International Journal of Global Science Research **ISSN (Online): 2348-8344**
Vol.1, Issue 1, April 2014, pp. 87-95
***Available Online* at www.ijgsr.com**

Short Communication

Effect of heavy metals (Cu, Zn) on the growth of Catharanthus roseus

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Received: 21/04/2014
Revised: 24/04/2014
Accepted: 30/04/2014

Abstract: Experiments were conducted to test the effect of heavy metals (Cu, Zn) on seed germination and root growth in medicinal plant *Catharanthus roseus*. A randomly selected fifty seeds in three replicates from C. roseus was used. Test solutions were prepared from each metal at three concentrations (50 µM, 100 µM and 500 µM). Distilled water was used as a control treatment. The objective of this work was to evaluate C. roseus for heavy metal tolerance. Effect of heavy metals, Cu and Zn on the plant growth parameters, Fresh weight (FW), dry weight (DW) and water content (WC), alkaloid content and antioxidant enzymes activity, peroxidase (POX), catalase (CAT) was evaluated. The plant growth (FW and DW) and POX activity increased in presence of low concentration (50 µM, 100 µM) in both metals. But in presence of high concentration (500 µM CuCl2), just opposite trend was observed. CAT activity was found to decrease in presence of both metals. Root growth was more readily affected by the heavy metals than seed germination. Further, it was observed that Zn caused higher plant growth as well as antioxidant activity than Cu. Hence, C. roseus showed better tolerance to Zn than Cu.
Keywords: *Catharanthus roseus*, $CuCl_2$, Heavy metals, Indole alkaloids, Stress physiology, $ZnCl_2$.

INTRODUCTION

Heavy metals are naturally present in the environment. Their occurrence, however, has gradually been increasing with the increase of industrialization. Copper (Cu) and zinc (Zn) are the most abundant heavy metals in the agricultural soils (Förstner, 1995). Copper and Zn, when present in low concentrations, are important micronutrients, while in high concentrations, these two metals become toxic to plants. The ability of plants to accumulate heavy metals is used in the process of phytoremediation where the green plants are employed to cleanse contaminated soils. Medicinal and aromatic plants appear to be a good choice for phytoremediation since these species are mainly grown for secondary products (essential oil) thus the contamination of the food chain with heavy metals is eliminated.

Aromatic and medicinal plants also have a demonstrated ability to accumulate heavy metals (Schneider and Marquard, 1996; Scora and Chang, 1997; Zheljazkov and Nielsen, 1996). Research has shown that heavy metals accumulated by aromatic and medicinal plants do not appear in the essential oil (Scora and Chang, 1997; Zheljazkov and Nielsen, 1996) and that some of these species are able to grow in metal contaminated sites without significant yield reduction. Alyssum has closely related species (A. lesbiacum L., A. murale L., A. bertolonii, A. pintosadilvae L.) known as nickel hyper accumulators (Kabata-Pendias and Pendias, 1992). Anise has shown ability to accumulate heavy metals when present in the medium (Shalaby et al., 1996) and is an aromatic plant grown for essential oil. Sage (Salvia officinalis L.) belongs to the same genera as clary sage (Salvia sclarea L.), was able to grow on heavy metal contaminated soils (Zheljazkov and Nielsen, 1996) and the obtained essential oil had no heavy metals. Heavy metals in soil present a stress condition for growth of the plants. Heavy metal stress can induce conditions of oxidative stress (Zhu, 2000). Changes in the activity of antioxidant enzymes in response to heavy metals (Faltein et al., 1999; Meneguzzo et al., 1999; Sharata and Tal, 1998) were reportedly different in tolerant and sensitive cultivars (Meloni et al., 2003; Olmos et al., 1994; Sairam et al., 2002). Recently, it has been suggested that the heavy metal tolerant cotton cultivar may exhibit better protection against AOS by increasing the activity of antioxidant enzymes under heavy metal stress (Meloni et al., 2003).

Under natural conditions of growth and development, plants are inevitably exposed to different types of stress, which may cause increased production of active oxygen species (AOS) (Smirnoff, 1993). AOS are highly reactive in the absence of any protective mechanism. Heavy metals tolerant plants, in addition to the ability to regulate ion and water movements, often have better antioxidative systems for effective removal of AOS (Rout and Shaw, 2001). The hydrogen peroxide produced is scavenged by catalase (CAT; EC 1.11.1.6.) and a variety of peroxidases (POX; EC 1.11.1.7). POX decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. Peroxidase enzyme has also been implicated in the coupling of vindoline and catharanthine into vinblastine (an antitumor agent) in leaves, and in the oxidation of ajmalicine to serpentine in roots of Catharanthus roseus (Misra et al., 2006). It has been reported that GPX increased in wild type as well as in transgenic Arabidopsis seedlings and in Citrus sinensis under salt stress (Borsani et al., 2001; Faltein et al., 1999). A group of seven GPX genes named AtGPX1-AtGPX7 in Arabidopsis was identified under abiotic stressors (Milla et al., 2003).

Antioxidant enzymes play important roles in adaptation to stress conditions. C. roseus, a medicinal plant, contains most important dimeric indole alkaloids currently known: vinblastine and vincristine found in leaves, and ajmalicine and serpentine found in roots (Misra et al., 1996). Although most aromatic and medicinal plants are directly sown in the field, the impact of the heavy metals on seed germination or the metal tolerance of seedlings during the early stages of development is not known. The objectives of this research were to test the ability of seeds of C. roseus to germinate in a heavy metal contaminated environment, screen as heavy metal tolerance. Consequently, the aim of this study was to examine whether various concentrations of heavy metals (Cu and Zn) on the C. roseus affect the plant

growth and antioxidant defense system, as well as the accumulation of alkaloids.

MATERIALS AND METHODS

Seeds of Catharanthus roseus (L.) G. Don (Pink flower, Family Apocyanacea) and wild type (control) were screened in the laboratory for their heavy metals tolerance. After surface sterilization with 1% (v/v) sodium hypochlorite solution for 10 min, followed by rinsing with distilled water, seeds were imbibed for 2 hrs in distilled water and sown in pots with sterilized sand (Misra and Gupta, 2006). After the test solutions were added, the Petri plates with seeds were randomly positioned in a controlled environmental chamber on a 24 hrs temperature cycle 24±1^{o}C and 18±1^{o}C (for 12 hrs each) for germination. Seed germination was measured every 24 hrs. Seeds were considered to have germinated at radical emergence of 1 mm. After germination, the seedlings were divided into four different groups and transplanted with each group watered every other day with one of the following solutions for one month: group I, distilled water (control: without metal solution), and with different concentration of $CuCl_2$ and ZnCl2 solutions, group II: 50 μM, group III: 100 μM, group IV: 500 μM). Three replicates were used for each concentration of both metal solutions.

After one month of the watering with metal solutions, the plant from each group were uprooted leaf pairs (apical, middle, and basal) were removed node by node and used for growth parameters (Dry weight, Fresh weight, water content), chlorophyll contents, alkaloid contents and enzymatic assays. The plant growth (dry weight and fresh weight) was evaluated using twenty plants from each group in triplicate. At specified periods of growth, all tissue parts (leaf pairs and root) were separated node by node and FW of these tissue parts was measured. For dry weight (DW) determination, these tissue parts were dried for three days in an oven at 70^{o}C (or till there is no decrease in weight). Water content (WC) as percentage of fresh weight (FW) was calculated using formula – WC (%) = [(FW-DW) / FW] × 100.

Preparation of enzyme extracts

A crude enzyme extract was prepared by homogenizing 500 mg of tissue (each leaf pair and root) in 0.1M Tris HCl buffer, pH 7.5, 0.5 mM EDTA and 1%PVP (MW 360,000), at 4^{0}C. The homogenates were centrifuged at 18,000xg for 30 min. The supernatant was used as the crude enzyme preparation.

Catalase (CAT, EC 1.11.1.6) activity was determined after the slight modification of (Upadhyaya et al., 1985). The assay mixture contained 20mM sodium phosphate buffer, pH 7.5, 0.025% H_2O_2 and enzyme extract. The decomposition of H_2O_2 was measured at 240 nm.

Peroxidase (POX, EC 1.11.1.7) activity was assayed in aliquots of crude enzyme preparation as described by (Putter, 1974) with some modifications. The assay mixture consists of 25 mM guaiacol and 0.020% H_2O_2 in 0.1 M sodium phosphate buffers, pH 6.5 at 30^{0}C. The product of the reaction was measured at 470 nm.

All the enzyme activities were expressed in katal.

Soluble protein was measure by the Bio-Red micro assay modification of the Bradford (1976) procedure using bovine serum albumin as standard.

Total alkaloid extraction and determination was carried out as described earlier (Misra and Gupta, 2006). Samples of leaf pairs and root were homogenized in 90% ethyl alcohol. The ethanol extract was evaporated to dryness. The residue was dissolved in distilled water and mixed with conc. HCl (final concentration of HCl was 3%). An equal volume of ethyl acetate was added.

The aqueous phase was collected, adjusted to pH 9.0 with ammonia and extracted with chloroform. The chloroform phase containing the alkaloids was collected and evaporated to dryness to get total alkaloid. The residue was dissolved in methanol and then analyzed by HPLC (Water, USA). The samples of alkaloids were loaded separately on a C^{18} reverse phase analytical cartridge and eluted at a flow rate of 1.0 ml/min with the elution buffer (methanol: acetonitrile: 0.01M ammonium acetate + 0.1% triethylamine in 1:1:2.251ratio) at pH 8.2. The alkaloids were detected at 254 nm.

Statistical Analysis

Each treatment was analyzed with at least three replicates and standard deviation (S.D.) was calculated. Statistical analysis was performed using the students t-test; $p<0.05$ and $p<0.001$ were considered statistically significant and highly significant, respectively.

RESULTS AND DISCUSSION

In presence of Zn, seed germination of Catharanthus roseus was about 98 % at low concentration (50, 100 µM), but very low (30%) in presence of higher concentration (500 µM) (data has not shown). Root growth was apparently promoted by low levels of Zn, as root growth was 30 percent higher in seedlings treated with Zn 100 µM as compared to control (data has not shown). Under all other tested conditions, root growth was significantly reduced by Zn. Copper concentration up to 100 µM had no effect on seed germination while Cu concentration of 500 µM greatly reduced seed germination as compared to control (20 %) (data has not shown). Root growth was significantly reduced in presence of higher Cu concentrations. Zinc at both tested concentrations (50 and 100 µM) significantly promoted seed germination and reduced seed germination in presence of 500 µM as compared with control.

Plant growth parameters in presence of ZnCl2

FW and DW in apical leaf pair increased with increasing concentration of $ZnCl_2$, 50 µM (lowest concentration) caused an increase in FW (≈1.16 fold), DW (≈1.16 fold), while 500 µM $ZnCl_2$ (highest concentration) caused a decrease in FW (≈0.46 fold) and DW (≈0.28 fold) (Table-1). Same pattern has been found in middle and basal leaf pair, the FW and DW increased with increasing concentration of $ZnCl_2$ 50 µM $ZnCl_2$ (lowest concentration) caused a increase in FW (≈1.13 fold, ≈1.51 fold), DW (≈1.28 fold, ≈1.48 fold), while 500 µM of $ZnCl_2$ (highest concentration) caused a decrease in FW (≈0.25 fold, ≈0.37fold) and DW (≈0.17 fold, ≈0.24 fold). In root, the FW and DW decreased with increasing concentration of $ZnCl_2$, 50 µM $ZnCl_2$ (lowest concentration) caused a decrease in FW (≈1.15 fold), DW (≈1.10 fold), while 500 µM $ZnCl_2$ caused a decrease in FW (≈0.24 fold), and in DW (≈0.18 fold).

Plant growth parameters in presence of $CuCl_2$

FW and DW in apical leaf pair increased with increasing concentration of $CuCl_2$, 50 µM (lowest concentration) caused an increase in FW (≈1.08 fold), DW (≈1.13 fold), while 500 µM $CuCl_2$, (highest concentration) caused a decrease in FW (≈0.42 fold), and DW (≈0.16 fold) (Table-2). Same pattern has been found in middle and basal leaf pair, the FW and DW increased with increasing concentration of $CuCl_2$, 50 µM $CuCl_2$ (lowest concentration) caused a increase in FW (≈1.01 fold, ≈1.20 fold), DW (≈1.04 fold, ≈1.20 fold), while 500 µM of $CuCl_2$ (highest concentration) caused a decrease in FW (≈0.22 fold, ≈0.25 fold) and DW (≈0.15 fold, ≈0.17 fold). In root, the FW and DW decreased with

increasing concentration of $CuCl_2$, 50 µM $CuCl_2$ (lowest concentration) caused a decrease in FW (≈1.02 fold), DW (≈1.03 fold), while 500 µM $ZnCl_2$ caused a decrease in FW (≈0.21 fold), and in DW (≈0.13 fold).

Enzyme activities and alkaloid contents

POX activity in apical, middle and basal leaf pairs increased with increasing concentration of $ZnCl_2$ (Tabale-3). 50 µM $ZnCl_2$ (lowest concentration) caused an increase in apical (≈1.62 fold), middle (≈1.38 fold), and basal (≈1.33 fold), while 500 µM $ZnCl_2$ (highest concentration) caused an increase in apical (≈2.51 fold), middle (≈2.17 fold), and basal (≈2.05 fold) leaf pairs.

Table-1: Growth parameters in terms of fresh weight (FW), dry weight (DW) and water content(WC) in *Catharanthus roseus* grown under four different treatments, group I: distilled water (control: without metal solution), and with various concentration of $ZnCl_2$ solutions, group II: 50, group III: 100, group IV: 500 µM). Each value represents mean of three replicates and SD determined.

Treatments	Growth parameters									Root		
	Leaf pairs											
	Apical			Middle			Basal					
	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)
Control	22.4±1.1	13.4±0.21	40.17±1.89	83.2±3.1	43.1±1.5	48.19±1.5	32.4±2.5	14.3±0.82	55.86±1.1	30.2±2.2	15.5±0.45	48.67±1.11
50µM	24.2±3.2	15.16±1.6	37.35±1.09	84.2±4.1	45.2±4.11	46.31±1.12	39.1±2.3	18.2±0.8	53.45±1.0	31.1±2.3	16.1±0.66	48.23±1.31
100µM	31.8±4.2	26.15±1.2	17.76±2.21	86.4±4.34	49.1±2.21	43.17±2.21	41.3±2.1	21.5±1.3	47.94±2.8	39.2±3.1	18.2±2.1	53.57±2.1
500µM	9.52±3.5	2.19±0.72	76.99±2.16	19.1±4.51	6.5±0.85	65.96±1.62	8.2±1.9	2.5±0.3	69.51±2.1	6.4±1.2	2.1±1.9	67.18±1.42

Table-2: Growth parameters in terms of fresh weight (FW), dry weight (DW) and water content (WC) in *Catharanthus roseus* grown under four different treatments, group I:distilledwater (control: without metal solution), and with various concentration of $CuCl_2$ solutions, group II: 50, group III: 100, group IV: 500 µM). Each value represents mean of three replicates and SD determined.

Treatments	Growth parameters									Root		
	Leaf pairs											
	Apical			Middle			Basal					
	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)	FW (mg)	DW (mg)	WC (%)
Control	22.4±1.1	13.4±0.21	40.17±1.89	83.2±3.1	43.1±1.5	48.19±1.5	32.4±2.5	14.3±0.82	55.86±1.1	30.2±2.2	15.5±0.45	48.67±1.11
50µM	24.2±3.2	15.16±1.6	37.35±1.09	84.2±4.1	45.2±4.11	46.31±1.12	39.1±2.3	18.2±0.8	53.45±1.0	31.1±2.3	16.1±0.66	48.23±1.31
100µM	31.8±4.2	26.15±1.2	17.76±2.21	86.4±4.34	49.1±2.21	43.17±2.21	41.3±2.1	21.5±1.3	47.94±2.8	39.2±3.1	18.2±2.1	53.57±2.1
500µM	9.52±3.5	2.19±0.72	76.99±2.16	19.1±4.51	6.5±0.85	65.96±1.62	8.2±1.9	2.5±0.3	69.51±2.1	6.4±1.2	2.1±1.9	67.18±1.42

Table-3: Alkaloid content (mg/g DW) and enzymes activity (POX and CAT) in *Catharanthus roseus* grown under four different treatments, group I: distilled water (control: without metal solution), and with various concentration of $ZnCl_2$ solutions, group II: 50, group III: 100, group IV: 500 µM). Each value represents mean of three replicates and SD determined.

leaf pairs									
Treatments	**Apical**			**Middle**			**Basal**		
	Alk.	**POX (katal)**	**CAT (katal)**	**Alk.**	**POX (katal)**	**CAT (katal)**	**Alk.**	**POX (katal)**	**CAT (katal)**
Control	4.2 ±0.4	23.3±1.2	18.4±1.5	6.6±0.6	30.9±1.6	29.8±2.3	5.2±0.6	28.7±1.5	26.3±2.4
50µM	7.7±0.11	37.9±2.7	17.3±0.8	9.9±0.8	42.6±2.2	27.8±1.9	7.2±0.19	38.3±1.8	19.9±1.3
100µM	8.1±0.22	44.7±3.7	12.9±0.9	12.5±0.3	64.9±0.7	18.8±2.1	8.5±0.17	55.8±2.4	16.6±0.4
500µM	9.2±0.45	58.5±1.3	8.3±0.8	14.9±0.6	67.2±0.3	10.9±0.8	10.6±0.4	58.9±0.6	9.2±0.3

Table-4: Alkaloid content (mg/g DW) and enzymes activity (POX and CAT) in *Catharanthus roseus* grown under four different treatments, group I: distilled water (control: without metal solution), and with various concentration of $CuCl_2$ solutions, group II: 50, group III: 100, group IV: 500 µM). Each value represents mean of three replicates and SD determined

leaf pairs									
Treatments	**Apical**			**Middle**			**Basal**		
	Alk.	**POX (katal)**	**CAT (katal)**	**Alk.**	**POX (katal)**	**CAT (katal)**	**Alk.**	**POX (katal)**	**CAT (katal)**
Control	4.2±0.5	19.3±1.3	15.4±1.9	6.6±0.9	20.9±1.2	26.8±0.9	4.2±0.8	18.7±1.9	28.3±2.1
50 µM	6.7±0.12	27.9±2.9	12.3±0.5	8.9±0.12	32.6±2.4	23.8±1.8	6.2±0.12	28.3±1.9	25.9±1.9
100 µM	7.1±0.23	34.7±3.9	10.9±0.5	10.5±0.21	57.9±0.9	16.8±1.9	6.5±0.11	35.8±2.4	18.6±0.9
500 µM	3.2±0.48	18.5±1.9	7.3±0.5	3.19±0.74	23.2±0.9	10.9±0.5	2.6±0.1	18.9±0.9	12.2±0.9

CAT activity in apical, middle and basal leaf pairs decreased with increasing concentration of $ZnCl_2$ (Table-3). 50 µM $ZnCl_2$ (lowest concentration) caused a decrease in apical (≈0.94 fold), middle (≈0.93 fold), and basal (≈0.76 fold), while 500 µM $ZnCl_2$ (highest concentration) caused a decrease in apical (≈0.45 fold), middle (≈0.36 fold) and basal (≈0.34 fold).

Alkaloid content in leaf pairs, apical, middle, and basal increased with increasing concentration of $ZnCl_2$. 50 µM $ZnCl_2$ (lowest concentration) caused an increase in apical (≈1.83 fold), middle (≈1.5 fold) and basal (≈1.38 fold), while 500 µM $ZnCl_2$ (highest concentration) caused an increase in apical (≈2.19 fold), middle (≈2.26 fold), and basal (≈2.04 fold).

POX activity in apical, middle and basal leaf pairs increased with increasing concentration of $CuCl_2$ (Tabale-4). 50 µM $CuCl_2$ (lowest concentration) caused an increase in apical (≈1.44 fold), middle (≈1.55 fold), and basal (≈1.51 fold), while

500 µM $CuCl_2$ (highest concentration) caused an decrease in apical (≈0.97 fold), middle (≈1.11 fold), and basal (≈1.01 fold) leaf pairs.

CAT activity in apical, middle and basal leaf pairs decreased with increasing concentration of $CuCl_2$ (Table-4). 50 µM $CuCl_2$ (lowest concentration) caused a decrease in apical (≈0.79 fold), middle (≈0.88 fold), and basal (≈0.91 fold), while 500 µM $CuCl_2$ (highest concentration) caused a decrease in apical (≈0.47 fold), middle (≈0.40 fold) and basal (≈0.43 fold).

Alkaloid content in leaf pairs, apical, middle, and basal increased with increasing concentration of $CuCl_2$. 50 µM $CuCl_2$ (lowest concentration) caused an increase in apical (≈1.59 fold), middle (≈1.34 fold) and basal (≈1.47 fold), while 500 µM $CuCl_2$ (highest concentration) caused an increase in apical (≈0.76 fold), middle (≈0.48 fold), and basal (≈0.61 fold).

Low amount of both heavy metals, Cu and Zn significantly promoted seed germination in C. roseus (data has not shown). Root growth was more readily affected by the tested heavy metals than seed germination. Roots are the primary plant organs that sense, become in contact with, and accumulate heavy metal(s) from the substrate. Root growth has been proven to be an indicator of metal tolerance in plants (Wilkins, 1978). Considering seed germination and root growth together, results from the present study suggested that C. roseus could be used for phytoremediation of soil contaminated with Zn or Cu if concentration of the metal in the soil solution does not exceed 100 µM.

Many changes have been found in the activities of antioxidant enzymes in plants under heavy metals stress. It has been reported that heavy metal tolerant plants, besides being able to regulate the ion and water movements, also exhibit a strong antioxidative enzyme system for effective removal of AOS (Rout and Shaw, 2001). The activity of antioxidant enzymes has been reported to increase under heavy metal stress in cucumber (Lechno et al., 1997), shoot cultures of rice (Fadzilla et al., 1997) and wheat shoot (Meneguzzo et al., 1999; Sairam et al., 2002), but decreased in wheat roots (Willekens et al.,1997), or was unaffected as in the case of SOD in cucumber (Lechno et al., 1997). The increased production of AOS in chloroplasts of plants under heavy metals stress has also been previously reported (Meneguzzo et al., 1999).

In the present study, changes in POX and CAT enzyme activity on one month old heavy metal stressed plants suggested that oxidative stress may be an influential component of possible environmental stresses on C. roseus. Higher enzyme activity was observed in all plant tissue parts under heavy metal stress (Table-3 and 4). When plants were treated with Cu and Zn, the activity of POX was increased significantly in all leaf pairs while CAT was decreased significantly in all leaf pairs. POX also plays an important role in the coupling of monomeric indole alkaloids, vindoline and catharanthine into vinblastine (antitumor agent) in leaves and ajmalicine to serpentine conversion in roots of C. roseus (Blom et al., 1991). The higher activity of POX in C. roseus plants, indicating that the increased antioxidative activity might reflect a damage response to stress factors, which was in agreement with the report of Mittal and Dubey (1991), who presumed that high anti-oxidative ability were parts of a damage response to heavy metals in plants.

ACKNOWLEDGEMENTS

We are thankful to the College of Natural and Applied Sciences (CONAS), Crescent University, Abeokuta, Nigeria, provided all

laboratory facilities for experiments as well as we are thankful to our Vice-chancellor, Prof. K. A. Okeleye for their support and encouragement to perform this project.

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