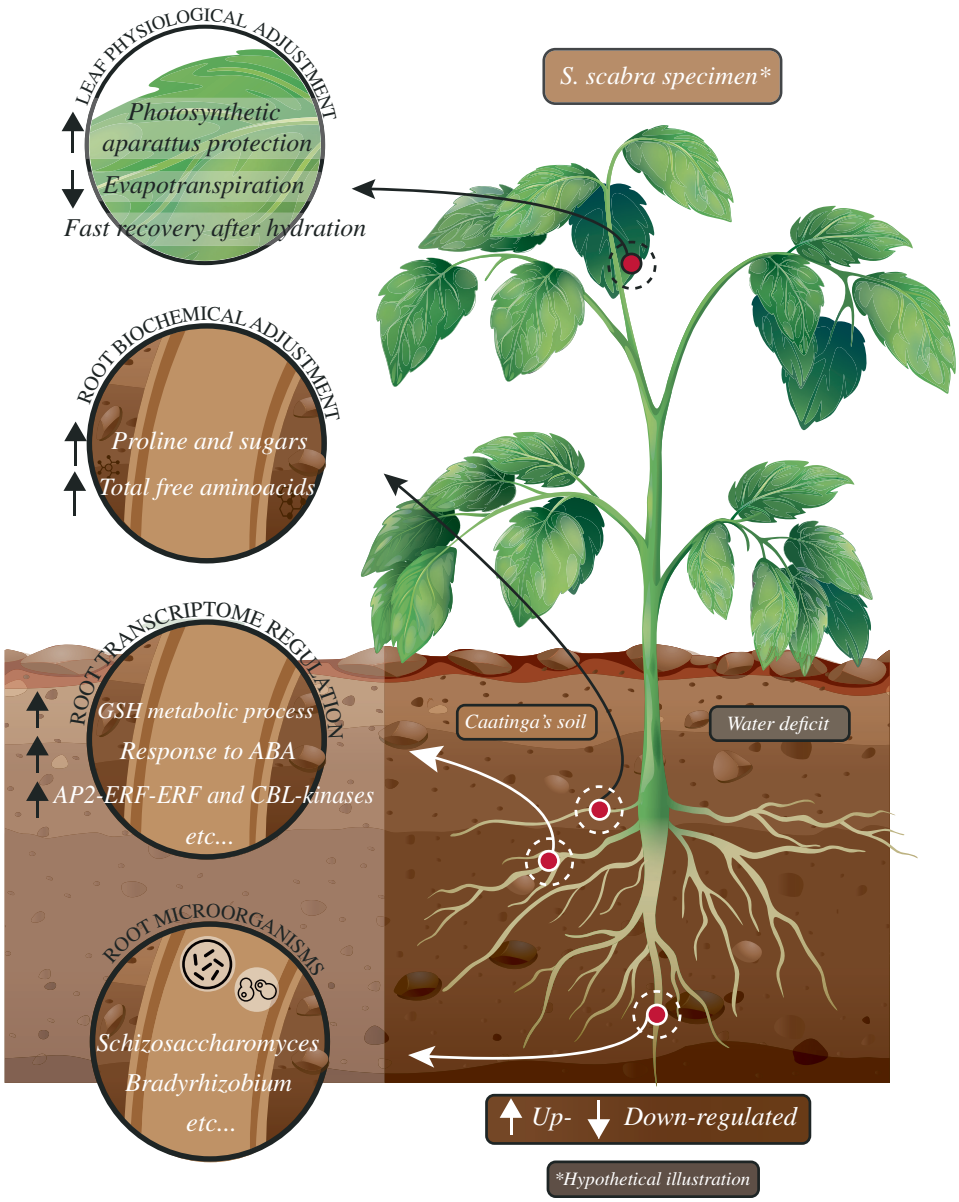

FIGURE 11 Schematic summary of some major findings of the present study. ABA, Abscisic acid; AP2-ERF-ERF, APETALA2/ETHYLENE RESPONSIVE FACTOR ERF subfamily; CBL-kinases, CBL-interacting serine/threonine-protein kinases; GSH, glutathione

the photosynthetic apparatus due to disturbances in photochemical reactions (Wu & Bao, 2011). Water deficit can reduce the ETR for photosynthesis and decrease the photochemical activity of PSII (qP). It causes overexcitation and photoinhibition damage to the PSII reaction centers (Meng et al., 2016). This type of damage results in weakened plants that are more susceptible to other environmental stresses, affecting plant growth and the plant establishment in the natural environment (dos Anjos et al., 2015). Species such as *S. scabra*—which showed a rapid ability to recover the photosynthetic apparatus activity—show increased phenotypic plasticity and greater ability to face unfavorable environmental conditions.

The maintenance of the photosynthetic pigment contents, chlorophyll "*a*" and chlorophyll "*b*," throughout the assay and the recovery and further maintenance (from WD.24h) of the carotenoids (auxiliary pigments in photosynthesis) content in the other water deficit times, may have contributed to a quick recovery of photosynthetic rates in the rehydration treatment. Collectively, the cited molecules, also called assimilation pigments, are some of the most important compounds in plants, as they affect photosynthesis intensity and the resultant biomass production (Zielewicz et al., 2020).

Regarding biochemical data, we observed that leaf and root TSC indexes, when different between treatments and their respective controls, showed distinct trends: an increase in roots (WD.24h and RD.6h) and a decrease in leaves (WD.6h and RD.6h). TSCs (such as sucrose, glucose, and fructose) play a relevant role in maintaining the overall plant structure and growth (Rosa et al., 2009). Lemoine et al. (2013) suggested the regulation of these compounds' content is complex. According to these authors, there are changes in the plant sugar distribution during unfavorable abiotic conditions, and root tissue is often favored. TSCs have diverse functions in plants. They act as metabolic resources and cell structural constituents and also as signaling molecules or

present effective participation (osmolytes accumulated in the roots, e.g.) in response to abiotic stresses, such as water deficit (Ahmad et al., 2020; Rosa et al., 2009).

The changes in leaf and root contents of TFA and proline proved to be quite informative. The root tissue actively invests in increasing TFA and proline syntheses in WD.24h and WD.48h treatments. This compound category (amino acids) has a robust availability of information, and its function in response to conditions causing dehydration is well known. In addition to sugars, certain TFAs, such as proline, act to adapt the osmotic relations in plants under dehydration (Izanloo et al., 2008; Souza et al., 2004). Increased root synthesis of such compounds aims to maintain turgor pressure and low cellular water potential, which are important to maintain metabolic functions and water uptake, respectively (Hayat et al., 2012). Furthermore, osmotic adjustment facilitates recovering metabolic activities after stress relief (Abid et al., 2018). In the leaf, during water deficit treatments, such parameters remained constant throughout the assay, except for WD.6h (where TFA is reduced and proline content is elevated). Maintaining this amino acid content in the leaf also helps decrease this organ's osmotic potential. It reflects the stability and functionality of the photosynthetic apparatus (Chun et al., 2018).

4.2 | Enriched biological processes and what they suggest

In addition to the physiological effort reported above, we molecularly found that *S. scabra* plays an active role in response to water deficit. When categorizing the GO terms of the UR transcripts for the 24 h water deficit treatment, nine biological processes (Figure 5) were enriched. These processes give us an overview of the molecular actions implemented by the species under study, complementing our understanding of the physiological and biochemical changes for the treatment under consideration.

The enriched "Glutathione metabolic process" term showed the highest volume of component elements. Interestingly, the vast majority of transcripts were associated with the GST enzyme (isoform presenting upregulation validated by qPCR; Figure 8). This enzyme counters a common effect of all cellular stresses except hypoxia: the increased production of reactive oxygen species (ROS) (Castro et al., 2021). ROS has a dual impact on the eukaryotic cell. They can act as signaling molecules, triggering signal transduction pathways in response to stresses. However, their strong oxidative properties can cause irreversible cellular damage at high levels (Huang et al., 2019). Recently, Srivastava et al. (2019) overexpressed a GST from rice (*Oryza sativa*) in Arabidopsis and studied the transgenic's tolerance to drought. These authors analyzed several parameters and, among them, found that levels of MDA (malondialdehyde; a marker of oxidative lipid damage caused by ROS in cell membranes) and ROS were reduced in the transgenic plant. The modified organism showed greater drought tolerance than its wild-type counterpart (Srivastava et al., 2019).

In turn, the enriched "Cellular amino acid biosynthetic process" and "Carbohydrate transport" GO terms may have molecularly contributed to the observed increase in the following root biochemical parameters in the WD24h treatment: TFAs and TSCs. For the first term, we found that the shikimate kinase (SK) enzyme showed the most modulation. SK, responsible for the chorismate synthesis, catalyzes the fifth step of the shikimate pathway (which produces aromatic amino acids) (Herrmann & Weaver, 1999). By studying wheat under irrigation suspension stress, Michaletti et al. (2018) found increased chorismate levels (in metabolomics analyses) and induction (from proteomics analyses) of the shikimate pathway. According to these authors, aromatic amino acids are susceptible to oxidation, and, in their free form, they may have a protective function against ROS.

Regarding the second GO term mentioned above, a SWEET protein (an acronym for "*Sugars Will Eventually be Exported Transporters*") (isoform presenting upregulation validated by qPCR; Figure 8) was the most prominent element. This protein group acts in sugar transport (Julius et al., 2017), and its participation in response to dehydration-causing stresses has recently been evaluated. Lu et al. (2019) observed that ectopic expression of MdSWEET17 (*Malus* × *domestica* cv. Royal Gala SWEET17) increased drought tolerance in tomato (*Solanum lycopersicum*). Overexpression of MdSWEET17 promoted accumulation of soluble sugar in leaf (root tissue was not analyzed), especially fructose and glucose, under drought conditions (Lu et al., 2019).

The "response to water" GO term is defined as "any process that results in a change in the state or activity of a cell or organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a stimulus reflecting the presence, absence, or concentration of water" (https://www.ebi.ac.uk/QuickGO/term/GO:0009415). For the term under consideration, a dehydrin enzyme (isoform presenting upregulation validated by qPCR; Figure 8) showed the most modulation. The UR expression of such proteins can be considered a marker associated with abiotic stresses, especially dehydration processes (Liu et al., 2017). The function of this protein group is related to the stabilization of membranes, enzymes, and nucleotides. Furthermore, the high content of antioxidant amino acids—such as lysine, histidine, and glycine in their primary structure—can counter ROS through oxidative changes (Liu et al., 2017). Recent data have shown that transgenic plants overexpressing dehydrins show superior physiological performance compared to the wild-type counterpart when subjected to dehydration-causing stresses, such as drought and low temperatures (Guo et al., 2019; Halder et al., 2018).

"Response to abscisic acid" was another GO term enriched and closely associated with the stress studied here. The referred hormone is considered a molecular actor of great importance, as it "orchestrates" several plant responses to abiotic stresses, such as drought and high salinity (Fernando & Schroeder, 2016), among others. The most modulated element for the term at issue was an HVA22-like protein (isoform presenting upregulation validated by qPCR; Figure 8). These proteins are still poorly studied. However, they are known as small membrane-anchored proteins (Chen et al., 2002). One of the few functional characterization assays for HVA22 has been performed in *Citrus cinensis*. The analyzed CcHVA22 (specifically CcHVA22d) was found to be associated with the regulation of vesicular traffic in

cells under dehydration. Overexpression of the CcHVA22d-coding gene resulted in a more tolerant transgenic tobacco plant to the mentioned stress, compared to non-transgenic ones. There was also a reduction in the damage caused by oxidative stress in transgenic individuals (Gomes-Ferreira et al., 2019).

In turn, NR enzymes (isoform presenting upregulation validated by qPCR; Figure 8) were the most modulated elements for the GO terms "Nitric oxide biosynthetic process" and "Nitrate assimilation." This enzyme is associated with the first step of the nitrate assimilation pathway in plants (Berger et al., 2020). Previous studies have reported that increasing nitrogen-associated metabolism increased drought tolerance in plants. This fact was verified by Xiong et al. (2018), who reported that drought increased nitrogen uptake and assimilation in *Brassica campestris* ssp. Chinensis roots. According to these authors, increased "N" availability may alleviate water stress by increasing osmolytes in the roots (as suggested by TFA and proline data presented in this work), also promoting increased root biomass, which would reduce the water restriction impact. In turn, Zhong et al. (2015) reported that high nitrate transport and assimilation levels contributed to increased drought tolerance of a transgenic *A. thaliana* strain.

Regarding PAs, they play an important role in regulating plant morphology and embryo development and participate in response to stressful conditions (Chen et al., 2019). For stress response, we found the enrichment of the "Spermidine biosynthetic process" term, which is an important PA. For this term, an element associated with an SAMDC enzyme (isoform presenting upregulation validated by qPCR; Figure 8) presented a high modulation. SAMDC is a key enzyme involved in spermidine and spermine (another important PA) biosynthesis (Hanfrey, 2006). Constitutive expression of an SAMDC gene (from *Capsicum annuum*) in Arabidopsis increased the transgenic's drought tolerance by inhibiting ROS accumulation (Wi et al., 2014). A similar fact (increased tolerance) was observed in transgenic Arabidopsis overexpressing an SAMDC from beetroot (monosomal strain M14) under salt stress. Increased tolerance was also associated with greater efficiency in ROS handling (Ji et al., 2019). The positive impacts of spermine in protecting against ROS in plants subjected to drought have also been reported (for a review, see Hasan et al. (2021)).

The last enriched term to be mentioned is "Phosphatidylethanolamine biosynthetic process." Phosphatidylethanolamine (PE) is an important phospholipid component of cell membranes that participates in plant signal transduction pathways (Nakamura, 2017). This phospholipid is synthesized from phosphatidylserine, and the reaction is catalyzed by the phosphatidylserine decarboxylase enzyme (PSS; Voelker, 1997). A PSS (isoform presenting upregulation validated by qPCR; Figure 8) was the most modulated element for the term at issue. This enzyme activity is associated with altered membrane phospholipid composition during acclimation to water stress, as found by Larsson et al. (2006) for oats (*Avena sativa*). According to these authors, membrane integrity is vital during water deficit conditions. Furthermore, it is also of fundamental relevance during subsequent rehydration to maintain compartmentalization and thus cell functionality (Larsson et al., 2006).

4.3 | Regulatory proteins in *S. scabra* under water deficit: Focus on TFs and kinase proteins

TFs and PKs are important regulators of gene activity and primary modulators of signaling pathways, respectively. Their actions underlie the proper functioning of many cellular processes, from cell development to stress response (Danisman, 2016; Falak et al., 2021; Lehti-Shiu & Shiu, 2012). Regarding TFs, AP2-ERF (ERF subfamily), MYB-related (isoform presenting upregulation validated by qPCR; Figure 8), and NAC (isoform showing upregulation validated by qPCR; Figure 8) were the groups with the highest number of induced representatives, suggesting the importance of their recruitment in the acclimation of *S. scabra* to WD24h treatment.

Genes of the AP2-ERF-ERF subfamily are involved in both the response to pathogenesis (Zang et al., 2020) and the response to unfavorable soil and climate conditions. Regarding abiotic parameters, Seo et al. (2010) reported that overexpression of BrERF4 (*Brassica rapa* ERF4) increased tolerance to salt stresses in transgenic Arabidopsis strains. Overexpression of another tomato AP2-ERF-ERF gene (JERF3) in transgenic rice organisms increased their tolerance to drought and osmotic stress (Zhang et al., 2010). Recently, the regulatory networks of Arabidopsis' AP2-ERFs were scrutinized, revealing the profound impact of these TFs on this model plant's physiology under stress (Xie et al., 2019).

In turn, MYB TFs are classified into at least three subfamilies: MYB-related, R2R3, and R1R2R3-MYB (He et al., 2019). Most reports of MYB genes involved in stress response are associated with the R2R3 group (e.g., He, Liu, et al. (2020); for review, see Li et al. (2019)). However, in addition to our transcriptomics data, some recent reports addressing the functional characterization of MYB-related TFs shed light on their role in plant response to dehydration-causing stresses. Zhao et al. (2019) proposed that a representative of the MYB group, LcMYB2 (*Leymus chinensis* MYB2), showed high expression levels for 24 h treatment under the effect of the ABA hormone or mannitol (osmotic stress) in *L. chinensis*, being expressed mainly in root tissue. Overexpression of LcMYB2 in Arabidopsis increased the transgenic's tolerance to water deficit stress by increasing osmoprotectant accumulation and promoting root growth.

Regarding the NAC TF family, many scientific studies indicate their involvement in the plant response to dehydration stresses. Functional characterization assays reveal that NACs can act as positive regulators that can be employed in genetic engineering strategies to obtain organisms with increased drought tolerance. Examples can be found in the works of Wang et al. (2021) and Ma et al. (2021). Both author groups obtained transgenic Arabidopsis strains that were more drought tolerant due to overexpression of NAC TFs originating from potato (*Solanum tuberosum*) and pepper (*C. annuum*), respectively.

Regarding UR kinome, the CAMK_CAMKL-CHK1, CMGC_MAPK, and RLK-Pelle_RLCK-VIIa-2 families (all presenting isoforms with upregulation validated by qPCR; Figure 8) stood out (Figure 7C). All of them showed high numbers of induced representatives in cowpea (a legume plant, as *S. scabra*) subjected to different root dehydration times (Ferreira-Neto et al., 2021). Representatives of the

CAMK_CAMKL-CHK1 family have also been induced in grapes in response to osmotically disruptive stresses, such as polyethylene glycol, salt (NaCl, 200 mM), and drought (Zhu et al., 2018). The major components of this group in the *S. scabra* UR kinome (WD24h treatment), CBL-interacting protein kinases, are closely related to the dehydration response in other plants already analyzed. It is exemplified in the recent work by Singh et al. (2020), in which a CBL-interacting protein kinase, named AdCIPK5 (*Arachis diogoi* CIPKK5), provided tolerance to osmotic or salt stress in transgenic tobacco strains. In addition, analyses of different physiological parameters revealed that the transgenic plants maintained higher chlorophyll content, higher catalase activity, and lower H_2O_2 and MDA levels during abiotic stress conditions (Singh et al., 2020).

Concerning CMGC_MAPKs, we observed that the cascade of events triggered by them relies on highly conserved modules of external signal transduction, resulting in adaptive plant responses (for review, see He, Wang, et al., 2020). Recently, Jeong et al. (2020) reported that a MAPK positively regulates responses to drought and ABA. The authors analyzed knockout pepper plants for the CaAIMK1 (*C. annuum* ABA Induced MAP Kinase 1) gene and transgenic Arabidopsis plants overexpressing the referred transgene. In the silenced lines, the resulting plants showed a drought-sensitive phenotype characterized by altered responses to ABA, including low leaf temperature and elevated stomatal opening. Regarding the transgenic lines, a drought-tolerant phenotype was observed, characterized by reduced water loss levels from transpiration and overexpression of stress-related genes in Arabidopsis (Jeong et al., 2020).

Regarding the RLK-Pelle_RLCK-VIIa-2 kinases, in addition to their association in response to root dehydration in cowpea by Ferreira-Neto et al. (2021), there are no explicit reports of their participation in response to other stresses. However, reports of their upregulation in legumes under dehydration stresses turn them into important biotechnological targets.

4.4 | Revealing some microbial "guests" of *S. scabra*

Usually, plants are inhabited by a variety of microbes. This relationship occurs both in plant organs located above and below ground and may result in mutualistic benefits or not (in the pathogens' case). Colonizing (mutualistic) microbes can be categorized as epiphytes (present on the plant surface), endophytes (present inside the tissues), and rhizospheric (inhabiting the soil, close to the roots). These organisms have plant growth promotion, disease suppression, removal of toxic compounds, and nutrient assimilation properties for their host (Harman et al., 2021). Due to the novelty of such an approach for the species under consideration, identifying probable hosts is enlightening.

Most of the identified microbes were fungi and bacteria representatives (the latter in a low sequence number), which are routinely identified in the plant clade. Regarding fungi, five genera stand out, with more than a hundred sequences identified. Among these, only the genera *Schizosaccharomyces* and *Filobasidiella* have no previous reports of interactions with plants. Interestingly, *Filobasidiella* is a genus of fungi that are pathogens of their peers (i.e., fungi that infect other fungi; Rodriguez-Carres et al., 2010).

Information for the relationship plants versus fungi of the *Saccharomyces* genus suggests a dual relationship: such microorganisms can be both pathogenic (Gognies et al., 2001) and mutualistic. In terms of their positive impact, it has been observed that *Saccharomyces cerevisiae* (one of the main representatives of the genus now under consideration) promotes plant growth under field or laboratory conditions (Karajeh, 2013).

For the *Neurospora* genus, there is information on the relationship "plants versus *N. crassa*" (considered a model fungus). Although this fungus is saprotrophic, it usually appears on vegetation or burned trees after forest fires. Kuo et al. (2015) found that *Pinus sylvestris* may be host to the mentioned fungus. In this plant species, *N. crassa* can exhibit both endophytic and pathogenic behavior. According to the cited authors, the lifestyles of *N. crassa* are probably controlled by environmental and plant host interaction factors.

Although species of the *Aspergillus* genus are known to act as plant pathogens, there are several indications of their mutualistic impact. *Aspergillus* members are used in various forms and treatments for their positive effects on plants, benefiting their hosts in several ways. In direct applications, *Aspergillus* spp. assist plants in phosphate uptake, in addition to promoting growth through the production of biologically active compounds (including auxins, gibberellins, and other phytohormone-like compounds, and a number of secondary metabolites; Hung & Lee, 2016).

Finally, bacterial sequences indicated the presence of another group of microorganisms found in our RNA-Seq libraries that deserve to be mentioned. As mentioned by Chakraborty et al. (2016), their presence in these libraries is not uncommon. Several reports in the literature indicate the bacterial presence in analyses where their detection is not the main focus of the implemented actions (Hadfield & Eldridge, 2014; Strong et al., 2014). Furthermore, in a recently published study, Sangiovanni et al. (2019) highlighted the importance of identifying these microorganisms in RNA-Seq data, adding value to the sequenced data and the information made available as an end product.

Because it is a legume plant, *S. scabra* notoriously exhibits a symbiotic relationship with nitrogen-fixing bacteria, as is the case of the *Bradyrhizobium* genus that comprises bacteria with this capacity, presenting the largest number of identified Eubacteria sequences. *Bradyrhizobium* is one of the most cosmopolitan and diverse bacterial groups promoting nodulation in various host legumes (Jaiswal & Dakora, 2019). The presence of *Bradyrhizobium* members was previously identified in *Stylosanthes* representatives (Delamuta et al., 2016).

The *Bacillus* genus was the second most abundant in terms of identified sequences. Several *Bacillus* taxa are responsible for significantly reducing the incidence or severity of several diseases in plant hosts (Choudhary & Johri, 2009). Some strains are considered true biological tools for crop improvement through biomolecular changes (Radhakrishnan et al., 2017).

Rickettsia, in turn, was the third genus with the highest abundance of sequences. The only other evidence of the natural occurrence of *Rickettsia* in plants was reported for papaya, where it was reported as pathogenic (Davis et al., 1998). However, in the past decade, a controlled assay (Caspi-Fluger et al., 2012) proved that individuals of this genus could also develop on cotton (*Gossypium hirsutum* "Acala") leaves.

Then, the *Escherichia* genus stood out. These bacteria can act as plant growth promoters, as Nautiyal et al. (2010) reported in analyses with maize. Finally, we highlight the presence of the *Streptomyces* sequences in the analyzed RNA-Seq libraries. *Streptomyces* spp. can act as both pathogenic or symbiotic bacteria. There is evidence that they colonize the rhizospheres of plant roots and internal tissues. It has been suggested that the production of antibiotics by these organisms may protect host plants against phytopathogens (for a review, see Tarkka et al. (2008); Olanrewaju and Babalola (2019)).

5 | CONCLUSIONS

This pioneering work provides relevant information about *S. scabra*, an orphan crop whose ecophysiological behavior is highly adapted to arid and semiarid environments with water scarcity, high salinity, and elevated temperatures. Our results showed that this species is able to quickly recover (rehydration treatment) and perceive fluctuations in the water regime (water deficit treatments), altering physiological, biochemical, and molecular parameters (i.e., gene expression). Physiologically, mechanisms that minimize evapotranspiration or protect the photosynthetic apparatus stood out. Biochemically, it was found that the root tissue actively invests in the synthesis of compounds (proline and sugars) that can act as osmolytes, emphasizing the importance of osmoregulation to water deficit acclimation. Consistently, transcriptome and qPCR analyses showed that a set of enriched biological processes with UR transcripts involved in protective functions against ROS, or encoding enzymes participating in important metabolic pathways, might contribute to *S. scabra* response to water deficit. Additionally, several UR kinases and TFs were identified. Such transcripts become important biotechnological targets for further studies in legume plants. Finally, sequences of some microbial clades were identified in the *S. scabra* root tissue, helping to understand the scenario under natural conditions without inoculation, especially considering fungi. Future works on metagenomics of the studied species are mandatory for a more complete picture of such microbial agents. Taken together, this study not only reveals insights into the physiological, biochemical and molecular mechanisms underlying the *S. scabra* response to water deficit but also provides candidate genes that may be useful in developing drought-tolerant crop varieties through biotechnological applications.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. Raw reads for RNA-Seq data have been deposited to the NCBI SRA (https://www.ncbi.nlm.nih.gov/sra) and are available under the BioProject accession number PRJNA837909.

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