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Influence of Cadmium on Water Relations, Stomatal Resistance, and Abscisic Acid Content in Expanding Bean Leaves[1]

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ABSTRACT

Ten day old bush bean plants (*Phaseolus vulgaris* L. cv Contender) were used to analyze the effects of 3 micromolar Cd on the time courses of expansion growth, dry weight, leaf water relations, stomatal resistance, and abscisic acid (ABA) levels in roots and leaves. Control and Cd-treated plants were grown for 144 hours in nutrient solution. Samples were taken at 24 hour intervals. At the 96 and 144 hour harvests, additional measurements were made on excised leaves which were allowed to dry for 2 hours. From the 48 hour harvest, Cd-treated plants showed lower leaf relative water contents and higher stomatal resistances than controls. At the same time, root and leaf expansion growth, but not dry weight, was significantly reduced. The turgor potentials of leaves from Cd-treated plants were nonsignificantly higher than those of control leaves. A significant increase (almost 400%) of the leaf ABA concentration was detected after 120 hours exposure to Cd. But Cd was found to inhibit ABA accumulation during drying of excised leaves. It is concluded that Cd-induced decrease of expansion growth is not due to turgor decrease. The possible mechanisms of Cd-induced stomatal closure are discussed.

Cadmium is a highly toxic element which has been the center of much research interest during the last 15 years. Studies concerning the phytotoxic effects of Cd principally have been focused on nutritional aspects (25), on enzyme activities (23), or on photosynthesis and related processes (6, 7, 26). Nevertheless, the mechanisms of Cd-induced plant growth inhibition are far from being understood.

Higher plants grown on Cd-containing substrates show disturbed water balance. Effects of Cd on stomatal function (7, 17), water transport (5, 19), and cell wall elasticity (6) have been reported. Cadmium may inhibit leaf cell expansion growth through alterations of the plant water balance. But as the effects of Cd on plant water relations generally have been studied after prolonged exposure, at only one determinate growth stage, the correlation between the effects of Cd on water relations and the Cd-induced decrease of cell size are not clear.

Studies on the mechanisms of Cd-induced inhibition of expansion growth, using complex multicellular higher plants, present severe difficulties. Under long-term exposure to Cd, almost all physiological processes will be affected, and the recognition of primary effects would be impossible. In short-term experiments, when entire plants are exposed to Cd for minutes, or for a maximum of a few hours, unusually high Cd-concentrations in the rooting medium generally are necessary to achieve a detectable growth reduction in upper plant parts. Under those conditions, undesirable side effects due to the imbalanced nutrient solution may occur. Moreover, any extrapolation to pollution effects in the field would be impossible. To cope with these difficulties, in studies on the causes of Cd-induced inhibition of expansion growth in whole higher plants, the selection of an appropriate experimental system is critical.

In the present study we used 10 d old bush bean plants for analyzing the effects of Cd on water relations and growth. The use of this plant material presents two main advantages: (a) Bean plants are highly sensitive to Cd (18). Therefore, a detectable growth inhibition may be achieved after relatively short exposure times, using low Cd concentrations. (b) In the primary leaves of bean plants, at this age, no more cell division occurs (24). So the effects of Cd on cell expansion growth will not be concealed by a Cd-induced impairment of cell division in this organ.

MATERIALS AND METHODS

Plant Material

Bush bean seeds (*Phaseolus vulgaris* L. cv Contender) (Les doigts verts, Catros-Gerand, Bordeaux) were germinated in trays of moist perlite, in a growth room illuminated by cool white fluorescent lamps. The irradiation was around 150 μmol m^{-2} s^{-1} near the primary leaves of the germinated plants. The photoperiod was 16 h light/8 h dark. The day/night temperatures and the day/night relative humidities were 25°C/21°C and 65%/75%, respectively.

Seven d after sowing, the seedlings were selected for uniformity and transferred to plastic beakers (5 L capacity, eight plants per beaker) filled with continuously aerated, modified, half-strength Hoagland nutrient solution. The composition of the nutrient solution was as follows: (in mM) 2.5 KNO_3, 2.5 $Ca(NO_3)_2$, 0.75 $MgSO_4 \cdot 7H_2O$, 0.5 KH_2PO_4; (in μM) 10 Mn as $MnSO_4 \cdot H_2O$, 10 Fe as FeEDDHA (sequestrene), 1 Cu as $CuSO_4 \cdot 5H_2O$, 0.95 Zn as $ZnSO_4 \cdot 7H_2O$, 23 B as H_3BO_3, 0.03

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Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The nutrient solution had a pH of 5 and a solute potential of 0.1 MPa.

After 3 d (plant age from sowing 10 d), the nutrient solution of all beakers was renewed, half of the plants receiving solution supplemented with 3 μM Cd as $CdCl_2 \cdot 2.5\ H_2O$.

The plants were grown for further 144 h. During this time samples were taken every 24 h for the determination of growth Cd-content, water relation parameters, and ABA concentration. If not otherwise stated, the plant material was harvested at the end of the dark period. At the 144 h harvest, a suitable amount of primary leaves of control and Cd-treated plants was excised. These leaves, adaxial surface down, were exposed to ambient air and light on a laboratory bench. After 2 h the water relation parameters and the ABA content were analyzed (146 h harvest). On leaves, excised at the 96 h harvest and exposed to ambient air for 2 h, additional measurements of the ABA content were performed (98 h harvest).

Growth and Cd-Concentration

The fresh weight of the primary leaves and the roots was determined immediately after each harvest. The dry weight of these organs was measured after 48 h desiccation at 70°C. The area of primary leaves was determined with a planimeter (Licor model 3100) and the root length with a ruler.

For Cd-concentration analysis, the oven-dried (70°C) plant material was wet-ashed with an acid mixture (HNO_3:$HClO_4$:H_2SO_4 = 10:1:1) and analyzed by atomic absorption spectrophotometry (Perkin Elmer model 503 with graphite furnace HGA 74).

Given values are the average of three determinations per organ and treatment.

Water Relations

The relative water content of primary leaves was determined on five leaf discs (diameter 9 mm) (16).

The water potential of the primary leaves was determined with a pressure chamber (Arimad 2, Israel). For measurement of the osmotic potential of primary leaves, the harvested plant material was immediately frozen in liquid nitrogen and stored at −40°C. After thawing, the osmotic potential of the expressed sap was determined with a Wescor HR-33 T Dew Point microvoltmeter.

The stomatal resistance of the abaxial surfaces of primary leaves of control and Cd-treated plants was analyzed with a porometer (Delta-T Devices MK 3 automatic transient porometer) on plants illuminated for 75 min.

All water relation parameters were determined at each harvest on six plants per treatment. The samples were taken from plants grown in different beakers.

Analysis of ABA Content

The ABA concentrations of both primary leaves and roots were analyzed by HPLC. The extraction and the quantification processes were as previously described (9). Sample recovery by this extraction method measured with 2-[^{14}C]ABA (Amersham) (25.6 μCi/μL) was 75% (liquid scintillation spectrometer, Beckmann LS 6800). For quantification we used an HPLC equipped with a variable wavelength detector (Spectra Physics SP 8440) set at 254 nm, a solvent delivery system (Spectra Physics SP 8700), an integrator (Spectra Physics SP 4970), a pump organizer (Spectra Physcis SP 8750), and an automatic sampler (Spark Holland, model SpH 125, fix) with a 50 μL loop for sample injection. The column was a Nucleosil C-18 120 A, 5 μm (Knauer). The operation pressure was 22 MPa and the flux was 1 mL/min. As solvent, a mixture of 45% methanol in 55% 0.2 N acetic acid was used. The retention time for ABA was 17 min. Given results are the average of three determinations per organ, treatment and harvest time.

Statistical Treatment

The whole experiment was conducted three times. The data presented here are from a representative experiment. The significance of differences between control and Cd-treated plants was determined by analysis of variance.

RESULTS

The plants grown with Cd-containing nutrient solution exhibited substantially increased Cd concentrations within both roots and leaves. In leaves, the Cd concentrations increased linearly with time up to 120 h after the start of the treatment (Fig. 1a). Roots attained their highest Cd concentration after 96 h of exposure to the metal (Fig. 1b).

Forty-eight h after Cd supply, a significant inhibition of plant growth was observed. The root length, the leaf area and the leaf fresh weight were significantly lower than in controls. The leaf dry weight and the root fresh and dry weights were not significantly reduced until 96 h exposure to Cd (Fig. 2).

In Cd-treated plants the leaf fresh weight was significantly more affected than the leaf dry weight and, from the 48 h harvest, these plants had significantly lower water contents than control plants. At the same time, in the Cd-treated plants lower relative water contents (Fig. 3a) and higher stomatal resistances than in control plants were found (Fig. 4a). Within both control and Cd-treated plants, the relative water content decreased during the period of maximum leaf growth rate (0–48 h). From 48 h to the end of the experiment, a progressive increase of the leaf relative water content was found in all plants (Fig. 3a). The plants exposed to Cd generally showed significantly lower values than control plants.

Although the plants exposed to Cd had lower water contents from the 48 h harvest, their leaf water potential was not significantly affected until 120 h of exposure (Fig. 3b). The solute potential of control plants (Fig. 3c) tended to increase with time. The Cd-treated plants showed lower solute potential values than control plants from 48 h of the start of the Cd-treatment. As a result of their lower solute potential, the turgor potential values (Fig. 3d) of Cd-treated plants were as high, or even nonsignificantly higher than those of control plants.

Although the plants grown in Cd-containing nutrient solution generally presented high leaf turgor, their stomatal resistances were significantly higher than those of control plants from the 48 h harvest (Fig. 4a). Control and Cd-treated plants did not show significant differences between their leaf ABA

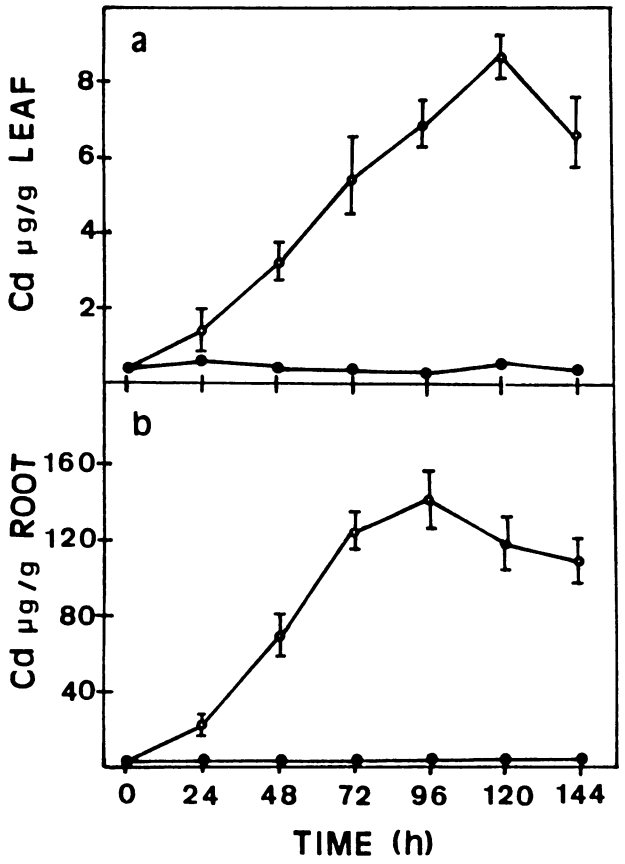

Figure 1. Time courses of Cd concentrations of bush bean plants grown in control (black circles) or 3 μM Cd nutrient solution (white circles). a, Leaf Cd concentrations (μg g^{-1} dry weight); b, root Cd concentrations (μg g^{-1} dry weight).

concentrations up to 96 h (Fig. 4b). At the 120 h harvest, in coincidence with a further increase of stomatal resistance, an almost 400% increase of the ABA concentration was found in the leaves of Cd-treated plants (Fig. 4b).

Significant differences between the root ABA concentrations of Cd-treated and control plants were observed at the 72 h, 96 h and 120 h harvests (Fig. 5). Nevertheless, no correlation between the ABA and Cd concentrations within roots could be established.

At the 146 h harvest, the excised leaves of all plants showed decreased relative water contents and lower water, solute, and pressure potentials. But only in control leaves was a significant increase of the ABA concentration found (Table I). Two h after excision, the leaves from Cd-treated plants, which at the 144 h harvest had high ABA levels, did not show any further rise but a decrease of the ABA level (Table I). A decrease of ABA levels 2 h after excision also was found in leaves from Cd-treated plants of the 96 h harvest. At the 96 h harvest these leaves contained 90 ng ABA g^{-1} fresh weight, whereas 2 h after excision (98 h harvest) only 40 ng ABA g^{-1} fresh weight were found. In control leaves the ABA concentrations were 63 and 139 ng g^{-1} fresh weight at the 96 h and the 98 h harvests, respectively. The excised leaves from both control and Cd-treated plants (146 h harvest) showed almost the same decrease of relative water content, but the turgor potential of the leaves from Cd-treated plants was significantly more decreased than that of control leaves (Table I).

DISCUSSION

The time courses of growth (Fig. 2) demonstrate that leaf expansion growth was earlier affected by Cd than the accumulation of dry weight.

According to the expanded Lockhart equation (13), cell expansion growth is a function of cell wall extensibility, hydraulic conductivity, osmotic potential and the threshold turgor, below which growth will not occur. Any inhibition of the expansion growth rate is due to a change in one or more of these parameters (11). In a strict sense, the Lockhart equation refers only to single cells, but experimental findings by Cosgrove (12) reassure the application to multicellular tissues and organs (13).

Under long-term exposure to high Cd concentrations, the stems of bean plants show increasing resistance to water flow (5), and visible leaf turgor loss occurs (3). But the results from the present study clearly indicate that decreased turgor is not the initial cause of reduced cell size. At the 48 h harvest, Cd-treated plants, in spite of higher stomatal resistance had lower water content than control plants, indicating decreased water flux to the leaves. At the same time, Cd-treated plants showed nonsignificantly higher turgor pressures but lower expansion growth rates than control plants. The observation that, in spite of high turgor the growth rate was decreased may suggest that the Cd supply decreased cell wall extensibility (10). But further studies measuring wall extensibility are required to prove this hypothesis. Moreover, there is no experimental evidence which proves that the decrease of cell wall extensibility would be due to a specific Cd effect in leaves. Other treatments which injure roots, such as root cooling or partial root removing, also induce a decrease of cell wall extensibility in primary leaves of *Phaseolus* (13).

Our results on excised leaves (Table I) also suggest a Cd-induced alteration of cell wall properties. The decreases of the relative water contents in excised leaves from both control and Cd-treated plants were quite similar, but the excised Cd-leaves showed much lower turgor pressure. Thus, for a certain decrease of the protoplast volume, the turgor pressure is maintained higher in control leaves than in Cd-treated ones. This result suggests that the leaves from plants grown with Cd have a higher bulk elastic modulus than control leaves, *i.e.* their cell walls are less elastic. This finding agrees with former observations on plants exposed to higher Cd concentrations for longer times (3, 4).

Cadmium had a marked influence on stomatal resistance. From the 48 h harvest, Cd-treated plants showed higher stomatal resistance than control plants. The rise of stomatal resistance and the decrease of the water content occurred at the same time. But at this harvest, Cd-treated plants had neither significantly higher leaf ABA levels nor lower leaf turgor pressures than control plants. Thus, initially, the stomatal closure seemed neither due to a general leaf turgor loss nor to a change in bulk leaf ABA levels. This early increase of stomatal resistance may be due to either a small pool of active ABA not detectable when bulk leaf ABA is analyzed (21) or other metabolic changes. In addition to ABA,

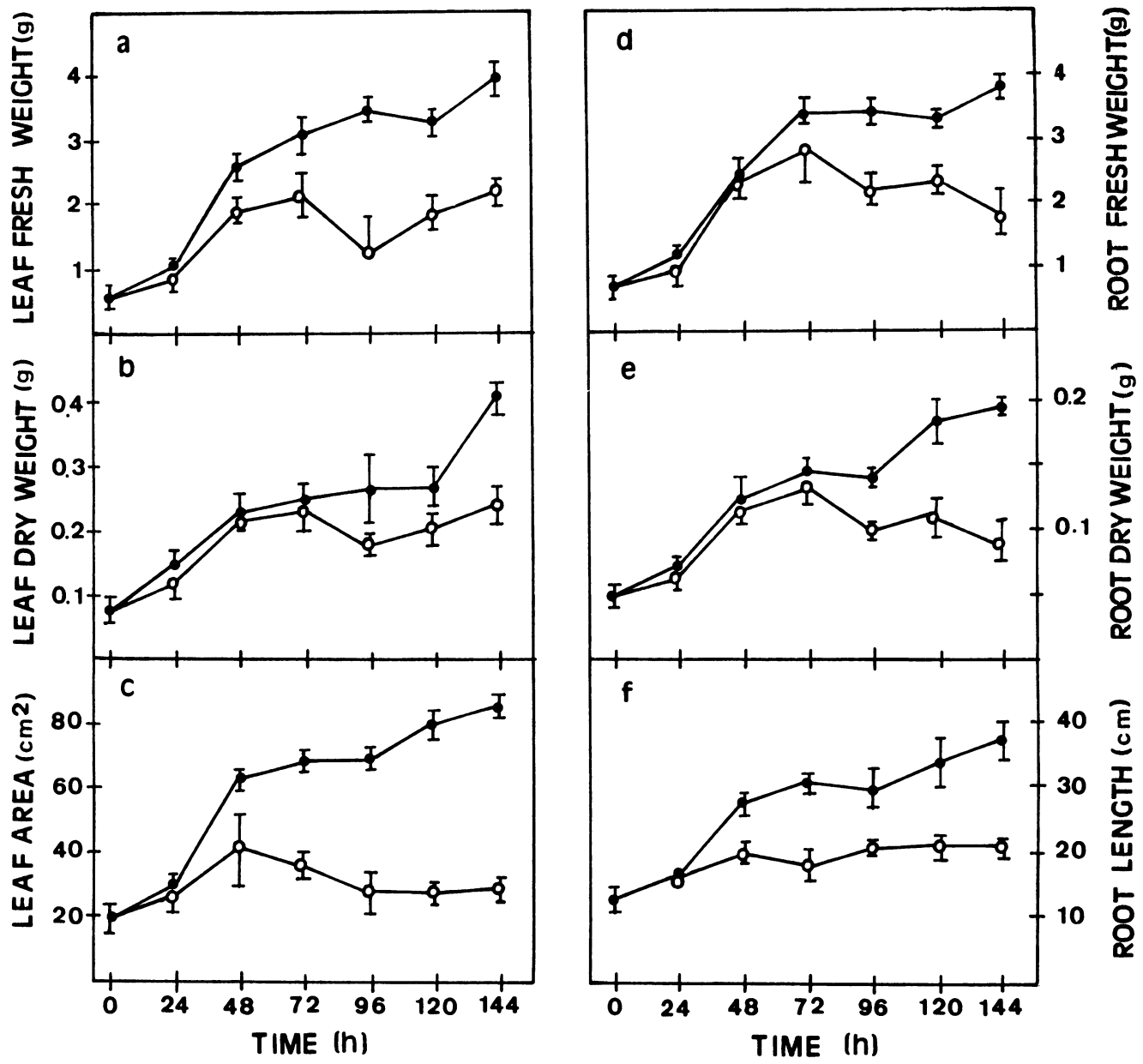

Figure 2. Time courses of root and primary leaf growth of bush bean plants grown in control (black circles) or 3 μM Cd nutrient solution (white circles). a, Leaf fresh weights (g); b, leaf dry weights (g); c, leaf areas (cm^2); d, root fresh weights (g); e, root dry weights (g); f, root length (cm).

other compounds may be involved in stomatal closure. Blackmann and Davies (8) have suggested that a decrease of cytokinin export from roots may restrict stomatal opening. No experimental data on the effects of Cd on cytokinin synthesis or transport are available. But any environmental factor, including the supply of mineral elements, that affects root growth seems to be related to changes in the export of cytokinins towards the shoot (22). In our experiment, a decrease of both root extension growth and stomatal resistance occurred between the 24 and the 48 h harvests.

A direct effect of Cd on the ion and water movement in the guard cells has yet to be proven, but can not be excluded. Both K (15) and Ca (14) play an important role in the regulation of stomatal opening. An interference of Cd with K^+ fluxes has been found in root cells (20). Recent studies in our laboratory suggest that Cd may inhibit the nyctinastic leaf movement of bean leaves by altering the K/Ca molar ratio in the pulvini (MD Vázquez, unpublished data). Provided that toxic amounts of Cd^{2+} reach to the guard cells, an interference of Cd with both K and Ca seems very likely.

The results presented here, in addition to former observations, indicate that in bean plants exposed to Cd the inhibition of stomatal opening may obey to three different mechanisms, depending on both Cd concentration supplied and exposure time, *i.e.* depending on the degree of toxicity suffered by the plants.

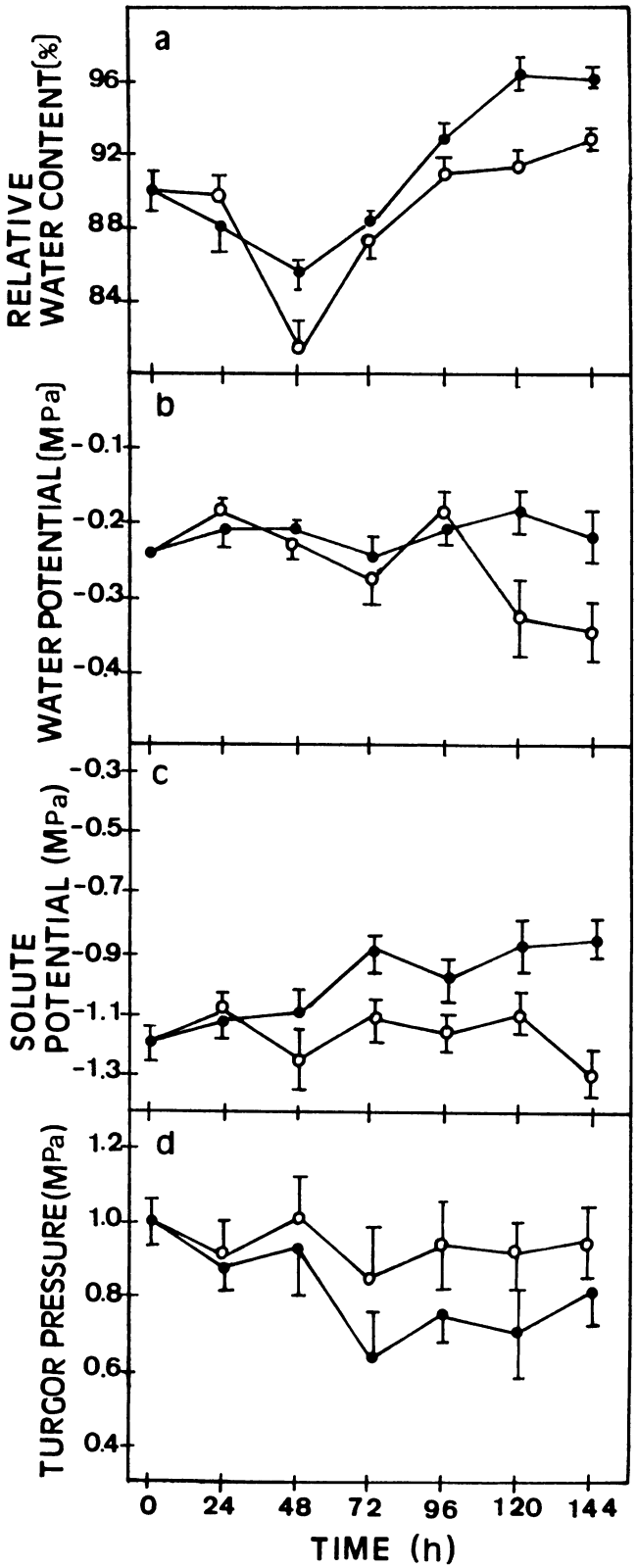

Figure 3. Time courses of water relation parameters in primary leaves of bush bean plants grown in control (black circles) or 3 μM Cd nutrient solution (white circles). a, Relative water contents (%); b, water potentials (MPa); c, solute potentials (MPa); d, turgor pressures (MPa).

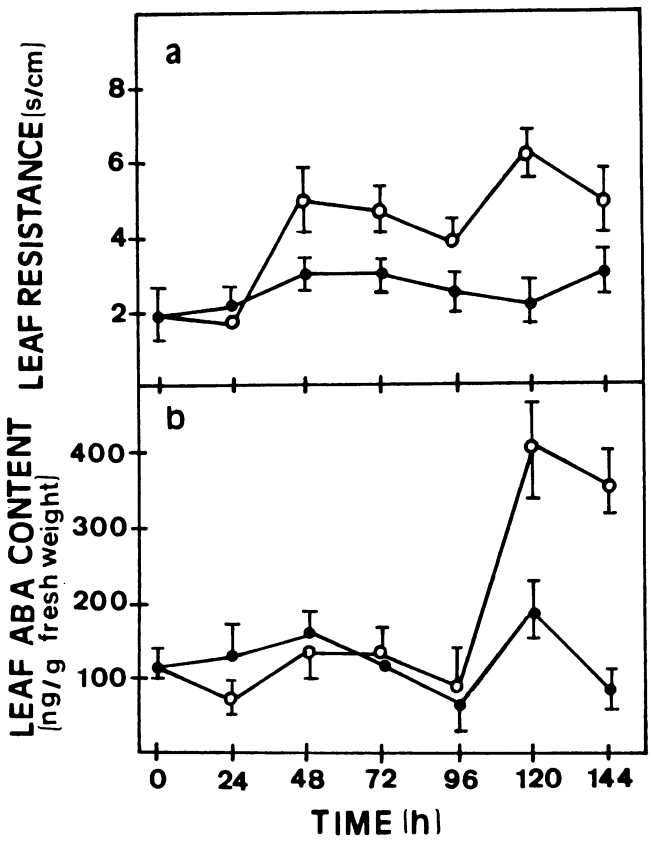

Figure 4. Time course of leaf resistances and leaf ABA levels in primary leaves of bush bean plants grown in control (black circles) or 3 μM Cd nutrient solution (white circles). a, Leaf resistances (s cm^{-1}); b, ABA levels (ng g^{-1} fresh weight).

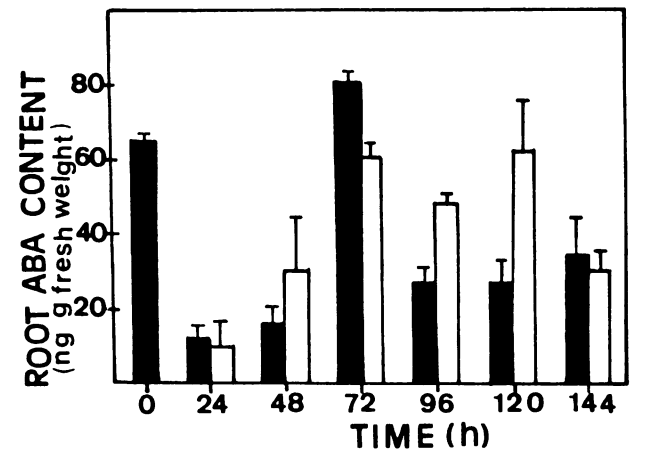

Figure 5. Root ABA contents (ng g^{-1} fresh weight) of bush bean plants grown in control (black columns) or 3 μM Cd nutrient solution (white columns).

Under relatively short exposure to a low Cd concentration, an increase of stomatal resistance occurred without both decrease of leaf turgor and increase of bulk leaf ABA (48 h harvest). This may be due to small changes in the pool of active ABA, effects of Cd on root metabolism, or direct interactions of Cd with guard cells. Longer exposure to Cd (120 h harvest) caused an increase of the bulk leaf ABA level,

Table I. *Effects of Cadmium on Water Relation Parameters and ABA Levels of Bean Leaves*

The plants were grown for 144 h in control or 3 μM Cd nutrient solution. The parameters were determined both immediately after harvest (144 h) and 2 h after excision (146 h). Values listed are the means of six or three (ABA) replications ± SD.

Parameter	Control		3 μM Cd	
	144 h	146 h	144 h	146 h
Relative water content (%)	95.77 ± 0.38	73.87 ± 1.72	92.70 ± 0.14	75.89 ± 2.14
Water potential (Mpa)	−0.27 ± 0.02	−0.71 ± 0.25	−0.33 ± 0.05	−1.05 ± 0.18
Solute potential (MPA)	−1.11 ± 0.29	−1.23 ± 0.15	−1.19 ± 0.16	−1.45 ± 0.10
Pressure potential (MPa)	0.82 ± 0.08	0.52 ± 0.11	1.04 ± 0.14	0.20 ± 0.13
ABA (ng/g fresh weight)	83.7 ± 25.6	267.2 ± 81.0	359.2 ± 84.1	172.9 ± 9.9

leading to further stomatal closure. Under longer exposure to higher Cd concentrations wilting and hydropassive stomatal closure may occur (2).

Many kinds of environmental stress are known to stimulate ABA synthesis, and ABA has been suggested to have a central function in cross-adaptation (1). In our experiment, the time courses of ABA concentrations within both roots and leaves were not consistent with a direct stimulating effect of Cd on ABA production. There was no correlation between Cd and ABA concentrations in roots. Leaves of Cd-treated plants showed increased Cd concentrations soon after the start of the treatment, but their ABA concentration was not significantly increased until the 120 h harvest. Excised leaves from Cd-treated plants did not show any further increase but a decrease of their ABA level during drying. This surprising result agrees with former observations which suggest that Cd may interfere with the ABA metabolism (2). As the intact plants, containing significant amounts of Cd, were able to increase their leaf ABA content (120 h harvest), this decrease of the ABA levels in drying excised leaves remains unexplained.

From our results we may conclude that Cd-induced inhibition of expansion growth of bean leaves primarily was not due to a decrease in turgor. Decreased cell wall extensibility may be a cause of reduced cell expansion. The Cd-induced increase of stomatal resistance was brought about by different mechanisms, depending on the degree of toxicity suffered by the plants. Although direct effects of Cd in leaves might account for both decreased cell expansion growth and increased stomatal resistance, the study of Cd-effects on root metabolism is clearly necessary for understanding the primary causes of Cd-induced growth inhibition.

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