Asian Journal of Chemistry Vol. 19, No. 3 (2007), 2161-2168

In vitro Phytoextraction Capacity of Blackberry for Copper and Zinc

UMRAN ERTURK, CEMILE YERLIKAYA† and NURAY SIVRITEPE*
Department of Horticulture, Faculty of Agriculture, Uludag University
Görükle Campus, Bursa-16059, Turkey
Fax (90)(224)4429098; Tel. (90)(224)4428970; E-mail: nuray@uludag.edu.tr

In vitro propagated three-node shoots of blackberry *cv*. Chester Thornless were subjected to different concentrations of $CuSO_4.5H_2O$ (control, 50, 100 and 150 ppm) and $ZnSO_4.7H_2O$ (control, 50, 100, 200 ppm) in ¼ strength MS medium, supplemented with 0.5 mg L^{-1} BAP, 30 g L^{-1} sucrose and 7 g L^{-1} agar, for 4 weeks. Excess levels of Cu on the growing media reduced dry biomass production of explants. In contrast to Cu treatments, dry biomass production was enhanced increasing Zn level up to 50 ppm in the growth medium. Moreover, relative growth rates of explants were not modified by 100 and 200 ppm Zn treatments. The relative chlorophyll content of leaves tended to decrease in blackberry with increasing supply of Cu and Zn. Thus, the predominant symptom appeared in metal treated explants was chlorosis. Significant changes were observed in mineral composition of the explants, however, concentrations were generally above mineral deficiency levels. The above-ground biomass of the explants exposed to different levels of Cu accumulated 498.5-1726.1 mg kg^{-1} in dry matter Cu and the levels were about 54-186 times higher than those of the control. Similarly, Zn content in above-ground biomass of explants treated with 50, 100 and 200 ppm Zn increased almost 10, 14 and 18 times compared with those of the non-treated ones. Based on these preliminary results, the possible usefulness of blackberry for phytoremediation technologies is discussed.

Key Words: Chlorophyll, Growth, Injury, Metal accumulation, Mineral Composition, Phytoremediation, *Rubus spp*.

INTRODUCTION

Heavy metal level of the biosphere has accelerated rapidly since the onset of the industrial revolution and heavy metal toxicity poses major environmental problems. The direct effects will be the loss of cultivated land, forest or grazing land and the overall loss of production. The indirect effects will include air and water pollution and siltation of rivers. These will eventually lead to the loss of biodiversity, amenity and economic wealth[1,2].

†Chemical Engineering Department, Istanbul Technical University, Istanbul-34465, Turkey.

Unlike organic pollutants, metals are not biodegradable and they are generally not mobile, therefore, their retaining time in the soil is thousands of years. Currently used clean-up strategies involve civil engineering practices (capping, excavation, soil washing and thermal treatment), which are expensive and destructive (destroying soil structure, fertility and productivity). Thus, the development of alternative, economically attractive, remediation technologies is imperative. The use of plant-based technologies (phytoremediation) have received much attention and are now considered to be promising alternatives to conventional techniques for diffuse or moderately contaminated soils[3].

Phytoremediation is a new area of research and is defined as the use of plants to remove or transform environmental contaminants. Phytoextraction, a subcategory of phytoremediation, is the practice of using plants to sequester metals from soil in harvestable plant material[4]. To date, at least 45 families have been identified to contain metal-accumulating species and more than 400 plant species of metal hyperaccumulators have been reported[5]. By definition, most hyperaccumulators are weeds, in that they reproduce rapidly, grow under conditions of low fertility and are adapted to a wide range of environmental (soil and climate) conditions. Thus, one of the most immediate concerns related to phytoextraction is the potential escape of hyperaccumulators from the site of remediation and the possibility that these plants will become environmental weeds[6]. On the other hand, most hyperaccumulators will not be used commercially where they evolved, thus import into other areas will be necessary. However, importing of a nonindigenous species into many countries can be a problem[6]. The ideal plant species to remediate a heavy metal contaminated site would be a rapidly growing, high biomass crop with an extensive root system that can tolerate and/or accumulate the contaminants of interest[3,7].

The use of metallophytes with lower shoot metal concentrations but higher biomass production has been proposed in order to overcome the limitations of hyperaccumulators[3]. Fast growing trees (*Salix spp, Populus*) and high biomass producing crops (*Brassica spp, Helianthus annuus, Spinacea olerecea*) have also been shown to have potential uses in phytoextraction, since higher biomass yields can compensate for a lower metal accumulation[3,8-10]. Therefore, a judicious election of plant species to be used in remediating metal contaminated soils must be made so that the effectiveness of phytoextraction can be maximized[7].

Blackberry as a horticultural crop is extremely tolerant of site and soil conditions. It is an easily propagated, fast growing and high biomass crop with an extensive root system. The ability of blackberry to re-sprout after pruning of above-ground biomass makes it an attractive crop for phytoextraction purposes.

Recently, a number of researches have been focused for screening and/or selection for metal tolerance in plants as well as developing metal stress-tolerant cell lines using *in vitro* techniques[11-14]. However, there are few reports on assessing *in vitro* phytoextraction capacity of plants[15,16]. Therefore here, we report on *in vitro* phytoextraction capacity of blackberry in the presence of high Cu and Zn in culture media as well as their characterization on the basis of dry biomass production, visual toxic response, chlorophyll content and other essential elements.

EXPERIMENTAL

The blackberry *cv*. Chester Thornless was propagated *in vitro* by the use of axillary buds on MS (M-5519, Sigma Chemical Co.) medium supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar. The media pH adjusted to 5.8 just before autoclaving at 121ºC for 20 min. The explants were proliferated on the above medium containing 1.0 mg L^{-1} 6-benzylaminopurine, 0.1 mg L^{-1} indole-3 butyric acid and 0.1 mg L^{-1} gibberellic acid. The proliferated cultures were subcultured at 4-week intervals and all cultures were maintained in a climatic chamber at 24ºC, under 51 µmol m^{-2} s^{-1} PAR of light intensity with a 16/8 h light/dark photoperiod.

Cu and Zn treatments

At the end of the third subculture, three-node shoots were excised from proliferating cultures and subjected to different concentrations of $CuSO_4.5H_2O$ (control, 50, 100 and 150 ppm) and $ZnSO_4.7H_2O$ (control, 50, 100, 200 ppm) in ¼ strength MS medium, supplemented with 0.5 mg L^{-1} 6-benzylaminopurine, 30 g L^{-1} sucrose and 7 g L^{-1} agar, for four weeks. The concentrations of $CuSO_4$ and $ZnSO_4$ in the control media were 0.006 and 2.15 mg L^{-1}, respectively.

After 4 weeks, the explants were collected and washed with distilled water for 2 min to remove medium, dried on filter paper and evaluated for their response to excess levels of Cu and Zn in the growth media.

Measurements

The growth response of blackberry *cv*. Chester Thornless to Cu and Zn treatments was measured in terms of dry biomass production. Dry biomass of explants was determined following heating at 70ºC for 24 h. Data are expressed as the percentage of control at each level of Cu and Zn treatments.

The relative chlorophyll content was measured with a portable leaf chlorophyll meter (SPAD 502, Minolta Co. Ltd., Japan) and the data represented were the means of one reading from each explant of each replicate.

Explants were also scored for visible symptoms of Cu and Zn toxicity on a 1-4 scale as follows: (1) no injury; (2) damage on leaf tips and margins; (3) necroses on the whole leaf; (4) dead. Following this, injury index was calculated according to the following formula:

Injury Index = $\sum$ (ni × i)/N, where ni is the number of explants receiving the mark 'i' (from 1 to 4) and N is the total number of explants in each metal concentration.

To determine the mineral composition, 0.3-0.4 g of previously dried, ground and homogenized plant material (shoot + leaves) was placed in a platinum crucible. The crucible was covered during the dry ashing as a precaution against contamination. Dry ashing was accomplished in a furnace at 550°C for 6 h. After 10 min cooling period, the ash was dissolved in 2 mL HNO_3 (65%) and heated on a hot plate for 5 min. The digested material was transferred into 25 mL volumetric flask and made up to 25 mL with distilled water, which was used for rinsing the crucible. A Unicam (Model 929 AA) flame atomic absorption instrument was used for the determination of Cu, Zn, Mn, Fe, Mg, Na, Ca and K in sample digests.

Statistics: The experiments were set up in a completely randomised design and repeated twice. There were 6 explants in each 250 mL jar (containing 50 mL medium), 3 jars in each replicate and 3 replicates in each treatment. Analysis of variance was performed on the data, and significant differences among treatment means calculated by LSD test at $p < 0.05$.

RESULTS AND DISCUSSION

The effect of Cu treatments on dry biomass production in explants of *cv*. Chester Thornless is presented in Table-1. Four week treatments on the MS medium supplemented with 50, 100 and 150 ppm Cu were sufficient to induce significant decreases in dry biomass compared to control. The reduction of dry biomass was between 36 and 47%, with increased Cu concentration. However, Cu induced growth inhibition did not statistically differ due to Cu concentrations. The reduced growth that occurs when blackberry explants are grown on medium containing 50-150 ppm Cu confirms other reports for other plants[17-22] which are able to tolerate elevated levels of metals[5]. Moreover, the reduced amount of dry biomass by excess levels of Cu in the growing media could not be an important aspect in terms of blackberry. Because blackberry *cv*. Chester Thornless is a semi-erect bushel, producing renewal shoots (canes) from the ground and the shoots grow up to 2 m per year.

In contrast to Cu toxicity induced growth inhibition in explants of blackberry *cv*. Chester Thornless, dry biomass production was enhanced with the increase in Zn level up to 50 ppm in the growth medium (Table-2). Moreover, compared to control, relative growth rates of explants were not modified by 100 and 200 ppm Zn treatments. Similar observations were made by Rout *et al.*[11], who reported that dry weight of tolerant calli from

Brassica spp. was enhanced with the increase in Zn concentration in the growth medium.

TABLE-1
EFFECTS OF Cu TREATMENTS ON DRY BIOMASS PRODUCTION, RELATIVE CHLOROPHYLL CONTENT AND VISUAL TOXIC SYMPTOMS OF BLACKBERRY *CV*. CHESTER THORNLESS GROWN *IN VITRO*

Cu Treatments (ppm)	Dry Biomass (% of control)	Relative Chlorophyll Content (SPAD)	Injury Index
Control	100.00 a*	35.67 a	1.00 d
50	63.79 b	30.24 b	1.76 c
100	62.65 b	26.41 c	2.44 b
150	53.02 b	17.69 d	3.31 a

*Values not associated with the same letter are significantly different ($p < 0.05$).

TABLE-2
EFFECTS OF Zn TREATMENTS ON DRY BIOMASS PRODUCTION, RELATIVE CHLOROPHYLL CONTENT AND VISUAL TOXIC SYMPTOMS OF BLACKBERRY *CV*. CHESTER THORNLESS GROWN *IN VITRO*

Zn Treatments (ppm)	Dry Biomass (% of control)	Relative Chlorophyll Content (SPAD)	Injury Index
Control	100.00 b*	35.67 a	1.00 c
50	113.96 a	25.92 b	2.18 b
100	100.37 b	24.22 b	2.54 b
200	99.44 b	18.60 c	3.16 a

*Values not associated with the same letter are significantly different ($p < 0.05$).

Relative chlorophyll content: The relative chlorophyll content of leaves tended to decrease in blackberry with increasing supply of Cu and Zn (Tables 1 and 2). This effect of increased heavy metal concentrations in nutrient solution on chlorophyll content was also reported for *Pinus pinaster*, *Fraxinus angustifolia* and *Holcus lanatus*[18,23], which were identified as metal hyperaccumulators[5]. However, Kaya and Higgs[24] showed that it is possible to ameliorate the negative effect of heavy metal toxicity on chlorophyll content by the use appropriate fertilization techniques.

Injury index: Copper and zinc treatments induced visible symptoms in explants and the severity of this injury varied depending on the metal concentrations (Tables 1 and 2). The predominant symptom appeared in 50 and 100 ppm Cu and Zn treated explants was chlorosis on leaf tips and margins of older leaves. However, the highest level of these two metals stimulated chlorosis on the entire leaf. Apart from Cu, Zn treatments induced reddening in older leaves and Zn treated explants have smaller leaves than those of the control ones. However, no viability loss was observed in blackberry *cv*. Chester Thornless due to Cu or Zn toxicity. These symptoms are similar to those reported by other workers[24,25].

Mineral composition

Increased Cu levels in the growing media caused significant decreases in Na, K, Ca and Mg contents of the explants (Table-3). However Fe, Mn and Zn contents of the explants were not significantly affected by excess levels of Cu. Moreover, supplement of 50 and 100 ppm Cu in MS medium caused significant increases in Mn and Fe uptake of explants, respectively.

TABLE-3
EFFECTS OF Cu TREATMENTS ON ION CONCENTRATIONS ($mg\ kg^{-1}$ DRY WEIGHT) OF BLACKBERRY *CV.* CHESTER THORNLESS GROWN *IN VITRO*

Cu Treatments (ppm)	Zn	Mn	Fe	Mg	Na	Ca	K
Control	79.09	73.85 b*	132.16 bc	936.36 a	1709.85 a	3096.79 ab	16107.18 a
50	69.59	100.80 a	140.96 b	885.44 a	1309.45 b	3283.68 a	16420.06 a
100	61.89	77.24 b	155.05 a	740.23 b	1308.92 b	2883.16 bc	15229.42 ab
150	61.10	78.66 b	121.98 c	732.51 b	1315.96 b	2738.02 c	12635.80 b

*Values not associated with the same letter are significantly different ($p < 0.05$).

Although no change was observed in Ca and Mn uptake of explants by Zn treatments, excess levels of Zn induced significant reduction in Na, K, Mg, Fe and Cu contents of the above-ground biomass (Table-4).

In the present study, the level of each mineral nutrient (except Mg) was consistently within the acceptable range, which suggests that blackberry plants did not suffer from nutrient deficiency[26,27]. Similar result was noticed previously when *Cistus ladanifer* plants were exposed to increasing concentrations of heavy metals including Cu and Zn[21].

Chlorophyll breakdown starts with the removal of the hydrophobic phytol chain, followed by the release of the central Mg atom[28]. Accordingly Cu and Zn induced Mg deficiency in blackberry could be the main cause of chlorophyll degradation in heavy metal treated explants.

Accumulation of metals

Accumulation of metals in the above-ground biomass (shoot + leaves) after a 4-week growth period with increasing Cu and Zn concentration in the medium was shown in Table-5. The above-ground biomass of the explants exposed to 50, 100 and 150 ppm Cu accumulated a large amount of Cu and the levels were about 54, 104 and 186 times higher than those of the control, respectively. Similar to the general results obtained in Cu, Zn concentration significantly rose with increasing Zn concentrations in the growth media. Zn content in above-ground biomass of explants treated with 50, 100 and 200 ppm Zn increased almost 10, 14 and 18 times compared with those of the non-treated ones.

TABLE-4
EFFECTS OF Zn TREATMENTS ON ION CONCENTRATIONS (mg kg^{-1} DRY WEIGHT) OF BLACKBERRY *CV.* CHESTER THORNLESS GROWN *IN VITRO*

Zn Treatments (ppm)	Cu	Mn	Fe	Mg	Na	Ca	*K*
0	9.30 a*	73.85 ab	132.16 a	936.36 a	1709.85 a	3096.79	16107.18 a
50	6.46 b	90.46 a	84.21 b	888.97 ab	1224.35 b	3172.73	14399.68 ab
100	7.51 ab	86.39 ab	88.91 b	814.19 bc	1082.84 b	2894.65	13148.30 b
200	6.54 b	68.33 b	68.63 b	779.72 c	1231.49 b	2977.88	13603.24 ab

*Values not associated with the same letter are significantly different ($p < 0.05$).

TABLE-5
Cu AND Zn ACCUMULATION IN THE ABOVE-GROUND BIOMASS OF BLACKBERRY *CV.* CHESTER THORNLESS GROWN *IN VITRO*

Cu Treatments (ppm)	Cu concentration in the biomass (mg kg^{-1} dw)	Times (h)	Zn Treatments (ppm)	Zn concentration in the biomass (mg kg^{-1} dw)	Times (h)
Control	9.30 c*	1.00	Control	79.09 c	1.00
50	498.54 b	53.60	50	822.30 b	10.39
100	968.94 ab	104.18	100	1090.82 b	13.79
150	1726.11 a	185.60	200	1450.01 a	18.33

*Values not associated with the same letter are significantly different ($p < 0.05$).

From the phytoextraction perspective an ideal metal accumulator should meet the following criteria: (i) it should be able to accumulate high level of metal concerned in its harvestable tissues (ii) it should be a fast growing, high biomass crop and (iii) it should possess well developed root system[3,7]. Based on these criteria, blackberry is an easily propagated, fast growing and high biomass crop with an extensive root system. Its re-sprouting ability after pruning of above-ground biomass is another advantage. If the major goal of the phytoremediation process is to remove the maximum amount of element in the shortest possible time, blackberry is a promising candidate for phytoextraction of Cu and Zn. On the other hand, according to a hyperaccumulator being regarded as plant which the concentrations of the heavy metal in its above-ground part is 10-500 times more than that in the control plant, the phytoextraction capacity of blackberry for Cu and Zn were satisfied for this standard value (Table-5). But comparing the absolute concentrations of hyperaccumulators (Cu > 1000 mg kg^{-1} and Zn > 10000 mg kg^{-1} in dry matter), blackberry could be regarded as Cu hyperaccumulator, while Zn accumulator. Additionally, *in vitro* assay of phytoextraction capacity was greatly reduced time and efforts needed to characterize the genetic potential of blackberry. However, future field study should be conducted to confirm the phytoextraction capacity of blackberry for Cu and Zn and the feasibility of the phytoremediation technology using this species.

REFERENCES

1. Z. Yanqun, L. Yuan, C. Schvartz, L. Langlade and L. Fan, *Environ. Int.*, **30**, 567 (2004).
2. Z. Yanqun, L. Yuan, T.C. Jianjun, C. Haiyan, Q. Li and C. Schvartz, *Environ. Int.*, **31**, 755 (2005).
3. J.D. Lazaro, P.S. Kidd and C.M. Martinez, *Sci. Total Environ.*, **354**, 265 (2006).
4. L. Xiaomei, W. Qitang and M.K. Banks, *Int. J. Phytoremed.*, **7**, 43 (2005).
5. M.N.V. Prasad and H.M.O. Freitas, *Electron. J. Biotech.*, **6**, 285 (2003).
6. L.S. Angle and N. Linacre, *Int. J. Phytoremed.*, **7**, 241 (2005).
7. V.J. Odjegba and I.O. Fasidi, *Ecotoxicology*, **13**, 637 (2004).
8. J. Lin, W. Jiang and D. Liu, *Biores. Tech.*, **86**, 151 (2003).
9. Y.A. Kuzovkina, M. Knee and M.F. Quigley, *Int. J. Phytoremed.*, **6**, 269 (2004).
10. C. Giordani, S. Cecchi and C. Zanchi, *Environ. Manage.*, **36**, 675 (2005).
11. G.R. Rout, S. Samantaray and P. Das, *Plant Sci.*, **137**, 89 (1999).
12. S. Samantaray, G.R. Rout and P. Das, *Plant Sci.*, **143**, 201 (1999).
13. A. Francini, L. Sebastiani and C. Vitagliano, *Acta Hort.*, **658**, 51 (2004).
14. L. Lombardi and L. Sebastiani, *Plant Sci.*, **168**, 797 (2005).
15. J. Rabier, P. Prudent, B. Szymanska and J.P. Mevy, *J. Phys.*, **107**, 1115 (2003).
16. G. Gyulai, M. Humphreys, A. Bittsanszky, K. Skot, J. Kiss, L. Skot, G. Gullner, S. Heyword, Z. Szabo, A. Lovatt, L. Radimzky, H. Roderick, H. Rennenberg, M. Abberton, T. Komives and L. Heszky, *Bioscience*, **60**, 300 (2005).
17. M.A. Utriainen, L.V. Karenlampi, S.O. Karenlampi and H. Schat, *New Phytol.*, **137**, 543 (1997).
18. I. Arduini, D.L.Godbold, A. Onnis and A. Stefani, *Chemosphere*, **36**, 739 (1998).
19. N.A. Ali, M.P. Bernal and M. Ater, *Plant and Soil*, **239**, 103 (2002).
20. Z.H. Ye, A.J.M. Baker, M.H. Wong and A.J. Willis, *Chemosphere*, **50**, 795 (2003).
21. P.S. Kidd, J. Diez and C.M. Martinez, *Plant and Soil*, **258**, 189 (2004).
22. C. Gisbert, R. Clemente, J.N.-Avino, C. Baixauli, A. Giner, R. Serrano, D.J. Walker and M.P. Bernal, *Environ. Exp. Bot.*, **56**, 19 (2006).
23. L. Symeonidis and S. Karataglis, *J. Argon. Crop Sci.*, **168**, 108 (1992).
24. C. Kaya and D. Higgs, *J. Plant Nutr.*, **25**, 1881 (2002)
25. F. Gharbi, S. Rejeb, M.H. Ghorbal and J.L. Morel, *J. Plant Nutr.*, **28**, 379 (2005).
26. J.M. Spiers, *J. Plant Nutr.*, **16**, 297 (1993).
27. J.M. Spiers and J.H. Braswell, *Acta Hort.*, **585**, 659 (2002).
28. U. Eckhardt, B. Grimm and S. Hörtensteiner, *Plant Mol. Biol.*, **56**, 1 (2004).

(*Received*: 18 May 2006; *Accepted*: 26 October 2006) AJC-5231