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The Correlation between Physiological and Structural Alterations Induced by Copper and Cadmium Stress in Broad Beans (*Vicia faba* L.)

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ABSTRACT

Physiological and structural alterations in broad beans (*Vicia faba* cv. Giza Blanka) in response to 10^{-5} M $CuSO_4$, 10^{-6} M $CdSO_4$ and a mixture of both solutions were recorded in 15- and 25-day-old plants. These treatments caused significant reductions in shoot height, leaflet area, fresh and dry weights, stomatal frequency, cell area in abaxial epidermis of leaflets, length and width of stomatal guard cells, size of parenchyma in all seedling organs, diameter of metaxylem vessels, concentration of photosynthetic pigments (Chl-a, chl-b, carotenoids), photosynthetic activity, activities of antioxidant enzymes (CAT, POX, SOD), as well as a major re-shuffle of the protein patterns. Treatments causing reductions in these parameters were mostly in the order: Cu+Cd > Cu > Cd. The disappearance of a 25-kDa polypeptide by all heavy metal treatments seems to suggest that they reduce cell enlargement and/or the synthesis of the heavy-metal-binding phytochelatins (PCs) through inactivation of α-expansin and/or a phytochelatin synthase (each with MM 25 kDa), respectively. The outcome of reductions in stomatal frequency, size, leaflet area, efficiency of narrower xylem vessels, and photosynthetic pigments is a significant decrease in photosynthetic activity and, consequently, the observed reductions in growth criteria.

KEYWORDS: antioxidant enzymes, Cd, Cu, Cu+Cd interaction, growth criteria, photosynthesis, proteins, vegetative anatomy, *Vicia faba*.

INTRODUCTION

Essential and non-essential heavy metals are known to cause curtailing of nearly all growth parameters (An 2004) and productivity (Wu *et al*. 2004, Kasim 2005) of plants, with varying degrees of severity. They also cause some structural (Lux *et al*. 2004) and ultrastructural (Papadakis *et al*. 2004) alterations in the vegetative organs of plants. Heavy-metal-induced decreases in cell wall elasticity have also been recorded (Barceló *et al.* 1989).

The physiological effects of heavy metals on plants include: (i) perturbation of stomatal functions leading to changes in water relations and rates of gas exchange (Poschenrieder *et al.* 1989, Papadakis *et al*. 2004); (ii) reduction of photosynthetic pigments and activity (Kasim 2001, Lou *et al.* 2004) and replacing the central atom of chlorophyll (Mg) to produce photosynthetically inactive HM-chlorophylls (Küpper *et al*. 1998), thus diminishing the photosynthetic activity (Alaoui-Sossé *et al.* 2004); and (iii) disruption of the integrity of cellular membranes (Saber *et al.* 1999, Tari *et al.* 2002).

Plants exposed to the impact of toxic levels of heavy metals respond by initiating a number of defense mechanisms. These include: (i) sequestration in cell vacuoles, on cell walls and in intercellular spaces (Souza & Rauser 2003); (ii) chelation with various agents such as the sulphur-rich peptides phytochelatins and metallothionins (Hall 2002); (iii) appropriate changes to the activity of antioxidant enzymes (Clijsters *et al*. 1999); and (iv) the synthesis of stress proteins mostly similar in structure to metallothionins (Prasad 1995, El-Aref & Hamada 1998).

Since heavy-metal-polluted soils and irrigation water usually contain several in mixtures (Adriano 2001), plants are seldom exposed in nature to the impact of a single heavy metal. Interaction between two or more heavy metals has consistently been shown to result in additive or synergistic detrimental effects on plants (Chaoui *et al*. 1997).

The present study was undertaken to analyze the impact of structural alterations induced by Cu (as an essential micronutrient) and Cd (as a non-essential heavy metal), either singly or in mixture, on growth criteria, antioxidant enzyme activities, photosynthetic pigments and activity, and protein patterns of broad beans (*Vicia faba* cv. Giza Blanka), an economically important crop in Egypt.

MATERIALS AND METHODS

Seeds of broad beans supplied by the Ministry of Agriculture (Cairo, Egypt) were selected for uniformity of size and shape, washed in distilled water and sown in pots containing acid-washed quartz sand at 25 ºC ± 1, under 16 h light: 8 h dark**,** and irrigated with distilled water for approximately one week until full germination. This was followed by irrigation every other day for 25 days with half-strength Hoagland solution supplemented with 10^{-5} M copper sulphate, 10^{-6} M cadmium sulphate, or a mixture of both (1:1 v/v). Control plants were grown on half-strength Hoagland solution only. The soil was washed weekly with distilled water. The applied concentrations of heavy metals were chosen after a preliminary experiment in which they caused moderate inhibition of germination.

The growth criteria were recorded in 4 replicate plants for each treatment after 15 and 25 days of germination. These variables were the shoot height (cm), fresh and dry weights per plant (g), and the average leaflet area (cm^2) of all bifoliolate leaves per plant.

For each treatment, 3 plants were selected for anatomical studies. Permanent cross-sections of stems, roots, leaf petioles and leaflet blades of 25-day-old plants were cut above the region of secondary root emergence, across the middle of the second basal internode of stem, and in the middle of the petiole and leaflet blade of the third basal leaf. As a measure of the effect of heavy metal treatments on cell expansion, the parenchyma cell diameter (PCD) in μ was measured in the cortex of the root and stem, in the leaf petiole and in the leaflet midrib. The average diameter of the widest 10 metaxylem vessels (in μm) in the stem and root of each of 3 replicate plants was recorded.

Variations in epidermal features were recorded from peels of the abaxial (lower) epidermis of leaflets of 15- and 25-day-old plants. They represented the epidermal cell frequency (defined as the number of epidermal cells mm^{-2}), length and width of stomatal guard cells (in μm), and stomatal frequency (number of stomata mm^{-2}). The average surface area of an epidermal cell (in μm^2) was calculated as the reciprocal of the epidermal cell frequency, while the total number of stomata/leaflet was calculated as the stomatal frequency x leaflet area in mm^2. Each value of epidermal cell area or stomatal frequency is the mean of 81 counts (3 counts x 3 peels x 3 leaflets x 3 replicate plants).

Leaves of three replicates of 15- and 25-day-old plants from each treatment were used for the determination of photosynthetic pigments (chlorophyll-a, chlorophyll-b, and carotenoids), rate of photosynthetic electron transport (photosynthetic activity), and the activities of catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD). The pigments were measured in mg g^{-1} dry weight according to the method described by Metzner *et al.* (1965). Intact chloroplasts were isolated as described by Osman & El-Shintinawy (1988), and photosynthetic activity in the thylakoids of isolated chloroplasts was measured as a function of photoreduction of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) according to Biswal & Mohanty (1976), and expressed as mM of reduced DCPIP min^{-1} g^{-1} fresh weight.

Antioxidant enzymes were extracted using the method of Beauchamp & Fridovich (1971). CAT (EC 1.11.1.6) and POX (EC 1.11.1.7) activities were assayed according to the method of Kato and Shimizu (1987), while that of SOD (EC 1.15.1.1) was measured following the procedure of Beauchamp & Fridovich (1971). SDS-PAGE of total proteins in 15- and 25-days-old plants was carried out according to the method of Laemmli (1970) as modified by Studier (1973). The resulting gel was scanned using Gel Doc-2001 Bio-Rad densitometric system.

All variables were subjected to two-way ANOVA statistical analysis using the SPSS 12.0.1 package.

RESULTS

Treatments with Cu^{2+}, Cd^{2+} and Cu^{2+}+Cd^{2+} caused a number of measurable alterations in some growth criteria, structural characters and physiological parameters of the vegetative parts of the treated plants. Results of statistical analyses showed that the changes in all parameters measured in the 15- and the 25-day-old plants followed closely similar trends. Except for the SDS-PAGE protein patterns, therefore, only the results from the 25-day-old plants will be presented.

Shoot height, leaflet area and the fresh and dry weights were significantly decreased by all heavy metal treatments (Fig. 1). The severity of the impact of these treatments on shoot height and dry weights is in the order Cu > Cu+Cd > Cd, while the combined treatment with Cu+Cd caused the greatest decrease in fresh weight and leaflet area. The reduction in leaflet area is considerably higher than the corresponding value of reduction in the other growth criteria. There is no evidence of non-additivity in the action of the two heavy metals, except in the case of dry weight, where the presence of cadmium made the impact of copper negligible (Fig. 1). All parameters concerning cell size in stems, roots and leaves showed significant reductions in response to the heavy metal treatments relative to their corresponding control values (Figs. 2 ,3 & 4), where the degree of reduction was mostly in the order: Cu+Cd > Cu > Cd. There was strong evidence of non-additive effects in the action of the two heavy metals on parenchyma cell diameter (Fig. 2), but only non-significant or small interactions for other cell measures (Fig. 3). While stem and root parenchyma lost only 20-23% of their size, reduction in the size of foliar parenchyma ranges between 25-40%. Furthermore, within the two parts of the leaf, the impact on parenchyma of the leaflet midrib was greater than that on cells of the leaf petiole, with Cu exerting the strongest effect on both tissues. Abaxial epidermal cells lost 24, 22 and 33% of their surface area by Cu, Cd and Cu+Cd, respectively. Reductions in length and width of stomatal guard cells are comparable to the decreases in the area of epidermal cells surrounding them. Metaxylem vessels of the root and stem suffered similar reductions in their diameter.

Stomatal frequency and total number of stomata/leaflet in the abaxial epidermis suffered significant reductions in the treated than in the untreated plants (Fig. 4). Severity of reductions was in the order: Cu+Cd > Cu > Cd, with only additive or small non-additive effects of both the two heavy metals.

Figure 5 illustrates highly significant reductions in the concentrations of photosynthetic pigments and in the photosynthetic activity of the 25-day-old plants. As might be expected, the order of severity (Cu+Cd > Cu > Cd) of reductions in all photosynthetic parameters was the same as that of the structural factors contributing to them (such as leaflet area, stomatal frequency, total number of stomata/leaflet, stomatal dimensions, diameter of metaxylem vessels). Chlorophyll levels showed significant non-additive effects of the two heavy metals: when cadmium was present, the impact of copper was reduced.

Under conditions of the present experiment, reductions induced by the heavy metal treatments in the activities of the three antioxidant enzymes catalase, peroxidase, and superoxide dismutase of the 25-day-old plants were highly significant (Fig. 6). There was no evidence of non-additivity in the case of peroxidase. For superoxide dismutase there was a very strong interaction, showing as in other measures that in the presence of cadmium, the impact of copper was much educed. For catalase there was a significant and different interaction, whereby the presence of cadmium increased the impact of copper.

The scanned image of the SDS-PAGE (Fig. 7) shows an array of proteins with molecular masses ranging between 14.06 kDa and 124.42 kDa. Some of these proteins (58.28-59.01, 44.60, 42.81, 25.63, and 14.06-14.82 kDa) are restricted to 15-day-old plants and disappear with age, while others (104.08 - 109.05, 96.49 - 97.40, 54.35 - 55.12, 18.91-19.54 and 15.21-15.62 kDa) are synthesized anew in the 25-day-old plants.

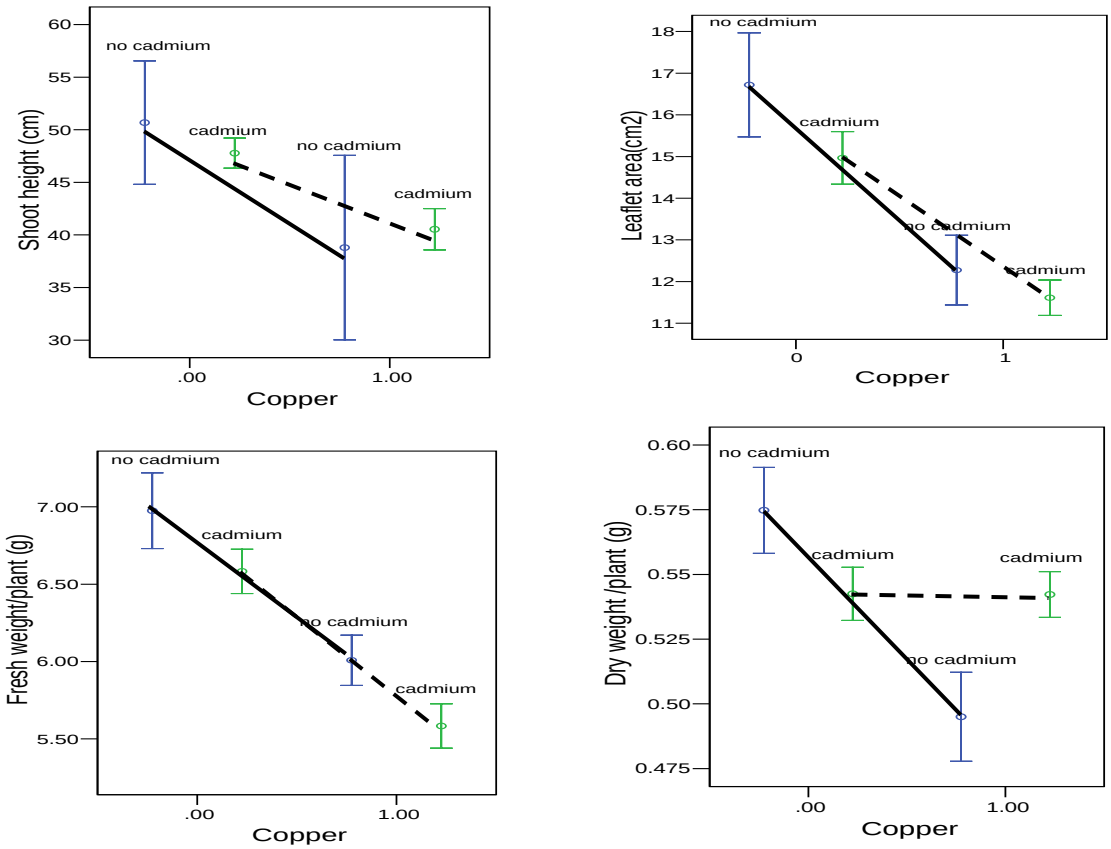

Figure 1 : Effects of Cu^{2+}, Cd^{2+} , and Cu^{2+} + Cd^{2+} on growth criteria of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

$F_{1,12}$ values (** = significant at p≤0.001; * = significant at p≤0.05; n.s. = non-significant)

Treatment	Shoot height	Leaflet area	Fresh weight	Dry weight
Cu	31.5**	398**	307**	85.8**
Cd	n.s.	38.2**	52.9**	n.s.
Cu+Cd	n.s.	7.7*	n.s.	84.7**

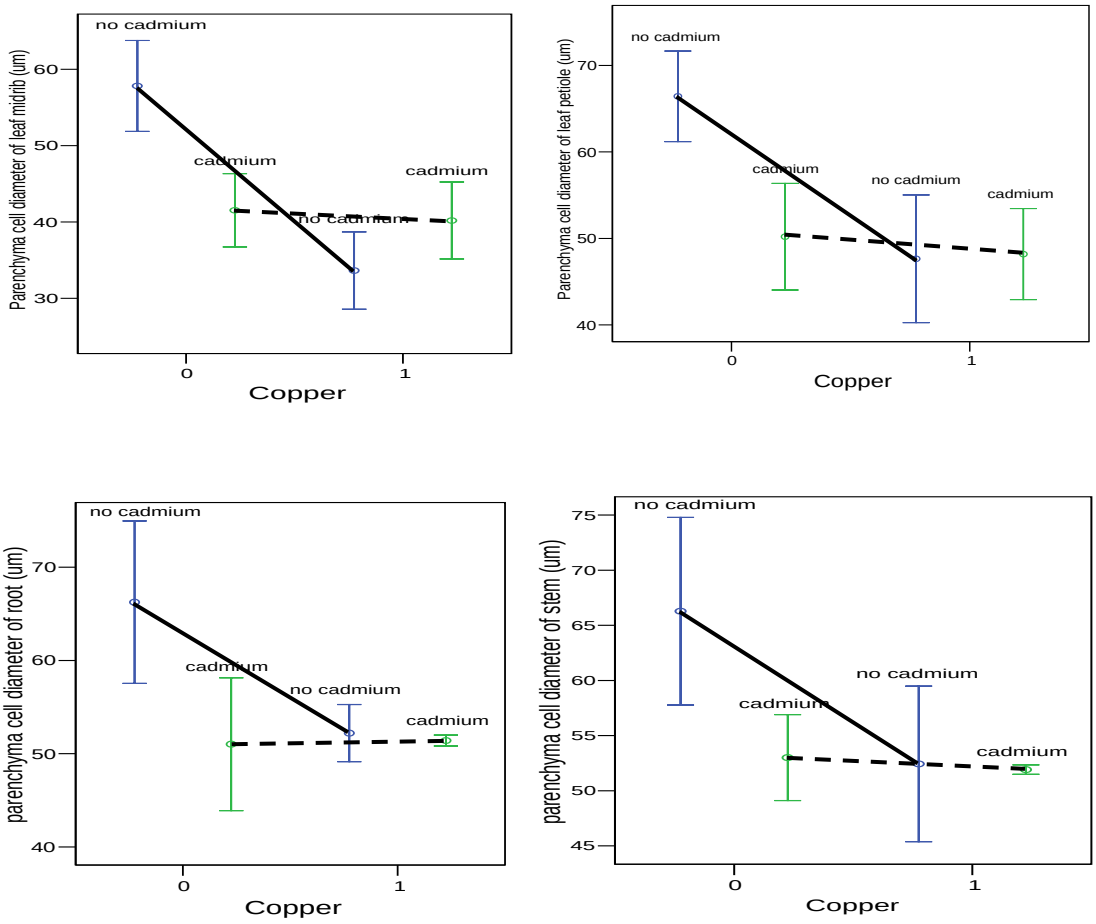

Figure 2 : Effects of Cu^{2+}, Cd^{2+}, and Cu^{2+} + Cd^{2+} on parenchyma cell diameter of of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

$F_{1,8}$ values (** = significant at p≤0.001; * = significant at p≤0.05)

Treatment	Parenchyma Cell Diameter			
	Leaf midrib	Leaf petiole	Root	Stem
Cu	110**	54.2**	25.3**	30**
Cd	16*	30.9**	34.8**	25.6**
Cu+Cd	88.1**	35.2**	28.3**	21.9*

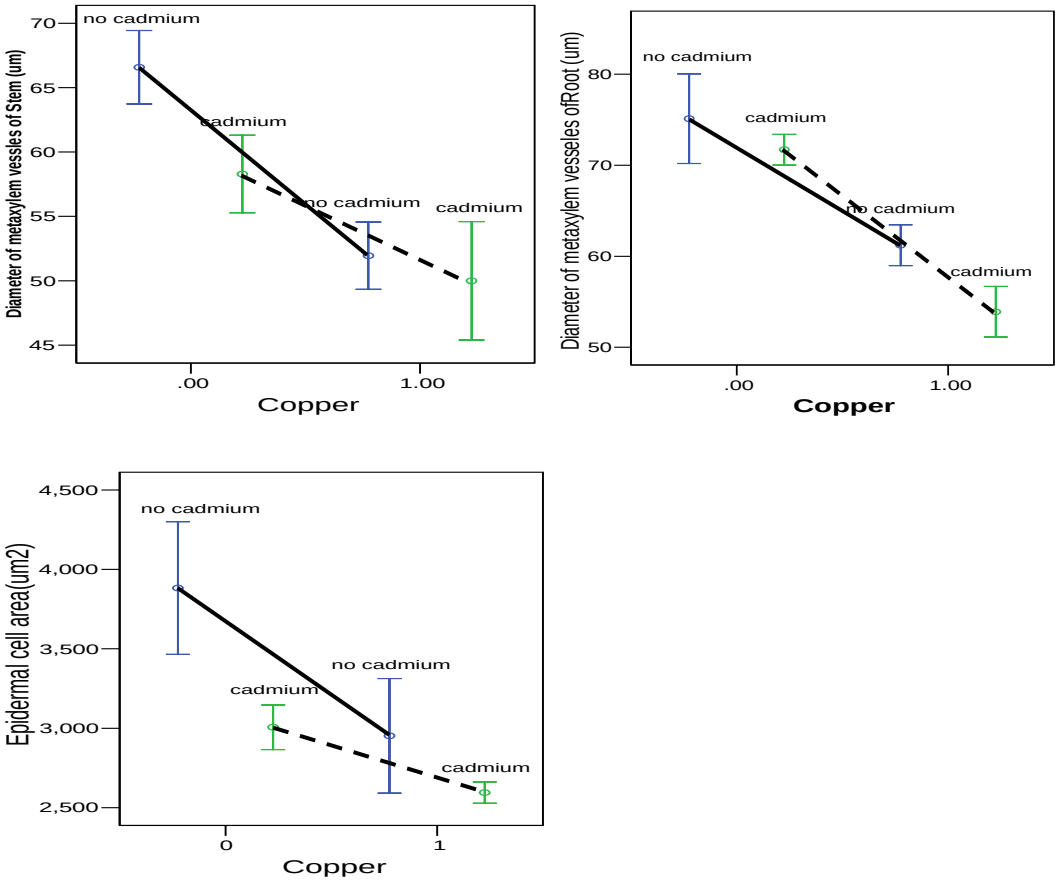

Figure 3 : Effects of Cu^{2+}, Cd^{2+}, and Cu^{2+} + Cd^{2+} on diameter of metaxylem vessel in stem and root and on epidermal cell area of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

	$F_{1,8}$ values (** = significant at p≤0.001; * = significant at p≤0.05; n.s. = non-significant)		
Treatment	Diameter of Stem	Metaxylem vessel Root	Epidermal cell area
Cu	59.5**	129.1**	101.4**
Cd	11.9**	14.8**	85.7**
Cu+Cd	4.6*	n.s.	15.2*

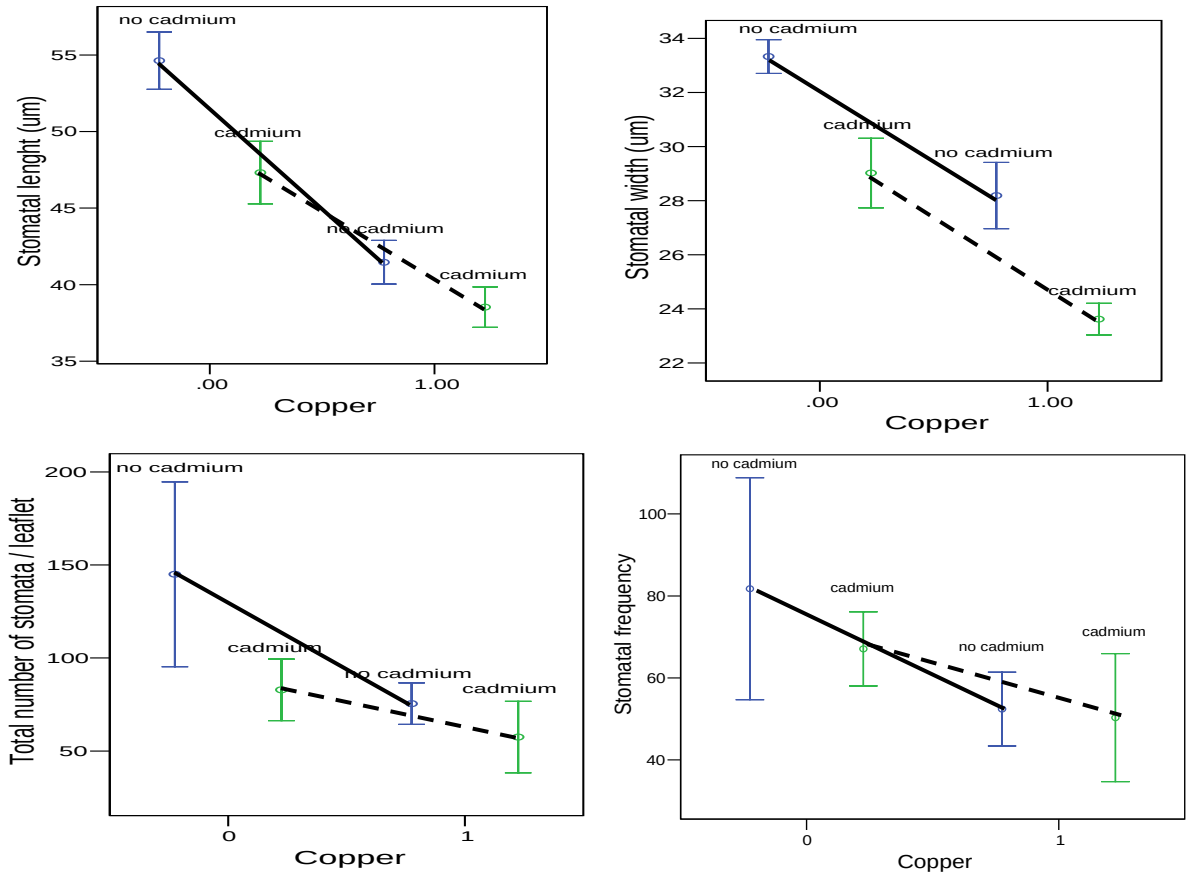

Figure 4 : Effects of Cu^{2+}, Cd^{2+}, and Cu^{2+} + Cd^{2+} on length and width of stomatal guard cells, stomatal frequency and the total number of stomata/leaflet (no. $x10^{-3}$) of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

$F_{1,8}$ values (** = significant at $p \leq 0.001$; * = significant at $p \leq 0.05$; n.s. = non-significant)

Treatment	Stomatal Length	Stomatal Width	Stomatal Frequency	Total number/leaflet
Cu	215.1**	145.9**	34.6**	51.6**
Cd	46.8**	103.4**	n.s.	36.7**
Cu+Cd	8.6*	n.s.	n.s.	11.2*

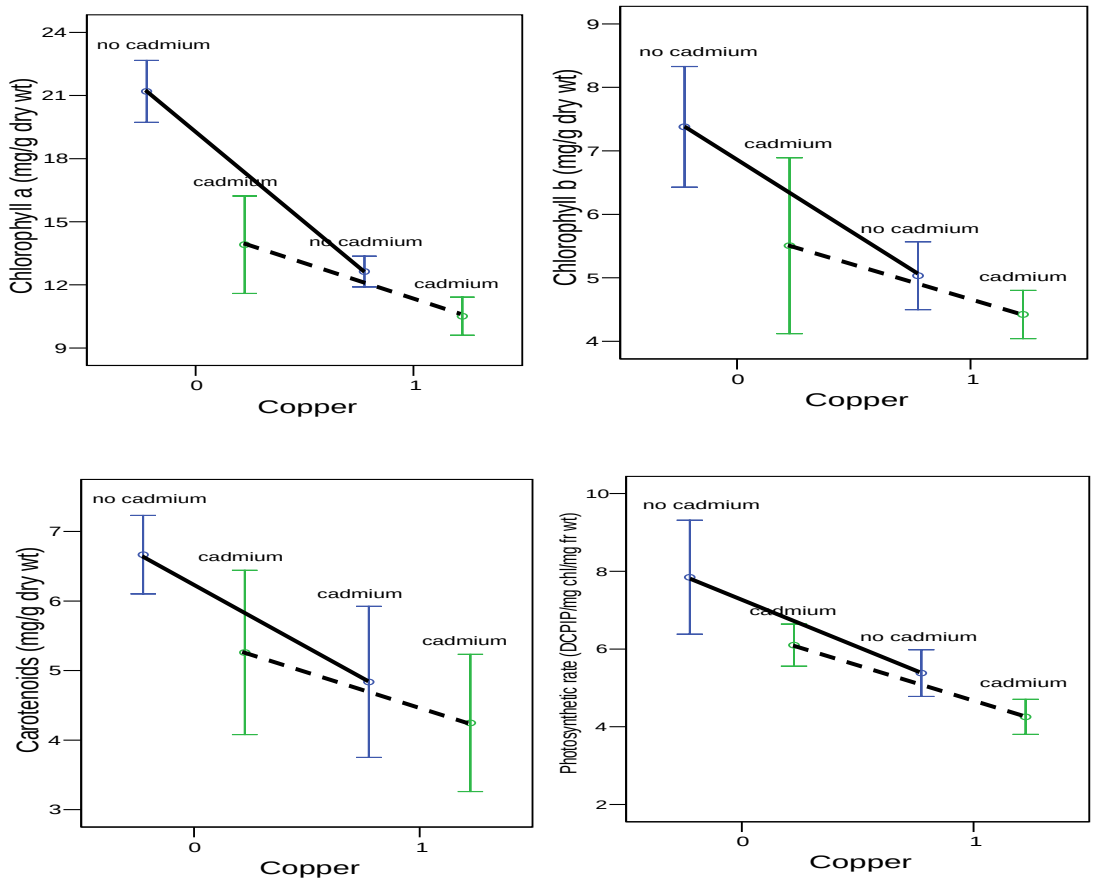

Figure 5 : Effects of Cu^{2+}, Cd^{2+}, and Cu^{2+} + Cd^{2+} on the concentration of the photosynthetic pigments and the rate of photosynthetic activity of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

Treatment	$F_{1,8}$ values (** = significant at p≤0.001; * = significant at p≤0.05; n.s. = non-significant)			
	Chl-a	Chl-b	Carotenoids	Photosynthetic rate
Cu	298.2**	66.9**	38.73**	114.5**
Cd	184.5**	35.1**	19.1*	50.8**
Cu+Cd	55.5**	9.1*	n.s.	n.s.

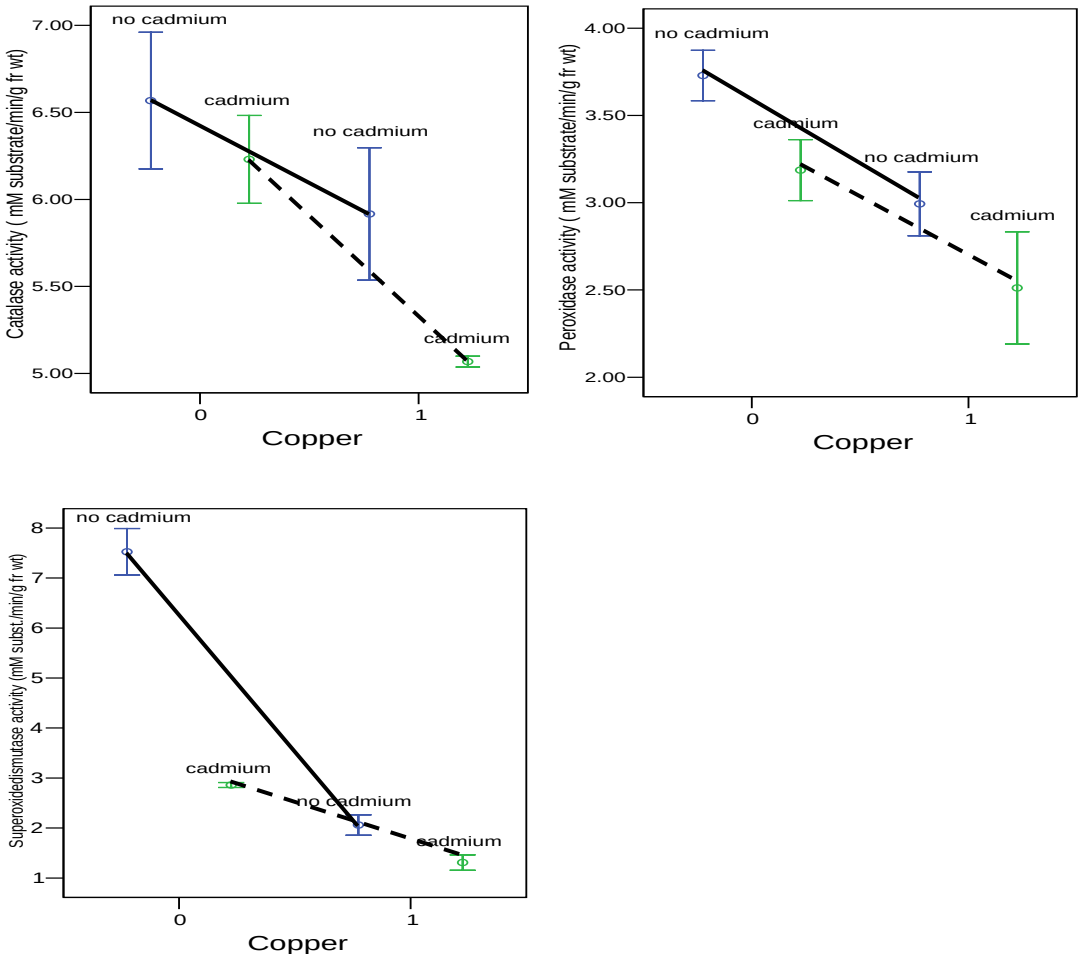

Figure 6 : Effects of Cu^{2+}, Cd^{2+}, and Cu^{2+} + Cd^{2+} on the activity of antioxidant enzymes of 25-day-old *Vicia faba* cv. Giza Blanka plants. Results of 2-way ANOVA statistical analysis are given in tabular form.

	$F_{1,8}$ values (** = significant at p≤0.001; * = significant at p≤0.05; n.s. = non-significant)		
Treatment	Catalase	Peroxidase	Superoxide dismutase
Cu	168.6**	195.1**	3190**
Cd	71.1**	103 **	1902**
Cu+Cd	13.3*	n.s.	993**

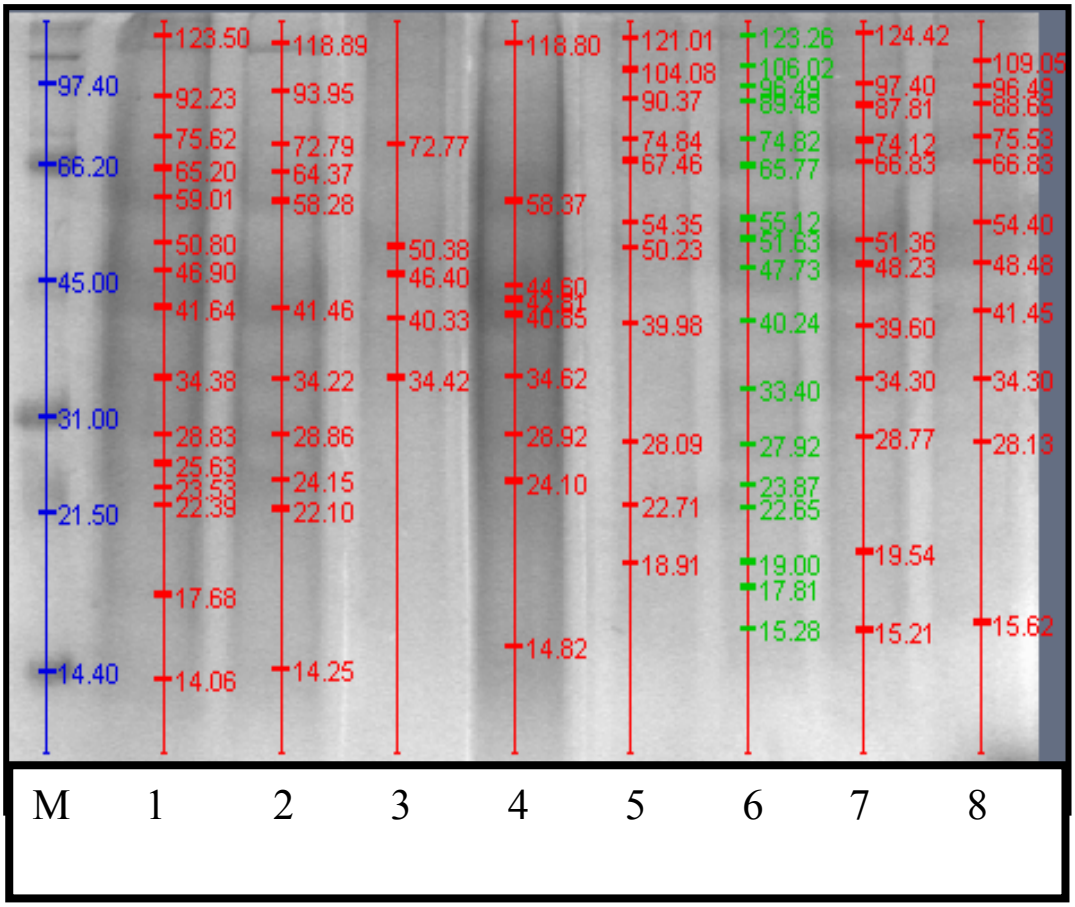

Figure 7: Protein banding patterns of 15- (lanes 1-4) and 25- (lanes 5-8) days-old plants of *Vicia faba* cv. Giza Blanka treated with 10^{-5} M Cu^{2+}, 10^{-6} M Cd^{2+} and Cu^{2+}+Cd^{2+} (1:1 v/v) as revealed by SDS-PAGE. Lanes 1 & 5, 2 & 6, 3 & 7, 4 & 8 represent the control, Cu^{2+}, Cd^{2+} and Cu^{2+}+Cd^{2+} treatments, respectively. Molecular masses of individual bands as determined by the densitometer are printed on their respective lanes. M = marker.

DISCUSSION

The limitations of stomata on photosynthesis are well-documented (Mediavilla *et al.* 2002). Therefore, reductions in stomatal dimensions and frequency acquire greater physiological significance when coupled with reductions in leaflet area (Fig. 1). The outcome of both reductions may lead to an increase in the detrimental effect on transpiration, gas exchange and photosynthesis. This is clearly illustrated in Fig. 3, where it can be seen that the reductions caused by Cu, Cd and Cu+Cd in the total number of stomata/leaflet relative to the control values are much greater than those caused by the same treatments in the stomatal frequency. The photosynthetic implications indicated by the effects of Cu, Cd and Cu+Cd on leaflet area, stomatal dimensions and stomatal frequency are clearly substantiated by the measured reductions of photosynthetic pigments (chl-a, chl-b, carotenoids) and the rate of electron transport (Fig. 4).

The changes in antioxidant enzyme activities in response to heavy metal stress are known to be dependent on heavy metal concentration (Shah *et al.* 2001). Activities of these enzymes might increase in order to cope with the oxidative stress imposed by heavy metals on plants, as was repeatedly found in other experiments (Ianelli *et al.* 2002). Alternatively, they might be diminished if the toxic effects of higher concentrations of heavy metals were greater than can be tolerated and combated by the antioxidant enzymes, as is the case in the present experiment. The decreases in antioxidant enzyme activities may result in the accumulation of reactive

oxygen species (which can cause severe damage to thylakoid membranes; Sandalio *et al.* 2001); thus leading to the recorded reductions in photosynthetic pigments and activity as shown in Fig. 4.

The disappearance of some proteins and the *de novo* synthesis of others, in response to heavy metal treatments, indicated that such treatments are highly effective in causing a major re-shuffle of the protein profiles of broad bean plants (Fig. 6). A similar conclusion was reported by El-Aref & Hamada (1998) who revealed 6 protein bands (58.99, 44.62, 42.83, 40.75, 25.78 and 14.78 kDa) as a result of Cu stress in three tomato genotypes, with either identical or closely similar molecular mass to 6 of the proteins recognizable in Fig. 6. Three of these proteins (44.60, 42.81 and 14.82 kDa) were assessed in the 15-days-old bean plants in response to Cu+Cd treatment. None of the proteins shown in Fig. 6 was purified and characterized. However, according to Prasad (1995), the heavy-metal-binding polypeptides called phytochelatins, whose molecular mass is mostly in the region of 7-18 kDa, are synthesized in higher plants by a 25 kDa protein PC synthase (γ-glutamyl-cysteine dipeptidyl transferase). It is remarkable that the only protein in Fig. 6 which disappears from the control 15-day-old plants in response to the applied heavy metal treatments has a molecular mass of 25.63 kDa. This assumption might be further interpreted on the basis that PC synthase is a constitutive enzyme requiring post-translational activation by some heavy metals including Cu and Cd (Schat *et al.* 2002). The proteins having molecular masses of 96.49 and 97.40 kDa might represent molecular chaperons of HSP 90 (Bray *et al.* 2000), which interfere in sequestration of heavy metals from metabolically active sites and storage in inactive compartments (Cosio *et al.* 2004). In this instance, Polle & Schützendübel (2004) stated that despite different uptake routes and properties of Cu and Cd, both stimulate the same signaling cascades induced by abiotic stress factors as drought, heat and oxidative damage. Herein, it might be tentatively suggested that the occurrence of the 96.49, 97.40 proteins, in response to Cu and Cd, would have resulted indirectly from a metal-induced oxidative stress as shown in certain hyperaccumulating plants (Freeman *et al.* 2004).

Furthermore, the molecular mass of the mature protein α-expansin responsible for 'loosening' of cell walls and cell enlargement is ~25 kDa (Cosgrove 1999). The molecular mass (25.63 kDa) of the only protein found in the untreated 15-day-old plants that disappears with age and in response to heavy metal treatments is remarkably close to that of α-expansin. Therefore, it is tempting to suggest that the irreversible reduction in cell size of all cell types in broad bean plants by the heavy metal treatments used in this experiment might well be the result of the inhibition of a phytochelatin synthase and/or a member of the α-expansin family.

Sizes of all cell types are reduced, probably through irreversible inhibition of proteins regulating the processes of cell wall extensibility. Narrower xylem vessels would have a reduced capacity to transport water and other solutes. Together with reduced turgor, smaller and fewer stomata would be less efficient in transpiration and gas exchange. The combination of reduction in the photosynthetic pigments and diminished efficiency of the stomatal apparatus would lead to a marked reduction in the photosynthetic activity. Hence, reductions in dry matter accumulation become a natural consequence.

It is evident that each of Cu and Cd can separately cause significant reductions in most of the recorded structural parameters with detrimental physiological consequences. However, when in combination the impact of the two heavy metals varies between strongly non-additive (where the presence of Cd tended to partially ameliorate the effect of Cu), to clearly additive. This additive effect was clear in the case of shoot height, fresh weight, leaf area and the activity of the two antioxidant enzymes POX and CAT, indicating a similarly additive effect on the water content of the seedlings. Various degrees of non-additive impact were evident in the case of dry weight, parenchyma cell diameter, metaxylem vessel diameter, stomatal

frequency, photosynthetic pigments and activity, and the antioxidant enzyme SOD. This non-additive effect on the factors involved in the photosynthetic processes (i.e. photosynthetic pigments, metaxylem vessel width, and the stomatal apparatus) is ultimately reflected as a similar non-additive impact on dry matter accumulation.

It might be concluded that exposure of *Vicia faba* cv. Giza Blanka seedlings to toxic levels of Cu and Cd, either singly or in combination, triggers a number of closely inter-related structural and functional events in the stressed plants.

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الملخص العربى

العلاقة بين التغيرات الفسيولوجية والتركيبية الناجمة عن الاجهاد بالنحاس والكادميوم في نبات الفول صنف جيزة بلانكا

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تم تسجيل التغيرات الفسيولوجية والتركيبية الناجمة عن معاملة نبات الفول صنف جيزة بلانكا عند عمر 15 يـوم و 25 يوم بمحاول كبريتات النحاس (10^{-5} مولار) أو كبريتات الكادميوم (10^{-6} مولار) أو بمخلوط منهما معاً. أحـدثت هـذه المعاملات انخفاضات معنوية في طول الساق، مساحة سطح الوريقة، الوزنين الطازج والجاف للنبات، معدل الثغور على السطح السفلي للوريقات، مساحة سطح خلية البشرة السفلي للوريقة، طول وعرض الخلتين الحارستين للثغر، حجم الخلايا البرانشيمية في كل أجزاء جسم النبات، قطر أوعية الخشب التالي في الجذر والساق، تركيز أصـباغ البنـاء الـضوئي (كلوروفيل-أ، كلوروفيل-ب، الكاروتينويدات)، نشاط البناء الضوئي ونشاط ثلاثة مـن الإنزيمـات المـضادة للأكـسدة (الكاتاليز، البيروكيديز وفوق أوكسيد الديزميوتيز)، كما تسببت في تغير كبير في أنماط البروتينات. وكان ترتيـب هـذه المعاملات من حيث شدة إحداثها لهذه التغيرات غالباً هو: كادميوم + نحاس > نحاس > كادميوم. ويشير اختفاء البروتين ذو الوزن الجزيئي 25 كيلودالتون نتيجة لكل المعاملات بالعناصر الثقيلة إلى أنها سببت النقص في حجـم كـل أنـواع الخلايا و/أو في تخليق الفيتوكيلاتين الذي يقيد هذه العناصر عن طريق تثبيط البروتين ألفا إكسباندسين (الـذي يـسبب استطالة جدر الخلايا) و/أو تثبيط تخليق الإنزيم فيتوكيلاتين سينثيز الذي يحفز تخليق الفيتوكيلاتين (ولكـل منهمـا وزن جزيئي 25 كيلودالتون)، على التوالي. والمحصلة الإجمالية للنقص في معدل وحجم الثغور ومساحة الوريقات والكفـاءة التوصيلية لأوعية الخشب التالي الضيقة وفي محتوى النبات من أصباغ البناء الضوئي هي النقص الكبير في معدل البناء الضوئي ومن ثم في مقاييس نمو النبات.