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Cadmium Toxicity Induces Production of H_2O_2 and Ethylene in Tomato Suspension Cell Culture

Research Article

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Abstract

Cadmium (Cd) is a non-essential toxic heavy metal and its interaction with plants causes toxicity to them with an overall reduction in plant productivity. Several physiological and biochemical changes occur when plants are exposed to Cd. The aim of the present work was to investigate the production of H_2O_2 and ethylene induced by toxicity of Cd in suspension cell culture of tomato. Upon exposure to Cd, a high amount of cell death was observed in suspension cell cultures of tomato. This cell death induced by Cd was accompanied by a significant increase in H_2O_2 and ethylene production. When inhibitors of H_2O_2 and ethylene were applied in the suspension cell cultures a significant decrease in the amount of cell death was observed following exposure to Cd. Thus the findings of the present investigation indicate that both H_2O_2 and ethylene are key players in mounting the toxic effects of Cd in suspension cell cultures of tomato.

Keywords: Cadmium; Cell death; Inhibitors; Stress; Tomato; Toxicity

Abbreviations

AVG: Aminoethoxy Vinyl Glycine; Cd: Cadmium; Cu: Copper; H_2O_2: Hydrogen Peroxide; ROS: Reactive Oxygen Species; TCA: Trichloroacetic Acid

Introduction

Plant stress is usually defined as an external factor that exerts a disadvantageous influence on it. Stresses trigger a wide range of plant responses, from altered gene expression and cellular metabolism to changes in growth rates and crop yields. The duration, severity and rate at which a stress is imposed, they all influence a plants response to a particular stress. A response may be triggered directly by a stress or may result from stress-induced injury. Plants respond to stress through a variety of mechanisms and at times failure to compensate for a severe stress above the threshold or tolerance capacity of the plant may result in plant cell death.

Excess uptake of heavy metal ions into the symplast is deleterious to most plant species [1,2]. Metal elements play an essential role as micronutrients, for example copper (Cu), or lack an essential function, for example cadmium (Cd).

Cadmium is a non-essential element with negative effects on growth and development of plants. Various sources like nickel-cadmium batteries, electroplating process, pigment manufacturing, power stations, metal working industries, urban traffic and cigarette smoke are responsible for its release in the environment [3]. Because

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of its high toxicity and great solubility in water, Cd is considered as an extremely significant pollutant in the environment [4]. Minerals uptake in plants is hampered by Cd [5]. The presence of Cd also affects many physiological processes like stomatal opening, transpiration and photosynthesis resulting into symptoms like chlorosis, leaf rolls, stunting and even necrosis [3,6,7]. Cadmium inhibits chlorophyll biosynthesis and reduces the activity of enzymes of CO_2 fixation process thereby producing alterations in the chlorophyll metabolism [7-9]. Cadmium induces lipid peroxidation and hinders the functionality of membranes [10]. Cadmium toxicity produces oxidative stress by either increasing the production of Reactive Oxygen Species (ROS) or by decreasing the activity of enzymatic and non-enzymatic antioxidants [3,11-13]. The ROS are produced in the cell as by-products of several metabolic steps. Depending on its concentration H_2O_2 is one such ROS which acts both as toxic by-product of cellular metabolism and as an important signaling molecule [14].

Reactive oxygen species are involved in the response of plants to all kind of biotic as well as a biotic stresses [15,16]. These ROS can trigger unlimited oxidation of various cellular structures, leading to oxidative destruction of the cell and the common ROS include Singlet Oxygen ($^1O_2$), Superoxide Radical ($O_2^{\bullet-}$), Hydrogen Peroxide (H_2O_2) and Hydroxyl Radical ($^{\bullet}OH$) [17].

When plants are subjected to Cd stress, a variety of ROS, such as superoxide, hydrogen peroxide and hydroxyl radicals are generated. These ROS cause oxidative damage in plants [16]. Free radicals are toxic to living organisms unless removed rapidly, destroyed or inactivated by various cellular components. In the absence of effective mechanisms that remove or scavenge free radicals, they can seriously damage plant by lipid peroxidation, protein degradation, breaking of DNA and cell death [18,19].

The simple gaseous olefin ethylene is a classical plant hormone with vital roles in many aspects of plant growth and development [20]. The role of ethylene as a stress hormone during various stressful conditions faced by plants is of prime interest [21].

Ethylene is known to produce serious effects on growth and development of plants [22,23]. Amongst the best known effect of ethylene is the induction of ripening of fruits, especially those of tomato fruits. Several other processes like seed germination, promotion or inhibition of flowering, abscission of various organs and senescence are also known to be effected by ethylene. Biosynthesis of ethylene is a highly regulated process and is under both positive and negative feedback control [24]. Ethylene production is induced under various external stress conditions such as wounding, pathogen invasion, heavy metal toxicity and flooding [22,25,26]. Ethylene synthesis is also increased upon treatment with Cd, Cu, Fe, Zn, and in the case of Cd and Cu, this increase is due to an upregulation of ACC synthase transcription and enhanced activity [27].

Here in the present study the objective was to evaluate the toxicity of Cd in the suspension cell culture of tomato and its ability to induce H_2O_2 and ethylene production and the possible involvement of these molecules in the cell death of suspension cell cultures challenged with Cd by application of certain inhibitors.

Materials and Methods

Generation of the suspension cell culture of tomato

Suspension cell culture of tomato (Lycopersicon esculentum cv. CO-3) was generated on MS medium supplemented with 3% sucrose, 1 mgL^{-1} 2,4-D and 0.2 mgL^{-1} BAP from the friable callus obtained from the leaf explants generated on MS medium supplemented with 3% sucrose and 2 mgL^{-1} BAP [28]. The friable callus obtained was suspended in 50 ml of liquid MS basal medium in a 250 ml flask supplemented with appropriate growth regulators and was kept on an orbital shaker at 100 rpm with 14 h light and 10 h dark cycle and temperature was adjusted to 25±2 °C.

For subculture, 8 days old suspension cell cultures were centrifuged and sedimented down to remove the old medium. The sedimented cells were then resuspended in 15 ml of fresh medium and then 5 ml from this was transferred to a 250 ml flask containing fresh and sterile 45 ml medium. All this process of subculturing was carried out under aseptic conditions under a laminar flow hood. After 5 days of subculture the suspension cell cultures were used for experimental purposes.

Exposure of tomato suspension cell culture to Cadmium

Treatment with the heavy metal Cadmium in form of $CdCl_2$ was given to the suspension cell culture of tomato. Stock solution of $CdCl_2$ was prepared by dissolving appropriate amount of $CdCl_2$ in sterile deionized water to obtain a concentration of 50 mM. From this stock solution different concentrations of $CdCl_2$ were prepared for treatment.

Treatment of the suspension cell culture of tomato was carried out 5 days after subculture. Different concentrations of the Cd (50,100,150,200 and 250 µM) were used for treatment. The required amount of the Cd was added to 50 ml of suspension cell culture under aseptic conditions in a laminar flow hood and the suspension cell culture were then placed back on the shaking incubator for growth. The amount of cell death induced by the Cd treatment in the suspension cell culture was measured 24 h after Cd treatment. Suspension cell cultures kept without any Cd treatment was used as control. In case of treatment of the suspension cell cultures with catalase (50 U/ml; H_2O_2 inhibitor) and Aminoethoxy Vinyl Glycine (AVG) (10 µM; ethylene biosynthesis inhibitor) these inhibitors were added 10 min before exposure to Cd.

Cell viability staining

For visualizing the effect of treatment with Cd in the suspension cell culture, the suspension cell cultures were centrifuged at 5000 *g* for 10 min and then the suspension cell cultures were resuspended in 10 ml of 50 mM phosphate buffer (pH 7.5). To 1 ml of this suspension cell culture was added 10 µL of Evans blue stock solution (100 mg Evans blue dissolved in 10 mL sterile distilled water) and was incubated for 15 min at room temperature. Following incubation the suspension cell culture was centrifuged at 2000 *g*. The pellet was washed once with 1 mL of 50 mM phosphate buffer (pH 7.5) to remove any excess Evans blue. The cells were again centrifuged and resuspended in 1 mL of 50 mM phosphate buffer (pH 7.5). Microscopic examination of the cells was carried out under a light microscope. Dead cells stained deep blue where as healthy live cells showed only faint blue coloruation.

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Estimation of cell death

To assess the amount of cell death in the suspension cell cultures treated with Cd, the cells were centrifuged at 2000 *g* for 5 min and the fresh weight was recorded. The cells were then resuspended in 10 mL of 50 mM phosphate buffer (pH 7.5) and 25 µL of Evans blue stock solution was added to it followed by incubation for 15 min at room temperature. After incubation the cells were centrifuged at 2000 *g* for 5 min and the pelleted cells were washed with sterile distilled water three times for 15 min each to remove any excess and unbound dye. After this the pelleted cells were resuspended in 2 ml of 50% methanol containing 1% (w/v) SDS and the dye bound to dead cells was solubilized at 60 °C for 30 min, this solubilization step was carried twice. Following each solubilization the cells were centrifuged and the resulting supernatants were pooled and were diluted with sterile distilled water to a final volume of 7 mL. The absorbance of this solution was recorded at 600 nm against blank containing distilled water. The control and inhibitor treated suspension cell cultures were also processed in the similar way.

Estimation of H_2O_2 production

For measurement of the amount of H_2O_2 in the suspension cell cultures treated with Cd, the suspension cell cultures were harvested by centrifugation at 5000 *g* for 5 min. Then the amount of H_2O_2 in the pellet was determined according to Sagisaka [29]. The treated suspension cells were homogenized in 5% cold Trichloroacetic Acid (TCA) and the homogenate was centrifuged at 17000 *g* for 10 min at 0 °C. The reaction mixture contained 1.6 ml of supernatant, 0.4 ml of 50% TCA, 0.4 ml of 10 mM ferrous ammonium sulphate and 0.2 ml of 2.5 M potassium thiocynate. The absorbance was recorded at 480 nm after 15 min of incubation. The amount of H_2O_2 was estimated by a calibration curve prepared with known concentrations of H_2O_2.

Ethylene production assay

Ethylene production was determined as reported by Harrison [30]. In the suspension cell cultures treated with Cd, the amount of ethylene produced was measured by withdrawing 2 ml air samples from the head space of the flask. Air samples were then analyzed for ethylene content on gas chromatograph equipped with a flame ionization detector.

Statistical analysis

All experiments were done thrice in triplicates for each analysis. Data represented here are mean of a single experiment done in triplicates with ±SE represented as bars. Variance analysis (ANOVA) was performed on experimental data and statistical significance at $p<0.05$ level was judged by the Least Significant Differences (LSD) method. Statistical analysis were performed using statistical program SPSS (SPSS Inc, Chicago, IL, USA).

Results

Cell viability in tomato suspension cell cultures exposed to Cd

Cellular toxicity caused by exposure to Cd was observed by staining with Evans blue. Viable cells were mostly colourless or stained only very faint blue, and exhibited an intact protoplast with dense cytosol (Figure 1A), while a deep blue coloration and protoplast shrinkage were observed in cells challenged with Cd (Figure 1B). The cell death rates were determined using a spectrophotometric assay (Figure 2). A very significant increase in the percentage of dead cells was observed after 24 h of Cd treatment. In the control untreated cells the percentage of dead cells in the population was about 11%. In contrast, the rate of cell death reached 27%, 34%, 42%, 80% and 81% after 24 h of treatment with various concentrations of Cd respectively. Cadmium induced cell death in a concentration dependent manner and based on the results obtained 200 µM of $CdCl_2$ was found and selected for treatment in all further experiments as a very significant amount of cell death (80%) was observed after 24 h of treatment.

H_2O_2 production in suspension cell culture following Cd treatment

A gradual increase in H_2O_2 production was observed in the suspension cell cultures of tomato following exposure to 200 µM Cd (Figure 3). Exposure of the suspension cell culture to Cd stimulated the production of H_2O_2 by 2 h post treatment. During the peak phase of oxidative burst a very pronounced and large amount of H_2O_2 was produced between 4-7 h post treatments (Figure 3). In case of control cells a basal level of H_2O_2 production was observed which was very insignificant quantitatively in comparison to the Cd exposed ones. Pretreatment of suspension cell cultures with H_2O_2 scavenger catalase significantly reduced the amount of H_2O_2 produced in the suspension cell culture and it was very less quantitatively in contrast to the Cd exposed cells (Figure 3).

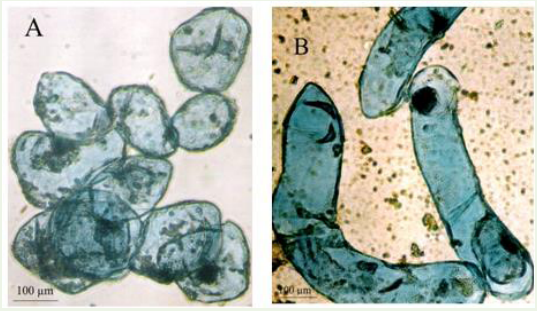

Figure 1: Evans blue staining for cell death in suspension cell culture of tomato after treatment with Cadmium (A) control; (B) Cd treated.

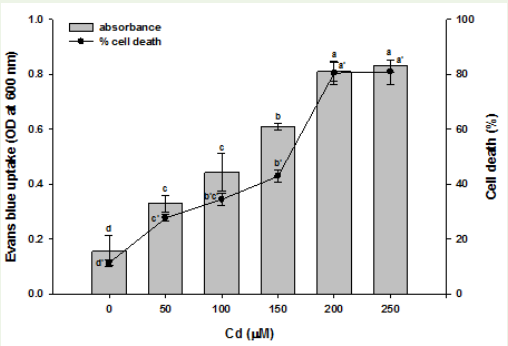

Figure 2: Effect of treatment with various concentrations of Cd on cell death in suspension cell culture of tomato. Bars represent ±SE. Different letters indicate that values are significantly different ($P<0.05$).

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Ethylene production in suspension cell culture following Cd treatment

Ethylene production was measured in the head space volume of the flasks containing the suspension cell cultures of tomato treated with 200 µM Cd, and also in the untreated control suspension cell cultures. Ethylene production was observed to increase sharply on exposure to Cd starting from 2 h post treatment and continued up to 8 h where maximum ethylene production was observed. At 8 h post treatment the amount of ethylene produced was almost 5-6 folds higher than that of control after which ethylene production started declining and a significantly declined production was observed at 14 h post treatment (Figure 4). In case of control cells an extremely low level of ethylene production was observed. Pretreatment of suspension cell culture with ethylene biosynthesis inhibitor AVG significantly reduced the amount of ethylene produced by the suspension cell culture. The amount of ethylene in AVG pretreated suspension cells was quantitatively equivalent to control untreated cells (Figure 4).

Effect of Inhibitors on Cd toxicity induced cell death

After establishing the production of H_2O_2 leading to an oxidative stress in the suspension cell cultures of tomato following exposure to Cd a potent antioxidant enzyme catalase was applied 10 min before the application of Cd in the suspension cell cultures to asses for a possible role of oxidative burst in the cellular toxicity of Cd inducing cell death. The amount of cell death was reduced to a significant level following application with 50 U/ml catalase in the suspension cell culture 24 h post treatment and was very much comparable to control cells as was evidenced by lower amount of Evan's blue uptake (Figure 5).

Similarly it was also observed that exposure of suspension cell cultures to Cd led to ethylene production. To asses for the involvement of ethylene in cellular toxicity of Cd and its possible involvement in the cell death signaling cascade inhibitor of ethylene biosynthesis was tested. Application of 10 µM AVG significantly reduced the amount of cell death induced by Cd exposure 24 h post treatment and the amount of cell death was almost comparable to control where no treatment was given (Figure 5). This inhibition of cell death by application of AVG indicates for possible role of ethylene in the cellular toxicity and cell death cascade induced by Cd in the suspension cell cultures of tomato.

Discussion

The cell morphology of tomato suspension cultures exposed to Cd and stained with Evans blue was screened to assess the toxicity of Cd in the suspension cell population. Dead cells showed typical features of cell death such as cytoplasm shrinkage and nuclear condensation (Figures 1A,1B). These changes in morphological features of dead cells, severe shrinkage of nuclei and cytoplasm have also been observed earlier in tomato suspension cells treated with $CdSO_4$ and other cell death-inducing chemicals [31,32].

Evans blue uptake method was used to monitor cell death induced by Cd in the treated suspension cell cultures. It was very much apparent from the results obtained that Cd induced a severe cell death in the treated suspension cell cultures as was observed by increased uptake of Evan's blue (Figure 2). Cadmium induced cell death in a concentration-dependent manner similar to its effect in a number of other plant as well as animal systems.

Independently of the type of stress influence (biotic or abiotic) the accumulation of ROS is an undeniably established fact. It is well known that water deficit, low temperatures, application of pesticides,

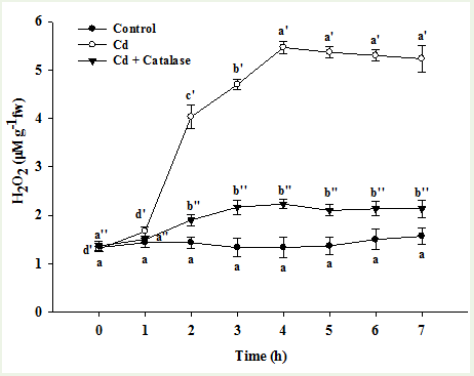

Figure 3: Effect of treatment with Cd and antioxidant catalase on H2O2 production in suspension cell culture of tomato. Bars represent ±SE. Different letters indicate that values are significantly different (P<0.05).

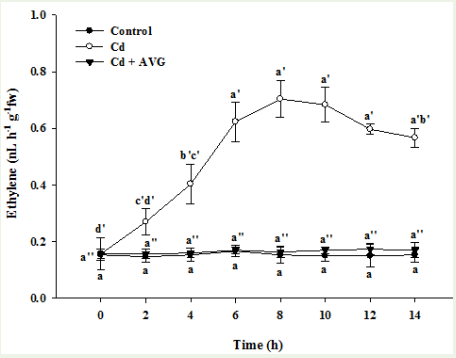

Figure 4: Effect of treatment with Cd and ethylene biosynthesis inhibitor AVG on ethylene production in suspension cell culture of tomato. Bars represent ±SE. Different letters indicate that values are significantly different (P<0.05).

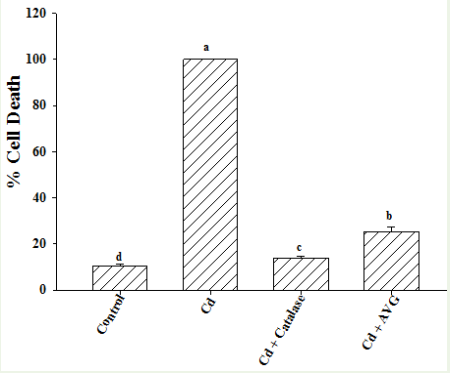

Figure 5: Effect of application of antioxidant catalase and ethylene biosynthesis inhibitor AVG on cell death in suspension cell culture of tomato treated with Cd. Bars represent ±SE. Different letters indicate that values are significantly different (P<0.05).

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noxious gasses, harmful radiations, heavy metals, acid rains, high solar irradiation, pathogen attack etc. all cause an increased production of toxic ROS (O^{2-}, H_2O_2, OH^-, $^1O_2$), which are highly detrimental to all biological systems, i.e. an oxidative stress occurs [33]. Generation of ROS including H_2O_2 production occurs typically during stress response processes associated with plant-stress interactions.

Treatment with Cd produced an enormous oxidative burst in the suspension cell cultures (Figure 3). Cadmium induced H_2O_2 production has also been observed in tobacco BY-2 suspension cell cultures [34]. The increased production of ROS is a key event in Cd toxicity in plants and it mediates by inhibition of antioxidant system [35]. Studies have shown that the ROS production induced by Cd is catalyzed by a NADPH oxidase-like enzyme in the plasma membrane [18,34]. The roles of cell wall peroxidases or polyamine oxidases have also been suggested to contribute to H_2O_2 production [36]. Regardless of the Cd-induced mechanisms of ROS generation, their increased level is always toxic to the system [17,34].

To check whether this oxidative burst was instrumental in cell death cascade induced by the Cd, the antioxidant catalase was applied and then the effect of Cd on H_2O_2 production and subsequent cell death induction was studied. The application of this antioxidant greatly reduced the amount of H_2O_2 produced (Figure 3) and also produced a significant decrease in the amount of cell death induced by Cd (Figure 5). This result suggests that oxidative burst is one of the possible causes of cell death induced by Cd in suspension cell culture of tomato. It has been reported earlier that H_2O_2 scavengers prevented H_2O_2 accumulation and reduced the symptoms of Cd toxicity in pea leaves [37]. Similarly, cell death inhibition by catalase has been reported in animal cells exposed to Cd. Co-incubation of rat glioma cells with catalase and Cd strongly inhibited Cd-induced DNA ladder formation, indicating that H_2O_2 is at least partially involved in apoptotic activity of Cd in animal systems [38]. Application of ascorbic acid and catalase has also been found effective in decreasing the amount of cell death induced by Cd in tomato suspension cell cultures [39]. Production of H_2O_2 was effectively blocked in suspension cells treatment with the antioxidant catalase following exposure to Cd, indicating that the increase in H_2O_2 is causatively related to Cd toxicity and cell death in tomato suspension cell cultures (Figure 5). Our results are in line with the earlier findings where H_2O_2 scavengers prevented H_2O_2 accumulation and reduced the symptoms of Al and Cd toxicity in suspension cell cultures of tomato [32]. Similar effects of Cd on H_2O_2 production and antioxidant treatment on lowering the H_2O_2 production was also observed in *Arabidopsis* leaves [40]. Apart from its role in the induction of cell death H_2O_2 from the oxidative burst is also a central molecule in coordinating the local and systemic defense signaling. Several unique properties of H_2O_2, such as its relative stability, ability to diffuse, and ability to cross membranes, enable it to fulfill this role. H_2O_2 moves from the point of generation forming a concentration gradient, and exhibits different concentration dependent effects. At its threshold concentration H_2O_2 triggers cell death while lower doses lead to defense activation [14].

Ethylene is a simple gaseous hormone that has been reported to play an important role in regulating and modulating plant responses including cell death, to both biotic and abiotic stresses [23,41]. Production of the plant phytohormone ethylene was observed to be stimulated by treatment with Cd in the suspension cell culture of tomato (Figure 4). This observation leads to the conclusion that ethylene production is one of the biochemical responses of tomato suspension cell culture following treatment with Cd.

Production of ethylene has also been reported to be enhanced in tomato suspension cells treated with $CdSO_4$. To assess the possibility for a role of ethylene in the induction of cell death by Cd, the ethylene biosynthesis inhibitor AVG was applied to check the effect of Cd on cell death induction in suspension cell culture of tomato. The applications of AVG lead to a significant reduction in the amount of cell death induced by Cd in the suspension cell culture (Figure 5). This result suggests that ethylene is another major player involved in Cd induced cellular toxicity in suspension cell cultures of tomato.

Cadmium treatment induced a transient increase in ethylene production up to 8 h post treatment (Figure 4). This ethylene apparently plays an important role in Cd toxicity. Suppression of ethylene production with AVG pretreatment reduced the amount of Cd-induced toxicity and cell death in suspension cell culture of tomato (Figure 5). A similar inhibitory effect of AVG was established in Al and Cd induced cell death in tomato suspension cells [32]. Our results are in line with these earlier findings and also show that ethylene production is an important component of heavy metal toxicity in tomato suspension cell cultures.

In a number of other systems like plant - pathogen interactions, following ozone application, treatment of suspension cell culture with fungal toxins and in a diversity of developmental processes, ethylene has been observed to be closely associated with increased cell death [42,43].

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