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PAPER

Contribution of phytochelatins to cadmium tolerance in peanut plants†

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Cadmium (Cd) is a well known heavy metal considered as one of the most toxic metals on Earth, affecting all viable cells that are exposed even at low concentration. It is introduced to agricultural soils mainly by phosphate fertilizers and causes many toxic symptoms in cells. Phytochelatins (PCs) are non-protein thiols which are involved in oxidative stress protection and are strongly induced by Cd. In this work, we analyzed metal toxicity as well as PCs implication on protection of peanut plants exposed to Cd. Results showed that Cd exposure induced a reduction of peanut growth and produced changes in the histological structure with a deposit of unknown material on the epidermal and endodermal cells. When plants were exposed to 10 μM Cd, no modification of chlorophyll, lipid peroxides, carbonyl groups, or hydrogen peroxide (H_2O_2) content was observed. At this concentration, peanut leaves and roots glutathione (GSH) content decreased. However, peanut roots were able to synthesize different types of PCs (PC2, PC3, PC4). In conclusion, PC synthesis could prevent metal disturbance on cellular redox balance, avoiding oxidative damage to macromolecules.

Introduction

Cadmium (Cd) is a non-essential metal and belongs to the group of heavy metals that do not have any known biological functions, such as chromium or mercury, and is highly toxic even at low concentrations. Naturally, it is found in the biosphere at concentrations of 0.01 to 1.8 ppm and its presence in agricultural soils is related to sewage sludge supply, atmospheric fallout from industrial processes, and mainly by use of phosphate fertilizers.[1,2] Cd is a well-known environmental stressor, especially in countries where agriculture is intensive, such as Australia and China. In Argentina, so far there is no evidence of contaminated agricultural soils with this metal, but it is known that there is an increase of phosphate fertilizer applications that would slowly increase the levels of Cd in soils, affecting not only crops but also human health.[3,4]

Metal uptake by plants is influenced by soil components and physicochemical characteristics such as minerals, metal hydroxides, organic matter, pH and buffer capacity, redox potential, water content and temperature, ion exchange, and radical exudates.[5,6] Moreover, in heavy metal experiments, the dose and type of metal used, as well as duration of exposure,[7] and also the environmental variables, should be considered.[8] Exposure of plants to Cd can cause many toxic symptoms, such as interference in the input, transport, and utilization of essential elements (calcium, magnesium, phosphorous and potassium) and water, causing nutrition and water imbalance.[9–11] Besides, other symptoms are reduction of nitrate absorption, transport from root to stem, reduction of nitrate reductase activity in stem;[12] alteration of photosynthesis and transpiration;[10] chlorosis caused by a deficiency of iron,[13] phosphates, or by a reduction of manganese transport.[14] It is well known that this metal can induce oxidative damage to membranes, such as lipid peroxidation by exacerbated reactive oxygen species (ROS) production,[10,15–17] protein damage by carbonyl group formation,[18] and activation or inhibition of antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX), peroxidase (POX) and catalase (CAT).[19]

Plants have developed different defence strategies against heavy metal toxicity, such as immobilization of Cd by cell wall pectin or carbohydrates[13] and reduction of the influx of Cd by cation transporters of the plasma membrane.[20] However, the intracellular sequestration of Cd by phytochelatins (PCs) is one of the best known defence mechanisms involved in metal detoxification. PCs are synthesized by phytochelatin synthase (PCS) from reduced glutathione, the major non-protein thiol in plants, and key for many functions in plants metabolism.

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Although it is constitutively present, its synthesis is strongly postranscriptionally activated by Cd ions.[21] Once Cd binds to the enzyme, it catalyzes glutathione (GSH) to PCs conversion.[22]

Accumulation of heavy metals and metalloids in agricultural soils has become important due to food safety issues, potential health risks and detrimental effects on soil ecosystems.[23]Peanut (*Arachis hypogaea* L.) is a self-pollinating, indeterminate, annual, herbaceous legume and a unique plant that produces pods underground. It is an agriculturally valuable plant with widespread distribution worldwide, serving as a subsistence food crop as well as a source of various food products.[24] All soils have a basal content of Cd which varies according to the parent rock from which they are derived. Since the peanut plant produces its fruits underground, and is in intimate contact with soil particles, fruits can incorporate high Cd concentration from contaminated substrates, introducing the metal into food chains and causing serious human health problems. Studies on peanut plants exposed to Cd are scarce, some of them are focused on evaluating enzymatic antioxidant systems;[23] however, to date no research has been conducted on the role of non-enzymatic antioxidant systems, such as GSH and PCs. Taking this into account, the aim of this study was to evaluate the contribution of PCs on Cd detoxification and tolerance in peanuts. Thus, Cd effects on plant growth, and its accumulation and participation in ROS generation as well as non-protein thiol contents were analysed.

Results

Plant growth in response to Cd

Exposure of peanut plants to Cd, even at the lowest concentration tested (10 μM Cd), decreased shoot dry weight and length after 30 days of treatment. No significant differences in root dry weight were observed in plants exposed up to 40 μM Cd compared to control treatment, nevertheless root length showed decreases in 20 and 40 μM Cd treatments (Table 1). Moreover, the exomorphological observation of peanut root exposed to Cd did not show any changes, except for a darkening and thickening in the root system that was clearly detected (Fig. 1).

Changes in histological structure of peanut root caused by cadmium

To investigate the effect of Cd on root structure, transverse cuts were made on peanut plants. In control roots the three tissue systems constituting three zones were recognized: the epidermis (dermal tissue system), the cortex (ground tissue system), and the central cylinder (vascular tissue system). In the epidermis, a single cell layer with small cells and very thin walls was observed. In the parenchymal cortex, intercellular spaces were small and scarce; in the innermost layer (the endodermis), all the cells were at the Casparian strip stage. The central cylinder showed a triarch or tetrarch arrangement with three or four protoxylem points abutting directly to the pericycle (Fig. 2A). However, some modifications were observed in 10 μM Cd treated roots at the cortex, being the cells of the endodermis the most affected by the metal, in which an unknown material was detected on the external cell wall. This material was also observed in the epidermis (Fig. 2B). Significant changes were observed in plant exposed to higher Cd concentrations. At 20 μM Cd, epidermal cells increased their size, causing rupture and disintegration. This phenomenon could be also found in cells conforming cortex (Fig. 2C). At 40 μM Cd, cortices with very few layers had most of their cells deformed and/or broken (Fig. 2D).

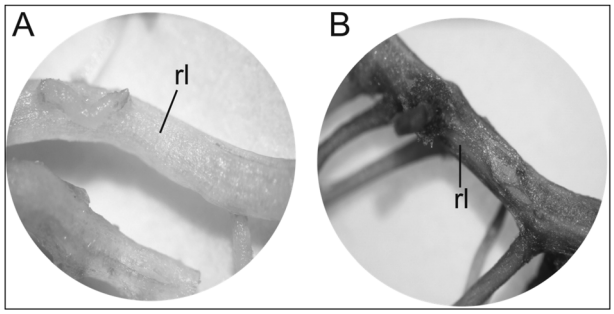

Fig. 1 Effects of 10 μM cadmium addition on exomorphological view of peanut root. A: Control; B: 10 μM Cd; rl: lateral root. Magnification 2.5×.

Stress indicators on peanut plants

Chlorophyll leaf levels, lipid peroxidation (estimated as malondialdehyde, MDA, content), oxidative modification of proteins and H_2O_2 content were used as oxidative stress indexes in shoots and roots of peanut plants treated with Cd.

Based on peanut growth and histological results, we decided to analyze Cd impact on peanut plants exposed to the minimal concentration that induced changes on plants, *i.e.*, 10 μM Cd. In peanut treated plants, chlorophyll, MDA carbonyl-groups and H_2O_2 contents remained unchanged (Fig. 3A–D). At this metal concentration, peanut treated plants accumulated 233 μg g^{-1} Cd, out of which 163.1 μg g^{-1} was found in root, 69.9 μg g^{-1} in leaves, and only 30 μg g^{-1} retained on perlite inert substrate. The results obtained in peanut control plants indicated the absence of Cd.

Peanut biothiols induced by cadmium

Peanut control showed that the major non-protein thiol content corresponded to GSH and its concentration was around 200 and 80 nmol g^{-1} fresh weight in leaves and roots, respectively. Cd-treated plants revealed a significant GSH decrease in concentration (23%) on both roots and leaves.

Table 1 Cadmium effects on peanut growth

Cd addition (μM)	Shoot dry weight (g)	Root dry weight (g)	Shoot length (cm)	Root length (cm)
0	1.25 ± 0.06 c	0.21 ± 0.02 a	22.07 ± 0.60 c	32.44 ± 1.47 c
10	0.67 ± 0.05 b	0.23 ± 0.02 a	17.85 ± 0.44 b	37.60 ± 2.16 c
20	0.48 ± 0.05 ab	0.20 ± 0.04 a	12.03 ± 0.66 a	13.00 ± 0.98 a
40	0.41 ± 0.08 a	0.21 ± 0.02 a	10.73 ± 0.77 a	19.16 ± 1.04 b

Data represent the mean ± SE (n = 10). Different letters in each column indicate significant differences ($P < 0.05$) according to the Duncan's test.

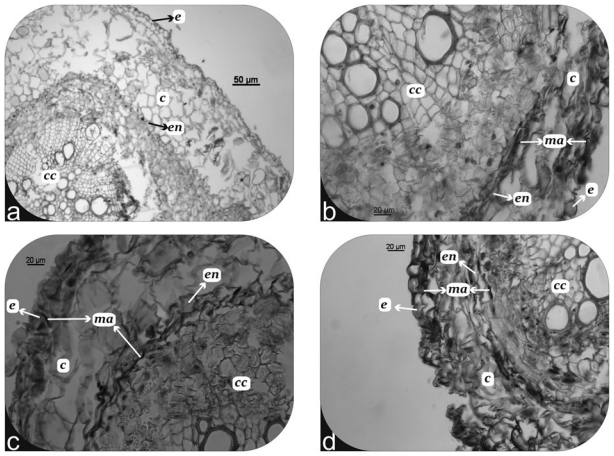

Fig. 2 Cross section of peanut root. (A) Control; (B), (C) and (D) exposed to cadmium at 10, 20 and 40 µM, respectively; c: cortex, cc: central cylinder, e: epidermis, en: endodermis, ma: unknown material.

In control conditions, no PCs were detected in these organs, however at 10 µM Cd, roots were able to induce PC2, PC3, and PC4 synthesis (Table 2). Among different PCs, PC4 showed the highest concentration followed by PC3 and PC2 (Fig. S1, ESI†).

Discussion

It is well known that the alteration of plant growth is one of the many symptoms caused by Cd, and the most sensitive one.[15,25–29] Our results revealed that the lowest Cd concentration tested not only reduced peanut growth as reported for other legume species,[30,31] but also induced histological root changes. The accelerated development of cells that form the endodermis and exodermis is also a known process that alters plant root histology exposed to Cd.[32,33] In addition, rhizodermis, exodermis, and endodermis zones act as barriers for movement *via* apoplast of toxic elements such as Cd.[32–36] In our study system, analysis of histological sections allowed the observation of a deposit of an amorphous material of unknown composition found in the cortex, endodermis, and mainly on the epidermis of the peanut plants exposed to Cd. Dermal tissues limit the passage of certain molecules to the vascular tissue, so that considering the damage in Cd-induced root cells, it is possible to suggest that the epidermis and endodermis would be acting as barrier defences preventing the entry of the metal and its translocation to the aerial part of the plant. Other effects of heavy metal exposed plants are associated with the alteration of the metabolic processes such as a decrease in the photosynthetic rate that leads to a reduction of plant productivity.[37] Several toxicity mechanisms were proposed in order to explain Cd effects on chlorophyll content.[38] Cd can cause an imbalance in the chloroplast metabolism, inhibiting chlorophyll content and reducing CO_2 fixation implicated enzymes.[39–40] Additionally, heavy metals can cause disturbances in the mineral metabolism of plants as Cd is involved in Fe deficiency (co-factor related to chlorophyll biosynthesis) by the inhibition of the Fe (III) reductase enzyme,[41] or by the inhibition of Fe uptake to aerial parts.[42] Another disturbance reported is the reduction in Mn transport caused by Cd.[43,44] Moreover, Cd can cause an active formation of free radicals, leading to oxidative stress.[45] This metal does not cause ROS production in a direct way but acts as a pro-oxidant through reduction in GSH and PC levels.[46] In addition, Cd affects antioxidant enzymes[10,13] that have the specific role of interacting with ROS. As ROS are subproducts of the aerobic metabolism and their production occurs in different cellular compartments,[47–49] an increase in their number produces oxidative stress, which can oxidize macromolecules[50] such as cellular lipids[10] and proteins.[18] In contrast, as observed in other legumes,[31,51,52] 10 µM Cd addition did not modify chlorophyll

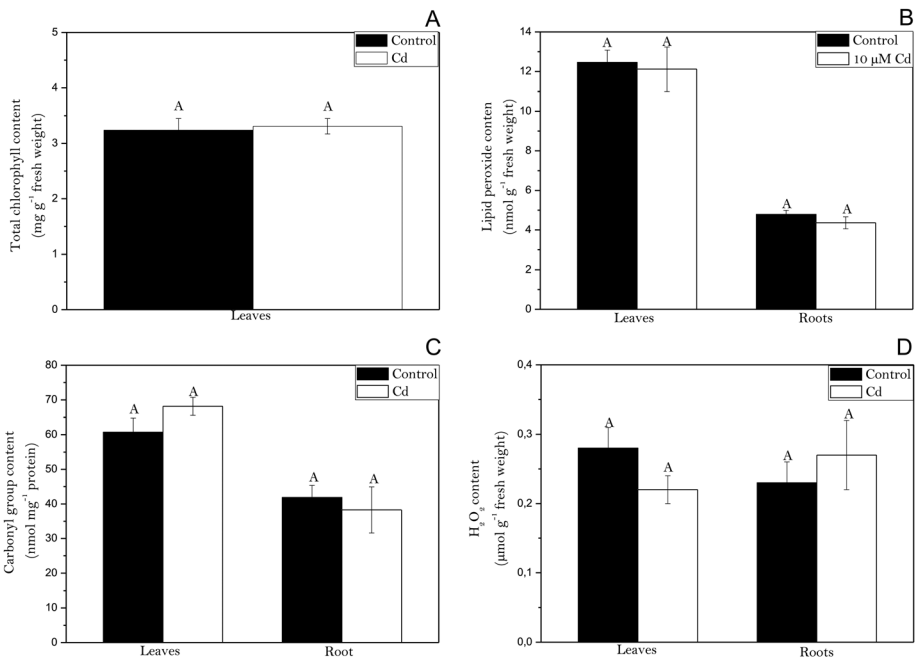

Fig. 3 Effects of 10 µM Cd addition on the contents of (A) chlorophyll, (B) lipid peroxides, (C) protein carbonyl, and (D) hydrogen peroxide. Different letters in each column indicate significant differences in each organ ($P < 0.05$) according to Duncan's test.

Table 2 Non-protein thiol contents on peanut plants exposed to Cd

	Cd addition (μM)	GSH	PC2	PC3	PC4
		(nmol g^{-1} fresh weight)			
Leaves	0	211.02 ± 16.56 a	nd	nd	nd
	10	162.67 ± 10.95 b	nd	nd	nd
Roots	0	92.07 ± 7.05 a	nd	nd	nd
	10	70.81 ± 5.73 b	27.07 ± 3.24	46.39 ± 7.93	48.91 ± 2.43

Data represent the mean ± SE ($n = 7$). Different letters in each row indicate significant differences ($P < 0.05$) according to the Duncan's test.

content and lipid peroxidation. These results are in agreement with those obtained previously in alfalfa and lupin plants.[31,53] These authors did not report any changes in MDA levels when *Lupinus albus* L. cv. Multolupa and *Medicago sativa* cv. Aragon were grown in a semi-hydroponic system exposed to 10 μM Cd. In addition, no lipid peroxidation was observed in Cd-exposed plants and the hairy roots of *Daucus carota*.[54] Oxidatively modified proteins have been implicated in a variety of physiological and pathological processes.[55] Oxidative modification typically causes the introduction of carbonyl groups into amino acid side chains of the protein. Thus, carbonyl determination can be used as an oxidative stress indicator.[56] The peanut plant did not show any significant change in carbonyl group content in roots or leaves exposed to 10 μM Cd. Several studies were performed to determine Cd-induced protein damage based on the increase of carbonyl group content found in different legumes (*Vigna mungo* L., *Pisum sativum* L. and *Medicago sativa*).[10,18,51,57] On the other hand, Cd did not induce any change in H_2O_2 concentration in peanut plants. Unlike this legume, a high level of H_2O_2 was found in many researches where plants were exposed to Cd.[26,58–60] Moreover, a significant increase of H_2O_2 levels was observed in pea plants exposed to Cd with respect to control plants.[60,61] As it is known, Cd can induce membrane NADPH oxidase and produce ROS which can be rapidly eliminated by the antioxidant system.[54] In peanut plants, the antioxidant system could prevent the alteration of H_2O_2 and chlorophyll contents, reducing lipid and protein damage.

Several responses to Cd-induced oxidative stress are probably related to both the level of Cd supplied and the concentration of thiolic groups already present or induced by Cd treatment.[54] Thiols possess strong antioxidative properties, and they are consequently able to counteract oxidative stress.[62] It is known that GSH is involved in ROS control.[63–65] This molecule has been studied because it can detoxify heavy metals and its concentration decreases in response to Cd.[66] Some authors agree that this decrease is related to PC, because GSH is involved in PC biosynthesis.[64,67,68] On the other hand, PC synthesis is catalyzed by PCS enzyme which is strongly activated by Cd.[69] Studies carried out with PCS, *in vitro* and *in vivo* in *Rubia tinctorum*, showed that Cd is an inductor of PC synthesis.[70,71] In alfalfa plants, PC synthesis induced by Cd has been demonstrated.[31,57] Similar results were obtained in other legumes such as *Lotus japonicus*[72] and *Vicia faba*,[73] exposed to Cd. Our findings revealed that Cd decreases the GSH content in peanut leaves and roots. This decrease could be related to the increase in PC synthesis observed in roots exposed to the metal. Thus, PCs could sequester Cd and therefore prevent alteration in the cellular redox balance that produces lipid and protein oxidation as well as chlorophyll degradation.

Inhibition of plant growth is one of the first parameters affected by heavy metals[26,27] and PCs increase with duration of metal exposure.[74] Our findings showed that although peanut growth decreased at the lowest concentration of Cd tested, PCs were strongly induced by this metal so that these thiols could play a significant role against Cd toxicity on cellular components such as lipids and proteins.

Conclusions

PCs play an important role in peanut detoxification and defence against Cd toxicity. Although plant growth decreased, no modification in chlorophyll content, lipid peroxidation, or protein oxidation was observed at the lowest concentration of Cd tested. Since PC synthesis is increased when plants are exposed to Cd, PCs could be used as stress indicators and their production could prevent metal disturbances in cellular redox balance, inhibiting oxidative damage to macromolecules.

Experimental

Plant material and growth condition

Arachis hypogaea L. cv. Granoleico (El Carmen S.A; General Deheza, Córdoba, Argentina) seeds were surface sterilized following the method previously described[75]. Sterilized seeds were germinated at 28 °C in Petri dishes on a layer of Whatman No. 1 filter paper and moist cotton until the radicle reached 3–5 cm. Afterwards, individual seedlings were aseptically placed on a perlite inert substrate—a semi-hydroponic system in which root development resembles that of plants grown in soils—in plastic containers and watered once a week with Hoagland medium[76] supplemented with Cd (0–40 μM). Plants were grown in a greenhouse under a controlled environment (light intensity of 200 μmol m^{-2} s^{-1}, 16 h day/8 h night cycle, constant temperature of 28 °C, and relative humidity of 50%) for 30 days. At harvest, plant length was measured and shoot and root were stored at −80 °C until analysis.

Microscopy studies

For histological studies, the root system was separated into main and lateral roots. From the main roots, portions of up to 5 mm in length were cut at approximately 1 cm of root tip. Roots were fixed in formol/acetic acid/ethanol/water at 30 : 5 : 50 : 15 v/v, dehydrated in an ascending series of ethyl alcohol, xylol and finally embedded in histowax. Seriate sections, ranging from 8 to 10 mm in thickness, were cut with a rotary microtome. They were stained with hematoxylin–safranin–fast green and mounted in distyrene, tricresyl phosphate and

xylene (DPX).[77,78] The photomicrographs were taken with an Axiophot Carl Zeiss microscope.

Stress indexes

Chlorophyll content: the amount of total chlorophyll was determined by the method previously described.[79] Briefly, about 0.1 g of peanut leaves was placed into a mortar and the tissues were grinded to a fine pulp after the addition of 80% acetone. The resulting extract was transferred to a Buchner funnel containing a pad of Whatman filter paper. While filtering the extract, the grinding of the leaves pulp was repeated to adjust the final volume of the filtrate to 10 ml. The optical density of the chlorophyll extract was read with a spectrophotometer set at 652 nm. The amount of total chlorophyll present in the extract was calculated on the basis of μg of chlorophyll g^{-1} of leaf tissue, according to the following equation:

$$\text{Total chlorophyll} = [(OD652 \times 1000)/3.45] \times 0.01\ \text{ml} \times 0.1\ \text{g}$$

Lipid peroxidation was determined by estimation of malondialdehyde (MDA) content, which is normally considered the major thiobarbituric acid (TBA) as previously described.[80] Plant material (0.1 g) was homogenized in 20% of trichloroacetic acid (TCA) and then mixed with 0.5% of TBA. The extract was heated for 25 min in a cold bath (95 °C). After cooling, the samples were centrifuged for 6 min at 6200 *g* and the supernatant was used to determinate MDA content at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm.

Protein oxidation was measured as the carbonyl group content by derivatization with 2.4-dinitrophenylhydrazine according to some modifications.[55] Proteins were extracted from 0.25 g of roots or leaves with 2.5 ml of 100 mM potassium phosphate (pH 7.0), 0.1% (v/v) Triton X-100, 1 mM Na_2EDTA, and 2.5 mg of leupeptin to prevent proteolysis of oxidized proteins during sample preparation. After precipitation of possible contaminating nucleic acids in the samples with 1% (w/v) streptomycin sulfate, an aliquot of 0.4 ml of the extracts was reacted with 0.1 ml of 20 mM dinitrophenylhydrazine in 2 M HCl, and another aliquot (control) with 0.1 ml of 2 M HCl for 1 h, with vigorous shaking every 10 to 15 min. Proteins were then precipitated with 10% (w/v) TCA, and the pellet was washed four times with 1 : 1 (v/v) ethanol/ethyl acetate. Precipitated proteins were solubilized in 6 M guanidine–HCl (pH 4.5) by incubation for 30 min with shaking. The insoluble material was removed by centrifugation, and the absorbance of the hydrazones (derivatized carbonyls) was measured at 370 nm. To obtain more accurate results, the amount of protein analyzed for carbonyl content was adjusted to 0.5 mg in all samples.

H_2O_2 determination was measured spectrophotometrically after reaction with KI following the procedure described.[81] The reaction mixture consisted of 0.1% TCA root or leaf extract supernatant, 100 mM potassium phosphate buffer, and 1M KI (w/v) in fresh double-distilled water. The blank probe consisted of 0.1% TCA in the absence of leaf extract. The reaction was carried out for 1 h in darkness, and the absorbance was then measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2.

Cd tissues concentration

Leaves and roots of peanut plants exposed to Cd were washed with distilled water and dried at 70 °C for 7 d. Samples were digested in an acid mixture of $H_2O/HNO_3/H_2O_2$ (5 : 3 : 2) for 30 min in closed containers under pressure.[15] Analysis of Cd in plant tissues was carried out using inductively coupled plasma mass spectrometry (ICP-MS).

Analysis of biothiols by HPLC

HPLC (high performance liquid chromatography) was used to quantify non-protein thiols in acidic extracts. Peanut leaves and roots were analyzed following procedures described.[82] Plant tissue was ground in liquid N_2, and 0.1 g of frozen powder was thoroughly mixed with 0.125 N HCl. The homogenate was centrifuged for 10 min at 10 000 *g* and 4 °C. Then, 100 μL was injected on a reverse phase HPLC and the detection was achieved after post-column derivatization with Ellman's reagent. The reaction took place in a thermostated 1.8 ml reactor at 37 °C, as previously described.[83] The derivative compound had an absorption maximum at 412 nm. An internal patron of *N*-acetyl-cystein (NacCys) was used for PC quantification and commercial PCs were used as standards for identification.

Statistical analysis

Differences among treatments were analyzed by one-way analysis of variance, and $P < 0.05$ was considered significant, according to Duncan's test.

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