Influence of Some Heavy Metals on Growth, Alkaloid Content and Composition in *Catharanthus roseus* L.

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Shoot biomass production, alkaloid content and composition as influence by cadmium, manganese, nickel and lead at uniform dose of 5 mM were investigated in *Catharanthus roseus* plants grown in sand culture. Treatment with Mn, Ni, and Pb significantly enhanced total root alkaloid accumulation. Cd and Ni treatment resulted in two-fold where as Pb treatment resulted in three fold increase in serpentine content of roots. The non-significant affect on biomass suggests that plants can withstand metal stress at the level tested with positive affect on root alkaloid content.

Key words: Alkaloids, *Catharanthus roseus*, elicitation, metals

As a source of medicinally active terpene indole alkaloids, the plant *Catharanthus roseus* (L) G Don is an important industrial crop. The plant derives its economic importance from its highly valued anticancer leaf alkaloids (vincristine and vinblastine) and antihypertensive root alkaloids (ajmalicine and serpentine). Because of high price, (e.g., value of ajmalicine has been calculated to be $ 37000/kg)[1] and low alkaloid content, the plant has attracted considerable attention of researchers[2,3]. These approaches include chemical semi-synthesis, cell and tissue culture and biotechnological approaches for alternative methods of production of these alkaloids[4]. Majority of these investigations revealed a low productivity of the desired compound as a result, the plant thus remains the only source of valuable TIAs[5]. Hence, investigations are essential for deeper understanding of factors regulating alkaloid accumulation at whole plant level.

Recent studies have revealed that metals have great potential in enhancing biosynthetic pathway in cell cultures. Addition of vanadyl sulphate to cell culture promoted higher content of ajmalicine and catharanthine[6]. Various rare earth elements as cerium, yttrium, and neodymium elicited differential response on ajmalicine and catharanthine in cell cultures[7]. Cd treatment enhanced the excretion of ajmalicine into the culture medium[8]. Addition of tetramethylammonium bromide induced positive response by increasing ajmalicine content in shake flask and bioreactor[9]. Effects of a number of elements as Co, Ni, Cr, Cu, B, Mo, Fe, and Vn produced variable response on alkaloid content of 4-6 day old seedlings of *Catharanthus*[10]. It is not clear whether metal elicitation studied at cellular level may actually replicate at whole plant level. Another aspect of metal study is that because of rapid industrialization significant amounts of heavy metals as Pb, Ni, and Cd due to long biological life remain in soil and water regimes and thus influence plant growth[8,11]. In the present investigation, the same approach of metal elicitation was adopted at whole plant level. Catharanthus plants, grown in sand culture were treated with metals as Cd, Mn, Ni, and Pb for 15 days before harvest. After the treatments, fresh and dry biomass of leaf, stem and root and their total alkaloid content and composition of leaf and root alkaloid were determined.

Plants raised from seeds of *Catharanthus roseus* (cv. Dhawal) obtained from germplasm bank of Central Institute of Medicinal and Aromatic Plants, Lucknow were grown in 5 l plastic pots filled with acid washed clean silica sand[12,13]. Balanced nutrient solution of Hoagland and Arnon[14] (except Fe, which as FeEDTA[15]) was supplied to the plants and these served as control. Three pots with one plant per pot were maintained for each treatment. Plants were maintained in glasshouse at ambient temperature of 30-35° and average light intensity of 800-1000 $\mu E/m^2/s$.

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TABLE 1: EFFECTS OF METAL TREATMENTS ON BIOMASS PRODUCTION IN CATHARANTHUS

Treatment	Leaf fresh biomass	Leaf dry biomass	Stem fresh biomass	Stem dry biomass	Root fresh biomass	Root dry biomass
Untreated	7.29	0.53	2.54	0.51	2.89	0.57
Cd 5 mM	7.67	1.00	2.50	0.49	2.99	0.66
Mn 5 mM	6.58	1.04	2.75	0.50	3.62	0.81
Ni 5 mM	6.46	0.94	1.67	0.38	1.29	0.53
Pb 5 mM	7.13	0.86	2.62	0.52	2.55	0.64
CD						
5%	4.53	0.95	2.14	0.42	2.88	0.69
1%	6.20	1.30	2.92	0.57	3.94	0.95

Fresh and dry biomass of Catharanthus plant parts, all values in g/plant

Four months old plants in which flowering has just initiated were additionally given metal treatments of Ni ($NiCl_2$), Mn ($MnCl_2$), Cd ($CdNO_3$) and Pb ($PbNO_3$) at uniform dose of 5mM along with Hoagland nutrient solution for 15 d. One liter of each treatment (Cd, Mn, Ni and Pb) was prepared in Hoagland solution. 250 ml of this solution was given daily to the plants. On 7th day the pot were flushed with distilled water. Three replicates were maintained for each treatment.

After the treatment, the plants were uprooted from sand, separated into leaf stem and root for fresh and dry biomass determination and further analysis into total alkaloids and its constituents. Fresh biomass of plant parts was determined and for dry biomass, the tissues were dried in hot air oven to a constant weight. For determination of alkaloid content freshly harvested leaf, stem and root samples of treated and untreated plants were oven dried at 60° for 48 h and powdered. A known weight of each plant material was extracted in 90% ethanol (3 times) filtered and concentrated to dryness. Dried residue, redissolved in ethanol, diluted with equal volume of water, and acidified with 3% hydrochloric acid. The mixture was extracted with hexane (3 times), hexane fraction discarded, and aqueous extract cooled to 10° and basified with ammonium hydroxide 3% to pH 8.5. This portion further extracted with chloroform (3 times). The combined chloroform extract washed with distil water, evaporated to dryness, and weighed. The total alkaloid content expressed as percent dry weight of samples (i.e. leaf, stem and root)[16]. Alkaloid constituents- vindoline in leaf were determined based on method of Gupta *et al*.[17]. The HPLC analysis was carried out on Waters equipment; pump 600E; auto injector-717 plus; column oven; detector-996 PDA. Data acquisition and computation were carried out with Waters Millennium software. Standard graphs and analysis were carried out on Merck Chromolith RP18e column, 5 µm (100×4.6 mm i.d.). Ajmalicine and serpentine in root determined by HPLC (validated but unpublished data). The results presented are mean values of three extractions and statistically analyzed for significance by analysis of variance.

TABLE 2: EFFECTS OF METAL TREATMENTS ON TOTAL ALKALOID CONTENT IN CATHARANTHUS

Treatment	Leaf	Stem	Root
Untreated	5.47	1.01	0.41
Cd 5 mM	5.63	5.98	0.57
Mn 5 mM	4.59	3.98	1.00
Ni 5 mM	5.11	9.05	1.04
Pb 5 mM	4.56	6.89	1.07
CD			
5%	3.43	8.91	0.45
1%	4.69	12.17	0.62

Total alkaloid contents in leaf, stem and root, all values in % dry weight

TABLE 3: EFFECTS OF METAL TREATMENTS ON ALKALOIDS CONSTITUENTS OF CATHARANTHUS

Treatment	Vindoline (leaf)	Ajmalicine (Root)	Serpentine (Root)
Untreated	0.1240	0.0512	0.0056
Cd 5 mM	0.0909	0.0348	0.0117
Mn 5 mM	0.0780	0.0223	0.0095
Ni 5 mM	0.0696	0.0233	0.0124
PB 5 mM	0.0653	0.0571	0.0172
CD			
5%	0.0879	0.0213	0.0155
1%	0.1241	0.0302	0.0220

Compositional variations in leaf and root alkaloid content, all values in % dry weight

Metal treatments were given to plant at a stage when plants were at flowering stage and alkaloid content is maximum[18]. The effect of heavy metals on plant growth was monitored, by recording the changes in biomass production of leaf, stem, and root. Table 1 represents these growth data. As a result of metal treatment no significant decrease in leaf fresh and dry biomass was observed. Similarly, there was no significant change in stem fresh and dry biomass except lower values of 1.67 and 0.38g/ plant respectively in Ni treatment. Similar affect of

metal treatment on root fresh and dry biomass also recorded where variations were observed, that were not significant. In many medicinally important plants micronutrient availability of Mn, B, Fe, and Zn play important role in plant growth. Higher supplies of B at 2.5-ppm and Fe at 22.4-ppm significantly increased herb yield in *Artemisia annua*[19]. Opium poppy (*Papaver somniferum*) is highly sensitive to deficiency of B (<0.05-ppm) beyond that level significant increase on biomass content observed[20]. In *Cineraria maritima,* higher doses of B at 1.0 mg/l and Zn at 0.1 mg/l were beneficial for shoot biomass production[21].

The total alkaloid content in leaf did not vary significantly with metal treatment (Table 2). In stem, though the alkaloid content was higher than untreated but these were not significant (Table 2). However, treatment of Ni, Mn, and Pb resulted in significantly higher contents of root alkaloids (Table 2). Thus in roots treatments of Ni, Mn, and Pb resulted in enhancement of biosynthetic pathway resulting in increased accumulation of alkaloids. Metal treatment resulted in reduced content of vindoline in leaves (Table 3). Ajmalicine content in Pb treatment in roots was similar to untreated control, nevertheless total alkaloid content were significantly higher (Table 2). However, Cd, Mn, and Ni treatment resulted in decrease in Ajmalicine content (Table 3). Cd, Mn, and Ni resulted in 2-fold and Pb in 3-fold increase in content of serpentine (Table 3).

The exact mechanisms responsible for higher alkaloid accumulation by metal treatments are not very clear, though several reasons and explanations have been put forward. It is of significance to mention that many metals, particularly micronutrients, belonging to the essential category, act as cofactors of enzymes involved in biosynthetic pathway of alkaloid accumulation (and at the same time also in the primary metabolic pathways that provide initial metabolic building blocks for alkaloid biosynthetic pathway to proceed). These include, Fe that is cofactor of enzymes as, geraniol-10-hydroxylase, peroxidase, tabersonine-10-hydroxylase, and deacetoxy vindoline hydroxylase. Mn is a cofactor of isopentenyl diphosphate hydroxylase[4]. Even elements, which have no, identified metabolic functions in plants such as vanadium, result in increase in transcripts of tryptophan decarboxylase and cellular tryptamine concentration leading to secretion of ajmalicine in culture media[8]. Zn finger proteins have been reported to act as repressors in the fungal elicitation induced secondary metabolism in *C. roseus*[22]. Metals may also act as secondary chemical signals[7]. However, it needs to be understood that cell cultures, when exposed to metals in culture media, are in direct contact with metals and factors as membrane permeability along with readily available C sources are not limiting. At whole plant level however, such mechanisms might not be responsible. The most probable justification could be mediated through root to shoot photo-assimilate metabolites transport. In peppermint (*Mentha piperita*) where essential oils are of economic value during Mn deficiency and subsequent recovery, the levels of primary carbon metabolites and their partitioning between leaf and stem significantly influence essential oil biogenesis[23]. Efforts are underway to determine transfer of carbon assimilates between shoot and root under metal treatment.

The results indicate that *C. roseus* plants treated with metal as Cd, Mn, Ni, and Pb for 15 days showed significantly high alkaloid accumulation in roots. Decreases in ajmalicine content, and 2-3 fold increases in serpentine content indicate influence of metal treatment on biosynthetic inter conversions. At the same time non-significant effect on biomass production revealed that, plants have the capability to withstand metal tolerance (upto the level tested) of Mn, Ni, and Pb.

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