Copper and zinc tolerance in two Montana grass species growing on copper mill tailings
by John Edward Surbrugg

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Land Rehabilitation
Montana State University

Abstract:
Copper smelter operations have been in existence in Anaconda, Montana since the late 1800's. The tailings material, deposited just east of the city, is essentially devoid of vegetation. However, Deschampsia cespitosa and Aarostis tenuis grasses were found colonizing areas of the tailings. Tailings from a vegetated area were analyzed and upon examination it was determined that copper and zinc were possibly toxic to the plants. The purpose of this study was therefore to determine the tolerance of Deschampsia cespitosa and Aarostis tenuis to copper and zinc. .

A greenhouse study using a sand, drip culture apparatus was used to determine metal tolerance. Commercial seed populations of the same genus and species were used as controls. An "in parallel" technique utilizing 4 concentrations of each metal allowed regression analysis of root growth on test concentration. Statistical analysis was used to identify differences in metal tolerance between the tailings and the control populations.

Results indicated that the mill tailings population of Deschampsia cespitosa was significantly more tolerant of copper and zinc than the commercial population. Agrostis tenuis from the mill tailings was found to possess significant copper tolerance only. Commercial and mill tailings populations of Agrostis tenuis were statistically similar in their response to zinc. A stimulating effect on root growth was observed at moderately low levels of zinc in the tailings plant species. This often observed stimulus may itself be related to tolerance.

An economic and viable method for stabilizing metalliferous sites is vegetative stabilization involving naturally occurring metal tolerant species. The confirmed tolerance to zinc and copper of the grass species colonizing the copper mill tailings at Anaconda, Montana indicates these species may be suitable for use in revegetating copper and zinc contaminated sites.

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GROWING ON COPPER MILL TAILINGS

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Approved:

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ABSTRACT

Copper smelter operations have been in existance in Anaconda, Montana since the late 1800's. The tailings material, deposited just east of the city, is essentially devoid of vegetation. However, Deschampsia cespitosa and Agrostis tenuis grasses were found colonizing areas of the tailings. Tailings from a vegetated area were analyzed and upon examination it was determined that copper and zinc were possibly toxic to the plants. The purpose of this study was therefore to determine the tolerance of Deschampsia cespitosa and Agrostis tenuis to copper and zinc.

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INTRODUCTION

Anaconda, Montana has been the site of copper smelter operations since the late eighteen hundreds (Figure 1). Nearly 75 years of waste material from the smelting process has been deposited just east of the city of Anaconda in a tailings dump (Anaconda Copper Company 1979). The extraction process removes much of the mineral from the ore, but may leave behind as much as one percent of the primary mineral. This may be accompanied by other metals in the ore which were not worth extracting (Bradshaw 1971).

Typical reclamation techniques for tailings dumps have included regrading, veneering the surface with crushed limestone, covering the tailings with topsoil and seeding (Anaconda Copper Company 1979). This traditional revegetation effort may be adequate under some spoil physical and chemical conditions but has potential to fail under the adverse conditions of tailings dumps. Tailings characteristically have high acid-generating potential and, without continuous applications of lime, vegetation may not survive.

The European method of mine reclamation may be quite different than the modern American approach. For almost 30 years, investigators in the United Kingdom have been successfully establishing metal tolerant vegetation on metalliferous mine sites. They have found that populations of certain species growing in soils containing large amounts of heavy metals may be tolerant of the metal, and will grow

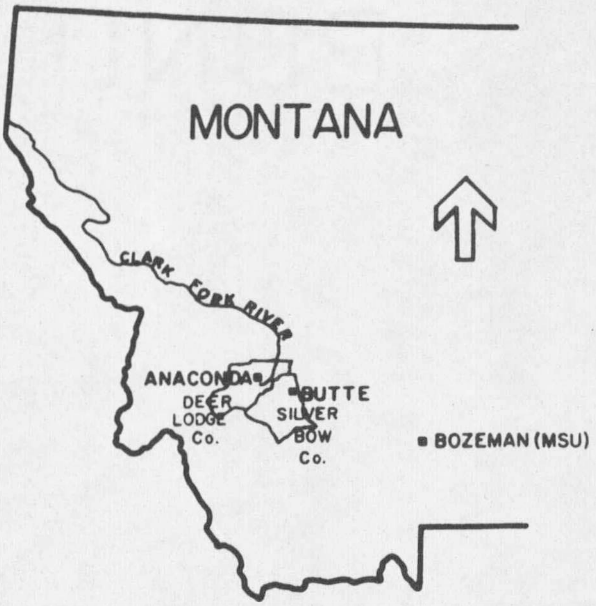

Figure 1. Location of Anaconda, Montana

better on such soils than transplants from uncontaminated soils (Bradshaw 1952).

Techniques for identifying tolerant vegetation have been developed. These techniques measure the degree to which plants will tolerate concentrations of heavy metals in solution culture (Wilkins 1957; Jowett 1958). These measurements along with field trials have produced commercial varieties of grass with specific tolerances to certain metals.

There is a great need to stabilize tailings dumps and other metalliferous sites in the United States with vegetation. Plants can act to minimize erosion by wind and water and improve the asthetic appearance of mining areas. The establishment of metal tolerant vegetation is considered advantageous in terms of cost and permanence over other methods of stabilizing mine sites (Bradshaw et al. 1978).

Naturally occurring grasses were found colonizing areas of the Anaconda tailings dump (Figure 2). This plant material may be suited for specific use in revegetating metalliferous sites in this area. Soil from the vegetated areas was analyzed and found to contain high levels of copper and zinc.

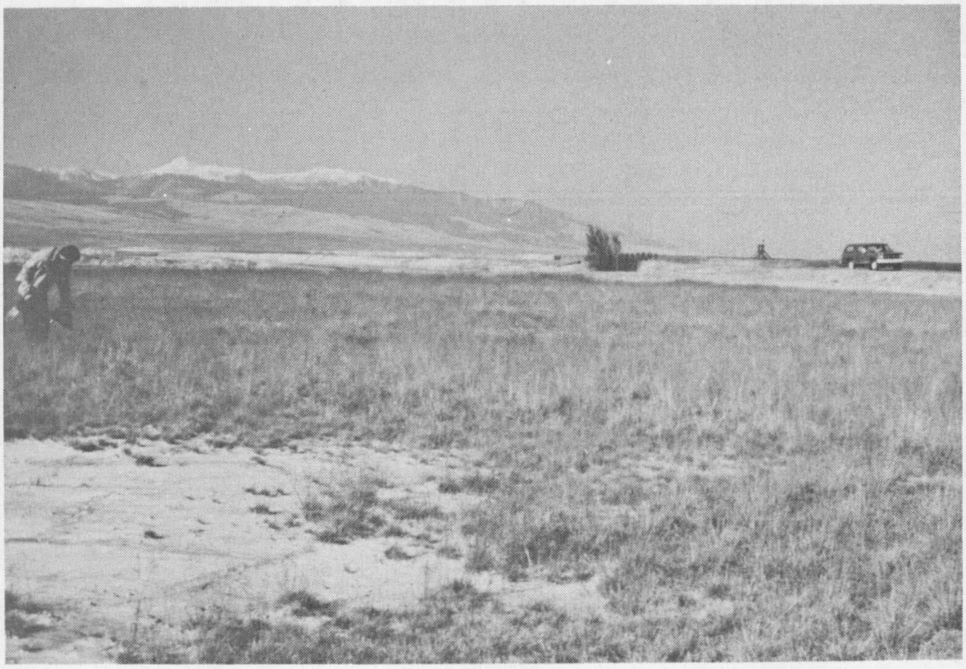

Figure 2. Naturally occurring grasses colonizing Anaconda tailings dump.

The purpose of this study was to measure the tolerance of grasses *Agrostis tenuis* and *Deschampsia cespitosa* from the Anaconda tailings dump to high concentrations of copper and zinc.

Specific objectives of this research included:

1. Determine the tolerance of *Agrostis tenuis* and *Deschampsia cespitosa* found growing on copper tailings to high concentrations of copper and zinc.
2. Determine the tolerance of *Agrostis tenuis* and *Deschampsia cespitosa* grown from commercial seed to high concentrations of copper and zinc.
3. Compare the metal tolerance of the copper tailings populations to the commercial seed populations.

LITERATURE REVIEW

The Genetics and Evolution of Tolerance.

Plant species that occupy environments where there is evidence of toxicity due to heavy metals do so because they have evolved metal tolerant populations (Bradshaw 1977). Areas contaminated by heavy metals have provided an excellent example for the study of evolution. Examination of metal tolerance on both sides of mine boundaries have shown sharp differences in metal tolerance at these locations (Jain and Bradshaw 1966; McNeilly and Antonovics 1968; Antonovics 1968; Antonovics and Bradshaw 1970). The characteristics of the tolerant population are maintained by very severe selection which allows only tolerant individuals to survive, while similar severe selection may be operating against tolerance on normal soils (Bradshaw et al. 1965).

Tolerance is specific for individual metals. Jowett (1958) showed that a population tolerant of one metal was not tolerant of another unless the second was also present in its original habitat. Metal tolerance may not be found in populations growing on soils in which high levels of heavy metals are not available because of high pH or high organic matter content, and therefore not toxic (Bradshaw 1977). When high levels of metals are available and toxic to plants, they cause a virtual cessation of root growth and the formation of short stumpy laterals and ultimately, although not immediately, the death of the plants (Bradshaw et al. 1965).

Many investigations have concentrated on defining the genetic basis of metal tolerance in plants. Bradshaw et al. (1965) defined tolerance as genetically controlled since it is constant in vegetative material and is passed on to seed material. Species that evolve tolerance appear to be those that contain genes for tolerance in their normal populations (Bradshaw 1977). Genetic control of zinc tolerance in Agrostis tenuis appears to involve a number of genes with additive and dominant effects (Gartside and McNeilly 1974a).

Many plant species may be found around heavily contaminated sites but not on contaminated soils. These are species that presumably have failed to evolve tolerance. The failure may be caused by a lack of the appropriate genetic variability in those species (Bradshaw 1977). Normal populations of many plant species have been screened for metal tolerance and a significant number of tolerant individuals were found only in those species which do evolve tolerance (Khan 1969). The occurrence of species in contaminated areas is therefore determined by genetic diversity rather than innate physiological tolerance (Bradshaw 1977).

Where did metal tolerance originate? Many mine sites are quite recent so it would be tempting to believe that mine populations are also quite young. However, many mines were preceded by ore bodies exposed at the soil surface. These may have existed since the last glacial period (Bradshaw 1971). Seed material from areas surrounding the mine may contain genes for tolerance. When non-tolerant seed is sown on metalliferous soil, although nearly all die, one or two plants per thousand may survive and grow well and a few more may grow rather weakly (Khan 1969). Metal tolerant individuals in Agrostis are found in normal non-tolerant populations although at very low frequency (Bradshaw 1971). With very severe selection, a tolerant population can therefore be produced from a non-tolerant one in only one or two generations (Bradshaw 1971).

Physiological and Biochemical Aspects of Tolerance.

Plants accumulate heavy metals either from foliar deposits or by uptake through their roots (Ernst 1976). Aerial lead pollution from automotive engines has operated on plant populations to produce lead tolerant individuals (Briggs 1972). Industrial emission of zinc, cadmium, nickel and copper can also cause evolution of heavy metal tolerance (Bradshaw et al. 1965; Wu and Bradshaw 1972). The ability to evolve tolerance is species specific and independent of the source of contamination (Gartside and McNeilly 1974b).

There are two basic mechanisms whereby plants can combat toxic levels of heavy metals. Avoidance, as defined by Levitt (1958, as referenced in Ernst 1976), is an exclusion mechanism by which metal uptake is prevented. Tolerance, also defined by Levitt (1958, as referenced in Ernst 1976), results when uptake occurs but resistance is due to exclusion of metals from metal-sensitive sites by formation of specific metal-resistant enzymes or alteration of metabolic pathways.

Heavy metal avoidance has never been identified as the mechanism for metal tolerance by plants (Bradshaw et al. 1965; Ernst 1976). External and edaphic conditions such as presence of organic compounds, (Turner and Gregory 1967; Ernst 1976) high amounts of calcium (Jowett 1964) and phosphorus (Ernst 1976) and low cation exchange capacity of the roots (Ernest 1976) can significantly reduce the absorption rate of copper and zinc. The selection for cation exchange capacity is genetically independent of heavy metal tolerance (Ernst 1976). No mechanism is known to exist which can prevent heavy metals from entering resistant plants (Ernst 1976).

Plants growing on metalliferous soil must be absorbing metals. Copper and zinc tolerant strains of Agrostis tenuis take up more copper and zinc respectively into the roots than do non-tolerant plants (Wainwright and Woolhouse 1975). Metal tolerant plants resist this toxic effect by removing these ions from the physiological process, by rendering them innocuous or by changing enzyme structures (Ernst 1976).

Metals may be disposed of by cells by storage in cell walls as revealed by fractionation and analysis of root homogenates; the greater the degree of metal tolerance, the greater the metal binding capacity of the cell-wall fraction (Turner and Marshall 1971; 1972). In addition to this protective role of the cell wall, the organelles of zinc tolerant cells in Agrostis tenuis are adapted to higher levels of zinc nutrition (Turner and Marshall 1971). Even though cell walls of metal tolerant strains of many species bind appreciable quantities of metal, this binding does not significantly prevent the metal from being taken up by the roots, translocated and accumulated in the aerial parts (Wainwright and Woolhouse 1975). The mechanism of tolerance in any individual may involve a variety of physiological processes each of which contribute to the total metal tolerance (Wainwright and Woolhouse 1975; Foy et al. 1978).

Laboratory Methods for Metal Tolerance Determinations.

The most rapid and convenient method of testing for metal tolerance in plants is the comparison of root growth in culture solutions with and without metal additions (Gregory and Bradshaw 1965). An index of tolerance can be derived from the length of root growth in toxic solutions expressed as percentage of growth in the control solution (Jowett 1958; 1964). The technique for measuring tolerance (Wilkins 1957) was originally described for the measurement of one plant species to one metal. It is now generally accepted as a tool for measuring tolerance of a wide range of other species and other toxic ions. Most of the plant species studied have been grasses, which are both morphologically convenient to work with, and widespread on toxic soils, but many other plants have also been investigated successfully (Wilkens 1978).

The first technique for measuring heavy metal tolerance was developed for measuring lead tolerance in Festuca ovina (Wilkens 1957). He used a series of lead concentrations as lead nitrate in culture solutions containing one gram per liter of calcium nitrate. Preliminary experiments indicated the calcium ion was clearly reducing the toxicity of lead. It was decided to use calcium nitrate so that higher concentrations of lead could be used without stopping all growth (Wilkens 1957). For routine testing of large numbers of plants one concentration of lead was chosen, as this allowed some growth in the least tolerant plants, but still produced a slight check in that of the most tolerant (Wilkens 1957).

Jowett's method, (1958) of testing was derived from that of Wilkens (1957). Jowett's method was employed for measuring Agrostis spp. tolerance to copper, nickel, lead and zinc. Multiple concentrations of each metal were used in the analysis. Tolerance was expressed as root growth in toxic solutions as a percentage of normal growth (Jowett 1958). These data obtained from a range of concentrations can be analyzed by calculating a regression of root growth on test concentration. This method can be a valuable research tool, however it may be limited in its usefulness for mass screening by large material requirements (Wilkens 1978).

Many modifications of the original technique have emerged. Perhaps the most noted alteration is the use of separate sets of roots in the treatments and control solutions. The original technique entailed making a first growth measurement in the control solution, transferring the roots to the treatment solution, and making additional measurements on the same roots (Wilkens 1957). In further experiments, it was shown that there was a high degree of parallelism in the behavior of plants tested for metal tolerance (Wilkens 1978). The alternative of using parallel controls, versus sequential controls, better allows comparisons between populations. This is due to each individual plant having a tolerance ratio instead of a ratio for each root. Also, environmental and independent variables have less influence on the measurements because tolerance indices are internally comparable (Wilkens 1978).

Another variation of assessing heavy metal tolerance was described by Gadgil (1969). She enclosed each tiller in a glass tube and trickled the culture solution down the suspended root. This drip culture technique was devised to overcome two problems which arise if roots are grown in tubes immersed in solution. The first problem was lack of aeration and the second was inadequate mixing (Gadgil 1969). The drip culture method also facilitated the use of large volumes of solution which helped maintain the concentration of solutes as the tillers grew. Gadgil's method is discussed in detail in the Material and Methods section of this thesis.

Other laboratory methods for measuring metal tolerance have been described. One simple screening method that easily identified tolerant plant species involved sowing seed samples in toxic substrate prepared by mixing metalliferous waste with a certain amount of ordinary potting soil. After a period of growth, the tolerant individuals were still growing whereas non-tolerant individuals had died (Weston et al. 1965; Walley et al. 1974; Humphreys and Bradshaw 1977). Another variation of this same technique utilized normal soil with various heavy metal additions (Miles and Parker 1979). These studies incorporated germination, emergence and seedling survival data with metal tolerance to obtain a chemical and physical tolerance index.

A more sophisticated method investigated metal tolerance in tissue cultures (Wu and Antonovics 1978). The results of two investigations (Wu and Antonovics 1975, 1978), indicated tissue culture experiments were comparable with results of growth and uptake experiments of the same clones grown in water cultures. Specific features of metal tolerant plants were maintained in tissue culture (Wu and Antonovics 1978) which confirmed metal tolerance as an inherited trait.

Benefits of Metal Tolerance in Revegetation.

It is desirable that rational procedures be developed for the economic and permanent revegetation of all types of toxic mining wastes (Jeffrey et al. 1974). Derelict land produced by mining for heavy metals and processing of crude mineral ores are both visually unattractive and possible sources of environmental contamination. These areas need to be stabilized and the establishment of vegetation is considered advantageous in terms of cost and permanence (Bradshaw 1978). Investigations on mine wastes in England and Wales have shown that naturally occurring metal tolerant plant populations will grow and reproduce provided that fertilizer is supplied (Smith and Bradshaw 1972).

The aim of most plant metal tolerance tests is to explain the results of experiments in test culture in terms of adaptation to some identifiable soil factor (Wilkens 1978). Greenhouse experiments may indicate that heavy metal toxicity is one of the factors inhibiting growth on a toxic site, however, nutrient shortage or unfavorable temperature and water regimes may also be involved. Field trials using greenhouse populations of known metal tolerance can assess plant tolerance of physical and chemical conditions of the metalliferous site.

A number of environmental factors, other than metal toxicity, limit plant growth on metaliferous wastes (Bradshaw et al. 1978).

Mine populations of metal tolerant plants generally appear to have a slower growth rate than non-mine plants (Humphreys and Bradshaw 1977). There is evidence to suggest that low growth rate is an adaptation to nutrient limitations and other stress factors (Parsons 1968; Grime and Hunt 1975). Other observations indicate that metal tolerant plants are more drought resistant than non-tolerant plants on non-toxic soils. Possible morphological characteristics such as cuticle thickness, leaf size and pubescence may be of importance (Bradshaw et al. 1978).

Three methods of vegetative stabilization of metal contaminated areas are being used. These include procedures based on agricultural practice and methods dependent on plants especially selected for tolerance to toxic materials. A third approach, based on the amelioration of edaphic conditions and interpretation of many ecological parameters at a site, has proven successful (Jeffrey et al. 1974). Both old and modern metal mines produce wastes of principally two physical types: coarse waste rock and fine-grained tailings. Each presents important restrictions to plant growth. The coarse spoils retain very little water whereas the fine-grained spoils are devoid of clay sized minerals and organic matter which contribute to the water-retaining properties and cation exchange capacity of normal soils (Bradshaw et al. 1978). Metalliferous spoils are invariably deficient in nitrogen and phosphorus (Johnson and Bradshaw 1978). Acidic mine spoils are more toxic than calcareous spoil heaps, especially if the spoils contain high levels of pyrite (Gemmell 1973). The success of the revegetation technique depends on correct appraisal and treatment of the site factors that limit plant growth (Johnson and Bradshaw 1978).

The conditions which restrict plant growth on mining wastes are evidently complex but there are indications that heavy metal toxicity, soil nutrient status and soil physical factors are all involved (Gadgil 1969). Revegetation of older tailings which contain high levels of metallic minerals have shown the superiority of using tolerant plant varieties in the reclamation plan. Modern mine tailings have lower levels of heavy metals than tailings from earlier extraction processes. In some cases metal tolerant varieties have no growth advantage compared to non-tolerant varieties even in the long term (Bradshaw et al. 1978). However, metal tolerant populations tend to translocate fewer amounts of heavy metals into their aerial parts than non-tolerant populations. This would be advantageous if the reclaimed area was used for grazing (Smith and Bradshaw 1972).

The establishment and maintenance of a vegetative cover on metalliferous sites is difficult and requires management. Ideally, maintenance and aftercare costs should quickly reduce to zero, but in many situations it is unrealistic to expect this to occur (Down 1974). Steps must be taken to maintain a high level of organic matter such as by periodic additions of domestic refuse or sewage sludge or by allowing grass cuttings to rot in situ (Gemmell and Goodman 1978). Maintenance fertilizer treatments are essential to counteract leaching of nitrogen, complexing of phosphate and the accumulation of nutrients in undecomposed organic residues (Johnson et al. 1977). Retarded decomposition of metal tolerant vegetation as compared with that on uncontaminated sites has been indicated (Williams et al. 1977). An essential characteristic of permanent revegetation of a metalliferous site is a low level of productivity (Johnson et al. 1977).

A logical and ecologically sound revegetation program using metal tolerant plant material should be incorporated into the scheme for reclaiming metalliferous mine sites (Jeffrey et al. 1974). Metal tolerant vegetation has been extensively studied for use in revegetating metal contaminated sites in various countries around the world. It has great potential for revegetating such sites in the United States.

MATERIALS AND METHODS

Collection of Plant Material

Plants of *Agrostis tenuis* and *Deschampsia cespitosa* were collected from a naturally revegetated site on a copper tailings dump near Anaconda, Montana. Three distinct grass bunches, presumably genotypes, for each two plant species were collected from this site, as it has been found that different genotypes from the same habitat may differ in metal tolerance (Gregory and Bradshaw 1965). The grass genotypes were collected at an interval spacing of approximately 30 m during the month of February. February was chosen as it was believed that transportation some 180 km to the greenhouse would be less detrimental to the plants during their dormant phase, and also because of convenience. The 6 clones were immediately transplanted to 20 cm diameter plastic pots using a potting mixture of 50% Bozeman Silt Loam, (fine, loamy, mixed, Argic Pachic Cryoboroll), and 50% river-washed sand. Plants were allowed to multiply in the greenhouse, to provide enough material for the experiment. A period of growth in normal, non-toxic soil does not diminish heavy metal tolerance if it is gene controlled (Bradshaw 1952). Other experimental work has shown that prolonged cultivation (up to 13 years) on non-toxic soils lead to no decline in tolerance of vegetatively propagated material (Urguhart 1971). During the entire multiplication phase, plants were watered with city tap water to further reduce the chance of somatic alteration of the plants metal tolerance during the greenhouse phase.

Plant material, used as control populations for the experiment, was grown from seed obtained from commercial seed suppliers. *Agrostis tenuis* seed was obtained from the Oregon State Seed Laboratory, Corvallis Oregon while *Deschampsia cespitosa* seed came from a commercial seed source in Montana. Both commercial varieties were assumed to survive and reproduce successfully under climatic conditions similar to those of Anaconda, Montana. The seed was planted in aluminum greenhouse flats using the same potting mixture used for the tailings populations. After stands were established, plants were transplanted to 20 cm diameter plastic pots for the duration of the multiplication phase. This seed material was also watered as needed with tap water.

Collection and Analysis of Soil Material

A sample of the rooting medium was collected from the tailings dump near the site of plant collection. The method of analysis was water saturated paste extract (United States Soils Salinity Staff 1969). Results of the tailings analysis are found in Table 1.

Analysis of the root medium was undertaken to provide an indication of metal levels affecting plant growth on this site and to help select the most probable metals for tolerance testing. Low pH can affect the availability of micronutrients and may have some

Table 1. Soil analysis[1] from Anaconda copper mill tailings.

Test	Tailings Material (0-5 cm)
pH	3.3
Fe	3.3
Al	155
Zn	340[2]
Cu	364[2]
Mn	1000[2]

[1] Metal values in mg/l of water saturated extract.

[2] Exceeds established suspect levels by DTPA extraction, Montana State Regulations (Harrington 1977).

influence on extract values (Viets 1962). Levels of zinc, copper and manganese in water saturated extracts were high when compared to DTPA extractable levels established by Montana State Regulations (Harrington 1977). DTPA extracts the water soluble ions plus some additional ions thus relative comparisons between the two techniques is applicable when water saturated extract values are higher.

From the soil analysis, copper and zinc were chosen as the metals which were probably limiting plant establishment most severely. Due to time constraints and available funds, it was not possible to determine the tolerance of the plant material to aluminum and manganese. Aluminum and manganese have some influence on plant growth in the tailings material. However, without further testing, their toxicity is unknown in this study.

Metal Tolerance Testing

1. Experimental Technique

The method used for this experiment was an "in parallel" technique similar to the method described by Jowett in 1958 and later revised in 1964. This technique used separate sets of roots to compare root growth of grass tillers grown simulataneously in a control nutrient solution and three higher concentrations of the particular metal being tested. The control solution was standard Hoagland and Arnon's (1938) solution which contained small amounts of both copper and zinc. Jowett's method, utilizing a series of concentrations, allowed statistical analysis of root growth in varying levels of the metal being investigated. Other facets of Jowett's method were modified to control some of the possible shortcomings of his technique specifically, lack of adequate root aeration and changes in test concentrations.

Tillers from each clonal plant were assumed to be of the same genotype. Uniform sized tillers were separated from the clonal plant and rinsed with water to remove any foreign material. Roots were gently removed from the meristematic base of each tiller and the plant base immediately submerged in standard Hoagland and Arnon's solution buffered to pH 5.5 with NaOH. Air was bubbled through the solution to promote adequate aeration and root initiation. Tillers remained in this solution for 4 days and most had produced roots approximately 1 cm in length by this time.

Healthy tillers were selectively chosen from the previous phase and their longest root measured and recorded. Tillers from each of the 4 genotypes were transferred to the sand, drip culture apparatus used to test for metal tolerance (Figure 3). This apparatus was improvised from a drip culture apparatus described by Gadgil (1969).

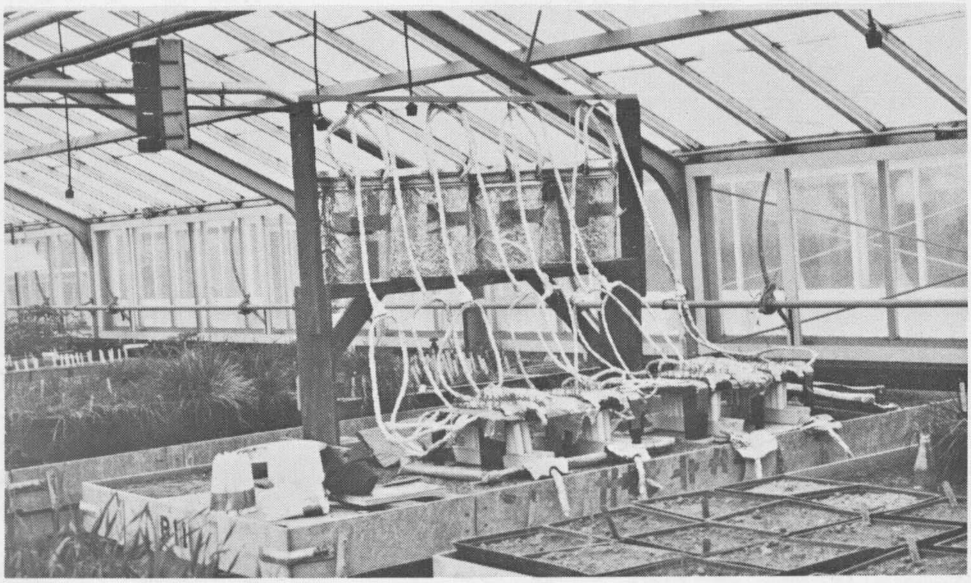

Figure 3. Sand, drip culture apparatus used in metal tolerance measurements.

The apparatus will be discussed in more detail in the next section. Tillers were allowed to grow for eleven days. The nutrient solution was monitored for changes in pH every two days and renewed on the fifth day of the test. Concentrations of copper and zinc were checked at the beginning and end of each renewal.

Changes in pH varied less than 0.2 pH units while copper and zinc concentrations changed less than 10% from their original concentrations. Solutions could have been changed more frequently to maintain more exact levels of pH and metals, however, the time, labor and expense required to gain such accuracy seemed futile for the precision needed to detect metal tolerance.

2. Apparatus

As previously stated, the basic idea for the sand drip culture apparatus was adapted from Gadgil (1969). Her sophisticated apparatus enclosed each tiller in a glass T-piece and trickled the culture solution down the suspended roots. The much less expensive apparatus used in this research utilized polyethylene tubes as the basic growth units. These tubes had a diameter of 2.5 cm and a length of 15.6 cm. Volume of each tube was 65.6 cm^3. The apparatus supported 64 tubes, or cells, which were separated equally into 4 treatments of 16 cells. A specially designed tray containing two treatments is illustrated in Figure 4. Each test concentration of metal, or treatment, had its own reservior from which the nutrient solution could flow by gravity to each individual cell. The flow to each cell was controlled by a screw-clamp and adjusted to a steady drip rate. Receiver bins, placed under each treatment, collected the nutrient solution and allowed it to be recycled.

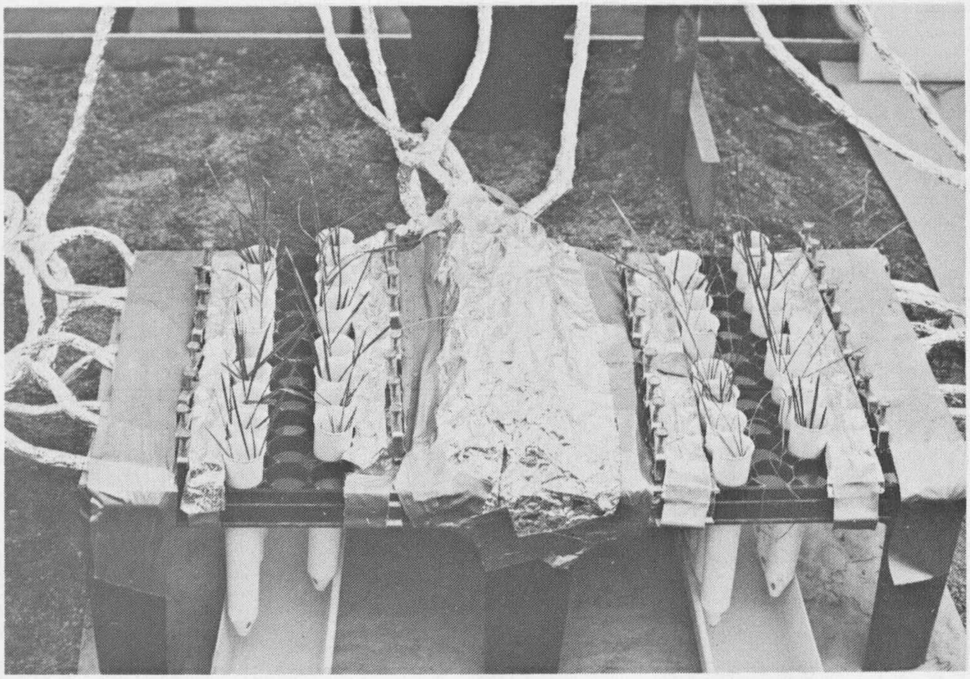

Figure 4. Apparatus tray containing two treatments and individual cells.

3. Aeration

Root aeration has been a problem in past metal tolerance techniques (Wilkens 1957; Jowett 1958). Wilkens, in 1957, felt that lack of aeration did not seriously interfere with root growth in *Festuca ovina* (Wilkens 1978). Most versions of tolerance testing techniques have placed the experimental plants in long glass tubes which were submerged in their specific test concentrations. Two problems have resulted using this technique. In addition to poor aeration, the test concentrations of toxic metal in the 3 mls of solution within the tube might be lowered because of plant root uptake or absorption (Wilkens 1978). The drip culture techniques designed by Gadgil were devised to overcome these two problems. The roots in the drip culture are ensured of adequate aeration and a fairly uniform concentration of toxic metal, since large volumes of solution can be used (Gadgil 1969). Gadgil's technique was modified as it was found through trial investigations that plant roots suspended in air were susceptible to desiccation in the larger growth tubes used for this experiment. The modification, which proved successful, consisted of placing the plant roots in acid-washed coarse sand. Course sand allowed adequate root aeration by remaining unsaturated during the testing phase. Hewitt (1966) found that cultures supplied with continuously renewed solution might require coarser sand than those given solution intermittently. Sand with a grading of 80-90% between 1.0 and 0.5 mm was satisfactory for a wide range of crop plants given nutrient solution intermittently (Hewitt 1966). The sand fractionation data found in Table 2 points out that the sand for these cultures was considerably more coarse than what Hewitt prescribed for intermittent supplied cultures. Nutrient solution presumably flowed through this sand at a rate which precluded the establishment of concentration gradients.

Table 2. Mechanical analysis of sand.

Size (mm)	% Fraction
>2.00	1
1.00-2.00	54
0.50-1.00	42
0.25-0.50	2
<0.25	1

4. Sand Preparation

Root medium was 99.7% SiO_2 river washed sand. Preparation entailed three basic processes. The sand was first soaked in 1.0 Normal nitric acid for twenty-four hours. The second step involved rinsing the sand with distilled water until the percolating effluent was similar in pH to the distilled water. Finally, the sand was saturated in nutrient solution at pH 5.5 until the pH of the effluent solution remained at that level. Sand needed for each test concentration was treated separately by immersing the sand in nutrient solution plus the concentration of metal involved. This procedure was adopted from Hewitt (1966).

5. Nutrient Solution and Test Concentrations

Four concentrations of copper and zinc were used to derive the metal tolerance for each plant species. The lowest concentration for copper tolerance testing was the concentration used by Hoagland and Arnon (1938) in their nutrient solution (Table 3). The lowest concentration for zinc measurements was 10 mg/l. Hoagland and Arnon's nutrient solution, often known as just Hoagland's solution, is a balanced solution incorporating four basic macronutrients, five micronutrients and iron.

There was some evidence of interactions between the toxic ion and other macro and micronutrients in the culture solution. Such interactions do not inevitably interfere with tolerance measurements, provided suitable concentrations are selected (Wilkins 1978). Realizing that interactions were occurring necessitated analysis with the atomic absorption spectrophotometer for actual metal concentrations.

Concentrated stock solutions were prepared from reagent grade chemicals and used to mix large volumes of solution. Sixteen liters of each metal concentration were prepared for each treatment of the experiment. Copper and zinc concentrations for each treatment are

Table 3. Composition of Hoagland and Arnon nutrient solution (1938).

Nutrient	g/l	mg/l
KNO_3	0.612	
$Ca(NO_3)_2$	1.312	
$NH_4H_2PO_4$	0.115	
$MgSO_4$	0.49	
H_3BO_3		2.86
$MnCl_24H_2O$		1.81
$CuSO_45H_2O$		0.08
$ZnSO_47H_2O$		0.22
H_2MoO4H_2O		0.09
$FeSO_4$	0.5%	} 0.6 ml/l 3 x weekly
Tartaric acid	0.4%	

shown in Table 4. It should be noted that actual metal concentrations for copper tolerance determinations were usually considerably less than the labelled level assigned to each treatment. This reduction was attributed to precipitation of the metal when the pH of the solution was raised to 5.5 (approximately 20 ml concentrated NaOH/161). Nutrient solution was not buffered for zinc tolerance

Table 4. Copper and zinc concentrations used for tolerance measurements.

Metal	Concentration (mg/l)		
	Labelled	Actual	
		Agrostis	Deschampsia
Copper	0.02	0.04	0.02
	0.5	0.28	0.38
	2.0	1.61	1.76
	4.0	3.01	3.42
Zinc	10.0	9.3	9.3
	50.0	51.0	51.0
	100.0	95.8	95.8
	300.0	286.0	286.0

determinations as it was found to drastically affect the higher zinc levels. The pH of nutrient solutions used in the zinc measurements was 4.3 ± 0.1.

The choice of heavy metal concentrations used to assess metal tolerance in plants was critical (Wilkens 1978). Ideally, a single concentration may be sufficient if it was properly detected. This level should sufficiently stunt root growth of the non-tolerant species while only slightly restricting growth of tolerant plant roots. This concentration may not exist for every metal and every species (Wilkens 1978). An alternate approach was to use a series of concentrations from which metal tolerance could be extrapolated. This latter method was chosen because these data can be analyzed statistically in many ways. In order to obtain a good relationship between root growth and metal concentration, the range of concentrations must be subjectively chosen (Wilkens 1978). This involved trial experiments for each species with varying metal concentrations to find levels both above and below the ideal level that Wilkens described. If levels of metals are properly chosen, a response curve can be graphed for each plant species and metal.

Statistical Analysis

The purpose of this research was to provide a test of metal tolerance for each plant species for each metal tested. The method of analysis chosen utilized relative root growth, rather than metal tolerance indices. Correlation of plant metal tolerance with soil metal levels was not a facet of this experiment because no other site with vegetation was involved. Relative root growth comparisons provide a sensitive test of tolerance (Wilkens 1978), thus satisfying the objectives of this experiment.

A variation of the split-plot design was used for this experiment because of difficulty in handling treatment combinations with the experimental apparatus and because of its merit of increased precision in estimating subplot effects and interactions (Little and Hills 1978). The main effects for Metal or Grass were of little concern, but a significant Metal x Grass interaction was of interest. This interaction, if significant, would indicate that the grass genotypes were reacting differently to the addition of the toxic metal, and thus that they differ in tolerance.

Response curves depicting the regression of root growth associated with increased metal concentration were generated for each section of this experiment. Each plant/metal relationship was composed of one curve for the tailings population and one for the commercial seed population. The response curve for the tailings population was formed from the mean of the three tailings population genotypes. Each genotype was replicated four times at each metal concentration. The statistical rational was that even though the tailings genotypes have varying degrees of tolerance, the information would be more representative of the entire population. This grouping of the tailings population was checked in the analysis of variance to ensure accuracy and to verify differences in the data.

The response curve for the commercial seed population was formed using data from only one seed source. It was calculated from the mean of four clones for each of the four metal concentrations.

RESULTS AND DISCUSSION

Copper Tolerance in *Deschampsia cespitosa*

Marked copper tolerance was found in *Deschampsia cespitosa* from Anaconda copper mill tailings in contrast to the copper tolerance of the commercial population used as a control. The relationship between root growth and copper concentrations of the two populations is plotted in Figure 5. Vertical lines refer to standard deviations around each mean. A statistical summary of relative root growth in response to four levels of copper is presented in Table 5.

Significant differences were found in the subplot interaction Copper x Grass and the subplot comparison of Grass genotypes. To pinpoint the source of variation within the Grass comparison, the sum of squares for this comparison was partitioned into sum of squares due to the tailings population and sum of squares due to the commercial population. The F-tests for this comparison indicates that most of the variation between grass genotypes was due to the difference between the mill population and the commercial population.

Significant differences were also found for the Copper x Grass interaction. This source of variation indicated that the grasses had variable responses at different levels of copper. The partitioning of the sum of squares for the Copper x Grass interaction into variation due the mill population and commercial population, (Table 5), indicated that the dominant source of variation was due to differences

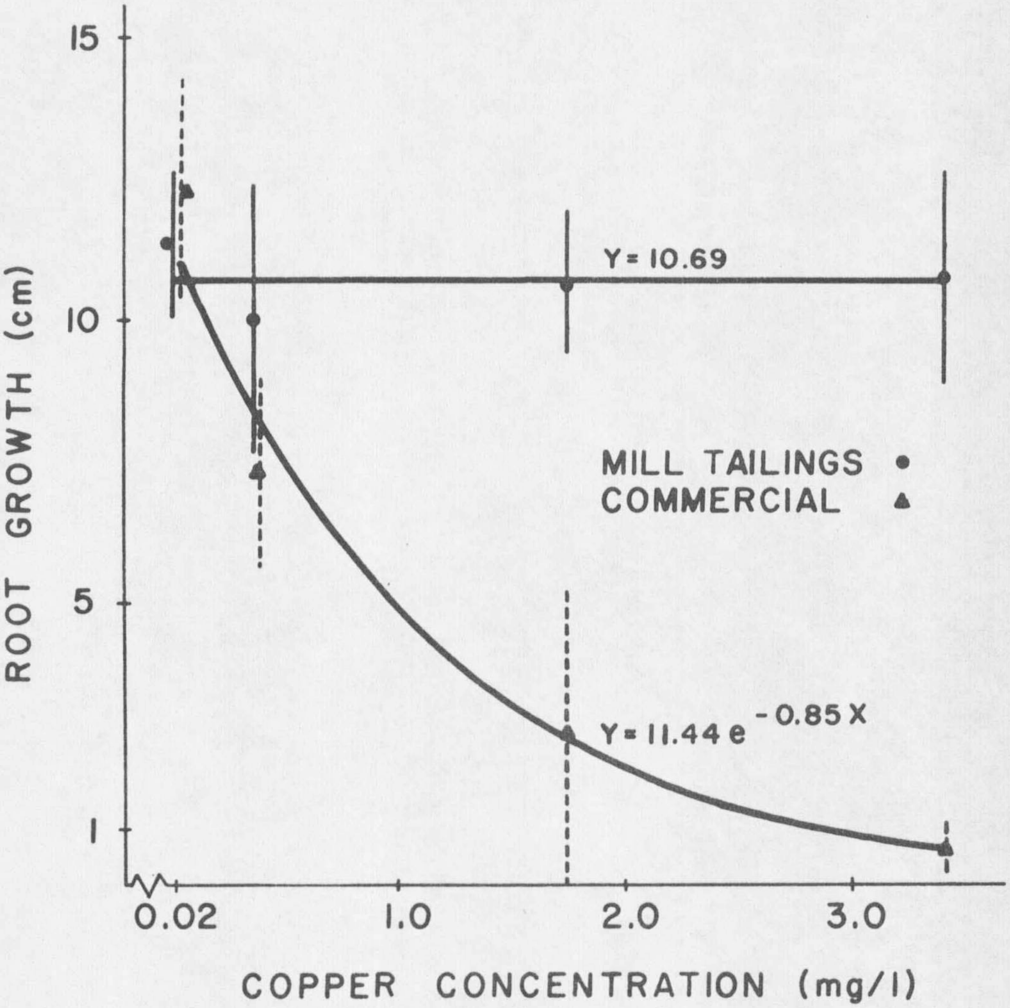

Figure 5. Root growth of mill tailings and commercial seed populations of Deschampsia cespitosa in response to 4 levels of copper.

in tolerance of the two populations. The three tailings genotypes were statistically similar in their response to copper. Normally, different genotypes from the same habitat differ in performance. This difference did not occur for these genotypes when testing the effect of copper concentration on length of root growth. Figure 5 illustrates that the copper tailings population sustained a higher rate of root growth when compared to the commercial population over the range of copper concentrations used for this assessment. The copper tailings population was more tolerant to copper than the commercial population.

Problems were encountered when standard regression techniques were applied to the relationship between root growth in the tailings population and copper. Calculations for the coefficient of determination (r^2) indicated a very poor linear regression. Other curves were applied to the data, resulting in either very poor fits or so complex to be artificial and devoid of physical and biological meaning. A second analysis of variance table was calculated to facilitate the understanding of the graphical representation (Table 6). The proportion of the total treatment sum of squares accounted for by linear regression was determined. Tests of significance verified that a linear regression of root growth was not occurring for the tailings population in its response to copper at the levels used in this experiment. Because the outer region of the response curve was reasonably flat and that standard deviations could account for much of the fluctuations, a linear line was believed to best represent the relationship. A horizontal line was plotted for the mean of all points, however, it can be assumed that at some higher copper concentration the tolerant populations would have reduced root growth.

Copper Tolerance in *Agrostis tenuis*

Agrostis tenuis from the tailings population was more tolerant to elevated levels of copper than the commercial seed population of the same species. The linear and curvilinear response curves for these populations and their standard deviations are presented in Figure 6. The analysis of variance may be found in Table 7.

Significant differences were found for all main separations of this test, these being the Copper, Grass, and Copper x Grass comparisons. Interest was focussed on the Grass and Copper x Grass comparisons as these indicate different responses between genotypes and genotypic relationships with copper. Both comparisons were partitioned into the sum of squares due to differences associated with the two populations.

Significant differences were found in both partitions of the Grass comparison, however, it was observed that the Tailings vs. Commercial partition accounted for most of the variation. This indicated that the two populations did behave quite differently in the

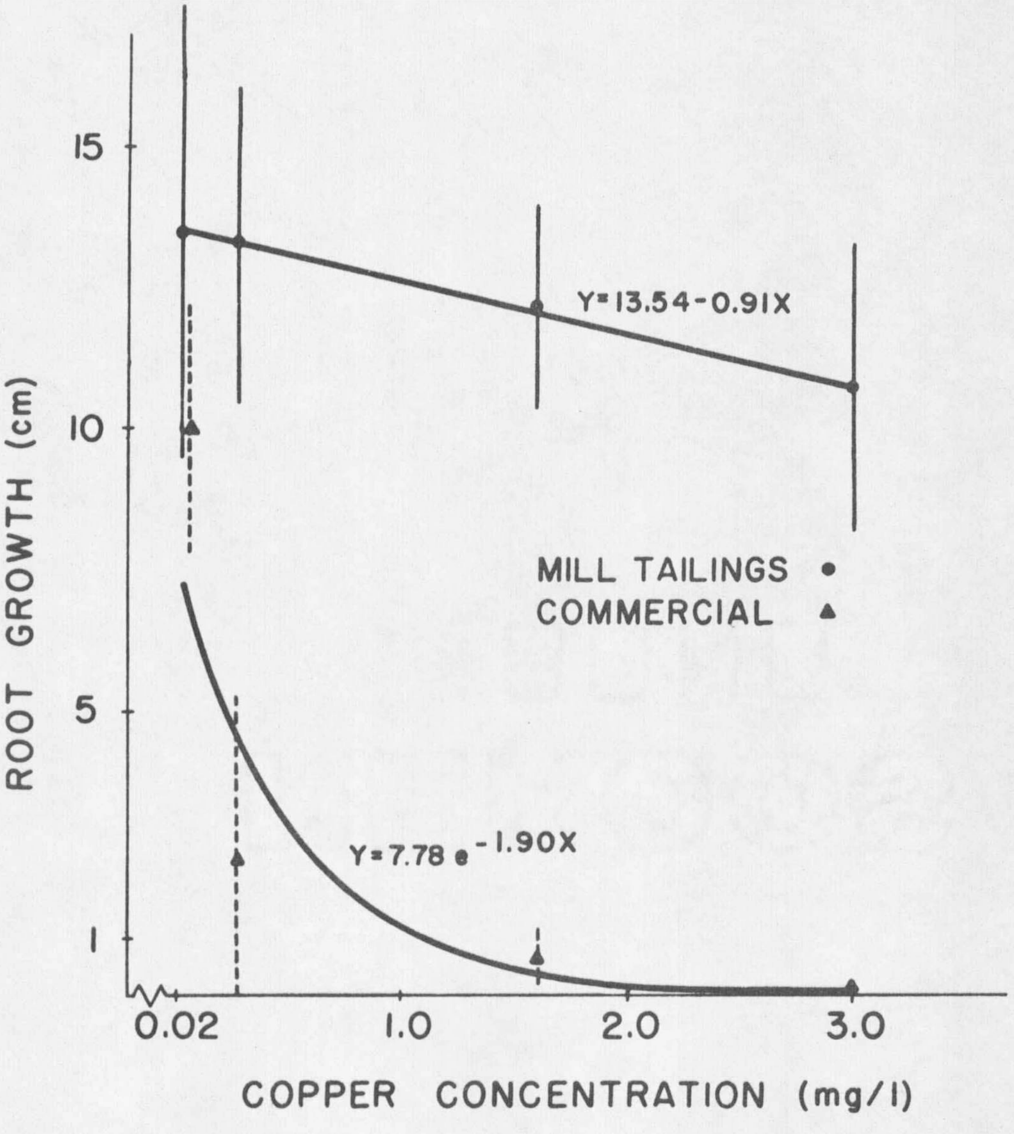

Figure 6. Root growth of mill tailings and commercial seed populations of *Agrostis tenuis* in response to 4 levels of copper.

experiment. The significant residual partition for the Grass comparison indicated there were genotypic differences between the tailings plants, however, they were not on the same scale as the differences between the two populations.

The Copper x Grass interaction was also found to be significant, thus indicating a difference in copper tolerance between the four Deschampsia cespitosa genotypes used in this assessment. Variation due to differences between the two populations accounted for the calculated significance of this comparison. The tailings population was found to tolerate higher concentrations of copper than the commercial population and, thus, was recognized as having conditional copper tolerance. Its known occurrence on copper mill tailings can be partially explained by this tolerance.

The non-significant residual term for this Copper x Grass interaction can be interpreted as meaning the three tailings genotypes were responding similarly to additions of copper. For this experiment, using these copper concentrations, it may be assumed that one response curve for each population would adequately depict root growth on copper relationships.

The response curves in Figure 6 illustrate the relationships for these two populations. The relationship for the tailings population was best described by a straight line. Standard deviations were quite large as is often encountered when working with biological data, however, the means of twelve tillers, used for this response curve, were found to be well represented by linear regression with a coefficient of determination (r^2) value of 0.99. The commercial population's response to copper was best represented by an exponential curve. This regression, had a coefficient of determination (r^2) of 0.96.

Zinc Tolerance in Deschampsia cespitosa

The tailings population of Deschampsia cespitosa was found to be significantly more tolerant to zinc than the commercial population used as the control. The regressional response curves are presented in Figure 7. The analysis of variance table may be found in Table 8.

Genotypic difference were found in this test and were found to be associated with differences between the tailings and commercial populations. The major concern was focussed on the Zinc x Grass interaction. This interaction was found to be non-significant, however, upon partitioning the sum of squares into sum of squares due to differences between populations and the remainder, it was found there were significant differences. Root growths for the two populations were reacting significantly different in their response to zinc. The tailings population was significantly more tolerant to zinc than the commercial population over the range of zinc concentrations used in this test. The Residual term was not significant, thus the

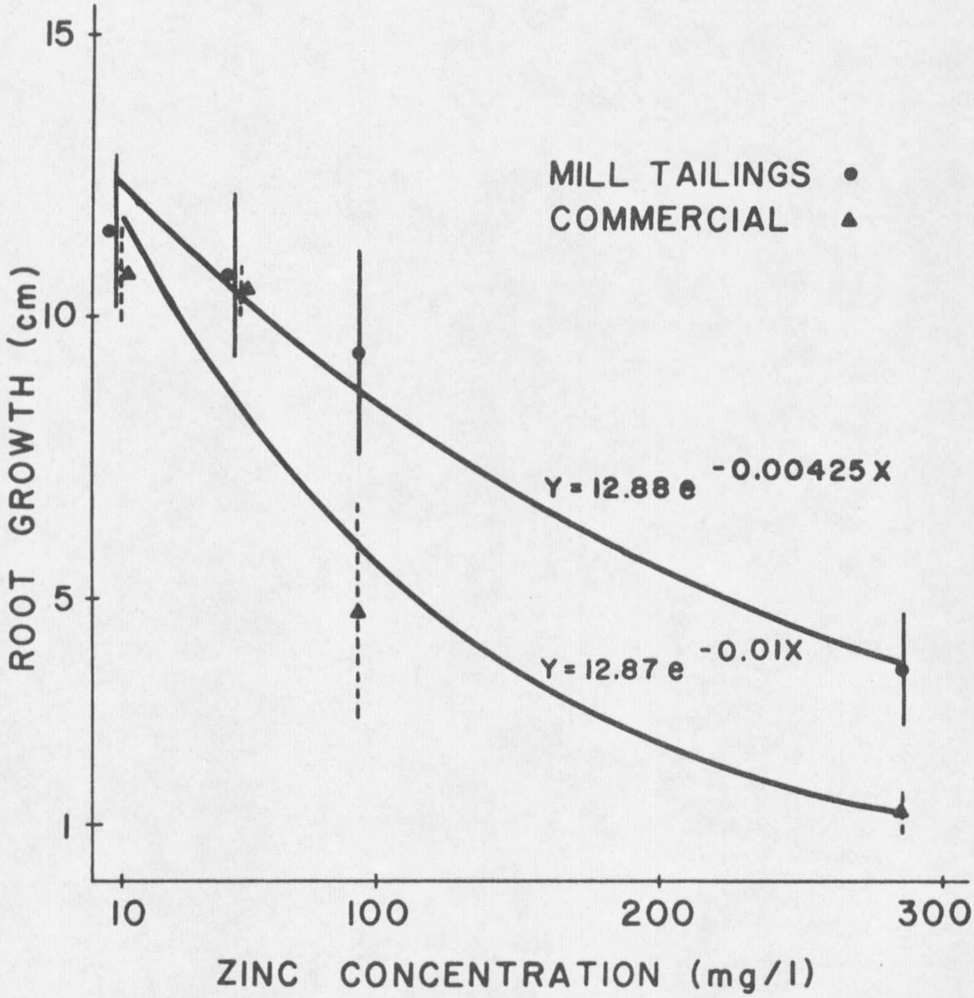

Figure 7. Root growth of mill tailings and commercial seed populations of <u>Deschampsia cespitosa</u> in reponse to 4 levels of zinc.

three tailings genotypes could be assumed to be responding to zinc similarly.

The response curves in Figure 7 for the tailings and commercial populations possess coefficients of determinations (r^2) values of 0.94 and 0.97 respectively. Statistically, these curves gave a good indication of how root growth was influenced by zinc. However, upon close examination, one or more points appear to deviate some distance from the regression line. Realizing that one equation may be unable to accurately describe a complex relationship like metal tolerance, complications will be discussed.

One complication to this relationship is the fact that low concentrations of potentially toxic substances may cause an increase rather than a decrease in root growth (Allen and Sheppard 1971). The means and standard deviations for the 51 mg/l zinc concentrations in Figures 7 and 8 show this phenomenon had occurred. Multiple concentrations can be used to adjust for this stimulative effect, by concentrating attention on the other parts of the response curve. These stimulated regions of root growth were not adjusted for in this experiment, however, because they did not appear to substantially alter the graphic meaning of the relationship.

Another complication found in solution culture experiments is the occurrance of less precise demarcations of the parameter being measured. For this experiment, the complete nutrient solution, supplied continuously, allowed grass tillers to tolerate the toxic effects of the ions. The cessation of root cellular elongation is the ultimate consequence of heavy metal toxicity. In natural situations, the failure to produce adequate root biomass would cause mortality by drought. In solution cultures mortality is postponed, with graphical results of the toxicity represented as an exponential curve.

Zinc Tolerance in *Agrostis tenuis*

Zinc tolerance in the two populations of *Agrostis tenuis* was not conclusive. The populations were statistically similar in their response to zinc. Response curves for both populations are presented in Figure 8 while the analysis of variance table may be found in Table 9.

The statistical summary presented in Table 9 contains some interesting determinations. Significant differences were found for the Zinc x Grass interaction, which indicates there were differences in zinc tolerance within the four genotypes measured. When the genotypes were grouped by populations, significant differences were not encountered. The three tailings genotypes, when averaged, were statistically similar to the commercial population.

Agrostis genotypes from the tailings population differed significantly in their tolerance to zinc. Each tailings genotype could have been compared separately to the commercial population and

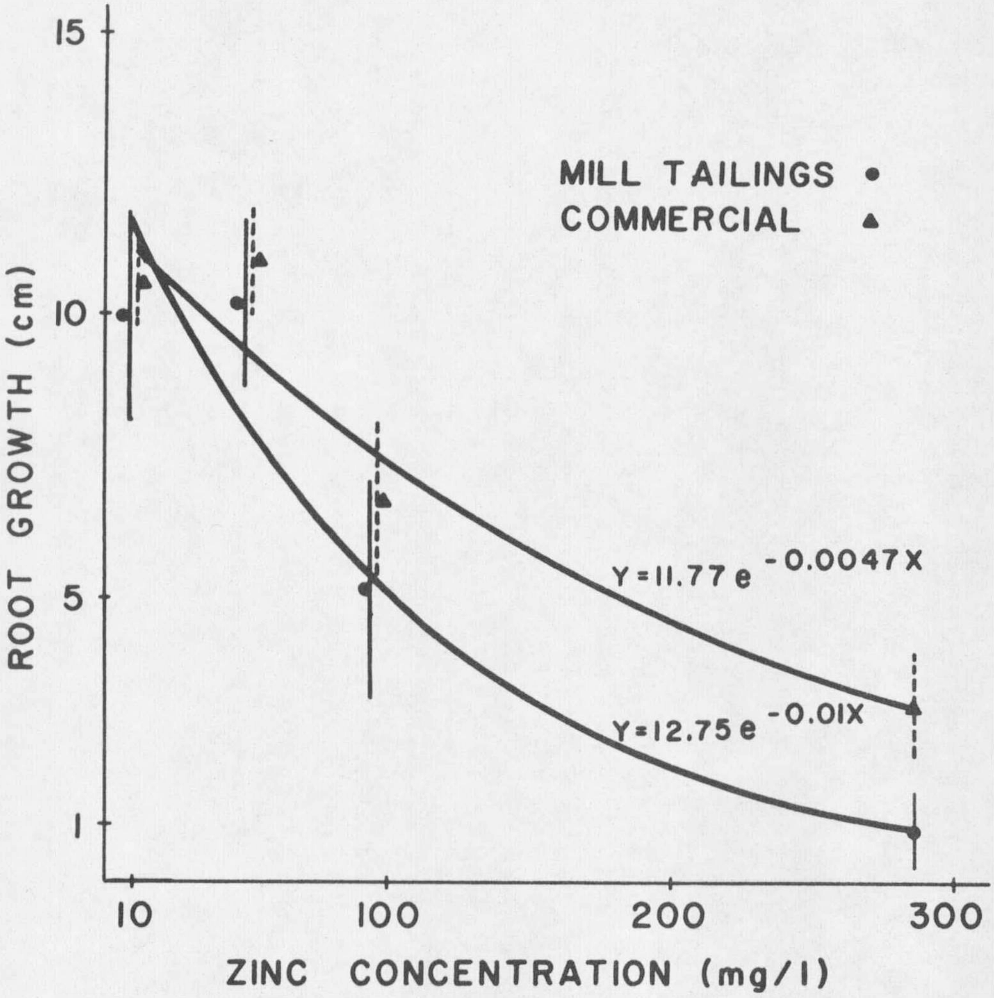

Figure 8. Root growth of mill tailings and commercial seed populations of Agrostis tenuis in response to 4 levels of zinc.

to the other genotypes of the same population. Results of this analysis would have merit if they were relevant to answering the objectives of this experiment. They are not. Genotypes of the tailings population were used to gather information on a range of metal tolerance possibly encountered within the population. They were treated as samples and their intrinsic value is bound to the population, not to each sample.

One response curve for each population is shown in Figure 8. The stimulative effect of low concentrations of an essential element like zinc is again prevalant at the 51 mg/l level. This stimulus may itself be related to tolerance. Tolerant plants which in some way exclude toxic but essential elements from the cytoplasm at high concentrations, may conversely take up the element with difficulty at low concentrations (Wilkins 1978). At low concentrations the non-tolerant plants may have a growth advantage which could partially explain how competitive forces may be excluding tolerant plants from normal soil.

Multiple Metal Tolerance in Tailings Populations

Metal tolerances may evolve as a response to genetic selection caused by the inhibitory effect of elevated metal levels in the soil. A theory states that (a) evolution of tolerance to one metal does not confer tolerance to other metals and (b) multiple metal tolerances only evolve in response to the appropriate combinations of metals in the soil (Jowett 1958; Antonovics et al. 1971). This theory has been questioned but generally is still reliable. Examples of tolerance to more than one metal has been reported for Agrostis sp. (Jowett 1958), Festuca ovina (Wilkins 1960), Anthoxanthum odoratum (Antonovics 1966; Bradshaw et al. 1965), and Deschampsia cespitosa (Cox and Hutchinson 1980). In most cases, multiple metal tolerance has been well correlated with high soil levels of the metals from which tolerance was assessed. An exception was reported in an Agrostis tenuis population which was zinc tolerant but also nickel tolerant, despite the lack of nickel in its soil of origin (Gregory and Bradshaw 1965).

Comparison of results from metal tolerance experiments using root growth measurements are risky. Although a standardized technique was used, other physiological and climatic variables were allowed to fluctuate. By design, this technique required no control over photoperiod and only moderate control of temperature. It was noted that greenhouse temperatures often fluctuated 20° C.

Parallel controls used in this experiment are less affected by changes in conditions than sequential controls. Values obtained in this way should be reproducible in successive experiments and should have value in comparisons between metal tolerances of the same plant species (Wilkins 1978). For comparisons of metal tolerances of different species, tolerance indices or ratios must be calculated for each population. Ratios were not determined for this experiment, since response curves would produce more valuable information for evaluating metal tolerance. Comparisons from the data were restricted to tolerance of copper and zinc for the same plant species.

Results of tolerance testing in *Deschampsia cespitosa* appear to indicate multiple metal tolerance. In separate experiments using these metals, the tailings populations produced significantly more root growth than the commercial population used as the control. Statistical analysis confirmed a significantly higher tolerance to copper and zinc for the tailings population of *Deschampsia cespitosa*.

Results for *Agrostis tenuis* tested for tolerance to copper and zinc did not confirm multiple metal tolerance. Tailings populations were significantly more tolerant to copper than the commercial ecotype. Evidence of increased tolerance to zinc was not conclusive. Without significant analysis, some tolerance to zinc can be assumed because *Agrostis tenuis* is growing on a metalliferous site containing appreciable levels of available zinc. Evidence which helped to support this assumption suggested that copper tolerance in a population of *Agrostis tenuis* may confer some ability to survive on high zinc soils (Walley et al. 1974).

SUMMARY AND CONCLUSIONS

Many derelict metalliferous mine sites in the United States are presently devoid of a protective vegetational cover and are potential sources of environmental pollution. Stabilization of mine wastes which contain high levels of metals is needed to control adverse effects on terrestrial and aquatic ecosystems and improve their visual appearance. The optimum solution to these problems is vegetative stabilization involving naturally occurring metal tolerant species. Metal tolerant vegetation has been reported to be more adapted to metal toxicities, macronutrient deficiencies and unfavorable soil physical properties.

Greenhouse experiments have indicated that naturally occurring populations of *Deschampsia cespitosa* and *Agrostis tenuis* from mill tailings possess metal tolerance. The *Deschampsia cespitosa* population was found to be tolerant to copper and zinc while *Agrostis tenuis* possessed only copper tolerance. Commercial populations of each species were used in this assessment as controls.

An "in parallel" measurement technique utilizing separate tillers in four metal concentrations was used for tolerance testing. Multiple concentrations allowed the calculation of regression of root growth on test concentrations. Confirmation of metal tolerance was made by statistical analysis. The apparatus used for testing was adapted from one described by Gadgil (1969). This drip culture apparatus was modified by placing tillers in coarse acid-washed sand to provide a more humid environment for root growth.

The need for metal tolerant plant material for use in reclaiming metalliferous sites has been made known. The grass species found growing on copper mill tailings at Anaconda, Montana have shown tolerance to elevated levels of copper and zinc. An analysis of tailings revealed high plant available levels of copper and zinc thus the mill tailings populations must be tolerating the metals in the tailings.

This technique is an economical and applicable method for measuring heavy metal tolerance in plants. Confirmed tolerance is the primary step in facilitating a comprehensive reclamation program for metalliferous areas. Further investigations, preferably in the field, are needed to substantiate the results from the greenhouse and to select the more promising species for seed propagation and large scale trials.

APPENDIX