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Cadmium-induced ultramorphological and physiological changes in leaves of two transgenic cotton cultivars and their wild relative

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ABSTRACT

The present study describes cadmium-induced alterations in the leaves as well as at the whole plant level in two transgenic cotton cultivars (BR001 and GK30) and their wild relative (Coker 312) using both ultramorphological and physiological indices. With elevated levels of Cd (i.e. 10, 100, 1000 μM), the mean lengths of root, stem and leaf and leaf width as well as their fresh and dry biomasses linearly decreased over their respective controls. Moreover, root, stem and leaf water absorption capacities progressively stimulated, which were high in leaves followed by roots and stems. BR001 accumulated more cadmium followed by GK30 and Coker 312. Root and shoot cadmium uptakes were significantly and directly correlated with each other as well as with leaf, stem and root water absorption capacities. The ultrastructural modifications in leaf mesophyll cells were triggered with increase in Cd stress regime. They were more obvious in BR001 followed by GK30 and Coker 312. Changes in morphology of chloroplast, increase in number and size of starch grains as well as increase in number of plastoglobuli were the noticed qualitative effects of Cd on photosynthetic organ. Cd in the form of electron dense granules could be seen inside the vacuoles and attached to the cell walls in all these cultivars. From the present experiment, it can be well established that both apoplastic and symplastic bindings are involved in Cd detoxification in these cultivars. Absence of tonoplast invagination reveals that Cd toxic levels did not cause water stress in any cultivars. Additionally, these cultivars possess differential capabilities towards Cd accumulation and its sequestration.

1. Introduction

Cadmium (Cd) is probably the most significant pollutant due to its high toxicity and large solubility in water [1]. It is the outcome of various anthropogenic activities like mining, fertilization, industrialization, etc. [2,3]. Both animals and plants are directly and indirectly being affected by Cd, howsoever, plants are more prone to the attack of Cd because it has been accumulated in different soils for many decades. Resultantly, it can enter into a plant very rapidly accumulating in roots [4,5] with a variable amount being translocated to the upper parts of the plant [6,7].

The phytotoxic effects of Cd could probably be a consequence of its interference with a number of metabolic processes associated with normal development of plant [8]. However, plants can develop tolerance mechanisms to reduce the concentration of free Cd^{2+} in the cytosol. These worth mentioning tolerance strategies are detoxification, accumulation due to plant chelators and compartmentalization of Cd^{2+} ions in the vacuoles [9]. Literature reports are available about the interaction of Cd with diverse biochemical processes in plants; however, very few studies describe structural and ultrastructural modifications induced by Cd, which may be either consequences or causes of physiological disfunction [10]. An increase in 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, plasmalemma and mitochondrial membranes are some of the most concernable effects of Cd at ultrastructural levels [11–14].

Among its cytotoxic activities, Cd causes oxidative stress [15–17], which can contribute to aging of chloroplasts and cells [18]. Moreover, it stimulates the synthesis of ethylene, whose involvement in

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events of plant cell senescence is well known [19]. Baszyński et al. [20] in tomato, Reese et al. [21] in tobacco, Barceló et al. [10] in bush bean and Rascio et al. [22] in maize have documented that disorganization of grana and an increase in the number and size of plastoglobuli in chloroplasts as well as increased cell and vacuole size and induced vesiculation was the main consequence of Cd stress. Ouzounidou et al. [23] found wavy appearance of grana and stroma thylakoids and dilation of thylakoid membranes in chloroplast of wheat under Cd stress.

Transgenic crops have almost occupied a significant portion of agricultural land in most parts of the world. Cotton, globally an important economic crop mainly utilized for fiber and oil production, has been exploited in a number of ways by using new plant biotechnology techniques. Now we have a number of transgenic cotton cultivars possessing foreign genes, which are being grown on a wide acreage throughout the world. In view of the growing importance of transgenic cotton in both developed and developing world, it is utmost important to exploit them against various environmental stresses. For the above-stated reasons and the near unavailability of studies regarding their possible roles against cadmium stress, we considered two transgenic cotton cultivars namely BR001 (herbicide resistant), GK30 (insect resistant) as well as their wild relative cotton genotype (Coker 312).

The main objectives of our present study were to explore the effect of Cd at the cellular level of leaf mesophyll cells and the possible cellular mechanism being involved in the Cd sequestration in these cells. Moreover, to find out the possible toxic effects of Cd on morphology and physiology at the whole plant level as well as on leaf. In addition, the potential of two diverse nature transgenic cotton cultivars for Cd detoxification in comparison to their wild relative cotton genotype has been investigated.

2. Materials and methods

2.1. Seed surface sterilization and treatment process

Mature seeds of two transgenic cotton cultivars (i.e. BR001 and GK30) and their wild relative cotton genotype (i.e. Coker 312), having uniform grade were first immersed in 70% ethanol for 3 min and then in 0.1% $HgCl_2$ for 8–10 min. They were 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, ten 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. To each petri plates, a 2–3 ml of the tested solution was applied for first 3 days. Then on day 4, the germinated seedlings were transferred to another set of sterilized petri dishes with double-layered filter papers and 7–9 ml tested solution was applied to each petri dish.

Independent experiments were run for measurements of qualitative and quantitative traits of whole plant and leaves as well as for microscopic studies. The petri plates were sealed with parafilm and placed in dark for 48 h followed by 4-day exposure to a 16 h photoperiod of 50 μmol m^{-2} s^{-1} under white fluorescent light with 28 ± 2 °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. Plant growth parameters and Water Absorption Capacity (WAC)

A number of qualitative and quantitative plant growth parameters, namely, root–shoot lengths, leaf length and diameter, fresh and dry biomasses were determined in two independent experiments.

The Water Absorption Capacity (WAC) of roots, stems and leaves was calculated in another of set of experiment according to Kim et al. [24] using the following formula;

$$\text{Water Absorption Capacity}\,(\%) = \left[\frac{(\text{FB} - \text{DB})}{\text{FB}}\right] \times 100$$

where FB and DB are fresh biomass and dry biomass of the plant materials, respectively.

Moreover, the relative increase or decrease (%) in different qualitative and quantitative traits at various Cd levels over their respective controls was calculated as follows;

$$\frac{\text{Relative Increase}}{\text{Decrease}}(\%) = \left[\frac{(\text{Mean value in treatment} - \text{Mean value in control})}{\text{Mean value in control}}\right] \times 100$$

2.3. Determination of Cd content

To determine the bioavailability of Cd, the seedlings were thoroughly washed first with distilled water and then with 20 mM Na_2-EDTA for about 15 min in order to remove excess Cd adhering to the surfaces. 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

Table 1
Mean length of root, stem and leaf of two transgenic cotton cultivars (BR001, GK30) and their wild relative (Coker 312) grown for 6 days under various Cd treatments.

Cultivars	Cd Treatments (μM)	Mean values and their relative increase/decrease over the controls			
		Root Length (cm plant^{-1})	Stem Length (cm plant^{-1})	Leaf Length (cm plant^{-1})	Leaf Width (cm plant^{-1})
BR001	0	2.42 ± 0.03a (100)	3.47 ± 0.12a (100)	1.47 ± 0.01a (100.00)	2.52 ± 0.01a (100.00)
	10	1.99 ± 0.07b (−17.49)	2.97 ± 0.12b (−14.42)	1.43 ± 0.02ab (−2.72)	2.42 ± 0.01b (−3.97)
	100	1.20 ± 0.15c (−50.55)	2.52 ± 0.02c (−27.40)	1.33 ± 0.01c (−9.52)	2.11 ± 0.05c (−16.14)
	1000	0.73 ± 0.03d (−69.83)	1.83 ± 0.02d (−47.12)	1.40 ± 0.02b (−4.76)	2.37 ± 0.02b (−5.95)
GK30	0	2.99 ± 0.05a (100)	3.67 ± 0.28a (100)	1.52 ± 0.02a (100.00)	2.56 ± 0.02a (100.00)
	10	2.85 ± 0.04b (−5.01)	3.06 ± 0.05b (−16.61)	1.45 ± 0.02b (−4.62)	2.47 ± 0.01b (−3.65)
	100	2.43 ± 0.04c (−18.91)	2.74 ± 0.02b (−25.32)	1.36 ± 0.01c (−10.33)	2.25 ± 0.02c (−12.11)
	1000	1.61 ± 0.03d (−46.27)	2.08 ± 0.05c (−43.29)	1.34 ± 0.02c (−11.87)	2.17 ± 0.02d (−15.36)
Coker 312	0	1.69 ± 0.04a (100)	2.76 ± 0.11a (100)	1.38 ± 0.01a (100.00)	2.28 ± 0.02 a (100.00)
	10	1.58 ± 0.02b (−6.52)	2.69 ± 0.02a (−2.42)	1.34 ± 0.01a (−2.91)	2.14 ± 0.01b (−5.86)
	100	1.33 ± 0.03c (−20.95)	2.31 ± 0.02b (−16.20)	1.27 ± 0.01b (−7.99)	2.23 ± 0.02a (−2.05)
	1000	1.18 ± 0.01d (−29.84)	1.98 ± 0.02c (−28.17)	1.21 ± 0.02c (−11.86)	2.17 ± 0.01b (−4.83)

Values are the means of three replications ± SE. Variants possessing the same letter are not statistically significant at $P < 0.05$. Values in the parenthesis show relative increase (+)/decrease (−) over the respective controls.

after adding a 2:1 (v:v) mixture of HNO_3 and $HClO_4$. After heating the mixture at 80 °C on water bath for about 2 h, Cd was quantified using an atomic absorption spectrometry (PE-100, PerkinElmer).

2.4. Transmission electron microscopy

Leaf fragments without veins (approximately 1 mm^2) 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 10 min interval between each washing. Then with 15–20 min interval, they were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) 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 ultra-thin 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.

2.5. Statistical analysis

One-way ANOVA was performed 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. Pearson correlation coefficients were calculated to determine relationship among qualitative and quantitative traits of all the three cultivars.

3. Results

3.1. Plant growth responses

Table 1 shows the mean values of root, stem and leaf lengths as well as leaf width and their relative increase or decrease over the respective controls in BR001, GK30 and Coker 312. Typical phenotypic responses were evident in all the three cultivars with concomitant increase in Cd levels. In comparison to their relevant controls, their mean values of root, stem and leaf lengths as well as leaf width progressively declined except BR001, which showed a minor increase in the mean values of leaf length and width at 1000 μM Cd over the two low levels of Cd. As far as the mean values of root and stem lengths are concerned, the decreasing trend was much pronounced in BR001 followed by GK30 and Coker 312 as revealed by relative increase or decrease over their respective controls. On the contrary, GK30 was badly affected as compared to BR001 and Coker 312 in case of mean values of leaf lengths and width. Moreover, within treatments, noticeable differences in the mean values of root length at 5% probability level were evident in our experimental materials. However, only the mean values of stem length in BR001 were statistically significant ($P < 0.05$) at all Cd levels, while in GK30 and Coker 312, they showed significant difference at 1000 μM Cd over their respective controls. Additionally, by considering the mean values of leaf length and width, statistically significant variations could be noticed at 1000 μM Cd level over their relevant controls in all the three cultivars.

3.2. Biomass production

The mean values of fresh and dry biomass of root, stem and leaf of both transgenic cotton cultivars (BR001 and GK30) and their wild relative (Coker 312) along with their relative increase or decrease are presented in Table 2. The tabulated data established that fresh and dry biomasses of the adapted root, shoot and leaf were significantly different at 1000 μM Cd concentration over the respective controls in case of all cultivars, however, the responses at other two

Table 2
Fresh and dry biomass production and their relative increase/decrease in 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})		Stem Biomass (g plant^{-1})		Leaf Biomass (g plant^{-1})	
		Fresh weight	Dry weight	Fresh weight	Dry weight	Fresh weight	Dry weight
BR001	0	0.03 ± 0.002a (100)	0.02 ± 0.001a (100)	0.09 ± 0.002a (100)	0.08 ± 0.001a (100)	0.23 ± 0.001a (100)	0.18 ± 0.002a (100)
	10	0.02 ± 0.002b (−27.84)	0.02 ± 0.001b (−24.03)	0.08 ± 0.001a (−3.86)	0.07 ± 0.001b (−10.17)	0.21 ± 0.001b (−5.62)	0.17 ± 0.002b (−6.72)
	100	0.01 ± 0.000c (−49.82)	0.01 ± 0.001c (−55.79)	0.08 ± 0.001b (−9.27)	0.06 ± 0.002c (−21.19)	0.21 ± 0.002b (−6.51)	0.16 ± 0.002c (−12.52)
	1000	0.01 ± 0.001d (−73.26)	0.01 ± 0.001d (−77.25)	0.07 ± 0.001c (−18.92)	0.05 ± 0.002d (−30.93)	0.17 ± 0.001c (−23.22)	0.14 ± 0.002d (−24.86)
GK30	0	0.03 ± 0.002a (100)	0.03 ± 0.001a (100)	0.10 ± 0.005a (100)	0.10 ± 0.002a (100)	0.28 ± 0.004a (100)	0.24 ± 0.002a (100)
	10	0.03 ± 0.002ab (−12.62)	0.02 ± 0.002b (−18.05)	0.10 ± 0.002ab (−8.01)	0.09 ± 0.001a (−4.12)	0.27 ± 0.002a (−2.30)	0.21 ± 0.002b (−11.56)
	100	0.02 ± 0.003b (−27.44)	0.02 ± 0.002c (−42.24)	0.09 ± 0.002b (−15.38)	0.08 ± 0.001b (−21.65)	0.25 ± 0.001b (−8.48)	0.19 ± 0.002c (−19.78)
	1000	0.02 ± 0.003c (−51.74)	0.01 ± 0.001d (−67.51)	0.07 ± 0.002c (−30.13)	0.06 ± 0.002c (−33.68)	0.18 ± 0.002c (−33.09)	0.14 ± 0.002d (−40.39)
Coker 312	0	0.02 ± 0.001a (100)	0.02 ± 0.002a (100)	0.04 ± 0.002a (100)	0.03 ± 0.001a (100)	0.21 ± 0.009a (100)	0.20 ± 0.015a (100)
	10	0.02 ± 0.002a (−9.23)	0.02 ± 0.001ab (−11.32)	0.04 ± 0.002ab (−10.17)	0.03 ± 0.001a (−7.92)	0.20 ± 0.009a (−4.37)	0.16 ± 0.009b (−16.95)
	100	0.02 ± 0.001ab (−15.38)	0.01 ± 0.001b (−32.08)	0.03 ± 0.003bc (−21.19)	0.03 ± 0.002b (−22.77)	0.15 ± 0.007b (−27.50)	0.12 ± 0.009c (−40.68)
	1000	0.01 ± 0.001b (−32.31)	0.01 ± 0.001c (−56.60)	0.03 ± 0.002c (−30.51)	0.02 ± 0.001c (−44.55)	0.12 ± 0.012c (−43.75)	0.09 ± 0.002c (−55.42)

Values are the means of three replications ± SE. Variants possessing the same letter are not statistically significant at $P < 0.05$. Values in the parenthesis show relative increase (+)/decrease (−) over the respective controls.

Table 3
Water Absorption Capacity (WAC) 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)	RWAC (%)	SWAC (%)	LWAC (%)
BR001	0	14.21 ± 1.41b (100)	8.88 ± 0.89c (100)	16.88 ± 0.78b (100)
	10	10.23 ± 0.94c (−28.02)	14.46 ± 1.13b (62.88)	19.68 ± 1.49ab (16.63)
	100	24.30 ± 1.21a (71.02)	20.67 ± 1.09a (132.81)	23.13 ± 0.88a (37.07)
	1000	27.93 ± 1.20a (96.60)	22.14 ± 0.96a (149.32)	20.80 ± 1.07a (23.13)
GK30	0	12.50 ± 1.27d (100)	6.64 ± 0.63b (100)	12.97 ± 0.77c (100)
	10	17.95 ± 1.02c (43.54)	2.82 ± 0.81b (−57.46)	21.11 ± 1.45ab (62.83)
	100	29.86 ± 1.23b (138.84)	12.26 ± 1.29a (84.68)	23.50 ± 1.24a (81.21)
	1000	40.78 ± 1.31a (226.13)	14.48 ± 1.71a (118.13)	19.69 ± 0.72b (51.88)
Coker 312	0	18.15 ± 1.03c (100)	14.24 ± 1.86b (100)	7.51 ± 1.81c (100)
	10	21.34 ± 1.29c (17.58)	12.78 ± 1.12b (−10.25)	19.04 ± 0.94b (153.42)
	100	34.98 ± 1.01b (92.71)	16.35 ± 0.99b (14.79)	23.33 ± 1.91ab (210.47)
	1000	45.60 ± 0.95a (151.22)	31.22 ± 1.26a (119.21)	26.97 ± 1.01a (258.92)

RWAC: Root Water Absorption Capacity; SWAC: Stem Water Absorption Capacity; LWAC: Leaf Water Absorption Capacity. Values are means ± SE of three replications. Variants possessing the same letter are not significantly different at $P < 0.05$ as determined by LSD test. Values in the parenthesis show relative increase (+)/decrease (−) over the respective controls. Values are the means of three replications ± SE. Variants possessing the same letter are not statistically significant at $P < 0.05$. Values in the parenthesis show relative increase (+)/decrease (−) over the respective controls.

Cd levels (10 and 100 μM) over their controls were almost different among these cultivars.

Moreover, the relative increase or decrease in the mean values of these biometric parameters in comparison to their respective controls depict that Cd stress profoundly affected the dry biomass of root, stem and leaf as compared to the fresh biomass of these plant parts. Taken together the relative increase or decrease data regarding the aforementioned parameters, it can be interpreted that root fresh and dry biomasses greatly reduced in BR001 followed by GK30 and Coker 312.

3.3. Water Absorption Capacity (WAC)

The mean values of Water Absorption Capacity (WAC) of different parts of the seedling and their relative increase or decrease over the controls are summarized in Table 3. The results clearly demonstrated that the water absorption capacity of root, stem and leaf at 1000 μM of Cd were significantly different ($P < 0.05$) compared with controls in all studied cultivars. In comparison to their controls, the WAC of root in cultivars GK30 and Coker 312 gradually enhanced while in cultivar BR001, it was first inhibited at 10 μM and then stimulated at other higher Cd levels but was not higher than the control.

In comparison to their respective controls, the WAC of stem was linearly increased at all Cd levels in cultivar BR001, but in cultivars GK30 and Coker 312 a non-significant reduction ($P > 0.05$) at 10 μM of Cd could be witnessed. Furthermore, the WAC of leaf in these cultivars progressively enhanced over their relevant controls. However, in BR001 a decline in its mean value was evident at 1000 μM as compared to 100 μM Cd level, while in GK30 the mean value of WAC of leaf at 1000 μM of Cd was lower than the other two low levels of Cd (10 and 100 μM).

Moreover, regarding relative increase or decrease in the mean values WAC of root, stem and leaf, it was found that the water absorption capacity of these parts relatively increased over their controls in all cultivars except at 10 μM at which the root WAC of cultivars BR001 and stem WAC of GK30 and Coker 312 were subsequently decreased. Also further inhibition in stem WAC of GK30 could be observed at 1000 μM over its control.

3.4. Cd accumulation in various parts of plant

The mean values of Cd uptake by roots and shoots of two transgenic cotton cultivars (BR001, GK30) and relative increase or decrease over the controls are shown in Table 4. It reveals that all cultivars responded differently regarding cadmium uptake and the response was in the order of Coker 312 < GK30 < BR001. Increasing concentration of Cd caused a linear enhancement of Cd uptake in different parts of the plant; however, its accumulation in roots was much higher than shoots in all these cultivars. Similarly, there was a progressive relative increase over the respective controls of all three cotton cultivars regarding Cd.

3.5. Correlation among qualitative and quantitative traits of the seedlings

Correlation study among qualitative and quantitative traits of the seedlings of BR001, GK30 and Coker 312 is shown in Table 5. The

Table 4
Cd uptake by root and shoot 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 (100.00)	0.09 ± 0.002d (100.00)
	10	149.57 ± 5.99c (17993.55)	76.66 ± 2.23c (85077.78)
	100	280.46 ± 2.07b (33826.21)	145.74 ± 1.06b (161829.63)
	1000	429.69 ± 6.29a (51878.63)	235.36 ± 2.42a (261411.11)
GK30	0	0.43 ± 0.02d (100.00)	0.032 ± 0.002d (100.00)
	10	108.95 ± 2.82c (54375.00)	54.50 ± 3.45c (170549.48)
	100	162.94 ± 2.28b (107645.00)	93.14 ± 3.77b (355848.45)
	1000	302.89 ± 5.74a (162275.00)	182.03 ± 6.42a (562879.38)
Coker 312	0	0.39 ± 0.01d (100.00)	0.02 ± 0.002d (100.00)
	10	100.37 ± 1.90c (25418.64)	36.39 ± 1.92c (153660.56)
	100	153.54 ± 3.12b (38936.44)	68.58 ± 2.92b (289674.65)
	1000	267.39 ± 3.84a (67879.66)	157.77 ± 1.36a (666519.72)

Values are the means ± SE of three replications. Means followed by different letters indicate significant differences among the treatments as determined by LSD test at 5% level of significance. Values in parenthesis show relative increase (+)/decrease (−) over the respective controls.

Table 5
Pearson correlation coefficients among qualitative and quantitative traits of the seedlings of two transgenic cotton cultivars BR001, GK30) and their wild relative cotton genotype (Coker 312).

	RL	SL	LL	LW	RFW	RDW	SFW	SDW	LFW	LDW	RWAC	SWAC	LWAC	RCd	SCd
SL	0.842**														
LL	0.685**	0.762**													
LW	0.651**	0.688**	0.825**												
RFW	0.877**	0.862**	0.572**	0.587**											
RDW	0.879**	0.911**	0.720**	0.675**	0.907**										
SFW	0.663**	0.550**	0.765**	0.635**	0.440**	0.522**									
SDW	0.744**	0.603**	0.790**	0.662**	0.517**	0.588**	0.977**								
LFW	0.829**	0.781**	0.774**	0.577**	0.717**	0.770**	0.772**	0.806**							
LDW	0.799**	0.806**	0.817**	0.614**	0.729**	0.817**	0.687**	0.731**	0.959**						
RWAC	−0.556**	−0.782**	−0.808**	−0.670**	−0.551**	−0.750**	−0.521**	−0.511**	−0.720**	−0.772**					
SWAC	−0.688**	−0.550**	−0.593**	−0.418*	−0.586**	−0.584	−0.553**	−0.679**	−0.689**	−0.657**	0.391*				
LWAC	−0.314	−0.486**	−0.536**	−0.392*	−0.403*	−0.506**	−0.118	−0.172	−0.432**	−0.615**	0.574**	0.395*			
RCd	−0.717**	−0.825**	−0.460**	−0.468**	−0.837**	−0.857**	−0.132	−0.216	−0.561**	−0.646**	0.626**	0.446**	0.602**		
SCd	−0.674**	−0.815**	−0.448**	−0.448**	−0.803**	−0.841**	−0.096	−0.173	−0.547**	−0.627**	0.659**	0.408*	0.570**	0.988**	

**Significance at 1% probability level; *Significance at 5% probability level; RL: Root length, SL: Stem length, LL: Leaf Length, LW: Leaf width, RFW: Root Fresh Weight, RDW: Root Dry Weight, SFW: Stem Fresh Weight, SDW: Stem Dry Weight, LFW: Leaf Fresh Weight, LDW: Leaf Dry Weight, RWC: Root Water Content, SWC: Shoot Water Content, LWC: Leaf Water Content, RCd: Root Cd uptake, SCd: Shoot Cd uptake.

highest positive correlation was present between root cadmium uptake (RCd) and shoot cadmium uptake (SCd), followed by stem fresh weight (SFW) and stem dry weight (SDW), leaf fresh weight (LFW) and leaf dry weight (LDW), and Stem length (SL) and root dry weight (RDW). Moreover, highest negative correlation could be observed between root dry weight (RDW) and root cadmium uptake (RCd) and root dry weight (RDW) and shoot cadmium uptake (SCd), which were followed by highest negative correlation between root fresh weight (RFW) and root cadmium uptake (RCd) and between stem length (SL) and root cadmium uptake (RCd).

3.6. Ultrastructural changes under Cd treatments

Alterations in the fine structures of leaf mesophyll cells were found to be concentration-dependent in both transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) (Figs. 1–4). These changes became more and more prominent with the increase in Cd concentration over their respective controls.

The leaf mesophyll cells of the non-adapted plants in all cultivars were having typical ultramorphology (Fig. 1A–E). Cell wall was clean and thin. The intercellular spaces were almost absent. Also cytoplasm was dense possessing flattened plasma membrane, nearly centrally located well-shaped nucleus with one or two nucleoli and chromatin materials and round-shaped mitochondria. Moreover, elliptical-shaped chloroplast having closely-arranged and properly-aligned granum thylakoids along with few starch grains and plastoglobuli, could also be observed in the electron micrographs.

Changes in the ultra-fine structures of the leaf mesophyll cells started at 10 μM Cd concentrations in all three cultivars, however, the degree of severity and sites of changes were cultivar-specific (Fig. 2A–E). Chloroplast was the common organelle in all the three cultivars, which became eventually smaller and nearly round-shaped. Another Cd-toxicity target site was nucleus, which was severely affected in BR001. Nucleolus was divided into a number of small nucleoli, while in case of Coker 312 the shape and number of nucleoli increased but their size was relatively smaller as compared to control. However, in GK30 the number and shape of the nucleolus did not alter at the specified concentration of Cd.

Other noticeable changes or modifications that occurred in all three cultivars at 10 μM were the increase in number of starch grains, plastoglobuli, lipid bodies and swollen and loosely packed thylakoids grana. The number of starch grains and plastoglobuli in BR001 noticeably increased followed by GK30 and Coker 312. Thylakoids grana in BR001 became swollen and loose as compared to its control and other two cultivars. Also electron dense granules, although insignificant in number, inside the vacuoles and attached to the cell walls in both transgenic cotton cultivars could be seen in these electron micrographs. These dense granules might be Cd. Moreover, the cell wall was clean, intercellular spaces were nearly absent and the plasma membrane was almost adjacent to the cell walls in all the three cultivars.

At 100 μM Cd level, damage to the fine structure of leaf mesophyll cells of all three cotton cultivars was more scattered as compared to 10 μM (Fig. 3A–E). The toxic effects of Cd were more evident in the symplastic organelles, namely, nucleoli and chloroplast in all the studied cultivars. However, collectively BR001 cellular set up was badly damaged followed by GK30 and their wild relative cotton genotype (Coker 312). Size and the numbers of nucleoli were progressively decreased and internal structure of chloroplast was roughly smashed along with the increase in the size of starch grains. Furthermore, their cell wall was clean, plasma membrane was flattened, vacuolar size was normal and mitochondria were almost round. Cd deposition in the form of electron dense granules inside the vacuoles and attached to the cell walls could be seen in both BR001 and GK30 and their wild relative (Coker 312).

Severe modification in ultrastructural features of the leaf mesophyll cells in both transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) were noticed at the highest Cd concentration of 1000 μM (Fig. 4A–H). Greatest alterations occurred in the plastid regions of the leaf mesophyll cells followed by changes in the nuclear set up and plasma membrane in all cultivars. Briefly, the size of chloroplast decreased and their outer membrane broken at many places. Thylakoids became swollen, shorter and broken on different points. Plastoglobuli and starch granules increased in size and number. The size and number of the lipid bodies also subsequently increased. Furthermore, the vacuolar compartmentalization enhanced, and plasmolytic shrinkage became severe. Conclusively, at this highest concentration the ultramorphological modifications were more pronounced as compared to other low levels of Cd, and cultivar BR001 was severely damaged followed by GK30 and Coker 312.

4. Discussion

Contamination of arable land of the world due to heavy metals such as Cd, Pb, Cr, Zn has now become a major global threat. Because of their immutable nature, these heavy metals have got a greater concern [25] all over the world. Among them, Cd toxicity syndrome is a well-established phenomenon in most of the living organisms. The present study was conducted with a view to highlight the Cd

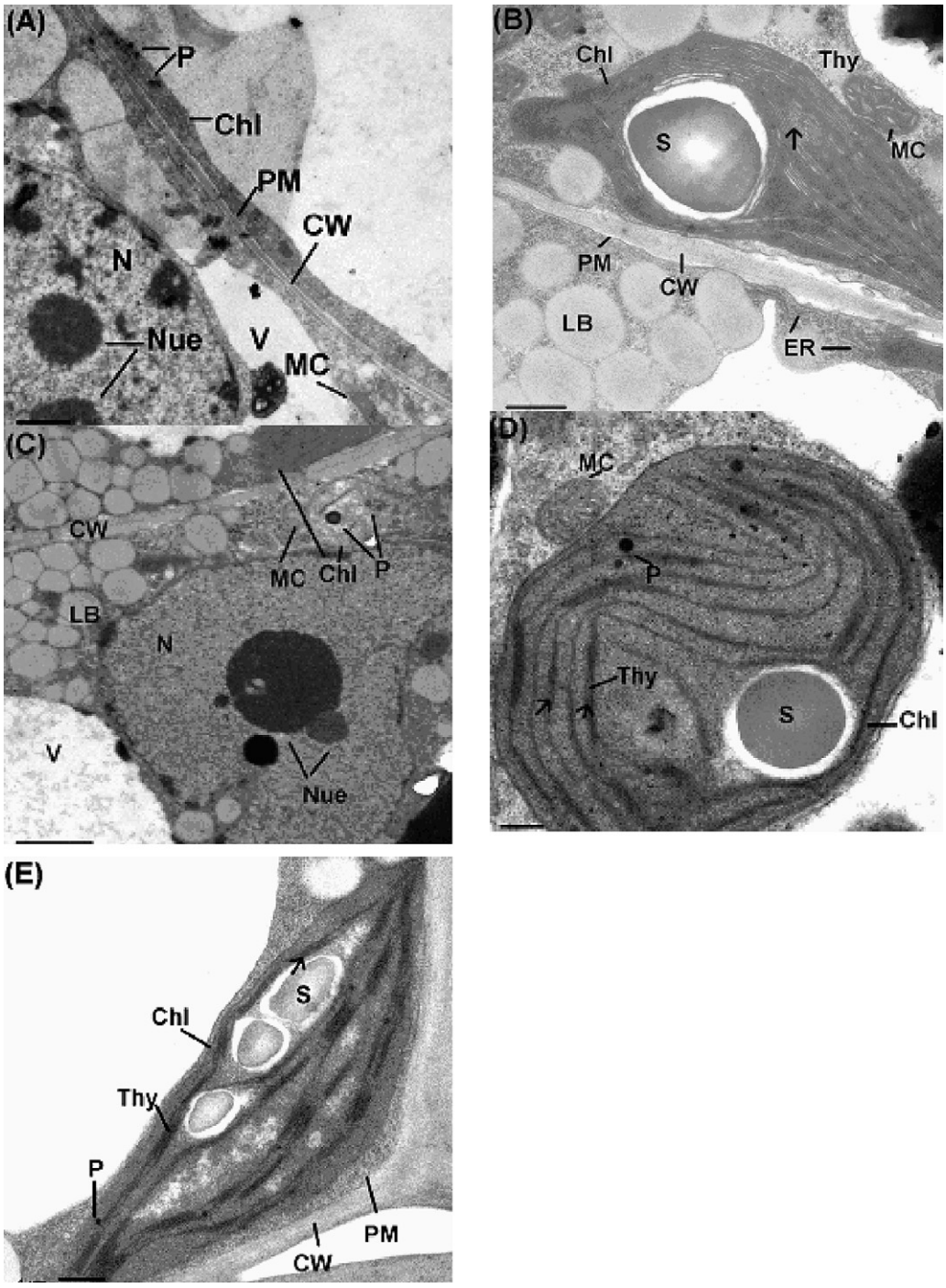

Fig. 1. Electron micrographs of leaf mesophyll cells of 6-day old germinated seedlings of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days at 0 μM Cd concentration. (A–B) TEM micrographs of control leaf mesophyll cells of BR001 respectively at low and high magnifications showing well-developed nucleus (N) with two nucleoli (Nue) and a distinct nuclear membrane, mitochondria (MC), endoplasmic reticulum (ER), plasma membrane (PM), cell wall (CW) as well as a number of lipid bodies (LB). Also chloroplast (Chl) is elliptical with well-developed grana and stroma thylakoids (Thy) having starch grains (S). (C–D) TEM micrographs of control leaf mesophyll cells of GK30 respectively at low and high magnifications showing a prominent nucleus (N) with two nucleoli (Nue) and a distinct nuclear membrane (NM). Also a large vacuole (V), well-shaped mitochondria (MC), endoplasmic reticulum (ER), plasma membrane (PM), cell wall (CW) and a number of lipid bodies (LB) can be seen. Chloroplast (Chl) is elliptical possessing well-developed stroma and grana thylakoids (Thy), starch grains (S) and plastoglobuli (P). (E) TEM micrograph of the control leaf mesophyll cells of Coker 312 showing plasma membrane (PM) attached with cell wall (CW) along with elliptical shaped chloroplast (Chl) with well-developed thylakoids (Thy) and a number of starch grains (S) and plastoglobuli (P) centrally located nucleus (N) possessing one nucleoli (Nue), a number of small vacuoles (V) and mitochondria (MC). Bars A = 2 μm; B = 1 μm; C = 2 μm; D = 0.5 μm; E = 1 μm.

phytotoxic effect on normal function of whole plant as well as ultramorphology of the leaf. To the best of our knowledge, this is the first research study about Cd toxicity syndrome on leaf morphology and its ultrastructure in two transgenic cotton cultivars and their wild relative cotton genotype.

4.1. Plant growth responses

Plant growth is a function of cell wall extensibility, water conductivity, osmotic potential, and threshold turgor among other factors. When these agents are insufficient, growth does not occur

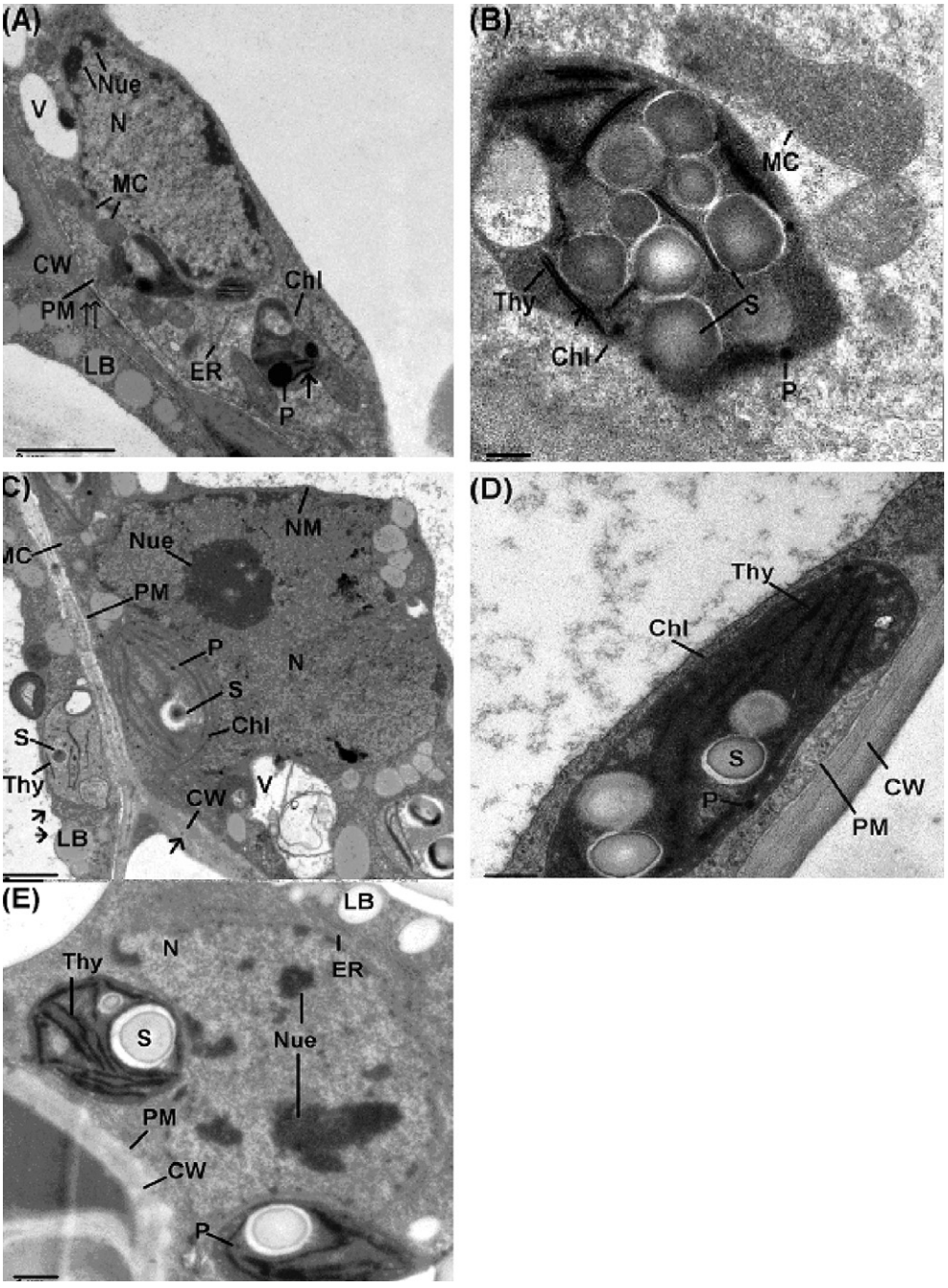

Fig. 2. Electron micrographs of leaf mesophyll cells of 6-day old germinated seedlings of two transgenic cotton cultivars (BR001, GK30) and their wild relative (Coker 312) grown for 6 days at 10 μM Cd concentration. (A and B) TEM micrographs of leaf mesophyll cells of BR001 respectively at low and higher magnifications showing irregularly shaped nucleus (N) with a number of nucleoli (Nue) along with prominent nuclear membrane (NM), plasma membrane (PM) well integrated with cell wall (CW). Also chloroplast (Chl) becomes round shaped and thylakoids (Thy) are of nearly swollen-shaped. Moreover, the number of starch grains (S) and plastoglobuli (P) increase. Cd deposition (double arrows) can also be seen in the micrograph. (C and D) TEM micrographs of leaf mesophyll cells of GK30 respectively at low and higher magnifications showing nucleus (N) with almost regular shape and a number of nucleoli (Nue), enveloped by a prominent nuclear membrane (NM). Also cell wall (CW) and plasma membrane (PM) are well integrated with each other. Chloroplast (Chl) is nearly of regular shape with a number of starch grains (S) and plastoglobuli (P). Arrow (→) indicates Cd deposition inside vacuole (V) and attached to the cell wall (CW). (E) TEM micrograph of leaf mesophyll 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 chloroplasts (Chl) are nearly of elliptical shape with well-developed thylakoids and less number of starch grains (S) and plastoglobuli (P). Bars A = 0.5 μm; B = 0.5 μm; C = 2 μm; D = 0.5 μm; E = 1 μm.

[26]. In the present investigation, we observed a decreasing trend in the mean values of root, stem and leaf lengths and leaf width over the controls in all cultivars. Root length underwent a significant reduction with the elevated levels of Cd. The same trend was not found in case of the mean values of stem and leaf lengths as well as leaf width. Moreover, the mean lengths of root, stem and leaf and mean values of leaf width of cultivar BR001 were pronouncedly influenced followed by GK30 and Coker 312.

Our results confirm the findings of Ouzounidou et al. [23], who observed inhibition in root and shoot lengths of wheat seedlings under Cd. The sensitivity of early growth seedlings has also been previously reported in wheat [27], barley [28] and cotton [29]. However, Sandalio et al. [30] found greater growth inhibition in leaves and stems of pea than roots under the toxic stress of Cd.

The growth inhibition of roots in the Cd stressful conditions can possibly be due to a direct action of Cd on the nucleus

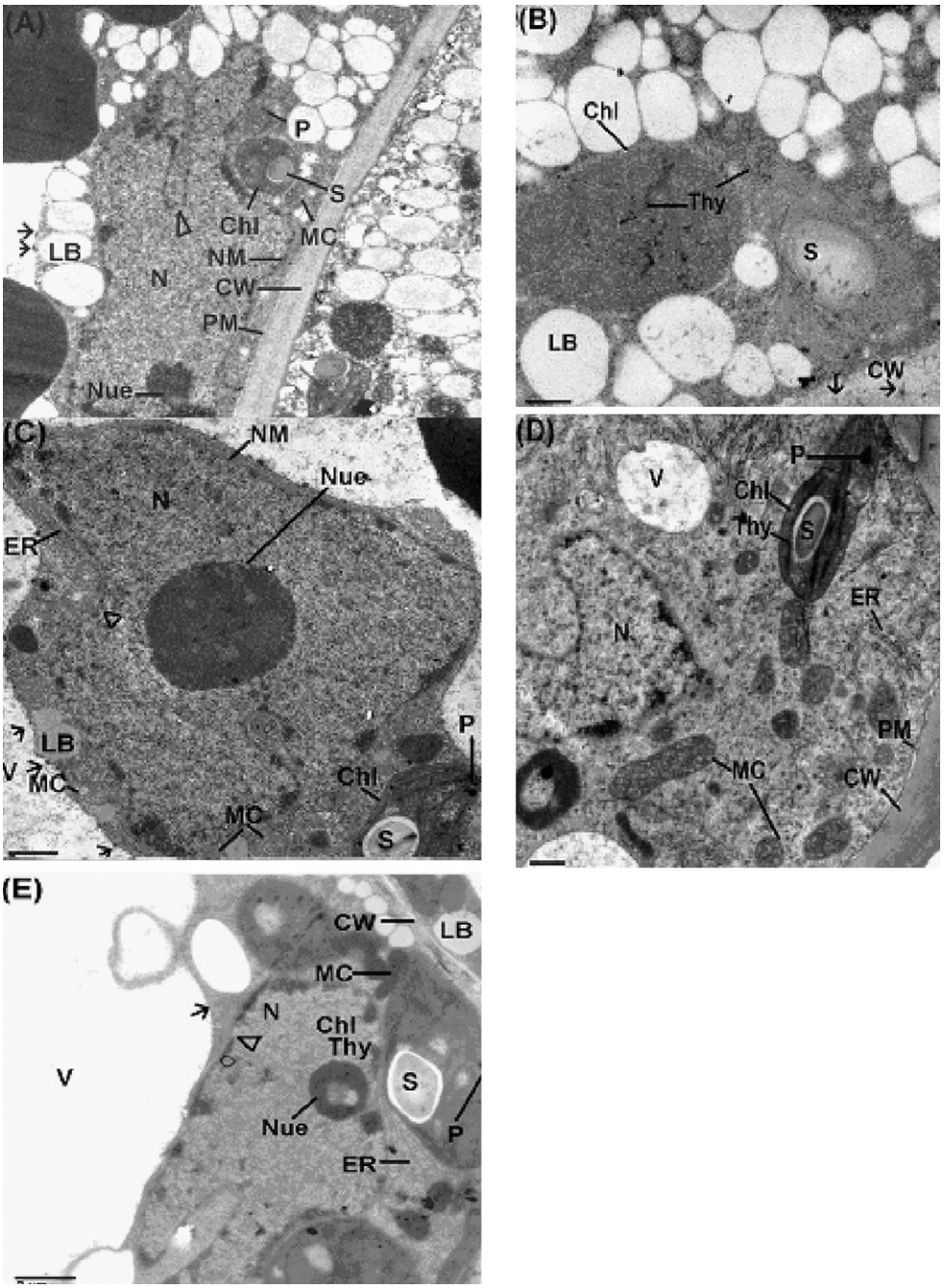

Fig. 3. Electron micrographs of leaf mesophyll cells of 6-day old germinated seedlings of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days at 100 μM Cd concentration. (A and B) TEM micrographs of leaf mesophyll cells of BR001 respectively at low and higher magnifications showing irregularly shaped nucleus (N) with a number of nucleoli (Nue). Rupturing (▽) of the nuclear membrane (NM) is also obvious. Also there is an increase in the lipid bodies (LB) along with the increase in number of starch grains (S) and plastoglobuli (P) as well as disruption in the membranous structure of the chloroplast (Chl) and its shape. Moreover, arrow (→) indicates increased accumulation of Cd in the form of electron dense granules inside vacuoles and attached to the cell walls. (C and D) TEM micrographs of leaf mesophyll cells of GK30 respectively at low and high magnifications showing less irregularly shaped nucleus enclosed by ruptured (▽) nuclear membrane along with almost well-shaped chloroplast (Chl) having less swollen thylakoids (Thy) and less number of starch grains (S) and plastoglobuli (P) as compared to BR001. Arrow (→) indicates Cd accumulation in the form of electron dense granules inside vacuoles. (E) TEM micrograph of leaf mesophyll cells of Coker 312 at high magnification showing an enlarged vacuole (V), disrupted (▽) nuclear membrane (NM), swollen but almost well-shaped chloroplast (Chl) possessing starch grains (S) and plastoglobuli (P). Cd deposition (→) in the form of electron dense granules inside vacuoles (V) can also be seen in the micrograph. Bars A = 2 μm; B = 1 μm; C = 2 μm; D = 1 μm; E = 2 μm.

or due to the interaction with hormones [31], while that of aerial parts of plant could be due to inhibition of photosynthesis [32], inactivity of both photosystem II and the enzymes of carbon reduction cycles [33,34] as well as disturbed functionality of stomata [31,35]. Moreover, shorter root than stem could be due to its tendency to be accumulated more in roots than shoots [36,37]. Also growth reduction in the upper parts of Cd-treated seedlings might be the consequence of disturbed sink force.

4.2. Biomass production

The biomass production of whole plants or of plant parts is used as an essential measurable parameter to monitor the effects

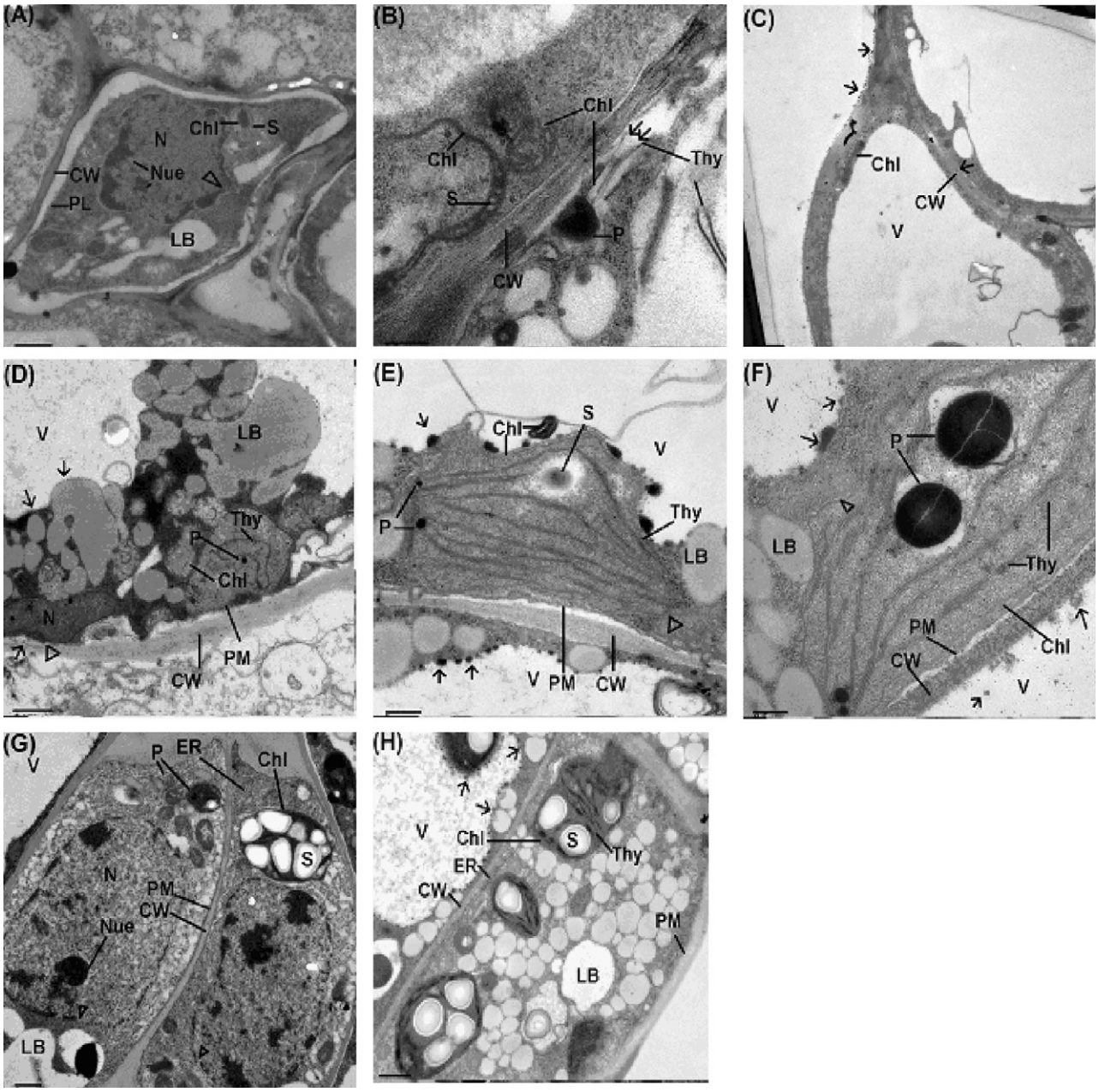

Fig. 4. Electron micrographs of leaf mesophyll cells of 6-day old germinated seedling of two transgenic cotton cultivars (BR001, GK30) and their wild relative cotton genotype (Coker 312) grown for 6 days at 1000 μM Cd concentration. (A–C) TEM micrographs of leaf mesophyll cells of BR001 at low and high magnifications showing small-sized and irregularly shaped nucleus (N) along with detrimental plasmolytic (PL) shrinkage and enlarged vacuoles (V). Cd deposition (→) in the form of electron dense granules inside the vacuoles and attached to the cell wall (CW) is obvious. Also the disruption of the nuclear membrane (▽) as well as of outer membrane (double arrows) of the chloroplast (Chl) can be seen in electron micrographs of BR001. Moreover, almost the size and shape of the chloroplast detrimentally change. (D–F) TEM micrographs of the leaf mesophyll cells of GK30 at low and high magnifications showing less irregularly shaped and small-sized nucleus (N) along with less severe plasmolysis (PL) of the plasma membrane (PM) and enlarged vacuoles (V). Moreover, shape of the chloroplast is less irregular as compared to BR001, while thylakoids become swollen and broke (▽) at many places as well as the size of the lipid bodies (LB) and starch grains (S) increases. (G–H) TEM micrographs of the leaf mesophyll cells of Coker 312 at high resolution showing centrally located nucleus (N) with a number of nucleoli (Nue) and well-shaped plasma membrane (PM) integrated with cell wall (CW). Cd deposition (→) can be seen inside the vacuoles (V). Moreover, chloroplast (Chl) morphology also alters due to swollen thylakoid (Thy) structures along with the increase in number of starch grains (S). Bars A = 2 μm; B = 0.5 μm; C = 2 μm; D = 1 μm; E = 1 μm; F = 0.5 μm G = 2 μm; H = 2 μm.

of various abiotic stresses. In our present study, we also evaluated the potential role of both transgenic cotton cultivars (BR001 and GK30) and their wild relative (Coker 312) regarding the biomass production submitted to cadmium stress. As a consequence of Cd treatment, the fresh and dry biomasses of root, stem and leaf were invariably decreased in our experimental materials. Cd stress profoundly affected the root fresh and dry biomasses followed by fresh and dry biomasses of stem and leaf. Also the dry weight sharply reduced as compared to fresh weight at all Cd levels (10, 100 and 1000 μM) in all these cultivars as well as in all parts of the seedlings. The data further reveal that the order of decline in fresh and dry biomasses of plant parts was BR001 > GK30 > Coker 312. Our present results are in line of the findings of Moya et al. [38] in rice under cadmium and nickel stresses, Ouzounidou et al. [23] in wheat seedlings under cadmium stress and those of Jin et al. [39] in hyperaccumulator and non-hyperaccumulator species of *Sedum alfredii* treated with Cd. They all found that obvious reduction occurred in the fresh and dry weights of root and aerial parts of the treated plants.

4.3. Water Absorption Capacity (WAC)

In order to investigate whether Cd stress imposed a secondary stress, i.e. water stress or not, the water absorption capacity of different plant organs was determined. We noticed quite interesting responses of these plant parts to different Cd levels. As a whole, in all cultivars, leaf absorbed more water followed by root and stem with the increase in Cd stress. These results are not in agreement with the findings of Fuhrer et al. [40] in beans, Barceló et al. [10] in bush bean, Sandalio et al. [30] in pea, Vitória et al. [4] in radish roots, Larsson et al. [6] in *Arabidopsis thaliana*, Pandey [41] in cabbage, Pereira et al. [5] in *Crotalariajuncea* and Ramos et al. [7] in *Lactuca* sp., who have suggested that Cd caused water stress conditions by inhibiting water uptake and transport because of the tendency of Cd to be accumulated in roots in more quantity and only a small quantity may be translocated to the aerial parts of the plant. The reasons in case of our findings might be that (1) we observed no visible or accountable wilting situation in any parts of the seedlings, (2) the internal cellular structure revealed that turgidity was almost maintained, (3) a number of induced mechanisms like prevention of water loss by stomatal closure, increased cell proliferation in the root cambial region [12] might have been developed which lead to increase the root surface areas [14].

4.4. Cd accumulation in different plant parts

Metal uptake in plants is correlated with increasing metal concentration in medium or soil [42–44]. In the present study, the Cd uptake by different plant parts of all these cultivars was concentration dependent. Moreover, Cd uptake by roots was more than aerial parts of the plant and BR001 captured more Cd followed by GK30 and Coker 312 [14]. These findings are in conformity with other studies like Jensen and Adalsteinsson [45] in winter wheat and Wu et al. [29] in cotton. It is important to mention 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 [14].

4.5. Correlation among qualitative and quantitative traits of the seedlings

The data regarding the correlation studies among qualitative and quantitative traits manifested that the highest cadmium uptake by roots would directly increase the shoot cadmium uptake. Additionally, we found significant as well as direct correlation between the root and shoot cadmium uptakes and the root, stem and leaf water absorption capacities. Such findings clearly demonstrate that under the Cd stressful conditions increase in the water absorption capacities of roots, stems and leaves would stimulate the root and shoot cadmium uptake.

4.6. Ultrastructural changes under Cd stress

In our present study, one of our main objectives was to explore the cellular mechanism being involved against Cd detoxification in two transgenic cotton cultivars (BR001 and GK30) and their wild relative (Coker 312). According to the microscopic investigation, the ultramorphological changes in the mesophyll cells were dose-dependent. With the increase in Cd levels, changes in the fine structures of leaf mesophyll cells also became evident. However, BR001 was the most affected cultivar followed by GK30 and Coker 312. The degenerative effects of various Cd levels were marked on nucleus and chloroplast in all the three cultivars.

Nucleus, which has a genetic organization and is responsible to control the action of cell through selective expression by genes [46], became irregular and number of nucleoli increased with the increase in Cd concentration. This modification was more pronounced in BR001 followed by GK30 and Coker 312. Increase in number of nucleoli indicates huge production of ribosomes and mRNA, which ultimately enhance the production of new proteins being involved in the heavy metal tolerance [47].

Another obvious consequence of Cd stress at ultrastructural level was deformed chloroplasts with nearly disorganized thylakoids systems in the adapted cells of leaf mesophyll. As compared to control, the chloroplast was round-shaped and small. The grana thylakoids became distended and short. The outer membrane of the chloroplast at the highest Cd level (1000 μM) was ruptured at many places in both transgenic cultivars, however, no such observation could be made in their wild relative. Also, the number and size of the starch grains increased. Additionally, there were observed no clear enhancement in the size of the plastoglobuli, although their number increased with the increase in Cd concentration. The accumulation of starch in leaves either in case of our study or that of Sandalio et al. [30] might be due to either nutrient deficiencies [48], decrease of the sink force or disturbed vein loading system [49]. Howsoever, Ouzounidou et al. [23] obtained different results during their studies on wheat under Cd stress.

Some other prominent changes like the size of vacuoles, the number of lipid bodies and thickness of the cell walls also increased with the elevation in Cd levels. Moreover, the plasmolytic shrinkage of plasma membrane at the highest level of Cd (1000 μM) was much pronounced in BR001 than GK30 while it was almost absent in their wild relative genotype (Coker 312). Increase in the number of lipid bodies in our present study confirms the findings of Čiamporová and Mistrik [50] and Ouzounidou et al. [51], who found greater number of lipid bodies in cytoplasm of maize cell under water and Cu stress conditions, respectively.

In our present experiment, deposition of Cd in the form of electron dense granules inside the vacuoles and attached to the cell wall was also found. However, at the lower concentration of Cd (10 and 100 μM) only transgenic cotton cultivars had Cd deposition, which was higher in BR001 over the GK30. Unlike the other two levels of Cd, at 1000 μM greater deposition of Cd could be observed in all three cultivars, which was highest in BR001 followed by GK30 and Coker 312. The Cd sequestration in vacuoles as well as in cell walls of leaf mesophyll cell confirms the findings of Ma et al. [52], Jiang et al. [46] and those by us [14].

Furthermore, the electron micrographs of the leaf mesophyll cells in the present study showed that nearly inconspicuous intercellular spaces became prominent with the elevation of Cd toxicity, which have also been previously observed by Barceló et al. [10] in bush bean, Sandalio et al. [30] in pea and Vitória et al. [12] in radish seedlings. The near absence of the intercellular spaces conveys an idea that Cd stress might have affected the leaf expansion. The ultrastructural studies on leaf mesophyll cells of two transgenic cotton cultivars and their wild relative reveal that as a whole Cd did not drastically alter the ultramorphology of different cell organelles, which might be due to the differences in the genetic make up of these materials and it needs further investigation.

5. Conclusion

- The above-stated physiological and structural changes in leaf mesophyll cells in transgenic cotton cultivars (i.e. BR001and GK30) and their wild relative cotton genotype (i.e. Coker 312) demonstrate that they have the potential to provide a highly valuable means to detect Cd stress in the earliest stages of plant development, and may also provide opportunities for screening different cultivars for their adaptation to cadmium stress.
- The differences observed particularly regarding the water absorption capacity in case of our findings and those of others might be due to the differences in plant species and their abilities to accu-

mulate and sequester Cd as well as the plant growth conditions and the adopted methodology.

- The absence of tonoplast invagination in the present study as opposed to the findings of Sandalio et al. [30] is an important indication that Cd did not cause secondary stress (i.e. water stress).
- The differences studied in Cd accumulation and its sequestration suggests that both transgenic cotton cultivars and their wild relative cotton genotype have differential capability to face the Cd toxicity.
- Cd induced senescence-like situation in the leaves of both transgenic cotton cultivars and their wild relative genotype. In senescent leaves, chloroplasts show an increase in number and size of plastoglobuli, as well as disturbances in their membrane structure [53]. Other events take place during senescence include a reduction in the size of chloroplast, invagination of the tonoplast into the vacuole, chromatin condensation in the nucleus, and loss of cytoplasmic components [54,30].
- Moreover, our present study is specific to the investigation of Cd(II) toxication using lab-based approach. In order to study its toxic effects on these cotton cultivars in the real soil environment further investigation is needed.

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