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Changes in protein profile detected in seedlings of *Caesalpinia peltophoroides* (Fabaceae) after exposure to high concentration of cadmium

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ABSTRACT. Sibipiruna (*Caesalpinia peltophoroides* Benth) is a tree of the Brazilian Atlantic Forest. It is a flowering ornamental tree widely planted throughout Brazil and indicated for restoration of degraded areas. We examined protein profile changes in leaves of seedlings of *C. peltophoroides* grown in nutrient solution under greenhouse conditions, after exposure to cadmium (Cd; 32 mg/L). A two-dimensional gel was used to analyze proteins expressed in response to stress 24 and 72 h after initiation of treatment with Cd. Various protein bands were identified that were related to stress response and/or metabolic adjustments, including proteins involved with resistance to stress, including detoxification, degradation, antioxidant, transport, signal transduction, photosynthesis, electron transport, biosynthesis reactions, and

transcription regulation. After 24 h of Cd exposure, the genes of most of these proteins were upregulated. These putative proteins were associated with resistance to stress, including heat shock proteins, heat stress transcriptional factor and other transcriptional factors, aquaporins, glutathione transferase and choline monooxygenase. Most of the putative proteins observed after 72 h of exposure to Cd were downregulated. They were mainly photosynthetic process proteins, such as NAD(P)H-quinone oxidoreductase, photosystem I assembly, and photosystem II CP47 chlorophyll apoprotein. There were also proteins involved with degradation, biosynthesis and antioxidant activity, such as ATP-dependent Clp protease, methylthioribose-1-phosphate and glutathione peroxidase 2. Based on preliminary proteomic analysis, we conclude that proteins related to photosynthetic activity are inhibited, decreasing plant performance under stress conditions and that several proteins related to defense mechanisms are activated, inducing the plant defense response.

Key words: Abiotic stress; Heavy metals; Photosynthesis; Phytotoxicity; Stress resistance; Woody species

INTRODUCTION

Environmental contamination by heavy metals is a worldwide problem and poses a significant threat to ecosystems. Anthropogenic action is the main cause of the increase in this environmental pollution, mainly after the industrial revolution, along with the urban and agricultural growth of last decades (Tavares and Carvalho, 1992; Jadia and Fulekar, 2009). These highly toxic pollutants remain in the environment for a long time and may accumulate to toxic levels, resulting in the contamination of aquatic and terrestrial life, affecting mainly plants, animals and humans (Gratão et al., 2005). Among the heavy metals, cadmium (Cd) is the most phytotoxic, even at low concentrations, because of its high water solubility (Das et al., 1997; Pinto et al., 2004; Zacchini et al., 2009). Therefore, it is easily taken up and translocated to different plant parts (Oliveira et al., 2001; Souza et al., 2009). Once accumulated in the plant, Cd promotes several physiological and biochemical changes, such as in photosynthesis and in several enzyme activities, affecting plant growth and development (Sanità di Toppi and Gabbrielli, 1999; Clemens, 2006; Pietrini et al., 2010). Plant adaptation to environmental stresses is based on the signal transduction network, from the perception of stress signals to response from gene expression, resulting in metabolic changes (Saibo et al., 2009; Visioli et al., 2010). Cd can cause a decrease of up to 50% in dry matter production in some species (Almeida et al., 2010; Pietrini et al., 2010).

Plants are sessile organisms and they need to acclimate to changing environmental conditions (Schützendübel and Polle, 2002). Some plant species show variable ability to accumulate Cd in relation to the concentration found in the substrate in which they grow (Vassilev et al., 2002). Woody species are less effective than herbaceous species in taking up heavy metals, such as Cd. However, trees may be more efficient because they have a larger root surface for the uptake of these metals (Zacchini et al., 2009). Also, they have a high

capacity to allocate metals in the trunk permanently, tempering the bioavailability of metals in the environment (Almeida et al., 2010). Thus, the use of woody species is promising for phytoremediation, as they represent an important way of recovering contaminated areas viably (ecologically and economically).

The objective of this study was to analyze the protein profile of *Caesalpinia peltophoroides* (sibipiruna). This is a woody species with great adaptive capacity to adverse conditions such as flooded soils, with low pH and low fertility. This tree of the Brazilian Atlantic Forest is indicated for restoration of degraded areas, especially riparian areas (Lorenzi and Souza, 2001; Mielke et al., 2003). Proteomics is a valuable tool that is becoming increasingly important, complementing the understanding of biochemical and physiological mechanisms in stress response. Recent molecular advances have shown the expression of a wide variety of stress-inducible genes (Kieffer et al., 2009; Amudha and Balasubramani, 2011). Abiotic stress responses are correlated with the synthesis of stress proteins and signaling molecules (Sanità di Toppi and Gabbrielli, 1999). Different transcription factors regulate the expression of these genes (Zhang et al., 2006; Amudha and Balasubramani, 2011). The combination of the interesting characteristics of environmental adaptation of *C. peltophoroides* and proteomic analysis represents an important strategy to identify a variety of protein responses to stress.

MATERIAL AND METHODS

Plant material and growth conditions

The experiments were conducted in a greenhouse at the Universidade Estadual de Santa Cruz (UESC), Ilhéus, Bahia, Brazil (14°47' S, 39°10' W), between April and June of 2008. Seedlings of *C. peltophoroides* were obtained from the Instituto Biofábrica de Cacau, Ilhéus, Bahia, Brazil. Plants were grown in a monitored environment during the experimental period. SLIA-M003 quantum sensors, coupled to a HOBO weather station (Onset Computer, Bourne, Massachusetts, USA), were used to monitor photosynthetically active radiation (PAR), and Hobo H8 ProSeries sensors (Onset Computer) monitored air temperature (T_{air}) and relative humidity (RH). During the experimental period daily PAR was 4.12 $mol \cdot m^{-2} \cdot day^{-1}$, average air temperature (T_{air}) ranged from 19° to 31.5°C (T_{mean} 24.5°C), and RH ranged from 49.3 to 100% (mean RH 86.2%). The plants were grown for eight weeks in nutrient solution containing half-strength Hoagland's solution No. 2 (Hoagland and Arnon, 1950) before they were exposed to Cd for 96 h. The experimental setup was designed based on two groups: a control group and a group exposed to a final concentration of 32 mg/L Cd (in the form of $CdCl_2$) in the nutrient solution. Each group was composed of 3 biological replicates, and each replicate contained at 8 plants. After this period of exposure, leaves of each replicate were harvested, snap-frozen in liquid nitrogen and stored at -80°C until protein extraction.

Protein extraction

Leaves of seedlings exposed to Cd were harvested after exposure for 0, 6, 12, 24, 48, 72, and 96 h. Leaf samples were freeze-dried and ground in liquid nitrogen for total protein extraction. Leaf protein extraction was based on the protocol described by Pirovani et al. (2008) with modifications. The plant powder was washed three or four times with 10% TCA in

acetone/0.07% 2-mercaptoethanol (2-ME) until the loss of coloration. At every step described above, the pellet was completely resuspended by sonication (4 pulses of 10 s each at 10 s intervals on ice, at an output of 70%) using an Ultrasonic processor (GEX 130, 130 W), and then centrifuged at 13,600 *g* for 10 min at 4°C. Finally, the powder was washed once in 10% TCA in water and 0.07% 2-ME, sonicated (3 pulses of 7 s each at 5 s intervals on ice, at an output of 50%), and then washed once in 80% acetone. The final pellet was dried at room temperature and depending on the following use, the pellets were resuspended in the electrophoresis buffer or in distilled water. A spectrophotometer plate reader (VERSAmax) with the microplate format was used to quantify total protein (480 nm). Three repetitions for each sample were carried out using the 2-D Quanti kit (GE Healthcare), according to manufacturer recommendations.

SDS-PAGE and two-dimensional electrophoresis (2D-PAGE)

For 1-DE analysis (SDS-PAGE), protein samples were denatured at 95°C for 5 min and resolved on SDS-gels (12.5%), at 150 V, in a BioRad mini-Protean II apparatus. For visualization of proteins, gels were placed immediately in colloidal Coomassie G 250 solution (Neuhoff et al., 1988).

For two-dimensional electrophoresis (2-DE), first-dimensional electrophoresis was performed on an Ettan IPGphor system (GE Healthcare). Protein samples were applied in 250 µL 2-DE rehydratation solution by reswelling 13 cm Immobiline DryStrip (pH 4-7, GE Healthcare) for 12 h. Isoelectric focusing (IEF) was performed for 5 h on the same apparatus under the following conditions: step and hold at 500 V for 1 h, gradient 1000 V for 1 h, gradient 8000 V for 2.5 h, and step and hold 8000 V for 55 min. After IEF, the strips were stored at -80°C until second-dimensional analysis.

The second dimension in 12.5% SDS-PAGE was performed using a Ruby SE 600 system (GE Healthcare) with 80 V/200 mA. Before that, the strips were incubated for 15 min in equilibration buffer (7.5 mM Tris-HCl, pH 8.8, 29.3% glycerol, 6 M urea, 2% SDS, 0.002% bromophenol blue, ultrapure H_2O) with 1% (w/v) DTT, and for another 15 min in equilibration buffer with 2.5% (w/v) iodoacetamide. The strips were then transferred to a vertical 12.5% SDS-PAGE gel. After electrophoresis, proteins were visualized with 0.1% (w/v) colloidal Coomassie G 250 (Neuhoff et al., 1988).

Image acquisition and data analysis

Gel images were processed using Image Scanner II (GE Healthcare). Image analysis program, the Melanie 7.0 software (GeneBio), was used for finding proteins differentially expressed after Cd exposure for 24 and 72 h. The gels were matched, and spot detection and spot matching were performed. The criteria for defining the expression pattern were as follows: appear, newly detected; disappear, disappeared under the limit of detection; increase, increase in amount; decrease, decrease in amount.

The isoelectric point (pI) and relative molecular mass (Mw) of each protein were calculated using protein molecular weight markers as the reference proteins. The most intense spots were analyzed *in silico* using Expasy TagIdent (http://web.expasy.org/tagident/), a protein identification program based on Mw and pI. TagIdent allows the generation of a list of proteins using pI and Mw against proteins in SWISS-PROT/TrEMBL databases. The

estimated Mw and pI values were obtained for each targeted spot. These values were entered into the ExPASy TagIdent database to obtain a preliminary identification. The error ranges for Mw and pI were set at 20% and 0.5 pH unit, respectively. Taxonomic group and key words (Spermatophyta, Populus, stress) were used to search in the protein database. If no matches were found, a general search for proteins in the approximate pI x Mw range was conducted.

RESULTS AND DISCUSSION

Separation of the soluble leaf proteins from *C. peltophoroides* was performed for time periods of 6, 12, 24, 48, 72, and 96 h, using 1-DE (Figure 1). Preliminary analysis of proteins revealed multiple bands between 14 and 66.2 kDa, when compared to the control (no Cd). Both the large and small subunits of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) were visualized in the gel, approximately 56 kDa and 15 kDA, respectively.

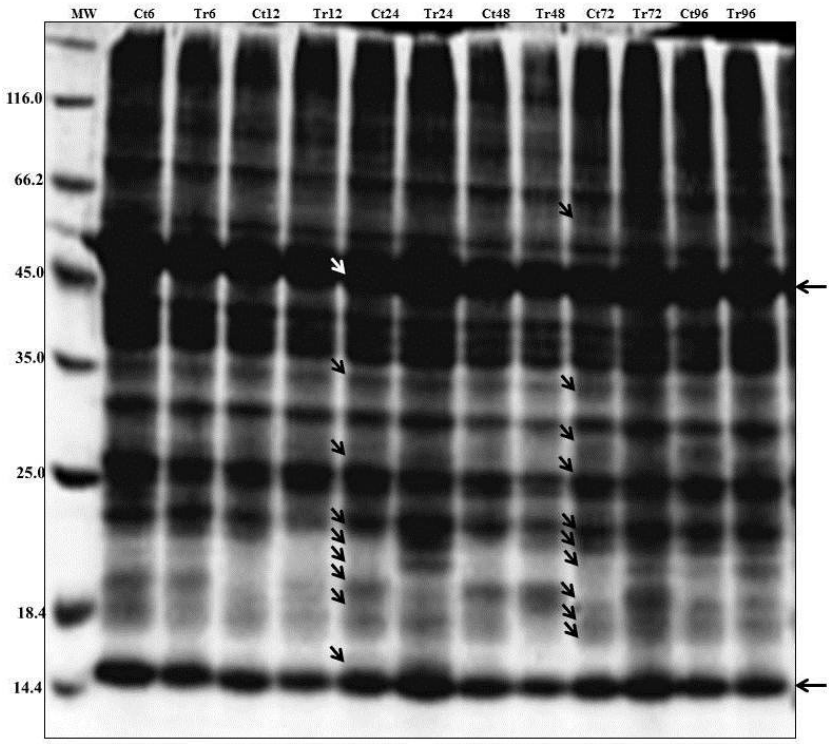

Figure 1. The one-dimensional gel electrophoresis (1-DE) analysis of leaf protein from *Caelsalpinia peltophoroides*, using varying treatments: control, no Cd (Ct) and treatment with Cd (Tr). The 1-DE was stained with Coomassie G 250 solution. *Lane MW* = molecular mass marker indicated on the left in kDa; *lane Ct6* = control (no Cd) performed for time 6 h after exposure start of experiment; *lane Tr6* = treatment (32 mg/L) for time 6 h; *lane Ct12* = control 12 h; *lane Tr12* = treatment 12 h; *lane Ct24* = control 24 h; *lane Tr24* = treatment 24 h; *lane Ct48* = control 48 h; *lane Tr48* = treatment 48 h; *lane Ct72* = control 72 h; *lane Tr72* = treatment 72 h; *lane Ct96* = control 96 h; *lane Tr96* = treatment 96 h. Arrows at right of figure indicate both the large and small subunit of Rubisco, respectively; write and black arrows indicate presence or absence of protein bands between 14 and 45 kDa, for time 24 and 72 h after exposure to Cd.

The goal for using 1-DE was to visualize the high or low abundance proteins, comparing samples of controls and Cd treatments. Samples from two different time periods regarding Cd exposure (24 and 72 h) were selected after this analysis and applied in two-dimensional electrophoresis (2-DE). Using the 2-DE separation technique, proteins were resolved for treated (32 mg/L Cd) and untreated (no Cd). The overall image of the proteome of sibipiruna leaves from seedlings *C. peltophoroides* after Cd stress is shown in Figure 2 (24 h) and Figure 3 (72 h).

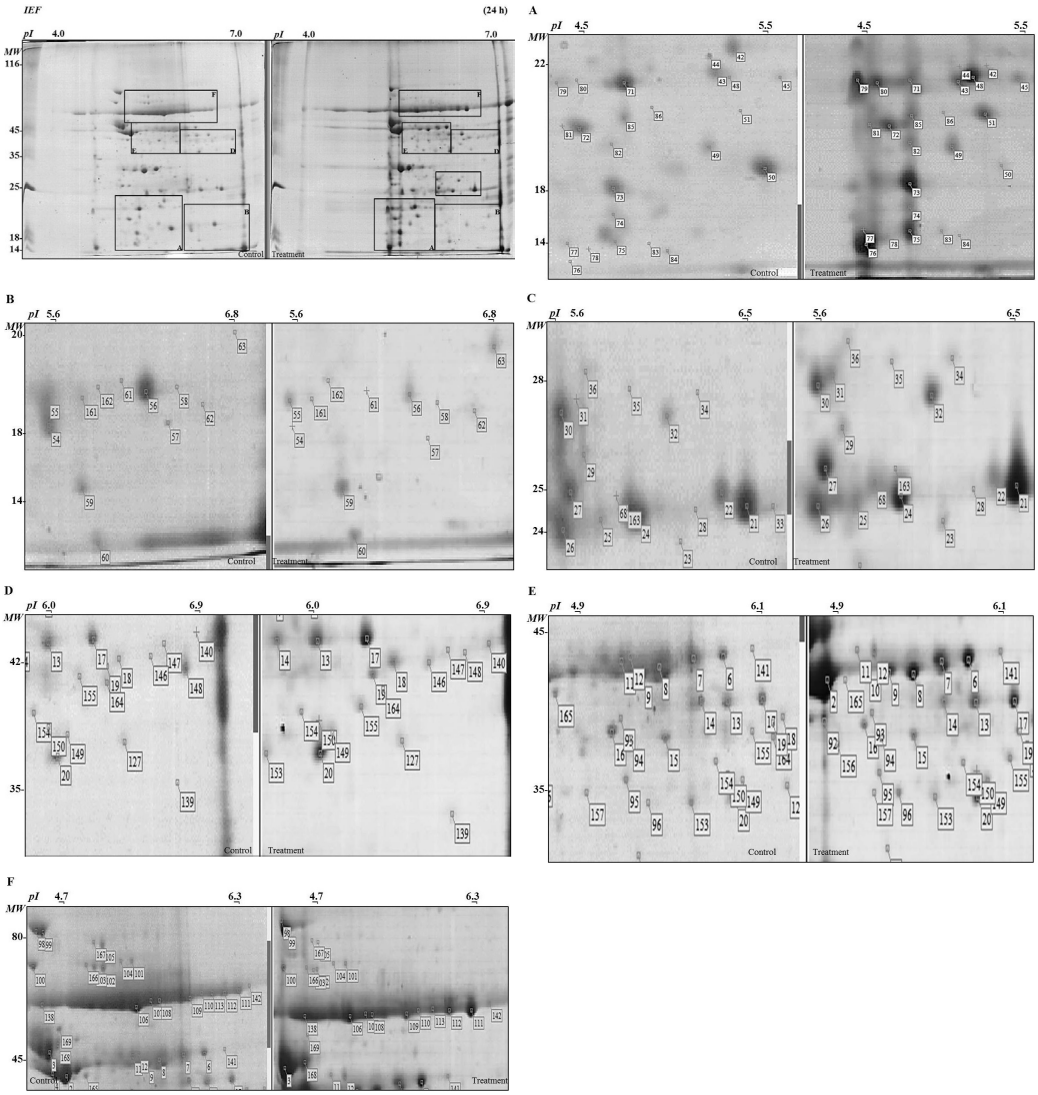

Figure 2. Comparison of 2-DE images for protein profile from seedlings of *Caelsalpinia peltophoroides* in absence and presence of Cd (32 mg/L) in nutrient solution after 24 h. IEF was from pH 4-7 and molecular weight ranges from 14.0-116.0 kDa. Master image corresponding to 24 h of Cd exposure (the top side) has been divided into areas, **A**, **B**, **C**, **D**, **E**, and **F**, which are displayed in detail, showing an expanded view of the indicated region on gel. Spots differentiating were exposed in detail in Table 1.

Analysis of the 2-DE gels using the Melanie software, followed by visual confirmation, revealed a variable number of spots. Several significant changes in protein expression profiles were observed comparing untreated and Cd-treated samples. A total of 132 differentially expressed spots were identified, obtained by cross-checking of the Mw/pI (molecular weight/isoelectric point) values acquired by gels, associated with information contained in the proteomic database (www.ExPASy.org).

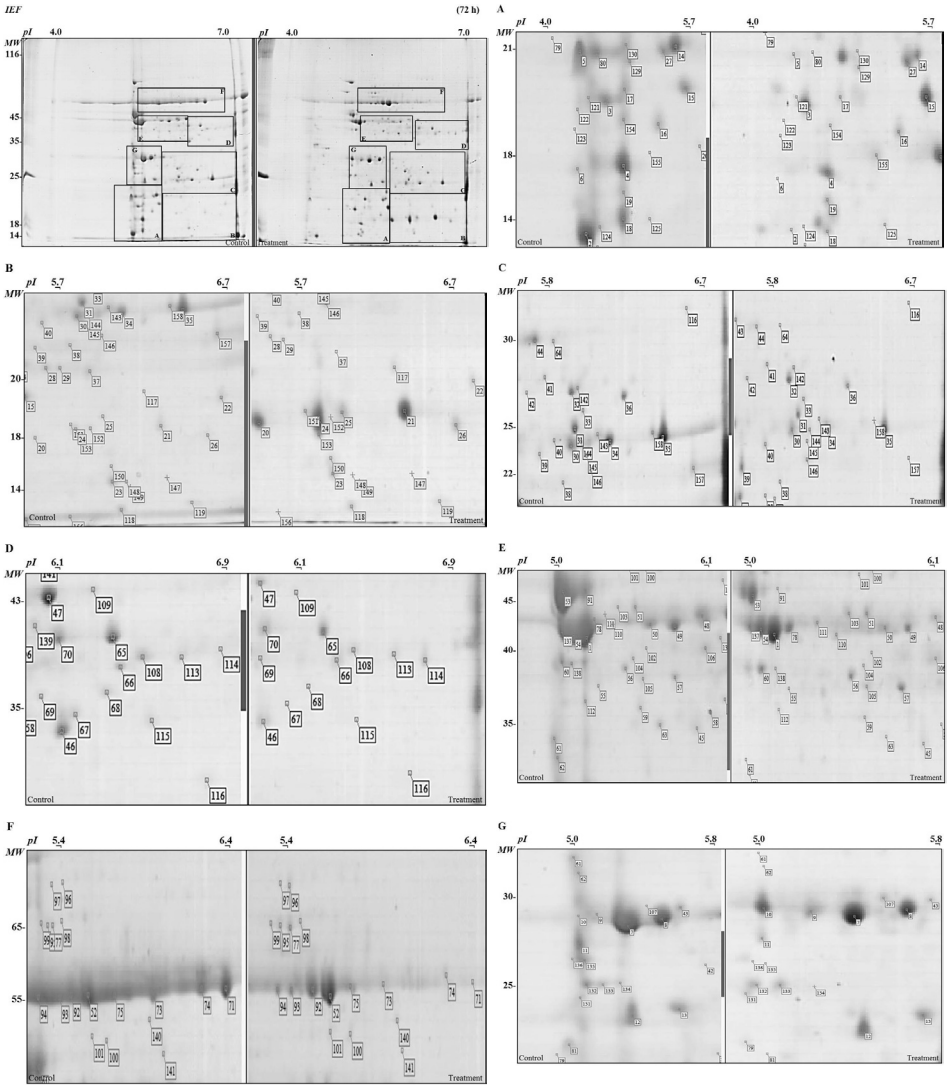

Figure 3. Comparison of 2-DE images for protein profile from seedlings of *Caelsalpinia peltophoroides* in absence and presence of Cd (32 mg/L) in nutrient solution after 72 h. IEF was from pH 4-7 and molecular weight ranges from 14.0-116.0 kDa. Master image corresponding to 72 h of Cd exposure (the top side) has been divided into areas, **A**, **B**, **C**, **D**, **E**, **F**, and **G**, which are displayed in detail, showing an expanded view of the indicated region on gel. Spots differentiating were exposed in detail in Table 2.

Many of the spots matched using TagIdent had potentially hundreds of candidate protein identities matched to a specific pI and Mw range. However, some systematic patterns were used in all analysis, considering, mainly similarity of the Mw and pI, conditions of abiotic stress and related species. Thus, it was possible deduce the categories and possible functions of the differential spots. These significant spots with their pI and Mw are summarized in Table 1 and Table 2. The result of TagIdent searching showed differential proteins related to stress response and/or metabolic adjustments. Ten deduced categories of significant spots were obtained: i) resistance to stress; ii) detoxification; iii) degradation; iv) antioxidant; v) transport; vi) signal transduction; vii) photosynthesis; viii) electron transport; ix) biosynthetic reactions, and x) transcription regulation (UniProtKB/Swiss-Prot and UniProtKB/TrEMBL databases).

In 2-DE gel analysis of seedlings treated with Cd for 24 h, 61 spots were selected (Figure 2). The expression of 44 spots increased or appeared (newly detected). All biological categories deduced for these spots were related to stress resistance in plants. As shown in Table 1, almost half of these proteins are heat shock proteins (HSP) or heat stress transcriptional factors (HSF). Increased transcription of a set of genes in response to abiotic stress such as heat or toxic agents is a highly conserved biological response (Waters et al., 1996). This response is mediated by HSF, which can bind to promoters of heat shock genes. In Table 1, different spots are related to HSFs (11 spots) and HSP (9 spots). In addition to the identification of HSPs and HSFs, identification of aquaporins corroborates the findings of (Kamal et al., 2010). Dehydration stress is Cd-induced due to biochemical impediments and effects on the function of aquaporins (transport pore), which play a vital role in the transport of molecules in plants (Kaldenhoff and Fischer, 2006; Visioli et al., 2010). Cd induces oxidative stress and activates a complex antioxidant defense system (Amudha and Balasubramani, 2011). An increase in antioxidant proteins was observed in these samples.

Among the 61 spots, only 17 spots decreased or disappeared (under the limit of detection), all related to different biological processes, mainly photosynthetic activity but also electron transport, protein degradation, biosynthetic processes or cell division (Table 1). Cadmium can interfere with numerous biochemical and physiological processes, including photosynthesis, respiration and protein metabolism (Zhang et al., 2010). Negative effects on protein activity, such as the photosystem I assembly protein ycf3 (spots 50 and 55), photosystem Q(B) protein (spot 16), NAD(P)H-quinone oxidoreductase subunit 3, chloroplastic (spots 22, 56, 57, 75, and 163), and cytochrome b6-f complex subunit 4 (spot 54), compromised the performance of photosynthetic activity. Reduction in net photosynthetic rate is one of the factors that cause a decrease in growth and productivity in plants (MacFarlane and Burchett, 2001; Almeida et al., 2007; Zhang et al., 2010). There may be changes in the growth of shoots and roots and in chlorophyll biosynthesis, lower rates of transpiration, photosynthesis and stomatal conductance, induction of premature senescence of leaves, and leaf chlorosis (Clemens, 2006). After 72 h of exposure to Cd, 71 significant spots differentially expressed were selected in 2-DE gel analysis (Figure 3). Only 19 spots increased or appeared, all associated with biological processes of stress resistance and oxidative stress, as shown in Table 2. Thirteen of these spots are related to HSP and HSF, according TagIdent database. This shows that Cd continues to stimulate the expression of these genes. It is known that a high concentration of Cd in plant cells induces the accumulation of reactive oxygen species (ROS). Cd^{2+} ions bind to sulfhydryl groups of proteins and replace divalent cations such as Zn^{2+} and Fe^{2+} in redox-reactive enzymes, which may cause release of these potentially harmful molecules (Yamaguchi et al.,

Table 1. Preliminary identification of protein spots from two-dimensional electrophoresis (2-DE) by the ExPASy TagIdent tool.

Spot No.[a]	Gel area[a]	MW[b] (kDa)	pI[b]	TagIdent ID[c]	Protein name[c]	Pattern	Biological process[d]
6	E	43	5.9	Q5N942	Serine/threonine-protein kinase SAPK4	Increase	Signaling pathway, stress response
7	E	43	5.6	O04121	Choline monooxygenase, chloroplastic	Increase	Stress response (expressed in leaves)
8	E	42	5.4	O49403	Heat stress transcription factor A-4a	Increase	Stress response and transcription regulation
9	E	42	5.2	Q10PR4	Heat stress transcription factor A-9	Increase	Stress response and transcription regulation
13	E	40	5.9	Q93XE1	Choline monooxygenase, chloroplastic	Increase	Stress response (expressed in leaves)
16	E	38	5.0	A4GYN9	Photosystem Q(B) protein*	Decreaase	Electron transport
17	D	40	6.2	P48407	Pinosylvin synthase 1*	Increase	Stress response and transferase activity
18	D	39	6.4	Q8H7Y6	Heat stress transcription factor A-2d	Increase	Stress response and transcription regulation
19	D	38	6.3	Q6VBA4	Isoform 2 of heat stress transcription factor C-1a OS	Increase	Stress response and transcription regulation
20	E	34	5.9	Q9SCW4	Heat stress transcription factor B-2a	Increase	Stress response and transcription regulation
21	C	24	6.5	Q7XBH4	Myb-related protein Myb4	Increase	Putative transcriptiron factor
22	C	24	6.4	B1VKF5	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic*	Decreaase	Transport
23	C	24	6.2	O80852	Glutathione S-transferase F9	Increase	Detoxification and stress response
25	C	24	5.8	P0C5C8	1-Cys peroxiredoxin A	Increase	Antioxidant protein
27	C	25	5.66	P50703	Osmotin-like protein OSML15	Increase	Stress response
29	C	25	5.74	Q9FRL8	Glutathione S-transferase DHAR2	Increase	Detoxification and stress response
30	C	26	5.6	P46421	Glutathione S-transferase U5	Increase	Detoxification and stress response
31	C	27	5.69	P48534	L-ascorbate peroxidase, cytosolic	Appear	Stress response
32	C	26	6.1	Q84RL6	Aquaporin TIP2-3	Increase	Stress response and pore transporter
34	C	27	6.23	Q6EUG4	Heat stress transcription factor C-2a	Appear	Stress response and transcription regulation
35	C	27	5.96	A2WL19	Dehydration-responsive element-binding protein 2A	Increase	Stress response and transcription regulation
42	A	23	5.1	A4GYT6	ATP-dependent Clp protease proteolytic subunit*	Desappear	Proteaolytic enzyme
48	A	21	5.1	P09886	Small heat shock protein, chloroplastic	Appear	Stress response
50	A	18	5.3	Q01545	Photosystem I assembly protein ycf3*	Desappear	Photosynthesis
51	A	20	5.2	Q39818	Heat shock 22-kDa protein, mitochondrial	Appear	Stress response
54	B	18	5.6	Q14FC6	Cytochrome b6-f complex subunit 4*	Desappear	Electron transport, photosynthesis and transport
55	B	19	5.6	A4GYR1	Photosystem I assembly protein ycf3*	Decreaase	Photosynthesis
56	B	19	6.3	A0ZZ38	NAD(P)H-quinone oxidoreductase subunit J, chloroplastic	Decreaase	Transport
57	B	17	6.4	A4GYR3	NAD(P)H-quinone oxidoreductase subunit J, chloroplastic*	Decreaase	Transport
58	B	19	6.5	Q53M11	21.9-kDa heat shock protein	Appear	Stress response
59	B	14	6.1	P22240	Abscisic acid and environmental stress-inducible protein TAS14	Increase	Stress response
62	B	18	6.7	P19037	18.1-kDa class I heat shock protein	Appear	Stress response
63	B	20	6.8	P27397	18.0-kDa class I heat shock protein	Appear	Stress response
68	C	24	5.87	A2WZI4	Dehydration-responsive element-binding protein 1F	Increase	Stress response and transcription regulation
71	A	21	4.7	A0A360	ATP-dependent Clp protease proteolytic subunit	Decreaase	Proteaolytic enzyme
75	A	13	4.7	A4GYR5	NAD(P)H-quinone oxidoreductase subunit 3, chloroplastic*	Increase	Transport
76	A	13	4.5	Q9ZNV8	Histidine-containing phosphotransfer protein 2	Increase	Stress response
79	A	21	4.4	P80022	Extracellular ribonuclease LE	Increase	Stress response
80	A	21	4.6	O64564	HSP 18.5 Class IV heat shock protein	Appear	Stress response
93	E	39	5.1	O80982	Heat stress transcription factor A-2	Increase	Stress response and transcription regulation
96	E	34	5.3	A2YNT8	Serine/threonine-protein kinase SAPK2	Appear	Signal transduction
102	F	66	4.8	Q70XZ4	Acetyl-Co-A carboxylase carboxyl transferase subunit β, chloroplastic	Decreaase	Fatty acid biosynthesis
103	F	65	4.8	Q9MTL3	Acetyl-Co-A carboxylase carboxyl transferase subunit β, chloroplastic	Decreaase	Fatty acid biosynthesis
105	F	73	4.8	A8MQR0	WPP domain-interacting tail-anchored protein 2	Desappear	Domain coiled coil
109	F	55	5.7	P25795	Aldehyde dehydrogenase family 7 member A1	Increase	Lipid peroxidation, DNA and protein modifications

Continued on next page

Table 1. Continued.

Spot No.[a]	Gel area[a]	MW[b] (kDa)	pI[b]	TagIdent ID[c]	Protein name[c]	Pattern	Biological process[d]
111	F	56	6.3	P35480	Chaperonin CPN60, mitochondrial	Increase	Stress response
112	F	56	6.1	Q94BZ5	Heat stress transcription factor A-5	Increase	Stress response and transcription regulation
113	F	56	5.9	O82289	Probable inactive poly [ADP-ribose] polymerase SRO1	Increase	Stress response
139	D	31	7.8	P30302	Aquaporin PIP2-3	Appear	Stress response and pore transporter
140	D	40	6.9	Q657C0	Heat stress transcription factor A-6a	Appear	Stress response and transcription regulation
141	E	44	6.1	P48408	Pinosylvin synthase 2*	Increase	Stress response and transferase activity
146	D	39	6.6	Q7XHZ0	Heat stress transcription factor B-4b	Appear	Stress response and transcription regulation
148	D	38	6.9	A9PFT0	ATP-dependent Clp protease proteolytic subunit*	Desappear	Proteaolytic enzyme
149	D	35	6.2	A0MES8	Ethylene-responsive transcription factor ABI4	Appear	Transcription regulation, plant defense, signaling
155	D	36	6.2	Q6VBA4	Heat stress transcription factor C-1a	Increase	Stress response and transcription regulation
161	B	19	5.7	P30236	22.0-kDa class IV heat shock protein	Appear	Stress response
162	B	19	5.8	B7EZJ7	23.6-kDa heat shock protein, mitochondrial.	Appear	Stress response
163	C	24	5.9	A0A389	NAD(P)H-quinone oxidoreductase subunit I, chloroplastic*	Desappear	Oxidorredutase
164	D	37	6.3	B9HCR2	Methylthioribose-1-phosphate isomerase*	Desappear	Amino-acid biosynthesis
165	E	41	4.8	A8Y909	D3-type cyclin*	Desappear	Cell cycle regulator
168	F	46	4.7	P41152	Heat shock factor protein HSF30	Increase	Stress response and transferase activity

[a]As given in Figure 2 (Melanie 7.0 software analysis); [b]values estimated from the 2-DE gel (Melanie 7.0 software analysis); [c]accession Nos. and possible function of the spots in downloaded UniProtKB/Swiss-Prot (Expasy); [d]deduce caategories based of ExPaSy TagIdent database. Differentially expressed protein spots between control [no cadimium (Cd)] and seedling of *Caelsalpinia peltophoroides* after exposure to Cd in nutrient solution for 24 h are shown. The spot numbers can be tracked to their location on the image in Figure 2. The table shows TagIdent IDs and protein names based on molecular weight (MW) and isoelectric point (pI). Deduced proteins related with woody species are preceded by asterisks.

Table 2. Preliminary identification of protein spots from two-dimensional electrophoresis (2-DE) by the ExPASy TagIdent tool.

Spot No[a]	Gel area[a]	MW[b] (kDa)	pI[b]	TagIdent ID[c]	Protein name[c]	Pattern	Biological process[d]
1	E	42	5.1	Q49KU3	NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic*	Decrease	Photosynthesis, electron transport
2	A	13	4.4	Q14FF2	NAD(P)H-quinone oxidoreductase subunit 3, chloroplastic*	Decrease	Photosynthesis, electron transport
4	A	16	5.1	P85801	Thioredoxin H-type*	Decrease	Electron carrier activity and oxidoreductase activity
5	A	21	4.3	A0A388	NAD(P)H-quinone oxidoreductase subunit 6, chloroplastic*	Decrease	Transport
7	G	29	5.2	A4GYP6	ATP synthase subunit a, chloroplastic*	Decrease	Ion transport, regulated photosynthesis
8	G	29	5.4	A4GYS3	Chloroplast envelope membrane protein*	Decrease	Hydrogen ion transport, photosynthesis
10	G	29	5.0	Q338B0-2	Isoform 2 of heat stress transcription factor A-2c OS	Increase	Stress response and transcription regulation
11	G	28	5.0	Q14FG9	ATP synthase subunit a, chloroplastic*	Decrease	Ion transport, regulated photosynthesis
12	G	23	5.3	Q09MF2	ATP-dependent Clp protease proteolytic subunit*	Decrease	Proteaolytic enzyme
13	G	24	5.5	O65084	Proteasome subunit beta type-3*	Decrease	Proteaolytic enzyme
14	A	21	5.5	A4GYR1	Photosystem I assembly protein ycf3*	Decrease	Photosynthesis
15	A	20	5.5	P30236	22.0-kDa class IV heat shock protein	Increase	Stress response, signal
16	A	19	5.4	Q14FF6	Photosystem I assembly protein ycf3*	Decrease	Photosynthesis
18	A	13	5.1	A9P8G6	Thioredoxin*	Decrease	Electron carrier activity and oxidoreductase activity
20	B	18	5.6	O04922	Probable glutathione peroxidase 2	Increase	Stress response, oxireductase, peroxidase
21	B	18	6.3	P29830	17.6-kDa class II heat shock protein	Increase	Stress response, signal
24	B	18	5.9	P04794	17.5-kDa class I heat shock protein	Increase	Stress response, signal
26	B	18	6.6	Q05832	18.3-kDa class I heat shock protein	Increase	Stress response, signal

Table 2. Continued.

Spot No[a]	Gel area[a]	MW[b] (kDa)	pI[b]	TagIdent ID[c]	Protein name[c]	Pattern	Biological process[d]
31	C	24	5.9	A9P9E4	Proteasome subunit beta type*	Decrease	Proteaolytic enzyme
34	C	24	6.2	Q9SLP8	Caffeoyl-CoA O-methyltransferase*	Decrease	Lignin biosynthetic process
35	C	24	6.4	Q7Y1U9	SVP-like floral repressor*	Decrease	Transcription regulation
36	C	26	6.2	P58155	Chloroplast envelope membrane protein	Decrease	Hydrogen ion transport, photosynthesis
37	B	20	5.9	B7EZJ7	23.6-kDa heat shock protein, mitochondrial	Decrease	Stress response, signal
39	C	22	5.5	Q9FIT9	21.7-kDa class VI heat shock protein	Increase	Stress response, signal
43	G	30	5.5	P52764	Chloroplast envelope membrane protein*	Decrease	Hydrogen ion transport, photosynthesis
46	D	34	6.0	Q9BBR8	Apocytochrome f	Decrease	Electron transport, photosynthesis and transport
47	D	43	6.0	B9HI36	Cytochrome P450*	Decrease	Electron transport, photosynthesis and transport
48	E	43	6.1	B9HCR2	Methylthioribose-1-phosphate isomerase*	Decrease	Amino-acid biosynthesis
49	E	42	5.7	A4GYX2	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Decrease	Photosynthesis, electron transport
50	E	42	5.6	Q14FA2	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic*	Decrease	Photosynthesis, electron transport
52	F	53	5.6	P25795	Aldehyde dehydrogenase family 7 member A1	Increase	Lipid peroxidation, DNA and protein modifications
53	E	45	5.0	Q09MC0	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic*	Decrease	Photosynthesis, electron transport
54	E	42	5.0	A4GYQ4	Photosystem II D2 protein*	Decrease	Photosynthesis
57	E	38	5.7	Q9LVW2	Heat stress transcription factor A-9	Increase	Stress response and transcription regulation
61	G	34	5.0	Q70KC5	MYB transcription factor*	Disappear	Regulation of transcription, DNA-dependent
62	G	32	5.0	A8Y905	D1-type cyclin*	Disappear	Cell cycle regulator
64	C	30	5.8	O47041	Light-indep protochlorophyllide reductase iron-sulfur ATP-binding protein*	Disappear	Chlorophyll biosynthesis, photosynthesis
65	D	40	6.3	B9HCR2	Methylthioribose-1-phosphate isomerase*	Decrease	Amino-acid biosynthesis
67	D	35	6.1	B9MVY0	Cytochrome P450*	Decrease	Electron transport, photosynthesis and transport
68	D	36	6.2	P52783	Glutamine synthetase cytosolic isozyme*	Decrease	Glutamine biosynthetic process
71	F	55	6.4	Q14FD1	Photosystem II CP47 chlorophyll apoprotein*	Disappear	Photosynthesis
73	F	55	5.9	A4GYR8	Ribulose bisphosphate carboxylase large chain*	Decrease	Photosynthesis
74	F	56	6.2	A4GYT7	Photosystem II CP47 chlorophyll apoprotein*	Decrease	Photosynthesis
75	F	54	5.7	Q49KY9	Acetyl-Co A carboxylase carboxyl transferase subunit β, chloroplastic*	Decrease	Fatty acid biosynthesis
78	E	42	5.2	Q9XQA8	Photosystem II D2 protein*	Decrease	Photosynthesis
80	A	21	4.4	Q09MC3	NAD(P)H-quinone oxidoreductase subunit 6, chloroplastic*	Decrease	Photosynthesis, electron transport
81	G	24	4.9	Q14FD2	ATP-dependent Clp protease proteolytic subunit*	Disappear	Proteaolytic enzyme
91	E	46	5.1	A4GYX2	NAD(P)H-quinone oxidoreductase subunit H, chloroplastic	Decrease	Photosynthesis, electron transport
92	F	54	5.5	Q41247	Aldehyde dehydrogenase family 7 member A1	Increase	Lipid peroxidation, DNA and protein modifications
96	F	69	5.4	P11143	Heat shock 70-kDa protein	Appear	Stress response, signal
97	F	69	5.3	P26413	Heat shock 70-kDa protein	Appear	Stress response, signal
101	F	49	5.6	Q9SYK9	UDP-glycosyltransferase 74E2	Increase	Stress response
104	E	40	5.6	Q09MI3	Photosystem II D2 protein*	Decrease	Photosynthesis
107	G	30	5.4	A4QM32	Chloroplast envelope membrane protein*	Increase	Hydrogen ion transport
112	E	36	5.1	P36491	Photosystem Q(B) protein	Decrease	Electron transport, photosynthesis
114	D	38	6.6	Q8H7Y6	Heat stress transcription factor A-2d	Increase	Stress response and transcription regulation
115	D	34	6.7	Q14FD1	Apocytochrome f*	Disappear	Electron transport, photosynthesis and transport
116	C	31	6.6	B9GST6	ATP-dependent Clp protease proteolytic subunit*	Disappear	Proteaolytic enzyme
117	B	20	6.3	Q07202	Cold and drought-regulated protein CORA	Appear	Stress response
129	A	22	5.1	P41609	ATP-dependent Clp protease proteolytic subunit*	Decrease	Proteaolytic enzyme
134	G	25	5.2	P15194	Chlorophyll a-b binding protein type 2 member 1B, chloroplastic*	Disappear	Photosynthesis
135	G	26	5.0	P93711	Caffeoyl-CoA O-methyltransferase*	Disappear	Lignin biosynthetic process
137	E	43	5.0	A4GYX1	NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic*	Decrease	Photosynthesis, electron transport
138	E	39	5.1	Q14FA3	NAD(P)H-quinone oxidoreductase subunit 1, chloroplastic*	Decrease	Photosynthesis, electron transport

Continued on next page

Table 2. Continued.

Spot No[a]	Gel area[a]	MW[b] (kDa)	pI[b]	TagIdent ID[c]	Protein name[c]	Pattern	Biological process[d]
140	F	50	6.0	Q94BZ5	Heat stress transcription factor A-5	Increase	Stress response and transcription regulation
141	F	46	6.0	B9HCR2	Methylthioribose-1-phosphate isomerase*	Disappear	Amino-acid biosynthesis
142	C	27	5.9	P20150	Chloroplast envelope membrane protein	Decrease	Hydrogen ion transport, photosynthesis
151	B	18	5.8	Q06652	Probable phospholipid hydroperoxide glutathione peroxidase*	Appear	Stress response, oxireductase, peroxidase
153	B	17	6.0	P02519	17.3-kDa class I heat shock protein	Appear	Stress response, signal
157	C	23	6.6	B9GW06	Cytochrome P450*	Disappear	Electron transport, photosynthesis and transport
158	C	24	6.4	Q84LN9	SVP-like floral repressor*	Disappear	Transcription regulation

[a]As given in Figure 3 (Melanie 7.0 software analysis); [b]values estimated from the 2-DE gel (Melanie 7.0 software analysis); [c]accession numbers and possible function of the spots in downloaded UniProtKB/Swiss-Prot (Expasy); [d]deduce caategories based of ExPaSy TagIdent database. Differentially expressed protein spots between control [no cadmium (Cd)] and seedling of *Caelsalpinia peltophoroides* after exposure to Cd in nutrient solution for 72 h are shown. The spot numbers can be tracked to their location on the image in Figure 3. The table shows TagIdent IDs and protein names based on molecular weight (MW) and isoelectric point (pI). Deduced proteins related with woody species are preceded by asterisks.

2009). In this study, four spots were related to aldehyde dehydrogenase (spots 52 and 92) and glutathione peroxidase (spots 20 and 151), enzymes associated with oxidative stress tolerance in plants (Amudha and Balasubramani, 2011).

Among the 71 spots (Figure 3), 55 were downregulated. By screening in the TagIdent databases, most of these spots were identified as photosynthetic process proteins (33 spots), but there were also proteins related to degradation (6 spots), biosynthesis (7 spots) and antioxidant proteins (4 spots) (Table 2). Thus, in this study, it appeared that Cd had a negative effect on the photosynthesis due to less abundant proteins such as those of the electron transport chain, carbon fixation and Calvin cycle, and Rubisco small and large subunits (spot 73). Photosynthesis plays an essential role providing an energy source for plant metabolism and abiotic stresses decreases its efficiency. Cd-induced phototoxic symptoms in *C. peltophoroides* such as chlorosis, wilting and leaf senescence and decrease in photosynthetic rate were observed in this study, which corroborates the findings in Table 2. Studies in several species exposed to Cd revealed inhibition of several proteins associated mainly with photosynthesis, including in Cd-tolerant woody species (Fagioni et al., 2009; Kieffer et al., 2009; Visioli et al., 2010).

CONCLUSIONS

Plants are exposed to various environmental stress conditions, such as exposure to Cd and other heavy metals or phytotoxic elements. In response, plants try to minimize the impacts with changes in a number of mechanisms to adapt to adverse conditions. Changes may include the signaling of a stress defense response, decrease in metabolic rate and/or activation of new metabolic pathways. In this study, specifically about adaptive response to stress, only a small percentage of this proteome was represented. Although preliminary, obtaining data from *in silico* analysis was a strategy that showed the more important putative proteins involved in stress response in *C. peltophoroides*. Cadmium was able to change the protein profile 24 and 72 h after stress exposure in nutrient solution. Malanie settings were adjusted and used to establish specific conditions for the identification of the spots. This approach for the identification of peptides and proteins is a preliminary exploration showing the possibility of using

2-DE gel image to predict proteins. The analysis of putative proteins showed the suppression of several proteins associated with photosynthetic activity, decreasing the plant's performance under stress conditions. However, there was an increase in proteins related to defense mechanisms for this species (HSP, HSF, aquaporins, and peroxidases). In tolerant plants submitted to stress, the expression of these genes is common. Further studies may help determine and characterize the genes expressed during exposure of *C. peltophoroides* to Cd. Comparing the protein profile in the absence and presence of heavy metals and understanding the changes in woody plants can facilitate decision-making and the deployment of species in multiple areas of phytoremediation in the future.

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