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Research Article

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Lead Phytoremediation Potential of Wild Type and Transgenic Tobacco Plants

Abstract

Genetically engineered plants may have a great potential to enhance translocation of lead (Pb) from root to the above ground parts. A pot experiment was conducted to investigate the effect of Pb uptake by non-transgenic (*Nicotiana tabacum* L. cv. Petit Havana SR1) and transgenic (p-cV-ChMTII GFP) tobacco plants, which carrying Chinese hamster metallothionein II. Transgenic and non-transgenic tobacco plants were grown in soils treated with 0, 1000, 2500, 5000 mg Pb kg^{-1} as $Pb(NO_3)_2$ up to the flowering stage for 6 weeks in a growth chamber under controlled conditions. The plant growth, chlorophyll content, mineral nutrient elements and reduced glutathione (GSH) concentrations were investigated along with the Pb uptake potential of the plants. A progressive decrease in above ground biomass production was observed due to the increase in Pb application for both transgenic and non-transgenic plants. Most of the leaf nutrient concentrations were negatively influenced by excessive Pb treatments, of which P showed the most drastic decrease. Shoot Pb concentrations reached up to 76.0 mg kg^{-1} in transgenic and 70.9 mg kg^{-1} in non-transgenic plants. Lead uptake was improved by transferring the p-cV-ChMTII GFP into the tobacco plant; however, it was not sufficient enough to be used in the Pb phytoremediation.

INTRODUCTION

Soils can have a high concentration of heavy metals due to the contamination caused by agricultural, industrial, and military activities and the pollution inherited from parent materials. Adverse effects of elevated metal concentrations may appear over both soil organisms and plants in the natural environments, and subsequently, threaten the health of human beings through nutrition chain (Pourrut et al., 2011; Kabata-Pendias, 2011; Li et al., 2016; Saghi et al., 2016). Heavy metal (cadmium (Cd), copper (Cu), lead (Pb), chromium (Cr), zinc (Zn), and nickel (Ni)) pollution is the common problem of industrial contaminated sites in the world. Heavy metal pollution can be regarded as a permanent problem of soils (Lestan et al., 2008) unless they are removed by either chemical or biological processes such as phytoremediation. Lead, unlike organic contaminants, cannot be decayed by biological processes in the soils, but oxidation states and/or bounded organic ligands may change. Thus, Pb pollution exerts a very high risk to the ecosystem and human health (Salama et al., 2016).

According to the Agency for Toxic Substances and Disease Registry of the United States (ATSDR), subordinating arsenic (As), Pb is the most toxic element in the list of all hazardous substances (ATSDR, 2019). Lead enters the plant systems through the soil or via atmosphere from different sources. The primary source of lead pollutions are anthropogenic activities such as agriculture (overuse of fertilizers and pesticides), industry (batteries, paints, coal burning, gasoline, etc.), exhaust emission, mining and smelting of Pb-ores etc. (Pourrut et al., 2011; Kabata-Pendias, 2011; Gupta et al., 2013; Li et al., 2016; Salama et al., 2016; Saghi et al., 2016). Lead is not an essential element for a living organism; even a small amount of Pb negatively affects an organism's tissues because it cannot be degraded or detoxified by any known biological process (Gupta et al., 2013; Yuan et al., 2015; Li et al., 2016; Saghi et al., 2016). Therefore, there is a need to develop effective and economical cleaning processes suitable for this type of contaminants.

Phytoremediation is a promising method which is a cost-efficient and ecologically friendly way of reclaiming heavy metal polluted soils, but there are limitations such as a longer time requirement, the specificity of each plant to a certain heavy metal, low biomass production, low translocation ability from roots to shoots, etc. (Daghan, 2004; Vangronsveld et al., 2009; Vamerali et al., 2010; Paz-Alberto and Sigua, 2013; Ali et al., 2013; Eren and Daghan, 2014). Some of the heavy metals (i.e. Cu, Zn, and Mn) essential for plant growth can be transferred to shoots in larger quantities comparing to non-essential ones. Nonessential heavy metals such as Pb usually accumulate in the roots, therefore, phytostabilized in the roots, and they are not translocated from roots to shoots in large quantities (Paz-Alberto and Sigua, 2013). However, the stabilization of heavy metals in the soil environment either by chemical or biological methods are not long lasting ways of detoxifying the heavy metals. After some period of time, these methods become inefficient due to the transformation of chemical precipitates and mineralization of plant roots in the soils. In fact, for sure, the contaminants should be removed from the soil environment by any or combination of chemical, physical, and biological methods. For this reason, plants with the high phytoextraction ability for heavy metals are to be screened in the contaminated land to find out naturally evaluated hyperaccumulant plants (Salt et al., 1995; Chaney et al., 1997; Kayser, 2000; Eren, 2014) or genetically engineered transgenic plants (Lefebvre et al., 1987; Liu et al., 2000; Song et al., 2003; Daghan, 2004; Daghan et al., 2013; Eren and Daghan, 2014).

Metallothionein, which is a metal binding protein, rich in cysteine content

with low molecular weight (6-7 kDa), may be obtained from various origins such as mouse, human, peas and yeast (Lefebvre et al., 1987; Suh et al., 1998; Krämer and Chardonnes, 2001; Daghan, 2004; Sakulsak, 2012; Daghan et al., 2013) which are proposed to have a genetic transformation of plants towards handling a phytoextractor plant. By this way, it was possible to increase the metal accumulation abilities of plants as much as 60-70%. The tolerance of transgenic plants to heavy metals can be increased by transferring MT gene. For example, MT-II gene transferred into *Nicotiana glutinosa* plants was tolerant as high as 200 µM with little toxicity symptoms while the respective non-transgenic one was able to tolerate only 50 µM Cd with serious toxicity symptoms and little growth in the hydroponics (Suh et al., 1998; Liu et al., 2000).

Metallothioneins (MTs) from different origins appear to be capable of binding, detoxifying and accumulating a greater range of metals such as Cu, Zn, Cd, Pb, and As (Sakulsak, 2012). Genetically engineered overexpression of MTs in plants can increase their performance to some extent for phytoremediation or bioremediation purposes. In the current study, we focused on the above ground parts of the plant, since above ground can be easily harvested and removed from the polluted environment for phytoremediation purposes. There are a few studies on how heavy metals affect the nutrition uptake of plants. Thus, we aimed to test Pb uptake and accumulation ability of MT-II gene bearing transgenic tobacco plant that improves the tolerance of plants against heavy metals. Besides that, plant biomass, chlorophyll content, nitrogen (N), phosphorus (P), potassium (K), iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn) uptake and reduced glutathione (GSH) concentration of shoots were also investigated in both plants.

MATERIAL and METHODS

Plant material

Wild type tobacco variety (*Nicotiana tabacum* L. cv. Petite Havana SR1) and genetically modified germplasms of the same variety was used in the pot experiment. The gene encoding for ChMTII under a constitutive promoter (Fig. 1) was introduced into tobacco plants via *Agrobacterium* mediated transformation. Details of p-cV-ChMTII gene and its transfer were given elsewhere (Daghan, 2004).

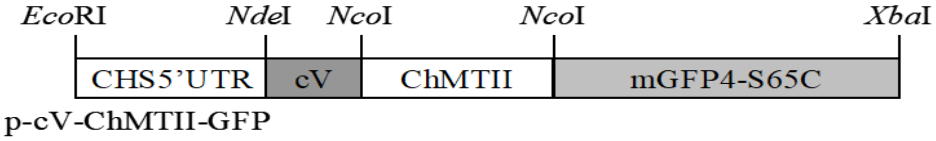

Fig. 1 Schematic presentations of p-cV-ChMTIIGFP construct. CHS 5′ UTR: 5′ untranslated region of *chalcone synthase*; cV: Vacuolar targeting signal from the *Catharantus roseus strictosidine synthase;* ChMTII: Chinese Hamster Metallothionein II; GFP: green florescent protein (mGFP4-S65C)

The leaf disc transformation method was used for generation of transgenic tobacco plants (Fraley et al., 1983; Horsch et al., 1985). Twenty five primary transformants from p-cV-ChMTIIGFP were selected on kanamycin and screened for the accumulation of recombinant protein. Transgenic plants typically exhibited non-altered morphology, indicating fully self-pollination. The T3 generation of p-cV-ChMTIIGFP encoding transgenic plants was tested for Pb uptake.

Soil material and analysis

Mahmutlu soil series of Amik Plain belonging to Mollisol order were used in this study (35° 47'-36° 24' E; 35° 48'-36° 37' N). The characteristics of soil were determined by the following standard procedures: Textural fractions by Bouyoucos hydrometer method

(Bouyoucos, 1962), organic matter by wet oxidation with $K_2Cr_2O_7$ (Nelson and Sommers, 1996), pH and EC in saturation paste by combined electrode and EC meter, the calcium carbonate equivalent by a manometric method (Soil Survey Staff, 1951), total nitrogen by the Kjeldahl method (Bremner and Mulvaney, 1982), plant available phosphorus by 0.5 M $NaHCO_3$ extraction (Olsen et al., 1954), potassium by 1.0 M ammonium acetate (NH_4OAc) extraction (Richards, 1954), and the bioavailable Pb, Fe, Cu, Mn and Zn by DTPA extraction (0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1M TEA) at pH 7.3 (Lindsay and Norvell, 1978). The USEPA 3051 microwave method was used to digest total Pb in soil (USEPA, 1995). Supernatants consisting of Pb, Fe, Cu, Mn, and Zn were analyzed by the ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry, Varian Series-II).

Physico-chemical properties of the experimental soil were shown in Table 1. The soil texture class was clay-loam. The experimental soil was slightly alkaline (pH 7.66) and non-saline (0.026%). The organic matter (2.16%) and $CaCO_3$ contents (11.4%) were moderate. The total concentration of Pb (3.92 mg kg^{-1}) was lower than the average of world soils (27 mg Pb kg^{-1}) according to Kabata-Pendias (2011).

Table 1. Some physical and chemical properties of the Mahmutlu soil used in the experiment

Soil Parameters	**Content**	**References**
Sand (0.02-2 mm) (%)	43.3	Bouyoucos 1962
Loam (0.002-0.02 mm) (%)	22.4	
Clay (<0.002 mm) (%)	34.3	
Soil Texture Class	CL (Clay Loam)	
pH	7.66	Soil Survey Staff 1951
Salt (%)	0.026	
$CaCO_3$ (g kg^{-1})	11.4	
Organic matter (g kg^{-1})	2.16	Nelson and Sommers 1996
Total N (%)	0.09	Bremner and Mulvaney 1982
Extractable P (mg kg^{-1} P_2O_5)	17.6	Olsen et al. 1954
Extractable K (mg kg^{-1} K_2O)	93.5	Richards 1954
Total Pb (mg kg^{-1})	3.92	EPA 1995
DTPA-Extractable Pb (mg kg^{-1})	0.29	Lindsay and Norvell 1978
DTPA-Extractable Cu (mg kg^{-1})	0.61	
DTPA-Extractable Fe (mg kg^{-1})	0.19	
DTPA-Extractable Mn (mg kg^{-1})	5.30	
DTPA-Extractable Zn (mg kg^{-1})	0.40	

Pot experiment

The pot experiments are very powerful and environmentally friendly way of testing metal accumulation abilities of plants in controlled growth conditions. Thus, the performance of a wild type tobacco and a transgenic one were tested in pots filled with two kg of soil. Each pot was amended with the basal fertilizer of 200 mg kg^{-1} N as NH_4NO_3 (this relatively high nitrogen was applied because of the N added from the $Pb(NO_3)_2$ treatments should be compensated to differentiate possible error variance related to Pb-Nitrate), 100 mg kg^{-1} P and 125 mg kg^{-1} K as KH_2PO_4, and 2.5 mg kg^{-1} Fe as FeEDTA.

Wild type tobacco seedlings were grown in peat-sand mixture (2:1, v/v) for 4 weeks, then transferred into the pots. An agar medium containing Murashige-Skoog salt (Sigma, Germany) supplemented with sucrose (2%), cefotaxime (200 µg mL^{-1}), glycin (0.4%), thiamine (0.4 µg mL^{-1}), pyridoxine (0.1%), nicotinic acid (0.1%) along with kanamycin (200 µg mL^{-1}) as the selecting agent were used for germination of transgenic tobacco plants and the first 4 weeks of growing stages of seedlings. The

seedlings were firstly transferred to the mixture of peat-sand for 2 weeks and then into the pots.

Transgenic and wild type tobacco seedlings were grown in soils amended with Pb 0, 1000, 2500, 5000 mg Pb kg^{-1} as $Pb(NO_3)_2$ up to the flowering stage for 6 weeks. Because of the widespread use of lead containing compounds such as paint, gasoline, batteries, and fertilizers; as well as the contamination from various industrial sources, urban soils often have lead concentrations well over the background levels. It has become apparent that extremely high exposures of lead (Pb) are common in some site specific soils. In this context soil Pb concentrations >10,000 mg kg^{-1} have been reported (Mellor and McCartney, 1994; Rooney and McLaren, 2000; Chen et al., 2001) due to Pb shoot deposition. On the other hand, lead is not biodegradable over time, but accumulates up in the time course. The excessive Pb exposures are generally buffered by precipitation of (hydroxy) carbonates, sulphates, phosphates in the soil environment at alkaline pH (Lindsay, 2001). Therefore the hazardous effect of Pb on plants can only be visual at relatively high loadings.

Each treatment was replicated three times. The plants were grown under controlled environmental conditions (6 Klux light intensity, 16 h light period, 25/20 ^oC light/dark temperature regime, and 60% relative humidity) in the growth chamber for 6 weeks.

Chlorophyll determination

The plant chlorophyll content of both young leaves and fully expanded old leaves was determined with a chlorophyll meter (Konica Minolta SPAD 502) as a SPAD unit before harvesting.

Harvest and plant analysis

The plants were cut one cm above the ground level at the end of 6 weeks of growth. Contaminants were eliminated by rinsing with de-ionized water. The plant samples were dried at 65 ^oC in oven for 48h to a constant weight. The dry plant samples were grinded in an agate mill (Retsch, RM 200). Then a scoop of 0.25 g sample was wet-ashed with 2 mL HNO_3 and 5 mL H_2O_2 mixture in a microwave oven (MarsXpress, CEM). Lead and total plant nutrient (Cu, Zn, Fe, Mn, P and K) concentrations of the digests were determined by the ICP-AES. Total N was determined using the Kjeldahl method (Jones et al., 1991). A certified reference material (Virginia tobacco leaves, CTA-VTL-2) was also used in order to test the accuracy and precision of the digesting and measuring procedures of nutrient concentrations.

Reduced glutathione (GSH) content of leaf samples was spectrometrically determined following the procedures of Cakmak and Marschner (1992). Glutathione standard series ranging 0-100 $\mu g\ mL^{-1}$ was used.

Statistical analysis

The experimental set-up was a completely randomised design with three replicates. The statistical analysis was performed using SAS computer software (SAS, 1997). Main effects of both Pb treatments and plant type were separated by the Least Significant Difference (LSD) test at $p< 0.05$.

RESULTS and DISCUSSION

To evaluate changes in Pb accumulation due to MTII overexpression, Pb uptake tests on the pots with the range of excess Pb concentrations (0, 1000, 2500 and 5000 mg kg^{-1}) were performed. Tobacco seedlings expressing MTII gene were not more tolerant to Pb than wild-type ones when tested in the excess Pb application**.** No necrotic lesions or chlorosis were evident on the leaves of both type plants. This result may indicate both plants can tolerate higher Pb doses or plant availability of Pb is very poor in calcareous soils due to precipitation reactions despite the very high Pb addition. Our results were similar with the results of Xiong et al. (2006). They investigated toxic effects of Pb on Chinese cabbage *(Brassica pekinensis* Rupr.) plants which are growing Pb contaminated soils. They reported that

the plants didn't show any toxicity symptoms with increasing Pb concentration in soil and plants.

Chlorophyll

The effect of Pb on young and old leaf chlorophyll content (in units SPAD) of transgenic and wild type plants were significant (Table 2). The chlorophyll contents of young leaves were higher than old leaves. The chlorophyll content showed a decreasing trend of increasing Pb exposure in both plants (Table 2). Our results were similar with the results of some researchers (Xiong et al., 2006; Akinci et al., 2010; Eren and Daghan, 2014). The lack of chlorophyll formation corresponding with excessive Pb exposure may be due to its role in the stress conditions (Sharma and Dubey, 2005).

Table 2. Effects of excess Pb application on chlorophyll content of old and young leaves, dry weight, Pb and GSH concentrations of transgenic and wild type tobacco

Cultivars	Doses (mg kg^{-1})	Old Leaf Chlorophyll (SPAD Unit)	Young Leaf Chlorophyll (SPAD Unit)	Dry Weight (g $plant^{-1}$)	Pb (mg kg^{-1})	GSH (µg g^{-1})
Transgenic Tobacco	0	39.57±0.06	51.53±1.00	15.27±0.45	0.18±0.015	977±99.0
	1000	39.90±0.66	44.27±1.36	10.59±0.66	40.47±7.28	1095±8.50
	2500	37.00±0.62	46.47±0.49	7.10±0.19	55.90±1.35	1025±47.0
	5000	32.37±0.80	38.50±1.45	3.46±0.04	76.00±10.50	989±10.4
Wild Type Tobacco	0	39.10±1.18	52.07±0.68	19.50±0.21	0.36±0.01	1098±49.0
	1000	41.97±1.04	52.40±1.13	16.22±1.07	36.17±4.14	1810±29.0
	2500	34.97±0.55	49.63±1.14	9.15±0.23	49.80±1.21	1419±141
	5000	33.60±1.47	45.50±0.66	3.77±0.28	70.90±6.67	1372±183
Probality of F [§]		**	*	*	n.s.	*

[§]: significance of interaction PbxCultivars *: ($p<0.05$) **: ($p<0.01$) n.s.: non-significant

The total chlorophyll content of both plant types increased up to 1000 mg L^{-1} of Pb treatment. The effect of the further increase in the Pb concentration was more detrimental in old leaves (Table 2). This suggests, up to a critical concentration, Pb may inhibit carbohydrate or nitrogen assimilation metabolisms and darker green colour may occur upon accumulation of some metabolites. However, the further increase may inhibit chlorophyll formation or mediate chlorophyll breakdown in the leaves. This finding is corresponding to the detrimental effect of excess Pb on chloroplast (Sharma and Dubey, 2005; Succuro, 2010; Gupta et al., 2011; Lamhamdi et al., 2013).

Dry Matter

The results of the dry matter amount of wild type and transgenic tobacco plants are presented in Table 2 and Fig. 2. In terms of dry biomass production, there were significant differences ($p<0.01$) between transgenic and wild type plants (Table 2). Wild type plants had higher dry biomass than transgenic plants. The highest dry weight (19.50 g $plant^{-1}$) was obtained in the wild type tobacco plants in the control treatment (0 mg Pb kg^{-1}) and the lowest dry weight (3.46 g $plant^{-1}$) was obtained in the transgenic tobacco plant at 5000 mg Pb kg^{-1} dose. Wild type and genetically engineered plants exposed to higher Pb (1000-5000 mg kg^{-1}) doses exhibited a significant decrease in dry weight compared to the control plants (Table 2).

The reduced dry weight with increasing Pb applications was similarly reported by Sahi et al. (2002) for *Sesbania drummondii*, Akinci et al. (2010) for tomato; Lamhamdi et al. (2013) for spinach and wheat; Gupta et al. (2008) for *Vigna mungo;* Xiong et al. (2006) for *Brassica pekinensis* Rupr.; and Eren and Daghan (2014) for transgenic and wild type tobacco. In contrast, Sahi et al. (2002) reported a tolerance up to 1500 mg L^{-1} for *Sesbania drummondii* and accumulation ~ 40 g kg^{-1} shoot dry weights.

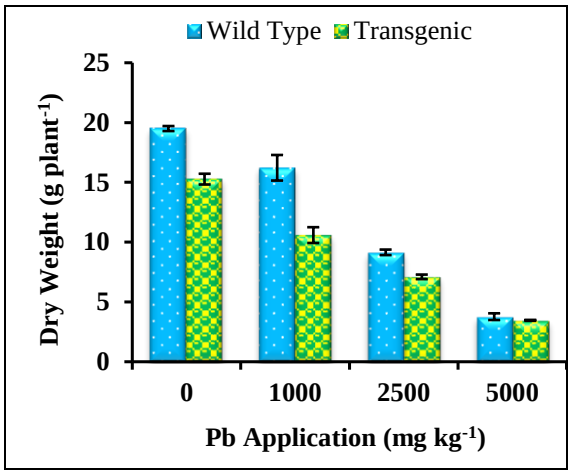

Fig. 2. The effect of Pb doses on shoot dry weights of transgenic and wild type tobacco plants. The results are the mean of three replicates ±Standard deviation (SD)

According to researchers either fresh or dry biomass decrease could be attributed to water balance disturbance, reduction in water uptake, Pb induced-mineral nutritional disorders, and reduction in photosynthesis and chlorophyll synthesis, soluble proteins and proline contents (Sharma and Dubey, 2005; Gupta et al., 2008; Lamhamdi et al., 2013).

The lead causes detriment of the chloroplast, limitation of photosynthesis and damaging cell membrane, inhibition of shoot and root growth, and therefore, it might decrease biomass production (Sharma and Dubey, 2005; Xiong et al., 2006; Gupta et al., 2011; Pourrut et al., 2011; Lamhamdi et al., 2013). The decrease in biomass along with chlorophyll content and mineral composition of leaves indicated that the outcome is rather very complex in excess Pb (Table 2).

Lee et al. (2005) reported that AtPDR12-overexpressing *Arabidopsis* plants showed less well growth performance and higher Pb contents than its respective natural form under excessive Pb (II) containing growth media. On the other hand, plants having AtPDR12 showed higher Pb resistance (II) and accumulated lower Pb than the wild-type plants. Despite the fact that a Pb concentration below 500 mg Pb kg^{-1} adversely affected *Rapistrum rugosum* and *Sinapis arvensis*, dry weight of *Rapistrum rugosum* and *Sinapsis arvensis* did not respond to Pb treatments (Abolghasem et al., 2016).

Pb uptake

Despite no significant difference, the transgenic tobacco plant accumulated more Pb than the wild type tobacco plant (Table 2, Fig. 3). The aboveground Pb concentration of both plants progressively increased due to increasing Pb treatments. The Pb concentration of the transgenic plants was always higher than the wild one (Fig.3). The maximum Pb concentrations were 76.0 and 70.9 mg Pb kg^{-1} for 5000 mg Pb kg^{-1} treatment.

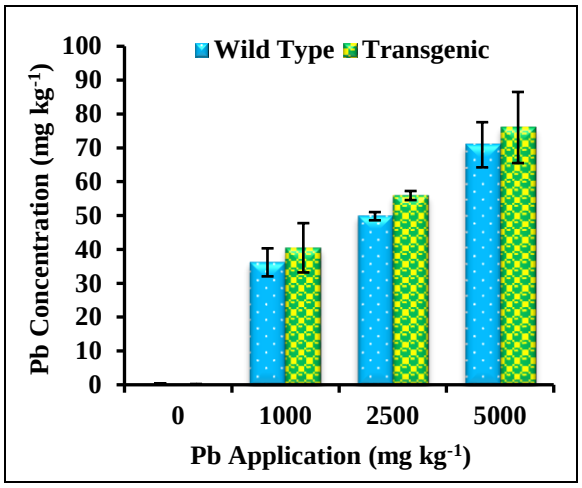

Fig. 3. The effect of Pb doses on shoot Pb concentrations of transgenic and wild type tobacco plants. The results are the mean of three replicates ± Standard deviation (SD)

Kabata-Pendias and Pendias (2011) indicated that the normal concentrations of Pb in plant tissues were between 0.1-10 mg kg^{-1} dry weight (DW) and toxic concentration levels of Pb were between 30-300 mg kg^{-1} DW. In the current study, the Pb concentrations in the shoot dry matter were in the toxic levels. The results of the present study are inconsistent with the findings regarding the transgenic plants and their corresponding wild-types (Arazi et al., 1999; Lee et al., 2005; Bhuiyan et al., 2011; Eren and Daghan, 2014; Ramesh et al., 2015; Du et al., 2015). Arazi et al. (1999) improved tobacco plant's Pb uptake and tolerance ability by introducing calmodulin-binding protein (NtCBP4) which enabled Pb to be transported through the membrane of calcium ion channels. On the other hand, transplanting a gene encoding phytochelatin synthase *(TaPCS1)* from wheat into shrub tobacco (*Nicotiana glauca* R. Graham) enhanced phytoremediative potential by improving both seedling root length and Pb accumulation potential compared to the wild-type (Gisbert et al., 2003). Similarly, the expression of the glutathione-Cd vacuolar transporter YCF-1 which mediates vascular compartmentalization of Cd and Pb from the cytoplasm in *Arabidopsis* moderately increased the Pb tolerance, and slightly improved the Pb accumulation (Song et al., 2003). The YCF-1 gene was successful to fortify the Pb tolerance and uptake up to 2.2 folds its corresponding wild type in *Brassica juncea* (Bhuiyan et al., 2011). These findings suggest that transgenic plants expressing YCF1 may have a potential for phytoremediation of Pb. In contrast, wild-type plants can also uptake and accumulate Pb. For example, Xiong et al. (2006) reported that Chinese cabbage can uptake 14.3, 202, and 418 mg Pb kg^{-1} (DW) in soils containing 0, 4, and 8 mmol Pb kg^{-1}, respectively. Therefore, it is necessary to better understand Pb uptake, translocation, accumulation, and hazard mechanisms of plants under excessive Pb exposure in order to select successful plants for Pb phytoextraction.

Reduced Glutathione (GSH)

There was a significant difference in the reduced glutathione (GSH) concentration between the transgenic and wild type tobacco plants ($p<0.001$). The GSH concentrations in the leaves of wild type were low, ranging from 977 to 1095 µg g^{-1}, whereas in the transgenic tobacco: 1098-1810 µg g^{-1} (Table 2). At 2500 mg Pb kg^{-1} application, the GSH concentration of both transgenic and wild type plants began to decrease. This tendency was more pronounced in plants grown at 5000 mg Pb kg^{-1} (Table 2). These results are in consonance with the observations of

Daghan and Köleli (2012) and Eren and Daghan (2014).

Glutathione, playing a multifaceted role in plant metabolism, is considered as highly important signal molecule strongly acting between stress factors and adaptive responses of the plants; consequently, the GSH system has been considered as an indicator in plant ecophysiological studies (Anjum et al., 2012). It is well known that glutathione accumulates under heavy metal exposure and it is considered as an indicator of stress resistance (Noctor and Foyer, 1998; Ahmad et al., 2010). Environmental stresses such as heavy metals activate the defence mechanism of plants (Yuan et al., 2015). However, the efficiency of GSH that is responsible for the detoxifying oxidative damage is plant specific (Ahmad et al., 2010). GSH, found in all compartments of the cell in reduced form, may be the most important intracellular defence mechanisms against ROS damages. The tripeptide (γ-GluCysGly) glutathione is one of the vital metabolites in the plants (Noctor and Foyer, 1998; Ahmad et al., 2010; Yuan et al., 2015). Elevated level of Pb also changes water balance, inhibits enzyme activities, alters membrane permeability and disturbs mineral nutrition (Sharma and Dubey, 2005). Lead toxicity, as for the other heavy metals inhibits the activity of enzymes at the cellular level by reacting with the sulfhydryl groups (Yadav, 2010) and heavy metal toxicity, including Pb, stimulates GR activity in plants (Verma and Dubey, 2003; Gupta et al., 2010; Huang et al., 2010). However, non-Pb accumulating ecotype exhibited a higher increase in shoot-GSH level compared to Pb-accumulating ecotype (Gupta et al., 2010). Transgenic plants bearing different genes may show higher metal accumulation and tolerance than their wild-types. Bennet et al. (2003) engineered three transgenic Indian mustards, named γ-ECS, (overexpressing the glutathione synthesizing enzymes γ-glutamylcysteine synthetase), GS (glutathione synthetase) and APS (overexpressing adenosine triphosphate sulfurylase) to enhance phytoremediation potential. All genetically engineered plants had 1.5-3.0 fold higher accumulation ability for Cd, Zn, Cr, Cu, and Pb. Yuan et al. (2015) investigated the effects of exogenous reduced glutathione (GSH), cysteine (Cys) and increasing Pb application (100 and 500 mg L^{-1} Pb) on *Iris lactea* var. Chinensis plant growth and lead (Pb) accumulation. They reported that shoot biomass was dramatically declined at 500 mg L^{-1} Pb application. On the other hand, the Pb accumulation of plant shoot and root was increased with the application of exogenous GSH and Cys.

Nutrient uptake under excessive Pb exposure

The effects of increasing Pb doses on macro (N, P and K) and micro (Cu, Fe, Mn and Zn) nutrient element concentrations of transgenic and wild type tobacco plants were presented in Table 3. However, N concentration of plants increased due possibly to Pb-induced inhibition of N assimilation or growth. As adequate amounts of nutrient element supplied to plant along with abiotic stress conditions such as heavy metals, excessive heat, water scarcity, salinity can result in accumulation effect. The other point could be related to relatively faster plant uptake of NO_3 than Pb due possibly to adsorption and/or precipitation reaction which may induce accumulation of N under increasing Pb pollution. The nitrogen content of roots is to decrease under Pb toxicity which may be related to limited nitrate uptake due to Pb induced moisture stress in the roots (Eren, 2010). In contrast, P uptake and translocation to the shoots were significantly hindered with increasing Pb treatments ($p<0.005$). This, in fact, can be related to insoluble precipitates of Pb with P in soils (Kabata-Pendias and Pendias, 2000). Potassium concentrations were slightly increased compared to control plants (0 mg Pb kg^{-1}). This was likely to be dependent on inhibition of dilution-effect of Pb induced-growth decline. Despite Cu concentrations showed a very narrow range 6.78-8.81 mg kg^{-1}, the changes occurred

with Pb treatments were significant ($p<0.01$). Iron (Fe), Mn and Zn concentrations did not change with the increasing Pb treatments (Table 3).

Table 3. Effects of excess Pb application on N, P, K, Cu, Fe, Mn, Zn concentration of transgenic and wild type tobacco

Cultivars	Doses (mg kg^{-1})	N	P	K	Cu	Fe	Mn	Zn
		(%)			(mg kg^{-1})			
Transgenic Tobacco	0	3.16±0.32	0.29±0.006	1.85±0.34	8.81±0.69	65.27±3.99	40.92±1.47	4.78±0.33
	1000	4.05±0.40	0.19±0.017	2.19±0.10	7.80±0.57	77.31±5.53	37.90±2.71	4.17±0.16
	2500	3.89±0.33	0.12±0.00	2.29±0.13	7.25±0.01	65.79±4.42	54.05±0.19	5.70±0.25
	5000	4.32±0.27	0.11±0.006	2.20±0.11	6.78±0.53	67.60±9.85	65.13±4.49	7.95±0.45
Wild Type Tobacco	0	2.49±0.09	0.26±0.006	1.63±0.18	6.95±0.45	62.03±3.11	34.93±1.54	5.22±0.69
	1000	3.39±0.34	0.18±0.01	1.91±0.17	7.77±0.23	58.52±3.53	35.42±1.22	4.48±0.65
	2500	3.77±.035	0.11±0.00	2.03±0.13	7.69±1.22	54.74±4.64	46.66±0.41	4.83±0.18
	5000	4.04±0.27	0.10±0.00	2.02±0.16	8.63±0.52	52.21±2.58	62.53±2.16	8.34±0.50
Probality of F §		n.s	**	n.s.	*	n.s.	n.s.	n.s.

§: significance of interaction PbxCultivars *: ($p<0.05$) **: ($p<0.01$) n.s.: non-significant

The high concentration of Pb in soil can cause an imbalance of plant mineral nutrition. Many researchers have shown that mineral nutritional imbalances in the cell occur because of excessive Pb (Kabata-Pendias and Pendias, 2000; Sharma and Dubey, 2005). Kabata-Pendias and Pendias (2000) reported mineral nutrition content and ratio were significantly changed under Pb toxicity. An excessive level of Pb is firstly damaging plant roots and mineral nutrients could not uptake by roots. After that, all physiological (photosynthesis, enzyme activity, nutrient uptake, etc.) and morphological (reduce growth, toxicity/deficiency symptoms, etc.) disorders become apparent in plants (Sharma and Dubey, 2005; Succuro, 2010; Pourrut et al., 2011; Lamhamdi et al., 2013). In many cases, Pb inhibits some cations (K^+, Ca^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Cu^{+2}, and Fe^{+2}) and anions to enter in root systems (Sharma and Dubey, 2005; Pourrut et al., 2011). When plants are exposed to Pb, some of the cationic nutrient elements (K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+}) are physically blocked by Pb through competition, reducing or even eliminating their absorption (Godbold and Kettner, 1991). Similarly, Walker et al. (1997) pointed out the excessive Pb related reduction in uptake of K, Ca, Mg, Fe and NO_3^- in *Cucumis sativus* seedlings and Ca, Mg, K, P uptake in maize. An inverse relation between Pb and other nutrient element concentrations were also reported for transgenic and wild type tobacco plants (Eren, 2010) and for spinach and wheat in hydroponic culture (Lamhamdi et al., 2013). On the other hand, mineral deficiencies well corresponded with chlorophylls a and b and proline contents (Lamhamdi et al., 2013). It was previously demonstrated that Pb stress causes a detrimental reduction in chlorophyll synthesis with disorganization and decreases in the magnitude of thylakoids and grana, as well as the stereo structure of chlorophyll-induced by replacement of Pb for key nutrients (Sengar and Pandey, 1996; Haider et al., 2006; Akinci et al., 2010). Lead can also influence the translocation, partition and allocation of nutrient elements among different plant organs. Manganese and S partition between shoots and roots can largely change under Pb toxicity (Eren, 2010).

CONCLUSION

Transgenic (p-cV-ChMTII-GFP) and wild type (*Nicotiana tabacum* L. cv. SR-I) tobacco plants were compared with regards to Pb accumulation. Transgenic plants didn't demonstrate higher Pb accumulation compared with wild type plants. Despite no visual necrotic symptoms were evident on the leaves, the growth performances of both plant types were reduced upon excessive Pb

exposure. The shoot Pb concentration even below 70 mg kg^{-1} had significant disturbances in nutrient uptake by plants which resulted in profound metabolic changes (e.g. in photosynthetic capacity) and consequently a strong inhibition of plant growth. The most detrimental decrease in nutrient uptake was observed for P, meanwhile, some macro and micro nutrients were also negatively affected by excessive Pb treatments. Shoot Pb concentration was slightly higher in the transgenic plant than the wild type, but this progress was not promising enough. The translocation of Pb from the soil/root into shoots remained insufficient even at 5000 mg kg^{-1} soil Pb concentration. This might be an indication of soil induced low Pb availability of the plant due to precipitation reactions of Pb with carbonates and phosphates or inhibited translocation from roots to shoots.

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