Heavy metal resistance in *Salix*

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Dedicated to my father, **Harry Punshon**

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Contents

Glossary

The following glossary gives details of terms used throughout this work accompanied by accepted definitions. CSTD = The Chamber Science and Technology Dictionary, (Ed. P.M.B. Walker 1988, Chambers, Edinburgh).

acclimation [acclimatisation] Becoming adapted to environmental stress; *physiological* adjustments in an organism moved to a new environment, usually taking a short period of time. (CSTD)
Phenotypic adaptation to environmental fluctuation: the gradual and reversible adjustment of physiology and morphology to changes in environmental conditions (Crawford 1990).

adaptation Any morphological, physiological or behavioural characteristic which fits an organism to the conditions under which it lives; the *genetic* or developmental processes by which such characters arise. (CSTD)
The process that allows an organism to adjust to a changed environment (Beeby 1993). Stresses may cause a change in physiology, or behaviour (in the case of vascular plants this adaptation may involve migration, *see* Bradshaw & Hardwick 1989). These changes are genetic and heritable.

amelioration To improve the condition of, to make or become better (Collins Concise Dictionary).
Amelioration of metal polluted soil is used in this thesis in the context of removal of the plant-available fraction of heavy metals from the soil using resistant ecotypes.

amendment Chemical or physical treatments applied to polluted soil intended to restore fertility and reduce availability of heavy metals to plants (Smith & Bradshaw 1972; Sims & Kline 1990).

biological availability The proportion of the total metal concentration that can be assimilated in to the tissues of living organisms (Beeby 1993).

bioremediation [incl. biotreatment technologies] The use of tolerant vegetation; in some cases in association with a symbiotic fungi (mycorrhizae), to stabilise a polluted substrate, improve structure and to ameliorate the condition of the soil by active uptake and accumulation of plant-available pollutants. Also termed 'phytoremediation' (Brown, Chaney, Angle & Baker 1994).
The development of systems that use biological catalysts to degrade, detoxify or accumulate environmental pollutants (McEldowney, Hardman & Waite 1993).

clone Organisms all derived from a single progenitor by asexual means, having an identical genotype. In plants, this includes those derived by vegetative propagation such as grafting or taking cuttings (CSTD).

hyperaccumulator Brookes (1977) coined the term 'hyperaccumulator' or 'nickel plants' to describe plants which naturally contain more than 1000μg g^{-1} nickel (0.1%) in dried aerial parts of the plant. This definition has been extended to include other metals.

metal resistance The ability of plant populations to withstand elevated concentrations of heavy metals. This can involve either 'true', constitutional tolerance (Baker 1987) or avoidance (Turner &Dickinson1993b). Ernst *et al,* however, (1992) defines *both* tolerance and resistance as an heritable increase in the ability to cope with excessive metal, usually naturally or artificially selected under the pressure of a toxic level of metal exposure.

metal tolerance An inherited gentotypic adaptation to metal stress (Baker 1987; Bradshaw 1991).

Antonovics *et al* (1971) uses the term in two main senses;

(i) generally any species (or ecotype) found occurring in an area of toxicity from which other species appear to be excluded.

(ii) specific individuals of a species which are able to withstand greater amounts of toxicity than immediate

	relatives on normal soil; genotypes with the ability to evolve tolerant ecotypes.
metallophyte	Plants which naturally prefer to inhabit a metal-enriched environment (Ernst, Schatt & Verkleij 1990).
phenotypic plasticity	A variation in phenotypic expression of a genotype that occurs in response to particular environmental conditions and which enhances the capacity of the individual to survive and reproduce under those conditions (Sultan 1987)
pollution	Any increase in the concentration of matter or energy generated by human activity which degrades a living community or its abiotic environment (Beeby 1993).
reclamation	[incl. restoration, rehabilitation]: The establishment of a self-sustaining plant community (or communities) on unstable or barren substrates, to either recreate the original ecosystem, or to create a specific communtiy to meet the requirements of the site (Beeby 1993). Stabilisation by covering metal polluted sites with vegetation (Smith & Bradshaw 1972).
stress	Any environmental factor which restricts growth and reproduction of an organism or population (Crawford 1990).
variety	A race, strain, breed or ecotype. A category of individuals within a species which differ in constant, transmissible characteristics from the genotype but which can be traced back to the genotype type by a complete range of gradations, a geographical or biological race (Modified from Cambridge Science & Technology Dictionary 1988).

Abstract

The resistance of a selection of British *Salix* spp. primarily to Cu, Cd and Zn was tested in solution culture as part of a search for suitable bioremediation shrubs. Tolerance, estimated from comparative root growth was found within the genus but differed widely between species and clones. In repeated tests *Salix caprea* was the most resistant and *S. alba* and *S. purpurea* were most susceptible. *S. viminalis, S. fragilis* and the hybrids *S.* x *sericans* and *S.* x *calodendron* showed intermediate resistance. Considerable interspecific and interclonal variation was evident, a characteristic which was prominent in resistant forms of *S. caprea.* Root length successfully predicted resistance or toxicity, but adventitious root production was unaffected by metals. All clones were resistant to elevated cadmium; growth of *S. viminalis* and *S. burjatica* was stimulated in the presence of Cd. Resistance of clones to dual metal treatments was lower than singular treatments, although *S. caprea* was resistant to Cu+Cd and Cd+Zn.

Resistance induction using short-term Cu pre-treatments was largely unsuccessful. Long-term exposure to cumulative metal treatment, however, reduced phytotoxicity and increased resistance. Both induction experiments strongly support the hypothesis that established cuttings are more resistant to impingent metals than unstable 'juvenile' material. Clones varied in their response to acclimation, with the largest increase in resistance observed for *S. viminalis* in response to Zn.

Metal analysis of dried plant tissues revealed that patterns of metal accumulation are characteristic to individual metals. Cu accumulated primarily within root systems and translocation to the stem and leaves was restricted. Cd and Zn were detected in higher concentrations within aerial tissues and more consistent distribution of these metals was observed between tissue compartments. Significant levels of removable metals (μg cutting^{-1}) could only be found for Cd; up to 40% of total accumulated Cd could theoretically be removed by coppice. Cuttings accumulated higher concentrations of metals when exposed to dual combination treatments but toxicity was also higher, indicating interaction between metals.

Pollen germination was affected by metals but no convincing evidence of tolerance indication was found. Attempts to optimise media conditions for *in vitro* propagation of *Salix* indicated that species and clones require specific formulations and that the ease of rooting *in vivo* did not readily translate to *in vitro* studies. It is suggested that the variable resistance of *Salix* may be the result of high levels of phenotypic or genetic mosaicism enhanced by clonal propagation and metal stress. This variation, however, is essential for the development of metal resistant bioremediation shrubs.

Chapter I
Introduction

1.0. Introduction

The context of this study is the potential of the genus *Salix* (willows) for stabilising and ameliorating soils contaminated by heavy metals. The genus shows a wide variation of morphological types ranging from fast-growing biomass shrubs such as *Salix viminalis* (common osier) to hardy species such as *Salix caprea* (goat willow) that are able to grow on nutrient poor and industrially polluted soils (Grime, Hodgson & Hunt 1988). Suitable willows species for bioremediation require high innate metal resistance and resistance-trait stability. The ability to take up toxic metals from the soil and effectively ameliorate soil conditions by reducing the plant-available metal concentration may be another desirable characteristic.

The objectives of the study were to screen a wide range of *Salix* species in solution culture for resistance to copper, cadmium and zinc, using root mensuration techniques (Wilkins 1978), and also to determine the effects of metal pre-treatments on the stability of resistance traits in selected clones. Additionally, the study aimed to establish metal accumulation characterstics in the tissues of *Salix* cuttings. Methodology was to be based upon a suitable hydroponics system in which *Salix* cuttings could be exposed to known metal concentrations in nutrient solution. This was to be supported with soil-based resistance testing using different types of contaminated substrate. Additional objectives included investigation of alternative methods of measuring resistance based on pollen grain germination, and an investigation of *in vitro* micropropagation of clonal material.

1.1. Background to the study

Anthropogenic activities such as mining, smelting, refining and disposal of sewage sludge contribute metals to the biosphere in large quantities (Alloway 1995). Areas adjacent to metal processing industries may receive toxic concentrations of metals through release of metalliferous aerosols and particulate matter from chimney stacks.

Mine waste or 'tailings' typically contain high concentrations of toxic metals, up to 10% in some cases (Smith & Bradshaw 1979), and may also contain a range of 'guest' elements. Sewage sludge is rich in the plant nutrients N, P and K and is often applied to agricultural land as a fertiliser (Moffat 1989), but it is also a major source of toxic concentrations of persistent heavy metals (McGrath 1987). Potentially toxic concentrations of heavy metals are now ubiquitous in industrial and urban areas in the atmosphere, soil and water affecting wildlife at every trophic level (Nriagu & Pacyna 1988).

All metal ions are toxic to plants when present at high available concentrations in the soil (Epstein 1972); although many are plant-essential micronutrients, they also become toxic at supra-optimal concentrations (Davies 1991). In woody plants elevated concentrations of metals inhibit growth (Clijsters *et al,* 1991; Kahle 1993) and reproduction (Chaney & Strickland 1984; Searcy & Mulcahy 1985a,b,c; Holub & Zelenakova 1986). Some soils are geogenically enriched with toxic metals as a result of the weathering of mineral deposits, and plant species associated with these substrates are often specifically adapted to withstand the high concentrations of metals. For example serpentine (ultramafic) soils typically contain extremely high concentrations of Ni, Co and Cr (Brooks 1987; Baker & Proctor 1990) to which a large number of species from the genera *Alyssum* (45 species) and *Thlaspi* (12 species) have been found to be tolerant (Brooks 1987). In addition, the entire genus *Calochortus* has also been found to possess constitutional tolerance to Ni, Co and Cu (Fiedler 1985). Natural enrichment of the soil by toxic metals can also occur as a result of changes in soil conditions. For example Al, Fe and Mg are ubiquitous in most soils in insoluable forms, but changes in the pH of the soil or anaerobic conditions brought about by flooding can bring the available concentration of these metals up to toxic levels (Woolhouse 1983; Ponnamperuma 1972).

Conventional methods of reclaiming or restoring metal-contaminated sites usually involve soil excavation or site capping. Excavation entails the removal of the polluted substrate either for incineration, or for further processing to remove the constituent metals. Chemical reclamation usually involves acid washing of material to lower the pH, making the metals more amenable to extractants (Rulkens *et al* 1995). Immobilisation of polluted soil is more widely known as site capping. This is where polluted ground is sealed with concrete, or heavy gauge plastic, which is then covered with clean topsoil. Other reclamation methods include the application of chemical amendments such as lime and sewage sludge to reduce metal availability. These methods are expensive, do not provide long-term solutions to metal pollution and, in addition, site re-vegetation is still necessary (Smith & Bradshaw 1972; Pitchel *et al* 1994; Mench *et al* 1994).

Examples of plant populations able to survive on soil contaminated by toxic metals from anthropogenic sources began to appear in 1934; observed initially in herbaceous species occurring on metalliferous mine spoils (Prat 1934). It was later established that many plant populations have the ability to develop tolerance to heavy metals due to anthropogenic selection pressure (Antonovics *et al* 1971; Baker 1987; Bradshaw 1991). As the existence of metal tolerance in mine populations became established, it was proposed that other metalliferous mine wastes could be stabilised by planting seed from these tolerant ecotypes (Smith & Bradshaw 1972; McCormack & Steiner 1978). Remediation schemes using resistant herbaceous plants have been suggested as early as 1969 (Gadgil 1969) with the sole intention of re-vegetation. In more recent work Baker *et al* (1988) suggested using hyperaccumulating plant species in bioremediation to reduce the metal content of the soil. Re-vegetation of mine spoils using resistant plants is now an established technique of reclamation (Rulkens *et al* 1995), but current interests have progressed such that 'bioremediation' and 'phytoremediation' (Brown *et al* 1994) constitute the additional amelioration of the soil by plant uptake of heavy metals.

Research into the metal tolerance of woody plants began much later (McCormack & Steiner 1978; Denny & Wilkins 1987a,b; Eltrop *et al* 1991; Dickinson *et al* 1991a,b) although initial bioremediation trials using trees were largely unsuccessful; possibly due to the lack of beneficial mycorrhizal infection on the roots of reclamation shrubs (Danielson 1991). Under normal circumstances trees benefit greatly from association with a mycorrhizal fungus which greatly enhance water absorption and nutrient acquisition (Marx 1975). Furthermore, naturally non-mycorrhizal plants are rare and constitute less than 1% of described plant species (Harley & Harley 1987). Infection of roots with a complementary mycorrhizal fungi can promote the growth of certain woody plant species in metal-polluted soils (Bradley *et al* 1982; Dueck *et al* 1986; Denny & Wilkins 1987c; Jones 1988a).

Other suggestions as to the apparent lack of success of tree establishment on metalliferous waste is the level of competition between tree seedlings and herbaceous plants. Borgegård & Rydin (1989) found that in trials investigating growth and metal uptake of *Betula* spp. on copper tailings, there was a negative correlation between tree- and field-layer biomass, and also between tree density and soil thickness which suggested that the rapid development of a herbaceous layer prevented tree establishment. It was also noted in this study that the application of soil cover of up to 1m on top of copper tailings did not prevent upward movement of metals, and the fine roots of trees of up to 4 years old had penetrated the copper tailings beneath the soil cover. Young trees tend to produce greater vertical root biomass (40-50%) than older trees (20-30%), (Borgegård & Rydin 1989) which may bring seedlings in to contact with the metal pollutants further down the soil profile.

Bioremediation studies using fast-growing biomass trees began in the late 1980's and early 1990's. Species from the genera *Salix* and *Populus* have been tested for their ability to increase yield by utilising the high concentrations of plant nutrients (N, P & K) in sewage sludge and waste waters while withstanding high metal concentrations

(Sabey *et al* 1990; Perttu 1993; Labrecque *et al* 1994; Mench *et al* 1994a). Willows in particular have many attributes that would prove beneficial in the bioremediation of metalliferous soils; the most important being their rapid growth and ability to produce large quantities of light, flexible wood which has many economic uses (Stott 1992). A recent symposium on *Salix* (Watling & Raven 1992) brought together a wide range of research including their affinity for hybridisation and the resultant taxonomic problems this causes (Rechinger 1992; Meikle 1992), the associated macrofungi (Watling 1992), economic uses (Stott 1992), their ecological role as pioneer species for a wide range of ecosystems (Sommerville 1992) and their ability to withstand nutrient-poor and metal-rich soils (Grime *et al* 1988; Eltrop *et al* 1991; Mang & Reher 1992 *abstract*).

Salix spp. have been used effectively in the past for motorway slope stabilisation and rapid production of natural screens (Gray 1992) and have also been used successfully in Sweden for the production of biomass and biofuel for over 15 years (Vahala & Eriksson 1991). *Salix caprea* and *S. cinerea* have been observed growing on metalliferous mine spoils (Eltrop *et al* 1991; Kahle 1993) and other nutrient poor, disturbed industrial sites; Grime *et al* (1988) documented mine spoils as a popular ecological niche of these species.

There is considerable scope within *Salix* for screening and development of vigorous, metal-resistant species and hybrids for use in the bioreclamation of polluted soils, considering the wealth of morphological and ecological variation within the genus. Work on bioremediation using fast-growing willow trees is a developing area of study (Aronsson & Perttu 1994), but is hindered by a confinement of testing to established 'biomass' clones, such as *Salix viminalis* and *S.* x *dasyclados* which have a high nutrient requirement and little natural ability for tolerating high metal concentrations (Riddel-Black 1994). Willow species such as *S. caprea* and *S. cinerea* have a notable ability to colonise nutrient-poor soils and although they do not produce as much woody biomass as the common osiers and osier hybrids, there are natural hybrids between

fast-growing and resistant *Salix* species which may provide a compromise suitable for bioremediation.

1.1.1. Aims of the study

1. To screen a wide range of *Salix* genotypes for resistance to Cu, Cd and Zn and to select resistant clones suitable for use in bioremediation programmes.

2. To investigate the effect of low-concentration metal pre-treatments and cumulative metal concentration increments on resistance in selected willow clones.

3. To examine uptake and accumulation patterns of Cu, Cd and Zn in metal-resistant and sensitive clones grown both in solution culture and in soil.

4. To explore the resistance of willows growing in soil obtained from metal-contaminated sites and to investigate whether this confirms results obtained in solution culture.

5. To investigate concentration of trace metals in willows naturally occurring on metal contaminated substrates.

6. To investigate whether analysis of pollen germination in metal-amended solution can be used as a more sensitive indicator of the metal resistance status of willows compared with whole plant testing.

7. To develop an effective micropropagation media and technique for bulking up selected willow clones and associated mycorrhizal cultures.

1.2 Definition and classification of potentially toxic metals.

Until recently toxic metals have been classified as elements having a density greater than between 4.5g cm^{-3} (Lapedes 1974) and 6g cm^{-3} (Phipps 1991), and have therefore been referred to as 'heavy' metals. Certain of these metals such as Cu and Zn are essential to living organisms in minute amounts and are referred to as 'trace' elements, although they may become toxic at higher concentrations (Alloway 1995). The term 'heavy metal' is now generally considered to be unsatisfactory because it refers only to the density of the element and is meaningless to their behaviour in biological systems (Nieboer & Richardson 1980). The term 'potentially toxic element' (or PTE) has been suggested instead (Alloway 1995) but 'heavy metal' is still used widely. Current classifications of potentially toxic elements are based on the ligand forming preferences of the ions and the stability of the metal complexes they form (Nieboer & Richardson 1980; Phipps 1991). This gives more meaningful information on their toxicity, by taking their availability in to consideration. This separates the ions into the following categories;

- Class A elements = show a preference for ligands containing O.
- Class B elements = show a preference for ligands containing N or S.
- Borderline elements = having preferences which are intermediate, lying between class A and B.

The metals investigated in the present study are Cu, Cd and Zn and to a lesser extent Ni and Pb, all of which are 'borderline' elements according to the new classification system. These elements are most commonly studied in ecotoxicology and have been chosen for this study due to their prominent status as pollutants, the growing problem of their deposition in soils as a result of industrial activity and their availability and toxicity to plants. Each metal has certain differences that affect their availability and toxicity to biological systems; such as their cation exchange values, organic affinity and behaviour in biological systems. The equilibrium constants (pK) of the reaction $M^{2+} + H_2O = MOH^+ + H^+$, determines the adsorption behaviour of different metals and the stability of the resultant metal-complexes. A metal most able to form hydroxy

complexes is sorbed to the greatest extent and is less 'available' or active within a biological system (Alloway 1995). Specific adsorption increases with decreasing pK values, but in the case of Pb and Cu which have the same value, Pb is more strongly adsorbed due to its greater ionic size. The equilibrium constants are given below in Table 1.0 and includes the characteristics of the toxic metals used and information relevant to their behaviour in biological systems. (Adapted from Alloway 1995, Lepp 1981; Baker & Senft 1995; Kiekens 1995; McGrath 1995 and Davies 1995).

1.3. Sources of toxic metals.

1.3.1 Natural sources of metal elements

Over 99% of the earth's crust is made up from ten major elements; O, Si, Al, Fe, Ca, Na, K Mg, Ti and P with the remaining elements in the periodic table (including toxic metals) present in much smaller quantities. The physical and chemical weathering of parent rock causes the formation of a soil of similar elemental content (Borovik 1989). Sedimentary rocks such as clay and shale generally have a higher metal concentration than igneous rocks due to their ability to absorb metal ions and their ease of weathering (Brooks 1987) therefore the soils which form upon them are often enriched with heavy metals.

Localised ore bodies or 'anomalies' containing high concentrations of one or more potentially toxic metals are the main commercial source of these metals. Prior to industrial inputs of toxic metals in to the atmosphere the main natural source of metals was from volcanic eruptions and, to a much lesser extent, forest fires and crustal material (Friedland 1989; Alloway 1995). From volcanoes alone the emission of toxic metals is relatively high; for Ni 330 ng m^{-3}; Cd 8-92 ng m^{-3}; Cu 200-3000 ng m^{-3}; Pb 28-1200 ng m^{-3} and for Zn 1000 ng m^{-3} have been detected (Hawaii/ Etna: Bowen 1979).

Table 1.0. Information about the toxic metals used in this study

Metal Element	*Natural source*	*Pollution Source*	*Normal levels (mgl^{-1})*	*pK*	*Predominant species in soil (pH)*	*Effects of metal on woody plants (source)*
Copper †	Occurs in pure metal state; also minerals chalcite (100% Cu content); chalcocite.	Mining and smelting; fertilisers, sewage sludge application	1 - 20	7.7 strong	$[Cu(H_2O)_6]^{2+}$ at pH <7 $Cu(OH)^0_2$ in neutral and alkaline soils	Stunting of growth (Foy *et al* 1978) & inhibition of root growth (Daniels *et al* 1972).
Cadmium	Black shales	Phosphatic fertilisers, Zn ores, sewage sludge and incineration of plastics.	0.1-1.0	10.1 weak	Free ion Cd^{2+} predominant but neutral $CdSO_4$ or $CdCl_2$ at pH > 6.5	Damage to photosynthetic function; (Bazzazz & Govindjee 1974a Li&Miles 1975; Clijsters & Van Assche 1985; Godbold *et al* 1991a)
Zinc†	Sphalerite & wurtzite.	Burning of coal and other fossil fuels; aerial fallout set to rise to 11 x 10^6 t by 2000. Sewage sludge, agrochemicals.	50	9.0 weak	Zn^{2+} below pH 7.7; $ZnOH^+$ up to 9.11 above this the neutral $Zn(OH)_2$ is predominant.	Photosynthetic electron transport (Baker *et al* 1982); vacuolation in root meristem cells (Davies *et al* 1991); root meristem size and root hairs (Powell *et al* 1988)
Nickel	Serpentine or ultramafic substrates	agricultural materials; burning of fuel & residual oils; diesel exhaust, Ni mining sites, sewage sludge.	20 (100-7000 serpen-tine.)	9.9 weak	$NiFe_2O_4$ most stable; above pH 8 $Ni(OH)^+$ and Ni^{2+}; in acid soils $NiSO^0_4$ and $NiHPO_4$ occur depending on levels of SO_4^{2-} and PO_4^{3-}	Induced Fe deficiency and foliar necrosis (Hutchinson 1981); decreased dry matter, abnormal starch accumulation (Rauser 1978).
Lead	Pure metal; Black shales.	Vehicle exhaust fumes; mining and smelting; agricultural materials.	17-29	7.7 very strong	Calcareous soils $PbCO_3$; non-calcareous $Pb(OH)_2$, $Pb(PO_2)_2$	Inhibition of growth and mineral nutrition (Breckle &Kahle 1992; Nakos 1979)

† indicates that the element is plant-essential

1.3.2. Anthropogenic sources of metal pollution

The main anthropogenic activities which contribute toxic concentrations of metals in to the soil and atmosphere are shown in Table 1.1. The main metal pollutants considered here are Ag, Au, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Sb, Se and Zn although by far the most important of these in terms of the quantity which is released are Cu, Cd, Zn, Pb and Ni (Alloway 1995).

Table 1.1. Sources of anthropogenic metal entry into ecosystems.

Source	*Information*	*References*
Metalliferous mining and smelting of metal ores.	Dumping of metalliferous mine waste causes a reduction in vegetation diversity; leaching and movement of metals into environment.	Gregory & Bradshaw (1965); Smith & Bradshaw (1972); Alloway (1995); Merrington (1995).
Agriculture and sewage sludge disposal.	Phosphatic fertilisers contain high levels of **Cd.** Pig and poultry manure contain **Cu** and **Zn.** Sewage sludge contains high levels of various metals. Sludge application reduces populations of soil micro-organisms.	Alloway (1995); McGrath (1987); Antonovics (1971) Chaudri, McGrath & Giller (1992) Koomen, McGrath & Giller (1990)
Combustion of fossil fuels	Burning leaded petrol contributed significant levels of **Pb** into the biosphere; reduced by introduction of unleaded petrol (1986-1989)	Alloway (1995)
Metallurgical and specialist industries	Production of chlorine; batteries; paints/pigments, catalysts, printing, medical uses produces a wide range of metals pollutants.	Alloway (1995)
Waste disposal	Landfill sites; risk of metal leaching; metals can enter ground water as chloride complexes.	Alloway (1995)

1.4. The effect of toxic metals on woody plants

Toxic metals have a variety of detrimental effects on all aspects of tree growth, including inhibition of root and shoot growth and reproductive processes such as pollen grain viability and germination. A literature search shows numerous published works involving woody species and their response to toxic concentrations of heavy metals (Table 1.2).

Table 1.2. A list of published works on woody plants and toxic metals

Species	*Pollution type*	*Author (year)*
Betula pendula.	Cd.	Asp *et al* (1994)
Pinus spp.	Coal spoil	Berry (1982)
Betula pendula, pubescans	Cu, Cd, Zn, Pb.	Borgegård & Rydin (1989)
Calluna vulgaris.	Cu, Zn.	Bradley, Burt & Read (1982)
Fagus sylvatica	Cd, Pb.	Breckle & Kahle (1992)
Betula papyrifera	Zn	Brown & Wilkins (1985a)
*Betula papyrifera**	Zn	Brown & Wilkins (1985b)
Picea sitchensis	Cu, Cd, Pb, Zn	Burton, Morgan & Roig (1983)
Picea sitchensis	Cd, Cu, Ni.	Burton, Morgan & Roig (1986)
Picea sitchensis	Cu, Cd, Pb, Zn	Burton & Morgan (1984)
Pinus rubra	Heavy metals	Chaney & Strickland (1984)
Pinus sylvestris.	Cd.	Colpaert & Van Assche (1993)
Pinus banksiana.	Al.	Cummin & Weinstein (1990)
Pinus banksiana.	Coal spoil	Danielson (1991)
Betula papyrifera.	Zn.	Denny & Wilkins (1987a,b,c)
Acer pseudoplatanus	Cu.	Dickinson,Turner & Lepp (1991b)
Pinus banksiana.	Cd, Cu, Ni, Zn.	Dixon & Buschena (1988)
Betula pendula,	Pb.	Eltrop *et al* (1991)
Salix caprea.		
Picea abies.	Al.	Geburek & Scholz (1989)
Picea abies	Al, Pb.	Godbold & Kettner (1991)
Populus tremula.	Cd.	Godbold, *et al* (1991)
Salix spp	Open cast coal sites	Good, Williams-& Moss (1985)
Betula spp.		
Salix spp	Cd.	Goransson & Phillipot (1994)
Betula pendula.	Cu, Cd.	Gussarson & Jensen (1991)
Salix spp*	Metallic mine tailings	Harris & Jurgensen (1977)
Populus spp*		
Forest trees	Heavy metals	Holub & Zelenakova (1986)
*Betula papyrifera.**	Ni.	Jones & Hutchinson (1986), (1988a, b,c)
Aesculus hippocastaneum,	Zn.	Jordan (1975)
Quercus spp.		
Woody plant roots.	Heavy metals	**Kahle (1993)**
Deciduous forests.	Heavy metals	Kohler *et al* (1995)
Fagus spp.*	Zn.	Kumpfer & Heyser (1988)
Salix spp,	Sewage sludge &	Kutera & Saroko (1994)
Populus spp.	waste waters	
Salix spp	Sewage sludge	Labrecque, Teodorescu & Daigle (1994)
Salix spp.	Cd.	Landberg & Greger (1994)
Wood.	Pb.	Lepp & Dollard (1974)
Salix spp.	Metal-polluted river silts	Mang & Reher (1992)
Alnus glutinosa,	Al.	M^{c}Cormick & Steiner (1978)
Betula alleghaniensis,		
B. papyrifera,		
B. populifolia,		
Eleaganus umbellata,		
Pinus rigida,		
P. sylvestris,		
P. virginiana,		
Populus hybrid NE-388,		
Quercus palustris,		
Q. rubra.		

Table 1.2. (Continued.)

Species	*Pollution type*	*Author (year)*
Trees	Heavy metals	McNeill & Glimmerveen (1993)
Picea rubra.	Al.	McQuattie & Schier (1990)
Pinus halepensis	Pb.	Nakos (1979)
Shrubs & trees	Cu.	Norem (1982)
Salix spp.	Cd.	Östmann (1994)
Salix spp.	Cu.	Punshon *et al* (1995)
Salix spp.	Heavy metals.	Riddel-Black (1994a)
Quercus rubra.	Pb.	Rolfe (1973)
Populus deltoides.	As.	Rosehart & Lee (1973)
Shrubs	Sewage sludge	Sabey, Pendleton & Webb (1990)
Salix spp.	Sewage sludge	Simon *et al* (1991)
Q. alba, *Loriodendron tulipifera,* *Robinia pseudoacacia,* *Pinus echinata,* *Eleaganis umbellata.* *Picea glauca* *Picea* spp., *Fagus* spp.,	Cd, Cu, Fe, Mn, Pb.	Schroeder & Franzle (1992)
Betula papyrifera.	Al.	Steiner & M^{c}Cormack (1979)
Acer pseudoplatanus.	Cu	Turner, Dickinson (1993a,b)
Trees*	Heavy metals	**Wilkins (1991)**

* indicates that trees tested were mycorrhizal; bold type indicates a literature review.

1.4.1. Effects on root growth

The physiological responses of plant root growth and development in response to toxic metals have been investigated in detail, although more information is available for herbaceous than woody plants. The main effects of toxic metals on tree roots can be divided into three categories: inhibition of root growth (Wilkins 1978; Breckle 1991; Davies 1991; Kahle 1993), root membrane damage (Asp *et al* 1994) and alteration of enzyme activities (Van Assche, Cardinaels & Clijsters 1988). Of these, root growth inhibition has been the most studied. For example Turner & Dickinson (1993b) observed selective root foraging in *Acer pseudoplatanus* L. growing in a layered soil profile consisting of copper-contaminated and uncontaminated soil. Roots proliferated in the uncontaminated regions of the soil profile while being inhibited in the contaminated regions. This experiment demonstrated clearly the inhibition of root growth by copper as well as demonstrating the ability of the tree to modulate root growth according to soil contamination. Davies (1991) pointed out that despite the

extensive use of root growth as a measure of metal toxicity very little is known about the cellular and molecular basis of metal-induced root growth inhibition. Pritchard (1994) states that root growth is the result of the interaction between cell elongation and cell division where the inflow of solutes in to growing cells is the limiting factor. The following sub-sections reviews work carried out on the effect of toxic metals on these aspects of root growth.

1.4.1.1. Root cell elongation and differentiation

Toxic metals affect both the elongation and differentiation of root cells (Wilkins 1978; Davies 1991; Kahle 1993), acting directly upon the roots causing biochemical and physiological changes (Barceló & Poschenreider 1990). Expansion of root cells is controlled by enzymes which in turn activate chemical cross-linkages within the cell wall (Pritchard 1994). Toxic metals have been found to complex strongly with organic molecules such as enzymes and co-factors (Nieboer & Richardson 1980; Phipps 1981; Borovik 1989); therefore metal-substitution of cross-linkage enzymes could inhibit root growth by decreasing the elastic properties of the cell wall. Evidence for this has been found using grasses and legumes, although there are no published examples for trees. Barcelo *et al* (1986) showed that elevated levels of Cd decreased cell wall elasticity in *Phaseolus vulgaris* L. and Powell *et al* (1986a) described a reduction in the size of cells proximal to the root meristems of *Festuca rubra* in response to Zn, as well as a reduction in the size of the nucleus and nucleolus. Work by Wainwright & Woolhouse (1977) and Denny and Wilkins (1987b) found similar effects of zinc on root growth in *Agrostis tenuis* and *Betula* spp.

Arduini *et al* (1994) studied the effects of Cu and Cd on root elongation, lateral root growth, root density and thickness in *Pinus pinea* and *P. pinaster*; finding not only that high concentrations of these metals inhibited elongation, but that lateral root growth was also reduced, and the apical cortex of treated roots showed some thickening. They report yellow discolouration of treated root systems which were attributed to increased

suberisation; a common response of roots to toxic metals (Kahle 1993). The cortical thickening observed in treated roots has also been observed by Breckle (1991) in *Fagus sylvatica* exposed to Pb^{2+} and by Turner & Dickinson (1993b) in *Acer pseudoplatanus* L. in response to various metal ions in contaminated soil. This response suggests that these metals affect cell elongation in the extension zone rather than inhibiting mitosis in the meristem.

1.4.1.2. Cell Division

Overall growth of roots is attributed to the production of new cells within the apical meristem as well as the expansion of existing cells, and so metals which cause a reduction in the amount of meristemmatic cell division will also inhibit root growth. Several workers using herbaceous plants have observed that Zn can reduce the percentage of mitotic cells present in the root apical meristem; this is referred to as the mitotic index (MI) (Swieboda 1976; Powell *et al* 1986b; Kocik *et al* 1982). Other toxic metals such as Al and Ni have also been found to have similar effects on MI in herbaceous plants (Horst *et al* 1983; Robertson & Meakin 1980). Arduini *et al* (1994) found that a combined Cu and Cd treatment significantly reduced the MI in *Pinus pinaster*, suggesting that metal-toxicity in roots of herbaceous and woody plants are relatively comparable.

Davies (1991) suggests a degree of caution when using MI as an indication of mitotic activity as there is a paucity of detailed work on the duration of the cell cycle which itself is a product of a series of distinct cell division phases. Powell *et al* (1986b) observed a lengthening of the cell cycle in a Zn-sensitive cultivar of *Festuca rubra* L. by 132% at 0.2 $\mu g\ cm^{-1}$ Zn, but found no significant change in the duration of mitotic phase (M). A constant value of M makes the MI more useful and indicates a change in the duration of other phases in the cell cycle; without this the index is questionable. Davies (1991) suggested the increase in cell cycle in this case is due to an increase in the G1 phase (presynthetic interphase stage) of the cell cycle. This particular stage has

some significance in mitotic cells undergoing stress; not only is it the longest phase (Clownes 1976), but has been observed to respond to other stresses such as low temperature by this characteristic lengthening (Francis & Barlow 1988). This phase is considered to be the ideal stage for arresting mitosis of a somatic cell undergoing stress, and can be maintained until conditions improve. It is proposed that a reduction in root growth by toxic metals such as Zn may be an indirect effect caused by the protective suspension of the cell cycle in the root apical meristem.

1.4.1.3. Membrane damage and water relations

There have been further reports on cell membrane damage and alteration of water relations within higher plants responding to toxic metals (Barceló & Poschenreider 1991). Membrane damage is a direct effect of metals on plant roots as the root system is the main contact point for metal entry in plants growing in polluted soil. A change in water relations as a response to metal toxicity arises as a result of root cell membrane damage and is a secondary effect of metal toxicity. Membrane damage in response to toxic metals often involves K^+ balance of the root cell membrane (Asp *et al* 1994; Gussarson & Jensen 1991; Lindberg & Wingstrand 1985) and has been studied in response to Cu and Cd. Although there are a great deal of reports of membrane damage arising from Al toxicity (Haug & Caldwell 1985; Barceló & Poschenreider 1991) further investigation of this metal is beyond the scope of the present study.

Metal interaction with the root plasma membrane can cause increased ion permeability which results in a leakage of internal K^+ (Wainwright & Woolhouse 1977; Gussarsson & Jensen 1992). Gussarson & Jensen (1992) and Asp *et al* (1994) both describe an temporary inhibition of K^+ influx in Cu and Cd treated tree roots. Active transport of inorganic ions such as K^+ across the root cell plasma membrane are driven by transmembrane electrochemical gradients, which in turn are controlled by ATPases. Active K^+ uptake is therefore a target for metal complexation due to the dependence on

metal-attracting biomolecules. This has also been confirmed for Cd-toxicity in the roots of *Beta vulgaris* L. by Lindberg & Wingstrand (1985).

Gussarsson & Jensen (1992) proposed that metal ions depolarise the electrical potential of the plasma membrane and that a net efflux of K^+ is intended to re-polarise the membrane (Hedrich & Schroeder 1989). They also noted that despite a recovery in K^+ influx after several hours exposure to Cd, the levels were never fully restored, and therefore efflux of K^+ causes permanent damage to the cell. The partial recovery in plasma membrane function observed in plant roots may be a stress response, and a way in which plants can deal with long term environmental stresses; Thompson (1985) and Steer (1988) maintain that a rapid turnover of the plasma membrane is a means by which direct stresses upon the root system may be modulated.

There has been additional evidence, using herbaceous plants, to suggest that sulphydryl reactions and lipid peroxidation may be responsible for increased membrane permeability caused by toxic metals. De Vos *et al* (1991) studied the effect of Cu on the root cell plasmalemma in Cu-tolerant and sensitive ecotypes of *Silene cucubalus* Wib. finding that with increasing Cu concentrations ion leakage increased proportionally as did lipid peroxidation products in root samples.

1.4.2. Other effects of toxic metals on plants

1.4.2.1. Photosynthesis

In addition to growth reduction and damage to root cells, toxic metals also effect enzyme activity (Van Assche, Cardinaels & Clijsters 1988; Van Assche & Clijsters 1990). Metals can inhibit the production of certain enzymes in higher plants, as well as promoting the production of others. Changes in enzyme activity can be detected prior to the appearance of visual toxicity symptoms such as stunting, chlorosis and necrosis (Van Assche & Clijsters 1990); affecting normal photosynthetic function and may be considered a more sensitive indicator of phytotoxicity.

The main point of interest to the present study is the effect toxic metals may have on the amount of above ground biomass produced by plants, as this affects the productivity, economic and ecological success of bioremediation shrubs. A reduction in normal photosynthetic function will directly reduce the productivity of the plant; decreasing biomass. The appearance of chlorosis may also indicate that metals have been translocated in to the aerial parts of the plant where metals can potentially enter the food chain through insect herbivory.

In a general summary of metal-induced inhibition of photosynthesis Van Assche & Clijsters (1990) regard the key effects of metals to be:

(i) binding to functional ligands (mainly SH-groups) that regulate enzyme structure or involved in catalytic action.

(ii) induction of metal deficiency in metalloproteins, and substitution of an essential metal ion for a toxic metal ion.

These basic reactions of metals with enzymes and proteins affect general photosynthetic functioning by inhibiting chlorophyll biosynthesis (Baszyñski *et al* 1980), electron transport (Clijsters & Van Assche 1985) and CO_2 fixation; specifically RubisCo and the integrity of the Calvin Cycle.

1.4.2.2. The effect of toxic metals on reproductive processes in trees

Germination of pollen and pollen germ tube elongation *in vitro* have been used as sensitive indicators of atmospheric pollution (Cox 1988). The sensitivity of pollen to pollution is attributed to its response to lower pollutant concentrations in comparison with other plant structures such as leaves (Varshney & Varshney 1981). There has been extensive work on the effect of ozone and SO_2 on pollen growth in higher plants (DuBay & Murdy 1983; Feder 1981) with studies showing inhibited germination and stunting of pollen tube growth. Changes in the pH of the stigmatic surface is thought to be the main cause of inhibition in the case of the above air pollutants (Cox 1988). However there is less published research on the effects of toxic metals upon

reproductive processes in trees. The effects of metals and acidity on pollen germination and tube growth has been investigated (Cox 1988; 1992), showing that there is an antagonistic effect between pH and Cu on pollen growth characteristics of the broad-leaved trees *Acer saccharum* Marsh and *Betula alleghaniensis* Britt.. It was also found that there were no inhibitory effects observed for Cu alone; which is in contrast with the findings of Chaney & Strickland (1984) who observed inhibition when Cu was present above 0.036 mg l^{-1} although pH was not studied in this case. Searcy & Mulcahy (1985a) found parallel expression of tolerance to Cu and Zn in the growth characteristics of pollen in *Silene dioica* and *Salix alba.* This prompted further work using pollen for the selection of tolerance in plants (Searcy & Mulcahy 1985b). However, maintaining a constant pH of the test solutions would elucidate the effects of a range of important metal pollutants on pollen germination, and the degree to which this effect reflects the tolerance status of the tree could potentially also be established.

1.5. Metal resistance in woody plants

It has long been established that certain plant species subject to inputs of toxic metal can respond by evolving metal tolerant ecotypes (Gregory & Bradshaw 1965; Baker 1987; Eltrop *et al* 1991; Dickinson *et al* 1991a). This response to anthropogenic selection pressure was first observed in the herbaceous species *Silene dioica* growing on a copper mine (Prat 1934). When the mine population was grown in copper-amended media with plants from an uncontaminated site, greater root growth was observed in the mine populations; Prat (1934) suggested that these populations had developed tolerance to copper by a process of natural selection. Work continued to focus on herbaceous plants that occurred naturally on metalliferous mine sites, and established their tolerance to metals (Gregory & Bradshaw 1965; Antonovics *et al* 1971; Baker 1987). As studies continued, much progress was made on the tolerance of herbaceous plants to metals and the cellular mechanisms that were responsible (For a review *see* Ernst *et al* 1992). However studies involving trees were not published until 1978, over 40 years after research in to metal tolerance first began. McCormack & Steiner (1978) carried out

broad-range screening experiments on the aluminium tolerance of eleven species of trees in solution culture. Their work was the first of its kind in that it tested non-mine populations to determine their natural, or innate level of aluminium tolerance. The results successfully separated resistant species from sensitive ones and made important recommendations for the use of trees in revegetating mine spoils of varying kinds. This study was followed up by the same researchers (Steiner, McCormack & Canavera 1980) concentrating on the variation in Al-tolerance between *Betula* trees from 49 different provenances. Despite varying responses, some tolerant clones were able to survive in concentrations of available Al of up to 120 mg kg^{-1}. This revealed that tolerance varied intraspecifically as well as interspecifically, and suggested that screening ranges of different clones of the same species of tree could potentially reveal those with high innate metal tolerance capabilities.

Brown & Wilkins (1985a) tested the metal tolerance of *Betula pendula* Roth. and *B. pubescans* from a Zn-contaminated mine site, and compared them to reference populations from uncontaminated sites. They found that the tree ecotypes taken from the Zn-contaminated site had a greater ability to withstand elevated concentrations of Zn than the reference ecotypes. This experiment mirrored those carried out for grasses over 50 years before. They found that the trees from the contaminated site were more tolerant to high concentrations of Zn; findings which were similar to the tolerance characteristics identified for herbaceous species. They stressed the need for more studies on woody plants, commenting that the majority of experimental data for metal tolerance had been supplied from 'two or three grass species'.

Denny & Wilkins (1987a) observed a tolerance response in ecotypes of *Betula papyrifera* collected from a zinc-contaminated sites, and also noted an accumulation of zinc in the vacuolar compartment of root cells in the tolerant ecotypes and lower concentrations of zinc in the aerial compartment (Denny & Wilkins 1987b). These findings indicated that tolerant ecotypes of trees, in common with tolerant grasses, have

different zinc uptake and translocation characteristics and avoid direct contact with zinc by accumulation in vacuoles (Davies, Davies & Francis 1991).

Turner *et al* (1991) used several different methods to detect tolerance in *Acer pseudoplatanus* L. (sycamore) growing on Cu- and Pb-contaminated sites; with solution culture, soil and pollen studies giving conflicting results. None of the growth parameters used showed tolerance abilities, but it was suggested that the results were strongly influenced by the duration of the tolerance tests. Dickinson *et al* (1991a) reports development of metal resistance in sycamore from a Cu-contaminated site, where the trees on the site pre-dated the pollution episode. They stated that no tolerant offspring were produced by the trees, and identified resistance in adult trees using tissue culture techniques. They suggested that this type of resistance is a result of a facultative adaptation, and that the long-lived nature of trees makes a flexible phenotypic response to stress a distinct advantage. Dickinson *et al* (1991a) reviewed the survival of trees and other long-lived plants in polluted environments and suggested that tolerance may be the result of an orchestrated response, combining innate tolerance, avoidance strategies; both genotypic and phenotypic adaptation. They argued that the ability to acclimate to short-term environmental stresses is a distinct evolutionary advantage for long-lived plants.

1.5.1. Phenotypic plasticity

Phenotypic plasticity (acclimation) is another means by which a tolerance response may be expressed in plants experiencing environmental stress. Despite earlier theories that phenotypic plasticity buffered the effects of natural selection due to the lack of heritability of the trait (Thompson 1991), there is now great interest in this type of adjustment to environmental stress and it is considered to be a fundamental component of evolutionary change (Thompson 1991). Workers now realise that although acclimation to an environmental stress may not be passed on to progeny, the potential to acclimaye may itself be inherited. Plastic responses have been demonstrated in

transplant experiments using modules of vegetatively propagated plants; this was shown by Turkington & Harper (1979) who observed a particularly strong genetic adaptation of ecotypes of *Trifolium repens* to their local environment; most specifically; their ability to grow best with the grass species with which they were originally associated. This type of acclimation can occur on a very small scale (Dickinson *et al* 1991a) amongst different plant species growing together and also with clonal plants in response to environmental pollution (MacNair 1981). Plasticity in plants is made possible by modular growth (Schmid 1992); where new plant parts can constantly be added to the actively growing plants, with an individual plant having a 'population-like' structure of phenotypes. This type of modular, vegetative growth is adopted by many herbaceous plants, such as grass species which were among the first to develop metal tolerance on mine sites. It also occurs in a modified form in woody species; where the individual develops new meristems and new branches, with selection pressure favouring the well acclimatised parts over those that are susceptible to environmental stress.

The development of a genetic 'mosaic' was suggested to occur by Gill (1986; 1995) with reference to trees. Theoretically, selection pressures developing over a short period of time could act upon the developing meristematic regions giving rise to new roots and branches, altering the phenotypic expression and enabling the prolonged survival of trees despite changing environmental conditions. This theory draws on similarities between the modular growth of tree meristems and the vegetative growth of ramets that occur in clonal herbaceous plants, in that tree development involves the addition of new parts on to the body of the individual (Schmid 1992).

1.5.2. Exploitation of metal resistance traits

Metal tolerance or resistance, whether genotypic or as a result of phenotypic plasticity is an essential trait of bioremediation shrubs. Screening programmes for bioremediation shrubs should not only seek to identify clones with higher innate metal tolerance; but

also identify those with a higher degree of phenotypic plasticity: in the present study it was considered that the latter could be investigated by using resistance induction and metal pre-treatments to estimate approximate tolerance ranges.

Tree-planting trials on polluted soils are already under way (Göransson & Phillipot 1994; Landberg & Greger 1994) primarily using the fast-growing *Salix* and *Populus* clones that are also useful as a source of woody biomass. These studies indicate that willows can accumulate high concentrations of metals, especially cadmium, within their aerial tissues. This accumulation may result in the reduction of the plant-available fraction of metal in the soil.

1.6. Uptake and accumulation of toxic metals by woody plants

Uptake of toxic metals in trees in relation to tolerance was first investigated by Denny & Wilkins (1987a) in *Betula* spp. and the relationship between external and plant tissue concentrations was examined. This study was a comparison between trees known to be tolerant to Zn and a reference non-tolerant population, and found that while external concentrations of Zn increased, Zn uptake by tolerant *Betula* trees levelled off after an initial increase. This pattern of uptake has also been observed in herbaceous species (Barry & Clarke 1978) and was later characterised as a strategy of metal tolerance (Baker & Walker 1989).

1.6.1. Metal uptake strategies

Baker & Walker (1989) noted that data on external: tissue metal concentrations under varying circumstances revealed three distinct strategies of tolerance. The first strategy, an **excluder** strategy as described by Denny & Wilkins (1987a), entails the control of internal metal concentrations by limited uptake. The second, known as the **indicator** strategy does not involve active control of metal uptake and the internal concentration accurately reflects concentration external metal levels. The third, **accumulator** strategy occurs in distinctly adapted plants that preferentially absorb metals from the

soil. Their tissue concentration is typically higher than the surrounding soil; these plants have been termed 'hyperaccumulators', occurring on naturally enriched, ultramafic substrates. The development of hyperaccumulating plants has taken place over evolutionary time (Brooks 1987), but their development may have begun with increased uptake and metal resistance similar to that seen in species responding to anthropogenic pollution. In the present study it was thought to be feasible to attempt to screen for, and induce metal tolerance in trees, with a view to possible removal of toxic metals from soils.

Strategies of tolerance to metals used by higher plants can be distinguished using uptake and accumulation characteristics. For example avoidance, the ability of resistant plants to avoid localised metal pollution in the soil by selective root proliferation (Turner & Dickinson 1993b) or alternatively by exclusion, where metals are absorbed but isolated from the symplasm by sequestration in vacuoles. This has been observed to occur in plants exposed to zinc; which has been found sequestered in vacuoles in certain tolerant plant roots (Denny & Wilkins 1987a; Taylor 1987; Cumming & Taylor 1990; Brune, Urbach & Dietz 1995).

Studies on metal uptake by woody plants all show a similar trend; the majority of strongly sorbed metals taken up by plants are located in the roots (Rolfe 1973; Denny & Wilkins 1987a; Gussarson & Jensen 1991; Breckle & Kahle 1992; Dickinson *et al* 1994; Punshon *et al* 1995). However, translocation of toxic metals within plants varies widely between different metals. Studies using more available toxic metals such as Cd and Zn have identified large concentrations of this metal in the aerial parts of trees; Landberg & Greger (1994) identified clones of *Salix* which transported up to 72% of their total metal uptake in to the shoots. These clones could theoretically remove metals from the soil if a regular shoot harvest was performed. Östman (1994) studied the uptake of Cd in *Salix* shoots and stems and found that uptake of Cd by willow trees was very high. The calculated annual uptake amounted to 3-4% of plant-available Cd

in the soil. Extrapolations of these calculations suggest that in a 20-25 year rotation cycle the concentration of Cd in the soil could be significantly reduced.

1.7. The role of mycorrhizae in tree survival

Micro-organisms associated with plant roots play an important role in many physiological processes, involving saprophytism, pathogenicity and symbiosis (Marx 1975). The most common symbiosis involving plant roots is in conjunction with a mycorrhizae-producing fungus. The term 'mycorrhizae' literally translated means 'fungus-root'; the majority of plants are naturally mycorrhizal and non-mycorrhizal plants are in a distinct minority (Harley & Harley 1987). Relatively few plants including sedges, crucifers and certain aquatic species grow naturally without mycorrhizal association. Dixon & Marx (1987) state that forest trees are obligately dependent upon mycorrhizal association for normal growth and development. Association with a mycorrhizal fungus increases nutrient acquisition (Bowen 1973; Harley & Smith 1983), water absorption (Duddridge *et al,* 1980) and can protect against soil pollutants (Bradley, Burt & Read 1982) and are therefore important in establishment of successful bioremediation programmes (Danielson 1991).

There is evidence to suggest that some species of mycorrhizal fungi may not be inhibited by toxic metals in the soil; for instance the arbuscular mycorrhizal fungus *Scutelospora dipurpurescans* was identified growing in the soil around a zinc refinery (Griffoen 1994). It was suggested in this case that metal pollution has acted as a form of evolutionary selection producing a tolerant strain of fungus. Griffoen (1994) drew together a list of arbuscular mycorrhizas found in association with higher plants on toxic metal enriched soil. By far the majority of these fungal species were *Glomus* spp. and were found growing in soils enriched with a wide variety of toxic metals, including Zn, Mn, Fe, Cd, Cu, Pb and Ni.

1.7.1 Bioremediation of metal polluted soil using mycorrhizal infection of trees

It is important in bioremediation programmes using trees to identify strains of metal tolerant mycorrhizal fungi that are vital to the growth and survival of seedlings. Marx (1975) proposed the tailoring of bioremediation shrubs with appropriate mycorrhizae, and despite the risk of competition with native mycorrhizal populations present in more developed spoils (Watling 1994 pers comm.) initial infection may provide the necessary support for seedling establishment. The type of mycorrhizal fungus involved in the association is also important in determining the fate of toxic metals. In cases where the fungus prevents plant uptake of metals there will theoretically be no reduction in the metal concentration of the soil and the site is only stabilised by vegetation. The goal of bioremediation is amelioration as well as stabilisation, which involves active removal of metals from the soil in forms that cannot pass through the food chain. Therefore the tolerance of both mycobiont and phytobiont is important and must involve absorption and sequestration of metals.

Studies using higher plants in combination with mycorrhizal fungi to vegetate metalliferous mine spoils have been in progress for some time (Marx 1975; Preve *et al* 1984; Koomen, McGrath & Giller 1990; Hetrick, Wilson & Figges 1994), but they concentrate on site stabilisation only, with the mycorrhizal fungus considered to act acting as a barrier to absorption of metals by plants. In one study using trees Berry (1982) used the ectomycorrhizal fungus *Pisolithus tinctorus* to establish hybrids of pitch pine (*Pinus rigida*) and loblolly pine (*P. taeda*) on coal spoil. Although coal spoil does not contain the same abundance of toxic metals as metal mine tailings there are often some associated toxic metals. In this case Mn and to a lesser extent Zn and Cu were important toxic elements. Berry (1982) noted that association of the pines with *Pisolithus tinctorus* reduced the concentration of these elements in plant tissues in comparison with non-mycorrhizal trees. Shetty *et al* (1994), from the same research group, also tested other soil microbes for improving spoil revegetation. The microbes used in this test were only effective in uncontaminated soil, and could not improve

growth to the same extent as inoculation with a mycorrhizal fungus. They found that inoculation with a mycorrhizal fungus altered translocation patterns of Zn only in the obligate mycobiont *A. gerardii.*

Future testing of the beneficial effects of mycorrhizal symbiosis on bioremediation using woody plants may have many complicating factors, including the tolerance of the plant, the tolerance of the fungus, the type of mycorrhizae, the normal mycorrhizal status of the test plant, the metals tested as well as soil factors such as pH. The first important stage in understanding such interactions is to establish the resistance of non-mycorrhizal trees prior to tmycorrhizal clones, so that resistance and uptake characteristics in trees can initially be clarified.

1.8. Non-mycorrhizal bioremediation using trees.

Initial bioremediation screens were established using herbaceous species, due to the early findings in metal tolerance research (Gadgil 1969; Smith & Bradshaw 1972). In 1982 Norem *et al* published findings from a study established in 1970 which tested the ability of a range of trees and shrubs to revegetate a copper mine spoil. They found that *Dodonea viscosa* and *Baccharis sorothroides* were the most successful in terms of survival, although no metal tolerance traits were implicated. It has been suggested that the use of herbaceous plants for metal removal from the soil may be most effective when hyperaccumulating plants are used (Baker *et al* 1988; 1991; 1994a,b; Bernal & McGrath 1994; Brown *et al* 1994; Kramer *et al* 1996). Hyperaccumulators can remove high concentrations of metals (upto 1%) from the soil and translocate them to the aerial tissues which can then be harvested. The use longer-lived, woody species is also a viable option (Mench *et al* 1994a), not only for aestethic purposes but for the merits of a useful end product. However, very little work has been carried out prior to the 1990's on the interactions of trees and heavy metals with direct regard to their use as bioremediators.

One recent suggested use of trees is for the disposal of metalliferous sewage sludge specifically using willows (Perttu 1993; Aronsson & Perttu 1994; Dickinson *et al* 1994; Östman 1994; Landberg & Greger 1994; Kutera & Soroko 1994; Labrecque *et al* 1994), although preliminary studies have been confined to clones with high nutrient requirements and high rates of biomass production. It would seem there is a wealth of more adaptable clones within the genus which have received little attention so far.

The use of trees as 'collectors' of heavy metals remains a contentious issue; some workers claim to be able to remove large quantities of metal using trees (Östman 1994; Goransson & Philipot 1994) and some consider tree planting as a way of improving the site without uptake of heavy metals into the tree and through the food chain (Beckett *et al* 1995). Relatively few studies based on woody species have looked at the uptake and removal of metals by trees, and less still have considered the ability of mycorrhizal trees to remove metals from contaminated soils, indicating that much important research has yet to be done. Borgegård & Rydin (1989) studied the growth and metal content in a range of species planted in the soil cover over copper tailings, testing a conventional form of site remediation using trees. Their findings indicated not only that there was considerable upward movement of metals through the covering layer, but that the fine roots of young trees (less than 4 years old) had penetrated the spoil material. Levels of various metals within the leaves were elevated as a result and was found to be correlated with the thickness of the soil covering. The continued survival of the trees investigated in this study indicate that tree-planting is a viable bioremediation practice.

It is hoped, therefore, in the present work that *Salix* may have considerable application to bioremediation programmes. Recent studies have shown their ability to withstand repeated fertilisation with sewage sludge, which is typically enriched with metals. Studying the natural metal resistance of willows will provide valueable experimental data on their suitability for bioremediation and their ability to remove metals from polluted soil.

Chapter II
Salix

2.1. *Salix*.

2.1. Introduction

The genus *Salix* contains one of the most diverse range of vigorous, fast-growing, resilient tree species and hybrids known . The name *Salix* is derived from the Celtic word *sallis*: *sal* meaning 'near', and *lis* meaning 'water' (Newshomle 1992) and indicates the strong association of the genus with riversides, lowland and wetland habitats although this is not a necessary requirement of many species (Sommerville 1992). The genus contains over 300 woody species and innumerable hybrids (Meikle 1982) with a wide range of habit; from 30 metre tall trees to semi-prostrate shrubs (Stott 1992). Currently there are 18 species of willow regarded as native to the British Isles (Clapham *et al* 1989), and at least 60 hybrids, most between two species although some have three or four parent species (Stace 1975). Willows form a significant part of the native habitat of Britain; supporting a wide range of invertebrates and other animals, giving them considerable conservation status (Sommerville 1992).

2.1.1. A synopsis of Salix *systematics*

Systemmatically the position of *Salix* within the Plant Kingdom is clear. On the subgenus level the systematics of willows become particularly difficult; this has been the case since Linné established the existence of 29 species in 1753 (Pohjonen 1991). The difficulty arises from the unclear boundaries between species, the introduction of hybrids, subspecies, varieties, forms, populations and clones. Pohjonen (1991) summarises the specific problems which confuse willow systematics, giving reasons why these problems have arisen within the genus. Their wide distribution, perennial habit, polyploidy, ability to hybridise, wide ecological amplitude and morphological plasticity all contribute towards the taxonomical difficulties. Pohjonen (1991)

proposed that these complications occur firstly because *Salix* is one of the youngest broad-leaved genera in the time frame of evolution. Secondly that true hybrids between the willows exist and, thirdly, insect pollination of willows brings about considerable geographical isolation and the creation of numerous local populations. An understanding of willow systematics is central to any kind of experimentation on the genus. This must include a knowledge of the origin, sex and correct identification of each clone, which is considered in the present work to be the basic unit of practical application. It is now widely accepted that willows posses remarkable natural variation *within* species and there are 6 levels of within species variants; i) subspecies, ii) varieties, iii) forms, iv) populations, v) clones and vi) cultivars.

Nomenclature of species, subspecies and forms of *Salix* follows certain rules most commonly used in identification of clones for breeding and biomass purposes. A population can be given an number such as 'R-53-075' for *Salix burjatica* 'Aquatica No 56' for instance, or in the case of a selected individual from a botanical collection an accession number can be used. The latter is used in the present work. Each clone is derived from a particular population of a particular species, subspecies or variety. The sequence of nomenclature appearing in this thesis is as follows: species or hybrid name, sex, cultivar name, source, (hybrid parentage if applicable) and the number of the clone within the collection.

The experiments utilised the resources held at Ness Botanic Gardens, Wirral, Merseyside which holds a collection of over 450 clones, subsampled in 1985 from the National Willow Collection which was established by Kenneth Stott at the Long Ashton Research Institute. A synopsis of the species and subspecies of *Salix* featured in this study is given in Table 2.0.

2.1.2 *Willow classification*

Table 2.0. European tree and shrub species of Salix *according to the system of Skvortsov (1968) after Rechinger (1992).*

SUBGENUS *SALIX* (tree willows)	
Section Amygdalinae Koch	**Section Pentandrae**
S. triandra L.	*S. pentandra* L.
Section Salix	**Section Subalbae Koidz.**
S. alba L.	*S. babylonica* L.
S. fragilis L.	
S. neotricha Görz	
SUBGENUS *VETRIX* Dum. (shrub willows)	
Section Hastatae A. Kerner	**Section Arbuscella Seringe ex Duby**
S. hastata L.	*S. phylicifolia* L.
	S. basaltica Coste
Section Glabrella A. Skv.	*S. arbuscula* L.
S. crataegifolia Bert.	*S. foetida* Schleich.
S. glabra Scop.	*S. waldsteiniana* Willd.
Section Nigricantes A. Kerner	**Section Vimen Dum.**
S. nigricans Smith	*S. viminalis* L.
S. apennia A. Skv.	*S. dasyclados* Wimm.
S. mielichhoferi Sauter	*S. burjatica* Nasarov
Section Vetrix Dum.	**Section Canae A. Kerner**
S. silesiaca Willd.	*S. elaeagnos* Scop.
S. Appenidiculata Vill.	
S. pedicellata Desf.	**Section Villosae Rouy**
S. caprea L.	*S. lanata* L.
S. aegyptiaca L.	
S. cinerea L.	**Section Incubaceae A. Kerner**
S. atrocinerea Brot.	*S. repens* L.
S. aurita L.	*S. rosmarinifolia* L.
S. salviifolia Brot.	
S. tarraconnsis Pau. ex Fontquer	**Section Helix Dum.**
S. starkeana Willd.	*S. caesia* L.
	S. purpurea L.
Section Daphnella Seringe ex Duby	*S. amplexicaulis* Bory & Chaubard
S. daphnoides Vill.	
S. acutifolia Willd.	

To date no single, satisfactory method has been found that gives a comprehensive classification of this genus. Meikle (1992) described the scientific confusion created by early attempts to classify these trees both because of their great propensity for hybridising with almost any other species in the genus, and their morphological

variation with regard to age, light regime, environment and sex. This results in a great deal of different synonyms for the same species, and considerable uncertainty concerning whether some trees are species in their own right, or the product of hybridisation.

2.2. The use of willows for short-rotation forestry

Short rotation forestry (SRF) exploits the rapid juvenile build-up of biomass of broad-leaved tree and shrub species, and their ability to coppice from cut stumps. This method of biomass production utilises solar energy, is renewable, and is environmentally sensitive with regard to the production of greenhouse gases (Dawson 1992). Production of woody biomass for energy production also avoids some of the problems associated with the collection and storage of solar energy. Biomass energy is stored in the form of cellulose and lignin, and species which rapidly produce large amounts of new material, such as *Salix* and *Populus* (and to a lesser extent *Fraxinus*, *Alnus* and *Eucalyptus*) will be ideal for use as biomass trees. *Salix* has three major characteristics which make it particularly suitable for use in SRF; firstly it grows on poor, wet soils; secondly it roots easily from cuttings and finally it has a recurring capacity to resprout from cut stumps, giving high yield of soft, flexible wood (Dawson 1992). *Salix* hybrids have also been tested for their suitability for use as pulp (Deka, Wong & Roy 1992).

Salix viminalis (Common Osier) is the most popular species for use in SRF, and is also grown as a source of material for basket making (specifically 'Bowles Hybrid' and 'Mullatin'). It is notable in that it is one of the tallest-growing species of willows, although it does have a considerably higher nutrient requirement than other species (Stott 1992). Hybrids between *S. viminalis* and *S. caprea* L. (Goat Willow) and *S. cinerea* L. (Grey Willow) have also been shown to be good biomass producers

(Pohjonen 1991). These particular hybrids such as *S.* x *sericans* Tausch ex A. Kern and *S.* x *calodendron* Wimm. produce more lateral branches and fewer, shorter rods per stool, than the common osier but the rod diameters are greater and compensate by giving similar yields.

Alternative non-food cropping cropping systems are now becoming desirable in the UK, as an estimated 1.0-1.5m ha of land will have been removed from conventional agriculture by the year 2000, and this figure is set to increase to 5.0-5.5m ha by 2010 if agricultural overproduction is not halted (Dawson 1992). The criteria for alternative cropping systems to maintain these disappearing rural communities are an economically viable enterprise that can be easily established on relatively poor land. SRF may provide the solution to problems in the UK, with many workers using the successes of Scandinavian willow biomass production as a major influence (Sennerby-Forsse 1994).

Willows have been economically important as a source of bioenergy in Sweden for over 15 years (Vahala & Eriksson 1991; Christersson, Sennerby-Forsse & Zsuffa 1993; Sennerby-Forsse 1994), and research by the National Swedish Energy Forestry Programme (NSEFP) has produced a new agricultural crop with high potential for sound ecological and economic outcome. Sweden now has commercialised energy plantations of approximately 10,000 ha to replace oil imports (Sennerby-Forsse 1994); with a developed requirement for 200,000 ha to meet greater energy needs. The UK has set-aside land in excess of this, and it is feasible to assume that energy forestry may become a economically viable option.

Willow biomass trials were carried out between 1974 and 1984 in Northern Ireland (NI Horticultural and Plant Breeding Station, Loughgall) and Long Ashton Research

Station, to test agronomic aspects such as pre-planting treatments, planting densities and harvesting frequency. In these trials *Salix burjatica* 'Korso' showed most promise as a biomass tree (Dawson 1992). With optimum spacing, an annual yield of 17.0 tonnes ha^{-1} was obtained (McElroy & Dawson 1986). Programmes of willow planting for biomass continue in Sweden (Pohjonen 1991) but have also spread to Canada (Kenny, Gambles & Zsuffa 1993) and the U.S. (Kopp *et al* 1993).

2.3 Biological waste disposal using willows.

The use of willows as vegetation filters is another, much more recent development, and one which has only been in progress in the past five years. Vegetation filters such as those required for disposal of sewage sludge have the main purpose of removing nutrients and toxic metals from the sludge while potentially containing the metals in a non-available form within the woody tissues (Riddel-Black 1994; Neilsen 1994). The activity combines the increasing interest in energy crops and an increasing concern about the disposal of sewage sludge (Heding 1994). In-land disposal of sewage sludge is likely to increase following the banning of North Sea dumping in 1998. This has led to increased interest in the ability of willows to survive sludge application and the dynamics of heavy metal removal from the soil.

Several recent studies have shown that certain members of the genus are able to absorb significant amounts of cadmium from polluted soils (Östman 1994; Landberg & Greger 1994), although some workers have found that many species and hybrids commonly used for biomass production do not perform well as vegetation filters (Riddel-Black 1994a), a fact which indicates the need for a wider ranging investigation into the selection of appropriate species, hybrids and varieties. Trees intended for bioremediation have many similar requirements to those used as

vegetation filters; they must be fast-growing, metal-resistant trees that are able to remove metals and provide physical stabilisation.

2.4. Ecological aspects of willows

One aspect which is neglected by willow planting for biomass and for waste disposal is the role which willow trees play as components of a developing ecosystem. Table 2.1 gives details of the morphology, ecology and bioremediation potential of the main British willow species.

Willows fulfil all of the criteria required of a bioremediation shrub. There is great variety in both habitat preference, morphology and growth characteristics within the genus. Coupled with the ease with which willows hybridise, identification of the ideal bioremediation clone may also be possible through plant breeding, although prior to this the innate metal resistance of pure species and main hybrids must be established. The evolutionary history of the genus indicates that *Salix* is a young and rapidly evolving genus. Grime *et al* (1988) noted the increasing hybridisation between the hardy species *S. caprea* and *S cinerea*, both of which occupy disturbed and polluted environments. The genus is therefore an obvious starting point for screening and development of metal resistance shrubs for bioremediation.

Table 2.1. Characteristics, use and bioremediation potential of willow species.

Species (Common Name)	*Morphology*	*Ecological Information*	*Economic Uses*	*Potential for bioremediation.*
S. alba (White willow)	Tree: large central stem, can grow up to 25m	By river banks; streams and ponds.	Wood source for cricket bats.	Poor; nutrient requirement high.
S. fragilis (Crack willow)	Tree: up to 25m	By river banks, ponds and streams. Propagates by fallen branches	No economic uses	Some; stabilising river banks; undemanding in terms of soil type.
S. viminalis (Common Osier)	Tall shrub: 5-10m. Multiple stems, responds well to coppice. Grows rapidly.	Requires wet, nutrient rich soil; occurs by streams and osier beds.	Biofuel (Vahala & Eriksson 1991); biomass, basketry.	Poor: needs nutrient rich soil.
S. purpurea (Purple Osier)	Shrub: 3m multiple stems, characteristic purple stem colour	As above	Basket-making rarely used in biomass	Very poor; more discreet distribution, sensitive to harsh soil conditions.
S. triandra (Almond Willow)	Shrub: 7m	Grows by sides of streams; mostly S. and E. England; rare elsewhere	Basket-making	Poor, testing sparse; no natural occurrence on poor soils.
S. daphnoides (Violet Willow)	Shrub or tree 10m multiple stems; characteristic red coloured stems	Introduced from Europe; planted by ponds and rivers	Basket-making	Variable. Undemanding in terms of soil type.
S. caprea (Goat Willow a.k.a Great Sallow)	Tall shrub/small tree; no more than 10 m high	Woods, hedgerows, industrial, poor or disturbed soils.	No economic uses	Very good. Some species found on lead spoil with up to 4000ppm Pb (Eltrop *et al* 1991)
S. cinerea (Grey Willow)				
ssp ***cinerea***	Tall shrub/small tree, no more than 10m high.	Wet, mineral soils, fen carr; E.England	No economic uses	Poor; limited distribution
ssp ***oleifolia***	Shrub to 10m	Woods, by streams and ponds. Widespread & common.	No economic uses	Good, associated with a wide range of substrates
S. nigricans (Dark-leaved Willow)	Shrub to 4m.	Mountains, N. England, Scotland, N.Ireland.	No economic uses	Good; survives on thin, rocky substrates.

Table 2.0. (Continued...)

Species (Common Name)	*Morphology*	*Ecological Information*	*Economic Uses*	*Potential for Bioremediation*
S.burjatica	Tall shrub:5-10m. Multiple stems, responds well to coppicing. Grows rapidly. Erect growth habit.	Requires wet, nutrient-rich soil.	Used as a biomass shrub in Sweden (Pohjonen 1991); not used in basketry due to its coarse stems.	Poor; roots well from cuttings but requires high nutrient status.
S. pentandra (Bay Willow)	Shrub to small tree; grows 18m in optimum conditions.	Grows in swampy forests and known to tolerate high water table, capable of growing on organic soils of pH down to 4.5 (Stott 1984).	Productive biomass willow in Finland, comparable to *S. fragilis*. (Pohjonen 1991)	Moderate; records of tolerating extreme pH may be useful (Stott 1985).
S. phylicifolia (Tea-leaved Willow)	Bushy shrub to 3m high.	Native of N. Europe and W. Siberia. Common throughout Finland.	None established but has considerable breeding potential due to its winter hardiness and adaptability to poor conditions.	Moderate; can adapt to poor soil conditions.

(Table compiled from Keble Martin 1982; Meikle 1984, 1992; Newscholme 1992; Stott 1992; Coombes 1992.)

Chapter III
Materials and Methods

3.0. Materials and Methods

3.1. The collection of experimental material

The source of all experimental material for this study was obtained from the Stott willow collection at Ness Botanic Gardens, Neston, Merseyside. The collection contains approximately 450 clones of a wide range of temperate *Salix* species and hybrids. The collection was established in 1985 using material supplied by the Long Aston Research Station, under the auspices of Dr. Kenneth Stott. The willows are identified in terms of sex, hybrid parentage and ploidy; much of which work was carried out by Dr. Hugh McAllister at the Ness Botanic Gardens.

The willow plantation at Ness is spread over three main areas at the edge of the gardens and follows a clear planting scheme, comprising of distinct groups of six ramets derived from the same root stock, with two metre walkways separating each group. The collection was well mapped out and accurately labelled.

3.1.1. Time of year

Cuttings were collected and successfully rooted throughout a wide growth season, with sampling occurring as late as November producing satisfactory propagation. Houle & Babeux (1993) found that cutting viability and rooting vigour of *Salix balsamifera* showed seasonal variation characteristics, with the greatest overall magnitude of growth occurring between May and June, although viability remained high for a much longer period of time. They observed considerable variation in viability and growth between and within clones, which continued to change throughout the growth season. The relationship between root number and root length also changed, with root number remaining more consistent throughout the growth season.

3.1.2. Cutting size

Willow whips of at least one years growth were sampled, with material for each experiment consistently cut from the same ramet within the clone group in order to decrease variation. All foliage was removed to prevent water stress and the whips were cut into 18 cm lengths. Cuttings with a diameter of between 3-14 mm were used in the present studies. Measurements of diameter were taken in all but Test 1 where suitable metal concentrations for future tests were being determined. Burgess *et al* (1990) tested the difference in growth performance of *Salix alba* cut to different lengths and of differing diameters, finding that for this particular species ideal diameter was 0.6-1.3 cm and ideal length was 30 cm. Responses to different initial cutting size may vary between species and was therefore kept constant in the following solution culture tests, taking into consideration the design of the test system and the availability of willow resources.

Cuttings were placed in 3.5 litre black polypropylene containers with 1 litre of distilled water at room temperature in the laboratory until white callus material appeared on the submerged surface of the cutting, indicating root primordia had developed and roots were beginning to emerge. The cuttings were then placed into nutrient solutions in a suspension hydroponics system before any substantial root growth had occurred.

3.2. Root elongation tests in solution culture.

3.2.1. System design

The determination of toxic metal resistance in *Salix* was carried out using a suspension hydroponic system purpose built for the study. The roots of each cutting were suspended into an aerated, circulating nutrient solution. The cuttings were supported from above and the whole system was constructed from inert materials including translucent polypropylene and silicone (plastics are often coloured using dyes made from toxic metal containing compounds, such as cadmium) (Hewitt, 1966) and were sprayed black on the outside in order to prevent algal growth. The system consisted of

a number of independent hydroponic units, each of which was made up of a 20 litre polypropylene nutrient solution container tray (dimensions: 75 x 45 x 20 cm) and a support tray of identical dimensions, with 18 mm^2 perforations organised in a grid layout. Each unit contained one treatment and could accommodate *up to 400 cuttings* (Plate 1.0).

Nutrient solution was pumped from a 25 litre reservoir under the greenhouse bench to the container tray by means of a low-power peristaltic pump. The pump operated at 60 rpm, supplying 125 ml solution min^{-1}; therefore nutrient solution circulated once every 3 h 18 mins. A drainage tube was fitted to the container tray 2 cm down from the lip of the tray to maintain a constant level of solution in the tray, and to allow passive diffusion of air into the solution. The drainage tube was constricted using cable ties, so that the drainage rate was similar to the rate of nutrient solution supply, keeping the level of solution constant.

When the units were set up, a sheet of heavy gauge black polythene was secured tightly over the holes in the support tray with double sided tape. Small slits were cut in the plastic above each hole, so that when cuttings were pushed through in to the nutrient solution, a tight seal was formed. Additional support was provided by a sheet of green plastic garden mesh, which was stretched over the lip of the support tray. The support tray could be lifted off completely at each measurement date, without disturbing the cuttings, thus avoiding damage to root systems.

The perforations in the support tray were arranged in a grid, allowing each clone to be co-ordinated within the tray, avoiding the need for individual labelling. There were in total, 45 groups of 9 perforations, and therefore cuttings were always set out in clone-groups of nine. Replicate number exceeded 27 in all hydroponic tests, with clone-groups randomised throughout the tray using a trial plan.

Plate 1. The hydroponics system.

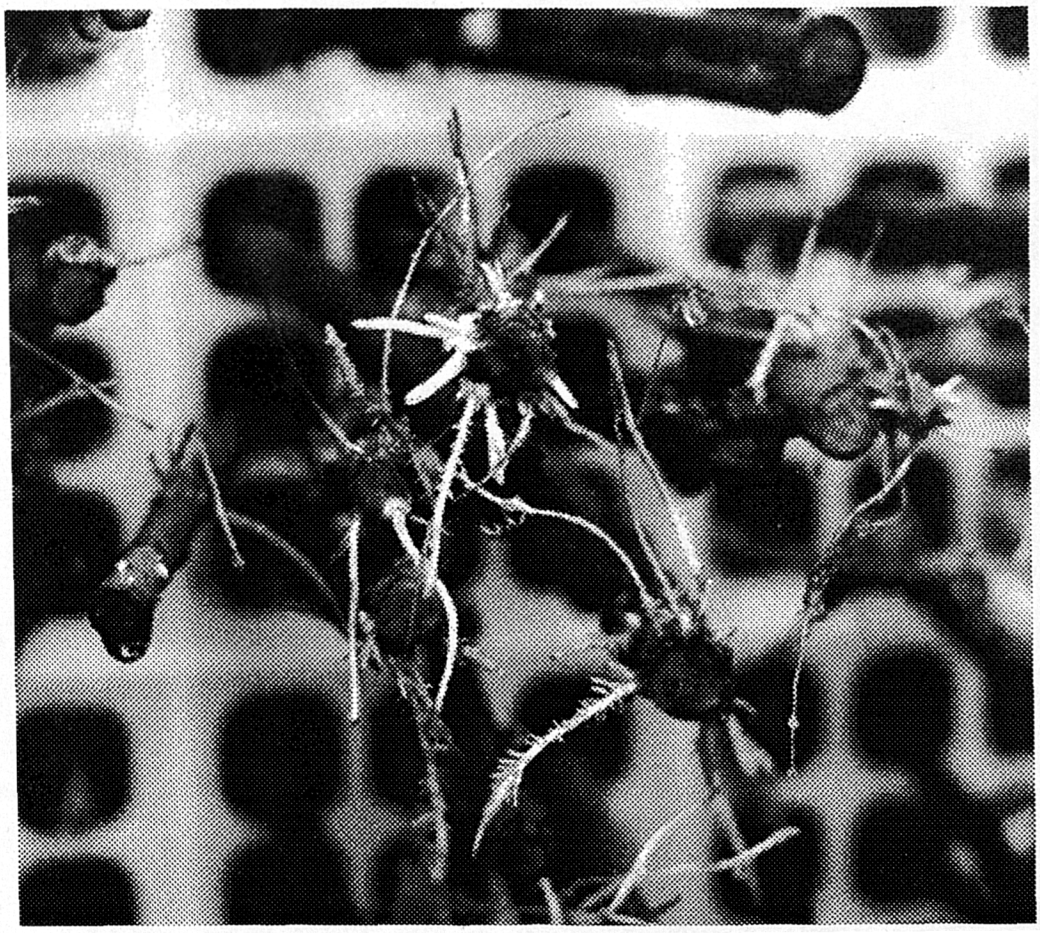


Plate 2. Adventitious root growth in Salix cuttings in solution culture.

3.2.2. The use of solution culture in research.

Solution culture, often referred to as 'hydroponics' or 'nutrient film technique' is an established technique for the experimental investigation of plant nutrition (Hewitt 1966). Its use for the study of metal tolerance and toxicity date back to the first metal tolerance experiments (Prat 1934), and it has provided many important insights in to plant growth and uptake in polluted substrates since then. There are a variety of different types of solution culture system designs to choose from, the choice of design depending on the nature of the experimental work being carried out. The following sections deal with the different aspects of solution culture system design, giving reasons for the design preferences of the system used in the present study.

Many systems incorporate the use of a support medium, added to the nutrient solution as anchorage for plant roots. Usually this is sand, (Hewitt 1966) although other substrates such as silica sand, river sand, rock crystal, pure calcium carbonate and sugar charcoal have also been used. Other, more recent substrates have been developed, such as perlite, vermiculite and plastic granule media, and these types of substrate are inert and relatively free of surface contamination. Support substrates were not used in the present system because organic debris from growing plants may accumulate on the support media and present a risk of complexation with some of the more strongly sorbed metals, such as copper. Furthermore Hewitt (1966) pointed out that pH control is more difficult in sand cultures, due to the restriction of diffusion and reduction in volume of solution surrounding the roots.

Loss of water by transpiration is an important factor in solution culture studies, this is especially important when growing willow cuttings, because uptake and transpiration rate can be very high in some species. This makes the use of single-cutting units difficult due to the risk that some cuttings, especially those in the untreated controls, will dehydrate completely before solution is due to be changed. Replacement of nutrient solutions must be standardised throughout all treatments. If the replacement

frequency is dictated by the healthier, untreated plants, over dosing of the treated plants can occur. Testing large numbers of cuttings in one container allows cuttings with greater transpiration rates to survive, and enables nutrient replacement to be standardised.

Hewitt (1966) suggested that smaller culture containers require replenishment of nutrients approximately twice a week. With the system used in the following study, 20 litres of nutrient solution was held in each treatment container, affording larger intervals between changes. The frequency of nutrient solution replacement depended on the number of cuttings included in each unit, being more frequent when the unit was full.

Aeration of nutrient solutions are essential to avoid the rotting of plant roots; in the present system each hydroponic unit is supplied by four air stones, connected to aquarium pumps. Natural aeration is also made possible by leaving a small gap of approximately 2 cm between the surface of the nutrient solution and the support tray.

Solution culture systems ensure propagation of test plants in a chemically defined medium, where nutrient status, pH and organic content of the solution can be manipulated. The first standard nutrient solution was formulated in 1860 and consisted of KNO_3, $Ca_3(PO_4)_2$, $CaSO_4$, $MgSO_4$ and NaCl (Hewitt 1966) and research in to the development of plant-specific nutrient solution has continued to the present day. Hewitt (1966) gave details of typical nutrient solutions and their original uses, the majority of which were used for the improvement of the growth performance of commercial plants. Early research in to plant nutrition used solution culture techniques to determine micro- and macro-nutrient requirements by omitting certain nutrients and inducing deficiency symptoms (Piper 1942; Gregory 1943; Hoagland 1941 (type B)).

Nutrient solutions for the hydroponic growth of trees appeared initially for fruit trees (Weinberger & Cullinan 1936; Blake *et al* 1937), but solutions for the growth of

coniferous and forest seedlings (pine, spruce and birch) were successfully formulated by Ingestad in 1962. One of the most successful nutrient solution formulations for a wide range of herbaceous and woody plant species was put forward by Hoagland and Arnon (1938), and is still used today (Wang *et al* 1994).

Table 3.0. Chemical composition of Hoagland's Nutrient solution

Chemical	**Stock (g l^{-1})**	**100% solution (ml l^{-1})**	**Final elemental concentration (mg l^{-1})**
KNO_3	101.10	6	235 [K], 224 [N]
$Ca(NO_3)_2.4H_2O$	236.16	4	160 [Ca]
$NH_4H_2PO_4$	115.08	2	62 [P]
$MgSO_4.7H_2O$	246.49	1	24 [Mg], 32 [S]
Micronutrient stock:		1	
Constituent chemicals:			
KCl	3.728		1.770 [Cl]
$MnSO_4.H_2O$	0.338		0.110 [Mn]
H_3BO_3	1.546		0.270 [B]
$ZnSO_4.5H_2O$	0.338		0.131 [Zn]
$CuSO_4.5H_2O$	1.540		0.032 [Cu]
H_2MoO_4	0.081		0.050 [Mo]
Fe-EDTA	6.922		1.120 [Fe]

All root elongation tests were carried out in 25% Hoagland's solution (Hoagland & Arnon 1938) formulated with distilled water and reagent grade chemicals. Studies by Turner (1991) showed that a higher strength nutrient solution ameliorated effects of metal toxicity. In the present study 25% strength solution was found to be adequate for the healthy growth of *Salix* cuttings and was therefore used throughout.

Hoagland's solution was also suitable in the present study because the addition of nitrogen is in the form of both nitrate (NO_3^-) and ammonium (NH_4^+). As NO_3^+ is absorbed, which usually occurs rapidly in solution culture, H^+ ions are also absorbed and OH^- ions are excreted, the net effect being a rise in pH (Jones 1982); the presence

of NH_4^+ as an alternative nitrogen source counteracts the pH rise. Changes in pH can affect root growth as well as altering the availability of metal added in the treatments (Davies, Lear & Lewis 1987; Sauerbeck 1991). Most nutrient solutions have a pH between 5.0 and 7.0 when constituted, and pH-unadjusted Hoagland's typically has a pH of 5.5 which is ideal for the growth of *Salix* cuttings (Newscholme, 1992). Metals were added to the nutrient solution from 1000 mgl^{-1} stock solutions. The metal concentration of the nutrient solution were checked regularly using the atomic absorption spectrophotometer.

3.2.3. Measuring heavy metal resistance in Salix cuttings

The ability of *Salix* cuttings to withstand elevated concentrations of toxic metals was assessed experimentally by comparing root elongation in metal-treated cuttings with those grown in an unamended control solution (Wilkins 1978). Maximum root elongation specifically is indicated by measurement of the longest root on each cutting tested. In addition to this the total number of roots per cutting was also counted in order to give a more accurate indication of root growth on the whole; i.e. in situations where root elongation is inhibited but production of new roots continues, the increase in root biomass is not overlooked. In long-term tests, where lateral root branches began to develop and root complexity increased all lateral roots ≥ 30 mm in length were included in root counts.

Metal resistance is expressed by means of a tolerance index (TI%) as follows:

$$TI\%_{[M]} = \frac{\text{Length of longest root in metal solution}}{\text{Length of longest root in control solution}} \times 100$$

The index expresses the total root growth of the treated cuttings as a percentage of cuttings growing in background solution. Where changes in metal tolerance are investigated (i.e. the response of plants to pre-treatments or increasing metal concentrations was carried out) the length of the longest root measurement used in the equation was substituted by the relative growth rate (**RGR**) (Hunt 1978) of treated

plants throughout the test period. The resultant index expressed the RGR (in mm root elongation day^{-1}) of the test-cuttings as a percentage of the RGR of plants growing in background solution.

3.2.4. Harvesting procedure

After solution culture tests were completed the roots and bases of cuttings were rinsed in fast-flowing deionised water for 2 hours after which leaf and stem material were removed and roots and woody tissue were maintained for 10 days in distilled, deionised water to remove extraneous metals bound to the plant surface. The harvesting procedure was carried out in the same order as the previous measurements, ensuring that all plants were harvested after the same period of time.

Cuttings that had not grown throughout the test period were discarded and clones within each treatment were combined for analysis of tissue metal levels, with samples replicated three times. Combination of plants for digest was carried out to ensure there was sufficient material for digest in metal-treated plants; where production of new material was inhibited, and also reduced between-cutting variation of metal concentration. Cuttings that had produced root, new stem and leaf material throughout the test period were separated into wood, roots, leaves and new stem portions. These separate portions were dried, weighed and prepared for analysis as described in section 3.6.1.

3.2.5. Single-flask technique for testing metal resistance in willow cuttings.

Following large-scale studies on metal resistance using the hydroponics system, selected clone-metal combinations were tested in single flask culture. This method was similar in nature to the hydroponics system, in that aerated, 25% Hoagland's solution amended with metals was used as before, but each cutting was grown in a separate flask. The tests were intended to establish the limit of innate resistance to toxic metals in cases where this had not been clarified during hydroponic testing; i.e. where notable

cases of metal resistance were observed. Measurements of **RGR** (root, shoot and overall fresh weight) were taken. The tests also investigated the level of cutting growth variation in an experimental system where cuttings were independent of each other compared to the hydroponic technique.

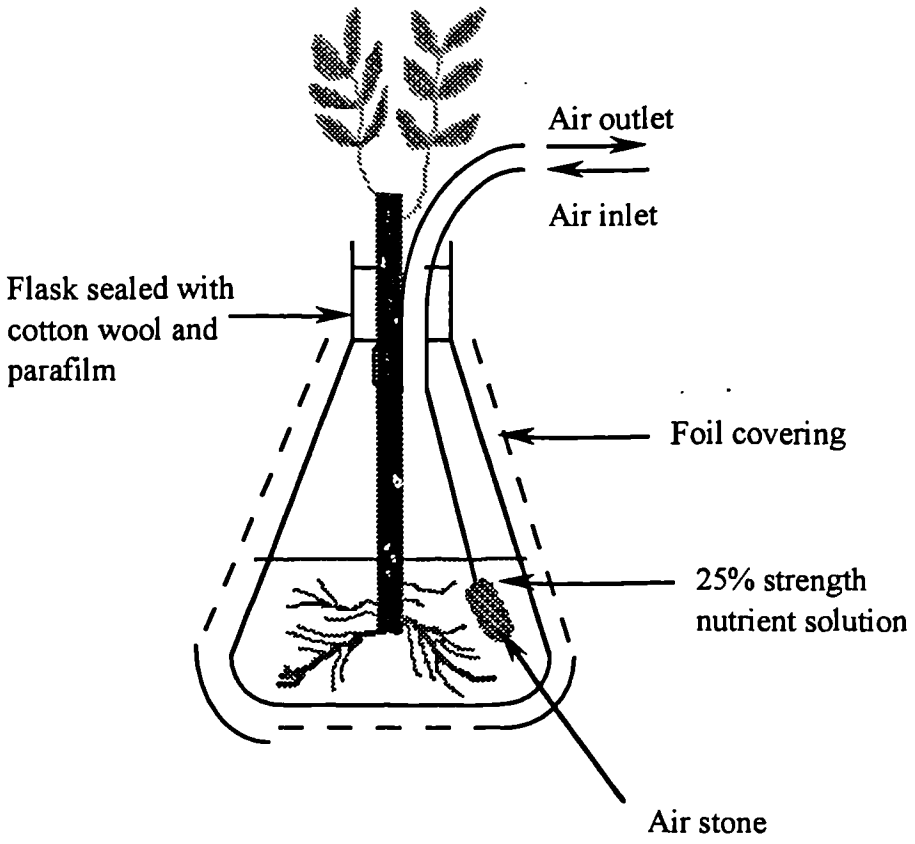


Fig. 1.0. Set up of single flask solution culture units.

Each growth unit consisted of a 250 ml pyrex Erlenmeyer flask, containing 200 mls of 25% Hoagland's solution; (treatments amended with metal), an air stone connected to a Hoffman aquarium pump operating at full power (5 growth flasks per pump) and an air outlet tube. Cuttings were prepared for the tests by growth for 4-5 days in 3.5 litre polypropylene buckets containing 1 litre of glass distilled water until root growth commenced, and small shoots appeared. Each cutting was measured prior to testing, with cuttings selected that were within 4.20 - 7.70 mm in diameter, each having several adventitious roots and at least one shoot. Flasks were sealed with non-absorbent cotton

wool wrapped in parafilm to prevent contamination. The constant air flow from the air outlet tube prevented entry of microorganisms, and the flasks remained free of external contamination. The flasks were wrapped in foil to prevent algal growth in the nutrient solution. Solution was changed every 5 days after the experiment commenced, with growth parameters and the amount of nutrient solution monitored at the same time. Growth parameters measured were: number of adventitious roots, length of longest root, length of longest shoot and fresh weight. The experiment ran for 30 days in total.

3.3. Statistical Analysis of resistance-test results.

All data obtained from growth tests in hydroponic and single flask culture were analysed using the general linearised model of the MINITAB statistical package. The growth data of individual willow species and hybrids showed a characteristic rooting viability; where a similar percentage of cuttings tested failed to root irrespective of metal treatment. Pojhonen (1991) draws attention to the differences in the ability of willow species to root from cuttings, in a review on species suitability for biomass forestry. He describes the characteristically high rooting viability of cuttings of vigorous, fast-growing species such as *Salix viminalis* and the low viability of *S. caprea* and *S. cinerea* cuttings. The growth data obtained from the present study demonstrated the different inherent rooting abilities, and as a result contained a large number of zero values for those species with lower rooting viabilities. The large numbers of zero values on the data affected normality, increased the standard deviation and standard error, and reduced the mean. The inclusion of the zero values was thought to give artificially low mean growth values, and therefore an inaccurate presentation of the data. The zero values were therefore separated from the data. This allowed growth characteristics of successful cuttings to be analysed, supported by an additional value for root viability.

3.4. Propagation of selected Salix *clones in vitro.*

Axenic plantlets of *Salix caprea* Green Dicks (clone: NESS 3287), *Salix caprea* Sidelands (NESS 3288), *Salix* x *aquatica gigantea* (NESS 3349), *S.* x *sericans* (NESS 3305) and *S.* x *calodendron* (NESS 3311) were established in culture from single-node stem explants during the spring. Several basal salt media formulations were tested including DCR (Gupta & Durzan, 1985), WPM (Woody plant medium; Lloyd & McCown, 1981), SH (Shenck & Hildebrandt, 1972) and MS basal salts (Murashige & Skoog, 1962). Prepared media was adjusted to pH 5.8 and was autoclaved at 121°C and 15 psi. Molten media were allowed to cool and poured into sterile glass boiling tubes sealed with stainless steel closures (Oxon). All culture work and pouring of sterile media was carried out under sterile conditions in a laminar flow cabinet.

Explants were collected from fresh cutting material grown in solution culture under greenhouse conditions. Young shoots with leaves removed were cut into single-node sections and shaken for 20 minutes in distilled water. The explants were transferred to sterile conical flasks and shaken for a further 20 minutes with a solution of sterile distilled water and 0.02 mgl^{-1} Benomyl fungicide to reduce the incidence of fungal infection. The benomyl solution was decanted off and a 10% NaOCl + Tween (1 drop 100 ml^{-1}) was added to the conical flask, and the explants were shaken for 15 minutes, during which time the condition of the explants was carefully monitored, to avoid excessive tissue damage. The NaOCl solution was decanted off and the explants were washed three times with sterile double-distilled water; shaken for 5 minutes each washing. The explants were transferred onto solidified medium and incubated in a temperature controlled growth room with P.A.R. of 99μM at 20°C with a 16/8 photoperiod. The cultures were bulked up by regular monthly subculturing, although the frequency varied with the species or hybrid being used.

3.4.1. Measuring the success of Salix explants in vitro.

Throughout the experiments on *in vitro* growth of *Salix* explants, the success of the different media formulations were assessed by counting those cultures that had survived, in addition to the number of explants that had developed both root and shoot material. This was expressed as a percentage of the explants initiated. The criterion for a successful media formulation was the highest percentage survival alongside the greatest percentage of new tissue production. Formulation inducing an abnormal balance of shoot or root production were not considered successful in these tests.

3.5. Analysis of soil characteristics.

H_2O-extractable and HNO_3-extractable heavy metal concentrations were determined for several test soils, including the soil from the willow plantation at Ness Botanic Gardens (See Chapter 6.0), bare spoil and substrate from the lead/zinc mine spoil in Trelogan, in which *S. caprea* and *S. cinerea* were found growing, Prescot soil and a standard potting compost (John Innes No.1) which was used as a reference, uncontaminated soil. Analysis of physical soil characteristics of the bare spoil from Trelogan, Prescot soil and the reference soil were also determined, they consisted of:

- pH
- % weight loss at 105°C (moisture content)
- % weight loss on ignition (organic matter content)
- exchangeable cations (Ca, K and Mg)

3.5.1. pH

The pH of test soils was determined using a glass-bulb pH electrode, (soaked in 10% HCl prior to use) and a Jenway hand-held pH meter. A 50 ml beaker was half filled with soil and filled with distilled deionised water, giving a soil:water ratio of approximately 1:2 by volume. The mixture was stirred thoroughly using a glass rod and allowed to stand for 10 minutes before reading the pH. Readings were replicated three times and the arithmetic mean was determined via antilogarithms of the readings.

3.5.2. Moisture content

20 g of fresh soil was weighed into a dry evaporating basin of known weight. Samples were placed in an air-circulation oven at 105°C and dried to a constant weight; where successive weighings did not differ more than 1-2 mg. The samples were cooled in a dessicator and weighed, with moisture content calculated from the loss in weight.

3.5.3. Loss on ignition (Organic matter content).

5 g of oven-dried soil (from the moisture determination) was weighed into a dry crucible of known weight. Samples were placed in a muffle furnace and the temperature was allowed to rise to 550°C, samples were maintained at this temperature for 3 hours before removing and cooling in a dessicator. Samples were weighed and organic matter was calculated as percentage weight loss.

3.5.4. Exchangeable cations

10 g of air-dried sieved soil (2 mm) was extracted with 250 ml of 1M NH_4OAc (pH 7) and filtered using Whatman N°44 paper. The concentration of displaced cations (principally Ca, K and Mg) were determined by flame photometry and expressed as $cmol_c 100g^{-1}$.

3.5.5. Water-extractable metal concentration.

The fraction of the total metal content of soil that can be extracted with water may be taken as a simple and appropriate representation of the concentration of metals available for uptake by plants. The method described below has been shown to be successful for a variety of metal-contaminated soils, and correlates well with plant uptake of Cu, Ni and Zn (Ure, 1990;Watmough 1994). 5 g of air dried soil was shaken with 25 ml of distilled deionised water for 2 hours. The sample was filtered through Whatman N°1 filter paper into 30 ml glass universals and prepared to volume. Metal concentration was determined immediately by atomic absorption spectrophotometry using water only blanks.

3.5.6. HNO_3-extractable metal concentration using microwave digestion

All soil samples used were digested by microwave digestion technique using the MDS-81D microwave digestion instrument, Teflon PFA vessels (120 ml size) with pressure relief valve and capping station (CEM Corporation), as this allowed more complete digest and reduced the risk of losing sample contents. The method below was optimised using the manufacturers recommendations as a base line.

Triplicate soil samples were dried in an air-circulation oven to a constant weight at 105°C and ground to a fine powder using a mechanical mill. 1.0 g of sample was added to the Teflon digestion vessels and 10 mls of 5*M* HNO_3 added. The safety valve and caps were placed on and tightened in the capping station. After placing the samples in the turntable the exhaust fan was switched on and the rotating turntable was activated. The microwave was programmed as follows: step 1: 2 minutes 20 seconds at 100% power, step 2: 10 minutes at 80% power. The solutions were the allowed to cool for 5 minutes and then manually vented to release pressure. After cooling in a fume cupboard the solutions were filtered (Whatman N°1 paper) into 25 ml volumetrics and prepared to volume using deionised, distilled water. The samples were analysed by atomic absorption spectrophotometry. Samples were replicated three times with one HNO_3 blank per run. All samples were analysed using a Pye Unicam Atomic Absorption spectrophotometer at standard operating conditions; using a deuterium flame with background correction.

3.6. Preparation of plant tissue samples for metal analysis.

Material for digestion was sampled randomly from the hydroponics system, washed in fast-flowing glass distilled water for 2 hours, roots and wood were maintained for 10 days in distilled deionised water, before being dried at 80°C for 48 hours. Material was separated in to leaf, root, new stem and woody tissue prior to drying and were then ground to pass through a 1 mm stainless steel sieve. All material was ground in a mechanical sample grinder.

Table 3.1. Atomic absorption spectrophotometer operating conditions.

Instrumental Parameters	***Copper***	***Cadmium***	***Zinc***	***Lead***
Light source	Hollow cathode	Hollow cathode	Hollow cathode	Hollow cathode
Lamp current (mamps)	5	3	5	5
Wavelength (nm)	324.7	228.8	213.9	217.0
Slit width (μm)	320	320	320	320
Flame description	lean, blue	lean, blue	lean, blue	lean, blue
Sensitivity (at 0.0044 A = 1% absorption)	0.04μg ml^{-1} (1μg ml^{-1}= 0.1A)	0.01μg ml^{-1} (0.25μg ml^{-1} = 0.1A)	0.02μg ml^{-1} (0.5μg ml^{-1} = 0.1A)	0.1μg ml^{-1} (2μg ml^{-1}= 0.1A)
Linear range	upto 4μg ml^{-1}	upto 2μg ml^{-1}	upto 1μg ml^{-1}	upto 15μg ml^{-1}
Comments	Cu signal often depressed in high Zn/Cu ratios.	None	Non-atomic species absorb strongly at 213.9nm; background correction is especially important	Pd absorbance is depressed by presence of: Al, Co, Ni, Pt, Rh, Ru & HF. Eliminated by making solution in 0.5% (w/v) 0.1M EDTA or use a more oxidising flame or take measurements higher in the flame. N_2O flame more sensitive

Source: Perkin Elmer Standard Operating Conditions Manual.

3.6.1. Microwave digestion of plant material.

The methods described are optimised conditions for the plant material analysed in this study using manufacturers recommendations as a base line. The samples were separated into non-lignified material i.e. leaves (easy to digest) and lignified (recalcitrant) material i.e. roots, new stem and original cutting material for ease of digest.

3.6.1.1. Leaves

0.5g of finely ground sample were weighed into each Teflon digest vessel after which 10 mls of 70% HNO_3 was added in a fume cupboard. The vessels were allowed to stand for 30 minutes and a further 5 ml of HNO_3 was added to each sample. A safety valve and cap were placed on each vessel and tightened using the capping station. Each vessel was

numbered, a venting tube attached and placed in the turntable with fan and turntable activated. The oven was programmed for 4 minutes at 100% power (step 1) and 8 minutes at 50% power (step 2). Samples were removed and checked for any venting or loss of material, then allowed to cool to room temperature. The vessels were manually vented and digested samples were transferred to 25 ml volumetrics. Using this programme, the samples were digested until completely clear, and the filtration stage was omitted. The samples were prepared to volume using distilled, deionised water and analysed using atomic absorption spectrophotometry. Triplicate samples were prepared with HNO_3 blanks.

3.6.1.2. Woody material

0.5g of dried, ground (1 mm) material was prepared for digest as described in the previoius section. For 12 vessels the program was set at 20 minutes at 35% power after which the samples were removed, and allowed to cool to room temperature. Vessels were tightened using the capping station, and replaced. The oven was programmed for a further 10 minutes at 65% power. The samples were allowed to cool to room temperature and the sample was transferred to 25 ml volumetrics and prepared as above. Again, triplicate samples and blanks were digested.

Between digests the Teflon vessels, caps, safety valves and venting tubes were decontaminated by washing with tap water and Decon followed by HNO_3 and distilled water after which they were dried at 60°C. Vessels were additionally decontaminated by microwaving at full power for 20 minuted with 15 mls HNO_3 between experiments.

Chapter IV

Metal resistance screens

4.0. Rationale

A range of *Salix* species and hybrids were screened in metal-amended nutrient solutions to assess innate metal resistance. McCormack & Steiner (1978) screened a variety of woody plant species for innate tolerance to aluminium in solution culture. Their findings indicated that screening for innate metal resistance may be useful in identifying species of plants for bioremediation programmes. The impetus behind screening *Salix* for metal tolerance in the present study was based on pollution resistance characteristics already reported for species such as *Salix caprea* (goat willow) and *S. cinerea* (grey willow) (Grime *et al* 1988; Eltrop *et al* 1991). Nutrient poor and metal-contaminated soils appear to provide a niche for these apparently naturally resistant trees. Furthermore, the genetic variability and propensity for hybridisation that exists within the genus suggests these valuable characteristics may also exist in other willow species and may be transferable to subsequent hybrids.

This chapter reports a pilot study to evaluate variability for metal resistance in *Salix* and also investigated the working range of metal concentrations for use in further experiments. This was an obvious starting point to determine upper non-toxic, sub-lethal and lethal concentrations of heavy metals.

4.1. Screening for metal resistance: pilot study to develop methodology

Background to methodology: TI and EC_{100}

The following experiments aimed to identify and quantify the level of susceptibility of a range to willow species and hybrids to copper, cadmium, zinc and nickel. It was planned to use screening techniques to select willow species and hybrids with potential for use in bioremediation of heavy metal polluted soil.

Testing trees for tolerance to toxic concentrations of heavy metals requires an accurate and reliable mensuration technique. The most widely used indicator of metal tolerance in plants has been based on root growth inhibition (Bradshaw 1952; Wilkins 1957, reviewed by Wilkins 1978) in which the maximum root elongation of plants growing in elevated metal solutions is expressed relative to that of plants in background solution (Section 3.2.3 Materials and Methods). There have been criticisms of the index, however, and one alternative method put forward is the use of EC_{100} values (MacNair 1983). EC_{100} is defined as the effective concentration at which there is 100% mortality. The method is based on the assumption that the ability of plants to produce roots at certain fixed concentrations of metals reflects their heavy metal tolerance. Macnair (1983) and Schatt & Ten Bookum (1992) used EC_{100} to investigate whether metal tolerance was under polygenic control (i.e. a large number of genes, each having a small effect) or the control of a single major gene.

Schatt & Ten Bookum (1992) provided a valuable critique of both the EC_{100} and *TI* values as accurate indicators of metal tolerance in plants. They pointed out the main problem with using *TI* is the high level of 'statistical noise' that it contains because it is a quotient of two variables (growth in metal amended and control solutions), each of which have differently skewed probability distributions; greater inherent variation in control data distorts the *TI* calculation. The direction of skewness of data used in *TI*

calculations is also dependent on the metal tolerance of the plant tested and the concentration at which it is tested. Furthermore, *TI* does not eliminate all forms of innate variation in root growth unrelated to tolerance. One example of this especially pertinent to the present study, is the inherent differences in the ability of different species to root from cuttings. EC_{100} values also contain inherent errors, due to the fact that measurement relies upon 100% mortality of the test plants which means the value may fail to perceive quantitative variation in metal tolerance; for example, how plant growth is affected at concentrations approaching the EC_{100} value.

Several workers have recommended the alternative use of multiple concentration tests (Nicholls & McNeilly 1979; Schatt & Ten Bookum 1992) in which metal concentrations are increased in a stepwise manner over a period of time, until the EC_{100} is reached. The regression of the growth response can then be calculated and used to indicate metal tolerance (Nicholls & McNeilly 1979). However, the time period over which metal concentrations are increased can be an influencing factor. The EC_{100} value may be more accurate if individual plant clones are exposed to a wide range of metal concentrations simultaneously, discounting the influence of metal acclimation. Clearly the use of only one growth parameter as an indication of whole-plant tolerance should be questioned and for this reason the total number of roots produced and the longest stem height are monitored in addition to length of the longest root in the present study.

The experiment reported in this part of the study tested the root and shoot growth of nine native British *Salix* species and one hybrid to a wide range of metal concentrations.

4.1.1. Aims

- To screen a range of *Salix* cuttings in solution culture to Cu, Cd, Zn and Ni and select those which are most metal resistant.
- To use both *TI* and EC_{100} values to estimate tolerance and compare the usefulness of these measures.
- To identify the upper non-toxic, sub-lethal and lethal concentrations of Cu, Cd, Zn and Ni for future use.

4.1.2. Methods

The large numbers of metal treatments and willow used imposed restrictions on the hydroponic unit size and replicate number. Instead of 20 litre hydroponic units (described in Section 3.2.1.) 3.5 litre black polypropylene buckets were used. Cuttings of nine species and one hybrid (Table 4.0) were supported from above by pushing each cutting through a slit in heavy gauge black polythene which was stretched over the lip of the container and secured with water-proof tape. An air stone, connected to an aerator pump was immersed in 3 litres 25% strength Hoagland's nutrient solution (formulated with glass distilled water and reagent grade chemicals) added to each bucket. Cuttings were used at the stage where adventitious roots and leaf buds were just beginning to appear, but no substantial root or new stem growth had occurred. Viable cuttings ($N = 5$) of each willow species were tested, with 50 cuttings per bucket, with one bucket for each metal treatment (Table 4.1), giving twenty four buckets altogether. The buckets were arranged randomly in a controlled-temperature glasshouse without artificial lighting at approximately 19°C (daily fluctuations no greater than ± 5°C). The experiment was set up in March 1994 and ran for 28 days with nutrient solution replaced every seven days. Throughout the duration of the test 30 ml samples of metal-amended and control nutrient solution were taken from each of the treatment buckets; control, 0.6 mg Cu l^{-1}, 0.6 mg Cd l^{-1}, 10 mg Zn l^{-1} and 1.0 mg Ni l^{-1}. Samples of nutrient solution were refrigerated in foil-wrapped universals until there

were seven days samples and metal concentration was determined using AAS on days 7, 14, 21 and 28 of the test.

Table 4.0. Salix clones used in the pilot study

Species / hybrid Details(Source)	Ness Gardens Accession No.
S. caprea L. (Trelogan[a]) (♂)	n/a
S. nigricans Sm. "Myreside Fen" (♀)(Donald)	3438
S. phylicifolia L. "Malham" (♀)(Sinker)	3450
S. viminalis L. "Ivy Bridge" (♂)(Rogers ex LARS)	3369
S. triandra L. "Black Maul x Dutch Light Bark" (♂)	3064
S. pentandra L. "Dark French" (?) (Stott F/C)	3274
S. purpurea L. "Jagiellonka" (♀)(Long Ashton)	3018
S. caprea L. "Loughgall" (♀)(Loughgall)	3288
S. fragilis L. "Russelliana Kew" (?) (Kew Gardens)	3235
S .x calodendron[b] Wimm. (♀)(Unknown)	3311

[a]Material collected from a lead/zinc mine site in Clwyd and identified by Liverpool University Botanic Gardens. [b]Constituent species of hybrid *S.* x *calodendron* = *S. cinerea* x *S. caprea* x *S. viminalis.*

The final length of longest root, number of roots and height of tallest new stem of each cutting was measured at the end of the experiment. The six levels of metal treatment used in this study are shown in Table 4.1.

Table 4.1. Metal treatments (mg l^{-1})

Treatment Level	Concentration (mg l^{-1})			
	Cu	Cd	Zn	Ni
1	0.0005*	0.0	0.002*	0.0
2	0.2	0.2	10	1.0
3	0.4	0.4	20	2.0
4	0.6	0.6	30	3.0
5	0.8	0.8	40	4.0
6	1.0	1.0	50	5.0

* denotes metals are essential micronutrients that are supplied at background levels in nutrient solutions.

Metal levels used in the experiment were based on toxic, critical tissue concentrations identified for *Picea sitchensis* (Burton, King & Morgan 1985) and also using information from Watmough (1994) and Alloway (1995) on metal availability. A modified form of the widely accepted *TI* (Wilkins 1978) was formulated using a combination of indices calculated from the above measured growth parameters, length of longest root (L_R) root number (N_R)and stem height (H_S). Tolerance of willows was expressed as:

$$TI_{[M]}\ \% = \frac{L_R + N_R + H_S}{3}$$

where $TI_{[M]}$ is the tolerance index at a specified metal concentration [M] and where:

L_R = mean length of longest root of cuttings in test solution / mean length of longest root of cuttings in background solution x100

N_R = mean root number of cuttings in test solution / mean root number of cuttings in background solution x100

H_S = mean height of the longest stem of cuttings in test solution / mean height of the longest stem of cuttings in background solution x 100

EC_{100} values were defined as the metal concentration at which there was 100% cutting mortality or failure to grow. This was confirmed by continued mortality or lack of growth at subsequent test concentrations above this. Where mortality or lack of growth occurred at the higher end of the range of metal concentrations the results were taken to be less certain. The results from this test were analysed using the general linearised model analysis of variance using the Minitab statistical package.

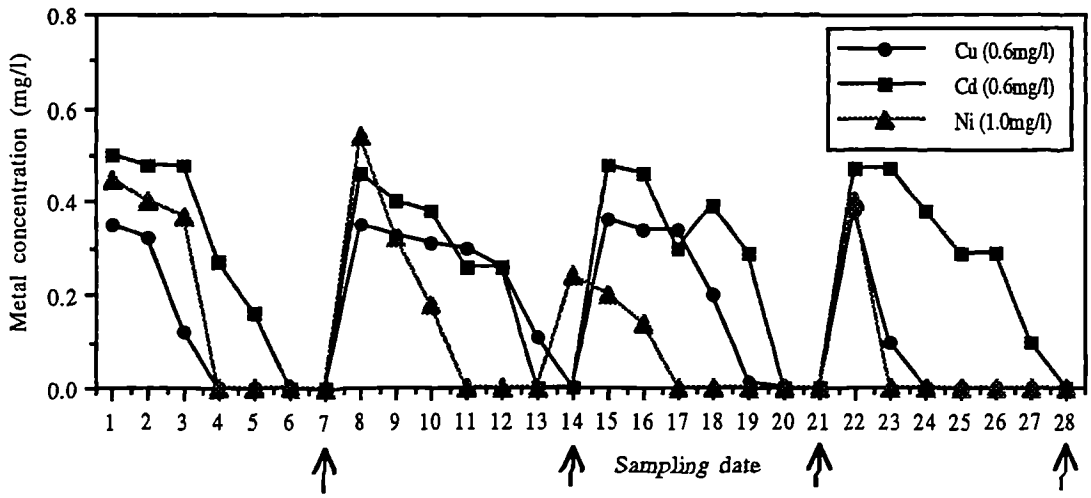
4.1.3. Results

4.1.3.1. Metal concentration in nutrient solution

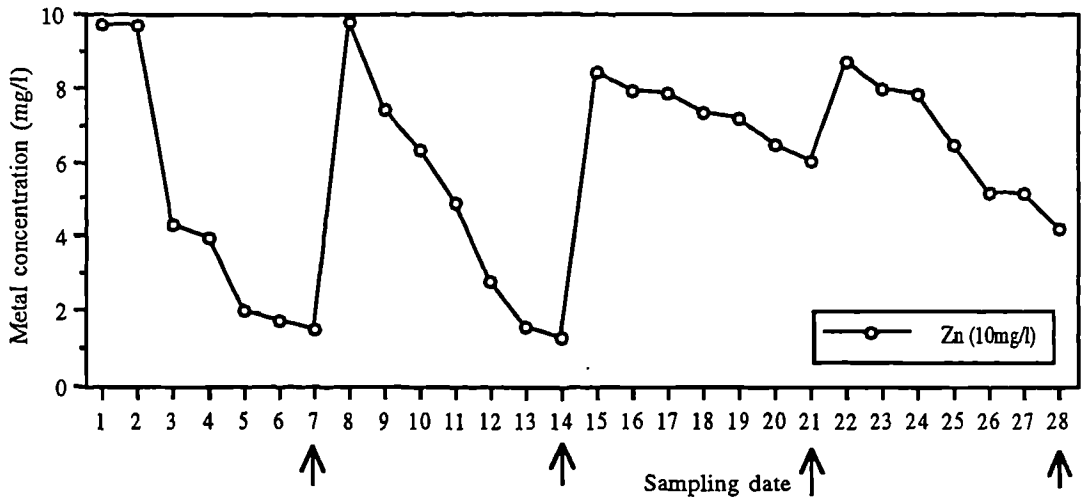
Figure 4.0 shows the metal concentration of nutrient solution monitored daily throughout the duration of this experiment. The metal concentration in control solution is not included in these figures because they were below detection limits. The

concentration of all metals fell throughout the 7 day test period between replacement of the nutrient solutions. When metals had fallen below detection limits a zero value is indicated on the figure. The concentration of copper detected in the nutrient solution was always much lower than the concentration originally added. The concentrations of the other metals showed that target concentrations were reached in the formulated nutrient solutions, with the exception of Ni which rapidly dropped in concentration shortly after addition. The fluctuations in metal levels were similar for all metals although nickel and copper appeared to be removed or bound rapidly. These results indicate that the uptake of cadmium, zinc and nickel is more gradual.

Fig. 4.0. Concentration of Cu, Cd, Zn and Ni in 25% Hoagland's solution containing 50 willow cuttings over 28 days. Arrows indicate the point in the test period where nutrient solution was replaced.
(a) Copper, Cadmium and Nickel



(b) Zinc



Experimental results are summarised in Table 4.2. using data presented for root elongation, root number and shoot height of each of the clones tested (Figs 4.02-4.13). Abbreviations denote the observation of growth stimulation (⇧), inhibition (⇩) or no difference in comparison to control plants (0). For the purpose of this table if the error bars of treated plants overlapped with those of the control plants (taken from standard errors of the mean) the two growth responses were not considered to be different.

Table 4.2. Summary of results shown in Figures 4.02-4.13.

Clone	Copper			Cadmium			Zinc			Nickel		
	L_R	N_R	H_S	L_R	N_R	H_S	L_R	N_R	H_S	L_R	N_R	H_S
S.caprea (Trelogan)	0	0	0	0	0	0	⇩	⇩	0	0	0	0
S. nigricans	⇩	0	⇩	0	0	0	⇩	⇩	⇩	⇩	⇩	⇩
S. phylicifolia	0	0	0	⇧	0	⇧	0	0	0	⇩	⇩	⇩
S. viminalis	⇩	0	⇩	⇩	0	⇩	⇩	⇩	⇩	⇩	⇩	⇩
S. triandra	⇩	0	⇩	0	0	0	⇩	⇩	⇩	⇩	⇩	⇩
S. pentandra	⇩	⇩	⇩	0	⇩	0	⇩	⇩	0	⇩	⇩	⇩
S. purpurea	⇩	⇩	⇩	⇩	0	⇩	⇩	⇩	⇩	⇩	⇩	⇩
S. caprea	⇩	⇩	⇩	⇩	⇧	⇩	⇩	0	⇩	⇩	⇩	⇩
S. fragilis	⇩	⇧	0	⇩	⇩	⇧	⇩	⇩	⇩	⇩	⇩	⇩
S. x *calodendron*	⇩	⇧	0	0	0	0	⇩	⇩	⇩	⇩	⇩	⇩

Figure 4.02 Mean root length of ten clones (a)-(j) exposed to six different copper concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

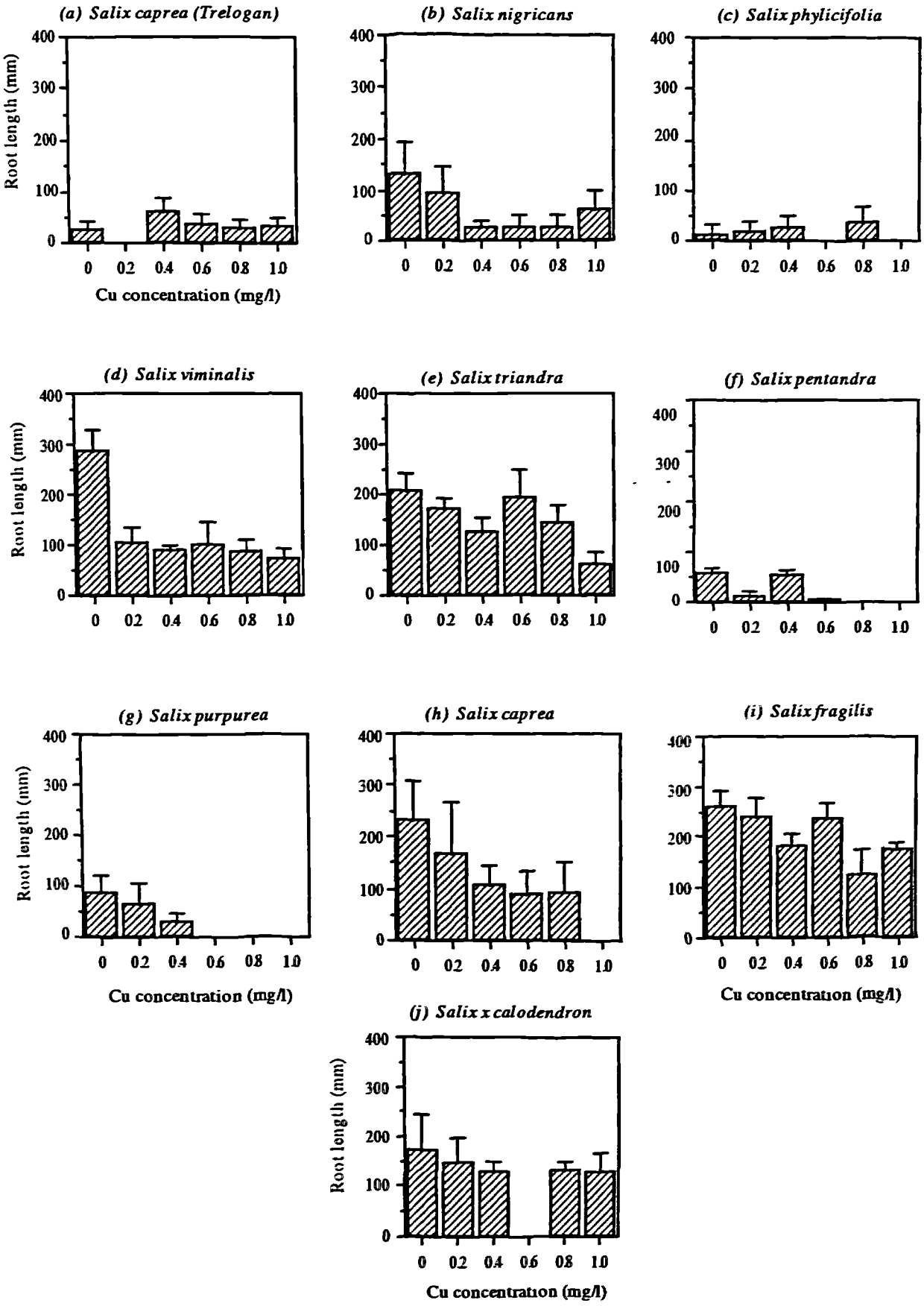


Figure 4.03. Root number per cutting of ten clones(a)-(j) exposed to six different copper concentrations Means and standard errors of zero-adjusted data. (Section 3.3)

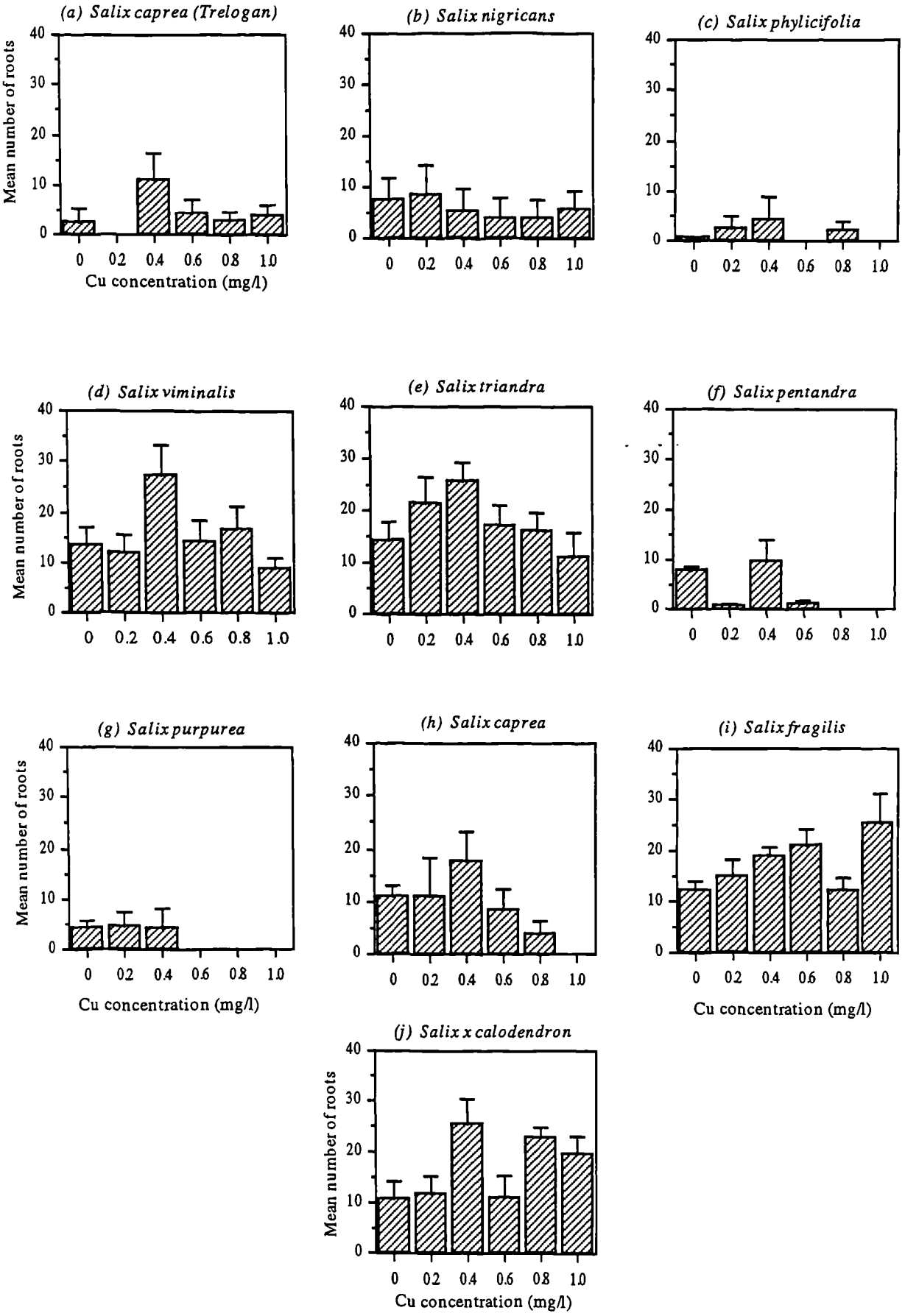


Figure 4.04. Stem height of ten clones(a)-(h) exposed to six different copper concentrations for 28 days

Means and standard errors of zero-adjusted data.(Section 3.3)

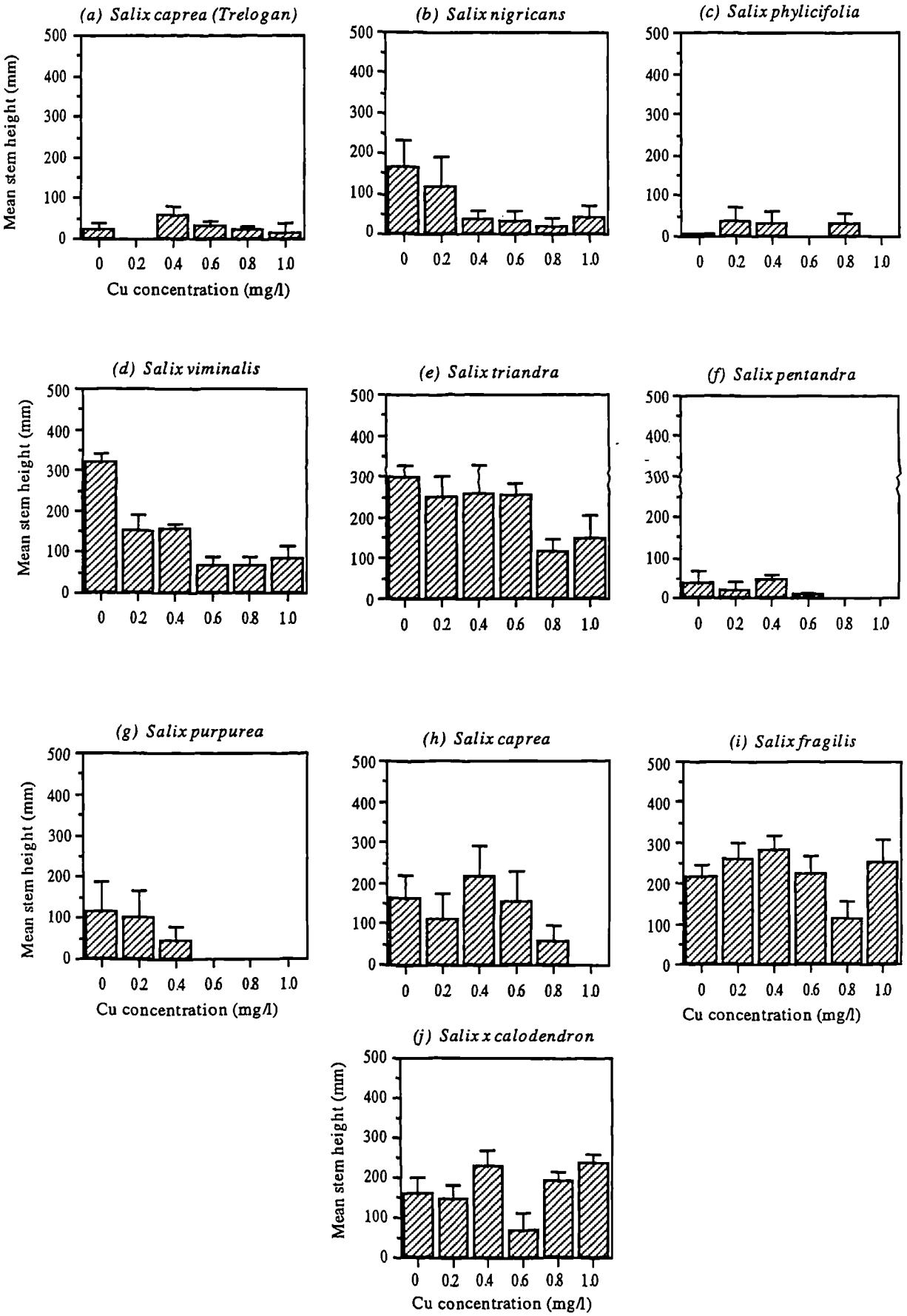


Figure 4.05. Mean root length of ten clones (a)-(j) exposed to six different cadmium concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

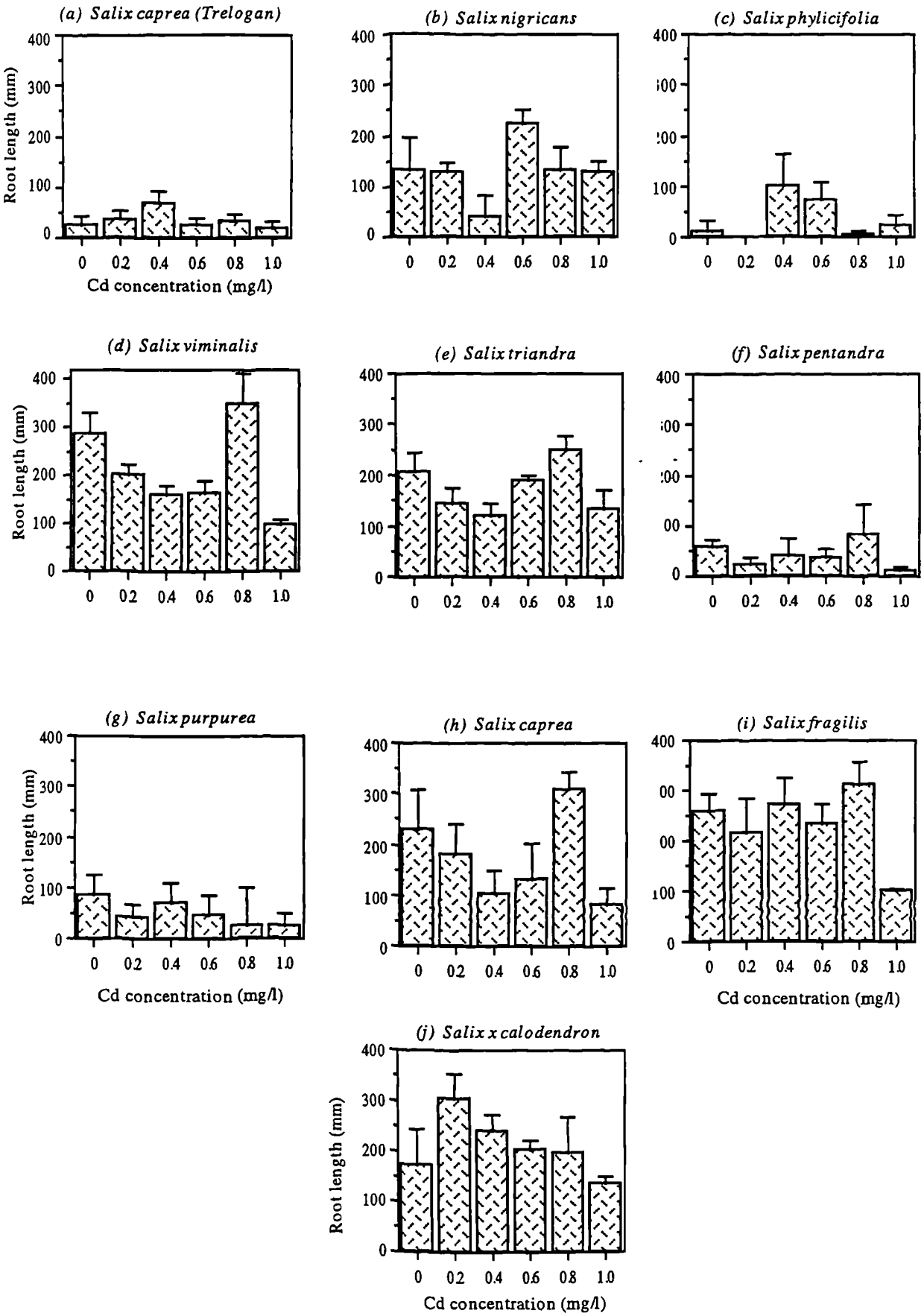


Figure 4.06. Root number per cutting of ten clones (a)-(j) exposed to six different cadmium concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

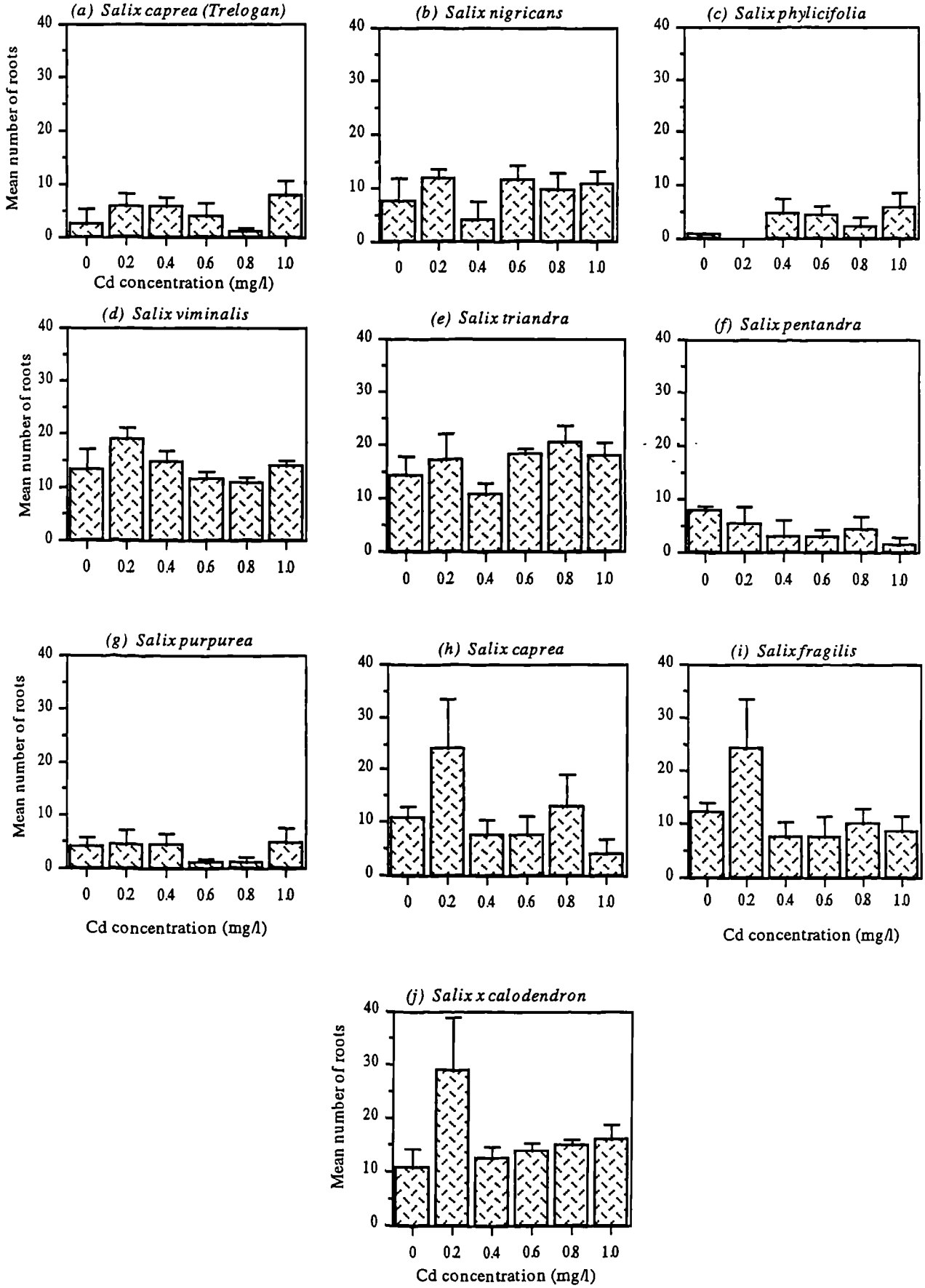


Figure 4.07. Stem height of ten clones (a)-(j) exposed to six different cadmium concentrations for 28 days. Means and standard erros of zero-adjusted data. (Section 3.3)

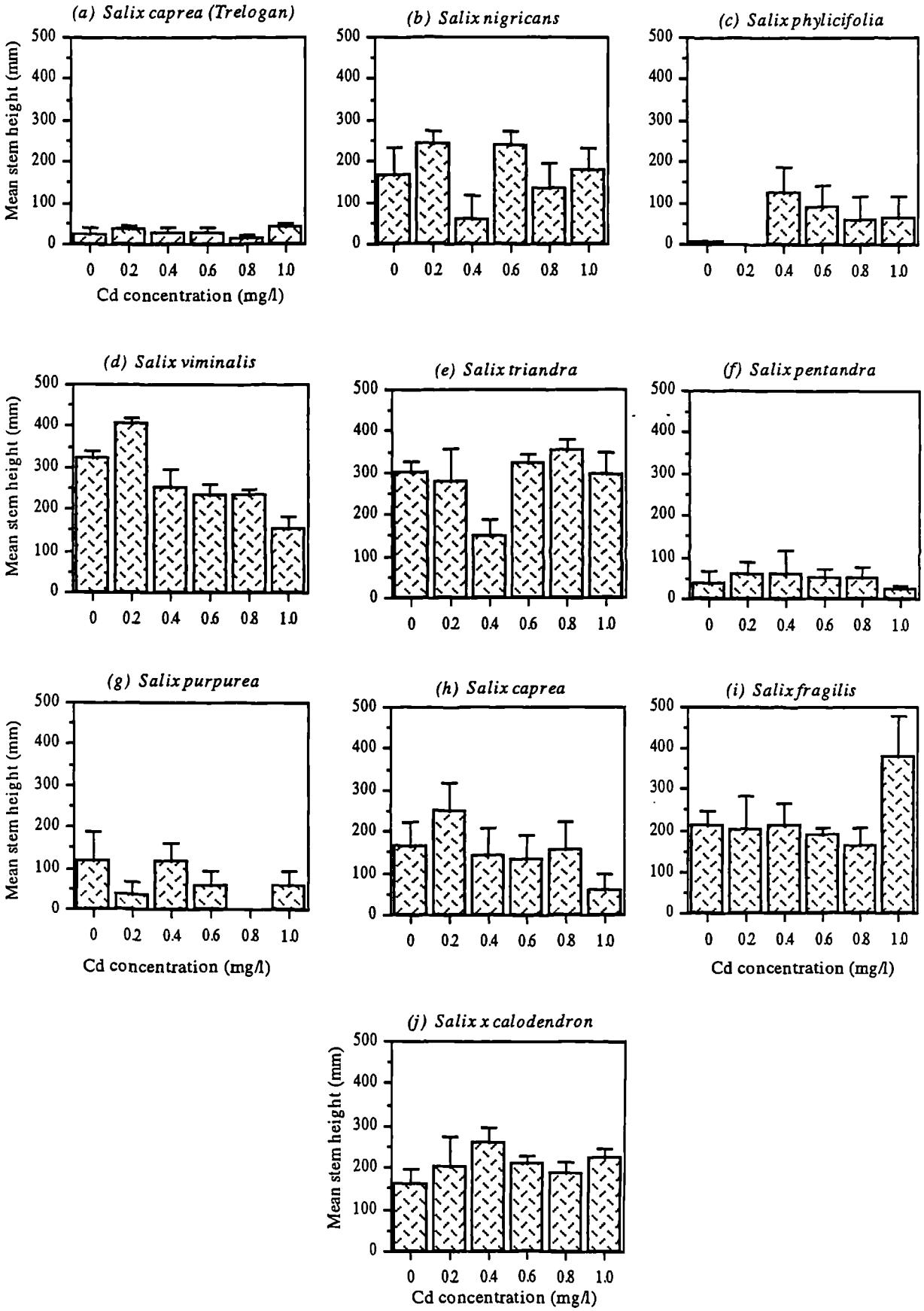


Figure 4.08. Root length of ten clones (a)-(j) exposed to six different zinc concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

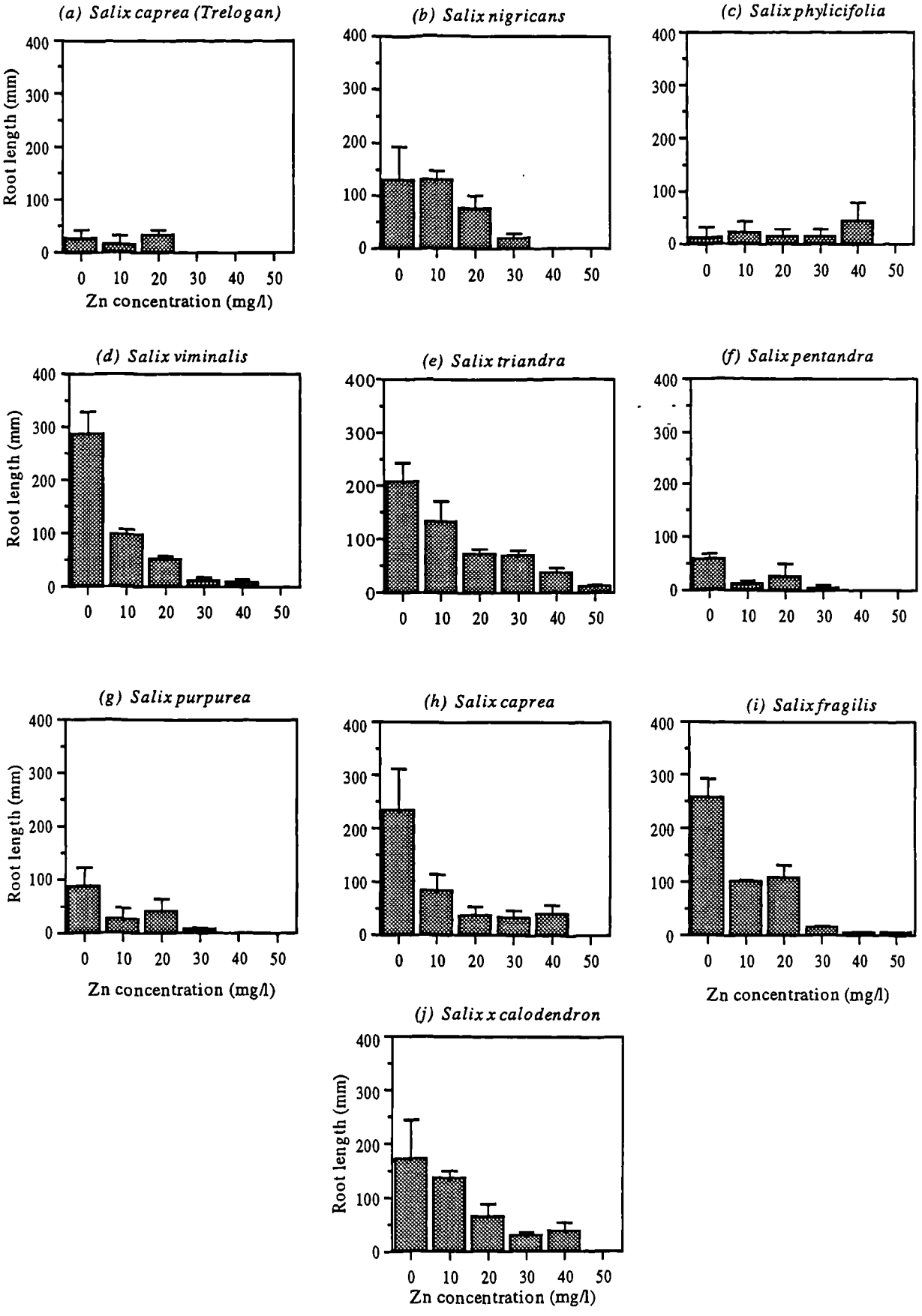


Figure 4.09. Root number of ten clones (a)-(j) exposed to six different zinc concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

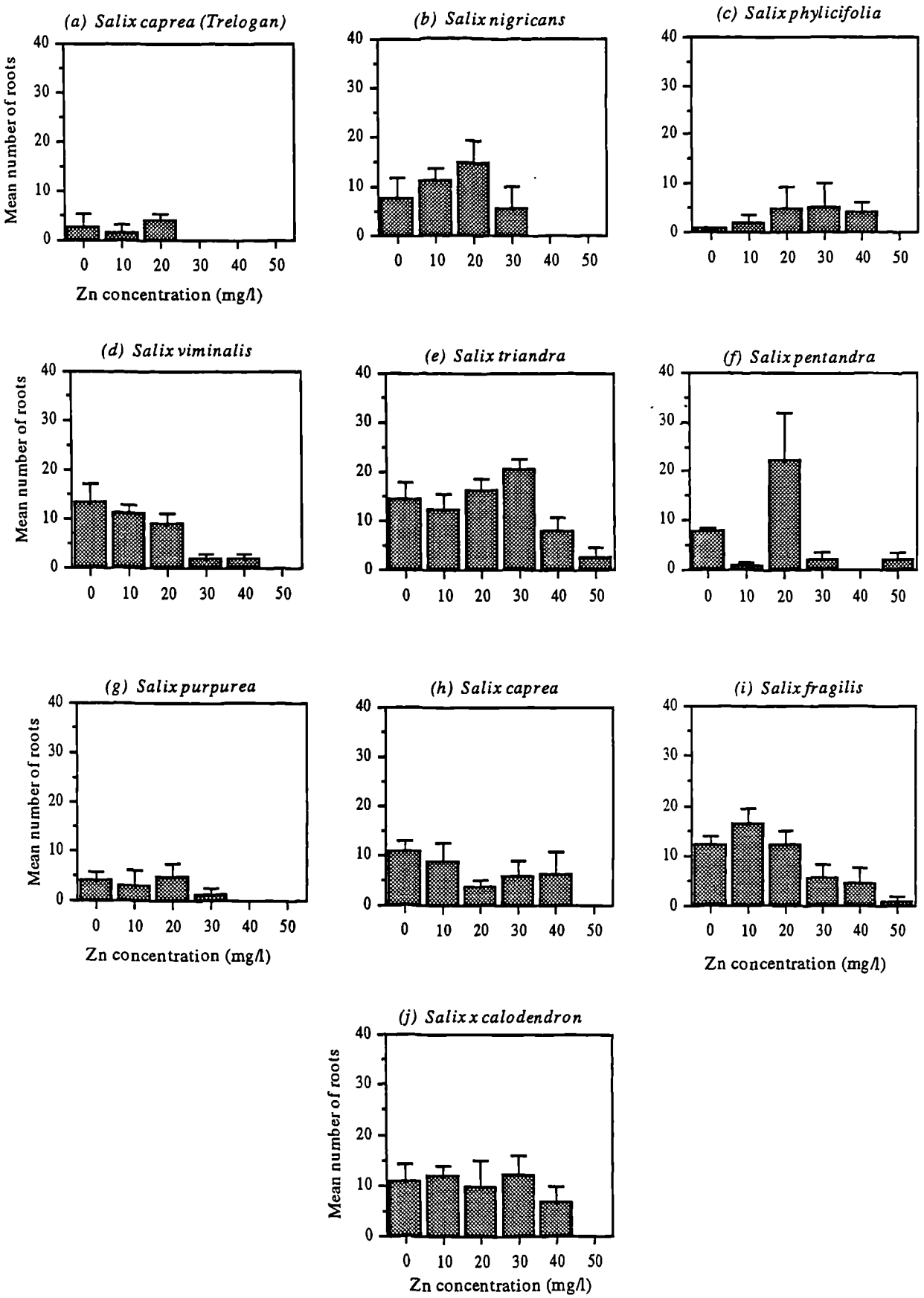


Figure 4.10. Shoot height of ten clones (a)-(j) exposed to six different zinc concentrations for 28 days

Means and standard errors of zero-adjusted data. (Section 3.3)

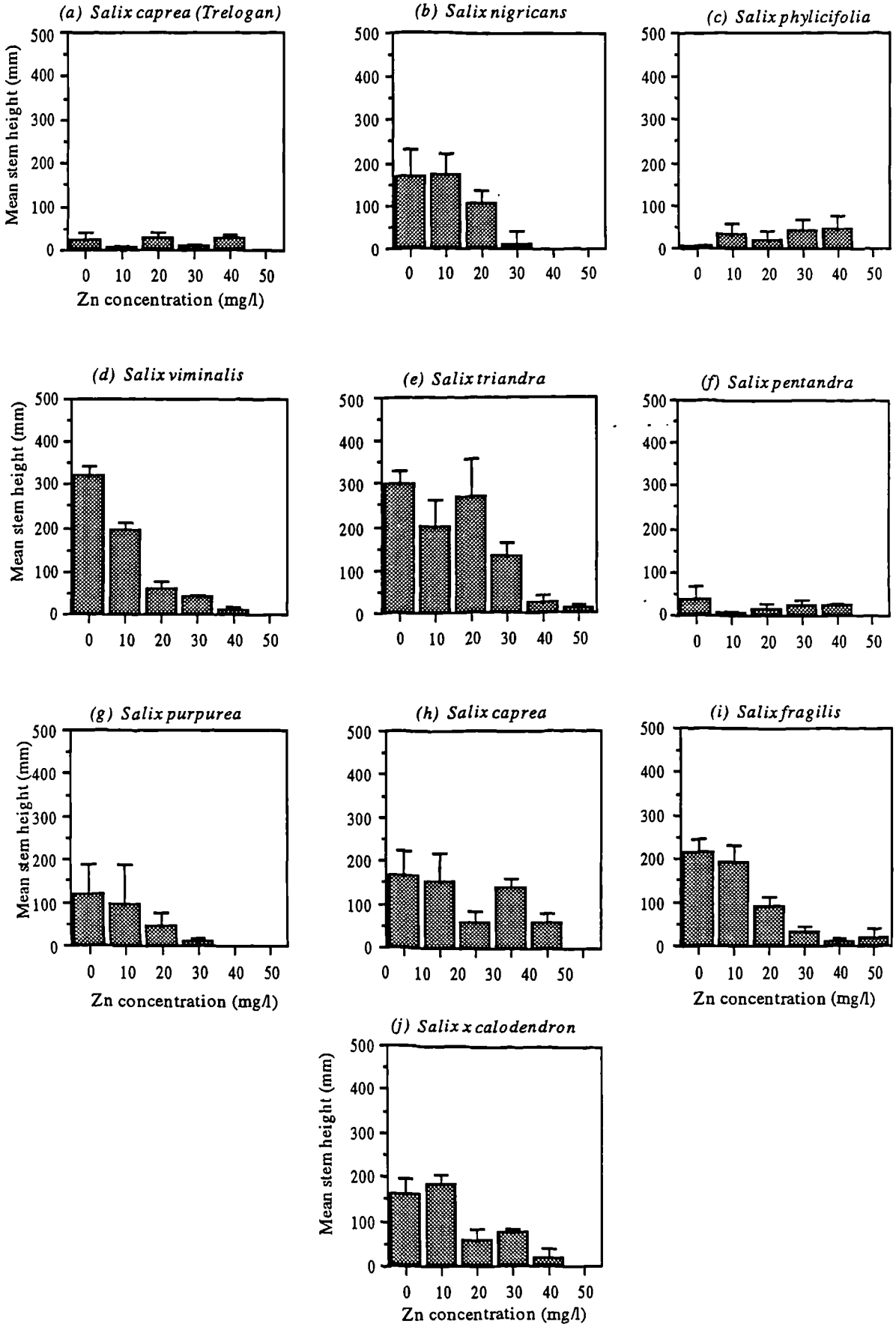


Figure 4.11. Root length of ten clones (a)-(j) exposed to six different nickel concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

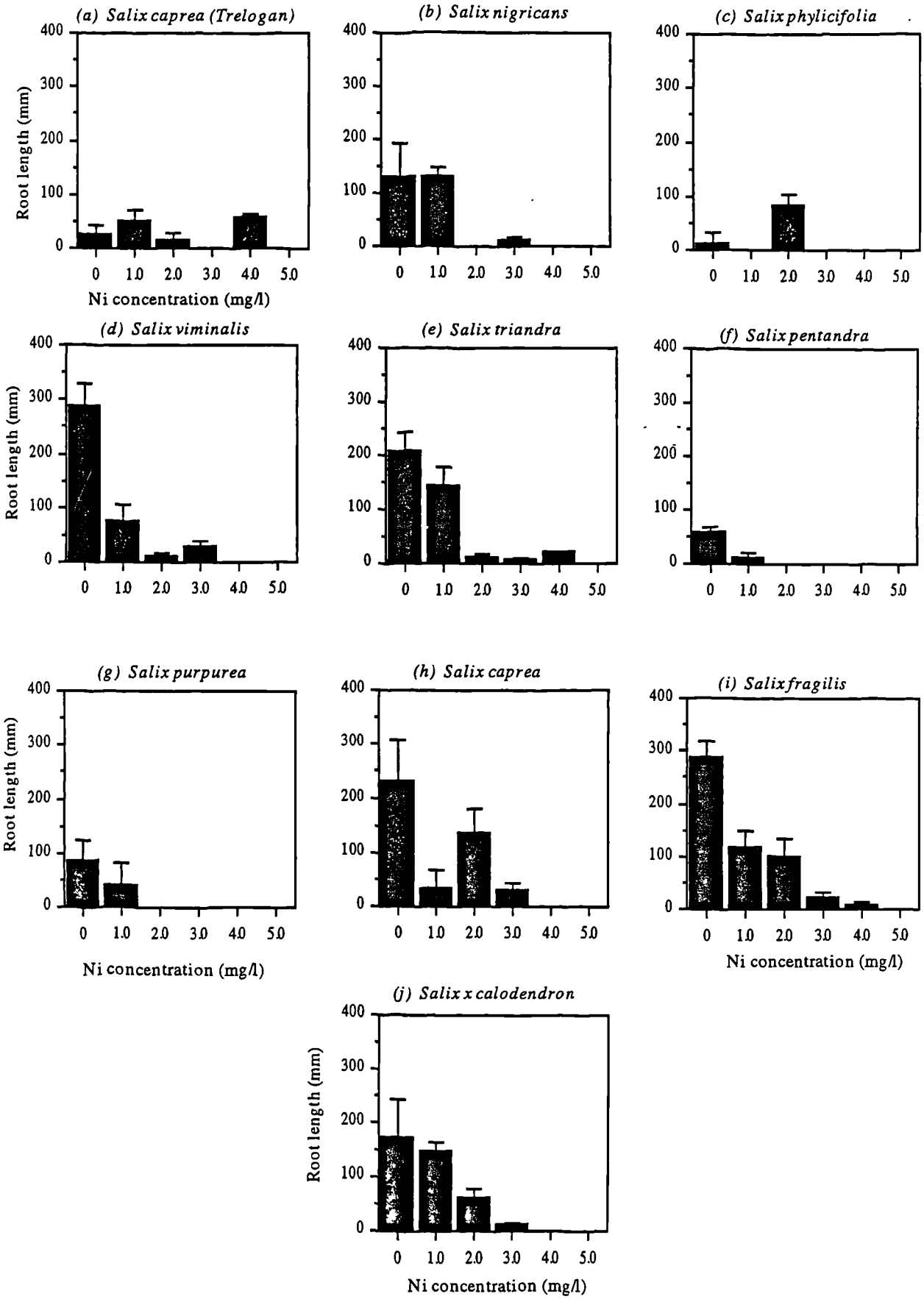


Figure 4.12. Root number of ten clones (a)-(j) exposed to six different nickel concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)

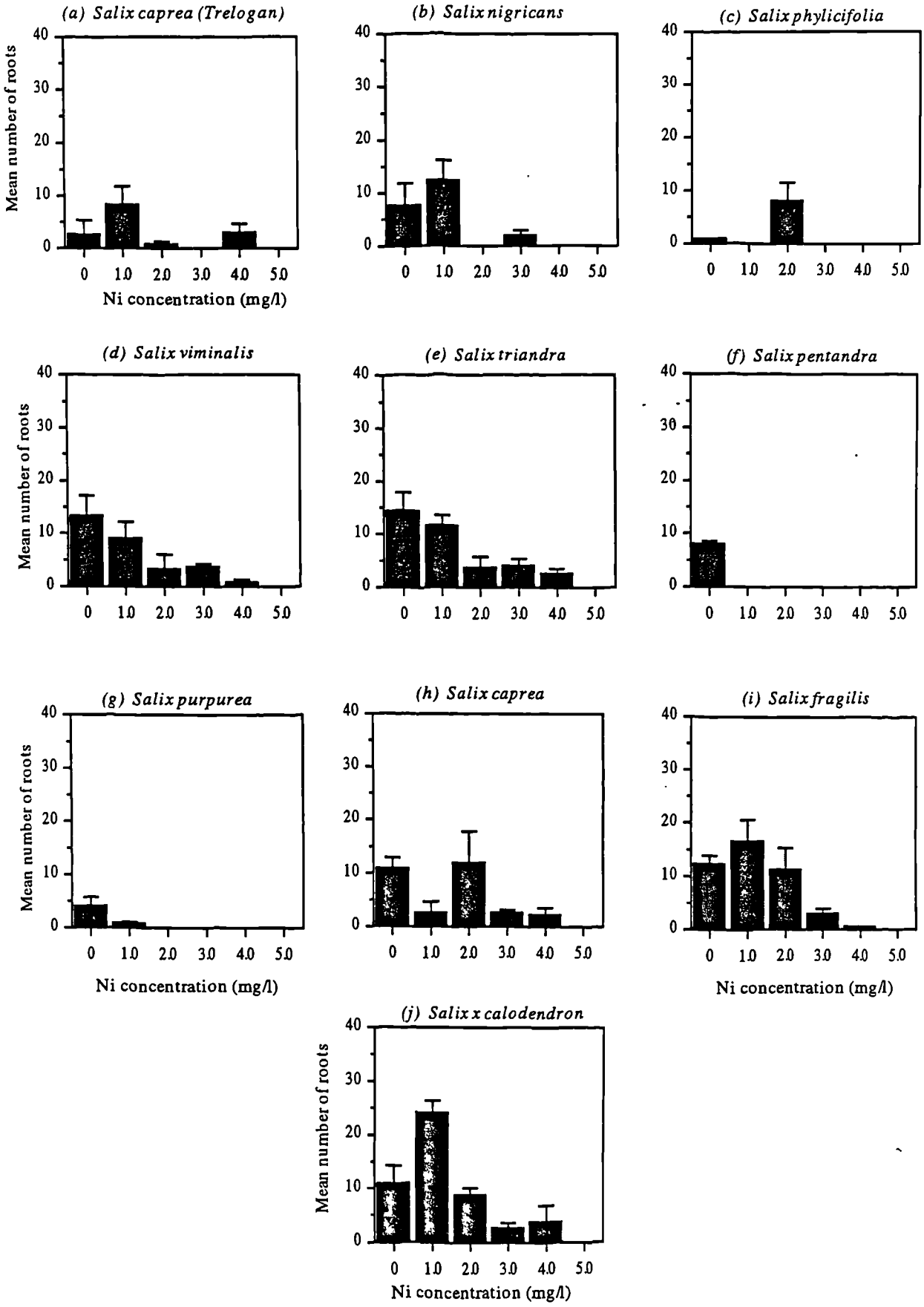
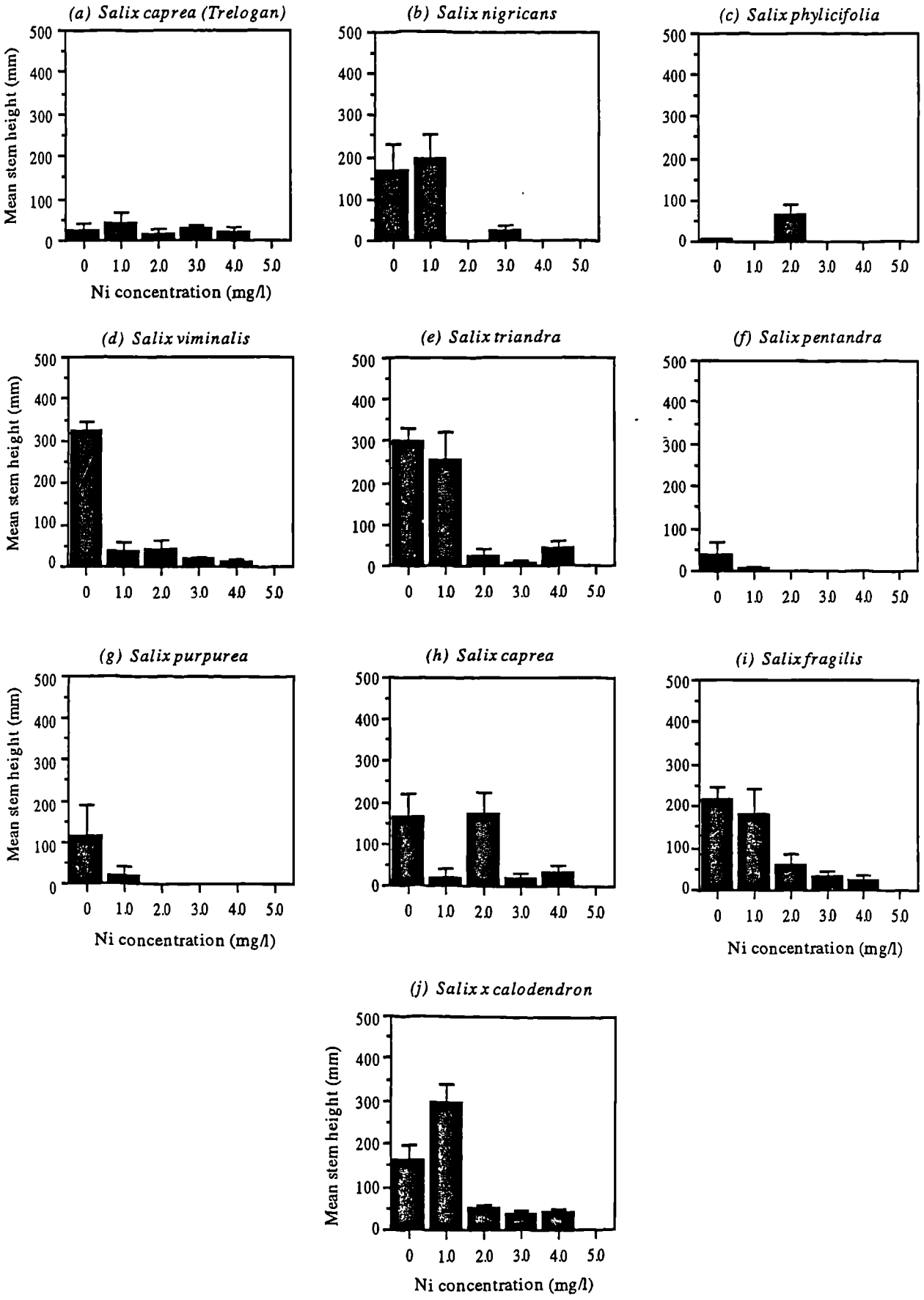


Figure 4.13. Shoot height of ten clones (a)-(j) exposed to six different nickel concentrations for 28 days. Means and standard errors of zero-adjusted data. (Section 3.3)



4.1.3.2. Root and shoot growth responses

There was a high degree of inter- and intra-specific variation in root elongation, which differed between the metals. Root growth was least affected by cadmium. Copper caused marked inhibition of root elongation eight species, zinc and nickel in nine. Zinc and nickel were by far the most clear cut in producing progressive root inhibition. Copper induced changes in root morphology including reduced branching and discolouration; a response typical of roots under metals-stress (Kahle 1993), but this was not observed for cadmium. Cadmium-induced root length inhibition occurred in *S. viminalis, S. purpurea, S. caprea* and *S. fragilis*. Root elongation of *Salix caprea* from the lead/zinc contaminated spoil at Trelogan remained consistently low; with a maximum root length of 50 mm. Zn and Ni inhibited root growth to the greatest extent.

The root number of *S. viminalis, S. triandra, S. fragilis* and *S.* x *calodendron* increased in response to different levels of copper treatment despite a suppression of root elongation. This was not observed for the other metals. Root number declined in four species in response to Cu and in only two species in response to Cd. Root number declined in all species exposed to zinc and nickel, with the exception of *S. caprea* (Trelogan) exposed to nickel.

Elongation of new shoots was also most notably inhibited by nickel and zinc, being accompanied by leaf chlorosis and necrosis. Inhibition of stem growth was observed in 3 species of cadmium- and eight species of nickel-treated plants.

In general the concentrations of zinc and nickel used had a marked effect on all perameters. *S. caprea* (Trelogan) was only inhibited by exposure to zinc, and the measurement of different growth perameters have given different results, for example the root length of *S. fragilis* was inhibited by copper but the rot number increased.

4.1.3.3. Calculated TI *values*

TI values and have been calculated using all growth peramaters of cuttings exposed to 1.0 mg l^{-1} Cu, 1.0 mg l^{-1} Cd, 20 mg l^{-1} Zn and 1.0 mg l^{-1} Ni (Table 4.3.).

Table 4.3. Modified TI (%) values of willow species. † denotes no growth at this metal treatment level.

Species/Hybrid	Metal treatment (concentration)			
	Cu 1.0 mg l^{-1}	Cd 1.0 mg l^{-1}	Zn 20 mg l^{-1}	Ni 1.0 mg l^{-1}
Salix caprea (T)	162.2	265.2	239.3	129.3
S. nigricans	51.9	137.0	102.6	126.0
S. phylicifolia	†	401.2	464.2	†
S. viminalis	41.3	66.40	34.0	33.3
S. triandra	53.2	102.3	77.6	90.3
S. pentandra	†	212.0	9.6	112.6
S. purpurea	†	62.0	26.0	65.6
S. caprea	†	60.5	16.0	27.0
S. fragilis	130.4	128.3	86.3	59.3
S. x calodendron	134.4	139.7	165.0	53.3

Table 4.4 The EC_{100} *values indicated by 100% cutting mortality or failure to grow in metal amended nutrient solution; concentration given as mg* l^{-1}.

Species / Hybrid	Cu	Cd	Zn	Ni
S. caprea (T)	>1.0	> 1.0	30	5.0
S. nigricans	>1.0	> 1.0	40	4.0
S. phylicifolia	1.0	> 1.0	50	3.0
S. viminalis	>1.0	> 1.0	50	4.0
S. triandra	>1.0	> 1.0	>50	5.0
S. pentandra	0.8	> 1.0	40	2.0
S. purpurea	0.6	> 1.0	40	2.0
S. caprea	0.8	> 1.0	50	4.0
S. fragilis	>1.0	> 1.0	>50	5.0
S. x calodendron	>1.0	> 1.0	50	4.0

Table 4.5. shows the F and corresponding p values for statistical analysis of root length, number and stem height for all species tested. Between species differences have been analysed using an overall GLM analysis. Inherent growth differences that form the basis for selection can be obtained from Table 4.3. indicating inherent tolerance differences. Natural variation in the magnitude of root and stem growth is discussed in Chapter 2; The genus *Salix*.

Table 4.5. Analysis of variance table showing F values of growth peramaters in response to different metals and different metal concentrations. (N=5)

*** denotes $P < 0.001$; ** denotes $P < 0.05$; NS means there were no significant differences.

Source (Degrees of Freedom)	L_R	N_R	H_S
Species$_9$	23.15***	41.54***	48.10***
Metal$_3$	34.85***	43.25***	53.49***
Concentration$_5$	23.76***	18.04***	34.62***
Species x Metal$_{27}$	2.25***	3.42***	4.60***
Species x Concentration$_{45}$	2.72***	1.56**	3.06***
Metal x Concentration$_{15}$	5.14***	5.08***	5.45***

4.1.4. Discussion

This pilot study provides important information about the development of the methodology for future tests. Firstly, variation in growth responses within propagules of *Salix* demonstrates a need for greater replication. Large variation in the growth responses of *Salix* has been observed by Good *et al* (1978), Good *et al* (1985) and Good & Williams (1986) and is therefore to be expected, although clear responses to heavy metals could still be picked out in this test. Secondly the concentrations of metals used in this study indicate that for cadmium the critical concentration for willow may lie above the range used, although this metal has been widely accepted to be phytotoxic at levels below those used in this study, clearly further tests using cadmium

must be carried out. For zinc and nickel the critical concentrations are within the lower end of the ranges used.

Calculation of the regression of mean root growth in a range of metal concentrations to indicate tolerance, suggested by Nicholls & McNeilly (1979), could not be applied in the present study because the data were too variable and because the pattern of growth differed widely between species. *TI* and the EC_{100} both gave indications of the responses of willows to metal stress, the former gives more information of the individual plant response, whereas the latter is more indicative of maximum metal toxicity thresholds. The modified *TI* value can be greatly distorted by species with a small growth habit. Species of willow with inherently low growth rates such as *S. caprea* and *S. pentandra* should be treated with care when interpreting *TI* values. A very small actual growth increment can increase the *TI* value tenfold as a result of the proportionate comparison with growth in control solutions. Similarly the EC_{100} was distorted by the sporadic rooting observed in some species; where mortality or lack of growth at the higher concentration range may be mistaken for the EC_{100} but is in fact a product of unpredictable viability. Inclusion of both measures may reduce the influence of these species-specific distortions.

One surprising finding of the present study was the high *TI* values for cadmium; exposure of willows to cadmium did not appear to result in serious growth inhibition. *TI* was above 100% in seven species tested except *S. viminalis, S. purpurea* and *S. caprea. S. phylicifolia* showed the highest *TI* value (400%) at 1.0 mg l^{-1} cadmium, but grew very little overall in control solutions. Copper, zinc and nickel all elicited extreme phytotoxic responses from all willow species at the concentrations used in this study.

S. caprea sampled from the lead mine site at Trelogan showed a lack of tolerance to Zn which was one of the major pollutants at this site. This may be the result of the sporadic rooting observed in this species (Pojhonen 1991), or else suggests that the survival strategy adopted by this particular individual is one of avoidance, and that it does not possess constitutional zinc or nickel tolerance.

This investigation has shown marked differences between the response of a range of *Salix* species and hybrids to heavy metals in solution culture, successfully identifying a clone which was sampled from a metalliferous mine site by its elevated resistance. The results suggest that trees from this genus have a surprisingly high innate resistance to cadmium without prior exposure. The ranges of concentrations used in this study successfully identified sub-lethal and phytotoxic concentrations of copper an zinc; although the response of plants to nickel suggest that in future lower concentrations should be used.

Both resistance estimates (*TI* and EC_{100}) have made distinctions between susceptible and resistant species, but *TI* appears to be a more informative measure because it quantifies the growth response of the test plant at a number of different concentrations and an EC_{100} value can automatically be calculated when the index is zero. The selection of metal resistant species from this test is problematic because the response of each individual species differs for each metal, further screening is therefore necessary to identify resistant species.

4.2. Interspecific resistance to copper.

Background to the study

The resistance screening experiment described in the previous section (Section 4.1.) showed that *Salix* spp. are of particularly varying sensitivity to copper in solution culture. Responses to this metal are examined further in the present experiment. This screening experiment had much greater replication, with a view to additional determination of the optimum replicate number.

4.2.1. Aims

- To elucidate in detail the resistance characteristics of willows to copper in solution culture.
- To establish an optimal replicate number for further studies.
- To test further willow species, hybrids and clones and varieties for metal resistance.

4.2.2 Methods

Although the choice of willow material was partly subject to availability, a similar range of species to those were used in the present study (using different clones) and a hybrid between *S. caprea* and *S. viminalis* were tested (Table 4.5.).

Table 4.5. Salix *species/hybrids used in the present study*

[a] *S.* x *sericans* = *S. caprea* x *S. viminalis;* also known as *S. smithiana* Forbes (Pohjonen 1991).

Species or hybrid (Source)	Accession No.
S. x *sericans* Tausch. ex A. Kern[a] "Sericans" (♀) (Unknown)	3305
S. caprea L. "Baston Fen" (♂) (White)	3283
S. pentandra L. "Dark French" (?)	3274
S. viminalis L. "Palkane E6 708" (?) (Pohjonen)	3375
S. cordata "Purpurescans" (?) (Tuinzing)	3280
S. glaucophylloides x *viminalis* "Glenmark NZ1216 (♂) (Hathaway)	3387
S. purpurea L. "Augustifolia" (♀) (Long Ashton Collection)	3010
S. caprea L. "Sutton" (♀) (Donald)	3285

The suspension hydroponic system (Section 3.2.1) was used in this part of the study. Cuttings were placed in hydroponic units in late summer 1993 containing aerated, circulating 25% Hoagland's solution, as background solution, or test solutions amended with 0.25, 0.50 and 0.75 mg Cu l^{-1}. Fifty cuttings of each clone were replicated in separate unit. Nutrient solution reservoirs were changed every seven days, and any debris that had accumulated in the units was removed at this time. Hydroponic units were monitored in a controlled temperature glasshouse (19°C ± 5) without artificial lighting. The experiment was run for 21 days, after which the length of longest root and root number were measured.

Copper tolerance was estimated using the index described in Section 4.1. but modified to encompass root length, number and viability:

$$\Pi_{[M]} = \frac{L_R \pm N_R \pm V_R}{3} \times 100$$

Viability is defined as the percentage of the test population successfully rooted divided by the control population.

4.2.3. Results

Growth data are presented as the means and standard errors, excluding zero values (cuttings which failed to root throughout the experiment) with rooting viability presented separately. There was a highly variable response to copper of root length (Fig. 4.15), number (Fig. 4.16) and viability (Fig. 4.17) between and within clones. Root elongation was inhibited in all species except *S. caprea* when cuttings were grown in copper-amended nutrient solution, but the number of roots per cutting increased. Rooting viability varied interspecifically (from 60-90%) and also with exposure to copper; viability declined with increased copper concentration in all clones. The *S. caprea* clone 'Baston Fen 'and the hybrid between *S. caprea* and *S. viminalis* had the greatest tolerance indices. The data indicates that the number of the roots is least affected by treatment with copper in comparison with the other parameters measured in this experiment. There was a noticable difference in the

growth response of the two goat willow clones tested (*S. caprea* 3283 and 3285). Clone 3285 showed a general lack of growth, and in successfully rooted cuttings the magnitude of growth is very low. Figure 4.14. provides an over view of all parameters measured for all species at the highest level of copper treatment.

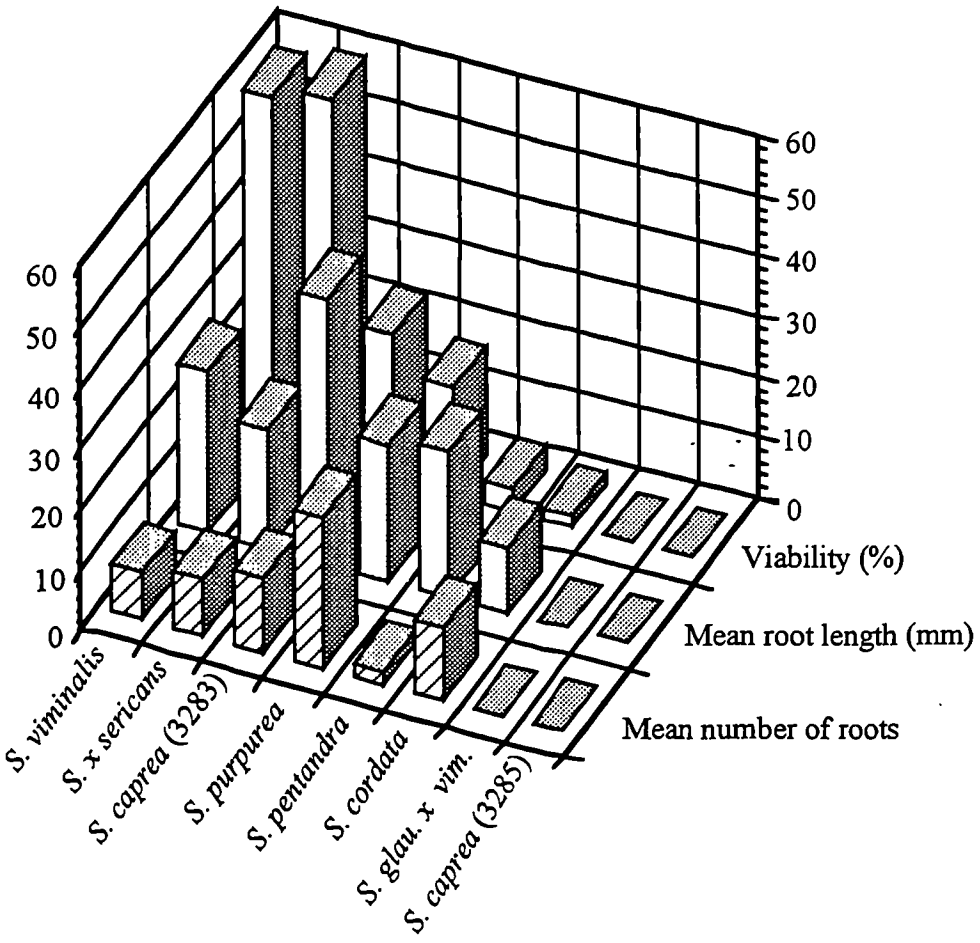
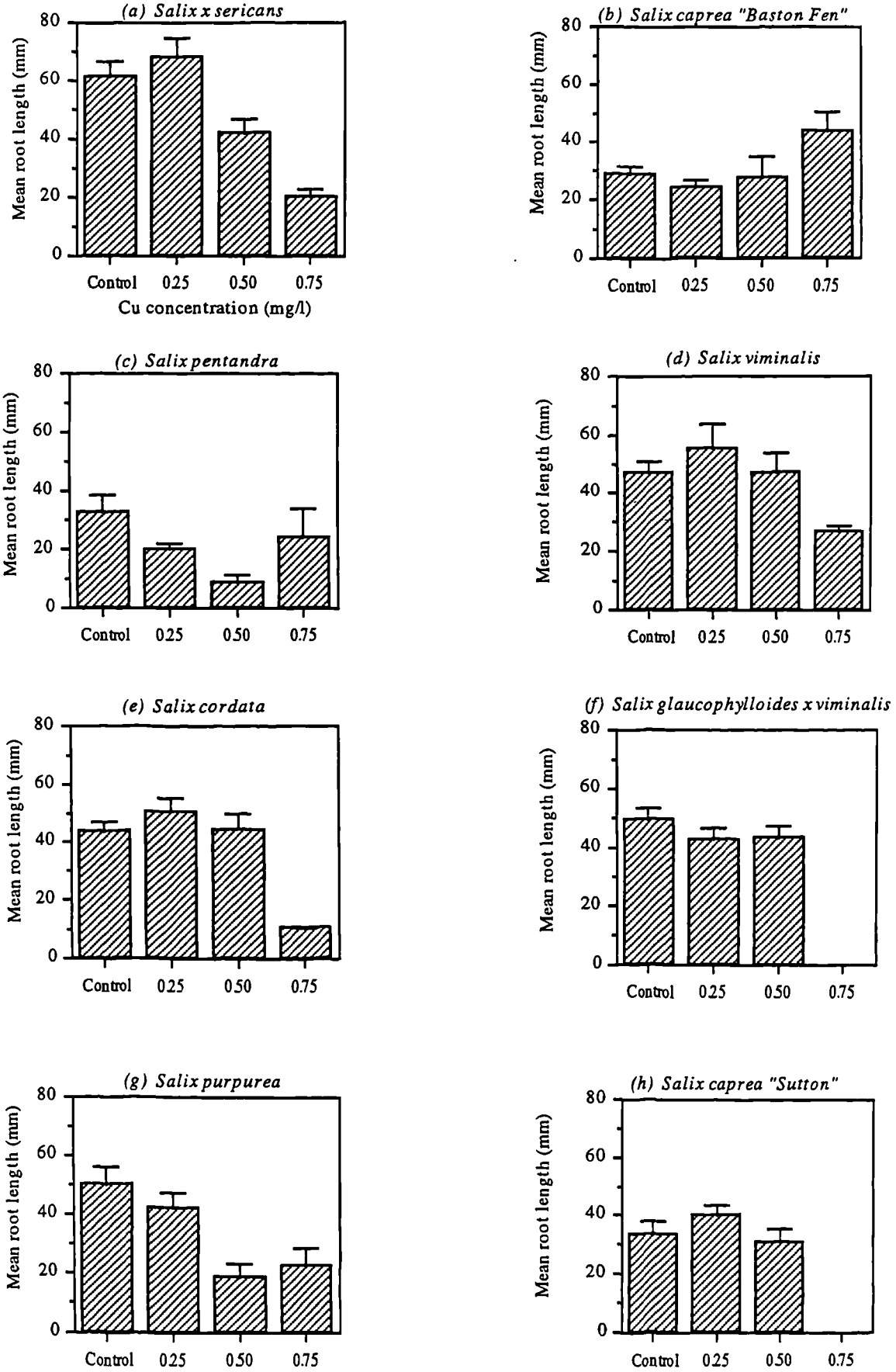


Figure 4.14. Response of willows to treatment with 0.75 mg l^{-1} copper for 28 days

There were highly significant statistical differences for both length and number data, between species and between different copper concentrations with significant interactions (Table 4.7). These differences were further reflected in a calculated *TI* for each clone is shown in (Figure 4.16). The highest test concentration (0.75 mg l^{-1}) was used for this calculation because this showed the greatest distinction between species with different resistance capabilities could be seen more clearly.

Figure 4.15. Length of longest root of eight clones (a)-(h) exposed to four different copper concentrations (Means and standard errors of zero adjusted data where n = 50).



Note: *S. glaucophylloudes x viminalis and S. caprea "Sutton"* failed to grow at 0.75 mg Cu l^{-1} throughout.

Figure 4.16 Number of roots per cutting of eight clones (a)-(h) exposed to four different copper concentrations. (Means and standard errors of zero-adjusted data where n = 50).

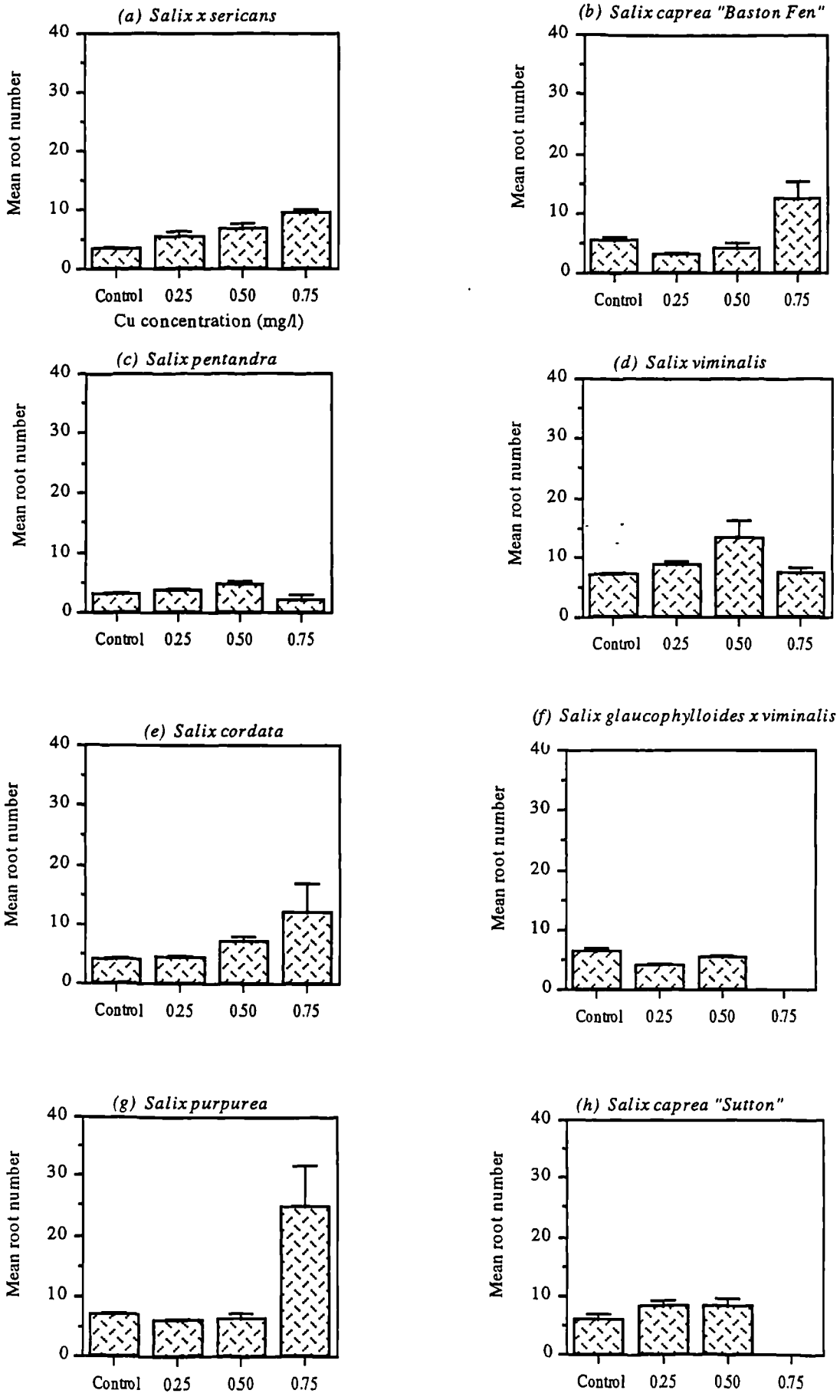


Figure 4.17. Cutting viability of eight clones (a)-(h) exposed to four different copper concentrations (% survival of cuttings tested).

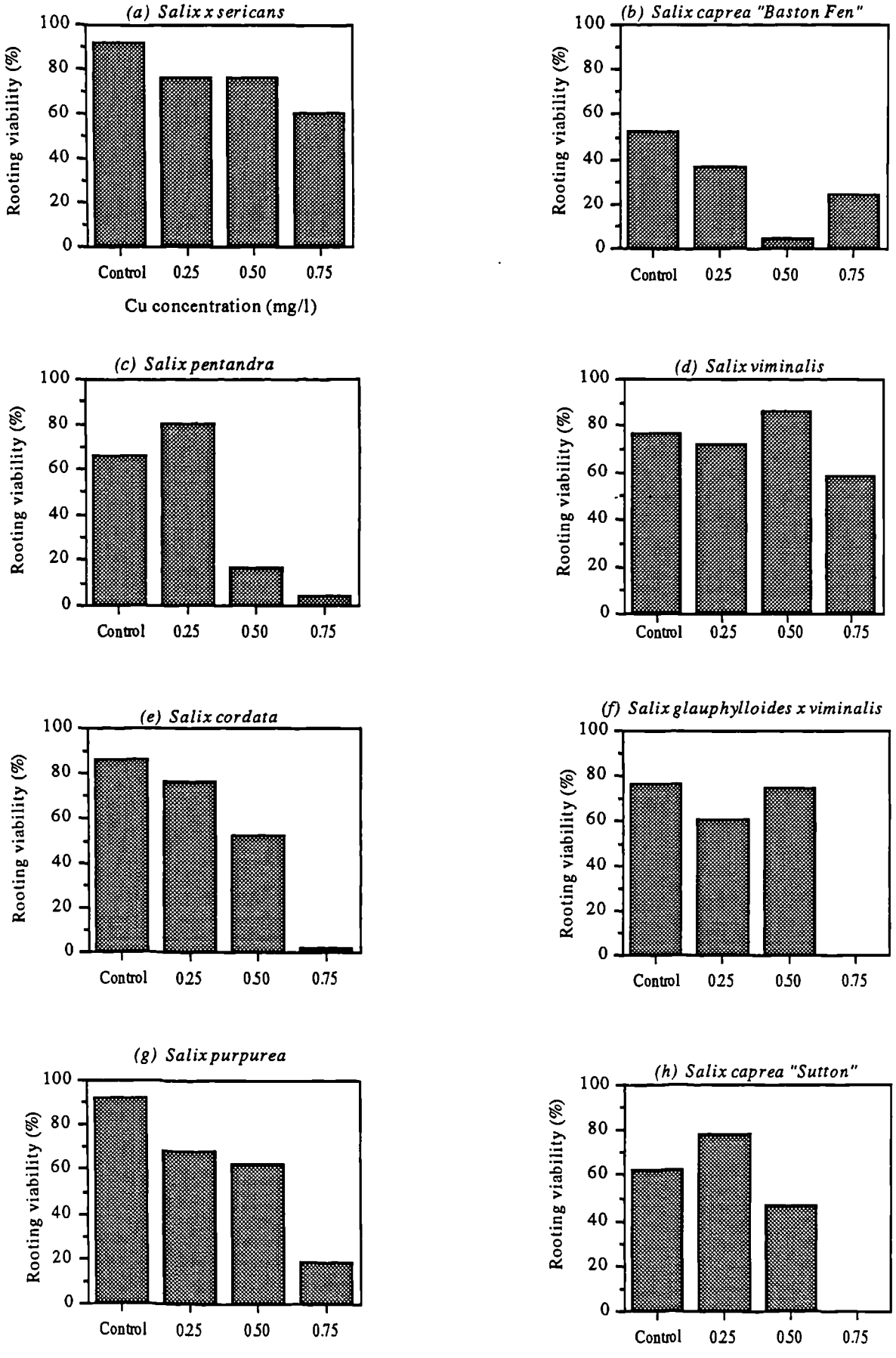
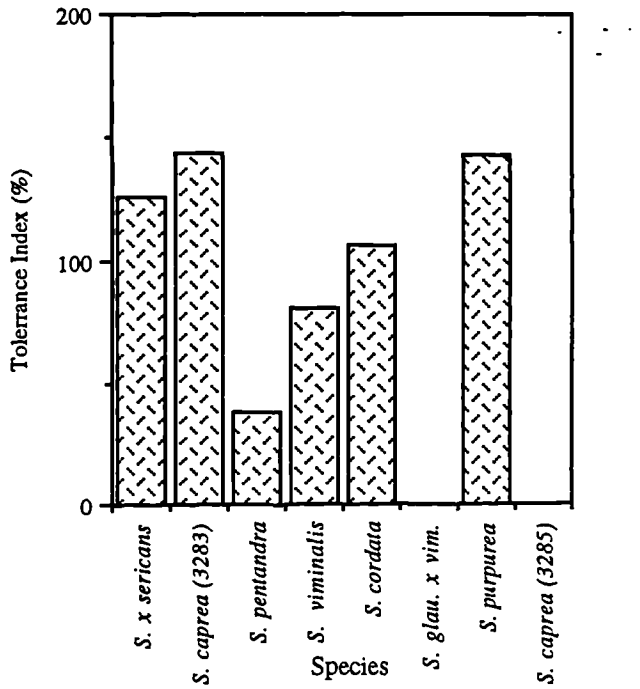


Table 4.6. Results of two-way ANOVA carried out on root length and root number of eight Salix *spp. after 21 days exposure to copper in solution.*

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	*P*
Root Length						
Treatment	3	173803	173803	57934	43.77	0.0001
Species	7	136691	136691	19527	14.75	0.0001
Interaction	21	108482	108482	5166	3.90	0.0001
Error	1568	2075226	2075226	1323		
Total	1599	2494201				
Root number						
Treatment	3	1299.99	1299.99	433.30	16.81	0.0001
Species	7	3305.31	3305.31	471.19	18.32	0.0001
Interaction	21	3276.04	3276.04	156.00	6.05	0.0001
Error	1568	40423.24	40423.24	25.78		
Total	1599	43304.48				

Fig. 4.18. Modified TIs *of* Salix *spp. in response to 21 days exposure to 0.75 mg l^{-1} copper.* Salix glaucophylloides x viminalis *and* Salix caprea (3285) *failed to grow at this concentration of copper.*



The optimum replicate number based on control length data, was calculated using the following formula:

$$N = \frac{(t \times s.e.)^2}{p \times \text{sample mean}}$$

Where N is the replicate number giving statistical precision, t is the t-test value; s.e. is the standard error of the mean and p is the probability value. N has been calculated where P= 0.01, 0.05 and 0.10 and is shown in Table 4.7.

Table 4.7. Optimum replicate number (using control mean root length data)

Species / Hybrid	*N*		
	p=0.01	*p=0.05*	*p=0.10*
S. x *sericans*	869	35	9
S. caprea	1431	79	14
S. pentandra	3191	127	32
S. viminalis	846	34	3
S. cordata	713	29	8
S. glaucophylloides x *viminalis*	677	27	3
S. purpurea	1447	58	15
S. caprea	1758	70	18

4.2.4. Discussion

The results show greater innate copper tolerance of *S. purpurea, S. caprea* 'Baston Fen' and S. x *sericans*. The elevated resistance of *S. purpurea* is attributable to the large increase in production of new roots at 0.75 mg l^{-1} copper. This disagrees with the findings in the previous test which showed that *S. purpurea* was one of the least metal-resistant species. Clearly further testing of this species may be necessary to clarify its response. Similarly this may account for the anomalous results observed for *S. caprea* clones 3283 and 3285 and may indicate that variation in growth responses of willows differs markedly between clones of the same species derived from different sources. It is also possible that the variation has arisen from the different sexes of the clones under test; Pohjonen (1991) points out that female *S. caprea* L. cuttings are non-rooting although this is unsubstantiated and is challenged by these findings. It is interesting to note at this stage that the most resistant willow species tested so far in this study show the greatest variation in their growth characteristics and viability. The high standard errors reflect the requirement for

much greater replication (Table 4.7). This suggests an inherently greater level of phenotypic plasticity and may be supported by the significantly larger national distribution of *S. caprea* compared to that of the others studied here (National Vegetation Classification 1989). These calculations suggest that for the majority of species tested a replicate number of approximately 50 should give satisfactory data. *S.* x *sericans* and *S. viminalis* both have low optimal *N* values, and are both characteristically fast growing willow shrubs. Their fast growth and high rooting viability may contribute to their reliability as test species in this case. It is also important to note the response of *Salix viminalis* in this test in view of its importance as a biomass shrub. The calculated *TI* value is approximately 80% at a copper concentration of 0.75 mg Cu l^{-1} despite being one of the lowest *TI* values obtained in this test this level is still high for cuttings for an individual previously unexposed to metals. Establishing the level of pollution resistance in this economically useful, fast-growing biomass shrub may be of benefit bioremediation schemes. Landberg & Greger (1994) and Östmann (1994) have already focused almost solely upon this willow species as the subject of research into cadmium removal from sludge treated soils.

The *TI* of *S.* x *sericans* to copper in the present study is also noteworthy (approximately 125% at 0.75 mg Cu l^{-1}). Previous reports on this hybrid have indicated that it possesses favourable growth and viability characteristics (Stott 1992). Pohjonen (1991) states that hybrids such as this can be useful in combining the high growth potential and winter hardiness of *S. caprea* with a species that possesses a high rooting viability. It would seem quite possible that such a clone may also incorporate a favourable trait with respect to metal resistance.

These results also suggest some experimental modifications to further testing of metal resistance in the present study; namely that growth characteristics should be monitored regularly throughout the duration of the test to clarify the factors

influencing rooting viability; and to separate the natural level of cutting viability from metal-induced mortalities. Non-viable cuttings could then be distinguished from susceptible cuttings.

The results of this test indicate that certain clones of *S. caprea* and hybrids with *S. viminalis* are copper resistant at concentrations up to 0.75 mg l^{-1}. Root elongation was reduced by copper but root number increased. The resistant clones such as *S. caprea* are also more variable and require greater replication for reliable testing. Recommendations made, therefore, are that *S. caprea*, *S. viminalis* and hybrids between them show considerable metal resistance potential and should be included in further resistance tests.

4.3. Copper resistance and uptake.

Background to the study

Previous tests have shown that elevated concentrations of copper in nutrient solutions inhibit root growth and viability of *Salix* cuttings, although there was clear evidence of differential susceptibility between clones, species and hybrids. This experiment investigates resistance to, and accumulation of copper within the different tissues of willow cuttings with more detailed screening over time. It was hoped that this would enable relationships between accumulation and resistance characteristics to be examined. An important assumption in these experiments is that processes governing accumulation and transport of metal *within* plants remains the same irrespective of the medium in which the metal is supplied, and that solution culture is a reasonable predictor of behaviour in soil. This experiment was also an initial investigation into how much copper willows could accumulate over time, to explore whether it is possible that willows can be used to remove metals from polluted soils.

Copper is a particularly problematic metal pollutant; being one of the least mobile metals and posing a challenge for bioremediation, it is the focus of several studies within this work. Previous experiments have demonstrated that at similar concentrations copper is more toxic to willows than cadmium, and is accumulated primarily in the roots.

4.3.1. Aims

- To measure root growth response to copper over a 28 day growth period for a range of species.
- To re-test 'copper-resistant' clones from previous experiments and to examine reproducibility of previous experimental findings.
- To investigate the relationship between copper resistance and accumulation.

4.3.2. Methods

Table 4.8. Salix *species and hybrids used in the present study*

Species or Hybrid (Source)	Accession No.
S. purpurea L. "Jagiellonka" (♀) (Long Ashton Collection)*	3018
S. alba L. "Caerula Long Ashton Tree 78" (?) (Unknown)	3204
S. fragilis L. "Latifolia Kew" (?) (Kew Gardens)	3234
S. caprea L. "Sutton" (♀) (Donald)*	3285
S. cinerea spp *oleifolia* L. "Bude (E)" (♀) (Stott)	3296
S. x *sericans* Tausch. ex A. Kern.[a] "Coles" (♂) (Coles)	3303
S x *forbyana* Sm.[b] "Reabrook" (♂) (Sinker)	3340
S. viminalis L. "Ivy bridge" (♂) (Rogers ex LARS)*	3369

[a] *S.* x *sericans* = *S. viminalis* x *S. caprea* also known as *S.* x *smithiana* Forbes.

[b] *S.* x *forbyana* = *S. purpurea* x *S. cinerea* x *S. viminalis*

* indicates clones used in previous tests.

Willow cuttings were grown in hydroponic units filled with re-circulating, aerated 25%-strength Hoagland's nutrient solution, as described previously (Section 3.2.1.), in February 1994. Plants were grown in unamended background nutrient solution (supplied with 0.005 mg l^{-1} Cu as a micronutrient as controls) and treatment solutions amended with 0.25 mg l^{-1} and 0.50 mg l^{-1} copper. The higher concentration of 0.75 mg l^{-1} was not used in the present experiment as this concentration caused severe phytotoxic effects. Each willow clone was represented by 36 replicates per treatment randomised within the unit, with duplicate units (eight species x 36 replicates x 2 blocks). All hydroponic units were maintained in a controlled temperature glasshouse (19°C, daily fluctuation ±5°C) without artificial lighting. Nutrient solutions were replaced and trays cleared of debris every seven days. The test ran for 28 days in total, and length of longest root, root number and root viability were monitored every seven days. Each cutting was separated into leaf, new stem, wood (the original cutting material including the bark) and roots. Following the test period the excised roots and woody tissues were washed in flowing glass-distilled deionised water and then maintained in distilled de-ionised water for 10 days, after which they were

removed and prepared for acid digestion. Triplicate samples of each tissue were digested in HNO_3 and analysed for copper (Section 3.6).

Growth data were subjected to statistical analysis, after removing zero values (Section 3.3.). Growth rates were calculated due to the sequential nature of the measurements and were calculated using linear regression. Resistance was estimated using root elongation rate and rate of new root production. This is given by the equation:

$$TI_{[M]}\ (\%) = \frac{R_L \pm R_N}{2} \times 100$$

where R_L (mm day^{-1}) = rate of root elongation in test solution / rate in background solution

and R_N (number produced day^{-1}) = rate of new root production in test solution / root production rate in background solution.

Rooting viability was not incorporated into the *TI* calculation, as it was not considered a parameter which varied in direct response to copper treatment, although changes in viability are shown alongside root growth responses.

4.3.3. Results

Root growth

The effect of copper on root elongation, root number and rooting viability are shown in Figs 4.19-4.21. There was a clear pattern of inhibition of root elongation with increasing copper concentration in all species and hybrids tested except *S. caprea* 'Sutton'. Root number generally increased over the test period. *S. cinerea* ssp. *oleifolia* and showed greater rooting viability in copper-amended nutrient solution (Fig 4.21 d and e), and this also occurred in *S. caprea* (Fig 4.21d). Plants treated with copper amended solutions showed a distinctive root discoloration and root thickening with small densely packed lateral roots. This observation is similar to those made by other workers studying the effects of copper on root system development (Arduini *et*

Figure 4.19. Root elongation of eight clones (a)-(h) exposed to three different concentrations of copper. (Means and standard error of zero-adjusted data, where n=72).

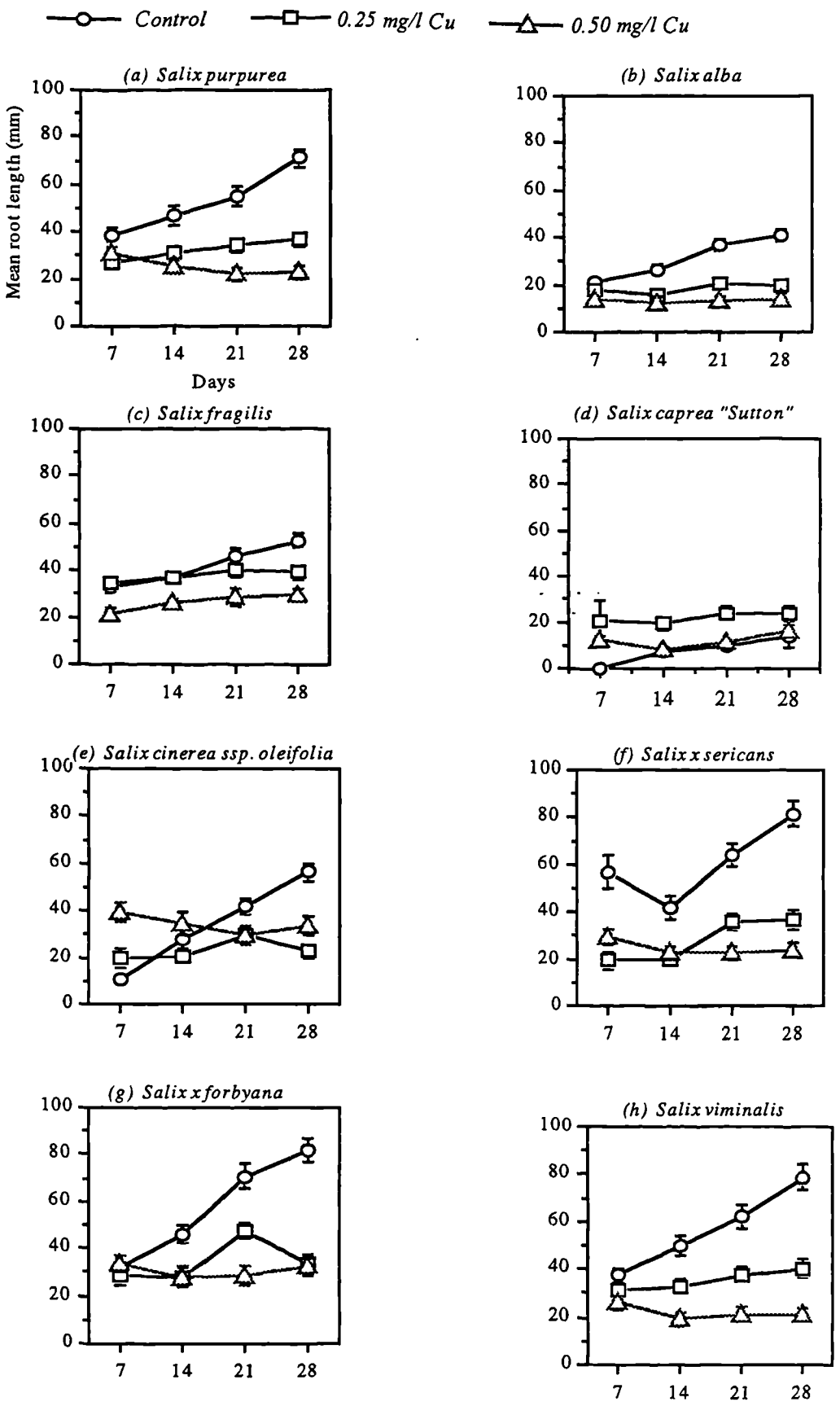


Figure 4.20. Root production per cuttings of eight clones (a)-(h) exposed to three different concentrations of copper for 28 days. (Means and standard errors of zero-adjusted data where n=72)

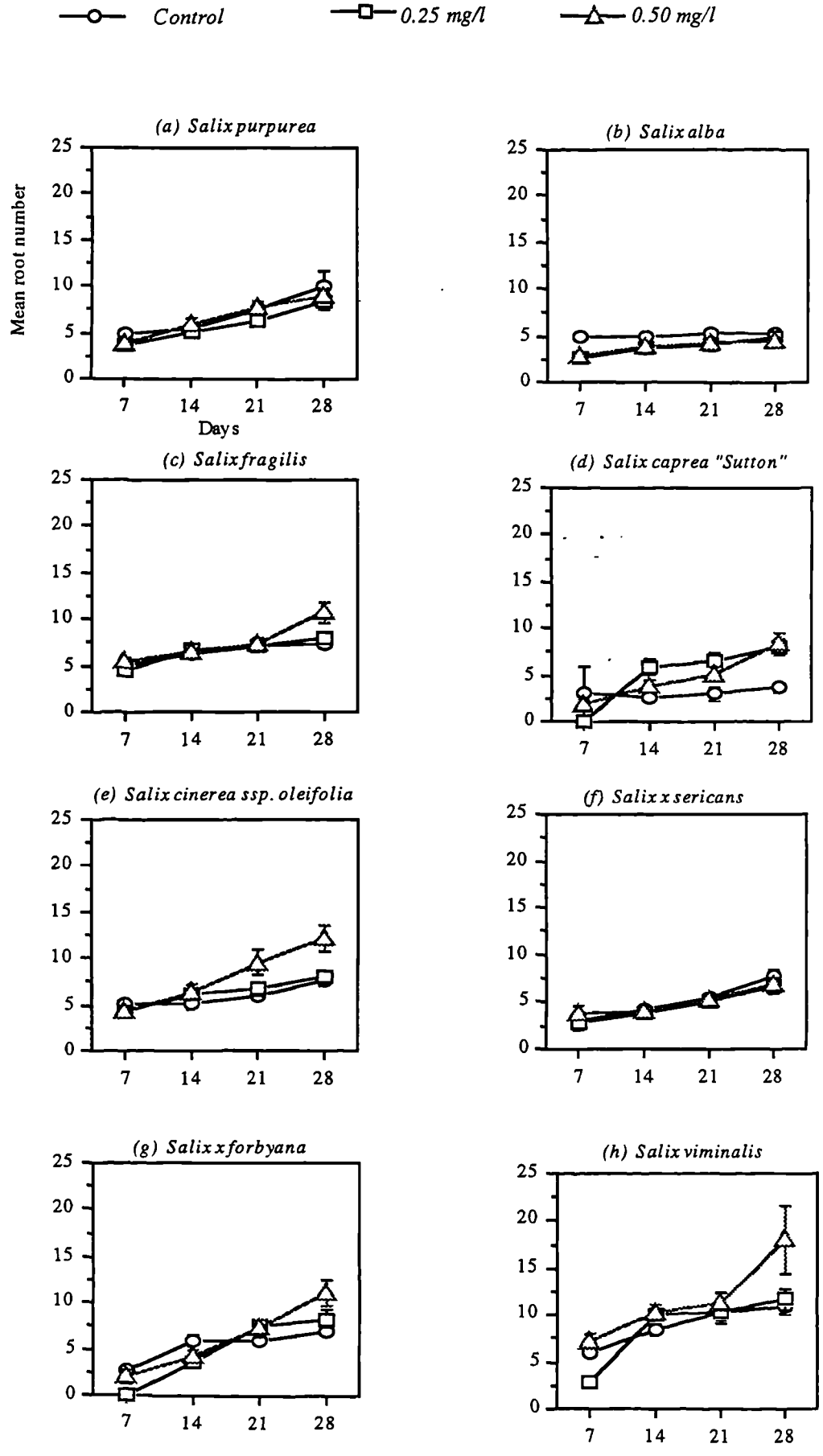
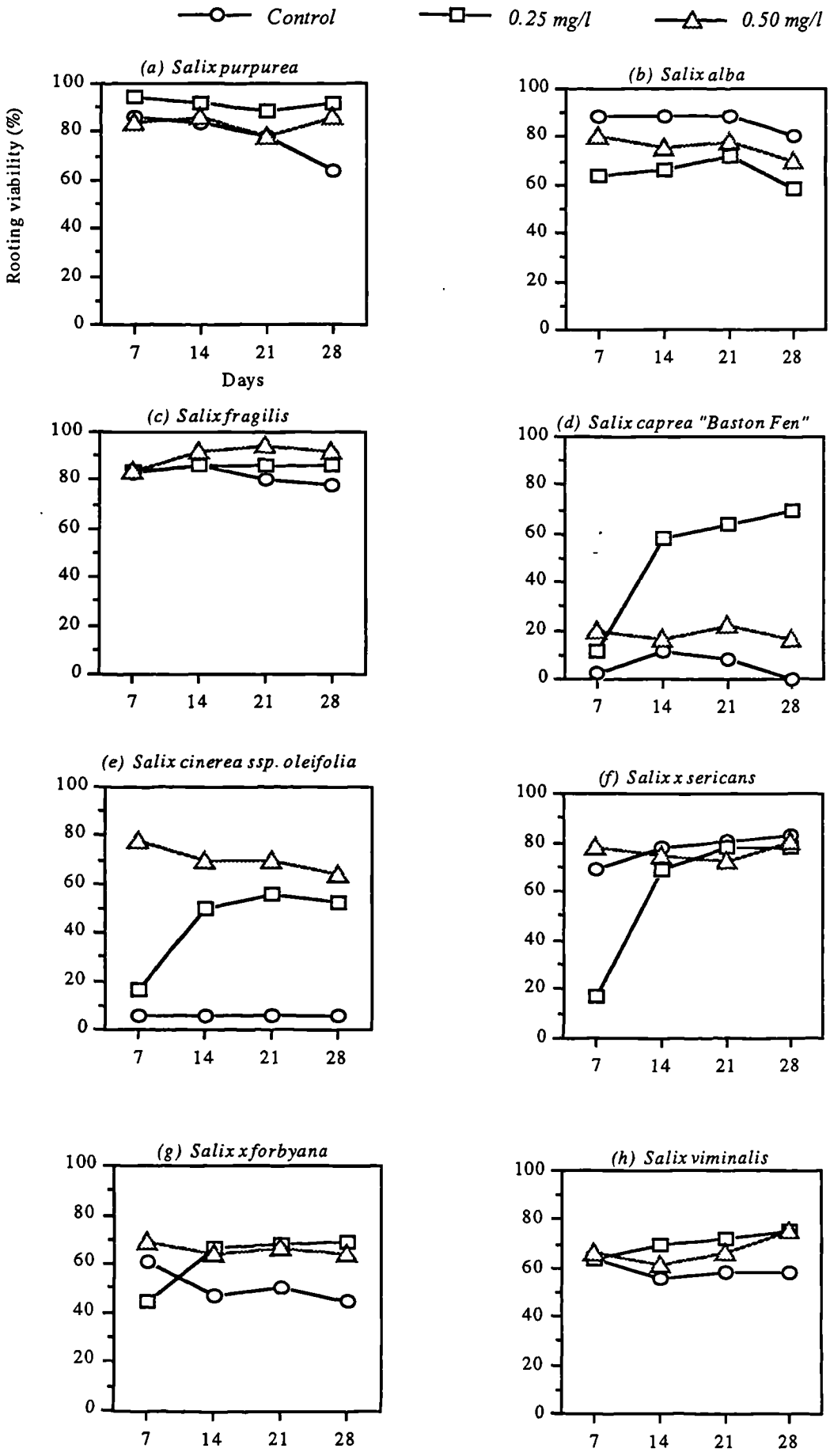


Figure 4.21. Cutting viability of eight clones (a)-(h) exposed to three different concentrations of copper for 28 days (% viable cuttings from test population).



al 1994, 1995). Differences in root length and number were highly significant (Table 4.12).

Table 4.9. F values from GLM analysis of variance of root growth data, showing differences attributable to a species effect and copper concentration.
*** indicates $P < 0.0001$; ** indicates $P < 0.05$ and NS indicates $P > 0.05$ (not significant).

Source$_{(\text{Degrees of Freedom})}$	Root length	Root number
Willow species$_7$	54.65***	157.00***
Duplicate blocks$_1$	37.25***	15.99**
Cu concentration$_2$	203.50***	6.68**
Sampling date$_3$	56.39***	128.61***
Species x Blocks$_7$	3.62***	9.92***
Species x concentration$_{14}$	14.17***	14.38***
Species x sampling date$_{21}$	2.06**	5.25***
Blocks x concentration$_2$	8.07***	18.24***
Blocks x sampling date$_3$	3.88**	1.00ns

Differences in root number were more significantly attributed to clonal variation than to the different concentration of copper used. Statistical analysis using GLM detected significant differences between blocks and showed significant interactions.

Uptake and accumulation characteristics

Levels of copper accumulation were much lower in leaf material than either wood or roots (Figs. 4.22-4.24). *Salix caprea* leaf samples contained the highest concentration of copper in all treatments. Copper accumulation in the leaves of willow cuttings was influenced by the external copper concentration, but this relationship varies inter-specifically. Copper concentration in woody tissue was generally low and below 50µg g^{-1}, except in *S. alba, S. fragilis* and *S. viminalis* at 0.50 mg Cu l^{-1}. The levels of copper accumulated within woody tissue of *S. alba* and *S. fragilis* were 2-3 times greater than background levels but in *S. viminalis* this was several orders of magnitude greater than background levels. Accumulation of copper in the roots was

the greatest observed of all the tissue compartments analysed. Whilst this appeared to be influenced by the external copper concentration, it was not a straightforward relationship and varied inter-specifically. The concentration of copper within roots of cuttings (2,000 μg Cu g^{-1} in roots of *S.* x *sericans* treated with 0.25 mg Cu l^{-1}; Fig. 4.24) appeared high compared to the concentration supplied throughout the experiment, but given the total mass of the dried root systems these figures can be expected. For example, cuttings supplied with 0.25 mg Cu l^{-1} (of which 25 litres were supplied every 7 days for 4 weeks); could theoretically accumulate anything up to 25 mg copper by then end of the test. Cuttings exposed to elevated copper concentrations produced drastically stunted, deformed roots which often weighed less than 2g when oven-dried. In parts per million the amount of copper in root systems of this weight could be up to approximately 2, 000 ppm.

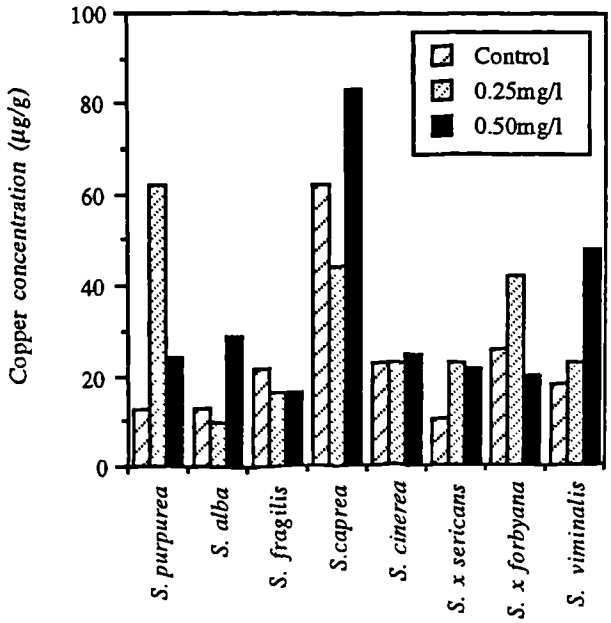


Fig. 4.22. Total copper concentration ($\mu g\ g^{-1}$) of leaf material of eight willow species grown in background and copper-amended nutrient solution for 28 days. Note: range 0.-100$\mu g\ g^{-1}$.

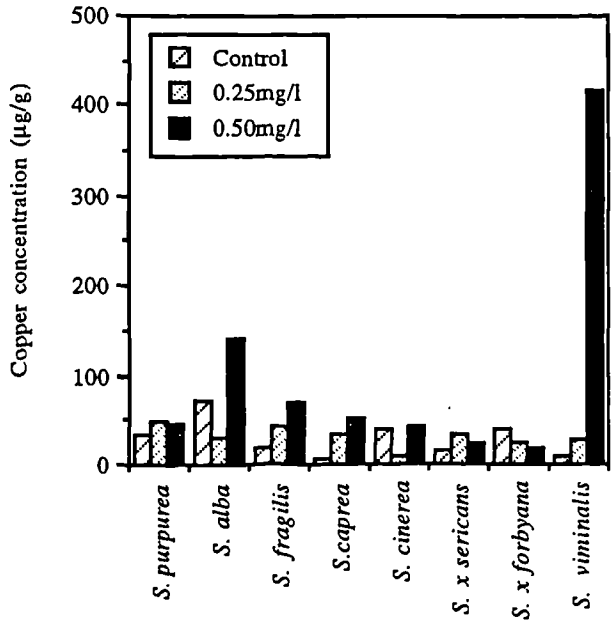


Fig. 4.23. Total copper concentration (µg g^{-1}) of wood material of eight species of willow exposed to background and copper-amended nutrient solution copper for 28 days. Note range 0-500µg g^{-1}

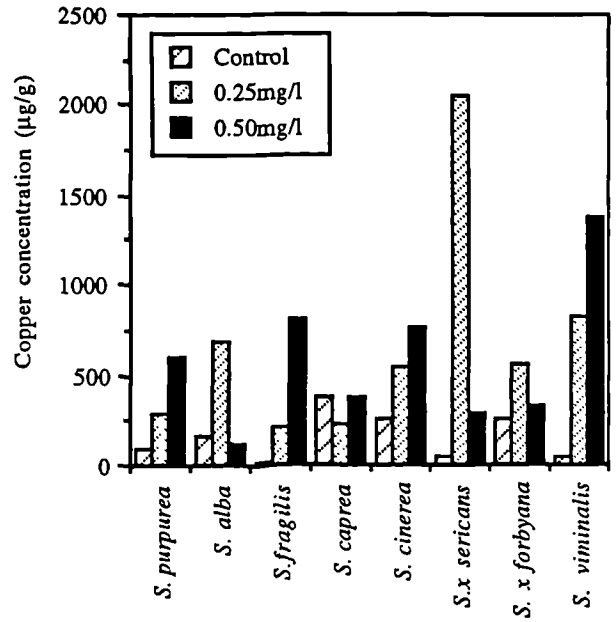


Fig. 4.24. Total metal concentration (µg g^{-1}) of root material of eight willow species exposed to background and copper-amended nutrient solution for 28 days. Note range 0-2500µg g^{-1}.

Growth-rate based tolerance indices

Root growth rate-based *TI* values were calculated for both 0.25 and 0.50 mg Cu l^{-1} and are shown in Figure 4.25. Five of the clones tested had greater tolerance to the higher copper concentration meaning that they benefited from the addition of copper. These include *S. fragilis*, *S. caprea*, *S. cinerea* ssp. *oleifolia*, *S.* x *forbyana* and *S. viminalis*.

Levels of tolerance were exceptionally high in *S. caprea*, with all other species and hybrids reaching an maximum tolerance limit of approximately 200%.

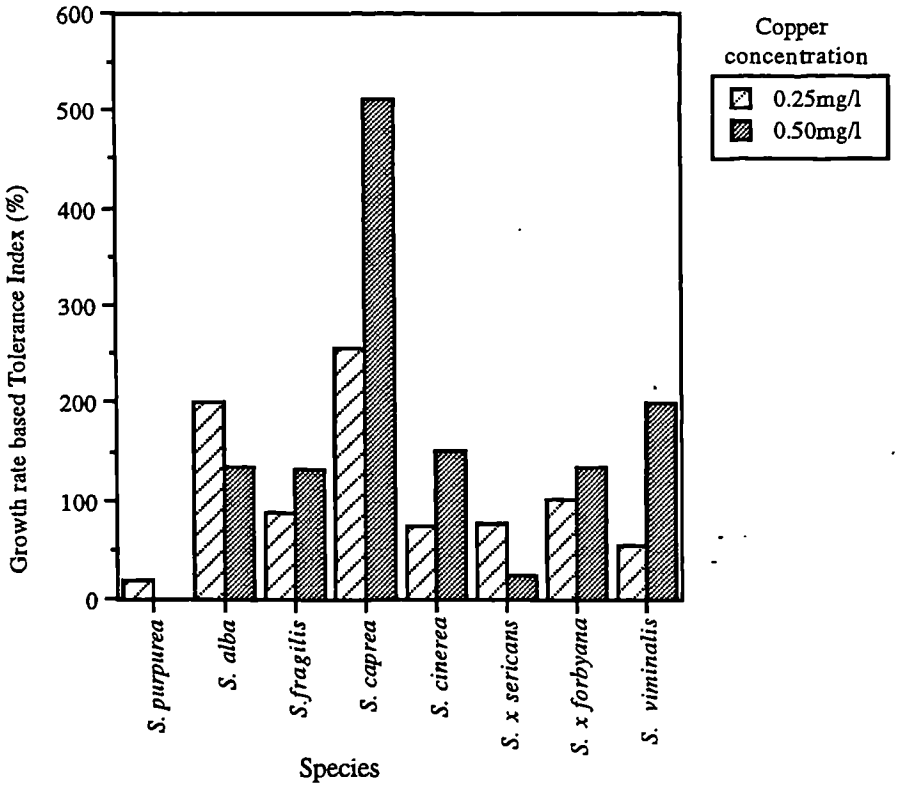


Fig. 4.25. Modified root elongation rate-based TI *of eight willow species exposed to two levels of copper amended nutrient solution for 28 days.*

4.3.4. Discussion

Variation between duplicate blocks of willows is large and in some cases produced greater differences within the growth data than other factors (Table 4.9). Metal treatment remained the most significant affect on root elongation (F_2= 203.5 P = 0.0001), although there was considerable inter- and intra-clonal variation in root elongation. In recent studies on clarification of *Salix* phylogenies using RAPD analysis (Allnutt 1996) it was demonstrated that genetic variation within clones and ramets willows was as great, or greater than variation within species.

This growth data does, however, imply that root elongation is more sensitive to copper than root number, in agreement with Arduini *et al* (1994, 1995). In five clones the number of roots produced was significantly greater in the highest copper treatment than background nutrient solution; notably in *Salix fragilis*, *S. caprea*, *S. cinerea oleifolia*, *S.* x *forbyana* and *S. viminalis*. In the previous screening experiment the ability of cuttings to root in solution appeared to be affected by copper treatment (see Section 4.2.). Monitoring growth and viability regularly over a 28-day period in the present study showed that rooting viability did not change substantially throughout the duration of the test, nor did it appear to be affected by treatment with copper supporting the observation that copper does not affect cell division. Cuttings tested in previous experiments showed differences in growth and copper resistance. *S. purpurea* was particularly metal resistant in Section 4.2. thought to be solely due to the high number of new roots produced, whereas in this test this clone was susceptible to copper. Growth trends remained consistent for *S. viminalis* but differed markedly for *S. caprea* 'Sutton' which grew very poorly in Section 4.2, whereas in this experiment this clone was the most copper resistant.

Accumulation of copper by willow cuttings from solution showed the characteristic pattern of the highest concentrations in the roots. The order of copper accumulation in different tissues tended to follow the order ROOTS>WOOD>LEAVES. This characteristic pattern is likely to be attributable to the high organic affinity of plant-available copper ions in solution causing the majority of copper to be bound on the surface of or within the roots. The changes in root morphology indicate that copper has passed across the root cell membrane and has not been isolated within the apoplasm (Taylor 1987). Root systems were thoroughly washed, after being desorbed for one week with distilled deionised water, and it was assumed that all extracellular copper was removed. Przemeck & Haase (1991) studied the bonding of several heavy metals, including copper, to xylem sap in plant roots and their findings indicated that copper binds to the heavier peptides found within xylem sap. From the metal analysis

data in this test it can be assumed that copper binds mainly to the tissues in which it is in immediate contact, reducing the total translocatable copper; the low concentrations of copper detected in aerial tissues of willow cuttings grown in amended solution could also be explained by findings of Przemeck & Haase (1991).

Although translocation of copper to the aerial portion of the plants was found to be restricted, the level of copper within *S. caprea* leaves was higher than any other species. *S. caprea* has continually shown anomalous results when compared to other willow species possibly as a result of its higher growth variability. This species also had a smaller concentration of copper in the roots compared to the other willows tested.

It is important to examine the relationship between resistance to copper and the uptake and accumulation of this metal within willow cuttings. The biomass willow *S. viminalis* produced roots of much greater length than *S. caprea* (Figs 4.19(h) and (d) respectively), which is suspected to have greater resistance to pollution (Grime *et al* 1988) However, the reduction in root length in the presence of elevated copper is greater in *S. viminalis* than *S. caprea*. A reduced, or slower growth rate in *S. caprea* may be a factor in its appearance on industrial spoils and metalliferous tailings (Grime *et al* 1988; Eltrop *et al* 1991); where a small growth habit and slow growth rate may be adaptation to hostile soil conditions.

S. viminalis accumulated more copper within its woody tissue than any other clone (Fig. 4.23), which may be indicative of greater translocation to aerial parts of the cuttings, inducing phytotoxic effects and leaving the woody tissue saturated with copper. Water utilisation characteristics may influence the amount of copper bound within woody tissue. The riparian willow species *S. alba* and *S. fragilis,* may require a greater supply of water than the more drought-resistant *S. caprea.* Similarly, *S. viminalis* is a fast growing shrub and allocates a greater proportion of its total biomass to above ground biomass under normal circumstances. *S. viminalis*

accumulated less copper in the leaves on a $\mu g\ g^{-1}$ basis compared to *S. caprea* (Fig. 4.22) which may indicate that copper uptake was suppressed in this clone, or conversely could be a result of a greater leaf production.

The hybrids *S.* x *forbyana* and *S.* x *sericans* showed similar levels of copper sensitivity as *S. viminalis*, which is a constituent parent of both hybrids (Fig. 4.25). *S.* x *forbyana* is hybrid between *S. cinerea, S. purpurea* and *S. viminalis* but does not appear to possess characteristics favouring its use in bioremediation. *S.* x *sericans* (a hybrid between *S. caprea* and *S. viminalis*) was resistant to lower concentrations of copper in this test, although root growth characteristics suggested that the dominant genetic contribution may from *S. viminalis*.

Copper inhibited the growth of different willow clones to a different extent throughout the growth period, with results indicating a severe suppression of root elongation for copper-sensitive clones *S. purpurea* and *S. alba*. In agreement with findings from previous tests, growth data for *S. cinerea* and *S. caprea* deviated from the expected pattern; and cuttings treated with copper grew better than control plants. Comparison in growth performance of clones tested in previous experiments gives consistent results for *S. viminalis* although large differences in viability were seen in the more variable *S. caprea.* The relationship between copper resistance and accumulation is complex and cannot be clarified without further testing. One notable finding was the higher leaf-bound copper detected in the resistant *S. caprea* clone, which also appeared to grow better in response to copper treatment.

4.4. Conclusions

The high levels of resistance to copper observed in *S. caprea, S. viminalis* and their hybrids tested in this chapter indicates that willows have potential use in the bioremediation of metal contaminated soil. However, results of metal accumulation

studies have demonstrated that copper remains in the roots of cuttings and comparatively less is translocated in to the aerial tissues.

This study raises the question of which species would be most suitable for bioremediation; a species which grows quickly and easily develops phytotoxicity or a species with a slower rate and a smaller magnitude of growth which may have a greater chance of survival and regeneration. The damage to root and leaves observed in cuttings treated with copper in this chapter demonstrated that selection may be more suitably based on the ability of trees to survive metal toxicity and potentially develop resistance gradually in the long term.

Results of experiment 4.1. suggested that willows may play an important part in remediating soils which are polluted with high concentrations of plant-available cadmium. Willow cuttings were more tolerant to cadmium than any of the other metals investigated. This ability must be re-examined in further experiments, not only to explore the anomalous effect of cadmium on willows in more detail, but to understand how much cadmium willows can tolerate and accumulate.

The study of resistance induction, which has been successfully applied to herbaceous species (Aniol 1984; Baker *et al* 1986) may provide a possible means of lowering the differential susceptibility of some valuable willow species to copper, and other toxic metals such as zinc. In addition, a more informed choice of willow hybrids based on the results of these resistance screens may provide an alternative solution.

The conclusions from this chapter clearly show that screening *Salix* for resistance to heavy metals was successful and can therefore be summarised:

- Large differences in metal resistance exist between species and clones
- The clones tested had a high innate cadmium tolerance.

- *S. caprea, S. viminalis* and their hybrids should be selected for further investigation due to their high resistance to heavy metals.
- Copper suppressed root elongation but not root number.
- The relationship between copper resistance and accumulation differ interspecifically and are especially complex in *S. caprea*.

Chapter V

Resistance induction

5.0. Introduction and rationale

Despite the widespread study of metal tolerance in various plant populations on contaminated soil, there have been only a few reports of induced tolerance in vascular plants (Baker 1987; Outridge & Hutchinson 1991) and even fewer that relate to woody species (Dickinson *et al* 1991a, b; 1992). Cox & Hutchinson (1980) induced metal tolerance in the grasses *Deschampsia caespitosa* and *Anthoxanthum odoratum* by pre-exposing them to copper and zinc respectively, and several other workers have shown that plants receiving low-dose metal pre-treatments show an increased ability to resist a further higher dose of metals. (Aniol 1984; Brown & Martin 1981; Baker *et al* 1986; Watmough *et al* 1995a). Brown & Martin (1981) showed that when non-tolerant genotypes of another grass *Holcus lanatus* were pre-exposed to 0.2 μg Cd ml^{-1}, their root growth was increased when subsequently exposed to 1.0 μg Cd ml^{-1} compared with non-acclimated controls. In addition to this positive effect that pre-exposure to metals may have on the resistance of plants, induced resistance can also be lost. Baker *et al* (1986) found that the tolerance index of a Cd-tolerant *H. lanatus* clone ('Hallen') fell by 17% (from *c*.88% to 71%), when the plants were grown in uncontaminated soil for six years. Although this reduction appears small, they found that this 'lost' tolerance could not be recovered when the clone was subsequently grown on its native soil.

Outridge & Hutchinson (1991) noted that tolerance induction is deemed unimportant in the literature because the majority of studies have been carried out on plant clones in which tolerance had arisen by natural selection, and that in comparison induced tolerance was inconsequential. They point out, however, that data on non-tolerant genotypes is lacking, and that in the context of previously unexposed plants, induced tolerance may be very important.

Some emphasis has also been placed on the efficacy of gradual acclimation of trees to low levels of metal pollutants in the soil for resistance induction (Dickinson *et al* 1992; Cumming & Taylor 1990; Outridge & Hutchinson 1991). Dickinson *et al* (1992) reported that in a woodland area receiving an input of copper from a nearby aerial source, pre-existing mature *Acer pseudoplatanus* L. (sycamore) were shown to be resistant to copper in *in vitro* experiments, whereas seedlings were not. Their findings imply that long term exposure to small, but increasing levels of copper had brought about the development of resistance. Seedling establishment was inhibited in this woodland, however, but seedlings may be exposed to a higher concentration of plant-available metals because they initially establish in the uppermost layers of the soil horizon, which in an aerially contaminated site will contain the bulk of the soil metal load (Patterson & Olson 1983).

Experimental attempts to induce resistance using pre-exposure to metals have been successful for some herbaceous species (Outridge & Hutchinson 1991; Baker *et al* 1986), and *de novo* induction of metal resistance in woody plants has been performed recently under experimental conditions using callus culture (Dickinson *et al* 1992; Watmough *et al* 1995a). The observations of Dickinson *et al* (1991a, b) clearly show that resistance to metals in woody species can be induced given appropriate selection pressure, although the duration and magnitude of the selection pressure may be an important factor. Herbaceous plants in possession of the appropriate genetic variation can respond to low-dose pre-treatment to metals, although longer-lived woody species may require long term acclimation in order to elicit a phenotypic response.

The experiments described in the present chapter were an attempt to gain a greater understanding of resistance induction in trees and constitutes one of the first *de novo* metal resistance induction tests carried out on rooted cuttings of trees. The following experiments use willow species chosen on the basis of the lowest susceptibility to metals (i.e. most metal-resistant), and two induction techniques as suggested by

findings in the literature. The first technique entailed pre-treating cuttings with copper, with both low and high (sub-lethal) concentrations, comparing the effect of these pre-treatments on root growth rate in a subsequent exposure to copper compared with untreated cuttings. The second induction technique involved exposing willow cuttings to gradually increasing treatments of copper, cadmium and zinc in nutrient solution over a much longer test period. This latter technique was also used to study the effect of metals applied both singly and in combination, due to the widespread occurrence of several metals in many metal-contaminated substrates (Smith & Bradshaw 1972). This chapter also focuses more closely on the uptake and accumulation of heavy metals in various plant tissues, and the effect of mixtures of metals on metal uptake.

5.1. Induction of copper resistance using short-term, low-dose pre-treatments.

Background

The previous chapter showed that several willow clones can grow in relatively high concentrations of heavy metals, however their resistance to copper is low in comparison with cadmium and zinc. Studies on grasses have shown that greater resistance can be achieved by exposing plants to low dose pre-treatments (Outridge & Hutchinson 1991; Baker *et al* 1986; Aniol 1984), and this experiment was an attempt to induce greater copper resistance in *Salix* clones with low susceptibility (high resistance) to copper in relation to other clones.

5.1.1. Aims

- To investigate whether the natural level of susceptibility of selected willow species to copper in solution can be reduced by short-term pre-treatments.
- To investigate any relationship between the pre-treatment concentration and the subsequent resistance response.
- To measure the uptake and accumulation of copper in willow cuttings treated with copper over a 63 day period, to further examine whether there is a relationship between copper accumulation and higher levels of resistance.

5.1.2. Materials and Methods

Table 5.1. Species used in the copper pre-treatment experiment

Species / Hybrid (Source)	Accession No.
Salix cordata "Purpurescans" (?)(Tuinzing)	3280
S. fragilis L. "Russeliana Kew" (?) (Kew Gardens)	3235
S. caprea L. "Sidelands" (♂)(Stott)	3289
S. caprea L. "Sutton" (♀) (Donald)	3285
S. cinerea ssp. *oleifolia* "Macreight (E)" (♀) (Stott)	3294

Pre-treatment of cuttings

Cuttings from 5 clones (Table 5.1.) were collected in November 1994 and maintained in 3.5 litre black polypropylene buckets with 1 litre distilled water for approximately 14 days until adventitious roots and shoots began to emerge. They were then placed in 6 contiguous hydroponic units, with 27 replicates of each willow accession randomly placed within each unit (2 blocks each of 3 treatments with 27 replicates of 5 species; 6 units in total). Pre-treatments consisted of 0.25 (low), 0.50 mg Cu l^{-1} (high) or background solution (no pre-treatment) in aerated, circulating quarter-strength Hoagland's nutrient solution for 28 days. Length of longest root, root number and cutting viability of each cutting was measured every seven days for 28 days, with solutions replaced every 7 days. The units were maintained in a controlled temperature glasshouse (19°C ± 5), with 16/8 hour day/night light regime under Osram (SON-T 400W) lamps.

Subsequent exposure to copper

Following the final measurements on day 28 of the test period, cuttings were carefully removed from the support trays ensuring those from duplicate units were kept separate. The roots and woody material were carefully washed for 2 hours in running distilled deionised water to remove residual nutrient solution and metals after which they were maintained for a further 10 days in distilled water. Remaining clones of each willow accession were then randomly divided in to three groups and re-allocated to one of the same treatments. This meant that all pre-treatments (x3) and treatment (x3) were represented in every combination (x9). Root growth was measured after one week and again after 28 days.

Zero-adjusted growth data are presented for the induction phase of the experiment as mean growth at each monitoring date, and the subsequent growth of pre-treated plants is expressed as growth rate, due to the effect of separating test populations into three further treatments. The cutting viability (% successfully rooted cuttings from each

test population) is presented alongside relevant growth data as before. It was ensured that growth data from each duplicate unit were not significantly different before data was combined.

Analysis of cutting material

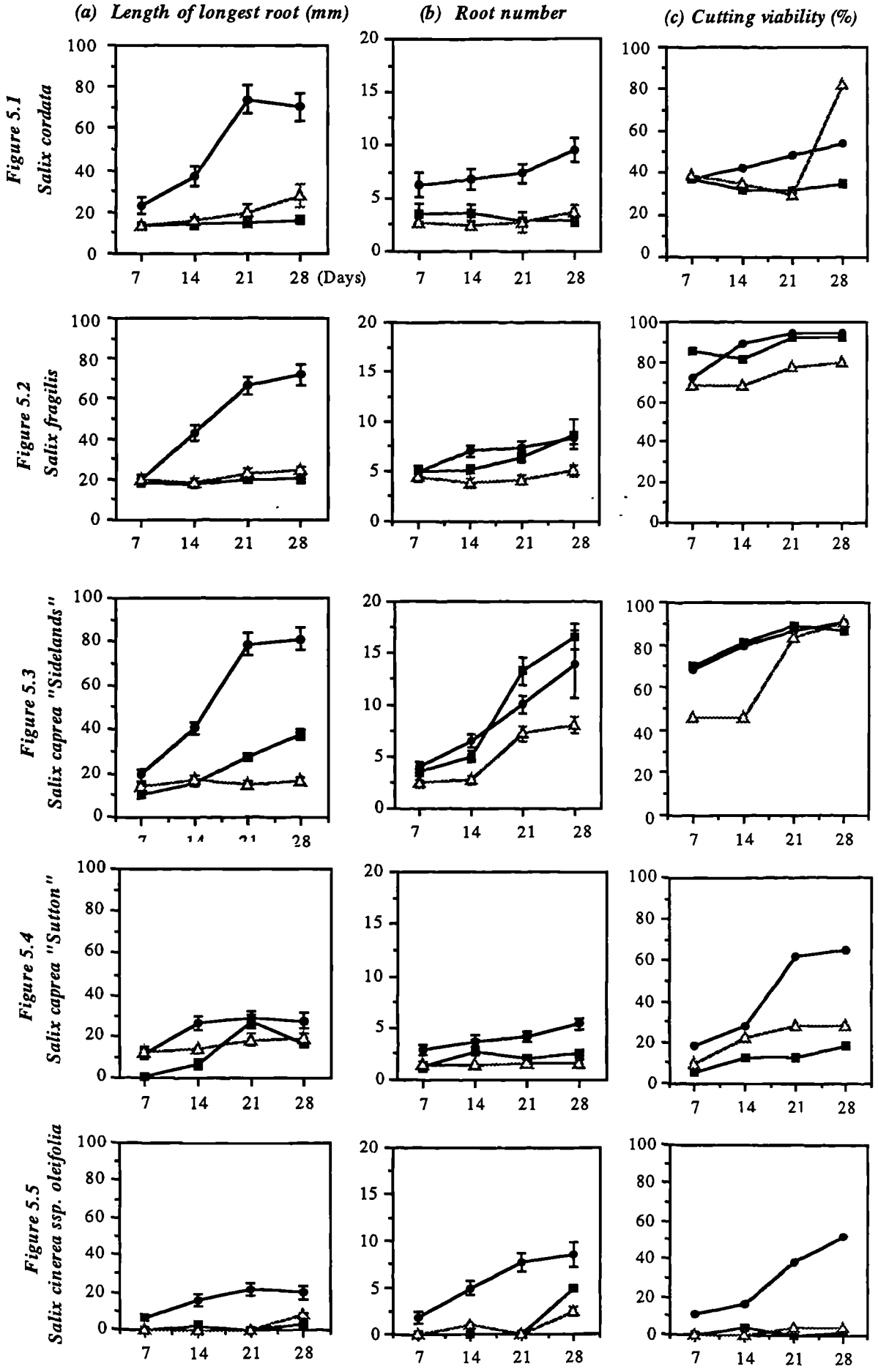
After the test period, the roots and woody material were separated from the plants and washed thoroughly in flowing distilled-deionised water for 2 hours after which they were maintained in distilled, deionised water for one week. Leaf, new stem, root and original woody tissue were dried at 80°C for 48 hours in a air-circulation oven. Samples were prepared, digested and analysed for metals as described in Section 3.6.

5.1.3. Results

5.1.3.1. Response of root growth to copper pre-treatments

Figures 5.1 to 5.5 show the growth response of the willow clones during a 28 day pre-exposure to copper. Each figure is subdivided into the three growth responses; (a) mean length of longest root; (b) mean number of roots per cutting and (c) percentage of successfully rooted cuttings (viability).

Figure 5.1-5.5. Growth responses (a)-(c) of 5 clones exposed to 3 concentrations of copper for 28 days.
(Means and standard errors of zero-adjusted data where n=54)
Copper treatments: —●— *Control* —■— *0.25 mg/l* —△— *0.50 mg/l*



Root elongation was inhibited by copper in all willow species, but *S. caprea* 'Sutton' showed the least inhibition relative to the control. Production of new root material was inhibited by copper, notable exceptions being *S. fragilis* (Fig. 5.2c) and *S. caprea* 'Sidelands' (Fig. 5.3c). The latter clone produced the greatest number of roots when exposed to 0.25 mg Cu l^{-1}. Cutting viability was affected by elevated copper in all clones, but *S. cinerea* ssp. *oleifolia* and *S. caprea* 'Sidelands' began to produce roots in response to elevated copper after the initial inhibition. Rooting viability of *S. cordata* was variable in response to 0.50 mg Cu l^{-1} and showed a marked increase in viable cuttings after 21 days of treatment (Fig. 5.1c).

5.1.3.2. Response of pre-treated cuttings to subsequent exposure to copper.

Figures 5.6-5.10 show (a) the root length- and (b) root number-based growth rates of the five pre-treated willow species re-exposed to copper. Growth rates varied in response to both pre-treatment and subsequent copper treatment. In most cases the highest root elongation rate resulted from a continuous treatment with background solution; only *S. caprea* 'Sutton' showed a greater rate of growth in 0.25 mg Cu l^{-1} than the control after pre-treatment with 0.50 mg Cu l^{-1}. Increases in the rate of new root production in response to a pre-treatment was observed in *S. fragilis* and *S. cinerea oleifolia* pre-treated with 0.25 mg Cu l^{-1}.

Figures 5.6-5.10 (a)-(b) Subsequent growth responses of 5 pre-treated clones exposed to 3 copper treatments for 28 days (Means and standard error values).

Pre-treatments: ■ *Control* ▨ *0.25 mg/l* □ *0.50 mg/l*

(a) Root elongation rate (mm/day) ***(b) Root production rate (no. per day)***

Figure 5.6 *Salix cordata*

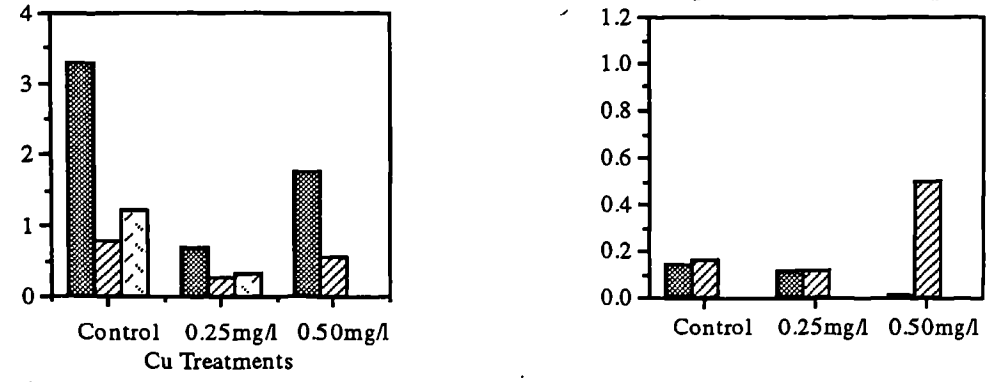


Figure 5.7 *Salix fragilis*

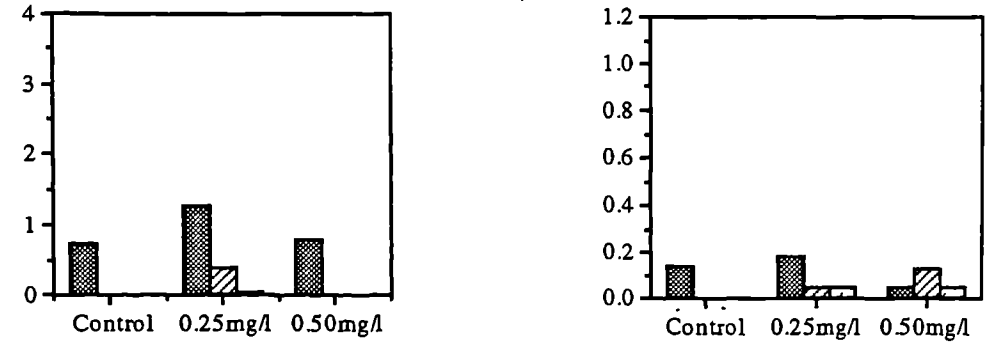


Figure 5.8 *Salix caprea "Sidelands"*

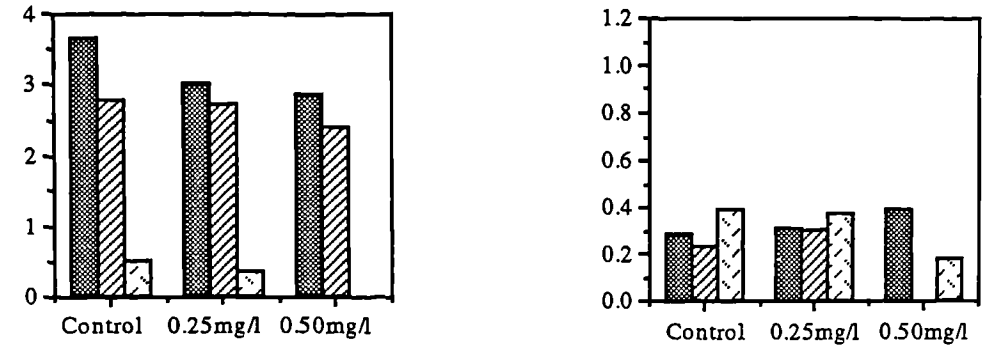


Figure 5.9 *Salix caprea "Sutton"*

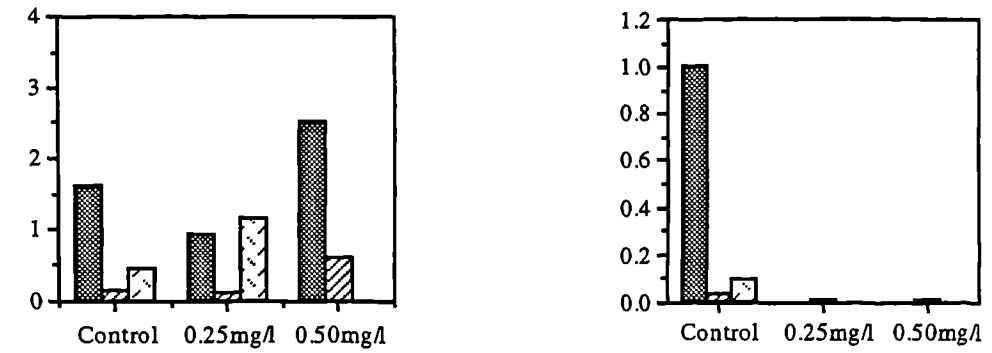
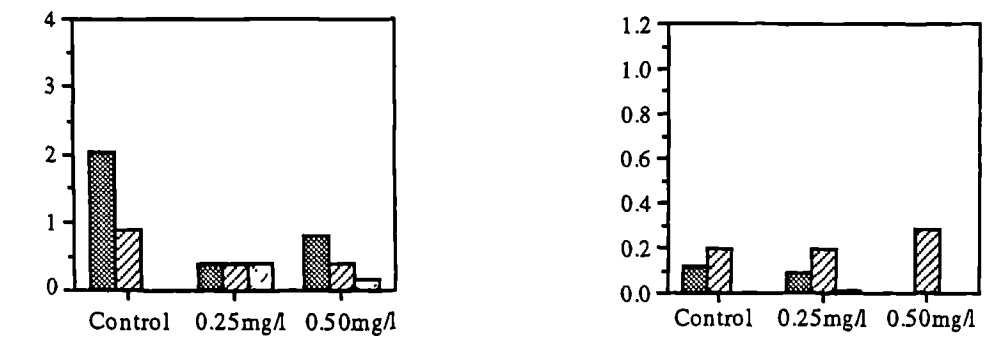


Figure 5.10 *Salix cinerea ssp. oleifolia*



Root elongation of cuttings pre-treated with background nutrient solution was generally higher than those pre-treated with copper. In *S. fragilis* in particular, cuttings pre-treated with background solution and then exposed to 0.25 mg Cu l^{-1} grew better than cuttings continually grown in background solution. Pre-treatment of *S. caprea* 'Sutton' with 0.25 mg Cu l^{-1} increased root elongation rate in response to a higher treatment of 0.50 mg Cu l^{-1}. Similarly the higher concentration of pre-treatment increased root elongation rates when cuttings of this clone were then re-exposed to a lower treatment (Fig.5.9a).

The subsequent root elongation and production rates of *S. caprea* 'Sidelands' were less affected by copper treatment regardless of pre-treatment (Fig 5.8a,b). Overall, root elongation and production rates were greatest in cuttings receiving no copper treatment, and non-pre-treated cuttings responded more favourably to subsequent copper treatment than pre-treated cuttings for the majority of species; for example *S. cordata* in Fig. 5.6a; *S. fragilis* in Fig. 5.7a; *S. caprea* 'Sidelands' in Fig. 5.8a and b; *S. caprea* 'Sutton' in Fig. 5.9a.

Table 5.2. F values from GLM analysis of root growth responses for willow cuttings after pre-treatment, excluding S. cinerea ssp. oleifolia *due to lack of growth for this clone.*
*** indicates $P<0.0001$; ** indicates $P<0.05$ and ns indicates no significant difference ($P>0.05$).

Source$_{(\text{Degrees of Freedom})}$	**Length of longest root**	**No. roots per cutting**
Cu concentration$_2$	216.08***	25.95***
Willow clones$_3$	17.12***	5.57**
Sampling date$_3$	49.50***	17.51***
Duplicate blocks$_1$	14.33***	27.06***
Cu concentration x Sampling date$_6$	44.18***	7.40**
Cu concentration x Willow species$_6$	19.21***	2.42**
Cu concentration x duplicate blocks$_2$	8.08***	5.06***
Sampling dates x duplicate blocks$_3$	3.70***	2.16ns
Willow species x duplicate blocks$_3$	2.92**	2.46ns

Table 5.3. F values from one-way ANOVA of length of longest root data from duplicate units after 28 days pre-treatment with copper. (D.F.=1)

ns indicates no significant differences ($P>0.05$).

Willow species	Control	0.25 mg l^{-1}	0.50 mg l^{-1}
S. cordata	1.92ns	0.79ns	0.01ns
S. fragilis	0.36ns	0.00ns	0.16ns
S. caprea 'Sidelands'	2.32ns	0.99ns	2.21ns
S. caprea 'Sutton'	3.65ns	1.44ns	1.61ns
S. cinerea ssp. *oleifolia*	0.28ns	0.46ns	2.14ns

Table 5.4. F values from GLM of root length and number data from willow cuttings pre-treated with copper, excluding S. cinerea ssp. oleifolia *due to lack of growth for this clone.*

*** indicates $P<0.0001$; ** indicates $P<0.05$ and NS indicates no significant difference ($P>0.05$).

Source(Degrees of Freedom)	Length of longest root	No. roots per cutting
Pre-treatment$_2$	9.46***	0.39ns
Treatment$_2$	18.89***	2.55ns
Willow clone$_3$	25.39***	60.10***
Sampling date$_1$	115.80***	34.82***
Duplicate block$_1$	1.70ns	3.21ns
Pre-treatment x treatment$_4$	1.18ns	1.50ns
Pre-treatment x Willow clone$_6$	1.15ns	0.75ns
Pre-treatment x Sampling date$_2$	0.68ns	0.07ns
Pre-treatment x duplicate block$_2$	1.65ns	2.54ns
Treatment x Willow clone$_6$	4.90***	1.16ns
Treatment x Sampling date$_2$	25.69***	0.72ns
Treatment x duplicate block$_2$	0.79ns	1.17ns

Both root length and number are significantly affected by copper concentration and there were significant differences between clones. Unfortunately, *F* values from the GLM analysis of zero-adjusted experimental data indicated that there were significant differences between duplicate blocks during the pre-treatment phase of the experiment (Table 5.2). However, when the growth responses of individual species were analysed using a simple one-way ANOVA test for data collected on the last

sampling date, no significant differences were found (Table 5.3). Analysis of results from the re-exposure of pre-treated clones (Table. 5.4) showed that elongation was affected but neither pre-treatment nor subsequent treatment significantly affected the number of roots produced. The effects of duplicate treatment blocks on growth data were not significant for either root length or root number for this phase of the experiment, nor were there any significant interaction between duplicate blocks and the other factors tested. Interactions between subsequent copper treatment and different willow species as well as copper treatment and sampling dates were significant for root length only.

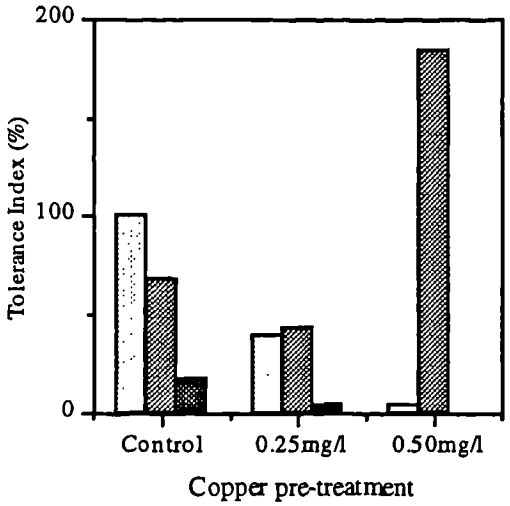
5.1.3.3. Tolerance Indices

The modified *TI* (Section 4.3.2) of willow cuttings is shown in Figure 5.11-5.15. Growth of cuttings maintained in background nutrient solution throughout the experiment were used as the control for calculating the indices. In *Salix cordata* there were positive pre-treatment effects observed in response to 0.25 mg Cu l^{-1} after pre-treatment with the 0.50 mg Cu l^{-1}; *TI* increased by >100% compared to cuttings without pre-treatment (Fig 5.11). For *Salix fragilis* only those that had been pre-treated with elevated copper subsequently grew in metal treatments (Fig. 5.12). *Salix caprea* 'Sidelands' exposed to 0.25 mg Cu l^{-1} after pre-treatment at this concentration showed an increased *TI* of 10.5% compared to cuttings without pre-treatment (Fig. 5.13). *Salix cinerea* ssp. *oleifolia* responded to 0.25 mg Cu l^{-1} following pre-treatment with 0.50 mg Cu l^{-1} with a 17.4% increase in *TI* compared to cuttings without pre-treatment (Fig. 5.15). There were no pre-treatment effects observed for *S. caprea* 'Sutton' although pre-treatment with metals did allow some growth in 0.50 mg Cu l^{-1} (Fig. 5.14).

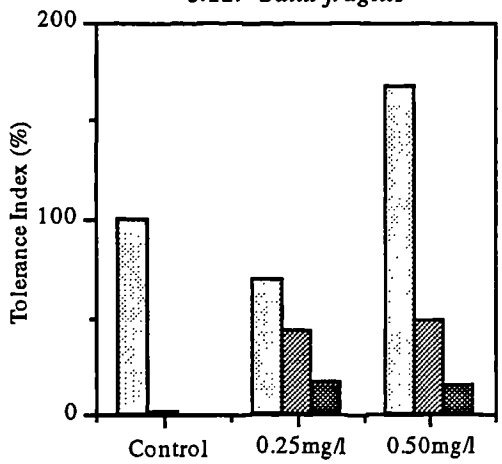
Figures 5.11-5.15 Modified Tolerance Indices (%) of five pre-treated clones re-exposed to copper for 28d.

Re-exposure treatments: ☐ *Control* ▨ *0.25 mg/l Cu* ■ *0.50 mg/l Cu*

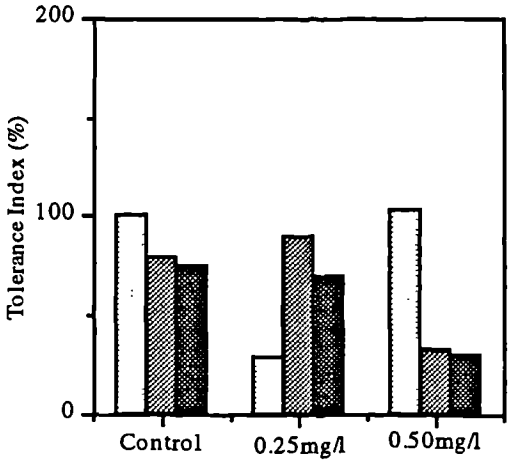
5.11. Salix cordata



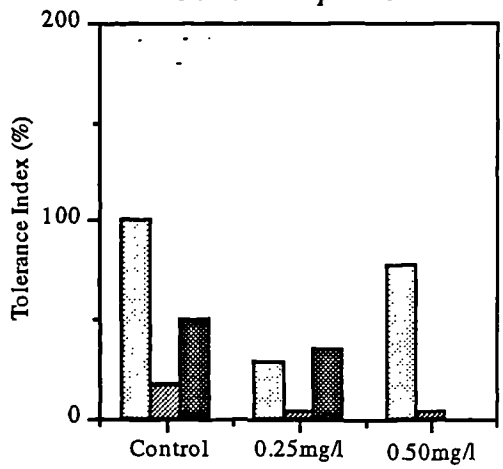
5.12. Salix fragilis



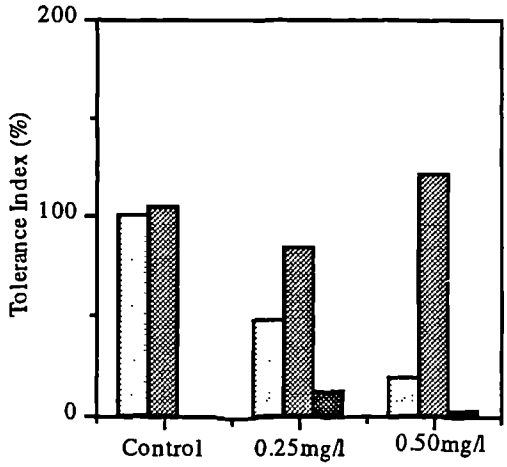
5.13. Salix caprea 'Sidelands'



5.14. Salix caprea 'Sutton'



5.15. Salix cinerea spp. oleifolia



5.1.3.4. Copper accumulation within the various compartments of willow cuttings.

Histograms showing copper concentrations in leaf, root new stem and woody tissue of pre-treated cuttings both before and after re-exposure are shown in Figs 5.16-5.19. The first three bars on each histogram represent the copper content of pre-treated cuttings with the remaining nine bars indicating metal levels in the re-randomised cuttings following subsequent exposure, with all pre-treatment:treatment combinations shown.

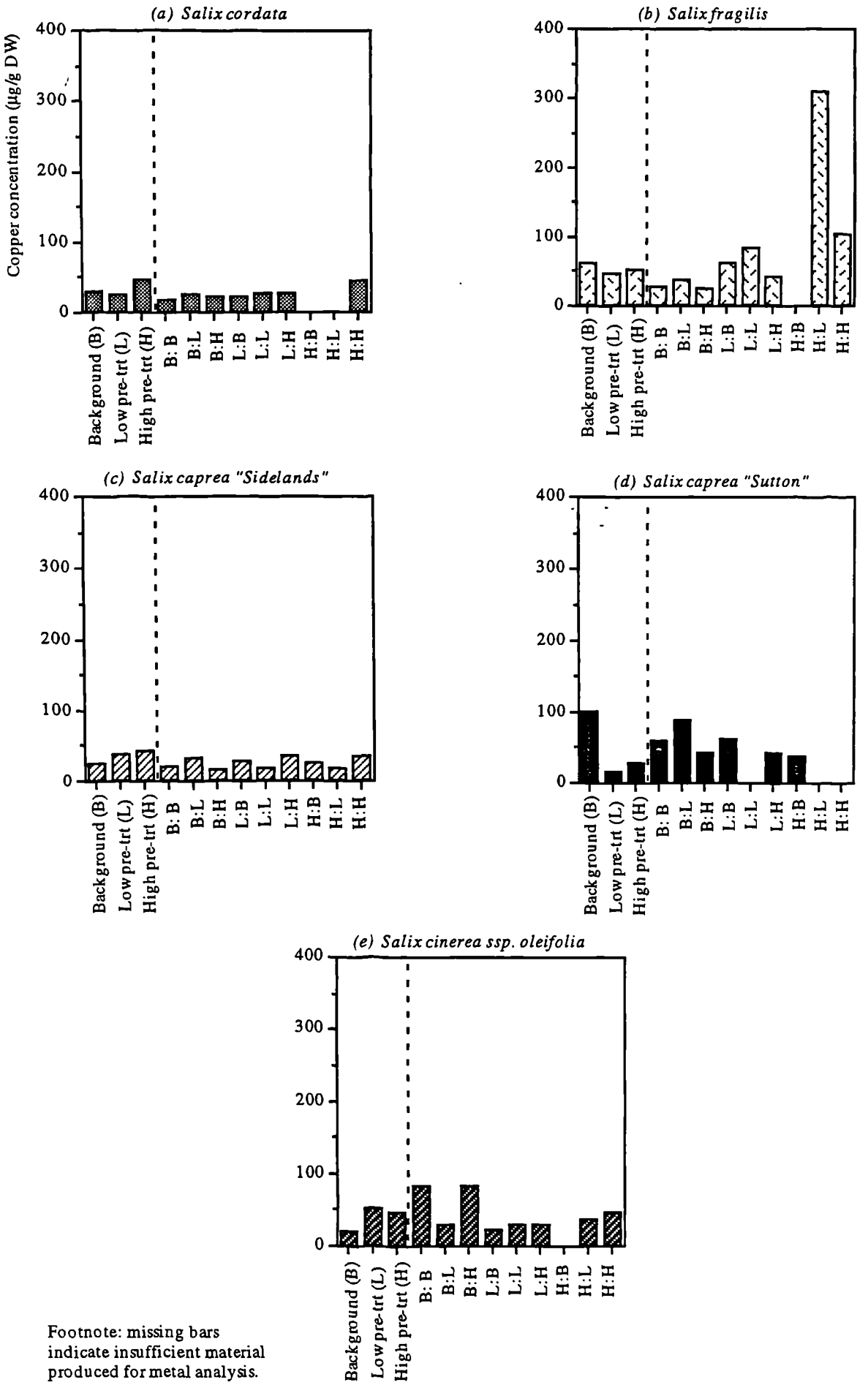
Overall the uptake of copper and translocation into specific plant tissues was variable. however. Continued treatment with copper did not necessarily cause a continued increase in uptake. The main trends are the higher accumulation of copper within the roots of treated plants. Concentrations within the wood, stem and leaves were typically much lower, generally following the order:

ROOTS > WOOD > NEW STEM > LEAVES

Several points can be made in addition to this, however. Copper concentration in cuttings grown for 63 days without any metal treatment (shown as B:B on Figs. 5.16-5.19) had a lower, but very similar concentrations to those after 28 days (B), with the exception of *S. caprea* 'Sutton', roots, due to little root growth in background nutrient solution after the initial pre-treatment phase. Copper concentrations in leaves remained more-or-less stable (Figure 5.16) after pre-treatment and subsequent exposure with the notable exception of *S. fragilis*. This clone contained a higher concentration of copper in treatments where cuttings had been continually treated with copper either as a pre-treatment or as a subsequent treatment. Clearly translocation of copper to leaves was particularly significant in this species. In *S. caprea* 'Sutton' and *S. cinerea* most leaf uptake occurred in control solutions. *S. fragilis* showed the greatest accumulation of copper within the leaf material after exposure to a high pre-treatment (H) and a low treatment (L).

Accumulation of copper in the woody component of the willow cuttings was low; *S. caprea* 'Sidelands', *S. caprea* 'Sutton' and *S. cinerea* ssp. *oleifolia* (3294) showed increases after 28 days pre-treatment and subsequent uptake appears to roughly reflect. Some clones, such as *S. fragilis* and *S. caprea* 'Sidelands', which produced comparatively larger quantities of new stem material did not contain similarly larger amounts of copper within this compartment. Overall copper accumulation trends for *S. caprea* 'Sidelands' showed that pre-treated cuttings re-exposed to copper contained lower concentrations of this metal within their roots than cuttings sampled immediately after the pre-treatments were applied.

Figure 5.16 (a)-(e) Concentration of copper (μg/g DW) in leaf tissues of five clones after 28-day pre-treatment (LHS dotted lines) and a further 35 day treament (RHS line) with copper.
B= background nutrient solution L= 0.25 mg/l Cu H= 0.50 mg/l Cu



Footnote: missing bars indicate insufficient material produced for metal analysis.

Figure 5.17 (a)-(e) Concentration of copper.(µg/g DW) in root tissues of five clones after a 28-day pre-treatment (LHS dotted line) and a further 35 day treatment (RHS line)with copper.

B = background nutrient solution L= 0.25mg/l Cu H=0.50mg/l Cu

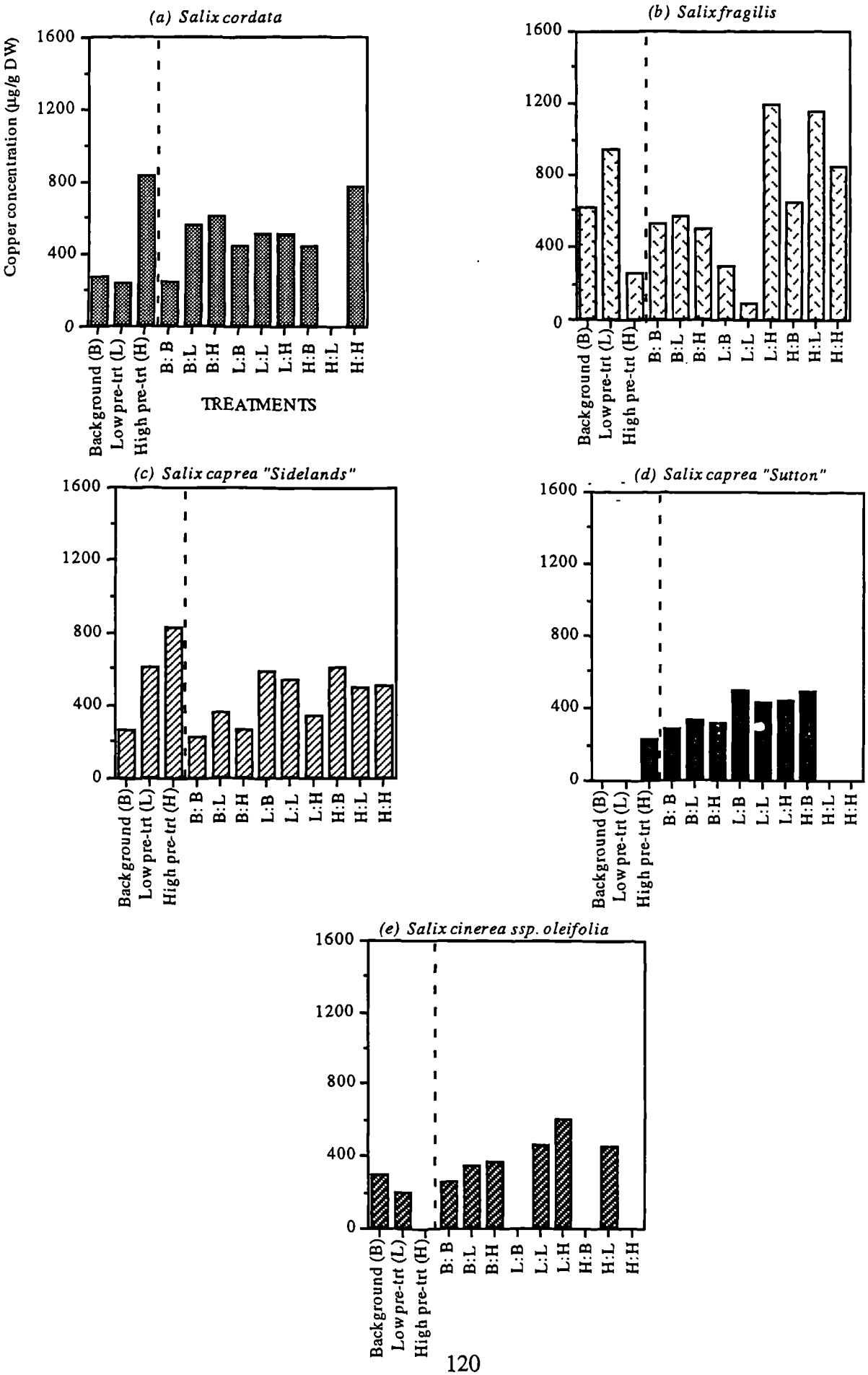


Figure 5.18 (a)-(e) Concentration of copper (µg/g DW) in new stem tissue of five clones and after a 28-day pre-treatment (LHS dotted line) and a further 35 day treatment (RHS line) with copper

B= background nutrient solution L= 0.25 mg/l Cu H= 0.50 mg/l Cu

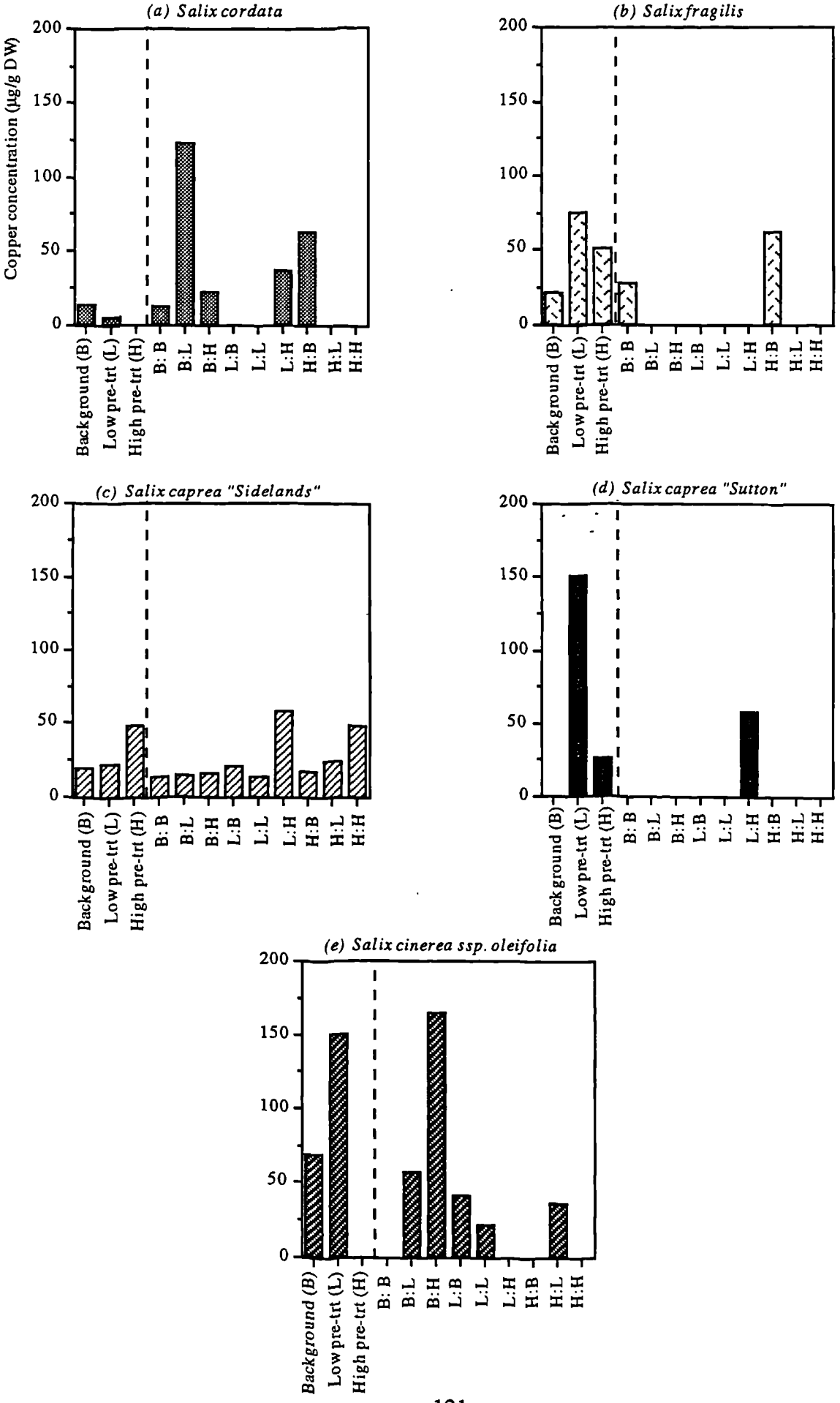
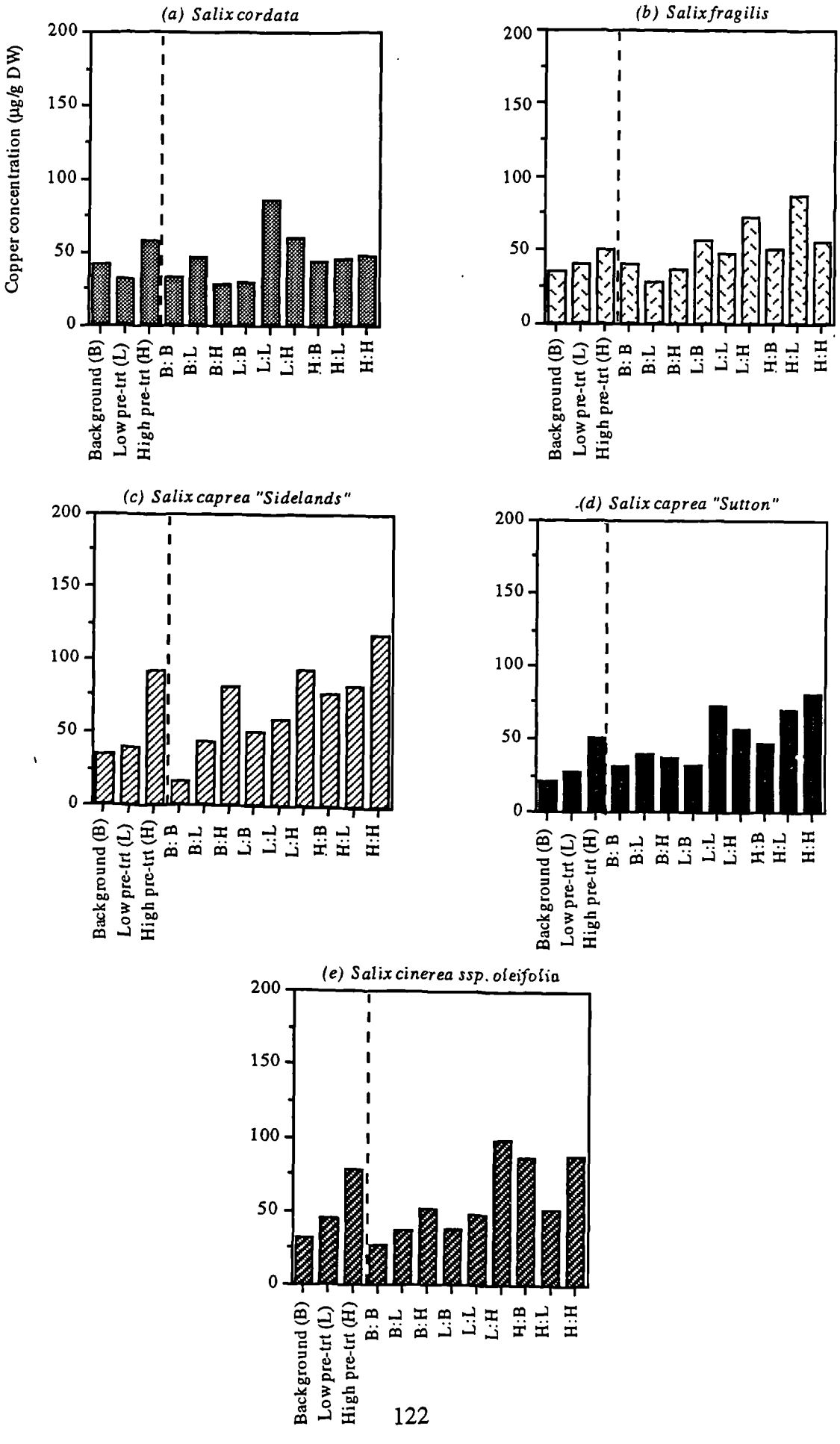


Figure 5.19 (a)-(e) Concentration of copper (µg/g DW) in woody tissue of five clones and after a 28-day pre-treatment (LHS dotted line) and a further 35 day treatment (RHS line) with copper.
B = background nutrient solution L = 0.25 mg/l Cu H = 0.50 mg/l Cu



5.1.4. Discussion

The main conclusions of this experiment is that short-term pre-treatment of willow cuttings with copper at the levels used in this study did not induce a convincingly greater tolerance to copper over the time period considered here; the treatment x pre-treatment interaction for root length (F_4= 1.18 P=0.317) and root number (F_4= 1.50 P=0.202) were not significant at the 95% level. Some increases in tolerance were observed, although these increases are relatively small in most cases and are largely the result of increased root production influencing the *TI* value. Nonetheless there is sufficient evidence in Figs. 5.11-5.15 to suggest that more subtle pre-treatment may be effective in inducing subsequent resistance. The treatment of cuttings with copper in advance of a larger dose was successful for *S. cordata* (Fig..5.11) with an increase in *TI* of 114.5%, whereas all other pre-treatment effects have increased *TI*s of no more than approximately 20%. However, in many cases willow clones pre-treated with background nutrient solution often showed a greater ability to withstand subsequent copper treatment; establishment of an extensive healthy root system proved to be more beneficial to continued growth and survival than pre-treatment. Despite this continuous treatment with a low concentration of copper was effective for *S. fragilis* and *S. caprea* 'Sidelands' (Figs. 5.12 and 5.13). This may indicate that certain willow species respond favourably to longer-term presence of a low, sub-lethal concentration of pollutant which does not cause serious phytotoxic effects.

Copper concentration data indicated that there was no clear relationship between tolerance and uptake of copper from the nutrient solution. The greater uptake of copper in *S. fragilis* in all cuttings pre-treated with a high concentration of copper may be a reflection of the greater new stem and foliage production typical of this clone. *S. cordata* showed a high rate of root production in response to pre-treatment with 0.25 mg l^{-1} Cu and subsequent treatment with 0.50 mg l^{-1} Cu, (Fig. 5.6b). This corresponded to a low concentration of copper within the root system compared with

cuttings which had been grown for 28 days at 0.50 mg l^{-1} copper (Fig. 5.17a). Pre-treated *S. caprea* 'Sidelands' had higher root production rates when pre-treated with the highest concentration of copper than the other willow species tested (Fig. 5.8b). Copper concentration within the roots of this species are highest in *Salix caprea* 'Sidelands' cuttings following the 28-day pre-treatment phase. The lowest uptake of copper into root tissues for this species were those that had been pre-treated with background solution (Fig 5.18) and this also occurred in *S. fragilis* (Fig. 5.16.).

These results strongly imply that willows which are not as susceptible to copper toxicity take up less copper into their tissues. *S. fragilis* and *S. caprea* 'Sidelands' grew more vigorously compared to the other species and accumulated less copper within the root system when pre-treated with background solution. Their growth exceeded that of clones pre-treated with copper, as well as those which were continually exposed to copper throughout the test. This further supports the suggestion that establishment of healthy cuttings may be important for surviving subsequent metal stress. This technique of resistance induction may have been less effective than was hoped because the pre-treatment concentrations of copper used elicited phytotoxic responses. The efficacy of pre-treatments may depend on stimulating a defence response; e.g. the production of metal-chelating compounds (Robinson & Jackson 1986; Tomsett & Thurman 1988) or restriction of the permeability of the cell membranes at specific sub-toxic concentrations (Wainwright & Woolhouse 1977; Strange & MacNair 1991) whereas the concentrations of pre-treatments used in this experiment may have caused too much shock and prevented any defence mechanisms being employed. Toxicity-induced damage and breakdown of metabolic function may have prevented a defence response; or the pre-treated clones may simply have been recovering from toxicity during the subsequent treatment period. Choosing the appropriate pre-treatment concentration may therefore involve identifying the concentration at which the first increase in enzymes involved in the stress response appears (Van Assche *et al* 1988).

These results suggest that a gradual exposure of willow cuttings to metals over a longer period of time may be more successful. The findings of statistical analysis on this data also suggest that the variability of growth for these species prevents accurate comparisons to be made between different treatments, and that rather than adopting the parallel experimental design used previously, a serial design may be more appropriate. Serial tests, where the same population is exposed to varying metal concentrations, may be a means of testing such variable responses more accurately.

5.2. Long-term acclimation to, and uptake of cumulative concentrations of Cu, Cd and Zn supplied singly and in combination.

Background

Previous experiments have demonstrated that resistance of willows to heavy metals, especially copper, varies both within and between species. Furthermore, variation in survivorship between clones and adventitious root growth within clones has complicated the interpretation of experimental data. The following tests were designed with these specific problems in mind. Previous attempts made to rapidly induce greater resistance in Section 5.1 were of limited success and resulted in significant growth inhibition and phytotoxicity. Dickinson *et al* (1991a, 1992) proposed that resistance induction by gradual acclimation was responsible for the continued growth and survival of long-lived woody species subjected to aerially deposited heavy metals from refineries. If resistance induction in trees can be achieved by long term treatment with low doses of heavy metals, then this may form a valuable part of phytoremediation procedure prior to shrub planting on sites superficially contaminated by heavy metals .

Occurrence of single heavy metal elements in a contaminated soil is, however, rare (Smith & Bradshaw 1972; Alloway 1995); and although testing the response of trees to single metals may give an indication of innate resistance in the absence of other environmental stresses, it will not give a true indication of how they respond to a mixture of heavy metals in a polluted soil. Heavy metals frequently occur with other 'guest' elements; especially those originating from mining and smelting sources. Alloway (1995) clearly documented interactions between heavy metals and the major elements within and adjacent to plant root surfaces (Kabata Pendias & Pendias 1992). Antagonistic effects *within plants* can result from combination of Cu + Zn and Cd + Zn. In addition antagonistic and synergistic interactions can occur when Cu + Cd is present adjacent to plant roots. These interactions can also affect uptake; elevated concentrations of copper, nickel, selenium, manganese and phosphorus have been

reported to reduce cadmium uptake (Page *et al* 1981). Although the interaction between cadmium and zinc in particular is less clear, there is evidence to suggest that zinc may have an antagonistic effect on cadmium uptake when this metal is present in the soil at low concentrations (Page *et al* 1981).

In some cases the presence of more than one metal has provided a selection pressure for multiple metal tolerance (Cox & Hutchinson 1980). Recently *in vitro* multiple metal tolerance traits have been induced in *Acer pseudoplatanus* L. (sycamore) (Watmough & Dickinson 1995c), and the following experiment is the first known example of attempted metal tolerance induction in woody cuttings *in vivo*.

This experiment tested the response of four clones to copper, cadmium and zinc supplied singly and in two-metal combinations in two separate hydroponic culture studies. The clones were chosen using information from previous studies showing good growth, high survivorship and low susceptibility to metals. A clone previously untested in this work, *S. burjatica* 'Aquatica Gigantea' Nazarov., was also included in these studies after positive reports in Pohjonen (1991) and Stott (1992) concerning biomass potential. Information about this clone is included in Chapter 2 (Table 2.1). Total concentration of copper, cadmium and zinc in the different plant tissues were measured at the end of the experiment. This enabled an estimation of the total metal accumulation per cutting to be calculated using yield data, helping to establish which clone may have the greatest potential for ameliorating soil conditions when used as part of a bioremediation program.

5.2.1. Aims

- To investigate whether the natural level of susceptibility of willows to copper, cadmium and zinc supplied singly and in combination, can be reduced (i.e. metal resistance increased) by gradual long-term cumulative exposure to these metals.

- To measure the uptake and accumulation of Cu, Cd and Zn in leaves, stem, wood and roots of willow cuttings exposed to single and mixed treatments of metals.
- To measure the concentration and estimate the total content of heavy metals than can be accumulated within cuttings, to indicate what percentage of this total could be removed if coppiced.

5.2.2. Materials and Methods

Four *Salix* clones were used in the present study (Table 5.5).

Table 5.5. Willow clones used in the cumulative induction experiment

Species /or Hybrid (Source)	Accession No
Salix caprea L. Higher Green Dicks (♀) (Stott)	3287
S. x *calodendron* Wimm.[a] (♂)	3311
S. burjatica Nazarov. Aquatica Gigantea Pavainen E7899 (?) (Pohjonen)	3349
S. viminalis L. Ivy bridge (♀) (Rogers ex LARS)	3369

[a] *S.* x *calodendron* = *caprea* x *cinerea* x *viminalis*

Experimental design

Willow cuttings were sampled from the National Willow Collection in October 1994 and prepared as described in Section 3.1.2. Cuttings were placed into test solution before any substantial root growth occurred (i.e. <1 mm). The study was divided into two separate tests; the first used an unamended 25% strength Hoagland's solution, and solutions amended with copper, cadmium and zinc in single treatments; the second test used unamended solution and three treatment solutions consisting of two-metal combinations. Both tests were set up in identical conditions in a temperature controlled glasshouse (19°C, with a daily fluctuation no greater than 5°C) and were monitored at the same time. The treatments were as follows:

Test One: Single metal treatments

1. control (25% Hoagland's solution only)
2. copper (supplied as $CuSO_4.5H_2O$),
3. cadmium (supplied as $8CdSO_4.2^1/_2H_2O$)

4. zinc (supplied as $ZnSO_4.7H_2O$)

Test Two: Mixed metal treatments

1. control (25% Hoagland's solution only)
2. copper + cadmium (supplied as $CuSO_4.5H_2O$ and $8CdSO_4.2^1/_2H_2O$)
3. copper + zinc (supplied as $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$)
4. cadmium + zinc (supplied as $8CdSO_4.2^1/_2H_2O$ and $ZnSO_4.7H_2O$)

Fifty four replicates of each of the four willow clones were randomised in six groups of nine within each treatment tray, with one tray per treatment. Treatments followed a schedule (Table 5.6) whereby the cuttings were initially given a low-concentration metal treatment for the first 28 days of establishment and growth, after which the concentrations of metals in the nutrient solution were increased by 100% and then increased further by approximately 50% every 14 days. The exact metal concentration increments (%) are shown in Table 5.6. with concentrations increased to the nearest round number. The schedule was based on previous knowledge of the highest no observable effect concentrations. Rather than being pre-determined, the progression of the test was dictated by the appearance of phytotoxic effects, such as chlorosis, necrosis and severe discolouration of the root systems. When phytotoxicity was observed, all solutions were replaced with background nutrient *solution on the date* when the next treatment increase would have been due. Length of longest root, number of roots of each cutting and the percentage of the test population successfully rooted (% survivorship) were monitored every 14 days, although no measurements were carried out during the rest period. Tolerance indices were calculated from these data using mean length of longest root and mean number of roots per cutting rather than a tolerance rate as in previous tests (in order to detect changes in tolerance responses). The index used was calculated at each sampling date as:

$$\% TI = \frac{I_L + I_N}{2} \times 100$$

where I_L is the mean length of longest root in metal-amended solution divided by that of control solution, and I_N is the mean number of roots per cutting in metal-amended solution divided by that of control solution.

Cutting harvest and analysis of plant material

Following the completion of the treatment schedule, the roots of the cuttings were washed for 2 hours in glass-distilled, deionised water by lifting the support tray and spraying the root systems continuously. After washing the roots and woody tissue of all cuttings was maintained in deionised water for 10 days to remove residual nutrient solution. Cuttings were separated into leaf (plus petiole), stem (new woody material), root and the woody tissue which constituted the original cutting, therefore removing all newly produced material from the original cutting. Non-viable cuttings were not included in tissue-metal analysis. Material was dried in an air circulating oven at 80°C for 48 hours and dry weight measurements were taken. All tissue material for replicate clones was combined and the number of viable cuttings noted, so that an estimate of dry weight production per cutting could be made. The material was then prepared and analysed for copper, cadmium and zinc as described in Section 3.6. Estimates of metal accumulation were calculated (in μg) from yield and metal analysis data, giving approximate figures for metal content in individual plant compartments and total uptake, expressed as μg of metal per 18 cm cutting over a 128 day period. The proportion of metal accumulated by clones which could theoretically be removed in a shoot harvest was calculated using total uptake figures and the concentration of metal translocated into the leaves and new stem material.

Table 5.6. Treatment schedule

Timing (days)	Tray number and metal concentration (mg l^{-1})	
	TEST 1	TEST 2
0-28 d initial low concentration pre-treatment during cutting establishment	(1) Control (2) Cu (0.15) (3) Cd (0.15) (4) Zn (2.5)	(1) Control (2) Cu (0.075) + Cd (0.075) (3) Cu (0.075) + Zn (1.25) (4) Cd (0.075) + Zn (1.25)
29-42 d 100% increase	(1) Control (2)Cu (0.3) (3) Cd (0.3) (4) Zn (5.0)	(1) Control (2) Cu (0.15) + Cd (0.15) (3) Cu (0.15) + Zn (2.5) (4) Cd (0.15) + Zn (2.5)
43-56 d 50% increase	(1) Control (2)Cu (0.45) (3) Cd (0.45) (4) Zn (7.5)	(1) Control (2) Cu (0.225) + Cd (0.255) (3) Cu (0.225) + Zn (3.75) (4) Cd (0.225) + Zn (3.75)
57-85 d "rest period"	*BACKGROUND NUTRIENT SOLUTION* Phytotoxicity (Chlorosis) observed - further treatment suspended.	
86-99 d 55.5% increase based on levels used on 43-56d treatment	(1) Control (2) Cu (0.7) (3) Cd (0.7) (4) Zn (11.5)	(1) Control (2) Cu (0.35) + Cd (0.35) (3) Cu (0.35) + Zn (5.6) (4) Cd (0.35) + Zn (5.6)
100-114 d 42.8% increase	(1) Control (2)Cu (1.0) (3)Cd (1.0) (4) Zn (17.2)	(1) Control (2) Cu (0.5) + Cd (0.5) (3) Cu (0.5) + Zn (8.4) (4) Cd (0.5) + Zn (8.4)
115-128 d 50% increase	(1) Control (2) Cu (1.5) (3) Cd (1.5) (4) Zn (25.8)	(1) Control (2) Cu (0.75) + Cd (0.75) (3) Cu (0.75) + Zn (12.6) (4) Cd (0.75) + Zn (12.6)

5.2.3. Results

Statistical analysis of data

Zero-adjusted data were analysed using the General Linearised Model; the results are shown for test one (Table 5.7) and test two (Table 5.8) separately.

Table 5.7. F values for GLM analysis of root length and number data for test one.

Source(Degrees of Freedom)	Root length	Root number
Willow species$_3$	46.98***	144.21***
Metal treatment$_3$	221.2***	22.81***
Sampling date$_7$	45.40***	66.04***
Species x Metal treatment$_9$	18.17***	15.52***
Species x Sampling date$_{21}$	1.40ns	2.31**
Metal treatment x Sampling date$_{21}$	5.37***	3.59***

*** denotes $P< 0.001$; ** denotes that $P< 0.05$; ns denotes $P>0.05$ (not significant)

Table 5.8. F values from GLM analysis of longest root and number data for test two.

Source(Degrees of Freedom)	Root length	Root number
Willow species$_3$	18.87***	86.29***
Metal treatment$_3$	38.47***	1.56ns
Sampling date$_7$	47.00***	49.47***
Species x Metal treatment$_9$	13.31***	17.68***
Species x Sampling date$_{21}$	1.33ns	1.98**
Metal treatment x Sampling date$_{21}$	2.25**	1.88**

*** denotes $p < 0.001$; ** denotes that $p < 0.05$; ns denotes $p>0.05$ (not significant)

5.2.3.1. Root elongation and production trends

Figures 5.21-5.24 show length of longest root and root number of four willow clones in response to single metal treatments throughout the experiment (test one), and Figures 5.25-5.28 show their response to combination metal treatments (test two).

Test one.

In most cases root elongation increased throughout the test period, despite steadily increasing metal concentration. In *S. burjatica* elongation was virtually halted following the first increase in copper concentration (Fig 5.23a). Root elongation of *S. burjatica* and *S. viminalis* were significantly enhanced when treated with cadmium (Figs. 5.23a and 5.24a). Copper caused the greatest root inhibition in all of the clones tested.

In all cases the number of roots produced increased throughout the test period, despite increasing metal concentrations. Greater increases in root production were observed after approximately 99 days of treatment. *S.* x *calodendron, S. burjatica* and *S. viminalis* in particular showed a greater root production towards the end of the test period in response to cadmium (Figs. 5.23b and 5.24b).

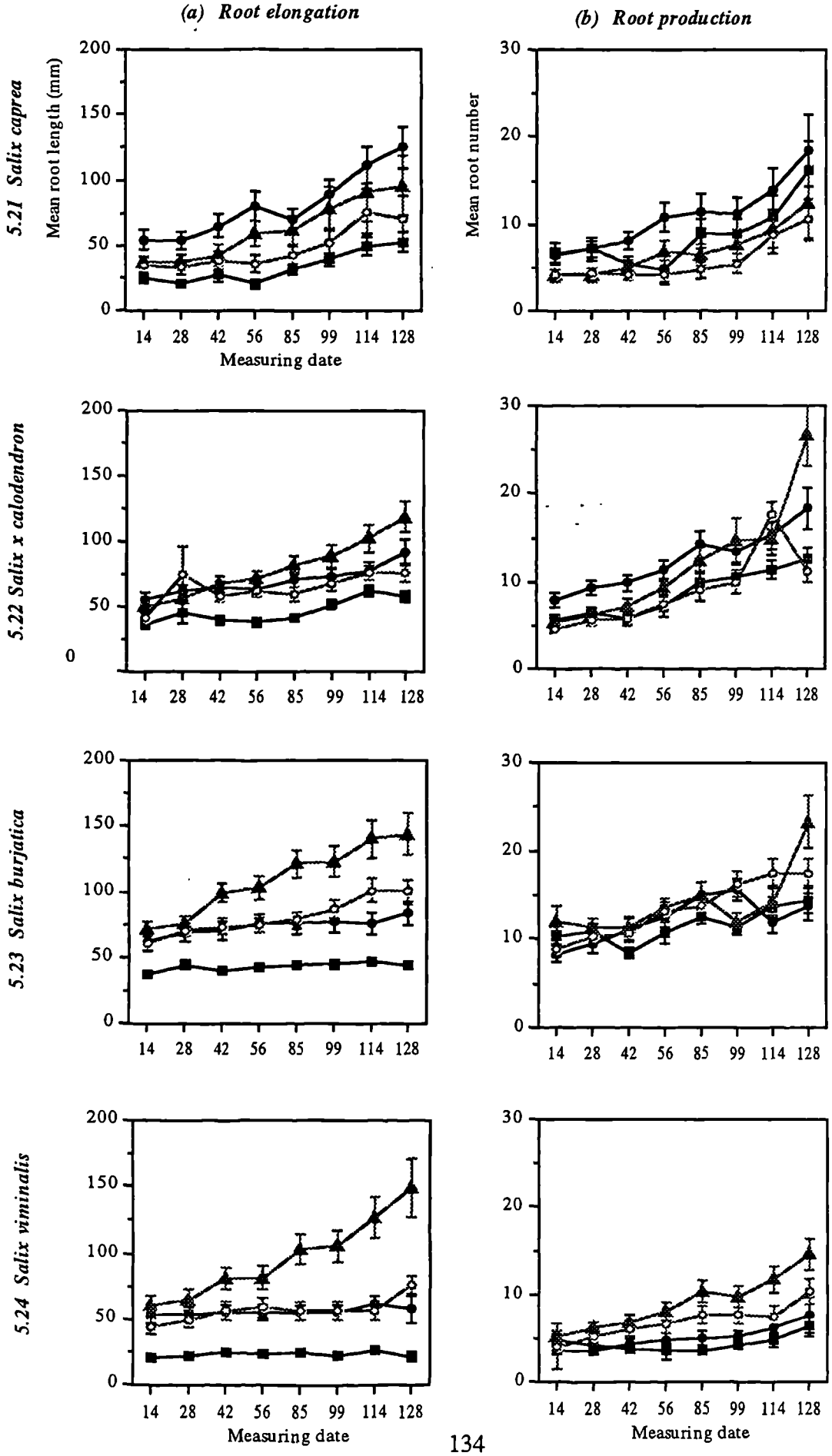
Test two

Root elongation of *S.* x *calodendron, S. burjatica* and *S. viminalis* grown in unamended nutrient solution tended to be greater than any of the treated plants but this was *vice versa* in *S. caprea.* Root elongation of *S. caprea* did not differ significantly between treatments until 42 days into the test (Figure 5.25a). Treatment with Cu+ Cd inhibited root elongation less in *S. caprea, S. burjatica* and *S. viminalis* cuttings exposed to Cu + Cd grew better than all other treated cuttings (Figs. 5.27a and 5.28a), but the Cu+ Zn treatment caused severe inhibition of root elongation in these species. All other clones showed a steady increase in growth in response to metal treatments compared to the controls despite increasing concentration.

Elevated metals caused increased production of new adventitious roots in *S. caprea* , but decreased production in all of the other clones. The overlap of the error bars on Figure 5.25b indicated that levels of growth inhibition of all treated *S. caprea* cuttings were not significantly different. Root production data for all plants were relatively closely grouped and greatest in *S.* x *calodendron* (Fig. 5.26b). Root production of *S. viminalis* was completely inhibited by treatment with copper + zinc (Fig. 5.27b).

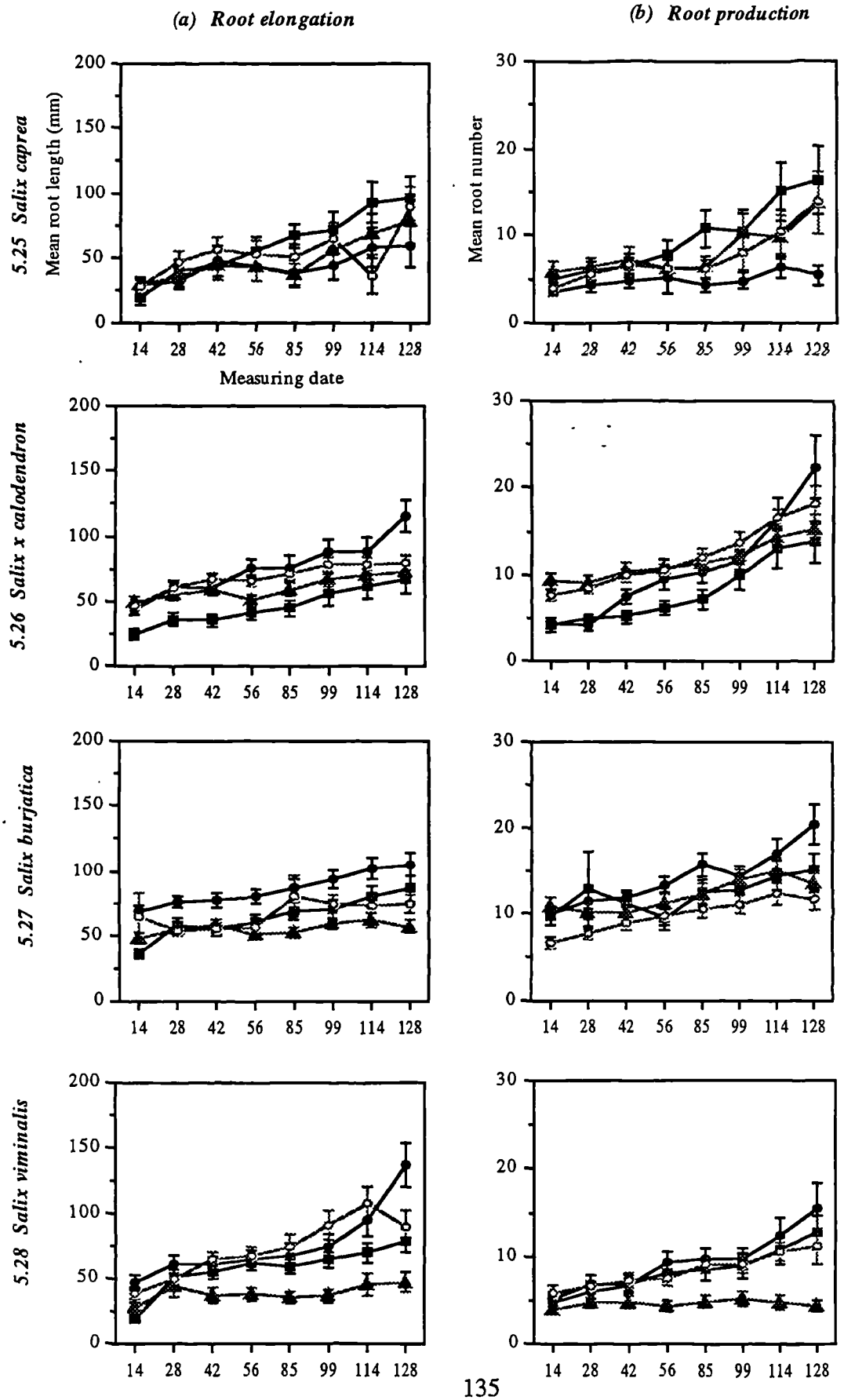
Figures 5.21-5.24. Root length (a) and number of roots (b) of clones exposed to copper, cadmium or zinc for 128 days in test one. (Means and standard errors of zero-adjusted data where n=54).

Treatments: Control Copper Cadmium Zinc



Figures 5.25-5.28. Root length (a) and number of roots (b) of clones exposed to combinations of copper, cadmium and zinc for 128 days in test two. (Means and standard errors of zero adjusted data where n=54).

Treatments: Control Cu+Cd Cu+Zn Cd+Zn



Cutting survival

The survivorship of willow cuttings in each treatment was recorded every 14 days, expressed as the percentage of the total number of willows in each treatment which had rooted and was growing actively (Tables 5.9-5.10).

Table 5.9. Mean survivorship (± standard deviation) of cuttings exposed to cumulative metal treatment over 8 successive measuring dates (128d) in test one.

Willow species	Control	Copper	Cadmium	Zinc
S. caprea	54.39 (±6.94)	37.93 (±6.98)	49.21 (±10.61)	45.08 (±9.03)
S. x *calodendron*	83.73 (±1.53)	84.53 (±2.20)	73.08 (±1.85)	73.57 (±3.56)
S. burjatica	74.73 (±3.45)	91.96 (±2.35)	74.96 (±1.31)	80.00 (±2.88)
S. viminalis	64.53 (±6.16)	49.76 (±5.45)	57.13 (±6.01)	71.95 (±3.64)

Table 5.10. Mean survivorship (± standard deviation) of cuttings exposed to cumulative mixed metal treatment over 8 successive measuring dates (128d) in test two:

Willow species	Control	Cu+Cd	Cu+Zn	Cd+Zn
S. caprea	48.35 (±8.32)	32.21 (±4.89)	32.80 (±4.20)	43.92 (±6.41)
S. x *calodendron*	53.44 (±4.95)	40.90 (±4.38)	74.70 (±3.45)	89.05 (±1.11)
S. burjatica	86.45 (±0.85)	65.23 (±7.51)	79.11 (±3.16)	87.90 (±1.60)
S. viminalis	61.77 (±6.19)	64.77 (±7.52)	53.90 (±3.65)	64.30 (±3.40)

Cutting viability of *S. burjatica* was generally higher in both tests in comparison with other willow clones. *S.* x *calodendron* showed a high survivorship in test one, and in response to Cu + Zn and Cd + Zn in study two. *S. caprea* had the lowest survivorship in both studies, a finding consistent with previous hydroponic experiments. When standard deviation of the survivorship is expressed as a percentage of the mean, the clone with the most variable rooting response can be clearly picked out. Variation in survivorship was consistently greater in *S. caprea*, with standard deviation approximately 12-21% of the mean whereas other clones showed a much lower variation no greater than 10% of the mean. Table 5.11 shows the results of statistical analyses of survivorship data for test one and two.

Table 5.11 Result of GLM analysis of arc-sine transformed % cutting survival data for tests one and two. *** denotes $P<0.0001$; ** denotes $P<0.05$; ns denotes $P>0.05$.

Source Degrees of Freedom	Test One	Test Two
$Species_3$	113.31***	435.27***
$Metal_3$	2.06ns	123.41***
$Date_7$	4.77***	6.17***
Species x $Metal_9$	11.20***	49.73***
Species x $Date_{21}$	0.76ns	1.46ns
Date x $Metal_{21}$	0.80ns	3.08***

Statistical analysis of cutting viability throughout the test period revealed that when supplied singly, heavy metals had no significant effect on viability (P=0.114), whereas when willow cuttings are exposed to combinations of heavy metals viability is significantly affected. Variation in rooting appears to be more strongly associated with species characteristics than metals. For instance, in test one *S. burjatica* survivorship was slightly higher in treated cuttings than in the control, but was not very variable.

5.2.3.2. Tolerance Indices

Tolerance indices were estimated using the equation described above, and are shown for study one in Figures 5.29-5.32, and for study two in Figures 5.33-5.36.

Test one.

Resistance to cadmium and zinc treatment was effectively increased by cumulative treatment in all of the willow clones tested; even a level line indicates increased resistance because metal levels increased throughout the 128 days. However, resistance in *S. viminalis* did show some evidence of decreased resistance with increasing copper concentration, and in general resistance to copper in all clones was lower at the end of the test, compared to after at 14d. *S.* x *calodendron* (Fig. 5.30) and *S. viminalis* (Fig. 5.32) showed a progressively increasing *TI* response to cadmium, whereas *S. caprea* (Fig. 5.29) and *S. burjatica* (Fig. 5.31) remained stable. *TI* values for zinc remained low but stable for *S. caprea* and *S.* x *calodendron*, but was more variable in the other species.

Figure 5.29.-5.32. Tolerance Indices of four clones exposed to copper, cadmium and zinc gradually over 128 days in Test one.

Treatments: —■— *Copper* —▲— *Cadmium* —○— *Zinc*

Fig. 5.29 Salix caprea

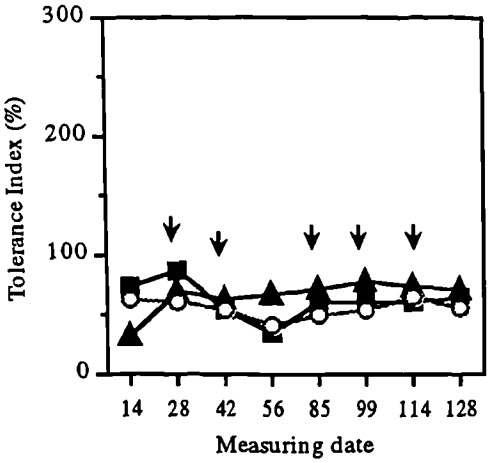


Fig. 5.30. Salix x calodendron

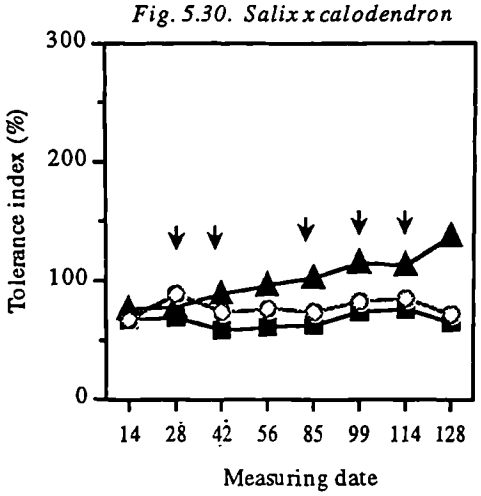


Fig. 5.31 Salix burjatica

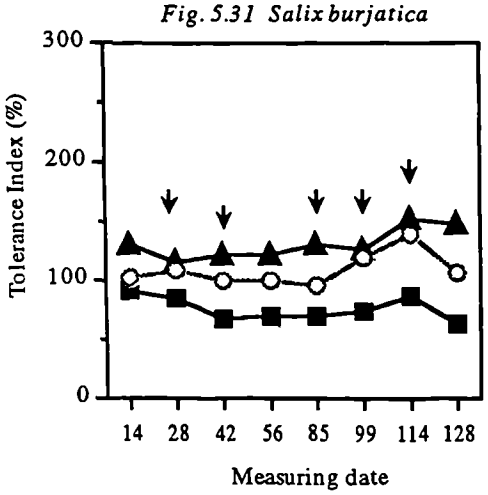
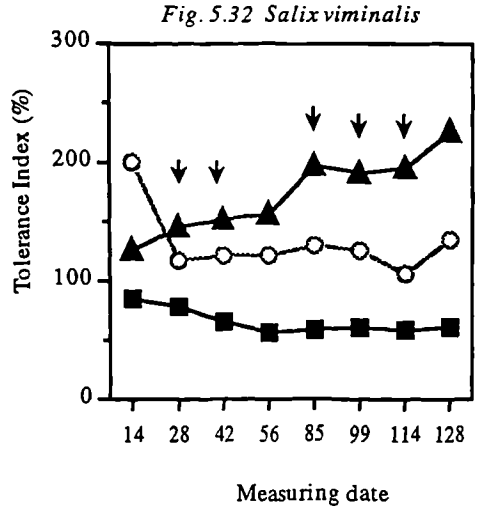


Fig. 5.32 Salix viminalis



Footnote: Tolerance index is calculated at different concentration at different sampling times. Metal concentrations increased at dates 28, 42, 85, 99 and 114 indicated by ↓ .

Test two

Tolerance to metals supplied in dual combination treatments remained more-or-less stable for three of the clones tested. In this test, *S. caprea* showed much higher Tolerance Indices to all metal treatments (Fig. 5.33) although this may be partly related to the variable growth of control plants there was still sufficient evidence to suggest elevated resistance. *S. caprea* cuttings treated with Cu + Cd showed an increase in *TI* of approximately 150% between initial and final measurements; a similar increase of 85% occurred for cuttings treated with Cu + Zn, and 100% for cuttings treated with cadmium + zinc. Resistance to mixtures of metals in all other clones was either stable (e.g. *S. burjatica* (Fig. 5.35) remained stable between 50-100% for all treatments), or was reduced by increasing metal treatment. However, it should be remembered that the plants were being to subjected to increasing concentrations of metals as the test progressed.

– *Figures 5.33.-5.36. Tolerance indices of selected clones exposed to combinations of copper, cadmium and zinc for 128 days for Test two.*

Treatments: —■— *Copper+Cadmium* —▲— *Copper+Zinc* —○— *Cadmium+Zinc*

Fig. 5.33. Salix caprea

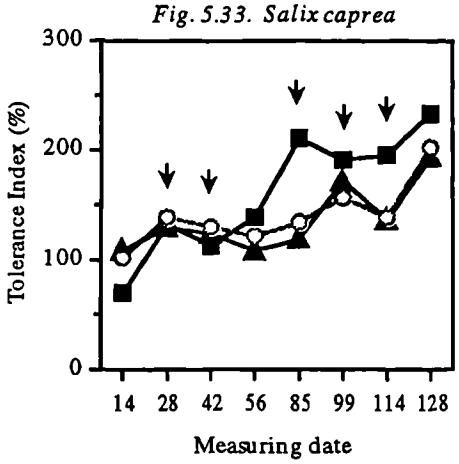


Fig. 5.34. Salix x calodendron

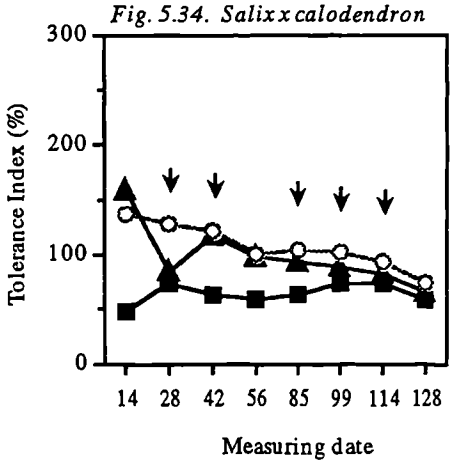


Fig. 5.35. Salix burjatica

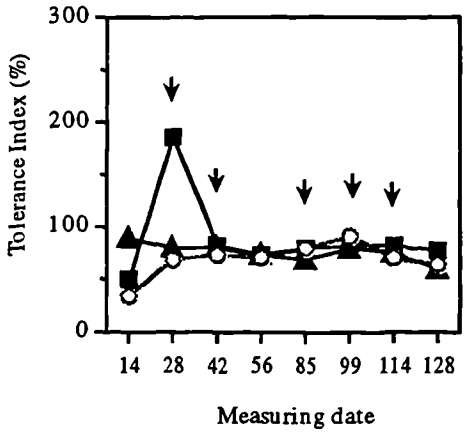
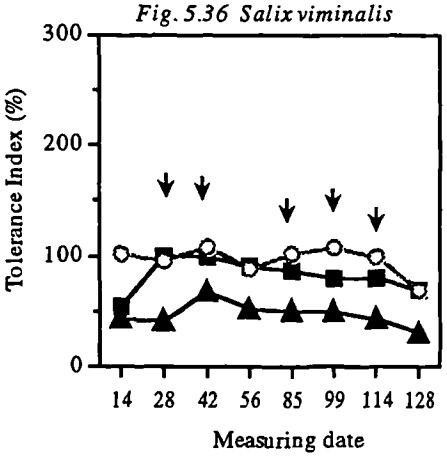


Fig. 5.36 Salix viminalis



Footnote: Tolerance Index is calculated at different concentrations and at different sampling times. Metal concentrations increased at dates 28, 42, 85, 99 and 114 indicatedby ↓

5.2.3.3. *Biomass production*

Test one

Harvest data from test one showed that in general, treatment with heavy metals reduced biomass of all plant tissue compartments, (Table 5.12) although there were some exceptions. When exposed to copper, both *S. caprea* and *S. burjatica* produced amounts of root biomass similar to untreated plants; furthermore leaf and new stem biomass of *S. burjatica* was likewise not reduced by copper. Reductions in root biomass of *S. caprea* in response to all metals tested were less severe than other clones; root biomass of *S. caprea* remained consistent around 0.4g DW per cutting in all treatments except zinc, which reduced root biomass by approximately 60% of controls. This occurred even with a generally lower overall biomass production by this species. Cadmium had a less inhibitory effect; *S.* x *calodendron*, *S. burjatica* and *S. viminalis* all produced more leaf, stem and root biomass than when treated with other metals and often more than the control. In background solutions production of stem material in *S.* x *calodendron* is also comparatively high compared with the established biomass shrub *S. viminalis*; with control plants producing 1.18g of stem material per cutting compared with 0.32g DW in *S. viminalis*.

Table 5.12 Biomass (g DW per cutting) of four clones after 128 days growth in single cumulative metal treatment. Note: 'stem' refers to new lignified shoots arising from the original woody cutting.

Metal	*S. caprea*			*S.* x *calodendron*			*S. burjatica*			*S. viminalis*		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Control	0.44	0.85	1.17	1.07	1.18	1.63	0.09	0.11	0.11	0.08	0.32	0.11
Cu	0.43	0.17	0.08	0.18	0.09	0.14	0.10	0.13	0.09	0.02	0.02	0.06
Cd	0.38	0.44	0.45	0.90	0.96	0.80	0.34	0.71	0.31	0.14	0.18	0.19
Zn	0.18	0.12	0.52	0.13	0.06	0.03	0.10	0.14	0.05	0.03	0.02	0.03

Test two

Biomass production by *S. caprea* was much lower in test two than in test one, despite being maintained under identical conditions; although once again biomass production by this clones was generally very low. Biomass reductions as a result of mixed metal treatments appear to have been more severe than those due to the addition of single

metals. No stimulatory effect of metals, observed previously for cadmium, were observed in test two.

Table 5.13 Biomass (g DW per cutting) of four willow clones in mixed cumulative metal treatments (study two).

Metal	*S. caprea*			*S.* x *calodendron*			*S. aquatica gigantea*			*S. viminalis*		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
Control	0.01	*	0.005	0.28	0.67	0.84	0.18	0.53	0.77	0.12	0.27	0.36
Cu+Cd	0.24	0.13	0.14	0.13	0.05	0.08	0.14	0.10	0.06	0.07	0.05	0.06
Cu+Zn	0.09	0.04	0.03	0.12	0.07	0.05	0.05	0.05	0.03	0.01	0.02	0.01
Cd+Zn	0.13	0.05	0.10	0.18	0.12	0.12	0.10	0.09	0.08	0.04	0.05	0.03

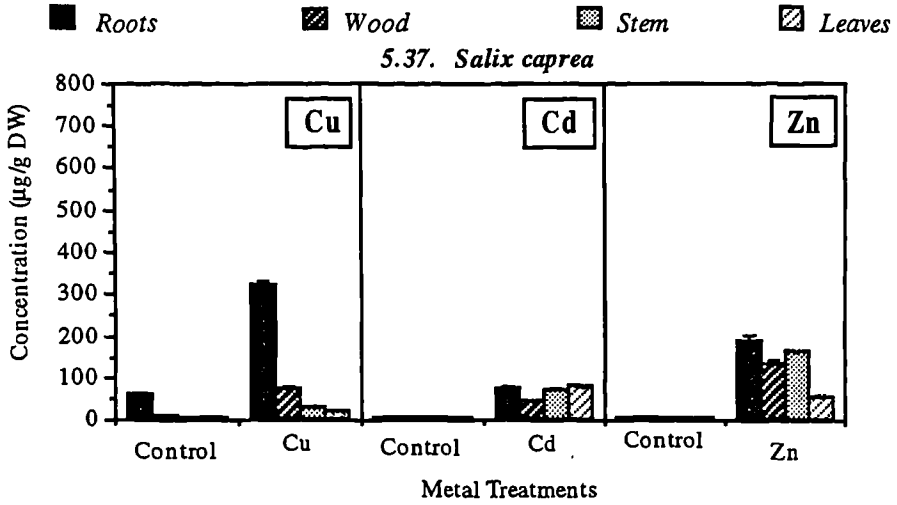
5.2.3.4. Metal analysis of plant material

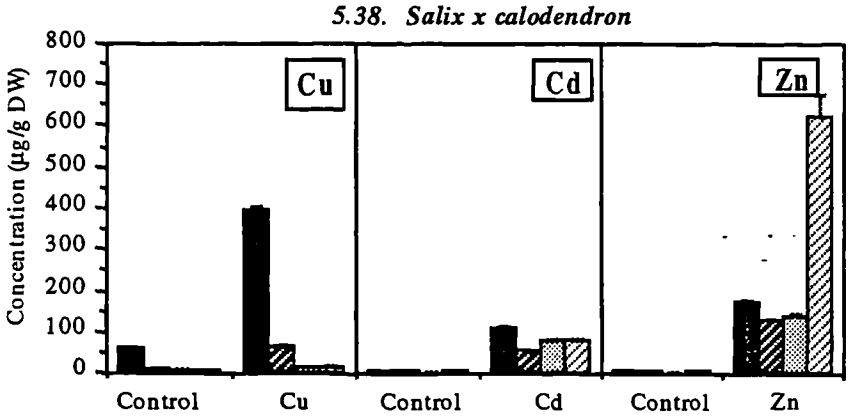
Figures 5.37-5.40 show the total metal content (in $\mu g\ g^{-1}$ DW) of the various plant tissue compartments of willow cuttings from study one, and figures 5.41-5.44 show concentrations of metals in cuttings from study two.

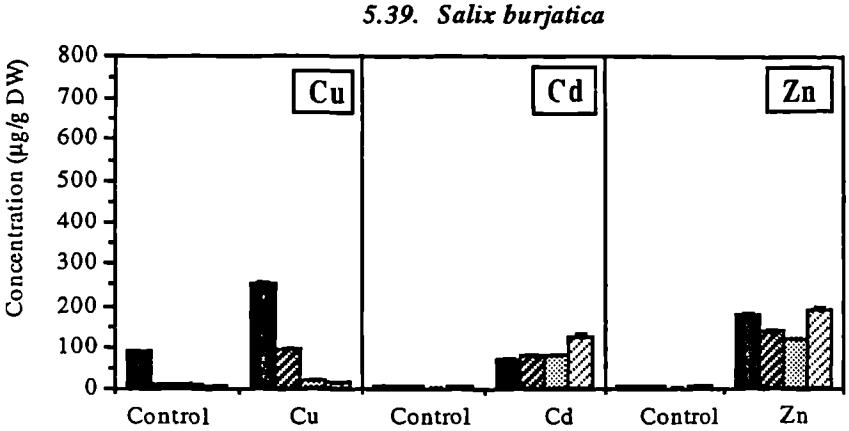
Test One

Uptake of copper was largely confined to the roots; followed by wood and stem tissues with the lowest concentration in leaf tissue. The highest concentration of root-bound copper was found in *S. viminalis* (Fig. 5.40) and *S.* x *calodendron* (Fig. 5.38); *S. burjatica* contained the smallest concentration (Fig. 5.39). Concentration in the stem and leaves of all clones were only slightly higher than in control solution. Statistical analyses of copper uptake (Table 5.14) showed no significant differences between copper uptake between different willow clones ($P = 0.332$), neither were there significant interactions between copper treatment and willow clones ($P = 0.510$) or between uptake into different compartments and willow clones ($P = 0.060$).

Figures 5.37-5.40 Concentration of Cu, Cd, and Zn in tissues of four clones exposed to cumulative single metal treatments for 128 days in Test One. (Means and standard deviation)







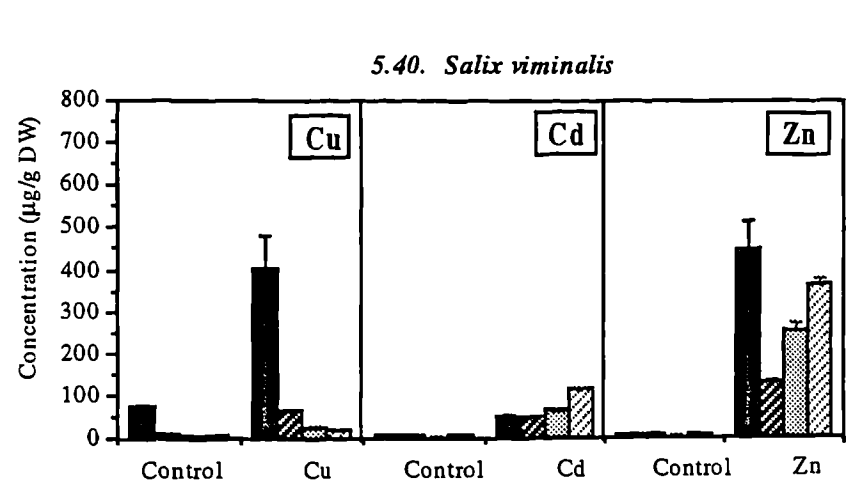


Table 5.14 F values from GLM analysis of metal analysis data.

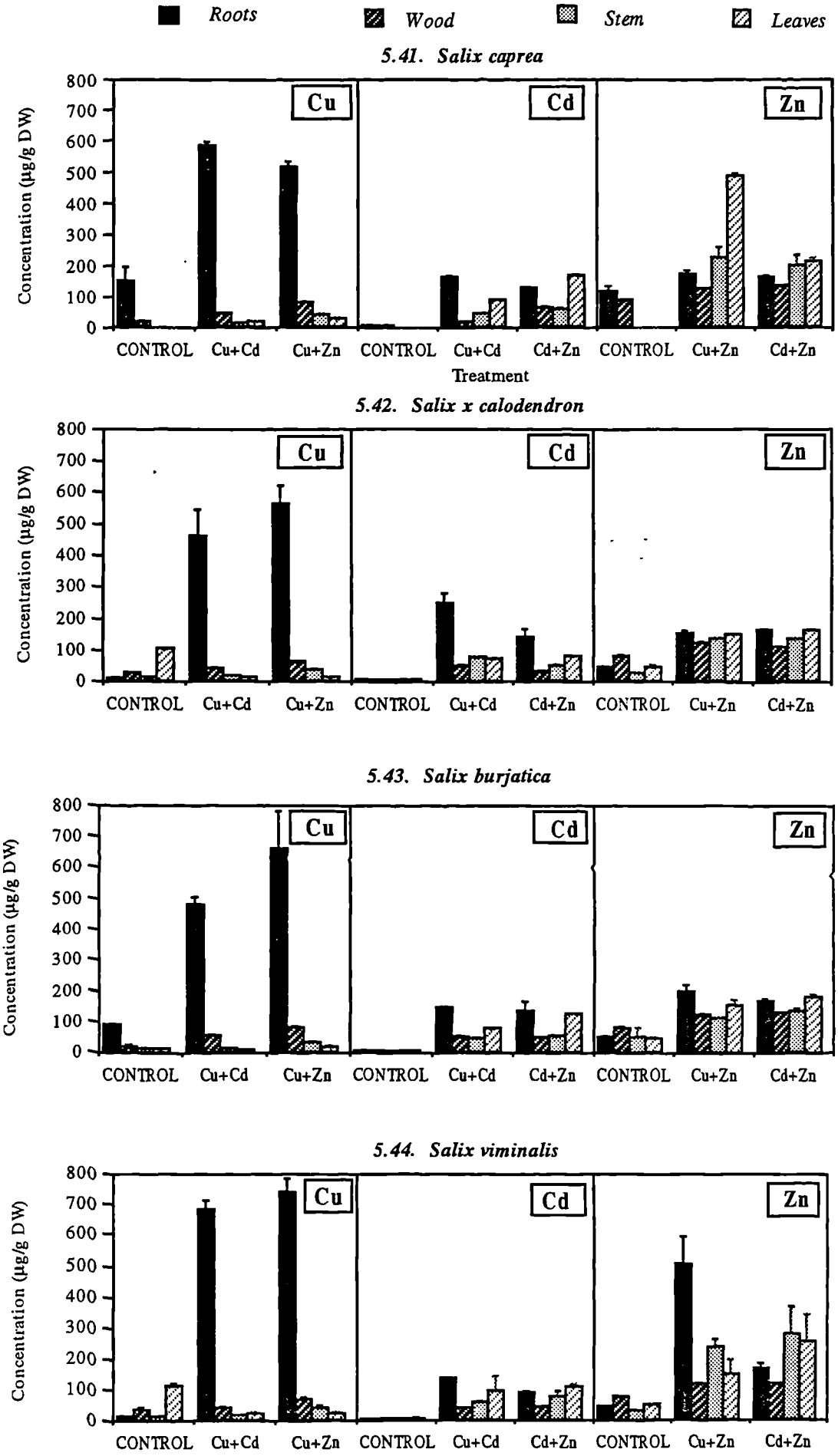
*** denotes $p < 0.001$; ** denotes that $p < 0.05$; ns denotes $p > 0.05$ (not significant)

Source (Degrees of Freedom)	Test Value		
	Copper	Cadmium	Zinc
Metal treatment$_1$	592.17***	1532.02***	552.32***
Tissue compartment$_3$	603.76***	21.21***	15.32***
Willow clones$_3$	1.16ns	6.52**	18.35***
Treatment x tissue compartment$_3$	280.46***	22.28***	33.13***
Treatment x species$_3$	0.78ns	7.24***	23.96***
Tissue compartment x clones$_9$	1.93ns	6.49***	6.07***

There was a more equitable distribution of cadmium between tissues compared to the other metals. *S. burjatica* and *S. viminalis* were found to have a higher proportion of cadmium in leaf tissue, and in these two species uptake into all other compartments occurred to a similar extent (Figs. 5.39 and 5.40). *S. caprea* and *S.* x *calodendron* (Fig. 5.38) both had a greater proportion of cadmium in the roots than in the woody tissue, and distinctly less in the leaves. Cadmium uptake was significantly different between treated and untreated cuttings, clones, tissue compartments and all interactions between these factors were significant (Table 5.14).

S. viminalis took up the greatest overall concentration of zinc into tissue compartments, with over 400μg g^{-1} DW in the roots and 350 μg g^{-1} DW in the leaves (Fig. 5.40). *S.* x *calodendron* was found to have approximately 600 μg g^{-1} DW zinc within leaf tissues, although low concentrations were found in the other compartments (Fig. 5.38). *S. caprea* and *S. burjatica* contained less zinc in all compartments except wood. In common with cadmium, uptake of zinc differed significantly between treatments, clones, compartments and all interactions between these factors (Table 5.14.)

Figure 5.41-5.44. Concentration of Cu, Cd and Zn in tissues of four clones exposed to cumulative dual-combination treatments for 128 days in Test Two. (Means and standard deviation)



Test Two

Cuttings treated with a metal combination that included copper had much higher concentrations of this metal within root tissues than those analysed from test one. The concentration of copper in the roots was generally slightly lower when the copper was accompanied by cadmium although the response of *S. burjatica* was more variable, making comparisons more difficult (Figures 5.41-5.43). There were significant differences between metals, tissue compartments and clones (Table 5.15).

Table 5.15. F values from GLM analysis of metal analysis data.
*** denotes $P< 0.001$; ** denotes that $P< 0.05$; ns denotes $P>0.05$ (not significant)

Source Degrees of Freedom	Test Value		
	Copper	Cadmium	Zinc
Metal treatment$_1$	85.79***	287.88***	42.55***
Tissue compartment$_3$	587.52***	114.50***	9.32***
Willow species$_3$	2.74**	2.92**	13.97***
Treatment x tissue compartment$_3$	82.73***	40.33***	4.42***
Treatment x species$_3$	1.61ns	5.87***	3.97**
Tissue compartment x species$_9$	1.88ns	7.47***	5.62***

Although statistically different, cadmium uptake in leaves and roots was similar in all species, with lower levels in the stem and wood (original cutting). In general uptake of cadmium was greater when in combination with copper than with zinc. Higher concentrations of zinc were taken up in *S. caprea* and *S. viminalis*. In *S. caprea* approximately 500 μg g^{-1} DW of zinc was detected in leaf material of cuttings treated with copper and zinc together. *S. viminalis* also took up a high concentration of zinc in the roots of copper and zinc-treated plants in addition, this species also took up a high concentration of zinc in the stem and leaf tissue in response to this treatment.

5.2.3.5. Metal accumulation in willow clones

Figures 5.45-548 (Test One) and 5.49-5.52 (Test Two) show the total metal content in μg per 18 cm cutting grown in background and metal-amended nutrient solution for

128 days. The shaded portion of the histogram bars represent the content taken up into leaf and stem material, which would theoretically be removed if the willows were harvested. The percentage of total metal uptake which would be removed by coppicing is shown above each column on the following metal accumulation figures. These calculations are extrapolations from solution culture data to the field situation and provide only a tentative indication of the amount and proportion of removable metals.

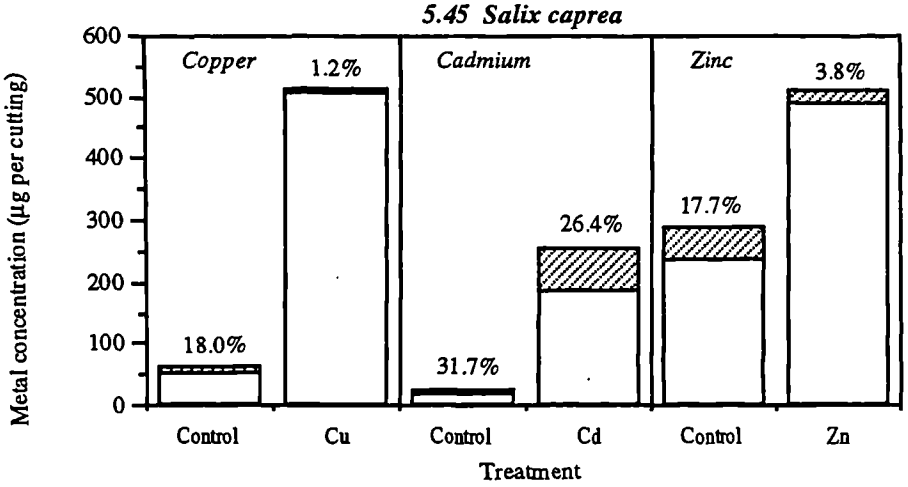
Test One

Accumulation of metals from metal-rich nutrient solution occurred to different extents both between different willow clones and different metal elements. Copper uptake was primarily confined to the wood and root material as mentioned previously, with a typically low removable metal portion found or all clones. Cadmium uptake has been shown to occur in higher concentrations in leaf and stem tissue than copper in almost all species tested (Figs. 5.37-5.40). The willow clones tested in this study responded to higher cadmium concentrations by a corresponding higher accumulation in the stem and leaf tissues. The percentage of total cadmium which was taken up into aerial tissues increased in both *S. burjatica* (Fig. 5.47) and *S. viminalis* (Fig. 5.48); by over 33% and approximately 20% respectively. This accumulation trend was also observed in *S. burjatica* in response to zinc; over 5% increase in zinc accumulation in the aerial parts was observed. *S. caprea* was found to accumulate the highest concentration of copper and zinc, although the concentration of cadmium found within cuttings was similar to other species tested.

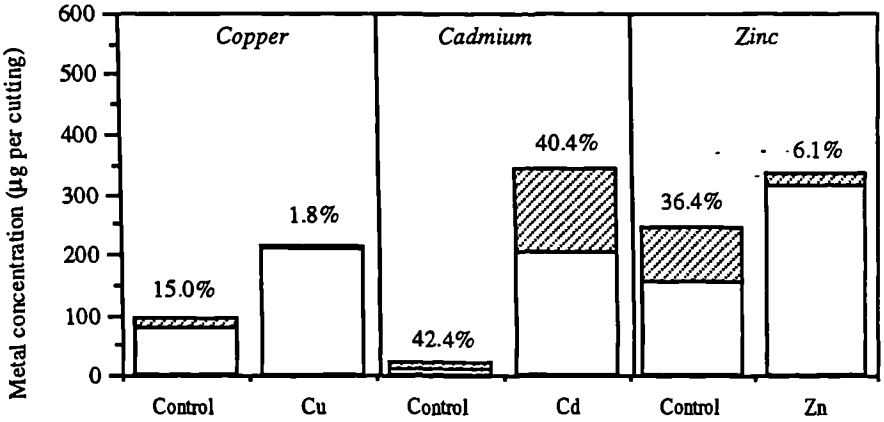
Figure 5.45-5.48. Metal accumulation in willows exposed to cumulative, single treatments for 28 days in Test One. (Calculated from metal concentration and yield data.)

▨ *Proportion removable by aerial harvest of leaves and new stem (excluding wood and roots).*

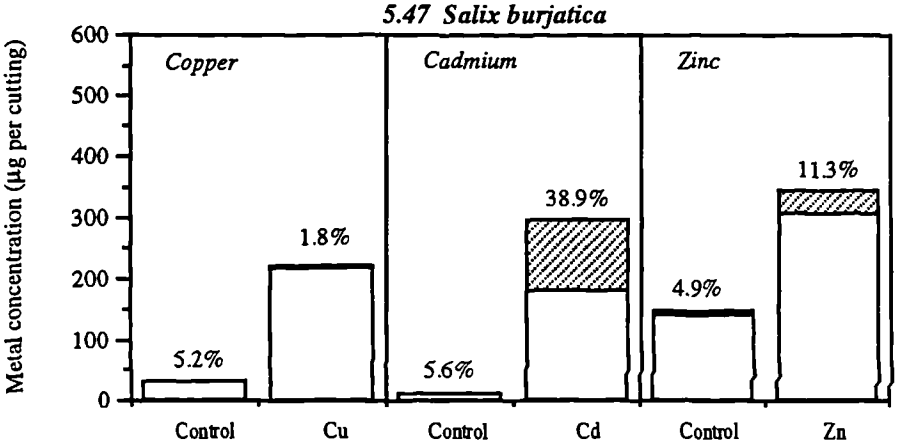
5.45 Salix caprea



5.46 Salix x calodendron



5.47 Salix burjatica



5.48 Salix viminalis

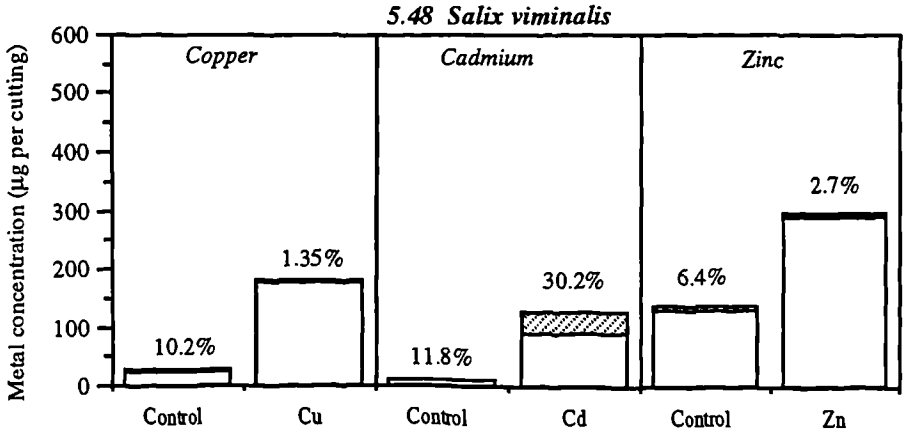
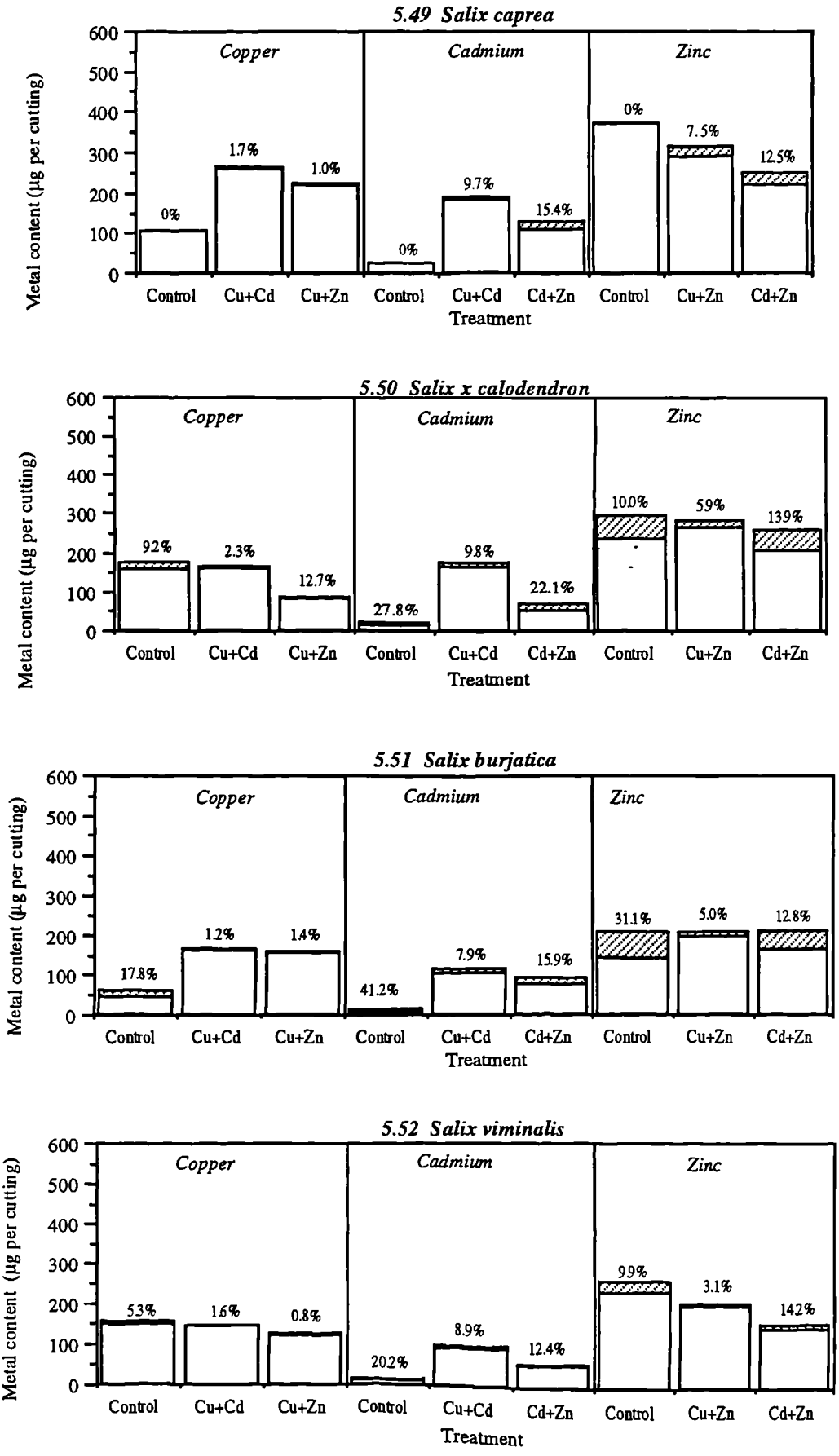


Figure 5.49-5.52. Metal content in clones exposed to cumulative, dual-combination treatments for 128 days in Test Two. (Calculated from metal concentration and yield data)

▨ *Proportion removable by aerial harvest of leaves and new stems (Excluding wood and roots).*



Test Two.

Uptake patterns in willows supplied with combination metal treatments were quite different to those seen in single-metal treated cuttings. *S. caprea* took up higher concentrations of copper in treated cuttings than other clones (Fig. 5.49), with a greater total uptake when Cu was supplied in combination with Cd. This clone also took up a large concentration of zinc; again the greatest uptake of any clone tested. The greatest removable portion of zinc was found in *S.* x *calodendron* (Fig. 5.50) and *S. burjatica* (Fig.5.51), in control and Cu+Zn treated cuttings.

The concentration of removable zinc increased in all species when supplied with another metal, although more so when zinc was accompanied by cadmium than with copper. This also occurred in *S.* x *calodendron* in response to copper, where cuttings accumulated a greater proportion of this metal in removable tissue compartments compared to controls. The percentage of removable zinc in *S.* x *calodendron* was greater in cuttings treated with cumulative concentrations of Cd+Zn than untreated cuttings (Fig. 5.50). In *S. burjatica* uptake of copper and cadmium was generally lower in untreated cuttings although the portion which could be removed by harvesting was higher. This was not the case for zinc however, and the total uptake of zinc in *S. burjatica* remained very similar in both treated and untreated metal treatments which were supplied at double the concentration.

In general the total uptake estimates from test two were greater in controls than treated cuttings; the greatest percentage of removable metal in combination was zinc; specifically in combination with cadmium. *Salix caprea* took up the greatest concentration of zinc and copper; and cadmium uptake was similarly high in *S. caprea* and *S. x calodendron.*

5.2.4. Discussion

The results of this experiment point to an important role of acclimation in metal resistance in woody plants. The scale of Tolerance Index values were much higher in the acclimated cuttings of the present study that in previous experiments where willows were exposed to elevated metal concentrations, many of which remained above the initial 100% level. The levels of cadmium tolerance, shown by the *TI* values (Figs. 5.29-5.32) indicate that willows posses some degree of innate cadmium resistance, and previous experiments in this study have failed to identify a threshold concentration for cadmium toxicity. *TI* values increased in response to increasing cadmium concentrations in all clones tested in this investigation. In study one *S. caprea* showed an increase in resistance to Cd of approximately 50% between initial and final measurements (Fig. 5.29). Resistance of *S.* x *calodendron* to cadmium increased by 78.5% after 14 days exposure, with a final TI of 137% (Fig. 5.30). The resistance of *S. burjatica* increased in response to Cd by approximately 20% over the duration of study one (Fig. 5.31) and in *S. viminalis* the increase was almost 100% (Fig. 5.32). Resistance to cadmium was suppressed by the presence of other metals such as copper and zinc, despite the addition of half the concentration *TI*s were low for all clones. The only exception being *S. caprea*, although it is likely that the greater resistance observed in this clone is partly attributable to the poor growth of the control compared to that of study one. Supplying metals in dual combination had similar toxicity effects on clones as the single metal treatments. This indicates an interaction between metals, as suggested by Alloway (1995), even though the extent of growth inhibition observed was similar in study two and study one, synergistic activity between these metals cannot yet be inferred. The only experimental evidence which may point to interaction between metals is the effect of combinations of metal on cutting survival. Single metal treatments did not reduced survival significantly whereas combinations of metals did (Table 5.11). Willows varied widely in their response to treatment with metal mixtures; although in common with test one the range of *TI* values was still high, possibly as a result of allowing cuttings to establish in nutrient solution amended with

very low concentrations of metals, thus ensuring a healthier basis for further growth. Treatment with copper + cadmium resulted in a range of resistance between 100-180% in *S. caprea* and approximately 100% for *S. viminalis*, which had a *TI* of around 50% at 14d, and this treatment appeared to be the least toxic of all the combination treatments.

Although there were no similar increases in resistance to either copper or zinc, in several cases the level of resistance to these metals was maintained at or near the level of the first measuring date despite a tenfold increase in metal dose between 14 and 128 days of treatment. Considering this, the *TI* values for both copper and zinc are still high. In test one the resistance of *S. caprea* to copper varied between approximately 30-80%; with a final *TI* of 64.5% at 1.5 mg Cu l^{-1} (Figure 5.29); this compared well with an initial *TI* of 73% when copper concentration was 0.15 mg l^{-1}. Total reduction in zinc resistance in *S. caprea* was 6.5% with a tenfold increase in treatment concentration. In *S.* x *calodendron* copper resistance was reduced by 1.5% and zinc resistance between 14 and 128d increased by 4.5% (Figure 5.30). Copper resistance in *S. burjatica* fell by 27.5% between 14 and 128d although zinc resistance increased by 5% (Figure 5.27). In *S. viminalis* copper resistance fell by 25.5% and zinc similarly fell by 65.5% overall (Figure 5.31) although the zinc resistance of this species was already very high (200%) at the outset of the experiment.

It is unclear in this study whether inclusion of a rest period offset more serious phytotoxic effects, although it did not appear to have significantly increased growth or reduced susceptibility to metals. Information in scientific literature relating to induction and loss of metal resistance indicates that removing the selection pressure may reduce resistance; Baker *et al* (1986) found that growing a Cd-resistant ecotype of *Holcus lanatus* L. on uncontaminated soil for 6 years reduced subsequent growth on its native soil. This 'lost' resistance could not be recovered by further induction attempts. In the present study suspending metal treatment for only a short period of time may have

hindered the subsequent development of resistance as well as offsetting phytotoxic effects, although there is no evidence for this. It is possible that woody plants may require longer periods of acclimation than herbaceous plants, shown to respond to short periods of pre-treatment with metals (Aniol 1984). This is in agreement with Dickinson *et al* (1991a; 1992) who proposed than tolerance in woody plants may be an orchestrated response resulting from the interaction of phenotypic adaptation and genotypic predisposition. This will be discussed further in Chapter 9 (*Discussion*).

The general trends observed in the uptake and accumulation data collected at the end of the experiment were that copper, and to a lesser extent zinc, applied singly or in combination were considerably more toxic to willows, causing chlorosis and alterations in root morphology as well as growth reduction, and were found to be taken up in larger quantities than cadmium. Copper was immobilised within root systems of willows, and at most only a hypothetical 4% of total copper uptake would reach aerial plant tissues. Zinc similarly caused reduction of leaf and stem biomass and in *S.* x *calodendron* and *S. viminalis* the leaf material was found to contain significantly higher concentrations of this metal. Copper concentrations in willow cuttings analysed in test two showed that uptake of this metal was greater when supplied in combination with either cadmium or zinc, and yet concentrations of cadmium in combination was very similar to those in test one. The increased copper uptake only occurred within roots and was therefore not removable.

Cadmium was supplied at the same concentration, and increased at the same rate as copper, but was not taken up to the same extent. This metal was present at much lower concentrations within dried tissue; concentrations within the various plant tissues were very similar between clones and generally did not exceed 100μg g^{-1}. This finding indicated that there may be control mechanisms influencing cadmium uptake; no loss of productivity (i.e. biomass reduction) was observed and once cadmium was taken up

there were no preferential sites of accumulation within the plant. A greater percentage of cadmium was potentially removable than both copper and zinc, possibly as a result of its weaker attraction to organic molecules and the apparently passive uptake of cadmium within willow cuttings. Combining cadmium with copper or zinc however, reduced the removable proportion when compared with test one. This reduced uptake may have in fact been due to the inhibition of leaf and stem production as a result of treatment with the more toxic metals.

Findings concerning metal uptake in the above two tests are in general agreement with those of Baker *et al* (1994); studying the response of a metallophyte *Thlaspi caeruelscans* J.& C. Presl. to a wide range of heavy metals. Among others they separated Zn and Cd (metals which were readily transported to shoots) from Cu which was predominantly immobilised within roots. This suggests that phytoamelioration of the soil is only possible for the more mobile metals, and that green clean-up techniques such as tree planting and coppicing will be ineffective for metals which are very strongly bound within the substrate. It would seem possible from these findings, that removal of immobile metals may involve digging out root stocks at regular intervals and re-planting the surviving whips; thus exploiting any *in situ* resistance development. It is unclear from solution culture tests such as this exactly how much copper could be removed by adherence to root systems, because copper is more available to roots in solution culture than in a soil matrix.

5.2.5. Conclusions

These experiments indicate that *Salix* cuttings can be acclimated to resist elevated concentrations of heavy metals when treatment occurs gradually over an extended time period. The results of experiment 5.1. showed that in the majority of cases cuttings pre-treated with background nutrient solution were more resistant to copper treatment than those treated with a higher concentrations. This may indicate that cutting establishment, and the subsequent organisation that occurs is important in metal

resistance. Prior to production of adventitious roots cuttings are metabolically unstable (Haissig 1986) and may be more susceptible to metal toxicity. Experiment 5.2. used this information to devise acclimation studies that allowed cuttings to develop adventitious roots and shoots in the presence of very low concentrations of metals, and as a result much higher exposure levels were reached before phytotoxicity became critical.

The maintenance of Tolerance Indices around or above a 100% level by gradual acclimation is the prominent finding of this chapter. Furthermore, the elevated resistance of *Salix* to cadmium is also confirmed. A further investigation into the resistance of *Salix burjatica* to elevated levels of cadmium is included in Appendix I. This study also finds that cadmium does not effect root elongation or production in this clone.

Resistance to, and accumulation of copper has not been successful in willows to the same extent as cadmium and zinc. Copper caused rapid phytotoxic effects in *Salix* cuttings in solution culture; it remained strongly bound to tissues in which it was in direct contact and was by far the most toxic metal tested in this work. Removal of copper via phytoremediation at present seems unlikely without the use of specific copper hyperaccumulating species (Brookes, Baker & Malaisse 1992).

Uptake and accumulation characteristics in *Salix* investigated in experiment 5.2 suggest that heavy metals are more toxic when supplied in combination; higher levels of metals were detected and more pronounced root inhibition occurred in cuttings grown in mixed metal treatments. This would not necessarily translate to the removal of larger quantities of metals from contaminated soil because of the greater toxicity. As well as showing a marked resistance to cadmium, *Salix* may also be able to remove high concentrations of cadmium from the soil, and a large proportion of total cadmium taken

Chapter VI

Growth and survival in polluted soil

6.0. Introduction and Rationale

Metalliferous substrates tend to be heterogeneous, varying both in terms of background edaphic characteristics and heavy metal content (Smith & Bradshaw 1972). Plant survival and tolerance are more difficult to predict when the metal species and concentration are variable because the severity of contamination differs according to the source of metal pollution, site topography, soil structure, fertility and vegetation. The most important background edaphic factors which directly affect heavy metal availability are pH, organic matter content (O.M.), exchangeable cations and nutrient status, as well as the presence of other pollutants (Livens 1991; Alloway 1995). In the present study, solution culture experiments have provided important information about the metal resistance of willows in a homogenous matrix where nutrient content, pH and metal concentration were controlled (Chapters 4 and 5). Exposure to mixtures of heavy metals in previous tests constitutes a more realistic approach to the field situation, but is still quite different. A further step, explored in the present chapter, is to test willow cuttings in soils originating from metal contaminated sources.

The following experiment investigated the ability of selected willow clones (all of which have been tested previously in solution culture) to survive and grow over a one year period in metal polluted substrates collected from a site aerially contaminated by copper and cadmium (Prescot, Merseyside) and a site contaminated with lead-zinc mine tailings (Trelogan, Clywd). Both contaminated sites in this investigation have been extensively studied in the past, and also specifically with reference to the metal tolerance of native plant ecotypes. There are profound differences between the two substrates; the first is a woodland ecosystem, which has received aerial deposits of heavy metals has an established soil structure, microbial community and decomposer system. The second site at Trelogan, however, consists of mine tailings; a processed mineral substrate having very few properties in common with soil (Smith &

Bradshaw 1972). Areas contaminated by mine tailings are a particular problem for bioremediation, and this type of contamination is most commonly dealt with by excavation, or by sealing the substrate with an impermeable layer and covering with topsoil. To successfully establish any vegetation at all, the addition of lime and fertilisers is usually necessary (Cox & Rains 1972; Mench *et al* 1994a; Pitchel *et al* 1994).

This experiment investigated the survival and biomass production of five selected willow clones grown on two different contaminated substrates from these two sites. pH, moisture content, organic matter content, principally exchangeable cations, H_2O- and HNO_3-extractable concentrations of Cu, Cd, Zn and Pb were measured at the beginning of the experiment. This study aims to understand how previous hydroponic studies translate into a field situation by investigation the resistance response of clones tested in contaminated substrates. This experiment also attempts to clarify the type of metal-polluted substrate bioremediation strategies may realistically improve.

6.1. Growth, survival and metal uptake of willows on contaminated soils.

6. 1.1. Background to the contaminated sites

Prescot (O.S. Grid reference number SJ 464 926)

Aerially-contaminated soil was collected from Prescot; a small town 9 km to the east of Liverpool. The main pollution input to the site is copper, and to a lesser extent cadmium and zinc from nearby metal refining industry. The plant began its operations in 1906, converting scrap copper into copper wire bars. It was superceded in 1932 by a copper fire refinery which produced copper and cadmium alloys and high grade copper for use as rods and anodes. Soils downwind of the plant received substantial inputs of copper and cadmium in the form of dust particles and aerosols until the plant ceased operation in 1991. The site has been reviewed extensively by Dickinson *et al* (1996); Dickinson *et al* (1996) showed that cessation of the activities of the refining plant may have led to a slow decrease in the levels of plant-available heavy metals.

Soil was collected from a woodland site adjacent to a churchyard which was populated mainly by *Acer pseudoplatanus* L. (sycamore) and to a lesser extent *Aesculus hippocastaneum* (horse chestnut). Previous studies at this site have shown that trees pre-dating the activities of the refinery have developed resistance to heavy metals (Dickinson *et al* 1991b; 1992; Turner & Dickinson 1993a,b; Watmough & Dickinson 1995a,b). Before this, metal resistance of grasses occurring in this area was reported by Wu & Bradshaw (1972); Wu *et al* (1975); Wu & Antonovics (1978) and Bradshaw & McNeilly (1981).

Trelogan (O.S. Grid reference SJ 185 774)

The mine site at Trelogan in Clywd, on the North Wales coast consists of a wide area of metalliferous mine tailings. The tailings appear to have been weathered and spread over surrounding vegetation, and have accumulated around the bases of several

mature sycamore trees on the site until only the upper most branches remain uncovered. Plate 3.0 shows the metalliferous substrate and the sparse vegetation at this site. Mining activities at Trelogan ceased around 1900, and grass species such as *Agrostis tenuis* and *A. stolonifera* have invaded in sparse patches around the spoil. A few tree species on the site include sycamore (*Acer pseudoplatanus* L.), several willows (of which Salix *caprea* and *S. cinerea* were studied) and a small number of young oaks (*Quercus* spp).

The parent rock underlying Trelogan spoil is limestone, typically rich in calcite giving the soil a neutral pH; Smith & Bradshaw (1979) found a pH of 7.1 at this site. Smith & Bradshaw (1979) found high calcium concentrations; between 41,200 and 69,400μg g^{-1}, with between 1,050-768 μg g^{-1} potassium, 392-125μg g^{-1} phosphorus and approximately 600 μg g^{-1} total nitrogen. The development of metal tolerance in native grass populations have been investigated by Cook *et al* (1971) and Gregory & Bradshaw (1965).

6.1.2. Aims

- To investigate the growth and survival of five *Salix* clones in metal-contaminated soils compared with a standard potting compost.
- To measure the concentration of copper, cadmium, zinc and lead within tissues of willows grown in contaminated soils for a year.
- To compare the concentration of heavy metals within the aerial tissues of *S. cinerea* from Trelogan to those found in selected *Salix* clones grown on Trelogan soils for one year.

6.1.3. Materials and Methods

Soil sampling

Soil for use in the pot experiment and for metal analysis was collected at the sites from the same area and profile depth (10 cm). Sub-samples were removed and

analysed for the H_2O- and HNO_3-extractable concentrations of Cu, Cd, Zn and Pb at the beginning of the experiment. Soils from Prescot and Trelogan were collected by cutting profiles into the soil, and removing samples from 10 cm below the surface, where concentrations of heavy metals were likely to be most elevated (Turner 1991). The following characteristics of the soil were also determined using the methods detailed in Allen (1989) (See Section 3.5). pH, % weight loss at 105°C (moisture content), % weight loss on ignition (organic matter content), Ammonium acetate-exchangeable cations (Ca, K and Mg), H_2O-extractable heavy metals (Cu, Cd, Zn and Pb) and HNO_3-extractable heavy metal concentration. All samples were analysed in triplicate.

HNO_3-extractable metal concentrations were also determined in soils sampled from the willow plantation at Ness Botanic Gardens and in Trelogan soil on areas where *S. caprea* and *S. cinerea* were growing. *S. caprea* at Trelogan was established in a large ditch on a steep incline in the centre of the spoil, and samples were taken higher up (A) and lower down (B) the incline around the roots (refer to Plate 4.0). Soil was also collected from soil in *S. cinerea* growing on the spoil edge (Plate 5.0) on substrate which was more similar in appearance to the bare spoil.

Plate 3.0. Lead/ Zinc mine tailings at Trelogan, Clywd, showing the trees (Acer sp. and Salix) growing on the eroded spoil.

Plate 4.0. Salix caprea L. growing on a steep incline at Trelogan, Clywd.

A and B show soil sampling points.

Plate 5.0. Salix cinerea L. growing on spoil edge at Trelogan, Clywd.

Table 6.1. Willow species and hybrids used in the soil experiment

Species/Hybrid (Source)	Accession Number
S. caprea L. (♀) "Higher Green Dicks" (Stott)	3287
S. caprea L. (♀) "Loughgall" (Loughgall)	3288
S. viminalis L. (?) "Ivy Bridge" (Rogers ex LARS)	3369
S. x *calodendron* (♀)Wimm[a].	3311
S. burjatica Nazarov (♂) Pavainen E7899 (Pohjonen)	3349

[a]*S.* x *calodendron* = *cinerea* x *caprea* x *viminalis*.

One year old willow rods were collected from the National Willow Collection in Ness Botanic Gardens in August 1994, with each clone sampled from the same ramet in the clone group. The rods were prepared for planting by removing all leaf material and cutting down to 20 cm lengths. The cuttings were maintained in 3.5 litre buckets containing 1 litre distilled water for 5 days prior to planting, to prevent drying out. Cuttings were planted in 5 litre plastic plant pots containing 4.5 litres of undiluted test soil, without fertilisers or lime. The three treatments were Prescot soil (P), Trelogan soil (T) and John Innes No. 1 Potting compost as a control (JI). Cuttings were planted with approximately 16 cm beneath the soil, with five cuttings of the same clone per 5 litre pot and four replicate pots per treatment (3 soils; 5 clones per pot; 4 replicate pots giving 20 cuttings per soil = 60 pots). The reference uncontaminated soil chosen for this experiment was John Innes N°1 potting compost; with a pH of 5.6, it allowed good growth of all willow clones, which typically prefer a pH of approximately 5.5 (Newscholme 1992).

The pots were placed randomly in an enclosure on the roof of the University Buildings and watered daily during the summer months, with any weeds removed by hand. Cuttings were allowed to grow for one year and plants were measured and harvested in August 1995. Plant material was removed from the pots and care was taken not to damage any fine roots, which were cleaned thoroughly with water to remove soil. Cuttings were separated into leaf (including petioles), new stem, root

and the original woody cutting material after which roots and wood were washed thoroughly and maintained in distilled deionised water for one week to remove surface-bound metals. Leaf, new stem, and root tissues from each replicate pot were combined, dried at 80°C for 48 hours and weighed to give a yield figure (g DW) of new material produced per pot. Woody material including bark from viable cuttings in each pot were also pooled, dried and weighed. Plant material was prepared, digested and analysed for metal concentration as described in Section 3.6. Leaf material (plus petioles) and woody stem material from mature *S. cinerea* shrub growing on the metalliferous spoil at the Trelogan site (Plate 5.0) were also analysed in triplicate for concentrations of Cu, Cd, Zn and Pb.

6.1.4. Results

Soil analysis

The physical soil characteristics are shown in Table 6.2, and metal concentration are shown in Table 6.3. All figures are means and standard deviation (n=3).

Table 6.2. Background edaphic factors in the three soils used in the pot experiment.

Soil	pH	Moisture Content (%)	Organic Matter (%)	Exchangeable Cations $cmol_c 100g^{-1}$	NH_4OAc-extractable Ca ($\mu g\ g^{-1}$)
Control	5.6 (±0.23)	67.3 (±0.92)	73.76 (±2.78)	288.86 (±17.56)	139.04 (±1.15)
Trelogan	7.0 (±0.06)	7.79 (±2.93)	4.72 (±0.17)	316.38 (±53.86	6,099.8 (±999.23)
Prescot	4.3 (0.01)	45.88 (±2.77)	28.50 (±1.19)	8.67 (±2.33)	1.26 (±1.14)

Table 6.3. H_2O-extractable and nitric acid-extractable (total) metal concentrations in samples of soils used in pot experiments. (Means ± standard deviation)

Soils	H_2O-extractable metals(μg g^{-1})				HNO_3-extractable metals(μg g^{-1})			
	Cu	Cd	Zn	Pb	Cu	Cd	Zn	Pb
John Innes Compost	0.11 (±0.026)	0.11 (±0.028)	0.63 (±0.00)	*	47.20 (±1.62)	3.43 (±0.31)	40.09 (±3.64)	20.13 (±2.61)
Trelogan bare spoil	0.012 (0.0089)	2.16 (±1.06)	11.06 (±2.86)	*	72.20 (±40.16)	116.60 (±7.19)	1,468.25 (±88.59)	13,488.0 (±527.9)
Prescot	0.37 (±0.098)	0.02 (±0.028)	0.49 (±0.28)	*	1,278.33 (±117.8)	14.14 (±1.69)	101.21 (±8.22)	417.45 (±57.55)

* indicates the metal concentrations were below detection limits (See Table 3.1)

Table 6.4. Normal ranges of heavy metals concentrations in soils and plants (from Alloway 1995).

Element	Normal range in soils*	Critical soil total concentration (mg kg^{-1})	Normal range in plants*	Critical concentration in plants (mg kg^{-1})
Cu	2-250	60-125	5-20	20-100
Cd	0.01-2.0	3-8	0.1-2.4	5-30
Zn	1-900	70-400	1-400	100-400
Pb	2-300	100-400	0.2-2.0	30-300

* Kabata Pendias & Pendias (1992).

The soils differed both in their physical characteristics (Table 6.2) as well as in concentration of metals (Table 6.3). John Innes compost contained a greater moisture and organic matter content than both contaminated substrates, although the organic matter content of Prescot was till relatively high. Previous studies on soil from Prescot have shown that rates of leaf litter decomposition were slower due to the toxicity of metals to decomposer organisms (Coughtrey *et al* 1987) and this has also been found for other forest ecosystems (Zwolinski 1994; Kohler *et al* 1995). Trelogan spoil also had a low moisture content; the substrate was a mixture of fine sandy silt with a high clay content; suggesting that percolation of water through the spoil is less important than run-off over the surface.

The concentration of exchangeable cations (an indication of CEC) of the three soils used also differed. There are typically large differences between the CEC of mineral and organic soils; in the former CEC can range from a few to 60 $cmols_c kg^{-1}$ and in the latter levels can be as high as 200 $cmols_c kg^{-1}$ (Alloway 1995). The cations measured to determine the concentration of total exchangeable cations were Ca, K and Mg. The control soil (John Innes No.1) contained a high percentage of organic matter, which also contributes to CEC (due to high absorptive capacity above pH 5), and the concentration of exchangeable bases in control soil was close to normal levels for organic soils. The concentration of exchangeable cations in Trelogan spoil was much higher than the control due to abundance of calcium in the substrate, with Prescot having the lowest concentration. The concentration of NH_4OAc-extractable calcium has also been included in Table 6.2 due to the ameliorating effect Ca can have on metal toxicity under normal circumstances (Wilkins 1978). Results of soil analysis carried out in this study are generally in agreement with Smith & Bradshaw (1979).

The total copper content of Prescot was particularly high; with $\approx 1{,}300 \mu g\ g^{-1}$ present, of which only $\approx 0.3 \mu g\ g^{-1}$ was extractable by H_2O. Differences between the metal concentrations in the HNO_3-extractable and H_2O-extractable soil fractions were greatest for lead. Concentrations of lead removed using water extraction were below detection limits (approximately 1.0 $\mu g\ ml^{-1}$).

Table 6.5. Total (HNO_3 -extractable) concentrations of heavy metals in soil from Ness Gardens and Trelogan. (Table shows soil source and the species of willow occurring on the site; where A or B indicate the position on the slope from which samples were taken; see Plate 4.0. Values are means ± standard deviation where n=3)

Soils	Cu	Cd	Zn	Pb
Ness Gardens *S. cinerea* (3294)	14.78 (± 4.82)	3.23 (± 0.31)	45.73 (± 3.88)	47.79 (± 7.72)
Trelogan *S. cinerea*	197.23 (± 3.30)	254.81 (± 9.33)	1,259.54 (± 88.89)	12,840.0 (± 4009)
Trelogan *S. caprea* 'A'	25.98 (± 8.53)	15.28 (± 2.56)	447.01 (± 66.36)	522.50 (± 127.7)
Trelogan *S. caprea* 'B'	282.16 (±21.66)	17.38 (± 3.41)	692.72 (± 33.5)	465.36 (± 79.9)

Total concentrations of heavy metals measured in soil from Ness gardens were low, and below critical levels (see Table 6.4). There was considerable variation between the metal concentration in spoil collected from beneath vegetation at the Trelogan site. Soil from beneath *S. cinerea* was found to have much higher concentrations of cadmium, zinc and lead, whereas soil on the lower slope under *S. caprea* contained more copper. The data indicates that there was significantly more copper, cadmium and zinc lower down the slope in which *S. caprea* was growing, although the concentration of lead was greater on the higher part of the slope.

Biomass production and cutting survival

Figures showing yield of individual plant compartments per pot (5 cuttings) are shown in Figure 6.1-6.3, expressed as means and standard errors, with cutting survival in Figure 6.4. There were substantial growth and survival differences between the five willow clones tested in this experiment (and between the three test soils in each case). All of the clones grew well in control compost, and produced a extensive root system. Leaf biomass in both *S. caprea* clones was greater than *S. viminalis* which produced a larger amount of stem material. Biomass production of all clones except *S. caprea* Loughgall in Prescot soil was negligible in comparison

with that of uncontaminated soil, and *S. viminalis* failed to grow altogether. One notable observation was the high, consistent growth of *S. caprea* Loughgall in Prescot soil, which produced similar leaf and stem biomass as control cuttings although root biomass was reduced. *S. caprea* Higher Green Dicks, *S. caprea* Loughgall and *S. burjatica* survived in Trelogan mine spoil and produced small amounts of biomass (Fig. 6.2). In addition, the *S. caprea* clones produced greater biomass in Trelogan spoil than other clones. Cutting viability of both *S. caprea* clones was higher in both contaminated soils than any other clone. Viability was generally lowest in plants grown in Trelogan mine spoil, with the exception of *S. caprea* clone 3288.

There were significant differences between soil type, plant tissue and clones, with significant interaction between all factors except plant tissue and species (Table 6.6).

Table 6.6. Results of GLM analysis of yield data in the pot experiment (g DW per pot=5 cuttings).

Source	D.F.	Seq.SS	Adj. SS	Adj. MS	F	*P*
Soil type	2	7026.07	7026.07	3513.03	173.89	0.001
Plant tissue	2	360.62	360.62	180.31	8.93	0.001
Willow clone	4	772.66	772.66	193.16	9.56	0.001
Soil x tissue	4	791.63	791.63	197.91	9.80	0.001
Soil x species	8	991.21	991.68	123.90	6.13	0.001
Tissue x species	8	190.68	190.68	23.84	1.18	0.315
Error	151	3050.55	3050.55	20.20		
Total	179	13183.42				

The only non-significant interaction was between the plant tissue and the particular clone.

Figure 6.1. Dry weight of leaves, stem and roots produced per pot by five willow clones grown in potting compost for one year (Means ± standard errors; n=4).

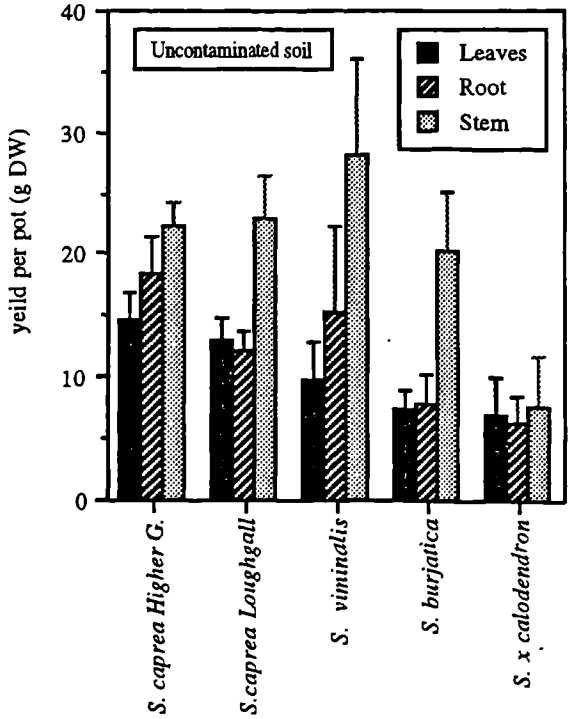


Figure 6.2. Dry weight of leaves, stem and roots produced per pot by five willow clones grown in Trelogan Pb/Zn mine spoil for one year (Means ± standard errors; n= 4).

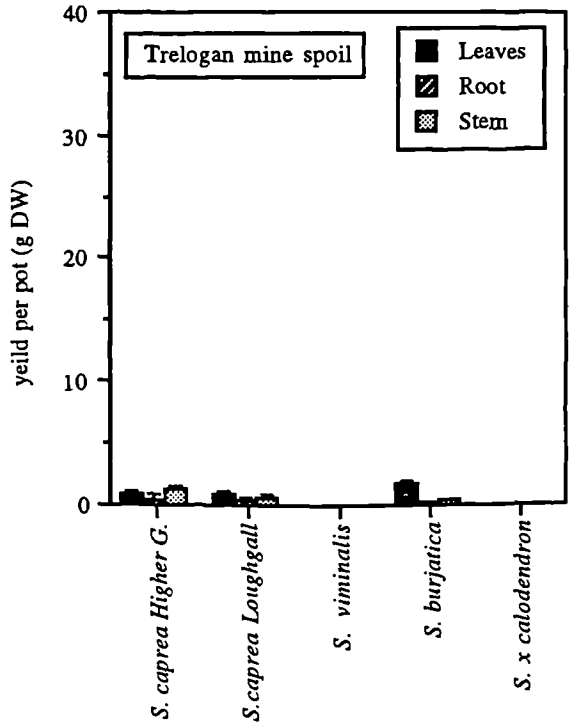
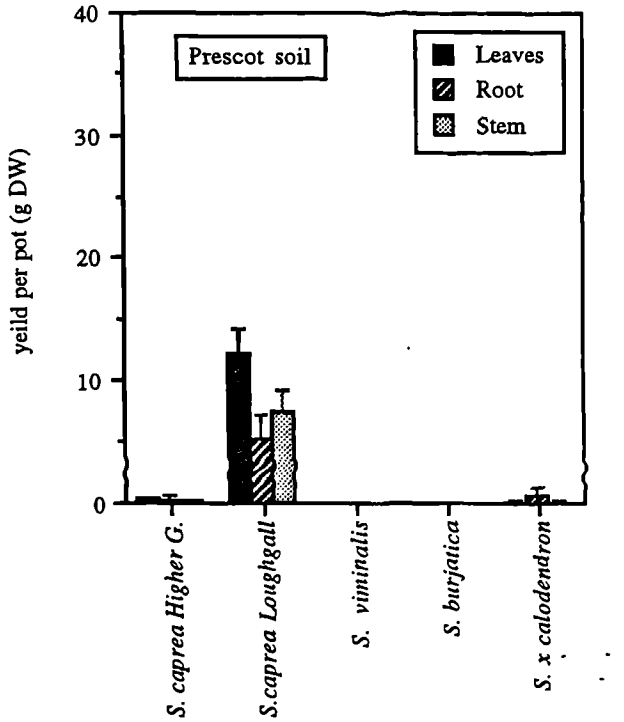
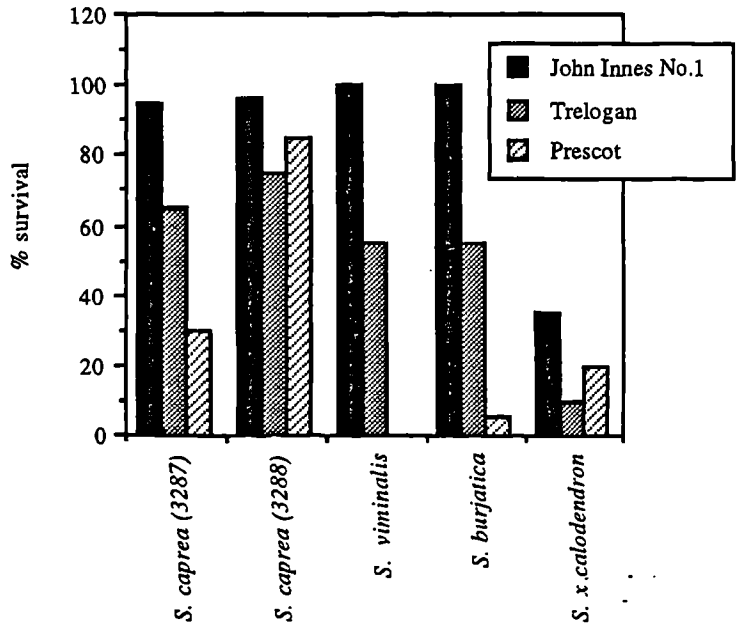


Figure 6.3. Dry weight of leaves, stem and roots produced per cutting by five willow clones grown in Prescot soil for one year (Means ± standard errors; n= 4).



All willows growing in Trelogan spoil were chlorotic and showed distinctive reddening of new stem material, which is also a characteristic of native Trelogan willows. Only *S. caprea* Loughgall grew successfully on Prescot soil; and showed comparable growth to plants grown in John Innes Compost (Fig. 6.3). Survival of all clones was highest in control compost (Fig. 6.4), although *S. caprea* Higher Green Dicks (3287), *S. viminalis* and *S. burjatica* showed greater survival in Trelogan mine spoil than in Prescot soil whereas *S. viminalis* did not survive. Despite over 50% cutting viability for *S. viminalis* grown in Trelogan spoil the amount of biomass produced was negligible.

Figure 6.4. Viability of cuttings grown in three different substrates for one year. The surv val of clones in response to the three test soils expressed as the percentage of all cuttings planted which grew successfully.



Metal concentration in Salix *tissue compartments*

In *S. cinerea* from the Trelogan spoil showed that there were low levels of copper in leaf and stem material, although levels of other metals were much higher (Table 6.7). The concentration of cadmium and lead in *S. cinerea* were higher in the woody stem material than within the leaves, whereas accumulation of zinc was slightly higher in the leaves. Concentration of lead in woody stem material was found to be significantly elevated above that of leaf material; approximately 160 $\mu g\ g^{-1}$. The critical concentrations of heavy metals within both soil and plant material are given in Table 6.4, indicating that there are levels of cadmium, zinc and lead within the aerial tissues of *S. caprea* ex. Trelogan which would theoretically cause phytotoxicity symptoms in other plants.

Table 6.7. Concentrations of heavy metals in aerial tissues of S. cinerea *ex. Trelogan* ($\mu g\ g^{-1}$) *collected from plants established on mining spoil. (Means ± standard deviation, where n=3).*

Compartment	Copper	Cadmium	Zinc	Lead
Leaves + petioles	4.24 (± 0.56)	43.86 (±10.20)	87.14 (±0.90)	17.25 (±4.25)
Woody stem	4.62 (±0.52)	76.43 (±7.55)	77.30 (±3.19)	157.35 (±2.53)

In the pot experiments *S. viminalis, S. burjatica* and *S.* x *calodendron* failed to produce enough material in contaminated soils for metal analysis, and therefore only the *S. caprea* clones were analysed. Table 6.8. shows the results of a single GLM analysis on all metal analysis data of the two *S. caprea* clones, investigating the significance of clone, soil, tissue compartment, metal element and all interactions between these factors.

Table 6.8. F values from GLM analysis on data from metal concentration of plant tissues of the two S. caprea clones grown in the pot experiment (n=3). *** denote $P< 0.0001$; ns denotes $P>0.05$ (not significant)

Source (Degrees of Freedom)	F values (Significance)
Clone$_1$	0.47ns
Soil type$_2$	43.71***
Plant tissue$_3$	22.97***
Metal element$_3$	24.63***
Clone x Soil type$_2$	1.13ns
Clone x Plant tissue$_3$	1.38ns
Species x Metal element$_3$	0.70ns
Soil type x Plant tissue$_6$	12.67***
Soil type x Metal element$_6$	29.51***
Plant tissue x Metal element$_9$	10.39***

Metal concentrations in the two *S. caprea* clones was not significantly different (P=0.494), although the soil type, tissue compartment and metal elements were all significantly different. There was significant interaction between the soil type and

both the tissue compartment and the metal element. Metal concentrations measured in plant tissues of both *S. caprea* clones are shown in Figures 6.5-6.8.

Fig. 6.5 (a) Concentration of copper in tissue of S. caprea "Higher Green Dicks" following one years growth in control compost and contaminated soil (μg g^{-1} DW)

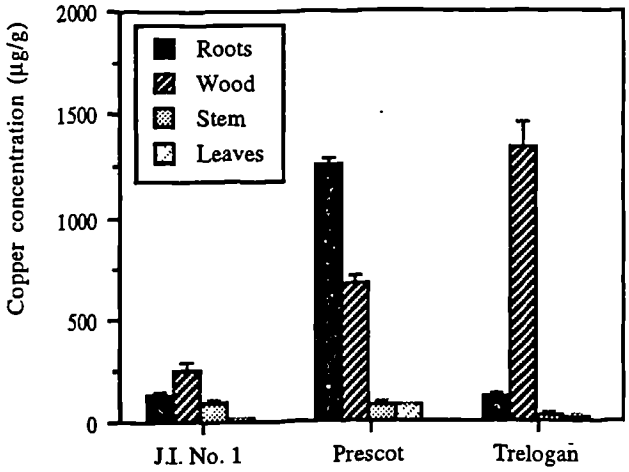
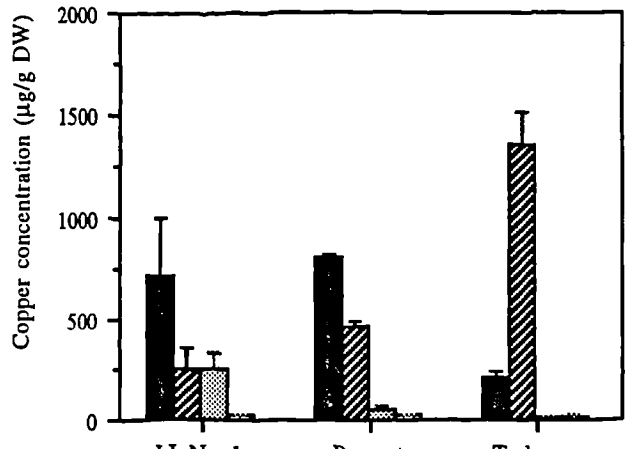


Fig. 6.5 (b) Concentration of copper in tissues of S. caprea "Loughgall" following one years growth in control compost and contaminated soil (μg g^{-1} DW).



(Means ± standard error where n = 3)

In control compost uptake of copper into roots was greater in Loughgall (3288) than Higher Green Dicks (710.24 μg g^{-1} and 124.52 μg g^{-1} respectively) (Fig. 6.5a.). Both clones showed similar patterns of uptake in Prescot and Trelogan soil. In the latter, concentration of copper in root tissues were very high, in both clones whereas root bound copper was lower compared to Prescot.

Fig. 6.6 (a) Cadmium concentration in tissues of S. caprea "Higher Green Dicks" following one years growth in contol compost and contaminated soils (μg g^{-1} DW).

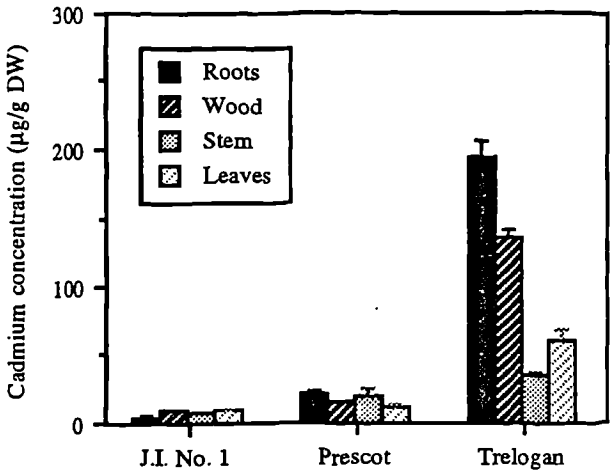
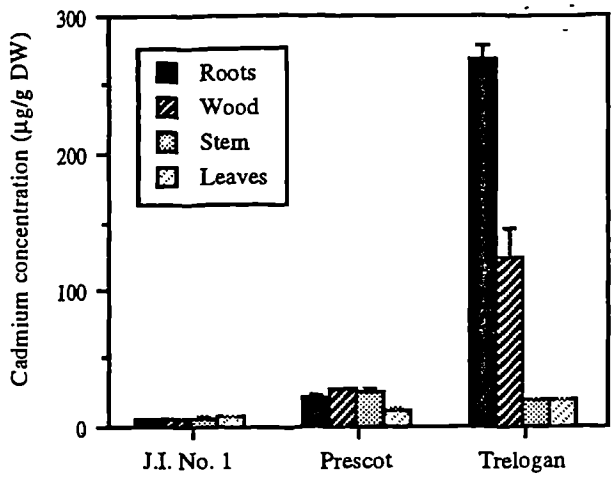


Fig. 6.6 (b) Cadmium concentration in tissues of S. caprea "Loughgall" following one years growth in contol compost and contaminated soils (μg g^{-1} DW).



(Means ± standard error where N = 3)

Cadmium

The concentration of cadmium taken up from Prescot soil was slightly elevated above that of controls in both clones, although there were no significant differences between tissue compartments. High concentrations of cadmium were measured in the roots of both clones grown in Trelogan spoil. Higher Green Dicks grown in Trelogan spoil had significantly higher cadmium accumulation within the leaves (Fig. 6.6).

Fig. 6.7 (a) Zinc concentration in tissues of S. caprea "Higher Green Dicks" following one years growth in control compost and contaminated soil (μg g^{-1} DW).

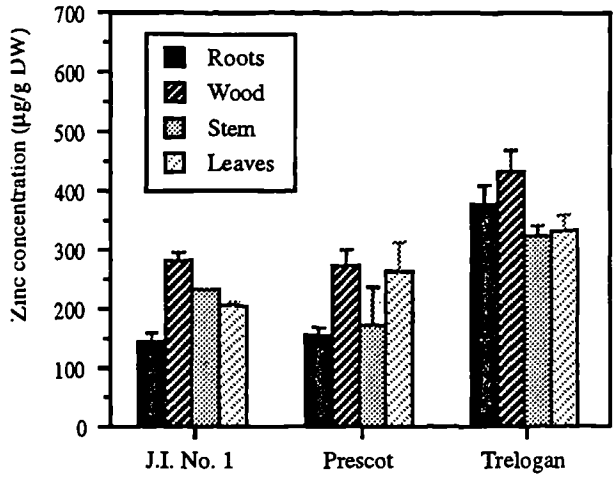
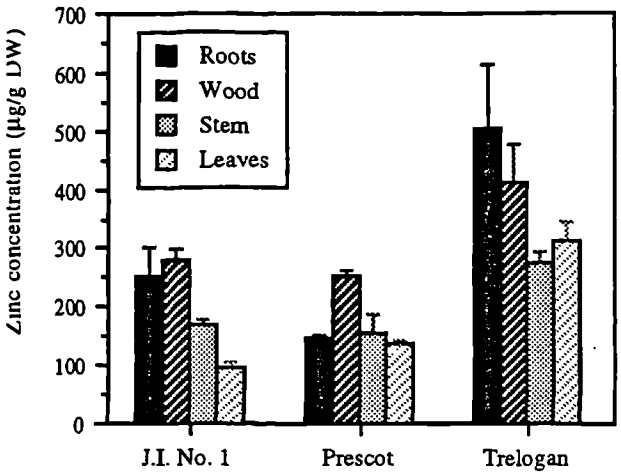


Fig. 6.7 (b) Zinc concentration in tissues of S. caprea "Loughgall" following one years growth in control compost and contaminated soil (μg g^{-1} DW).



(Means ± standard error where N = 3)

Similar concentration of zinc were measured in the tissues of both clones. Trelogan spoil contained the greatest total concentration of zinc, and significantly higher concentrations were measured in root and woody tissues. Uptake of zinc from Prescot was much lower, and was similar to background levels (Fig 6.7a-b).

Fig. 6.8 (a) Lead concentration in tissues of S. caprea "Higher Green Dicks" following one years growth in control compost and contaminated soil (μg g^{-1} DW).

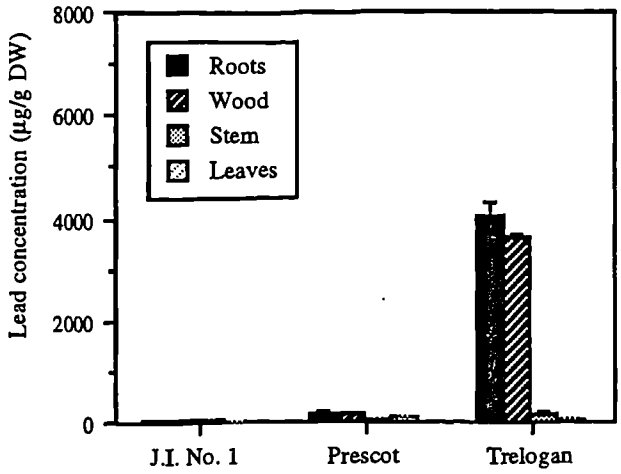
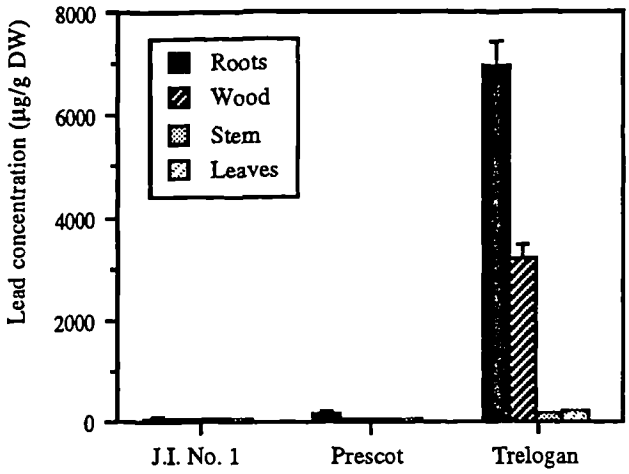


Fig. 6.8 (b) Lead concentration in tissues of S. caprea "Loughgall" following one years growth in control compost and contaminated soil (μg g^{-1} DW).



(Means ± standard error where n = 3)

Lead concentration in tissues of both clones grown in Trelogan spoil were much higher than in Prescot or control compost. Elevated concentrations of lead were measured in the wood and stem compartments of both clones (Fig 6.8a-b). The concentration of lead in the leaves and stem of Trelogan clones were only slightly elevated above that of control grown plants.

Cuttings grown in contaminated spoil contained similar concentrations of copper, cadmium and lead as *S. cinerea* sampled from the edge of the spoil. There were, however, much higher concentrations of zinc in the aerial tissues of both *S. caprea* clones grown on Trelogan spoil than in tissues of the native *S. cinerea.* In the pot experiment, clones had accumulated over 200 μg Zn g^{-1} in leaves and stem alike but *S. cinerea* leaves contained under 100 μg Zn g^{-1}.

6.1.5. Discussion

The results of this investigation support the findings of hydroponic studies in showing that there are considerable constitutional differences in the ability of *Salix* to grow in metal contaminated soil, both between species and clones. *S. caprea* was the only species tested which produced a comparable amount of biomass in contaminated and uncontaminated soil, but there were also significant clonal growth differences within this species. Differential uptake of the four heavy metals into the two *S. caprea* clones were not significant, indicating that the more successful growth of the Loughgall clone in Prescot soil was not due to metal exclusion or selective uptake. Cadmium was only slightly higher in the aerial tissues of the native Trelogan willow, with the exception of the high accumulation of Cd in the leaves of Higher Green Dicks (60.27μg g^{-1} DW) (Fig. 6.6b), although this may have been due to the small sample weight for this clone. These findings suggest that *S. cinerea* from Trelogan is either avoiding the areas of the spoil with have elevated metal concentrations, or there is a metal- exclusion mechanism involved.

Trelogan spoil contained higher concentrations of cadmium, zinc and lead than Prescot, and also presented a harsher edaphic environment, which may have contributed towards the poor growth of the majority of clones. The high concentration of NH_4OAc-extractable calcium is likely to be responsible for the neutral pH of the substrate which would reduce the availability of metals for uptake by plants. The successful establishment and growth of *Salix caprea* "Loughgall" on

Prescot soil without any prior acclimation was notable, and confirms the higher constitutional resistance that exists within this clone.

Copper uptake into leaf tissues was found to be particularly low for all of the clones measured, and was also below critical levels in *S. cinerea* ex. Trelogan (see Table 6.5). The low uptake of copper by plants has been discussed by other workers studying behaviour of heavy metals in the soil; in particular Baker & Senft (1995) state that the availability of copper is related to soil chemical potentials, analogous to pH and the species or form of copper predominant within the soil. In acid soils such as Prescot, the predominant form of copper may have been the available hexaquo copper ion $[Cu(H_2O)_6]^{2+}$ although this will depend on the form of copper which impinged upon the site. However, clones grown in Prescot soil contained more copper into the leaves than those in Trelogan spoil. The plant available copper concentration in Prescot soil was approximately 30 times greater than Trelogan (Table 6.3), whereas uptake of copper in aerial tissues (i.e. the sum of leaf and new stem material) of clones grown in Prescot soil were 2.5 times greater for the Loughgall clone and 3 times greater in Higher Green Dicks than cuttings in Trelogan spoil. The reasons why copper is confined to the roots despite being present in much greater available concentrations in the soil is most likely to be explained by the chemistry of the element, and its strong affinity for organic molecules, although this itself is strongly linked to the physical characteristics of the respective soils. The high concentrations of copper found in the wood and root compartments indicate that the majority of copper may be bound to the cell walls and the external membranes of the cuttings.

Concentration of copper in plants grown on John Innes compost was elevated above expected levels (refer to Table 6.4 for critical concentrations); and the concentration of copper in Ness Gardens soil from which the clones were sampled is also above

critical concentrations; in this case the high values may either be attributed to experimental error, or an indication of the preferential uptake of copper by this clone.

The H_2O-extractable concentration of cadmium in Trelogan spoil was approximately 100 times that of Prescot soil (total Cd was eight times higher); whereas the concentrations of cadmium within the aerial tissues of the Higher Green Dicks clone grown in Trelogan spoil were only 3 times greater than Prescot, and in the Loughgall clone the concentrations were similar to within $1\mu g\ g^{-1}$. This indicates that *some form* of metal exclusion mechanism may be operating within this clone. The concentration of H_2O-extractable zinc in Trelogan spoil was 2.1 times greater than Prescot soil, with 2.2 times more zinc in the aerial tissues of Trelogan-grown Loughgall clone, and 1.4 times more zinc in the Higher Green Dicks clone. The chemistry of the interactions between different heavy metal elements in soils with different physical characteristics is complex, but this suggests that the mobility and uptake of zinc reflects the H_2O-extractable metal concentrations .

Similar comparisons could not be made for lead, because concentrations extracted with water were below detection limits. It is actually quite unlikely that high concentrations of lead can be extracted with water, considering the strength of the bonds lead forms with organic molecules. Despite this, the concentrations of lead within the aerial tissues of both clones were elevated above that of control plants, with far greater concentration in Trelogan grown plants, indicating that there is some relationship between external and internal lead concentrations. The high concentrations of lead measured in root and woody tissue are enormously elevated in clones grown in Trelogan soil; approximately $3{,}000\mu g\ g^{-1}$ in the wood and between $4{,}000\text{-}7{,}000\mu g\ g^{-1}$ in the roots. In common with copper, this element would also appear to be strongly bound to cell walls and external membranes of the cuttings; a finding which is supported by the chemistry of the metal.

Contrary to results from hydroponic culture, the growth and survival of *S. caprea* clones was very similar to that of the fast-growing willows *S. viminalis* and *S. burjatica* in normal compost. This may have been attributable to the longer time period over which this experiment was run; *S. caprea* is typically slow rooting (Pohjonen 1991) and may require longer periods of time to produce the same number of surviving cuttings as the fast-growing *S. viminalis*. Productivity estimates obtained from field planted *Salix* usually refers to the growth achieved over a four year period following the year of establishment during which this experiment was carried out.

Uptake of heavy metals examined in the two successful clones is in agreement with data from previous hydroponic experiments. Copper uptake in both clones remained largely confined to the root system, in common with previous findings for this metal, whereas cadmium and zinc were present in much higher concentrations in the aerial tissues than copper.

The findings of this soil study have various implications for bioremediation programmes. Soils contaminated by heavy metals must be analysed in order to characterise the range of metal elements and the forms in which they predominate. Solution culture studies of mixed metal treatment effects (Section 5.2) have indicated that there are interactive, antagonistic effects of heavy metals both on growth and uptake in trees. The differences in metal uptake by the clones tested in this experiment may also be attributed to the presence of other metals, in particular lead, which has not been studied in solution culture. The restricted uptake of both copper and lead identified in this experiment, may appear problematic because these elements are not translocated into aerial tissues and cannot easily be removed when the willows are coppiced. However, the greater concentration of cadmium and zinc in aerial tissues may be considered more of a problem because they may move through the food chain more easily. The concentrations of copper and lead bound to roots was

high enough to make a considerable reduction in levels in soils if willow stumps were removed periodically.

The results of the above soil study supports findings from solution culture experiments that *Salix caprea* is the most metal resistant of all the clones tested. Furthermore, despite the normally slow growth of this clone, data from one years growth in uncontaminated soil indicates that in certain clones biomass production is comparable to that of the fast-growing *S. viminalis*; a clone which has been extensively studied in the past in attempts to establish vegetation filter plantations for the disposal of sewage sludge (Riddel-Black 1994; Aronsson & Perttu 1994). Clone selection within *S. caprea* may now be as important for bioremediation as the selection of *S. viminalis* clones was for biomass. The successful growth of *S. caprea* Loughgall in Prescot soil combined with the concentration of copper measured in the various compartments is very encouraging and should provide impetus for further research.

6.1.6. Conclusion

The following points can be made in conclusion of this chapter:

- Growth and survival of different *Salix* clones on contaminated soil varied but resistant and susceptible clones were successfully separated.
- *S. caprea* (clone 3288 "Loughgall") grew successfully on copper contaminated soil from Prescot which contained three times the background level of plant available copper.
- High concentrations of copper and lead were accumulated within the roots of cuttings in contaminated soil, whereas cadmium and zinc were more mobile and translocated to aerial tissues.
- Metal content in aerial tissues of test plants in Trelogan spoil were similar to that of the native mine ecotype, with the exception of zinc which was present in much higher levels in test plants.

Chapter VII
Pollen analysis

7.1. Evaluating heavy metal resistance of *Salix* using *in vitro* pollen germination.

Background

The effects of heavy metals on pollen viability and pollen tube growth was introduced in Section 1.4.2.2. Pollen grain germination has previously been used to give an indication of the resistance of plants to heavy metals (Searcy & Mulcahy 1985a) and is based on evidence from studies of *Lycopersicum esculentum* (tomato) which showed that the genes expressed in pollen represent 60% of the genes expressed in the sporophytic phase. This potentially makes pollen an effective tool for screening sporophytic characteristics (Tanksley *et al* 1981). Recent research on the effect of heavy metals on reproductive processes of woody plants has shown that heavy metals can alter pollen morphology, quality, reduce its relative dimensions and fertility. Bessanova (1992) studied the effects of Cu, Cr and Zn on pollen germination of *Betula* sp. (birch) and *Aesculum hippocastaneum* (horse chestnut) and found a correlation between the accumulation of heavy metals in flower buds and the number of meiotic aberrations, sterility and variation in size, indicating that reproductive processes are also subject to anthropogenic selection pressure. Most studies concerned with heavy metals and pollen have been carried out on herbaceous species and it has been shown that considerable changes in pollen tube growth and pollen ultrastructure occur in response to heavy metals (Handique & Baruah 1995).

There are no known studies which deal specifically with the effect of heavy metals on *Salix* pollen. Considering the widespread occurrence of polyploids and the propensity for spontaneous hybridisation within *Salix* (Section 2.1.1) the inclusion of pollen in a study of metal resistance was thought to be particularly important because it constitutes the genetic contribution to the next generation. If an individual plant possesses tolerance to heavy metals, the trait may well be represented in the gamete. If the behaviour of pollen in response to heavy metal parallels that of the whole plant, testing pollen may then enable the tolerance status of the parent tree to be estimated

within a short period of time. The possession of an allele which encodes resistance to environmental stress in diploid plants has a 50% chance of being passed on to the gametes. Considering the high degree of heterozygosity in plants such as *Salix*, which are typically out-crossing, dioecious and insect pollinated (Loveless & Hamrick 1984) the possession of a metal resistance allele by a polyploid plant, which has more than double the number of copies of the allele, would seem likely to increase the likelihood of its expression in the pollen grain.

7.1.1. Aims

- To determine whether pollen can be used as a bioindicator of metal resistance in willows by comparing pollen germination of clones from contaminated and uncontaminated sites in metal-amended and background germination solutions.

7.1.2. Materials and Methods

Samples of *Salix* pollen from two metal contaminated sites and a selection from the National Willow collection at Ness Botanic Gardens were collected in February 1995 (Table 7.0). The chromosome number for each clone (Love 1976) has been included in the following clone information, indicating the ploidy of the clones, where the basic chromosome number is 19 in most cases although a chromosome number of 11 is also common.

Table 7.0. Willow clones used in the pollen germination study

♂ **Willow clones (Source)** $_{\text{Chromosome number}}$	**Accession No.**
S. aurita L. (Dunlop - contaminated site)$_{4n=38*}$	n/a
S. caprea L. (Trelogan - contaminated site)$_{2n=38}$	n/a
S. caprea L. "Higher Green Dicks" (Stott)$_{2n=38}$	3287
S. x *dasyclados*[a] Wimm. Finland (Zwinnenberg)$_{4n=76}$	3314
S. daphnoides Vill. "Ovaro" (Paiero)$_{2n=38}$	3399

[a]*S.* x *dasyclados* = *caprea* x *cinerea* x *viminalis* *Love (1976) reports two different chromosome number for this species, following examination by different workers; the species may also occur as a polyploid.

Pollen samples from species of *Salix* were collected from the following metal contaminated sites:-

Trelogan (O.S. Grid reference SJ 185774)

Samples were collected from *S. caprea* growing on lead/zinc mine tailings at Trelogan; see Section 6.1.1. for further information on this site.

Walton, Liverpool (O.S. reference SJ370955)

Samples were collected from *S. aurita* L. (Eared Willow) growing on a site which previously received heavy metal contamination from a Dunlop tyre manufacturing factory which has now been demolished. Total concentrations of metals in the soil at the site were elevated above background and EC guidelines, although they are not as elevated as concentrations at Trelogan. Analysis showed elevated total HNO_3-extractable soil concentrations of cadmium (2.5μg g^{-1}), copper (40μg g^{-1}), and zinc (230μg g^{-1}) (Strain 1995).

Pollen collected from different willow species and sites were tested separately, according to the time in the season when the pollen was produced. Five tests were carried out in total. Storage of pollen is possible at -4°C for a maximum of 24 hours, but using material that is not fresh causes a decrease in viability of the pollen grains, and therefore pollen was collected and tested in the same day. Pollen was germinated in incubation chambers which consisted of a sterile petri dish containing moistened Whatman N°1 filter paper, a glass U-tube with a cavity microscope slide placed on top. In each individual pollen study there were 4 incubation chambers per treatment. To ensure that a comparable concentration of pollen grains were applied to each slide, a suspension of germination solution and pollen was prepared using 30 mls of solution and fresh pollen collected from 5 catkins so that a thick layer of pollen was formed on the meniscus of the solution which was then spun on a vortex mixer. 5 drops of the suspension were added to the cavity of each microscope slide. The lid of the petri dish

was replaced and the pollen growth chamber was incubated for 24 hours at 25°C in the dark.

After incubation, slides were removed from the growth chamber and a cover slip was placed over the cavity in the slide, spreading the pollen grains over a greater area to make them easier to count and inhibiting further pollen germination. The slides were examined immediately at x100 magnification using a light microscope. A total count of germinated and ungerminated pollen grains in each field was taken and the percentage of germinated grains was calculated. Five fields from each incubation chamber were counted. Successful germination was denoted by the growth of a pollen tube that was similar to, or greater than the diameter of the pollen grain (Du Bay & Murdy, 1983). Germination solution consisted of 15% sucrose and 100 ppm H_3BO_3 (to inhibit fungal growth) (Searcy & Mulcahy 1985a) with unamended controls and treatments of 1.0 mg l^{-1} copper (supplied as $CuSO_4.5H_2O$), 1.0 mg l^{-1} cadmium (as $8CdSO_4.2^1/_2H_2O$),and 10 mg l^{-1} zinc (as $ZnSO_4.7H_2O$). The pH of each germination solution was adjusted to 5.0. from an original level of approximately 5.5-5.8 using 0.1*M* HCl. Data from the pollen experiment was arc-sine transformed and analysed using the general linearised model of the Minitab statistical package. Resistance of pollen to heavy metals was estimated using the following index:

$$RI_{[M]} = \frac{\text{Mean germination (\%) in metal amended solution}}{\text{Mean (\%) germination in background solution}} \times 100$$

where *RI* is the resistance index for the concentration of metal [M] (Cu, Cd and Zn).

7.1.3. Results

The results of the statistical analysis indicated significant germination differences due to the metal supplied and the particular species of willow used. There were no significant differences in pollen germination between four replicated incubation chambers; considered as blocks in this experiment, indicating the reliability of the experimental data.

Table 7.2. Results of GLM analysis of pollen germination data

Source	D.F.	Seq. SS	Adj. SS	Adj. MS	*F*	*P*
Metal treatment	3	17183.0	17183.0	5727.7	83.57	0.000
Willow species	4	10563.3	10563.3	2640.8	38.53	0.000
Blocks (chambers)	3	339.3	339.3	113.1	1.65	0.177
Treatment x Species	12	21697.4	21697.4	1808.1	26.38	0.000
Treatment x Replication	9	2101.0	2101.0	233.4	3.41	0.000
Species x Replication	12	1776.7	1776.7	148.1	2.16	0.013
Error	356	24397.9	24397.9	68.5		
Total	399	78058.7				

Germination of pollen after 24 hours in metal-amended sucrose solution are shown in Figures 7.1.-7.5. The results are expressed as mean percentage germination ± the standard error. Results of a one-way ANOVA on the pollen germination within each individual willow clone is indicated on each figure.

Figures 7.1-7.5. Pollen grain germination from Salix spp. from Walton, Trelogan and an uncontaminated site in response to copper, cadmium and zinc. Means, standard errors and results of one-way ANOVAs where n = 20

Figure 7.1. Salix aurita (Walton)

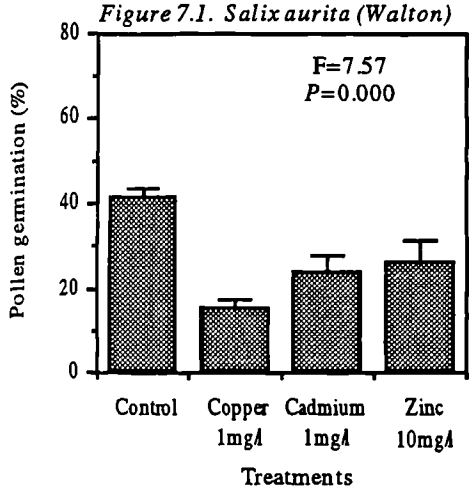


Figure. 7.2. Salix x dasyclados

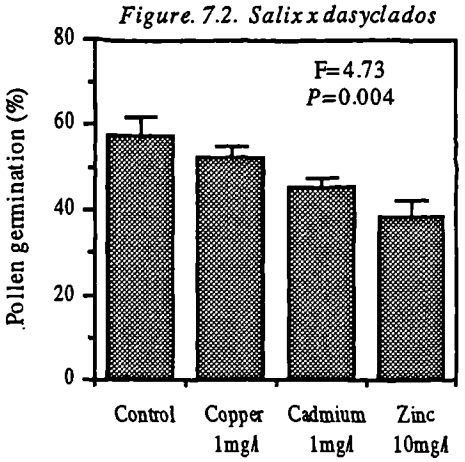


Figure. 7.3. Salix caprea "Higher Green Dicks"

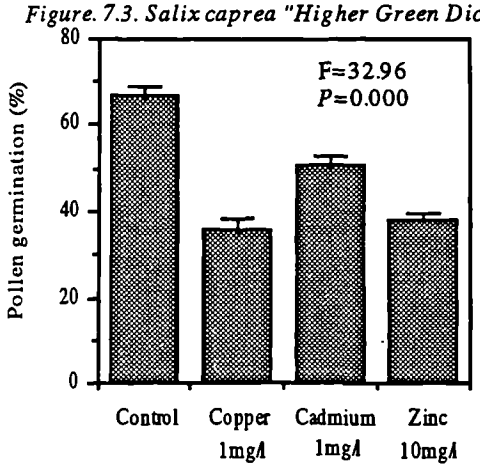


Figure. 7.4. Salix daphnoides "Ovaro"

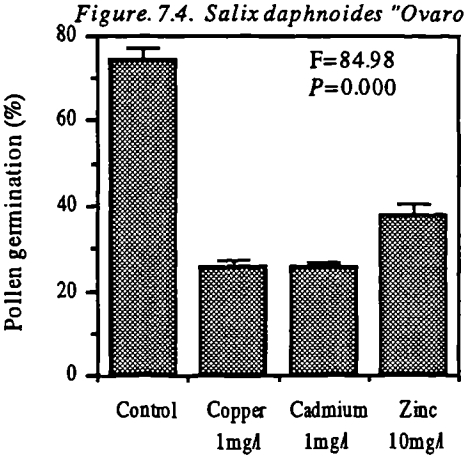
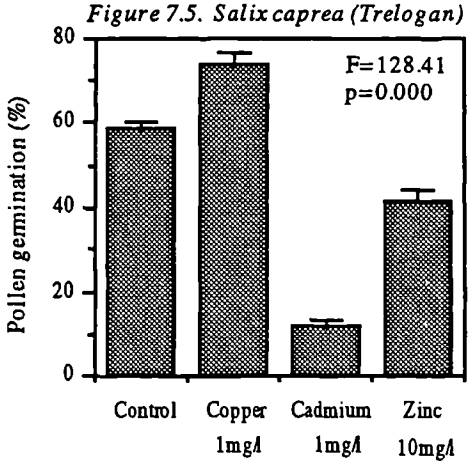


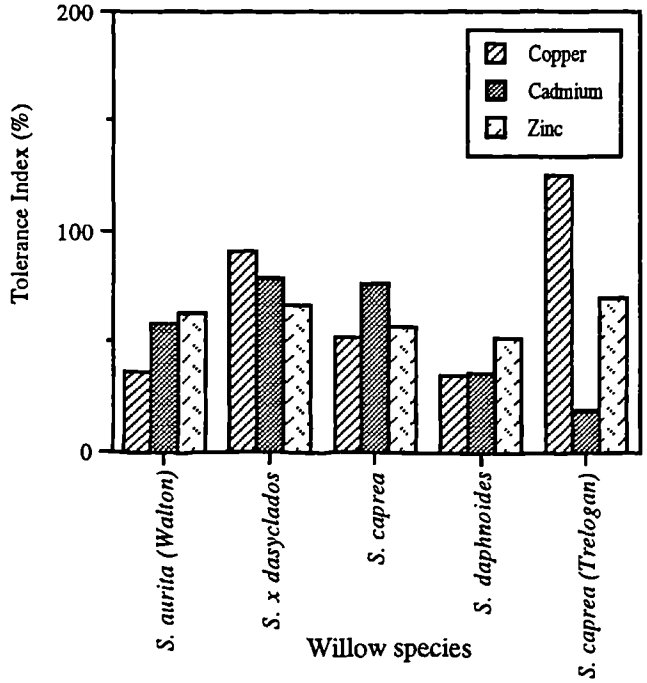
Figure 7.5. Salix caprea (Trelogan)



Germination of *S. aurita* from Walton in background solution was the lowest at 41.2%, and pollen from this clone germinated the least in copper-amended germination solution (Fig. 7.1). Less inhibition of germination was observed for *S.* x *dasyclados* (Fig. 7.2.); zinc inhibiting germination the most and copper the least for this clone. *S. caprea* 'Higher Green Dicks' pollen germinated significantly better in response to cadmium than the copper and zinc treatment (Fig. 7.3) whereas *S. daphnoides* germination responses in copper and cadmium solution were low (25.7% and 25.6% respectively) with marginally greater germination in response to zinc (Fig. 7.4). Finally, *S. caprea* pollen collected from the lead/zinc mine site at Trelogan germinated significantly better when the solution was amended with copper, whereas germination in cadmium-amended solution was particularly low at 11.6%.

Figure 7.6. shows the calculated tolerance indices for the five clones tested. Tolerance to both copper and zinc was highest in pollen sampled from the *S. caprea* growing on the Trelogan mine spoil site (126.5% and 70.14% respectively), although tolerance to cadmium was highest in *S.* x *dasyclados* and *S. caprea* from the uncontaminated source at Ness Botanic Gardens (87.6% and 75.9% respectively). Pollen from *S. aurita* growing on the Walton site did not appear to be particularly tolerant to the levels of metals supplied, with higher levels of tolerance found in clones from uncontaminated sites.

Figure 7.6. Calculated tolerance indices of willow pollen exposed to heavy metals



7.1.4. Discussion

The results from the pollen experiments indicate that germination studies can be used to detect interspecific differences in metal resistance, but where clones had also been tested in solution culture, there were strong disagreements between the two types of tests. The high resistance of *S. caprea* ex. Trelogan pollen to copper is supported by initial hydroponics screening data (Table 4.4. p75) although analysis in of the aerial tissues of willows growing on the most polluted parts of the mine spoil, carried out in Chapter 6 (Table 6.7 p172) showed that copper concentrations in leaf and stem material are very low. Given the consistent finding that copper remains bound to the roots and is not translocated to leaves, the observed resistance seems erroneous. Pollen data on cadmium and zinc data are also contrary to expected results; the concentrations of these metals in aerial tissues of Trelogan willows is elevated above critical levels, and greater degrees of resistance would therefore be expected.

Resistance of *S. caprea* 'Higher Green Dicks' in solution culture to single metals was approximately 70-80% throughout and in the present study the resistance of the pollen is similar, although pollen was more tolerant to cadmium than adventitious

roots (see Figure 7.3 and 5.29 respectively) The remaining clones were not tested in solution culture experiments, and comparisons cannot be made.

There is evidence to suggest that reproductive processes are particularly sensitive to cadmium; Chaney & Strickland (1984) tested the effect of a number of heavy metals, including the three studied here, on the germination of *Pinus resinosa* (red pine) pollen. Based on the lowest concentration to significantly inhibit germination, they found that Cd^{2+} was the most toxic, followed by Cu^{2+} and Zn^{2+}. In the present study apparent tolerance of *S.* x *dasyclados* and *S. caprea* to cadmium is unusual considering the apparent toxicity of this metal. Previous solution culture tests have showed that some willows appear to posses a notable ability to tolerate cadmium; accumulating upto 100 $\mu g\ g^{-1}$ in the aerial tissues (Figures 5.37-5.40). The effect of cadmium in particular on pollen is still unclear; although Strickland & Chaney (1979) have ruled out the influence of cadmium on respiratory gas exchange in *Pinus resinosa* pollen, it is likely that the numerous enzymes involved in germination and tube growth, e.g. those which catalyse amino acid synthesis and carbohydrate metabolism, may be affected.

The clones tested in this study show a variety of responses, and there is no single consistent tolerance or toxicity response of *Salix* to any of the metals studied here, nor do ecotypes sampled from metalliferous sites germinate significantly better than those from an uncontaminated source. *S. caprea* from the mine site at Trelogan is the only clone to show a greater pollen germination in response to copper, whereas *S.* x *dasyclados*, a clone with no known recent exposure to metals shows only small reductions in germination. The germination characteristics of pollen sampled from this clone are different to the others; in addition *S.* x *dasyclados* is polyploid, whereas all of the other clones are diploid. The ploidy of plants has particular evolutionary significance; polyploid plants in particular have been studied in relation to their evolutionary dynamics (Bretagnolle & Thompson 1995; Thompson & Lumaret 1991).

Polyploidy is the occurrence of plants having a chromosome number more than twice that of the haploid genome of related taxa (Bretagnolle & Thompson 1995) and is particularly important in the evolutionary dynamics of *Salix*. In terms of pollen studies, polyploid plants have twice as much genetic information within the gametes as haploid plants. A 2n gamete, such as *S.* x *dasyclados* has arisen by hybridisation with another species and is known as an alloploid. The larger amount of genetic information present within a 2n gamete may have a direct influence on its ability to survive stress from heavy metals. Theoretically, a pollen grain which has twice the number of chromosomes may be able to cope with heavy metal disruption of the enzymes required for germination and tube production, because it has twice the amount of DNA from which to continue amino acid production.

In a review, Thompson & Lumaret (1991) stated that the occurrence of natural polyploids can be affected by the environment, and that newly formed polyploids can spread to colonise habitats which are hostile to their diploid parents. The polyploid nature of *S.* x *dasyclados* may have been instrumental in the expression of a resistance response, although the extrapolation of this response into that typical of a whole plant growing in metal-amended solution is extremely tenuous.

These results suggest that pollen germination studies possibly could be used to indicate differences between metal resistance in reproductive processes in *Salix*, but they do not relate to the ability of particular clone to grow roots and survive in polluted soil. Further work is required to clarify the usefulness of pollen germination techniques.

Chapter VIII
Salix in vitro

8.0. *Salix in vitro*

Background

The applications of plant tissue culture was applied to tree improvement initially for the production of larger numbers of identical clones for both coniferous (Mott & Amerson 1981) and broad-leaved tree species (Kerns & Meyer 1986). There are three main phases in tree improvement, involving selection, breeding and progeny testing. Tissue culture can amplify a selected genotype by rapidly producing a large number of clones. Several stages are involved in improvement of tissue culture in trees; they are as follows:

1. Selection of the desired genotype
2. Production of large numbers of clones from internodal sections or 'explants', or meristemmatic regions such as tips and leaf petioles.
3. Acclimation and out-planting of explants.

Tissue culture conditions are largely very different from normal conditions; with increased humidity, low O_2, high temperatures and a highly regulated day length (George 1993). Acclimation involves gradually decreasing the exposure of the fragile explants to these conditions, in a similar manner to 'hardening off' of seedlings. Durzan (1988) reviewed the current biotechnologies for the improvement of woody perennials, including somatic embryogenesis; where plant embryos are *initiated from* a vegetative, non-gametic cells. New genetic engineering techniques are also being applied to woody plants to introduce the desired genes directly in to the developing embryo which can then be cloned and propagated.

The following section aimed to develop a standard nutrient media formulation that could be used to rapidly propagate *Salix* clones in order to create replicates of resistant individuals, or to manipulate metal resistance *in vitro.* Identification of metal resistance traits *in vitro* has proved successful in previous studies (Wu &

Antonovics 1978; Qureshi *et al* 1981; Poulter *et al* 1985), however more recent work using callus and cell suspension cultures derived from *Acer pseudoplatanus* L (sycamore) growing in the vicinity of a copper refinery has identified tolerance to copper (Watmough & Dickinson 1995c) and cadmium (Dickinson, Turner & Lepp 1991b; Dickinson *et al* 1992). Furthermore manipulation of resistance traits *in vitro* has also been successful (Watmough *et al* 1996). The appearance of resistance traits in cell culture enables tolerance in trees to be manipulated more easily, although prior to investigations of this nature, optimum culture conditions must be established. In the following study a collection of willow clones was established *in vitro*, in order to study the success of several different basal salts and hormone concentrations in propagating *Salix* explants.

The application of tissue culture techniques to *Salix* in the past has mainly been involved with biomass crop improvement (Vahala & Eriksson 1991; Gronroos *et al* 1989a,b) although other there are studies dealing with technique optimisation (Neuner & Beiderbeck 1992). This chapter examines the hypothesis that growth of fast-growing trees such as *Salix* can be increased yet further *in vitro,* and that slower growing species such as *S. caprea*, which have shown higher metal resistance in previous investigations but have a reduced ability to root from softwood cuttings, can also be bulked up much faster than *in vivo* propagation.

8.1. Aims

- To identify a nutrient medium formulation for the rapid propagation and optimal growth of *Salix* single node explants *in vitro.*

8.2. Materials and Methods.

Explants were sampled from young shoots of selected *Salix* clones which were growing in unamended 25% Hoagland's solution for 3 weeks in the spring of 1994. Explant sampling, preparation and media formulations were carried out as described

in Section 3.4. The optimisation of media formulation was divided into two studies; the first being a screen for the suitability of different basal salt, and the second testing different hormone concentrations on the success of willow explants.

8.2.1. Basal salt screen

Establishment and growth of selected willow clones (*S. caprea* "Loughgall" [3288], *S.* x *sericans* [3305] and *S. viminalis* [3369] were used to determine the optimal basal salt preparation for willow propagation and growth. Single node explants were established on hormone-free, solid nutrient medium containing a standard preparation (1x) of basal salt, plus 30 g l^{-1} sucrose, 500 mg l^{-1} casein hydrolysate, 500 mg l^{-1} *myo*-Inositol and 7 g l^{-1} technical agar. The basal salts used were MS (Murashige & Skoog 1962), Woody Plant Medium (WPM) (Lloyd & McCown 1981); DCR (Gupta & Durzan 1985) and SH media (Shenck & Hildebrandt 1972). Sterilised explants were maintained on 20 ml solid medium in 60 ml Sterilin universal containers under fluorescent lights (25 μmol $m^{-2}s^{-1}$) and were maintained in incubated cabinet at 25 ± 2°C with a 16h day for 28 days after which the number of viable explants were counted and expressed as a percentage of the total number of explants initiated. Losses due to contamination were also recorded.

Table 8.1. Willow clones used in the in vitro study

Species/Hybrid (Source)	Accession No.
S. caprea L. (♂) " Loughgall" (Loughgall)	3288
S. x *sericans* Tausch. ex A. Kern[a](♀) "Sericans"	3305
S. viminalis L. (?) "Ivy Bridge" (Rogers ex LARS)	3369

[a]*S.* x *sericans* = *caprea* x *viminalis*

8.2.2. *IBA concentration screen*

Single-node explants were established onto 20 mls solid media, containing either MS or SH basal salt preparations (1x). The media formulations were the same as those used in the previous nutrient screen (Section 8.2.1) with the addition of different concentrations of Indole-3-Butyric Acid (IBA) for root growth promotion (George 1993). BAP (Benzyl Amino Purine) was supplied at 0.5 mg l^{-1} throughout the experiment, due to the ease with which shoots were produced in hormone-free media, and concentrations of rooting hormone were varied. Treatments consisted of a low concentration of IBA (0.5 mg l^{-1}) and a higher concentration (1.0 mg l^{-1}). Explants were initiated onto 20 mls of test media and allowed to grow for 28 days. The number of rooted explants were scored as a percentage of the total viable explants on the particular media formulation.

Table 8.2. Willow clones used in the IBA concentration screen

Species/Hybrid (Source)	Accession No.
S. caprea L.(♂) "Higher Green Dicks" (Stott)	3287
S. caprea L. (♂) " Loughgall" (Loughgall)	3288
S. x *sericans* Tausch. ex A. Kern[a](♀) "Sericans"	3305
S. x *calodendron* Wimm[b] (♀)	3311
S. burjatica Nazarov (♂) Pavainen E7899 (Pohjonen)	3349

[a]*S.* x *sericans* = *caprea* x *viminalis*

[b]*S.* x *calodendron* = *caprea* x *cinerea* x *viminalis*

8.3. *Results*

Study one: Basal Salt Screen

Salix explants initiated on SH and MS media were generally the most successful, however, *S. caprea* showed a slightly higher rate of establishment on WPM. Establishment of *S.* x *sericans* was high on all medias, with the exception of DCR. *S. viminalis* similarly failed to establish on WPM, although it has one of the highest establishment rate on DCR.

Table 8.3. Explant success (% viable explants) of willow clones grown on four different basal salt preparations. Initial number of explants established is given in parentheses.

* denotes no growth success.

Clone number	MS	SH	DCR	WPM
S. caprea (287)	50% (40)	45% (30)	30% (30)	68% (35)
S. x sericans (305)	87% (38)	85% (34)	11% (35)	86% (38)
S. viminalis (369)	79% (43)	83% (30)	92% (40)	*

Study Two: IBA concentration

Table 8.4. Rooting success of willow explants in response to IBA concentration. Initial number of explants established is given in parentheses.

* denotes no growth success.

Clone	MS basal salts		SH basal salts	
	0.5 mg l^{-1} IBA	1.0 mg l^{-1} IBA	0.5 mg l^{-1} IBA	1.0 mg l^{-1} IBA
S. caprea (287)	13% (29)	37% (27)	10% (20)	12% (23)
S. caprea (288)	17% (17)	28% (12)	19% (24)	25% (19)
S. x sericans (305)	52% (30)	60% (25)	35% (34)	40% (25)
S.x calodendron (311)	* (35)	* (28)	* (40)	* (27)
S.burjatica (349)	19% (24)	26% (25)	* (26)	12% (21)

There was a generally poor rooting response of *Salix* explants on solid media, with *S.* x *calodendron* in particular producing no roots whatsoever despite healthy growth of shoot material. Shoot growth for all clones tested was vigorous, although in many cases the explants were observed to be chlorotic by the end of the test period, attributed to the lack of root growth. Where root growth was established explants appeared green and showed increased growth production. Root production in *S. caprea* (3287) was generally very poor and roots appeared sheathed in a dark endophytic bacteria. Despite a lower percentage of rooted explants *S. caprea* (3288) did produce viable explants which were subsequently bulked up on MS media, although viability of subsequent cultures showed reduced vigour and chlorosis. By

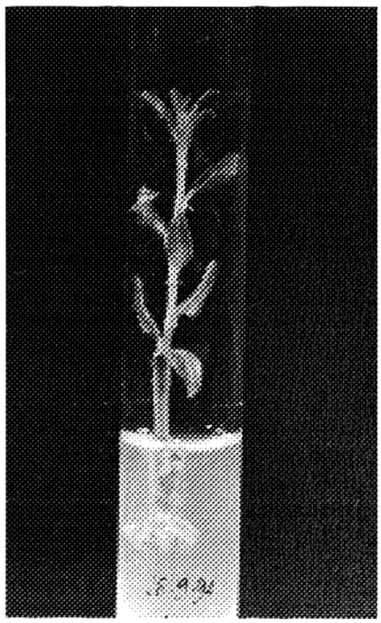


Plate 6.0. *Salix* x *calodedron* grown for 14 days on MS + 1.0 mg l^{-1} IBA showing shoot growth but no root growth.

Plate 7.0. *S. x sericans* growing on MS media supplemented with 1.0 mg l^{-1} IBA (21d).

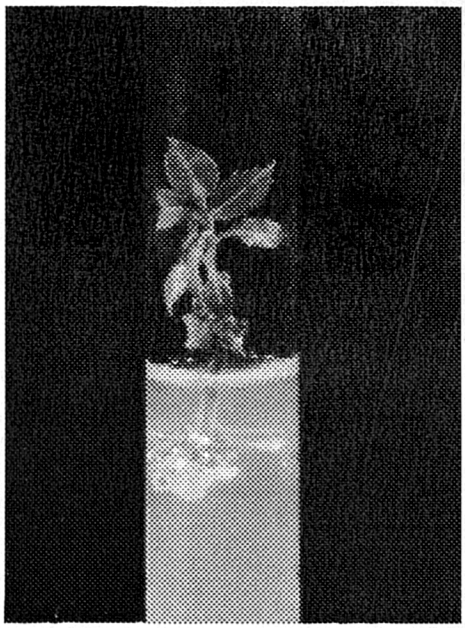


Plate 8.0. *S. caprea* (3288) established on MS media + 1.0 mg l^{-1} IBA (18d).

far the most successful response in this study was that of *S.* x *sericans* supplied with 1.0 mg l^{-1} IBA (Plate 7.0)

8.4 Discussion

Despite vigorous growth of many willow clones in solution culture, the response of single node explant material to the above culture conditions was variable and sparse. No further experimental protocols using resistant clones were carried out on cultured materials due to the large growth variation, and lack of rooted material. Successful growth of clones was not consistent throughout treatments, and was therefore attributed to variability of initial explant material. The large variation in the clonal growth of willows established in solution culture appeared to be greatly amplified *in vitro*; with similar findings for callus culture studies (Allnutt 1996). The main successes of the *in vitro* study, which suggest that further work should be carried out, was the speed at which successful explants became established; on average successful explants could be distinguished from moribund explants after only 7 days. This indicates that growth factors within the media are incorrect; and finding optimal conditions for growth of willows will entail further more detailed screening. SH basal salts were most generally successful, and are also adopted by Neuner & Beiderbeck (1992). Levels of rooting hormone may present further complications; root primordia developed on the majority of *S.* x *calodendron* explants, although the roots did not develop further (See plate 6.0).

This experiment showed that rapid propagation of willows is possible, although standard conditions may not be applicable due to clonal differences. The successful growth of the nodal segments into explants, in many cases within only 7 days indicates that the method may be able to effectively half the amount of time taken for propagation, which would be especially advantageous for *S. caprea*. However growth was arrested shortly after establishment in all explants, strongly indicating inappropriate media conditions. The poor results of subsequent subcultures of *S.*

caprea (clone 3288) (not shown) indicate that the media used here served only as an initiation media, and that further research *in vitro* in necessary to establish a suitable multiplication media.

The main advantages of micropropagation are that it allows rapid vegetative multiplication of plants with a desired genotype. Studies have shown that selection and induction of metal resistance characteristics can be successfully carried out in tissue culture (Qureshi *et al* 1981; Turner & Dickinson 1993a). However phenotypic and genotypic variation can result from certain tissue culture manipulations; those involving complete disintegration of intercellular organisation such as suspension and callus culture can result in changes in the genetic stability of the plant, and cause mutation to occur. (George 1993; Dodds & Roberts 1985). In cases where a plant having certain important traits are being propagated, preserving genetic and phenotypic integrity is important. However, research has also shown that the greater the period of time an explant spends in culture, the greater the chance of the explant becoming specifically adapted to culture conditions; for example explants can become acclimatised to certain growth hormones supplied in the media, and may lose the inherent ability to produce growth hormones themselves (George 1993).

The feasibility of establishing an *in vitro* willow collection for producing clones of desirable genotypes is questioned in this study. The ease by which willows can be propagated *in vivo* renders *in vitro* propagation largely unnecessary. In theory such a collection would only be necessary if the cellular growth changes of resistant and susceptible was being investigated in dose-response experiments.

Other uses of *Salix in vitro* investigated in preliminary studies were establishing dual cultures of willows and mycorrhizal fungi in order to study metal resistance in mycorrhizal explants. This is particularly important considering the increases in resistance symbiotic associations can provide (Bradley, Burt & Read 1982). Work

carried out as part of the present study (not included) made several attempts to establish dual cultures of *Salix* and mycorrhizae-forming fungi *in vitro*. *Hebeloma crustuliniforme* and *Pisolithus tinctorus* cultures from Sheffield University were inoculated onto a variety of granulated media formulations where media was extruded through a syringe to create air spaces to increase the surface area for fungal growth. Media formulations were identical to those used in the above *in vitro* study although substituting 30g l^{-1} sucrose with 3.5g l^{-1} glucose in order to encourage the fungi to derive carbohydrate from the host explant and thus establish an association. This however, was largely unsuccessful as the fungi became saprophytic and consumed the explant. Suggestions as to why this was the case may involve the explant itself, which was either too small or not established enough to support a fungal association. Alterations to the methodology may improve the survival of a mycorrhizal association. For example a sterile Peat:Vermiculite media supplemented with nutrient media (PVM) in which rooted cuttings are planted is an established method of establishing mycorrhizal seedlings. However, a further drawback of attempts to induce dual cultures with willow cuttings is the demand for water; nutrient media was rapidly used up in vessels containing 250 mls PVM; often in only a matter of days depending on the vigour of the cutting and had to be constantly replaced. This replacement put the sterility of the fungal culture at risk.

The optimisation of the media formulation is therefore even more important and specific when attempting to establish mycorrhizal associations, as the fungi can become saprophytic and overwhelm the explant. However, the poor rooting of many willows in the media formulations used must be resolved before any mycorrhizal work can be carried out, and it is suggested that further mycorrhizal work be carried out using more conventional PVM cultures.

Chapter IX

General discussion

9.0. General discussion

9.1. Metal resistance in Salix.

The experimental work reported in this thesis shows the existence of variability of heavy metal resistance traits within *Salix*. The expression of these traits in certain species, clones and varieties was complex. Resistance characteristics of species and clones in repeated tests was both variable and inconsistent. If an arbitrary *TI* value of 60% is used to eliminate the more susceptible clones, however, some plants showed high levels of metal resistance compared with others. *S. caprea* expressed metal resistance most consistently, in agreement with published reports of the affinity of this species for disturbed, polluted sites (Grime *et al* 1988; Eltrop *et al* 1991). This species expressed elevated copper resistance in five of the six solution culture studies, and the soil experiment. In addition, this species expressed resistance to cadmium but not zinc in solution culture. This species also possessed the greatest interclonal variation. Only one of the five *S. caprea* clones from Ness Gardens was not resistant to copper in solution but grew successfully on soil aerially contaminated with copper. *S. caprea* sampled from the mine spoil at Trelogan) (Plate 4.0 p173) had *TI* values over 100% for copper, cadmium, zinc and nickel.

The use of different types of tolerance tests gave conflicting results, in common with other workers (Turner *et al* 1991). These previous differences were thought to have arisen because the innate resistance of parent and seedling may have been different (Pye 1988; Turner 1991). Searcy & Mulcahy (1985a) also found disagreements in the expected resistance of pollen from copper tolerant and sensitive clones of *Mimulus guttatus*, which they attributed to variations in germination requirements and reduced viability. Cox (1988) also detected variable responses of coniferous and broad-leaved tree pollen to copper observing both inhibition and stimulation effects, attributed to variation in pollen requirements for copper, the level of copper nutrition in the pollen or the parent plant. Resistance characteristics from different types of tolerance test

may differ because the way in which different plant organs come into contact with heavy metals influences the level of exposure. In pollen metal exposure occurs either by translocation of heavy metals from the roots to the aerial parts, or direct exposure from aerial deposition. In root systems growing in contaminated soil exposure is invariably direct. Resistance characteristics from plants aerially contaminated and edaphically contaminated may therefore disagree.

Elevated resistance of *S. viminalis* to copper was observed in three of four solution culture tests, and elevated cadmium resistance was a consistent response. Resistance characteristics between the different clones of *S. viminalis* used were very similar, with high resistance to copper. Inhibition of root elongation tended to show a characteristic continuous pattern in this species which was also successfully acclimated to zinc. *S. viminalis* was in general less metal resistant than *S. caprea*, although it responded more favourably to acclimation to elevated zinc and cadmium concentrations.

S. cinerea (including the subspecies *cinerea* and *oleifolia* Macreight. synonymous with *atrocinerea* Brot.) showed elevated and variable resistance characteristics and shared the characteristic low rooting viability with *S. caprea*, to which it is taxanomically close. This confirms the interspecific rooting differences mentioned by Pohjonen (1991) and may indicate that more variable, resistant clones have sporadic rooting characteristics. Although both of these species were similar, *S. cinerea* was generally less metal resistant and less amenable to resistance induction than *S. caprea*. Clones from both of these species have a small growth habit, do not produce a large amount of new stem material and are both often found growing in disturbed, polluted soils (Grime *et al* 1988; Eltrop *et al* 1991). There is limited ecological evidence to suggest a tendency for hybridisation between *S. caprea* and *S. cinerea* (Grime *et al* 1988) which may be an example of the continuing evolution of this

genus. Increasing industrial pollution may be an important selection pressure for the formation of new, resistant species (Cullis 1984).

The production of adventitious roots for the majority of willow clones was not affected by metal exposure but appeared to be influenced by the age of the ramet or the position relative to the apex from which the cutting was taken (Van der Krieken, Kodde & Visse 1996).

Variation in the response of *Salix* hybrids to heavy metals was also observed. Two *S.* x *sericans* clones were tested for resistance to copper and both showed elevated resistance. *S.* x *sericans* is a hybrid between *viminalis* and *caprea* and possessed several characteristics typical of the parent species when tested in solution culture. For example the viability of this clone is similar to that of *S. caprea.* However, the root elongation characteristics of *S.* x *sericans* were similar both in magnitude and in response to copper treatment to *S. viminalis* . *S.* x *calodendron* was tested for resistance to copper, cadmium and zinc on two occasions and were resistant in both experiments.

The performance of S. x *calodendron* grown in contaminated soils was poor in common with *S. viminalis*. Comparisons between resistance characteristics in soil and solution culture are questionable and there are many reasons why they differ. Above all, the rate and magnitude of plant growth is generally greater in hydroponic culture (Hewitt 1966), due to greater nutrient availability. The strength of the nutrient solution dictates overall metal toxicity; in general the higher the calcium concentration the less toxic the metals in solution become (Wilkins 1978). The level of plant nutrients in contaminated soils varied; with Trelogan mineral spoil presenting the harshest soil environment being derived from mine waste. The duration of resistance tests may also influence the final outcome (Bradshaw & McNeilly 1981; Schatt & Ten Bookum 1992); both of these factors were different in solution culture

and soil-based tests. The concentration of H_2O-extractable heavy metals in the test soils were comparable to the concentrations of metals used in shorter term solution culture tolerance tests. Plants in the soil-based study, however, were grown for one year in contaminated soil substrates, whereas in solution culture the maximum exposure time was 128 days which may have brought about more drastic separation of susceptible and resistant clones in the soil-based study.

Despite the differences between solution culture and soil-based tests *S. caprea* clones remain the species which was most often found to have elevated *TT* values. In addition, the interclonal and intraclonal variation of this species was higher than any other.

The results gained from the solution culture tests suggest that identification of a resistant clone should depend upon the highest frequency of resistance expression in a series of repeated tests. This indicates that metal resistance may be expressed differentially within clonal material sampled from the same tree, and can be supported by several key theories. The first is the Genetic Mosaicism Hypothesis proposed by Gill (1986; 1995) and is particularly pertinent to *Salix*. The hypothesis suggests that spontaneous mutations can occur among proliferating meristems on a growing perennial, with the modular basis of plant development assuring that certain novel mutations are preserved as the plant grows. The modular growth form is also important in phenotypic plasticity (Sultan 1987) (see Glossary for definition). Gill (1995) states that clonal units are subject to higher rates of mutagenesis due to the generation of a large number of new meristems.

If clonal plants such as *Salix* are genetic mosaics, the cuttings used in solution culture tests will have been sampled from a mosaic of different phenotypes, which may explain the growth and resistance differences of cuttings sampled from the same individual. In addition, the experimental techniques used to investigate the presence

of metal resistance in willows; i.e. propagating and stressing cuttings from clonal material, fits into Gill's criteria for the generation of phenotypic and genotypic mosaics, which may explain the large variation observed within test populations; often up to 20% (Gill 1995). Gill (1986) originally applied the genetic mosaicism hypothesis to clonal plants under stress from herbivory, although the generalisations of the hypothesis can be applied to plants under other types of stress, such as chemical and nutritional stresses. McClintock (1984) suggested that the rate of somatic genome modification may be affected by environmental stress and there are examples of potentially adaptive changes in plant genomes in response to nutrient or temperature conditions which have resulted in detectable changes in nuclear DNA (Schneeberger & Cullis 1991; Cullis 1987).

Phenotypic plasticity (Sultan 1987; Schmid 1992) is presumably also an influencing factor on the induction and expression of metal resistance in trees, although it is not likely to have been induced within the scope of the above experiments using clonal material. Sultan (1987) noted that the repertoire of an individuals' phenotypic responses is genetically based, and may be to a large extent an adaptation, which has evolved in response to changing environmental conditions. *S. caprea* and *S. cinerea* tend to favour stressed environments; therefore they may posses a more adaptive expression of phenotypes, hence the variation and unpredictable nature of cuttings from these species.

9.2. The induction of metal resistance

Induction of metal resistance in herbaceous plants has been investigated previously by several workers using metal pre-treatments (Aniol 1984; Brown & Martin 1981; Baker *et al* 1986) although there remains a paucity of research using similar techniques applied to trees. Coughtrey & Martin (1981) carried out work using cadmium and found that by removing the roots of pre-treated plants any significant effect of pre-treatment on subsequent growth and development was also removed.

They also noticed a more favourable response of the tolerant plant population in response to the cadmium treatment. Based on this data they suggested that for cadmium at least, tolerance mechanisms may lie within the roots. Of the published literature on induction and acclimation of metal resistance the majority have concentrated on cadmium (Brown & Martin 1981; Baker *et al* 1986; Outridge & Hutchinson 1991), and there are less reports on the induction of copper and zinc resistance.

In the copper-resistance induction experiments of the present study a low-dose pre-treatment was unsuccessful for the majority of clones tested. Induction of elevated copper resistance capabilities may be more difficult to induce because willows were particularly susceptible to this metal. In long-term acclimation experiments using ranges of metal concentrations which began low and were progressively increased ten fold, plants showed either a stable resistance response to copper or a slight decrease; suggesting that more effective acclimation to copper can be carried out using lower concentrations, with smaller increments.

The induction of cadmium tolerance was more successful than copper. Brown & Martin (1981) also found significant increases in Cd tolerance in *Holcus lanatus* and suggested that tolerant plants may have a requirement for heavy metals (Mathys 1975). However, the preponderance of induction studies with cadmium, and the ease by which Cd resistance has been induced in other grasses (Baker *et al* 1986) and clonal ferns (Outridge & Hutchinson 1991) may support recent proposals that cadmium is plant-essential (Leavitt, Dueser & Goodell 1979). This is a contentious issue and Leavitt *et al* (1979) suggested methodological shortcomings which may have led to this conclusion. The results obtained in the present study suggest that low concentrations of cadmium have a stimulatory effect on the growth of *Salix* cuttings in common with Brown & Martin (1981) and Coughtrey & Martin (1977) studying *H. lanatus*.

A further short-term investigation cadmium resistance of *S. burjatica* in single-flask solution culture was carried out to clarify unexpected findings of cadmium resistance. This study confirmed the trends observed in previous hydroponic tests. The levels of cadmium used in this study were higher than those reported in many published findings which have claimed to separate Cd-resistant ecotypes from normal grass populations (Coughtrey & Martin 1977) and caused significant growth reduction in other studies (Burton *et al* 1984; Godbold *et al* 1985a; Asp *et al* 1994). Data indicated that non-acclimated *S. burjatica* were able to tolerate elevated concentrations of Cd and may indicate constitutional resistance present within members of the genus.

Acclimation of willows to copper and zinc was in general much less successful than cadmium. Copper was the most toxic metal studied and root systems of treated plants were morphologically different to controls. Roots and lateral branches were stunted, discoloured, and appeared thickened. However, there were no obvious differences between the root systems of control plants and those of Cd-treated plants. Tolerance to copper has been observed in many herbaceous mine ecotypes, although willows seem particularly susceptible to this metal, perhaps as a consequence of the disruption in water relations that result from copper toxicity. Copper has been found to induce leakage of K^+ in the root cells of higher plants such as *Agrostis capillaris* (Wainwright & Woolhouse 1977); *Silene vulgaris* (De Vos *et al* 1991) and *Mimulus guttatus* (Strange & MacNair 1991). It is reasonable to assume that tree roots may also suffer K^+ leakage in response to copper which has several important consequences for water, mineral and pollutant uptake. Excessive leakage of K^+ creates an artificially high negative internal electrical potential; and disrupts ion transport mechanisms and water uptake (Markert 1993). There are no reports of the effect of copper on the water relations of *Salix* and the ecology of certain members of the genus suggest that many species are heavily dependant on a copious water supply (Grime *et al* 1988). Non-riparian species such as *S. caprea* and *S. cinerea*, however,

are not as strongly associated with very moist soils, and can survive on very thin layers of substrate (Meikle 1984), in theory these species may be more resistant to water stress and may therefore be able to tolerate metal-induced water stress. This may be a factor in the susceptibility of many willow species to high concentrations of plant-available copper; and may also explain why hardier, non-riparian species have greater copper resistance characteristics.

Several clones tested in the present study also showed resistance to zinc, including *S. fragilis* and *S.* x *calodendron* . In general *Salix* spp. acclimation to zinc was successful; in some cases growth responses of plants exposed to zinc were similar to control grown plants. Zinc acclimation was most successful for *S. viminalis* . Zinc may also be connected with the induction of water stress (Barceló & Poschenreider 1990) and there is evidence to suggest that zinc influences the size of meristems and the formation of root hairs (Powell *et al* 1988). Root dry weights of the majority of species tested were reduced in response to zinc although both root and stem growth of *S. burjatica* increased when treated with zinc.

Willows exposed to dual combinations of metals in solution culture were much more susceptible than to single metals. This suggests that the metals tested have different mechanisms of toxicity in willow roots, therefore supplying more than one metal may bring about more than one toxicity response. Metal combinations were supplied at half the concentration of single metals and slightly greater growth inhibition effects were observed. This suggests that the particular metal ions supplied is just as important, if not more so, than the concentration at which they are supplied. Furthermore, there is evidence that certain metal combinations are more toxic than others. Supplying clones with elevated cadmium in solution culture caused less root inhibition than in combination with either copper or zinc. The exception to this general finding was again *S. caprea.* Greater *TI* values were observed in the mixed metal-treated plants than those exposed to single metals; this may have been distorted

by the poor growth of control plants, nonetheless treated plants showed a steady growth increase throughout the duration of the test.

Given appropriate acclimation conditions *Salix* clones can be induced to grow in the presence of a combination of heavy metals. Resistance induction to more than one metal presents a greater challenge, but is more indicative of field conditions. The long-lived nature of trees may explain the success of the longer-term acclimation experiments and the failure of the short-term pre-treatment experiment. In this study the cuttings which were grown for 28 days in un-amended background solution were more able to tolerate subsequent metal exposure than those previously exposed to copper; suggesting that cuttings which have been allowed to establish normal adventitious roots are more able to survive metal contamination than those which are poorly established in the presence of metals. This supports the findings of Dickinson *et al* (1991a&b, 1992) who detected copper-tolerance in mature *Acer pseudoplatanus* L. (sycamore) which were established prior to aerial contamination of the site by a nearby copper smelter, and noted a lack of seedling establishment. Cutting establishment may be a particularly susceptible stage for metal toxicity, although this has not been studied so far. The effect of metals on the development of viable *Salix* cuttings was also investigated both in the short-term and long term induction experiments. Findings indicated that there was greater variation in rooting viability in short-term tests than in the longer tests, indicating that the earlier a woody plant cutting is exposed to metals during the course of its establishment, the more susceptible it is to toxicity.

9.3. Uptake and accumulation of heavy metals by Salix.

Considerable differences were observed in both the uptake and accumulation of heavy metals within the various plant compartments of *Salix*. The main trends which emerged from accumulation studies were that very high concentrations of copper were found within the roots of plants treated with copper either singly or in

combination. The accumulation of greater metal concentrations within the roots of treated plants is a typical finding, and has been attributed to the fact that roots are the first point of metal entry into plants growing on polluted soil (Breckle 1991; Barceló & Poschenreider 1990). However, when the uptake of copper was investigated in more detail and compared to cadmium and zinc, results suggested again that the characteristics of the individual metal is critical to uptake and accumulation.

There was also a great deal of interspecific variation in metal accumulation; in Section 4.3 *S. caprea* accumulated a greater concentration of copper in the leaves than any other clone tested whereas *S. viminalis* and *S. purpurea* accumulated more copper in the woody component (which in this test also included new stem material).

Investigations of metal concentrations in the various plant organs indicate that at similar concentrations copper transport to aerial tissues is limited compared to cadmium. The individual characteristics of the metal elements studied in this work are summarised in Table 1.0 and are of particular significance in relation to accumulation characteristics. As indicated by the pK value, copper typically forms more stable complexes with organic molecules, and is therefore less mobile once a bond is formed. Cadmium forms less stable complexes and is therefore more mobile; it is taken up into the plant where it can then be translocated to the aerial tissues. The natural impedances to copper uptake may play a large role in successful induction of resistance and subsequently bioremediation, because copper may not enter the symplast.

Statistical analysis of metal analysis results showed that there were no significant differences in copper accumulation between difference clones although there were significant interspecific differences between accumulation of both cadmium and zinc. The non-specific compartmentalisation of copper by *Salix* in this investigation indicates that it is the bioavailability of copper which controls its movement through

plant tissues, as a result of its behaviour when in contact with biomolecules and organic compounds in general (Borovik 1989).

When copper is supplied in dual-combination treatment with either cadmium or zinc, however, there are significant clonal differences. In all clones except *S. caprea* more copper was detected in the roots of cuttings exposed to copper + zinc than copper + cadmium. The concentration of copper in woody tissue, stem and leaf material were also higher in cuttings treated with copper + zinc. In some cases up to twice as much copper was detected in the aerial tissues of cuttings when zinc was also applied. It would appear from this study that copper quickly becomes bound to available sites within the plant and since the first organ encountered is the root system, this typically contains the majority of bound copper. This may also explain why very little copper is translocated into the aerial parts of the cuttings. The concentration of copper bound within the roots may prevent further uptake by stunting and reducing the efficiency of the roots system. Alloway (1995) notes that uptake of a metal element is not only determined by the quantity of that metal present within the soil solution, but also depends upon the amount of root produced.

Zinc is a more biologically mobile metal compared to copper. Both copper and zinc are plant-essential micronutrients and a pathway already exists for their uptake. The results of the metal analyses of various *Salix* tissues after exposure to dual combinations of heavy metals is in contrast to accepted theories (Alloway 1995) where ions such as copper and zinc, which are thought to be absorbed via similar mechanisms, will actively complete with each other at the plant interface and one will inhibit the other. The results of these studies are therefore unexpected.

Cadmium uptake was greatly restricted in *Salix*, and there were no dramatic differences between the concentrations detected in leaf, stem, woody tissue and roots as there are in plants treated with copper. Uptake patterns for cadmium indicated that

in *S. caprea* there is no preferential accumulation in the root tissues, despite early findings that cadmium binds preferentially to the roots (Pettersson 1976). The much lower uptake of cadmium into the plant tissues may be result of its non-essentiality (Epstein 1972; Petit, Ringoet & Myttenaere 1978). The results for other *Salix* clones however, showed different compartmentalisation characteristics. For example, in general Cd uptake into cuttings was lower in *S. caprea*. When cadmium was supplied with either copper or zinc, the concentrations of cadmium effectively doubled in *Salix caprea, S.* x *calodendron* and *S. burjatica*. *S. caprea* was found to have a greater concentration of Cd within roots in single metal tests whereas the addition of zinc increased the uptake into leaf material a pattern which is similar to copper. The reasons why uptake increased in cuttings treated with two heavy metals rather than one, may lie in the different toxicity mechanisms. Mixtures of metals were more toxic to *Salix* despite being supplied at a reduced rate in comparison with single metal tests. This indicated that the higher resultant toxicity may have been the result of interaction, or antagonism; where each metal element has a different toxicity mechanism. The increased uptake may be a result of membrane damage and an increase in susceptibility caused by metal interaction. Alloway (1995) notes that interaction and antagonism between metal elements can occur both adjacent to and within the cells.

Like copper, uptake and compartmentalisation of zinc occurred to a much greater extent than cadmium for all the clones tested. In contrast with copper, however, zinc was taken up into all of the tissue compartments in more comparable amounts. Unlike the other species-metal combination analysed the pattern for zinc varied to a much greater extent with the specific clone under test. The presence of an additional metal in solution also changed the compartmentalisation characteristics of zinc. Supplying *S. caprea* with copper in addition to zinc increased the levels of zinc in the leaves ten fold and when cadmium was added leaf concentrations were increased six-fold compared to singly treated plants. In *S. viminalis* the zinc compartmentalisation

characteristics remain the same but the concentration of zinc in plants treated with copper + zinc and cadmium+ zinc are also much greater.

From all of the trends described above the most prominent is the enhanced metal uptake by *Salix* tissues when more than one metal is supplied. In the soil test, all substrates were tested for metal concentration, where the analysis took only four metals into consideration, although there may conceivably have been more guest elements present in the soils; especially in Trelogan spoil. The response of *Salix* to heavy metals has been encouraging. Extrapolated values calculated for the total metal concentration found in *Salix* cuttings, indicate the amount of metal theoretically removable during coppicing. In cuttings exposed to single metal treatments the most significant finding is the larger amount of cadmium which can be removed in all of the *Salix* clones. Exposure to cadmium did not cause a marked phytotoxic response in any of the cuttings tested; it was not accumulated within tissue compartments to the same extent as copper and zinc, the combination of which appears to have facilitated a greater uptake of cadmium into the aerial tissues. Significant concentrations of cadmium and zinc are realistically removable using *Salix*.

In soil studies, which were ran over a longer period of time, higher concentrations of metal were detected in plant tissue. Harvest data show that leaf and new stem production in contaminated substrate was negligible for all but *S. caprea* 'Loughgall' (3288). Plants grown on soil from Trelogan accumulated slightly larger concentrations of Cd than those grown in solution culture tests, however, once again uptake of cadmium in to individual compartments was restricted.

If the amount of metal accumulated within the aerial tissues are calculated as a percentage of the total metal accumulated an extrapolation of the concentration of metal which may effectively be removed by *Salix* can be made. Extrapolations of total metal uptake by plants exposed to mixtures revealed that the enhanced uptake is

combined with reduced root biomass. Levels of metals may be higher on a $\mu g\ g^{-1}$ basis in plants given two metals rather than one; although the greater inhibition in growth that this causes translates to lower total metal concentrations per plant and the harvestable proportion of metal taken up is also reduced in solution.

9.4. The implications for use of Salix *in bioremediation*

The results of uptake and accumulation studies in solution culture indicate that there are limitations to the use of non-hyperaccumulating plants in bioremediation of contaminated substrates. The first limitation is that the more heavy metals present in the soil, the more growth will be inhibited and the more difficult induction will become. Secondly, resistance studies indicated that plants which are naturally resistant to heavy metals are usually slow growing, often with a smaller growth habit. Small growth habits may facilitate metal resistance (Ernst *et al* 1992) but faster growth is more desirable for bioremediation. Fast-growing shrubs which produce a large amount of above ground biomass are preferable, but are generally less tolerant. Observations of the growth of *S. viminalis* clones in solution culture in this study indicated that although these clones had a higher rooting viability and initially grew longer roots and shoots, they rapidly became chlorotic and died. In contrast, *S. caprea* clones grew very slowly, producing shorter root system and new stems, and remained healthy, showing only slight chlorosis on leaf tips. The compromise then is to choose between a fast growing shrub with high mortality, or a slower-growing hardier shrub.

The variation in the growth responses of *Salix* in this study have given rise to some experimental uncertainty; often clones which have shown resistance, fail to do so in further tests. This variability, however inconvenient for scientific analysis, may be the driving force behind the invasion of metal contaminated areas by the hardier members of the genus, as observed by Eltrop *et al* (1991). The work of Wu, Bradshaw & Thurman (1975) sought to quantify the proportion of a normal

population which when screened showed an ability to tolerate heavy metals; this proportion was approximately 0.02%, a figure which is statistically insignificant. In natural selection (and perhaps artificial selection) the small number of naturally resistant individuals must be significant if they can form the basis of a resistant population. The results of this thesis indicate that this important natural variation, whether genotypic or phenotypic, is abundant within *Salix*, making them valuable bioremediation shrubs.

9.5. Conclusions

- Heavy metal resistance in *Salix* was avraible between and within species.
- Elevated cadmium concentrations did not reduce the growth of *Salix* cuttings.
- The order of toxicity of the metals tested to *Salix* was: Ni>Cu>Zn>Cd.
- *Salix caprea* was the most resistant species, showing elevated resistance to Cu and Cd in both solution culture and soil experiments.
- The resistance of *S. viminalis* to zinc was poor in initial experiments ($TI_{[20mg/l]}$=34%), although after successful acclimation using cumulative dosing resistance was greatly increased ($TI_{[25.8mg/l]}$>100%).
- Metal resistance of hybrids between *S. caprea* and *S. viminalis* was intermediate between the parent species and there was some evidence of hybrid vigour.
- Differences in metal resistance of some species tested in both solution and soil culture were attributed to differential availability of heavy metals in solution and soil, the presence of additional toxic 'guest' metals in contaminates soils, the different lengths of test period in soil and solution culture and the enhanced growth of cuttings in solution culture.
- Accurate identification of metal resistance in variable clonal trees such as *Salix* is only possible by repeated tests of a wide range of clonal material.
- Growth variability in *Salix* may be a product of genetic mosaicism which is enhanced by vegetative propagation and stress.

- Resistance induction in *Salix* was successful after long-term acclimation to cumulative metal concentration, but not after short-term pre-treatments; this failure was partially attributed to the importance of healthy establishment of cutting material for subsequent resistance.
- Dual combinations of Cu, Cd and Zn were more toxic to *Salix* than single metal treatments and resulted in enhances metal accumulation in plant tissues.
- Copper accumulated primarily in the roots of treated plants with evidence of restricted uptake in to aerial tissues.
- There were no significant differences in copper accumulation in long-term experiments, but interspecific differences were evident in short-term tests.
- When supplied singly, zinc was accumulated to a greater extent than cadmium, with comparable accumulation between different tissue compartments for both of these metals.
- Exposure to dual-combination metal treatment resulted in greater accumulation of Cu in root tissues and Cd primarily in root but also in leaf tissue. Reduced accumulation of Zn in aerial tissues accurred whrn in combination with Cd.

9.6. Recommendations

- Selected *Salix* species such as *S. caprea* should be used in bioremedation programmes, especially those on land contaminated with cadmium.
- Less resistant, economically important species such as *Salix viminalis* can only be used successfully in bioremediation programmes following periods of acclimation to metals likely to be encountered in contaminated soil.
- The resistance of *Salix* spp. to cadmium; the levels at which Cd becomes toxic and possible mechanisms for the observed resistance should be considered further.
- A more detailed investigation into zinc and nickel resistance is necessary as these metals have been dealt with only briefly in this study.
- The production of mycorrhizal *Salix* cuttings is of great importance to the success of bioremediation schemes. Attempts to produce mycorrhizal explants *in vitro*

proved to be time-consuming and presented problems with high rooting viability; further manipulations of mycorrhizal *Salix* should be carried out using a peat: vermiculite system.

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Appendix I

Response of S. burjatica to elevated cadmium

Appendix I

Further investigation: innate Cd resistance of *Salix burjatica*

Background

Further tests on the responses of *Salix* clones to elevated cadmium in solution were carried out after findings of both studies 4.1 and 5.2 where clones grew in solutions containing up to 1.0 ppm and 1.5 ppm respectively without any substantial reduction in growth. The following test investigated the response of *Salix burjatica* Nazarov. "Aquatica gigantea" (Clone 3349) a vigorous clone with high survivorship in solution which previously showed no growth inhibition responses to increasing concentrations of cadmium (Section 5.2; figure 5.31). There have been no toxic threshold, or EC_{100} values established for this metal in *Salix*, and the high innate resistance and growth stimulation observed when willow cuttings are exposed to cadmium disagrees with previous findings for other species.

Coughtrey & Martin (1977) investigated cadmium tolerance in the grass *Holcus lanatus* ecotypes sampled from both clean and contaminated sites; using Cd concentrations of 1 and 2 ppm to separate resistant and susceptible populations. Brown & Martin (1981) used lower cadmium concentrations in tests several years later on the same ecotypes; only 0.2 and 1.0 ppm in solution. Furthermore, Baker *et al* (1986), also using the *Holcus lanatus* 'Hallen Wood' ecotype studied by previous workers, once again separating resistant and susceptible populations at approximately 1 ppm of cadmium. Outridge & Hutchinson (1991) induced cadmium tolerance in the winter fern *Salvinia minima* by exposing plants to 25 and 50 $\mu g\ Cd\ l^{-1}$ (equivalent to 0.025 and 0.05 ppm). Using these concentrations as a basis for separating plants into Cd-resistant or Cd-susceptible populations strongly indicates that many non-acclimated willow clones are naturally cadmium resistant.

Several workers have observed the stimulation of plant growth in response to cadmium (Brown & Martin 1981; Coughtrey & Martin 1977) and the non-selective uptake of cadmium by tree species such as *Quercus* spp. has led workers in the past to conclude that cadmium may be plant-essential (Leavitt *et al* 1979) although cadmium is still widely regarded as being toxic. The concentrations of cadmium used in the following investigation are deliberately elevated above those used in previous solution culture tests to identify the toxic threshold and to confirm the results of previous studies.

10.1. Aims

- To establish whether resistance to cadmium in solution is a consistent response of the willow clone *Salix burjatica*.
- To find the toxic threshold for cadmium exposure to *Salix burjatica*.

10.2. Materials and Methods

Cuttings of *Salix burjatica* were collected from Ness Botanic Gardens in late April 1995 and prepared for solution culture as described in Section 3.0. The cuttings were grown in distilled water until roots were approximately 4 mm in length to ensure that only viable cuttings were included in the test. Root number, length of longest root, shoot height and fresh weight of each cutting were recorded at the outset of the experiment, which used cuttings with a diameter between 3.8-7.7 mm. The experiment used independent growth units which consisted of a foil-wrapped 250 ml Erlenmeyer flask containing 200 mls of control or cadmium amended 25% Hoagland's solution (Hoagland & Arnon 1950). Aeration was provided by an aquarium aerator stone and each flask was sealed with non-absorbent cotton wool covered with parafilm. Full experimental details are included in Section 3.1.5. The experimental set up of each unit is shown in Figure 1.0 (p46). Cadmium treatments consisted of a control (containing 25% strength Hoagland's solution), 2 ppm, 4 ppm and 6 ppm Cd (supplied as $8CdSO_4.2^1/_2H_2O$) with five replicate units per treatment.

Growth parameters were measured 5 and 10d after the start of the test. Cadmium concentrations in the nutrient solution were checked regularly by atomic absorption spectrophotometry. Growth data was subjected to analysis using the general linear model of the Minitab Statistical Package (Table 10.1.).

10.3. Results

Table 10.1. F values from GLM analysis of growth data.
*** denotes $P < 0.0001$; ** denotes $P < 0.05$ and ns = not significant ($P > 0.05$)

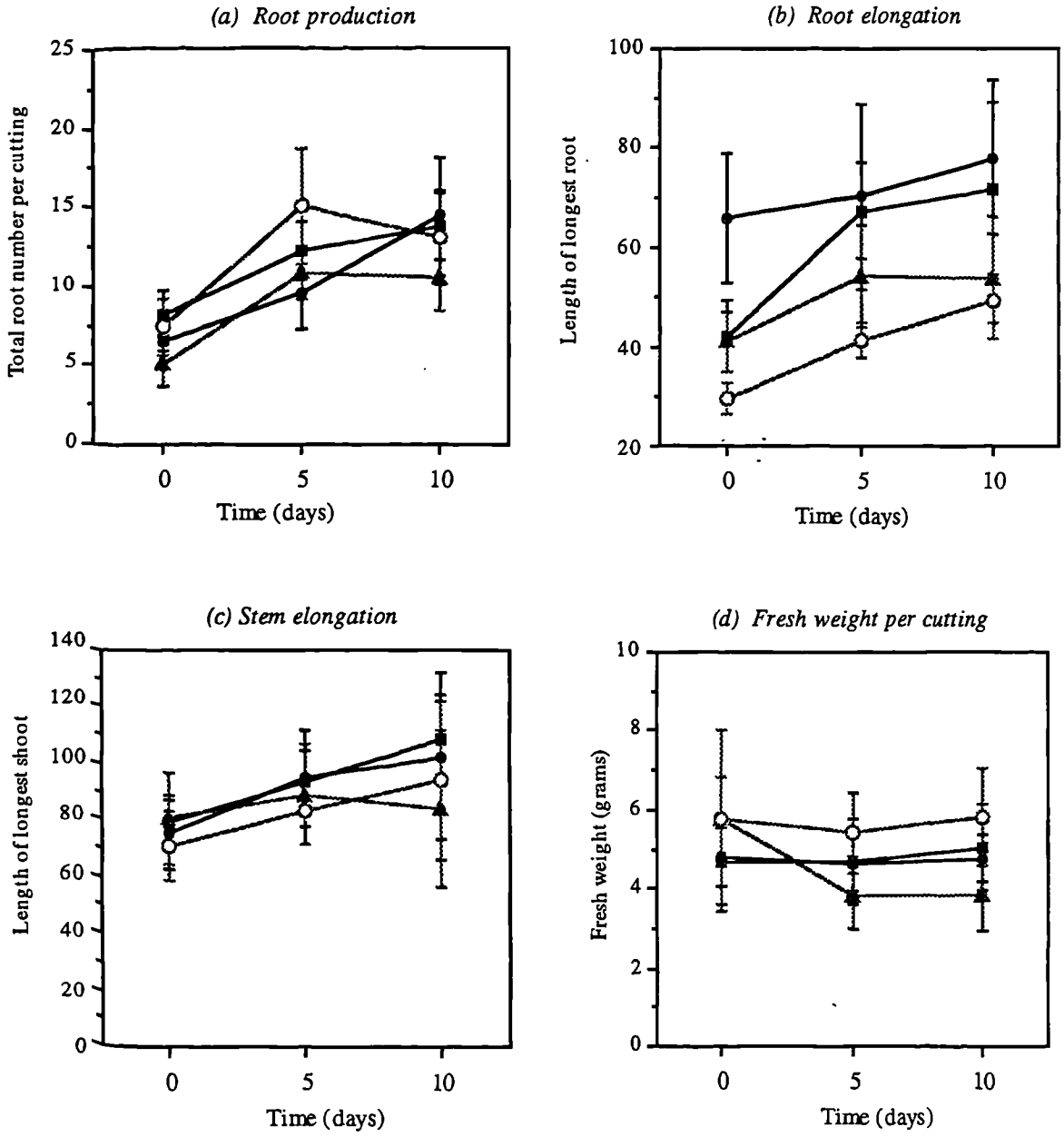
Growth response	Source(Degrees of Freedom)		
	Cd treatment$_3$	**Time$_2$**	**Interaction$_6$**
Root number	1.02ns	8.16**	0.42ns
Root length	4.32**	2.94ns	0.21ns
Shoot height	0.27ns	1.48ns	0.992ns
Fresh weight	1.663ns	0.05ns	0.02ns

Growth responses of *S. burjatica* to elevated cadmium are shown in Figure 10.1 and show that only root elongation is significantly affected by elevated cadmium concentrations. However, plants treated with both 2 and 4 ppm of cadmium show a greater rate of growth between the start of the test and day 5. Furthermore the root elongation of plants treated with 6 ppm Cd still show a steady increase, with a similar growth rate to that of control plants. The changes in fresh weight of the cuttings was minimal in the time period over which the test ran, suggesting that this parameter was not viable. Root production changed significantly over time, but was not affected by cadmium treatment.

Figure 10.1 Growth responses of Salix burjatica exposed to highly elevated cadmium concentrations for 10 days

Cadmium treatments: Control 2ppm 4ppm 6ppm

(Means and standard deviation where n=5)



10.4. Discussion

Phytotoxicity in response to elevated cadmium in solution was not identified in this test and results suggest that the toxic threshold lies considerably higher than 6ppm. The results of this additional test indicate that non-acclimated willow clones posses resistance to cadmium in solution culture. The inhibitory effect of cadmium on root elongation is, however, questionable when rates of elongation are calculated. For example in control-grown plants the root elongation rate is 0.64 mm day^{-1}; whereas in Cd treated plants rates were much higher; 0.8 mm day^{-1} for plants exposed to 2 ppm Cd; 1.16 mm day^{-1} for 4 ppm Cd and 1.52 mm day^{-1} for exposure to 6 ppm. This shows that the concentrations of cadmium used did not have any serious deleterious effects on the growth of *Salix burjatica*.

The innate resistance of *S. burjatica* and other willows to elevated cadmium in solution is an unexpected result, and is in contrast with many published findings. Cadmium is a non-essential element which is strongly phytotoxic (Clijsters & Van Assche 1985). The effects of cadmium on plant tissues is well documented; one of the most sensitive parameter being root elongation; concentrations of between 5-60 μM Cd (approximately 0.5-6.0 ppm) significantly reduced elongation of *P. abies* seedlings (Godbold *et al* 1991). Burton *et al* (1984) observed a reduction in root length of *Picea sitchensis* (sitka spruce) when Cd concentration in the soil exceeded 2.5 ppm, taken to be the toxic threshold for this metal. Asp *et al* (1994) studied the effect of cadmium on K^+ uptake in K^+-starved *Betula pendula* and found that when concentration of 2 or 5 μM (approximately 0.2 or 0.5 ppm) were present in the nutrient solution the normal pattern of K^+ recovery was suppressed, although they noted some recovery after prolonged exposure (20 hours). In addition studies by Arduini *et al* (1994) demonstrated that root elongation in *Pinus pinea* and *P. pinaster* was more sensitive to cadmium than cell division by examining root system density and cortex width.

As well as root growth, cadmium is reported to significantly affect photosynthesis (Baszynski *et al* 1980; Clijsters & Van Assche 1985). Baszynski *et al* (1980) noted that 0.2 ppm cadmium caused degeneration in chloroplast structure in *Lycopersicum esculentum*, and Bazzaz & Govindjee (1974b) found a 20-30% decrease in chlorophylls in chloroplasts exposed to 0.5 ppm Cd. Whereas the willow cuttings exposed to elevated cadmium concentrations in this experiment were only very slightly chlorotic.

Resistance mechanisms are thought to involve energy expenditure, or some metabolic cost (Brown & Martin 1981; Wilson 1988), which may involve a compensatory reduction in growth; however, so such reductions were observed after 10 days exposure to cadmium in this test. Ernst (1976) estimated that tolerant plants have 20-50% lower total biomass production than normal ecotypes, once again reduction in growth or biomass (see Section 5.2i) was repeatedly not found in this experiment.

Evidence that *Salix* in particular are cadmium resistant has arisen from the recent use of willows as vegetation filters for sewage sludge and waste waters. Findings by several workers (Göransson & Phillipot 1994; Landberg & Greger 1994; Östman 1994; Ericson 1994) strongly support the findings of this study and suggest that *Salix* is a useful tree species for amelioration of polluted soil. Göransson & Phillipot (1994) claimed that fast-growing trees could theoretically remove 1.5 kg Cd ha^{-1} yr^{-1} without disturbance in growth. Landberg & Greger (1994) also observed general tolerance of *S. daphnoides, S. triandra, S. purpurea* and *S. viminalis* at Cd concentrations upto 10μM (approximately 1.12 ppm) as well as considerable interclonal variation in response to heavy metals.

Theories on metal resistance which apply specifically to cadmium include the production of metallothionein-like metal complexes which sequesters the metal ion in a form which is harmless to the cell (Jackson *et al* 1987; Tomsett & Thurman 1988;

Robinson 1989). Theories concerning the role that phytochelatin compounds play in metal resistance is debatable; Schat & Kalff (1992) tested copper tolerance and phytochelatin production in *Silene vulgaris* and concluded that the amount of phytochelatin was indicative only of the amount of toxicity experienced by the plant under test. They proposed that phytochelatins are not related to tolerance, rather they are a metal-imposed strain.

The methodology used in this experiment was different to the majority of solution culture studies performed in that independent hydroponic units were used instead of the large 20 litre containers. The single-flask method gave successful results, although the replicate numbers were much lower due to restrictions on space. Furthermore, some individual exhausted the nutrient solution sooner than others, therefore the solutions had to be changed after shorter intervals to prevent plant injury from water stress. The different rate of solution uptake was a potential source of experimental error because solution replacement and metal dosing should be identical for every individual. Therefore this investigation strongly supports the methodology of the other solution culture tests.

Appendix II

Publications

Proceedings of the British Ecological Society Conference "Trees and Bioremediation" Glasgow 1995, Institute of Chartered Foresters (*in press*).

The potential of *Salix* clones for bioremediating metal polluted soil.

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Abstract

Bioremediation involves stabilisation and amelioration of soils contaminated with high concentrations of toxic metals such as copper, cadmium and zinc. This paper investigates which *Salix* species and hybrids have the potential to effectively bioremediate metal polluted soil. Twenty-three clones (eleven species and five hybrids) were screened in nutrient solution amended with toxic concentrations of metals to determine their innate metal resistance. Selected clones were then used in induction experiments to attempt to increase resistance levels. The study showed that hybrids of *S. caprea* (naturally resistant) and *S. viminalis* (fast-growing biomass shrub) have considerable potential for use in bioremediation. Certain clones were also able to take up metals and compartmentalise them within their woody tissues; most notably in the case of cadmium; this may be beneficial if the metals are to be removed from the soil on a long-term basis.

Key words: heavy metals, tolerance, bioremediation, Salix, copper, cadmium, zinc, uptake.

Introduction

Contamination of the biosphere by heavy metals is an established environmental problem (Alloway 1995; Nriagu & Pacyna 1988). Metal extraction, smelting of metalliferous ores and disposal of sewage sludge to land all contribute to the increasing heavy metal load of soil in particular, which constitutes a major sink for these pollutants. There is now an estimated 4000 km^2 of contaminated agricultural land in the UK as a result of these activities (Thornton 1980). Furthermore, sparsely vegetated contaminated areas can be the source of further environmental pollution as a result of weathering, erosion and leaching of dissolved pollutants (Merrington 1995).

Plant populations surviving in heavily contaminated soils have been extensively studied. Early laboratory-based experiments showed distinct heavy metal tolerance characteristics in mine populations of herbaceous plants (Prat 1934; Bradshaw 1952) and this has also been observed in woody species more recently (Brown & Wilkin 1985; Eltrop *et al* 1991). The tolerance traits of these

ecotypes is a well established example of evolution to anthropogenic selection pressure (Baker 1987; Ernst *et al* 1992). Several workers have realised the potential of metal-resistant plants in remediating contaminated areas (Gadgil 1969; Smith & Bradshaw 1972, 1976). McCormack & Steiner (1978) suggested woody plants may be suitable for use in bioremediation after screening various tree species in solution culture for aluminium resistance. Their results were particularly encouraging and revealed that many woody species, for example *Betula,* possessed levels of tolerance well above those observed in some crop species. The concept of reclaiming polluted sites using plants was carried further by Baker (1988) who reported the existence of plants that are able to hyperaccumulate large concentrations of toxic metals from serpentine, and other edaphically-extreme soils. The uptake and translocation of heavy metals by some species may ameliorate the condition of the soil by reducing the plant-available metal content. This combination of soil stabilisation and metal removal by plants constitutes a potentially worthwhile and economic biological remediation technique, commonly termed 'bioremediation'. Rulkens, Tichy & Grotenhuis (1995) reviewed bioremediation as well as widely-used chemical remediation techniques. This work highlights some important comparisons between biological and chemical techniques. Many chemical techniques; involving soil excavation, acid washing and treatment with a chemical extractant are only effective on sandy soils, and leave a sterile, unvegetated and structureless substrate. An alternative biological method would be a cheaper, safer *in situ* remediation technique which does not affect the soil microflora or structure. However, the main disadvantage of bioremediation is the long time scales that may be involved; Ernst (1988) predicted that several centuries would be required to complete site reclamation, although Baker *et al* (1991) proposed that this could be reduced to a few cropping periods.

Effective bioremediation is heavily dependent on the choice of plant species used and the present study was based on the assumption that bioremediation species should have the following characteristics:

- posses a metal resistance trait.
- posses a deep root system to stabilise metalliferous substrate and improve soil structure.
- be vigorous and fast-growing
- have an ability to grow on nutrient-poor soil.
- have an economically viable secondary use; e.g. biofuel.

Trees and shrubs in the genus *Salix* fulfil all of the above criteria. They are adapted to a variety of environmental conditions and ecological habitats, including industrially contaminated and nutrient poor soil in the case of *Salix caprea, S. cinerea* and associated hybrids (Grime, Hodgson & Hunt 1988). They are also genetically variable trees with a distinct propensity for hybridisation with almost any other species in the genus (Stott 1992), allowing for selective plant breeding for hybrids with beneficial bioremediation characteristics.

The common osier (*Salix viminalis*)and its associated hybrids are valuable fast-growing biomass shrubs which have been used extensively in Sweden for the production of biofuel for the past fifteen

years, enabling a reduction in oil imports (Sennerby-Forsse 1994). In the past five years a new branch of research using willows has developed which is particularly pertinent to this study, involving the use of fast-growing biomass trees for the disposal of sewage sludge. The high concentrations of toxic metals in sludge (Alloway 1995) creates the need for trees with similar metal resistance characteristics to those required for bioremediation. The present paper presents the results of screening and resistance induction experiments carried out on a variety of native British willow species and hybrids.

Materials and Methods

Willow material was obtained from the National Willow Collection, held at the Liverpool University Botanic Gardens at Ness, Merseyside, in spring and summer 1993. The botanical collection follows a strict planting scheme, and is well labelled; identification of willow species was confirmed in the laboratory before tests began (using Meikle 1984). Willow stems, at least one year old, were repeatedly removed from the same individual within the planting scheme. All leaf material was removed, and the rods were cut in to 18cm lengths and then placed in 3.5 litre black polypropylene buckets containing 1 litre of distilled water in a glasshouse (19°C ± 5) without artificial light for one week until small leaf buds and root primordia began to develop. Viable cuttings were then transferred to a suspension hydroponic system, (Figure 1.0). Each hydroponic unit consisted of a black container tray (75 x 25 x 15cm, 20 litre capacity) constructed from inert polypropylene. A perforated support tray of identical dimensions was fitted on top, and black, heavy gauge plastic was stretched over the perforations through which willow cuttings were pushed, forming a tight but flexible seal. Each container tray was filled with 25% strength Hoagland's solution (Hoagland & Arnon 1941) adjusted to pH 5.5, aerated with four air stones connected to an aeration pump.

Metal treatments were supplied using the sulphate salts of copper, cadmium and zinc. The nutrient solution was circulated using a low-power peristaltic pump, thus solution was constantly replenished from a 25 litre reservoir of fresh solution, which was replaced every week. Each unit accommodated one metal solution treatment, and up to 400 cuttings could be planted in each unit at any one time. The cuttings were randomised within the unit. The units were placed in a controlled temperature glasshouse (19°C ± 0.5) without artificial light.

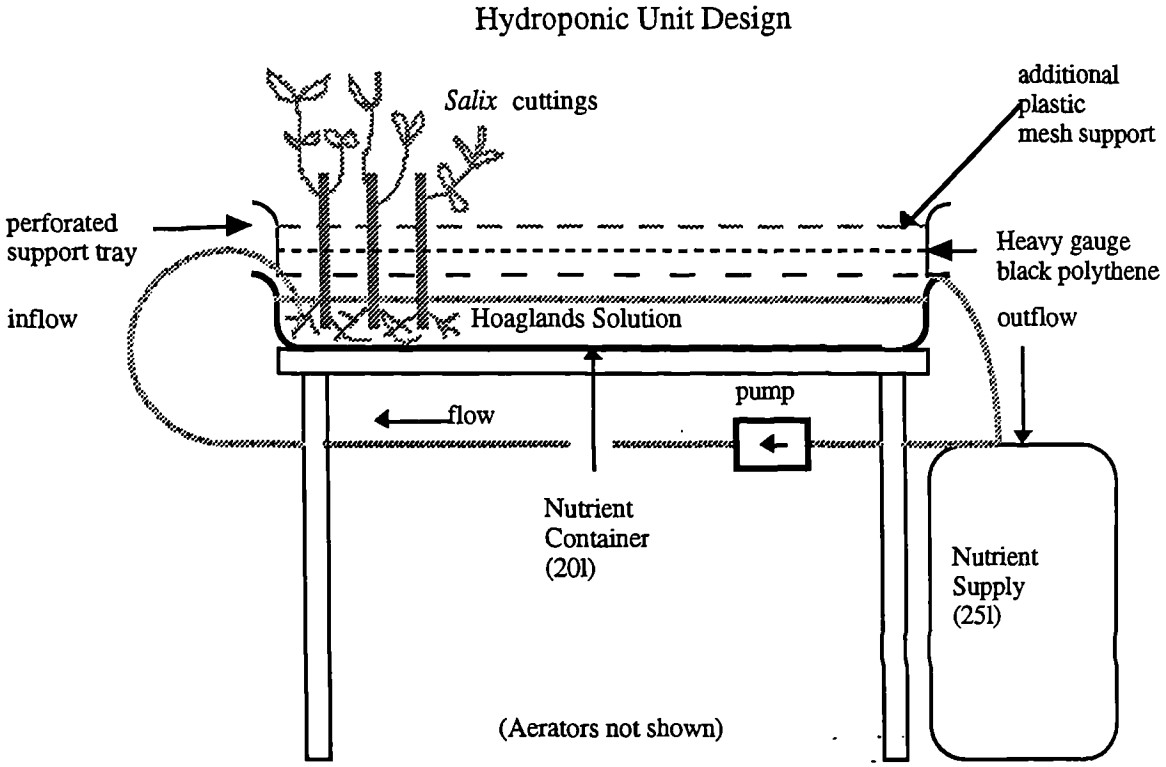

Figure 1. The suspension hydroponic system used in the following study.

Tolerance to heavy metals was estimated using a Tolerance Index (*TI*) (Wilkins 1978), based on the measurement of the longest root in metal-amended solution expressed as a percentage of the root length in background nutrient solution:

$$TI_{[M]} = \frac{\text{length of longest root in metal-amended solution}}{\text{length of longest root in background solution}} \times 100$$

Metal concentrations in plant tissues were analysed using standard atomic absorption spectrophotometry techniques (AAS). Root, wood, leaf and new stem material was dried at 80°C for 24 hours, ground to a fine powder using a mechanical grinder, and weighed aliquots digested in 10mls HNO_3 using microwave digestion techniques until samples were clear. The samples were diluted to 25ml using distilled deionised water, and analysed using a Pye Unicam AAS with deuterium lamps, an acetylene flame and background correction under standard operating conditions.

A large number of willow species and hybrids were tested in a number of solution culture experiments over a period of three years. The species and hybrids reported in the present paper are included in Table 1 (a) and (b).

Table 1. Salix *clones tested for resistance to copper, cadmium and zinc. (The accession denotes the numbering system used to identify different clones of willow at Ness Botanic Garde ns.)*

(a) Species

Species	Common Name	Accession Number
Salix caprea L.	Goat willow	Trelogan, Clywd
S. caprea L.	"	3283
S. caprea L.	"	3285
S. caprea L.	"	3288
S. caprea L.	"	3287
S. caprea L.	"	3289
S. nigricans Sm.	Dark leaved willow	3438
S. phylicifolia L.	Tea leaved willow	3450
S. viminalis L.	Common osier	3369
S. triandra L.	Almond willow	3064
S. pentandra L.	Bay leaved willow	3274
S. fragilis L.	Crack willow	3234
S. fragilis L.	"	3245
S. purpurea L.	Purple willow	3010
S. purpurea L.	"	3018
S. alba L.	White willow	3204
S. cordata	Sand dune willow	3280
S. cinerea L.	Grey willow	3294

(b) Hybrids

Hybrid name	Origin/ Parentage	Accession Number
S. x *sericans* Tausch ex. A Kern	*cap* x *vim*	3305
S. x *calodendron* Wimm.	*cap* x *cin* x *vim*	3311
S. x *aquatica gigantea* Sm.	*dasyclados* x *cap.*	3349
S. x *forbyana* Sm.	*purp.* x *cin.* x *vim.*	3340
S. x *multinervis* Doell.	*aurita* x *cin.*	3338

cap = *S. caprea*; *vim* = *viminalis*; *cin* = *cinerea*; *purp* = *purpurea.*

Experiment One: Screening *Salix* species for resistance to copper, cadmium and zinc.

The first experiment in this study tested cuttings from ten species of willow to increasing concentrations of metals in solution. The cuttings were placed in solution culture in the spring of 1993 and grown for 28 days in copper (0.2, 0.4, 0.6, 0.8 and 1.0mg l^{-1}), cadmium (0.2, 0.4, 0.6, 0.8 and 1.0mg l^{-1}) and zinc (10, 20, 30, 40 and 50mg l^{-1}) and a control solution. with background concentration of plant essential trace metals. There were five replicates of each clone and treatments were replicated twice. The units were positioned randomly throughout the glasshouse. After 28 days the cuttings were removed and measured as described above.

Results

There was considerable inter-specific variation in response of willows to metals; generally species such as *S. alba* and *S. purpurea* were very sensitive to all the metals tested. The following table shows the willow species and hybrids with elevated *TI* values :

Table 3. Willow species and hybrids with elevated metal resistance (≥70%).

Copper (0.8mgl^{-1})	Cadmium (1.0mg l^{-1})	Zinc (20mgl^{-1})
S. caprea	*S. nigricans*	*S. pentandra*
S. phylicifolia	*S. phylicifolia*	
S. pentandra	*S. pentandra*	
S. x *sericans*	*S. purpurea*	
	S. x *sericans*	

Experiment two: induction of metal resistance using pre-treatment and acclimation

The first induction tests involve a short term pre-treatment of willow cuttings to 0.25mg l^{-1} (low pre-treatment) and 0.50mg l^{-1} (high pre-treatment) of copper, after which cuttings were re-exposed to all combinations of copper pre-treatments together with control cuttings that had received no pre-treatment. The clones used in this experiment were *S. cordata* (3280) *S. fragilis* (2325) *S. caprea* (3289), *S. caprea* (3285) and *S. cinerea* (3294). Root length and root number of the cuttings was monitored at 7 day intervals throughout both pre-treatment and treatment phases so that a mean growth rate value could be used in *TI* calculations. This enabled changes in growth characteristics as a result of different metal treatments to be detected.

The second type of resistance-induction experiment involved growing cuttings in solution culture for 112 days, where concentrations of copper, cadmium and zinc were increased by 50% of the initial dose every 14 days. The starting concentrations were 0.15mg l^{-1} for both copper and cadmium and 3.5mg l^{-1}

for zinc. Cuttings were replicated fifty-four times and randomised within each unit. Length and number of roots were measured every 14 days with 28 days between the fifth and sixth measurements. *S. caprea* (3287), *S. viminalis* (33269). and their associated hybrids *S.* x *calodendron* (3311) and *S.* x *aquatica gigantea* (3349) were tested.

Results of short-term pre-treatment experiment

S. caprea and *S. fragilis* showed a definite response to pre-treatment; growth rates of pre-treated plants were higher than plants that had received no pre-treatment. Cuttings with the highest root growth rate were those continuously grown in 0.25mg l^{-1} copper throughout the experiment. This is shown below in Figures 2 and 3.

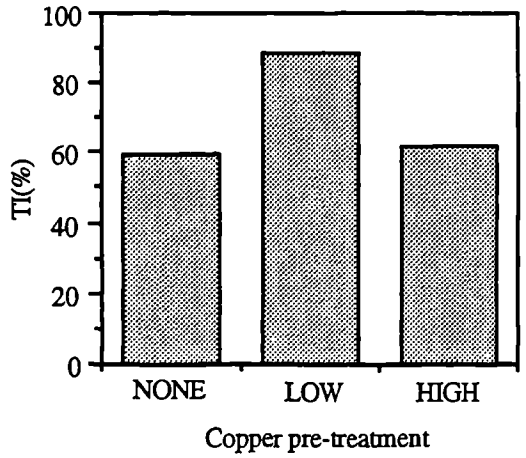

Figure 2. Copper tolerance of S. caprea *in 0.25mg l^{-1} copper in response to three pre-treatments.*

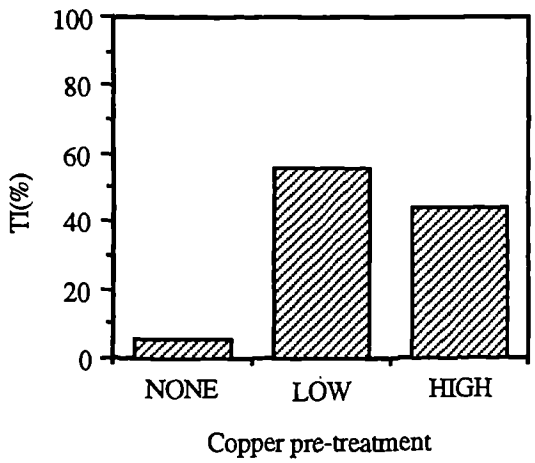

Figure 3. Copper tolerance of S. fragilis *in 0.25mg l^{-1} copper in response to three pre-treatments.*

Results of long-term acclimation test.

The *TI* values of cuttings grown in increasing copper, cadmium and zinc-amended solution are shown below in Figures 4-7. The *TI* values for copper remain more or less constant, whereas *TI* values for cadmium and to a lesser extent zinc, increased with prolonged exposure to these metals. Marked increase in *TI* values was observed for cadmium in all willow species except *S. caprea* and there was no inhibition of root growth observed for cadmium treated plants.

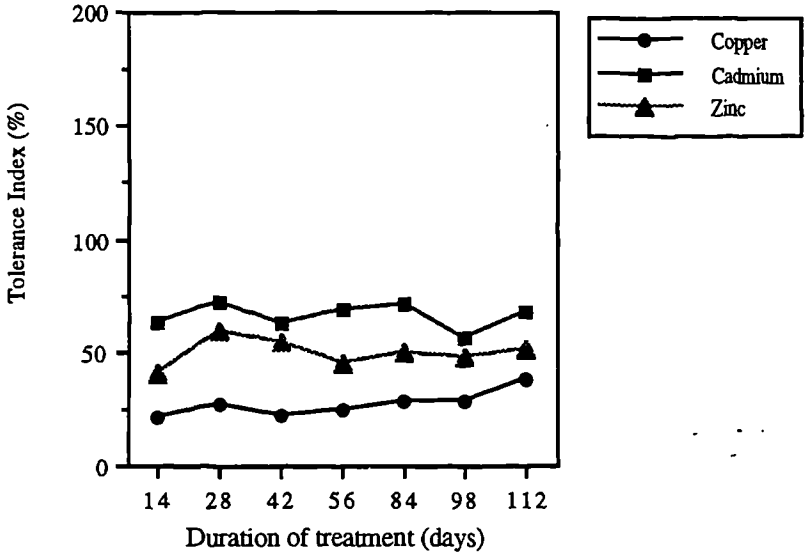

Figure 4. TI *of* S. caprea *to cumulative treatments of Cu, Cd and Zn over 112 days*

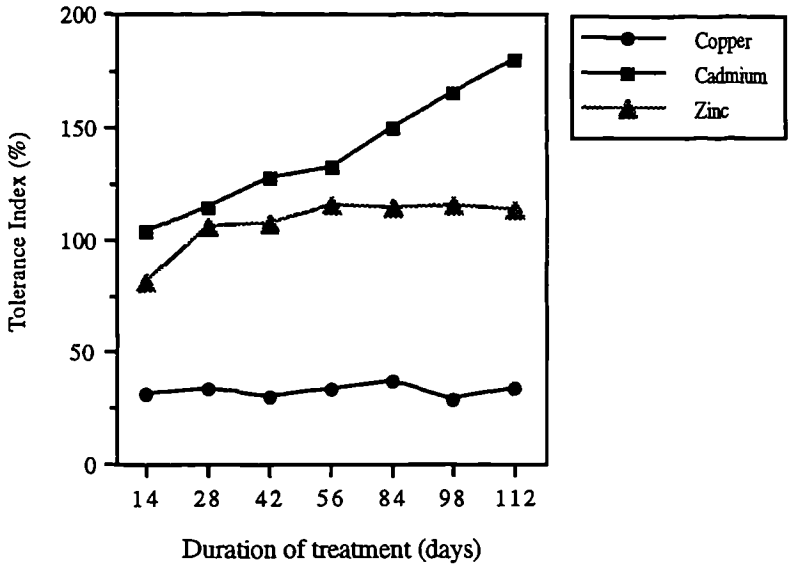

Figure 5. TI *of* S. viminalis *to cumulative treatments of Cu, Cd and Zn over 112 days.*

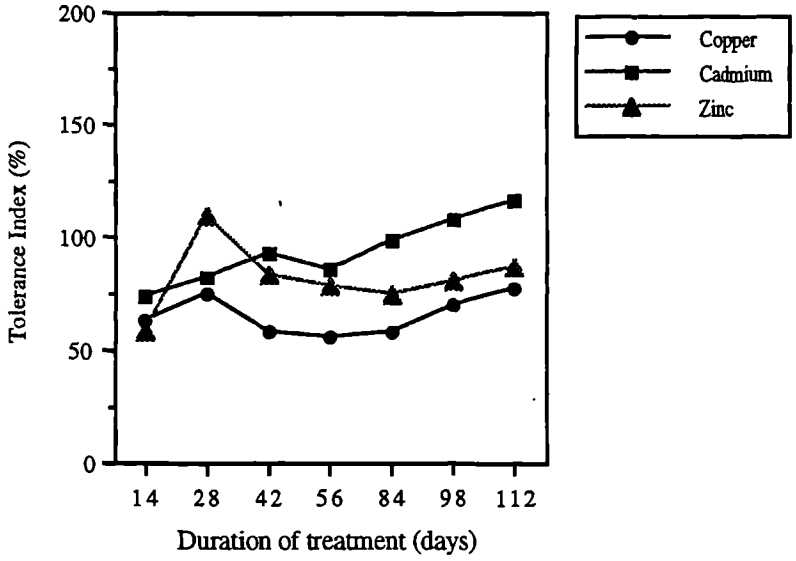

Figure 6. TI *of* S. x calodendron *to cumulative treatments of Cu, Cd and Zn over 112 days*

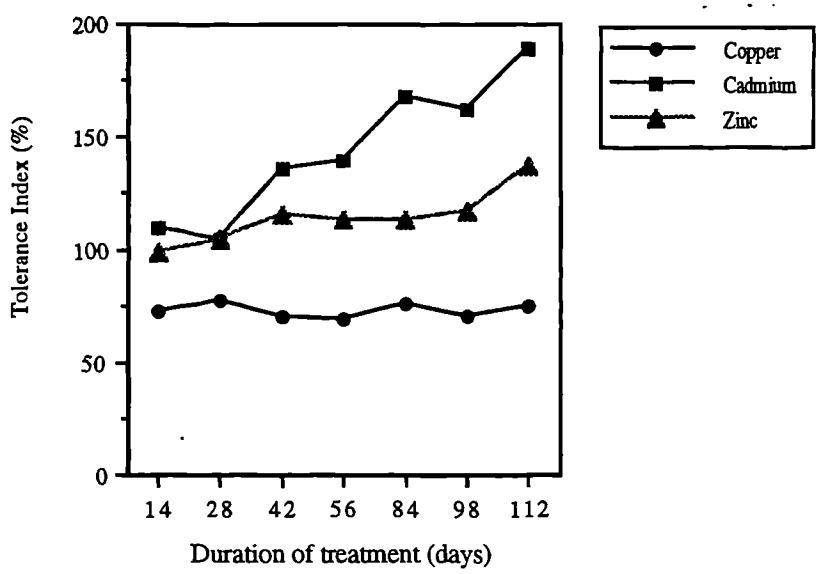

Figure 7. TI *of* S. x aquatica gigantea *to cumulative treatments of Cu, Cd and Zn over 112 days.*

Uptake and accumulation of heavy metals in willow

There was greater uptake of copper and zinc in *S. caprea*, and *S.* x *calodendron* had the greatest concentration of cadmium. In all cases, metal uptake was lowest in *S. viminalis* . The uptake of heavy metals for the different willows tested is summarised in Figure 8.

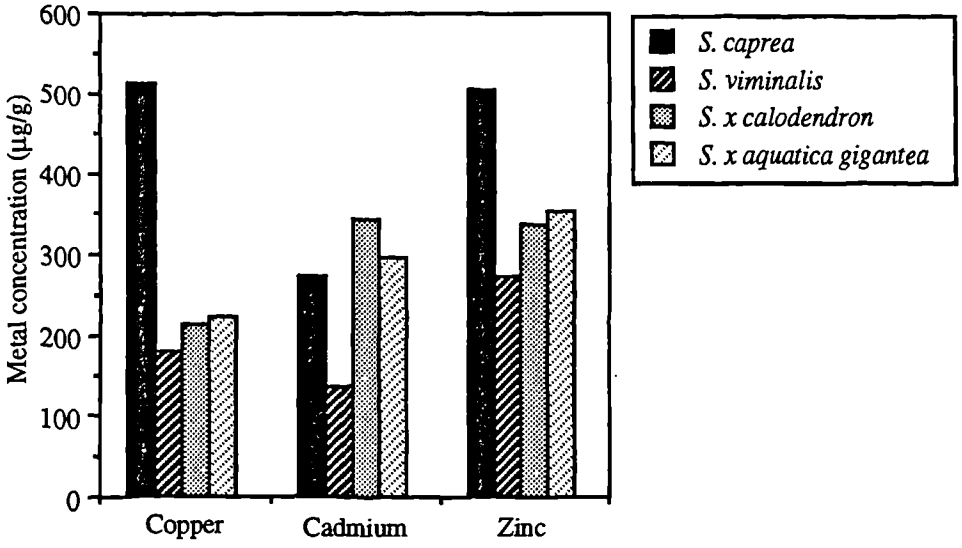

Figure 8. The total heavy metal uptake of willow cuttings growing in increasing metal treatments for a period of 112 days.

The accumulation characteristics of different metals within plant compartments was found to be consistent for all willow species and hybrids tested and are summarised in the following points.

- **Copper:** ROOTS >WOOD > STEM > LEAVES
- **Cadmium:** LEAVES ≥ STEM > WOOD ≤ROOTS
- **Zinc:** LEAVES > STEM = WOOD <ROOTS

Discussion

Exposing different willow species and hybrids to heavy metals highlighted important inter-specific differences in innate metal resistance. *S. caprea* and *S.* x *sericans* are particularly tolerant to copper, although *S. caprea* has consistently shown a lack of tolerance to cadmium. *S. pentandra* was resistant to all the metals tested although its growth was generally poor. The occurrence of willows on metal polluted mine spoils (Grime *et al* 1988; Eltrop *et al* 1991) suggests the presence of a metal tolerance trait, and these results strongly support this theory. One finding of this study is the unusually high natural resistance of willows to cadmium. Although phytotoxic effects were observed, results for *TI* and uptake of cadmium suggest that willows could potentially be used to remove cadmium from polluted soil. The large concentration of cadmium accumulated in the leaves and stems could be removed by regular shoot harvests. This suggestion was also made by Landsberg & Greger (1994), who tested a wide range of *Salix viminalis* biomass clones in cadmium treated soil. In addition,

Östman (1994) recommended the use of willows for removal of cadmium from polluted soils and extrapolated from field data that 3-4% of plant available cadmium could be removed. Although possible seasonal and age-related changes in uptake and accumulation may occur, the data are still encouraging. Pre-treating willow cuttings with heavy metals can increase *TI*s, and a longer period of acclimation is most effective. Periods of acclimation to metals could precede out-planting of willow cuttings in contaminated soils, alternately stock plants could be cultivated on moderately contaminated soils.

The uptake and accumulation of metals over the 112 day period of the acclimation experiments suggests that *S. caprea* and the hybrids *S.* x *calodendron* and *S.* x *aquatica gigantea* can take up significant concentrations of metals from solution, and tests growing these hybrids on a variety of polluted soils and mine tailings is currently in progress. Uptake of copper was generally lower than cadmium and zinc, possibly due to the high affinity of this element for organic matter causing it to bind strongly to the roots. The latter, more mobile metals were taken up in greater quantities and stored in the aerial compartments of cuttings, potentially making them easier to remove. This may, however present a risk of transfer of metals into the food chain, nevertheless it is clear that there is potential for bioremediation of metal-contaminated soils using willows; but more research is needed to examine the long-term pattern of metal uptake and allocation of translocated metals into various plant compartments. Many bioremediation studies have highlighted the benefits of mycorrhizal fungi normally associated with woody plants (Danielson 1985), and a protective effect of mycorrhizas has been put forward for some types of fungal symbiont (Bradley *et al* 1981). Bioremediation should now move towards integrated field studies using metal-resistant mycorrhizal trees and shrubs, to elucidate the actual time scales involved and assess the efficacy of bioremediation.

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Resistance to copper toxicity in some British willows

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Abstract

Sixteen *Salix* clones of known origin were screened for resistance to copper in solution culture, using copper concentrations of 0.25, 0.5 and 0.75 mg l^{-1} in 25% strength Hoagland's nutrient solutions. Significant differences were found in root length increase, number of lateral roots produced and patterns of metal uptake between species, hybrids and clones. The potential use of selected willow clones for reclamation and restoration of contaminated soils is discussed.

1. Introduction

Considerable advances have been made in understanding both metal toxicity in soils (Lepp, 1981; Alloway, 1990) and the phenomenon of metal tolerance in herbaceous plants (Baker, 1987; Ernst, 1990; Shaw, 1990; Ernst et al., 1992). Using this knowledge, vegetation can be established on soils formerly devoid of plant life either by chemical and physical amelioration of soils (Johnson et al., 1977) or else by planting metal tolerant species (Bradshaw and McNeilly, 1982). These techniques have been particularly successful in greening metalliferous mining spoils (Smith and Bradshaw, 1979), thus improving their aesthetic and economic value. In more recent years, two other factors have added renewed interest to these studies in Britain and elsewhere. Firstly, afforestation programmes are under way to encourage more widespread tree planting on the urban fringe (Forestry Commission, 1991; Anon., 1993) and on surplus agricultural land (MAFF, 1991) which are sometimes contaminated by metals, from industrial fallout or sewage sludge fertilizer usage respectively. Secondly, with more stringent restrictions placed on marine dumping, there is increased interest in more extensive land disposal of sewage sludge, which invariably contains elevated levels of metals.

One enlightened approach to remediation of metal-contaminated soils put forward recently has been to grow crops of herbaceous species that are known to accumulate high

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concentrations of metals in their tissues (Baker et al., 1991). It has been demonstrated that within a few to several harvests, soil metal levels can be significantly reduced in this way (McGrath et al., 1993). It would appear that fast-growing trees with the same traits may provide an even better solution, either in terms of more permanent revegetation of sites for conservation purposes or short-rotation forestry for biomass. Recent studies have demonstrated the existence of metal tolerance traits in other common species of trees (Borgegård and Rydin, 1989; Dickinson et al., 1992a, b; Turner and Dickinson, 1993a, b). The high productivity and invasive growth strategies of several willow species (Cannell et al., 1987; Grime et al., 1988), their ability to colonise edaphically extreme soils (Eltrop, Brown et al., 1991; Mang and Reher, 1992) and the known genetic variability of *Salix* (Vahala et al., 1991 Eriksson et al., 1991; Meikle, 1992; Newsholme, 1992) may make them particularly suitable for these purposes. There may be an opportunity to use willows in bioremediation programmes where soils are metal contaminated as a result of mining activities, industrial fallout or sewage sludge application to land.

The aims of the present work were to develop a technique to screen willows in solution culture, to identify concentration of copper toxic to common species of willow in Britain, and to investigate the variability of the toxic response between species, hybrids and clones. The study was a first attempt to investigate the possibility of selecting willows for the purposes described above.

2. Materials and methods

Willow cuttings were obtained from the UK National Willow Collection of over 500 clones at Ness Botanic Gardens, South Wirral, which itself mainly originated from the Stott collection at Long Ashton Research Station. The collection is well documented and of known provenance. Shoot cuttings of uniform size (approximately 18cm lengths) were selected from the previous years growth of known clones on two occasions in February and July 1993. The lower part of each cutting was immersed in water, and after two weeks cuttings with obvious root initials were selected and then placed in an experimental hydroponics system. This consisted of the cuttings being supported with the lower part submerged in darkened 25 l polypropylene trays (dimensions $100 \times 50 \times 10$ cm) supplied with 50 l recirculating (125 ml min^{-1}) 25% strength Hoagland's nutrient solution (Epstein, 1972), completely replaced with fresh solutions every 7 days. Copper was supplied in the solutions as $CuSO_4$ to different trays in varying concentrations, and solution copper levels were monitored daily for the duration of the first experiment. Background solutions (described here as 0 mg l^{-1}) actually contained 0.06 mg Cu l^{-1} to supply essential growth requirements of this micronutrient. The hydroponics system was maintained under standard glasshouse conditions.

In the experiments described below, a range of different species, hybrids and clones were tested using the hydroponics system (Table 1). In the first experiment, four concentrations of copper were supplied (0, 0.25, 0.5 and 0.75 mg l^{-1}) to separate trays, using 50 cuttings per clone which were measured for growth after 21 days. In the second experiment 72 replicates of each clone were tested in a duplicated block design using 3 copper concentra-

Table 1
Salix clones used in the two experiments

Experiment 1		Experiment 2	
Species or hybrid [a]	Accession number [b]	Species or hybrid [a]	Accession number[1]
S. purpurea	3010	*S. purpurea*	3018
S. caprea	3283	*S. alba*	3204
S. caprea	3285	*S. fragilis*	3234
S. viminalis	3375	*S. caprea*	3283
S. cordata	3280	*S. cinerea*	3296
S. glaucophylloides [c]	3387	*S. viminalis*	3369
S. pentandra	3274	*S.* × *multinervis* [d]	3338
S. × *sericans* [e]	3305	*S.* × *forbyana* [f]	3340
		S. × *sericans*	3303

[a] Constituent species of hybrids: *S.* × *sericans* = *S. caprea* × *S. viminalis*; *S.* × *multinervis* = *S. aurita* × *S. cinerea*; *S.* × *forbyana* = *S. purpurea* × *S. cinerea* × *S. viminalis*.
[b] Accession numbers follow those used in the Ness Gardens collection.
[c] Hybrid of *S. glaucophylloides* × *S. viminalis*.
[d] *S. cinerea* × *S. multinervis* (= *S. aurita* × *S. cinerea*).
[e] Hybrid of *S. cinerea* × *S.* × *sericans*.
[f] *S. cinerea* × *S. forbyana* (= *S. purpurea* × *S. cinerea* × *S. viminalis*).

tions (0, 0.25 and 0.5 mg l^{-1}) and measuring the cuttings every 7 days over a 28 day period.

Growth was determined by measuring both the length of the longest lateral root and the number of lateral roots on each cutting. After Experiment 2 was completed, all solutions were replaced with water for 7 days after which samples of stem, leaf and root were removed for metal analysis. Washed and oven-dried samples were digested in 5 M HNO_3 at 80°C following standard procedures, and copper was determined using AAS under standard operating conditions (Turner and Dickinson, 1993b). Data were analysed using the general linearized model analysis of variance in the Minitab statistical package.

3. Results

There was considerable variation in root growth between species in background nutrient solutions and in response to elevated copper concentrations (Fig. 1, Table 2). Root length increase of the two most productive clones in uncontaminated solutions, *S. pentandra* and *S.* × *sericans*, was progressively inhibited by increasing copper levels, the former species being particularly sensitive. At concentrations of 0.75 mg Cu l^{-1} root growth of four of the clones was severely or totally inhibited. *S.* × *sericans* produced more roots and longer roots than other clones in 0.75 mg Cu l^{-1}, together with *S. viminalis* and one clone of *S. caprea*; the two component species of this hybrid. However, the two *S. caprea* clones responded quite differently to elevated copper. Root length increase of *S. purpurea* was significantly improved by the addition of copper at the lower concentrations, even though the number of roots produced was less.

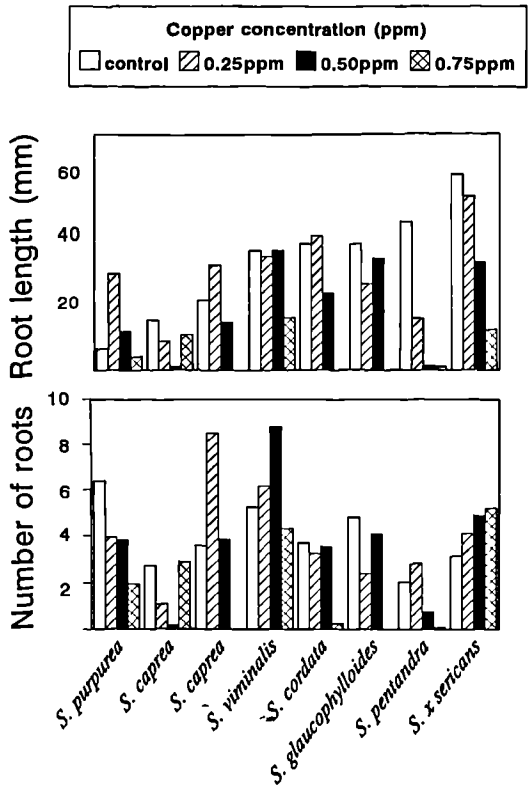

Fig. 1. Growth of roots of *Salix* clones after 21 days in Experiment 1.

Table 2
Analysis of variance tables for 21 day root length and root number data in Experiment 1 (Fig. 1). Root number data were square root (+0.5) transformed before analysis

Source	DF	SS	MS	F	p
Root length					
Concentration	3	172 525	57 508	43.42	<0.001
Clone	7	135 295	19 328	14.59	<0.001
Interaction	21	108 085	5147	3.89	<0.001
Error	1568	2 076 900	1325		
Total	1599				
Root number					
Concentration	3	134.561	44.854	50.98	<0.001
Clone	7	191.141	27.306	31.04	<0.001
Interaction	21	160.918	7.663	8.71	<0.001
Error	1568	1379.553	0.880		
Total	1599				

Significant differences in root length and root number also existed (at $p<0.001$) between clones at each metal concentration, and between metal concentrations for each clone, with only two exceptions [effects of metal concentration on root length of *S. pentandra* ($p=0.05$), and effects of metal concentration on root number of *S.* × *sericans* (not significant)].

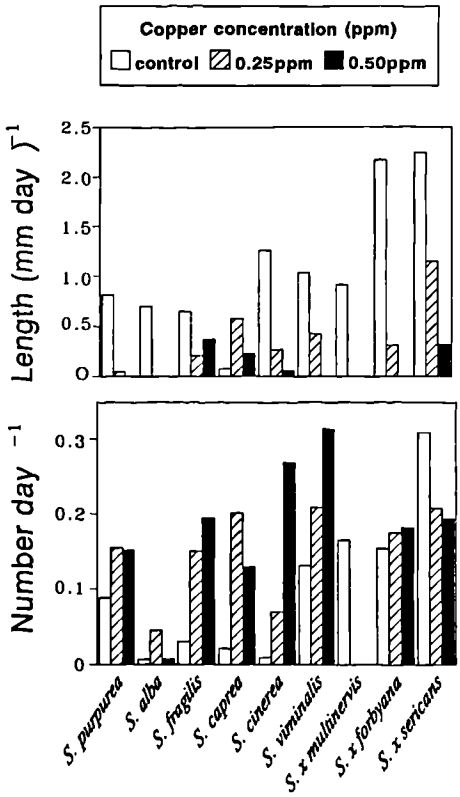

Fig. 2. Growth of roots of *Salix* clones during 28 days in Experiment 2.

A similar high degree of variation was found between species and hybrids in the second experiment (Fig. 2). Growth differences recorded in these experiments were highly significant between species and between metal treatments within species (Table 3). The same *S. caprea* clone as one used in the first experiment (left-hand side of Fig. 1) produced more roots that grew better at elevated copper levels; in the first experiment its growth was not significantly inhibited at 0.75 mg Cu l^{-1}. The pattern of response of *S.* × *sericans* was also the same as in the first experiment; together with *S.* × *forbyana* roots continued to be produced at elevated copper levels but length increase was progressively inhibited. These two hybrids were also the most productive clones in uncontaminated solutions. *S.* × *multinervis* was particularly sensitive to copper. In *S. purpurea*, *S. alba* and the three hybrid species, root length increase was inhibited by elevated copper but there was little effect of the numbers of roots produced. Contrary to this, *S. fragilis*, *S. cinerea* and *S. viminalis* produced more roots in the presence of elevated copper, whilst root length increase was inhibited. Of the 6 non-hybrid clones, production of roots was generally higher but length increase was inhibited in elevated copper solutions. At 0.5 mg Cu l^{-1}, root length increase occurred in only four of the 10 clones.

Uptake patterns of metals also varied between species (Fig. 3). Much higher concentrations occurred in the roots, even though the plants were kept in metal-free solutions for one week prior to the analysis, and relatively small amounts were translocated to leaf and stem

Table 3
Analysis of variance tables for root length and root number data (all sampling dates combined) in Experiment 2 (Fig. 2). Root number data were square root (+0.5) transformed before analysis

Source	DF	SS	MS	F	p
Root length					
Concentration	2	313 004	156502	265.3	<0.001
Clone	8	684 592	85574	145.0	<0.001
Interaction	16	182 054	11378	19.29	<0.001
Error	7749	4 572 496	590		
Total	7775				
Root number					
Concentration	2	12.87	6.434	6.54	<0.01
Clone	8	1778.92	222.37	226.0	<0.001
Interaction	16	227.85	14.24	14.47	<0.001
Error	7749	7623.90	0.984		
Total	7775				

Significant differences in root length and root number also existed (at $p<0.001$) between clones at each metal concentration, and between metal concentrations for each clone at all sampling dates, with only three exceptions for root number differences between copper concentrations (*S. purpurea*, $p<0.05$; *S.* × *sericans*, not significant; *S. viminalis*, $p<0.01$).

tissues; concentration ranges were 9.5–63 μg Cu g^{-1} in leaves and 10–47 μg Cu g^{-1} in stems. The clones appear to fall in two groups according to the concentrations of copper found in the roots; four clones had accumulated concentrations above 500 μg Cu g^{-1} (*S. viminalis* accumulated 830 μg Cu g^{-1}) and 3 clones had concentrations below 250 μg Cu g^{-1}. However, this pattern does not correspond to those described above for root production or growth.

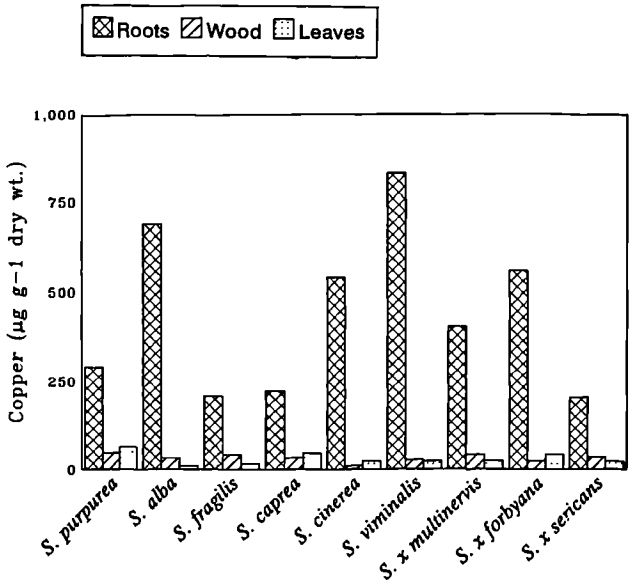

Fig. 3. Uptake of copper into plant tissues in 0.25 mg Cu l^{-1} in Experiment 2.

4. Discussion

Clonal variation in rooting of willows (Good et al., 1978) and differential growth of clones have been described previously (Lumme and Tormala, 1988); the results of the present study have shown that this also extends to their response to excess copper. *Salix caprea*, *S. cinerea* and their hybrids with *S. viminalis* (which include *S.* × *forbyana* and *S.* × *sericans*) are known to be the species suited to polluted and hostile environments (Good et al., 1985; Sommerville, 1992; Stott, 1992); metal tolerance has been found in *S. caprea* on lead mine spoils (Eltrop et al., 1991) and in hybrids of the three species on metal contaminated river silts (Mang and Reher, 1992). In the present study the same species and hybrids appear to grow best in elevated copper solutions. However, there is a considerable range of variation within individual species, as shown by the varying response of the two *S. caprea* clones; the same clone of this species in both experiments showed an ability to grow in elevated copper. Repeated testing of different clones of the same species or hybrids produced a different pattern of response in *S. purpurea* and *S. viminalis*, but much the same response in *S.* × *sericans*. Clearly, variability in copper resistance exists between different willows at all levels; between species, hybrids and populations. Further study is required to evaluate the stability of metal resistance traits and the degree to which such responses are genetically determined or else can be induced by environmental pre-treatment (see Dickinson et al., 1992a, b).

With further research, there is an opportunity to identify productive clones of *Salix* with resistance to copper and to other metal contaminants. The possibility of establishing willows on poorly vegetated metal-contaminated soils is an obvious application of the work, and studies are currently under way to evaluate the degree to which this has occurred through natural selection following invasion of long-exposed mine spoils by seeds. We are investigating whether metal tolerance is already established in some field populations.

There are also wider application to these studies, in view of the differing uptake patterns of metals. Willows which survive in metal contaminated soils by metal avoidance, without significant uptake of metals into aerial tissues, will obviously be most appropriate in situations where nature conservation is a priority; for example, to avoid food chain transfer of metals. However when the priority exists to restore or reclaim contaminated soils, for example on sewage sludge contaminated agricultural land, a species which accumulates and tolerates large concentrations of metals in above-ground harvestable tissues would clearly be more appropriate. Repeated harvest would offer the potential amelioration of toxic soils. Growth trials in soils contaminated with metals from different sources are currently in progress.

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