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Research Article

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Influence and distribution of lead nitrate on growth and secondary metabolite accumulation in *Withania somnifera* (L.) Dunal *in vitro* shoots

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ABSTRACT

Withania somnifera is a medicinal ayurvedic plant native to India. Lead (Pb) is a toxic heavy metal which has no biological function in plants. The aim of the present study is to investigate the influence of heavy metal Pb on *W. somnifera* at cellular level under controlled conditions. The role of Pb on shoots of *W. somnifera* was determined in terms of biomass, secondary metabolite production (High Performance Thin Layer Chromatography) and phytochemical quantification. Influence and accumulation of Pb at the cellular level were recognized using UV visible spectroscopy, field emission scanning electron microscope and x-ray diffraction analysis. The current result showed that the concentration up to 2.4 mM Pb for 7 days of exposure showed higher biomass and withaferin A yield compared to control shoots. On the other hand, other secondary metabolites such as flavonoids, phytosterols and phenols levels were reduced compared to control shoots. In the morphological study, Pb concentration in the analysed sample was determined as 2%. The probability of the nanoparticle nature of the bioaccumulated lead also verified using spectroscopy and x-ray diffraction analysis. To date, we are the first to report on the influence of Pb on *in vitro* shoot cultures of *W. somnifera*.

KEYWORDS: *In vitro* shoots, Lead nitrate, *W. somnifera*, Secondary metabolites, Field emission scanning electron microscopy

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INTRODUCTION

Heavy metals are generally present in the soil, plant bodies and aquatic ecosystems at a higher level, however, smaller proportion are also found in the atmosphere. Many heavy metals (Nickel, Iron, Copper and Manganese) are considered as essential metals for plant growth, but the toxicity of these metals in plants is species dependent (Jan & Parray, 2016). Certain non-essential heavy metals have been used as abiotic elicitors to elicit the accumulation of specialized metabolites in plants. It has been reported that plants which undergo heavy metal stress produce higher secondary metabolite content which is positively correlated to heavy metal concentration but only to a certain point (optimum concentration), beyond which the metabolite production decreases (Rout *et al.*, 2019). Generally, Pb causes a significant reduction in seed germination rate, thickens cell walls, growth inhibition and chlorosis in plants (Yolcu *et al.*, 2021; Collin *et al.*, 2022) Certain plants have hyper accumulation capability to withstand and grow in metal rich soils *viz.* mining area or at industrial area (Castañares & Lojka, 2020). These hyper accumulators have evolved a few specialized capabilities to withstand heavy metal stress and toxicity such as bioaccumulation of metals in their greener regions, sequestering metals to vacuoles/vesicles of the cells, binding to phytochelatins or metallo enzymes and activation of numerous antioxidants (Hakeem, 2015; Collin *et al.*, 2022).

W. somnifera is a well-known Indian medicinal and Ayurvedic plant with adaptogenic properties and hyperaccumulating capability (Maharia *et al.*, 2010). *W. somnifera* has a higher metal bioaccumulation ability towards Lead (Pb), Chromium (Cr) and Cadmium (Cd). Among analysed metals, Pb was the highly accumulated metal and it was found mainly in the aerial parts (stem & leaves) of *W. somnifera* (Khan *et al.*, 2007; Balafrej *et al.*, 2020). The report shows that Pb mainly precipitates in the cell wall of the root and only free Pb ions can be transported to other parts of the plant via xylem and phloem cells (Mathur & Chauhan, 2020). Therefore, *in vitro* liquid medium and shoot

cultures (Parameswari *et al.*, 2017) were used for the Pb exposure study which facilitates free availability of Pb to the shoots. Hence the present study was focused to study the uptake of lead nitrate ($Pb(NO_3)_2$) by *in vitro* shoot cultures of *W. somnifera* and its influence on withaferin A (an important secondary metabolite native to *W. somnifera*) and the accumulation of lead in leaves.

MATERIAL AND METHODS

Plant Material

Seeds of *Withania somnifera* (L.) Dunal Var. JA20 (Jawahar-20) were collected from the University of Agricultural Sciences, Bangalore. Surface sterilized seeds were germinated under *in vitro* conditions and seedlings were maintained on half strength MS (Murashige & Skoog) medium containing 2% sucrose and 0.8% agar. The shoot obtained from the germinated seedlings was used as the source of explants and has been inoculated onto the full-strength MS liquid basal medium, cultured at 25±2 °C and a photoperiod of 16 hours for 30 days. For shoot multiplication, 30 days old shoots were inoculated onto MS medium supplemented with 4.44 µM BAP (6-benzylaminopurine) hormone (Vinod *et al.*, 2022).

Treatment with Lead Salts

The nodal sections of *W. somnifera* were excised, trimmed at both ends and inoculated in culture bottles containing MS supplemented with BAP medium. For Pb treatment, one month old *in vitro* shoots of *W. somnifera* from MS supplemented with BAP medium were transferred to MS media containing different concentrations (T1-T5) of $Pb(NO_3)_2$ salts (Table 1). Media without $Pb(NO_3)_2$ served as a control (T0). Each experiment had 3 replicates with three explants in each. The shoots were harvested after 7 and 14 days of exposure period, weighed, shade dried at room temperature and extracted using methanol (Vinod *et al.*, 2022). The fresh shoot tissues were weighed and the growth index was calculated using the formula:

$$GI = Fresh\,weight\,of \quad \{\frac{harvested\,biomass - inoculum}{inoculum}\}$$

Quantification of Secondary Metabolites

Withaferin A stock solution was prepared in the concentration of 1 mg/mL using HPLC grade methanol. From the stock solution, the working solutions were diluted to a 1:1 ratio and stored at -4 °C until further use. High Performance Thin Layer Chromatography (HPTLC) was performed on precoated silica gel aluminium plate 60F254 (MERCK, Germany) using CAMAG HPTLC for withaferin A quantification. For HPTLC analysis, the procedure reported by Vinod *et al.* (2022) was followed. The concentrated methanol extract was further used for the phytochemical analysis. Phytochemicals such as flavonoids, phenols and steroids were quantified using the procedure reported by (Kumar *et al.*, 2014; Clemensen *et al.*, 2022).

Table 1: Concentration and treatment period for Pb exposure to *in vitro* shoots of *W. somnifera*

Treatments	$Pb(NO_3)_2$ concentration	
	7 days	14 days
T0	0 mM (control)	
T1	0.6 mM	
T2	1.2 mM	
T3	1.8 mM	
T4	2.4 mM	
T5	3.0 mM	

UV-visible Spectroscopy

The methanol extracts were further subjected to spectral scan in a UV-Visible Spectroscopy from the range of 200 to 1000 nm (UV-vis 1800 Shimadzu). HPLC methanol was used as a reference control.

X-Ray Diffraction

The X-ray Diffraction (XRD) patterns of the samples were recorded (PANalyticalX'Pert PRO XRD) using Cu Kα radiation (λ = 0.15406 Å). Line broadening analyses using the Debey-Schherer formula after accounting for instrumental Broadening revealed the presence of crystalline size in the samples.

$$D\ XRD = 0.90\lambda / \beta\ \cos\theta$$

Where, D- crystal size; λ- wavelength of the X-rays; β- full width at half maximum of the diffraction peak; θ- diffraction angle (Sahayaraj *et al.*, 2012).

Electron Microscopy Analysis

Leaves from Pb treated shoots were excised, cut into small portions (3 × 3 mm) and fixed for 2 hours at 4 °C in 0.1% (weight/volume) buffered sodium phosphate and 3% (weight/volume) glutaraldehyde at pH 7.2. The leaves were stored in buffer solution until FESEM EDAX analysis.

Statistical Analysis

The data on the effect of Pb on shoot biomass in control and treated shoots were analysed using SPSS and expressed as mean ± standard deviation.

RESULTS

Morphological Changes and Biomass Production

W. somnifera shoot cultures were treated with lead nitrate at different concentrations for 7 days and 14 days period. T1 shoots showed a slightly low growth index compared to control shoots (T0). However, among all the treated cultures, a gradual increase in growth index was observed up to T4 of 7 days and T3 of 14 days exposed shoots. In T5 shoots of 7 days exposure and T4 & T5 shoots of 14 days of exposure results in a decreased growth index compared to T0. An increase in culture period to 14 days

further reduced the growth rate at earlier stages (T1); however, a maximum growth of 1.32 was observed in T3 (2.4 mM) of 14 days (Figure 1) and further increase in Pb concentration significantly decreased the growth index (T4 & T5).

Phytochemical Analysis

The concentration of flavonoids was estimated using the method described by Clemensen *et al.* (2022). The flavonoid content of shoots grown on media supplemented with varying concentrations of lead nitrate is presented in Figure 2. Compared to control shoots (T0), the flavonoid content in Pb treated shoots was (T1-T5) found to be significantly reduced at both periods. However, among Pb treated shoots, a gradual increase in flavonoid levels was observed (T1- T4) which decreased with an increase in the concentration of Pb (T5).

The phytosterol content estimated in Pb-treated and control shoots is presented in Figure 2. Among control shoots, the concentration of phytosterols in 14 days old *in vitro* shoots of *W. somnifera* recorded significantly higher levels than in 7 days treated shoots. Among treatment groups, a significant decrease in levels of phytosterol was observed with an increase in concentration of $Pb(NO_3)_2$ when compared to control (T0).

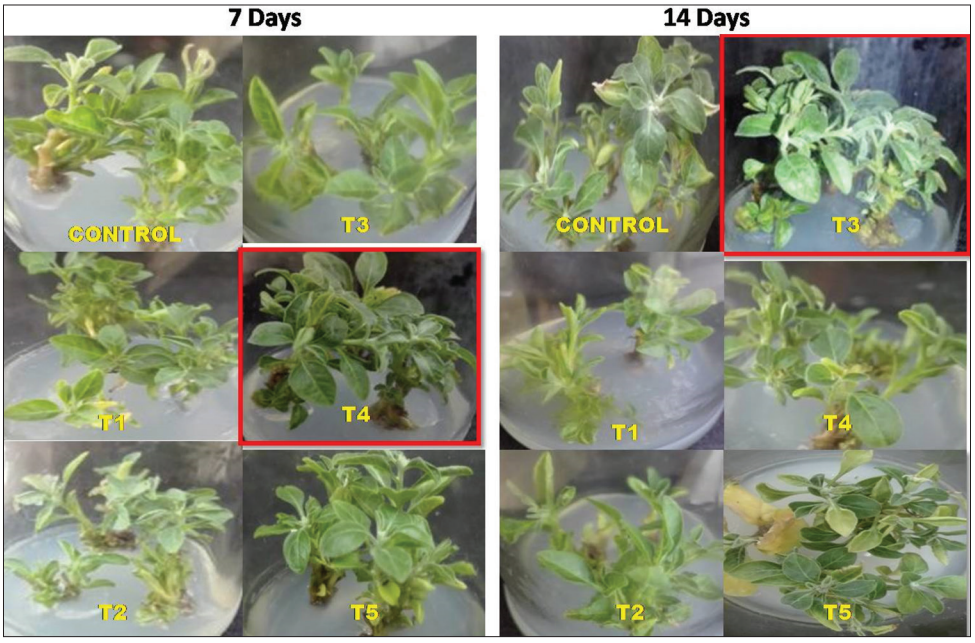

Figure 1: *In vitro* shoot cultures of *W. somnifera* exposed to different concentration of $Pb(NO_3)_2$ for 7 & 14 days

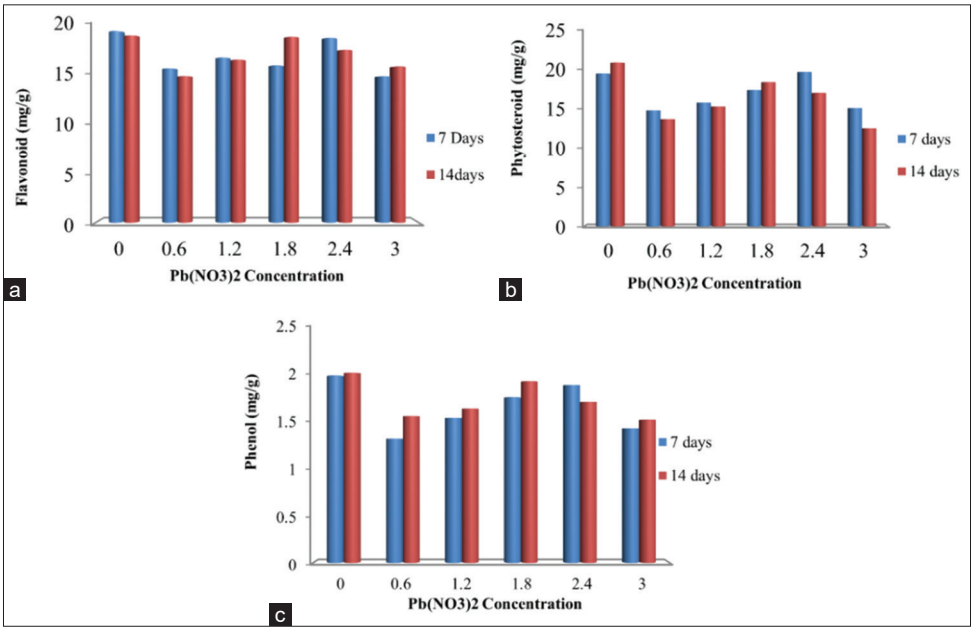

Figure 2: The figure shows the concentration of a) flavonoid, b) phytosteroid and c) phenol concentration in Pb treated shoots of *W. somnifera*

Among treated groups, a gradual increase in phytosterol content was observed in T1-T4 (14.84- 19.75 mg/g) in 7 days and T1-T3 for 14 days (13.72-18.45 mg/g) after which the levels were decreased.

The total phenol content (TPC) estimated in Pb treated and untreated (control) shoots is presented in Figure 2. Among treated groups, a gradual increase in phenol content was observed in T1-T3 (1.56-1.93 mg/g) after which the levels decreased with the maximum content of 1.93mg/g (T4) in shoots grown for 14 days. Compared with control the levels of phenol decreased significantly, but among Pb treated shoots, there was a gradual increase from 1.32 to 1.89 mg/g (T1-T4) which decreased to 1.43 mg/g (T5) for 7 days of exposure. A similar trend was observed for 14 days of exposure, with the increase in TPC levels up to T3 (1.93 mg/g) after which the TPC levels decreased significantly.

Quantification of Withaferin A

Withaferin A concentration was positively correlated to the Pb concentration up to T4 of 7 days exposure. On 14 days exposure to Pb, T3 had the higher withaferin A content (1.37 mg/g) after which (T4 & T5) a reduction in withaferin A content was observed (Figures 3 & 4). The accumulation of withaferin A was found to be 6.33-fold higher in T4 for 7 days and 4.87-fold higher in T3 for 14 days exposure shoots compared to control *in vitro* shoots.

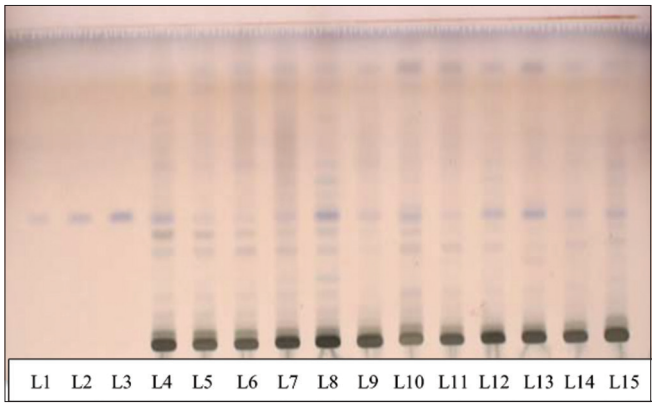

Figure 3: HPTLC separation of *W. somnifera* Pb treated and control shoot samples. Lane 1-3 = Withaferin A (standard); Lane 1-6 = Pb7 days treated shoot samples (T1-T5); Lane 6-12 = Pb14 days treated shoot samples (T1-T5)

UV-visible Spectroscopy

The results showed significant changes in the metabolite peaks of test samples compared to control. Both control and test samples exhibited similar distributions of peaks at 290 nm, 295 nm, 410 nm, 465 nm, and 665 nm. However, the intensity of these peaks was differed among test samples. Compared to control, Pb treated test samples especially at high concentration (T3-T5) had higher peak intensity at both treatment period (7 & 14 days).

Field Emission Scanning Electron Microscopy (FESEM)

FESEM micrograph (Figure 5) shows the presence of several elements which are essential for the growth of the shoots cultured *in vitro* among which 2% Pb was identified in the leaves of analysed sample which was further confirmed by the EDAX spectrum. Elemental mapping also showed the presence of carbon 41%, oxygen 33%, magnesium 2%, potassium 12%, chlorine 4%, phosphorus 3% and cobalt 1% along with Pb. Further, there were no nanostructures or any distinct surface morphology was observed in FESEM micrographs.

X – Ray Diffraction Analysis

The XRD image of lead nitrate treated *in vitro* leaves of *W. somnifera* (T5) is shown in Figure 6. The major peak at 2θ value of 27.35 was confirmed as Pb. The crystalline size of the powder is calculated to be about 8.778 nm using the formula.

DISCUSSION

Approximately 2 g of one month old shoots grown in MS supplemented with BAP medium were transferred to MS basal medium. This was done to ensure that the explants used for the present study do not have any residual influence of hormones and whatever response we observe will be solely due to the treatment of $Pb(NO_3)_2$. The biomass accumulation patterns in Pb exposed shoots (T2-T4) were significantly increased compared to control (T0).On treatment with Pb salts, the *W. somnifera* shoots exhibited increased metal tolerance and resistance which resulted in the increased growth index at both 7 days and 14 days treatment periods. However, our observation is contrary to many studies which proved that Pb was negatively correlated with plant growth after a certain concentration (Collin *et al.*, 2022). Although lead (Pb) is a toxic

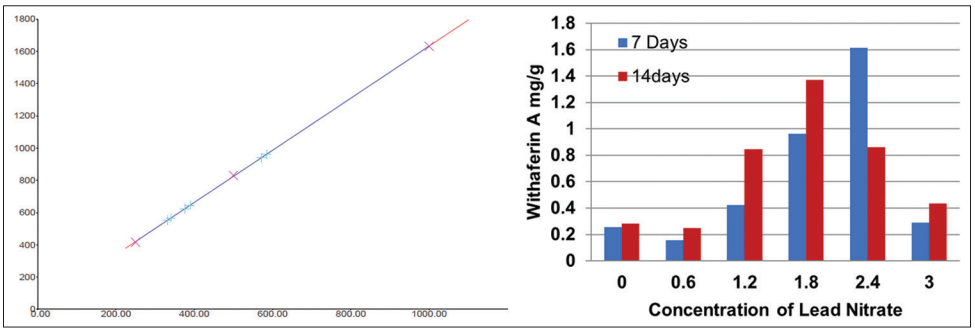

Figure 4: Withaferin A accumulation in Pb exposed and control shoots of *W. somnifera* for 7 and 14 days

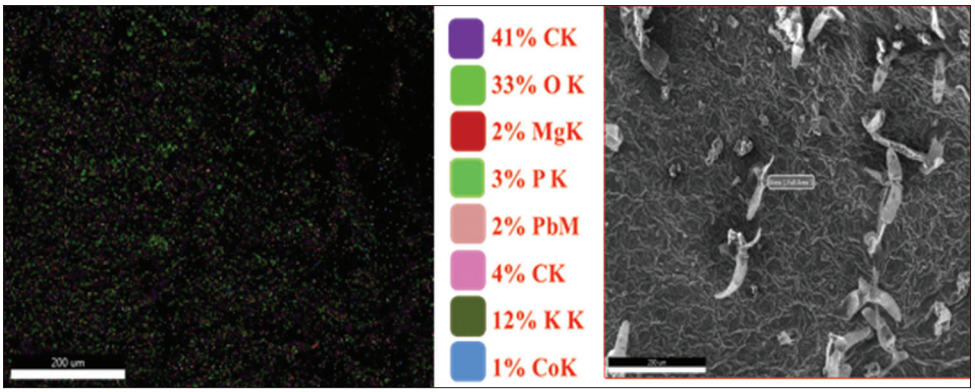

Figure 5: Elemental mapping and Pb accumulation pattern in T5 shoot of *W. somnifera*

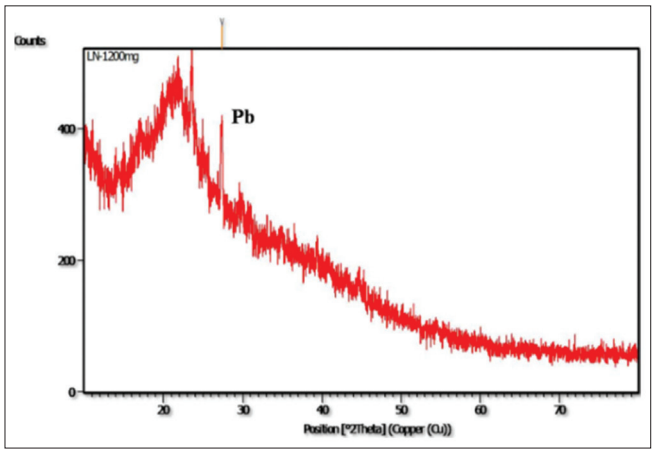

Figure 6: XRD spectrum of T5 shoots for 14 days exposure

heavy metal, *W. somnifera* showed significant growth in media containing Pb up to a concentration of 2.4 mM (T4) for 7 days and 1.2 mM (T2) for 14 days of exposure (Table 2). A similar response was observed in *in vitro* cultures of *Datura inoxia* which showed endurance up to 45 mg/L lead concentrations with a slight decrease in the number of shoots with increasing concentrations (Waoo *et al.*, 2014). The ability of these plants to tolerate stress might be due to their potential to selectively absorb the necessary nutrients and sequester non-essential metals to vacuoles or other organelles for maintaining the system homeostasis (Peng & Gong, 2014).

Following the isolation of the active ingredients, they can be incorporated into the modern medicine system to develop effective formulations for therapeutic purposes. Several specific reactions operating communally are responsible for the production of secondary metabolites in plants (Mansoori *et al.*, 2020). The medicinal value of these secondary metabolites is due to the presence of reactive components that produce a definite biochemical reaction in humans (Natarajan *et al.*, 2022). Flavonoids comprise the most common group of plant polyphenols and provide much of the flavour and colour to fruits, vegetables and have potent antioxidant and free-radical scavenging activities.

During Pb treatment, Pb increases the flavonoid content at earlier concentrations but decreased flavonoid content was

Table 2: Growth index of Pb exposed shoots of *W. somnifera*

Treatment	7 days	14 days
T0	1.25±0.011	1.44±0.015
T1	1.10±0.015	1.04±0.002
T2	1.67±0.030	1.31±0.005
T3	1.89±0.039	1.32±0.010
T4	1.93±0.031	1.15±0.019
T5	1.03±0.012	1.09±0.011

observed at higher Pb concentration (T5). Similar results were observed in the flavonoid content of shoots subjected to 14 days treatment. Ibrahim *et al.* (2017) has reported a decrease in the phenol and flavonoid content with an increase in the concentration of cadmium and copper. A similar result was also observed in the phytosterol content of Pb treated shoots in the present study. The toxicity of Pb affects the phytosterol level at higher concentrations and prolonged periods. TPC level was also increased during Pb treatment and increased TPC content was observed at T4 of 7 days exposure and T3 of 14 days exposure. TPC analysis on leafy vegetables and fruits was done by Chandra *et al.* (2014). They reported that basil (32.50 mg/g) and chard (41.15 mg/g) contain higher TPC than other leafy vegetables. In a study reported by Saini and Gupta (2017), the seedlings of mung beans were treated with Zn, there was an initial decrease till 100 ppm followed by a step increase at 300 ppm (1.793 mg/g) and followed by a further decreased (2.215 mg/g) at 1000 ppm of TPC were observed, when compared with untreated seedlings.

HPTLC analysis was performed in the current study to quantify withaferin A content in the methanol extract of $Pb(NO_3)_2$ treated *in vitro* shoots of *W. somnifera*. One of the important secondary metabolites in *W. somnifera* is withaferin A has higher pharmaceutical value than other withanolides (Natarajan *et al.*, 2022). Pb treatment was positively correlated with withaferin A content till a specific concentration. Increased concentration of withaferin A was observed at T4 of 7 days and T3 of 14 days with T4 of 7 days exposure being higher compared to control (T0) and other treatments. The present result was in correlation with Sivanandhan *et al.* (2012), in their study approximately 7-fold increase in the withaferin A contents was observed when *W. somnifera* cultures treated with aluminium chloride as an elicitor. Salicylic acid was used as an elicitor for *W. somnifera in vitro* callus cultures where 20-fold increases in withaferin A

was reported. Thus, the results show the heavy metal stress on the *in vitro* cultures of *W. somnifera* increases the secondary metabolite content, especially withaferin A.

UV visible spectrophotometer was used to identify if the Pb was absorbed and accumulated as ions or particulates in shoots of *W. somnifera*. The standard Pb ion in the solution showed a peak at 295 nm. The samples of Pb treated shoots also showed peaks at the same wavelength; however, it might be due to the presence of high protein content (absorbance at 290 nm) in the Pb treated shoots or it might be from a protein molecule that is present on the surface of the reduced Pb element. The probability of the presence of nanoparticles in the Pb treated samples is also investigated in further studies. It has been reported that the Pb nanoparticles were synthesized through green synthesis using *Zingiber officinale* extract and gave peaks around 239 nm and 335 nm during UV visible spectroscopy analysis (Delma & Rajan, 2016). Another study on *Cocos lucifera* extract and Pb nanoparticles synthesis followed by spectroscopic analysis showed peaks at 212 nm (Elango & Roopan, 2015). Thus, the presence of peaks varied from 200-350 nm for green synthesized Pb nanoparticles which is due to their size and shape. Therefore, it might be possible that the accumulated Pb may reduce to nanoparticles in live plants of *W. somnifera* which could give its UV absorption at 295 nm (Figure 7). The peaks found at 465 nm and 665 nm were found to be chlorophyll a and b, peak identified at 410 nm shows the presence of β-carotene. Chlorophyll a & b and β-carotene levels were increased in Pb treated shoots for 14 days of exposure than in control shoots (Figure 7) which may be due to the Pb stress on the tissues of *W. somnifera*. However, on 7 days of Pb exposed shoots, there was no significant difference was observed in the control shoots except T4 shoots had stronger chlorophyll a & b and β carotene peaks (Figure 7).

Electron microscopy images were measured and topographical analysis was performed based on the surface analysis of leaf tissues of a T5 shoots of 14 days exposure. The analysed samples were found to contain 2% Pb along with other elements like O, Mg, K, Cl, P, and Co. The determined 2% Pb was found to be scattered all over the sample which further confirms that the Pb was absorbed, transported and stored in the leaves of *W. somnifera* (Figure 5). While, the nature of the accumulated Pb in the leaf could not be studied using FESEM. However, the Pb peak at 2.4 kev in the EDAX spectrum shows the probability of nanoparticle formation in the live plant samples (Figure 8). A similar result was observed in (Miri *et al.*, 2018; Diba *et al.*, 2021). In conclusion, the presence of Pb in leaf tissues of *W. somnifera* might be from diffusion, transportation and accumulation of Pb from the media to the leaves.

The report on *Sesbania drummondii* confirms that the absorbed Pb cannot be leeched out from the plant even if they put in the control condition (Hu *et al.*, 2015). Alfaraas *et al.* (2016)

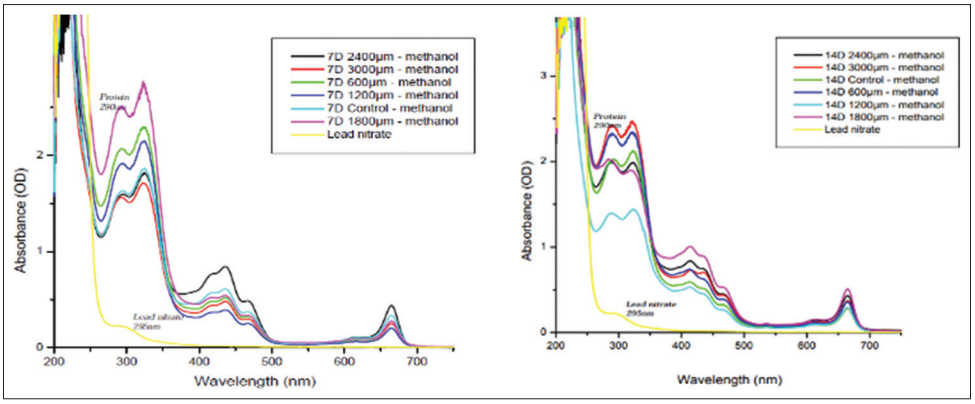

Figure 7: UV-Visible Spectrum of Pb treated shoots for 7 days (left) and 14 days (right) exposure

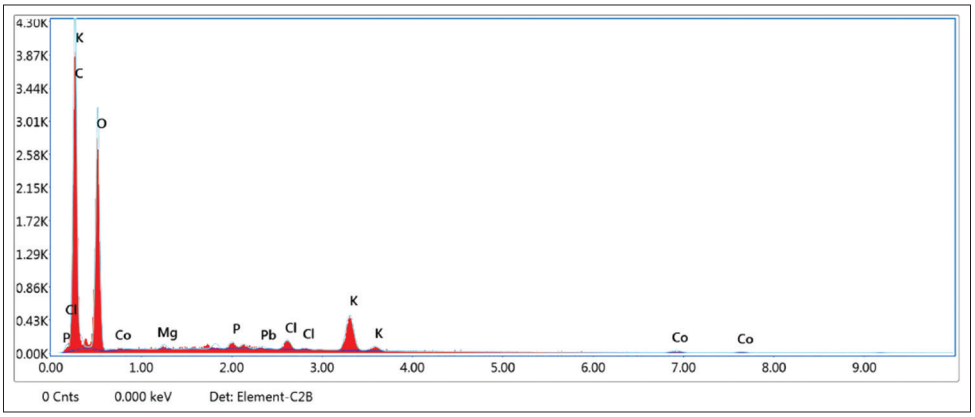

Figure 8: EDAX spectrum of T5 shoot

reported that the effect of heavy metals such as Pb and Cd on the leaf and root of paddy plants via cross sections. They reported that accumulation of Pb and Cd in paddy plants was in a concentration dependant manner and also at higher concentrations, metal causes physiological changes like chlorosis of leaf and reduction in growth. Further, Pb adsorption, translocation and hyper accumulation were detailed in crop species by Wang *et al.* (2021).

Crystalline size and structural properties of accumulated Pb were revealed using X-ray diffractions. XRD studies were carried out with Cu – κα radiation (κ – 0.154 nm) and 2 theta ranged from 20° to 80°. From Figure 6 Pb peak was observed 2θ values of 27.3 and using 2θ value crystalline size was calculated to be 8.78nm. A similar result was reported by (Delma & Rajan, 2016), and reported that Pb nanoparticles synthesized using *Zingiber officinale* extract had diffraction peaks at 2θ values of 38.140, 19.720 and 32.380 and the average particle size of Pb nanoparticles was found as 3nm according to Debye Scherrer equation. In addition, 2θ values for Pb nanoparticles synthesized by bacterial strains of *Bacillus toyonensis* were 26, 30, 43 and 51 (Mathew & Krishnamurthy, 2018). Similar result was identified from our study that the Pb from *in vitro* shoot of *W. somnifera* showed 2θ values at 27.35.

CONCLUSION

In the present study, the maximum biomass and withaferin A content was observed in 7 days $Pb(NO_3)_2$ exposure when compared to control and 14 days treated shoots of *W. somnifera*. High withferin A content (1.615 mg/g) was quantified in T4 (2.4 mM) shoots of 7 days exposure and showed 6.33 fold increase of withaferin A. UV visible spectroscopy confirmed the presence of Pb peak at 295 nm when compared with standard $Pb(NO_3)_2$ solution. Electron micrograph confirms the absorption, transportation and accumulation of Pb (2%) in the leaf tissues of Pb treated shoots (T5 at 14 days). However, the elemental nature of the Pb in the sample was not identified in either instrumentation. However, according to EDAX and XRD results, there is a high probability for the presence of Pb nanoparticles in the live leaf tissues. To conclude, *W. somnifera* can survive heavy metal stress. Though toxicity affects its growth on prolonged exposure it also acts as a good elicitor for withaferin A production. Further, ultra-structural analysis on leaf/stem tissues are needed to analyse the presence of nanoparticles in live tissues.

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