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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

Table 1
Seed germination, radical and hypocotyl lengths of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments

Cultivars	Cd treatments (μM)	Germination (%)	Radical and hypocotyl lengths (cm plant^{-1})			
			After 24 h		After 6th day	
			Radical	Hypocotyl	Radical	Hypocotyl
BR001	0	85.00 ± 1.15b	0.32 ± 0.02b	0.15 ± 0.02b	2.42 ± 0.03a	3.47 ± 0.12a
	10	91.53 ± 1.07a	0.40 ± 0.02a	0.17 ± 0.02ab	1.99 ± 0.07b	2.97 ± 0.12b
	100	92.67 ± 0.88a	0.42 ± 0.01a	0.23 ± 0.02a	1.20 ± 0.15c	2.52 ± 0.02c
	1000	79.61 ± 1.29c	0.20 ± 0.01c	0.17 ± 0.02ab	0.73 ± 0.03d	1.83 ± 0.02d
GK30	0	93.07 ± 1.55bc	0.43 ± 0.01d	0.19 ± 0.01d	2.99 ± 0.05a	3.67 ± 0.28a
	10	96.58 ± 0.47ab	0.57 ± 0.01c	0.24 ± 0.01c	2.85 ± 0.04b	3.06 ± 0.05b
	100	98.37 ± 0.86a	0.81 ± 0.01a	0.33 ± 0.01a	2.43 ± 0.04c	2.74 ± 0.02b
	1000	89.67 ± 1.60c	0.72 ± 0.01b	0.29 ± 0.01b	1.61 ± 0.03d	2.08 ± 0.05c
Coker 312	0	88.67 ± 3.18a	0.50 ± 0.02a	0.31 ± 0.01b	1.69 ± 0.04a	2.76 ± 0.11a
	10	81.00 ± 1.73b	0.41 ± 0.01bc	0.25 ± 0.02bc	1.58 ± 0.02b	2.69 ± 0.02a
	100	84.33 ± 1.45ab	0.45 ± 0.02ab	0.38 ± 0.02a	1.33 ± 0.03c	2.31 ± 0.02b
	1000	78.79 ± 0.90b	0.35 ± 0.02c	0.23 ± 0.02c	1.18 ± 0.01d	1.98 ± 0.02c

Values are the means of three replications ± SE. Variants possessing the same letters (a, b, c, d) are not statistically significant at $P < 0.05$.

in order to assess the adverse effects of cadmium (Cd) on seed germination and radical emergence. Table 1 shows the means ± SE of seed germination percentage and relative lengths of radical and hypocotyl after 24 h and at the time of termination of experiment. It reveals that germination rate in both transgenic cultivars had an upward trend at 10 and 100 μM Cd levels over their respective controls; while 1000 μM Cd concentration showed inhibitory influence on seed germination as compared to their relevant controls. However, their wild relative cotton genotype showed quite different response towards Cd stress regarding seed germination. The germination percentage at 0 μM was significantly higher ($P < 0.05$) as compared to 10 and 1000 μM Cd levels, while non-significant difference ($P > 0.05$) was observed between 0 and 100 μM Cd concentrations.

Moreover, after 24 h the radical emergence in BR001 was dramatically increased at 0–100 μM but decline was observed at 1000 μM concentration as compared to control. However, in GK30, radical length was stimulated over control at 10 and 100 μM, and at 1000 μM its mean length was decreased over 100 μM but still it was higher as compared to control and 10 μM Cd level. As compared to transgenic cultivars, the mean radical length of Coker 312 at 0 μM Cd concentration was significantly higher over other Cd levels (10, 100, and 1000 μM). Also, after 24 h hypocotyls in both the transgenic cotton cultivars grew faster at all Cd levels in comparison to their respective controls. Regarding hypocotyl lengths of BR001, non-significant differences ($P > 0.05$) were observed between control and other treatments except at 100 μM Cd concentration; while in GK30, significant differences were observed at all Cd levels. In comparison to transgenic cotton cultivars, the mean values of hypocotyl length in Coker 312 were greater at all Cd levels except at 1000 μM concentration at which its mean length was shorter than GK30.

Table 1 further depicts the mean radical and hypocotyl lengths for both the transgenic cotton cultivars and their wild relative cotton genotype after a 6-day treatment of Cd concentrations. After 6th day treatment, for cultivar BR001 both the radical and hypocotyl lengths at various Cd concentrations were significantly different ($P < 0.05$) as compared to control and they showed decreasing trends towards increasing Cd concentration. The same decreasing pattern and significant differences at various Cd concentrations in the mean radical and hypocotyl lengths was also recorded for transgenic cultivar GK30 and their wild relative genotype Coker 312, although the mean hypocotyl length in Coker 312 was not significantly different between 0 and 10 μM Cd concentration. In comparison to BR001 and GK30, Coker 312 showed decreased radical lengths at all the Cd levels except at 1000 μM where it was greater than BR001. Moreover, the mean hypocotyl lengths of Coker 312 were smaller as compared to BR001 and GK30 at varying Cd concentration except at 1000 μM where it showed comparatively greater hypocotyl length than BR001.

3.2. Biomass production, tolerance indices and root tip percentage

The effects of different Cd concentrations on root fresh weight, root dry weight, root tolerance index, plant height tolerance index, and root tip percentage of two transgenic cotton cultivars and their distant wild relative are listed in Table 2. It was observed that different Cd concentrations posed inhibitory effects on all the studied parameters of cultivar BR001 and were significantly different ($P < 0.05$) as compared to their respective controls. It revealed that inhibition in all these parameters of BR001 was started at 10 μM of Cd level. The same inhibitory effects of Cd treatments were also recorded for cultivar GK30. However, its root fresh weight, root length's tolerance index and root tip percentage at 10 μM of Cd were not significantly different ($P > 0.05$) with respect to their controls, while others showed significant differences at 5% level of probability.

Their wild relative genotype (Coker 312) of these transgenic cotton cultivars showed dramatic response to various Cd levels (Table 2). The root fresh weight at 1000 μM was significantly ($P < 0.05$) different over control but showed no significant ($P > 0.05$) differences at 0, 10, and 100 μM of Cd concentrations. A significant inhibition in the root dry weight and root tip percentage for the elevated levels of Cd was started at 100 μM over the control. However, root length (TI) significantly reduced over control at 10, 100, and 1000 μM of Cd treatments and plant height (TI) obviously reduced over control at 100 and 1000 μM of Cd levels.

3.3. Root morphological traits

The average measurements of root morphological traits for BR001, GK30 and Coker 312 are listed in Table 3. It reveals that root surface area, volume and average diameter of both the transgenic cotton cultivars were significantly influenced by various Cd levels as compared to their relevant controls. In cultivar BR001, a significant decrease ($P < 0.05$) up to 100 μM Cd level over their respective controls was observed in the root volume and average diameter, while at 1000 μM Cd level, the surface area, volume and average diame-

Table 2
Biomass production, tolerance indices and root tip percentage of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments

Cultivars	Cd treatments (μM)	Root biomass (g plant^{-1})		Tolerance index (%)		Root tip (%)
		Fresh weight	Dry weight	Root length	Plant height	
BR001	0	0.03 ± 0.002a	0.02 ± 0.001a	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a
	10	0.02 ± 0.002b	0.02 ± 0.001b	82.51 ± 2.99b	79.96 ± 2.72b	70 ± 5.77b
	100	0.01 ± 0.000c	0.01 ± 0.001c	49.45 ± 2.00c	59.83 ± 1.03c	50 ± 0.00c
	1000	0.01 ± 0.001d	0.01 ± 0.001d	30.17 ± 2.33d	41.30 ± 3.23d	20 ± 5.77d
GK30	0	0.03 ± 0.002a	0.03 ± 0.001a	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a
	10	0.03 ± 0.002ab	0.02 ± 0.002b	94.99 ± 1.46a	92.50 ± 1.76b	90 ± 5.77ab
	100	0.02 ± 0.003b	0.02 ± 0.002c	81.09 ± 2.58b	84.71 ± 3.40c	60 ± 5.77c
	1000	0.02 ± 0.003c	0.01 ± 0.001d	53.73 ± 2.99c	49.83 ± 2.05d	70 ± 11.55bc
Coker 312	0	0.02 ± 0.001a	0.02 ± 0.002a	100 ± 0.00a	100 ± 0.00a	100 ± 0.00a
	10	0.02 ± 0.002a	0.02 ± 0.001ab	91.96 ± 1.47b	96.13 ± 1.27a	92 ± 1.53ab
	100	0.02 ± 0.001ab	0.01 ± 0.001b	77.49 ± 2.12c	81.73 ± 2.10b	77 ± 8.89bc
	1000	0.01 ± 0.001b	0.01 ± 0.001c	68.63 ± 2.63d	64.19 ± 6.93c	62 ± 4.36c

Values are the means of three replications ± SE. Variants possessing the same letters (a, b, c, d) are not significantly different at $P < 0.05$.

ter of roots significantly increased as compared to control. The root surface area in cultivar BR001 at 10 and 100 μM Cd concentrations was significantly different from control but were non-significant from each other. However, root volume and average root diameter of cultivar BR001 plants showed significant differences over the control and varying Cd levels. Regarding root volume, significantly evident differences at all Cd levels and among themselves were noticed in BR001. But in case of average root diameter, significantly noticeable differences at all Cd levels compared with control but non-significant differences between 10 and 100 μM, and 10 and 1000 μM were found.

Moreover, in cultivar GK30, root morphological features such as root surface area and root volume significantly decreased ($P < 0.05$) at 10 μM over controls and then increase at 100 μM, 1000 μM Cd levels over their relevant 10 μM Cd concentration was observed. Also in case of root volume, significant differences were recorded over the control and among all Cd treatments. But in case of root surface area as compared with control the response was significant among various Cd stress levels but were non-significant between 10 and 100 μM. Furthermore, the average diameter of cultivar GK30 significantly affected at all Cd levels over control but non-significant variable responses were noticed between 10 and 1000 μM, and 100 and 1000 μM Cd concentrations.

In case of non-transgenic cotton genotype (Coker 312), over their relevant controls the mean values of root surface area, volume and average diameter were non-significantly decreased at 10 μM and then subsequently increased at 100 μM. However, the trend was not the same regarding the above mentioned root parameters at 1000 μM. The mean values of root surface area and average diameter were decreased but significant increase ($P < 0.05$) over the control was detected in the mean value of root volume at 1000 μM Cd concentration.

3.4. Bioaccumulation of cadmium in roots and shoots of plants

The present investigation revealed that Cd uptake in plant tissues was concentration-dependent. According to Table 4, roots accumulated Cd in more quantity as compared to aerial part of the seedlings in case of both transgenic cotton cultivars (BR001, GK 30) and their wild relative cotton genotype (Coker 312). Moreover, BR001 captivated Cd in more quantity as compared to GK30 and Coker 312 and variations were quite significant ($P < 0.05$) at all Cd treatments.

3.5. Ultrastructural observations and features

The ultrastructural alterations in the root tip cells of both transgenic cotton cultivars *viz*. BR001 and GK30 and their wild-relative *viz*. Coker 312 were concentration-dependent (Figs. 1–4). The damage to the root tip cells became severe with increasing concentration of Cd as compared to control.

Table 3
Root characteristics of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments

Cultivars	Cd treatments (μM)	Surface area (cm^2 plant^{-1})	Volume (cm^3 plant^{-1})	Ave. diameter (mm plant^{-1})
BR001	0	1.68 ± 0.13a	1.68 ± 0.04a	3.59 ± 0.13a
	10	1.38 ± 0.02b	1.20 ± 0.06c	2.58 ± 0.11bc
	100	1.46 ± 0.05b	1.01 ± 0.07d	2.21 ± 0.03c
	1000	1.53 ± 0.03ab	1.38 ± 0.04b	2.88 ± 0.38b
GK30	0	1.97 ± 0.05a	2.21 ± 0.06a	4.31 ± 0.07a
	10	1.55 ± 0.06c	1.54 ± 0.05d	3.80 ± 0.09b
	100	1.63 ± 0.05c	1.74 ± 0.06c	3.36 ± 0.23c
	1000	1.78 ± 0.03b	1.91 ± 0.04b	3.66 ± 0.09bc
Coker 312	0	0.64 ± 0.05a	1.50 ± 0.07b	1.36 ± 0.06a
	10	0.57 ± 0.06a	1.49 ± 0.09b	1.23 ± 0.15a
	100	0.78 ± 0.15a	1.75 ± 0.09ab	1.41 ± 0.28a
	1000	0.49 ± 0.07a	1.85 ± 0.09a	0.86 ± 0.13a

Values are means ± SE of three replications. Variants possessing the same letter (a, b, c, d) are not significantly different at $P < 0.05$ as determined by LSD test.

Table 4
Cd uptake by different plant parts of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments

Cultivars	Cd treatments (μM)	Roots (mg kg^{-1} dw)	Shoots (mg kg^{-1} dw)
BR001	0	0.83 ± 0.03d	0.09 ± 0.002d
	10	149.57 ± 5.99c	76.66 ± 2.23c
	100	280.46 ± 2.07b	145.74 ± 1.06b
	1000	429.69 ± 6.29a	235.36 ± 2.42a
GK30	0	0.43 ± 0.02d	0.032 ± 0.002d
	10	108.95 ± 2.82c	54.50 ± 3.45c
	100	162.94 ± 2.28b	93.14 ± 3.77b
	1000	302.89 ± 5.74a	182.03 ± 6.42a
Coker 312	0	0.39 ± 0.01d	0.02 ± 0.002d
	10	100.37 ± 1.90c	36.39 ± 1.92c
	100	153.54 ± 3.12b	68.58 ± 2.92b
	1000	267.39 ± 3.84a	157.77 ± 1.36a

Values are the means ± SE of three replications. Means followed by different letters (a, b, c, d) indicate significant differences among the treatments as determined by LSD test at 5% level of significance.

In controls of all the treated cultivars, root tip cells were having typical ultrastructures (Fig. 1[A–E]). Dense cytoplasm, flattened plasma membrane, centrally located well-shaped nucleus with one or two nucleoli and chromatin materials, sparsely distributed small vacuole, round-shaped mitochondria were some of the features, which were prominent in the electron micrographs. Moreover, cell walls were clean and intracellular spaces were almost absent.

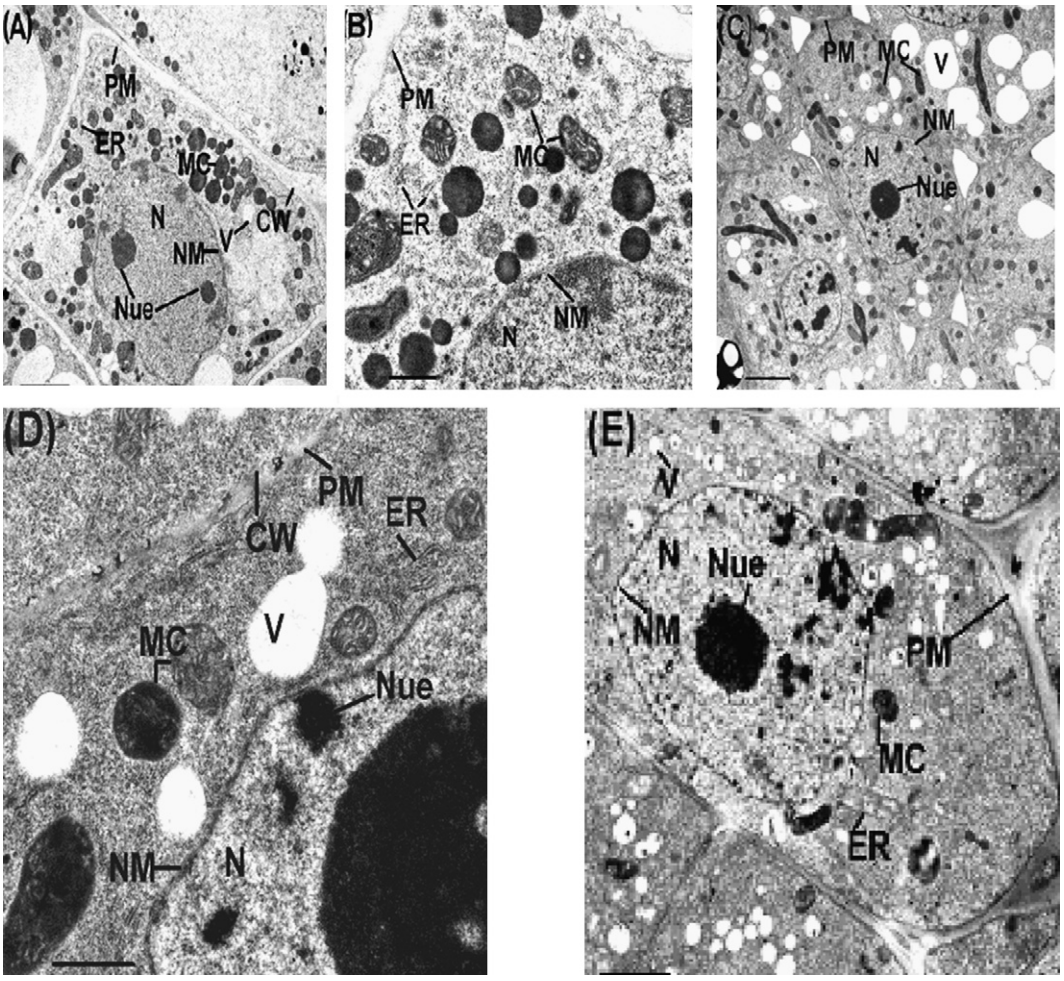

Fig. 1. Electron micrographs of root tip cells of 6-day-old germinating seeds of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days at 0 μM Cd concentration. (A and B) TEM micrographs of control root tip cells of BR001 respectively at low and high magnifications showing well-developed nucleus (N) with two nucleoli (Nue) and a distinct nuclear membrane, a number of vacuoles (V), mitochondria (MC), endoplasmic reticulum (ER), plasma membrane (PM) and cell wall (CW). (C and D) TEM micrographs of control root tip cells of GK30 respectively at low and high magnifications showing a prominent nucleus (N) with one nucleolus (Nue) and a distinct nuclear membrane (NM), a number of vacuoles (V), mitochondria (MC), endoplasmic reticulum (ER), plasma membrane (PM) and cell wall (CW). (E) TEM micrograph of the control root tip cell of Coker 312 showing a complete cell with centrally located nucleus (N) possessing one nucleoli (Nue), a number of small vacuoles (V), mitochondria (MC). Bars A = 2 μm; B = 1 μm; C = 5 μm; D = 1 μm; E = 2 μm.

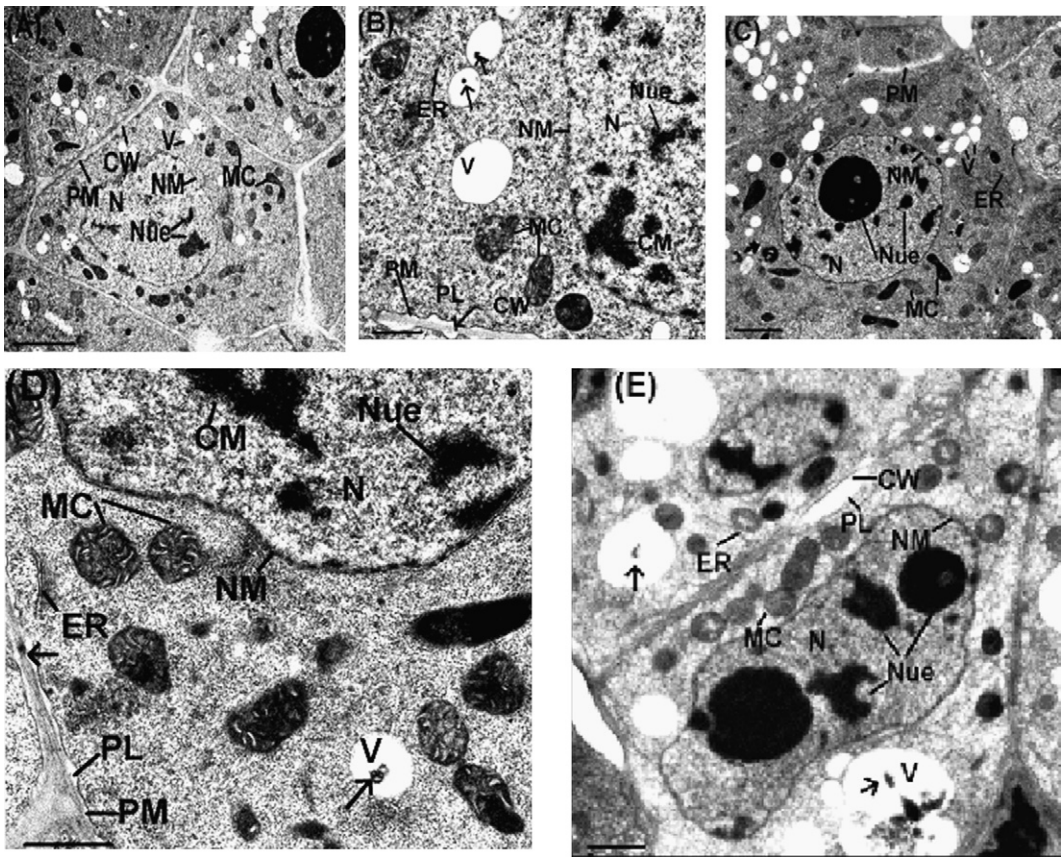

Fig. 2. Electron micrographs of root tip cells of 6-day-old germinating seeds of two transgenic cotton cultivars (BR001, GK30) and their wild relative (Coker 312) grown for 6 days under various Cd treatments at 10 μM Cd concentration. (A) TEM micrographs of root tip cells of BR001 at low magnification showing irregularly shaped nucleus (N) with a number of nucleoli (Nue) and condensed chromatin materials (CM) along with prominent nuclear membrane (NM). (B) TEM micrographs of the root tips of BR001 at high magnification showing increased vacuolation (V), plasmolysis (PL) along with Cd deposition (→) in the form of electron dense granules in vacuoles and attached to the cell wall (CW). (C) TEM micrographs of root tip cells of GK30 at low magnification showing nucleus (N) with almost regular shape and a number of nucleoli (Nue), condensed chromatin materials (CM) enveloped by a prominent nuclear membrane (NM). (D) TEM micrographs of the root tips of GK30 at high magnification showing well-developed nuclear membrane (NM), plasmolysed (PL) plasma membrane (PM) along with Cd deposition (→) in the form of electron dense granules inside vacuoles (V) and attached to the cell wall (CW). (E) TEM micrograph of root tip cells of Coker 312 at high magnification showing well developed centrally located nucleus (N) with a number of nucleoli (Nue) and well-structured nuclear membrane (NM).Also the size of vacuoles (V) and number of mitochondria has increased along with Cd-deposition (→) inside the vacuoles. Bars A = 5 μm; B = 1 μm; C = 2 μm; D = 1 μm; E = 2 μm.

The ultramorphological changes began to appear at 10 μM Cd concentrations with obvious differences in all the treated cultivars (Fig. 2(A–E)). Foremost and visible alterations like increase in number of nucleoli and vacuoles could be observed in root tip cells of both the transgenic cultivars. However, both cultivars greatly differed in the number of nucleoli, shape of nucleus, plasmolysis of cytoplasm, density of cytoplasm and nucleus, and number and size of vacuoles. As compared to GK30, the number of nucleoli was less, shape of nucleus was irregular, plasmolysis was severe, cytoplasm and nucleus was less dense and the size of vacuoles was bigger in BR001. Moreover, Cd in the form of electron dense granules accumulated more in BR001 as compared to GK30, which could be observed in the vacuoles and attached to the cell wall. In comparison to ultramorphological features of these transgenic cotton cultivars, the electron micrographs of their wild relative cotton genotype (Coker 312) showed an increase in number of mitochondria and nucleoli and vacuoles became enlarged in size. Moreover, the presence of electron dense globules inside the vacuoles could also be seen (Fig. 2(E)).

At 100 μM Cd level, damage to the root tip cells of all three cotton cultivars was more extensive as compared to 10 μM (Fig. 3(A–E)). Increase in number of nucleoli and vacuoles, shrinkage of cytoplasm, irregularity in structure of nucleus and more dense cytoplasm and nucleus were some of the features, which could be observed more evidently in BR001 as compared with GK30. Moreover, in BR001 vacuoles were larger in size and Cd accumulated more in the form of crystals and electron dense granules in vacuoles and attached to cell walls as compared to GK30.

The ultramorphological modifications in Coker 312 were more pronounced at 100 μM Cd level as compared to other two low Cd levels. Briefly, the size of the vacuoles became bigger and bigger and number of mitochondria increased. Moreover, plasmolysis of the plasma membrane was evident and the damage to the mitochondria was more severe as compared to other lower Cd levels. Cd deposition in the form of electron dense granules and crystals could be seen in side the vacuoles and mitochondria. As a whole less alterations to the cellular structures in Coker 312 happened as compared to other two transgenic cotton cultivars (BR001, GK30).

The highest Cd concentration (1000 μM) in our present experiment severely damaged the cellular structures of the root tip cells over the other low levels of Cd (10 and 100 μM) in all the treated cotton cultivars (Fig. 4(A–G)), however, root tip cells of BR001 were comparatively badly damaged. Detrimental plasmolytic shrinkage, increase in vacuolar size to the point of damage, shortening of the size of nucleus and rupturing of nuclear envelop were some of the obvious variations, which were more evident in BR001 than GK30.

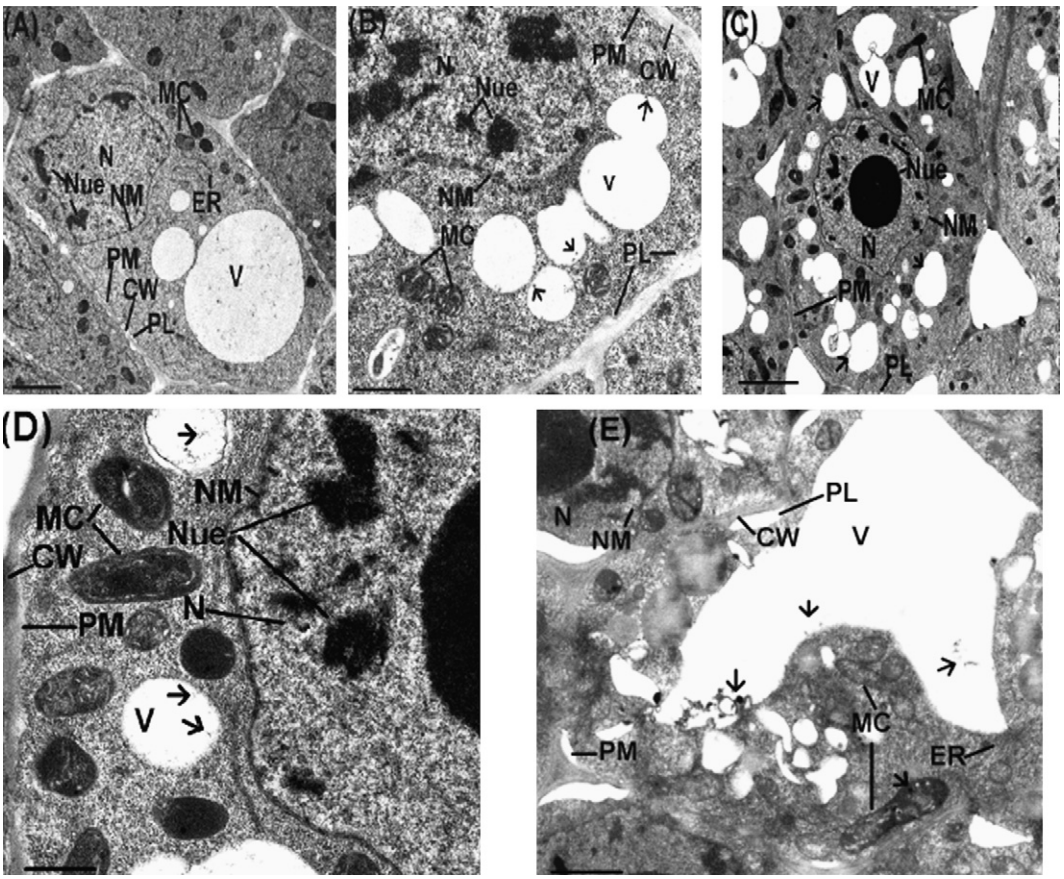

Fig. 3. Electron micrographs of root tip cells of 6-day old germinating seeds of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments at 100 μM Cd concentration. (A) TEM micrographs of root tip cells of BR001 at low magnification showing irregularly shaped nucleus (N) with a number of nucleoli (Nue). (B) High magnification of TEM micrographs of root tips of BR001 showing prominent plasmolytic (PL) shrinkage of the plasma membrane (PM) along with the increase in number and size of vacuoles (V). Moreover, Arrow (→) indicates increased accumulation of Cd in the form of electron dense granules inside vacuoles. (C) TEM micrographs of root tip cells of GK30 at low magnification showing less irregularly shaped nucleus and greater number of nucleoli. (D) TEM micrographs of root tip cells of GK30 at high magnification showing less severe plasmolytic (PL) shrinkage of the plasma membrane (PM) and small-sized vacuoles (V) as compared to root tip cells of BR001. Arrow (→) indicates Cd accumulation in the form of electron dense granules inside vacuoles. (E) TEM micrograph of root tip cells of Coker 312 at high magnification showing an enlarged vacuole (V), a number of mitochondria (MC), less plasmolysed (PL) plasma membrane (PM) and well-developed cell wall (CW). Cd deposition (→) in the form of electron dense granules inside vacuoles (V) and attached to the mitochondria can be seen in the micrograph. Bars A = 2 μm; B = 1 μm; C = 5 μm; D = 1 μm; E = 2 μm.

Moreover, the number of nucleoli increased, and cell wall structure was badly damaged in BR001 as compared to GK30. Also, Cd sequestration was found in the form of crystal and electron dense granules in both vacuoles and attached to cell wall in both cultivars. Furthermore, these electron micrographs showed that BR001 sequestered more Cd as compared to GK30.

In comparison to these transgenic cotton cultivars, their wild relative cotton genotype (Coker 312), kept the integrity of plasma membrane with cell wall along with some prominent ultrastructural modifications like increase in the size of vacuoles to the highest level, shortening of nucleus and damage to the mitochondria during the course of Cd treatment. However, the ultramorphological changes occurred in root tip cells of Coker 312 were less pronounced as compared to other two transgenic cotton cultivars (BR001, GK30)

4. Discussion

Cd toxicity is a well-established phenomenon in most of the living organisms. A thorough knowledge of the toxic effects of this element in plants may contribute to the general understanding of the primary toxicity mechanism and of the tolerance trend in living organisms [25]. In present investigation, we studied the noticed effects of elevated levels of Cd on seed germination, radical and hypocotyl lengths, root fresh and dry weights, root tip percentage and morphological and ultrastructural features of roots on 6-day-old seedlings of two transgenic cotton cultivars. To the best of our knowledge, this is the first research study about Cd toxicity syndrome on root morphology and its ultrastructure in transgenic cotton cultivars and their wild relative.

4.1. Effect of Cd treatments on plant's qualitative and quantitative parameters

Plants exposed to various stresses may experience an inhibitory effect at various stages of growth. Metal tolerant and metal sensitive plants can be distinguished by their growth performance on metal contaminated substrates [26]. Although, the adverse influence of heavy metals particularly that of Cd on growth of seedlings has been reported for several plant species [27,28], the variability index of different species and genotypes in their sensitivity to cadmium at germination stage is not fully available.

In order to determine heavy metals toxic effects on plants, germination assay is a basic procedure [29–31]. In the present study, regarding seed germination dramatic response of both transgenic cultivars and their wild relative could be witnessed. Both trans-

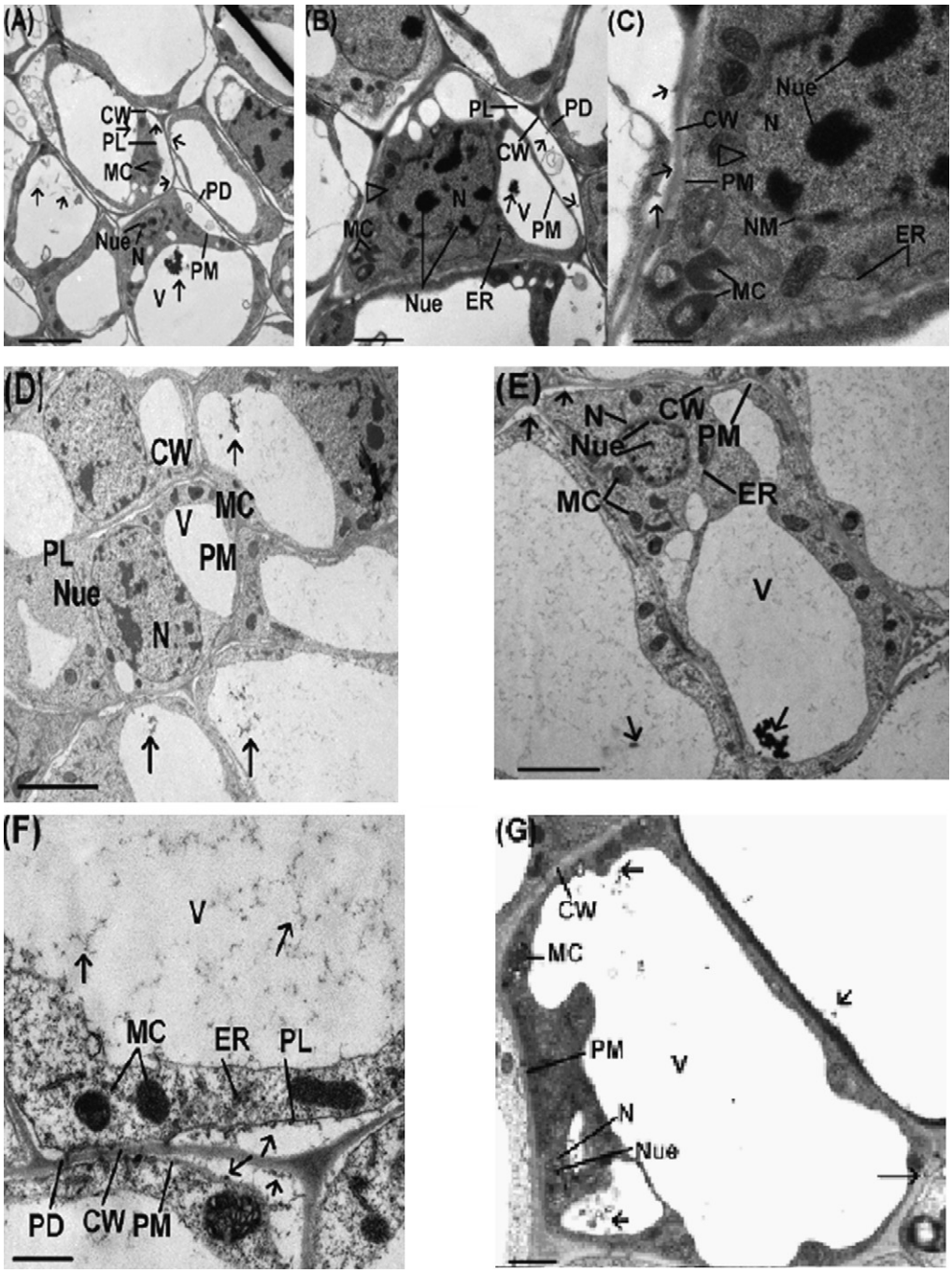

Fig. 4. Electron micrographs of root tip cells of 6-day-old germinating seeds of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days under various Cd treatments at 1000 μM Cd concentration. (A) TEM micrographs of root tip cells of BR001 at low magnification showing small-sized and irregularly shaped nucleus (N) along with detrimental plasmolytic (PL) shrinkage and enlarged vacuoles (V). Cd deposition (→) in the form of crystals inside the vacuoles is obvious. (B) High magnification of TEM micrographs of the root tip cells of BR001 showing increase in number of nucleoli (Nue) and rupturing (▽) of nuclear membrane (NM). Also plasmolysis (PL) of the plasma membrane (PM) and Cd deposition (→) in the form of electron dense granules and crystals inside vacuoles and attached to the cell wall (CW) are apparent. (C) The highest magnification of TEM micrographs of the root tip cells of BR001 showing the rupturing (▽) of nuclear membrane and Cd deposition (→) in the form of electron dense granules attached to the cell wall. (D) TEM micrographs of the root tip cells of GK30 at low magnification showing less irregularly shaped nucleus (N) with a small sized nucleus and a number of nucleoli (Nue). Moreover, plasmolysis (PL) is less severe and Cd deposition (→) in vacuoles and attached to the cell wall (CW) can be observed. (E) TEM micrographs of the root tip cells of GK30 at high magnification showing increased vacuolation (V) and Cd deposition (→) in the form of electron dense granules and attached to the cell wall. (F) The highest magnification of TEM of the root tip cells showing severe plasmolytic shrinkage (PL) and greater deposition of Cd in the form of electron dense granules attached to the cell wall (CW) and present in vacuoles. (G) TEM micrograph of the root tip cell of Coker 312 at high resolution showing centrally located an enlarged vacuole (V), a small-sized nucleus (N) with three small nucleoli (Nue). Moreover, plasma membrane (PM) and cell wall (CW) are well shaped and Cd deposition (→) could be seen inside the vacuoles(V) and attached to the cell wall. Bars A = 5 μm; B = 2 μm; C = 1 μm; D = 5 μm; E = 2 μm; F = 1 μm G = 2 μm.

genic cultivars and their wild relative did not respond in the same manner. Lower Cd concentrations (10 and 100 μM) had stimulatory effects whereas, the highest concentration of cadmium (1000 μM) badly affected the seed germination in both transgenic cultivars. Chugh and Sawhney [32] also observed similar influence of cadmium toxicity on germinating seeds of pea. Not only Cd but also low levels of other heavy metals like Cu and Pb have stimulating effect on seed germination [33–36]. However, in case of their wild relative, although 100 μM of Cd slightly enhanced the seed germination percentage but as a whole Cd did not positively influence the seed germination percentage as compared to control.

The cadmium effect on the whole plant, particularly on radical and hypocotyl was also highly significant. The radical and hypocotyl measurements after 24 h reveal that radical grew faster than hypocotyl with the exceeding levels of Cd over their respective controls in case of both the transgenic cultivars. A similar trend was also observed after 6-day exposure to cadmium stress. However, Coker 312 responded in a similar manner like that of both transgenic cultivars in case of radical length at the time of termination of experiment. Taking into account of the overall Cd toxic effect on both the transgenic cotton cultivars and their wild relative, it is noteworthy that cultivar BR001 was badly affected as compared to GK30 and Coker 312. The sensitivity of early growth seedlings has also been previously reported in cotton [37], barley [38], wheat [39], pea [40], *Phaseolus vulgaris* [41] and *Nicotiana* [42]. One possible reason of longer radical as compared to hypocotyl in the present study may be the tendency of cadmium to accumulate in higher amounts in roots than shoots [43,44].

Moreover, our investigation showed that root fresh weight and dry weight, tolerance indices of both roots and whole plant and root tip percentage were significantly affected by elevated levels of cadmium in both the transgenic cultivars and their wild relative. Taking into account the root fresh and dry biomass, cultivar GK30 excel well followed by Coker 312 and BR001. Regarding the tolerance indices of root length and plant height, and root tip percentage, as a whole Coker 312 proved to be much efficient towards Cd stress followed by GK30 and BR001. The relative decrease in root dry weights under Cd toxicity syndrome has been previously investigated in cotton [45] and wheat seedlings [46].

4.2. Effect on root morphological parameters

Root is that part of a plant, which is in direct contact with nutrient medium. Its morphology can directly affect the uptake of water, minerals and heavy metals [54]. Various morphological parameters like root surface area, volume and diameter are under direct influence of heavy metals. They can play an important role in the accumulation and distribution of heavy metals like Cd [55]. Root surface area of plant directly affects ions in the soil solution up taken by plant root. Root volume is an important parameter for assessing root physiological function [56].

In the present study, we observed that both transgenic cotton cultivars were having apparent root morphological traits. Both had diversed variations, however, BR001 was badly affected as compared to GK30 by Cd treatment at all levels. As compared to controls, in case of both transgenic cultivars, the exceeding levels of Cd were having inhibitory influence on root tip percentage, root surface area, volume and diameter. However, with the elevation of Cd levels, the root surface area in case of both the cultivars first decreased and then gradually increased which is in conformity with Berkelaar and Hale [57], who reported an increase in root surface area and root tip percentage during study on Cd toxicity in wheat, which ultimately led to greater accumulation of Cd in roots.

However, the response of Coker 312 regarding root surface area was quite different as compared to its distant relative transgenic cotton cultivars, BR001 and GK30. Resultantly, the bioaccumulation of Cd by roots and shoots was relatively less in comparison to BR001 and GK30. Moreover, other traits like root volume and average diameter in both the transgenic cotton cultivars and their distant relative were obviously influenced by all Cd levels.

4.3. Effect on Cd accumulation in roots and shoots

Metal uptake in plants is correlated with the increasing metal concentration in the soil or in the medium [30,47] and in whole plants, roots are the primary sites to which heavy metals gain access. In most cases, heavy metals are readily taken up by plant through root and transported to the aerial parts by xylem vessel. However, much amount of them is stored in roots as compared to shoots [48]. In general, a large fraction of Cd is retained by the roots and only comparatively small amounts (about 10%) are transported to the shoots [49–51].

We observed that the bioavailability of Cd was concentration-dependent in all parts of seedlings, and cultivar BR001 accumulated greater amount of Cd in roots and shoots than GK30, while their wild relative cotton genotype (Coker 312) was the least responsive towards the Cd accumulation. An increase in Cd concentration in both root and shoots was also observed both in maize [52] and soybean [53] after a short exposure to Cd stress. Moreover, the Cd accumulation at greater amount in roots as compared to shoots in present investigation confirmed the findings of other workers [37,48] who respectively observed greater accumulation of heavy metals in roots than aerial parts of cotton and winter wheat. It is here noteworthy that high retention of heavy metals in root is desirable in a dual-purpose crop like cotton, which is mainly utilized as a fiber and oil crop.

4.4. Effect on root ultrastructure

A thorough exposure of seedlings of two transgenic cotton cultivars and distant wild relative for 6 days to increasing concentration of Cd also showed visible symptoms of Cd toxicity at root ultrastructural level. Absorption, transportation and cellular localization of heavy metals including Cd directly count for their toxicity.

In the present investigation, ultrastructural alterations in root tip cells of both transgenic cotton cultivars were mainly concentrated on membranes like plasma and nuclear membrane, vacuoles and nucleoli (Figs. 2–4). With the increase in Cd concentrations, modifications like detrimental plasmolytic shrinkage, enlarged and greater number of vacuoles, increase in the number of nucleoli etc. also became prominent. Even low concentrations of Cd could induce ultramorphological alterations and lead to increase the number of vacuoles and nucleoli in both transgenic cotton cultivars. Moreover, the size of vacuoles became bigger and bigger, number of electron dense granules and crystalline structures attached to the cell walls and present in the vacuoles increased and nucleoli produced in greater number with exceeding levels of Cd. Increased vacuolation in present study might play a significant role in Cd detoxification and tolerance, thus preventing the circulation of free Cd ions in the cytosol and forces them into a limited area [5].

However, in case of their wild relative cotton genotype (Coker 312), the ultramorphological modifications in the root tip cells of 6-day-old seedlings were occurred principally in vacuoles, nucleus and mitochondria. With the elevated levels of Cd, increase in number of nucleoli was almost like that of both transgenic cultivars, while the vacuolar size became enlarged instead of their number (Figs. 2–4). Also plasmolysis of plasma membrane was either absent or insignificant and nuclear envelope was almost in good shape, although its size was greatly reduced at the highest Cd level

(i.e.1000 μM). Moreover, in comparison to control, with increase in the toxic levels of Cd, number of mitochondria also increased. Increase in the size of vacuoles in the present study reveal that Coker 312 might be helpful in accumulating more cadmium in the dead part of the cell. Furthermore, greater number of mitochondria means the production of large amount of energy inside the cell, which is needed in order to combat with cadmium toxicity.

Further more, the presence of Cd in the form of crystals and electron dense granules in vacuoles and attached to cell wall reveal that in these cultivars both apoplastic and symplastic binding of Cd might be performed, which is supported by the findings of other research workers in bread and durum wheat cultivars [8], *Arabidopsis halleri* [12], *Allium cepa* [15], and *Zea mays* [58]. Also the production of greater number of nucleoli with the elevated levels of Cd might increase the production of ribosomes and mRNA, which ultimately enhance the production of new proteins being involved in the heavy metal tolerance [59]. Differences studied at both ultrastructural and functional levels in roots of both transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) under cadmium stress might be due to their diversed genetic nature towards Cd sensitivity, which needs to be further confirmed.

5. Conclusions

In the light of present investigation, we can conclude our findings as follows:

- Comparatively better growth of the aerial parts of seedlings over roots and greater accumulation of Cd in roots than shoots in both transgenic cultivars and their wild relative seem that Cd does not enter into the xylem transport system.
- The presence of Cd in the form of crystals and electron dense granules both in the vacuoles and attached to the cell walls reveals that its sequestration might possibly be done by dead parts of the cell.
- Increase in number and size of vacuoles and greater number of nucleoli convey the possible role of all these genotypes in Cd tolerance.
- As the functional and ultrastructural modifications were observed in these cultivars, it may be argued that Cd induced functional and ultrastructural senescence-like effects in these cultivars.
- And moreover, the substantial differences at the whole plant and cellular levels among these cultivars simply point to a variable scope of these cultivars for Cd-detoxification.

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