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Spectral changes in the leaves of barley plant due to phytoremediation of metals - results from a pot study

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Abstract

This research studied the changes in leaf reflectance spectra (350-2500 nm) due to metal phytoextraction into barley plants grown in metal-spiked soils (3 levels of Cd, Pb, As and their metal-mixture treatments). Growth of barley was adversely affected due to 100 mg As kg^{-1} and metal-mixture (10 Cd+150 Pb+100 As; mg kg^{-1}) treatments. Metal phytoextraction were in order of: root >straw ≥leaves >grains. Results of reflectance spectra of leaves show the influence of As-treatment only, causing spectral changes in visible and infrared domains mostly, as apparent from the significant correlation between leaf-As and leaf-spectra. Chlorophyll and water stress indices and band depths analyses showed significant correlations to leaf-As, and can be used to distinguish metal-stressed plants. Finally, regression models demonstrate the potential use of hyperspectral reflectance data to monitor plant health during phytoremediation process and to estimate leaf-As in barley, particularly in this study.

Keywords: Phytoextraction, metal-contamination, barley, spectral reflectance, vegetation indices, regression analysis.

Introduction

Heavy metals are known to be persistent in environment, and the anthropogenic activities viz., mining, ore processing, smelter's emission, industrial effluents/wastes disposal, are progressively alleviated the metals' concentrations in soil and river sediments. Unlike organic contaminants, metals cannot be degraded biologically and hence, require either immobilization or removal. Phytoremediation (i.e., utilizing of plants to remediate contaminated soils; now termed 'phytotechnology') is a green-clean technique [Flathman and Lanza, 1998]. For this long-term plant-based remediation approach, the continuous monitoring of metal bio-accumulation and plant health status is foremost requirement [US-DOE, 2000]. This can be done by assessing plants' growth parameters and metal-induced stresses.

Adverse effects of excessive concentration of soil-metals on plant growth are well documented [Kabata-Pendias and Pendias, 2001; Prasad, 2004]. They inhibits chlorophyll biosynthesis, decreases total chlorophyll in higher plants, and are known to substitute the central Mg-ion in chlorophyll molecules, resulting in a breakdown of photosynthesis. Structural and ultra-structural changes in vegetative tissues are also common effects of metal-stresses [Barceló and Poschenrieder, 2004]. Such metal-induced physiological and biochemical stresses in vegetation subsequently influence the vegetation reflectance spectra especially in visible (400-700 nm), near-infrared (NIR, 700-1300 nm), and shortwave infrared (SWIR, >1300 nm) spectrum domains. A healthy vegetation shows bimodal reflectance pattern, characterized by strong absorption of blue (~450 nm) and red (~680 nm) wavelengths by chlorophyll, and strong reflectance of NIR wavelengths due to light scattering by leaf mesophyll tissues. The spectral reflectance (in SWIR regions at ~1400 and ~1900 nm) also influences by plant water content. Researchers have studied these spectral changes to relate them with phytoaccumulation of metals [Horler et al., 1980; Clevers and Kooistra, 2003; Schuerger et al., 2003; Clevers et al., 2004; Kooistra et al., 2004; Dunagan et al., 2007; Sridhar et al., 2007a,b; Su et al., 2007]. Most studies used various vegetation indices (ratios or linear combinations of two or more spectral wavelengths [Bannari et al., 1995]) and red-edge position (a sharp transition between red and NIR wavelengths; positively related to chlorophyll concentration; [Horler et al., 1983; Dawson and Curran, 1998]) as an indication of plant stress.

To this context, studying the spectral characteristics of plant (which mainly governed by pigments, cellular structure, water content) is found to be useful in rapid diagnosing metal-induced vegetation stress before any stress-related damage occurs [Horler et al., 1980; Carter and Knapp, 2001]. Such study could provide a theoretical basis for remote sensing of metal-contaminated sites. The aim of present study is to examine the changes in spectral reflectance of barley grown into various metal-spiked soils, and thus to monitor the plant health and to estimate metal bio-accumulation for optimizing phytoremediation system.

Materials and Methods

Experimental set-up

A pot experiment with artificially contaminated soil was carried out in glass-chambers at a test facility of Institute of General Ecology, TU Dresden in Tharandt, Germany from May-Aug 2011. The study with metal-spiked into one type of soil has an advantage of constant soil conditions than those of naturally contaminated soils. Furthermore, metal concentrations in soil solution in metal-spiked soil are larger (i.e., more metal-bioavailability, so to study the spectral changes in leaves upon bio-accumulation) than in real contaminated soils at similar total metal concentration [Mertens and Smolder, 2010]. Barley (*Hordeum vulgare L.*; *cv. KWS Bambina E*) was used as a test crop and seeds were obtained from Lochow, Bergen. Barley is a common crop in Saxony region, and even more commonly grown on contaminated agricultural sites due to its robustness and tolerance to the soil-metals. Yet, low translocation of Cd and other metals from soils to the barley grain makes it even more promising on metal-contaminated sites.

Bulk of uncontaminated soil substrate (0.00 µg Cd g^{-1}, 3.81 µg As g^{-1}, and 23.96 µg Pb g^{-1}) was brought from permanent monitoring site of Saxon State at Melptiz to the test facility at Tharandt. Bulk of soil was sieved through 0.5 cm using mechanical sieve. Soil at Melptiz

was sandy (82% sand, 13% silt, and 5% clay) in texture, with medium cation exchange capacity (9.0 cmol$_c$ kg^{-1} in top soil), 18-46% base saturation, 5.3-5.6 pH, and moderate-low water retention capacity (13-18%).

Treatment details

Soil contamination was simulated by spiking three metal-salts viz., cadmium as Cd(NO$_3$)$_2$·4H$_2$O, lead as Pb(NO$_3$)$_2$, and arsenic as As$_2$O$_3$, individually each at 3 levels and also in mixture at their respective levels (Tab. 1). These metals were studied because they are the ubiquitous contaminants in agricultural soils in Saxony region [IBA, 2010]. Spike solutions of Cd and Pb were prepared by sequential dilutions of Cd(NO$_3$)$_2$·4H$_2$O and Pb(NO$_3$)$_2$ in deionized water, while As$_2$O$_3$ dissolved in a NaOH-solution adjusted to near neutral pH. The prepared metal-spike solutions were then added to the respective bulk of soil of treatments (i.e., 40 kg soil for 4 replicate pots for each treatment; 13 × 4 = 52 pot were prepared for 13 treatments set). The spiked soils mixed homogenously, and kept for two wetting-drying cycles for 10-12 days to equilibrate metal spiking. Each 40 kg metal-spiked soil was then divided on weight basis (10 kg pot^{-1}) into pot, and pots were thereafter randomized between four glass-chambers i.e., 13 pots per glass-chamber. Initial soil sample was collected from each pot before sowing barley seed for the analysis of total metals. Pre-concentration of metals in soil is given in Table 1.

Sowing of barley (30 seeds pot^{-1}) was done on 18 Apr 2011. Recommended rate of major-nutrients (N, P, K, Ca, and Mg) was given after compensating nitrate-nitrogen added through spiking of nitrate-salts as in case of Cd and Pb treatments. N, P and K solution was prepared from analytical grade chemical respectively NH$_4$NO$_3$, K$_2$HPO$_4$ and KCl. Plants were irrigated daily with 200-300 ml deionized water. Barley was harvested completely in 2nd week of September.

Table 1 - Treatment details, initial pre-concentration of metals in soil*, and biometric parameters of barley grown with metal-spiked soil.

Details of metal-spiking treatments	Pre-concentration of metals** in soils (mg kg^{-1})			Plant stand (# per pot) at 31 DAS§	Plant height (cm) at 31 DAS§	Total dry biomass$^\epsilon$ weight (g)
	Cadmium	**Lead**	**Arsenic**			
Control: No metal	0.00 (±0.0)	24.0 (±0.70)	3.8 (±0.31)	32.5^a (±1.20)	25.7^{bc} (±0.50)	43.7^b (±4.14)
Cd$_1$: 1 mg kg^{-1}	1.2 (±0.11)	-	-	29.8^{ab} (±1.10)	26.9^{ab} (±0.28)	42.4^b (±4.91)
Cd$_2$: 5 mg kg^{-1}	5.4 (±0.28)	-	-	27.5^b (±0.50)	28.7^{ab} (±1.79)	38.0^b (±4.82)
Cd$_3$: 10 mg kg^{-1}	11.1 (±1.79	-	-	28.0^{ab} (±0.40)	28.0^{ab} (±1.13)	43.2^b (±3.24)
Pb$_1$: 25 mg kg^{-1}	-	51.0 (±3.29)	-	27.8^{ab} (±1.10)	28.4^{ab} (±0.52)	48.7^b (±3.85)
Pb$_2$: 75 mg kg^{-1}	-	103.2 (±2.99)	-	25.0^b (±0.70)	28.3^{ab} (±1.31)	38.4^{bc} (±4.02)
Pb$_3$: 150 mg kg^{-1}	-	180.0 (±7.52)	-	28.3^{ab} (±0.50)	32.3^a (±0.80)	33.8^b (±1.65)
As$_1$: 5 mg kg^{-1}	-	-	8.9 (±0.50)	27.8^{ab} (±1.25)	25.3^{bc} (±1.72)	48.8^b (±1.45)
As$_2$: 50 mg kg^{-1}	-	-	47.1 (±6.76)	25.5^b (±0.65)	20.6^{cd} (±1.19)	9.7^a (±1.25)
As$_3$: 100 mg kg^{-1}	-	-	76.1 (±3.29)	28.3^{ab} (±0.75)	19.9^{cd} (±1.36)	3.9^a (±1.13)
Mix$_1$: Cd$_1$+Pb$_1$+As$_1$	1.2 (±0.15)	55.4 (±6.09)	8.9 (±0.27)	27.0^a (±0.80)	27.6^{ab} (±1.32)	41.8^c (±4.69)
Mix$_2$: Cd$_2$+Pb$_2$+As$_2$	5.5 (±0.46)	94.3 (±4.53)	57.4 (±1.04)	26.0^b (±1.50)	19.0^d (±1.56)	4.7^a (±1.24)
Mix$_3$: Cd$_3$+Pb$_3$+As$_3$	11.0 (±0.47)	194.0 (±10.3)	88.6 (±3.75)	25.8^b (±1.25)	15.0^d (±1.33)	3.0^a (±1.32)

*Aqua regia extraction; **values represent mean (±SE) of 4 replicates.
In each column values not connected by same letter are not significantly different at α=0.05 according to Post hoc Tukey HSD test; §DAS = Days after sowing; $^\epsilon$Total dry biomass includes dry weight of straw + leaves + spikes + grains at each spectral measurement and at harvest, were then sum-up with respect to each treatment and averaged over the 4 replicates.

Spectral reflectance measurement of leaves

Total four spectral reflectance measurements done on 20 May, 22 July, 24 Aug and 5+6 Sept 2011. For measuring leaf spectra, 4-5 plants from each pot were cut at ~2.5 cm above soil using steel scissor. Immediately, leaf reflectance spectra was measured in a laboratory with ASD FieldSpec portable spectroradiometer (350-2500 nm at resolution of 1 nm; ASD, Inc., Boulder, CO) using a plant probe leaf-clip assembly fitted with an internal halogen light. The reflectance spectra were measured by holding barley 2-3 leaves (alongside without overlapping) into leaf clip holder. Leaf-clip assembly has got two-sided rotating head: a black panel face for reflectance and a white for transmittance measurement. Reflectance spectra were calibrated against a white Spectralon panel face.

Table 2 – Vegetation indices calculation and absorption regions for which continuum removal was applied in present study.

Vegetation Indices		
Chlorophyll indices	**Calculation**	**Reference**
Normalized Difference Vegetation Index (NDVI)	$(R_{864} - R_{671}) / (R_{864} + R_{671})$	Rouse Jr et al. [1974]
Leaf Chlorophyll Index (LCI)	$(R_{850} - R_{710}) / (R_{850} + R_{710})$	–
Chlorophyll Index SR705	R_{750} / R_{705}	Sims and Gamon [2002]
Red Edge Inflection Point (REIP) – *Savitzky Golay* **Filter** 1st *order*	–	Cho and Skidmore [2006]
Water stress indices		
Normalized Difference Water Index (NDWI)	$(R_{864} - R_{1245}) / (R_{864} + R_{1245})$	Gao [1996]
NDWI – Mid Infrared (NDWI_MIR)	$(R_{864} - R_{2161}) / (R_{864} + R_{2161})$	–
Moisture Stress Index (MSI)	R_{850} / R_{1680}	Hunt Jr and Rock [1989]
Leaf Water Vegetation Index (LWVI2)	$(R_{1094} - R_{1205}) / (R_{1094} + R_{1205})$	Galvão et al. [2005]
Reflectance wavelengths between which the continuum removal applied		
Location of absorption features	**Wavelength (nm) at which Band Depth computed**	
415 – 545 nm	495	
545 – 750 nm	680	
900 – 1060 nm	970	
1120 – 1275 nm	1165	
1370 – 1570 nm	1435	
1740 – 1825 nm	1780	
1825 – 2170 nm	1925	

Individual spectral measurement for barley leaves was averaged (*n*=4-5 plants) by treatment to reduce variation and spectra were corrected for ASD jump (drift correction; additive) using AS-toolbox package add-in [Dorigo et al., 2006] installed in ENVI+IDL version 4.3. First derivative (FD) calculation was done on corrected spectra with 5-point moving average to intensify spectral features. Several VIs have been developed [Zarco-Tejada et al., 2001]

and in this study, VIs relevant to the metal-induced plant stress, particularly in chlorophyll and water absorption regions, were computed: chlorophyll indices NDVI, LCI, SR705, REIP and water stress indices NDWI, NDWI_MIR, MSI, LWVI2 (Tab. 2). Continuum removal (CR) was applied to corrected spectra which normalizes the reflectance spectra by applying convex hull and amplifies the individual absorption feature [Clark and Roush, 1984]. Difference in BDs between control and metal-treatment was statistically compared for each absorption region and at specific wavelength (Tab. 2).

Biometric measurements and chemical analyses
Plant stand (#plants pot^{-1}) was recorded on 1-month after sowing. Whereas, plant height and dry weight of plant parts (i.e., leaves, straw, and spike grain) were measured at the time of each spectral measurement and at harvest. The biometric data measured at each interval + at harvest were then sum-up with respect to the treatments and averaged over the 4 replicates.

At each spectral measurement and harvest, the plant samples were dried at 80 °C for 24 hrs and milled. For analyzing total metal concentration, 250 mg of milled sample was digested with 5-mL concentrated HNO_3 in Teflon tubes in pressurized microwave accelerated reaction system for 30-40 min. The digested substrate was then diluted with 10-mL deionized water and analyzed for total Cd, Pb and As using inductively coupled plasma-atomic emission spectrometry. Similarly, initial soil samples were also analyzed for total metal by digesting 500 mg air-dried soil in 5-mL concentrated HNO_3. Standard reference soil and plant samples were digested for recovery test. Precision for all standards were better than 5%. All chemical used in chemical analysis were of analytical grade.

Statistical analyses
Statistical analyses of spectral and chemical data were performed in MS Excel spreadsheets 2007 with XLSTAT 2009.1.02 add-ins and reported at α=0.05 significance level. Mean and standard error (±SE) were computed for all data. Data were statistically tested for significant effects through analysis of variance and *Post hoc* Tukey HSD test was done to compare the treatment mean. Correlation coefficient (Pearson's 'r') was computed between spectral variables (i.e., corrected spectra, VIs, BDs of CR spectra) and leaf-metal concentration for each treatment. Regression models (PLSR, partial least square regression and MLR, multiple linear regression; Unscrambler 9.7, CAMO ASA, Norway) for predicting leaf-metal concentration from spectral data were computed by leave-one-out cross validation technique. Coefficient of determination (R^2) between measured and predicted value and RMSE value were used to examine the relationship found.

Results and Discussion
Effect of metals on barley growth
The result presented in Table 1 shows that treatments As$_2$, As$_3$, Mix$_2$, and Mix$_3$ caused significant (*p*<0.05) reduction in plant stand (by 13-20% reduction), plant height at 1-month (by 25-42%), and total dry biomass weight (by 78-93%) compared to untreated barley plants. The inorganic arsenic species (i.e., arsenite, As-III; arsenate, As-V) are generally phytotoxic [Kabata-Pendias and Pendias, 2001] and found to reduce biomass and yield significantly at higher concentration [Carbonell-Barrachina et al., 1997]. Present result is in consistent

with study of Jiang and Singh [1994] wherein author reported reduction in barley yield due to 50 mg As kg^{-1} soil. Barley is As-sensitive and phytotoxicity depends on As concentration in the rooting medium [Shaibur et al., 2008]. For As-sensitive plant, soil concentration of 5 mg As kg^{-1} is toxic, while 50 mg As kg^{-1} could reduce growth by 50% [Lepp, 1981]. The As-phytotoxicity to barley is also expected because of sandy soil substrate used in present study (82% sand) and according to Sheppard [1992], inorganic-As is 5-times more toxic in sandy soils (thresholds 40 mg kg^{-1}) than in clayey soils (thresholds 200 mg kg^{-1}).

In contrast, barley grew steadily in Cd and Pb-spiked soils and no significant growth reduction was observed compared to untreated plants. This might be a result of relatively low concentration of Cd (1, 5, and 10 mg kg^{-1}) and Pb (25, 50, and 150 mg kg^{-1}). Barley cultivars are reported to have greater degree of tolerance to high Cd and Pb concentration in soils [Vassilev, 2003; Pirselová, 2011]. Conversely, low concentration of metal could have growth promoting effects [Nyitrai et al., 2003]. Furthermore, no effect of Cd and Pb on barley growth is obvious as a fact of NO$_3$-metal salts spiking, which supplied NO$_3^-$ ions (readily available N) at early stage of barley growth and thus aided to alleviate an immediate Cd- and Pb-phytotoxic effects. According to Berry [1924], Pb(NO$_3$)$_2$-salt at certain low concentration could have manurial properties and stimulating effect. It implies that metal-induced stress varies with soil-metal concentration and physico-chemical properties of metal-salts [Vassilev and Yordanov, 1997].

Metal accumulation into the plant parts

Result of chemical analyses for Cd, Pb and As concentration in the leaves (average of 4-intervals + at harvest), root, straw and grain of barley is shown in Figure 1. The amount of all three metals in plant parts increased with increasing levels of metal treatment, and was found significant ($p<0.05$). For all three metals, noticeable difference in metals' accumulation in different plant parts was observed, with preferentially higher metal accumulated in roots, followed by straw plus leaves, and while grains contained much lower concentration of metal (Fig. 1). Of the total metal accumulation in plant, ≈94% Pb, ≈82% Cd, and ≈64% As were concentrated in roots only, advocates an existence of different metal sequestration mechanism, which hampered the metal translocation from root to the above-ground plant parts. Several studies [Guo et al., 2007; Karimi et al., 2013; Žaltauskaitė and Šliumpaitė, 2013] had confirmed hyper-accumulation of Cd into barley roots, could partly be explained by the Cd-loading into cell vacuole as non-active form and immobilization by cell wall [Vassilev et al., 2002]. Phytoaccumulation of Pb into above-ground plant parts is principally rare due to low solubility of most Pb-compounds, and ready precipitation of Pb by sulphate and phosphate at root system [Dushenkov et al., 1995; McGrath et al., 2002]. Arsenic translocation from root to shoot is as well rare phenomenon in most terrestrial plant species, except certain hyper-accumulators species of Brake ferns, *Pteris vittata* [McGrath et al., 2002; Banejad and Olyaie, 2011].

Spectral reflectance analysis
Cadmium and lead treatment

The spectral changes due to Cd and Pb (except Cd$_3$ and Pb$_3$ treatments) do not show visible differences; spectra are nearly indistinguishable from the spectra of untreated plant, and the shape of these curves is consistent over the time (data not presented). This might be as a

result of NO_3-salts spiking, which could favor chlorophyll biosynthesis, and hence spectra were not much deviated from control spectra, rather relatively a deepen absorption features at ~495 and ~680 nm were noted in case of Pb_3 at 1-month (Fig. 3a). Non-significant Pearson's correlation between leaf-Cd, -Pb and leaf-spectra was noticed in both single and multi-metals treatment except for 1-month of sowing (Fig. 2a-b). On 1-month, significant negative correlation was noted between leaf-Pb (in Pb-treatment) and 513-645 nm ($r = -0.58$ to -0.72; $p=0.007$), 691-721 nm (-0.58 to -0.73; $p=0.008$). This could be either due to of straight effect of $Pb(NO_3)_2$-salt spiking into soil or relatively low level of Pb that itself stimulates photosynthetic machineries and pigment biosynthesis [Nyitrai et al., 2003], causing increased absorption at these wavelengths (Fig. 2a-b).
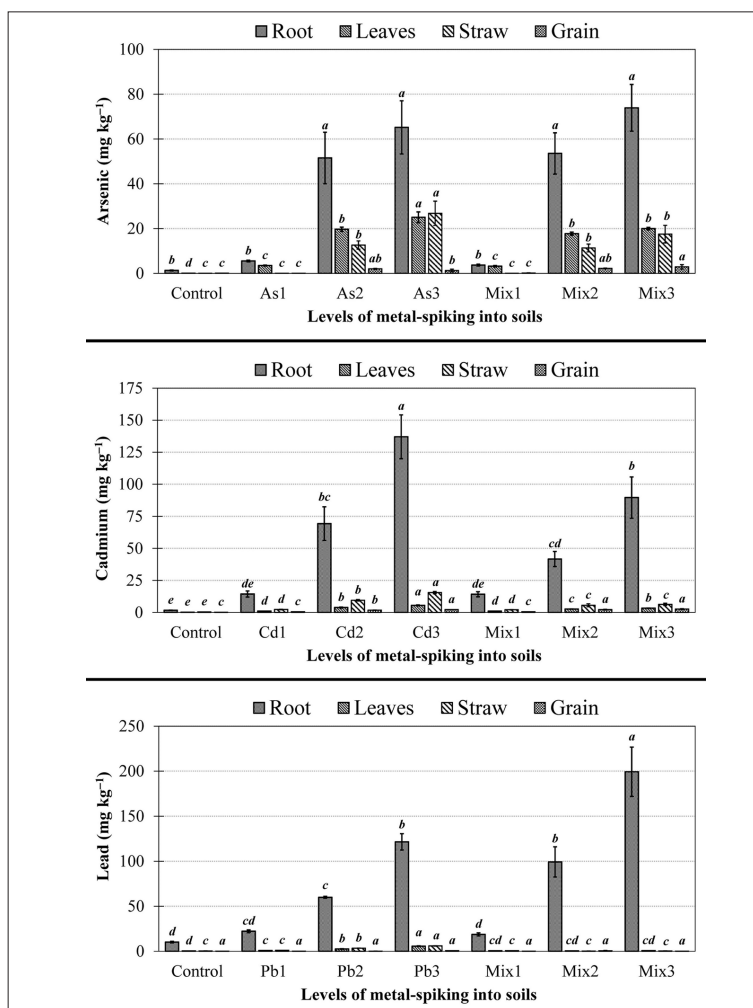

Figure 1 – Cadmium, lead and arsenic concentration (in mg kg^{-1}; dry weight basis) in roots, leaves*, straw and grains of barley. Error bars are values of ±SE. The column not connected by same letter are significantly different at α=0.05 according to *Post hoc* Tukey HSD test. *Average value of all measurements done on 31, 65, 92, 126 days after sowing, and at harvest.

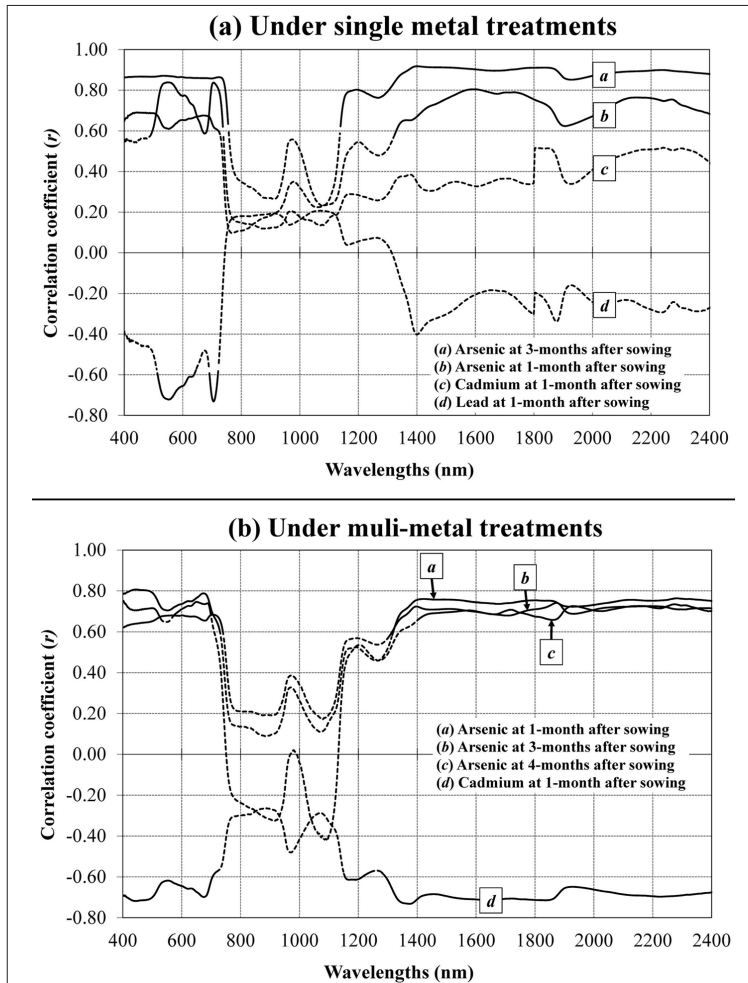**(a) Under single metal treatments**

(*a*) Arsenic at 3-months after sowing
(*b*) Arsenic at 1-month after sowing
(*c*) Cadmium at 1-month after sowing
(*d*) Lead at 1-month after sowing

(b) Under muli-metal treatments

(*a*) Arsenic at 1-month after sowing
(*b*) Arsenic at 3-months after sowing
(*c*) Arsenic at 4-months after sowing
(*d*) Cadmium at 1-month after sowing

Figure 2 – Pearson's correlation (*r*) of the leaf-metal concentration and leaf reflectance spectra under (a) single metal treatment and (b) multi-metals treatments. Solid line (—) represents significant correlation between wavelength and metal concentration at *α*=0.05 level of significance and Dotted line (----) represents non-significant correlations.

Arsenic and multi-metals treatment

In contrast to Cd- and Pb-treatments, barley grown in As- and multi-metals spiked soils had increased reflectance in visible and SWIR regions, while small changes occurred in NIR compared to control spectra. As it can be seen in Figure 3a and 3c, spectral change was clear with the highest level of treatment over control spectra, and spectral amplitude in visible and SWIR regions was prominent with As_3 and Mix_3 treatments. Reflectance difference between treated and control plant is significant ($p<0.0001$) in the visible and SWIR regions with the greatest difference observed at ~700-715 nm for As_3 and Mix_3 treatments (Fig. 3c-d). Other potential changes due to metal treatment includes absorption features around 970, 1160, 1430, and 1920 nm, are likely related to leaf water stress.
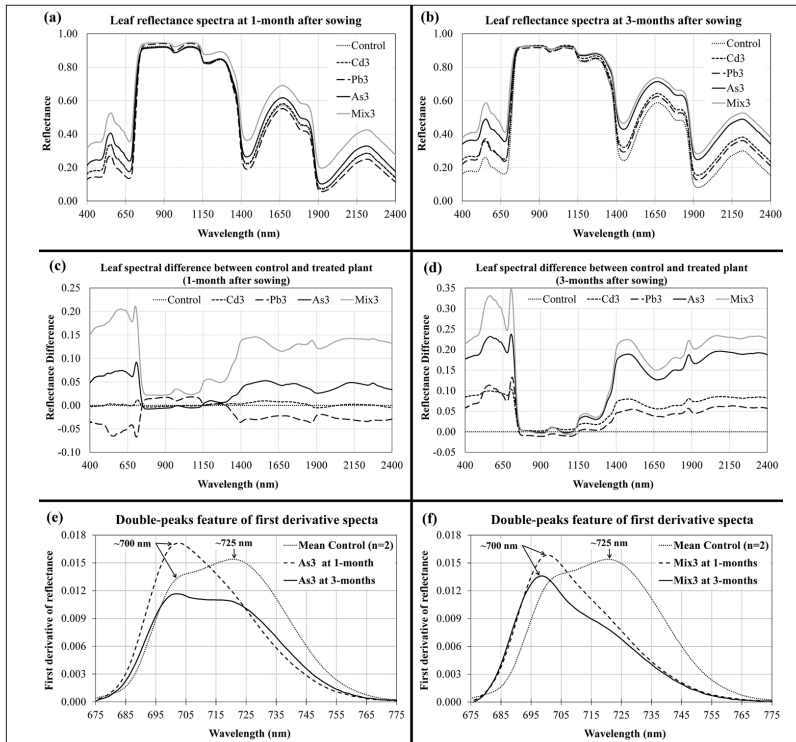
Figure 3 – Comparison of leaf reflectance spectra for higher level of Cd, Pb, As, Mix treatments on 1- and 3-months after sowing (a, b). Leaf spectral difference between control and untreated plants for higher level of Cd, Pb, As, Mix treatments on 1- and 3-months after sowing (c, d). First derivatives of leaf reflectance in the 475 – 775 nm wavelength regions for higher level of arsenic and multi-metals treatments on 1- and 3-months after barley sowing (e, f).

FD spectra between 475 and 775 nm for As_3 and Mix_3 treatments are shown in Figure 3e-f. The magnitude of the derivative peaks decrease with increasing level of metal and demonstrate a shift towards shorter wavelengths, i.e., *blue-shift*. Moreover, double-peaks feature at ~700 and ~725 nm observed in FD spectra respectively attributed to the chlorophyll absorption and light scattering by leaves tissues [Horler et al., 1983]. The double-peaks feature has been identified by several researchers [Smith et al., 2004 and references therein], and it has a definite significance to stressed vegetation with second peak(s) of derivative spectra being less apparent (or absent) in the stressed vegetation. In present study, it is evident that the magnitude of second peak of FD spectra at ~725 nm decreased with stress progressed (as on 3-months, second peak disappeared in Mix_3 treatment; Fig. 3e-f).

Pearson's correlation analyses also showed that leaf-As had strong positive correlation with reflectance in visible and SWIR regions (Fig. 2). On 1-month, wavelengths 351-727 and >1325 nm were significantly correlated with leaf-As, with the highest r values of 0.69 ($p=0.013$) and 0.80 ($p=0.002$) respectively at 443-473 and 1585-1603 nm for single-metal treatments; r values of 0.81 ($p=0.002$) and 0.76 ($p=0.004$) respectively at 443-449 and 2275-2289 nm for multi-metals treatments. Similarly, on 3-months, significant and the

highest correlation coefficient values (r=0.87; p=0.0005 and 0.92; <0.0001) were obtained for leaf-As with wavelengths at 531-537 and 1399-1403 nm, respectively.

The present results of As-stress induced spectral changes in visible regions can be linked to the fact of reduction in chlorophyll due to As-phytotoxicity. Previous works have shown that high As damages the photosynthetic machinery and pigment biosynthesis [Peñuelas and Filella, 1998; Garg and Singla, 2011; Sanal et al., 2014], which leading to a decrease in light absorption and henceforth increases reflectance in visible region [Milton et al., 1989; Slonecker et al., 2009; Bandaru et al., 2010]. This phenomenon is well-documented and used to monitor vegetation stress [Horler et al., 1980]. Small variations in NIR region (750-1300 nm) in As- and Mix-treatment could be due to a decrease in the leaf water upon As-bioaccumulation (Fig. 1). This spectral change is similar to the results of Sridhar et al. [2007b] who found variations in leaf NIR reflectance in barley treated with cadmium and zinc.

Vegetation indices and continuum removal spectra
Chlorophyll and water stress indices

In present study, looking at the effect of metals especially on chlorophyll and water absorption features (Fig. 3a-d), indices related to them were computed (Tab. 2). The trend of some of spectral indices for all the treatments is presented in Figure 4a-d. Cadmium and lead treated barley showed non-significant changes in the considered indices on 1- and 3-months, without observing any significant (p<0.05) correlation of indices with leaf-Cd and -Pb concentration. The probable reasons of non-significant results, as discussed in earlier sections, are: (i) relatively low concentration of Cd and Pb-spiking into soil, (ii) higher degree of tolerance of barley to Cd- and Pb-toxicity, and (iii) positive effect of NO_3^- metallic-salt. Sridhar et al. [2007b] reported that Cd at 1 mM (112 mg kg^{-1}) and 10 mM (1120 mg kg^{-1}) concentration (respectively 10- and 100-times higher Cd concentration than the Cd applied in present study) had phytotoxic effect on barley and such Cd-induced stress was revealed by NDVI analysis.
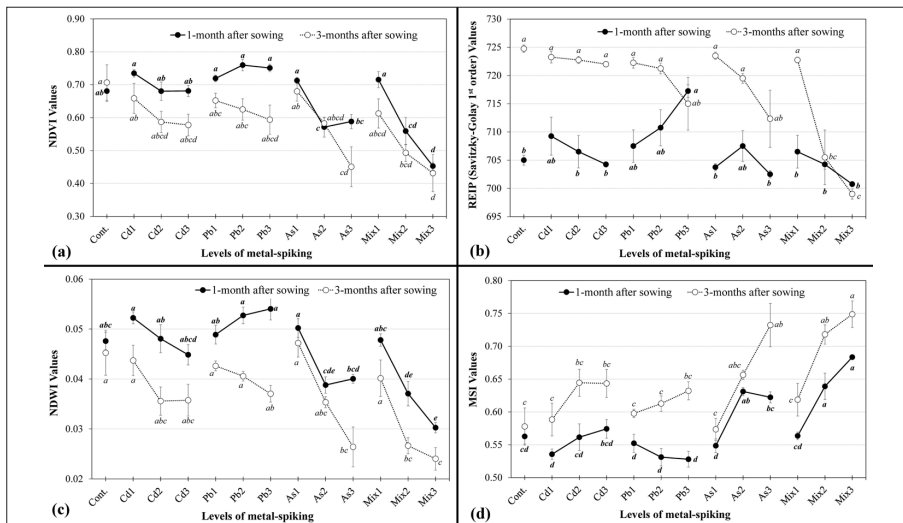

Figure 4 – Comparison between values of vegetation indices at 1- and 3-months after sowing. Error bars are values of ±SE of 4 replicates. The markers not connected by same letter are significantly different at α=0.05 according to *Post hoc* Tukey HSD test.

Arsenic and multi-metals (As_2, As_3, Mix_2, and Mix_3) treated plants, in contrast, can statistically be distinguished from untreated plants. For NDVI, SR705 and LCI on 1-month, least values were obtained with Mix_3 (0.45, 1.72 and 0.17, respectively) and they differed significantly ($p \leq 0.0001$-0.0003) from control treatment. Consistently, also on 3-months, a significant ($p < 0.0001$) decrease in the indices value was observed in Mix_3 treatment (NDVI, 0.43; SR705, 1.71; LCI, 0.17) followed by Mix_2 and As_3 treatments. These result clearly shows the metal-induced stress caused by reduction in chlorophyll absorption (Fig. 3a-d) with increased As-accumulation in plant parts (Fig. 1). It is confirmed by significant negative correlation between chlorophyll indices and leaf-As for Mix-treatments ($r = -0.81$, -0.75, -0.70 on 1-month and $r = -0.68$, -0.72, -0.71 on 3-months, respectively for NDVI, SR705, LCI). For As-treatment, similar to Mix-treatments, significant negative association between leaf-As and chlorophyll indices on 3-months was observed ($r = -0.86$, -0.86, -0.81 respectively for NDVI, SR705, LCI). Unusually, no significant correlation of chlorophyll indices with leaf-Cd and -Pb was observed in Mix-treatments. Results revealed that in comparison to either control or Cd or Pb treatments, chlorophyll indices are highly indicative of metal-induced stress in barley for As- and Mix-treatments. Moreover, stress caused by higher levels of As- and Mix-treatments had more impact than lower levels (As_1, As_2, Mix_1, and Mix_2). A decrease in the values of chlorophyll indices could be the indirect effects of As-bioaccumulation, specifically into the roots, that decrease photosynthetic pigment content, influence chloroplast membrane structure and disturb the functions of integral photosynthetic process [Garg and Singla, 2011 and references therein; Appenroth, 2010].

In many studies [Horler et al., 1980; Horler et al., 1983; Clevers and Kooistra, 2003], researchers have focused on red-edge feature (red-edge inflection points; REIP) characterized by incline between red and NIR wavelengths. The REIPs (Savitzky-Golay Filter first order derivative and smoothing technique [Savitzky and Golay, 1964]) were derived for each treatment (Fig. 4b). No significant changes occurred in the REIPs on 1-month except Pb_3 treatment (717 nm; *red-shift* by 12 nm). The *red-shift* of REIP towards longer wavelength in Pb_3-treatment is confirming the broadening of chlorophyll absorption feature around 680 nm (Fig. 3a) and the probable causes are discussed in earlier sections. On 3-months, REIPs for Mix_2 (705 nm) and Mix_3 (699 nm) significantly differed from control plant (724 nm), and it shows *blue-shift* respectively by 19 nm and 25 nm with reference to control. The *blue-shift* of REIP strongly linked to the inhibition of chlorophyll biosynthesis due to As-induced stress, as confirmed with significant negative correlation ($r = -0.63$; $p = 0.027$) between REIP and leaf-As. As-bioaccumulation in plant leaves caused the changes in visible and NIR spectra [Bandaru et al., 2010] and similar *blue-shift* results due to metal-induced stress have been reported by Milton et al. [1989]; Clevers et al. [2004]; Dunagan et al. [2007]; Liu et al. [2008]; Ren et al. [2010].

The water index uses reflectance in the NIR (light scattering) and SWIR (water absorption feature) regions to monitor water-stress conditions in vegetation. Alike chlorophyll indices, Mix_3-treated barley showed significant ($p < 0.0001$) differences in values of water indices from the control plant on 1- and 3-months (Fig. 4c-d). Treatments Mix_2, As_2, and As_3 were also differed statistically ($p < 0.05$) from the control. Barley grown in multi-metals spiked soil had started to show significant changes in the water indices on 1-month onwards. In Mix-treatments, merely leaf-As concentration had significant correlation with studied water indices, NDWI, NDWI_MIR, MSI, and LWVI2 (respectively, $r = -0.81$, -0.80,

0.81, and −0.83 on 1-month and r = −0.74, −0.75, 0.73, and −0.73 on 3-months). This shows that As-accumulation in plant parts, particularly into roots, is solely responsible for indirect inhibition of root growth and enzymatic activities [Sanal et al., 2014] and ultimate consequences are the water stress and disturbed nutrients absorbance [Appenroth, 2010].

Continuum removed spectra and band depths
Continuum removal (CR) analysis of particular spectral absorption features is one of the popular normalization technique to amplify variation in reflectance spectra from a common baseline [Kokaly and Clark, 1999]. In this study, CR was applied to seven absorption features and BDs at each and selected wavelength in the absorption features (Tab. 2) were calculated by subtracting the CR reflectance from 1. The Cd and Pb-treated plants at 1-month had the deeper BDs at each wavelength within all absorption features compared to untreated plants, and as a result, a negative difference (i.e., larger band depths than the control) was observed between untreated and Cd-, Pb-treated plants (Fig. 5a). Whereas, at 3-months, patterns changed (i.e., shallower BDs for Cd- and Pb-treated plants than control), and hence positive difference observed (Fig. 5c). A negative difference in BDs at 1-month infers that irrespective of levels, Cd and Pb-spiking into soil had no phytotoxic effects on the growth of barley, but certainly barley growth was enhanced (Tab. 1), and so it causes strong absorption at each wavelength. However, non-significant variation was observed between control and Cd-, Pb-treatments for BD at selected wavelengths (Tab. 2, Fig. 6). According to Curran [1989], Boyd et al. [2006], absorption features in visible region (at 495 and 680 nm) can be related to chlorophyll, 970 nm to water, at 1165 nm to biochemical of lignin, 1435 nm to water or nitrogen, 1780 nm to cellulose or lignin, and 1925 nm to cellulose or water.
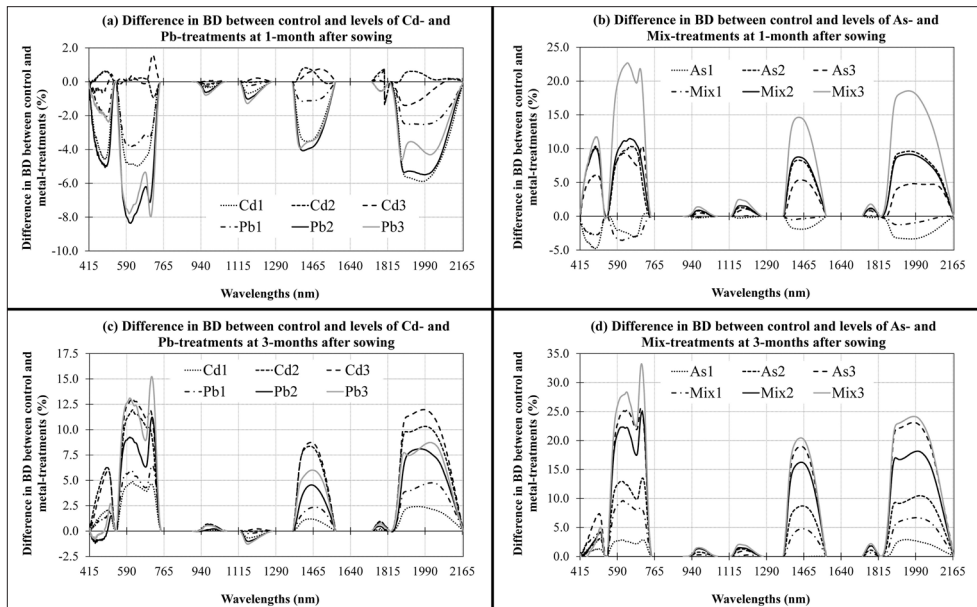

Figure 5 – Difference (%) in band depth values between control and levels of metal-treatments on 1-month and 3-months after sowing. A positive difference refers to the larger band depth in the control treatment than the metal-treatments at given wavelengths.

This was not the case, however, for As- and multi-metals treated plants, which had shallowest BDs relative to control on 1- and 3-months except low levels on 1-month (Fig. 5b, 5d). It is evident from figures that the greatest positive difference (relative to the control) was observed with Mix_3 at each wavelength of absorption regions, predominantly in red region (550-740 nm) and water absorption regions (1380-1550, 1850-2140 nm). Over the time, the BDs for As- and Mix-treatments at each wavelength show greater distinction from control (Fig. 5b, 5d). Statistical analysis of BDs at selected wavelengths showed that Mix_2- and Mix_3-treatments had caused significant decrease in BDs compared to the control (Fig. 6). Consequently, BDs showed significant negative correlation with only leaf-As concentration in Mix-treatments ($r = -75$ to -92, $p<0.05$), demonstrating phytotoxic effects of As-stress on barley. In plant, As is mainly accumulated in the root system (~ 75 mg kg^{-1} in barley root for Mix_3 treatment, Fig. 1), which cause physiological changes including chlorophyll reduction and water imbalance in the plant tissues [Garg and Singla, 2011]. Result of CR analysis is in agreement with finding of Noomen et al. [2006] who noted reduction in BDs in the chlorophyll and water absorption regions in CR spectra of maize plants treated with various hydrocarbon gases.

Regression analysis

Prediction model for leaf-metal concentration from leaf-spectra, was one of the aim of present study and it could be a monitoring tool for phytoremediation process. The PLSR technique is widely used to build prediction model [Carrascal et al., 2009]. Since it is evident from correlation result (Fig. 2) that only leaf-As (in both As- and Mix-treatments) had significant association with leaf spectra, the PLSR and MLR analysis was run using significantly correlated spectral features, explanatory variables and leaf-As concentration, dependent/response variables. Table 3 summarizes the set of spectral regions that yielded the best R^2 (close to 1) and RMSE (the lowest) for the prediction of leaf-As concentration. On 1-month, the best model for leaf-As estimation was obtained with significant spectra (selected through Marten's uncertainty significance testing implemented in Unscrambler 9.7; see Tab. 3 *foot-note*) from 415-750, 1370-1570, 1825-2170 nm range, which yielded R^2, 0.98 and 0.95 and RMSE, 0.95 and 1.66, respectively for calibration and cross-validation (Tab. 3). The cross-validation results showed that this PLS model with PC# 7 explained 95.22% variance of response variable (leaf-As). The PLSR models using mean normalized spectra from 415-2170 nm also showed best result with R^2, 0.94 and RMSE, 1.93 for cross-validation, wherein PC# 10 explained 93.70% variance in leaf-As. Results revealed that PLS regression involving chlorophyll and water absorption wavelengths appeared to be relatively the best predictors of leaf-As, particularly when including spectra from visible (415-750 nm) and/or SWIR (1370-1570 and 1825-2170 nm) regions. Alike, on 3-months, the best model obtained using significant wavelengths of water absorption regions (i.e., 1370-1570, 1825-2170 nm), with R^2, 0.90 and RMSE, 3.62 for cross-validation. However, raw and FD of water absorption spectra, also gave relatively good results with cross-validation R^2, 0.89 and 0.88 and RMSE, 3.89 and 3.92, respectively (Tab. 3). Other best fit models for leaf-As estimation on 1- and 3-months can been seen in Table 3. However, the selection of wavelengths for obtaining best fit model were inconsistent.
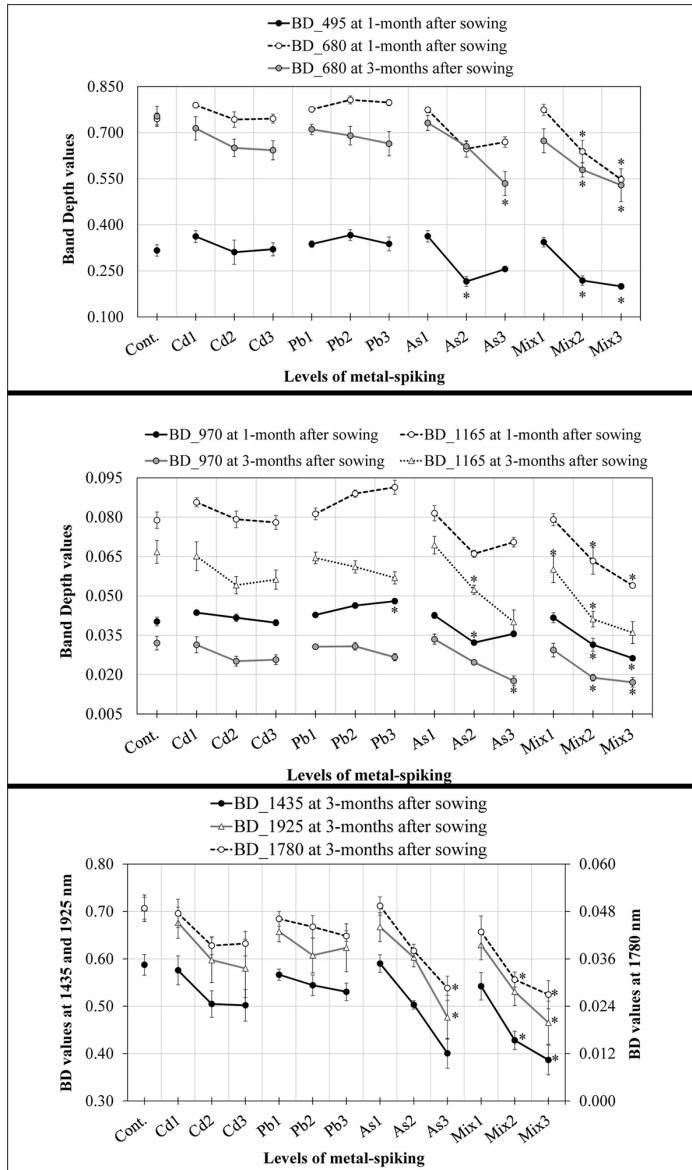
Figure 6 – Band depth at selected wavelength for different levels of treatment. Error bars are values of ±SE of 4 replicates. The markers with asterisk (*) are significantly differed from control treatment at α=0.05 according to *Post hoc* Tukey HSD test.

Multiple linear regression (MLR) model, done with Unscrambler 9.7, for estimating leaf-As using the VIs and BDs showed good results. On 1-month, the model using all VIs and BDs yielded R^2, 0.89 and RMSE, 2.50 for cross-validation (Tab. 3). The MLR model showed the best R^2 (0.97) and RMSE (1.28) for cross-validation upon excluding non-significant variable viz., LIC, REIP, and BDs at 680, 970, 1435 nm. Similarly on 3-months, MLR

using NDWI, MSI, and BD at 680, 1435, 1925 nm is to be the best predictors (R^2, 0.93 and RMSE, 2.90 for cross-validation) for leaf-As estimation.

Table 3 – Best fit PLSR and MLR models for estimation of leaf-As concentration in barley for As- and Mix-treatments set (*n=19-22*) on 1- and 3-months after sowing, expressed in terms of coefficients of determination (R^2) and root mean square error (RMSE) of calibration, *cal* and cross-validation, *val* models.

	Spectral regions selected for regression models	R^2 cal.	R^2 val.	RMSE cal.	RMSE val.
Sr	*For As- and Mix-treatment set (n=22; on 1-month after sowing)*				
1	MLR without LCI and BDs at 680, 970 nm	0.99	0.91	0.81	2.28
2	Mean normalized spectra of 415-2170 nm; PC# 10	0.99	0.94	0.64	1.93
3	*Spectra ranges as of CRR absorption features; PC# 10	0.99	0.92	0.80	2.13
4	**Abs feature of 415-750, 1370-1570, 1825-2170 nm; PC# 7	0.98	0.95	0.95	1.66
5	Spectra of 1370-1570 nm; PC# 6	0.95	0.90	1.64	2.39
6	Spectra of 1825-2170 nm; PC# 5	0.92	0.86	2.02	2.60
7	Spectra of 1370-1570 and 1825-2170 nm; PC# 7	0.94	0.88	1.72	2.70
8	CR spectra of 1370-1570 and 1825-2170 nm; PC# 6	0.97	0.93	1.27	2.00
9	Spectra of 415-2170 nm; PC# 10	0.99	0.89	0.60	2.50
10	MLR with all calculated VIs and BDs	0.99	0.94	0.66	1.86
11	MLR without REIP and BDs at 1435 nm from (*n=21*)	0.99	0.97	0.59	1.28
	For As- and Mix-treatment set (n=19; on 3-months after sowing)				
12	***Significant spectra of 1370-1570, 1825-2170 nm; PC# 7	0.95	0.90	2.50	3.62
13	Spectra of 1370-1570 nm; PC# 7	0.97	0.89	1.99	3.89
14	Spectra of 1825-2170 nm; PC# 5	0.91	0.84	3.21	4.63
15	FD spectra of 415-2170 nm; PC# 5	0.91	0.83	2.31	4.81
16	FD spectra of 1370-1570, 1825-2170 nm; PC# 6	0.98	0.85	1.61	3.92
17	MLR with NDWI, MSI and BDs at 680, 1435, 1925 nm	0.96	0.93	2.03	2.89
18	MLR with all calculated BDs	0.96	0.90	2.13	3.47
19	MLR with BDs at 495, 680, 1165, 1435, 1780, 1925 nm	0.96	0.90	2.16	3.35
20	MLR with NDVI, LCI, REIP, NDWI, NDWI_MIR, MSI	0.96	0.88	2.20	3.74

PC = Principal Component; FD = first derivatives, no. of smoothing points 5; CR = continuum removed
*Spectra range from: 415-750, 900-1060, 1120-1275, 1370-1570, 1740-2170 nm;
**Selected significant wavelengths: 528-563, 627-634, 637-638, 705-719, 1392-1403, 1487-1505, 1867-1893, 1938-1970, 1999-2006, 2073-2170 nm;
***Selected significant wavelengths: 1370-1391, 1473-1476, 1561-1570, 1846-1897, 1948-1976 nm.

PLS regression models, in general, shows that the best spectral regions for barley leaf-As estimation in the present study positioned in chlorophyll and/or water absorption regions of spectrum, which were highly influenced either directly or indirectly by As-toxicity as can be seen from the results presented in previous sections. Similarly, MLR models, which typically include VIs and BDs based on wavelengths in visible and SWIR regions of spectrum, also present good results. Results of both regression analyses were similar

to those reported by Slonecker et al. [2009]; Bandaru et al. [2010]; Hong et al. [2010]. According to them, regression results in present study put forward the possibility of using visible and SWIR reflectance to predict leaf-As concentration in barley grown with As- and Mix-treatments.

Conclusions

Results of present study demonstrate that hyperspectral reflectance data, as well as studied VIs and BDs appears to have potentials for assessing metal-induced stress (particularly, As-stress in present study) and for monitoring the phytoaccumulation of arsenic into above-ground parts of barley. For Cd and Pb-metal, further studies are recommended with higher concentration and with metal-salt other than Nitrate-salt, may give different spectral signatures to be used for monitoring phytoremediation process. In this view, the spectral reflectance and so the relevant spectral features offered an easy and simple way to assess plant health and metal-concentration. Therefore, the ground-based spectral sensing may be an effective alternative technique for screening and monitoring the metal hyperaccumulators to determine the levels of plant stress towards the efficient phytoremediation management.

Acknowledgement

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