Apoptosis like PCD, autophagy, and necrosis; different types of cell death induced by cadmium in tobacco cells

Mohammad Pormehr[1], Faezeh Ghanati[1]*, Farzaneh Mohammadi[1], and Atefeh Payez[2], Sepideh Mirzaei Rad[1]

1. Department of Plant Biology, Faculty of Biological Science, Tarbiat Modares University (TMU), POB 14115-154, Tehran- Iran.

2. Department of Biological Science, Farhangian University, Tehran- Iran

Abstract

Cadmium (Cd) is an inessential and harmful element, exposure to which is fatal for plants. Nonetheless, the type(s) of death are far less well understood. In the present study, suspension-cultured tobacco (*Nicotiana tabacum* cv. Barley21) cells were exposed to 5 to 150 µM $CdCl_2$ for 12 hours. The viability of the cells, the content of reactive oxygen species (ROS), apoptosis, and necrosis were examined by flow cytometry. Autophagy, as a pro-survival mechanism against Cd, was evaluated using the autophagy inhibitor wortmannin. The antioxidant system and the gene expressions involved in programmed cell death (PCD) were assessed by semi-quantitative RT-PCR. At 5 µM Cd treatment, despite the accumulation of 0.7 mg/g Cd in the cells, no adverse effects were observed in viability, fresh weight, or dry weight. At concentrations above 5 µM, the increase in internal Cd levels provoked the antioxidant system and initiated different types of cell death. At a concentration of 50 µM, Cd mostly induced PCD. In 100 µM and 150 µM Cd treatments, however, autophagy and necrosis were respectively the most prominent types of cell death. The expression pattern of Hsr203J and ATG9 at different Cd concentrations was consistent with the pronounced type of death in each treatment. The results suggested that the intensity of Cd toxicity for tobacco cells depends on the level of its internal concentration and that the type of death is related to the outcome of the antioxidant system activity.

Keywords: Apoptosis; Autophagy; Cadmium; Necrosis; Tobacco cells

Abbreviations: ATG, autophagy-related gene; CAT, catalase; CdCl2, cadmium chloride; FRAP, ferric reducing antioxidant power. HPLC, high performance liquid chromatography; Hsr, hypersensitive response; MDA, malondialdehyde; PAL, phenylalanine ammonia-lyase; PCD, programmed cell death; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase

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Introduction

Programmed cell death (PCD) has been defined as a sequence of potentially interruptible events that lead to the controlled death of cells. It is a critical component of developmental and defense responses. Cell death is generally classified as apoptosis, autophagy, and necrosis (Yan et al. 2020). Apoptosis in plant cells is characterized by chromatin condensation, DNA degradation, and condensation of the cytoplasm, which causes the separation of the cell wall and the plasma membrane (Kacprzyk et al. 2014).

In autophagy, chromatin condensation does not occur; instead, the cytoplasm is massively

* *Corresponding Author*
E-mail Address: ghangia@modares.ac.ir
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vacuolized (Kroemer et al. 2005). This type of cell death is necessary for cellular differentiation and development (Levine and Kroemer 2008). Meanwhile, autophagy can be involved in cell survival depending on the rate of stress and the type of cells (Moreira et al. 2007).

By definition, necrosis is an unorganized destruction of cells that usually occurs after overwhelming stress. Due to the loss of osmoregulatory ability, the cell absorbs large amounts of water and ions, causing its membrane and organelles to swell (Lockshin and Zakeri 2004). Cadmium (Cd), as an environmental pollutant with a long biological half-life, represents a serious risk to all organisms, including plants (Gallego et al. 2012). Cd toxicity is often accompanied by oxidative bursts, programmed cell death, and necrosis (Lehotai et al. 2011). However, the mechanism and type of Cd-induced death in plant cells are not clear. Expression of caspase-like proteases has been observed in $CdSO_4$-treated suspension-cultured tomato cells, showing morphological and biochemical similarities to hypersensitive responses (Iakimova et al. 2008). In Cd-treated Arabidopsis thaliana, altered patterns of distribution and mobility of mitochondria have been observed, accompanied by cell death (YuHua et al. 2009). In Cd-treated BY-2 cultured cells, the cytoplasm was extensively vacuolized, the plasma membrane lost its integrity, the protoplast was wrinkled, cellular components were unprocessed, and the cells ultimately died (Kutik et al. 2014).

It has been shown that cell-death responses to Cd depend on its concentration. For instance, when tobacco cells were exposed to 50 µM $CdSO_4$, a decrease in viability started on the second day, gradually continued, and the cells ultimately died after one week, when specific oligonucleosomal fragments were clearly observable. However, when these cells were exposed to 1 mM $CdSO_4$, protoplast wrinkling occurred within a few hours, and the cells died soon after, with no fragmentation of DNA detected (Kuthanova et al. 2008).

Similar to other heavy metals, Cd toxicity is often related to the overproduction of reactive oxygen species (ROS). In addition to exacerbating cellular damage, ROS play a critical signaling role in the adaptation of plants to stress (Dat et al. 2000). Crosstalk between signaling molecules, such as ROS and other signaling cascades, triggers cell death execution in plants (Emanuele et al. 2018). Through this role, ROS functions as a regulator that determines the type of cell death (Villalpando-Rodriguez and Gibson 2021). The objective of the present study was to clarify the types of cell death in tobacco cells exposed to different concentrations of Cd.

Materials and Methods

Cell Culture and Treatment

In this study, suspension cultures of a rapidly growing cell line of tobacco (*Nicotiana tabacum* L. cv. Burley 21) were used. The suspensions contained a modified LS medium without glycine, with sucrose (3% w/v), auxin (NAA and IAA, 3 mg/L each), and 1 mg/L kinetin, at pH 5.8 (Ghahremani et al. 2014). The cells were grown on a rotary shaker at 123 rpm in darkness and were sub-cultured weekly. Cells in their exponential growth phase were treated with 0, 5, 25, 50, 100, and 150 µM Cd ($CdCl_2$) for 12 hours. Cd was added after filter sterilization (0.2 µm) under sterile conditions.

Viability, Apoptosis, Autophagy, and Necrosis Analysis

Apoptosis was monitored using the Annexin V-FITC kit (BioVision, USA) and flow cytometry analysis (FACSAria II, BD Biosciences, USA) (Pormehr et al. 2019).

Autophagy in the cells was detected by staining with Acridine Orange (Filippi-Chiela et al. 2013). Briefly, the cells were stained with Acridine Orange (1 µg/mL) for 15 minutes, then analyzed by flow cytometry. FlowJo software (Version 7.6.1) was used for data analysis.

The involvement of autophagy in tobacco cell death under different concentrations of Cd was evaluated using wortmannin as an inhibitor of autophagy. Wortmannin is a fungus-derived steroid metabolite that acts as a non-specific, covalent inhibitor of phosphoinositide 3-kinases. Wortmannin solution was prepared in dimethyl

sulfoxide (DMSO). The concentration of DMSO was less than 0.05% in all treatments. Preliminary experiments were conducted to determine the appropriate concentration of wortmannin, and based on these results, wortmannin was applied at a final concentration of 12 µM. The cells were treated with 0, 5, 25, 50, 100, and 150 µM Cd with or without wortmannin for 12 hours.

Cd, MDA, ROS, and Antioxidant Assays

The cells were ashed at 250 °C for 2 hours and then at 600 °C for 2 hours, followed by digestion of the ash in high-concentration HCl: deionized water (1:1 v/v), drying on a sand bath (110 °C), and dissolving in 1N HCl. The Cd content was detected by atomic absorption spectrophotometry (Shimadzu AA-670, Japan). Membrane lipid peroxidation was monitored by quantifying malondialdehyde (MDA) (Ghahremani et al. 2014).

The content of ROS was determined based on the deacetylation of 2',7'-dichlorofluorescein diacetate and the production of 2',7'-dichlorofluorescein. The product was monitored through flow cytometry at excitation and emission wavelengths of 485 and 535 nm, respectively. Background subtraction was performed, and the data were expressed as a percentage of the control.

Activities of catalase, superoxide dismutase (SOD), and peroxidase (POD) were measured based on the methods described by Hajnorouzi et al. (2011).

For analysis of non-enzyme antioxidants, flavonoids, and phenolic acids, the samples were extracted with MeOH: HOAc (100:0.3 v/v) followed by evaporation under reduced pressure. The residue was re-dissolved in MeCN, extracted three times with hexane, dried, and then re-dissolved in MeOH before injection into the HPLC system (Waters e2695, USA). The system was equipped with a C18 column (Perfectsil Target ODS-3, 250 × 4.6 mm; MZ Analysentechnik, Mainz, Germany) and a 2489 UV/Visible detector. Phenolic compounds were eluted with a gradient of 30-100% MeOH against 2% acetic acid containing deionized water. Total phenolic content was determined by the Folin-Ciocalteu method, with gallic acid used as a standard (Akkol et al. 2008).

Molecular Analysis

Unless otherwise mentioned, all materials were purchased from Cinna Gen (Iran). RNA was extracted using a Hybrid-RTM kit and its quality was monitored on agarose gel electrophoresis and NanoDrop. The RNA was stored at -80 °C. cDNA was synthesized in a mixture containing oligo-(dT) primer, reverse transcriptase (RevertAid™ M-MuLV, Fermentas, Korea), RNase inhibitor, and dNTPs. RT-PCR primers were made based on the protein-conserved domains of Nicotiana tabacum PAL (NM001325017.1), Hsr203J (AF212184.1), CAT (NM001325093.1), and ATG9 (NM001325763/1) from NCBI. Actin was used as a housekeeping gene (Table 1). The reaction was performed at 42 °C for 55 minutes, followed by a 15-minute step at 75 °C.

Separation of products was achieved on 1% agarose gel electrophoresis, followed by staining of bands with ethidium bromide and measurement of their intensity by UV imaging using ImageJ software (version 1.52b).

Data Analysis

Algorithms from the web-based MetaboAnalyst (http://www.metaboanalyst.ca) were used for hierarchical cluster analysis (HCA) and principal component analysis (PCA). Three independent repetitions of all experiments and observations were conducted, each with at least three samples. Data were analyzed by multiple mean comparisons at a significance level of $p \leq 0.05$ using the Duncan test in the SPSS package (version 16, Chicago, IL, USA).
Test at $P < 0.05$. Data were analyzed using SPSS version 16.0 statistical software.

Results

Cadmium content and growth parameters

Along with the increasing Cd supply, its absorption by tobacco cells increased so that the highest Cd

Table 1
Sequences of primer pairs used in the quantitative RT-PCR analysis.

Genes	Primer Sequences	Amplicon size (bp)
Actin	Forward 5'- TCTGGAGATGGTGTGAGCCACAC - 3' Reverse 5'- GGAAGGTACTGAGGGAGGCCAAG -3'	600
CAT	Forward 5'- GTCCCACATTCAGGAGAACTGG - 3' Reverse 5'- CTGGGAGCTGCAGATAGTTTGG -3'	600
PAL	Forward 5'- CATCAGATTTGAAATCTTGGAAGC - 3' Reverse 5'- GCCATGGCGATTTCAGCTCCCTTGAA-3'	800
Hsr203J	Forward5'- GTGATAGAGGAAGTATCCGGCTGGCTTAG-3' Reverse 5'- ACGCTTGTTGATGAACTCTGCAACGGCTTC -3'	1000
ATG9	Forward5'- TTCTCCTATCTCCGTTTCTCGT Reverse 5'- CTCATCTGTTATTGCAGCCCTG	1100

Table 2
Growth parameters and Cd contents of tobacco cells under different Cd supply. Data present averages of three independent experiments in triplicate each ± standard error. Different letters indicate significant differences according to the Duncan test ($P \leq 0.05$).

Cd treatment (µM)	Cd content (mg/g FW)	Fresh Weight (g)	Dry Weight (g)
0	0.34±0.001 f	8.8±0.04 a	0.8±0.003 a
5	0.75±0.004 e	8.1±0.03 a	0.8±0.004 a
25	1.51±0.023 d	7.6±0.01 b	0.7±0.005 ab
50	3.06±0.036 c	7.3±0.03 b	0.7±0.004 b
100	5.89±0.042 b	6.7±0.01 c	0.6±0.001 c
150	7.45±0.057 a	6.1±0.07 c	0.5±0.007 d

content was detected in 150 µM Cd-treated cells (Table 2). Although at 5 µM Cd supply, its internal content was twice of the controls, no significant alteration was observed in the growth of cells, whereas higher Cd concentrations adversely affected the fresh and dry weights of the cells, compared to the control (Table 2).

Viability, Apoptosis, Autophagy, and Necrosis rate

As shown in Fig. IA, Cd treatment at concentrations above 5 µM significantly reduced the viability of tobacco cells. The lowest viability (7% of the control) was detected in the cells after treatment with 150 µM Cd (Fig. IA).

Presence of autophagy inhibitor wortmannin together with Cd showed no significant effect on viability of the cell up to 25 µM Cd, but at higher concentrations of Cd resulted in significant decrease of cell viability (Fig. IA).

In comparison with control group, concentrations above 5 µM of Cd significantly increased the percentage of PCD of tobacco cells, compared to the control (Fig. Ib). The tendency of changes of PCD in the presence of wortmannin was similar to that of Cd treatment (Fig. Ib). Treatment with Cd at concentrations above 5 µM significantly increased the percentage of autophagy in tobacco cells, compared to the control (Fig. Ib). Similar autophagy pattern was observed in tobacco cells in the presence of wortmannin (Fig. Ib). Maximum percentage of autophagy was measured in tobacco cells after treatment with 100 µM Cd with or without wortmannin (Fig. Ic).

The percentage of necrotic tobacco cells significantly increased at concentrations above 5 µM Cd, compared with the control group (Fig. Id). At these concentrations the presence of wortmannin also increased the percentage of necrosis of tobacco cells (Fig. Id).

Antioxidant status

Treatment with Cd significantly increased SOD, CAT, and POD activities in tobacco cells, compared to the control (Fig. II). The highest enzymes

Table 3
The contents of phenolic compounds, ROS, and MDA content and PAL activity of tobacco cells under different Cd concentrations. Data present averages of three independent experiments in triplicate each ± standard error. Different letters indicate significant differences according to the Duncan test ($P \leq 0.05$).

Cd supply (µM)	Cinnamic acid (µg g^{-1} DW)	Salicylic acid (µg g^{-1} DW)	Ferulic acid (µg g^{-1} DW)	Caffeic acid (µg g^{-1} DW)
0	0.73±0.05 d	8.36±0.02 e	1.43±0.05 e	9.9±0.08 e
5	0.80±0.04 d	8.85±0.52 e	1.45±0.02 e	10.03±0.05 e
25	1.36±0.06 b	28.4±0.37 b	2.66±0.03 b	21.33±0.06 b
50	1.65±0.05 a	35.04±0.48 a	3.27±0.07 a	28.43±0.30 a
100	1.27±0.02 bc	25.51±0.66 c	2.45±0.04 c	19.18±0.23 c
150	1.19±0.04 c	23.18±0.32 d	2.15±0.03 d	17.95±0.0 d
Cd supply (µM)	Quercetin (µg g^{-1} DW)	Catechin (µg g^{-1} DW)	Epicatechin (µg g^{-1} DW)	Vanillin (µg g^{-1} DW)
0	16.83±0.09 e	9.96±0.08 e	10.21±0.45 e	5.25±0.24 e
5	17.05±0.12 e	10.05±0.4 e	10.23±0.36 e	5.1±0.07 e
25	23.17±0.06 b	14.18±0.27 b	12.94±0.21 b	14.89±0.09 b
50	27.18±0.21 e	15.18±0.64 a	13.16±0.56 a	18.62±0.29 a
100	20.33±0.11 c	13.56±0.80 c	11.87±0.38 c	12.55±0.32 c
150	18.65±0.31 d	12.53±0.41 d	11.37±0.21 d	10.67±0.07 d
Cd supply (µM)	Total phenolics (mg. g^{-1} DW)	PAL activity (mM CA mg protein^{-1} h^{-1})	ROS (Percent)	MDA (nM/g FW)
0	10.51±0.08 c	0.21±0.005 c	11.2±0.47 e	0.51±0.04 d
5	11.4±0.03 c	0.21±0.005 c	12.8±0.18 e	0.58±0.05 d
25	18.15±0.04 b	0.35±0.007 b	35.5±0.87 d	0.61±0.56 d
50	23.8±0.26 a	0.45±0.006 a	67.1±0.67 c	1.03±0.10 c
100	17.37±0.91 b	0.34±0.002 b	83.5±0.78 b	1.91±0.09 b
150	15.47±0.18 b	0.32±0.002 b	91.8±0.73 a	2.40±0.12 a

activities were detected at 25-100 µM Cd treatments (Fig. II).

In comparison with control cells, exposure to Cd, particularly at 25-100 µM, significantly increased the contents of caffeic-, salicylic-, ferulic-, and cinnamic acids (Table 3). The contents of flavonoids derivatives i.e., catechin, epicatechin, and quercetin, and phenolic aldehyde vanillin were increased by Cd treatment. The pattern of increase of these compounds was similar to that of aforementioned phenolic acids (Table 3).

The concentration of phenolics was also increase by Cd and its maximum was detected at 50 µM Cd treatment (Table 3).

The activity of PAL, the rate limiting enzyme in phenolics biosynthetic pathway, in Cd concentrations above 5 µM was significantly higher than the control (Table 3).

Treatment with Cd at concentrations above 5 µM significantly increased both ROS content and the rate of peroxidation of tobacco cells membrane lipids, compared with the control groups (Table 3).

Expression of antioxidant, autophagy and PCD-related genes

Expression of *CAT* gene, encoding the most effective scavenger enzyme for H_2O_2, was carried out. A significant increase, up to 2.2-fold of the control, was observed in the expression of *CAT* gene. However, at other Cd concentrations, *CAT* gene expression was identical to the control (Fig. IIIa).

Relative gene expression of *PAL* in 25-100 µM Cd-treated cells was remarkably higher than control (Fig.IIIb).

Treatment of tobacco cells with Cd at concentrations above 5 µM remarkably increased the expression of autophagy-related *ATG9* gene,

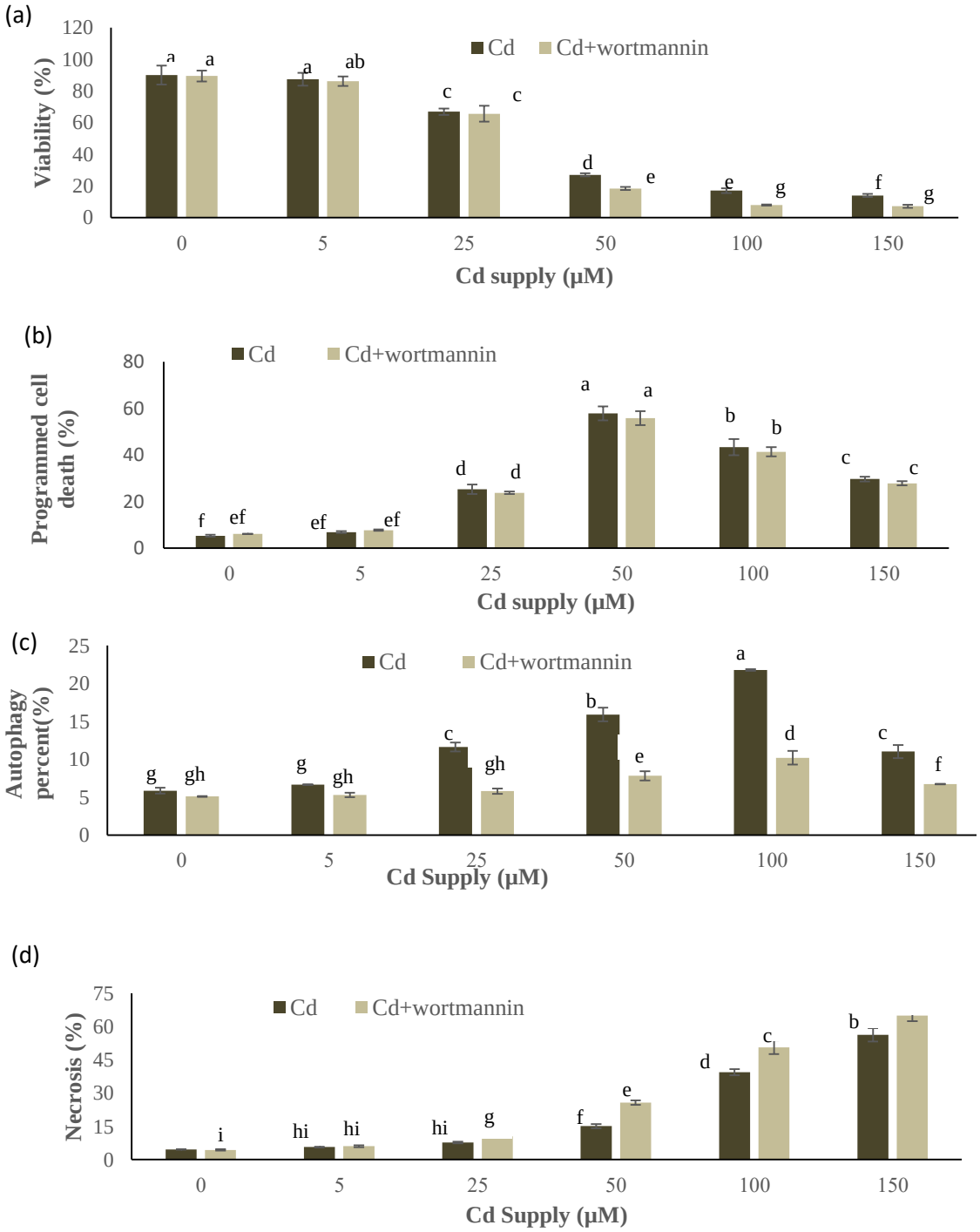

Fig. I. Effect of different concentrations of Cd with or without autophagy inhibitor wortmannin on tobacco cells. (a) Viability, (b) PCD, (c) Autophagy, (d) Necrosis. Data present averages of three independent experiments in triplicate each ± standard error. Different letters indicate significant differences according to the Duncan test (P ≤ 0.05).

compared to the corresponding controls (Fig. IIIc). The maximum *ATG9* gene expression (ca 25-fold of the control) was observed in 100 µM Cd-treated cells (Fig. IIIc). In 150 µM Cd treatment the expression of *ATG9* was lowered, but still was higher than the control.

The expression of PCD-related *Hsr203J* gene at 25-100 µM Cd was significantly higher than the control, with the maximum at 50 µM. In other Cd concentrations, *Hsr203J* gene expression was identical to the control (Fig. IIId).

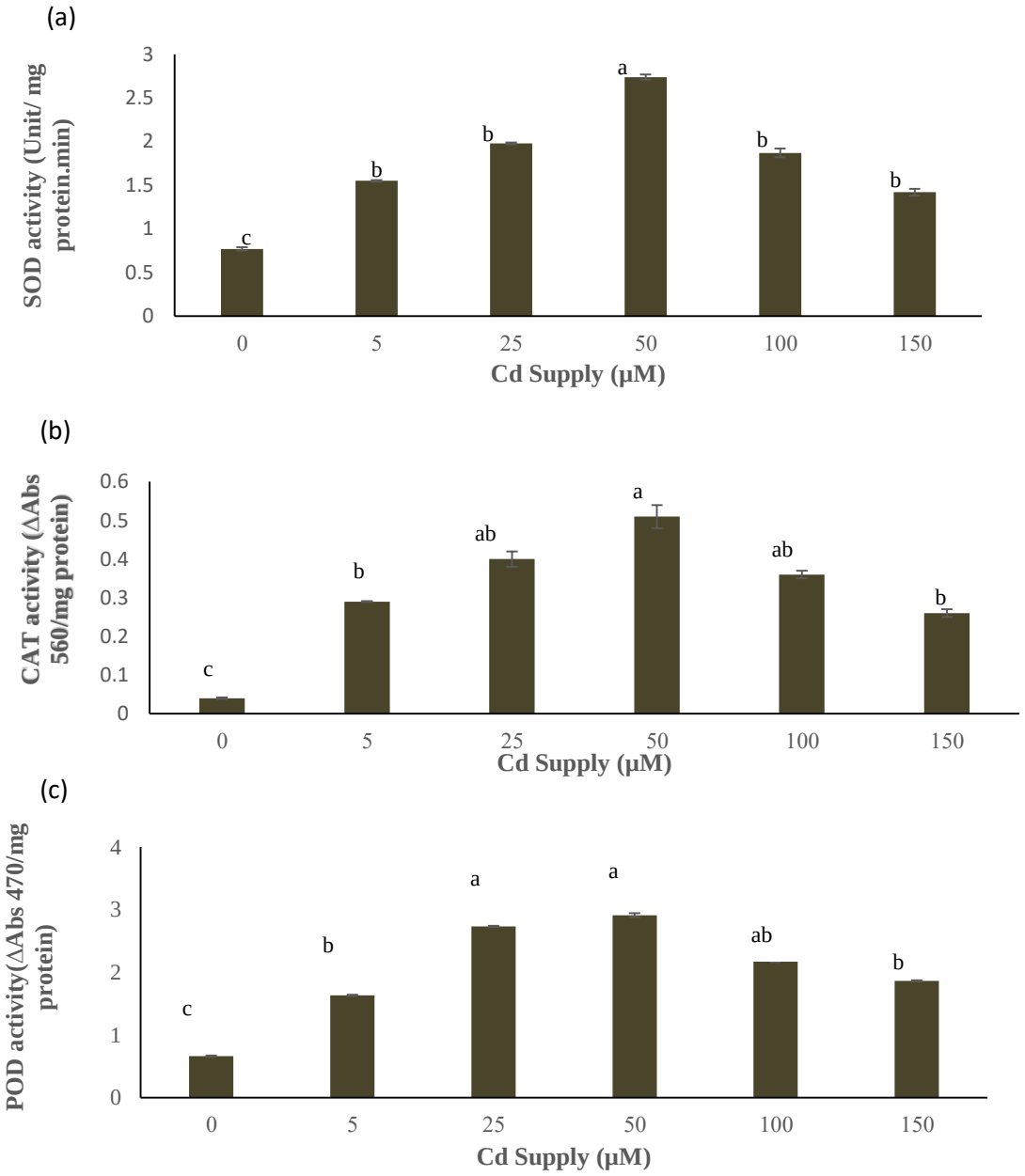

Fig. II. Effect of different concentrations of Cd on antioxidant enzymes activity of the tobacco cells. (a) SOD, (b) CAT, (c) POD. Data present averages of three independent experiments in triplicate each ± standard error. Different letters indicate significant differences according to the Duncan test ($P \leq 0.05$).

Correlations

Correlation between Cd concentrations and measured parameters was shown in Fig. IV (blue to red indicate increased content). It shows a correlation between viability, PCD, autophagy, and necrosis percent, SOD, CAT, POD, and PAL enzyme activity, phenolics content and *CAT*, *PAL*, *Hsr203J*, and *ATG9* gene expression along with the different Cd treatments. Classes 0 to 5 represent 0, 5, 25, 50, 100 and 150 µM Cd treatments, respectively.

There were two main clusters in this image. The first cluster indicated a negative correlation between all parameters at 0 and 5 µM Cd treatments except for dry weight, fresh weight, and viability. The second cluster represented a

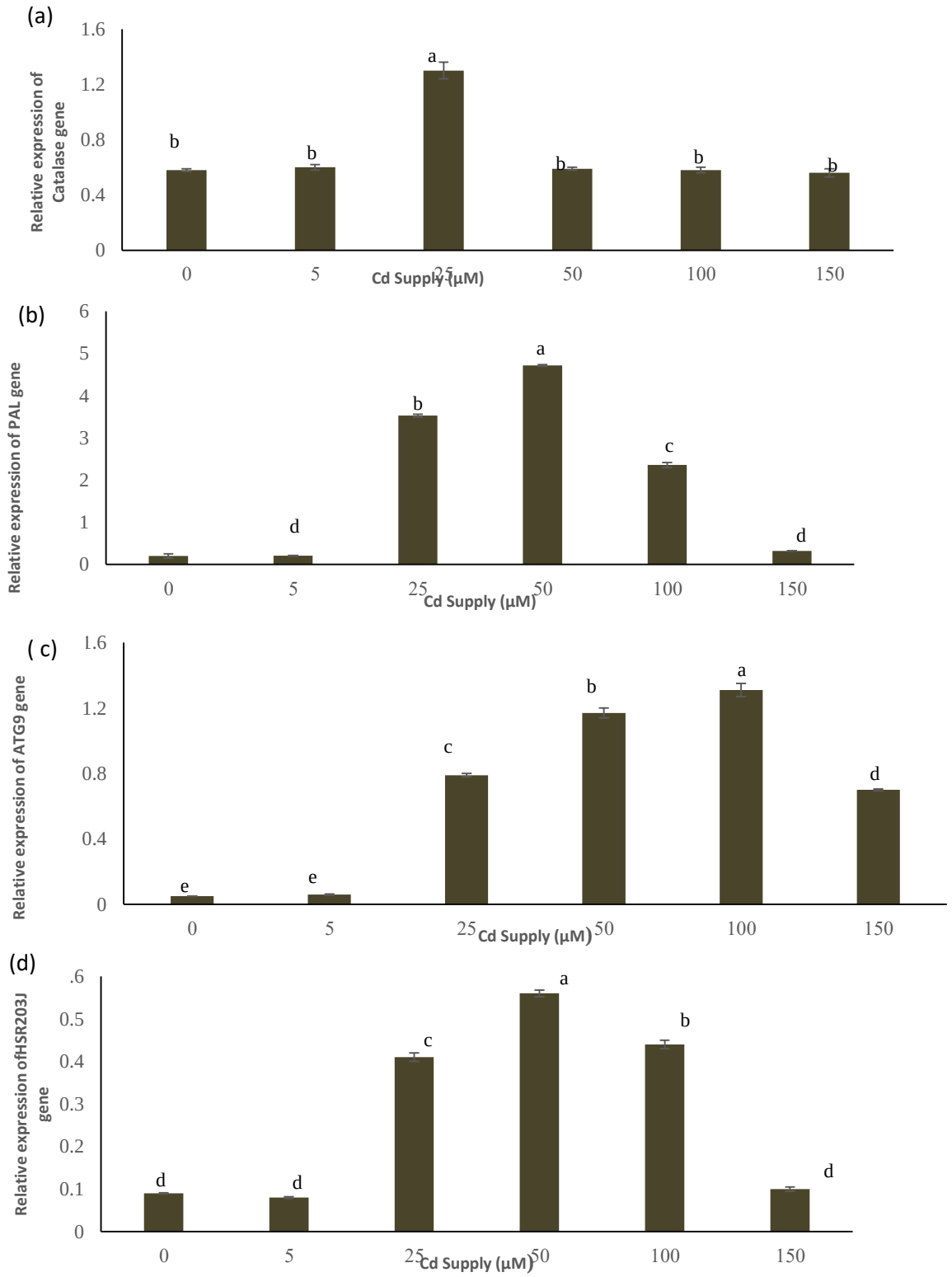

Fig. III. Effect of different concentrations of Cd on the expression of CAT (a), PAL (b), ATG9 (c), and Hsr203J (d) genes in tobacco cells. Data present averages of three independent experiments in triplicate each ± standard error. Different letters indicate significant differences according to the Duncan test ($P \leq 0.05$).

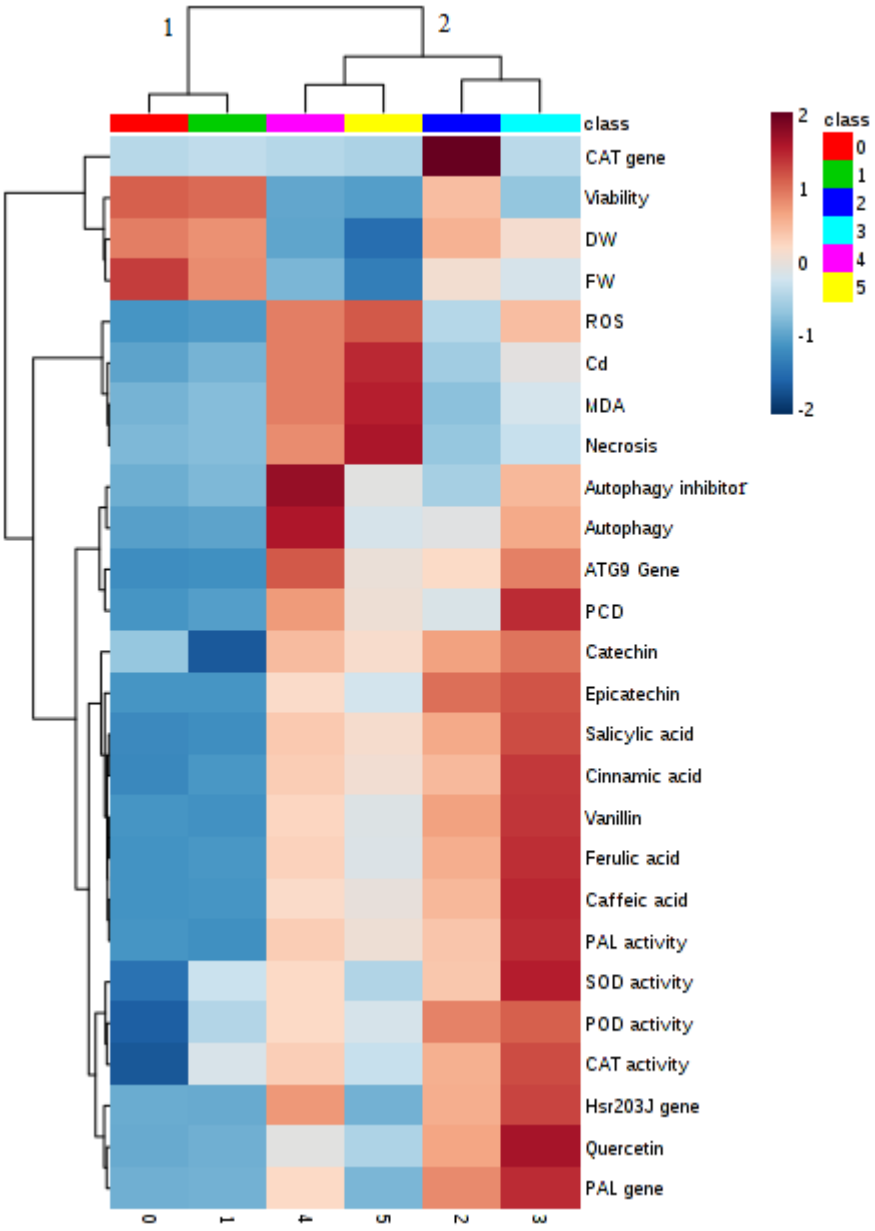

Fig. IV. Correlation between Cd concentrations, growth parameters, antioxidant system, and cell death of tobacco cells. Colors in matrix boxes show direction of correlation: intense red denotes strong positive and blue denotes strong negative correlations.

positive correlation among antioxidants, PCD, autophagy, autophagy inhibitor and their related gene expressions in 25, 50 and 100 µM Cd treatments. There was also a strong correlation between Cd accumulation, ROS, MDA and necrosis level in 150 µM Cd treatment.

Discussion

Cadmium is not a redox-active element; however, it has been supposed that this element can take the place of redox-active metals in the structure of certain proteins and can also activate NADPH oxidases, thereby increasing ROS (Cuypers et al., 2010). The extent of Cd toxicity is completely dependent on the extent of Cd accumulation in the cells and the subsequent level of ROS increase. There is much evidence showing that excess ROS accumulation could induce antioxidant genes (Liu

et al., 2018). Exposure of tobacco cells to 5 µM Cd did not have an adverse effect on cell viability, fresh weight, and dry weight, despite the accumulation of 0.7 mg/g fresh weight Cd inside the cells. It seems that this level of internal Cd only triggered the enzymatic antioxidant system and resulted in the reduction of ROS levels and the maintenance of membrane integrity. With increasing Cd supply to 25 µM and higher concentrations, a subsequent increase in its accumulation in tobacco cells occurred and triggered an increase in SOD activity, which catalyzed the conversion of superoxide anions to hydrogen peroxide. Since the activity of CAT did not change, the content of ROS increased, causing oxidative stress and subsequent cell death, such as apoptosis, necrosis, and autophagy. There is evidence that suggests the critical role of ROS as signaling molecules in the cell death pathway. The increase of ROS at 25 µM Cd treatment also induced the non-enzymatic antioxidant system in turn, providing the cell with the maintenance of the membranes. This may be interpreted as a hallmark of apoptosis (Minina et al., 2014; Emanuele et al., 2018; Liu et al., 2018; Godwin et al., 2019).

The accumulation of 3 mg/g FW Cd in tobacco cells at 50 µM Cd supply remarkably reduced cell viability (to 27% of control) and pushed the cells toward autophagy (16%) and necrosis (15%). As soon as oxidative stress occurs, proteins that are oxidized and damaged by reactive species must be degraded rapidly. Many reports have shown that in plants, autophagy is a vital process that eliminates oxidized proteins and acts as a pro-survival mechanism (Xiong et al., 2007). Improvement in growth and resistance against oxidative stress was reported in Arabidopsis thaliana, where autophagy-related ATG5 and ATG7 genes were overexpressed (Minina et al., 2018).

There is, however, a possibility that autophagy itself mediates cell death (Navarro-Yepes et al., 2014). Reports have shown that inhibition of autophagy genes by RNA interference resulted in a switch from autophagy-related cell death to rapid cell death with typical signs of necrosis (Minina et al., 2014). Similar to these reports, the application of the autophagy inhibitor wortmannin was accompanied by an increase in necrosis, while the percentage of PCD was constant and the highest level of Hsr203J gene expression was observed. It is likely that under the toxicity of 50 and 100 µM Cd, autophagy is an irreversible situation in the death pathway.

Under 150 µM Cd treatment, the remarkable accumulation of Cd inside the cells resulted in a reduction in the antioxidant defense system, thereby causing a significant shift from PCD (30%) to necrosis (56%). Interestingly, this was accompanied by a decrease in autophagy, in comparison with 100 µM Cd treatment.

Conclusion

In conclusion, the results presented here suggest that the intensity of Cd toxicity in tobacco cells primarily depends on the level of its internal accumulation and the subsequent extent of induced ROS. The type of cell death that occurs is determined by the balance between the scavenger system's activity and the ROS induced by Cd.

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