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Varietal differences for cadmium-induced seedling mortality, foliar toxicity symptoms, plant growth, proline and nitrate reductase activity in chickpea (*Cicer arietinum* L)

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Abstract
Cadmium is a highly toxic, metallic soil contaminant, having no metabolic use, which adversely affects the plant growth especially at early stages and has resulted in the loss of crop productivity. The objective of this study was to find varietal differences in Chickpea (*Cicer arietinum* L.) for seedling survival, growth, foliar toxicity symptoms i.e. leaf chlorosis and leaf necrosis, proline and nitrate reductase activity of six cultivars namely ICC 1069, ICC 12422, ICC7589, ICC 4969, ICC 4835 and ICC 4958. The pots arranged in glass house, were supplied with 0, 25, 50 and 100 mg Cd kg^{-1} soil in the form of cadmium chloride and assessed for the mentioned characteristics on 30 and 60 days after each treatment. With substantial varietal difference, applied Cd enhanced the seedling mortality more notably at higher levels. Leaves of all the varieties showed increased signs of chlorosis and necrosis, stunting of shoot and reduction of dry matter and leaf area per pot. Drawing parallels between various symptomatic and growth attributes revealed that Cd-sensitivity of chickpea in survival and growth of plants was closely related to enhanced chlorosis and necrosis of leaves, reduced number and area of leaves per pot. Changes have occurred in the physiological and biochemical activities which are observed even at low Cd levels (25 mg Cd kg^{-1}). Cd has imposed drastic decrease in nitrate reductase whereas Cd treated plant tissues showed an increase in proline. This study concludes that cadmium stress causes reduction in physiological and biochemical activities which ultimately affects plant survival and inhibits growth. This intensity of damage was proportionate with the concentration of the metal. Values for the observed parameters increased as the growth of seedling progressed on 30 and 60 days of sampling.

Keywords: Cadmium; Chickpea; Foliar Toxicity Symptoms; Seedling Mortality.

Introduction

Over the last few years, heavy metals have received considerable attention as a consequence of increasing environmental pollution from industrial, agricultural, energy and municipal wastes. Their non-degradability creates a hazard when they are discharged into organisms (Ammar et al., 2007).

Cadmium (Cd) is one of the highly toxic metal pollutants present in the environment (Wagner, 1993). Although Cd occurs naturally in low concentration in agricultural soils, its level has been steadily increased due to the application of sewage sludge, city waste and Cd-containing phosphatic fertilizers (Williams and David, 1973). Cadmium inhibits root and shoot growth and yield production, affects nutrient uptake and homeostasis, and is frequently accumulated by agriculturally important crops and then enters the food chain with a significant potential to impair animal and human health (Toppi and Gabbrielli, 1999; Baker et al., 1994).There is general agreement that high Cd concentration retards metabolic activities and causes plant death (Toppi and Gabbrielli, 1999), inhibits chlorophyll synthesis, activity of PS I and PS II (Bharadwaj and Marcarenhas, 1989; Wojcik and Tukiendorf, 2005; Khan et al., 2006; Mobin and Khan, 2007). Contrarily, low Cd concentration has been reported to stimulate growth (Gussarsson, 1994; Arduini et al., 2004). The reduction of biomass by Cd toxicity could be the direct consequence of the inhibition of chlorophyll synthesis and photosynthesis (Padmaja et al., 1990). Excessive amount of Cd may also cause decreased uptake of nutrient elements, inhibition of various enzyme activities, induction of oxidative stress including alterations in the enzymes of the antioxidant defense system (Sandalio et al., 2001).

The sensitivity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms such as (i) uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents; (ii) efflux of heavy metals from cytoplasm to extranuclear compartments including vacuoles; (iii) complexation of heavy metal ions inside the cell by various substances, for example, organic acids, amino acids, phytochelatins, and metallothioneins; (iv) accumulation of osmolytes and osmoprotectants and induction of antioxidative enzymes; (v) activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (Cho et al., 2003).

Indeed, heavy metals are known to involve a breakdown of cells membrane lipid due to the increased accumulation of reactive oxygen species (ROS) mediated-oxidative stress (Rellan-Alvarez et al., 2006). ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Cakmak, 2000), may lead to unspecific oxidation of proteins and membrane lipids, may lead to unspecific oxidation of proteins and membrane lipids (Cho and Seo, 2005).

Unlike redox-active metals, like Cu, Fe, etc., Cd is unable to induce the production of ROS through a Fenton-like reaction. It can induce oxidative stress indirectly by causing a disturbance in chloroplasts (Baszynski et al., 1980). Thus, Cd causes degradation of chlorophyll and carotenoids as well as inhibition of their biosynthesis (Bazzaz et al., 1992), which can result in disturbances in electron transport rates of PS I and PS II leading to the generation of oxygen free radicals (Asada et al., 1991). Moreover, Cd is known to induce a transient loss of antioxidative capacity, perhaps accompanied by a stimulation or inhibition of oxidant-producing enzymes, resulting in the generation of ROS (Sandalio et al., 2001; Smeets et al., 2005).

Available reports show marked differences for Cd-tolerance in various plant species including wheat (Zhang et al., 2002), cotton (Wu et al., 2004), pea (Metwally et al., 2005) and rice (Wu et al., 2006). This is because Cd is readily bioavailable during initial hours of exposure (Martin and Kaplon, 1998) and its hyperaccumulation causes the leaf chlorosis (Baryla et al., 2001; Smeets et al., 2005; Wang and Zhou, 2006). Such symptoms on various plant parts directly determine the severity of stress, and therefore may be useful in the diagnosing stress effects and adopting appropriate strategies to increase stress tolerance (Ahmad et al., 2005).

Despite scattered information existing (Rout et al., 1999), varietal differences in chickpea for Cd-tolerance, merits thorough investigation with specific focus on foliar toxicity symptoms and seedling survival. We surmise that symptoms of Cd-toxicity and seedling survival have close association with each other and determine the final plant stand. In view of this hypothesis, this study reports association of Cd-induced foliar chlorosis, necrosis and seedling mortality in some elite varieties of chickpea which is an ancient crop of India and contains 18-20% protein, 62% carbohydrate, 4% fat and is a rich source of Ca, Fe and niacin.

Materials and Methods

The seeds of different cultivars of chickpea (*Cicer arietinum* L.) were obtained from National Seed Corporation Ltd., New Delhi. Seeds were surface sterilized with 0.1% sodium hypochlorite solution for 10 min. and rinsed with distilled water. Seeds of all the six cultivars namely ICC1069, ICC12422, ICC7589, ICC4969, ICC4835 and ICC4958 were sown in 10-inches diameter earthen pots containing thoroughly mixed soil and organic manure in 3:1 ratio. The soil was treated with 0, 25, 50 and 100 mg $CdCl_2$ kg^{-1} soil. The treatments were arranged in a randomized design with three replicates and five plants per pot. The pots were kept in a naturally illuminated greenhouse. Distilled water was used to water the plants as and when required.

All determinations were made 30 and 60 days after Cd treatments were applied. Number of surviving plants was counted to determine the seedling survival. Chlorosis and necrosis of leaves were quantified and expressed as percentage of total area. Number of green photosynthetic leaves was counted on each plant. Leaf area per plant was determined of intact plant by taking the image of leaf on a paper and area determined subsequently on a leaf area meter (LA-211, Systronics, India). Shoot length was measured on meter scale. Shoot dry weight was recorded by drying the plants in an oven at 80°C till constant weight. Proline content was estimated following the procedure used by Bates et al. (1973) whereas the nitrate reductase activity (NR activity) was determined in fresh leaf samples by the procedure explained by Jaworski (1971).

Analysis of variance was performed using SPSS computer package (SPSS v 17) to find differences among chickpea varieties. Cd treatments and their interactions, trend lines and correlation coefficients were drawn of the degrees of leaf chlorosis and necrosis with shoot dry matter.

Results

Data revealed significant ($P \leq 0.01$) differences among varieties, Cd-levels and their interactions for seedling survival and symptoms of leaf chlorosis and necrosis. Higher Cd-levels were more damaging than the lower ones. Among the varieties, ICC4958 exhibited the lowest seedling survival, while it was the greatest in ICC1069 followed by ICC12422 on both 30 and 60 days of sampling (Fig. 1). Scattered signs of chlorosis and necrosis on the leaf lamina, between the veins and marginal chlorosis on leaves were well evident although leaf chlorosis was with greater frequency than necrosis. Leaf chlorosis and necrosis were nominal in control plants of all the varieties. However, applied Cd produced these symptoms in all the varieties, being substantially greater in ICC4958 than ICC1069 at 100 mg kg^{-1} $CdCl_2$ on both the samplings (Fig. 1).

Shoot length and its dry mass, and number and area of leaves per pot indicated significant ($P \leq 0.01$) differences among varieties and Cd-levels with an interaction ($P \leq 0.01$) of these factors for shoot dry mass and leaf area per pot but no interaction was evident for shoot length and number of leaves. Under control conditions, maximum shoot length and shoot dry weight were produced by ICC1069 followed by ICC12422, while a lowest one by ICC4958. However, at 100 mg kg^{-1} Cd level these attributes were reduced in all the varieties being the lowest in ICC4958, but highest on ICC1069 on both 30 and 60 days of sampling (Fig. 2).The number of leaves per pot and leaf area did not differ much under control, but was reduced in all the varieties, although substantial varietal difference was evident. At highest Cd-level, maximum leaf number and leaf area was evident in ICC1069 whilst the minimum in ICC4958 at both 30 and 60 days of growth stage (Fig. 3).

The level of proline in leaves shows an increasing trend at 25 and 50 mg Cd kg^{-1} soil and is found to be maximum at 100 mg Cd kg^{-1} soil in all the varieties at both 30 and 60 days of growth stage (Fig. 4). The order of performance of cultivars in terms of percent increase was ICC1069 > ICC12422 > ICC7589 > ICC4969 > ICC4835 > ICC4958. Under control conditions, maximum nitrate reductase activity (NRA) was produced by ICC1069 followed by ICC12422, while a lowest one by ICC4958 (Fig. 4).

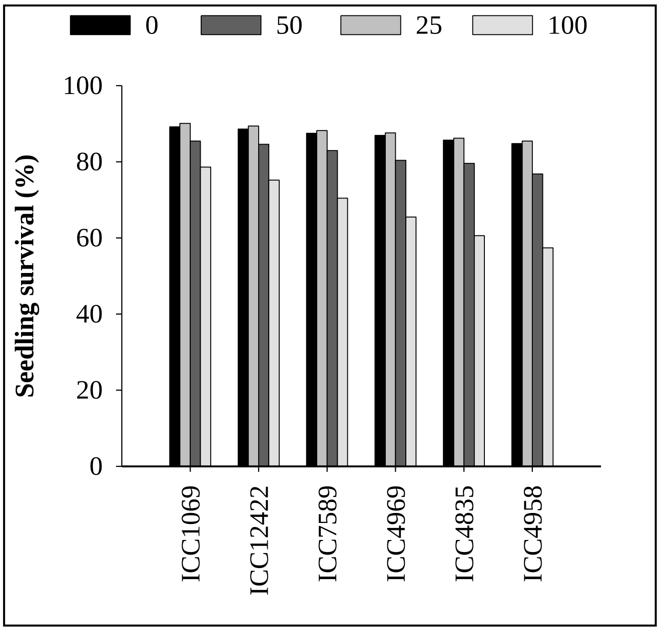
Parallels were drawn of plant survival and shoot dry mass with foliar toxicity symptoms and plant growth attributes separately at 0 and highest (100 mg kg^{-1}) Cd level (Table 1) at both 30 and 60 days of sampling. None of these relationships was evident under no-Cd applied. However, at the highest Cd-level, seedling survival was positively related to shoot dry mass per pot. Furthermore, leaf chlorosis and necrosis was negatively related to plant survival and its dry mass and positively related to number and area of leaves per pot (Table 1).

Discussion

This study showed that despite substantial varietal differences, increased Cd-levels were detrimental to chickpea as evident from gradual reductions in the plant survival, increased symptoms of foliar toxicity (Fig.1). The reduction in the plant survival has been described due to the fact that heavy metals are able to interact with essential macro and microelements, thus exerting a significant influence on plant nutrient uptake (Pal et al., 2006). The uptake of Cd ions seems to be in competition for the same transmembrane carrier with nutrients, such as K, Ca, Mg, Fe, Mn, Cu, Zn and Ni (Clarkson and Luttage, 1989; Rivetta et al., 1997) thus increasing amount of Cd concentration in growing media limits the nutrient transport to shoots (Yildiz, 2005) therefore inhibits germination processes and the development of seedlings (Rascio et al., 1993).

Visual symptoms of Cd-damage as noted here are reported in certain other plant species e.g. radish (Khan and Frankland, 1983), pea (Hernandez and Cooke, 1997) and of leaf chlorosis in oilseed rape (Baryla et al., 2001; Carrier et al., 2003) and rice (Adhikari et al., 2006) grown in Cd-contaminated soils. These effects appeared to result from an antagonistic effect of Cd accumulation on the levels of essential nutrients in leaves (Epstein and Bloom, 2005; Adhikari et al., 2006). The symptoms of Cd-damage noted in this study were similar to the deficiency of essential nutrients including potassium, magnesium, manganese and iron (Epstein and Bloom, 2005), which is important in view of the fact that all these elements are either structurally or functionally related directly in the loss of chlorophyll, the reduction in growth is a likely reason for the crippled nutritional status of leaves.

Fig. 1: Changes in seedling survival (%), leaf chlorosis (%) and leaf necrosis (%) in chickpea varieties under increasing Cd levels at two growth stages [legends are levels of $CdCl_2$ (mg kg^{-1} soil)].



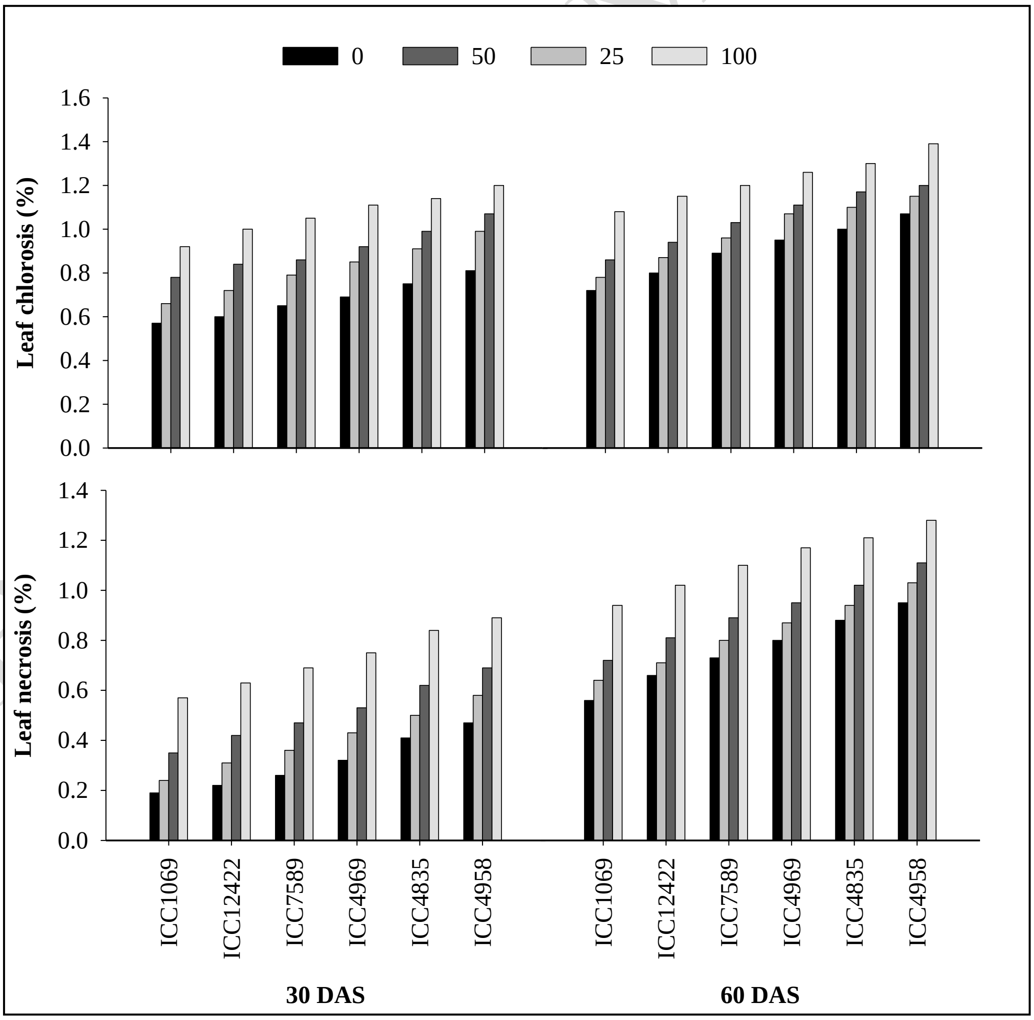


Fig. 2: Changes in shoot length (cm) and shoot dry weight (gm) in chickpea varieties under increasing Cd levels at two growth stages [legends are levels of $CdCl_2$ (mg kg^{-1} soil)].

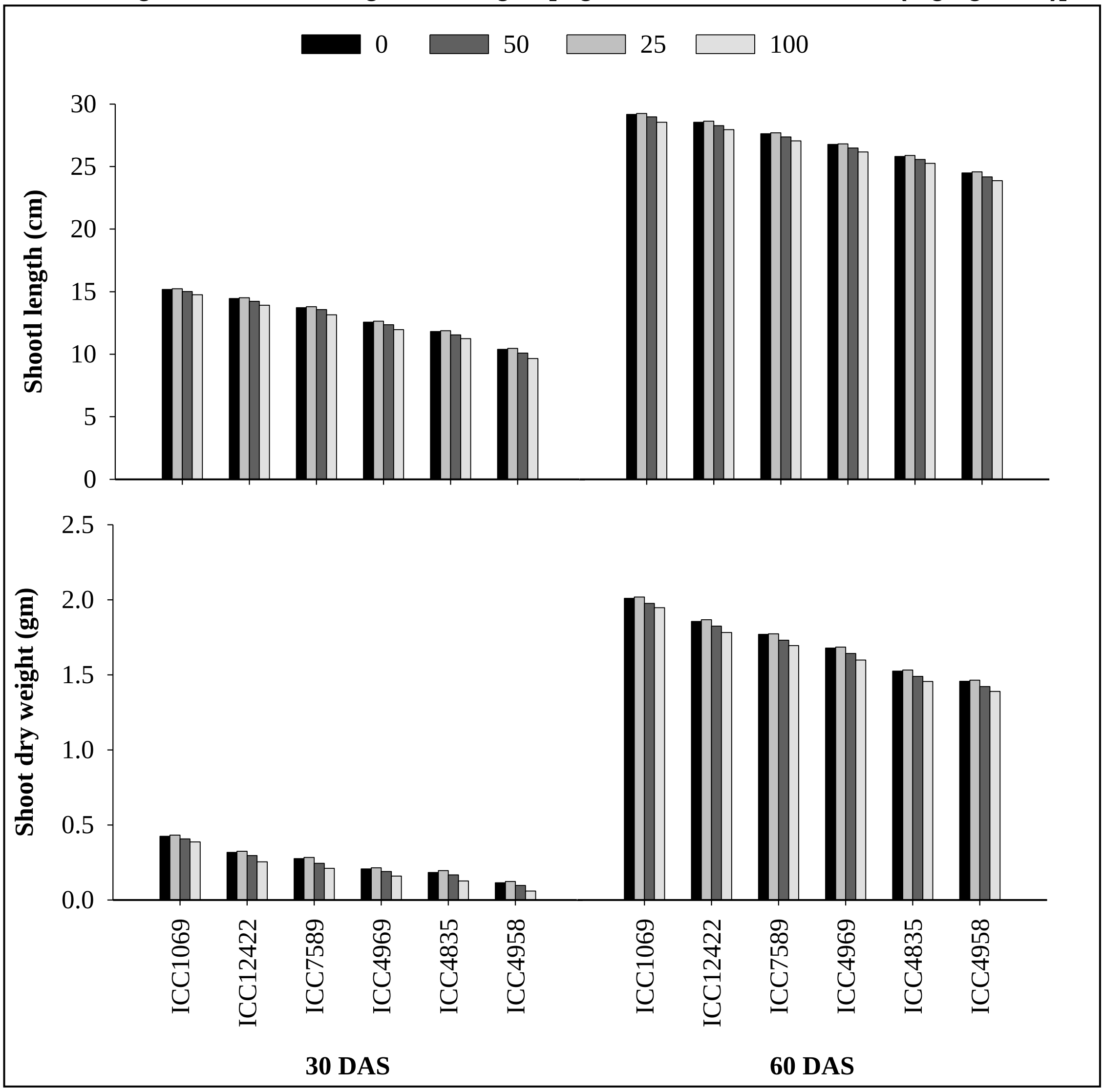


Fig. 3: Changes in leaf number pot^{-1} and leaf area (cm^2) in chickpea varieties under increasing Cd levels at two growth stages [legends are levels of $CdCl_2$ (mg kg^{-1} soil)].

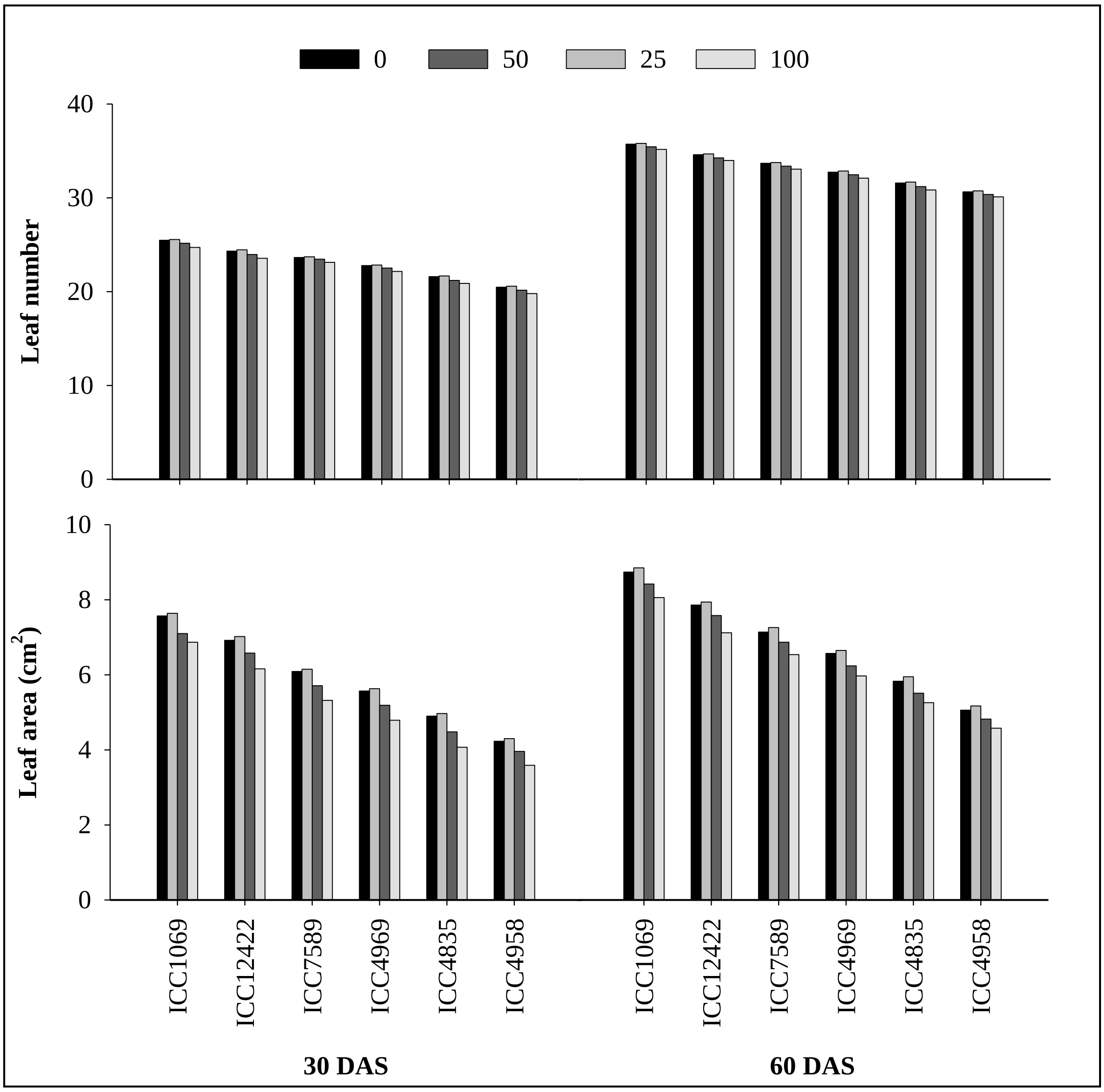


Fig. 4: Changes in proline content (mg g^{-1}) and nitrate reductase activity [nMNO$_2$ g^{-1} (F.M.) n^{-1}] in chickpea varieties under increasing Cd levels at two growth stages [legends are levels of $CdCl_2$ (mg kg^{-1} soil)].

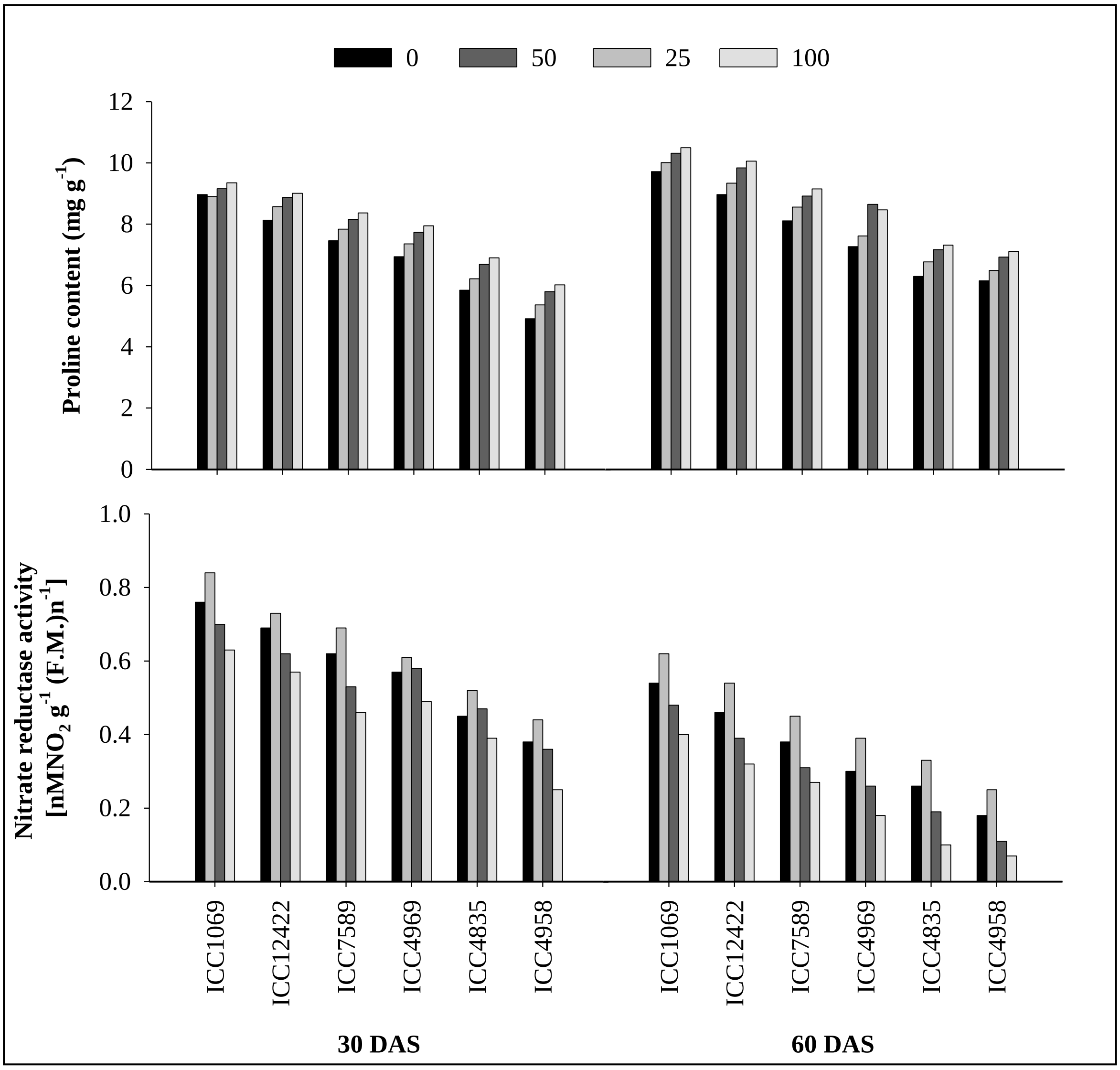


Table 1: Correlation coefficient (r) of seedling survival and shoot dry weight with Cd-induced leaf chlorosis, leaf necrosis, shoot dry weight, number of leaves and leaf area under 0 and 100 mg $CdCl_2$ kg^{-1} soil at 30 and 60 days after treatment.

X-variable	Y-variable	0	100
	30 days		
Seedling survival	Leaf chlorosis	-0.998**	-0.987**
	Leaf necrosis	-0.991**	-0.998**
	Shoot dry weight	0.966**	0.961**
	Number of Leaves per pot	0.995**	0.988**
	Leaf area	0.996**	0.996**
Shoot dry weight	Leaf chlorosis	-0.963**	-0.991**
	Leaf necrosis	-0.945**	-0.961**
	Number of leaves per pot	0.980**	0.962**
	Leaf area	0.984**	0.976**
	60 days		
Seedling survival	Leaf chlorosis	-0.988**	-0.988**
	Leaf necrosis	-0.992**	-0.991**
	Shoot dry weight	0.990**	0.991**
	Number of Leaves per pot	0.996**	0.997**
	Leaf area	0.994**	0.992**
Shoot dry weight	Leaf chlorosis	-0.990**	-0.986**
	Leaf necrosis	-0.998**	-0.991**
	Number of leaves per pot	0.997**	0.998**
	Leaf area	0.996**	0.997**

* = P<0.05; ** = P<0.01; ns = not significant

The most common effect of Cd toxicity in plants is stunted growth, leaf chlorosis and alteration in the activity of many key enzymes of various metabolic pathways (Arduini et al., 1996). Normally, Cd ions are retained in the roots and only small amounts are transported to the shoots (Cataldo et al., 1983). Cd in cells gets associated with cell walls and middle lamella and increases the cross-linking between the cell wall components, resulting in the inhibition of the cell expansion growth (Poschenrieder et al., 1989). Moreover, Cd also alters the water relation in plants, causing a physiological drought (Barcelo and Poschenrieder, 1990) and causes metabolic dysfunctions such as production of reactive oxygen species (Asada, 1999), photosynthesis (Krupa et al., 1993; Chugh and Sawhney, 1999) and nutrient uptake (Obata et al., 1996). These and other such altered processes lead to the decrease in the length and dry mass of the plant subjected to Cd stress (Fig. 2). Hasan et al. (2007a) also reported that the presence of cadmium in the soil decreases the growth of chickpea plants.

Higher concentration of Cd significantly reduced number of leaves and leaf area in all the cultivars (Fig. 3). This may be attributed to be due to the senescence and death of older leaves and appearance of injury symptoms on the younger leaves at high Cd stress, thereby reducing number of leaves and leaf area per pot (Ghani and Wahid, 2007).

Proline, an amino acid, is well known to get accumulated in wide variety of organisms ranging from bacteria to higher plants on exposure to abiotic stress (Saradhi et al., 1993). Plants have shown proline accumulation under environmental stress (Ahmad and Jhon, 2005; Ahmad et al., 2006; Ahmad et al., 2008). It has been often suggested that proline accumulation may contribute to osmotic adjustment at the cellular level and enzyme protection stabilizes the structure of macromolecules and organelles. Increase in proline content may be either due to de novo synthesis or decreased degradation or both (Kasai et al., 1998). Proline accumulation in *B. juncea*, *Triticum aestivum* and *Vigna radiata* in response to Cd^{++} toxicity has been demonstrated by Dhir et al. (2004).

Among various enzymes, nitrate reductase (NR) is a key enzyme in the conversion of nitrate to nitrite and its sustained activity is crucial to N assimilation (Gouia et al., 2000; Ghnaya et al., 2005, 2007). Reductions in NR activity, reduced nitrogen fixation and ammonia assimilation in nodules have been reported in legumes with applied Cd (Balestralse et al., 2004). The presence of Cd in the soil affected the assimilation of NO_3 in *Cicer arietinum* (Ali et al., 2007; Hasan et al., 2007b).

To substantiate the validity of the above findings, the parallels were drawn between plant survival, foliar toxicity symptoms and growth attributes at 30 and 60 days of sampling (Table 1). Existence of positive correlations of plant survival with shoot elongation, its dry mass and number and area of green leaves, while negative ones of leaf chlorosis and necrosis revealed that plant growth was crippled primarily due to reduced photosynthetic area (Carrier et al., 2003; Adhikari et al., 2006). Therefore, selection of varieties capable of producing greater photosynthetic area under Cd-stress may be a lasting solution to the problem in view.

Conclusion

In crux, despite substantial genetic variability for its tolerance, effects of Cd-damage on the seedling survival and subsequent growth are complex, which appeared to mainly involve the deficiencies of certain essential nutrients and reduced photosynthetic area for dry matter yield. Nevertheless, findings of this study carry great implications for accruing better chickpea stand in marginally Cd-contaminated soils.

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