Comparing Effects of Copper and Chromium Treatments on Growth of *Cyperus esculentus* L. in Field and i*n Vitro* Studies and Further Explanation by Restriction Fragment Length Polymorphism Analysis

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Abstract: The contamination of agricultural soils by heavy metals following long-term use of fertilisers, urbanisation and industrialisation raise concerns. The effects of copper ($CuSO_4$) and chromium (Cr_2O_3) salt solutions on *Cyperus esculentus* tubers and plants were studied under *in vitro* and potted-field conditions. The objective of the study was to evaluate the effects of copper and chromium ions on the growth of *Cyperus esculentus*. The concentrations of copper and chromium salt solutions prepared and applied as treatments were 0 ppm, 100 ppm, 200 ppm, 300 ppm, and 400 ppm. *In vitro* study was set up using petri dishes. Parameters measured were percent germination, number of plumules and radicles, plumule and radicle lengths. In the field study, percent germination, plant height, fresh and dry weights were measured. The *in vitro* and field experiments were laid out as a completely randomised design with four replications. Germination in the *in vitro* study was observed in copper and chromium treated tubers 3 and 6 days after treatment (DAT) respectively. Multiple radicle and plumule formation per tuber was recorded and this was observed to be metal concentration-dependent. Results of the field experiment showed that tubers germinated in both copper and chromium treated soils 5 and 7 days after planting, respectively. Copper solutions stimulated plant height as compared to control whereas chromium concentrations greater than 100 ppm inhibited plant height. These differences were significant ($\alpha = 0.05$). The micrograph from the RFLP analysis revealed alterations of DNA bands obtained for 400 ppm Cu-treated and all (except 100 ppm) Cr-treated plants as compared to DNA bands of control plants. It is concluded that copper inhibited *Cyperus esculentus* at 400 ppm only whereas concentrations above 100 ppm chromium affected the plant negatively.

Keywords: Copper; Chromium; Treatments; RFLP; *Cyperus esculentus*

1. Introduction

Cyperus esculentus L. of the family Cyperaceace is an erect, grass-like perennial plant. It is one of the underutilised or "orphan" crops with enormous benefits to man especially the edible tubers. This plant is cosmopolitan and its cultivation can be hampered by the degradation of arable lands by heavy metal pollution. *Cyperus esculentus* (also called tiger nut) is popular because of its nut-like tubers eaten raw by many residents of Benin City, Nigeria. It is cultivated in many northern States of Nigeria where the use of fertilizers and pesticides for crop cultivation is common and are strongly championed by farmers and Governments. Fertilizers and pesticides contain traces of heavy metals. The presence of heavy metals in soil is significant because they are non-degradable and can persist for a long time (Iwegbue *et al*., 2013). Detection of moderate to high concentrations of heavy metals in soil will make that environment unsuitable for agricultural purpose. There is a risk associated with high soil concentration of toxic metals to plants, natural waters and humans (Adriano, 2001).

In Nigeria, the pace of urbanisation, industrialisation and other various anthropogenic pressures have introduced high contamination incidences by heavy metals in several cities (Ladigbolu and Balogun, 2011; Nubi *et al*., 2011). Contamination of soils by heavy metal is dependent on their bioavailability as well as their mobility in the environment. Heavy metals are absorbed by plants when grown in polluted sites and when consumed deliver these heavy metals into the human body (Zurera-Cosano *et al*., 1989; Cambra *et al*., 1999). The presence of heavy metals in many agricultural farming regions has been heightened by yearly widespread flooding incidents of localities that are under pressure from unconventional mining of solid natural minerals and deposits in many communities of the northern states of Nigeria.

Reports of pollution by heavy metals in Nigeria have suggested chromium and copper (Azumi and Bichi, 2010; Garba *et al*., 2010; Ibeto and Okoye, 2010) to be widespread. Soil concentrations of heavy metals range from less than 1 mg/kg to 100,000 mg/kg, either due to geologic origin or anthropogenic activity of the soil (Iwegbue *et al*., 2013). Soil contents of heavy metals can be viewed as a double edged sword because of the role of some in normal plant growth and living organisms to the toxicity associated with presence of others (Pb, Cd and Hg etc). Copper and chromium are amongst the most commonly occurring heavy metal pollutants and major constituents of industrial effluents (Thilakar *et al*.,

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2012). Both copper and chromium are essential trace elements required for the normal functioning of human physiology whereas only copper remains essential as a micronutrient in plants. Chromium is not required by plants though it is constantly present in the soil. These metals are toxic and deleterious at higher concentrations to both plants and animals, and the essential ones are beneficial at lower concentrations. Their absence in humans also causes deficiency condition like decreased fertility and abnormal bone formation for chromium and copper, respectively (Ayodele and Ajala, 2009).

Deficiency or excess copper in plants causes anomaly by altering important physiological process like photosynthetic electron transport. Copper is a structural component of proteins and enzymes that participate in vital growth processes. Copper concentrations exceeding 20μg/g can be toxic (Wright and Welbourn, 2002). The presence of excess copper during germination and growth reduces seed germination and inhibits growth by interfering with cellular processes like respiration and photosynthesis (Prasad and Strazalka, 1999), as well as pigment content, development of plant organelles, protein synthesis, biomass, root, leaf and stem growth. Due to nutrient-metal interaction, the pattern of nutrient uptake is disturbed when high concentration of chromium is present in the soil. The phytotoxicity of chromium includes root growth reduction, seed germination inhibition, depressed biomass, necrosis and chlorosis. Chromium stress in plants has been demonstrated to cause damage to DNA, pigments, protein and also initiates lipid peroxidation. Chromium toxicity reduces seed germination and radicle growth. Inhibition of growth in plants during chromium stress results from cell division inhibition and induction of chromosomal aberrations (Zeid, 2001). Excess copper, chromium and cobalt produced adverse effects on biomass, iron concentration, chlorophyll a and b contents, protein, and catalase enzyme activity in cauliflower (Chatterjee and Chatterjee, 2000).

The exposure of living organisms to heavy metals at high concentrations creates oxidative stress that generates toxic oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH*) and superoxide radical (O_2^-) (De Vos *et al.*,1993). Toxic oxygen species cause changes in membrane lipids, proteins and nucleic acids. DNA damages such as nitrogen base degradation, single- and double-strand breakage and cross-linkage to proteins are induced by toxic oxygen species (Imlay and Linn, 1986). Assessment of the toxic effects of heavy metals in living organisms is one most commonly studied subjects in environmental science. DNA finger printing techniques like RAPD and AFLP have clearly shown DNA alterations in animals, bacteria and plants induced by pollutants. The application of molecular techniques in metal toxicity studies were reported by Korpe and Aras (2011), Mengoni *et al.* (2000), De Wolf *et al.* (2004) and Liu *et al.* (2009). These molecular techniques were engaged because of their sensitivity.

The toxicity of individual elements to a plant species will be better understood when data on population (growth) parameters are supported by results of molecular analysis. This will give correct interpretations of the effects of individual elements on the plant species. Copper was selected as one metal essential for normal growth of plants and chromium as non-essential element but ubiquitous in the soil. There is dearth of information on the use of *Cyperus esculentus* in heavy metal studies. Plant responses to *in vitro* and field-based metal treatments differ. There is gain in knowledge if such information is provided by empirical studies. The objective of this study was to evaluate effects of copper and chromium ions on growth of *C. esculentus,* supported by data from restriction fragment length polymorphism (RFLP) analysis.

2. Materials and Methods

2.1 Plant Material and Preparation of Copper and Chromium Solutions

Healthy looking tubers of *Cyperus esculentus* L. were obtained from northern farmers using "Hausa Quarters" along Sokpomba road, Benin City, as base with latitude of 6.32893^o N and longitude of 5.62803^{o}E. The test of viability was carried out using the floatation method by gently putting the tubers in a bowl containing deionised water for 3-5 minutes. Tubers that sank down were taken as viable and used for the experiment. Analytical grade salts of $CuSO_4$ and Cr_2O_3 were purchased and used for the preparation of copper and chromium solutions. The concentrations applied were 0, 100, 200, 300, and 400 ppm. These were based on ecological screening values of metals. Preparation was done by dissolving the appropriate weight of the salts required to prepare any particular concentration in one litre of deionised water.

2.2. *In vitro* Experimental Setup

Forty cleaned petri-dishes were fitted with filter papers (Whatman No.1) and correctly labelled according to the salt solutions (treatments) to be applied. Five millimetres of treatment solutions were used to wet the filter paper. Ten (10) viable tubers were put into each petri dish. Thereafter, another five millilitres solution was added to the petri dishes. Each petri-dish was augmented with an appropriate solution when the need arose. Each treatment was replicated four times. This set up was monitored for germination, radicle and plumule production daily.

2.3. Field Experimentation

Top loamy soil (0-15 cm depth) was collected as a composite sample from a fallow space in the Botanic Garden, Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria. Forty experimental pots were prepared by filling 3 kg soil into each and correctly labelled according to the specific treatments in the study. The experimental pots were perforated underneath. These pots were watered

with the respective treatment solution and left in the field overnight. Sowing was done the following day in the morning. Five tubers were sown per experimental pot at 2-3 cm depth into the soil. Each pot was watered with 200 ml of the respective treatment solution immediately after sowing. This volume was below water holding capacity of soil and sufficient to make the soil sample wet for the metal ions to be in contact with the plant root. Each treatment was replicated four times. The subsequent application of copper and chromium treatment solutions to the soil was done every fourth day for nine weeks after planting. Field data collected were percent germination, plant height, fresh and dry weights. Germination record was taken daily for 18 days after planting. Plant height measurements (during vegetative stage of growth) were taken using a measuring tape. Nine weeks after of planting (9 WAP), the plants were harvested from the soil along with roots. The roots were washed to remove all soil particles from the root region. The plants were air-dried and their fresh weights recorded. Then each plant was correctly labelled and taken to the oven for drying at the temperature of 50^{o}C for a period of 4-5 days to obtain the dry weight. The plant samples were measured continually until a constant dry weight was obtained.

2.4. Molecular Analysis of Plants

Three weeks after sowing, leaf samples of plants from each concentration were collected and used for molecular analysis. DNA extraction was carried out using kit.

2.4.1. DNA Extraction

One gramme (1g) fresh leaf samples were obtained for DNA extraction and analysis. DNA was isolated using DNA extraction kit (Norgen Biotek, Canada) as directed by the manufacturer (Okoror *et al*., 2015).

2.4.2. Restriction Fragment Length Polymorphism Analysis

The reaction mixture for the RFLP analysis was prepared by adding 5µL of assay buffer, 10µL of BSA and 3µL of restriction enzyme (Hind I) to 10µL of the DNA extract. For 1 hour at 37^{o}C, the vials were incubated to completely digest the DNA. The silver stain of polyacrylamide gel was used to visualize the products digested by the restriction enzyme. Then, 50 ml of a fixing solution diluted with 9.5 ml ethanol and 30.5 mL double distilled water were used for gel fixation for 30 minutes and for another 30 minutes impregnated with silver staining solution. The gels were then washed for 1-3 minutes in double-distilled water.

After the staining solution was removed, for 10 minutes, the gels were kept in the developing solution in the dark, such that the developing solution was poured out when the bands were dark enough and immediately, the stopping and preserving solution was added to stop and preserve the patterns formed (Parani *et al*., 1997; Sahu *et al*., 2012).

2.5. Percent Phytotoxicity and Metal Tolerance Index

Phytotoxicity percentage and metal tolerance index were calculated on days 3 and 9 for Cu ion treatments and on days 20 and 35 for Cr ion treatments in the *in vitro* experiment using the formula by Bauddh and Singh (2011).

$$\text{Percent Phytotoxicity} = \frac{(\text{Radicle length of control} - \text{Radicle length of test})}{\text{Radicle length of control}} \text{X100} \quad (1)$$

$$\text{Metal Tolerance Index (MTI)} = \frac{\text{Radicle length of seed in test}}{\text{Radicle length of seed in control}} \text{X100} \quad (2)$$

2.6. Statistical Analysis

Mean and standard deviation were calculated for the data recorded in the study. Two-factor analysis of variance was used to analyze the data obtained. Separation of means was carried out using Duncan Multiple Range test or Tukey's test with the aid of GEN STAT version 8.

3. Results

The results obtained in this study are shown in Tables 1-6, Figures 1-8 and Plate 1.

3.1. Germination

Germination was recorded for *Cyperus esculentus* tubers both in the laboratory and field experiments. The percent germination records showed that *C. esculentus* tubers germinated in all concentrations of Cu^{2+} solution applied as treatments during the *in vitro* experiment. Delay in germination was very pronounced in 300 and 400 ppm Cu treated tubers where the first indication of germination occurred 9 and 12 days after treatment (DAT) respectively (Table 1). The least percent germination was recorded in 400 ppm Cu^{2+} treatment. For chromium (Cr^{3+}) solution treated tubers, germination was observed 6 and 20 days after treatment in 100 ppm Cr^{3+} and 400 ppm Cr^{3+}, with a mean percent germination of 12.5% and 5% respectively. Further delay in germination was observed in 200 ppm Cr^{3+} and 300 ppm Cr^{3+} treated tubers (Table 2). The viability test of tubers carried out prior to the setting up of *in vitro* experiment did not prove their quality and is suspected to be responsible for the low germination observed in control treatments.

Germination in the field was entirely different from what was obtained in the laboratory. Five days after planting (5 DAP) in the field, germination was recorded in all treatments except for 300 ppm $Cr^{3+}$and 400 ppm Cr^{3+}. There was improvement by 7 DAP where germination was observed in all treatments. The application of the Cu^{2+} and Cr^{3+} solutions to the soil did not affect germination of *C. esculentus* tubers significantly when compared to values obtained for control and treated 18 DAP (Table 3). Generally, the range of

germination in the field was 65-97%. This indicated that the tubers were viable and showed resistance to metal treatments applied in soil.

Table 1. Percentage germination of *Cyperus esculentus* tubers treated with $CuSO_4$ solution during *in vitro* experiment.

Cu^{2+} solution(ppm)	3 DAT	6 DAT	9 DAT	12 DAT	15 DAT	18 DAT
0	15.50±7.50	32.50±25.00	35.00±23.80	35.00±23.80	37.50±22.17	40.00±21.60
100	7.55±5.17	15.50±5.57	17.32±7.50	17.32±7.50	20.62±7.58	27.50±7.08
200	7.55±5.17	11.57±5.57	11.57±5.57	14.14±4.14	14.14±4.14	18.93±5.90
300	0.00±0.00	0.00±0.00	9.50±2.50	14.14±4.14	14.14±4.14	15.60±5.12
400	0.00±0.00	0.00±0.00	0.00±0.0	15.50±5.16	15.50±5.16	15.50±5.16

Note: Values = mean ± S.D; DAT = Days after treatment; ppm = parts per million.

Table 2. Percentage germination of *Cyperus esculentus* tubers treated with Cr_2O_3 solution during *in vitro* experiment.

Cr^{3+} solution (ppm)	6 DAT	20 DAT	25 DAT	30 DAT	35 DAT
100	12.50±5.00	12.50±5.00	12.50±5.00	15.50±2.50	15.50±2.50
200	0.00±0.00	0.00±0.00	12.50±5.00	12.50±5.00	12.50±5.00
300	0.00±0.00	0.00±0.00	0.00±0.00	12.50±5.00	15.00±5.77
400	0.00±0.00	5.00±1.77	11.50±5.57	11.50±5.57	11.50±5.57

Note: Values= mean ± S.D; DAT= Days after treatment; ppm= parts per million

Table 3. Percentage germination of *Cyperus esculentus* tubers in soil treated with $CuSO_4$ and Cr_2O_3 solutions.

Treatments	Conc.(ppm)	5 DAP	7 DAP	9 DAP	11 DAP	15 DAP	18 DAP
Control	0	18.10^{a}±7.74	25.00^{a}±11.09	50.00^{a}±12.43	70.00^{a}±34.64	92.50^{a}± 5.00	92.50^{a}± 5.00
Cu^{2+}	100	20.00^{a}±10.00	32.50^{a}±5.00	45.00^{a}±19.50	67.50^{a}±25.94	70.00^{a}±28.30	82.50^{a}±14.03
	200	20.00^{a}±5.00	27.00^{a}±8.28	57.50^{a}±11.13	60.00^{a}±22.43	65.00^{a}±26.46	65.00^{b}±26.46
	300	22.50^{a}±5.00	27.00^{a}±4.14	42.50^{a}±9.57	70.00^{a}±21.60	77.50^{a}±26.30	80.00^{a}±18.28
	400	25.00^{a}±7.10	32.50^{a}±9.62	82.50^{b}±12.58	95.00^{a}±10.00	95.00^{a}±10.00	97.50^{a}±2.00
Cr^{3+}	100	17.50^{a}±5.00	47.50^{a}±12.17	77.50^{b}±18.93	90.00^{a}±8.16	92.50^{a}±5.00	95.00^{a}±5.77
	200	15.00^{a}±1.00	32.50^{a}±5.00	57.50^{a}±15.00	80.00^{a}±14.14	90.00^{a}±4.14	97.50^{a}±2.00
	300	0.00^{a}±0.00	32.50^{a}±8.93	47.50^{a}±27.54	67.50^{a}±22.17	77.50^{a}±22.17	82.50^{a}±13.63
	400	0.00^{a}±0.00	32.50^{a}±5.00	47.50^{a}±27.54	72.50^{a}±26.30	75.00^{a}±23.80	85.00^{a}±13.80

Note: DAP = Days after planting; ppm = parts per million, Values are presented as mean ± S.D, means with similar alphabets as superscript in one column are not different significantly at 0.05 level of significance using Tukey's test.

2.3. Number and Length of Plumules Produced

Number of plumules produced was recorded only in the *in vitro* experiment. Multiple plumule formation was observed in tubers of *Cyperus esculentus* during the *in vitro* experiment. The mean number of plumules produced by the tubers in the control treatments was 6 obtained 18 DAT applications. Copper ion solutions depressed plumule production in *Cyperus esculentus* tubers. Numbers of plumules produced by Cu^{2+}-treated tubers were higher than that of Cr^{3+} - treated tubers. The negative effects of Cu^{2+} solutions applied were observed at 300 ppm and 400 ppm concentrations. Plumule production was not observed in 400 ppm Cu^{2+} until 10 DAT (Figure 1). Plumule production by Cr^{3+} treated tubers was first observed in 100 ppm 6 DAT. Plumule production in Cr^{3+} concentrations higher than 100 ppm were recorded at least 20 DAT and average of 1 plumule/ tuber was observed. Thirty-five DAT, the average number plumule/tuber was 3, 2, 1 and 1 for 100, 200, 300 and 400 ppm Cr^{3+}-treated tubers respectively (Figure 2). The observation of control treatments ended 18 DAT and this wide disparity in time of response by tubers treated with Cr^{3+} solutions made control plot not to fit into Figure 2 appropriately.

The average length of plumule recorded 18 DAT for tubers in control treatments was 19 cm. Other treatments gave lower values. For example, the average lengths of plumule recorded for 100, 200, 300 and 400 ppm Cu^{2+} treated tubers were 9.7, 7.3, 5.4, and 2.8 cm respectively (Figure 3). Similarly, the average lengths of plumule recorded 35 DAT for 100, 200, 300 and 400 ppm Cr^{3+} treated tubers were 8.6, 2.1, 1.5 and 2.1 cm respectively (Figure 4). The wide disparity in time between records taken for control and Cr^{3+} treated tubers did not permit the addition of a plot for control. But the control can be read from Figure 3.

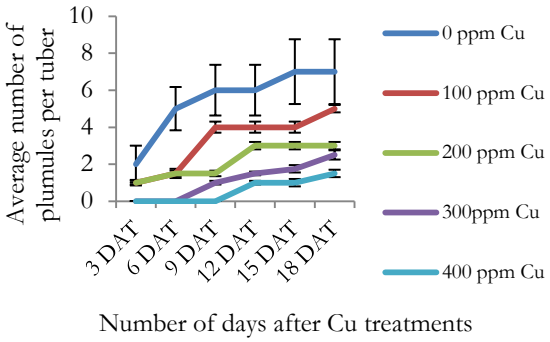

Figure 1. Number of plumules produced by *Cyperus esculentus* tubers treated with $CuSO_4$ during *in vitro* experiment. (DAT= Days after treatment. Error bars represent standard deviation).

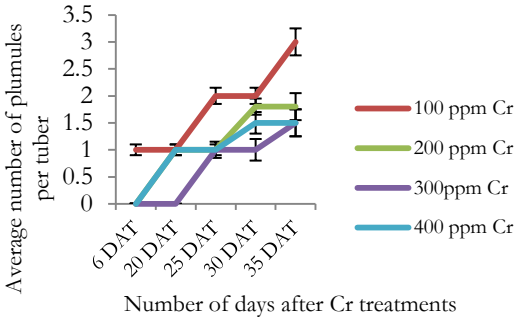

Figure 2. Number of plumules produced by *Cyperus esculentus* tubers treated with Cr_2O_3 during *in vitro* experiment. (DAT= Days after treatment. Error bars represent standard deviation).

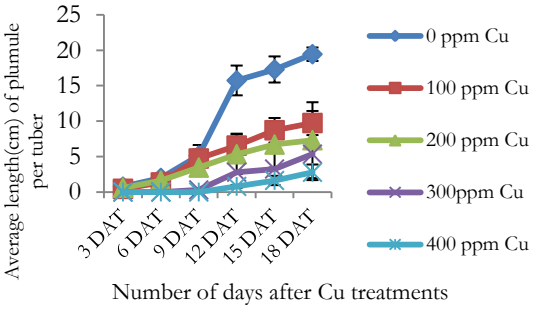

Figure 3. Average length (cm) of plumule produced by *Cyperus esculentus* tubers treated with $CuSO_4$ during *in vitro* experiment. (DAT = Days after treatment. Error bars represent standard deviation).

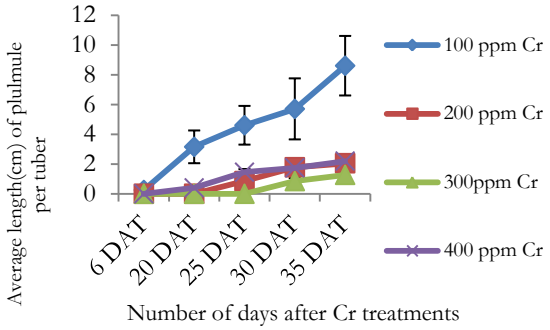

Figure 4. Average length (cm) of plumule produced by *Cyperus esculentus* tubers treated with Cr_2O_3 during *in vitro* experiment. (DAT= Days after treatment. Error bars represent standard deviation).

3.3. Number and Length of Radicles Produced

Number of radicles per tuber was recorded only in the *in vitro* experiment. Eighteen DAT, the mean number of radicles in control treatment was 98. The numbers of radicles per tuber under Cu^{2+}treatments were 43, 27, 9 and 0 for 100, 200, 300 and 400 ppm respectively (Figure 5). Radicle production was inhibited at the highest Cu^{2+} solution applied (400 ppm). Radicle production in Cr-treated tubers began 20 DAT. Radicle initiation was recorded in 200 and 300 ppm Cr^{3+} 23 DAT. Thirty-five DAT, average numbers of radicles produced for Cr treatments were 11, 5, 3 and 5 for 100, 200, 300 and 400 ppm respectively (Figure 6). Radicle length records taken showed that Cu and Cr treatments of *C. esculentus* tubers affected extension growth negatively. For example, 18 DAT, mean radicle length for control, 100, 200, and 300 ppm Cu^{2+}-treatments were 15.70, 6.09, 2.87, and 1.30 cm respectively (Figure 7). Mean values of radicle length for 100, 200, 300 and 400 ppm Cr-treated tubers were 2.35, 0.95, 0.80 and 1.96 cm respectively 35 DAT (Figure 8). The value for average radicle length of 400 ppm Cr^{3+} treated tubers was higher than that obtained for either 200 or 300 ppm Cr^{3+} treated tubers. This observation is not unconnected with viability of the tubers used in the *in vitro* experiment.

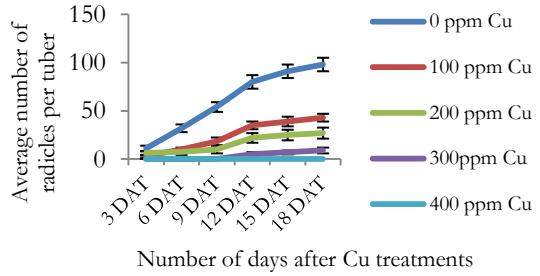

Figure 5. Number of radicles produced by *Cyperus esculentus* tubers treated with $CuSO_4$ during *in vitro* experiment. (DAT = Days after treatment. Error bars represent standard deviation).

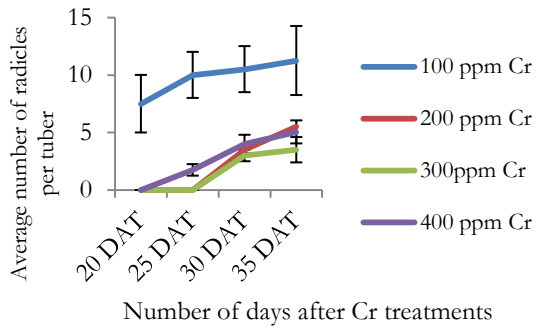

Figure 6. Number of radicles produced by *Cyperus esculentus* tubers treated with Cr_2O_3 during *in vitro* experiment. (DAT = Days after treatment. Error bars represent standard deviation).

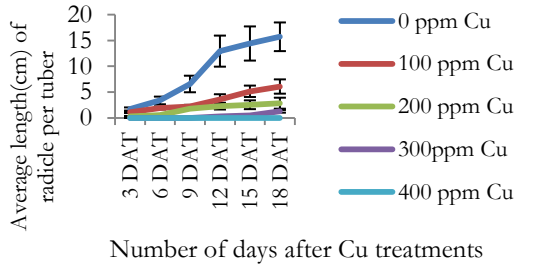

Figure 7. Average length (cm) of radicles produced by *Cyperus esculentus* tubers treated with $CuSO_4$ during *in vitro* experiment. (DAT= Days after treatment. Error bars represent standard deviation).

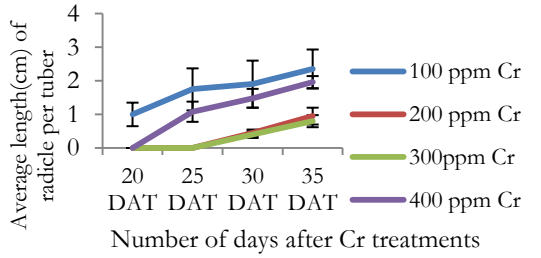

Figure 8. Average length (cm) of radicles produced by *Cyperus esculentus* tubers treated with Cr_2O_3 during *in vitro* experiment. (DAT = Days after treatment. Error bars represent standard deviation

3.4. Restriction Fragment Length Polymorphism micrograph

The result obtained for the RLFP analysis shows DNA bands obtained from plant samples grown in Cr- and Cu-treated soils (Plate 1). Eight DNA bands were indicated in the micrograph. Comparing the bands formed using the band obtained from 0ppm treated plants as standard; the effects of the different treatments were visible. Three out of eight bands clearly show distortions. These were DNA bands 5, 7 and 8 obtained from plant samples treated with 400ppm Cu, 400ppm Cr and 200ppm Cr respectively.

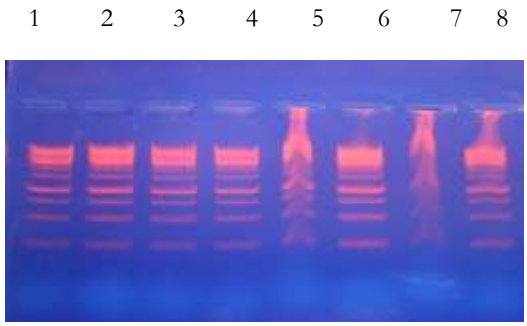

Plate 1. A micrograph showing the DNA bands obtained from a RFLP analysis of *Cyperus esculentus* treated with different concentrations of $CuSO_4$ and Cr_2O_3 solutions. *Note:* 1= Cr 100 ppm, 2 = Cu 200ppm, 3 = Cu 300ppm, 4 = Cu 100ppm, 5 = Cu 400ppm, 6= 0ppm, 7 = Cr 400ppm, 8= Cr 200ppm, the last was completely digested and came up with no band.

3.5. Plant Heights, Fresh and Dry Weights

Progressive increase in height of *Cyperus esculentus* plants grown in Cu^{2+} and Cr^{3+} treated soils was observed from 3 WAP to 9WAP (Table 4). After 9 weeks of growth, mean height of plants recorded in control,100, 200, 300 and 400 ppm Cu^{2+} treated soils were 69.03, 66.00, 71.00, 79.70, and 80.60 cm respectively. From the values observed, Cu^{2+} treatments applied supported increase in plant height. Plant height values recorded in soils treated with 100, 200, 300 and 400 ppm Cr^{3+} solutions were 85.10, 59.63, 60.73, and 44.03 cm respectively 9WAP. Comparing these values to that of control, one observes enhancement of plant height in 100 ppm Cr^{3+} treated soils and depression in plant height in 200 - 400 ppm Cr^{3+} treated soils (Table 4).

Fresh and dry weight values obtained in the study are shown in Table 5. The average values for fresh weight (FW) of plants grown in control, 100, 200, 300, and 400 ppm Cu^{2+} treated soils were 2.14, 2.89, 1.22, 2.04 and 1.41 g respectively. Fresh weight for 100, 200, 300 and 400 ppm Cr^{3+} treated plants were 1.22, 1.59, 1.20 and 1.57 g respectively. The differences in fresh weights were significant (α= 0.05). The mean FW of plants grown in 100 ppm Cu^{2+} treated soils was higher than that of the control (0 ppm) while mean FW for 100 ppm Cr^{3+} was lower than control. Dry weights (DW) of plants obtained for control, 100, 200, 300 and 400 ppm Cu^{2+} treated soils were 0.72, 0.80, 0.52, 0.69, and 0.61 g respectively. Also, 100, 200, 300 and 400 ppm Cr^{3+} treated plants gave 0.61, 0.53, 0.46, and 0.57 g respectively as DW. The mean DW of control plants was lower than that for 100 ppm Cu^{2+} plants but higher than that obtained for 100 ppm Cr^{3+} treated plants (Table 5).

3.6. Phytotoxicity and Metal Tolerance Indices

The estimates of phytotoxicity and metal tolerance indices during the *in vitro* experiment are shown in Table 6. The phytotoxic effects estimated at 3 DAT and 9 DAT for tubers treated with 100 ppm Cu^{2+} solutions were 37.14 and 65.81% respectively. This indicates that

the toxic effects were more pronounced 9 DAT than at 3 DAT as compared to control. The longer the exposure time, the weaker the average resistance induced by the tubers. The phytotoxic effects estimates for 300 and 400 ppm Cu^{2+} treated tubers were 100%. The estimates showed that the resistance induced by tubers in these treatments did not produce measurable responses. The estimates of phytotoxic effects for tubers treated 100 ppm Cr^{3+} 20 DAT and 35 DAT were 42.86 and 64.29% respectively. The higher estimate showed that the effects became more pronounced at 35 DAT when compared with the control. Higher estimated phytotoxic values at 35 DAT were obtained for 200 ppm Cr^{3+} and 300 ppm Cr $^{3+}$. The phytotoxic estimates at 20 DAT for 200, 300 and 400 ppm Cr $^{3+}$ were the same, i.e. 100%, and this represents total inhibition with no measurable response. The phytotoxic effects estimates should be interpreted as a gap between control and metal treated tubers. The estimated metal tolerance indices for 100 ppm Cu^{2+} at 3and 9 DAT were 62.86 and 34.14%. Similarly, values estimated for 200ppm Cu^{2+} were 17.14 and 27.36%. These infer the ability of the plant to tolerate the concentrations of metals applied as treatments. The higher values of metal tolerance indices show that the tubers at such concentrations exhibited some level of tolerance. For 100 ppm Cr^{3+} treated tubers, metal tolerance indices at 20 and 35 DAT were 57.14 and 35.71% respectively; while for 200 ppm Cr^{3+} treated tubers at 20 and 35 DAT were 0 and 14.44% respectively. A value of zero (0) indicates metal intolerance. The tubers were suspected to build up some level of tolerance for the metal as the exposure periods increased from 20 - 35 DAT for treatments > 200 ppm Cr^{3+}.

Table 4. Plant heights of *Cyperus esculentus* grown in different concentrations of $CuSO_4$ and Cr_2O_3 solutions treated soils.

Treatments	Conc.(ppm)	3 WAP	5 WAP	7 WAP	9 WAP
Control	0	$41.00^{a}\pm1.77$	$68.40^{a,b}\pm13.25$	$69.73^{a,b,c}\pm10.89$	$69.03^{a,b}\pm11.58$
Cu^{2+}	100	$44.00^{a}\pm4.19$	$68.03^{a,b}\pm2.55$	$67.17^{a,b,c}\pm2.97$	$66.00^{a,b}\pm0.70$
	200	$42.33^{a}\pm1.53$	$64.30^{a,b}\pm6.73$	$68.03^{a,b,c}\pm3.65$	$71.00^{a}\pm1.95$
	300	$38.97^{a}\pm1.01$	$69.10^{a,b}\pm11.65$	$74.60^{a,b}\pm6.36$	$79.70^{a}\pm10.64$
	400	$33.57^{a,b}\pm9.02$	$66.70^{a,b}\pm9.40$	$79.80^{a,b}\pm12.76$	$80.60^{a}\pm12.97$
Cr^{3+}	100	$43.37^{a}\pm3.98$	$77.43^{a}\pm11.25$	$83.87^{a}\pm13.98$	$85.10^{a}\pm13.33$
	200	$36.03^{a,b}\pm2.35$	$66.57^{a,b}\pm5.77$	$56.73^{b,c}\pm9.71$	$59.63^{a,b}\pm12.72$
	300	$35.47^{a,b}\pm2.84$	$69.70^{a,b}\pm14.92$	$55.57^{b,c}\pm7.40$	$60.73^{a,b}\pm7.41$
	400	$23.77^{a,b}\pm5.78$	$39.03^{b}\pm15.92$	$43.37^{c}\pm9.60$	$44.03^{b}\pm9.90$

Note: WAP= weeks after planting, values are presented as mean± S.D, mean with similar alphabets as superscript in one column are not different significantly at 0.05 level of significance using Tukey's test.

Table 5. Fresh and dry weights of *Cyperus esculentus* plants harvested from the different concentrations of $CuSO_4$ and Cr_2O_3 solution treated soils 9 weeks after planting (WAP).

Treatments	Conc.(ppm)	Fresh weight (g)	Dry weight (g)
Control	0	$2.1418^{a,b}\pm0.1362$	$0.7167^{a}\pm0.0784$
Cu^{2+}	100	$2.8933^{a}\pm1.0625$	$0.7954^{a}\pm0.3881$
	200	$1.2191^{b}\pm0.4785$	$0.5175^{a}\pm0.2451$
	300	$2.0439^{a,b}\pm1.2987$	$0.6896^{a}\pm0.3429$
	400	$1.4074^{b}\pm0.3853$	$0.6102^{a}\pm0.2388$
Cr^{3+}	100	$1.2169^{b}\pm0.3952$	$0.6102^{a}\pm0.1511$
	200	$1.5937^{b}\pm0.2535$	$0.5321^{a}\pm0.1195$
	300	$1.2026^{b}\pm0.5441$	$0.4600^{a}\pm0.1429$
	400	$1.5658^{b}\pm0.7364$	$0.5734^{a}\pm0.2005$

Note: Data are presented as mean± S.D, mean with similar alphabets as superscript in one column are not different significantly at 0.05 level of significance using Duncan multiple range test.

Table 6. Estimates of phytotoxicity and metal tolerance index of copper and chromium treatments on *Cyperus esculentus* tubers during the *in vitro* experiment.

Metal concentration	DAT	Phytotoxicity (%)	Metal tolerance index (%)
100 ppm Cu	3	37.14	62.86
100 ppm Cu	9	65.81	34.19
200 ppm Cu	3	82.86	17.14
200 ppm Cu	9	72.64	27.36
300 ppm Cu	3	100.00	0.00
300 ppm Cu	9	100.00	0.00
400 ppm Cu	3	100.00	0.00
400 ppm Cu	9	100.00	0.00
100 ppm Cr	20	42.86	57.14
100 ppm Cr	35	64.29	35.71
200 ppm Cr	20	100.00	0.00
200 ppm Cr	35	85.56	14.44
300 ppm Cr	20	100.00	0.00
300 ppm Cr	35	87.84	12.16
400 ppm Cr	20	100.00	0.00
400 ppm Cr	35	70.21	29.79

4. Discussion

Damages to growth and development like photosynthesis, lipid metabolism and nucleic acid synthesis in plants following exposure to high metal concentration are subject of ecotoxicological studies. These toxic effects of heavy metals can be shown with population and molecular parameters. In this study, high concentration of Cu and Cr ions affected *Cyperus esculentus* negatively.

The *in vitro* study revealed the direct effect of Cu and Cr on *Cyperus esculentus* plants. Germination percent obtained indicated inhibitory effects where Cr-treated tubers were more inhibited. The delay associated with germination of plants was very prominent in Cr-treated tubers. For example, 200 ppm, 300 ppm, and 400 ppm treated tubers germinated twenty days after treatment (20 DAT). By 12 DAT, germination has been recorded in similar copper concentrations. The field experiment gave a different insight into germination of *C. esculentus* in Cu and Cr-treated soils. Germination records show that the plant germinated in all concentrations of Cu-treated and Cr-treated soils within 5 and 7 days after treatment respectively. The delays in germination were obviously shorter with growth in the soil. This suggests that the effect of treatments on plants were weakened by the presence of soil particles. Morealso, the mean percent germination records did not show any significant differences in the effects of increasing metal concentrations in the soil. The direct contact of tubers with the treatment solutions *in vitro* was probably the "actual" effect of the treatments. Singh *et al* (2007) reported the inhibitory effects of Cu treatments on seed germination and seedling growth of wheat. Ashagre *et al.* (2013) reported that heavy metals affect seed germination by their ionic toxicity which caused decrease in the breakdown of starch by amylase and concomitant weak germination response recorded. Hema and Subramani (2013) reported decrease in germination of *Vigna radiata* following copper and chromium treatments. There is also the existence of genetic variability among crop species and cultivars for ionic stress. Plumule and radicle production by crop species subjected to metal treatments is used to assess the species response and tolerance. In this study, multiple plumule formation was recorded in tubers of *Cyperus esculentus*. Plumule production was observed in all concentrations of copper treatments, with 300 ppm Cu and 400 ppm Cu solutions producing delayed response of over ten days after treatments. Longer delayed response of plumule production of over 23 DAT was observed in 200 ppm, 300 ppm and 400 ppm Cr-treated tubers. The number and length of plumules produced were inhibited when the values of Cu-treated and Cr-treated tubers are compared with control. Cu-treated tubers exhibited the feature of multiple plumule formation whereas this feature was recorded in 100 ppm Cr and 400 ppm Cr-treated tubers only 35 DAT. Other treatments; 200 ppm and 300 ppm Cr-treated tubers produced only one plumule each. Multiple plumule development is a plant habit shown by species to survive threat from herbivores and stressful conditions. This habit gives *C. esculentus* advantage to compete with weeds during cultivation. Number and length of radicles produced by Cu-treated tubers decreased as the concentrations increased, 18 DAT. Tubers treated with 400 ppm Cu failed to produce radicle. Ashagre *et al.* (2013) reported that tomato seeds (*Lycopersicon esculentum* cultivar Roma VF) did not produce roots in 300, 400, 500 and 600 ppm Cu treatments. Akinci and Akinci (2010) reported that radicle lengths of *Cucumis melo* seeds were inhibited by chromium ion treatments. Similarly, Panda *et al.* (2002) stated that the inhibition of radicle growth of seeds by chromium ions varied with concentrations. In this study, length and number of radicles produced by *C. esculentus* tubers were less than 3 cm long and few in numbers. Hayyat *et al.* (2015) stated that chromium ions inhibited root growth in many crops.

The response of *Cyperus esculentus* in metal-treated soil samples showed some interesting variations. Mean plant heights obtained for 200 ppm, 300 ppm and 400 ppm Cu-treatments were higher than control, indicating some level of stem growth stimulation nine weeks after planting (9 WAP). The reason for this can be inferred from the application of treatments which was done every fourth day as against the all-time contact with the treatments during the *in vitro* experiments. We suggest that the space of days between one treatment application and the next gave the plants ample time to use the available copper ions in the soil for beneficial growth. Hans *et al.* (2004) reported that chromium retards plant growth. Mean plant height was inhibited by 200 ppm Cr and above treatments in the soil, nine weeks after planting. These values were 86.4%, 88% and 63.8% of control value for 200 ppm, 300 ppm and 400 ppm Cr-treated plants respectively. Mean plant height recorded for plants grown in soil treated with 100 ppm Cr was higher than the value for control plants. This value was 123% of the value for control. Zeid (2001) stated that low concentrations of chromium as ionic treatments in plants, cause increase amylase activity, germination and growth. Chromium as an element is not known to play a functional importance in plants. One is unable to explain the mechanism behind this increase in plant height (growth) recorded at 100 ppm Cr-treatment of soil. Fresh and dry weights records of whole plants showed that low concentrations of copper (100 ppm) facilitated the accumulation of matter. This is connected to the nutrient role played by copper at low concentrations. The roots of plants harvested from chromium treated soils showed some peculiar morphological features. The fibrous roots were coloured reddish or pinkish which were different from those of control or Cu-treated plants. The roots formed conspicuous and dark rings at intervals along

the length and from these points, other side roots that were thick and distinct were produced. This feature was not observed with control or Cu-treated plants.

The DNA band patterns obtained after RFLP analysis showed that the DNA contents from plants grown in 0 ppm (control), 100 ppm Cu, 200 ppm Cu, 300 ppm Cu and 100 ppm Cr exhibited similar patterns with no issue of missing bands. This indicates that the DNA structures were neither disrupted nor altered at these concentrations of Cu and Cr treatments. Vinod *et al.* (2012) stated that heavy metal stress in plants causes inefficiency of DNA synthesis. Furthermore, they stated that high concentration of Cu led to reduced synthesis of DNA in wheat. Heavy metals cause injury at the cellular level following the production of free radicals (Panda and Choudhury, 2005). DNA extracts obtained from plants grown in 400 ppm Cu, 200 ppm Cr, 300 ppm Cr and 400 ppm Cr-treated soils showed pronounced alterations of the band patterns. The band patterns suggest that the DNA structure and synthesis in the plants were affected by the treatments. The DNA band patterns of plants treated with 300 ppm Cr was not conspicuous and this suggests that the restriction enzyme (HIND 1) applied may have cleaved it disproportionately, resulting in complete digestion and meaningless band pattern. Choudhary and Panda (2005) stated that the production of ROS under chromium ion stress leads to DNA and protein damage. It is possible to connect the growth responses of *Cyperus esculentus* in Cr- treated soils with the micrograph showing the DNA bands. From RFLP result, chromium can be described as more inhibitory or toxic than copper.

5. Conclusion

Generally, the effects of copper and chromium on germination, and growth of *Cyperus esculentus* were majorly inhibitory and inhibition increases as the concentrations increased. The most damaging concentration of copper to germination and growth was at 400 ppm, whereas chromium was detrimental at concentrations above 100 ppm. The effects of copper and chromium were studied both morphologically and at the molecular level using RFLP techniques. The toxicity observed in both cases show that chromium is more harmful to *Cyperus esculentus* than copper. This study has empirically showed the differences in response of *C. esculentus* tubers *in vitro* and potted field conditions. It is clear that the response *in vitro* where the tubers were in direct contact with the metal solutions should be reckoned and interpreted as the sole and uninterfered effects of Cu^{2+} and Cr^{3+} metals. Also, the Cr^{3+} exhibited higher phytotoxic effects than Cu^{2+} solutions as treatments. In the field, the roots of chromium treated plants were found to be reddish or pinkish coloured, which were peculiar to them. Poor yields of crop plants grown in agricultural soils exposed to anthropogenic risks should be given critical investigation to gain sufficient understanding. In future, the effects of other heavy metals would be considered to ascertain the tolerable concentrations to the plant and highlight associated environmental risks.

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