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Cadmium induced changes on proline, antioxidant enzymes, nitrate and nitrite reductases in *Arachis hypogaea* L.

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Abstract: *The groundnut , Arachis hypogaea seedlings, when grown in pot cultures for 10–25 days at 25, 50, 100 µM $CdCl_2$, showed a marked decline in growth compared to control. Similar trend was observed for nitrate reductase (NR) and nitrite reductase (NiR) activities whereas proline, peroxidase (POD) and catalase (CAT) showed increasing trend when observed on the 10th day of the experiment. Changes have occurred in the physiological and biochemical activities which are observed even at low Cd levels (25 µM). At 100 µM concentration, with increase in experimental days, Cd has imposed drastic decrease in leaf and stem respectively, where nitrate reductase has varied from 20.87 – 79.41 and 29.11 – 72.91 % and nitrite reductase 21.66 – 79.41 and 43.58 – 75.92% respectively. Contrastingly Cd treated plant tissues showed an increase in proline 111.2 – 159.87 % (percentage changes) and 131.23 – 212.16 % for leaves and stems respectively, In addition cadmium caused significant changes in the activity of antioxidative enzymes, peroxidase 48.12 – 72.19 % in leaf and 37.71 – 75.25 % in stem and catalase 64.86 – 143.92 % in leaf and 129.13 – 214.74 % in stem as compared to control. The study concludes that the activities of NR, NiR, proline, POD, CAT are inhibited suggesting that Arachis hypogaea seedlings are under Cd stress affecting their growth.*

Key words: *Cadmium stress, Growth, Proline, Antioxidant enzyme activities*
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Introduction

Anthropogenic inputs associated with agricultural practices, mineral exploration, industrial processes and solid waste management are important contributors to heavy metal contamination of natural ecosystems (Alumaa *et al*., 2002; John *et al.,* 2007 and Pandey *et al.,* 2007). Heavy metal toxicity, especially by lead, cadmium, arsenic, and mercury, constitute serious threat to human health (Wenneberg, 1994). Accumulation of heavy metals and metalloids in agricultural soils has become important due to food safety issues, potential health risks and detrimental effects on soil ecosystems.

Humans are basically responsible for environmental contamination by heavy metals, for cadmium (Cd) alone is estimated to be 29,190 tones $year^{-1}$ worldwide (Sanita di Toppi and Gabbrielli, 1999). The presence of Cd in soil leads to crop yield losses and human health hazards (Seregin and Ivanov, 2001). An impressive number of studies on Cd as an environmental pollutant have been performed, but the mechanisms causing toxic effect in plants are not yet fully understood (Sanita di Toppi and Gabrielli, 1999). In plants, Cd is a non essential element with an intermediate mobility in the phloem and strong interactions with thiols (Seregin and Ivanov, 2001). Cd in plants increases with elevated Cd concentration in the medium (Lagriffoul *et al*., 1998; Aeri and Rana, 2002). Up to 96% of the absorbed Cd accumulates in the cell wall and the vacuoles; only a small quantity of Cd reaches nuclei, chloroplasts, and mitochondria (Seregin and Ivanov, 2001).

Cadmium pollution causes several toxic symptoms in plants *viz.* growth retardation, inhibition of photosynthesis, altered water relations, disturbances in the uptake and distribution of macro and micronutrients, efflux of cations and generation of free radicals (Siedlecka and Krupa, 1999; Sandalio *et al*., 2001; Ramos *et al*., 2002 and Sahu *et al.,* 2007). Long term Cd stress inhibits the formation of the photosynthetic apparatus in young plants of barley (*Hordeum vulgare* L.) and decreases the content of soluble proteins (Vassilev *et al*., 1997). A drastic reduction of the enzyme Rubisco and an induction of stress related proteins are reported in rice leaf segments stressed with copper, cadmium and mercury (Hajduch *et al*., 2001).

Several reports have proved to cause oxidative stress with Cd (Sandalio *et al*., 2001; Pereira *et al*., 2002; Milone *et al*., 2003; Wu *et al*., 2003). Cadmium is a redox inactive metal that weakens the antioxidative protection by depleting glutathione pool, interaction with essential thiol groups in proteins, Zn displacement, altered calcium and sulfhydryl homeostasis, or *via* generation of reactive oxygen species by redox cycling quinones (Stohs and Bagchi, 1995). The antioxidant protection in plant cells is complex and highly compartmentalized, and includes both enzymatic and non enzymatic components (Noctor and Foyer, 1998; Mittler, 2002). Superoxide dismutases belongs to metalloenzymes that catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2. The bulk of H_2O_2 is removed by catalases, localized in the peroxisomes, and by peroxidases, localized in the vacuoles, the cell wall, and the cytosol (Mittler, 2002). Ascorbate peroxidases

scavenge small amounts of H_2O_2 in subcellular compartments (Mittler, 2002). Ascorbate is the major primary antioxidant reacting directly with ROS ($OH^{\bullet}$, $O_2^{\bullet-}$). It also acts as a secondary antioxidant preventing membrane damage. Glutathione is in general the predominant non protein thiol, redox buffer, and phytochelatin precursor (Noctor and Foyer, 1998). Oxidative stress may occur due to overproduction of ROS and/or of a disfunction of the scavenging systems.

These studies examines the influence of the heavy metal Cd on groundnut, *Arachis hypogaea* by determining the root and shoot length, proline, nitrate and nitrite reductase enzymatic activities and the behaviour of antioxidant enzymes such as catalase and peroxidase in leaf and stem.

Materials and Methods

Arachis hypogaea L seeds were obtained from Achrya N.G. Ranga Agricultural Regional Research Atation Tirupati, Andhra Pradesh, India. Seeds were sterilized with 0.1% sodium hypochlorite solution for 10 min and rinsed with double distilled water. Seeds were sown in earthen pots (30 x 25 cm) containing red sandy soil and farmyard manure in 3:1 proportion. The soil moisture ranged from 4 to 8% and the moisture was maintained in green house conditions, soil pH was 7.58, electrical conductivity(EC) 0.16 dSm^{-1} and soil organic carbon (SOC) was 0.31%. The pots were kept under natural photoperiod and each pot contained 10 seedlings. The plant was treated with three different concentrations of $CdCl_2$ solutions 0(control), 25, 50 and 100 µM and observations were made after 10, 15, 20 and 25 days of treatment. The pots were treated with three different concentrations once in two days with above solutions. The leaves and stems were taken from plants at 10, 15, 20 and 25 days after cadmium treatment and used for determinations of physiological and biochemical studies. At different days the growth of root and shoot lengths (cms) were determined. Each parameter was carried out in triplicates.

Proline: Proline was determined according to Bates *et al*. (1973). Leaves and stems (500 mg) were extracted with 3% aqueous 5-sulphosalicylic acid, centrifuged at 5000 rpm. The sample of the supernatant was used for the proline assay and measured at 520 nm. Proline content was expressed as µg g^{-1} fresh weight.

Nitrate reductase activity (EC 1.6.6.1): The Nitrate reductase (NR) activity of plant tissue (leaf and stem) was determined by (100 mg) grinding in a chilled mortar at 4 ºC with 0.1M potassium phosphate buffer, pH 7.4 (8 ml g^{-1} fresh weight) containing 1mM EDTA, 7.5 mM cysteine, 2.5% (w/v) casein. The homogenate was centrifuged (for 15 min at 4ºC) and the supernatant was assayed for NADH–NR activity as determined by method of Wray and Filner (1970). The supernatant was measured spectrophotometrically at 540 nm wavelength. NR activity was expressed as µmol NO_2 $h^{-1}g^{-1}$ fresh weight.

Nitrite reductase activity (EC 1.6.6.4): The Nitrite reductase (NiR) extraction was undertaken in the same conditions as those described for nitrate reductase. Nitrite reductase activity was measured as the reduction in the amount of nitrite in the assay mixture by incubating 0.1 ml of supernatant for 30 min at 30ºC with 100 mM of potassium phosphate buffer (pH 7.4), 15 mM $NaNO_2$ and 5mM methyl viologen as electron donor (final pH 7.4 and total volume 1.0 ml). The absorbance of the supernatant was determined spectrophotometrically at 540 nm by the

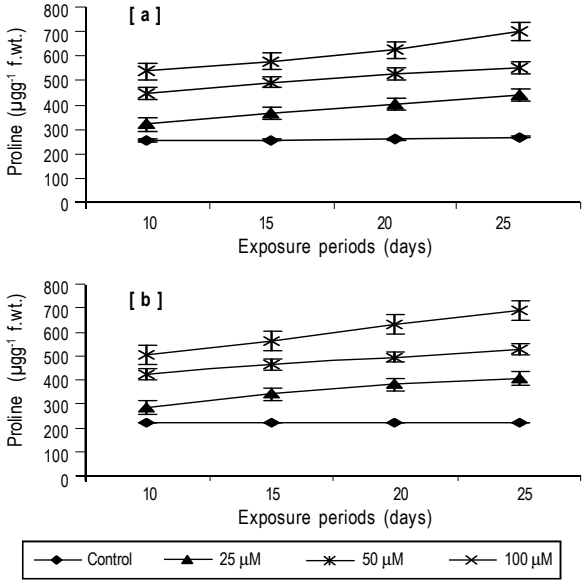

Fig. 1: Effect of cadmium on proline content in (a) leaf (b) stem of *Arachis hypogaea* L. Values are mean of three replicates ± SD and bars indicate standard deviationCx. All values are significant at p<0.05 compared to control

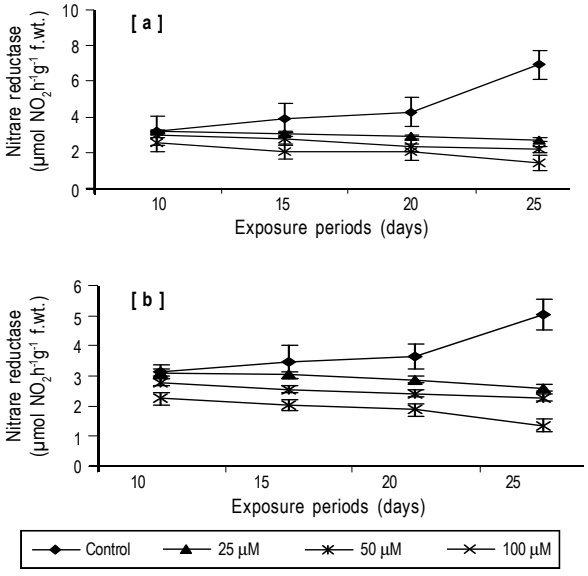

Fig. 2: Effect of cadmium on nitrate reductase (a) leaf (b) stem of *Arachis hypogaea* L. Values are mean of three replicates ± SD and bars indicate standard deviation. All values are significant at p<0.05 compared to control

Table - 1: Effect of Cd on growth of shoot length (cm) and root length (cm) of *A. hypogaea* at different concentrations and exposure periods

Exposure (days)	Control		25 µM		50 µM		100 µM	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
10	10.36±1.05	4.76±0.98	7.1±0.83**	3.86±1.07*	6.4±2.11*	3.33±1.01**	5.0±1.3*	3.16±1.0*
15	15.53±1.4	6.93±1.7	10.43±1.1*	5.6±0.99**	6.96±1.08*	4.86±1.9*	5.73±1.0**	3.43±1.21**
20	19.76±2.05	9.1±1.56	14.6±1.29*	6.36±0.89*	10.5±1.44**	6.13±1.05**	6.43±1.39	3.6±1.22*
25	25.16±1.01	11.96±1.29	16.23±1.41*	8.3±1.93*	12.66±2.75*	6.9±1.08**	6.93±1.0*	3.7±1.07*

All values are means of three replicates ± SD. ** Significant (p<0.01), * Significant (p<0.05) compared to control

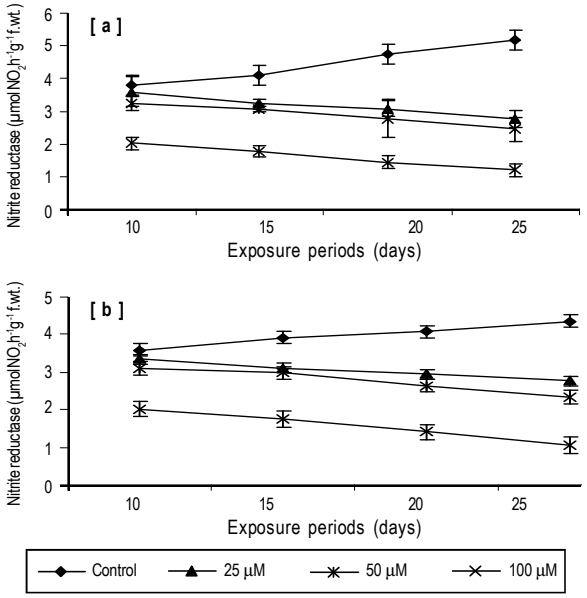

Fig. 3: Effect of cadmium on Nitrite reductase in (a) leaf (b) stem of *Arachis hypogaea* L. Values are mean of three replicates ± SD and bars indicate standard deviation. All values are significant at p<0.05 compared to control

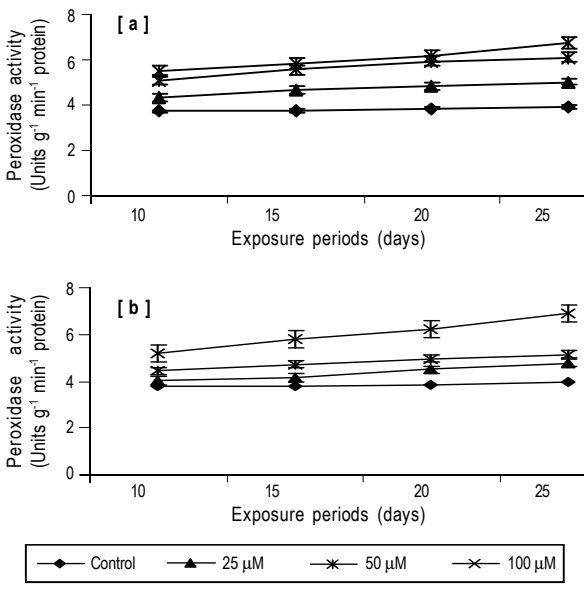

Fig. 4: Effect of cadmium on POD in (a) leaf (b) stem of *Arachis hypogaea* L. Values are mean of three replicates ± SD and bars indicate standard deviation. All values are significant at p<0.05 compared to control

method of Losada and Paneque (1971). NiR activity was expressed as µmol $NO_2 h^{-1} g^{-1}$ fresh weight.

Peroxidase (EC 1.11.1.7): Peroxidase in fresh leaves and stem samples (100 mg) was homogenized in 50 mM Tris-HCl buffer, pH 7.0, the supernatant solution centrifuge for 20 min and peroxidase activity was assayed by the method of Kar and Mishra (1976). The reaction mixture contained 100 mM Tris-buffer (pH 7.0), 10 mM pyrogallol and 5 mM H_2O_2. The supernatant was measured at 425 nm in a spectrophotometer. Peroxidase activity was expressed as g^{-1} min^{-1} protein.

Catalase (EC 1.11.1.6): The activity of catalase was assayed according to Beers and Sizer (1952). Fresh samples leaves and stems (200 mg) were homogenized in 5 ml of 50 mM Tris/NaOH buffer (pH 8.0) containing 0.5 mM EDTA, 2% (w/v) PVP and 0.5% (v/v) Triton X-100. The homogenate was centrifuged for 10 min at 4 8°C and after dialysis supernatant was used for enzyme assay. Supernatant was measured at 240 nm wavelength in a spectrophotometer. The activity of catalase was expressed as µmol H_2O_2 oxidised min^{-1} mg^{-1} protein.

Statistical analysis: Mean values of the experiments done in triplicate. The data was analysed using t-test to compare means between different cadmium treatments and exposure periods at p<0.01 and p< 0.05 significant levels.

Results and Discussion

Arachis hypogaea L. seedlings grown over a period of 10-25 days with increasing concentrations of Cd (25, 50 and 100 µM) have revealed that there is a significant decrease in shoot and root length (Table 1). At 25 –100 µM and between 10^{th} – 25^{th} days, a decrement is observed in percentage change of shoot length with 31.47 – 72.46 % and root length 18.91 – 69.06 % respectively. At higher Cd concentration of 100 µM there is maximum reduction in root length (50.51, 60.44 and 69.06%) and shoot length (63.1, 67.46 and 72.46%) respectively during on 15, 20 and 25 days. It is clearly understood that Cd has induced stunted growth, chlorosis, blackening of the root systems. Possibly these changes in the plant system could be due to alteration in water and nutritional status of plants as well as various plant processes (Godbold and Kettner 1991; Kastori *et al.*, 1992). Similar observations of the blackening of

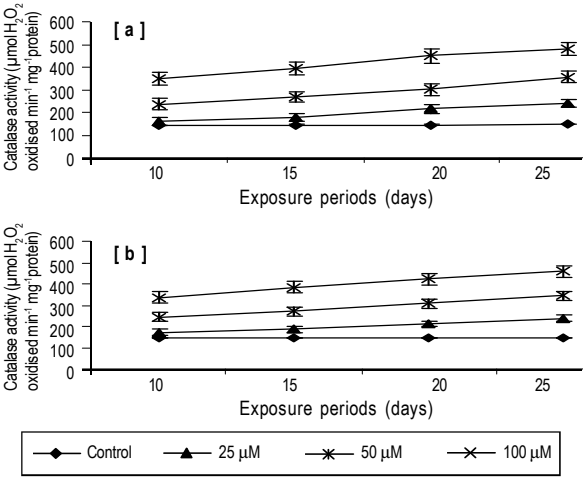

Fig. 5: Effect of cadmium on CAT in (a) leaf (b) stem of *Arachis hypogaea* L. Values are mean of three replicates ± SD and bars indicate standard deviation. All values are significant at $p<0.05$ compared to control

the root system was stated by Godbold and Kettner, 1991). The several authors has been observed in barley seedlings (growth inhibition, reduced pigment levels, necrosis) treated with 50 and 500 µM Cd are consistent with previously reported toxic symptoms (Vassilev *et al*., 1997, Vassilev and Yordanov, 1997).

Proline in plant tissues (leaves, stems) significantly increased from 10– 25 day with increasing molar concentrations (Fig. 1). At 25 µM the percentage change for leaves was 25.81 – 63.42 and for stem 29.7 – 83.42. At 50 µM the percent change was 75.36 –104.86 and 93.13 – 138.35 respectively. At 100 µM the percentage change was 111.2 – 159.87 and 131.23 – 212.16 for leaves and stems respectively. Among proline responds most sensitively to stress conditions (Nover, 1989). Proline accumulation in plants can serve as biomarker of heavy metal stress. Proline increased greatly in leaves and less in stem under Cd stress, more markedly under 100 µM Cd. Proline accumulation has also been proposed as a mechanism of storage of excess nitrogen (Rhodes *et al*., 1999). It is well known that putrescine and proline biosynthesis shares common precursors, arginine and ornithine. In plants, proline is generally synthesized from glutamate (Ireland, 1997). Many plants accumulate high concentrations of proline when treated with toxic heavy metals (Bassi and Sharma, 1993 a, b; Costa and Morel, 1994). Proline accumulation in plants also occurs in response to stresses such as salinity (Lutts *et al*., 1996), drought (Aspinall and Paleg, 1981), and low and high temperature (Chu *et al*., 1974; Naidu *et al*., 1991). Many researchers believe that proline accumulation is a symptom of injury which does not confer tolerance against metal or other stresses (Lutts *et al*., 1996). By contrast, suggestions have been made that proline might protect plants from heavy metal toxicity (Farago and Mullen, 1979; Smirnoff and Cumbes, 1989; Kavi Kishor *et al*., 1995).

A significant decrease in nitrate and nitrite reductase activity (Fig. 2, 3) was observed in leaves and stems between 10 – 25 day with increased cadmium application (25, 50, 100 µM). At 25 µM the percentage change for nitrate reductase in leaves was 0.94 – 60.66 and for stem 1.58 – 48.63. At 50 µM the percent change was 7.16 – 68.61 and 11.71 – 54.58 respectively. At 100 µM the percentage change was 20.87 – 79.41 and 29.11 – 72.91 for leaves and stems respectively. At 25 µM the percentage change for nitrite reductase in leaves was 5.53 – 46.63 and for stem 6.42 – 36.7. At 50 µM the percent change was 14.74 – 52.99 and 13.13 – 46.33 respectively. At 100 µM the percentage change was 21.66 – 79.41 and 43.58 – 75.92 for leaves and stems respectively. In the leaves and stem, the increase in Cd concentration is accompanied by a decrease in NR and NiR enzyme activity, which was inhibited significantly by the highest Cd concentration supplied in the experiment. The present study indicated a marked decline in NR and NiR activities in *Arachis hypogaea* L. seedlings when exposed to increasing levels of Cd. Similar to our observations, decline in NR activity has been observed in many plant species growing under toxic levels of Cd, Cu, Pb, Zn and Co (Dubey and Pessarakli, 2002). The heavy metals adversely affect plant growth, metabolism and inhibit the key enzymes of N assimilation pathway (Dubey and Pessarakli, 2002). Among the enzymes of N assimilation, NR is regarded as the most sensitive to heavy metals (Dubey and Pessarakli, 2002). A decline in NiR activity has been observed in soybean (Khan, 1996) and rice plants (Dubey and Pessarakli, 2002) grown under NaCl and Na_2CO_3 salts in the growth medium. Decreased activity of N assimilatory enzymes NR and NiR in *Arachis hypogaea* L. seedlings has been severly affected with Cd.

Significantly increased activity of POD was observed (Fig.4) in leaves and stems between 10– 25 day with increasing Cd molar concentrations (25, 50 and 100 µM). At 25 µM the percentage change was 16.67 – 28.06 for leaves and 7.1 – 21.37 for stems respectively. At 50 µM and 100 µM the percentage change in POD activity was 37.37 – 55.61 and 48.12 – 72.19 respectively for leaves and 18.54 – 30.49 and 37.71 – 75.25 respectively for stems. POD activity has drastically increased at all the Cd concentrations and experimental days. Peroxidases is located in cytosol, vacuole, cell wall as well as in extra cellular space and uses guaiacol as electron donor, utilizes H_2O_2 in the oxidation of various inorganic and organic substrates (Asada, 1994). The activity of POD increased by 250% in comparison to the control in shoots of plants treated with 10 mg kg^{-1} of As (Mascher *et al*., 2002). POD activity in pea genotypes increases with Cd sensitivity and is a biomarker for metal toxicity in plants (Radotic *et al*., 2000; Metwally *et al*., 2005).

Between 10 – 25 day and with increase in cadmium concentrations (25, 50 and 100 µM) the activity of CAT in (Fig.5) leaves and stems showed an significantly increasing trend. At 25, 50 and 100 µM and between 10–25 days, the percentage change was 11.88 – 65.02, 6.16 – 65.39 and 64.86– 143.92 respectively for leaves and similarly the percentage change for stems at various concentrations were 59.23 – 128.32 , 142.74 – 228.96 and 129.13 – 214.74 respectively. The increase in CAT activity in the leaves and stems of *Arachis hypogaea* growing seedlings with elevated Cd levels has been observed with exposure period. Brej (1998)

positively demonstrated a significant increase in CAT activity in the roots and leaves of some populations of *Agropyron repens* growing in highly contaminated soils. Similar results demonstrating an increase in CAT activity in radish leaves and roots, just after 24 hr exposure to the Cd metal is reported (Vitória *et al*., 2001). It can be clearly seen in *Crotalaria juncea* that there was a large increase in CAT activity induced by 2 mM $CdCl_2$ in the leaves and Pb stress in rice seedlings (Pereira *et al*., 2002; Jing *et al*., 2007). The major function of CAT activity in leaves is to metabolize the peroxide liberated in the peroxisome during photorespiration and the activity of the enzyme is greatly reduced when plants are grown in elevated atmospheres of CO_2 (Azevedo *et al*., 1998). This study concludes that the POD, CAT and praline contents have shown an increasing trend where as NR and NiR activities have decreased and suggest that *Arachis hypogaea* L. seedlings are under Cd stress, even at low concentrations, affecting the overall growth of the crop.

In conclusion, our results demonstrate that the NR, NiR levels decreased and proline, POD, CAT activities increased in leaves and stems with increasing cadmium concentrations at all exposure periods. The crop *Arachis hypogaea* L. is highly sensitive even at very low cadmium concentrations.

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