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Abstract: Drought is a major threat to coffee, compromising the quality and quantity of its production. We have analyzed the core proteome of 18 *Coffea canephora* cv. Conilon Clone 153 and *C. arabica* cv. Icatu plants and assessed their responses to moderate (MWD) and severe (SWD) water deficits. Label-free quantitative shotgun proteomics identified 3000 proteins in both genotypes, but less than 0.8% contributed to *ca.* 20% of proteome biomass. Proteomic changes were dependent on the severity of drought, being stronger under SWD and with an enrolment of different proteins, functions, and pathways than under MWD. The two genotypes displayed stress-responsive proteins under SWD, but only *C. arabica* showed a higher abundance of proteins involved in antioxidant detoxification activities. Overall, the impact of MWD was minor in the two genotypes, contrary to previous studies. In contrast, an extensive proteomic response was found under SWD, with *C. arabica* having a greater potential for acclimation/resilience than *C. canephora*. This is likely supported by a wider antioxidative response and an ability to repair photosynthetic structures, being crucial to develop new elite genotypes that assure coffee supply under water scarcity levels.

Keywords: acclimation; climate change; coffee; comparative proteome; water deficit response

1. Introduction

Drought is one of the major environmental constraints affecting plant growth and crop yield [1–3]. Global climate changes result in more frequent, prolonged, and severe drought periods, alterations that are expected to be particularly aggravated in tropical regions [4,5]. In plants, water scarcity affects morphological, physiological, and biochemical processes, including C-assimilation and partitioning, respiration, nutrient uptake, translocation, and whole metabolism [6–9]. Water deficit primarily affects the cell turgidity and promotes stomatal closure, controlling transpiration rates, and water loss, but will also unavoidably restrict CO_2 supply to photosynthesis, ultimately impairing leaf metabolism and plant growth [7,10]. Under longer periods and greater drought severity, the photosynthetic

machinery becomes increasingly impaired at the photochemical and biochemical levels due to impacts in pigment pools, photosystems functioning, enzyme activities (namely, ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO), and membrane integrity [10–14]. A secondary stress also occurs, related to an increase in reactive species of oxygen (ROS) and chlorophylls. Nonetheless, given that plants have often evolved under recurrent exposures to drought, they have acquired highly efficient stress defense systems, involving adjustments at several scales, from the gene to the whole-plant level. Plant defense mechanisms include those associated with the strengthening of the antioxidative system, comprising enzymes (e.g., catalase, superoxide dismutase, peroxidase) and non-enzymatic components (e.g., ascorbic acid, α-tocopherol, RFOs) [9,15–17], complemented with thermal dissipation mechanisms (e.g., photoprotective carotenoids) and the cyclic electron flow (CEF) involving photosystem (PS) I and/or II [18–20]. Altogether, these response mechanisms contribute to maintain energy balance and cell oxidative homeostasis.

Understanding the role of proteins in the response of plants to increasing water limitation is crucial to better characterize acclimation mechanisms and to contribute to assist in breeding programs for the development of tolerant cultivars [21,22]. Advances in next-generation proteomics offers a quick and accurate molecular approach to identify proteins and reveal pathways associated with the physiological responses of biological systems to abiotic stressful conditions [23]. For instance, a shotgun proteomic analysis of three wheat cultivars (*Triticum aestivum* L.) differing in their ability to maintain grain yield upon restricted water availability revealed significant differences in their response to drought through changes in the proteins involved in photosynthesis and the oxidative stress metabolism [24]. Similarly, label-free quantitative shotgun proteomic analysis of eight rice genotypes (*Oryza sativa* L.) upon exposure to water shortage, enabled the identification of 1253 non-redundant proteins across all genotypes, of which only eight increased significantly in abundance after drought, and being mainly associated with photosynthesis, oxidative stress response, proteolysis, and translation [25].

Coffee (*Coffea* species) is among the most important worldwide agricultural commodities, representing a significant source of income to many countries of the tropical region of South America, Africa, and Asia [9,20]. The *Coffea* genus comprises at least 125 species [26], but two clearly dominate the global trade: the allotetraploid *Coffea arabica* L. (Arabica coffee; 2n = 4x = 44) and one of its diploid ancestors, *C. canephora* Pierre ex A. Froehner (Robusta coffee; 2n = 2x = 22). *Coffea arabica* is by far the most significant in the global coffee production, but it is considered more sensitive to elevated temperatures than *C. canephora* [27–29]. Nevertheless, both inadequate temperatures and water availability conditions are the main environmental constraints to plant development and production [27]. However, some cultivars show a significant resilience under drought, extreme temperatures, and full sunlight, triggering a wide number of energy dissipation and antioxidant mechanisms [19,30–33] as well as by changing the lipid matrix of their chloroplast membranes [34–36]. For instance, genotypes of both *C. arabica* and *C. canephora* can display different drought tolerance levels, although in general, *C. canephora* is thought to be more tolerant than *C. arabica* to prolonged periods of drought, as empirically observed in coffee plantations [28].

Leaves of *Coffea* plants display a low stomatal conductance (g_s) even under optimal growth conditions and, thus, stomatal constraints—more than mesophyll or biochemical ones—have been shown to be the major limitations of photosynthesis under well-watered conditions [27,37,38]. Under increasing drought severity, non-stomatal constraints also occur, gradually becoming the most important limitation to photosynthesis, compromising mesophyll diffusional and photo/biochemical processes [20]. Drought-induced decreases in photosynthetic rates are further exacerbated by a decline in leaf development and enhanced shedding, thus reducing the photosynthetically active area, which ultimately affects plant growth and productivity [27]. Additionally, water shortage in the phase of fruit expansion and filling can also lead to appreciable productivity losses and decreased bean quality [39,40]. As an example, two consecutive years of severe drought (2014 and 2015) in the Espírito Santo State (Brazil) promoted an average decline of *C. canephora* yields close

to 15% in 2014 and to 48% in 2015. Strong aftereffects were still reported in the following year due to the fragile plant status after such prolonged water deficit periods (and high temperatures) [41]. Therefore, the identification and development of drought-tolerant genotypes is of crucial importance to maintain the sustainability of this crop and fulfill coffee demands.

This study uses a label-free proteomic approach to deepen the knowledge regarding the underlying mechanisms by which two cropped genotypes of the two mostly cultivated *Coffea* species respond to moderate and severe water deficits. To achieve this aim, we used two different genotypes: *C. canephora* cv. Conilon clone 153 (CL153), a late maturation/ripening diploid clonal variety created from Emcapa 8131 (also known as Vitória 13) by Incaper (Vitória, ES, Brazil) that has already shown some relevant drought tolerance [42,43] and *C. arabica* L. cv. Icatu Vermelho (Icatu), an introgressed tetraploid variety originated from a cross between *C. canephora* and *C. arabica* cv. Bourbon Vermelho that was further crossed with *C. arabica* cv. Mundo Novo and has a very high tolerance to drought under environmentally controlled conditions [20,33,44] and under field conditions [32]. Understanding the proteomic response of these genotypes to drought complements physiological, biochemical, and transcriptional leaf studies, previously performed under the same plants and the conditions studied here [19,20,42,44]. To our knowledge, this is the first in-depth proteomic analysis that has been presented for coffee, establishing the core proteome of *C. canephora* and *C. arabica* genotypes, and providing new and timely insights of the coffee's performance under future climate scenarios, contributing to the selection of new elite coffee genotypes.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seven-year-old potted plants of two cropped genotypes were used in this study. A total of 18 plants (9 per genotype) were grown since the seedling stage, in 80 L pots, in walk-in growth chambers (EHHF 10000, ARALAB, Albarraque, Portugal) under controlled environmental conditions of temperature (25/20 °C, day/night), relative humidity (*ca.* 70%), irradiance (*ca.* 750 µmol m^{-2} s^{-1} at the third upper part of the plants), photoperiod (12 h), and air [CO_2] (*ca.* 380 µL L^{-1}). Plants were maintained without restrictions of water (until the imposition of different water deficit levels, see below), nutrients (provided as in [45]) or root growth space.

2.2. Water Stress Gradual Exposure and Leaf Water Status

The implementation of different water availability levels was set as detailed in [20]. Briefly, plants were divided into three groups, the first was maintained under well-watered (WW) conditions throughout the entire experiment, with a predawn leaf water potential (Ψ_{pd}) above −0.35 MPa. In the other two groups, water deficit was gradually imposed along two weeks by partially withholding irrigation (with a partial water replacement of the amount lost in each pot every two days) until Ψ_{pd} stability between −1.5 and −2.5 Mpa (moderate water deficit, MWD) or below −3.5 Mpa (severe water deficit, SWD). These conditions represented approximately 80% (WW), 25% (MWD), or 10% (SWD) of maximal water availability in the pots [46]. After reaching the desired Ψ_{pd} values for MWD or SWD conditions, pot moisture was kept for another two weeks before sampling. Leaf Ψ_{pd} was measured immediately after leaf excision, using a pressure chamber (Model 1000, PMS Instrument Co., Albany, OR, USA) to characterize water deficit.

2.3. Protein Extraction and Digestion

Protein extraction was performed using three different plants for each treatment following the protocol initiated by [47] but with some modifications described in [19]. Protein concentration was determined by Coomassie blue dye-binding method using BSA as a standard [48]. Protein samples were resuspended in 30 µL LDS sample buffer (Invitrogen, Carcavelos, Portugal) and incubated for 5 min at 99 °C. For each sample,

50 µg of proteins was loaded onto a NuPAGE 4–12% gradient gel (Invitrogen, Carcavelos, Portugal) and submitted to a short electrophoretic migration for 5 min. After staining the gel with SimplyBlue Safe Stain (ThermoFischer, Oeiras, Portugal), the whole protein content from each well was excised from the gel as a single polyacrylamide band and digested with trypsin gold (Promega, Carnaxide, Portugal) for 1 h at 50 °C using 0.01% ProteaseMAX surfactant (Promega, Carnaxide, Portugal) [49].

2.4. Nano Liquid Chromatography Coupled to High Resolution Mass Spectrometry

Peptides were resolved following [19] with an Acclaim PepMap100 C18 (3 µm, 100 Å, 75 µm id × 50 cm) nanocolumn with a 90-min gradient developed at a flow rate of 0.2 µL min-1 from 4% to 25% of buffer B (80% CH3CN, 0.1% HCOOH) for 65 min, and then 25% to 40% for 15 min. The UltiMate 3000 LC system (Dionex-LC packings) was directly coupled to the mass spectrometer (Q-Exactive HF, ThermoFisher, Illkirch-Graffenstaden, France) via an electrospray source and operated in similar conditions as previously described [50]. Full-scan mass spectra was acquired from m/z 350 to 1800 with a resolution of 60,000. The 20 most abundant precursor ions in each scan cycle were sequentially subjected to fragmentation through high-energy collisional dissociation. MS/MS scanning was initiated when the AGC target reached 105 ions with a threshold intensity of 17,000 and potential charge states of 2+ and 3+ after ion selection was performed with a dynamic exclusion of 10 s. The instrument resolution for the MS/MS scans was set at 15,000.

2.5. Protein Identification and Label Free Quantification

MS raw files were analyzed using Mascot DAEMON version 2.3.2. The *C. canephora* reference protein sequence database was used for peptide-to-spectra matching for both genotypes (downloaded from NCBI the 1 July 2019). This database contained 25,574 protein entries totaling 10,251,572 amino acid residues [51]. The following parameters were used in the search: trypsin as enzyme, maximum of two missed cleavages, mass tolerances of 5 ppm on the precursor ion and 0.02 Da on the MS/MS, fixed modification of carboxyamidomethylated cysteine, and oxidized methionine and NQ deamidation as variable modifications. All peptide matches with a MASCOT peptide score below a *p*-value of 0.05 were filtered and assigned to a protein according to the principle of parsimony. Only proteins with at least two peptides were retained in the present study. In this condition, the false discovery rate (FDR) assessed with the corresponding reversed decoy database search was less than 1% at the protein identification level. Peptide-to-spectrum matches (PSMs) were counted for each protein for label-free quantification (spectral counting). For each protein, the normalized spectral abundance factor (NSAF) was calculated as the sum of its spectral counts observed in all the samples, divided by its molecular mass expressed in kDa [52]. The NSAF values were then presented as a ratio (%NSAF) by dividing the NSAF of each protein to the sum of all NSAF values detected for the whole dataset. The "Strict core proteome" was defined as all the proteins systematically identified in all the samples, while the "Core proteome" included proteins identified in at least one replicate of each experimental condition. The "Pan proteome" consists of all the proteins identified in this study. The annotation of proteins through gene ontology (GO) terms was performed using a DIAMOND search against the UniRef50 database and the homologs found in this search were mapped to GO terms through the GO annotation database of the reference genome, *C. canephora*, downloaded from Coffee Genome Hub (http://coffee-genome.org/download, accessed on 1 February 2021). GOslim terms were obtained by using the Map2Slim option in OWLTools and the generic GOslim list of GO entries.

2.6. Identification of Differentially Abundant Proteins to Drought

The abundance of proteins among the different conditions in each genotype was compared via multiple *t*-tests considering samples with unequal variance and bilateral repartition of differential abundances of proteins. Proteins with a *t*-test *p*-value < 0.05 and a log2 fold change (FC) > 1.5 were identified as differentially abundant proteins (DAPs).

Proportional Venn diagrams were constructed using Deep Venn [53]. The identified DAPs were then annotated following the reference genome of *C. canephora* as referred above.

2.7. Enriched Gene Ontologies, KEGG Pathways and Protein Networks upon Drought

Enrichment analyses for Gene Ontologies (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were applied to responsive DAPs under FDR < 0.01. The STRING version 11.0 web interface (https://string-db.org/cgi/input.pl, accessed on 1 June 2021) was used to predict the protein interaction network of DAPs with confidence scores higher than 0.7 and the organism option set to *Arabidopsis thaliana*. Proteins that did not interact with any others were removed. Resulting protein interaction networks were exported and visualized using Cytoscape version 3.7.2 [54].

3. Results

3.1. Main Proteome Features of Coffea canephora and Coffea arabica Regardless of Water Availability

Label-free quantitative shotgun proteomic analysis based on 18 plants from the two coffee genotypes allowed to obtain a dataset with 1.4 million MS/MS spectra, of which a total of 520,806 could be assigned to peptide sequences from the protein database. This resulted in a 36% detection rate, with a total of 23,871 peptide sequences found within the whole dataset. A total of 3425 polypeptide sequences were identified and quantified. Spectral counts are indicated in Table S1. Taking together all water availability treatments, 2982 polypeptides were found in CL153 and 3046 polypeptides in Icatu, from which 2752 proteins (*ca.* 90% of proteins per genotype) were commonly identified in the two genotypes.

The cumulative percentage of the normalized spectral abundance factor (NSAF) revealed that a relatively low number of proteins contributed to most of the protein biomass (Figure 1). In fact, half of the proteome biomass, as assessed by the NSAF, was explained by 134 and 142 proteins in CL153 and Icatu plants, respectively.

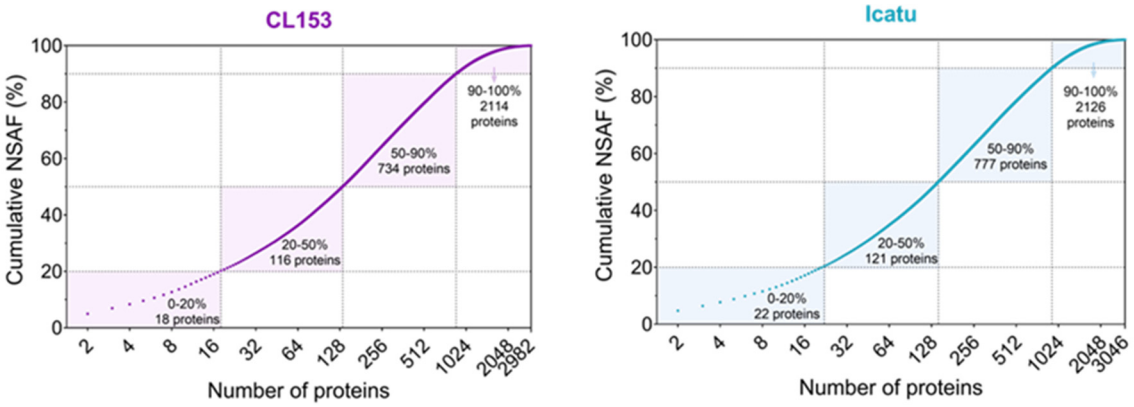

Figure 1. Normalized spectral abundance factor (NSAF) cumulative profiles for leaf proteome of *Coffea canephora* cv. Conilon Clone 153 (**left**) and *C. arabica* cv. Icatu (**right**) plants. The cumulative profiles were obtained after ranking proteins from the most to the least abundant, as estimated by the individual NSAF. For an easier visualization, the x-axis is in a log2 scale.

The components of the core proteome—proteins identified in at least one replicate of each experimental condition—were relatively common in CL153 (1210) and Icatu (1188), representing about one-third of the total proteins identified, the so-called pan proteome (Figure 2). The core proteome represented by far the greatest percentage of the total proteome measured by the NSAF values, reaching as much as 92–93% of the protein biomass. Additionally, when considering only proteins from the strict core proteome—proteins identified in all the samples—these represented 73% (CL153) and 47% (Icatu) of the global protein content (Figure 2).

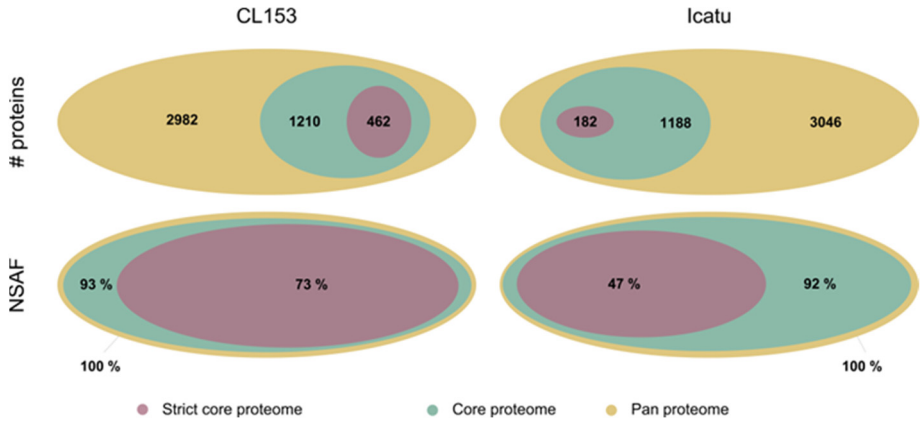

Figure 2. Core and pan leaf proteomes of *Coffea canephora* cv. Conilon Clone 153 (CL153: **left**) and *C. arabica* cv. Icatu (Icatu: **right**) plants considering all water treatments. The circles in the top diagrams represent the number of proteins that define each proteome category (Pan proteome, Core proteome, and Strict core proteome). The circles in the bottom diagram show how much these proteins contribute to the total proteome in terms of protein biomass assessed by NSAF contributions. The "Strict core proteome" was defined as all the proteins systematically identified in all the samples while the "Core proteome" included proteins identified in at least one replicate of each experimental condition. The "pan proteome" consists of all the proteins identified in this study.

Functional protein classification of the core-detected proteome showed a high similarity of biological functions between these genotypes, considering all water conditions tested (Figure 3). Among the main functions, proteins were mostly involved in biosynthetic processes, in response to stress, and in cellular nitrogen compound metabolic processes.

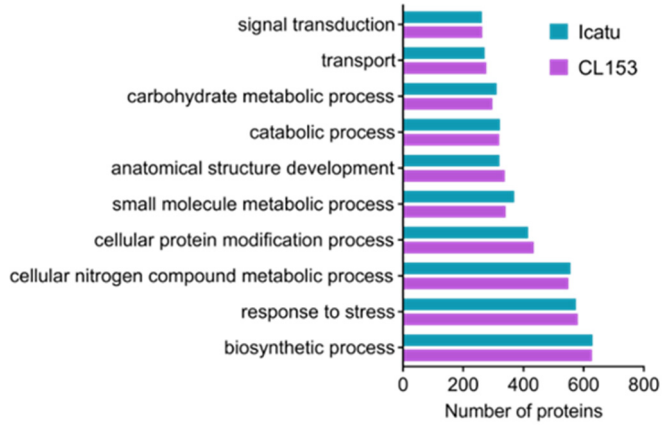

Figure 3. Distribution of the 10 most represented GOslim terms associated with the core proteome of *Coffea canephora* cv. Conilon Clone 153 and *C. arabica* cv. Icatu genotypes.

Because several closely related isoforms, identical in terms of functional annotation, were detected amongst the most abundant proteins, they were clustered into protein groups to compare their relative contribution to the global proteome (Table 1). Overall, 21 protein groups in CL153 and 16 in Icatu (representing *ca.* 0.7 and 0.5% of total protein numbers) were listed as the most abundant ones, since they contributed to the 20% cumulative NSAF, i.e., the proteome biomass being present in each genotype (Table 1). The auxin-binding protein ABP20 was the most abundant protein found in the two genotypes, followed by the Putative Bark storage protein A, although being much less abundant in Icatu.

Table 1. List of the most abundant leaf protein groups defined as contributing to the 20% cumulative NSAF for both *Coffea canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu plants and their respective Gene Ontology (GO) annotation considering biological processes. Proteins that shared the same functional description were merged into a protein group and their NSAF was summed. This was the case for two Putative bark storage protein A (Cc00_g11330 and Cc00_g11300) and two Oxygen-evolving enhancer protein 2 (Cc05_g00840 and Cc02_g11770) proteins. #: the protein is not on the top 20% NSAF for this genotype, the value is provided for comparison.

Protein Group	CL153 NSAF %	Icatu NSAF %	Biological Process
Auxin-binding protein ABP20	2.940	2.869	auxin-activated signaling pathway
Putative bark storage protein A	3.095	0.545 #	nucleoside metabolic process
Oxygen-evolving enhancer protein 1	1.978	1.868	Photosynthesis; photosystem II stabilization
Photosystem I reaction center subunit II	1.434	1.705	photosynthesis
Oxygen-evolving enhancer protein 2	1.793	2.015	photosynthesis
Histone H4	1.051	1.075	nucleosome assembly
Photosystem II 10 kDa polypeptide	1.009	0.678	photosynthesis
Plastocyanin	0.948	0.450 #	electron transport
Glyceraldehyde-3-phosphate dehydrogenase	0.934	0.898	glucose metabolic process
Ribulose bisphosphate carboxylase small chain SSU11A	0.928	1.095	carbon fixation
Phosphoglycerate kinase	0.881	0.759	glycolysis
Peroxisomal (S)-2-hydroxy-acid oxidase	0.729	0.679	metabolic process
Photosystem I reaction center subunit III	0.659	0.748	photosynthesis
Chlorophyll a–b binding protein CP29.2	0.654	0.748	photosynthesis
Polyphenol oxidase I	0.590	0.232 #	metabolic process
Carbonic anhydrase	0.565	0.386 #	carbon utilization
Ribulose bisphosphate carboxylase/oxygenase activase 1	0.510 #	0.713	photosynthesis
Photosystem II 22 kDa protein	0.472 #	0.693	photosynthesis
Chlorophyll a–b binding protein CP26	0.395 #	0.626	photosynthesis
Photosystem I reaction center subunit VI	0.463 #	0.619	photosynthesis
Putative Acidic endochitinase	0.147 #	0.577	carbohydrate metabolic process
Basic endochitinase	0.251 #	0.552	carbohydrate metabolic process
Uncharacterized protein At2g37660	0.315 #	0.495	metabolic process
Quinone oxidoreductase-like protein At1g23740	0.478 #	0.480	oxidation–reduction process
Peptidyl-prolyl cis–trans isomerase	0.336 #	0.470	protein folding

Functional categories of the most abundant proteins included biological processes mainly involved in photosynthesis, metabolic processes, and oxidation–reduction. However, of all abundant proteins found, 14 showed significant differences in their abundance between the two species ($p < 0.05$). Five of these proteins were more abundant in CL153 than Icatu and were mainly linked to metabolic processes (e.g., putative bark storage protein A, Photosystem II 10 kDa polypeptide, Plastocyanin, Polyphenol oxidase I, and Carbonic anhydrase). The remaining nine proteins were, in contrast, more abundant in Icatu, being mainly linked to photosynthesis, carbohydrate metabolic processes, and protein folding (e.g., Photosystem I reaction center subunit II, Oxygen-evolving enhancer protein 2, RuBISCO activase 1, Chlorophyll a–b binding protein CP26, Photosystem I reaction center subunit VI, Putative Acidic endochitinase, Basic endochitinase, Peptidyl-prolyl cis–trans isomerase, plus the uncharacterized protein At2g37660).

3.2. Altered Protein Abundance in Response to Water Deficit Severity

The two genotypes triggered a similar number of differentially abundant proteins (DAPs) in response to drought, but with higher numbers under SWD than MWD (Figure 4). MWD triggered 16 DAPs in CL153 and 15 in Icatu, of which almost half were down-regulated (nine and eight, respectively) in relation to well-watered (WW) plants. Under harsher drought conditions (SWD), the number of DAPs were three-times greater in relation

to the ones under MWD, reaching 33 in CL153 and 35 in Icatu, but being also predominantly down-regulated (22 for both genotypes). The full list of DAPs is indicated in Table S2.

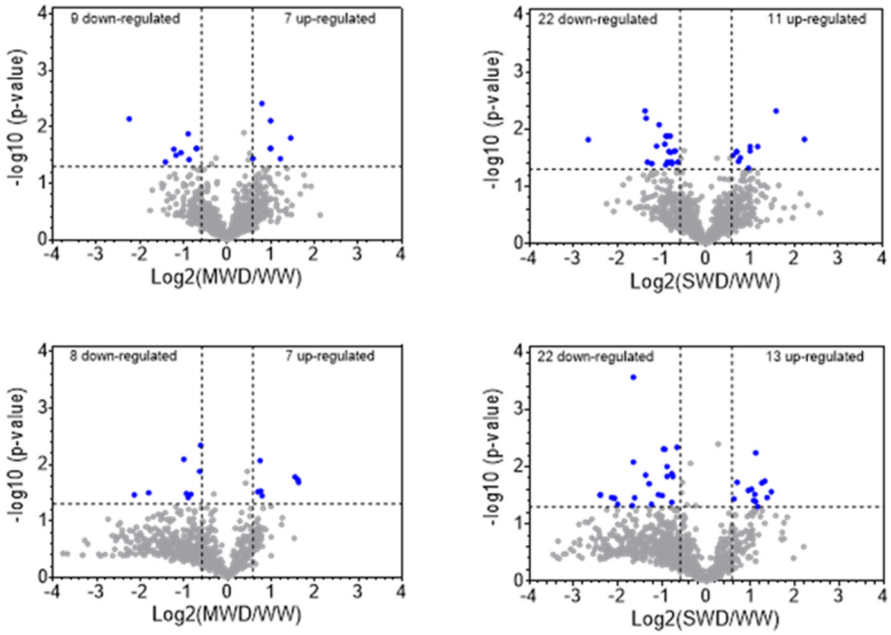

Figure 4. Volcano plots depicting the differentially abundant proteins (DAPs) found between well-watered plants (WW) and either moderate water deficit (MWD) or severe water deficit (SWD) for *Coffea canephora* cv. Conilon Clone 153 (**upper graphs**) and *C. arabica* cv. Icatu (**lower graphs**) genotypes. Significant DAPs are indicated with blue dots. The x-axis represents the log2 fold change in differences of protein abundance between treatments, while the y-axis represents the *p*-value (–log10).

Water deficit triggered a very specific response of DAPs in the two genotypes, since a low number of proteins was common to both drought treatments (Figure 5). In CL153, seven DAPs were commonly regulated by both MWD and SWD (six decreased in abundance), with the 29 kDa ribonucleoprotein A being the lowest abundant one and the Protein-L-isoaspartate O-methyltransferase being the highest abundant protein (Table S3). In Icatu, only five DAPs were simultaneously found in both MWD and SWD conditions (three decreased in abundance). A polygalacturonase and the L-ascorbate peroxidase 3 were, respectively, the least and the most abundant protein (Table S3).

From all DAPs, nine (CL153) and ten (Icatu) were uniquely found under MWD (Figure 5). The most abundant DAP in CL153 was the Pathogenesis-related protein 1B (fold change of 1.5) involved in plant defence. It is also worth mentioning the abundance of proteins in CL153 which were involved in the response to water deficit (as glycerate dehydrogenase) and in general cellular processes (as carbon utilization and cell wall biogenesis) (Table 2). In Icatu plants under MWD, the Polyphenol oxidase I (fold change of 1.6; Table 2) involved in metal ion binding was the most abundant DAP, followed by the Carotenoid 9,10-cleavage dioxygenase, and other proteins involved in ion homeostasis and photosynthesis (Table 2).

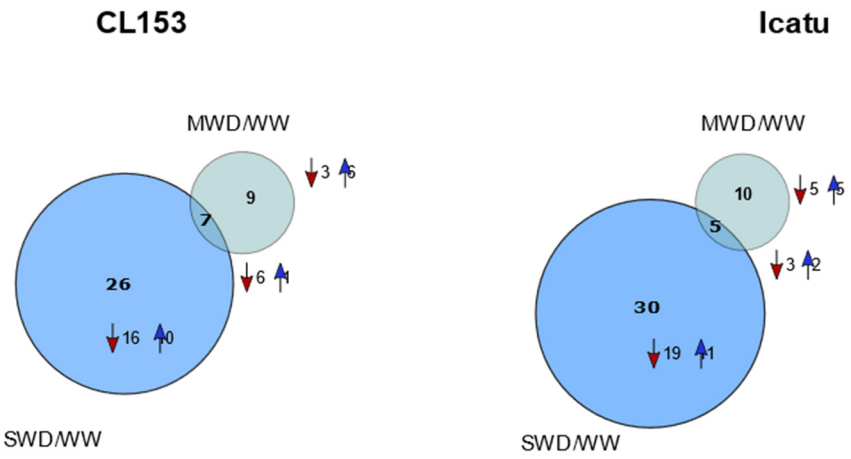

Figure 5. Proportional Venn diagrams showing the patterns of significant differentially abundant proteins (DAPs) found between moderate water deficit (MWD) and well-watered (WW) plants (MWD/WW) or between severe water deficit (SWD) and WW plants (SWD/WW) in *Coffea canephora* cv. Conilon Clone 153 (CL153) (**left**) and *C. arabica* cv. Icatu (**right**) genotypes. Numbers in bold indicate the total number of DAPs while arrows indicate the number of DAPs, either with decreased (red) or increased abundance (blue) in each comparison.

Table 2. Differentially abundant proteins that increased in abundance under moderate water deficit (MWD) in *Coffea canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu genotypes. log2 Fold Change (FC) values are indicated and their respective Gene Ontology (GO) annotation considering biological processes.

CL153	**log2 FC**	**Biological Process**
Glycerate dehydrogenase	0.594	cellular response to water deprivation; oxidative photosynthetic carbon pathway
Glutamate–glyoxylate aminotransferase 2	0.798	biosynthetic process; photorespiration
Carbonic anhydrase	1.000	carbon utilization
Putative Ubiquilin-1	1.000	ubiquitin-dependent protein catabolic process
Protein-L-isoaspartate O-methyltransferase	1.000	protein methylation; protein repair
Putative UDP-rhamnose	1.222	carbohydrate transport; plant-type primary cell wall biogenesis
Pathogenesis-related protein 1B	1.459	Plant defense
Icatu	**log2 FC**	**Biological process**
Putative Protein tas	0.696	cellular response to amino acid starvation
Fructose-1,6-bisphosphatase	0.746	fructose 1,6-bisphosphate metabolic process; starch catabolic process; sucrose biosynthetic process; photosynthesis
ADP, ATP carrier protein 3	0.763	mitochondrial transmembrane transport
L-ascorbate peroxidase 3	0.793	response to oxidative stress
Ferritin-1	1.547	leaf development; photosynthesis; iron ion homeostasis
Carotenoid 9,10(9′,10′)-cleavage dioxygenase	1.615	carotene, carotenoid catabolic process
Polyphenol oxidase I	1.627	ion binding

SWD triplicated the number of DAPs (26 in CL153; 30 in Icatu), being the majority down-regulated (Figure 5). The oxidoreductase NADP-dependent D-sorbitol-6-phosphate dehydrogenase was the most abundant DAP in both genotypes, although being almost twice abundant in CL153 than in Icatu (a fold change of 2.9 and 1.5, respectively). Nevertheless, the two genotypes also showed a specific response in the regulation of proteins to SWD. For instance, CL153 showed an abundance of proteins involved in detoxification responses as thioredoxin H-type 1 and the xylose isomerase, as well in photosynthesis as the PsbP domain-containing protein 4 (Table 3). In Icatu, SWD plants showed an abundance of proteins involved in responses to stress as Serpin-ZX, in detoxification processes as L-ascorbate peroxidase T and Malate dehydrogenase, as well as proteins related to the photosynthetic apparatus, as the chlorophyll *a–b* binding protein 4 and the RuBisCO activase 1 (Table 3).

Table 3. Differentially abundant proteins that increased in abundance under severe water deficit (SWD) in *Coffea canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu genotypes. log2 Fold Change (FC) values are indicated and their respective Gene Ontology (GO) annotation considering biological processes.

CL153	**log2 FC**	**Biological Process**
PsbP domain-containing protein 4	0.613	photosynthesis
Histone H2B	0.692	nucleosome assembly
NAD-dependent malic enzyme 62 kDa isoform	0.692	malate metabolic process; pyruvate metabolic process
40S ribosomal protein SA	0.737	cytoplasmic translation; response to osmotic stress
Xylose isomerase	0.778	carbohydrate metabolic process
Acidic endochitinase	0.963	cellular response to water deprivation; response to stress
Gamma carbonic anhydrase-like 2	0.999	photorespiration; response to stress
Thioredoxin H-type 1	1.000	cell redox homeostasis
UDP-glucose 4-epimerase GEPI48	1.170	metabolic process
Protein-L-isoaspartate O-methyltransferase	1.585	protein repair
NADP-dependent D-sorbitol-6-phosphate dehydrogenase	2.228	oxidoreductase activity
Icatu	**log2 FC**	**Biological process**
Chlorophyll a–b binding protein 4	0.625	photosynthesis
Putative Protein tas	0.697	cellular response to amino acid starvation
Probable lactoylglutathione lyase	0.951	catabolic process; response to cold
L-ascorbate peroxidase T	1.023	cellular response to oxidative stress
Short-chain dehydrogenase TIC 32	1.070	protein transport
Elongation factor 1-gamma 2	1.097	protein biosynthesis
Serpin-ZX	1.111	protease inhibitor; response to stress
L-ascorbate peroxidase 3	1.116	response to oxidative stress
Malate dehydrogenase [NADP]	1.164	carbohydrate metabolic process; malate metabolic process; tricarboxylic acid cycle
Ribulose bisphosphate carboxylase/oxygenase activase 1	1.256	photosynthesis
Probable glutathione S-transferase	1.322	auxin signaling pathway
V-type proton ATPase catalytic subunit A	1.376	Golgi organization; ion transport
NADP-dependent D-sorbitol-6-phosphate dehydrogenase	1.469	oxidoreductase activity

3.3. Functional Characterization of Proteomic Responses to Water Deficit Severity

The differential impact of drought severity was also reflected in the number of enriched GO terms, since a lower number was triggered under MWD (seven in CL153; two in Icatu) than in SWD (22 in CL153; 30 in Icatu) by the two genotypes (Figure 6). Under MWD, three GO-terms were up-regulated in CL153, being included in the categories of responses to stress, stimulus, and osmotic stress, as well as the photosystem, while two additional GO-terms were down-regulated: cell redox homeostasis and the generation of precursor metabolites and energy. In contrast, MWD had little effect in Icatu given that only two of the former GO-terms (responses to stimulus and osmotic stress) were up-regulated (Figure 6).

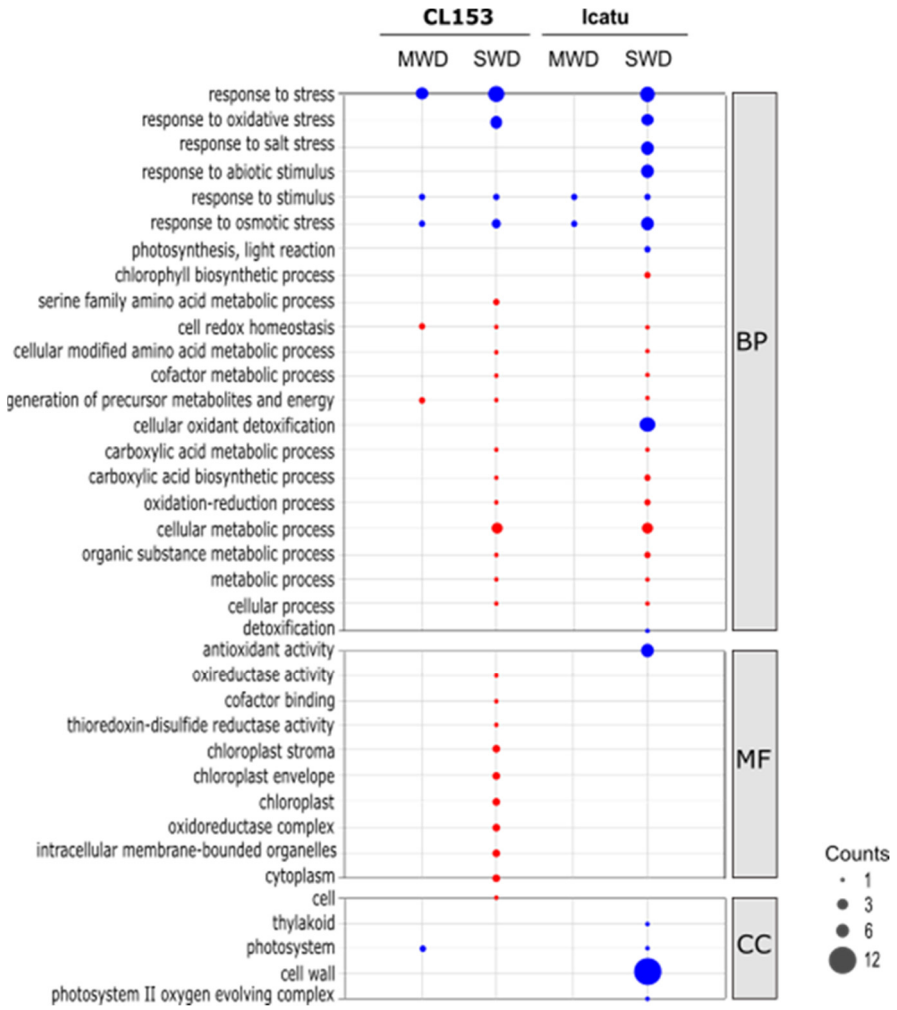

Figure 6. Enriched GO terms found in *Coffea canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu genotypes when comparing well-watered plants (WW) with those exposed to moderate (MWD) or severe (SWD) water deficit. GO terms indicate Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC). Colors indicate the number of GO-terms that decreased (red) or increased (blue) in abundance upon water deficit imposition.

SWD triggered more enriched terms in the two genotypes, albeit more in Icatu (30 GO-terms) than in CL153 (22 GO-terms). Furthermore, in the latter genotype, only four enriched terms were up-regulated in CL153, while almost half of them (14) were upregulated in Icatu (Figure 6). In both genotypes, up-regulated DAPs were commonly enriched in responsiveness proteins to abiotic stresses (e.g., response to stress, oxidative stress, stimulus, osmotic stress). However, Icatu showed a wide number of categories regarding stress response mechanisms, including the response to abiotic stimulus, cellular oxidant detoxification, and antioxidant activity. Additionally, this genotype further showed enriched GO-terms associated with the photosynthetic apparatus (photosynthesis–light reaction, thylakoid, photosystem II oxygen evolving complex), as well as with the cell wall that was the most enriched GO-term in Icatu (Figure 6).

Despite the observed number of GO-terms referred above, due to the reduced number of proteins involved in each one, only four significant KEGG pathways were found enriched upon water deficit exposure, and all of them being restricted to SWD, that is, no significant KEGG pathways were found under MWD (Figure 7). Up-regulated DAPs in CL153 were enriched in the KEGG pathway of glyoxylate and dicarboxylate metabolism, while, in Icatu, two other pathways were found: the superoxide metabolic process and the glutathione

metabolism (Figure 7). In contrast, the KEGG pathway of carbon metabolism was linked to down-regulated DAPs in the two genotypes, although at a minor magnitude (Figure 7).

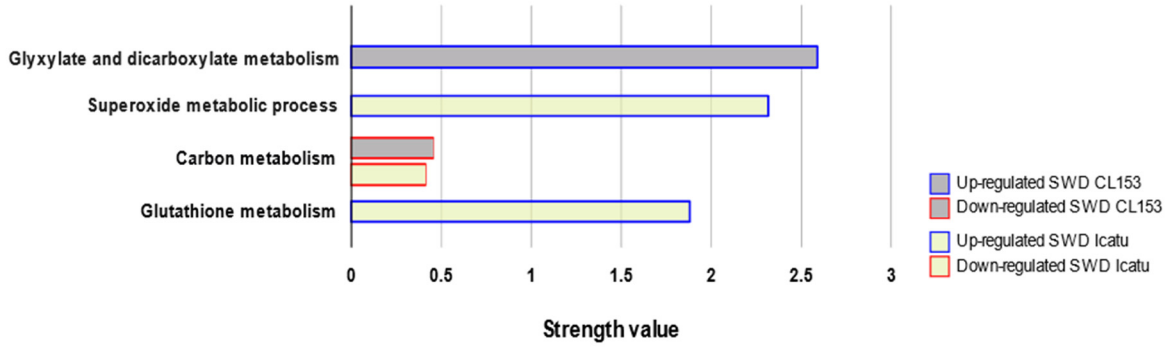

Figure 7. Enriched KEGG pathways found in *Coffea canephora* cv. Conilon Clone 153 (CL153) and *C. arabica* cv. Icatu genotypes when comparing well-watered plants (WW) with those exposed to moderate (MWD vs. WW) or severe (SWD vs. WW) water deficit.

4. Discussion

4.1. The Global Coffee Proteome

Finding and characterizing ubiquitous proteins that are regulated across plant species is of both fundamental and practical importance to understand their function, organization, and role in the plant's ability to cope with different environmental stimuli. In this study, a shotgun analysis using coffee leaves resulted in 1.4 million MS/MS spectra, from which 520,806 were assigned to peptides with an assignation rate of 36%, supporting similar shotgun proteomic results performed on both plants and animals [55]. Using the reference genome of *Coffea canephora* [51], almost 90% of proteins were identified in either *C. canephora* (CL153) or *C. arabica* (Icatu) genotypes, reflecting a higher number than usually reported in similar proteomic studies [25,56,57]. Interestingly, regardless of such a high number of proteins, only 0.7% (16 proteins) in CL153 and 0.5% (21 proteins) in Icatu contributed to *ca.* 20% of the proteome biomass (Table 1), in line with what was reported in other eukaryotes [58–60].

The core proteome represented 92–93% of the total protein biomass measured by NSAF values (Figure 2), indicating that most proteins were systematically found across all water conditions. The similar core proteome found in *C. canephora* and *C. arabica* (Figure 3) is likely due to the close evolutionary relationship between these species, as *C. canephora* is one of the progenitors of the polyploid *C. arabica* [61]. In CL153, the common proteins found among all samples (strict proteome) were higher (462) and more abundant (73%) than those in Icatu (182 and 47%, respectively). The lower number of common proteins (and their abundance) in Icatu could be due to the annotation of the reference genome used (*C. canephora*), but the numbers of assigned spectra for both genotypes are relatively similar, which apparently rules out such possibility. Even though novel proteogenomic studies should improve the level of annotation and use different *Coffea* genomes, our results suggest that Icatu has a greater responsiveness to the imposed water deficit levels than CL153, at least considering the proteomic level. This is in line with the wider triggering of defense mechanisms and production of metabolites found in Icatu than in CL153 under SWD, agreeing also with recent physiological analyses [19,42,62]. In fact, under changing conditions of air [CO_2], supra-optimal temperatures, or a moderate water deficit, these genotypes usually differ in their ability to cope with these limitations, especially under the harshest applied conditions [19,20,29,62,63].

The auxin-binding ABP20 was the most abundant protein in both genotypes, followed by the Putative Bark storage protein A, although the latest one had a low abundance in Icatu. Auxin is a key regulator of plant growth and development, integrated in signaling pathways that respond to environmental cues, and helping to regulate the level of glucosinolates [64]. Bark storage proteins (BSPs) accumulate in bark parenchyma and xylem rays in autumn

and decline in abundance when growth resumes, helping in the remobilization of stored nitrogen [65]. In addition, these proteins also respond to wounding and drought stimuli, apparently helping to preserve nutrients [66]. Recent studies have also suggested their involvement in the acclimation to salinity stress, involving nitrogen management because of the restricted nutrient uptake imposed by salt [67]. In fact, proteins related to cellular nitrogen, together with stress-responsive and biosynthetic proteins, were found to be a major component of the core proteome of coffee (Figure 3). This suggests an innate proteome adequacy to help these coffee genotypes facing environmental changes. An adequate supply of nitrogen is of utmost importance for plant growth and development, as well as other relevant processes such as the synthesis of DNA, RNA, proteins, and even for photosynthesis [68]. Most of the assimilated nitrogen is directly invested in photosynthesis, playing an important role in regulating plant responses to environmental changes, including water shortage [69–71]. Thus, the high abundance of proteins, in the core proteome of these two *Coffea* genotypes, involved in nitrogen metabolic processes suggests, at a first glance, a superior ability of their photosynthetic machinery to face environmental changes. This also explains their high resilience to elevated temperatures, which is greater than what is usually assumed for *Coffea* species [38,62], and especially to severe water constraints, where Icatu was found to be resilient [20].

4.2. Differential Proteome Responses to Water Deficit of C. canephora and C. arabica Genotypes

The number of differentially abundant proteins (DAPs) increased with the severity of the water deficit, being predominantly down-regulated upon water stress (Figure 4). Nevertheless, the triggered response was different according to the severity of drought, not only due to the greater number of DAPs observed under SWD, but also because a very low number of DAPs were commonly regulated by MWD and SWD treatments: seven in CL153 and five in Icatu (Figure 5). This could be explained by the clear resilience of both genotypes under MWD, but not under SWD, where significant negative impacts were almost exclusively observed in CL153 regarding the photosynthetic components [19,20].

Despite the reported resilience of both genotypes under MWD, proteomic drought responses were genotype-dependent. The most abundant proteins found under MWD are generally involved in response to abiotic stresses as the Pathogenesis-related protein (PRP)1B in CL153 and the Polyphenol oxidase I and Carotenoid 9-10-cleavage dioxygenase in Icatu (Table 2). PRPs play crucial roles in plant defense responses to biotic and abiotic stresses, being involved, namely, in the tolerance to salinity and drought in rice [72], drought in tomato [73], and freezing, salinity, and osmotic stresses in wheat [74]. Therefore, its important presence is likely associated with a tolerance to MWD conditions in CL153. Likewise, Polyphenol oxidase (PPO) is involved in plant responses to environmental stresses, controlling the regulation of the redox state of phenolic compounds under stress [75]. Its abundance in Icatu, together with the carotenoid protein, probably contributes to the repair of photosynthetic structures, since it uses chlorophyll and carotenoids for folding, assembly, and insertion into thylakoid membranes [76]. This further agrees with the performance of the photosynthetic components in Icatu under MWD [19,20].

The harsher water deficit (SWD) increased the number of DAPs in both genotypes. Under SWD, the most abundant protein was the NADP-dependent D-sorbitol-6-phosphate dehydrogenase (2.9-fold in CL153 and 1.5 in Icatu), being also the only one to be commonly expressed by both genotypes (Table 3). This protein is associated with a resistance to abiotic stresses such as drought and salinity [77], regulating the levels of polyols (small-chain carbohydrates) that act as osmolytes during drought exposure and recovery processes [78]. For instance, an intracellular reinforced accumulation of sorbitol under water deficit was found to reduce biomass loss in grapevine berries, acting as an effective osmoprotectant and cellular homeostasis buffer [79]. In accordance, this protein might also play an important role in abiotic stress tolerance in coffee plants through the regulation of polyol transport and metabolism.

Notably, except for NADP-dependent D-sorbitol-6-phosphate dehydrogenase, the response to SWD was also genotype-dependent, that is, each genotype tried to cope with the severe drought through a specific group of DAPs associated with photosynthesis. This included the PsbP domain-containing protein 4 in CL153, and the chlorophyll *a*–*b* binding protein 4 and the RuBisCO activase 1 in Icatu (Table 3). The increase in RuBisCO activase, a catalytic chaperone that modulates RuBisCO activity, might be a key factor in plant response to altered environmental conditions [80] due to its stress sensitivity, namely, to heat and drought [81]. Therefore, its abundance in Icatu might have contributed to the reported stability of the initial RuBisCO activity under SWD, which was observed in this genotype but not in CL153 [19,20]. Furthermore, since drought imposes constraints in the PSI and II, it is not surprising to find responsiveness proteins in the two genotypes.

This potential positive response could have attenuated the drought impact. However, this was not enough to improve plant acclimation capability in CL153 since it did not prevent either a significant decline in PSI and II activity and efficiency, nor a decrease in the content of cytochromes involved in thylakoid electron transport (Cyt b_{559}, Cyt b_{563}, Cyt *f*) [19]. This also highlights those different acclimation capabilities found between genotypes rely on the control of cellular oxidative conditions, which should be stronger in Icatu. In fact, while CL153 showed an abundance of proteins involved in general cellular responses to stress as thioredoxin H-type 1 and the xylose isomerase, Icatu up-regulated proteins involved in responses to abiotic stress such as Serpin-ZX and in detoxification processes such as L-ascorbate peroxidase T and Malate dehydrogenase (Table 3). Indeed, serpins have an important role under abiotic stress, as noted by their interactions with proteases that respond to desiccation [82]. Serpins are usually up-regulated in response to drought [83], as shown here for Icatu, and they likely play an important role in drought stress tolerance. ROS-detoxifying enzymes also play key roles under water deficit. Cells are exposed to high concentrations of ROS when plants experience drought stress [84]. The undesirable impacts of ROS in cells are usually counteracted through defense mechanisms for reducing or preventing oxidative damage while improving drought resistance [25]. The overproduction of ROS can be counteracted by a variety of mechanisms, including enzymatic antioxidant mechanisms, such as catalase (CAT), superoxide dismutase (SOD), Ascorbate Peroxidase (APX), peroxidase (POD), and PPO, which can minimize cellular damage [15]. In accordance, antioxidant molecules were found to be a crucial component in the coffee plants' acclimation to a wide number of environmental constraints by protecting the photosynthetic apparatus against high irradiance [85], cold [86], temperature [38], and drought [19,33,46]. For instance, the activity of enzymes such as CAT, SOD, APX, and glutathione reductase (GR) was enhanced under both drought and cold stress in several *C. arabica* genotypes, including Icatu [46].

4.3. Functional Characterization of Coffee Proteome Responses to Water Deficit

In line with previous analyses, functional annotations also revealed a stronger impact of SWD than of MWD on both genotypes regarding the number of enriched GO terms (Figure 6) and the enrichment of functional pathways only under SWD (Figure 7). This explains the absence of metabolic impacts on these genotypes under MWD [19,20]. By contrast, harsh drought conditions are known to trigger a cascade of metabolic, cellular, and physiological processes to enhance plant tolerance to stress [87–90].

A recent transcriptomic study has also detected the low impact of MWD in these two genotypes, in comparison with SWD, which triggered several protective genes (more in CL153 than in Icatu) associated with antioxidant activities, including genes involved in water deprivation and desiccation, such as *Lea* and aspartic proteases [44]. A high number of Transcription Factors, including ERFs, DREBs, and leucine zippers were found to be significantly up-regulated under drought, which together with a large number of phosphatases and protein kinases found, suggested the involvement of ABA signaling in the drought tolerance of these genotypes. However, because not all transcriptomic results

were in line with previous physiological and metabolic results, we suggested the existence of post-transcriptional regulations [44].

In this novel proteomic study, GO-terms were associated with biological processes involved in response to abiotic stress (e.g., oxidative, salt, osmotic stress) and subsequent molecular detoxification processes (e.g., cellular oxidant detoxification, antioxidant activity), but especially in Icatu. Under SWD, this genotype further presented a strong up-regulation of GO-terms associated with the cell wall, suggesting a major role of cell wall re-organization/repair in the Icatu response to drought. Despite the down-regulation of the carbon metabolism pathway in the two genotypes (Figure 7), both showed an enrichment in pathways involved in enhancing tolerance. For instance, the glyoxylate and dicarboxylate metabolism pathway enriched in CL153 is known to play an important role in reducing metabolic disorders and transporting energy to help plants cope with stress [91]. This pathway is involved in the dissipation of energy excess, as well as in the energy that could result in ROS production [92]. Remarkably, two important KEGG pathways contributing to cellular detoxification were also enriched in Icatu under SWD: the glutathione metabolism and the superoxide metabolic process, which are consistent with the reported enrichment of GO-terms associated with oxidative stress control (Figure 7). In fact, the ascorbate–glutathione (ASA–GSH) cycle is a major pathway of H_2O_2 scavenging and an effective mechanism of detoxification in plants [93], together with other defense enzymes already referred to (e.g., SOD, CAT, POX). In the ASA–GSH cycle, H_2O_2 is reduced to H_2O via ascorbate and reduced glutathione, being the resulting oxidized glutathione recycled back to this cycle, thus helping to scavenge ROS [94,95]. In this context, the enrichment of these detoxification pathways in Icatu, together with other protective mechanisms, including a greater thylakoid electron transport, the reinforcement of electron carriers' contents, and protective mechanisms around the cyclic electron transport involving both photosystems, would help to protect the photosynthetic machinery and to maintain the potential photochemical use of energy (and control ROS production) in this genotype, but not in CL153 [19,20]. This resulted in negligible impacts found under SWD for Icatu regarding photosynthetic functions and their components (e.g., photosynthetic capacity, maximal photochemical efficiency of photosystem II, electron carriers, photosystems I and II, and RuBisCO activities), in contrast with CL153. Therefore, several responses likely contributed to Icatu's better performance under SWD, including the triggering of mechanisms of (thermal) energy dissipation and ROS control over the photosynthetic machinery [19,20,46], as well as a greater dynamic in the lipid matrix of the chloroplast membranes, which are sustained by the proteins and their functions found in this study.

5. Conclusions

This study establishes for the first time, to our knowledge, the core proteome of *C. canephora* (cv. Conilon CL153) and *C. arabica* (cv. Icatu) and provides novel information regarding the changes in their proteomic profiles in response to water deficit. Drought promoted significant proteomic changes, triggering a similar number of DAPs in both genotypes, but with the enrollment of different proteins. Results showed that the impact of MWD was almost absent in the proteome, in agreement with the physiological tolerance previously reported at this drought level for CL153 and Icatu [20]. However, the response to drought was dependent on the severity of the applied stress, with SWD having a greater impact than MWD. This was reflected quantitatively, with a higher number of DAPs that were predominantly down-regulated, but also qualitatively since most proteins expressed under SWD were distinct from those found under MWD. However, a clear genotype-dependent response under SWD was also found, with Icatu showing a greater prevalence of proteins, enriched GO-terms, and enriched KEGG pathways associated with stress response and the control of oxidative stress categories than CL153. These results support previous findings, where it has been shown that Icatu plants are able to maintain the potential photosynthetic functioning under the imposition of SWD due to a greater antioxidative response.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12010148/s1, Table S1: List of proteins and their spectral counts. Table S2: List of differentially abundant proteins. Table S3: List of proteins commonly identified in both water deficit treatments.

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