Genetics and Plant Physiology – 2013, Volume 3 (1–2), pp. 98–108

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ROLE OF ETHYLENEDIAMINE TETRAACETIC ACID AND SALICYLIC ACID IN ALLEVIATING CYTOGENETIC TOXICITY OF COPPER IN ROOTS OF *ALLIUM CEPA* (L.)

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Received: *14 January 2013* Accepted: *09 October 2013*

Summary: The ameliorative impact of salicylic acid (SA) and ethylene diamine tetraacetic acid (EDTA) on cytogenetic and growth changes induced in *Allium cepa* by toxic concentration of copper (Cu) was investigated. Onion bulbs were treated with solutions prepared from different combinations of these substances: Cu (100.79 mmol l^{-1}), Cu + EDTA (1273.75 mmol l^{-1}), Cu + SA (1000.00 mmol l^{-1}), Cu + EDTA + SA for 5 days. It was observed that Cu toxicity inhibited root length and root biomass by 43% and 76% respectively, and induced a 71% decrease in the mitotic index. Chromosomal aberrations such as anaphase bridges, stickiness and vagrant were also induced in treated roots. The damaging effect of Cu on the mitotic index was reduced to 16% and 34% by EDTA and SA, respectively. Though SA was not as effective as EDTA in protecting root length and biomass, it demonstrated some degree of ameliorative effects on the sticky chromosomes. However, the impact of EDTA seemed to be hindered by the presence of SA. In all treatments the absorbance ratio ($A_{260/280}$) of DNA remained 1.8-2.0, indicating that the quality of DNA was not significantly affected by the tested Cu concentration. Chelating Cu with EDTA protected root growth and cytotoxic effects while SA corrected genotoxic effects caused by Cu toxicity.

Citation: Umebese C.E., T.A. Azeez, K.O. Adekoya, 2013. Role of ethylenediamine tetraacetic acid and salicylic acid in alleviating cytogenetic toxicity of copper in roots of *Allium cepa* (L.). *Genetics and Plant Physiology*, 3(1–2): 98–108.

Keywords: *Allium cepa*; copper, EDTA; salicylic acid; aberrations; DNA.

Abbreviations: Cu – copper; EDTA – ethylenediamine tetraacetic acid.

INTRODUCTION

Copper is an essential micronutrient in plants that supports many physiological processes including plant growth and it is a key to plant elasticity. It is present as a co-factor of many metalloproteins in several enzymes that play important roles in photosynthesis, respiration and other metabolic processes. It is

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important in chlorophyll formation and promotes seed production (Claire et al., 1991; Quartacci et al., 2000). Plants respond to the deficiency and excess of Cu in the soil. Cu deficiency in plants causes deleterious effects, such as chlorosis, necrosis, root inhibition, delayed flowering, reduction in starch production, plant vigour and nitrogen fixation in legumes (Chatterjee and Chatterjee, 2000). However, problems are known to occur due to excess Cu in cells. Toxic levels of Cu reduce seed germination, shoot vigour, iron availability and can suppress mitotic activity and induce chromosomal aberrations in plants (Liu et al., 1994, 1995; Ke et al., 2007; Jiang et al., 2001). Cu has been observed to promote oxidative DNA strand scission, mostly single strand breaks and base damage. In conjunction with other metals they induce more damage to pyrimidine than purine bases. These may be precursors of possible carcinogenic effects (Liang et al., 1999).

Salicylic acid is a compound that is chemically similar to aspirin. It functions as a phytohormone, an important factor in environmental stress tolerance in plants (Bosch et al., 2007). SA produces an ameliorative protective effect in plants in response to abiotic stress, such as metal toxicity, heat, chilling, osmotic and salt stress (Borsani et al., 2001; Janda et al., 2001; Singh and Usha 2003; Wang and Li, 2006). SA can stimulate flowering, increase flower life, promote ethylene synthesis (Singh and Usha, 2003) and enhance photosynthetic rate and growth rate (Khan et al., 2003). It acts as an endogenous signal molecule responsible for the induction of antioxidant responses that protect plants from damage (Hussein et al., 2007; Senaratna et al., 2000).

Another compound, ethylenediamine tetraacetic acid has been shown to have some protective ability on plant growth under metal toxicity. EDTA is a hexadentate (six toothed) ligand and chelating agent often found to be the most effective chelating agent with the ability to sequester metals such as Cu, Mn, Fe, Pb and Co (Blaylock et al., 1997; Haung et al., 2008). It improves metal build up in the shoot of the plant because it develops a metal chelate complex which enhances its movement within the plant, increasing its transport from roots to aerial parts (Turgut et al., 2004; Zhuang et al., 2007). Zn-EDTA treatment at the rate of 0.35g/plot led to the highest plant height, fresh weight of aerial plants leaf area, total chlorophyll content, NPK in leaves, fruit setting and total yield in tomato growth production in irrigation conditions (Salama et al., 2012).

Allium cepa, a classical, low budget and short term biological assay for evaluation of cytogenetic effects of various genotoxic substances has been proposed as a standard method for toxicity (Fiskesjo, 1987; El-shahaby et al., 2003 and Rank, 2003). The roots of *Allium* contain oxidase enzyme which activates the conversion of promutagens into mutagens. Hence, the objective of this study was to assess the ameliorative effect of SA and EDTA against Cu-mediated toxicity in *Allium cepa* utilizing the anaphase telophase chromosome aberration assay. The investigations include the impact on the root length and mass as well as attenuating the possible

oxidative DNA damage probably caused by excess Cu in cells.

MATERIALS AND METHODS

Plant material and *Allium* assay

Onion bulbs (*Allium cepa*) of the purple variety of average weight (22g – 27g) were used as the test system. The dry outer scales and the brownish bottom plates were removed from healthy onion bulbs leaving the rings of the root primordia intact. Bulbs of onions were first sprouted in tap water under ambient temperature as described by Friskesjo (1987). After 24 h, bulbs with good root growth were selected. Six replicate bulbs were selected, each with the base seated on 25 ml vials filled with the test solution. Distilled water served as the control. Preliminary investigations showed that concentrations of $CuSO_4.5H_2O$ as high as 3000 mmol l^{-1} (768 mmol l^{-1} of Cu) killed the root cells. Therefore, 393.73 mmol l^{-1} $CuSO_4.5H_2O$ (100.79 mmol l^{-1} of Cu) was used to study the effect of Cu toxicity with or without 1273.75 mmol l^{-1} EDTA or 1000.00 mmol l^{-1} SA. The 5 test solutions were Cu, Cu + EDTA, Cu + SA, Cu + EDTA + SA and the control. Onion roots were allowed to grow in the test solutions for 5 days and the solutions were renewed daily. The root length of onion bulbs from each solution was recorded daily and the dry biomass of the roots of each plant was determined after oven drying at 40°C for 3 days. Five bulbs were used for each concentration and treatment combinations as well as control. The length of the newly emerged roots was measured before transfer to the test solutions.

Cytological study

After the treatments, some of the emerged root tips were removed and fixed immediately using Clarke's fixative -95% ethanol:glacial acetic acid (3:1, v/v) 24 h after the 5th day of growth. The root tip was placed at the center of a clean microscope slide and cut into smaller bits using dissecting needles. The root tip was macerated in drops of 1N HCl for hydrolysis for 5 min. The root tip was then stained with two drops of aceto-orcein stain for 15 min. Afterwards the cells were squashed gently and evenly spread. Only one root tip was used per slide and five slides were prepared for each bulb. The stained material on the slide was carefully covered with a cover slip ensuring that no air was trapped in the process. The slide was covered with a sheet of filter paper and pressed down firmly to remove any excess stain. Good slides were preserved by sealing them with colorless nail varnish. Each treatment was repeated three times. The prepared slides were placed on a Zeiss light microscope for viewing. Three random fields (between 20 and 30 cells/field) were used per slide for recording mitotic cells and aberrations. Photomicrographs of some slides were made under the X40 objective lens or X100 objective (oil immersion lens). The mitotic indices were calculated by dividing the number of dividing cells per field by the total number of cells per field and multiplying the results by 100.

DNA extraction and quantification

DNA of the root tips from each solution was extracted adopting DNA Extraction Protocol of Dellaporta et al. (1983), modified using an Eppendorf

tube centrifuge. The extracted DNA samples were quantified using the spectrophotometric analysis at wavelengths of 260 nm and 280 nm. Each cuvette had a mixture of 95 µl of distilled water and 5µl DNA sample against distilled water as blank. The yield of DNA of onion root tips extracted was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm.

Means of 3 replicates were recorded with standard errors. The test of significance between treatments was done using analysis of variance (ANOVA) and Duncan's multiple range test.

RESULTS

Bulbs of *Allium cepa* placed directly on vials containing Cu, Cu + SA, Cu + EDTA + SA showed suppressed root growth when compared with the control and signs of wilting and clumping of roots appeared on the bulbs from the second day of exposure. Root growth of bulbs treated with chelated Cu (Cu + EDTA) were least affected by Cu toxicity (Fig. 1). The root cells of bulbs treated with a toxic concentration of Cu also showed light blue coloration at the root tips showing Cu uptake.

Onion bulbs subjected to chelated Cu had the highest mitotic index while the bulbs treated with Cu only, had the lowest mitotic index and the highest chromosomal aberrations (Table 1). Treatments containing SA (Cu+SA and Cu+SA+EDTA) reduced the number of chromosomal aberrations in the bulbs.

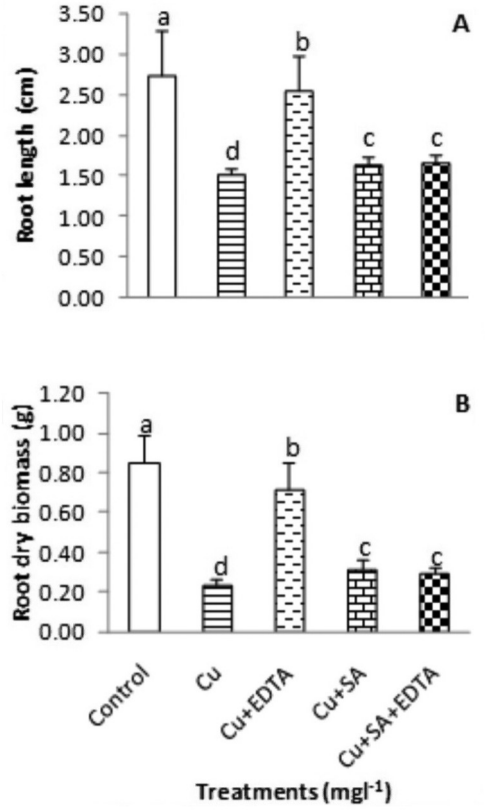

Figure 1. Root length (A) and root biomass (B) of 5-day-old onion bulbs subjected to a toxic concentration of Cu and the protective impact of SA and EDTA. Bars with similar letters are not significantly different at $P<0.05$ using the Duncan's test.

Thus, EDTA protected the mitotic index while SA reduced chromosomal aberrations. The control had the highest mitotic index with no chromosomal aberration. Spectrophotometric analysis of the extracted DNA from roots subjected to different treatments showed an absorbance ratio ($A_{260/280}$) of 1.80–2.00.

Root length and biomass were strongly inhibited by the toxic concentration of Cu (Fig. 1) but were significantly protected by chelating Cu

Table 1. Mitotic index, chromosomal aberrations and DNA absorbance ratio of the roots of 5-day-old onion bulbs subjected to a toxic concentration of Cu.

Treatments	No. of cells	Normal cells dividing			Mitotic index [%]	Chromosomal aberrations			DNA Absorbance Ratio [$A_{260/280}$]
		Metaphase	Anaphase	Telophase		Stickiness	Bridged	Vagrant	
Control	300	15	11	32	19.33±0.681	0	0	0	1.82
Cu	125	3	2	2	5.60±0.305	4	5	7	1.92
Cu + EDTA	284	17	13	16	16.20±0.513	2	6	4	1.87
Cu + SA	203	9	4	13	12.81±0.105	0	3	5	1.89
Cu + EDTA + SA	215	12	7	5	11.16±0.242	0	3	4	1.85

with EDTA. SA induced an increase in the length of roots subjected to the toxic concentration of Cu but retarded root growth in bulbs subjected to chelated Cu. Though SA also showed ameliorative ability, the combination of SA with chelated Cu reduced the protective ability of EDTA.

The mitotic stages in the root meristems of the untreated onion bulbs (control) showed no abnormal cell division (Plate 1). Plates 2–5 show the

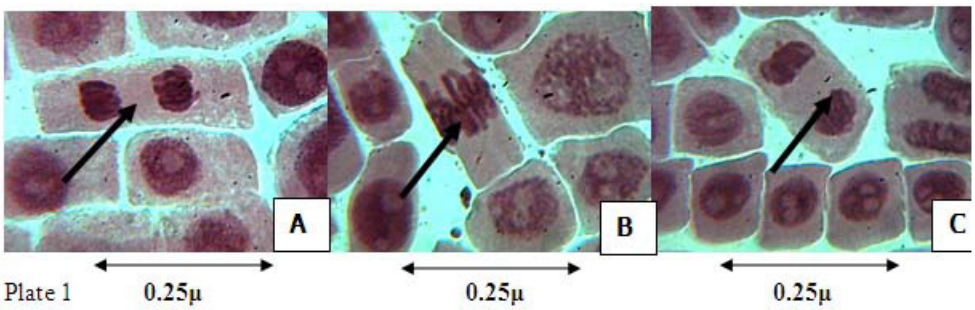

Plate 1. The mitotic stages in the root meristem of the untreated onion (control); Anaphase (A), Metaphase (B) and Telophase (C). Mag. x 100.

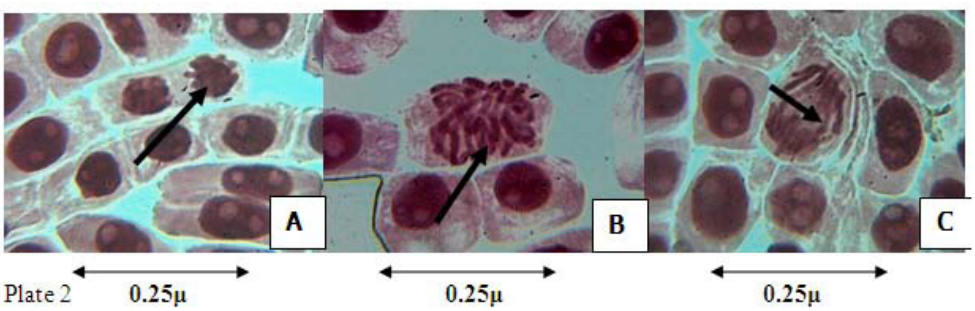

Plate 2. Chromosomal aberrations in onion root cells subjected to Cu toxicity: Sticky Chromosome (A), Vagrant (B) and Bridged Anaphase (C). Mag. x 100.

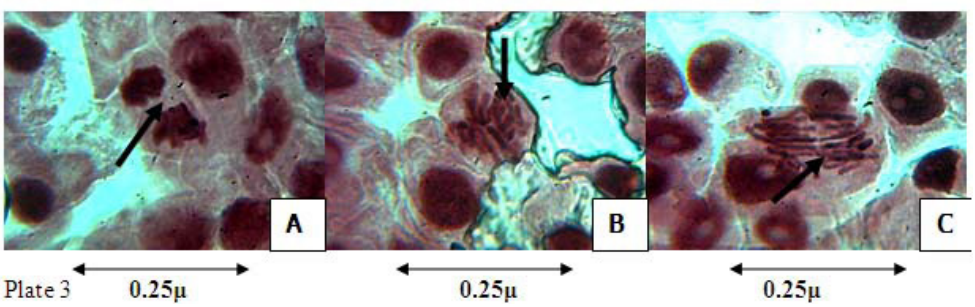

Plate 3. Chromosomal aberrations in onion root cells subjected to Cu toxicity chelated with EDTA: Sticky Chromosome (A), Bridged (B) and Vagrant (C). Mag. x 100.

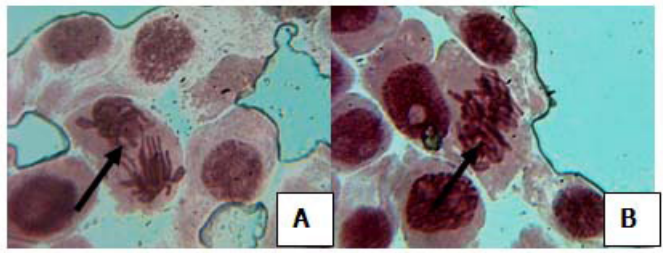

Plate 4. Chromosomal aberrations in onion root cells subjected to SA and Cu toxicity: Bridge (A), and Vagrant (B). Mag. x 100.

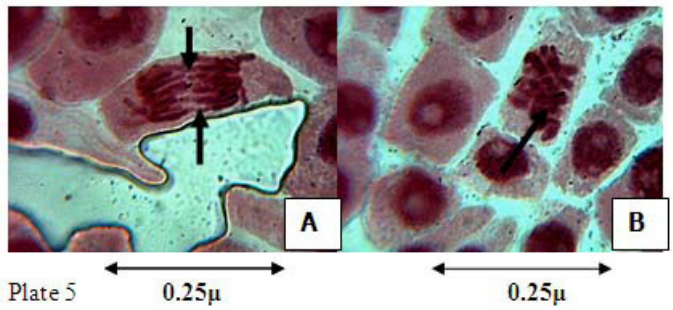

Plate 5. Chromosomal aberrations in onion root cells subjected to SA and Cu toxicity chelated with EDTA. Bridges (A), and Vagrant (B). Mag. x 100.

chromosomal aberrations in onion root cells subjected to different treatments. These include sticky chromosome, bridged anaphase, vagrant and c-mitosis. The most common aberration in all treated root cells of onion bulbs were bridged and vagrant. The sticky chromosome caused by Cu (Plate 2) and Cu + EDTA (Plate 3) was corrected by the addition of SA to the solution as shown in Cu + SA (Plate 4) and Cu + EDTA + SA (Plate 5).

DISCUSSION

Plant systems are sensitive biomonitors of the cytotoxic and genotoxic effects of different chemicals (Grant, 1999). Positive results monitored in higher plant systems like the *Allium* assay indicate the presence of cytotoxic and/or genotoxic attributes of some compounds. This can also be used to monitor ameliorative and protective effects of some other compounds. High concentrations of Cu (>768 mmol l^{-1}) kill root cells of onion. In *Allium* toxicity test, growth inhibition is indicated by the appearance of stunted root and wilting of root. This growth reduction and root wilting is followed by suppression of mitotic activity and occurrence of chromosomal aberrations (Odeigah et al., 1997; Grant, 1982). In the present study, onion bulbs subjected to a toxic concentration of Cu (100.79 mmol l^{-1} of

Cu) caused wilting of root, root length and biomass reduction, which may have resulted from binding of Cu to proteins, thereby inhibiting enzyme activity important in plant processes such as photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic processes (Küpper et al., 2000). Onyemaobi et al. (2012) have reported that the decrease in root length may be due to enhanced lignin production that tend to solidify the cell wall and hence restricts root growth. Earlier reports had stated that inhibited mitotic entry might also be the cause of growth inhibition in various plants (El-Deek and Hess, 1986).

When onion roots were subjected to Cu chelated with EDTA, there was an increase in the root length and biomass though its reorganizing ability was weakened by the presence of SA. This corroborates the reports of Ruley et al. (2006) that application of EDTA gives plants the potential to tolerate heavy metal toxicity. It can also be proffered that the presence of EDTA helps to ameliorate the toxic effect of Cu on root morphology and cell division process. However, stressed onion roots had chromosomal aberrations; bridged and vagrant chromosomes were present in all treatments and can be considered as indicators of clastogenicity. Furthermore, roots of onion bulbs exposed to toxic concentrations of Cu and chelated Cu showed sticky chromosomes; laggards and vagrant chromosomes are indicators of spindle poisoning (Onyemaobi et al., 2012). Chromosome stickiness may be caused by immediate reactions with DNA causing DNA-DNA or DNA-protein cross linking (Amin, 2002). According to Odeigah et al. (1997), sticky chromosomes are indicative of high toxicity which is usually irreversible physiological effect leading to cell death.

There is no single overall theory which can explain all aberrations since they are probably induced through different mechanisms. However, depression of energy systems, interference with DNA synthesis at the S-phase, protein synthesis and binding can affect the integrity of a chromosome. These may have a role to play in fragmentation, pulverization and clumping of chromosomes.

Salicylic acid, a natural signal molecule, has been shown to play an important role in regulating a number of physiological processes in plants. It helps plants to resist heavy metal stress (Bosch et al., 2007). The present study showed that SA improved the root length and biomass of bulbs and corrected the chromosome stickiness of bulbs subjected to the toxic concentration of Cu. The latter was evident in the presence of an increased number of normal cells as compared with those not supplemented with SA.

The DNA purity maintained in the present study (1.82 -1.92) based on the A260/280 absorbance was an indication that DNA-protein complex in *Allium cepa* cells was not affected by the tested substances.

In conclusion, the toxic concentration of Cu used in this study reduced the root cells, root growth and biomass of onion bulbs. Chelating toxic Cu with EDTA improved the root growth and biomass of the roots but some chromosomal aberrations were observed. SA also had

ameliorative effect but this was not as effective as EDTA. The presence of SA with chelated Cu weakened the protective impact of EDTA on growth. However, SA regulated the sticky chromosomes caused by the toxic concentration of Cu.

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