EVALUATION OF SEED AND SEEDLING RESPONSE TO AID REVEGETATION OF HAZARDOUS CHEMICAL WASTE SITES

by

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(ABSTRACT)

The response of several plant species to heavy metal contaminated soils was evaluated using plant bioassays with a soil substrate. A natural soil was collected from Dinwiddie County, Virginia and soil analysis was performed. The plant species, Lolium multiflorum, Setaria italica and Trifolium repens latum, Robinia pseudoacacia, Andropogon gerardi, Asclepias syriaca, Echinacea purpurea, Rudbeckia hirta and Festuca rubra were grown in to determine the response to cupric and cadmium chloride in soils (mg Cu/kg soil). A few plant species were grown in small pots in a plant growth chamber for 28 days using control, 10, 30, 100 and 300mg Cu or Cd/kg soil. Germination proved to be less sensitive than root length. S. italica had highest EC50s. In Cu 20.7 and 15.3 in Cd. All plant species were grown for 7 days in 0.3, 1.0, 3.0 10.0, and 30.0mg Cu/kg soil and in control. Germination was not effected by metal concentrations in most species (p=0.07-0.6), except

T.repens latum, R. hirta and F. rubra at 30mg/kg (p=0.0007). Root length was significantly effected by Cu concentrations for almost all species (p=0.0001-0.0112). Setaria italica had the highest EC50 at 10.86mg/kg. Robinia pseudoacacia root length was not significantly affected by Cu concentrations. The other species had EC50s ranging from 3.74-7.51mg/kg. Both inhibition and stimulation of root growth were observed.

Preliminary studies regarding germination rates, fungicides and rangefinding are included.

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CHAPTER I

INTRODUCTION

Many of the products and processes of modern living contribute to the increase of heavy metal content of our environment. Industrial processes such as electroplating, circuit board production and petroleum refining are processes that increase our comfort level, but the bi-products of which contaminate the biotic system (USEPA, 1991; Meltzer et al., 1990; Adriano, 1986). Ore smelting in the United States and abroad produces emissions containing heavy metals that contaminate the soil and vegetation for miles downwind causing a 'pollution shadow' analogous to a rain shadow caused by mountain interference with rainfall patterns. Pollution shadows are prominent on the landscape because they contain little or sparse vegetation if metal content is high enough (Little and Martin, 1972; Roberts and Johnson, 1978; Hunter et al., 1987; Steinnes et al., 1988). Additionally, the rock waste produced by smelters, referred to as tailings are fine grained rock particles containing unextractable metals bound tightly to the rock particles. Usually disposed of in dikes or settling ponds (Noem and Day, 1985; Reith, 1986), these wastes are subject to wind dispersal toward uncontaminated sites. Metal wastes also enter the ecosystem as household landfill items such as Ni-Cd

batteries or as fertilizers and pesticides which create non-point source pollution problems in lakes and streams.

Excess heavy metals negatively affect the environment in many ways. Aquatic ecosystems become simplified in biotic structure with input of heavy metals (Herricks et al., 1974). In terrestrial systems, metals interfere with most of the aspects of plant growth and development, reducing the potential to germinate and root apices become shortened, stunted and brown (Foy, 1978; Savage, 1981). Photosynthetic and transpiration rates are reduced by heavy metals for many reasons, one of which is that metal ions induce stomatal closure (Bazzaz et al., 1974). Defects occurred on developing chloroplasts in 100 M Cd (Ghoshroy and Nadakavukaren, 1989) and CO_2-fixation was inhibited (Weigel, 1985). Other effects such as discoloration and wilting of leaves are evident with the uptake of metal ions (Foy, 1978).

In general, metal stressed ecosystems tend to have lower species diversity (Odum, 1985) because few species are able to tolerate the conditions. Natural invasion of vegetation, however, occurs on sites with extremely high metal content. Plant communities are found in soils of post-mining sites with as much as 1500mg/kg Cd or Cu (Jordan, 1975). And though plant cover is usually sparse and patchy, about 25%, some vegetation is found (Thompson

and Proctor 1983). Kimmerer (1982) surveyed the flora of lead-zinc mines in Wisconsin and found most were early successional native species like Solidago spp., Potentilla intermedia, Melilotus alba and Plantago aristata. Distinct vegetation assemblages occur on naturally formed serpentine soils found worldwide that are high in chromium and nickel (Walker, 1954). Baker, et al. (1986) found that plant species successful at colonizing metal-rich sites were also more tolerant of the extreme edaphic and climatic conditions suggesting that the possession of certain general stress tolerant physiological characteristics may enhance establishment success in sites with high soil metal content. Using serpentine plants as an example, Kruckeberg (1954) found them to be tolerant of low calcium levels. Jowett (1959) found such an adaptation in Agrostis tenius to low calcium and phosphate levels. The problem may not be that revegetation cannot occur on disturbed sites but that natural processes are extremely slow and sources of plants able to persist under the conditions of the site may not be located near the site.

Vegetation is the biological element limiting wind and water erosion and immobilizes some wastes (Johnson and Bradshaw, 1977). Plants roots bind soil particles reducing the probability of erosion. Soil particles are also broken down by roots and vegetation increases the organic matter content aiding in the transformation of spoils over time to

productive soils (Russell, 1977). Rehabilitation of degraded soils will replace or duplicate the ecological function disrupted by metal stress (Cairns, 1979; Cairns, 1989).

Degraded soils, however, have properties that inhibit the establishment of vegetation. Large soil particles and a deficiency of organic matte result in low water holding capacity, low pH buffering capability and a reduced cation exchange capacity (Down, 1977; Ludeke, 1977; Paone et al., 1978). Direct sunlight on barren soil produces soil temperature extremes to which many temperate plants are not adapted (Bell and Ungar, 1980).

The Resource Conservation and Recovery Act of 1976 (RCRA) was enacted by Congress to reduce the potential of sites to become hazardous waste problems by regulating the management, production and disposal of hazardous wastes of companies while in operation. Abandoned sites fall under the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) commonly known as Superfund. It was established in 1980 to identify and clean up abandoned hazardous waste sites nationwide (USEPA, 1987b). There are 20 such sites in Virginia on the National Priorities List (NPL), about half of which are impacted with heavy metals (Department of Waste Management, 1990).

Metal contaminated soils have undergone an irreversible

soil by fixing it in a matrix of inert material such as concrete (The Hazardous Waste Consultant, 1992). Current efforts to chemically remove metals from soils by acid extraction have proven time consuming, expensive and damaging to soil characteristics rendering it unusable (Paff, 1991).

The methods used to revegetate mechanically disturbed areas are designed for fast and relatively easy coverage of large areas. The plants are selected for speed of germination, nitrogen-fixing capabilities and tolerance to harsh environmental conditions. The seeds are not necessarily native to the predisturbance area. Hydroseeding is one technique that employs pumping seeds and water over large areas. When a fertilizer and mulch are added, the procedure is referred to as hydromulching. Mulch cools surface temperatures and helps retain water (Wolf et al., 1984). A problem with spraying reseeding ingredients is mechanical damage to the seeds during the pumping process which may lower the percentage capable of germination (Kay 1977). The practice is sometimes referred to as 'spray and pray' (L. Daniels, Crop and Soil Environmental Sciences, VPI&SU, pers. comm.) because of its uncertain outcome.

Post-disturbance replacement of fertile soil over waste piles, called topsoiling, prepares wastes for revegetation and reintroduces native species (Wade, 1989). Stored

topsoil, however, has limited viability so as a strategy it must be selected during the planning phase. Over time the seeds degrade making germination less likely. The microorganism population changes as the soil dries and becomes less fertile overall (Schuman and Power, 1981). The seeds could also be grown under greenhouse conditions or in plant growth chambers to produce seedlings for transplantation (Vora, 1992).

Metal tolerance has been studied in a few plant species. Certain plants are able to form metal tolerant populations under some conditions. These species are invaluable in reclamation programs. Agrostis tenuis (Jowett, 1959; Herstein and Jager, 1986) and Holcus lanatus (Coughtrey and Martin, 1978; Baker et al., 1986) and Festuca rubra (Herstein and Jager, 1986) are three such plant species that tolerant populations to copper and cadmium. When the response by a plant species to a particular environmental toxin is known prior to its use, revegetation success can be increased (Hill, 1983). Theoretically, if a list of plants with known thresholds for various chemicals were available, reclamation managers could select plant species based on whether the species exhibited an ability to thrive under the site conditions. The information would be obtained from laboratory toxicity tests using natural soil close in physical and chemical characteristics to that

of the site.

Plant species are being tested in various ways for usefulness in mitigating metal wastes. A wild mustard that potentially bioaccumulates selenium is being tested to reduce the concentration in impacted areas (Mine Waste Management, 1992). Sphagnum dominated human-made wetlands are being designed to absorb iron and magnesium (Wieder et al., 1985; Gerber et al., 1985).

Risk assessment of hazardous waste sites is the main role of plant species in hazardous wastes, besides revegetation. Plant bioassays are designed to assess the relative toxicity of hazardous waste sites by evaluating plant response (USEPA, 1989a; Gorsuch et al., 1990). The plant species are selected for their demonstrated sensitivity to changes in the growing environment. The Environmental Protection Agency (EPA) recommends the use of lettuce (Lactuca sativa as a test organism because it has been shown to be sensitive to many potential toxicants (USEPA 1989a). Other species recommended by the Food and Drug Administration (FDA (1987) include oats (Avena sativa), wheat (Triticum aestivum L.), corn (Zea mays L.), cabbage (Brassica oleracea L.), soybean (Glycine max L.), tomato (Lycopersicon esculentum Mill.). Selection of these species is also based on demonstrated sensitivity, short germination time (FDA, 1987). While these tests of plant

little information regarding rehabilitation of the land in question is provided. Rather than evaluate the risk presented by the wastes, similar procedures could be developed to determine the usefulness of plant species in revegetating the land.

The aim of this research is to evaluate the response of various plant seeds and seedlings in soil contaminated with heavy metals. The protocol of the bioassay provides a basic framework for this evaluation. Several bioassay techniques have been developed. Plant bioassays have been performed using solution cultures or soil eluates of the toxicant and a variety of methods of exposing the seed to the solution. Petri dishes with seeds on filter paper (Wang, 1987) or seeds lying on glass plates with chromatography paper in glass tanks of solution (USEPA, 1983b; USEPA, 1989b) are two such techniques. Growth pouches made of absorbent paper which extends into the toxic solution is another variation. The seeds are placed in a fold of paper at one end with the other extending into the solution (Gorsuch et al., 1990). All methods are inexpensive, require little technology, are easy and produce results rapidly. Other techniques involved the use of pure silica sand in petri dishes sealed inside plastic bags to eliminate contamination (USEPA, 1989a; Thomas and Cline, 1985). The latter method is more environmentally

representative because of the soil substrate employed. This methodology will be used for evaluating plant responses in the current research.

A variety of growth characteristics have been used in plant bioassays to measure the effects of toxicants. Plant biomass, shoot height, root/shoot ratios, germination proportions and root length are mainly used in risk assessment. Root length, however, has been shown to be the most sensitive and plant characteristic to measure (Wang, 1987; Herstein and Jaeger, 1986; Gorsuch et al., 1990).

The purpose of this research is to adapt the plant bioassay protocol to evaluate plant response to environmental contamination in soils. The information will not be used to assess risk, but to supply reclamation managers with the limits of contamination a species can grow under. The test materials will consist of metal contamination of a natural soil and plant species native to Virginia that could be used in revegetation efforts. Assessment of the response will be determined by calculating an LC50 (the lethal concentration at which 50% of the seeds fail to germinate) and the EC50 (the effective concentration at which root length is 50% of the control root length). Other parameters to be determined will be the highest concentration used that produces no effect on an organism (NOEC) and the lowest concentration that produces an effect on an organism (LOEC) (Federal Register,

1980). The method used will follow that of Thomas and Cline (1985) which uses intact soil as the substrate. This research will use a natural soil as the substrate to increase the environmental applicability of the results (Cairns and Pratt, 1989).

CHAPTER II-A

INTRODUCTION

Preliminary testing was begun using 3 plant species. Robinia pseudoacacia L., black locust, Festuca rubra L., red fescue and Solidago nemoralis Aiton, gray goldenrod (Gleason and Cronquist, 1991) were chosen because they are considered natives of Virginia and because they represent different vegetation types. Additionally, black locust is already widely used in revegetation schemes because it is a legume and produces root nodules that fix nitrogen. It can survive in a wide range of environmental conditions and provides cover for later emerging shade-tolerant species (Ashby et al., 1985).

Gray goldenrod is found in most of the counties of Virginia on dry, rocky, shallow soils and is tolerant of a wide range of pH conditions. It has not been used as a reclamation species because of the belief that it causes hay fever (Bob Lyons, Horticulture Dept, VPI&SU, pers. comm.).

Creeping red fescue is a forage crop that is not as widely used in reclamation as its relative tall fescue Festuca elatior which is not native to Virginia (Harvill et al., 1986). Red fescue is mainly used for hillside stabilization such as highway slopes (Strausbaugh and Core,

1977).

The seeds were purchased from various merchants. Red fescue was purchased from a local seed supplier. Black locust was purchased from a tree supplier in Massachusetts and goldenrod from Wisconsin. All seeds were stored inside a plastic bag with a paper liner to absorb moisture and stored at 4°C (Young and Young, 1986).

The tests in this Chapter were undertaken to determine such things as time until germination can be considered complete, necessity of pretreatments and effectiveness of nutrient solutions.

CHAPTER II-B

PRETREATMENT OF FESTUCA RUBRA

INTRODUCTION

The literature suggested a cold-moist pretreatment for Festuca rubra to decrease the time in which full germination would occur (Williams, 1983). This seed was chosen to work with first because of its availability and high germination potential.

MATERIALS AND METHODS

One sheet of #1 Whatman filter paper circles were trimmed to fit the bottom half of 20 - 100mm diameter disposable petri dishes. The paper was wetted with 2 ml ddH_2 (deionized distilled) and approximately 50 seeds were randomly distributed over it. All lids were replaced on the dishes and 10 dishes were additionally covered with foil. All dishes were placed in a dark plant growth chamber set at $4^{o}C$. After 5 days cold moist treatment 5 of the dishes with foil and 5 without foil were randomly selected and removed to a second plant growth chamber set at germinating conditions - a 16/8 hour photoperiod with corresponding temperatures $20^{o}C/10^{o}C$. Because of dryness and edge browining, two more sheets of filter paper were added below the seeds in the plates. Germination counts

were begun on day 5 in the new conditions and made periodically for a total of 11 days.

Determining moisture levels was difficult so watering was done individually during the cold period. Four additional milliliters of ddH_2O were added to all dishes in both chambers the day before the earliest germination counts were made.

The remaining dishes were kept in cold moist conditions for 9 days and then placed in germinating conditions. These seeds received extra filter paper simultaneously with the 5-day cold treatment seeds. Again water was added as needed.

After germination conditions began, fungi or mold began appearing on several of the dishes. The affected seeds were discarded so as not to infect others. By the end of the 11 day period, 5 dishes of the 5-day cold treatment and 5 dishes from the 9-day cold treatment had been affected. No futher analysis regarding the fungi or mold was performed.

Since the germination proportions exhibited variance heterogeneity, the proportion that germinated after 11 days for both groups was arcsine transformed (in radians) and an analysis of variance was done to determined whether presence/absence of light during germination or the length of the cold treatment resulted in a difference in total germination.

RESULTS AND DISCUSSION

The analysis of variance provided statistical evidence that germination occurred equally well under both lighting conditions and both periods of cold treatment ($p = 0.5812$ and 0.05). There is slight evidence, however, that with lights the seeds began germinating earlier than without. Germination could have been affected by an ineffective watering schedule.

CHAPTER II-C

EFFECT OF NUTRIENT SOLUTION ON GERMINATION

INTRODUCTION

The ability to thrive under metal stress for a plant may be complicated by soils low in essential nutrients. Fertilizers can be used to ameliorate soil deficiencies in field experiments. In the laboratory, however, solutions containing the necessary nutrients can be controlled.

The experiment was designed as a two-way anova with a type of pretreatment crossed with and without nutrient solution to determine whether a specific pretreatment and/or nutrient solution would improve germination. Festuca rubra and Solidago nemoralis were used.

MATERIALS AND METHODS

Nutrient solution was prepared according to Hoagland and Arnon solution #1 (1950) in ddH_2O. Five days before seeds were to be placed in germinating conditions, six sterile petri dishes were fitted with three sheets of Whatman #1 filter paper wetted with 5ml of either ddH_2O or nutrient solution producing replicates of each treatment. Approximately 40 seeds were placed in each dish under 1 additional sheet of filter paper for stratification. With lids on, the dishes were wrapped in aluminium foil and placed in cold ($2^{o}C$) moist conditions for 5 days. This was

done for S. nemoralis and F. rubra.

On day 1 a second set of seeds in petri dishes were assembled for S. nemoralis and F. rubra without pretreatment and both sets were placed in the plant growth chamber set to germinating conditions, a 16/8 hour photoperiod with corresponding temperatures of 25° and 15°C (Williams, 1983). The aluminum foil from the pretreated group was removed. The seeds were grown for a total of 9 days before final germination counts were made.

Fungus was noted and quantified for each plate by multiplying the percentage area covered by the density of the fungal masses - light=1, medium=2, dark=3. The product was divided by 10 which provided quotients that were easier to manipulate. The quotient was used to estimate relative fungal invasion in statistical analysis.

To reduce variance heterogeniety the germination proportions were arcsine transformed (Sokal and Rohlf, 1981), (in radians)(SAS User's Guide, 1985). Statistical analyses were performed using analysis of variance of germination as affected by pretreatment and nutrient solution.

RESULTS AND DISCUSSION

The germination of Solidago nemoralis was significantly increased by pretreatment for 5 days in cold, moist

conditions (p=0.0115). Nutrient solution, however, did not effect germination (p=0.3658). Germination proportions were not affected by either pretreatment or nutrient solution in Festuca rubra (p values = 0.2885 and 0.2169). Fungus was not statistically associated with either the pretreatment or the nutrient solution for either seed (p values = > 0.2000).

An unidentifed light blue stain appeared on the filter paper of most of the plates by day 5 in germination conditions. It did not appear to be associated with the seeds, so seed stain was ruled out. It occurred in all F. rubra plates and is not believed to be fungus, either.

Pretreatment did not appear to enhance Festuca rubra germination and indicated the possiblity that such pretreatment could be dropped in future experiments with this seed. The significant difference in germination for S. nemoralis indicates the continued use of pretreatment for this seed.

Nutrient solution was not effective in increasing germination and will not be used in future studies. At the outset, it was felt that the nutrient solution might encourage the growth of fungi, but this turned out not to be the case.

CHAPTER II-D

VIABILITY STUDY

INTRODUCTION

Because of poor germination, viability studies were carried out on Festuca rubra and Robinia pseudoacacia. Solidago nemoralis was not used because it is too small a seed, approximately 2mm in length.

MATERIALS AND METHODS

A tetrazolium solution (0.1%) was made by mixing 1g 2,3,5 triphenyltetrazolium chloride in a 1000ml ddH_2O (Association of Official Seed Analysts, 1970). Festuca rubra and Robinia pseudoacacia seeds were presoaked in plain ddH_2O overnight by placing them on wet Whatman #1 filter paper in petri dishes. They were then sliced in half to expose the embryo. The seed halves were placed in petri dishes with their cut surfaces in contact with Whatman #1 filter paper that had been soaked in the tetrazolium chloride staining solution. The petri dishes were sealed inside plastic ziploc bags and placed in a plant growth chamber at 20^{o}C. After 11 hours of staining, the seeds were removed from the stain and scored. The criteria used to assess viability was successful staining of the radicle in R. pseudoacacia which is very prominent and staining of the radicle region for F. rubra

(Association of Official Seed Analysts, 1970). Percent viable was calculated as the ratio of stained seeds over number of seeds used.

RESULTS AND DISCUSSION

Successful staining of F. rubra averaged 93.5% in two replicates consisting of approximately 100 seed halves each. R. pseudoacacia viability was 87% in one replicate of approximately 100 seed halves.

In the F. rubra trials, 15 seed halves did not stain at all, possibly because the seed was not cut through the embryo. F. rubra seeds are extremely slender, less than 2mm, making an accurate longitudinal cut difficult even with a dissecting microscope. R. pseudoacacia seeds, on the other hand, are a bit larger and the embryo of R. pseudoacacia is extremely easy to identify with a dissecting microscope. While slicing through the seed, however, the root axile was easily broken off. This occurred so frequently that only one replicate petri dish was obtainable.

The F. rubra and R. pseudoacacia seeds obtained are viable. The staining of F. rubra averaged 93.5% and the viability of R. pseudoacacia 87%. This test determines that the embryos are alive but whether germination will occur is not certain. It is recommended that approximately 90% germination be obtainable in toxicity testing, and with

viability close to 90%, the probability of germination is more certain.

CHAPTER II-E

FILTER PAPER STUDY

INTRODUCTION

Use of terrestrial plants in the recovery or reclamation of hazardous chemical waste sites has focused on the evaluation of the toxicity present by measuring plant response (Gorsuch et al., 1990; Linder et al., 1988). To that end, many of the plant species used, such as lettuce (Lactuca sativa L.) or cucumber (Cucumis sativaL.) are favored because of their demonstrated sensitivity to adverse conditions (Wang, 1987). Their response, however, relates little information about how well a revegetation species might fare when seeded onto the site.

Single species toxicity tests have been used to assess and monitor environmental contamination problems (USEPA, 1987a). The testing protocol was originally developed for aquatic environments, but has since been applied to terrestrial systems (USEPA, 1989a; FDA, 1987).

The purpose of this study is to use the toxicity test protocol to determine the response of several plant species, which are potentially usable in revegetation efforts, to heavy metals. Two important life history stages will be evaluated, germination and early root growth, to cadmium and copper ions in solution.

MATERIALS AND METHODS

Seeds were obtained from local commercial seed suppliers. Black-eyed susan (Rudbeckia hirta L.), annual rye (Lolium multiflorum L.), German foxtail millet (Setaria italica (L.) P. Beauv.), crownvetch (Coronilla varia L.), creeping red fescue (Festuca rubraL.) and big bluestem (Andropogon gerardi Vitman.) (Gleason and Cronquist, 1991) were used.

Stock solutions of copper and cadmium chloride were prepared by dissolving ACS reagent-grade chloride salts in ddH_2O (deionized, distilled water) (Thomas et al., 1986). Dilution of the stock solution of $CuCl_2$ and $CdCl_2$ produced 10, 30, 100 and 300mg/l solutions. Control replicates were made with ddH_2O.

Forty seeds were planted on 3 sheets of Whatman #1 filter paper in the lower half of 100mm sterile plastic petri dishes (Scherbatskoy et al., 1987). The dishes were amended with 5ml of the appropriate concentration of $CuCl_2$ or $CdCl_2$ solutions or ddH_2O. Six replicates of each species/metal/concentration combination were assembled and sealed with parafilm to lower the evaporation rate. Red fescue and big bluestem were pretreated by chilling at $4^{o}C$ for 10 days before the start of the test (Young and Young, 1983; Williams, 1983).

All petri dishes were randomly distributed between two

identical plant growth chambers in a randomized complete block design. They were grown in a 16/8 hour photoperiod at constant 24°C for 5 days. The dishes were watered as needed. Germinated seeds, defined as having 1mm growth of radicle through the seed coat, were counted every other day and all seeds were allowed to grow for a total of 28 days. The roots of 10 plants per dish were randomly selected and measured directly. The coleoptile that developed on the grass seeds was measured.

The germination proportions displayed variance heterogeneity with the Wilkes-Shapiro test for normality so the proportions were arcsine transformed (in radians) prior to analysis of variance to reduce variance heterogeneity. blocked over chamber and replicates. The means from each concentration were compared using Student-Newman-Keuls procedure (Sokal and Rohlf, 1981). LOEC and NOEC values, (lowest observable effects concentrations and no observable effects concentrations, respectively) were determined for each species. LC50s (concentration at which 50% of the control failed to germinate) were calculated using Spearman-Karber method (Hamilton, 1977). Least squares regression analysis was used to evaluate dose-response relationships for germination using the log10 of the metal concentration. Polynomial regressions were used in a stepwise manner to evaluate the relationship of copper to root or coleptile length. EC50s (concentration that pro-

duced root length 50% of controls) were calculated for each species using inverse prediction where linear regressions were determined (Sokal and Rohlf, 1981).

RESULTS

Tables 1 and 2 contain the NOEC, LOEC, LC50 and EC50 values for copper and cadmium trials.

On the third day of the incubation period, a temperature spike occurred in one of the plant growth chambers. The parafilm on the dishes was destroyed, drying out the seeds prematurely. Germination means were not statistically affected, but root lengths were visually shorter and in most cases not present at all. Therefore the information on root lengths is based solely on three replicates from the unaffected plant growth chamber.

Germination responses for annual rye and German foxtail millet were quite similar. Both species attained nearly 99% germination in controls and exhibited no significant difference in germination in 30mg/l or less in either metal. Statistical differences did occur, however, at 100mg/l and at 300m/l in both metals. The NOEC and LOEC were 30mg/l and 100mg/l respectively. Germination LC50 for annual rye was 122.58mg/l Cd (112.96 - 133.00) and 144.08mg/l Cu. German foxtail millet LC50s were similar - 151.44mg/l Cd and 154.92mg/l Cu had EC50 of 6.71mg/l Cd and 5.21mg/l Cu.

Annual rye EC50 values could not be calculated because the coleptiles were measured instead of the roots.

German foxtail millet failed to sustain roots in any concentration higher than 10mg/l in either metal. The EC50 in Cu was 5.21mg/l and 6.71mg/l Cd. Both dose-response relationships are best described by linear equations; in Cd $Y = 6.82 - 2.0X$ with $r^2 = 0.91$ and in Cu $Y = 6.96 - 2.19X$ with $r^2 = 0.93$.

Black-eyed susan attained 89% germination in controls. Again no significant differences in germination occurred in concentrations below 100mg/l - the NOEC was 30mg/l and LOEC was 100mg/l. LC50 values were 59.7mg/l Cd and 196mg/l Cu. Root response in Cd was $Y=0.98 - 0.18X$ with $r^2=0.45$, a linear response and in Cu it was $Y = 1.17 - 0.46X + 0.45X^2$ with $r^2 = 0.84$, a quadratic response.

Red fescue attained less than 50% germination in control dishes. Previous testing with this seed produced germination much closer to 100% in controls. The only significant difference in germination from controls occurred at 300mg/l, the LOEC, for both metals. The LC50 in Cd were 92mg/l and 127mg/l in Cu. There was no regression correlation between Cd concentrations and root lengths, $p=0.1335$. A quadratic equation expresses the relationship in Cu, $Y=0.47+0.08X-0.026X^2$ with $r^2 = 0.78$. EC50 was 128mg/l Cu.

Crownvetch controls did not germinate above 20% in

either metal, which is not unusual for this species (Lee Daniels, Crop and Soil Environmental Sciences, VPI&SU, pers. comm.). Though germination was poor overall LC50 values were estimated at 8.43mg/l Cd and 17.78mg/l Cu. EC50 values could not be determined.

Big bluestem failed to germinate in appreciable amounts and was dropped from the study.

CONCLUSIONS

The dose-response curves for germination were linear for annual rye, German foxtail millet and black-eyed susan after transformation. Coefficients of determination for the least squares regressions were above 0.7 with p-values of 0.0001 for these species. Though linear relationships exist between concentration and germination counts, germination shows the same insensitivity to heavy metals as has been found in previous studies. Patterson and Olson, 1982 found no reduction in the germination of woody species grown on filter paper in concentrations ranging from 0 - 100mg/l Cu. Germination in 10mg/l Cu was not effectively reduced in conifers or hardwood species (Scherbatskoy et al., 1986). Miles and Parker, 1979, found no effect on the germination of little bluestem or black-eyed susan, grown in Cd contaminated soil. Previous studies on black locust produced no significant differences in germination below

100mg/l in solution studies. Some germination occurred even at 1000mg/l $CdCl_2$ solution.

Except for crownvetch and black-eyed susan, the test species appeared to respond quite similarly to Cd and Cu during germination. Crownvetch, however, normally exhibits low percentage germination and erratic growth. Black-eyed susan appeared more sensitive in Cu than Cd.

Annual rye germinated and produced longer roots than other species in this test design. German foxtail millet appeared to be fairly insensitive to both copper and cadmium during germination but extremely sensitive to both metals during root extension. The poor germination of red fescue indicates a problem with the test set-up. In previous tests by the author, red fescue germination was normally close to 100% within 7 days.

It is not possible to generalize about plant species responses to stresses. Responses may be taxon dependent (Mine Waste Management, 1992; Patterson and Olson, 1982; McCreight and Schroeder, 1982). Or a species response may be stress dependent (Godbold and Hutterman, 1985; John and van Laerhoven, 1976; Heale and Ormond, 1981).

Follow-up studies involving a soil medium will ensue to compare dose-response relationships using a more environmentally realistic growing medium. A natural soil with characteristics of a degraded soil will be collected and used in testing.

Table 1. Germination parameters of plant species grown for 28 days in Cu and Cd solutions (mg metal/l).

Cadmium	LC50	Confidence limits	NOEC	LOEC	SLOPE	CV(%)
Annual rye	122.58	(112.96-133.00)	30	100	2.73	35.41
German foxtail millet	151.44	(130.44-175.91)	30	100	2.70	20.17
Black-eyed susan	59.66	(54.61-65.19)	30	100	2.62	35.24
Copper						
Annual rye	144.08	(130.00-159.66)	30	100	3.18	23.33
German foxtail millet	154.92	(141.55-169.55)	30	100	2.73	25.57
Black-eyed susan	196.07	(173.24-221.93)	30	100	1.17	20.62

Table 2. Root length parameters of plant species grown for 28 days in Cu and Cd solutions (mg metal/l).

Cadmium	LC50	Confidence limits	NOEC	LOEC	CV(%)
Crownvetch	8.43	(2.92 - 21.25)	30	100	20.37
German foxtail millet	6.71	(1.48 - 22.81)	30	100	46.71
Black-eyed susan	None	None	---	---	-----
Copper					
Crownvetch	17.78	(8.42 - 36.60)	30	100	27.56
German foxtail millet	5.21	(0.83 - 20.75)	30	100	32.41
Black-eyed susan	7.40	(1.21 - 30.84)	30	100	63.70

CHAPTER II-F

THE SOIL FROM DINWIDDIE COUNTY

INTRODUCTION

A soil was obtained from Dinwiddie County, Virginia, taken from a break between a crop field and a forest which had been leveled and planted to White Pine. The soil is classified as an UCHEE soil (Mike Guenthner, CSES, VPI&SU, pers. comm.) which has as characteristics a loamy, silicaceous texture of thermic temperature class. Taxonomically it is an arenic hapludult, of fine loamy sandy texture approximately 20-40" thick. A ultisol with >35% base saturation of humid soil moisture regime (Agriculural Handbook #436).

Approximately 20 five-gallon buckets of B-horizon soil were obtained from a depth of less than 12in. The soil was air-dried, ground through a 1cm screen and stored in covered containers at room temperature. Soil analyses for physical characteristics were performed by the Soils Laboratory at Virginia Polytechnic Institute and State University. Chemical characterization was performed on an inductively coupled plasma spectrometry (ICP-AES)(Isaac and Johnson, 1983) for plant nutrients and cadmium. Results of all laboratory studies are in Table 1.

Table 1. Soil analysis generated by the ICP-AES in the Soil Testing and Plant Analysis Laboratory at VPI&SU (mg/kg soil).

Manganese	3.36	Calcium	72.7
Copper	0.09	Magnesium	7.5
Iron	10.90	Sodium	3.5
Aluminum	21.50	Sulphur	6.39
Boron	0.05	Zinc	0.97
Phosphorous	0.46	Potassium	113.64

Water-holding capacity 17.4g/100g soil.
pH 5.50
Base saturation 4.76%
Texture is sandy loam

CHAPTER II-G

FUNGICIDE TESTING

INTRODUCTION

Preliminary studies generated germination rate and germination potential information for Robinia pseudoacacia. It was selected as the best candidate for preliminary soil testing because of its easy pretreatment, rapid germination time, and high germination potential. The pretreatment, soaking the seeds in boiled water overnight, was suggested by the seed supplier as a method to break R. pseudoacacia's hard seedcoat. With this pretreatment more than 80% of the seeds germinated within 10 days of planting in ddH_2O.

During a preliminary soil test of germination rate, fungi appeared on R. pseudoacacia seeds. The source of the fungi was conjectured to be either the growth of normally occurring fungi on the seed coat induced by the hot water pretreatment or the growth of fungi from the soil, which was not sterilized. The purpose of this test was to determine whether a different pretreatment and/or a fungicide would reduce the occurrence of fungi. Though no problems had been found with Festuca rubra and Solidago nemoralis, they were also tested for germination response under various fungicide treatments in a second experiment. They were pretreated in cold moist conditions for 9 days,

though all other conditions in the two tests were similar.

MATERIALS AND METHOD

The literature and personal recommendations advised the use of three fungicides. Benomyl, an agricultural systemic fungicide, is normally applied in aqeous solution to soils. Two surficial fungicides, sodium hypochlorite (Chlorox bleach) in a 3:1 dilution and hydrogen peroxide in a 0.1% solution, were also recommended. For R. pseudoacacia the three treatments were crossed with 3 pretreatments. The hypothesis was that no significant difference in germination percentages due to fungicide treatment alone or combined with a type of pretreatment would be found.

These three treatments were crossed with 3 different pretreatments for R. pseudoacacia as follows: piercing each seed and soaking in deionized distilled water (ddH_2O) for 2 days on filter paper in 25°C or piercing alone without soaking. The third set, the control, was not pretreated nor was fungicide used on it.

Each fungicide was used alone on a set of R. pseudoacacia seeds and then the systemic fungicide was combined with each of the two surficial fungicides. A number of seeds were placed within several layers of cheesecloth and fastened with a rubberband to form a pouch. The pouch was suspended in a flask containing the surficial fungicide

with circulation provided by a stirbar for 3 minutes (Young and Young, 1986). The pouch was then placed in several successive flasks of ddH_2O to rinse. Forty seeds from the pouch were planted on soil in a petri dish to which 5ml of either ddH_2 or Benlate solution was added, providing approximately 85% water holding capacity. A replicate of each pretreatment/treatment combination was prepared for a total of six combinations including a control group. The petri dishes were placed in a plant growth chamber at 24°C for 10 days. Moisture was maintained by sealing each dish inside a ziploc bag. Data were recorded as the number of seeds germinated daily.

The test for R. pseudoacacia was designed as a completely randomized two factor model. The factors, fungicide and pretreatment method, are both fixed variables. Preliminary analysis using univariate normality plotting and analysis of variance were performed to determine whether the pretreatments, fungicides or a combination of the two would effectively aid germination. A one-way anova was used to analyze the germination of Festuca rubra and Solidago nemoralis blocking over replicates.

RESULTS

Univariate plotting, to assess the normality of the data and variances, was performed on arcsined germination

proportion data (reported in radians) over the three pretreatments and over the fungicide treatments. The results from the Shapiro-Wilkes test, for the pretreatments and the fungicide treatments ranged from 0.84 to 0.92 (p-values = 0.12 to 0.89) indicating the data values are a random sample with a normal distribution.

Analysis of variance of R. pseudoacacia response was performed using the transformed data. Both main effects, pretreatment and fungicide significantly affected germination, p-values=0.0005 and 0.0007, respectively at the 0.05 significance level. The combined effect of the pretreatment and fungicide revealed no signicant interaction, p-value=0.4719. The correlation coefficient for the model was 0.80. The Student Newman-Keuls procedure indicated differences between the means of the pretreatments. The pierced soaked seeds produced the highest germination and were significantly different from pierced only these were significantly different from control seeds. The germination means for the fungicide treatments were not significantly different except for both treatments using sodium hypochlorite, which also produced the lowest germination.

Fungus was present on 50% of the dishes in the study. Analysis of variance was performed on the Festuca rubra and Solidago nemoralis transformed data. There was no

statistical difference between the germination response of the fungicides used, p-values=0.45 and 0.22 for Festuca rubra and Solidago nemoralis, respectively.

CONCLUSIONS

Pretreating R. pseudoacacia by piercing and soaking improved germination significantly over both non-pretreated or piercing alone. This is expected because proper hydration is essential to all seeds. And because R. pseudoacacia has a hard seedcoat, this pretreatment provided access for water entry and allows time for imbibition. The lack of difference between germination means produced by control, hydrogen peroxide and Benomyl fungicides and their combinations, indicated that these fungicidal treatments did not improve germination in this test. The usefulness of a fungicide, at least during short-term testing is not apparent.

The fungicide treatments did not reduce the appearance of fungi in this test. Any affect may be hidden by the small replicate number. The method used to measure the fungus could be improved by quantifying the fungal biomass directly.

CHAPTER II-H

RANGEFINDING

INTRODUCTION

Three rangefinding tests were conducted to determine the concentration of cadmium that would inhibit the germination of three native Virginia plant species, Solidago nemoralis, Festuca rubra and Robinia pseudoacacia in degraded soil. The concentrations used were 0.6, 4.0, 6.0, then 10, 25, 50, 100 and finally 200, 300, 400, 500mgCd/kg soil as $CdCl_2$. The tests were conducted sequentially and each had its own set of control dishes.

MATERIALS AND METHODS

The soil was collected from Dinwiddie County, Virginia in the fall of 1990. Soil preparation and the results of physical and chemical analysis are in Chapter II-F.

For testing, the lower half of petri dishes were filled with soil. Solutions of the test concentrations of cadmium chloride were made by diluting ACS reagent grade cadmium chloride in distilled, deionized water. 10ml of the proper solution was distributed evenly over the surface of the soil. The soil was allowed to equilibrate for approximately 3 hours. Forty seeds of R.pseudoacacia, and approximately 60 of S. nemoralis and F. rubra were planted on top of soil. Each dish was sealed inside a plastic

ziploc bag to maintain moisture and prevent contamination of the plant growth chambers. The plant growth chambers were set on a 16-hour light and a corresponding temperature cycle ($20^{o}C/10^{o}C$). Germinating seeds were counted daily beginning day 2 during the 1 week test period.

To reduce variance heterogeneity, germination proportions were arcsine transformed (in radians). The Wilkes-Shapiro test for homogeneity of variance was used to determine the need for this step. Analysis of variance was performed to test for significant differences between the germination means of the cadmium concentrations.

RESULTS

All p-values from analysis of variance were greater than 0.05, ranging from 0.08 to 0.97 indicating no significant difference in germination between the concentrations. There was good germination at all cadmium levels and for all species.

DISCUSSION

It is difficult to draw conclusions from these experiments. The data indicate that there are no clear differences in germination among all concentrations of Cd applied.

Overall, F. rubra had the best and fastest germination which ranged between 48.3 and 76 percent. Growth for F.

rubra began within a day or two of planting. Pretreatment of this seed proved to be unnecessary. The best germination was obtained in soil dosed at 50 and 100mg/l and equalled the control germination. R. pseudoacacia was also very responsive in the treatment process. Its germination ranged from 23 to 71%. It too germinated well without pretreatment. S. nemoralis had slightly lower germination percentages ranging from 19 to 52%. The highest value was obtained, not in a control dish, but in the 6mg/kg soil test.

Because R. pseudoacacia is a large seed, it was easy to tell when germination took place according to criteria set in the prospectus. For the other two seeds this was not as simple due to the presence of the soil. It was difficult to tell exactly when the root penetrated the seed coat. Once the root penetrated the soil, the next clue to germination was the growth of the cotyledons or grass blade. In many cases, this became the criterion upon which germination was based.

Daily counts became difficult due to the number of seeds used. Forty seeds were used for the R.pseudoacacia, approximately 60 were used for S. nemoralis and F. rubra. With the large number of seeds per dish it was difficult to see exactly how many had germinated.

Germination is a vital but in this case not a very

sensitive portion of plant life cycle.

CHAPTER III

SEED AND SEEDLING RESPONSE TO CADMIUM AND COPPER I

INTRODUCTION

The three plant species to be used in the first of the definitive tests are Lolium multiflorum L., annual ryegrass, Setaria italica (L.) P.Beauv., German foxtail millet and Rudbeckia hirta L., black-eyed susan (Gleason and Cronquist, 1991). The first two species are non-native annual grasses widely used in reclamation efforts. Both provide quick cover to reduce erosion and provide protection for later emerging plants. Rudbeckia hirta is a native herb which could be used for revegetation purposes. Found throughout Virgina, a few of its varieties thrive in disturbed area.

This and the next two Chapters are the definitive tests performed on seeds using the natural soil obtained from Dinwiddie County (Chapter II-F). The range in which germination would display a response was determined by preliminary testing over 3 sets of ranges of $CdCl_2$ (Chapter II-H). The range was determined to be control, 10, 30 and 100mg/kg dry soil.

Trials with fungicides in preliminary testing determined it to be non-effective with a small sample size. Germination with Benomyl was only slightly higher than with

other fungicides (Chapter II-G) so the present test will use Benomyl, a systemic fungicide in half of the replicates in a 2-way analysis of variance.

MATERIALS AND METHODS

The soil was dosed with sufficient ACS reagent grade $CuCl_2$ or $CdCl_2$ from a stock solution to yield concentrations of 10, 30, 100 and 300mg metal/kg soil. The soil was allowed to air dry thoroughly before use, approximately 1 week. Sterile petri dishes were filled with 80g dry soil and brought to 100% field capacity with deionized, distilled water (ddH_2O). Forty seeds were placed atop the soil and covered with 10g additional soil of the same concentration. One half of the total of six replicates were begun two days later. Because of a problem with fungi, Benomyl, a commercial fungicide, was added to the ddH_2O according to manufacturers instructions to the first half. Each petri dish was placed inside a Ziploc bag and sealed. All dishes were randomly arranged in a plant growth chamber set for 24°C during the day (16 hours) and 15°C during night hours (8 hours).

One 20g sample from each batch of dosed but unused soil was mixed with ddH_2O in a 1:1 w:v slurry. After a 20 minute equilibration period with intermediate stirring, the pH was measured with a glass electrode (Peterson and Calvin, 1965).

Germination data were taken on the 5th day of the test and every third day until germination was complete. Germination was defined as 1mm growth of tissue protruding above the soil surface. The seeds were allowed to grow for approximately 28 days after which time a final count of germinated seeds was made. Watering was performed as needed. The seedlings were counted and removed intact from the soil. The roots were rinsed in ddH_2O and placed across a 1cm grid for photographing. Because the quality of the photographs did not allow for their use on a digitizer, the roots were measured indirectly. The number of grids each root intersected was counted and total root length was calculated using a correction factor according to Bohm (1979). A portion of the remaining soils from below the seed level was dried, reground and remixed. A 10g aliquot of dry soil was weighed and transferred to an acid-washed 50ml plastic centrifuge tube. The samples were agitated with 20ml DTPA (diethylen-triamine pentaacetic acid) solution (pH7.3) for 2 hours (Lindsay and Norvell, 1978). The samples were then centrifuged for 10minutes at 2000 rpm and poured through Whatman #42 filter paper (Lindsay and Norvell, 1978) into acid-washed 125ml plastic bottles. The extractants were diluted appropriately with ddH_2O and stored under refrigeration for metal analysis by aspiration into a Perkin-Elmer flame atomic absorption

spectrophotometer (model 240).

The Wilkes-Shapiro test for variance homogeniety determined the proportions to be of a non-normal distribution. The germination proportions for each concentration were arcsine transformed (in radians) to correct the variance heterogeneity. An analysis of variance was performed with the arcsined values against the log10 of the AA-determined soil metal concentration (mg metal/kg soil). The germination means were compared using the Student-Neuman-Keuls procedure (Sokal and Rohlf, 1981) which also produced the NOEC and LOEC values (Federal Register, 1980). LC50s plus confidence limits for germination were computed using the Spearman-Karber procedure (Hamilton et al., 1977; Hoekstra, 1991).

The root length mean of the 4 replicates produced at each concentrations per species was plotted against log10 of the AA-determined concentration. Least squares regression line parameters determined over the linearly declining portion of the dose-response curve were used to calculate EC50 values and confidence limits using inverse prediction (Sokal and Rohlf, 1981; Niederlehner, pers comm). Analysis of variance and the Student-Newman-Keuls procedure also produced NOEC and LOEC values for root length.

RESULTS

No fungus was noted on any of the experimental dishes with or without Benomyl during the duration of this test. The germination proportions of Cd treated seeds with Benomyl were significantly greater than seeds grown without Benomyl in all species. No significant difference in germination occurred between Benomyl treated seeds when compared to seeds not treated with Benomyl.

These data failed the Shapiro-Wilkes test for homogeniety of variance and were transformed by arcsining germination proportions. Analysis of variance of arcsined germination proportions performed for each species in separate metals were found to be significantly different at a significance level of 0.05 (p-values = 0.0001) over log10 of the concentrations. The NOEC, LOEC and LC50 estimates and confidence limits calculated using Spearman-Karber procedure are indicated for each species and metal in Table 1 for Lolium multiflorum and Setaria italica. The 28 day LC50 estimates for Lolium multiflorum were 22.99mg/kg (19.1 - 22.56) in copper and 30.24mg/kg (20.64 - 24.14) in cadmium. Setaria italica LC50 in copper was 35.3mg/kg (32.06 - 38.94) and in cadmium was 23.55mg/kg (21.768 - 25.48). Rudbeckia hirta controls had only 3% germination, so LC50 data could not be generated.

Dose-response relationships (Figure 1) for root lengths are linear for Lolium multiflorum and Setaria italica over the concentrations in which roots were present after 28 days. The EC50 for Lolium multiflorum in copper was determined to be 7.65mg/kg (4.36 - 11.72) and in cadmium 11.98mg/kg (5.0 - 21.08). Setaria italica EC50s were 20.74mg/kg (17.52 - 24.45) in copper and 15.27mg/kg (10.8 - 20.48) in cadmium. Rudbeckia hirta total root lengths in controls averaged 1.2cm and greater than 60cm in the lowest concentration of both metals making EC50 calculations impossible and probably meaningless. There were no living roots in any concentrations higher than 30mg/kg at the end of the test.

AA-determined concentrations for treated but unused soil for copper were 12.13, 36.18, 124.34 and 392.0mg/kg for nominal concentration of 10, 30, 100 and 300mg/kg, respectively (Table 3). For cadmium control soil concentrations were 16.56, 8.4*, 148.8, and 456mg/kg over the same nominal concentrations. These values are based on only 1 sample per level. Untreated control soil was not extracted so no comparisons could be made. *(The AA-determined concentration of the 30mg/kg cadmium soil is extremely low at 8.4mg/kg and is either a misreading of the absorption or the sample was taken from an area of soil not as well mixed).

Table 1. Germination parameters of plant species grown in soil Cu and Cd (mg/kg) for 28 days. (95% confidence limits).

Copper

Plant Species	LC50	NOEC	LOEC	CV%
Lolium multiflorum	22.99 (19.1 - 22.5)	10.8	34.7	48
Setaria italica	35.3 (32.1 - 38.9)	36.3	117.5	43

Cadmium

Lolium multiflorum	30.24 (20.6 - 24.1)	14.7	40.0	41
Setaria italica	23.55 (21.8 - 25.5)	14.8	36.8	45

Table 2. Root Length Parameters for plant species grown in soil for Cu and Cd (mg/kg) for 28 days. (95% confidence limits)

Copper

Plant Species	EC50	NOEC	LOEC	CV%
Lolium multiflorum	7.7 (4.36 - 11.7)	0.0	10.8	55
Setaria italica	20.7 (17.5 - 24.5)	1.0	13.5	18

Cadmium

Lolium multiflorum	12.0 (5.0 - 21.08)	1.6	14.7	55
Setaria italica	15.3 (10.8 - 20.5)	1.7	14.9	24

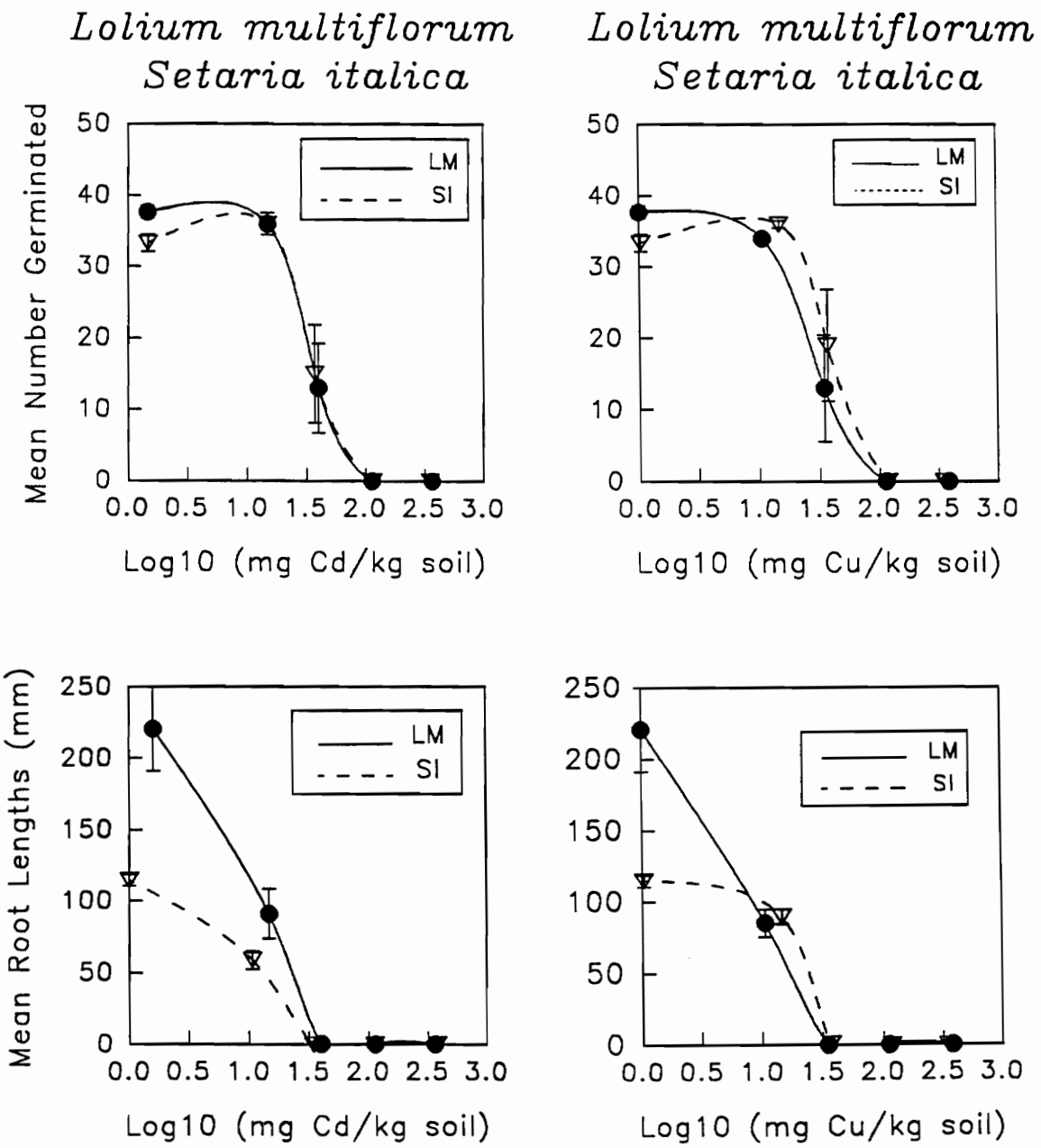

Figure 1. Germination and root length dose-response curves for plant species grown in copper and cadmium amended soil (mg metal/kg soil) for 28 days.

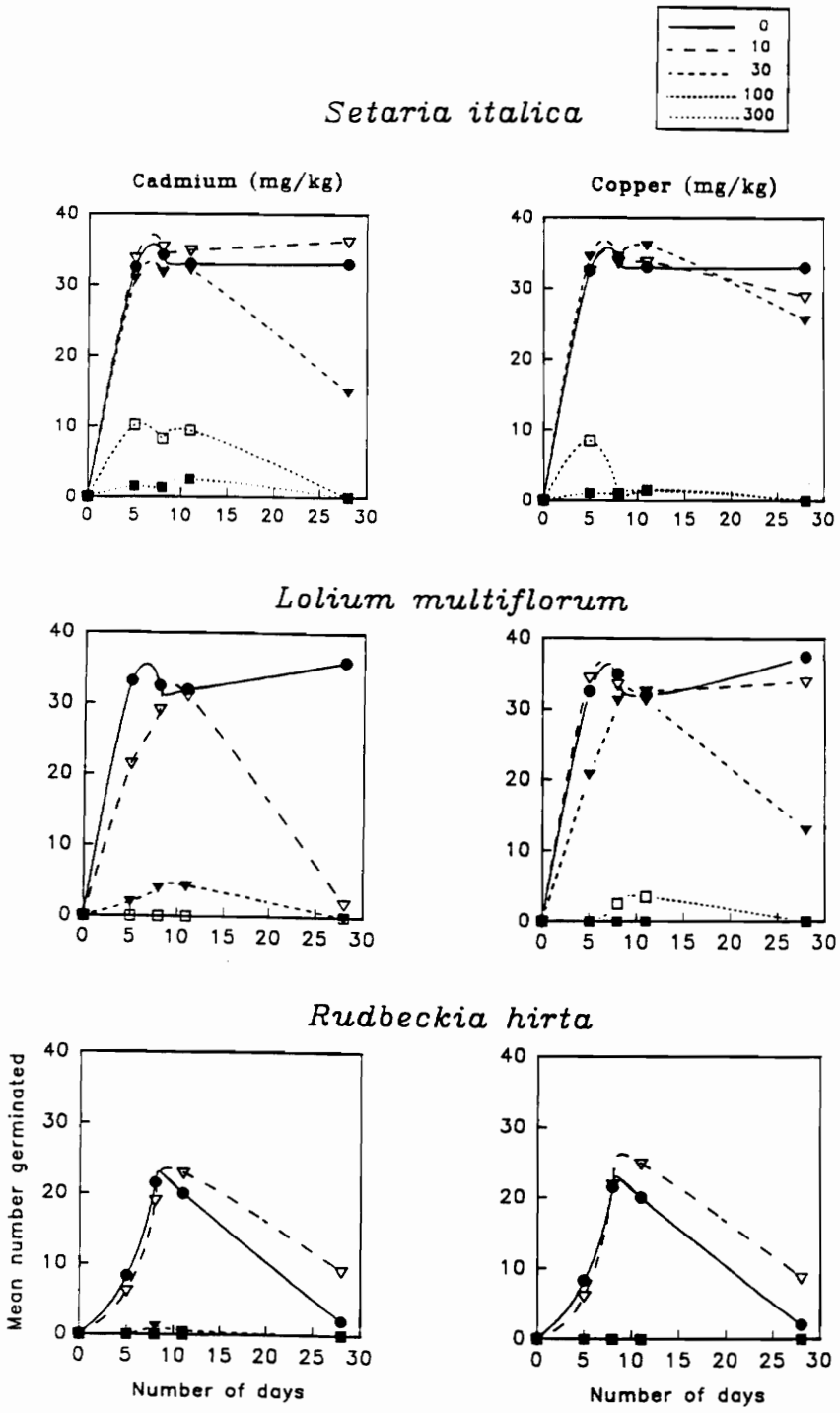

Figure 2. Germination counts of plant species grown in Cu and Cd amended soil for 28 days.

Germination counts over the test period are presented in Figure 2.

The pH measurements from the dosed but unused soil are listed in Table 4. The soil pH was not adjusted to neutral after the addition of $CuCl_2$ or $CdCl_2$ so the pH decreases with increasing Cu or Cd chloride solution addition.

The coefficients of variation for these species is in Table 1 and 2 for both germination and root length. The variability for the two species is equal for germination, but for root length Lolium multiflorum has twice the variability of Setaria italica.

DISCUSSION

Setaria italica has higher EC50 values in both metals than Lolium multiflorum after 28 days growth. Under similar conditions in the field, Setaria italica could be expected to thrive better. This species may simply be a slower grower than Lolium multiflorum.

Whether Benomyl prevented fungal growth is unlcear because no fungus was seen in any of the experimental units. But a significant increase in germination occurred due to Benomyl in all Cd treated replicates suggesting that Benomyl interacted with Cd somehow to synergistically effect plant growth. The preliminary tests indicated that germination was better but not that it reduced fungal

Table 3. AA-determined Cu and Cd concentration means from six replicate seedling dishes after 28 days (one standard deviation). The dosed but not used Cu and Cd samples (mg/kg soil) are from a single sample.

Nominal Conc.

Cd	*Lolium multiflorum*	*Setaria italica*	*Rudbeckia hirta*	Unused soil
0.0	0.60 (0.80)	0.96 (1.06)	0.33 (0.82)	
10.0	13.75 (1.29)	13.94 (0.93)	14.47 (1.75)	16.56
30.0	39.17 (6.25)	31.42 (11.50)	38.05 (6.81)	8.40
100.0	112.84 (3.20)	115.55 (6.82)	123.00 (19.24)	148.80
300.0	326.14 (87.78)	349.26 (29.68)	310.72 (82.53)	456.00

Cu	*Lolium nultiflorum*	*Setaria italica*	*Rudbeckia hirta*	Unused soil
0.0	0.00 (0.00)	0.04 (0.06)	0.0 (0.0)	
10.0	9.78 (0.72)	10.10 (0.43)	9.73 (0.49)	12.13
30.0	33.84 (2.39)	33.47 (4.34)	34.98 (2.85)	36.18
100.0	111.91 (10.19)	117.45 (6.08)	102.78 (30.66)	124.34
300.0	382.36 (38.96)	373.37 (44.65)	398.13 (43.30)	392.00

Table 4. pH values for Cu and Cd amended soils.

Nominal Concen.	Cd	Cu
0.0	5.50	5.29
10.0	5.07	4.93
30.0	4.78	4.47
100.0	4.61	4.08
300.0	4.47	3.94

growth.

The germination of Lolium multiflorum (Figure 2) in 9.8mg/kg Cu did not differ significantly from the germination of the control seeds over the first 11 days. In 33.8mg/kg Cu germination occurred at a slower rate but by day 11 mean germination was not significantly different from control and 9.8mg/kg germination. In 112mg/kg and 382mg/kg Cu germination was practically non-existent. Annual ryegrass germination in cadmium was similar.

Setaria italica germination in Cd and Cu was similar except that neither metal significantly reduced germination at approximately 30mg/kg as occurred with Lolium multiflorum. Also in 100mg/kg, though significantly reduced, mean germination was 25% of the number planted, which is approximately a 10-fold increase over Lolium multiflorum at this concentration.

Rudbeckia hirta germination was the slowest to occur. Control germination increased steadily to peak at day 8 and the lowest concentration of both Cd or Cu produced germination that exceeded the control by day 11. The two highest concentrations of Cd and Cu produced no germination during the measurement period.

The inability of Rudbeckia hirta to germinate in better proportions during this test is not clearly understood. In previous tests Rudbeckia hirta germinated at a slower

rate than other seeds so the extended length of the test period should have been amenable to it. On the other hand, the amount of water used during this test, 100% field capacity, may have posed a hazard to the seed because of its small size.

The range of concentrations used, 10 - 300mg/kg was chosen because germination was affected within this range in previous tests. The resulting dose-response curves for germination are expectedly asymtotic. The declining portion of the curve extends from approximately 90% germination to 0% germination with a midpoint where intermediate germination occurs. This is the minimum data necessary for the determination of dose-response relationships.

Setaria italica response to the heavy metals is interesting in that it germinated better than Lolium multiflorum in middle concentrations which suggests that it may be more tolerant during germination. By the end of 28 days, however, Lolium multiflorum and Setaria italica germination is very similar.

The response by the roots for the two species at the end of the test is similar (Figure 1). Root length produced in 10mg/kg Cu and Cd for Setaria italica was reduced by about 50%. Lolium multiflorum root length had been reduced by about 75%, so L. Multiflorum was affected more by the metal than S. italica. Lowered growth rates

and nutrient uptake are physiological responses of stressed plants which may be caused by changes in hormone balance (Chapin, 1991).

AA-determined metal concentrations from seedling pots are higher than intended, but lower than the concentrations from dosed but ususued soil samples for both Cd and Cu. Since all values are too high, the cause is consistent and operator error has not been ruled out. The only exception is the unused Cd sample taken from what was supposed to be 30mg/kg. The 8.4mg/kg value is not representative of the 30mg/kg batch since all seedling pots from this batch contain soil of approximately 35mg/kg. Though all values are higher than intended, the trend is consistent with expected uptake of metal ions by the plants reducing the concentration remaining in the soil.

Soil analysis performed in Chapter II-F revealed high concentrations of iron (10.9mg/kg) and aluminum (21.5mg/k). Both minerals are toxic to plants, but aluminum is particularily so. The toxicity of Al species is not fully understood. Toxicity has usually been associated with Al^{3+} at lower pH approximately 4.7, but less acidic forms such as $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ have also demonstrated toxicity (Parker et al., 1988). There is no way to know from this data the ratio of Al to Cu that may have been available at a given pH (Table 4). Nor is it possible to be certain of

the ion form of available Al. In general, the availability of the more toxic forms of Al ions to the plant roots increases as the pH decreases, so the effect on the plants may be due to a combination of Cu and Al or Cd and Al, rather than to the added metal ions alone. The symptoms of Al toxicity resemble Cu and Cd. Root extension is inhibited resulting in short, brown stubs that lack root hairs (Foy, 1978). The deformed morphology prevents proper uptake of water and nutrients (Karr et al., 1984).

Time is an important element to consider in determining toxicity parameters. Germination, which has been found to be less sensitive than root length, decreased by 50% over four weeks of growth. The germination data taken on the day 28 may be considered survival data when compared with earlier germination data (Figure 2).

Direct root measurements (i.e., a ruler or calipers) are more accurate than indirect methods. During practice counts, estimates made with the indirect grid counting method devised by Bohm (1979) were consistently shorter than direct measurements. The sample size during the practice trial, however, was very small.

The response of the roots should be the criteria upon which to base the range in subsequent studies with these species. Since no roots remained in 30mg/kg Cu in either metal the range of testing for L. multiflorum, S. italica and R. hirta should be lowered to below this concentration.

Also, there appears to be no difference between the response of roots for these species toward Cd and Cu for either species by the end of the test. Continued testing with a natural soil with Cu only will be considered.

CHAPTER IV

RESPONSES IN CADMIUM AND COPPER TEST II

INTRODUCTION

The plant species that will be used in this, the second of the definitive tests will be Festuca rubra L., red fescue, Solidago nemoralis Aiton, gray goldenrod and Robinia pseudoacacia L., black locust (Gleason and Cronquist, 1991). F. rubra and S. nemoralis are both native species to Virginia and presently have a wide distribution in open sites and sandy soils (Harvill et al., 1992). F. Rubra Cu tolerant populations have been identified (Herstein and Jaeger, 1986) although the population used in this test is not tolerant.

Because Benomyl, during the test in Chapter III, appeared to produce some synergistic effects with Cd, and because its value as a fungicide has not been demonstrated, it will not be used here. The range of concentrations used previously, control, 10, 30, 100 and 300mg metal/kg soil will be repeated in this test under the assumption that plant species may behave differently under similar stresses. Also this test was begun before the data in Chapter III was completely analyzed.

MATERIALS AND METHODS

The soil was collected from Dinwiddie County during the

summer of 1991. Soil physical and chemical characteristics are in Chapter II-F. Bulk-blending of soils was performed using a 5000mg/l stock solution made by mixing ACS reagent grade $CuCl_2$ with deionized distilled (ddH_2O). A 3000mg/l stock solution of $CdCl_2$ was made with ACS reagent grade $CdCl_2$. Sufficient diluted stock solution was applied to the soil to 1) obtain the desired concentration of copper by soil weight and 2) to not exceed the water holding capacity of the soil. Soils were wetted with the solutions in layers, mixed while wet, and allowed to air dry in 10% HCl acid-washed plastic tubs. Soils were reground with an acid washed porcelain pestle and thoroughly remixed with clean plastic utensils. All dish and glassware was soaked with 10% v:v HCl for 12 hours or rinsed with 20% v:v nitric acid before use (USEPA, 1989a).

The F. rubra, S. nemoralis and R. pseudoacacia seeds were of the same as those acquired and used previously. Each species was tested individually on a geometrically spaced log scale of copper concentrations including 0, 10, 30, 100 and 300mg/kg. Six replicates of each species/concentration combination were assembled. All petri dishes were filled with 80g treated soil and watered with 12ml ddH_2O to bring the soil to 76% field capacity. Half of the replicates were planted with 40 seeds of each species on day 1. The other half were

planted 3 days later because of the time needed to remove and measure the roots at the end of the test. The seeds were covered with an additional 10g soil of the same concentration. Each petri dish was placed in a plastic ziploc bag and sealed (Thomas and Cline, 1985). All dishes were randomly arranged in a plant growth chamber under fluorescent lights on a 16/8 hour photoperiod with temperatures set at $25^{o}/15^{o}$ to correspond to day/night cycle. Festuca rubra and Solidago nemoralis seeds were planted and placed in cold (constant $5^{o}C$) moist conditions for 7 days prior to germinating conditions. One 20mg aliquot from each batch of dosed but unused soil was mixed with ddH_2O to produce a 1:1 w:v slurry. After a 20 minute equilibration period with intermittent stirring, pH was measured with a calibrated glass electrode (Peterson and Calvin, 1965).

Germination was considered to have occurred when plant tissue grew to 1mm above the soil surface. Germination counts were begun 5 days after the day each half was planted. The seedlings were allowed to grow for a total period of 28 days. A final germination count was made on the final day of the test at which time all roots were removed from the soil and measured.

A portion of the soil below the seed layer from each petri dish was remixed and air dried. From each cup, a 10g aliquot of dry soil was weighed and transferred to an acid-

washed 50ml plastic centrifuge tube for agitation with 20ml DTPA (diethylene-triamine-pentaacetic acid) solution (pH 7.3) for two hours (Lindsay and Norvell, 1978). The samples were then centrifuged for 10 minutes at 2000 rpm and poured through Whatman #42 filter paper into acid-washed polypropylene bottles. The extracts were diluted appropriately with ddH_2O and stored under refrigeration until metal analysis performed by aspiration into a Perkin-Elmer Flame Atomic Absorption Spectrophotometer (model 240). The resulting concentrations (mg/l) were used to determine the actual Cu and Cd concentrations on a soil weight basis (mg/kg).

The means of the AA-determined Cu and Cd soil concentrations (mg/kg) were calculated per species and used in the analysis of variance performed on seed germination and root length. The NOEC and LOEC values were determined using the Student-Neumann-Keuls procedure for means comparison (Sokal and Rohlf, 1981). The Spearman-Karber method was used to determine the germination LC50 and confidence limits. Least squares regression analysis of the linear, declining portion of the dose-response curves for germination and root length were performed to determine the slopes. The regression parameters were also used to determine the root length EC50 plus confidence limits by inverse prediction (Sokal and Rohlf, 1981).

RESULTS

After 28 days growth in copper and cadmium amended soil, the EC50 values for F. rubra were 4.8mg/kg Cu (1.9 - 11.3) and 6.11mg/kg Cd (2.5 - 14.5). For S. nemoralis the EC50 values were 16.53mg/kg Cu (9.45 - 24.9) and 7.59mg/kg Cd (0.49 - 73.3). Spearman-Karber generated LC50 for F. rubra were 24.25mg/kg Cu (22.3-26.4) and 19.0mg/kg Cd (17.2-20.8). There was not enough germination data to perform Spearman-Karber procedure so the LC50 for S. nemoralis was calculated by interpolation without confidence limits. F. rubra control germination was approximately 70% of the number planted, S. nemoralis germination was about 30%.

F. rubra germination (Figure 1) is significantly stimulated by the addition of Cu to the soil at 10mg/kg above the control germination. The excess amount of Cu, however, inhibits root elongation at this concentration. At 10mg/kg Cd F. rubra germination is not significantly different from control. An affect is very dramatically seen in the roots, which grew only to approximately 20% of the length of control roots at this concentration.

Though F. rubra germination, after 28 days in Cu, exceeded the germination in Cd in all concentrations, root lengths were slightly longer in Cd than in Cu. The germination of S. nemoralis in Cu was also very slightly

Table 1. Germination parameters of plant species grown in soil with Cu and Cd (mg/kg) for 28 days. (95% confidence limits)

Copper

Plant Species	LC50	NOEC	LOEC	CV%
Festuca rubra	24.25 (22.3 - 26.4)	0	10.5	25.3
Solidago nemoralis	1.2	None	None	53.7

Cadmium

Festuca rubra	19.0 (17.2 - 20.8)	12.82	49.8	26.5
Solidago nemoralis	1.42	None	None	61.1

Table 2. Root length parameters for plant species grown in soil with Cu and Cd (mg/kg) for 28 days. (95% confidence limits)

Copper

Plant Species	EC50	NOEC	LOEC	CV%
Festuca rubra	4.8 (1.9 - 11.3)	0	10.47	52.7
Solidago nemoralis	16.53 (9.45 - 24.9)	10.23	30.9	90.2

Cadmium

Festuca rubra	6.11 (2.5 - 14.5)	0	12.82	46.2
Solidago nemoralis	7.59 (0.49 - 73.3)	0.30	13.09	127.6

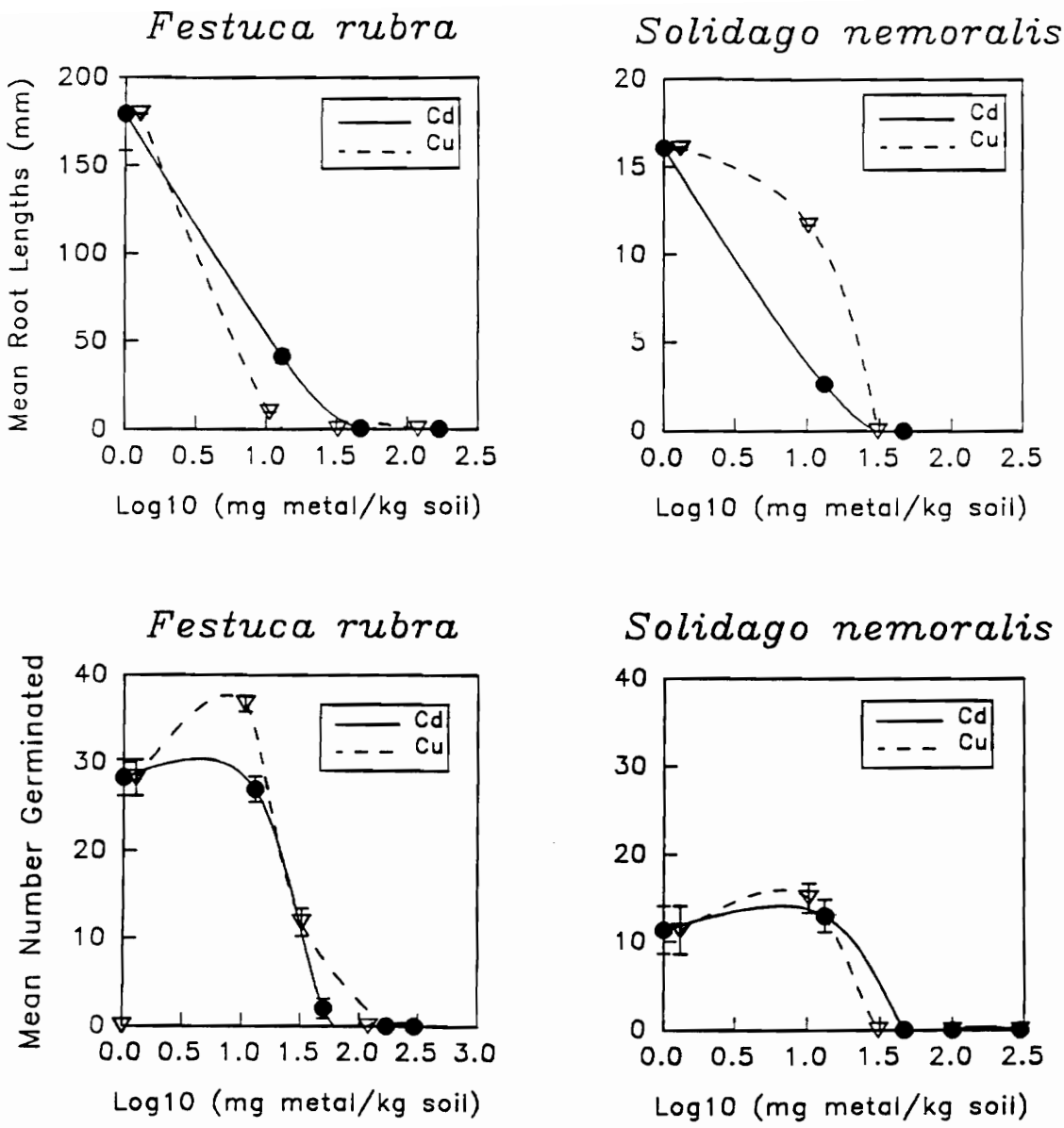

Figure 1. Germination and root length dose-response curves for plant species grown in copper and cadmium amended soil (mg metal/kg soil) for 28 days.

higher than in Cd, but the roots grown in Cu were about 4 times longer than in Cd.

The pH measurements of the dosed but unused soil are listed in Table 3. The pH of the soil was not adjusted to neutral so the pH declines steadily with increasing concentration of both metal solutions. The decrease in pH not only affected the availability of Cu but also other metal ions such as aluminum and iron that are also adsorbed to the soil particles.

AA-determined metal concentrations from seedling dishes are listed in Table 3. Keeping track of the dilutions of the samples from high Cd concentrations for both species was problematic and the resulting concentrations (mg/kg) are questionable. The dosed but unused 300mg/kg Cu soil sample was either not done or not recorded so no comparison can be made with results from seedling dishes.

DISCUSSION

At 10mg/kg Cu the two species appear to oppose each other in their response to the metal ions. F. rubra germinated better with Cu additions than without, but the additional Cu did not stimulate additional root growth; it was very inhibitory (Savage et al., 1981; Foy et al., 1978). S. nemoralis, on the other hand, germinated as well with Cu as without, but the roots were less sensitive to Cu

Table 3. AA-determined Cd and Cu concentrations from seedling dishes after 28 days (one standard deviation). The dosed but unused Cu and Cd (mg/kg soil) values are from a single sample.

Nominal Conc. Cd	*Festuca rubra*		*Solidago nemoralis*		Unused soil
0.0	0.00	(0.00)	0.00	(0.00)	
10.0	11.84	(1.18)	12.10	(0.63)	11.79
30.0	48.80	(7.56)	46.14	(5.20)	41.57
100.0	170.58	(20.25)	122.96	(43.84)	173.50
300.0	431.30	(100.60)	520.43	(92.25)	493.80

Cu	*Festuca rubra*		*Solidago nemoralis*		Unused soil
0.0	0.28	(0.14)	0.30	(0.15)	
10.0	9.55	(0.21)	9.27	(0.64)	10.43
30.0	31.05	(2.00)	30.38	(1.80)	29.64
100.0	118.29	(19.83)	141.33	(24.74)	111.31
300.0	360.68	(33.98)	358.14	(32.75)	-----

Table 4. pH values for Cu and Cd amended soils.

Nominal Concen.	Cd	Cu
0.0	5.00	5.00
10.0	5.20	4.82
30.0	4.51	4.54
100.0	4.60	4.05
300.0	4.39	3.86

compared to F. rubra.

AA-determined Cu and Cd concentrations from seedling dishes compare well with the concentrations determined for the unused soil. Uptake of metal ions by the plants is not suggested by these results. There is, in general, very little difference between the unused soil and seedling soil samples.

The decrease in pH with increasing concentration makes other ions adsorbed to the soil particles available for uptake in competition with Cu or Cd. Soil analysis (Chapter II-F) shows high concentrations of iron (10.9mg/kg) and aluminum (21.5mg/kg). Aluminum is particularly phytotoxic depending on which of its ion species is available. As in Chapter III, while there is no way to know which species of Al is present, the roots were probably affected by both metals and Al to some degree. Symptoms caused by Al toxicity, root stunting browning, (Karr et al., 1984) are similar to those caused by Cu or Cd.

The toxicity parameters determined from the results of this test are only estimates. Proper NOEC, LOEC, LC50 and EC50 data should be based on a minimum of three concentrations within the central region of the dose-response curve, without including any concentrations that produced 100% or 0% response. Since germination occurred in only one concentration other than the control for S.

nemoralis, no information could be obtained. The overall results concur with the previous test in suggesting that subsequent testing be performed on a range of lower concentrations based upon the root length information.

CHAPTER V

SEED AND SEEDLING RESPONSE TO COPPER

INTRODUCTION

Commonly used revegetation species such as Setaria ilalica (L.) Beauvois. German foxtail millet, Lolium multiflorum Lam., annual rye, and Trifolium repens latum L., ladino clover (Gleason and Cronquist, 1991) though not native to Virginia, have specific uses in land reclamation. These species are hardy and fast-growing, so they may be considered 'early successional species' in the process of reclamation. They serve to control soil erosion which is a major problem on devastated land and to provide early cover for other seeds. Since these seeds will most likely have initial contact with spoil or tailings, determining their ability to grow under heavy metal stress would also aid reclamation managers.

L. multiflorum Lam., annual rye, is an annual grass planted for erosion control of disturbed areas. It is similar to L. perenne, a European native that, mixed with other seeds, provides a nurse cover to aid the germination of shade tolerant seeds. Annual rye grows best in moist soils of slight acidity. S. italica is also an annual grass originating from the eastern hemisphere that is cultivated as fodder. Because it establishes quickly,

it is useful for erosion control, and can also provide cover for later germinating species. T. repens latum, is a legume used in pasture mixes. It provides nitrogen to nutrient poor soils by nitrogen-fixation. Robinia pseudoacacia, black locust, is a tree commonly used in revegetation work because of its nitrogen-fixing ability. It is a pioneer species and provides cover for later emerging plant species. Andropogon gerardi, big bluestem, Asclepias syriaco, common milkweed may have orginiated from the prairie and like many other prairie plants became naturalized with time in areas disturbed by settlers as they cleared land for agricultural purposes. They are potentially usable in reclamation where the purpose is other than agricultural. Knowing in advance how a plant species will respond under heavy metal stress will greatly increase revegetation success, reducing the need and cost of replanting.

Previous testing of Cu and Cd in equally spaced concentrations (on a logarithmic scale) ranging between 10mg/kg and 300mg/kg provided evidence that this range of concentrations could not produce responses in sufficient treatment levels to yield reliable toxicity parameters. The series of tests in this chapter represent an attempt to attain reliable data by narrowing the range of concentrations. The 30mg/kg was retained in the testing design because it was the lowest concentration in which

germination was totally inhibited. Equally spaced concentrations on a logarithmic scale were selected below this level.

This chapter is the summation of 4 tests performed on ten plant species using identical methodology. The first test was performed using Setaria ilalica, Lolium multiflorum and Trifolium repens latum. The second was performed using Asclepias syriaco, Andropogon gerardi and Echinecea purpurea. The third test was on Rudbeckia hirta, Festuca rubra and Robinia pseudoacacia.

A fourth test was attempted with 3 more species but germination was never above 1% after 6 weeks and so they were not be included in the data analysis.

MATERIALS AND METHODS

The soil used in these tests is the natural soil from Dinwiddie County, Virginia and is the same as that used in previous chapters.

Bulk blending of the soils was performed using a 5000ppm stock solution of $CuCl_2$ made with ACS reagent grade $CuCl_2$ and deionized distilled water (ddH_2O). Sufficient diluted stock solution was applied to the soil to 1) obtain the desired concentration of Cu by soil weight and 2) not exceed the water holding capacity for the weight of soil. Soils were wetted with the solution in layers to

evenly distribute the metal salt, mixed while wet, and allowed to air dry in covered acid washed plastic tubs. When dry the soils were reground with an acid washed porcelain pestle and thoroughly remixed with acid-washed plastic utensils. All plastic and glassware used was soaked for 12 hours in a 10% HCl bath or rinsed with 20% nitric (USEPA, 1989a).

Lolium multiflorum, Setaria italica and Trifolium repens latum seeds were obtained from a commercial supplier in Roanoke, Virginia. Andropogon gerardi, Asclepias syriaca and Rudeckia hirta seeds were obtained from a supplier in Wisconsin. Robinia pseudoacacia seeds were obtained from a tree seed supplier in Massachusetts. Festuca rubra and Echinecea purpurea were obtained from seed suppliers located near Blacksburg, Virginia.

Each species was tested individually over a geometrically spaced log scale of Cu concentrations including 0, 0.3, 1.0, 3.0, 10.0 and 30ppm. Four replicates of each concentration were used. Acid-washed plastic cups were filled with 300g of treated, dried soil and brought to 78% field capacity with ddH_2O. After 20 seeds were planted in each cup, an additional 25g of soil were used to cover the seeds. A lid consisting of a second plastic cup with a perforated bottom was inverted over the lower cup and the halves were sealed with parafilm. Because of the time needed to measure roots at the end of

the testing period, the replicates were assembled in two groups that were planted one day apart. The weight of each unit was recorded after assemblage and used to determine watering needs. The first group of replicates were placed in the plant growth chamber overnight. The next day, after the rest of the experimental units were assembled, all units were arranged randomly in a plant growth chamber using a random numbers table. Lighting was provided by fluorescent light bulbs adjusted for a 16/8 photoperiod. Temperatures were set to fluctuate between 24^{o}C (daytime) and 15^{o}C (nighttime) corresponding with the light regime. One 20g subsample from each batch of dosed but unused soil was mixed with ddH_2O to produce a 1:1 w:v slurry. After a 20 minute equilibration period pH was measured with a calibrated Accu-pHast probe (Petersen and Calvin, 1965).

Germination was considered to be emergence of plant tissue approximately 1mm in height or length above the soil. Counts were begun on day three of the test and were made every two days until total germination increased by less than 20% over the previous count. The first day of germination was determined as the mid-point between the start of germination and the day germination seemed complete (Miles and Parker, 1979) to account for different rates of germination. The seedlings were allowed to grow for about one week after the calculated first day of

germination. On the last day of growth, a final germination count was made and the roots of seven intact seedlings from each unit were carefully removed and measured directly. A petri dish lid with 7 marker points on it was fitted over each unit and the seedlings closest to the marks were selected for measurement. The selection method was perhaps not as random as it could have been. But it reduced the bias toward selection of only long shoots for measurement. The roots were dipped in water and blotted when necessary to expose root structures. The roots were measured from the apex to the root-shoot interface (Esau, 1977; Fahn, 1990).

After removal of roots a portion of the soil below the seed layer from each cup was remixed and left to air dry. From this, a 10g aliquot of dry soil was weighed and transfered to an acid-washed plastic centrifuge tube. The samples were agitated with 20ml of DTPA (diethylene-triamine-pentaacetic acid) solution for two hours (Lindsay and Norvell, 1978). The samples were then centrifuged for 10 minutes at approximately 2000rpm and poured through Whatman #42 filter paper (Lindsay and Norvell, 1978) into acid-washed plastic bottles. The extractants were diluted appropriately with ddH_2O and stored under refrigeration for Cu analysis by aspiration into a Perkin-Elmer Flame Atomic Absorption Spectrophotometer (model 240). The resulting absorbance values were used to determine the actual Cu

concentrations in each soil sample.

For each treatment level of Cu, the mean of concentrations determined by AA analysis per species were used in the analysis of variance performed on seed germination and root length. The NOEC, the no observable effects concentration which is the highest concentration which is not statistically different from control and the LOEC, the lowest observable effects concentration, which is the lowest concentration which is statistically different from the control were determined for each species using Student-Neumann-Keuls procedure for means comparison. The LC50, the lethal concentration at which 50% of the seeds failed to germinate, was determined using the Spearman-Karber procedure (Hamilton, et al., 1977). The preferred method of LC50 determination, probit analysis, failed to produce probabilities in many situations, particularily when data were non-monotonic (Hamilton et al., 1977).

Further analysis of the root length data was performed by fitting a regression line through the linear portion of the dose-response curve (USEPA, 1983b). The regression line parameters were used to estimate the EC50, the effective concentration at which growth of root lengths will be 50% of control root length, by inverse prediction (Sokal and Rohlf, 1981; Niederlehner, pers. comm.).

RESULTS

After 7 days growth in Cu amended soil, Setaria italica EC50 was 10.2mg/kg (3.02 - 123.0). EC50s for Lolium multiflorum, Asclepias syriaco, Andropogon gerardi, Rudbeckia hirta, Echinecea purpurea, Festuca rubra and Trifolium repens latum ranged from 4mg/kg to 7mg/kg Cu ($p=0.0001-0.0112$ compared to 0.05 significance level). No significant difference ($p=0.1353$) occurred in root length for Robinia pseudoacacia. Table 1 contains all root length data with confidence limits and toxicity parameters. Table 2 contains all germination information.

Figure 1 contains the dose-response curves for roots resulting from the three tests with data points and standard errors indicated on each graph. For clarification, Figure 2 contains the same information in three graphs depicting the different root response types.

What appears to be a threshhold (Woodwell, 1975) occurred at approximately 3mg/kg for L. multiflorum, F. rubra, R. hirta, and T. repens latum making this concentration the NOEC. A sharp decline in root lengths occurs above this concentration and no roots remain at approximately 10mg Cu/kg, the LOEC for these species. R. pseudoacacia, S. ilalica, A. gerardi, E. purpurea and A. syriaco displayed unusual trends in their dose-response relationships. The two types of responses (Figure 2)

Table 1. Root length parameters for plant species grown Cu (mg/kg soil) for 7 days.

Plant Species	EC50	(95% confidence limits)	NOEC	LOEC
Setaria italica	10.86	(4.98 - 17.8)	4.0	10.0
Lolium multiflorum	7.51	(5.14 - 7.9)	4.0	10.0
Trifolium repens latum	6.85	(4.39 - 7.41)	3.3	9.7
Rudbeckia hirta	6.35	(4.9 - 8.26)	4.0	10.2
Asclepias syriaca	5.76	(2.1 - 23.5)	3.7	10.3
Festuca rubra	5.37	(2.8 - 6.52)	4.2	10.3
Andropogon gerardi	5.24	(1.6 - 31.3)	3.9	10.9
Echinacea purpurea	3.74	(1.3 - 10.0)	1.1	1.3
Robinia pseudoacacia	None	None	None	None

Table 2. Germination parameters of plant species grown in Cu (mg/kg soil) for 7 days.

Plant Species	LC50	NOEC	LOEC	CV(%)
Festuca rubra	40.0	9.3	32.9	15.3
Rudbeckia hirta	17.7	9.2	33.9	34.6
Robinia pseudoacacia	None	None	None	39.7
Andropogon gerardii	None	None	None	61.8
Asclepias syriaco	None	None	None	49.4
Echinecea purpurea	None	None	None	22.6
Lolium multiflorum	None	None	None	14.9
Setaria italica	None	None	None	19.1
Trifolium repens latum	21.04	9.7	38.5	25.6

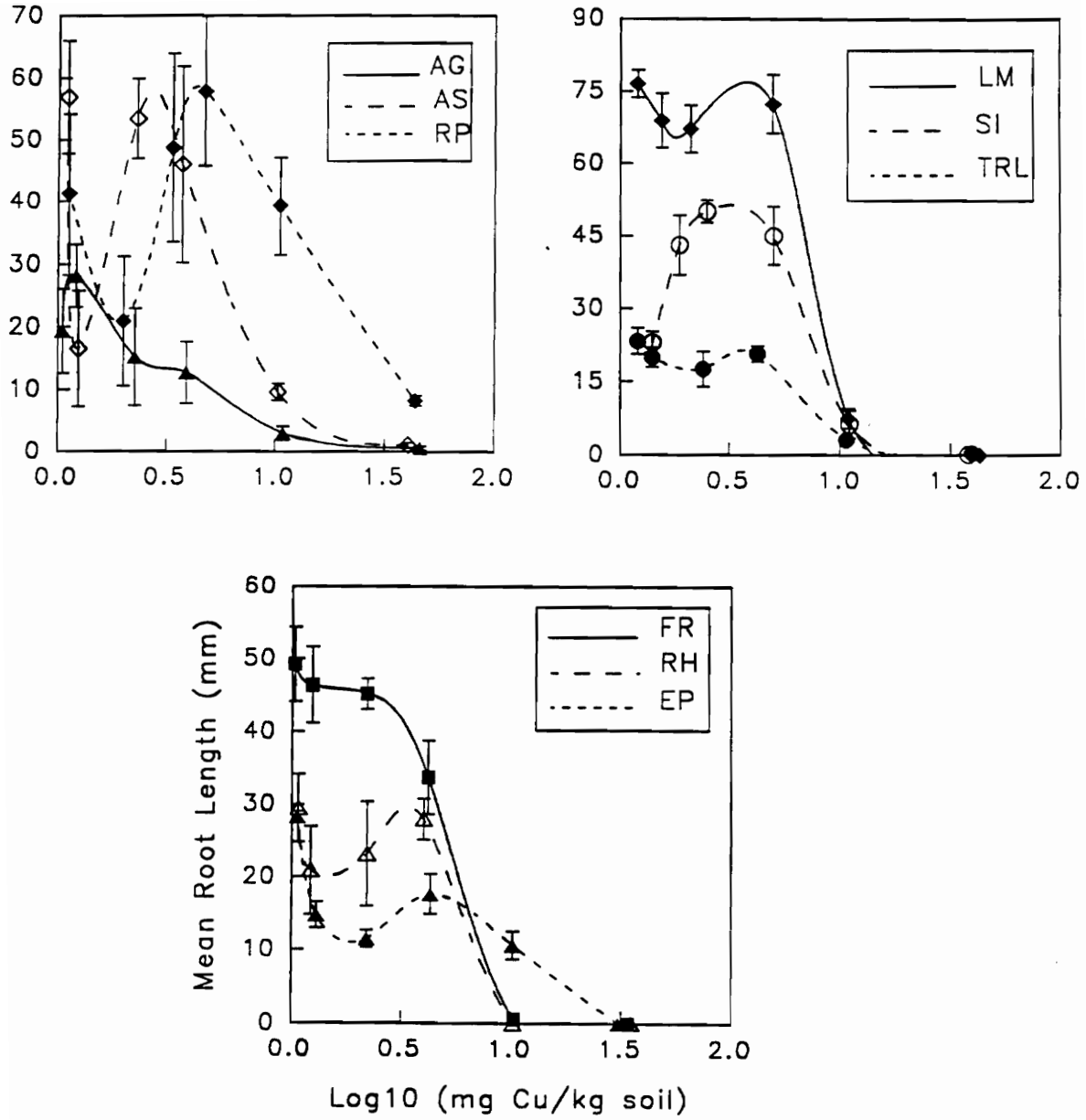

Figure 1. Root length dose-response curves for A. gerardi (AG), A. syriaca (AS), R. pseudoacacia (RP), L. multiflorum (LM), S. italica (SI), T. repens latum (TRL), F. rubra (FR), R. hirta (RH), and E. purpurea (EP) grown in Cu amended soil for 7 days (mg Cu/kg soil).

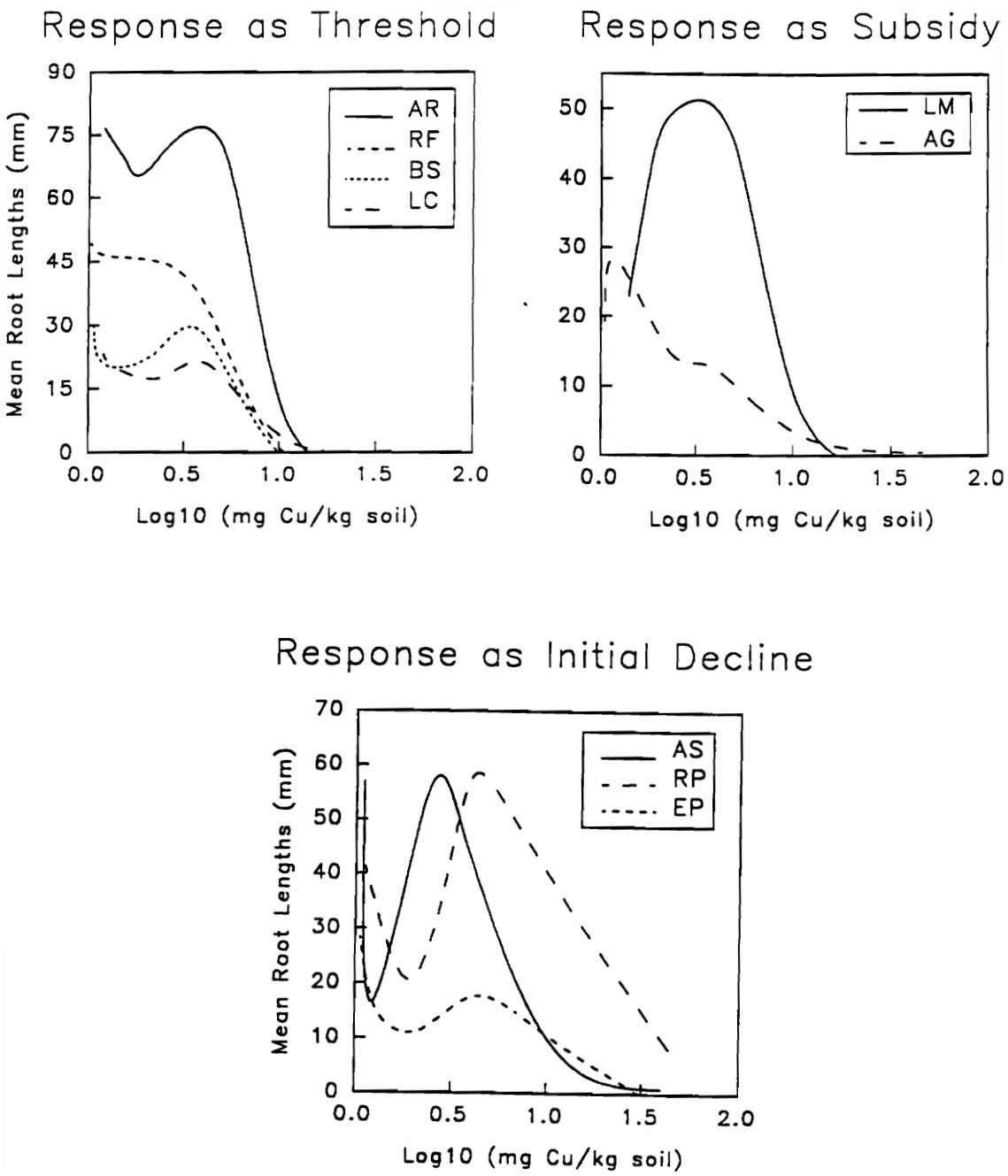

Figure 2. Different types of root responses by plant species to Cu amended soil (mg/kg soil) after 7 days. See Figure 1 for abbreviations.

depict either a decline or a stimulation in root length in the lowest concentration (approximately 0.3mg/kg). Those that decline in very low concentrations increase in root length either to the control level or greater in slightly higher Cu concentrations. S. ilalica root lengths grew to 200% over control root length in the 0.3, 1.0 and 3.0mg/kg Cu concentrations above which a decline occurred.

R. pseudoacacia root lengths grew 50% longer than control root lengths in 3mg/kg, but only after root length had been inhibited by 50% in 0.3mg/kg Cu. While not quite as dramatic a response as in S. ilalica, the resulting curve is similar, an increase in growth above control root length. Such is the case for A. gerardi in 3mg/kg Cu.

No significant differences in germination means (Figure 3) occurred between any Cu concentrations for R. pseudoacacia (p=0.0589), S. ilalica (p=0.4182), L. multiflorum (p=0.697), A. syriaco (p=0.1932), A. gerardi (p=0.0491), or E. purpurea (p=0.0736). Only the highest Cu concentration was significantly different from control for R. hirta (p=0.0001), F. rubra (p=0.0001) and T. repens latum (p=0.0003).

The mean AA-determined Cu concentrations used in the statistical analyses derived for each species by averaging the results at each treatment level. Table 3 contains this information with standard deviations. The AA-determined

concentrations of dosed unused soil from the final test was not determined.

Soil pH results are listed in Table 4 for the first and third tests performed. Soil pH was not performed on the middle set of species tested. The pH remained above 5 over most of the concentrations and was not significantly correlated with germination or with root length decrease in either study. The pH dropped to less than 5 at 10mg/kg and higher.

Visual symptoms noted were as follows. Algae grew on the soil surface of all units in the tests except the 2 highest concentrations. Leaves were produced in all Cu concentrations for all species. However, control leaves for S. italica were consistently more chlorotic and smaller by about 1/2 in height and width leaves in higher concentrations. In about 3mg/kg two R. pseudoacacia seedlings had totally white leaves after 7 days growth. Chlorosis was not noted in other species. Small shoot height was only noted in F. rubra in 30mg/kg Cu treatment. E. purpurea developed fungus in units before germination began. This seed was an extremely slow grower. The test was terminated after 3 weeks.

Wilted seedlings were found in particularily the 2 highest treatments. The seedling leaves were found lying across the soil surface and produced very short (approximately 0.5cm) or no exposed roots. Except where

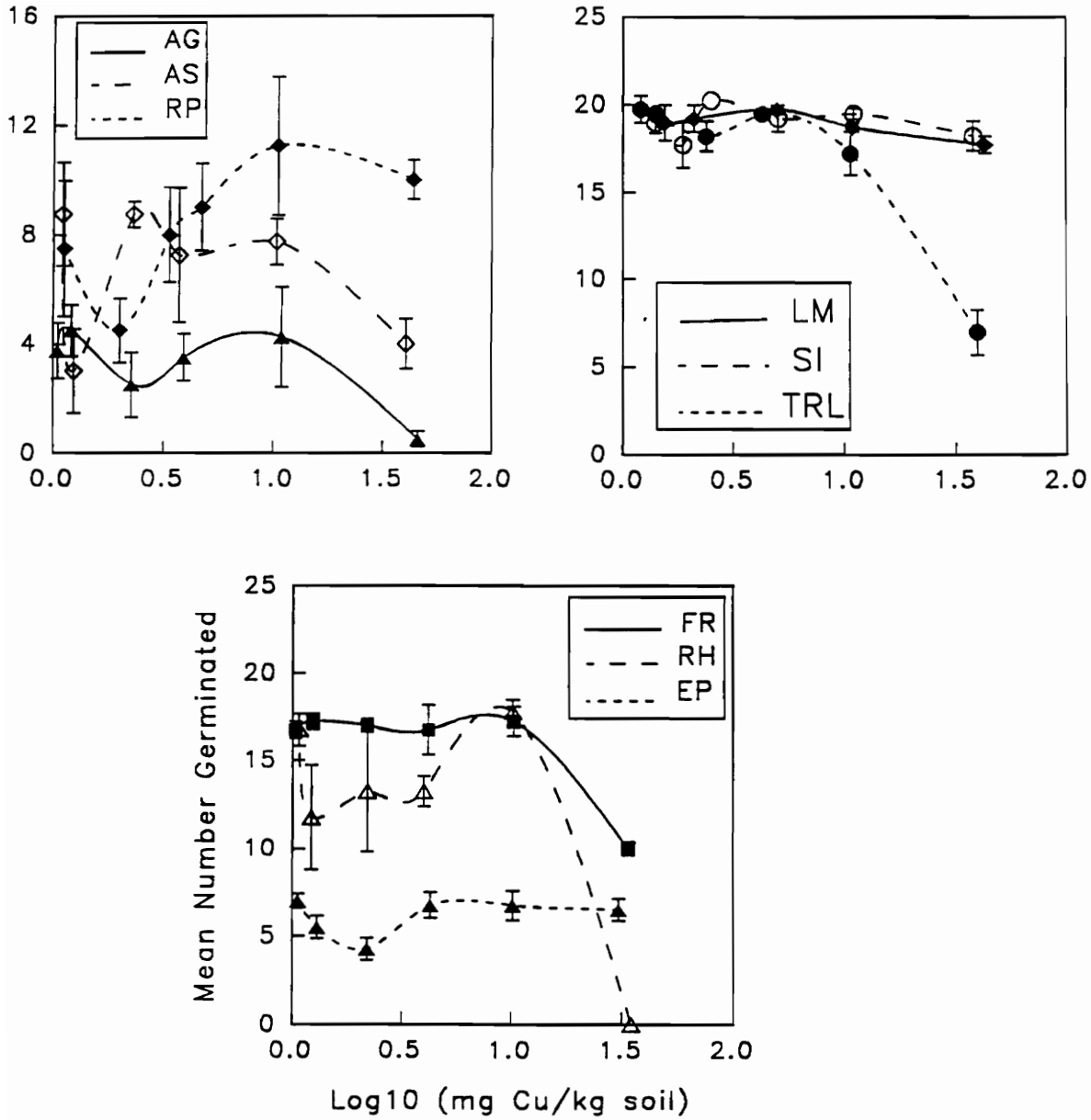

Figure 3. Germination dose-response curves for A. gerardi (AG), A. syriaca (AS), R. pseudoacacia (RP), L. multiflorum (LM), S. italica (SI), T. repens latum (TRL), F. rubra (FR), R. hirta (RH), and E. purpurea (EP) grown in Cu amended soil for 7 days (mg Cu/kg soil).

Table 3. AA-determined Cu concentrations from seedling cups (mg/kg soil) after 7 days (one standard deviation). The Cu concentrations from dosed but unused soil samples for two tests were from a single sample.

Nominal Conc. Test 1	*Robinia pseudoacacia*	*Asclepias syriaca*	*Andropogon gerardii*	Unused soil
0.0	0.12 (0.25)	0.10 (0.04)	0.06 (0.05)	
0.3	1.03 (0.57)	0.25 (0.73)	0.21 (0.09)	0.05
1.0	2.35 (1.26)	1.34 (0.17)	1.26 (0.06)	0.31
3.0	3.69 (0.54)	2.72 (0.22)	2.89 (0.19)	2.80
10.0	9.46 (0.92)	9.32 (0.55)	9.89 (0.64)	11.80
30.0	42.60 (4.75)	39.38 (1.04)	44.87 (5.51)	45.50

Test 2	*Lolium nultiflorum*	*Setaria italica*	*Trifolium repens latum*	Unused soil
0.0	0.23 (0.26)	0.40 (0.29)	0.23 (0.26)	
0.3	0.55 (0.17)	0.85 (0.17)	0.43 (0.05)	0.50
1.0	1.15 (0.29)	1.51 (0.11)	1.40 (0.29)	1.50
3.0	3.97 (0.60)	4.05 (0.16)	3.30 (1.36)	2.80
10.0	9.91 (0.18)	10.02 (0.62)	9.72 (0.35)	12.80
30.0	41.95 (8.89)	37.01 (5.17)	38.55 (0.40)	41.20

Table 3. AA-determined Cu concentrations from seedling cups (mg/kg soil) after 7 days (one standard deviation). The Cu concentrations from dosed but unused soil samples for two tests were from a single sample.

Test 3	*Festuca rubra*	*Rudbeckia hirta*	*Echinacea purpurea*
0.0	0.03 (0.06)	0.07 (0.03)	0.07 (0.04)
0.3	0.27 (0.06)	0.23 (0.04)	0.31 (0.02)
1.0	1.14 (0.18)	1.16 (0.16)	1.18 (0.10)
3.0	3.17 (0.19)	3.03 (0.15)	3.35 (0.30)
10.0	9.3 (0.20)	9.18 (0.37)	9.2 (0.57)
30.0	32.93 (1.55)	33.87 (1.40)	29.63 (0.64)

Table 4. pH values for Cu amended soils for two tests.

Nominal Concen.	Test 1	Test 3
0.0	5.62	5.17
0.3	5.27	5.46
1.0	5.36	5.38
3.0	5.26	5.27
10.0	4.89	4.96
30.0	4.27	4.45

noted, fungus was not a problem. R. pseudoacacia had wilted leaves in all treatment levels including controls. Fungus was noted in the soil of R. pseudoacacia units on the 3rd day after planting. The control units plus the units of the two adjacent higher concentrations were the first impacted by it. The last level to acquire fungus was the approximately 10mg/kg Cu. There was no attempt to formally quantify the fungus, which appeared on the seedlings, in soil cracks, and on the soil surface. It was not clearly determined whether the seed or the soil was the source. But since fungus was not problematic with other species that were planted in soil from the same treated batches, it is likely that the seeds were the source. Also noteworthy are the results of a very informal preliminary test with this seed using the present test design in which no fungus occurred until after approximately 3 weeks growth. The types of fungi noted during this test are explained in the final discussion and summary chapter.

Roots of all species grown in the two highest concentrations were either not present or severely reduced in size. Elongation terminated at the root-shoot interface and the root apex appeared burned. Some of these severely affected roots produced stunted lateral roots. All these are characteristic symptoms of Cu toxicity (Foy, 1978).

DISCUSSION

Setaria italica exhibited the highest EC50 of the seeds tested in Cu for 7 days at 10.86mg/kg. R. pseudoacacia displayed no difference in root length as a response to increasing Cu concentrations. The other species tested had EC50 values between 4 and 7mg/kg Cu. As noted from Table 1 the commonly used reclamation species fared a little better under these conditions than the native species.

Unusual responses to Cu were displayed by S. ilalica, R. pseudoacacia, A. gerardi and Echinecea purpurea root growth. In the 3 lowest treatment levels S. italica roots grew to approximately 200% of control root length, and reached a threshold at about 3mg/kg Cu. At 10mg/kg S. italica roots were very nearly totally inhibited. The exaggerated increase in root growth in these concentrations may be caused by the overproduction of growth hormones in the plant system to counteract the effects of the metal ion invasion. This response, called hormesis, has only been noted in algae responding to pesticides and other phytotoxins (Stebbing, 1987). To be certain that these responses are hormetic, very careful growth rate studies would need to be performed to determine whether fluctuations in the growth rate were present which is beyond the scope of this study. These fluctuations may only be due to the limited growth period of the test.

Tested for longer periods may smooth out such responses.

A second explanation for the abundant growth of roots in intermediate concentrations of Cu may be due to the requirements of Cu as an essential nutrient. S. italica and A. gerardi may be extremely limited by Cu deficiency and able to take full advantage of low levels of Cu better than the other species. Low concentrations of other heavy metals such as cadmium actually stimulate growth in some plant species by facilitating the uptake of other nutrients (John and van Laerhoven, 1976). E. purpurea's root length is reduced by low concentrations of Cu. Recovery occurs in slightly higher concentrations followed by a linear decline. This may also be a limited hormetic response. The type of responses displayed by these plant species are common responses found in dose-response relationships of other organisms (Cairns, 1992).

Predicting how a species will respond to heavy metals in soils is difficult particularily when the response is non-linear through the treatment levels. (Because most germination responses were not significant, only root growth responses will be used to consider prediction). This is the situation for A. syriaco and R. pseudoacacia, whose root lenths declined in 0.3mg/kg and increased in 1mg/kg until a threshold was reached. A threshold is defined as the highest concentration of a substance that

produces no adverse affects (Woodwell, 1975).

L. multiflorum, T. repens latum, F. rubra and R. hirta display classic dose-response curves. There is no significant difference from control in root length in lower concentrations until a threshold is reached after which root lengths are almost totally inhibited. Prediction would be possible over the central portion of the dose-response curve although a additional data point midway between the threshold and the next highest concentration would better define the slope of the curves for L. multiflorum, F. rubra and T. repens latum. The R. hirta curve requires at least two data points between the threshold and zero growth.

R. pseudoacacia germination, which shows no significant difference between the means, exhibits a positive trend between germination and Cu treatment. However, R. pseudoacacia was the only seed in this test in which fungus was abundant. The fungus was noted earliest and most abundantly in the three lowest concentrations, which also had the lowest germination proportions. The approximately 10mg/kg treatment level in which fungus was last to arrive and least abundant had the highest germination proportion. It is more likely that the occurrence of fungus produced the unusual dose-response curve.

Seed germination is a vital part of the life cycle of any plant, but may not be a good predictor of

establishment. There was no statistical difference in the number of seeds able to germinate in soil under Cu stress at approximately 10mg/kg or less for all seeds, while most of the seedlings in approximately 10mg/kg Cu displayed little root development. This is consistent with other findings regarding the sensitivity of plant life cycles to environmental stress. Germination has previously been shown to be a less sensitive life stage than root elongation (Patterson and Olson, 1982; Wang, 1987; Scherbatskoy et al., 1987).

Another notable trend in germination is the decrease in proportions at the lowest concentration, approximately 3mg/kg, while at the higher treatment levels germination increases to control levels or above. R. pseudoacacia, Asclepias syriaco and Rudbeckia hirta are examples of this trend.

The origin of the algae on the soil surface was undoubtly the soil. Some algae demonstrate a high tolerance to Cu (Levitt, 1980). In this case, however, the algae found on these soils were probably not exposed to much metal which was tightly adsorbed to the soil particles. A preliminary test (not included) to determine how much Cu was actually in the soil solution was performed by suctioning off the soil solution after dosing it with copper. The soil was allowed to equilibrate before

measuring the extract by AA analysis. The results were that neglegible amounts of Cu remained in the soil solution after dosing.

The physical appearance of the roots noted in this study were similar to findings noted by Heale and Ormrod (1981) and Pahlsson (1989) using excised tree roots in culture solution. These studies also confirm that small chlorotic leaves result from Cu toxicity along with stunted and burned root tips. More effects would have occurred if the test period were longer or concentrations used were higher.

Recommendations for future testing include increasing the concentration range for R. pseudoacacia to clarify its response to Cu. Increasing the growth period for the other species is the next step. The studies in Chapter III indicate that a more predictable dose-response curve may result when the growth period is increased.

The type of responses after 7 days for Lolium multiflorum, Festuca rubra, Rudbeckia hirta, and Trifolium repens latum are fairly classical and therefore predictability may be possible if the response is retained over time. Heavy metals slow the rate of root elongation and developemnt overall (Savage, 1981). With more time roots may appear in the higher concentration of these tests. The results for L. multiflorum indicate this possibility. Its EC50 determined after 7 days is nearly equal to its EC50 after

28 days (Chapter III).

CHAPTER VI

DISCUSSION AND CONCLUSION

After 7 days growth in Cu amended soil, the EC50 for Setaria italica was highest at 10.86mg/l (3.02 - 123.0). After 28 days growth S. italica had an EC50 of 20.7 (17.52-24.48). The increase in EC50 with an increase in time was the opposite of what I expected. I would think that as time increased the roots would eventually be overcome by the metal ions and die. The response by the root lengths of this species was not the normal monotonic dose-response curve after 7 days. Its root length at low Cu concentrations greatly exceeded control root growth.

The EC50s for the other species, Lolium multiflorum, Asclepias syriaco, Andropogon gerardi, Rudbeckia hirta, Echinacea purpurea, Festuca rubra and Trifolium repens latum ranged from 4mg/l to 7mg/l Cu which is substantially lower than that Setaria italica. These species displayed a fairly predictable declining dose-response curve. Considering the short testing period, 7 days, the low EC50s using this soil substrate may indicate the inability of these species to survive for a longer period of time. On the other hand, the EC50 for L. multiflorum (7.7mg/kg) and for F. rubra (4.8mg/kg) tested for 28 days in Chapters III and IV, respectively, are essentially the same as those for 7 days (7.51mg/kg and 5.37mg/kg for L. multiflorum and F.

rubra). Both species exhibited standard dose-response curves. This may indicate that prediction is possible after only a 7 day test - for some species. Testing S. italica in the lower range of Cu concentration for 28 days would be a great follow-up.

Approximately 50% of the plant species displayed a double peak in the root length dose-response curve. The phenomenon is similar to one noted by Wang (1986) in Lactuca sativa (lettuce) tested in various pesticides. Apparently this response is highly toxicant-dependent and makes prediction difficult.

Germination proportions for E. purpurea, L. multiflorum, R. pseudoacacia, A. gerardi, A. syriaca and S. italica are not significantly different between any of the concentrations used. R. pseudoacacia germination proportions tend to increase slightly. Because the lower four concentrations (including control) were invaded by fungus, the dose-response curve may indicate suppression of the actual growth potential of this species. Other trials with R. pseudoacacia where no fungus was present, produced approximately 90% control germination. Germination for A. syriaca may attain a threshold at approximately 10mg/l Cu, but germination at 0.3mg/l is much lower than control germination. Because of these wide variances there is no significant difference in germination in the highest concentration. F. rubra, T. repens latum and R. hirta

exhibit thresholds at approximately 10mg/l in germination. Higher than this, germination is totally suppressed in R. hirta, but only significantly reduced in F. rubra and T. repens latum. For these latter three species further testing between the threshold level and perhaps 100mg/l Cu would better define the shape of the dose-response curve.

Because of the soil make-up, it is not possible to say that all effects were produced by Cu or Cd. Especially when the pH of the soil became lower than 4.7 some form of Al was probably exchanging with Cu on the soil particles. The results produced by Al are very similar to those of Cu. Adjusting the pH to neutral would simplify interpretation as resulting from the intended metals only. However, adjusting the pH would also reduce the environmental realism of the test situation.

Bioassays are designed to determine, by organism response, the level at which a substance may be considered toxic or detrimental to human welfare. Eliminating all possible explanations for the affects noted is necessary to reach an unbiased conclusion. The tests in this study were not designed to be strict bioassays, but to determine how a plant responds in degraded soils contaminated with heavy metals. Part of the problem facing plants, indeed, aiding in the reduced growth potential of plants used for revegetation is the low pH.

The various fungi found on seeds and roots of the plants were identified by the Mycology Laboratory as normal inhabitants of seed coats, such as bacterias and yeasts. Other organisms from various families as Monilaceae, Penicillin and Paecilomyes were found. The literature on metal tolerance in fungi is mixed. Some mycorrhizal species are tolerant of heavy metals to the point of encouraging fruiting of the host plant (Turnau, 1990). Others (Tyler, et al., 1987) maintain that spore formation is sensitive to heavy metals in most fungi.

Algae grew on nearly all soil surfaces especially during the last three tests, did not seem distressed by the presence of Cu, but algae is not known to interfere with plant growth. For a preliminary test to determine how much Cu was in solution, the soil solution was removed from 4 aliquots of soil after dosing with $CuCl_2$ by suction and analyzed for the metal. Negligible amounts of metals were recovered with this method indicating the high adsorption of the metal to the soil particles (USEPA, 1993).

Though no increase in root growth was produced by the use of nutrient solution in preliminary testing, evaluating its effectiveness on root length may have provided a better indication of its overall usefullness. Its effect on metal precipitation and adsorption to soil would then have to be accounted for.

The bioassay methods under development for testing

terrestrial plants in soil as indicators of risk assessment have in common only methodological similarities. Parameters such as LC50 or EC50 for a plant species tend to vary with soil quality. The availability of metal ions depends on the cation exchange capacity (CEC) of the soil, which is influenced by soil texture, organic matter content and pH. Soils with high clay content will generally have higher CEC values therefore a greater number of exchange sites available to adsorb positively charged ions, such as metals. Organic matter, also negatively charged will retain cations, removing them from solution thereby reducing the impact of the metal on plants. Toxicology studies in solution cultures of chemicals produce much lower EC50s than studies using either sandy or silty loams because the metal, in soluble form, is more available to interact with the root (Adema and Henzen, 1989).

The method can account for differences in plant responses to environmental stress. Future testing can include longer testing periods and more species. Use of the data to predict a plant response is highly situation-dependent because of the abiotic characteristics involved. Complicated plant responses such as dose response curves with more than one peak also make prediction difficult but not impossible. The value may be in the knowledge that such a response is possible. From this basic test design,

one may be able to extrapolate to other soil types the likely response of plants to hazardous chemical wastes.

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Sigma Xi $350.00 - October 1991 - matched award
Society for Ecological Restoration, 1991 - present
American Institute of Biological Sciences, 1991 -present
Virginia Academy of Science, 1991 - present

RESEARCH INTERESTS

Restoration of impacted sites
Environmental toxicology
Phytotoxic effects on native plant species

TEACHING INTERESTS

College and university level biology and botany courses

RELATED EXPERIENCE

Research

Graduate Research Assistant
Department of Biology,
Virginia Tech
Blacksburg, Virginia
January - May, 1990.

-Began literature search related to metal phtotoxicity.
-Wrote thesis prospectus for research
-Performed preliminary plant growth studies in the plant growth chamber

Junior lab Technician
Department of Ecology, Evolution and Behavior.
University of Minnesota, Minneapolis, MN
August 1989-December 1989.

-Tested methods for extracting and quantifying fossil pollen.
-Analyzed vegetation history of the hardwood hollows in Northern Michigan.
-Chemically prepared surface and sediment pollen samples.
-Helped plan and carried our field trips to research site.
-Plotted pollen diagrams (depth vs. concentration) using IBM programs.

Research Assistant
Rocky Mountain Biological Laboratory.
Crested Butte, Colorado
June1989 - August 1989.

-Assisted in capturing and feeding butterflies
-Prepared butterfly specimens for genetic analysis using acrylamide gel electrophoresis.

Research Assistant
Department of Ecology, Evolution and Biology
University of Minnesota, Minneapolis, MN
June 1987 - June 1989.

-Analyzed the vegetation history of two forest hollows using pollen analytic techniques.
-Chemically extracted pollen from sediments for identification.
-Plotted pollen diagrams using IBM computer programs.

Teaching

Graduate Teaching Assistant
Department of Biology
Virginia Tech
Blacksburg, VA,
August 1990 - present.

-Taught 3 undergraduate Biology laboratory sections per semester.
-Advised undergraduate students during office hours.
-Wrote and graded test, quizzes and assignments.

PUBLICATION/PRESENTATIONS

Hill, Stephanie R. and John Cairns, Jr. A Preliminary Study of Revegetation of Hazardous Chemical Waste sites. Third Environment Virginia Symposium-Pollution Prevention and Economic Implications. April, 1992.

Hill, Stephanie and M.B. Davis. "Late Holocene Forest Changes around Poison Ivy Pond in Northern Michigan". Poster presented at the Third National Conference on Undergraduate Research, April 27-28, 1989.

ABSTRACTS

Hill, Stephanie R. and John Cairns, Jr. Virginia Polytechnic Institute and State University, Blacksburg, VA. "A Preliminary Study of Revegetation of Hazardous Chemical Waste Sites". Third Environment Virginia Symposium-Pollution Prevention and Economic Implication.

SPECIAL SKILLS

Perkin-Elmer Atomic Absorption Spectrophotometer
Zeiss Videoplan digitizer
Soil extraction techniques
Statistical Analysis Systems
MAC PC packages including MacWord, Deltagraphics, Cricketgraph and PC slide
IBM PC packages including WordStar, WordPerfect, Lotus 1,2,3 and plotting