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Research article

Proteomic profiles of young and mature cocoa leaves subjected to mechanical stress caused by wind

Graciele Santos Monteiro Reis, Alex-Alan Furtado de Almeida*, Nayara de Almeida Santos, Juliano Oliveira Santana, D'avila Maria de Souza Araújo, Irma Yuliana Mora-Ocampo, Dário Ahnert, Carlos Priminho Pirovani

Department of Biological Sciences, Santa Cruz State University, Campus Soane Nazaré de Andrade, Rodovia Jorge Amado, km 16, Ilhéus, BA, Brazil

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A B S T R A C T

Cocoa is a perennial and arboreal species intolerant to strong and frequent winds and, for this reason, is usually grown with windbreaks of trees. The mechanical alterations caused by the wind in the field have a great impact on the growth, development and productivity of cocoa. The present work had a main objective to understand the molecular mechanisms of responses to mechanical stress, caused by the action of constant wind flow in young plants of cocoa through alterations of the proteomic profile in young (YL) and mature leaves (ML). Plants were exposed to constant wind (CW) at a speed of 4.5 m s^{-1} for 12 h. There was a reduction in the accumulation of proteins in YL and a significant increase in ML submitted to CW in relation to the control. Differentially accumulated proteins, identified in YL and ML, belong to a broad functional group, related to energy production and carbon metabolism. Besides that, there was a higher efficiency in the protein relative abundance associated to energy production and the assimilation of carbon in the ML exposed to CW, in relation to the control. It was observed the appearance of new isoforms and, or post-transitional changes, which represent an acclimatization and tolerance response of these leaves to the stressor factor. In contrast, in YL, the energy production and the synthesis of gene products essential for their growth and development were affected by the mechanical stress caused by the wind, making them more intolerant.

1. Introduction

Plants growing in the field are subjected to adverse environmental variations such as temperature, rainfall, wind, sun radiation, etc., which can cause different types of stresses on the plant. In order to adjust themselves to these stresses, they developed molecular mechanisms that involve changes in gene expression able to alter their metabolism. Mechanical stress caused by wind, raindrops, subtle touches and friction, whether by the passage of animals or machines, induce tigmomorphogenesis in plants (Saidi et al., 2009). This term refers to morphophysiological changes, generally characterized by the decrease in plant height, change in mechanical properties of the stem, increase in root biomass and increase in radial growth of the stem, depending on the species (Telewski, 1995). Mechanical stress involves the synthesis of phytohormones, signaling molecules and other chemical components, in addition to gene expression (Saidi et al., 2009).

Some genes whose expression are induced by touch code for the Ca^{2+} protein-calmodulin ligand, suggesting that Ca^{2+} has a role as a mediator of growth responses (Allen et al., 1999). Several signaling molecules and phytohormones, including jasmonic acid, ethylene, abscisic acid (ABA), auxins, brassinosteroids, nitric oxide and reactive oxygen species (ROS) in addition to intracellular Ca^{2+} have been related to mechanical stimulus responses (Chehab et al., 2009). Mechanical damage caused by wind leads to the activation of genes involved in responses to water stress, cellular repair and metabolism, and defense against pathogens. On the other hand, mechanical stimulus can decrease the expression of several genes, such as genes that code for anthocyanin (Porter et al., 2009) and genes related to important enzymes in the photosynthetic apparatus, such as Rubisco. ROS are considered important signaling molecules used by cells to regulate gene expression in response to mechanical disturbances (Vellosillo et al., 2013). Increased gene expression of antioxidant metabolism enzymes in response to ROS accumulation, such as superoxide dismutase and peroxidases, was reported (Madanala et al., 2011).

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* Corresponding author.
E-mail address: alexalan@uesc.br (A.-A. Furtado de Almeida).

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The integration of genomic data with proteomic profiles can provide a better understanding of the molecular mechanisms of response to mechanical stress. The amount of proteins is not always related to the amount of mRNA, especially for low abundance proteins (Fernie and Stitt, 2012). In addition, many proteins undergo post-translational modifications, such as removal of signal peptides, phosphorylation and glycosylation, which are extremely important for protein activities and subcellular localization (Fernie and Stitt, 2012).

Young leaves (YL) and mature leaves (ML) of the CCN 51 cocoa clonal cultivar presented differential morphophysiological, biochemical, molecular and ultrastructural responses when exposed to intermittent and constant wind flow (Reis et al., 2018). In the field, YL are more intolerant to mechanical stress caused by wind because it can damage the leaves more easily (Reis et al., 2018). Furthermore, according to these authors, wind with moderate to high speed can: (i) interfere directly with leaf gas exchange, causing reduction in the photosynthetic rate in ML; (ii) promote rupture of the nuclear membranes in cells of the pulvinus and leaf mesophyll tissues, mainly in YL; (iii) cause lipid peroxidation and increase the activity of guaiacol and ascorbate peroxidases in YL and ML; and (iv) alter the gene expression of *psbA* and *psbO*, related to the photosynthetic apparatus, and of *Cu–Zn-sod and per 1*, related to antioxidative enzymes, in YL and ML.

This study was undertaken to analyze the molecular responses of mechanical stress caused by the action of wind flow on cocoa leaves, based on data from proteomic profile of young and mature leaves of CCN 51 cocoa clonal plants submitted to constant wind flow.

2. Material and methods

2.1. Plant material and growing conditions

The experiment was carried out under greenhouse conditions to protect the tested plants from external wind. The greenhouse used for this study is located at the campus of the State University of Santa Cruz, Bahia, Brazil (14° 47′S, 39° 02′W, 40 m asl). The clonal cocoa plants of the genotype CCN 51 were obtained from the rooting of stem cuttings from the ends of plagiotropic branches at the beginning of secondary growth, containing the apical bud, three auxiliary buds and three leaves, taken from five years old parent plants, multiplied and grown in the clonal garden at the *Biofábrica de Cacau* Institute (https://www.biofabrica.org.br/).

The bottoms of the cuttings (~3 cm), approximately 20 cm long, were dipped into chemically inert talcum powder containing indol-3-butyric acid (IBA) at 4 g kg^{-1}. Afterwards, each cutting was transferred to a 288-cm^3 tubelike, black plastic pot containing organic substrate (turf + grinded *Pinus* sp. barks and grinded coconut fiber at 1:1 ratio), enriched with macro and micronutrients, according to the recommendations for cocoa, and placed in plastic trays with capacity for 54 tubes. Then, the trays were transported to a nursery covered with plastic screens 50% shade, irrigated and cultivated according to the methodology described by Santos Júnior et al. (2008).

After rooting (4–5 months old), the clonal plants were transplanted into perforated plastic pots with capacity of 16 dm^3. The soil was fertilized with N, P and K and a mixture of $CaCO_3$ and $MgCO_3$. We have tried to reach the Ca^{+2}: Mg^{+2} 4: 1 ratio to increase the saturation value of the soil base to 30%, which results in pH increase for ideal growing conditions of cocoa seedlings. The fertilization of the plants was based on the needs of the crop during the eight months of the experiment (Santos Júnior et al., 2008).

During the experimental period, photosynthetically active radiation (PAR), temperature (T) and relative humidity (RH) of the air inside the greenhouse were monitored, using the same equipment as described by Reis et al. (2018). The mean values of PAR, T and RH were 953 ± 4 μmol photons m^{-2} s^{-1}, 27 ± 2 °C and 86 ± 3%, respectively.

2.2. Wind tunnel

When the CCN 51 clonal plants reached seven months of age, they were exposed to constant wind flow (CW) in a wind tunnel inside the greenhouse. Two wind tunnels made of wood were built according to the specifications provided by Reis et al. (2018). A fan at one extremity of the tunnel provided a constant air flow, with a fixed axis, inside the tunnel, with a speed of 4.5 m s^{-1}, measured by a mini-anemometer (CASSELA C 7748/Z). The average wind speed recorded in the cocoa growing region in Bahia, Brazil, corresponds to 2 m s^{-1}. YL and ML of six CCN 51 cocoa clonal plants were evaluated by treatment, after exposure to CW for 12 h, together with the control treatment (without wind and exposure time).

2.3. Extraction of proteins

Proteins were extracted from samples of YL and ML originated from CCN 51 cocoa clonal plants submitted to constant wind, for a period of 12 h, using phenol, followed by precipitation with 0.1 M ammoinum acetate in methanol, according to the methodological procedures described by Wang et al. (2003).

2.4. Two-dimensional SDS-PAGE

For the two-dimensional gel, the first dimension was conducted in an EttanIPGphor system (GE Healthcare). The protein sample (350 μg) in 250 mm^3 of rehydration solution was focused on 13 cm Immobiline Dry Strip Reswelling (pH 3–10, GE Healthcare) for 12 h. Then, the gel strips were incubated for 15 min in the equilibrium buffer solution 6 M urea, Tris-HCl (7.5 mM and pH 8.8), 29.3% glycerol, 2% SDS and 0.002% bromophenol blue] and with 1% DTT (p/v) and then in the equilibration buffer with 2.5% (w/v) iodoacetamide for another 15 min. The second dimension (SDS-PAGE) was performed in the SE600 Ruby system (GE Healthcare): 15 mA, for 45 min, 40 mA, for 30 min And 50 mA *per* gel, for 3 h for each strip, at constant temperature of 11 °C. The molecular weight marker used was the Amersham Low Molecular Weight SDS Calibration Kit for SDS electrophoresis (GE Healthcare). The gels resulting from 2-DE electrophoresis were fixed in a solution containing 40% ethanol and 10% acetic acid for 1 h and, finally, kept in 0.08% colloidal Coomassie blue G-250.

2.5. Image analysis of gels by the Image Master program

The images of the gels were digitized with the LabScanner scanner (Amersham Bioscience) and analyzed in ImageMaster 2D Platinum 7.0 (GE Healthcare), considering the area and intensity of the spots. Leaf samples from the control treatment were compared with leaf samples exposed to the wind. The analysis of differential spots was based on the calculation of ANOVA. Only values $p < 0.05$ and spots with variations in intensity (fold) ≥ 1.5 were considered as differentials.

2.6. Mass spectrometry

The selected protein spots were removed from the two-dimensional gel, equilibrated with 50% acetonitrile, containing 25 mM ammonium bicarbonate, followed by washes with distilled water. Protein digestion was performed according to Silva et al. (2013). The eluted peptides were fractionated by reverse phase chromatography in a nanoAcquity UPLC (WATERS), in a Micromass ESI-Q-TOF Micro System mass spectrometer (Waters, Manchester, United Kingdom). The spectra were analyzed using ProteinLynx Global Server 4.2 (WATERS) software. The NCBIprot database was used, this software has 82759882099 number of residues, 227181163 number of sequences and number of sequences for taxonomy: Viridiplantae (Green Plants) = 9630886. For the comparison with the NCBIprot, the MASCOT Server 2.7 MS/MS Ion Search tool (www.matrixsciesce.com) was used, which was configured for trypsin

Table 1
- Differentially accumulated proteins, identified by mass spectrometry (MS/MS) in young leaves of control plants and of plants of the clonal cocoa genotype CCN 51 submitted to constant wind for a 12 h exposure period.

SPOT ID	ACESSION NUMBER	PROTEIN NAME	Control	With wind	MM[a] (kDa)	pI[b]	SCORE[c]	ANOVA[d]	NUP[e]	FDR[f]	Mr (expt)[g]	Mr (calc)[h]	Organism
1	EOY02403.1	Carbonic anhydrase 1 isoform 2	–	+	35284	8.35	155	0.02420	K.NPALYAELAK.G	0.00% 6.67%	1088.5844	1088.5866	*Theobroma cacao*
2	EOY11078.1	GDP-mannose 3.5-epimerase 1	+	–	48472	6.88	629	0.00285	K.QLETNVSLK.E	7.69% 18.75%	1030.573	1030.5659	*Theobroma cacao*
3	XP_017977555.1	GDP-mannose 3.5-epimerase 2	+	–	48477	6.65	823	0.01272	R.LTMSDFR.E + Oxidation (M)	7.69% 5.88%	884.398	884.4062	*Theobroma cacao*
23	EOX91250.1	Photosystem II subunit O-2	+	–	35364	5.85	592	0.02805	K.RLTYDEIQSK.T	5.00% 8.33%	1251.622	1251.6459	*Theobroma cacao*
30	XP_020245676.1	Actin 7 isoform 2, partial	+	–	41973	5.31	764	0.00023	K.AGFAGDDAPR.A	0.00% 0.00%	9754.294	9 754 410	*Asparagus officinalis*
33	XP_007021667.1	Malate dehydrogenase 2, mitochondrial	+	–	35601	8.52	375	0.02817	K.LFGVTTLDVVR.A	0.00% 0.00%	1218.678	1218.6972	*Theobroma cacao*
40	EOY08281.1	Chloroplast signal recognition particle 54 kDa subunit	+	–	62806	9.52	506	0.02339	R.ALLEADVSLPVVR.R	0.00% 4.88%	1380.7866	1380.7977	*Theobroma cacao*
44	CBE70631.1	Glyceraldehyde 3-phosphate dehydrogenase, partial	+	+	13226	5.85	229	0.047877	R.VPTVDVSVVDLTVR.L	12.50% 11.11%	1497.8294	1497.8403	*Manihot esculenta*
47	ADO64898.2	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	+	–	53512	6.04	395	0.02251	R.DDFIEKDR.S	0.00% 0.00%	1036.4734	1036.4825	*Theobroma cacao*
48	AAB01597.1	Ribulose bisphosphate carboxylase (chloroplast)	+	–	53371	5.99	135	0.02872	R.DITLGFVDLLR.D	0.00% 5.26%	1260.6964	1260.7078	*Solanum tuberosum*

[a] MM, corresponds to molecular mass values (kDa).

[b] pI isoelectric point estimated by the program Image Master 2D Platinum 7.0.

[c] Score corresponds to the value of coverage calculated by Mascot.

[d] ANOVA.

[e] Number of unique peptides.

[f] FDR corresponds to peptide matches above identity threshold and peptide matches above homology or identity threshold.

[g] Experimentally calculated m/z values, monoisotopic mass (Mr expt).

[h] Theoretically calculated (Mr calculated).

Table 2
- Exclusive proteins identified by mass spectrometry (MS/MS) in young leaves of the clonal cocoa genotype CCN 51 submitted to constant wind for a 12 h exposure period.

SPOT ID	ACESSION NUMBER	PROTEIN NAME	MM[a] (kDa)	pI[b]	SCORE[c]	NUP[d]	FDR[e]	Mr (expt)[f]	Mr (calc)[g]	Organism
50	EOY22297.1	31-kDa RNA binding protein	36386	4.4	386	K.AGTVEIAEVIYNR.E	0.00% 4.76%	1433.7534	1433.7514	*Theobroma cacao*
53	EOX93083.1	Photosystem II subunit P-1	28666	8.65	574	R.KFVESTLGSFSVA.	0.00% 0.00%	1370.7018	1370.7082	*Theobroma cacao*
54	AUH23149.1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	19239	6.81	228	K.TFQGPPHGIQVER.D	0.00% 33.33%	1464.7312	1464.7474	*Antennaria dimorpha*
56	XP_017974861.1	40 S ribosomal protein S17	16106	10.15	308	K.IIEEVAIIPSK.R	0.00% 5.71%	1210.6274	1210.7173	*Theobroma cacao*
58	EOY04849.1	PfkB-like carbohydrate kinase family protein	35376	5.26	772	K.FANACGAITTTK.K	0.00% 19.05%	1253.5972	1253.6074	*Theobroma cacao*
59	AVQ04668.1	Phosphoglycerate kinase	42386	6.28	53	R.LSELLGVEVK.M	0.00% 100%	1085.8622	1085.6332	*Neolamarckia cadamba*
60 68	EOY21251.1	21 kDa seed protein	24263	5.94	1061	K.IENAGALGYK.F	0.00% 5.71%	1034.5832	1034.5396	*Theobroma cacao*
61	EOX93083.1	Photosystem II subunit P-1	28666	8.65	574	R.KFVESTLGSFSVA.-	0.00% 0.00%	1370.7018	1370.7082	*Theobroma cacao*
62	XP_007009267.1	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic isoform X2	48566	5.56	613	K.VPLILGIWGGK.G	8.33% 11.76%	1151.6116	1151.7067	*Theobroma cacao*
63	CAB00006.1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	52185	6.12	57	R.DITLGFVDLLR.D	0.00% 200%	1260.7108	1260.7078	*Dovyalis rhamnoides*
64	XP_017977430.1	2-phosphoglycolate phosphatase 1	41255	8.6	162	R.ENPGCLLIATNR.D	0.00% 0.00%	1356.6724	1356.682	*Theobroma cacao*
65	XP_007009267.1	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic isoform X2	48570	5.56	613	K.VPLILGIWGGK.G	8.33% 11.76%	1151.6116	1151.7067	*Theobroma cacao*
66	EOX93637.1	Chlorophyll *a-b* binding protein 3, chloroplastic	29725	6.85	275	R.YAMLGAIGAIAPEILGK.L + Oxidation (M)	0.00% 0.00%	1702.9376	1702.9327	*Theobroma cacao*
82	EOX91250.1	Photosystem II subunit O-2	35364	5.85	950	K.FCLEPTSFTVK.A	0.00% 0.00%	1327.662	1327.6482	*Theobroma cacao*
83	EOY02669.1	Glyceraldehyde-3-phosphate dehydrogenase C2 isoform 1	35816	8.51	61	R.NPEEIPWGESGAEYVVESTGVFTDKDK.A	0.00% 0.00%	2982.3964	2982.3771	*Theobroma cacao*
88	XP_007009267.1	Ribulose bisphosphate carboxylase/oxygenase activase	48566	5.56	1282	R.EGPPTFEQPK.M	4.00% 6.90%	1128.5444	1128.5451	*Theobroma cacao*
92	XP_007044141.1	ATP synthase subunit beta, mitochondrial	59857	6.01	1172	R.TIAMDGTEGLVR.G + Oxidation (M)	0.00% 0.00%	1277.6236	1277.6286	*Theobroma cacao*
93	YP_009570514.1	ATP synthase CF1 alpha subunit	55516	5.50	576	R.ADEISNIIR.E	5.88% 7.50%	1029.538	1029.5454	*Dioncophyllum thollonii*
101	KHN00016.1	40 S ribosomal protein S5	22624	9.62	91	K.TIAECLADELINAAK.G	12.50% 45.45%	1630.8076	1630.8236	*Glycine soja*
103	XP_008787626.1	DNA ligase 6-like	158222	6.94	54	K.AVTLSVPR.N	100% 100%	841.7532	841.5022	*Phoenix dactylifera*
105	EOY23716.1	Caffeic acid 3-O-methyltransferase 1	40531	5.32	521	K.NPDAPVMLDR.I + Oxidation (M)	0.00% 0.00%	1142.5524	1142.539	*Theobroma cacao*
106	EOY23716.1	Caffeic acid 3-O-methyltransferase 1	40531	5.32	603	K.NPDAPVMLDR.I	0.00% 6.25%	1126.5502	1126.5441	*Theobroma cacao*
107	TYJ00274.1	Hypothetical protein E1A91_A13G075900v1	14884	10.26	60	R.ASLNDFDR.F	0.00% 11.11%	936.6334	936.4301	*Gossypium mustelinum*
109	CAB00006.1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	52185	6.18	57	R.DITLGFVDLLR.D	0.00% 200%	1260.7108	1260.7078	*Dovyalis rhamnoides*
110	XP_007032095.1	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	43255	8.15	386	K.TFAEEVNAAFR.D	8.00% 10.71%	1253.6052	1253.604	*Theobroma cacao*

[a] MM, corresponds to molecular mass values (kDa).
[b] pI isoelectric point estimated by the program Image Master 2D Platinum 7.0.
[c] Score corresponds to the value of coverage calculated by Mascot.
[d] Number of unique peptides.
[e] FDR corresponds to peptide matches above identity threshold and peptide matches above homology or identity threshold.
[f] Experimentally calculated m/z values, monoisotopic mass (Mr expt).
[g] Theoretically calculated (Mr calculated).

Table 3

- Differentially accumulated proteins, identified by mass spectrometry (MS/MS) in mature leaves of control plants and of plants of the clonal cocoa genotype CCN 51 submitted to constant wind for a 12 h exposure period.

SPOT ID	ACESSION NUMBER	PROTEIN NAME	Control	With wind	MM[a] (kDa)	pI[b]	SCORE[c]	ANOVA[d]	NUP[e]	FDR[f]	Mr (expt)[g]	Mr (calc)[h]	Organism
7	EOY11151.1	Cysteine synthase	+	–	34424	5.22	788	0.0339181	R.YLSSVLFESVK.R	3.23% 5.41%	1270.6892	1270.680	*Theobroma cacao*
10	EOY30200.1	Chaperonin 20 isoform 2	–	+	26752	8.55	398	7.39E-04	K.TAGGLLLTEASK.E	0.00% 8.33%	1159.5962	1159.6448	*Theobroma cacao*
13	XP_017973236.1	Elongation factor TuA, chloroplastic	–	+	52371	6.45	76	3.69E-04	K.TAETVDIVGLR.E	0.00% 0.00%	1172.6348	1172.6401	*Theobroma cacao*
17	XP_022734369.1	21 kDa seed protein-like	–	+	24581	5.74	64	0.0379195	R.LSTDLNVEFVPIR.D	0.00% 5.71%	1501.794	1501.814	*Durio zibethinus*
18	EOY30200.1	HSP20-like chaperones superfamily protein	–	+	26752	8.55	398	0.0402307	K.TAGGLLLTEASK.E	0.00% 8.33%	1159.5962	1159.6448	*Theobroma cacao*
20	EOX93083.1	Photosystem II subunit P-1	–	+	28666	8.65	574	0.0340981	R.KFVESTLGSFSVA.-	0.00% 0.00%	1370.7018	1370.7082	*Theobroma cacao*
29	KHN00016.1	40 S ribosomal protein S5	–	+	22624	9.62	108	0.0119684	K.TIAECLADELINAAK.G	0.00% 0.00%	1630.8318	1630.8236	*Glycine soja*
32	AAB01597.1	Ribulose bisphosphate carboxylase (chloroplast)	+	–	53371	6.19	135	0.0012218	R.DITLGFVDLLR.D	0.00% 5.26%	1260.6964	1260.7078	*Solanum tuberosum*
35	XP_006401314.1	Chaperonin 60 subunit beta 3, chloroplastic	+	–	63590	5.63	918	0.0188844	R.KGVVTLEEGK.S	3.33% 2.50%	1058.5846	1058.5972	*Eutrema salsugineum*
38	EOY31529.1	NAD(P)-binding Rossmann-fold superfamily protein isoform	–	+	35532	6.32	81	0.008396	K.LQSLGFINLVLR.T	0.00% 20.00%	1371.9936	1371.8238	*Theobroma cacao*
39	OEL36727.1	2-Cys peroxiredoxin BAS1, chloroplastic	–	+	28081	6.45	420	0.0019193	K.EGVIQHSTINNLAIGR.S	0.00% 0.00%	1720.9108	1720.922	*Dichanthelium oligosanthes*
40	CAB90066.1	ATP synthase beta subunit, partial (chloroplast)	–	+	52979	5.14	435	0.008396	R.AVAMSATDGLTR.G	0.00% 0.00%	1191.5908	1191.5918	*Apium graveolens*
41 44	XP_007009267.1	Ribulose bisphosphate carboxylase/ oxygenase activase 2, chloroplastic isoform X2	–	+	48566	5.56	1112	5.76E-04	R.EGPPTFEQPK.M	0.00% 0.00%	1128.5408	1128.5451	*Theobroma cacao*
42	EOX99143.1	Glutamine synthetase 1,4	–	+	39269	6.02	94	2.03E-04	K.IFNHPAVAAEEPWYGIEQEYTLLQK.D	0.00% 0.00%	2945.4598	2945.4599	*Theobroma cacao*
43	EOY28076.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	–	+	46257	5.61	615	0.0424789	K.LQQQIASAR.S	3.23% 15.00%	1013.5408	1013.5618	*Theobroma cacao*
51	PIN00284.1	26 S proteasome regulatory complex, ATPase RPT3	–	+	47082	7.28	200	0.021637	K.ENAPAIIFIDEVDAIATAR.F	0.00% 9.09%	2028.0832	2028.0527	*Handroanthus impetiginosus*
52	XP_007012580.1	20 kDa chaperonin, chloroplastic	–	+	26979	8.55	398	0.0207853	K.TAGGLLLTEASK.E	0.00% 8.33%	1159.5962	1159.6448	*Theobroma cacao*
53	EOY02403.1	Beta carbonic anhydrase 3	–	+	35284	8.35	155	0.0039474	K.NPALYAELAK.G	0.00% 6.67%	1088.5844	1088.5866	*Theobroma cacao*
58	XP_017971285.1	Glutamine synthetase cytosolic isozyme 1	–	+	39241	6.02	94	0.022973	K.IFNHPAVAAEEPWYGIEQEYTLLQK.D	0.00% 0.00%	2945.4598	2945.4599	*Theobroma cacao*

[a] MM, corresponds to molecular mass values (kDa).

[b] pI isoelectric point estimated by the program Image Master 2D Platinum 7.0.

[c] Score corresponds to the value of coverage calculated by Mascot.

[d] ANOVA.

[e] Number of unique peptides.

[f] FDR corresponds to peptide matches above identity threshold and peptide matches above homology or identity threshold.

[g] Experimentally calculated m/z values, monoisotopic mass (Mr expt).

[h] Theoretically calculated (Mr calculated).

Table 4
- Exclusive proteins identified by mass spectrometry (MS/MS) in mature leaves of the clonal cocoa genotype CCN 51 submitted to constant wind for a 12 h exposure period.

SPOT ID	ACESSION NUMBER	PROTEIN NAME	MM[a] (kDa)	pI[b]	SCORE[c]	NUP[d]	FDR[e]	Mr (expt)[f]	Mr (calc)[g]	Organism
65	XP_017977172.1	Proteasome subunit beta type-4	27716	7.03	406	K.FNPLWNSLVLGGVK.N	0.00% 7.69%	1542.8508	1542.8558	*Theobroma cacao*
70	XP_017977430.1	2-phosphoglycolate phosphatase 1	41264	8.6	162	R.ENPGCLLIATNR.D	0.00% 0.00%	1356.6724	1356.682	*Theobroma cacao*
73	AJA30506.1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	49058	6.0	486	R.DDFIEKDR.S	0.00% 7.41%	1036.4788	1036.4825	*Duperrea pavettifolia*
74	XP_007034810.1	Proteasome subunit beta type-2-A -like protein	22638	5.86	591	K.LIAASGESGDR.V	0.00% 27.27%	1074.5170	1074.5305	*Theobroma cacao*
75	AUH23149.1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	19239	6.81	228	K.TFQGPPHGIQVER.D	0.00% 33.33%	1464.7312	1464.7474	*Antennaria dimorpha*
76	EOY34471.1	Glyoxalase I family protein isoform 1	27872	8,66	144	K.ESAANNPGLQSTPDEATK.G	0.00% 0.00%	1828.8442	1828.8439	*Theobroma cacao*
79	XP_007043300.1	Photosystem II stability/assembly factor HCF136	45257	7.75	726	R.GFGILDVGYR.S	0.00% 0.00%	1095.568	1095.5713	*Theobroma cacao*
84	XP_007027361.1	Adenosine kinase 2	37774	5.22	695	K.RPENWALVEK.A	2.56% 2.04%	1240.6684	1240.6564	*Theobroma cacao*
90	XP_007017586.2	Bifunctional dihydroflavonol 4-reductase	38737	5.74	120	K.YPEYNIPTK.F	0.00% 0.00%	1123.7674	1123.5549	*Theobroma cacao*
91	EOY06041.1	Lactate/malate dehydrogenase family protein	36097	6.6	703	R.VNIAVMVGGFPR.K + Oxidation (M)	0.00% 3.23%	1274.6754	1274.6805	*Theobroma cacao*
92	EOX99143.1	Glutamine synthetase 1,4	39269	6.02	94	K.IFNHPAVAAEEPWYGIEQEYTLLQK.D	0.00% 0.00%	2945.4598	2945.4599	*Theobroma cacao*
93	XP_007025436.1	Malate dehydrogenase 2, mitochondrial	37270	8.41	413	K.VYGLGLLSEFEK.Q	0.00% 8.16%	1353.6944	1353.718	*Theobroma cacao*
96	XP_007036696.1	Guanine nucleotide-binding protein subunit beta-like protein	36510	7.03	619	K.SIVEDLKVDLK.A	6.25% 9.52%	1257.725	1257.718	*Theobroma cacao*
100	EOY30200.1	Chaperonin 20 isoform 2	26752	8.55	398	K.TAGGLLLTEASK.E	0.00% 8.33%	1159.5962	1159.6448	*Theobroma cacao*
103	XP_007033923.2	Fructokinase-4	35367	5.19	966	R.TALAFVTLR.A	3.23% 5.41%	990.586	990.5862	*Theobroma cacao*
104	BBH03566.1	Actin-7	43099	5.25	510	K.AGFAGDDAPR.A	0.00% 0.00%	975.4292	975.441	*Prunus dulcis*
106	XP_007009267.1	Ribulose bisphosphate carboxylase/oxygenase activase 2, isoform X2	48566	5.56	613	K.VPLILGIWGGK.G	8.33% 11.76%	1151.6116	1151.7067	*Theobroma cacao*
107	XP_017977555.1	GDP-mannose 3,5-epimerase 2	48477	6.65	823	R.LTMSDFR.E + Oxidation (M)	7.69% 5.88%	884.398	884.4062	*Theobroma cacao*
108	EOY11078.1	GDP-mannose 3,5-epimerase 1	48472	6.88	629	K.QLETNVSLK.E	7.69% 18.75%	1030.573	1030.5659	*Theobroma cacao*
111	EOY08281.1	Chloroplast signal recognition particle 54 kDa subunit	62806	9.52	506	R.ALLEADVSLPVVR.R	0.00% 4.88%	1380.7866	1380.7977	*Theobroma cacao*
112	YP_009570514.1	ATP synthase CF1 alpha subunit (plastid)	55516	5.50	576	R.ADEISNIIR.E	5.88% 7.50%	1029.538	1029.5454	*Dioncophyllum thollonii*
114	OIV93058.1	Hypothetical protein TanjilG_20720	52571	7.04	64	K.VPDVFYCIK.L	0.00% 6.67%	1139.5506	1139.5685	*Lupinus angustifolius*
116	AAB39238.1	Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit	52833	5.99	66	R.EITLGMVDLLR.D + Oxidation (M)	0.00% 0.00%	1274.4742	1274.6904	*Monotoca scoparia*
118	EOY05144.1	Serine hydroxymethyltransferase 4 isoform 1	52261	7.15	389	K.FASSFDMPGFK.M + Oxidation (M)	0.00% 6.25%	1248.548	1248.5485	*Theobroma cacao*
126	AVQ04668.1	Phosphoglycerate kinase	42386	6.28	53	R.LSELLGVEVK.M	0.00% 100%	1085.8622	1085.6332	*Neolamarckia cadamba*
127	XP_006401314.1	Chaperonin 60 subunit beta 3, chloroplastic	63590	5.63	918	R.KGVVTLEEGK.S	3.33% 2.50%	1058.5846	1058.5972	*Eutrema salsugineum*
129	EOY32236.1	TCP-1/cpn60 chaperonin family protein	64513	5.62	1329	R.VEDALNATK.A		959.4822	959.4924	*Theobroma cacao*

(*continued on next page*)

Table 4 (*continued*)

SPOT ID	ACESSION NUMBER	PROTEIN NAME	MM[a] (kDa)	pI[b]	SCORE[c]	NUP[d]	FDR[e]	Mr (expt)[f]	Mr (calc)[g]	Organism
							10.00% 2.38%			
131	OEL36727.1	2-Cys peroxiredoxin BAS1, chloroplastic	28081	6.45	420	K.EGVIQHSTINNLAIGR.S	0.00% 0.00%	1720.9108	1720.9220	*Dichanthelium oligosanthes*
132 153	PSS07626.1	ATP-dependent Clp protease ATP-binding subunit ClpA CD4B like	103364	6.27	343	R.VLELSLEEAR.Q	0.00% 0.00%	1157.6784	1157.6292	*Actinidia chinensis* var. *Chinensis*
133	XP_007009267.1	Ribulose bisphosphate carboxylase/oxygenase activase 2, isoform X2	48566	5.56	1068	K.VPLILGIWGGK.G	0.00% 0.00%	1151.7078	1151.7067	*Theobroma cacao*
134	EOX93637.1	Chlorophyll *a-b* binding protein 3	29725	6.85	275	R.YAMLGAIGAIAPEILGK.L + Oxidation (M)	10.00% 11.76%	1702.9376	1702.9327	*Theobroma cacao*
138	AVQ04668.1	Phosphoglycerate kinase	42386	6.28	53	R.LSELLGVEVK.M	0.00% 100%	1085.8622	1085.6332	*Neolamarckia cadamba*
144	EOY02403.1	Carbonic anhydrase 1 isoform 2	35284	8.35	155	K.NPALYAELAK.G	0.00% e 6.67%	1088.5844	1088.5866	*Theobroma cacao*
150	EOX93083.1	Photosystem II subunit P-1	28666	8.65	574	R.KFVESTLGSFSVA.-	0.00% 0.00%	1370.7018	1370.7082	*Theobroma cacao*
164	XP_020245676.1	Actin-7	41973	5.31	764	K.AGFAGDDAPR.A	0.00% 0.00%	9754.294	9754.410	*Asparagus officinalis*
168	EOY02246.1	UDP-glucose pyrophosphorylase 2	51505	5.61	611	K.SIPSVIDLDSLK.V	0.00% 0.00%	1285.7058	1285.7129	*Theobroma cacao*
171	AAM28609.1	ATP synthase subunit 1, partial (mitochondrion)	43472	7.81	163	R.VVDALGVPLDGR.G	0.00% 0.00%	1209.6674	1209.6718	*Hydnora africana*
174	EOY28076.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	46257	5.61	615	K.LQQQIASAR.S	3.23% 15.00%	1013.5408	1013.5618	*Theobroma cacao*

[a] MM, corresponds to molecular mass values (kDa).

[b] pI isoelectric point estimated by the program Image Master 2D Platinum 7.0.

[c] Score corresponds to the value of coverage calculated by Mascot.

[d] Number of unique peptides.

[e] FDR corresponds to peptide matches above identity threshold and peptide matches above homology or identity threshold.

[f] Experimentally calculated m/z values, monoisotopic mass (Mr expt).

[g] Theoretically calculated (Mr calculated).

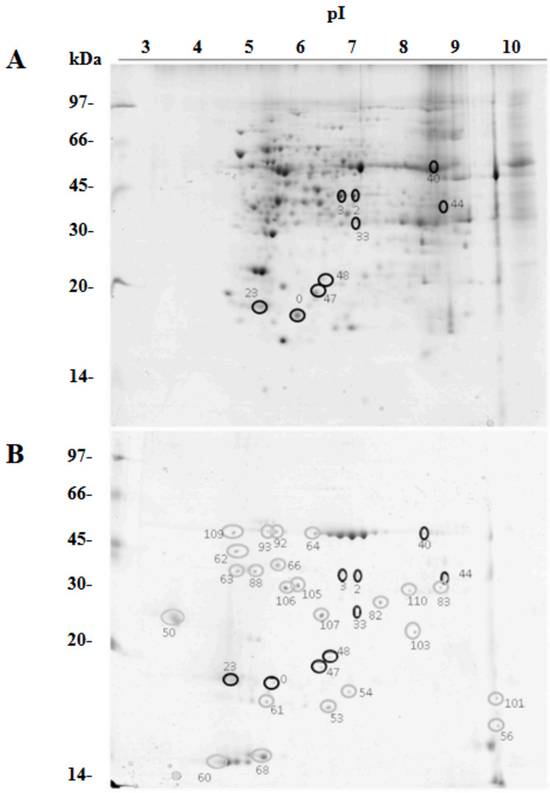

Fig. 1. 2D-PAGE protein profile of young leaf samples from plants of the CCN 51 clonal cocoa genotype, control (A) and exposed to constant wind for a 12 h exposure period (B). Differentially accumulated spots - black circle and exclusive spots - gray circle. The samples were focused on 13 cm strips with nonlinear pH 3–10 (NL).

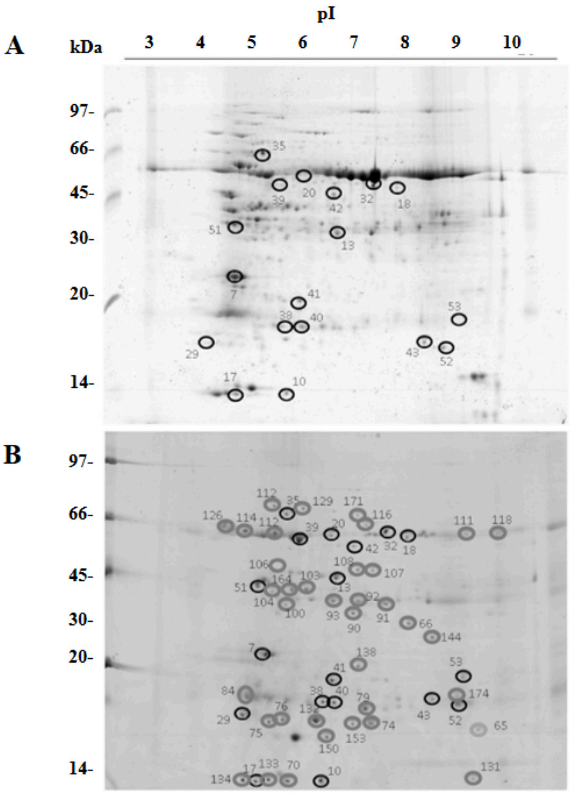

Fig. 2. 2D-PAGE protein profile of mature leaf samples from plants of the CCN 51 clonal cocoa genotype, control (A) and exposed to constant wind for a 12 h exposure period (B). Differentially accumulated spots - black circle and exclusive spots - gray circle. The samples were focused on 13 cm strips with nonlinear pH 3–10 (NL).

digestion, with a lost cleavage site, cysteine carbomidomethylation (Cys) as a modification and methionine oxidation (Met) as a variable modification. Following the MASCOT probability analysis, only significant hits ($p < 0.05$) were accepted. The generated FASTA sequences of identified proteins were obtained in the NCBIprot version 20191120 and date of download in 20200721 using the access number generated by MASCOT tool and taxonomy used (Viridiplantae - Green Plants). The FASTA sequences were analyzed using the BLAST2GO software (http://www.blast2go.com), which provides important information, such as: ontology, functions, biological processes and cell location.

2.7. *Systems biology*

Two networks were built involving proteins differentially accumulated in YL and ML submitted to the CW in relation to their respective controls. Proteins identified exclusively in the controls were considered to have their accumulations reduced in leaves treated with CW.

Proteins of *Arabidopsis thaliana*, homologous to those identified as differentially accumulated in YL and ML of the CCN51 genotype exposed to the CW, in comparison to their respective controls, were submitted to interactome analysis through the STRING database version 11.0 (https://string-db.org/). The proteins were analyzed individually using the following parameters: (i) meaning of network edges - confidence; (ii) active interaction sources - text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence; (iii) minimum required interaction score - high confidence (0.700) more than 50 interactions, significance level of 0.7; (iv) max number of interactors to show - 1st and 2nd shell and no more than 50 interactions. The file for each network was downloaded in TSV format and later the files were merged and analyzed using Cytoscape software version 3.7.1 (https://cytoscape.org/). The modularity and centrality properties (betweenness and node degree) of the network were calculated using the igraph package of the statistical tool RStudio. For each cluster, an enrichment analysis of gene ontology was performed using the BiNGO version 3.0.3 plugin (https://apps.cytoscape.org/apps/bingo).

2.8. *Statistical analysis*

The statistical evaluation of the relative point volumes allow detection of significant ($p < 0.05$) variables and less abundant spots in YL submitted to stress by CW for 12 h, in relation to the control. (Tables 1 and 2). On the other hand, there was a significant increase ($p < 0.05$) in the abundance of proteins differentially expressed in ML submitted to CW for 12 h, compared to the control (Tables 3 and 4) from the analysis of variance (ANOVA) to identify the differentially expressed spots ($p \leq 0.05$ and ≥ 1.5-Fold change) in software ImageMaster 2D Platinum 7.0 (GE Healthcare). The calculations of network modularity and centrality properties were obtained using the igraph package of the statistical tool RStudio version: 0.99.896 (https://rstudio.com/). Differentially abundant proteins were evaluated using the Heatmap 79 R package (Core Development Team, 2016) using RStudio version 4.0.0 (https://rstudio.com/).

3. Results

Proteomic profiles of young (YL) and mature (ML) leaves from CCN 51 cocoa clonal plants submitted to CW, for an exposure period of 12 h, and control treatment (without exposure to wind) were analyzed using triplicates of 2-DE gel protein samples. Variations in the accumulation of spots on gels in YL and ML and their treatments compared to control were assessed on pH gradient between 3 and 10 and molecular mass of 14–97 kDa (Figs. 1 and 2). Proteomic maps showed 120 and 198 spots in

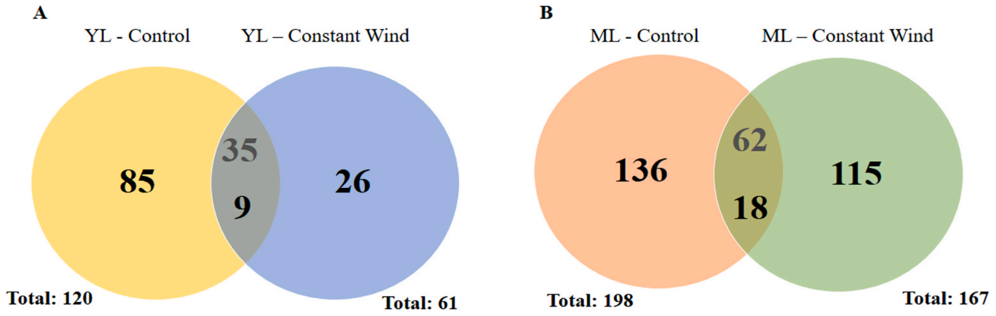

Fig. 3. Number of protein spots identified from 2-DE gel. Venn diagram of the total spots identified in the control treatment of young leaves of plants of the clonal cocoa genotype CCN 51, of young leaves submitted to constant wind for a 12 h exposure period (A), of the control treatment of mature leaves and of mature leaves subjected to constant wind for a 12 h exposure period (B).

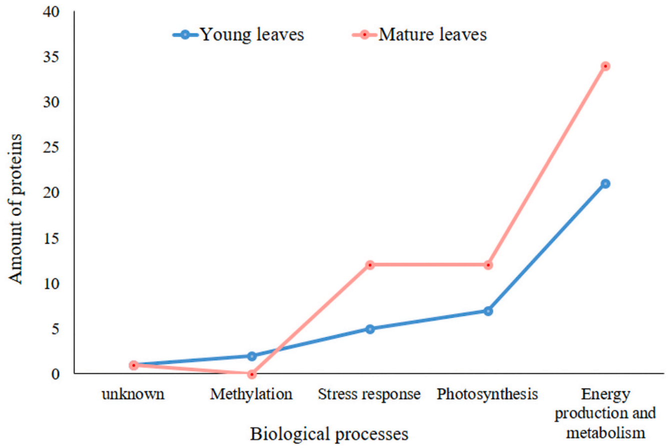

Fig. 4. Differentially accumulated and exclusive proteins according to their biological processes identified by mass spectrometry (MS/MS) in young leaves (blue line) and mature leaves (pink-red line) of the cocoa genotype CCN 51 submitted to constant wind for a 12 h exposure period. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

YL and ML on control gels, respectively, and 61 and 167 spots in YL and ML on gels exposed to CW for 12 h, respectively (Fig. 2). Of the 35 spots common between YL control and exposed to wind flow, nine reduced the abundance after wind stress exposure (Fig. 3A). On the other hand, in ML, 62 common spots were detected between control and leaves exposed to wind flow (Fig. 3B). Of these, 18 showed variation in protein accumulation, being 15 with increased protein relative abundance and three with reduced protein relative abundance. We identified 36 proteins in YL and 59 proteins for ML differentially accumulated and exclusive (Fig. 4). The identified proteins showed homology with protein sequences found in the NCBI, through the Mascot tool.

Proteins with significant variations ($p < 0.05$ and fold ≥ 1.5) of expression were categorized according to their physiological function and molecular processes. Most of the differentially expressed proteins identified in YL and ML were grouped into four main categories. Components involved in energy production and metabolism, such as (i) metabolic processes of organic substances, (ii) nitrogen compounds, (iii) biosynthetic compounds and (iv) oxidation reduction processes. These proteins corresponded to a total of 58% in YL submitted to CW (Fig. 5A). On the other hand, in ML, the sum of the four categories totaled 57.6% in the plants exposed to CW (Fig. 5B). Among the proteins identified in YL submitted to CW, about 18% and 5% were related to stress and methylation responses, respectively (Fig. 5A). In contrast, proteins related to methylation have not been identified in ML. In the category of responses to stress, about 20.33% were identified in ML submitted to CW plants (Fig. 5B). Regarding photosynthetic processes proteins, about 19% were found in YL and 20.33% in ML exposed to CW.

In YL exposed to CW we identified differentially abundant proteins in the category of metabolic processes of organic substances, but not proteins in the categories of biosynthesis and metabolism of nitrogen

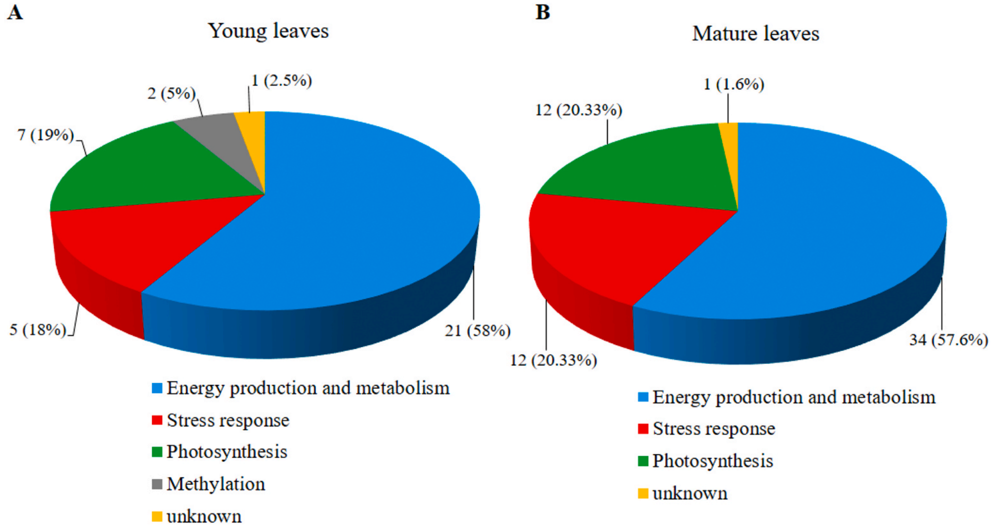

Fig. 5. Functional categorization of differentially accumulated proteins from plants of the clonal cocoa genotype CCN 51. In young leaves (A) and mature leaves (B) exposed to constant wind for a 12 h exposure period.

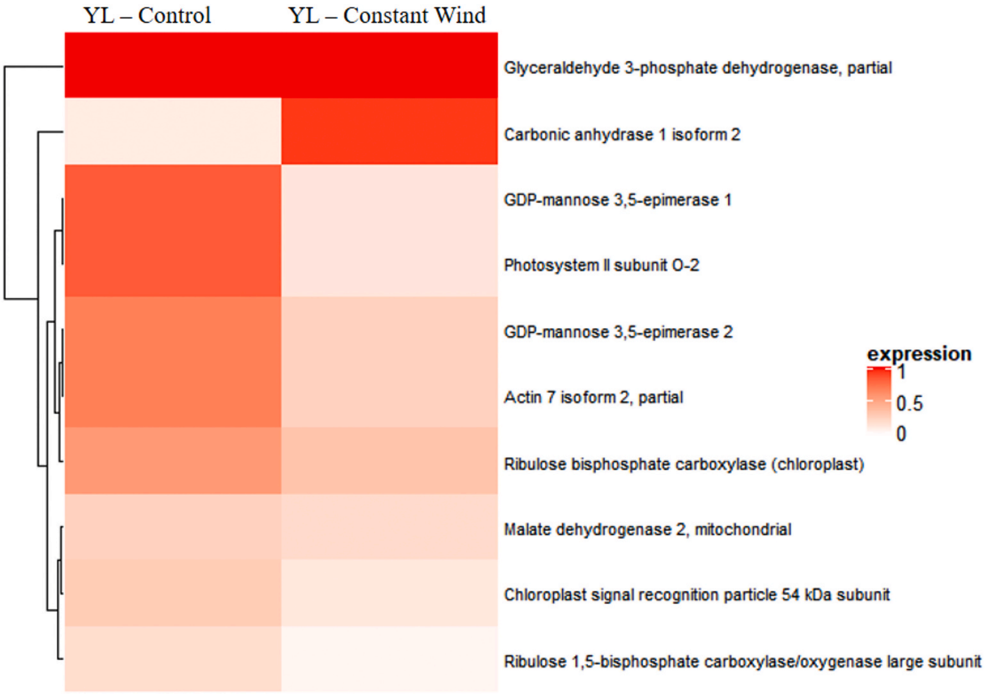

Fig. 6. Heat map graph of leaf proteins of the CCN 51 clonal cocoa genotype, differentially accumulated from the control treatment of young leaves and young leaves submitted to constant wind for a 12 h exposure period.

compounds. Proteins such as 3,5-epimerase 1 and 2 of GDP-mannose (spots 2 and 3), glyceraldehyde 3-P dehydrogenase (spot 44), malate 2 dehydrogenase (spot 33), larger subunit of ribulose 1,5- bisphosphate carboxylase/oxygenase (Rubisco) and ribulose bisphosphate carboxylase (spots 47 and 48), showed a significant reduction ($p < 0.05$) in the accumulation in YL compared to the control (Table 1). Among the proteins exclusive to the metabolic processes of organic substances were identified ribosomal proteins S17 and 40 S (spots 56 and 101) and the 3-O-methyltransferase enzymes of caffeic acid 1 (spots 105 and 106), ATPase β subunit - mitochondrial F1 (spot 92) and CF1 ATP synthase α subunit (spot 93), in addition to isoforms of Rubisco activase (spots 62, 65 and 88) (Table 2).

In the category of metabolic processes of nitrogenous compounds, spots 56, 92, 93, 101 and 103 were detected as accumulated only in YL exposed to CW. These spots were the same in the category of biosynthesis and metabolic processes of organic substances, except DNA ligase 6 (spot 103). For proteins related to oxidoreductase activities, four differentially accumulated ones were observed among the spots in common, which correspond to the same proteins mentioned in the category of metabolic processes of organic substances (spots 33, 44, 47 and 48) (Table 1). Proteins of the P-1 subunit of photosystem 2 (PS2) (spot 61), Rubisco's larger subunit (spot 63), isoform 1 of glyceraldehyde-3P C2 dehydrogenase (spot 83) were accumulated only in YL exposed to the wind and included in this same category (Table 2).

Proteins related to methylation were not significantly different between YL control and YL exposed to CW. The enzyme 3-O-methyltransferase from caffeic acid 1 (spots 105 and 106) was identified as accumulated only in YL submitted to CW. In the protein category of stress responses, two proteins in common were found in YL submitted to CW, 3.5 - GDP-mannose epimerase 2 and malate 2 dehydrogenase (spots 3 and 33), which are also a part of metabolic processes (Table 1). In addition, three proteins accumulated in that treatment were also identified, the RNA binding protein 31-kDa and DNA ligase 6 (spots 50 and 103, respectively). Folding proteins were found only in YL control plants (Table 2).

The following proteins were identified in ML exposed to CW: 40 S ribosomal protein S5 (spot 29), Rubisco's largest subunit (spots 32, 41 and 44), leaf isoenzyme glutamine synthase (spots 42 and 58), isoform 1 of the bound folding protein NAD (P) of the Rossmann superfamily (spot 38), β subunit of the chloroplast ATP synthase (spot 40), triosphosphate 1 isomerase isoform (spot 43), chloroplast TuA elongation factor (spot 13) and 26 S proteasome regulatory complex, ATPase (spot 51) (Table 3). They are related to metabolic processes of organic substances, presenting greater expression in relation to the control. The cysteine synthase proteins (spot 7) and the larger Rubisco subunit (spot 32) showed reduced expression in ML exposed to CW compared to control. In addition, in this same category, some proteins such as the β subunit of type-4 proteasome (spot 65), Phosphoglycerate kinase (spot 126), chloroplast ATP-dependent FTSH 2 metalloprotease (spot 132) and HCF136 stability/assembly factor PS2 (spot 79) were identified only in ML exposed to CW (Table 4).

Proteins related to oxidation reduction processes (spots 20, 32, 39, 40, 41 and 44), biosynthesis processes (spots 7, 13, 29, 32, 40, 41, 42, 43, 44 and 58) and processes related to nitrogenous compounds (spots 7, 13, 29, 42, 43 and 58) in mature leaves in their control and treatment were identified (Table 3). These differentially accumulated proteins were the same that are a part of the metabolic processes of organic substances, except for peroxiredoxin chloroplast proteins and PS2 P-1 subunit (spots 20 and 39), which belong to the category of oxidation reduction activities. In addition, other proteins have been identified, such as the PS2 P-1 subunit (spots 150 and 153), the stability/assembly factor HCF136 of PS2 (spots 79) and the superfamily protein of the HSP20 chaperones (spot 100) in the treatment ML submitted to CW (Table 4). The proteins from spots 79 and 126, identified only in ML exposed to CW, were also categorized within the processes of metabolism of nitrogen compounds and biosynthesis, as well as in the processes of metabolism of organic substances (Fig. 5B).

Among proteins related to stress responses, only isoform 1 of the folding protein linked to NAD (P) of the Rossmann superfamily (spot 38) showed greater abundance in ML exposed to wind, in relation to the control. In addition, the glyoxalase I protein isoform 1 (spot 76), peroxiredoxin BAS1 (spot 131), proteasome type-4 beta subunit (spot 65) and chloroplast ATP-dependent FTSH 2 metalloprotease (spot 132) were also accumulated in this treatment (Table 4). In contrast, in ML exposed

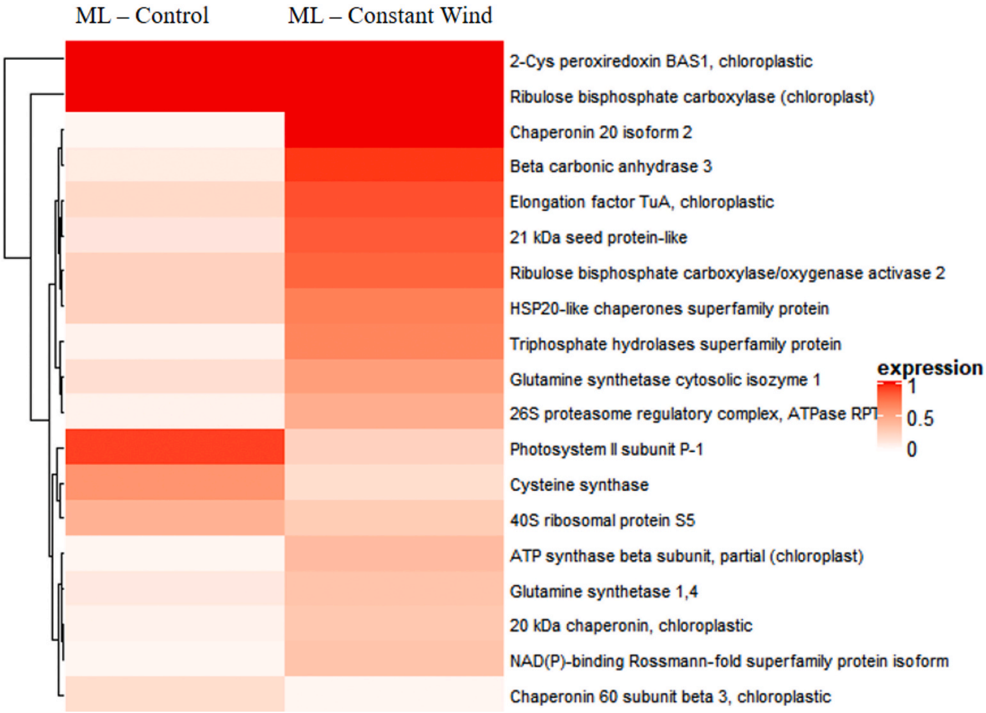

Fig. 7. Heat map graph of leaf proteins of the CCN 51 clonal cocoa genotype, differentially accumulated from the control treatment of mature leaves and mature leaves submitted to constant wind for a 12 h exposure period.

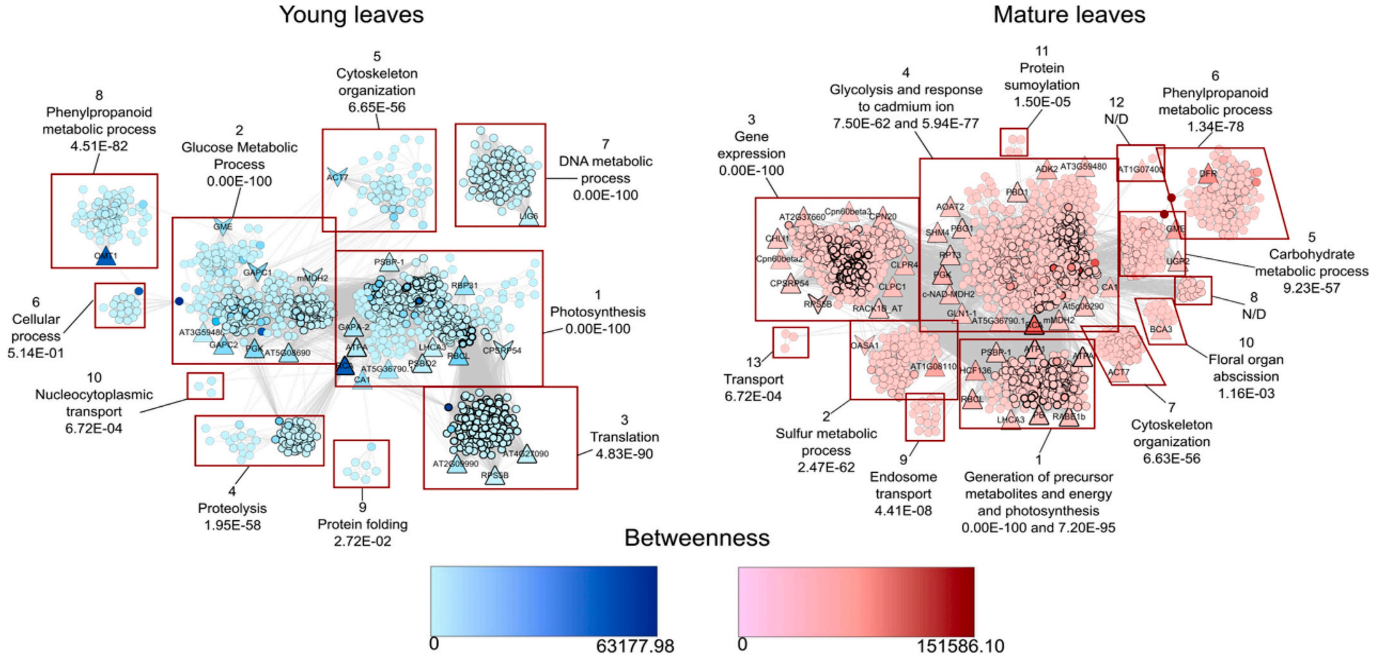

Fig. 8. *Arabidopsis thaliana* protein interaction networks homologous to those identified with increased accumulation (triangle) and decreased accumulation (inverted triangle) in young leaves (blue tones) and in mature leaves (pink-red tones) of plants of the CCN 51 cocoa genotype exposed to constant wind, in relation to their respective controls. Betweenness value represented by the color of the nodes, the lightest being the lowest value and the darkest the highest value. The width of the edge of the nodes represents the value of node degree, the greater the width of the edge, the greater the value and vice versa. Clusters (CL) are delimited by red frames. For each cluster, a biological process was assigned with the lowest corrected p-value, according to the BiNGO tool. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to CW, folding proteins were identified, such as the chaperone family (spots 10, 18 and 35), 26 S proteasome regulatory complex, (spot 51) and 20 kDa chaperonin (spot 52), whose abundance increased in relation to the control, in addition to proteins from spots 100, 126, 127, 129 and 138 (Table 4).

The amount of proteins in YL exposed to CW was quite reduced, when compared to the control, for most categories. Mechanical stress caused by the action of wind reduced the abundance of down-regulated proteins related to metabolic processes of organic substances in YL exposed to CW compared to its control (Fig. 6). A total of 21 proteins,

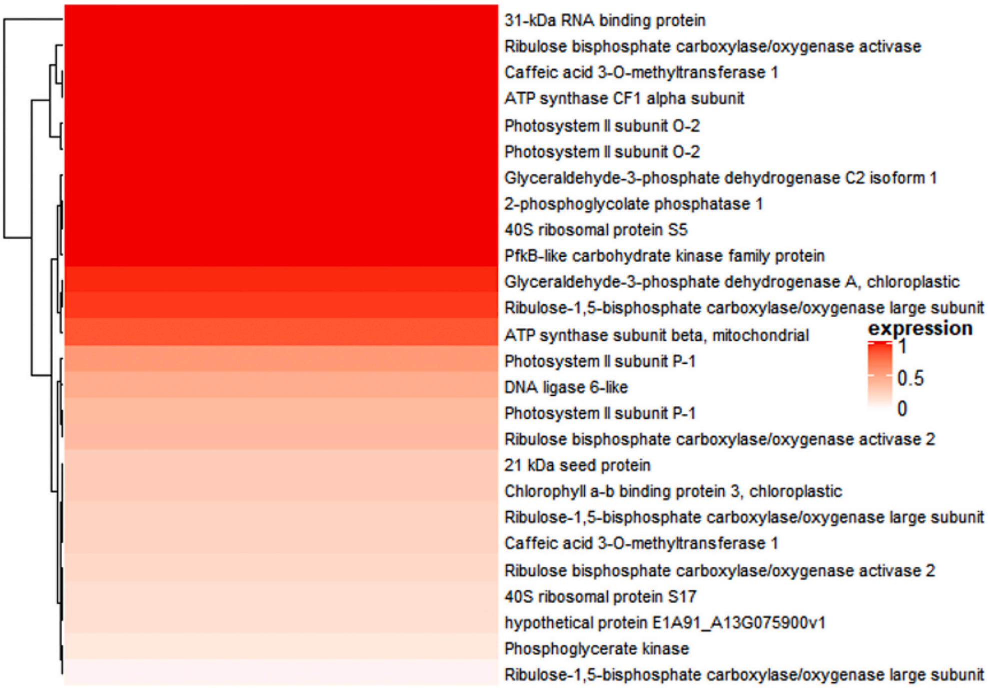

Fig. 9. Heat map graph of leaf proteins exclusive to the clonal cocoa genotype CCN 51, from the treatment of young leaves submitted to constant wind for a 12 h exposure period.

including proteins related to biosynthesis and oxidoreduction processes, were identified in YL exposed to CW. An increase in the abundance of up-regulated proteins related to methylation and stress responses, whose numbers were 2 and 5 in YL of plants exposed to CW. In ML submitted to CW, an increased number of up-regulated proteins was observed in the categories oxidoreduction and stress proteins (12). Regarding proteins related to nitrogenous metabolic compounds, there was a reduction in the amount of down-regulated proteins in ML of plants exposed to CW, compared to control (Fig. 7).

The representative network of YL exposed to the CW, illustrated in blue tones, is composed of 1.065 nodes (proteins), 28.656 connectors and 10 clusters (Fig. 8). The network contains 150 proteins considered bottleneck (betweenness value above average), of which 19 are homologous to those identified in *T. cacao*; 16 with an increase and 3 with a decrease in accumulation (Table S1). The network also contains 531 proteins considered to be hubs (node degree value above the average) of which 17 are homologous to the proteins identified in *T. cacao*; 15 with an increase and 2 with a decrease in accumulation (Table S1). In turn, 12 of the proteins homologous to those identified in *T. cacao* have characteristics, bottleneck and hubs, in the network. The homologous protein of 21 kDa seed (EOY21251.1) had confidence parameters below those established for the construction of the network.

In pink-red tones, the representative network of ML exposed to the CW has 1.590 nodes (proteins), 35.968 connectors and 13 clusters (Fig. 8). The network contains 301 proteins considered bottleneck, of which 30 are homologous to those identified in *T. cacao*; 29 with an increase and 1 with a decrease in accumulation (Table S2). The network also contains 598 proteins considered hubs, of which 27 are homologous to the proteins identified in *T. cacao*, 26 with an increase and 1 with a decrease in accumulation (Table S2). In addition, 17 of the proteins homologous to those identified in *T. cacao* have both bottleneck and hubs characteristics. On the other hand, the clusters with the highest number of proteins in the representative network of YL was cluster 1 related to photosynthesis and in the representative network of ML was cluster 4 related to glycolysis and the response to cadmium ion (Fig. 8; Tables S1 and S2). Sulfur metabolic process, response to cadmium ion and floral organ abscision are biological processes observed as attributed in the clusters of the ML network that were not observed in the YL network.

4. Discussion

4.1. Wind exposition causes changes in young and mature leaf proteome profile of the clonal cocoa genotype CCN 51

Proteomic analysis in YL and ML of CCN 51 cocoa clonal plants submitted to mechanical stress by CW allowed the identification of proteins whose abundance was altered in short time (12 h). A total of 10 and 19 proteins were differentially accumulated in YL (Fig. 6) and ML exposed to CW (Fig. 7), respectively. Proteomic analysis revealed a series of changes in the composition of important proteins involved in several metabolic pathways, mainly in photosynthesis; both in YL and ML exposed to CW for 12 h. Changes in protein relative abundance were well documented in proteomic studies of stressed organisms (González-Fernández et al., 2010). The difference in the concentrations of these proteins can occur through the synthesis of new proteins, through degradation or through post-translational modifications of existing proteins.

The analysis of systems biology showed, illustratively, that photosynthesis was one of the most affected processes in response to the mechanical stress caused by CW in both YL and ML (Fig. 8). This is due to the fact that the clusters related to this process (cluster 1) concentrated a greater amount of proteins (329).

Among the photosynthetic proteins, the enzyme Rubisco was the most abundant one in the plant tissues once it represents about 50% of leaf proteins. Despite the significant decrease ($p < 0.05$) of Rubisco isoforms (spots 47 and 48), observed in YL exposed to CW, compared to control, new Rubisco isoforms were found (spots 54, 63 and 109) for this treatment in the same plants (Fig. 9). According to Zou et al. (2005), genes that encode small subunits of Rubisco are down - regulated during biotic stresses. Previous work has shown that many genes involved in plant defense against pathogens and mechanical stress behave similarly.

ML showed similar responses for this enzyme including reduction in Rubisco accumulation (spot 41) and the appearance of new isoforms

Fig. 10. Heat map graph of leaf proteins exclusive to the clonal cocoa genotype CCN 51, from the treatment of mature leaves submitted to constant wind for a 12 h exposure period.

(spots 73, 75 and 116) (Fig. 10). In addition, ML showed a significant increase ($p < 0.05$) in the fragment of the larger subunit of Rubisco (spot 41). The decrease in the accumulation or repression of this enzyme in YL and ML submitted to wind, and the appearance of new isoforms, may result from the occurrence of post-translational modifications, which can affect the production of energy or, in the case of the appearance of new one isoforms, may represent an acclimatization response to the stressor. The increase in the accumulation of other isoforms, such as Rubisco activase in YL (spots 62, 65 and 88) and in ML (spots 106, 116 and 133), exposed to CW for 12 h, corroborate with the positive regulation of the activity of Rubisco to improve the efficiency of CO_2 fixation in *Theobroma cacao* plants (Reis et al., 2018) and in *Arabidopsis thaliana* plants exposed to abiotic stress (Rocco et al., 2013). According to Rocco et al. (2013), the up-regulation of this enzyme may be a part of the acclimatization response, in order to maintain CO_2 fixation under stress conditions.

The importance of Rubisco and Rubisco activase was confirmed by network analysis, since these proteins are considered bottleneck and hubs within the YL and ML networks. The importance of bottleneck proteins is related to their ability to connect several clusters that, in this case, represented other biological processes. In turn, hubs proteins play an important regulatory role within the network, since they are proteins with a greater number of connections. Additionally, Rubisco activase has the highest betweenness value within proteins homologous to those identified in *T. cacao*.

Photosystem 2 (PS2) is a protein complex involved in photosynthesis and composed of several subunits. In addition, it is one of the most affected components of photosynthetic machinery during various types of stresses (Gururani et al., 2015). One of the PS2 subunits (0–2) was identified in YL exposed to CW for 12 h, in two different spots (spots 23 and 82), one being down-regulated and the other exclusive, in addition to another important PS2 subunit (P −1) (spots 53 and 61). On the other hand, in ML exposed to CW for 12 h, the PS2 P-1 subunit was identified as up-regulated and exclusive (spots 20 and 150, respectively), along with other important photosynthesis proteins, such as the factor HCF136 of stability/assembly of PS2 (spot 79), EF-Tu (spot 13) and chlorophyll binding proteins ab 3 (spot 134), accumulated in YL exposed to CW for 12 h (spot 66). EF-Tu has been identified as a protein located in the chloroplast and involved in the regulation of protein synthesis (Table 3). Previous studies have shown that this protein was positively regulated after cold stress (Rocco et al., 2013). In general, the accumulation of these proteins may have occurred in an attempt to repair the mechanical damage caused by wind at the cellular level, to maintain photosynthetic activity.

Studies by Reis et al. (2018), with the same clonal cocoa genotype, demonstrated that the pulvine and YL and ML lamina, exposed to intermittent winds (IW) and CW, with different exposure times (3 and 12 h), presented macroscopic mechanical injuries and microscopic and ultrastructural changes involving rupture of cell membranes in the tissues of the pulvinus and mesophyll, followed by programmed cell death, mainly in YL tissues. According to these authors, there was also a change in the expression of the *psbA* and *psbO* genes, both in YL and in ML exposed to IW and CW at a speed of 4.5 m s^{-1}. The *psbA* gene encodes the D1 protein intrinsic to PS2, while the *psbO* gene encodes the *PsbO* protein extrinsic to PS2, which is considerably important for the evolution of oxygen during the photochemical phase of photosynthesis (Popelkova and Yocum, 2011). Outside that, the *PsbO* protein, along with *PsbP* and *PsbQ*, plays an important role in the accumulation of PS2 reaction centers, which may be involved as an assembly/stabilization factor of this Photosystem (Bricker and Frankel, 2011). The PsbO protein can perform this function through its ability to phosphorylate protein D1, thereby increasing its stability under stress conditions (Bricker and Frankel, 2011).

ML, exposed to CW for 12 h, showed an up-regulation of another isoform of the carbonic anhydrase protein. In addition, the 21 kDa subunit, a chloroplast recognition signal peptide, involved in post-translational targeting of members of the chlorophyll-binding protein family were down-regulated in YL (Fig. 9) and exclusive in ML (Fig. 10). It has been observed in ML, of CCN 51 cocoa clones, that CW and IW, with different exposure times, directly interfere with stomatal conductance, transpiration and water use efficiency, causing reduction in the photosynthetic rate (Reis et al., 2018).

The phosphatase of 2-phosphoglycolate I (spots 59 and 64), identified in FJ exposed to CW (Fig. 9), is an important nuclear enzyme, involved in different biosynthetic processes, such as the assimilation of photosynthetic carbon in plants, besides favoring the maintenance of ATP. Usually, chloroplasts synthesize very large daily amounts of 2-phosphoglycolate (2-PG), from the oxidation reaction of Rubisco (Schwarte and Bauwe, 2007). In addition, this enzyme participates in the elimination of metabolites from the Calvin & Benson cycle, which results in several negative effects on metabolism, such as the inhibition of the activity of the enzyme isomerase of the triose phosphate (ITP) (Schwarte and Bauwe, 2007). On the other hand, ITP plays an important role in glycolysis and is a source of energy production for the growth and maintenance of cellular processes, especially in conditions of environmental stress (Anderson, 1971). The accumulation of 2-PG may have compromised the action of ITP, decreasing energy production and affecting the synthesis of other enzymes. In contrast, in ML, there was a greater accumulation of 2-PG (spot 70) and ITP (spots 43 and 174).

Glyceraldehyde-3P dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway, which catalyzes the conversion of glyceraldehyde-3P to 1,3-bisphosphoglycerate with a concomitant reduction in NAD^+. In YL submitted to CW for 12 h, isoforms of GAPDH were identified in chloroplasts and cytoplasm (spots 83 and 110) (Table 2). Glycolytic GAPDH may be involved in the response to stress, since its concentration is increased by different environmental stresses (Santos et al., 2014). In ML exposed to CW for 12 h, no changes in GAPDH accumulation were identified. However, phosphoribulokinase (spots 126 and 138), an enzyme also important in the glycolytic pathway and categorized as a stress-related protein, was identified in ML submitted to mechanical stress caused by the wind (Table 4).

Proteomic analysis did not demonstrate a significant reduction in mitochondrial malate dehydrogenase mtMDH (spot 33) in YL submitted to CW for 12 h, compared to control (Fig. 5). In contrast, in ML, exposed to the same mechanical stress conditions, in addition to mtMDH, lactate/malate dehydrogenase and fructokinase were identified as exclusive enzymes (Fig. 10), which may have favored energy production. The mtMDH enzyme catalyzes the reversible reduction of oxaloacetate to malate, having an important role in the Krebs cycle in the production of NADH (Nunes-Nesi et al., 2005).

Regarding proteins related to carbon metabolism and energy generation, biotic and abiotic stresses have been shown to negatively affect the glycolytic pathway and reduce energy production in plants, causing a marked decrease in the concentration of different glycolytic enzymes or proteins involved in energy-generating reactions. For example, cultivars of *Zea mays* when exposed to the cold were intolerant, due to the reduction in oxidative phosphorylation (Mauro et al., 1997). Therefore, as a response of the cocoa plants to the mechanical action of the wind, there was interference in the electron transport chain in YL, inhibiting the production of ATP and carbon compounds (Reis et al., 2018). Essential enzymes for the production of ATP, from ADP + Pi, were found in very low amounts in YL compared to control (Table 1). The α CF1 subunit of the ATP synthase (spot 93) was identified only in YL exposed to CW (Table 2).

In ML, there was an up-regulation of α and β subunits of the chloroplast ATP synthase and the subunit 1 of the mitochondrial ATP synthase. In addition, there was the expression of other proteins related to the metabolism of nitrogen compounds, such as (i) ribosome recycling factor, (ii) glutamine synthase isoenzyme, (iii) cysteine synthase, (iv) hydroxymethyltransferase isoform 1 4 of serine, (v) glutamine 1 synthase and (vi) pyrophosphorylase 4 of UDP-glucose 2, showing a better response of these plants to the stimuli caused by the wind. On the other hand, proteins that perform functions related to signal transduction and biosynthesis, such as DNA ligase 6 (103), ribosomal proteins (spots 56 and 101) and mitochondrial ATPase – beta (spot 92) were accumulated in YL exposed to CW (Fig. 9).

The 3,5-epimerase 1 and 2 isoforms of GDP-mannose (spots 2 and 3) were down-regulated in YL submitted to mechanical stress caused by the action of CW for 12 h, in relation to control (Fig. 6), whereas in ML, exposed to the same conditions, there was an accumulation of these enzymes. GDP-D-mannose epimerase is the most highly conserved protein involved in ascorbate biosynthesis, which is of crucial importance for plants (Mounet-Gilbert et al., 2016). Ascorbic acid is one of the main antioxidants that guarantees the protection of plant cells against reactive oxygen species (ROS), generated during normal physiological processes, as well as by biotic and abiotic stresses (Mounet-Gilbert et al., 2016). In addition, GDP-D-mannose epimerase is considered a central enzyme, which links the main ascorbate biosynthesis pathway to primary cell wall metabolism in higher plants. D-mannose is a precursor for the synthesis of L-fucose and L-galactose and, therefore, for pectin and hemicellulose.

GDP-mannose is responsible for regulating the main development processes involving cell division and cell expansion. The down-regulation of proteins related to biosynthetic processes, such as GDP and also actin 7 (spot 30), favored YL intolerance to CW for 12 h (Table 1), since it interfered with the production of cell wall polysaccharides and in the production of ascorbate peroxidase, the latter found in YL of control plants. In addition to the accumulation of the enzyme GDP-mannose epimerase in ML exposed to CW for 12 h, there was an accumulation of actin 7 (spot 104) and isoform 2 of actin 7 (spot 164), which are proteins also associated with the structuring of the cell walls and in the organization and stabilization of the cytoskeleton (cluster 5 on the YL network and cluster 7 on the ML network). Actin has been linked to acclimatization to cold stress. In addition, this protein can function as a mechanosensitive structure regulated by phytohormones (Galkin et al., 2012). The cells respond to mechanical stimuli by transmitting the force to a mechanosensitive structure and later converted into a chemical signal (Blume et al., 2017), according to the observations made in the interaction networks (Fig. 8). This is due to the fact that in the ML network, where Act 7 has an increase in accumulation, there are also clusters that represent processes regulated by phytohormones such as cluster 10 (abscission). Interestingly, in stomatal cells, treatment with ABA induces the reorganization of actin filaments from radial to random and fragmented (Blume et al., 2017).

Rubisco, as well as other proteins such as PS2 subunits, peroxiredoxin, NAD (P) binding folding proteins of the Rossmann superfamily, related to oxidoreductase activities, can also act as metabolism regulating agents (Foyer and Noctor, 2003). The NAD-binding-folding protein (P) has also been identified with stress-related protein. Redox signals are initial warnings that control leaf's energy balance. Changes in the redox state of the compounds regulate the expression of several genes linked to photosynthesis, thus providing a more efficient adjustment of energy production for consumption (Foyer and Noctor, 2003).

4.2. Wind exposition affects protein of stress responses, folding and methylation in cocoa leaves

The proteins of the HSP chaperone superfamily are important in the folding, assembly, translocation and degradation of proteins in all cell compartments, in addition to directing defective proteins to the proteasome (Kosová et al., 2011). The increased activity of these proteins is related to responses to a wide range of environmental stresses, which have been reported in different plant species (Kosová et al., 2011). Contrary to what usually occurs in most plants exposed to biotic and abiotic stresses, in this work, HSP chaperone proteins were not detected in YL exposed to CW for 12 h, which may have hampered the maintenance of cellular homeostasis and favored the intolerance of plants to wind. In contrast, in ML, exposed to the same type of mechanical stress, there was, an up-regulation of HSP20 chaperones (spot 18), heat shock proteins 20 kDa (spot 10), β 3 subunit of chloroplast chaperonin 60 and 20 kDa chaperonin, chloroplastic (spot 52) (Fig. 7), in addition to the exclusive ones (spots 100 127 and 129).

Other proteins, such as potassium channel proteins are considered essential for cell ion homeostasis as well as for osmotic regulation and cell stability (Nakamura et al., 1995). These proteins were also categorized as folding proteins, which favored the tolerance of ML exposed to

CW for 12 h. The chlorome genome of terrestrial plants encodes proteins that play roles in photosynthesis or gene expression (Kupsch et al., 2012). Many factors necessary for cleaving, splicing or editing mRNAs have been described in recent years. RNA-binding proteins (RBPs) and several of these act in the editing of RNA of specific sites and in the protection of chloroplast mRNA against degradation and are still considered as specific stabilizers of chloroplast transcripts (Kupsch et al., 2012).

In this work, a large accumulation of RNA-31-kDa ligand proteins (spot 50) was identified in YL exposed to CW for 12 h. Interestingly, some studies have already shown high concentrations of mRNA under cold conditions, while the concentrations of all tested proteins have been reduced to 50% or less (Kupsch et al., 2012). In this present research, the results correspond to those demonstrated by these authors, since there was a decrease of about 50% of the total proteins in YL submitted to CW for 12 h and a significant increase in the RNA-31-kDa binding protein. The large accumulation of this protein is usually due to the demand for mRNA (Kupsch et al., 2012). What can also be attributed as a strategy used by plants exposed to wind to intensify the translation of proteins, since the stress promoted by the wind interfered in the accumulation of proteins.

In addition, other processing proteases were expressed, such as (i) DegP isoform 1 (spot 96), (ii) ATP-dependent FtsH metalloprotease bound to thylakoid membranes (spot 132), (iii) subunit-related protein proteolytic protease Clp dependent on chloroplast ATP 4 (spot 153) and (iv) β type-2-A and type-4 subunit of the proteasome (spots 74 and 65), which are considered critical for the biogenesis and maintenance of PS2 (Chi et al., 2012). More in-depth studies on proteases have already been carried out during seed germination, in which proteolytic activity is associated with seedling growth and development as well as with protein renewal (Rao et al., 1998). In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell, while intracellular proteases play an important role in regulating metabolism (Richardson, 1991). Aerobic protein degradation would be an efficient way to synthesize new proteins by recycling amino acids instead of using energy-consuming processes such as ion transport, nitrogen assimilation and synthesis of new amino acids (Zou et al., 2010). However, the occurrence of protease inhibitors in the plant kingdom represents a defense mechanism. Interestingly, trypsin inhibitors may perform other functions, such as protecting cells against free radicals, through the regeneration of ascorbic acid (Hou et al., 2000).

The accumulation of the enzyme 3-O-methyltransferase involved in caffeic acid biosynthesis may have been induced in stress caused by CW for 12 h in YL (spots 105 and 106), related to the biosynthesis of monolignols, the precursors of lignin. Lignin, in turn, is one of the main components of plant biomass and is involved in strengthening the cell wall of plant tissues under conditions of mechanical stress and biotic stress (Dauwe et al., 2007). Saidi et al. (2009) evaluating the effects of mechanical stress in *Solanum lycopersicum* observed morphological changes, such as stiffening of the cell wall, associated with induction in the lignification process. The accumulation of the enzyme 3-O-methyltransferase in caffeic acid may have been induced in order to stiffen the cell wall, since the YL was still in the development process and had little cell wall stiffness. In addition, this enzyme has also been categorized in the group of proteins involved in methylation processes, since it is associated with alternative methylation pathways in lignin biosynthesis, during plant resistance responses to diseases (Ye et al., 1994). Previous work has shown that the deficiency of this enzyme has a small effect on the content and quality of lignin (Do et al., 2007). In the present study, the accumulation of this enzyme, identified in YL exposed to CW for 12 h, may have induced an improvement in the quality of lignin.

YL with less lignified cell walls, compared to ML, are subject to greater intensity of stress imposed by mechanical damage to tissues caused by the wind. This stress leads to an increase in the accumulation of proteins involved in methylation, which can affect the methylation pattern of chromatin and histone proteins in YL submitted to CW. This, in turn, can promote reprogramming of gene expression in these plants, in order to improve their adaptation to the condition of wind stress. One hypothesis to be investigated is whether tolerance to stress caused by wind can be induced in parent plants to obtain new cocoa plantations more adapted to the wind, through inheritance transmitted by epigenetic mechanisms, such as those involving methylation patterns of chromatin.

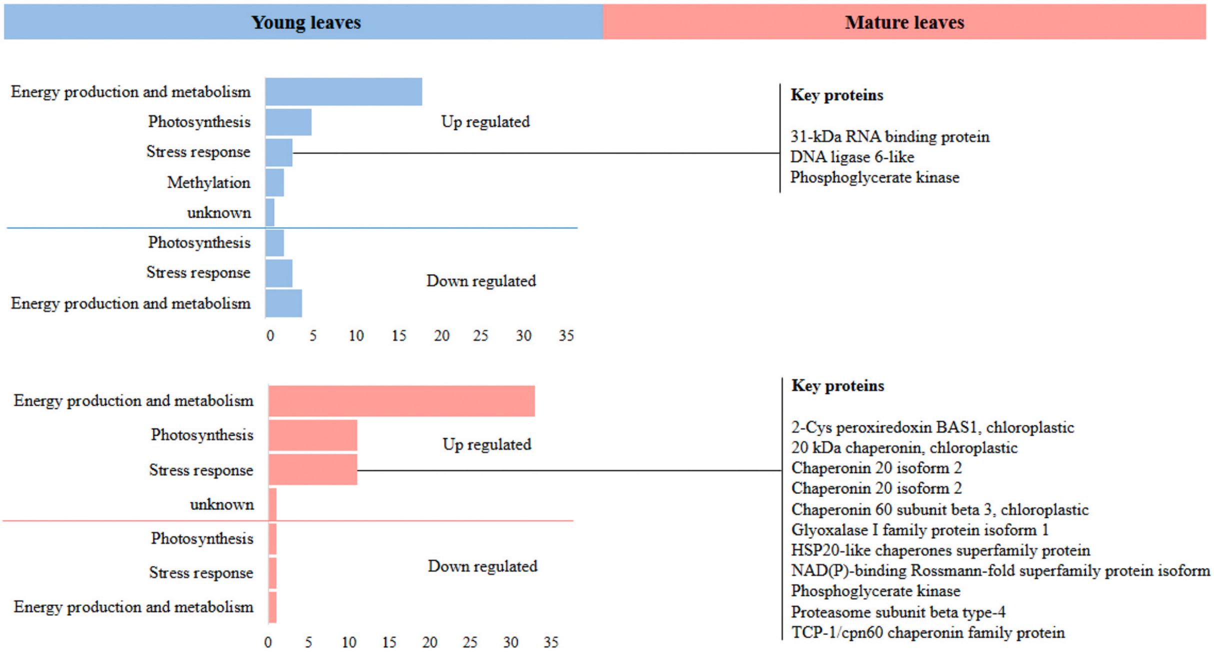

Fig. 11. Response schematic model of young and mature leaves of the clonal cocoa genotype CCN 51 submitted to the constant wind for a period of 12 h of exposure, through proteomic analysis. Accumulation levels (up and down regulated) reflect biological functions such as energy production and metabolism, photosynthesis, stress response and methylation. In mature leaves there was a greater accumulation of proteins related to the activation of defense and signaling pathways, demonstrating differential responses between young and mature leaves.

DNA ligase is an enzyme responsible for the physical reintegration of the ends of DNA during events of programmed replication and genetic recombination (Sunderland et al., 2006). In addition, DNA is constantly damaged by endogenous and environmental factors, which result in DNA breakdown in eukaryotic cells on a daily basis (Lindahl, 1993). DNA ligase, identified in YL exposed to CW for 12 h, was categorized as a stress-related protein and its accumulation may have occurred due to the damage caused to DNA molecules, at the cellular level, in response to induced mechanical stress on cocoa plants by the wind. Sunderland et al. (2006) demonstrated the consequences of deficiency of DNA ligase in plant cells in Arabidopsis plants, where such deficiency caused destabilization of the genome in vivo and loss of cell viability.

Peroxiredoxin present in cytoplasm, categorized as stress proteins, was identified in ML exposed to CW for 12 h (spot 131) (Fig. 7). Thiol-specific peroxidase catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols. According to Reis et al. (2018), the action of CW and IW, at different speeds and exposure times, on YL and ML of cocoa, promotes oxidative stress and, consequently, increases the activity of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX), in addition to altering the expression of the Cu–Zn--SOD gene at a speed of 4.5 m s-1. On the other hand, peroxiredoxins also participate in the defense process, comprising a large class of thiol-peroxidases that help in cleaning the cell against ROS. These proteins are capable of degrading H_2O_2, being sensitive to oxidation (Poynton and Hampton, 2013). The increased expression of peroxiredoxins in ML (Fig. 7), caused by mechanical stress, is related to the accumulation of H_2O_2 resulting from oxidative stress (Reis et al., 2018).

Other stress-related enzymes have been identified in ML exposed to CW for 12 h, such as glyoxalase I (spot 76) (Fig. 10), an enzyme that plays a critical role in the methylglyoxal detoxification system. Abiotic stress induces greater accumulation of methylglyoxal. This compound, produced as a non-enzymatic by-product of glycolysis, is considered cytotoxic, which can react and modify different molecular targets, such as DNA, proteins and other cellular components (Singla-Pareek et al., 2003). The overexpression of glyoxalase in transgenic plants prevents the increase in the concentration of methylglyoxal under stress, due to its efficient detoxification, thus reducing the toxicity of this compound (Singla-Pareek et al., 2003).

5. Conclusions

Mechanical stress caused by constant wind flow, with speed of 4.5 m s^{-1} and exposure time of 12 h, on young and mature leaves of young CCN 51 cocoa clonal plants grown in green house reduced accumulation of proteins in young leaves and increased in mature leaves, compared to the control.

Differentially accumulated proteins, identified in young and mature cocoa leaves, subjected to mechanical stress caused by the action of constant wind flow, belong to a broad functional group related to energy production and carbon metabolism.

In response to wind flow, there was a higher efficiency in the protein relative abundance associated with energy production and carbon assimilation in the mature leaves of CCN 51 clonal plants, evidenced by the emergence of new isoforms and, or post-transitional changes, as a process of acclimatization and tolerance to the stressor (Fig. 11).

In young leaves, the production of energy and the synthesis of gene products essential for their growth and development were adversely affected by the mechanical stresses caused by wind making them more intolerant to this stressor.

CRediT authorship contribution statement

Graciele Santos Monteiro Reis: Writing - review & editing. **Alex-Alan Furtado de Almeida:** Writing - review & editing. **Nayara de Almeida Santos:** Writing - review & editing. **D'avila Maria de Souza Araújo:** Data curation. **Irma Yuliana Mora-Ocampo:** Data curation.

Declaration of competing 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.

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Appendix A. Supplementary data

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