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QUARTERLY TECHNICAL PROGRESS REPORT

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"Identification and Validation of Heavy Metal and Radionuclide Hyperaccumulating Terrestrial Plant Species"

Progress Report for Period of December 20, 1994 - March 20, 1995

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I. Introduction

Although the period covered by this progress report began on December 20, 1994, which was the date that DOE approved the Interagency Agreement, the agreement was not approved by USDA until January 9, 1995 and the first scientists working on the project were not hired until February 1, 1995. The first goal of the research supported by the Interagency Agreement is to use hydroponic techniques to identify plant species and genotypes with potential for heavy metal hyperaccumulation for planting on a test site at Silverbow Creek and for radionuclide (^{90}Sr and ^{137}Cs) accumulation on a test site at INEL, Idaho, later this year. The second goal of this research is to identify soil amendment procedures that will enhance the bioavailability of heavy metals and radionuclides in the soil without increasing the movement of the contaminants of concern (COC's) into the groundwater. Our initial research covered in this report focuses on the first goal.

II. Soil analyses of test sites and design of nutrient solutions. Analyses were carried out on samples from Silver Bow Creek and a non-radioactive matched sample to INEL provided by MSE. Soil was received in a field moist state after thawing (approximately 20 % water content). Soil solutions were extracted by vacuum filtration, filtered through 0.45 μm membrane filters and analyzed directly by ICP emission spectroscopy for a range of elements (Table 1.) Samples of each soil were then dried, ground and extracted using two extractant methods: 1) 5 g of air-dry soil extracted with 25 ml of ammonium acetate/EDTA for 15 minutes and 2) 5 g of air-dry soil extracted with 25 ml of Spurway reagent (dilute acetic acid) for 1 minute. Both samples were filtered on 0.45 μm membrane filters prior to ICP analysis. These two methods provided some measure of the ability of the soils to supply the toxic metals and were a check on the soil solution analysis (Table 1.) The soil solution results should be viewed with caution as the samples had been stored moist for some time during transit. These analyses will be repeated on air-dry samples provided by MSE.

The soil analysis of Silver Bow Creek revealed that it is potentially fertile with high concentrations of Ca, K, Mg, and S. White crystals formed on the drying soil were probably gypsum. Sodium was also high in this soil. As high Ca is known to ameliorate toxicities of some metals and high ionic strength depresses activities of metals in solution we chose to screen plants in a high ionic strength nutrient solution. The full strength modified Johnson's solution we employed, while lower in ionic strength than the soil solution, will provide a more realistic background for screening a range of plants for suitability in the field. Any accumulation in the field will have to occur against the background of high macronutrients. Zinc, copper and cadmium were present at elevated concentrations (Table 1.) We chose concentrations of these COC's for the screening nutrient solution which were somewhat lower than the soil solution, intending to re-screen promising species at higher concentrations. The concentrations of the COCs in the screening nutrient solution were 100μM Zn, 5 μM Cu and 1μM Cd.

Additional analyses were carried out for arsenic which was present in insignificant quantities in the sample we were provided with (data not shown).

The matched samples for INEL also had high concentrations of macronutrients but with much lower sodium. This soil is also potentially fertile if water is not limiting. The concentrations of the COCs were low. Strontium was 8μM and cesium less than 1μM in the soil solution, which were analyzed by ICP-MS. We chose to use a full strength modified Johnson's solution for the first screen with a slightly higher Sr concentration (20 μM) but with the same Cs concentration as the soil solution.

Table 1. Soil extract data for Silver Bow Creek and INEL.

	Silver Bow Creek, Montana			INEL, Idaho		
	Soil Solution	Spurway	Ammonium acetate	Soil Solution	Spurway	Ammonium acetate
	μM	μg/g air dry soil		μM	μg/g air dry soil	
K	1600	235	520	1700	296	643
Ca	12225	3120	6080	4800	2080	11170
Mg	6917	427	770	1260	135	413
S	36125	3055	2770	428	14	151
Al	153	2	10	434	10	38
Na	41652	1195	1350	10.7	27	24
Mn	202	125	430	2.5	2	62
Fe	0.3	1.7	125	1.1	0.3	22
Cu	12	44	1495	2.5	0.3	3
B	55	4	4	51	1.4	2
Zn	571	488	2000	1.4	0.2	2
Mo	0.6	2.5	2	0.3	0.4	0.2
Co	0.1	0.4	3	0.1	0.1	0.1
Cd	1.4	2	12	0.2	0.1	0.4
Cr	4.8	2	3	1.6	1.1	3
Ni	2.3	0.2	4	2.4	0.1	3
Pb	1.5	10	118	1.2	0.8	4
Si	642	34	69	430	29	26
Cs				<1		
Sr				8		

III. Selection of candidate species for screening

A seed bank was established containing a wide variety of accessions of *Brassica* species and other species which have been referred to in the literature as potential indicators or accumulators. Seeds were obtained from the following sources:

USDA-ARS Plant Introduction Station, Iowa State University, Ames, Iowa.
Crucifer Genetics Cooperative, University of Wisconsin-Madison, Madison,Wisconsin.
USDA-ARS Plant Genetic Resource Center, Geneva, New York.
Granite Seed, Lehi, Utah.
Big Sky Wholesale Seeds, Shelby, Montana.
Treasure State Seed. Fairfield, Montana.
Valley Seed, Fresno, California.
F & J Seed Service, Woodstock, Illinois.
Frosty Hollow Ecological Restoration, Langely, Washington.
USDA Regional Plant Introduction Center, Pullman, Washington.

IV. First screen for Zn, Cu and Cd accumulation

In the first screen 285 test species were grown in 200 L tanks of full strength Johnson's solution with 100 μM Zn, 5 μM Cu and 1μM Cd. Dry seed was planted directly onto mesh in wells of a polystyrene float in direct contact with the nutrient solution. Seeds were sprayed with 200 μM $CaSO_4$ solution to aid germination. Plants were illuminated with supplemental lighting. Germination was slow and variable. The primary toxicity symptom was leaf chlorosis consistent with iron or manganese deficiency after the second week of growth. A range of foliar sprays, including $FeSO_4$, Fe-EDDHA, Fe-citrate and $MnSO_4$, were used with L-77 as a surfactant on selected plants in an attempt to identify and alleviate the deficiency. However, no response to foliar spraying was observed. Because the foliar spray was not applied until the plants were exhibiting severe symptoms, the treatment may have been too late to ameliorate any heavy metal-induced micronutrient deficiency. Plants were grown for 4 weeks and then whole shoots and roots were harvested. Shoots were digested and analyzed by ICPES.The mean and ranges of concentrations of the grouped species are shown in Table 2.

All *Brassica* species exhibited significant accumulation of Zn, Cd and Cu when compared with normal levels of these elements in crop *Brassica* plants. Other species tested such as crown vetch, hairy vetch and *Ipomea* species did not exhibit significant accumulation of these metals (data not shown). Candidate species for further investigation were those with both the highest biomass and accumulation of Zn, Cu and Cd. Figure 1 shows the total uptake of COCs by the individual best performers from each species. These results were presented at the Phytoremediation Symposium at the University of Missouri-Columbia in April. *B. napus* and *B. nigra* were the best performing species overall. Tissue analysis also confirmed that both Fe and Mn were at deficient concentrations in all species (Table 2). Individual plants which had been sprayed with Fe or Mn were excluded from the mean concentrations. In these cases, concentrations were indeed higher, but this was probably due to surface retention.

Table 2. Heavy metal and micronutrient concentrations of a range of *Brassica* species exposed to elevated Zn, Cu and Cd in solution.

		Shoot dry weight (g)		Concentration (μg/g dry weight)									
				Zn		Cu		Cd		Fe		Mn	
Species	No. Access. tested	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
B.napus	47	0.146	0.049-0.244	1838	1335-2216	51	31-71	43	33-57	28	12-45	24	20-28
B.nigra	5	0.116	0.040-0.299	1860	1421-2216	62	45-74	37	31-43	54	41-70	38	19-54
B.rapa	35	0.110	0.032-0.360	1823	1209-2711	68	28-127	41	26-72	65	35-134	24	15-32
B.oleracea	26	0.08	0.036-0.233	1801	959-3544	72	23-506	37	24-76	63	31-142	25	16-28
B.juncea	44	0.07	0.020-0.181	1942	256-6300	53	12-209	40	6-136	51	10-198	22	6-70
B.carinata	7	0.041	0.021-0.065	1684	1503-1866	95	14-397	29	26-32	49	32-66	18	16-21
C. abyss. *	5	0.067	0.045-0.109	1386	1038-1809	59	44-82	30	24-34	54	35-79	23	19-27
Comm.**	5	0.073	0.041-0.128	1448	1246-1742	48	31-78	27	24-29	46	32-74	19	16-24
Normal conc.					25-100		5-10				50-200		25-150

* *Crambe abyssinia.*

** A range of commercial varieties of cabbage, broccoli etc.

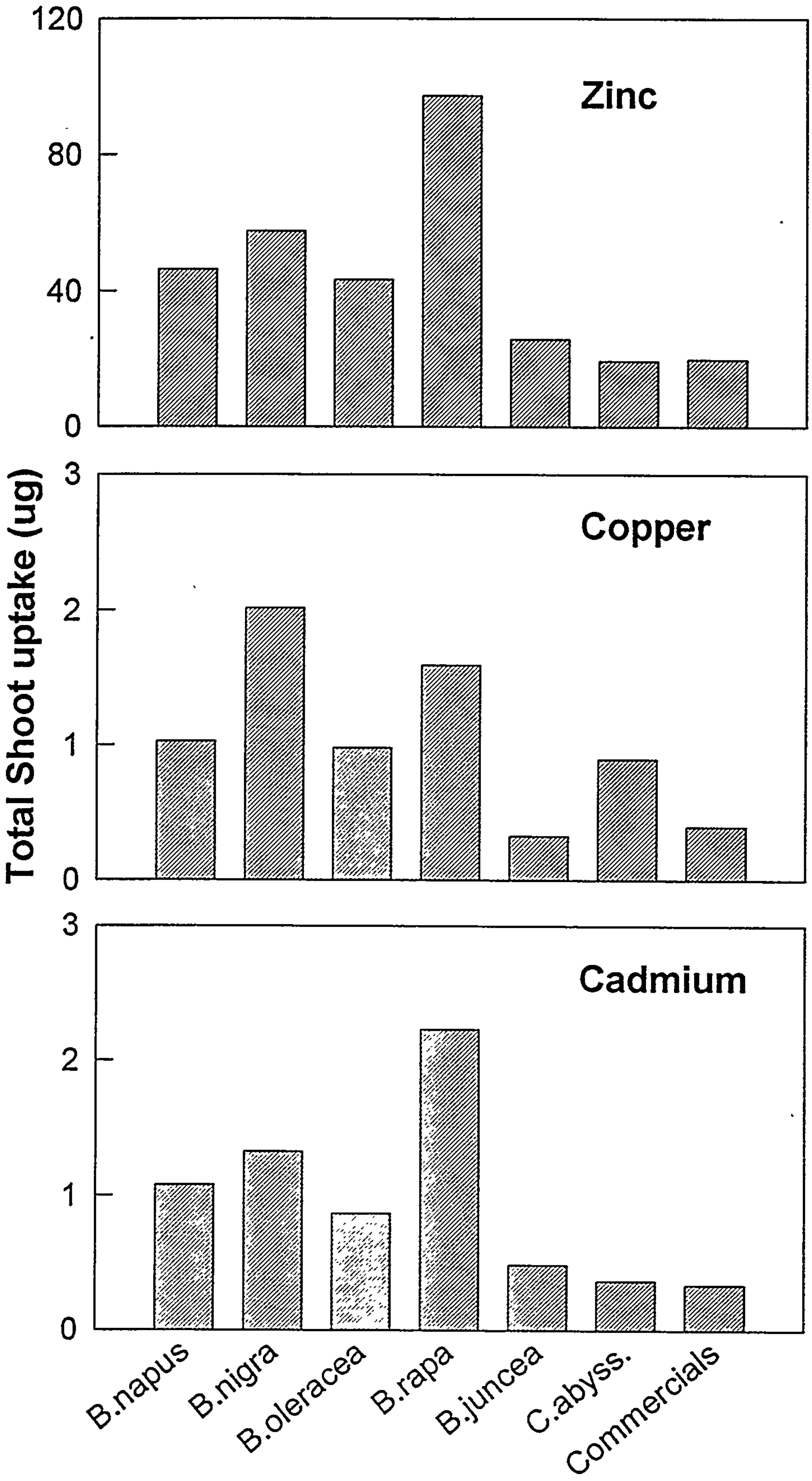

Figure 1. Total uptake of best individual biomass producer in each species.

V. Initial screen for Cs and Sr accumulation

Species investigated for their ability to accumulate Cs/Sr in shoots were *B. juncea, B .napus, B. nigra, B. carinata, B. rapa* and a variety of commercial *Brassicaceae* and *Vicia* spp. Also included were a variety of salt resistant species from the genera: *Kochia, Sarcobalus, Atriplex, Artemisia* as well as monocotyledonous species such as *Festuca, Agrostis, Agropyron, Elymus, Phalaris* etc. Plants were grown from germinated seeds in an aerated full strength modified Johnson's solution plus 20 μM $SrCl_2$ and 1 μM CsCl. Plant shoots and roots were harvested after two weeks. Shoots are currently being analyzed for elemental composition by ICP. Some results are available for the *Brassicaceae* species. Depending on the species, the range for Cs accumulation was between 12 and 25 μg/g dry weight. Interestingly, among the highest accumulators were commercial varieties such as broccoli and cauliflower. Accumulation of Sr in shoots ranged from 301 to 478 μg/g dry weight. The highest Sr accumulator was a commercial variety of broccoli (Arcadia).

VI. Rescreening of *Brassicas* at Zn and Cu concentrations comparable to those in the field

The Cu and Zn solution concentrations selected for the first heavy metal screen (5 and 100 µM, respectively) were conservative compared to soil concentrations determined by the soil analyses (Table 1). Therefore, before *Brassicas* from the first bulk screen could be used in field studies, an assessment of their performance at the Cu and Zn concentrations representative of those in the field was required. Since the first bulk screening experiment indicated that Cu and Zn uptake was fairly consistent across all the *Brassica* species tested, one accession each from *Brassica juncea* (184290), *B. napus* (535860), *B. nigra* (30837), and *B. rapa* (164468) were selected as the genotype to be tested in this experiment. Germinated seeds, one from each of the above *Brassica* species were planted together in 2.2 L pots (3 replicate pots per treatment) containing full strength modified Johnson's solution at pH 6.5. In an attempt to avoid the Fe and Mn deficiencies observed in the bulk screening experiment, the Fe-EDDHA concentration was raised to 15 µM while the Mn concentration was increased to 2 µM. A factorial design of 3 Zn concentrations (100, 200, and 400 µM), 3 Cu concentrations (5, 10, and 20 µM), and 3 replicates was employed. Plants were grown for 4 weeks, with the nutrient solution replenished after two weeks. Shoots were digested and analyzed by ICPES and ICPMS.

All four species tolerated the solution Zn concentrations, as long as the Cu concentration was only 5 µM. *B. juncea* grew in nutrient solution containing higher Cu concentrations (10 and 20 µM) only when the Zn concentration was 200 µM or less while only a single *B. nigra* plant grew in a solution containing 10 µM Cu with either 200 or 400 µM Zn. At 5 µM Cu and 100 µM Zn, all four species accumulated comparable levels of Zn (Figure 2). As solution Zn increased, Zn accumulation increased in all species (Zn accumulation by *B. juncea* was less than that of the other three species). Cu accumulation by *B. napus* and *B. rapa* tended to increase as the solution Cu was increased. At 5 µM Cu, the mean plant dry weight for all species except *B. rapa* decreased as the solution Zn concentration increased (Figure 3). *B. napus* generally had the greatest mean dry weight at the other Zn and Cu concentrations.

The Cu concentration appeared to be a limiting factor for growth in this study. Seedlings receiving the higher Cu concentrations had shorter roots with few, if any, root hairs. In addition, the mean dry weight for all species tended to decrease with increasing solution Cu. Furthermore, despite the additional Fe and Mn in the nutrient solution, the *Brassicas* tested here were Fe and Mn deficient (Table 3). Typical Fe and Mn concentrations for *Brassicas* are 50-200 ppm Fe and 30-200 ppm Mn. Means of alleviating this micronutrient deficiency must be developed if these species are to be used in the field, and we are initiating studies investigating the efficacy of foliar Fe and Mn fertilization to overcome the heavy metal-induced micronutrient deficiencies.

VII. Preliminary investigation of the forms of U taken up by plants

In order to devise a suitable screening system for uranium accumulation, information is required on the form of U available to plants in soil. There appears to be no published solution culture experiments on U and very few controlled experiments in soil where uptake has been measured in plant tissue. Geochemical studies on the Fernald site suggest that soluble U is present in pH 6.5 soil chiefly as the anionic carbonate species. This has been due to addition of alkaline, carbonaceous remediation and road construction materials. A considerable portion of U is also present as insoluble particulate matter. Geochemical modeling shows that carbonate complexes of U are very stable and provide potential for leaching of U from the environment. Phosphate complexes of U are also very stable and would be present where soils are fertilized with P to improve plant growth. It is not known which of these forms of U are taken up by plants. Free UO_2^{2+} ions are likely to be present in the soil solution only at pH less than 5.5. If this form were taken up by plants, considerable modification of the soil environment would be required for successful phytoremediation.

As a first step, an experiment was designed to investigate which forms of U might be taken up by plants from nutrient solution. The limitation of commencing a screen before this stage is that it will not be possible to ascribe failure of any plants to accumulate U to either their intrinsic properties or to unavailability of U to the plants in the soil solution. A half strength modified Johnson's solution containing 10 μM total U [supplied as $^{235}UO_2(NO_3)_2$] was used for the growth medium. . Peas (*Pisum sativum* cv Sparkle) were chosen as the test plant because this species was shown to have promise in terms of Pb accumulation in the shoot by by Dr. Jianwei Huang of DuPont at the recent Phytoremediation conference at the University of Missouri. As the most stable complexes of U are with P (to the extent that no free U is present in a typical nutrient solution), the initial experiment was carried out with plants which were precultured in high P solution and transferred to solutions containing U, but no P. After modeling of nutrient solutions with GEOCHEM-PC, three treatments were chosen which yielded nutrient solutions dominated by one of the 3 U species. At pH 5 a significant amount of U was present as a free ion, at pH 6.5 most U was present as hydroxide complexes and at pH 8 nearly all U was present as carbonate complexes. Plants were pre-cultured at the test pHs for 2 weeks in the absence of U and then transferred to U-containing solutions for 7 days. Control plants were continuously grown in identical solutions lacking U at the 3 test pH values. The experiment is currently being analyzed. Observations of roots at harvest revealed that U is quite toxic to roots with similar symptoms to Al toxicity such as stunting of lateral root growth and blackening of existing roots. The effect was most pronounced at pH 6.5. Future experiments will probably employ a lower concentration of U and will be conducted in the presence of P, thus reducing the activity of free U in solution.

Table 3. Heavy metal and micronutrient concentrations of the four *Brassica* accessions examined.

Nutrient Solution Concentration (µM)		# Surviving Plants (of 12)	Mean Shoot dry weight (mg)	Plant Tissue Concentrations (μg/g dry weight)							
Zn	Cu			Zn		Cu		Fe		Mn	
				mean	range	mean	range	mean	range	mean	range
100	5	7	35.4	2232	1994-2484	70	40-115	46	27-69	31	25-39
100	10	8	13.3	1412	784-2223	156	80-219	65	30-128	35	15-55
100	20	1	34.0	4364	-	63	-	39	-	28	-
200	5	9	31.0	3663	2469-4189	64	31-86	38	30-50	28	22-43
200	10	6	14.4	3054	2283-4312	131	78-161	79	52-165	35	25-44
200	20	2	20.0	3024	1867-4180	104	81-127	51	50.8-51.3	31	31.8-31.9
400	5	9	18.4	4996	1198-7509	145	41-530	65	26-143	29	25-48
400	10	3	20.3	3029	2722-3739	94	45-137	49	24-67	26	18-31
400	20	2	8.5	1675	1522-1698	131	115-146	48	42-53	25	20-29

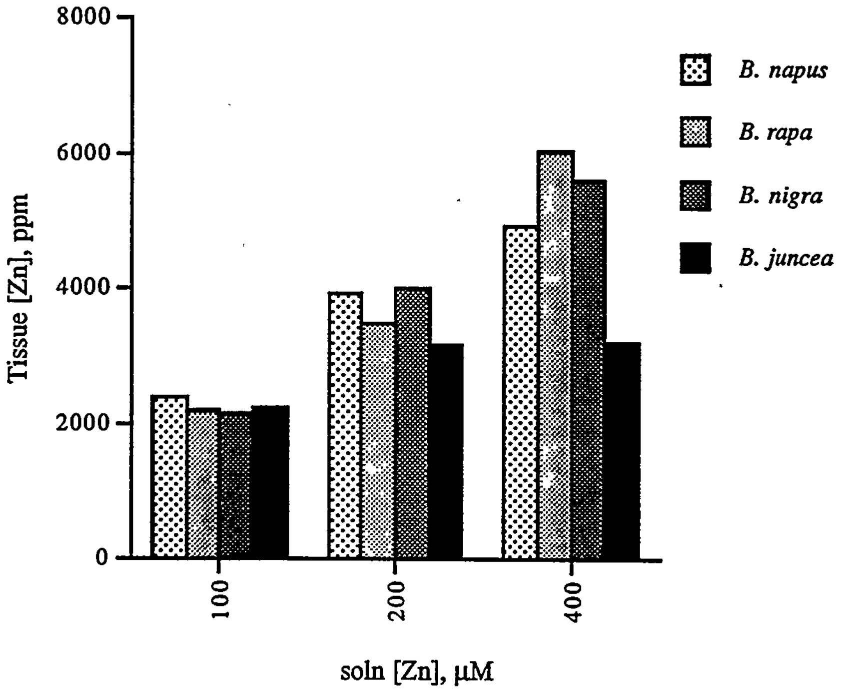

Figure 2. Zn accumulation by *Brassica* species at 5 μM Cu

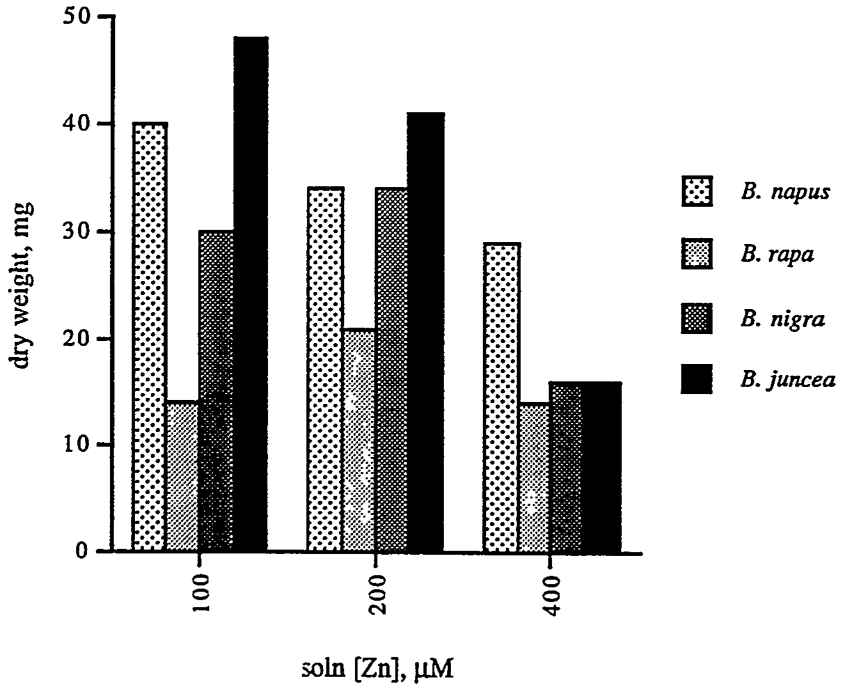

Figure 3. Mean dry weight for the four *Brassica* species at 5 μM Cu

VIII. Experiments in progress

The most promising *Brassica* species from the first screen are being rescreened in a solution culture experiment in which the COCs will be added after two weeks of pre-culture in control nutrient solution. In this experiment, the monocot seed bank will also be tested (including *Agrostis* and *Festuca).*The aim will be to determine whether better growth can be achieved if germination occurs in the absence of the COCs. In a field situation, plants could be transferred as seedlings. We will also further investigate foliar application of micronutrients in a controlled experiment employing sprays applied at different growth stages. Alleviation of this problem is the major impediment to achieving good biomass production in the presence of the COCs and will require a solution which is practical in the field. Prior to planting in the field we will grow a selection of our candidate species in a bioassay experiment using soil from Silver Bow Creek provided by MSE.

We have several thousand seedlings of *Thlaspi caerulescens* growing in sand and intend to transplant these to seed trays of Silver Bow Creek soil. Pre-culture of these slow growing hyperaccumulators and transfer to the field will maximize the probability of a reasonable growth during the short growing season in Montana. At this early stage, seed is not available for field planting from our best performing candidate species. Commercial seed has been obtained from Mycogen Ltd of Canadian cultivars of *B.napus, B.rapa* and *B.juncea* which should be suitable for the Montana site. Seed of a hyperaccumulating *B.juncea* accession has also been provided by collaborators at Phytotech Inc.