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Cadmium-induced functional and ultrastructural alterations in roots of two transgenic cotton cultivars

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ABSTRACT

The toxic effect of cadmium (Cd) at increasing concentrations was studied with special attention being given to the root morphological and ultrastructural changes in two transgenic cotton cultivars *viz.* BR001 and GK30 and their wild relative *viz.* Coker 312. In comparison to their respective controls, low concentration (10 and 100 μM) of Cd greatly stimulated seed germination, while it was inhibited by highest concentration of Cd (1000 μM) in case of two transgenic cultivars. However, in Coker 312 the seed germination percentage progressively decreased over the control at all Cd levels. Various physiological and morphological parameters of the root and whole plant in both transgenic cotton cultivars and their relative wild cotton genotype respond differently towards the Cd toxicity. Bioavailability of Cd was concentration-dependent where seedling root captured more Cd as compared to shoot. BR001 accumulated more Cd followed by GK30, while Coker 312 was less Cd accumulator. The ultrastructural modifications in the root tip cells of both the transgenic cotton cultivars and their wild relative were also dose-dependent. With the increase in Cd levels, the fine structures of their root cells also invariably changed. Increase in plasmolysis of the plasma membrane, greater number of nucleoli and vacuoles and enlarged vacuoles could be observed in both transgenic cotton cultivars. In comparison to them, Coker 312 showed relatively well developed ultrastructures of the root tips except enlarged vacuoles and greater number of mitochondria. Moreover, the accumulation of Cd in the form of electron dense granules and crystals both in vacuoles and attached to cell walls were visible in both transgenic cotton cultivars and their wild relative. These results suggest that both transgenic cotton cultivars and their wild relative cotton genotype responded positively towards Cd stress at seedling stage, the internal Cd-detoxification might be through apoplastic and symplastic binding. Moreover, as a whole BR001 proved to be sensitive whereas; GK30 and Coker 312 were found as tolerant.

1. Introduction

Cadmium (Cd) is an extremely significant pollutant due to its high toxicity and large solubility in water. It is wide spread in environment as a result of various anthropogenic activities [1,2] and has been mostly a "guest" metal in Pb:Zn mineralization because it never occurs in isolation in the natural environments [3]. Typically, the non-polluted soil contains Cd in the range of 0.04–0.32 mM, while moderately and highly polluted soils reach up to 0.32–1.00 mM Cd [4,5]. By virtue of its chemical and physical similarity to essential cations such as Fe, Cu, and Zn, Cd uptake in plants can be facilitated by the uptake systems of these cations [6–9]. Although having no known biological function [10], Cd can alter physiological and morphological features of both plants and animals. In plants, anatomic and structural changes are known to be some of the worst effects of Cd [11,12]. Liu et al. [13] and Shah and Dubey [14] found the occurrence of low mitotic index and pycnosis, cell division and cell proliferation, chromosomal aberrations, alteration in the synthesis of RNA and slowing down of ribonuclease activity in various crops. Moreover, an increase in the number of nucleoli and vacuoles, condensation of cytoplasm, reduction of mitochondrial cristae, severe plasmolysis, highly condensed chromatin materials, enlargement of vacuoles, disorganization of chloroplast structure, and disruption of nuclear envelope, plas-

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malemma and mitochondrial membranes at the ultrastructural levels of roots and leaves [15–18] can be attributed to phytotoxic effects of Cd. Among plant species and even genotypes of a given species, there is a great genotypic variation in Cd tolerance [19,20]. The reason for such a large genotypic variation is still not well understood. However, it is now well known that how plants have developed the tolerance mechanisms to reduce the Cd^{2+} influx at the cellular level. Briefly, restricted Cd influx through plasma membrane, exclusion of Cd (active pumping of Cd out of the cell), compartmentalization of Cd at the cellular level, detoxification of Cd-binding peptides or proteins and accumulation due to plant chelators [5,20–22]) are some of the tolerance strategies that have been developed by plants against Cd toxicity.

In present study, we considered the possible influence of Cd on root physiology and ultramorphology of two transgenic cotton cultivars *viz*. BR001 (herbicide resistant) and GK30 (insect resistant) in comparison to their wild relative cotton genotype *viz*. Coker 312.

Our main objectives were to establish an overall picture of Cd toxicity syndrome at different possible sites of the root ultrastructure and to find out its effect on morphology and physiology of root. Moreover, the potential of two transgenic cotton cultivars and their wild relative cotton genotype regarding Cd accumulations has been explored.

2. Materials and methods

2.1. Seed surface sterilization and treatment process

Mature seeds of two transgenic cotton cultivars, namely BR001 and GK30, and their wild relative genotype (Coker 312), were first immersed in 70% ethanol for 3 min and then in 0.1% $HgCl_2$ for 8–10 min. The seeds were then washed first with ddH_2O for three times and finally with distilled water. Subsequently, they were directly treated with tested solution for approximately 2 h. The treated seeds were spread over sterilized petri dishes (90 mm) lined with double-layered filter papers. In each petri plates, 10 seeds were placed. The tested solution was comprised of four treatments of Cd including 0, 10, 100, and 1000 μM. There were three replications per treatment, which were arranged in a completely random manner. During the first 3 days, 5 ml of the tested solution was applied to each petri plates. On day 4, the germinated seedlings were transferred to another set of sterilized petri dishes with double-layered filter papers and 10–12 ml tested solution was applied to each petri dish.

Three independent parallel experiments for germination assay and hypocotyl and radical lengths, root morphological traits and determination of Cd and TEM studies were run. The petri plates were sealed with parafilm tape and placed in dark for 48 h followed by 4-day exposure to a 16 h photoperiod of 50 $\mu mol\, m^{-2}\, s^{-1}$ under white fluorescent light with $28 \pm 2\,^{\circ}C$ culture temperature. Cadmium as $CdCl_2{\cdot}2.5H_2O$ of analytical grade was used. Control was provided with distilled water without Cd.

2.2. Seed germination assay and measurement of hypocotyl and radical lengths

Germination test and measurement of hypocotyl and radical lengths were performed after 24 h in a separate set of experiment. Five seeds per plate from each replication per treatment were randomly selected first for seed germination assay and then for measurements of hypocotyl and radical lengths. A 2 mm radical emergence from seed was considered as germinated seed. At the end of the experiment, five seedlings per replication for each treatment were used to measure the hypocotyl and radical lengths.

2.3. Plant growth parameters and tolerance index

A number of plant growth parameters, namely, root-shoot lengths, root fresh and dry weights, root volume, root surface area and diameter, and root tip percentage were determined in another set of experiment. Root automatism scan apparatus (MINMAC, STD1600^{+}), equipped with Win RHIZO software offered by Regent Instruments Company was used to measure root volume, root surface area and root average diameter. Total six plants per treatment, two from each replication, were used and their average values were taken as one replication. To determine the root tip percentage, only primary roots were taken into account. Percent values of 10 plants from each replication were averaged and considered as one replication. Tolerance Indices (TI) of root length and plant height against each concentration were calculated following Wilkins [23] and Baker et al. [24].

$$\mathrm{TI}(\%) = \frac{\text{mean length in metal solution}}{\text{mean length for the control}} \times 100$$

2.4. Determination of Cd content

To determine the bioavailability of Cd in different parts of the seedlings, the treated seedlings were thoroughly washed with distilled water and then with 20 mM Na_2–EDTA for about 15 min in order to remove excess Cd adhering to the root surface. After three times washing with distilled water, the plants were finally washed with ddH_2O. For quantification of Cd, the seedlings were separated into roots and shoots, and dried at 70 °C for 48 h. The samples were ground to fine powder and wet digested in a 5 ml mixture of strong HNO_3:$HClO_4$ (2:1, v/v). After heating the mixture at 80 °C on water bath for about two hours, Cd was quantified using an atomic absorption spectrometry (PE-100, PerkinElmer).

2.5. Transmission electron microscopy

Root tips (~2–3 mm in length) of randomly selected plants were fixed overnight in 4% glutaraldehyde (v/v) in 0.1 M PBS (Sodium Phosphate Buffer, pH 7.4) and washed three times with same PBS. The samples were post fixed in 1% OsO_4 (osmium (VIII) oxide) for 1 h, then washed three times in 0.1 M PBS (pH 7.4) with ten minutes interval between each washing. After that, they were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) with 15–20 min interval, and finally by absolute acetone for 20 min. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating the specimens at 70 °C for 9 h, the ultrathin sections (80 nm) were prepared and mounted on copper grids for viewing in the transmission electron microscope (JEOL TEM-1230EX) at an accelerating voltage of 60.0 kV.

2.6. Statistical analysis

One-way ANOVA was performed by using SAS v.9 software for statistical significance at $P < 0.05$. All the results were expressed as mean ± SE for three replications. Means were separated by least significant difference (LSD) test at 5% level of significance.

3. Results

3.1. Germination assay and radical and hypocotyl lengths

As a preliminary experiment, seeds of transgenic cotton cultivars (BR001, GK30) and their distant wild relative cotton genotype (Coker 312) were exposed to different concentrations of cadmium