Evaluation of Zinc Accumulation Ability of Transgenic and Non Transgenic Tobacco

Hatice Daghan
Mustafa Kemal University, Faculty of Agriculture,
Department of Soil Science, 31000 Hatay, Turkey
hdaghan@mku.edu.tr

Mehmet Arslan
Mustafa Kemal University, Faculty of Agriculture,
Department of Field Crop, 31000 Hatay, Turkey
marslan@mku.edu.tr

Nurcan Koleli
Mersin University, Faculty of Engineering,
Department of Environmental Engineering, 33342 Mersin, Turkey
nkoleli@mersin.edu.tr

Veli Uygur
Mustafa Kemal University, Faculty of Agriculture,
Department of Soil Science, 31000 Hatay, Turkey
vuygur@mku.edu.tr

Abdullah Eren
Mustafa Kemal University, Faculty of Agriculture,
Department of Soil Science, 31000 Hatay, Turkey
aeren@mku.edu.tr

Absract: The T2 generations of the ScMTII gene bearing transgenic and non-transgenic tobacco plants were grown on the nutrient medium with the addition of 0, 5 and 10 mg L^{-1} Zn to evaluate Zn accumulation capacity. Dry mass of shoot and roots, Zn concentration and Zn content were determined. In addition to Zn, glutathion (SH groups) concentrations, N, P, K, Cu, Fe and Mn of shoots and roots were determined. After growth for 15 days on media containing 10 mg L^{-1} Zn, non-transgenic plant showed slight chlorosis symptoms, including significant reduction in growth and chlorophyll. Transgenic and non-transgenic tobacco plants have been tested in terms of phytoremediator for Zn accumulations. The highest Zn concentration in shoots and also roots was tested in all Zn supply. The results showed that Zn accumulation ability of transgenic tobacco plant was higher than non-transgenic tobacco plants and Zn accumulation in the transgenic and the non transgenic tobacco plants were lower in the shoots compared to the roots. The Zn concentration in shoot of transgenic plant was increased by approximately 1.4 times compared to the non transgenic tobacco plants. Higher amount of Zn concentration in the shoot is a good indicator as a phytoremediation agent. The results showed that p-S-ScMTII gene bearing transgenic tobacco plant is not suitable for phytoextraction either detected lower amount of Zn concentration (<10000 mg Zn kg^{-1} DM) in the shoot or the translocation factor value was less than one.

Introduction

Heavy metal contamination of soils is a common problem in the world (Finzgar and Lestan, 2007). Many heavy metal cations such as Cu^{2+}, Zn^{2+}, Mn^{2+}, etc., are essential plant micronutrients, but when present in excess, these and non-essential metals, such as Cd^{2+}, Hg^{2+} or Pb^{2+}, can become extremely toxic

(Macek et al., 2002). Heavy metals, released to the environment from anthropogenic and natural sources, tend to persist in soils, sediments and water and are difficult to remove. Heavy metals in soils frequently remain in the upper horizons, causing adverse effects on soil microbial activities and crop productivity, with the added risk of contamination of the food chain. Remediation of contaminated soils is essential for sustainable soil use. Remediation technologies for contaminated soils can be grouped into three general categories: (1) extraction/removal, (2) destruction after separation, and (3) *in situ* processes. For third process, several technologies can be employed to clean up the soils contaminated by heavy metals, this process including thermal, biological, and physical/chemical procedures, or their appropriate combinations. These technologies usually require the removal of contaminants from contaminated soils, such as phytoremediation. Phytoremediation is defined as the use of green plants to remove pollutants from the environment or to render them harmless (Raskin et al., 1997). Phytoremediation technologies include phytovolatilzation, phytostabilisation and phytoextraction (Vangronsveld *et al*., 2000; Garbisu and Alkorta, 2001). The terms phytoremediation and phytoextraction are sometimes incorrectly used as synonyms, but phytoremediation is a concept while phytoextraction is a specific cleanup technology (Kumar et al. 1995). Phytoextraction is the most commonly recognized of all phytoremediation technologies, and is the focus of the research proposed in this prospectus. Phytoextraction is the most acceptable and applied phytoremediation technique that can successfully remove heavy metals from soils (Cunningham *et al*., 1995; Dushenkov *et al*., 1997; Ebbs *et al*., 1998; Huang *et al*., 1998). The removal of heavy metals from soil by plants, resulting in metal uptake, transport and concentration in plant tissues, is described as phytoextraction. These plants known as metal hyperaccumulators, frequently endemic to metalliferous soils with the capacity to accumulate unusually large concentrations of metals in their aboveground parts (shoots), are potentially most effective for this strategy (Baker and Brooks, 1989). Metal hyperaccumulator plants comprise species that accumulate (in mg/kg)>10000 (Mn or Zn), >1000 (Cu, Co, Cr, Ni, Pb) or >100 (Cd) in their shoots (Baker and Brooks, 1989; Wenzel and Jokwer, 1999). Of the over 450 plant species which have been identified as hyperaccumulators, 75% have been Ni hyperaccumulators (Clemens, 2001). Cropping metal-contaminated soils with species of these plants has demonstrated the potential of this technique as a low cost, low technology alternative to physical and chemical methods of soil remediation (McGrath *et al*., 1993), although it is limited by the rarity, slow growth rates and low biomass production of many hyperaccumulator species. Two most important characters include the ability to accumulate large quantities of biomass rapidly and the ability to accumulate large quantities of environmentally important metals in the shoot tissue (Kumar et al. 1995; Cunningham and Ow, 1996; Blaylock et al. 1997; McGrath, 1998). It is the combination of high metal accumulation and high biomass production that results in the most metal removal. In practice, metal accumulating plants are seeded or transplanted into metal contaminated soil and are cultivated using established agricultural practices. The roots of established plants absorb metal elements from the soil and translocate them to the above-ground shoots where they accumulate. If metal availability in the soil is not adequate for sufficient plant uptake, chelates or acidifying agents may be used to liberate them into the soil solution (Huang and Cunningham, 1996; Huang et al. 1997; Lasat et al. 1998). First, hyperaccumulator plants are usually specific for one particular metal (Baker and Brooks, 1989), and are adapted to precise climate and soil conditions (not really transferable). Furthermore, they cannot be managed as a conventional crop, have low biomass, and often a short life cycle. Therefore it seems more reasonable to search for non hyperaccumulator plants showing good features for phytoremediation and then transfer biotechnologically traits that make the modified plant even a more powerful tool than natural hyperaccumulators. Hence, the aim of the work reported in this paper was to engineer increased heavy metal absorption in a screen selected wild type plant species.

In recent years, genetic engineering is a technique that might be applied advantageously to the search for more suitable phytoremediation plants combining high metal accumulating capacity and high aboveground biomass yield (Kärenlampi et al. 2000). With genetic engineering, plants can be manipulated to accumulate, translocate and tolerate heavy metals, thus creating the ideal transgenic plant for environmental clean up in the shortest possible time (Pilon-Smits, 2005; Bennett, 2003; Persans *et al*., 2001). For instance, genes can be isolated from metal hyperaccumulators and inserted into fast growing high biomass plant species (Persans *et al*. 2001). It has been suggested that phytoextraction would become commercially available if metal removal and tolerance properties of hyperaccumulator plants, such as *Thlapsi caerulescens* (Brown *et al*., 1995; Bennett, 2003) or *Pteris vittata* (Ma *et al*., 2001), could be transferred into fast growing, high biomass producing crop species. The introduction of an additional metal binding domain to the implemented protein should further enhance the metal binding capacity (Macek et al. 1996, Kotrba et al. 1999). The goal of genetic modification is to develop fast growing, high biomass plants

with the metal accumulation traits of natural small biomass hyperaccumulators: 'engineered phytoremediators' (Ow, 1996). The advantage of this technique is the relatively short space of time and selective targeting of genes for improvement. Most recently, Cd accumulation was enhanced when a metallothionein gene from *Silene vulgaris* L. was over-expressed in tobacco (*Nicotiana tabacum* L.) (Gorinova *et al.,* 2006).

The main objective of this study was to evaluate Zn accumulation ability of transgenic (p-S-ScMTII) tobacco (*Nicotiana tobaccum*) cultivar Petit Havana and the non-transgenic tobacco cultivar Petit Havana (SR-1) grown on the nutrient medium with the addition of 0, 5 and 10 mg L^{-1} Zn. In addition to Zn accumulation, chlorophyll, glutathion (SH groups), N, P, Cu, Fe and Mn concentrations were also measured.

Material and Methods

Seeds of transgenic (p-S-ScMTII gene bearing) and non-transgenic tobacco (*Nicotiana tabaccum*) cultivar Petit Havana (SR-1) were obtained from RWTH-Aachen Molecular Biology Department. Seeds were germinated initially on Murashige and Skoog plates containing antibiotic (kanamycine), then transferred to a perlit and torf (1:1) mixture since having 2-3 leaves (after approximately 4 weeks). Then the seedlings were transferred into the pots included Hoagland nutrient solutions and were grown under controlled environmental conditions with a 16 h light/8 h dark period (light intensity of 10 klux or 120 μmol $m^{-2}s^{-1}$), a 25/20 ^{o}C temperature regime, and 60% relative humidity. Hoagland nutrient solutions consist of 3 mM KNO_3, 2 mM $Ca(NO_3)_2.4H_2O$, 0.25 mM $MgSO_4.7H_2O$, 1 mM KH_2PO_4, 0.001 mM $MnSO_4.H_2O$, 0.1 mM FeEDTA, 0.00025 mM $CuSO_4.H_2O$, 0.00025 mM $(NH_4)_6Mo_7O_{24}$, 0.001 mM $ZnSO_4.7H_2O$, 2.5 mM KCl, 0.0125 mM H_3BO_3; the medium buffered to pH 5.2. One in every 3 days the plants were transferred to fresh medium. Plants were treated with 0, 5 and 10 mg L^{-1} Zn as $ZnSO_4.7H_2O$. Zinc was added to the nutrient medium in doses of 0 (the control), 5 and 10 mg L^{-1} Zn. This Zn dosage was addition to initial Zn concentration (0.288 mg L^{-1}) in nutrient solution. The plants were harvested after 15 days of growth. Each treatment was replicated three times for each plant.

Before harvest, plants were assessed for the severity of leaf symptoms caused by Zn toxicity. In addition, chlorophyll in old and young leaves was measured using a Konica-Minolta SPAD-502 at the harvest. After harvest, the leaves and roots were separated. The roots were thoroughly washed with deionized water. For SH analysis in fresh tissues, approximately 0.5 g fresh mass (FM) of each organ was separately sampled three times, immediately frozen in liquid nitrogen and stored at -80 ^{o}C until analysis. In addition, fresh mass of all plant parts was weighted (data not shown).

The remaining tissues were oven-dried at 70 °C for determination of dry matter amount and then plant material was decomposed by microwave (MarsXpress) and the heavy metal concentration was determined by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry, Varian Series II). Dried root and shoot samples were ground and digested in 2 mL 30% H_2O_2 and 5 mL 65% HNO_3 in sealed vessels of a microwave apparatus. Cadmium, Zn, Fe and Cu were measured by ICP-AES. All sampling and measurements were carried out by using three independent replications. The accuracy of analyses was verified using blanks and the Virginia Tobacco Leaves (CTA-VTL-2) reference material.

Total N was determined using Kjeldahl metod, total P was determined also using blue color method and total K was analyzed also using method (Kacar, 1995). Phosphorus, K, Fe, Mn, Cd and Cu concentrations were also measured by ICP-AES.

SH-group content was determined using 5-5'-dithiobis (DTNB) under 5% *meta*-phosphoric acid as a reagent as described in Cakmak and Marschner (1992). Accordingly, 0.5 g fresh plant sample was homogenized in 5% meta-phosphoric acid and centrifuged at 4000 mg kg^{-1}. The reaction mixture contained 0.5 mL aliquot of the supernatant, 2.5 mL 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and EDTA 0.5 mL 6mM dithiobisnitrobenzoic acid (DTNB). After incubation at room temperature for 20 min reaction time, the color produced was measured at 412 nm using a Hitachi U-2000 Spectrophotometer. Reduced glutathione was used as a standard in the range of 0 to 100 μg L^{-1}. All measurements were carried out in triplicate.

The results of the experiments were analyzed statistically using Statistical Analysis System (SAS Institute, 1996). Comparisons between means were carried out using the Least Significant Difference (LSD) test at the significance level of $p < 0.05$.

Findings

After Zn application, visual symptoms were reported through the experiment. Both non-transgenic and transgenic plants (SR-1 and p-S-ScMTII) did not show any typically symptom for Zn toxicity, such as necrosis on oldest leaf, especially on the leaf parts close to stem (data not shown). But non-transgenic tobacco plant showed slight chlorosis at 10 mg L^{-1} Zn supply (Fig. 1). Increased Zn application was obviously decreased the shoot and root growth of both transgenic and non-transgenic tobacco (Fig. 1). After harvest of the tobacco plants the dry mass production of the shoots and roots were determined and is shown in Tab. 1. The dry mass of transgenic and non-transgenic tobacco plants decreased with the increasing concentration of Zn supply in the nutrient solution (Tab. 1) as shown also Fig.1. The highest shoot and root dry mass were obtained from the p-S-ScMTII gene bearing plants. The dry mass of transgenic and non transgenic tobacco plants were not significantly different (Tab. 1).

Figure 1: Effect of increasing Zn supply on shoots and roots growth of transgenic (p-S-ScMTII) and non-transgenic tobacco (SR 1) (*Nicotiana tabaccum* Petit Havana) grown in hydroponic nutrient solution for 15 days before harvest.

		Dry Weight		Zn Concentration		Zn Content		
Tested Tobacco Plant	Zn Supply	Shoot	Root	Shoot	Root	Shoot	Root	TF**
	(mg L^{-1})	(g $plant^{-1}$)		(mg kg^{-1} DM)		(µg $plant^{-1}$ DM)		
P-S-SCMTII	0	3.56	0.41	32	116	112	49	0.28
	5	3.78	0.48	784	3168	3009	1543	0.25
	10	2.12	0.09	1326	8145	2780	794	0.16
SR-1	0	3.46	0.35	31	70	106	25	0.44
	5	3.26	0.43	578	2215	1885	970	0.26
	10	1.86	0.06	980	7402	1846	488	0.13
LSD ($p<0.05$)		n.s.*	n.s.	86	306	560	232	

*n.s.: not significant
**TF: translocation factor

Table 1: Effect of increasing Zn supply on shoot and root dry weight, Zn concentration, Zn content and translocation factor of transgenic (p-S-ScMTII) and non-transgenic tobacco (SR 1) grown in hydroponic nutrient solution for 15 days.

The highest level of Zn concentration in the shoots and roots was detected in the transgenic tobacco plants. The concentration of Zn in the roots was higher than in the shoots in both transgenic and non-transgenic plants (Tab. 1). Compared to the control plants, increased Zn dosage resulted in higher Zn concentrations in plant shoots and roots grown in both transgenic and non-transgenic plants. However, the Zn concentrations were higher in the transgenic plants. Our results are in agreement with Paplikova et al. (2004). While the shoots of transgenic tobacco plant accumulated similar Zn concentration (31-32 mg L^{-1} Zn) with the control plant (SR-1) at 0 mg L^{-1} Zn supply, at 5 and 10 mg L^{-1} Zn supply accumulated approximately 1.4 times higher Zn. The highest Zn accumulation observed in roots was in transgenic tobacco plants at 10 mg L^{-1} Zn supply (8145 mg kg^{-1} DM), which was 1.1 times the concentration of the control plant. The highest Zn accumulation in shoots was observed at 10 µM Zn supply (1326 mg kg^{-1} DM), which was 1.4 times the concentration of the control plant. Hyperaccumulation implies concentrations in dry matter above 1% for Zn, i.e. 10000 mg L^{-1} (Reeves and Baker, 2000). Transgenic tobacco plant grown on 10 µM Zn supply can be not evaluated as hyperaccumulator. The distribution of the accumulated metals within the plant is important, for phytoremediation, especially for the rate of the translocation into the harvestable parts (Macek et al. 2002). In fact, to evaluate the potential of plants for phytoextraction the translocation factor (TP) was used. This ratio is an indication of the ability of the plant to translocate metals from the roots to the shoots of the plant (Marchiol et al., 2004). Translocation factor is calculated by the ratio of root metal concentration to shoot metal concentration. Metals that are accumulated by plants and largely stored in the roots of plants are indicated by the translocation factor values < 1 with values > 1 indicating that the metals are stored in the shoots. As this evaluation, the translocation factor values of tobacco plants were < 1 and these values value verified that the Zn is stored in the roots. The results showed that p-S-ScMTII gene bearing transgenic tobacco plant is not suitable for phytoextraction either detected lower amount of Zn concentration (<10000 mg Zn kg^{-1} DM) in the shoot or the translocation factor value was less than one.

Zinc content was calculated as dry mass x Zn concentration. Similarly, the highest level of Zn content in the shoots and roots was detected in the transgenic tobacco plants. The content of Zn in the roots was higher than in the shoots in both transgenic and non-transgenic plants (Tab. 1). In shoot and root, the total content of Zn (µg $plant^{-1}$ DM) (Tab. 1) in transgenic tobacco was larger than in non-transgenic tobacco plants. However, Zn content in shoot was higher in transgenic tobacco at 5 mg L^{-1}. Since Zn concentration at 10 mg L^{-1} Zn supply was the highest but dry mass production was the lowest. This indicated that transgenic tobacco was tolerant to 5 mg L^{-1} Zn supply. Content of dry matter of transgenic plant was not significantly different compared to non-transgenic tobacco (Tab. 1).

The concentrations of Cu, Fe, Mn in the shoots were lower than in roots in both transgenic and non-transgenic plants. The concentrations of Cu, Fe, Mn in the shoots were higher in non-transgenic tobacco plant than the transgenic tobacco plant. But this difference was not statistically significant (Tab. 2).

		Cu Concentration		Fe Concentration		Mn Concentration	
Tested Tobacco Plant	Zn supply	Shoot	Root	Shoot	Root	Shoot	Root
	(mg L^{-1})	(mg kg^{-1} DM)		(mg kg^{-1} DM)		(mg kg^{-1} DM)	
P-S-SCMTII	0	28	174	81	18265	40	209
	5	27	310	77	18900	46	53
	10	19	400	32	20322	15	38
SR-1	0	31	101	87	1900	42	440
	5	34	181	85	10126	56	147
	10	24	127	41	3752	24	561
LSD ($p<0.05$)		2.77	49	n.s.	2439	6.63	61

*n.s.: not signifigant

Table 2: Effect of increasing Zn supply on Cu, Fe and Zn concentration of transgenic (p-S-ScMTII) and non-ransgenic tobacco (SR 1) grown in hydroponic nurtient solution for 15 days.

		N		P		K	
Tested Tobacco Plant	Zn supply	Shoot	Root	Shoot	Root	Shoot	Root
	(mg L^{-1})	(%)		(%)		(%)	
P-S-SCMTII	0	4.65	n.d.**	0.59	21.07	7.54	4.11
	5	4.50	n.d.	0.59	1.28	8.01	4.13
	10	2.80	n.d.	0.44	3.00	5.90	2.47
SR-1	0	4.66	n.d.	0.59	18.42	8.30	3.94
	5	4.65	n.d.	0.59	1.25	8.08	4.12
	10	2.31	n.d.	0.41	3.13	5.56	2.77
LSD ($p<0.05$)		n.s.*	n.s.	n.s.	n.s.	n.s.	n.s.

*n.s.: not signifigant

**n.d.: not determined

Table 3: Effect of increasing Zn supply on shoot and root N, P and K of transgenic (p-S-ScMTII) and non-transgenic tobacco (SR 1) grown in hydroponic nutrient solution for 15 days.

Effect of increasing Zn supply on shoot and root N, P and K of transgenic (p-S-ScMTII) and non-transgenic tobacco (SR 1) grown in hydroponic nutrient solution for 15 days shown in Table 3. But these differences for N, P and K are not statistically significant (Tab. 3).

Plants have their own systems for binding heavy metals, mostly based on phytochelatins (Macek et. al., 2002). As a measurement of phytochelatins was determined spectophotometrically total glutathione (SH) in the fresh roots and shoots tissue of tobacco plants. Gluthation content of non-transgenic plant was higher in shoot at especially at 5 and 10 mg L^{-1} Zn application. But SH content of non-transgenic plant was not statistically significantly different compared to transgenic plant (Tab. 4).

The contents of chlorophyll on old and young leaf were severely reduced by the Zn applications relative to 10 mg L^{-1} Zn supply (Tab. 4), especially with the 10 mg Zn L^{-1} application. This value verified visual chlorysis symptom. Similar results in chlorophyll were obtained for each of the two tobacco plants.

		SH		Chlorophyll	
Tested Tobacco Plant	Zn Supply	Shoot	Root	Old Leaf	Young Leaf
	(mg L^{-1})	(mg kg^{-1} FM)		(SPAD)	
P-S-SCMTII	0	343	28	37	34
	5	136	25	37	31
	10	53	104	30	26
SR-1	0	269	25	39	35
	5	585	30	39	35
	10	462	71	32	24
LSD ($p<0.05$)		n.s.*	n.s.	2	n.s.

*n.s.: not signifigant

Table 4: Effect of increasing Zn supply on shoot and root SH and chlorophyll (on old and young leaf) of transgenic (p-S-ScMTII) and non-transgenic tobacco (SR 1) grown in hydroponic nutrient solution for 15 days.

Conclusion

The remediation of contaminated soil is necessary to preserve the soil resource (Li et al., 2005). There is an urgent requirement for develop of new techniques which are cheaper and more effective to remediate the contaminated soils. Using genetically modified plants is a new technique and has been introduced to remediate metal-contaminated soils. In the present study, the transgenic tobacco showed higher tolerance to Zn and Zn accumulation more than the non-transgenic tobacco plant. The ScMTII gene bearing transgenic tobacco showed not the possibility of its utilization for Zn phytoremediation. Phytoextraction is an environmentally sound method for cleaning up sites that are contaminated with heavy metals. However, the method has been questioned because it produces a biomass-rich secondary hazardous waste containing the extracted metals. Therefore, further treatment of this biomass is environmentally necessary.

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