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STUDIES ON THE DISTRIBUTION AND PHYSIOLOGY OF LEAD IN PLANTS

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STUDIES ON THE DISTRIBUTION AND PHYSIOLOGY OF LEAD IN PLANTS

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CONTENTS

	Page
ACKNOWLEDGEMENTS	i
SUMMARY	ii-iii
LIST OF FIGURES	iv-v
LIST OF PLATES	vi-vii
LIST OF TABLES	viii
CHAPTER 1	
INTRODUCTION	1-11
1.1 Lead in Soils	2
1.2 Lead Uptake by Plants	3
1.3 Growth of Lead-Contaminated Plants	5
1.4 Lead-Induced Physiological Changes	7
1.5 Aims of Present Study	9
CHAPTER 2	
MATERIALS AND METHODS	12-40
2.1 Growth Studies	12
2.1.1 Germination experiments	12
2.1.2 Single salt culture experiments	13
2.1.3 Hydroponic culture experiments	14
2.2 Analysis of Lead Content of Plant Tissue	17
2.3 Light Microscope Studies	19
2.4 Electron Microscopy	21
2.5 Electron Microprobe Analyses	23
2.6 Physiological Experiments	26
2.6.1 Cell division	26
2.6.2 Cell elongation	27
2.6.3 Cell wall elasticity/plasticity	28
2.6.4 Water uptake	29
2.6.5 Binding of lead to cell wall fractions	29
2.6.6 Estimation of endogenous auxins	30

Page

CHAPTER 3

THE EFFECT OF LEAD ON THE GERMINATION AND GROWTH OF *RAPHANUS SATIVUS* 41-69

3.1 Germination and Root Hair Development 41

3.2 Seedling Growth 42

3.3 Hydroponic Culture Experiments 44

3.4 Discussion 46

CHAPTER 4

THE UPTAKE OF ROOT-APPLIED LEAD BY *RAPHANUS SATIVUS* 70-86

4.1 Lead Content of Seedlings 72

4.2 Lead Content of Hydroponically-Grown Plants 73

4.3 Comparison of Plant Lead Contents with Plant Dry Weight Yield 74

4.4 Discussion 75

CHAPTER 5

LIGHT MICROSCOPE STUDIES ON THE DISTRIBUTION OF LEAD IN *RAPHANUS SATIVUS* 87-110

5.1 Lead Distribution in the Seed During Early Germination 88

5.2 Lead Distribution in Radish Seedlings 90

5.3 Discussion 95

CHAPTER 6

SUBCELLULAR DISTRIBUTIONS OF LEAD IN *RAPHANUS SATIVUS* ... 111-141

6.1 Glutaraldehyde-Fixed Specimens 114

6.2 Glutaraldehyde/Osmium Tetroxide-Fixed Preparations of Germinating Seed Material 115

6.3 Glutaraldehyde/Osmium Tetroxide-Fixed Preparations of Seedling Material 117

6.4 Discussion 123

CHAPTER 7

ELECTRON MICROPROBE EXAMINATION OF LEAD-CONTAMINATED TISSUE 142-162

7.1 Small Spot Analysis 143

Page

7.2 Small Area Analysis 144

7.3 Low Magnification Analysis of Root Cortex and Stele 145

7.4 Bulk Analysis of Lead-Treated Tissue using Electron Microprobe 147

7.5 Discussion 149

CHAPTER 8

EFFECTS OF LEAD ON THE HORMONAL REGULATION OF PLANT GROWTH 163-189

8.1 Preliminary Experiments 164

8.2 Studies on Cell Elongation 165

8.3 Lead-Induced Changes in the Physiology of IAA-Induced Cell Elongation 169

8.4 Discussion 173

CHAPTER 9

CONCLUDING REMARKS 190-199

REFERENCES 200-224

APPENDIX 1 Supplementary Data 225-245

APPENDIX 2 Reagents and Procedures 246-256

APPENDIX 3 Statistical Treatment of Data 257-273

DECLARATION

The work presented in this thesis was carried out by the candidate himself and due acknowledgement has been made of the assistance received.

This work has not been accepted for any other degree and is not concurrently being submitted in candidature for any other award.

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SUMMARY

The studies reported herein cover two main areas of research. The distribution of lead through the plant has been monitored using histochemical* and quantitative techniques, and physiological responses of the plant to lead contamination have been examined.

In seeds the testa prevents lead contamination of the embryo prior to germination. In seedlings root-applied lead is capable of essentially unrestricted movement through the plant, although anomalies in distribution exist. The metal is sequestered during its passage through the plant, and the endodermis offers a partial barrier to lead movement, restricting contamination of the aerial parts of the plant. In cotyledon leaves local accumulations may occur, causing necrotic lesions. Subcellular lead contamination again diminishes with distance from the root, but extensive exposure causes heavy contamination of cell walls, increasing wall fragility. Lead deposits occur in association with several cell organelles, but the primary response of plant cells to lead contamination lies in the formation of vesicles, apparently from the endoplasmic reticulum. These fuse into vacuoles and may totally disrupt cell ultrastructure. Lead distributions observed have been verified by X-ray microanalysis.

Responses to lead pollution occur in numerous physiological processes, but the most obvious effect lies in the reduction of plant growth. In the root this is at least partially attributable to reduced cell division, but in the stem growth inhibition results primarily from interferences in cell elongation.* This inhibition of elongation arises

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*Communications arising from these data have been published:
Lane S.D., Martin E.S., 1977. A Histochemical Investigation of lead uptake in Raphanus sativus, New Phytol., 79, 281-286.
Lane S.D., Martin E.S., Garrod J.F. 1978. Lead Toxicity Effects on Indole-3-acetic acid-induced cell elongation. Planta (Berl.) in press.

from lead-induced cell wall changes which increase wall rigidity. At high supply levels lead also influences water uptake, possibly via changes in membrane permeability. Endogenous IAA levels increase in response to lead contamination, and may partly alleviate the toxic effects of lead. The metal also promotes changes in chlorophyll synthesis, membrane permeability, ion uptake, water stress and respiration. Possible reactions and interactions involved in these responses are discussed.

LIST OF FIGURES

2.1.1 Chamber constructed for hydroponics experiments 32

2.1.3 Typical regimes in culture chamber during hydroponics experiments 34

2.1.5 Relationship between total lead added to culture solution and available lead 36

2.2.1 Performance curves for Orion lead-selective ion electrode 37

2.5.1 Peaks in typical electron microprobe trace of spot analysis 24

2.6.3 System employed in cell wall plasticity/elasticity studies 38

2.6.5 Cell wall fractionation procedure employed in experiments on the binding of lead to cell wall fractions 39

2.6.6 Extraction procedure employed in the estimation of endogenous auxins in lead-treated plants 40

3.1.1 The effect of lead on radish seed germination 57

3.2.1 The effect of lead nitrate on radish seedling growth .. 58

3.2.2 The effect of sodium nitrate on radish seedling growth 59

3.2.3 The effect of associated anions on lead-modified radish seedling growth 60

3.3.1 Effects of lead on fresh weights of hydroponically-grown radish plants 62

3.3.2 Effects of lead on dry weights of hydroponically-grown radish plants 63

3.3.3 Effects of lead on fresh weight/dry weight ratios of hydroponically-grown radish plants 64

3.3.4 Effects of lead on the linear dimensions of hydroponically grown radish plants 65

3.3.5 Effects of lead on leaf area of hydroponically-grown radish plants 66

3.3.6 Effects of lead on leaf formation in hydroponically-grown radish plants 67

3.3.7 Effects of lead on dry weight/unit length ratio for roots and stems of radish plants grown in hydroponic culture .. 68

Page

3.3.8 Effects of lead on dry weight/unit area for leaves of radish plants grown in hydroponic culture 69

4.1.1 Lead contents of radish seedlings subjected to various washing treatments 82

4.2.1 Lead contents of hydroponically-grown radish plants .. 83

5.1.2 Distribution of lead in radish testa after 12 hours exposure to lead 101

7.1.3 Relative peak heights observed in spot analyses for lead in cortical and stelar tissues of lead-treated radish seedlings 157

7.3.5 Root lead profiles copied from Plates 7.3.1 - 7.3.4 .. 160

7.4.1 Bulk analyses of lead-treated root material for several elements 161

7.4.2 Conventional analyses of hydroponically-grown radish plants for iron and calcium contents 162

8.1.1 Preliminary observations on the physiological basis for lead-induced changes in growth; changes in cell division and IAA-induced cell expansion in radish tissue 179

8.2.1 Effect of lead on IAA-induced cell elongation in wheat 180

8.2.2 Factorial experiment on the effect of lead on IAA-induced cell elongation in wheat 181

8.2.3 Chemical structures of synthetic auxins employed in elongation studies 182

8.2.4 Effect of lead on elongation induced in wheat coleoptile segments by synthetic auxins 183

8.2.5 Effect of synthetic auxins on lead-induced inhibition of coleoptile segment elongation in wheat 184

8.3.1 Effect of pre- and co-treatments on the lead/IAA interaction 185

8.3.2 Effects of lead on IAA-induced water uptake and cell wall elasticity/plasticity 186

8.3.3 Absorbtion of lead by cell wall fractions 187

8.3.4 Ultraviolet absorbtion spectra of cell wall fractions before and after addition of lead nitrate 188

8.3.5 Effect of lead on endogenous auxin levels in radish seedlings 189

LIST OF PLATES

		Page
2.1.2	Culture chamber constructed for hydroponics experiments	33
2.1.4	Plant culture arrangement with lightproof sleeve removed	35
3.2.4	Lead-induced discolouration in 7 day radish seedlings	61
5.1.1	Light micrographs of lead distribution in the testa of germinating seeds	100
5.1.3	Light micrographs of lead distribution in developing radish radicles	102
5.1.4	Light micrographs of lead distribution in developing radish hypocotyls and cotyledons	103
5.2.1	Light micrographs of lead distribution in radish roots - 1	104
5.2.2	Light micrographs of lead distribution in radish roots - 2	105
5.2.3	Light micrographs of lead distribution in radish stem tissue	106
5.2.4	Light micrographs of lead deposits associated with secondary thickening of vascular tissues	107
5.2.5	Whole mount preparations of lead distribution in radish cotyledon leaves	108
5.2.6	Light micrographs of lead distribution in radish cotyledon leaf material	109
5.2.7	Whole mount preparations of lead distribution in leaves of deracinated plants	110
6.1.1	Electron micrographs of lead distribution in glutaraldehyde	132
6.2.1	Electron micrographs of lead distribution in radish radicle cells	133
6.2.2	Electron micrographs of lead distribution in radish hypocotyl and seed cotyledon cells	134
6.3.1	Electron micrographs of lead distribution in radish root cap cells	135

Page

6.3.2 Electron micrographs of lead distribution in radish root tip cells 136

6.3.3 Electron micrographs of lead distribution in radish root cortical cells 137

6.3.4 Electron micrographs of lead distribution in radish root endodermis and pericycle cells 138

6.3.5 Electron micrographs of lead distribution in radish root stelar tissues 139

6.3.6 Electron micrographs of lead distribution in radish stem tissues 140

6.3.7 Electron micrographs of lead distribution in radish cotyledon leaf tissues 141

7.1.1 Electron microprobe analyses of lead deposits in radish root sections - 1 155

7.1.2 Electron microprobe analyses of lead deposits in radish root sections - 2 156

7.2.1 - 7.2.5 Microprobe Profiles and X-ray images of lead distribution in radish root cells 158

7.3.1 - 7.3.4 Microprobe Profiles and X-ray images of lead distribution in transects across the endodermis of radish root tissue 159

LIST OF TABLES

		Page
4.0.1	Some lead levels in plants	71
4.3.1	Comparisons of dimensions of seedlings grown in single salt culture with tissue lead concentrations	84
4.3.2	Comparison of dry weight yields of plants grown in hydroponic culture with tissue lead concentrations	85,86

There is also the question of ecological research, the importance of which is recognised but which has not yet been developed in a satisfactory manner. It is certain that the fate of lead in the soil, in vegetation and in other elements of the environment before it reaches man constitutes a new and difficult area of research.

Dr. P. Recht, concluding remarks, International Symposium on Environmental Health Aspects of Lead, Amsterdam 1972.

CHAPTER 1

INTRODUCTION

The occurrence of lead in the environment is widespread and well documented. Lead is found naturally as three ore forms - Galena (PbS), Cerussite ($PbCO_3$) and Anglesite ($PbSO_4$). In the South-West of England these ores are found mainly in North-South lodes occupying a tangential position relative to granite intrusions into the country rock (Dines, 1956). Dust from these ores forms the most natural source of lead in the atmosphere, other sources including volcanic activity, vegetation fires, and meteorites (Patterson, 1965). This dust is returned to the soil by gravitation and is washed from the atmosphere by rainfall (Lazrus et al., 1970; Harrison et al., 1975).

Natural sources of lead may be redistributed in the environment as a result of man's activities. Reports by Rühling and Tyler (1968) and Lee and Tallis (1973), based on the analysis of moss specimens from museum collections, reveal two recent periods during which lead has increased in concentration. First, an increase occurred during the eighteenth and nineteenth centuries, reflecting rapid industrial growth during this period, and second there has been an increase since about 1950, reflecting the increased popularity of the internal combustion engine. In the past lead in the form of lead arsenate has also been added to soils deliberately as an insecticide (Jones and Hatch, 1945; Chisholm and Bishop, 1967; Chisholm, 1972), and inadvertently in fertilizers (Zimdahl and Ardvik, 1973), and sewage sludge (Kirkham, 1975; Dowdy and Larson, 1975). It is of interest that lead contamination of cereals may have been reduced since 1962,

because ammonium sulphate fertilizer based on sulphuric acid produced by the lead chamber method, has been replaced by fertilizer produced by lead-free methods (Solgaard et al., 1978).

1.1 Lead in Soils

Redistributed lead is found mainly in the upper horizons of the soil (Swaine and Mitchell, 1960; John, 1971), but this distribution may be modified by high soil moisture levels (Swaine and Mitchell, 1960). Rainfall and/or irrigation may also cause limited leaching of lead, the amount being increased by ploughing and erosion (Tso, 1970). Literature concerned with lead levels in the soil falls broadly into two groups; lead contamination attributed to mining activity and lead dispersed by automobiles.

In the former case the problem appears to be a regional one (Lee and Tallis, 1973). Lead contents of up to 47,000 ppm have been recorded in mining areas in Wales with levels in excess of 5,000 ppm in local gardens and 3,000 ppm in local fields (Davies and Roberts, 1975). Ireland (1975) reported levels of approximately 1,800 ppm in the region immediately below a lead/zinc spoil tip, and suggested that the availability of lead in this situation may be markedly increased by earthworm activity. In the region of a working lead smelter lead contamination of the soil was found to diminish with increasing distance from the lead source, and the distribution of lead was strongly affected by prevailing wind conditions (Little and Martin, 1972; Burkitt et al., 1972).

Lead levels in soil as a result of automobile exhaust emission are far lower, with levels of up to 3,000 ppm being recorded in the U.S.A. (Hemphill et al., 1974). Actual quantities of lead vary with

traffic flow, and again with distance from the road and prevailing winds (Rühling and Tyler, 1968; Atkins, 1969; Davies and Holmes, 1972).

The retention of lead from these sources in soils is modified by pH, clay content and competing ions (Hahne and Kroontje, 1973; Jurinak and Santillan-Medrano, 1974; Farrah and Pickering, 1977) and also by the organic matter content of the soil (Hassett, 1974). Changes in these factors also lead to changes in the availability of soil lead to plants (MacLean et al., 1969; John, 1972; Zimdahl and Foster, 1976).

1.2 Lead Uptake by Plants

Over the years a number of authors have investigated the uptake of lead by plants (Prat, 1927; Hooper, 1937; Marten and Hammond, 1966; Ganje and Page, 1972; Tunney et al., 1972). Much of this interest has resulted from the long half-life of lead in biological systems and because plants facilitate the entry of lead into food chains which may eventually lead to man and his agricultural animals (Chisnall and Markland, 1971; Jones and Clement, 1971; Låg and Bølviken, 1975; Hanssen and Dams, 1975). In general lead uptake by plants may be divided into three types according to the lead source:

(1) Lead pollution from atmospheric sources;

(2) Lead pollution from soil sources;

(3) Lead supplied in solution.

Most of the literature concerning lead from atmospheric sources relates to automobile activity, although some reports are made in reference to fallout from lead smelters or battery works. It would seem that lead levels in plants in the vicinity of these sources demonstrate a similar distribution to those in soil samples (vide supra) (Cannon and Bowles, 1962; Page, Ganje and Joshi, 1971; Ganje and Page, 1972;

Graham and Kalman, 1974), although it has been reported that lead levels recorded in plant material vary from species to species (Hopkinson et al., 1972). In general these articles do not discuss whether the occurrence of lead is the result of direct aerial contamination of the vegetation, or a result of lead contamination of the soil caused by fallout. However, other results show that in the field, where foliage tends to shield the soil from fallout, atmospheric sources of lead result in higher lead levels in leaves than in roots (Motto et al., 1970; Dedolph et al., 1970). In parallel greenhouse experiments, where lead was available solely through the roots, the results were reversed. This would indicate that in the field direct contamination of the foliage was the major factor in lead accumulation.

Lead particle size is important in this context, smaller particles adhering to the leaf surface while larger particles bounce off or wash off easily (Roberts and Hutchinson, 1975). Although such contamination may cause the plant to be exposed to high lead levels, it appears to be only of marginal significance to the plant as most of the pollutant remains associated with the cuticle and superficial cell walls (Little, 1973).

Papers concerned with lead uptake from soil sources are manifold. In general it would appear that lead uptake increases with increasing lead supply (Marten and Hammond, 1966; Chisholm, 1972; Pollack and Fisher, 1973). Lead is accumulated primarily in the roots and translocated in smaller amounts to the aerial parts of the plant (Alloway and Davies, 1971; Rains, 1971; Lagerwerff et al., 1973). In one case higher levels were reported in leaves than in roots of beans and peanuts (Berg, 1970). Uptake is modified by a number of soil factors, including pH, (Lagerwerff, 1971; Dowdy and Larson, 1975), lime content

(John and van Laerhoven, 1972), organic content (MacLean et al., 1969; Zimdahl and Foster, 1976), and the concentration of other nutrients (Jones, Jarvis and Cowling, 1973).

Studies involving the uptake of lead by plants in hydroponic culture are rather less common. The available results indicate that levels of lead in the plant material increase with increasing lead supply (Rasmussen and Henry, 1963) and, in the case of root-applied lead, more lead is accumulated in roots than in the aerial parts of plants Broyer et al., 1972; Rolfe, 1973; Jones, Clement and Hopper, 1973; John, 1977). However, in these studies the actual amounts of lead accumulated vary considerably from species to species.

These distribution patterns of lead have been supported by histochemical studies, which also provide information about subcellular accumulation of lead. In a range of plant material, mostly restricted to lower plants, lead accumulations have been reported which are associated with cell walls (Hammett, 1928a and b; Brown and Slingsby, 1972; Malone et al., 1974), with the nucleus and with the nuclear membrane (Skaar et al., 1973), the cell vacuole (Gullvåg et al., 1974) and with the plasma membrane, chloroplasts and mitochondria (Ophus and Gullvåg, 1974). However, to date there have been no comprehensive histochemical investigations of lead distribution in higher plants.

1.3 Growth of Lead-Contaminated Plants

Data referring to the growth of plants exposed to lead are fairly prolific, and have been discussed in reviews by Zimdahl and Ardvik (1973) and Höll and Hampp (1975). Many of these reports are only concerned with soil-based experiments, and indicate that at low levels of application lead has a stimulatory effect on the growth of barley, buckwheat, corn and wheat (Stoklasa, 1913; Völker, 1914;

Keaton, 1937). According to Berry (1924) this positive effect is attributable to associated nitrate ions.

At higher levels of application (in excess of 0.09%) lead has produced a deleterious effect on the growth of oat plants (Berry, 1924). However, work by other authors has shown no significant effect of lead on corn growth at levels of 3200 Kg/hectare (Baumhardt and Welch, 1972).

Similarly experiments using nutrient solution culture have produced a variety of results. Bonnet (1922) found that lead nitrate at a concentration of 10^{-3}N caused a cessation of stem growth and a reduction in root growth in corn, peas and broad bean. Hooper (1937) noted no significant reduction in the growth of french bean in the presence of lead at levels of up to 30 parts per million lead sulphate, while Rasmussen and Henry (1963) found that lead supplied at 31.25 parts per million to orange seedlings inhibited growth. At lower levels there was a slight stimulation of growth. In the aforementioned study the associated anion appeared to have no effect on this response, but a variation in pH from 4.5 to 6.5 caused a sharp increase in the toxic effect.

The nature of the reaction of plant material to lead also appears to vary between varieties of the same species. Experiments on lettuce varieties using lead at levels of up to 50 parts per million showed significant differences in dry matter production with increasing lead supply, although no visible toxicity symptoms were recorded in the growing plants (John, 1977). A number of other authors have produced reports of varietal variation in lead tolerance (Wilkins, 1957; Wu and Antovics, 1976; Briggs, 1976).

A difference in lead sensitivity also exists between monocotyledonous and dicotyledonous species. Suchodoller (1967) demonstrated that an application of 10^{-1} M resulted in mortality in bean plants within 24 hours, while this occurred in barley plants only after 4 days.

Although many individual reports are available on the effect of lead on the dry weight yields of various plants, there are few comprehensive studies on the growth response of plants to lead. The published results also indicate that the application of lead to plants produces a wide range of responses depending on species and variety.

1.4 Lead-Induced Physiological Changes

While a number of papers are available relating to the outward responses of the plant to lead burden, little of this literature refers to modifications in the physiological status of the plant exposed to lead. Studies on the effect of lead on metabolic systems are also mainly concerned with the mitochondrion and the chloroplast. Miller and Koeppe (1970a) found that lead caused an inhibition of succinate oxidation and a stimulation of NADH oxidation in isolated corn mitochondria. In subsequent work they found that the inhibitory effect on succinate oxidation was ameliorated by the addition of extra phosphate (Miller and Koeppe, 1970b). Reports of a significant swelling in the mitochondrion in response to lead have been explained as the result of a non-specific increase in mitochondrial membrane permeability (Bittell *et al.*, 1974).

Other reports are available which refer to animal mitochondria. Two possible effects of lead on this organelle were suggested by Walton (1973). First, a reduction in adenosine triphosphate (ATP) synthesis in the presence of lead could arise from the diversion of

energy derived from the electron-transport chain to the active accumulation of lead, and second, lead contamination could cause the hydrolysis of ATP, the formation of complexes with sulphydryl groups of mitochondrial enzymes and the precipitation of matrical anions in the mitochondrion. A similar suggestion was made by Vallee and Ulmer (1972). They also reported that in cattle iron storage was modified by the presence of lead, resulting in the generation of ferritin and of ferruginous micelles in mitochondrial membranes.

In the chloroplast a significant inhibition of chlorophyll synthesis in response to lead has been reported by Hewitt (1948a), who found that the addition of iron as a foliar wash reduced the chlorosis. Fiusello (1973) also reported an inhibition of chlorophyll synthesis in *Lemna minor*, but was unable to induce this in terrestrial plants.

The photosynthetic process also appears to be adversely affected. Hampp *et al.* (1973) and Malanchuk and Gruendling (1973) reported a reduction in carbon dioxide fixation in the presence of lead. An inhibition of Photosystem II by lead has been reported by Miles *et al.* (1972), the site of inhibition being between the primary electron donor of the system and the site of water oxidation. Reduction in net photosynthesis has been reported, and also a reduction in transpiration attributable to an observed reduction in stomatal aperture (Bazzaz *et al.*, 1974).

Interpretations of the action of lead on the mechanisms of plant growth are even less common. Hammett (1928c) reported that lead reduced the percentage of cells in mitosis, and also suggested that a reduction in cell size may also be involved in the growth effects reported. In a subsequent paper Hammett and Justice (1928) concluded that the reduction in cell division was the result of a marked uptake

of lead by the mitotic nucleus. Levan (1945) reported that lead caused the dividing cells of Allium cepa roots to remain in a metaphase condition. More consideration has been given to lead-induced changes in cell division in animal cells, chromosome damage of the 'gap-break' type being reported by Muro and Goyer (1969) and by Vallee and Ulmer (1972).

The effect of lead on the hormonal regulation of plant growth is a topic which has received scant attention, although there is a recent report of an interaction between lead and the IAA-oxidase system (Mukherji and Maitra, 1977). These authors report that lead causes IAA-oxidase activity to increase in rice tissue and also that a number of different growth substances are capable of ameliorating the inhibitory effect of lead on plant growth.

1.5 Aims of Present Study

The ability of lead to pass along, and accumulate in, food chains (vide supra) stipulates that the study of the response of the intermediates of food chains is of great importance. The manner in which plants react to lead pollution must, of necessity, modify the passage of lead to the subsequent consumers.

While many surveys of lead-contaminated areas have involved the analysis of lead contents of plants, detailed experiments on the reaction of crop species to lead appear to be mainly limited to monocotyledonous species (e.g. Keaton, 1937; Miller and Koeppe, 1970a and 1970b; Broyer et al., 1972; Baumhardt and Welch, 1972).

The aim of the present investigation was to extend this existing information by designing experiments to ascertain the effects of lead on the development of a dicotyledonous crop species. For the

purposes of this study the radish, Raphanus sativus, was selected as the experimental plant material. While not a crop of vital economic importance, radish plants may be grown quickly in restricted conditions (Ware and McCallum, 1968; Chesnaux, 1972) and in previous reports radish has been used as a sampling species for heavy metal assimilation studies (Lagerwerff, 1971; John, 1972).

The results of the experiments carried out have been interpreted in terms of physiological responses, and other experiments have been carried out to evaluate the physiological changes which occur as a result of lead uptake. These results and associated interpretations are presented in the following chapters.

During the early part of this investigation soil samples were collected from local lead contaminated sites (Appendix 1.1). These were then dried, ashed and analysed for total lead content. Values ranged from 64 to 1360 micrograms per gram dry weight. Subsequently radish plants were grown on samples of the soils in a greenhouse. In all cases plants showed severe inhibition of growth and at higher concentrations there was a high incidence of mortality. After five weeks the surviving plants were dried, ashed and analysed for lead content (Appendix 1.2).

The results of this procedure proved rather unsatisfactory. While a relationship could be observed between lead content of plant material and total lead present in soil, the relationship was somewhat erratic. Any relationship between dry weight yield and lead supply was even more erratic. Observations by other researchers on lead in soils (vide supra) suggest that this variation arises as a result of variations in other soil parameters. Subsequent analysis of the soil samples showed marked variations in soil pH, cation exchange

capacity and levels of other heavy metals, particularly copper and zinc.

From these preliminary results it was concluded that to use environmentally-polluted samples for a physiologically-orientated study of this nature would prove highly unsatisfactory. Therefore, a wholly hydroponic mode of plant nutrient supply was adopted for this study. Lead was supplied using a logarithmic range of concentrations of up to 10 mEq/l to obtain the maximum range of responses by the plant to the presence of lead.

CHAPTER 2

MATERIALS AND METHODS

This study has involved the action of lead upon a number of systems within the plant and as a result a fairly wide range of techniques has been employed, details of which are presented below.

Throughout the study solutions were made up using distilled, deionised water and 'Analar' grade chemicals unless otherwise stated. All glassware was washed in detergent and tapwater, rinsed in Molar hydrochloric acid, then triple rinsed in deionised water. Prior to use seed material was stored in the dark at $4^{o}C$.

2.1 *Growth Studies*

2.1.1 *Germination Experiments*

Experiments on the effects of lead on seed germination were carried out on radish seeds, variety 'French Breakfast' supplied by Samuel Dobie and Sons Ltd., of Chester. These seeds demonstrated approximately 90% viability when subjected to the triphenyl tetrazolium chloride viability test (Lakon, 1949 - see Appendix 2). Seeds were sown in batches of 20 on to filter paper in petri dishes containing 10 ml aliquots of various lead nitrate solutions. A problem exists in the use of single-salt solutions in growth experiments because the differences in lead concentration cause changes in solution pH which may themselves alter the growth of the plant. Initially attempts were made to overcome this using buffers, but problems were encountered with lead precipitation. Finally each solution was adjusted to pH 6 using nitric acid and sodium hydroxide. This was repeated for all solutions used in the germination experiments and single-salt culture

experiments. Twenty replicates of each lead treatment were prepared. Initially lead nitrate concentrations of 0, 0.01, 0.1, 1.0, 5.0 and 10.0 mEq/l were examined, but subsequently experiments were also carried out using lead nitrate levels of 5, 10, 15, 20, 30, 40 and 50 mEq/l. The dishes were then transferred to a Fisons model 140-G2 environmental chamber and incubated in the dark at 20°C for 7 days. At 24 hour intervals the dishes were removed to a darkened room and the number of seeds which had germinated were counted. Germination was defined in this instance as the emergence of the radicle from the testa.

In addition counts were compiled on the formation of root hairs on the developing radicles.

In order to assess the effect of nitrate ions, as opposed to lead, on germination parallel experiments were carried out in which nitrates were applied at equivalent concentrations as the sodium salt.

2.1.2 Single Salt Culture Experiments

In this series of experiments the effect of lead on root, stem and cotyledon leaf development was examined during the early stages of seedling development.

To reduce the inherent variation which is normally observed in the growth of a random batch of seedlings a germination test was applied. Batches of approximately 500 radish seeds were sown on to filter paper soaked in deionised water, and placed in the dark at 20°C. After 24 hours the seeds were examined, and those which had not germinated were discarded. The remaining seedlings were allowed to develop for a further 24 hours, at which time seedlings were selected which appeared to be as near identical in dimension as possible. Thus the experimental period ran from day two to day sixteen of development.

Seedlings were placed, five at a time, on 30° inclined slopes covered in absorbent paper in plastic boxes. Lead solutions in 200 ml aliquots were poured into each box, and the boxes closed. They were then transferred to a Fisons model 140 G2 environmental chamber and subjected to a temperature of $26^{o}C$ during the day and $19^{o}C$ during the night. The seedlings received a photoperiod of 14 hours of light at an intensity of 11,840 lux and 10 hours of darkness. These regimes were selected on the basis of the results of Chesnaux (1972). The boxes were examined every 24 hours for a period of 14 days, and on each day measurements were taken of root length, stem length, leaf length and leaf breadth. Experiments were carried out to examine the effect of a range of lead nitrate concentrations (0, 0.01, 0.1, 1.0, 5.0 and 10.0 mEq/l) and a similar range of sodium nitrate solutions upon the above parameters, and experiments were also carried out to examine the effect of different lead salts on growth. In the last case problems were encountered in that isoequivalent solutions of lead nitrate, lead acetate and lead citrate demonstrate different pH values. To overcome this, pH adjustments were carried out using the appropriate acid (nitric, acetic or citric) and sodium hydroxide.

2.1.3 Hydroponic Culture Experiments

Radish plants were grown for periods of up to 5 weeks in a growth chamber constructed of wood and glass, and made airtight with adhesive tape (see Figure 2.1.1 and Plate 2.1.2). The chamber was lined with expanded polystyrene sheeting and aluminium foil to provide a light-reflective surface and to aid temperature control, and installed within a heated greenhouse. The chamber was cooled by a thermostatically-controlled fan drawing air through a muslin/glass wool/activated charcoal filter, resulting in a steady maximum day temperature

of 26°C and a minimum night temperature of 20°C with a mean relative humidity of 77% (see Figure 2.1.3). Milk bottles, purchased from the local reclamation depot and of known glass composition (see Appendix 2), were cut to approximately 300 ml capacity and used as the culture vessels. These were respectively steam-washed and acid-washed prior to each experiment, and enclosed in a cylinder of black paper to exclude light. The plants were supported on blackened expanded polystyrene discs coated with paraffin wax (Plate 2.1.4) and floated on Arnon and Hoagland's solution (Hewitt, 1966 - see Appendix 2), which was adjusted to pH 6 with nitric acid and sodium hydroxide. Solutions were renewed at weekly intervals. A continuous supply of air filtered through felt and washed in deionised water was fed to the culture vessels through a system of polythene tubing and Pasteur pipettes. Overhead illumination was provided by a mixture of 6 x 100 W tungsten bulbs and 4 x 20 W warm white fluorescent tubes, cooled by a 1 cm deep water filter situated beneath the lights. This system provided an even light intensity of 11,000 lux and untreated plants were found to grow to maturity in 4 to 5 weeks.

Problems exist in the use of hydroponic culture for the examination of heavy metal pollution, for while it is possible to control the chemical composition of the culture solutions and to vary with accuracy the amounts of lead added, the anionic composition of the medium causes lead precipitation. Although a number of investigators (e.g. Hooper, 1937; Broyer et al., 1972; Miller and Koeppe, 1972; Jarvis et al., 1977) have used hydroponic techniques in lead uptake studies, the problem of precipitation has rarely been discussed. Hooper (1937) recognised that precipitation occurred and as a result limited her experiments to the low ranges of concentrations of lead at

which precipitation is not apparent. However, precipitation represents one side of an equilibrium situation, and as the soluble fraction is depleted of ions, this loss is compensated for by the movement of ions from the precipitate back into solution. Also while it might be possible to reduce the problem of precipitation, at least outside the plant, by means of split-root techniques or by alternation of lead/culture solution treatments, the precipitation of anions by lead is a natural event and must occur normally in contaminated soils. Moreover the possibility exists that the changes in ion flux resulting from any system in which lead and culture solution treatments are separated may itself prove deleterious to the plant. In view of this it was decided to retain a simple culture system, lead and the nutrient solutions being supplied simultaneously. A similar conclusion has also been reached by John (1977) who found that lettuce plants were able to assimilate lead from culture solution precipitates even when free lead was not detected in solutions. However to obtain an approximate indication of the availability of lead in the culture solutions and at the different supply concentrations, measurements were made of available lead using a specific ion electrode system. (For details of this technique see below.) The results showed that in spite of precipitation the concentration of available lead increased with increasing lead supply (see Figure 2.1.5). Moreover agitation increased the availability of the lead in solution so that aeration of the cultures served two purposes - firstly to oxygenate the culture solutions, and secondly to minimise sedimentation of the precipitate.

The seedlings used in this part of the study were selected as before (see Section 2.1.2) except that a second selection procedure for

identical seedlings was carried out after 72 hours. Therefore in each case the experimental period commenced at day 3 of development and continued for a further 2, 3, 4 or 5 weeks. Five replicates were set up for each treatment and at the end of the experimental period the plants were harvested and washed in deionised water. A number of parameters were measured: fresh weight, dry weight, root length, stem length, stem breadth, and leaf area. Weights were measured on a Sauter model 404 analytical balance, calibrated to 5 places of decimals, while linear dimensions were measured with vernier calipers and leaf area with a KGM Vidiaids model 528 Area Quantifier with a model 113 camera.

2.2 *Analysis of Lead Content of Plant Tissue*

To obtain preliminary information about the uptake and distribution of lead within the plant, and to compare the effects of lead on growth with internal lead concentrations, plant material from the single salt culture and the hydroponic culture experiments was divided into stems, roots, and leaves and analysed for lead content. In the case of the single salt culture plants, and with the plants grown in hydroponic culture for 2 and 3 weeks it proved necessary to bulk the samples to provide sufficient amounts of tissue for analysis. The dried samples were weighed, and then ashed at 350°C for 4 hours in a Gallenkamp model FR-600 muffle furnace. The residue was next ground and then refluxed at 90°C in 1 mol/l nitric acid for 2 hours. Aspects of plant ashing procedures for lead analysis have been discussed by Webber (1972) who reported that combined ashing techniques of this type gave the best recovery of lead.

At the end of the extraction period the preparations were decanted and because the measurement technique employed is pH sensitive, (see Figure 2.2.1a) neutralised with 1 mol/l sodium hydroxide solution. The total volume was measured and the lead molarity of each solution measured with an EIL 7030 Laboratory pH meter used in conjunction with an Orion model 94-82 lead selective ion-electrode and a model 92-02 double junction reference electrode. The system was calibrated against a 3 decade range of lead nitrate molarities.

Ion selective electrodes are robust and sensitive tools for the measurement of ion concentration. The electrodes are sensors which when placed in a solution develop a potential related to the logarithm of activity of a given ion. At low concentrations this offers a very close approximation of ion concentration. Effectively the electrode consists of a non-conductive cylinder, which is sealed at its lower end by a membrane which allows only the transport of the anion or cation to be measured.

Inside the membrane is a filler containing a fixed amount of the ion under examination. Ions are accepted by, or released from, the membrane depending on the external ion concentration until an equilibrium state is achieved. In the course of this ion flux the membrane develops a potential, which may be measured in relation to a reference electrode in the same test solution using a millivoltmeter. With the EIL 7030 meter the procedure is simplified in that the instrument may be calibrated directly in molarities. Three types of ion-selective electrode are available:

1) Glass electrodes - e.g. sodium, potassium, pH.

2) Liquid junction electrodes in which the membrane takes

the form of a porous disc saturated with an organic ion exchanger, generally immiscible with water.

3) Solid electrodes where the membrane is a disc of crystalline material, either cut from a crystal and polished or pressed and fused from powder.

The Orion lead electrode is of the latter type and is sensitive to free lead concentrations as low as 10^{-7} mol/l (see Figure 2.2.1.b). Unfortunately other ions may interfere with the analysis. Copper, mercury and silver contaminate the membrane, forming a precipitate on the membrane and slowing down electrode response. If high levels of cadmium (> lead concentration) are present, this may also contaminate the membrane. Such contamination may easily be detected by the changes in response time of the electrode and the precipitate removed by gentle polishing using Orion electrode polishing strips. For further information on ion selective electrodes, see Simpson (1976).

2.3 *Light Microscope Studies*

In this part of the study a histochemical method was used to investigate the distribution of applied lead in the seed and seedling stages of radish. Examinations were also carried out on older plant material exposed to lead for short periods of time, but these were of a fairly curtailed nature as growth studies had shown previously that plants grown in solutions containing relatively high concentrations of lead tend to remain in the seedling stage of growth.

Radish seeds were exposed to a 10 mEq/l lead nitrate solution for varying periods of time, then rinsed in deionised water. The seeds were treated with hydrogen sulphide, which in the presence of lead gave rise to a dark brown or black stain. Initially specimens were dehydrated to 70% alcohol prior to hydrogen sulphide treatment,

but it was found that substantial loss of lead occurred during the dehydration procedures. Subsequently specimens were placed directly in a saturated aqueous solution of hydrogen sulphide for 20 minutes, then transferred to 70% alcohol, dehydrated and embedded in paraffin wax (see Appendix 2). While the hydrogen sulphide technique is not specific for lead, it is rapid and gives good contrast for photography (Crowdy and Tanton, 1970). That the stain distributions observed were representative of lead distribution was confirmed by staining with sodium rhodizonate (see Appendix 2). This compound has been found to give good results even with sparingly soluble lead compounds and may be made specific for lead, forming a scarlet lead rhodizonate complex at pH 2.8 (Glater and Hernandez, 1972). Sections of the prepared blocks were cut using a Leitz rotary microtome set at 10 μm, and then dewaxed in xylol and mounted in Canada Balsam.

The seedling and whole plant material was treated in a similar manner to that described for the seeds, except that lead solutions were supplied at concentrations of 0.1, 1.0 and 10.0 mEq/l. The plants were grown in single salt culture or, in the case of the older specimens, in hydroponic culture followed by exposure to aqueous lead solutions, these treatments being carried out in the chambers and under the regimes described in section 2.1. Material was rinsed and exposed to saturated hydrogen sulphide solution as before, and then dehydrated, embedded and sectioned. Resin-embedded sections of root material were also examined after sectioning on a Porter-Blum ultramicrotome model MT2-B (Ivan Sorvall Inc., Newtown, Connecticut, U.S.A.) set to a section thickness of approximately 1 μm. In each case comparisons were made with sections of untreated plant material.

The sections were examined under a Zeiss Photomicroscope II

and photographs taken with the same instrument using Ilford Pan F film at A.S.A. 50. Negatives were developed using 'Acutol' and 'Acufix' reagents following the manufacturer's recommendations and printed on Ilfospeed grade 2 paper.

In addition histochemical studies were carried out using the methods of Chayen et al. (1969) to identify the chemical nature of layers within the testa. Details of these procedures are presented in Appendix 2.

2.4 *Electron Microscopy*

The plants examined in the course of the ultrastructural studies were grown under conditions identical to those used for plants subjected to light microscopic examination.

Lead is electron dense and so its visualisation in the electron microscope requires no staining procedure. However, in the course of preliminary examinations using conventional fixation, dehydration and embedding techniques (see Appendix 2) the problems of lead leaching which had been observed in the light microscope studies were found to exist also in the electron microscope preparations. In fact the problem is more acute, as the incubation periods at the various stages of the standard preparatory procedures are much longer than those encountered in light microscopy, and also the sections examined are much thinner. Rapid preparatory procedures have been described by Glauert (1975), and modifications of these procedures were employed in subsequent experiments to improve lead retention (see Appendix 2). It was also found that a hydrogen sulphide pretreatment tended to aid lead retention. Unfortunately hydrogen sulphide is itself toxic, and was found to cause extensive breakdown of the tissue, but this was

overcome by applying the sulphide treatment as a 0.5% solution of ammonium sulphide in cacodylate buffer (see Appendix 2). Investigations were also carried out on material fixed only in glutaraldehyde and on unfixed material, but in the latter case the lack of preservation rendered any structural evaluation of the material impossible.

Comparison of glutaraldehyde-fixed and double-fixed material showed that the second fixation in osmium tetroxide also substantially reduced the lead content of tissue. Attempts to overcome this by using potassium permanganate as an alternative second fixative proved ineffective. As the second fixation procedure is essential if good subcellular detail is required, it was decided to accept the limitations of osmium tetroxide and retain this in the second fixation procedure.

In all cases embedded material was sectioned on a Porter-Blum MT2-B Ultramicrotome on to water. The sections were expanded with chloroform vapour and gold sections transferred to uncoated 100 μm copper grids (Taab Laboratories, Reading). Ultramicrotomy of tissue exposed to high concentrations of lead proved difficult, since crystals in the tissue caused damage to the knife edge after a short period of use, and subsequent sections were therefore badly scored. Knives were therefore changed at frequent intervals.

The grids were examined under a Philips EM300 Transmission Electron Microscope, at 80 KV. Observations were also carried out at lower accelerating voltages (40 and 60 KV) in cases where contrast was low and cellular detail indistinct. Photographs were taken using Kodak 4489 Electron microscope film, processed using Kodak D19 developer and Kodafix fixative and printed on Ilfospeed grade 2 paper. Control material, grown and prepared in an identical manner to the lead-treated specimens, was also examined for comparative purposes. These sections

were examined in an unstained condition, and also after post-staining for one hour in Reynold's lead citrate stain (see Appendix 2).

2.5 *Electron Microprobe Analyses*

These were carried out at two locations, and using two different types of instrument. Small spot analyses were carried out at the Department of Zoology, Cardiff University, while area analyses and X-ray images were constructed at JEOL U.K. Ltd., London. However, specimen preparation was identical for both systems.

Radish seedlings were grown in deionised water for 4 days and transferred to 10 mEq/l lead nitrate solution for 3 days. Roots were then washed, fixed and embedded. Since osmium interferes with energy-dispersive X-ray analysis of lead, and because osmium fixation had been seen to cause loss of lead from the specimen, analysis was only carried out on glutaraldehyde-fixed material. Accordingly specimens were fixed, dehydrated and embedded as in section 2.4 (see Appendix 2). Because of the specimen distortion which may occur during microanalysis and because it was desired to minimise interference due to the grid bars, sections were mounted on carbon-coated Formvar films supported on 500 µm copper grids (EMscope Laboratories, London) (see Appendix 2). The small-spot analyses were carried out on gold sections using a Philips EM300 Transmission Electron Microscope, at an accelerating voltage of 80 KV with a 165 eV-resolution silicon detector and an EDAX 505 multichannel X-ray analyser. Spectral traces were displayed on a TV monitor and photographed on Ilford Pan F film. A typical trace, with peaks identified, is shown in Figure 2.5.1.

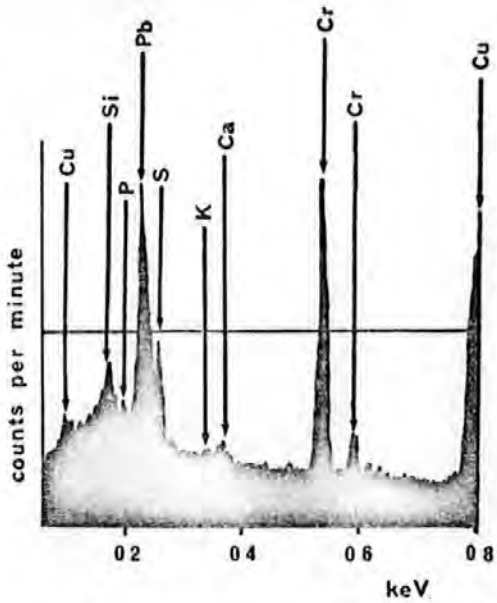


Figure 2.5.1. Peaks in typical electron microprobe trace of small spot analysis carried out on lead-treated root tissue. Copper peaks result from proximity of analysis area to grid bar; chromium peak is caused by specimen holder.

Measurements were also taken of relative lead content at several point sites in the inner cortex/stele region of the root. As the results recorded in this type of analysis may be modified by variations in specimen thickness and density, a correction was introduced. When electrons strike a specimen, X-ray radiation is released from two sources. Firstly, when the incident electrons interact with the electron shells of the specimen atoms, they may cause electrons to migrate from a lower energy shell to a higher energy shell. When the displaced electron drops back, energy is released in the form of 'hard' or short-wavelength radiation; this is the radiation detected in elemental analysis. In addition radiation may be released due to the slowing down of incident electrons as they pass through the specimen. This radiation is of a longer wavelength and is

referred to as 'soft' or 'white' radiation. It is also referred to as 'Bremsstrahlungen' or 'breaking rays', and its magnitude varies according to specimen thickness and density. Therefore by dividing the counts per minute of 'hard' radiation by the counts per minute for 'soft' radiation a correction for specimen thickness and density may be applied.

Using the same analysis system semi-quantitative analyses for several elements were carried out on small samples of radish material following lead treatment at 0, 1.0 and 10.0 mEq/l lead nitrate in hydroponic culture (see section 2.3). Portions of root material and whole plant material were washed in distilled water, dried and weighed. Samples were bulked to provide approximately constant aggregate sample weights and the aggregate samples ashed at low temperature (less than 100°C) in a reactive oxygen plasma ('Plasmod' - Nanotech (Thin Films) Ltd.) for 10 minutes. The ash was dissolved in 10 μl 0.5 N nitric acid and sprayed on to carbon coated grids. For full details of this technique see Davies and Morgan (1976). The grids were then analysed for several elements, including calcium, sodium, potassium, magnesium, phosphorus and lead, using the analytical system described above.

In addition to the microprobe elemental analyses, plant material was analysed in a more conventional manner for iron and calcium contents. Whole plants, grown in lead contaminated culture solutions (see section 2.1.3) were harvested when 4 weeks old and washed, dried, weighed and ashed using the procedures described in section 2.2. Extracts were analysed for calcium content using an EEL flame photometer calibrated against a range of calcium nitrate solutions, and for iron using an 'Atomspek' atomic absorbtion spectrophotometer, calibrated against a range of ferric chloride solutions.

Area analyses were carried out using a JEOL Temscan - 100 CX microscope in the scanning transmission (STEM) mode at 100 KV. The STEM mode allows the examination and analysis of thicker specimens than the transmission mode, and so blue sections were used for this part of the study. Analysis was carried out using a silicon detector of 145 eV resolution and a Link 290 X-ray analyser. Results were displayed on a TV monitor in the form of lead profiles measured along a horizontal line across the field of view, and also in the form of lead X-ray images of the field of view. These were recorded on Polaroid film. In addition belt transects of lead-impregnated roots were compiled by analysing adjacent areas of the root at low magnification. These were carried out in an approximately radial orientation and with machine settings kept constant.

2.6 *Physiological Experiments*

2.6.1 *Cell Division*

Batches of approximately 250 radish seeds were sown on filter paper in petri dishes and wetted with deionised water. The seeds were incubated in darkness at 25°C for 72 hours, and during this time subjected to the selection process described in section 2.1.3. Following this 6 batches of 10 seedlings were transferred to filter paper in clean petri dishes and supplied with 10 ml lead nitrate solutions at 0, 0.01, 0.1, 1.0, 5.0 and 10.0 mEq/l. After a further incubation of 12 hours in darkness at 25°C the root tips were excised and subjected to the fixation and staining procedure outlined in Appendix 2. The preparations were finally dehydrated and mounted in Euparal (Raymond A. Lamb Ltd., London) as it was found that this final procedure reduced cytoplasmic staining and greatly enhanced contrast.

Counts were carried out using a Baker microscope fitted with a mechanical stage, at a magnification of x400. A 10 x 10 eyepiece graticule was fitted to facilitate counting. Because of difficulties in distinguishing prophase and anaphase counts were restricted to metaphase and telophase.

2.6.2 Cell Elongation

In initial studies elongation experiments were carried out on 7 day old radish stem material from plants which had been grown in deionised water in the dark at 25°C. Segments 10 mm in length were cut 5 mm behind the stem apex, and transferred to a range of solutions containing various concentrations of lead nitrate and indol-3yl-acetic acid (vide infra).

However in subsequent experiments coleoptiles of Triticum aestivum were used as the experimental material as these were found to produce more reliable results. Caryopses of uniform size were selected and soaked in running tap water for two hours, then sown embryo uppermost at regular intervals in plastic petri dishes lined with Whatman no. 1 filter paper and wetted with 5 ml of distilled water. Regimented sowing of this type has been found to produce the most uniform coleoptile material and also the most consistent results in coleoptile elongation tests (D.N. Price, personal communication).

The dishes were incubated in darkness at 25°C for 36 hours, at which time the petri dish covers were removed and an additional 10 ml of distilled water were added to each dish. After a further incubation period of 48 hours uniform coleoptiles were selected and cut from the seeds under low intensity illumination from a 40 W tungsten bulb screened with 3 layers of dark green cinemoid gel sheet no. 24 (Rank Strand Ltd., Brentford). A 10 mm segment was

excised 3 mm from the tip of each coleoptile and washed in deionised water. Segments, supported on filter paper, were incubated in petri dishes containing 10 ml aliquots of mixtures of lead nitrate, IAA, mannitol and calcium nitrate as necessary. Five segments were placed in each dish and 10 replicate dishes were used for each treatment. The replicates were incubated in darkness at 25°C for 48 hours and the increase in segment length measured with a pair of vernier calipers. Some variation was observed in control treatments which was attributed to variation in seed batches.

2.6.3 Cell Wall Elasticity/Plasticity

Measurements of cell wall elasticity/plasticity were made using the system devised by Heyn (1931). Wheat coleoptiles were prepared using the methods described in section 2.7 and incubated in batches of 10 in solutions containing 1 μg/ml IAA and lead nitrate at 0, 0.01, 0.1, 1.0, 5.0 or 10.0 mEq/l. The segments were incubated in the dark at 25°C for 24 hours and then removed and rinsed. The primary leaf was withdrawn from each coleoptile and the segments transferred one at a time to the apparatus shown in Figure 2.6.3.a. During this process the coleoptiles were kept drenched in deionised water and were positioned on the support rod in such a manner that the rounded ends of the pointer and the support rod were just touching. A 250 mg rider was attached to the pointer and the change of inflexion of the pointer noted at one minute intervals until the pointer had stabilised. The weight was then removed and readings resumed until the pointer had stabilised once more. The total deflection was recorded as a measure of tissue rigidity, and the angle through which the pointer returned on removal of the rider was recorded as a measure of tissue elasticity. Tissue plasticity was computed as the difference between

these figures. A typical response curve is shown in Figure 2.6.3.b.

2.6.4 Water Uptake

Measurements of IAA-directed water uptake were made in the presence of lead using the technique described by Masuda (1965). Discs 10 mm in diameter and 1 mm thick were cut from freshly-harvested Jerusalem artichoke (Helianthus tuberosum) tubers and washed in aerated distilled water for 24 hours, then blotted under constant pressure and placed in petri dishes containing 1 μg/ml IAA and lead nitrate at 0, 0.01, 0.1, 1.0, 5.0, and 10.0 mEq/l. Each dish contained 5 discs of tuber tissue, and each treatment was replicated 5 times. After incubation at 25°C for 72 hours the discs were removed, blotted and reweighed. Measurements were also taken of final disc diameter using vernier calipers.

2.6.5 Binding of Lead to Cell Wall Fractions

In order to determine more about the effect of lead on the cell wall, the ability of various wall fractions to bind lead was assayed. Wall fractions were prepared from 4-day old coleoptile material using a combination of the techniques of Siegel (1962), Barratt and Northcote (1965) and Preston (1974). The complete procedure is shown in Figure 2.6.5. The resulting extracts were precipitated, washed, and taken up in deionised water. In some cases (non-cellulosic polysaccharides, hemicellulose C and cellulose) the precipitates could not be redissolved in deionised water and in these cases measurements were taken using a fine aqueous suspension of the cell wall fraction. Aliquots of these solutions and suspensions were added to known volumes of standard lead nitrate solutions, and changes in the concentration of available lead measured with the lead-selective electrode system described in section 2.2. In this instance the

system was linked to a Bryans 28000 chart recorder so that end-points could be detected accurately. As well as the cell wall extracts, samples of commercially-prepared α-cellulose were examined for their ability to bind lead ions.

In addition the ultraviolet absorbtion spectra of certain of the fractions were monitored using a Pye Unicam SP 1800 spectrophotometer. Samples of the fractions, dissolved in their respective solvents, were scanned in the spectrophotometer through the ultraviolet spectrum, using the solvent system alone as a reference. The samples were then contaminated with lead nitrate and re-examined. An equal amount of lead nitrate was also added to the reference solutions to obliterate any spectra due to the nitrate ions.

2.6.6 Estimation of Endogenous Auxins

Five hundred radish seeds were sown on filter paper in plastic boxes, and lead nitrate was provided in culture solution (see section 2.1.3) at concentrations of 0, 0.01, 0.1 or 1.0 mEq/l. Levels in excess of these were not applied because the resultant growth inhibition would have reduced the amount of tissue available for analysis. Seeds were placed in an environmental chamber under the conditions detailed in section 2.1.2, 5 replicates being set up for each treatment. After 7 days the seedlings were harvested and the roots excised. The stems and leaves from each replicate were bulked and weighed, and stored immediately in liquid nitrogen. The tissue was then extracted using a technique similar to that described by Knegt and Bruinsma (1973). Wherever possible during the extraction procedure samples were kept at reduced temperature ($< 4^{o}C$) and in a nitrogen atmosphere to reduce losses, and when extracts had to be left overnight they were stored in liquid nitrogen. The complete procedure is shown in Figure 2.6.6.

Chromatography was carried out using an isopropanol:ammonia:water (9:3:1) solvent system (Weaver 1972), and bands localised against IAA standards using a 248 nm ultraviolet lamp. The bands were re-extracted with methanol, reduced and estimated using the wheat coleoptile elongation test as detailed in section 2.7, and also with Ehrlich's reagent (see Appendix 2).

For comparison, experiments were carried out using tissue to which standard amounts of IAA had been added. From these it was found that recovery using this technique was approximately 65%.

In all experiments where numerical data were collected means, standard deviations and standard errors of replicates were calculated. Appropriate significance tests were also carried out, using either t-tests or analysis of variance (see Appendix 3).

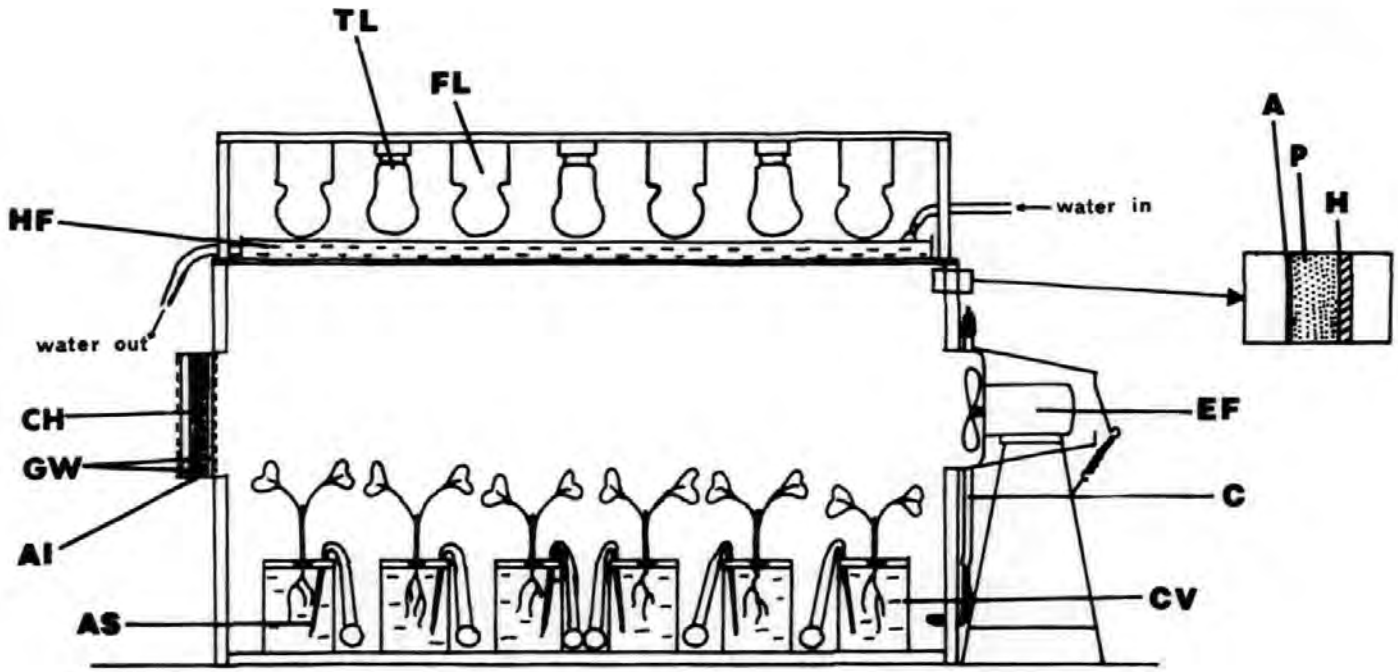


FIGURE 2.1.1. Chamber constructed for hydroponics experiments.

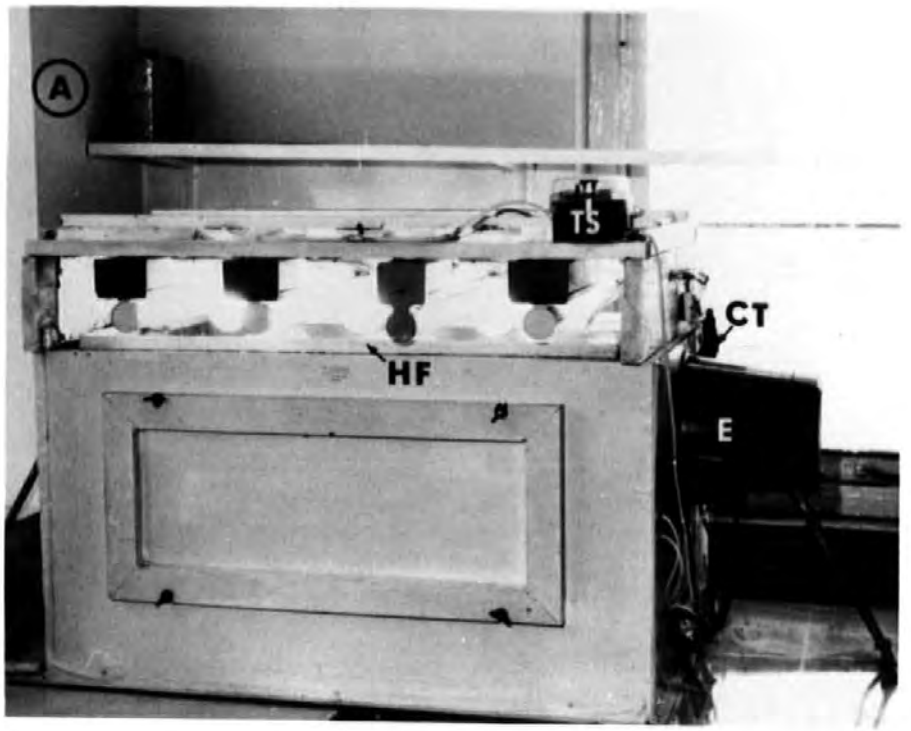
A - aluminium foil; P - expanded polystyrene; H - hardboard; EF - extractor fan; C - contact thermometer; CV - culture vessel; AS - air supply; AI - air intake; GW - glass wool; CH - activated charcoal; HF - heat filter; TL - tungsten lamp; FL - fluorescent lamp.

Plate 2.1.2 Culture chamber constructed for hydroponics experiments

A. External view

B. Internal view

TS - time switch; CT - contact thermometer; E - extractor fan; HF - heat filter; AS - air supply; LS - lightproof sleeve


A
TS
CT
HF
E

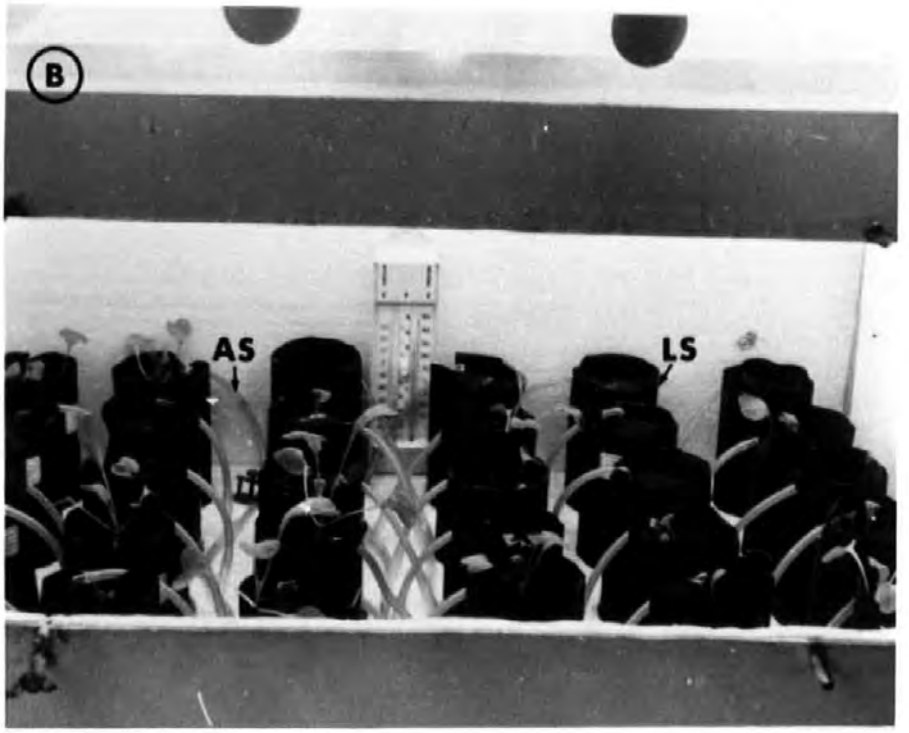

B
AS
LS

Figure 2.1.3 Typical regimes in culture chamber during hydroponics experiments

A. Temperature regime

B. Relative humidity regime

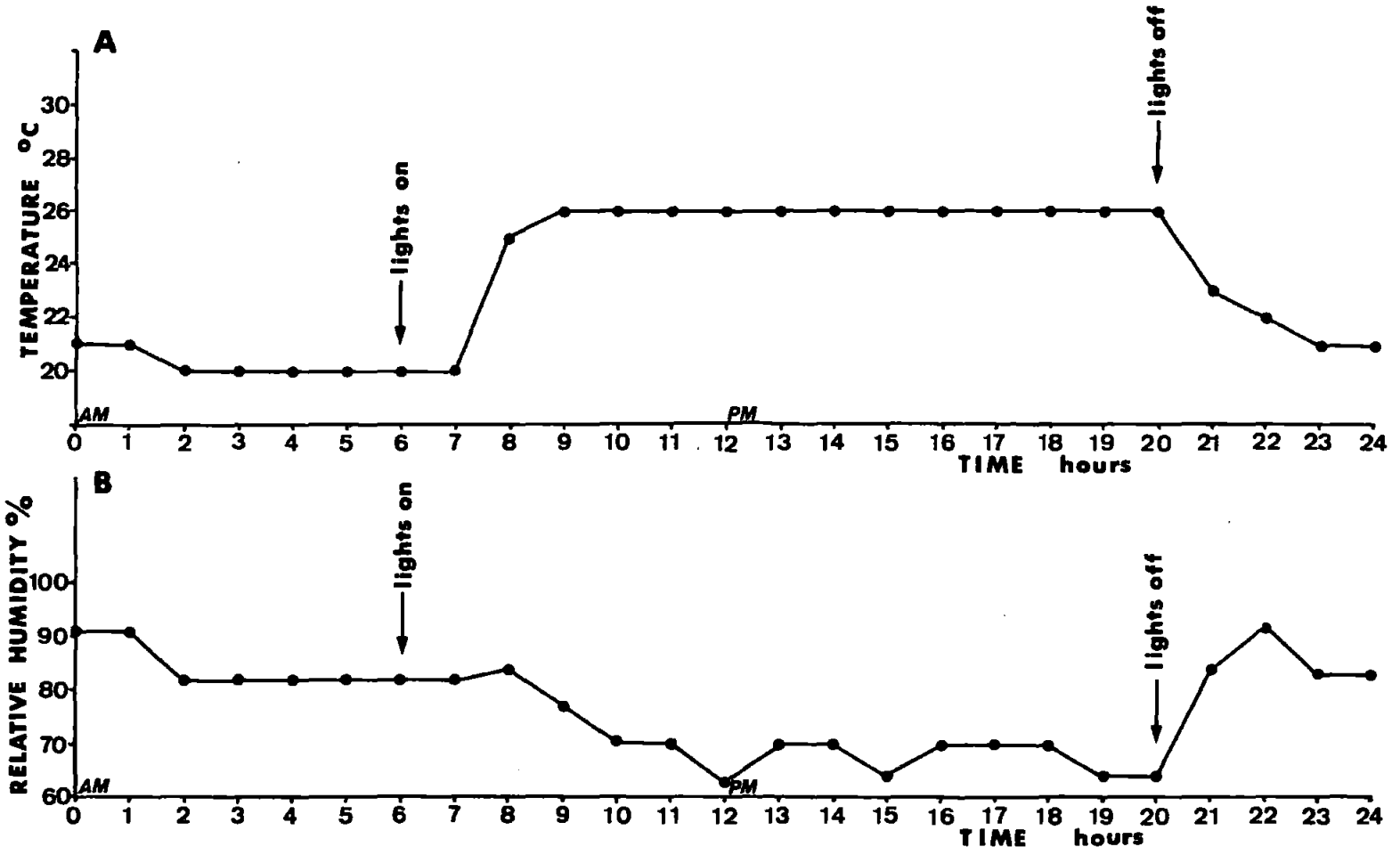

A
TEMPERATURE °C
30
28
26
24
22
20
lights on
lights off
AM
PM
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
TIME hours
B
RELATIVE HUMIDITY %
100
90
80
70
60
lights on
lights off
AM
PM
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
TIME hours

Plate 2.1.4 Plant culture arrangement with lightproof sleeve removed

C - reshaped Pasteur pipette delivering air to culture solution

P - plug of synthetic sponge to allow for lateral expansion of stem

F - float of expanded polystyrene blackened on top and sealed with paraffin wax

B - milk bottle cut to approximately 300 ml capacity.

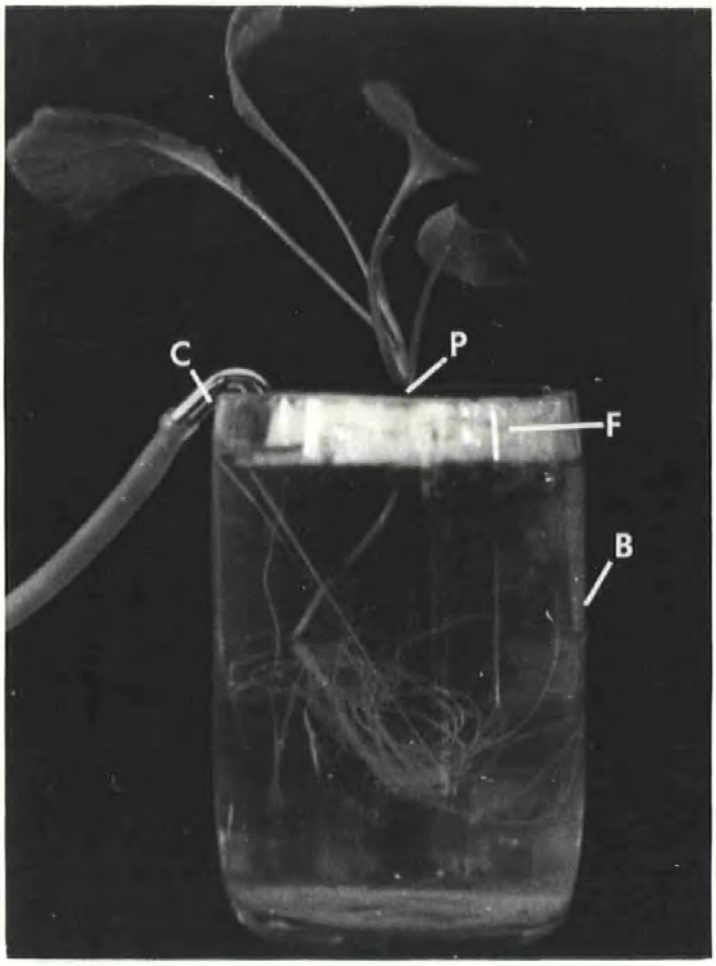

C
P
F
B

Figure 2.1.5 Relationship between total lead added to culture solution and available lead at start of experimental period, as measured by lead-specific electrode system (solution agitated).

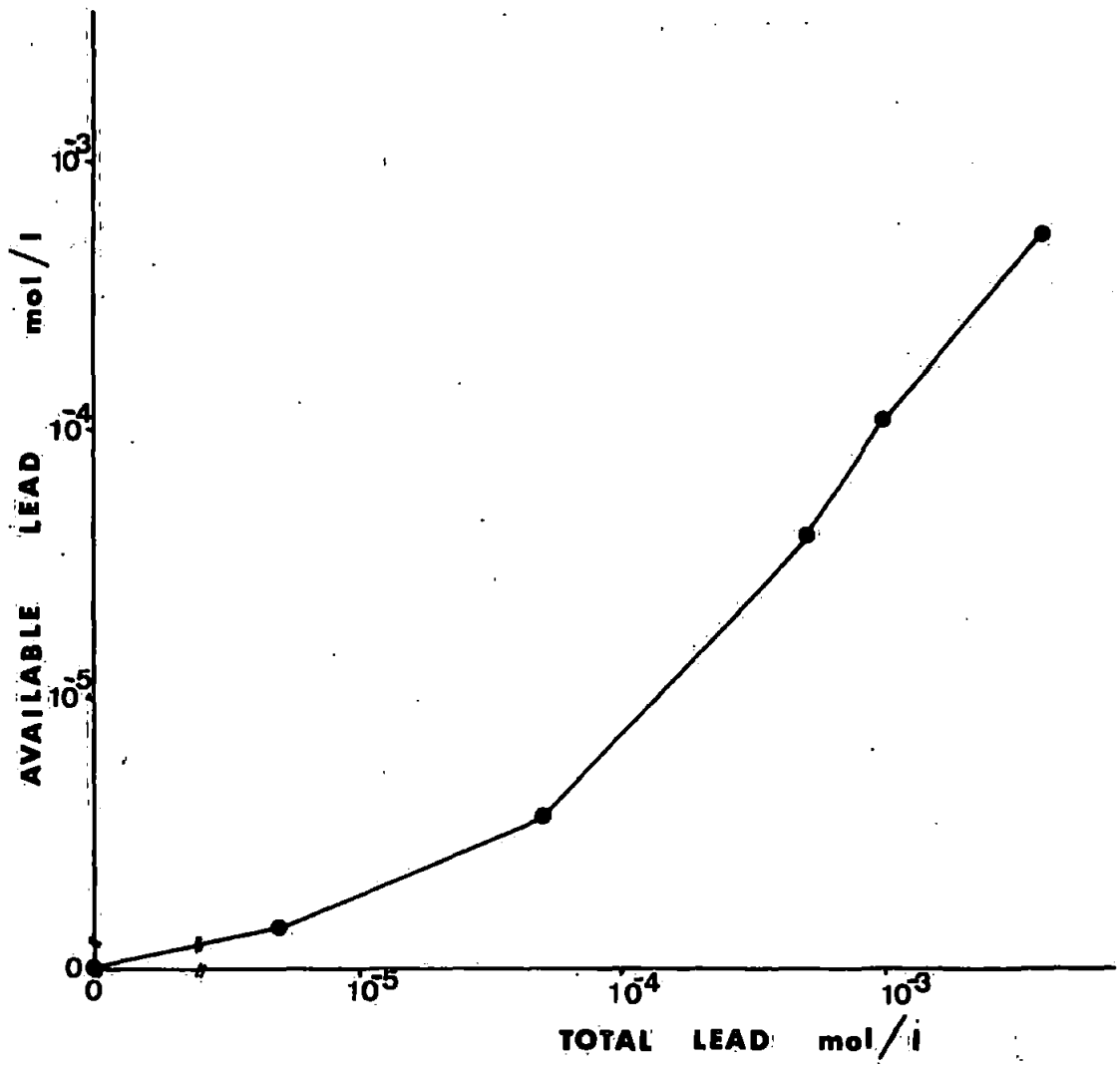

AVAILABLE LEAD mol/l
10^{-3}
10^{-4}
10^{-5}
0
0
10^{-5}
10^{-4}
10^{-3}
TOTAL LEAD mol/l

Figure 2.2.1 Performance curves for Orion lead selective ion electrode

A. Effect of solution pH on electrode potential in pure lead perchlorate solutions at 25°C

B. Typical electrode response to changes in lead ion activity (solid line) and free lead concentration (broken line) in pure lead perchlorate solutions at 25°C

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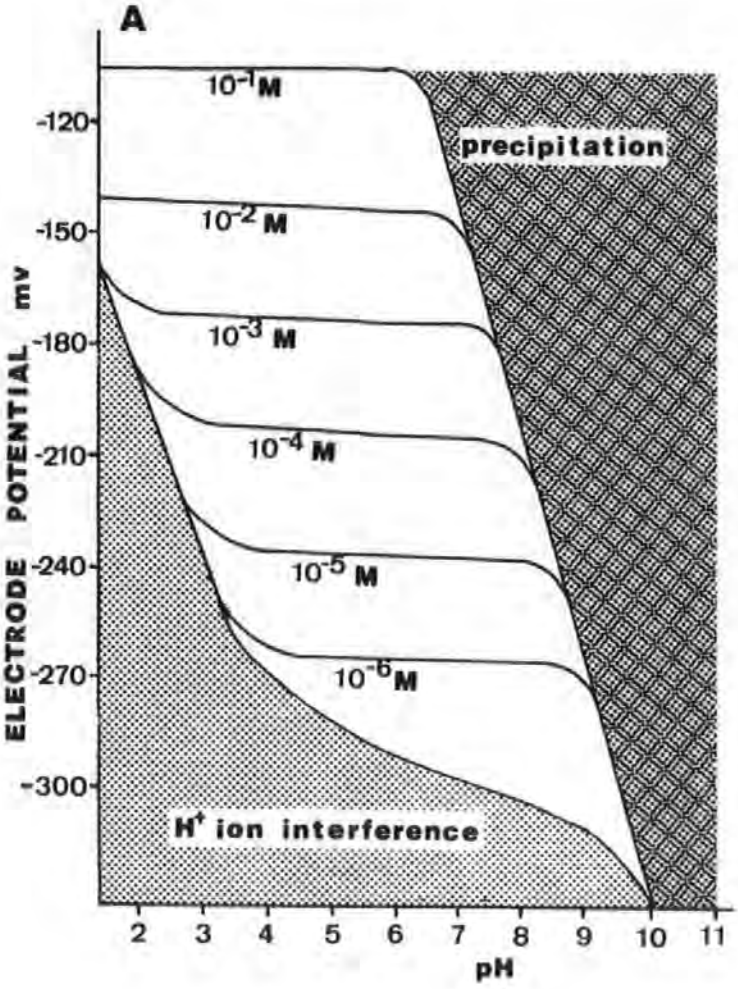

A
10^{-1}M
10^{-2} M
10^{-3} M
10^{-4} M
10^{-5} M
10^{-6}M
precipitation
H^+ ion interference
ELECTRODE POTENTIAL mv
-120
-150
-180
-210
-240
-270
-300
2 3 4 5 6 7 8 9 10 11
pH

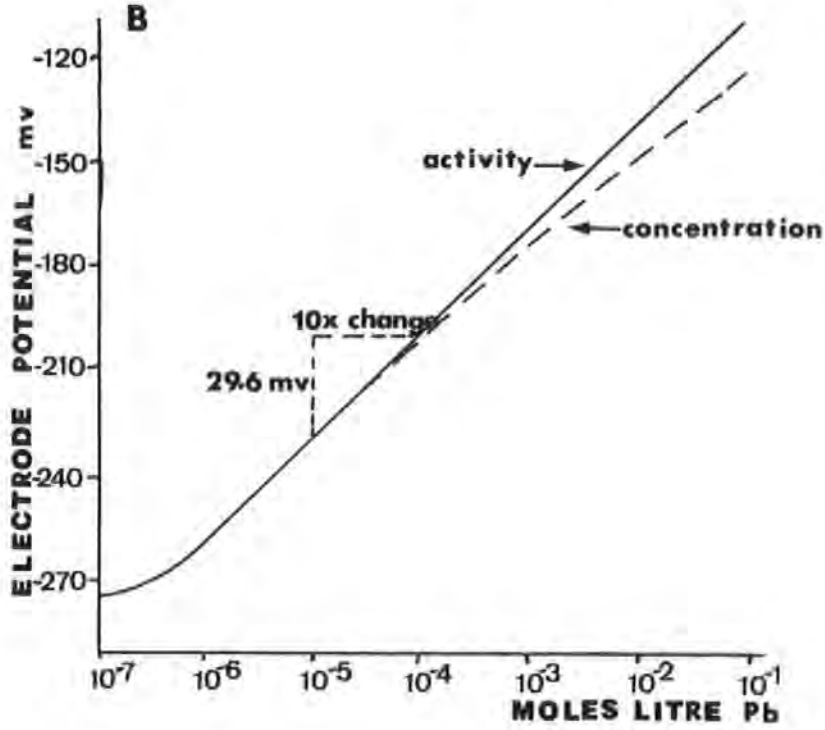

B
activity
concentration
10x change
29.6 mv
ELECTRODE POTENTIAL mv
-120
-150
-180
-210
-240
-270
10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
MOLES LITRE Pb

Figure 2.6.3 System employed in cell wall plasticity/elasticity studies

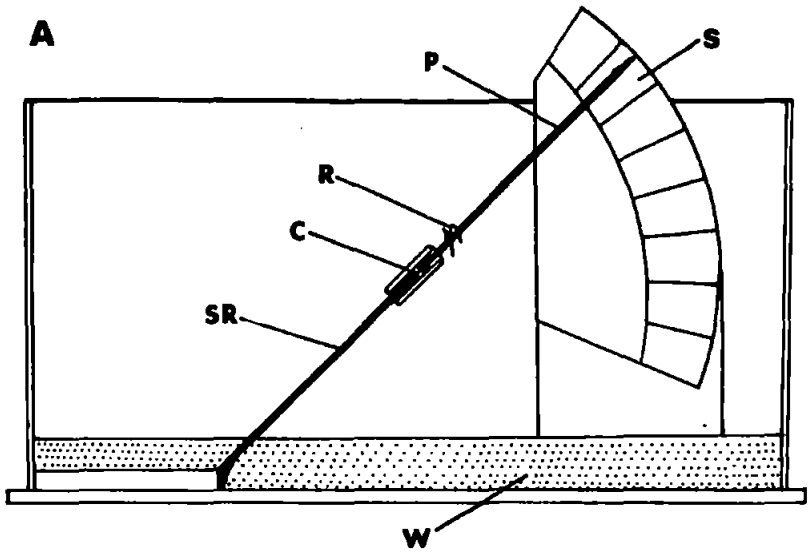
A. Apparatus used to measure coleoptile curvature, based on the design of Heyn (1931). In use the apparatus is enclosed in a glass cover to reduce water loss.

B. Typical response curve of coleoptile section under inflexion stress.

SR - support rod; C - coleoptile segment; R - 250 mg rider;

P - capillary glass pointer; S - scale in degrees;

W - water soaked sponge to increase humidity


A
P
S
R
C
SR
W

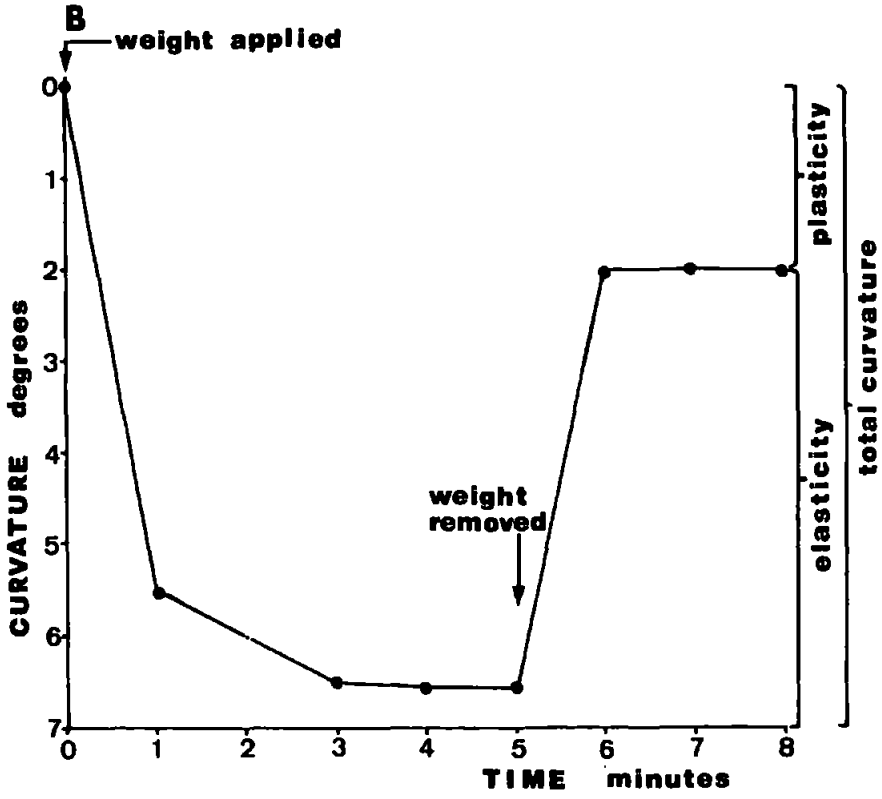

B
weight applied
weight removed
CURVATURE degrees
TIME minutes
0
1
2
3
4
5
6
7
8
plasticity
elasticity
total curvature

Figure 2.6.5 Cell wall fractionation procedure employed in experiments on the binding of lead to cell wall fractions

After Siegel (1962), Barratt and Northcote (1965) and Preston (1974)

CELL WALL FRACTIONATION

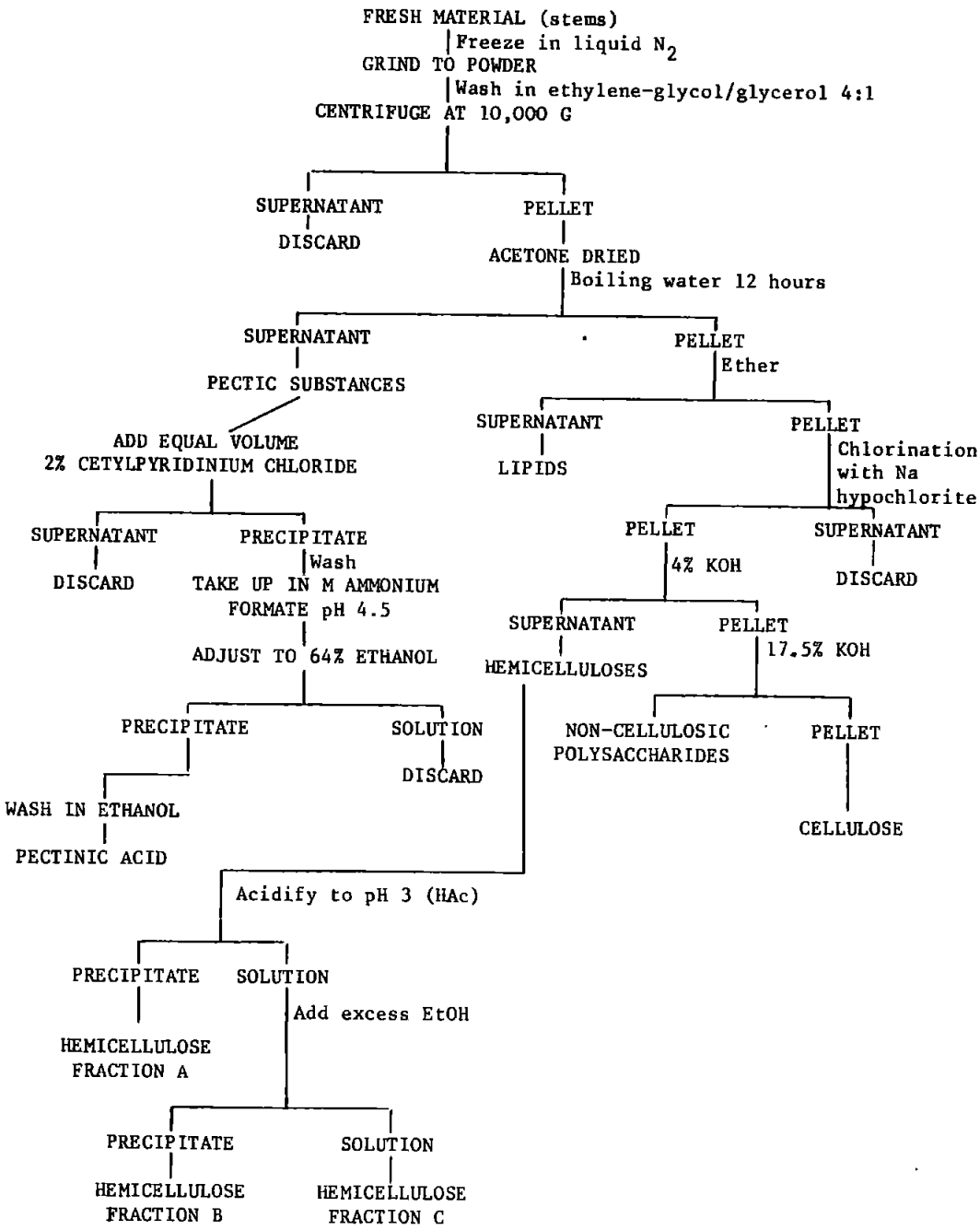
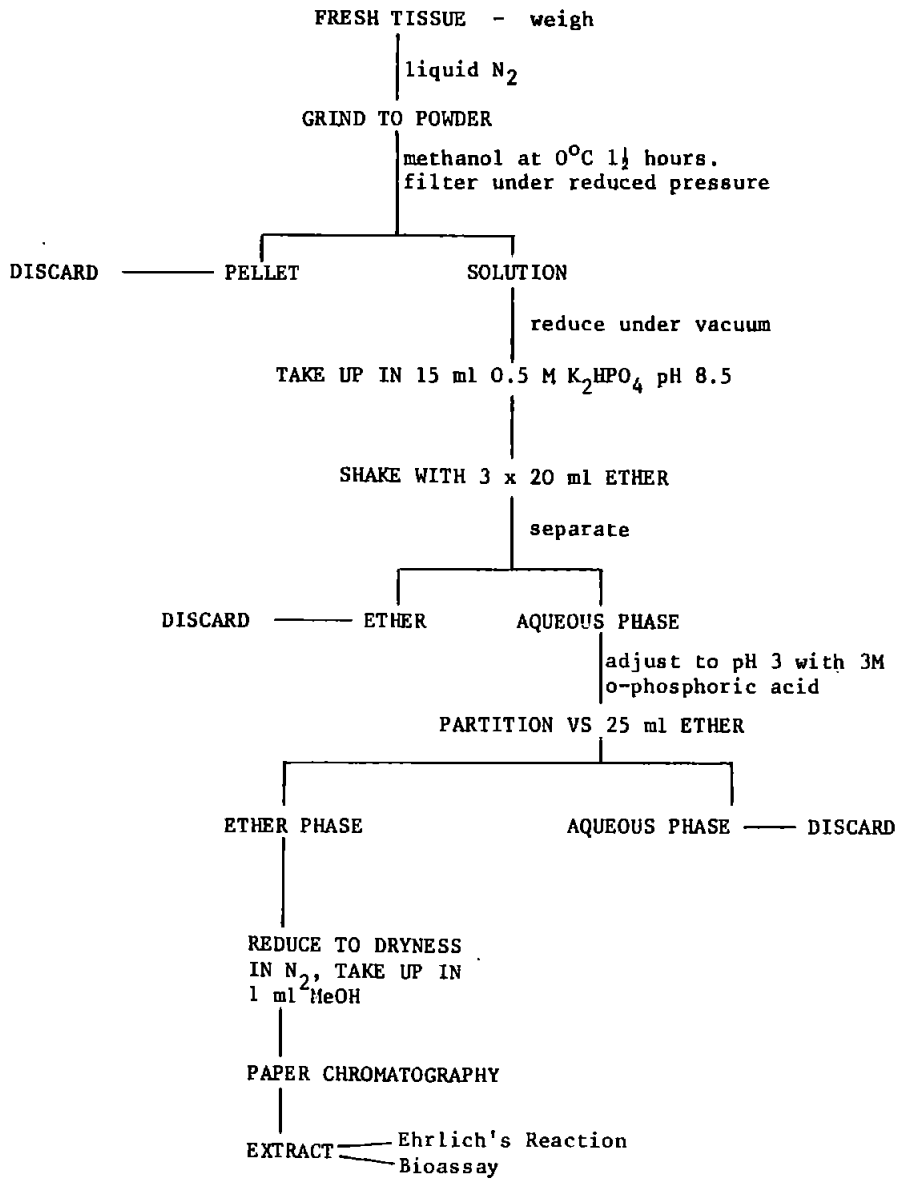


Figure 2.6.6 Extraction procedure employed in estimation of endogenous auxins in lead-treated plants

IAA EXTRACTION PROCEDURE



CHAPTER 3

THE EFFECT OF LEAD ON THE GERMINATION AND GROWTH OF *RAPHANUS SATIVUS*

Previous investigations (e.g. Stoklasa, 1913; Völcker, 1914; Stutzer, 1916) have demonstrated that at low concentrations lead nitrate may produce a slight stimulation of plant growth, while at higher concentrations growth is inhibited. These studies have been simple, and concerned solely with the effect of lead on the total weight of matter produced by the plant. The following experiments were carried out to evaluate in detail the effects of lead on the differential development of plant organs with time.

3.1 Germination and Root Hair Development

Radish seeds exposed to lead nitrate solutions at concentrations of up to 10 mEq/l showed no significant alteration in germination during the first seven days of incubation, although at the highest concentration lead appeared to cause some inhibition of radicle emergence. Subsequent trials in which lead nitrate solutions were supplied at higher levels (up to 50 mEq/l) produced a marked decrease in the percentage of seeds germinating (Figure 3.1.1.a and b). Contrary to the results of Dilling (1926), seeds in which the radicle failed to emerge in the seven day period of incubation showed no signs of recovery of their germination potential when transferred to deionised water, although it is of interest that the seeds exposed to 50 mEq/l $Pb(NO_3)_2$ still showed positive results in the triphenyl tetrazolium chloride germination tests at the end of the trial period.

In a review article Köller et al. (1962) discussed the inhibition or promotion of germination by nitrates. To elucidate the

possible role of the associated nitrate ions in a germination test of this type, a parallel experiment was carried out in which sodium nitrate was supplied to radish seeds at levels of up to 10 mEq/l. This provided no evidence for a significant nitrate ion effect at these levels (Figure 3.1.1.c).

The development of root hairs on the emerging radicle was, however, significantly inhibited by lead exposure ($P < 0.001$) (Figure 3.1.1.d). This was most evident at levels in excess of 1 mEq/l and at a concentration of 10 mEq/l of lead few secondary roots and very few root hairs were apparent after seven days. It also appeared that these seedlings suffered orientation problems, becoming twisted and malformed. This could be attributable to the lack of root hairs and secondary roots since this could reduce the seedling's ability to anchor itself on its filter paper substrate, and so render it difficult for the stem to maintain a vertical orientation.

3.2 Seedling Growth

Measurements of developing radish seedlings were taken during the first 14 days of growth. These measurements included root and stem length, and cotyledon leaf length and breadth. Exposure of these seedlings to lead nitrate solutions brought about statistically significant reductions in all growth parameters of the plant, these reductions increasing with time and with increasing lead supply (Figure 3.2.1). An exception was apparent at the 0.01 mEq/l level of treatment, where the presence of lead nitrate resulted in an increase in root length, and leaf length and breadth. Statistical analysis at the 14 day stage showed this to be significant ($P < 0.05$) in the case of root length, but not for leaf length or breadth. It was suspected

that such increase might be attributable to a stimulatory effect of the associated nitrate ions rather than to the lead itself. After 14 days the most marked reductions were those apparent in root length. Some of the specimens had also developed a multiple root system while others remained single. At the higher concentrations (5 and 10 mEq/l) lead exposure was typified by cotyledon chlorosis and the formation of necrotic lesions on the cotyledons. In addition a characteristic dark area was visible at the junction of the root and stem (Plate 3.2.4 a and b).

As in the germination experiments, control experiments were carried out to determine the effect of the associated nitrate ions on growth. These revealed that treatment with sodium nitrate brought about an enhancement of root growth, except at supply levels of 10 mEq/l sodium nitrate, when root length was approximately equal to that observed in specimens growing in deionised water. No significant variation was apparent in stem growth, but a sharp promotion of cotyledon expansion was apparent at supply levels in excess of 1 mEq/l sodium nitrate (Figure 3.2.2).

In addition experiments were carried out in which lead was supplied to developing seedlings as nitrate, citrate or acetate in order to evaluate the effect of the associated anion on the growth response of the seedling to lead. Growth inhibition in roots was most marked in the case of lead acetate, slightly less in the case of lead nitrate and least with lead citrate. Stem measurements also revealed little difference between the nitrate and acetate salts, but the provision of lead as the citrate resulted in the best growth rates. In cotyledon measurements little difference could be seen between treatments except that the lead nitrate solution caused a

slight reduction in leaf breadth when compared with the other salts.

3.3 *Hydroponic Culture Experiments*

In plants allowed to continue their development, the effects of lead on the growth parameters of the plant became more pronounced. Experiments were carried out in which plants were harvested at 2, 3, 4 and 5 week stages, and in the case of the 5 week treatments it was found that at the time of harvest plants grown at supply levels of lead of up to 0.1 mEq/l had produced expanded tubers of marketable proportions (while no official recommendations are available with respect to minimum size of radishes for retail sale, Sainsbury's chain of stores require tubers to be at least one centimetre in diameter) but the expansion which occurred in plants treated with 1.0, 5.0 and 10.0 mEq/l was minimal.

Analyses of fresh and dry weights of roots, stems and leaves showed definite inhibition of these growth parameters (Figures 3.3.1 and 3.3.2). This inhibition increased with increasing lead supply in a parabolic fashion.

Comparison of the fresh and dry weights in the form of a fresh weight/dry weight ratio provides a measure of the water content and hence the turgor of the tissue. Calculation of this ratio for the data from the hydroponic culture experiments revealed definite changes with lead supply, values of the ratio becoming progressively lower with increasing lead supply (Figure 3.3.3). This would suggest increasing water stress in the plants as lead supply increases. This view was supported by the observation that plants grown under lead burdens of 5 and 10 mEq/l were distinctly flaccid. This observation was also supported by pressure bomb measurements of lead water potential (see Appendix 1.2).

Similar reductions to those seen in fresh and dry weights were also seen in root length, stem length and breadth, and leaf area. It is interesting to note however that there was little increase in leaf area in control specimens between four and five weeks when compared with the increases observed in other treatments. It may be that at this stage of growth other factors were becoming limiting in the culture system (Figures 3.3.4 and 3.3.5). As in the 14 day analysis, at the 5 and 10 mEq/l treatment levels the leaves appeared chlorotic, this appearing first in an intervenal position and becoming more pronounced in progressively younger leaves. This would suggest a possible interference in iron metabolism. Investigations of chlorophyll synthesis in radish seedlings have shown that in short term experiments if lead solutions were fed directly to the cotyledons then chlorophyll synthesis was retarded; if the solution were supplied via the stems, then synthesis was slightly enhanced (see Appendix 1.3).

The reduction in leaf area of the plant was not only due to a reduction in leaf expansion, but also arose as the result of a reduced rate of leaf formation. At the time of harvesting the 5 week old specimens, the control plants were developing their third or fourth pair of true leaves, whereas those plants grown in 5 or 10 mEq/l lead nitrate solution possessed only 1 or 2 pairs of true leaves (Figure 3.3.6).

Examination of the results in terms of dry weight per unit length or unit area showed that lead caused a reduction in dry weight per mm for roots and stems, and an increase in dry weight per cm^2 for leaves. However, in the case of roots and stems these results tended to be rather difficult to interpret, since the ratio does not take into account variation in stem breadth, or variation in the proliferation

of lateral roots in the root system. The increase in dry weight per cm^2 observed in the leaves probably arose from the increased relative contribution of the cotyledon leaves to the overall leaf area, as the cotyledon leaves are appreciably thicker than the true leaves (Figures 3.3.7 and 3.3.8).

Throughout the trial period it was apparent that the toxic effects of lead became more pronounced with time. This did not seem to be the result of a lessening of the rate of growth of the lead treated plants, but merely the result of an increased difference between control and lead-treated specimens resulting from the faster growth of the control plants.

In general terms lead appears to cause reductions in the growth of the plant as a whole. However, this effect seems to be most pronounced in the root as in this organ prolonged exposure to levels of 5 and 10 mEq/l resulted in a virtual cessation of growth, and at levels of 10 mEq/l loss of root material may occur through breakdown of the root structure. Also it may be seen that the effects of lead exposure increase with increasing lead concentration. These relationships tend to adopt a parabolic form, suggesting that the effects observed vary in proportion to the square of the logarithm of concentration.

3.4 *Discussion*

Studies on the growth responses of dicotyledonous plants to lead burden are relatively few (Hooper, 1937; Rasmussen and Henry, 1963; Lagerwerff, 1971; Broyer et al., 1972; John, 1977). In this series of experiments it was found that lead had no visible effect on seed germination at levels of up to 10 mEq/l, although at higher

levels (up to 50 mEq/l) germination rates were severely reduced. Fleming (1943) reported only slight reduction in the germination of a range of vegetable seeds in soil treated with up to 2000 lbs per acre of lead arsenate, but it is difficult to evaluate such data as there is no way of knowing how much of this lead was available to the seed (see Chapter 1). Dilling (1926) reported the inhibition of germination of cress and mustard seed at levels in excess of 0.01% (≈ 0.97 mEq/l). In this study lead had no effect on germination of radish seeds, which may be attributed to a higher ability of the radish embryo to withstand lead toxicity, or to a physical or chemical barrier to its entry into the seed. Moreover in Dilling's experiments it was found that at lead supply levels of up to 0.2% (≈ 19.4 mEq/l) inhibited seed recovered its ability to germinate when returned to lead-free solutions. In the case of radish no such recovery of germination ability was seen. The reversibility of the lead-induced inhibition of germination in Dilling's experiments would suggest that the inhibition arises either from the entry of lead into low affinity sites in the seed, or from purely osmotic considerations. The toxic response in radish is irreversible and it must therefore be assumed that the inhibition is of a more substantial nature in this case. It is possible that the lead ions, on entering the embryo, interfere with the enzymes and inorganic salts - e.g. precipitation of phosphates may interfere with synthesis of adenosine triphosphate (Köller, 1962).

The action of lead on the formation of root hairs and secondary roots was conspicuous from an early stage, and may be important to the plant in two ways: firstly it may reduce the plant's ability to anchor itself in its substrate, and secondly the absence of root hairs and secondary roots results in a reduction in the root surface area,

which may cause a serious decrease in the root's absorptive abilities. This is especially important when considered in conjunction with the subsequent inhibition of root development.

From the results of the seedling growth experiments it was obvious that lead exerted a deleterious effect on all the growth parameters of the plant. This appeared to be most severe in roots, where there was an almost immediate cessation of growth on exposure to the highest concentration of lead. In the stems, and even more so in the cotyledon leaves, inhibition of growth was less severe and some growth continued even in the 10 mEq/l treatment. Such results are indicative either of a greater ability of the leaf and stem to withstand lead exposure than the root, or of the root providing a barrier restricting movement of the lead ions into the aerial parts of the plant. The latter mechanism has also been suggested by Jones et al. (1973). In radish this may be partly due to the accumulation of lead in the region at the top of the root which shows dark staining in the presence of lead.

In general the reduction in growth of the root, stem, and cotyledon leaf increased with increasing lead supply, a threshold being evident in the region of 1 mEq/l. However, root length, leaf length and leaf breadth were stimulated at the 0.01 mEq/l treatment level. Berry (1924) also recognised this stimulation in experiments with oats, and found that in single salt culture an optimal response occurred with lead nitrate solutions of 12.5 ppm (≈ 0.075 mEq/l). In soil experiments he found that optimum levels were much higher, in the region of 900 ppm (≈ 5.43 mEq/l), presumably because of the soil's ability to bind ions in a low available form (see Chapter 1). Such

stimulation was also reported by Stoklasa (1913), Stutzer (1916) and Keaton (1937).

Parallel experiments with nitrate supplied as the sodium salt also showed a substantial enhancement of root elongation up to a concentration of 0.1 mEq/l of nitrate, at which point the root length response to nitrate began to plateau. At the 0.01 mEq/l treatment level, an increase in root length over control treatments approximately equal to that observed with 0.01 mEq/l lead nitrate was observed. It seems likely, therefore, that the stimulation observed at 0.01 mEq/l may be attributed to the associated nitrate ion rather than to the lead itself. No such improvement at low levels was observed in the cotyledon leaves of plants exposed to sodium nitrate, although enhanced cotyledon expansion occurred at treatment levels in excess of 1 mEq/l. In spite of this, improvement was clearly visible in cotyledon leaf development at low levels in the lead nitrate treated specimens. It may be that this improvement is a reflection of the improvement in growth observed in the root; or perhaps more likely that lead ions are adopting sites in the root and stem, and in the process release other ions which are then able to move into, and be utilized by, the developing cotyledons.

From the results shown in Figure 3.2.3 it was clear that at the supply levels involved (1 mEq/l) the associated anion, or 'counterion', exerted an appreciable influence on the growth response of the plant. In the root the order of decreasing toxicity appeared to be lead acetate > lead nitrate > lead citrate, while in the stem the nitrate salt appeared to be more toxic than the acetate. In view of the observations of Hiatt (1968) on the counterion effect, it would

seem feasible that this variation in toxic response results from variation in the accumulation of the compounds by the root. Such variation would require the involvement of the 'high concentration mechanism' of ion uptake as described by Hiatt, and which in the case of monovalent cations he found to come into operation at supply concentrations of the order of 1 mM. Such a system would be supported by the growth responses of the rest of the seedling as any variability in the uptake of lead due to the counterion effect would tend to be decreased as lead becomes sequestered into non-motile sites during its passage through the plant. Such variations arising from the counterion effect could, at least in part, explain the wide variation in lead uptake and yield response observed by different authors (c.f. e.g. Keaton, 1937; Baumhardt and Welch, 1972; Broyer et al., 1972; Hassett et al., 1976; Miller et al., 1977).

The responses observed in radish seedlings in single salt culture were found to be repeated in plants grown in hydroponic culture, notable reductions occurring with increasing lead supply in all parameters measured.

Difficulties exist in the determination of fresh and dry weights. Fresh weights vary with several factors, such as time of day and state of development of the plant, as well as with the size of the plant. Dry weights also vary with time of day, rate of respiration and mineral status. In addition great care must be exercised during the weighing process; with fresh weight determinations, it is of paramount importance that the periods between harvest and weighing are constant and as short as possible; with dry weight determinations the period and temperature of drying are critical. Subsequent to water loss, weight loss may continue as a result of

the volatility of resins, fats, etc., in the material. Alternatively, weight gains may occur as a result of oxidation (Evans, 1972). In spite of this, measurements of dry, and to a lesser extent, fresh weights remain the most commonly-used parameters in the comparison of the growth of plants.

Various authors have reported the effect of lead on plant fresh and dry weights. Berry (1924) found that in soil experiments slight toxic effects of lead nitrate were recorded at lead levels of 0.09% by weight of soil, and that the response increased with concentration. Slight increases in weight were recorded at lower treatment levels. Although in some cases additional increments of superphosphate of lime and muriate of potash were supplied, no analysis was carried out of the nitrate status of the soil prior to the experiment. Keaton (1937) also found slight increases in dry weight production at soluble lead ranges between 0.1 and 0.4 ppm lead oxide. No deleterious effects were seen at levels of up to 7190 lbs of lead carbonate per acre. Recently Miller, Hassett and Koeppe (1977) reported similar reductions in corn shoots with increasing lead supply, and found that the toxic effect was increased by the presence of another heavy metal (cadmium).

Other reports have considered weight gains of plants grown in culture solution. Miller and Koeppe (1970a) found that in sand cultures growth was retarded when 60 μmoles of lead nitrate per 500 g of sand were supplied. In the presence of phosphate ions 600 μmoles were required to elicit a similar response. Broyer et al. (1972) and Hooper (1937) both reported no significant reduction in weights of plants grown in hydroponic culture; but in these cases the lead was supplied at relatively low levels (50 μM lead nitrate and 30 ppm lead sulphate respectively). A more recent report by John (1977) indicated

that in culture solution low concentrations of lead (10 ppm) may improve dry weight yields slightly during the early stages of growth of certain lettuce varieties. He did not observe visual symptoms of toxicity at higher concentrations, but found that there were reductions in the dry weight production of plant tops at supply levels of 50 ppm.

It can be seen that such reports as are available are varied in their nature, and one of the most obvious shortcomings lies in the narrowness of the concentration range supplied. In the present study lead was supplied in a log. range of concentrations from 0.01 to 10.0 mEq/l. There was no indication of a low concentration stimulation, but both fresh and dry weights showed a reduction with increasing lead supply. This relationship tended to adopt a parabolic form in leaves, stems, and roots. In view of the wide variation which exists in the literature it is difficult to compare these results with existing data from other sources, but in general terms the reactions observed appear to be compatible with those observed by other authors.

Consideration of the ratio of fresh to dry weights provides an indication of the water content of the tissue. This is not merely a reflection of the turgor of the tissue, but also provides a measure of the plant's ability to maintain metabolic activity (Zimmerman, 1977). In an enclosed system of the type employed in this study, where the plants are in water culture and therefore each plant has available an abundant supply of water, it would be suspected that fresh weight/ dry weight ratios would remain fairly constant. However this is not the case (Figure 3.3.3). Instead the ratio is reduced with increasing lead supply, indicating a progressive lessening of water content. Other authors have failed to consider this parameter in

examinations of the effect of lead on plant growth. However, Fiusello and Molinari (1973) found that treatment of Avena sativa with 10^{-4} M lead nitrate solution caused a 23% reduction in dry weight over controls, but fresh weight was decreased by 34%, suggesting a similar reduction in the proportional water content of the tissue. Suchodoller (1967) also described the folding downwards of bean leaves on exposure to lead as resulting from a loss of turgor pressure.

The observed loss of water could be explained either by increased water loss through transpiration, or reduced water uptake. The former possibility seems unlikely, as the influence of lead on transpiration has been examined by Bonnet (1922) and by Hampp (1973), who found that the presence of lead resulted in a pronounced inhibition of transpiration. The observed reductions in water content may therefore result from a reduction in water uptake, probably arising from the arrest of root development. Such a process would be in agreement with the results of Hampp (1973), who reported reductions in water uptake with increasing lead supply.

Consideration of the development of roots, stems, and leaves of hydroponically grown plants further emphasised the deleterious effects of lead on growth. These effects included interference with root and stem elongation, and leaf formation and expansion. The interference was most marked in roots, where at high levels of lead supply growth ceased completely; then in leaves. It is interesting to note that there was a critical concentration at which the stem failed to expand to acceptable market proportions.

Other authors have noted lead-induced interference with root development. Bonnet (1922) observed short thick roots with abnormally long root hairs after lead treatment. Such a result appears somewhat

anomalous, as other authors (Griffith, 1919; Rasmussen and Henry, 1963; Hassett et al., 1976; Mukherji and Maitra, 1977; etc.) have reported thinning and stunting of roots, even at very low supply concentrations of lead (Bell, 1924). Considerations of the effect of lead on the aerial parts of the plant are rather less definite. Baumhardt and Welch (1972) reported that soil-applied lead at levels of up to 3200 Kg/ha reduced corn height in a manner which tended to increase with increasing lead supply; Rasmussen and Henry (1963) also reported lead-induced inhibition of stem development, which in their study was found to be of a similar magnitude to that observed in roots.

While direct linear measurements and weight measurements give an indication of the state of development of the plant, further information may be gained by considering the ratio of dry weight to linear and areal dimensions of the plant. By comparing the dry weight per unit length of roots, a measure is achieved of root proliferation and hence of root surface area and absorbtive capacity. When dry weight per unit length of stem is considered, an indication is provided of stem enlargement and hence of the development of food reserves, which is perhaps more relevant than the simple measurement of the widest part of the stem. The computation of dry weight per unit leaf area reflects the physiological development of leaves more accurately than leaf area alone, as in the young plant leaf dry weight per unit area becomes less as the contribution of cotyledon leaves to dry weight becomes proportionally less. Later this increases with the development of a more massive system of vascular tissue to provide support and a route for the passage of photosynthates from the leaf. This latter process may continue after leaf expansion has ceased (Evans, 1972). The values of these ratios computed from the basic growth data in

these experiments clearly support the basic results obtained, and strengthen the view that the disturbances promoted by the supply of lead to the plant becomes more acute with time.

It is also worth noting that the visual symptoms observed in the single salt culture experiments were still obvious in hydroponic culture, although the formation of necrotic areas on the leaves was less common, and was restricted mainly to the cotyledon leaves. Also the dark region at the top of the root was less clearly delineated. This would strengthen the suggestion that the symptoms arose from contact with the lead itself and not merely through the absence of nutrient ions from the single salt culture system.

No mention has been made in previous literature of the dark banding apparent at the top of the root of plants exposed to lead; but similar observations have been made of chlorosis and necrosis. Hewitt (1948a and b) referred to intervenal necrosis in plants exposed to a range of heavy metals, although he found that damage was minimal in lead-contaminated specimens of sugar beet, oats, tomatoes, potatoes and kale. Reports of lead-induced chlorosis come from several sources (Hewitt, 1948a and b; Miller and Koeppe, 1970a and b; Fiusello, 1973), and other heavy metals have been observed to promote similar chlorosis (Hewitt, 1948a and b; Forster, 1954; De Kock, 1956). The authors link the symptom to iron deficiency, as the provision of additional iron to the plant was seen to correct the chlorosis.

Such an iron deficiency could arise either through reduced uptake of iron via the (stunted) roots to the plant, or through a competition for sites within the chlorophyll synthesis system. It seems unlikely, however, that chlorosis could arise solely through a

reduction in uptake as in these studies it has been found that chlorosis occurred even in the younger stages of seedling growth, where it would be suspected that adequate supplies of minerals would be available within the cotyledons themselves. It is feasible that the response in this case may be similar to that suggested by De Kock (1956) who proposed that heavy metals may compete with and displace iron from chelating centres including phosphoproteins in the chloroplast.

It is obvious that the supply of lead to the plant rapidly produces adverse effects which are both manifold and severe. The most obvious effect is a substantial reduction in growth, which becomes more obvious with exposure time. It would be expected that such modifications of plant development would be the result of alterations in cell division and/or in the processes of cell expansion, both factors possibly mediated through hormonal interactions.

Figure 3.1.1 Germination Studies

A. Germination of radish seed in the presence of 0 - 10 mEq/l lead nitrate solution

1 - day 7; 2 - day 3; 3 - day 1.

B. Germination of radish seed in the presence of 10 - 50 mEq/l lead nitrate solution

1 - day 7; 2 - day 3; 3 - day 1.

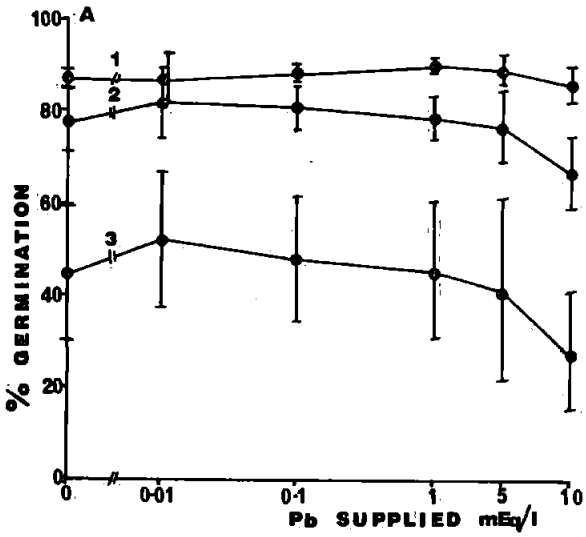
C. Germination of radish seed in the presence of 0 - 10 mEq/l sodium nitrate solution

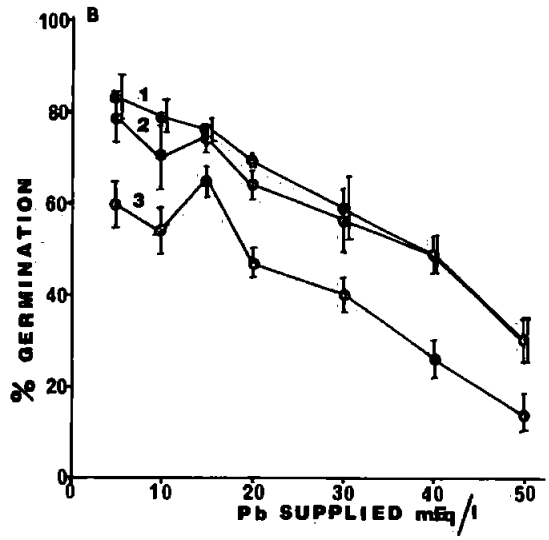
1 - day 7; 2 - day 3; 3 - day 1.

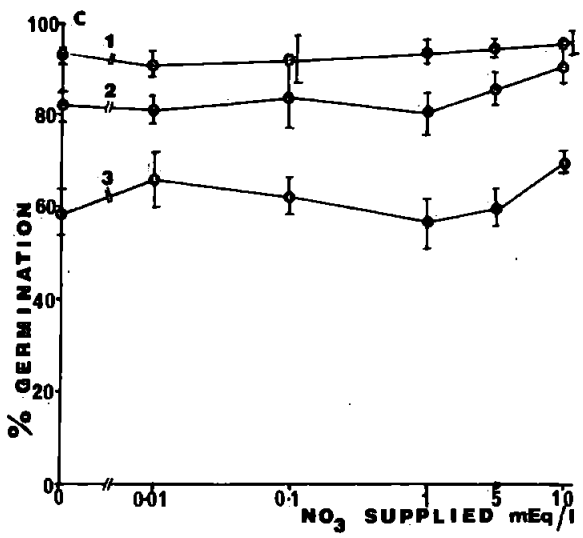
D. Root hair development in radish seedlings in the presence of 0 - 10 mEq/l lead nitrate solution

1 - day 7; 2 - day 3; 3 - day 2; 4 - day 1.

Vertical line = $\pm$ standard error


A
% GERMINATION
Pb SUPPLIED mEq/l
0
0·01
0·1
1
5
10
20
40
60
80
100
1
2
3


B
% GERMINATION
Pb SUPPLIED mEq/l
0
10
20
30
40
50
60
80
100
1
2
3


C
% GERMINATION
NO3 SUPPLIED mEq/l
0
0·01
0·1
1
5
10
20
40
60
80
100
1
2
3

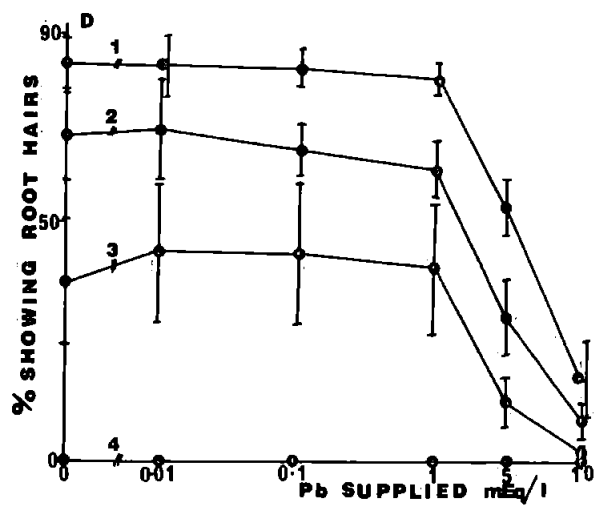

D
% SHOWING ROOT HAIRS
Pb SUPPLIED mEq/l
0
0·01
0·1
1
2
10
50
90
1
2
3
4

Figure 3.2.1 Seedling growth in the presence of 0 - 10 mEq/l lead nitrate solution

A. Root length

B. Stem length

C. Cotyledon leaf length

D. Cotyledon leaf breadth

1 - day 14; 2 - day 7; 3 - day 1.

Vertical line = $\pm$ standard error

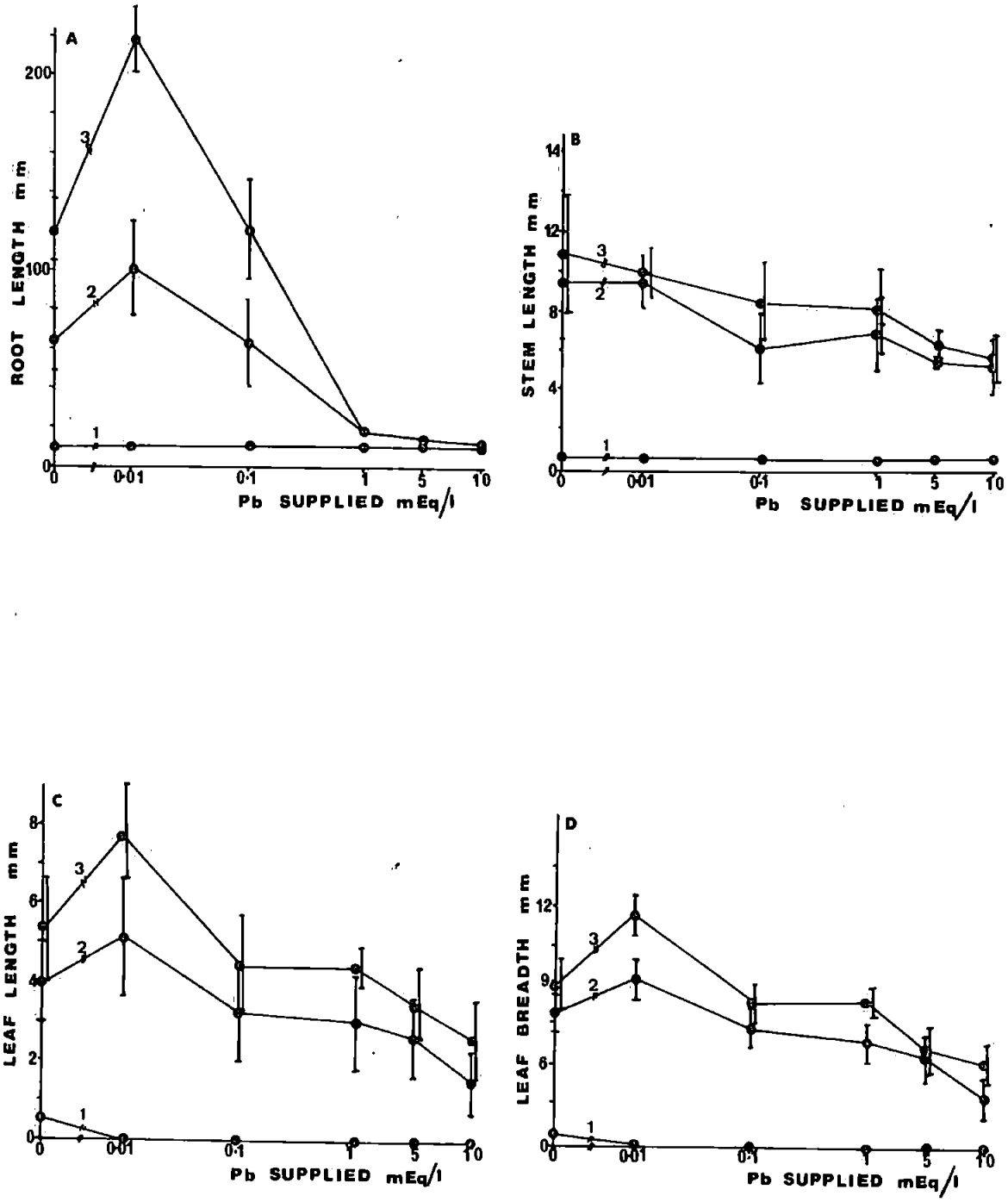

A
ROOT LENGTH mm
200
100
0
3
2
1
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l
B
STEM LENGTH mm
14
12
8
4
0
3
2
1
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l
C
LEAF LENGTH mm
8
6
4
2
0
3
2
1
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l
D
LEAF BREADTH mm
12
9
6
0
3
2
1
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 3.2.2 Seedling growth in the presence of 0 - 10 mEq/l sodium nitrate

A. Root length

B. Stem length

C. Cotyledon leaf length

D. Cotyledon leaf breadth

1 - day 14; 2 - day 7; 3 - day 1.

Vertical line = ± standard error

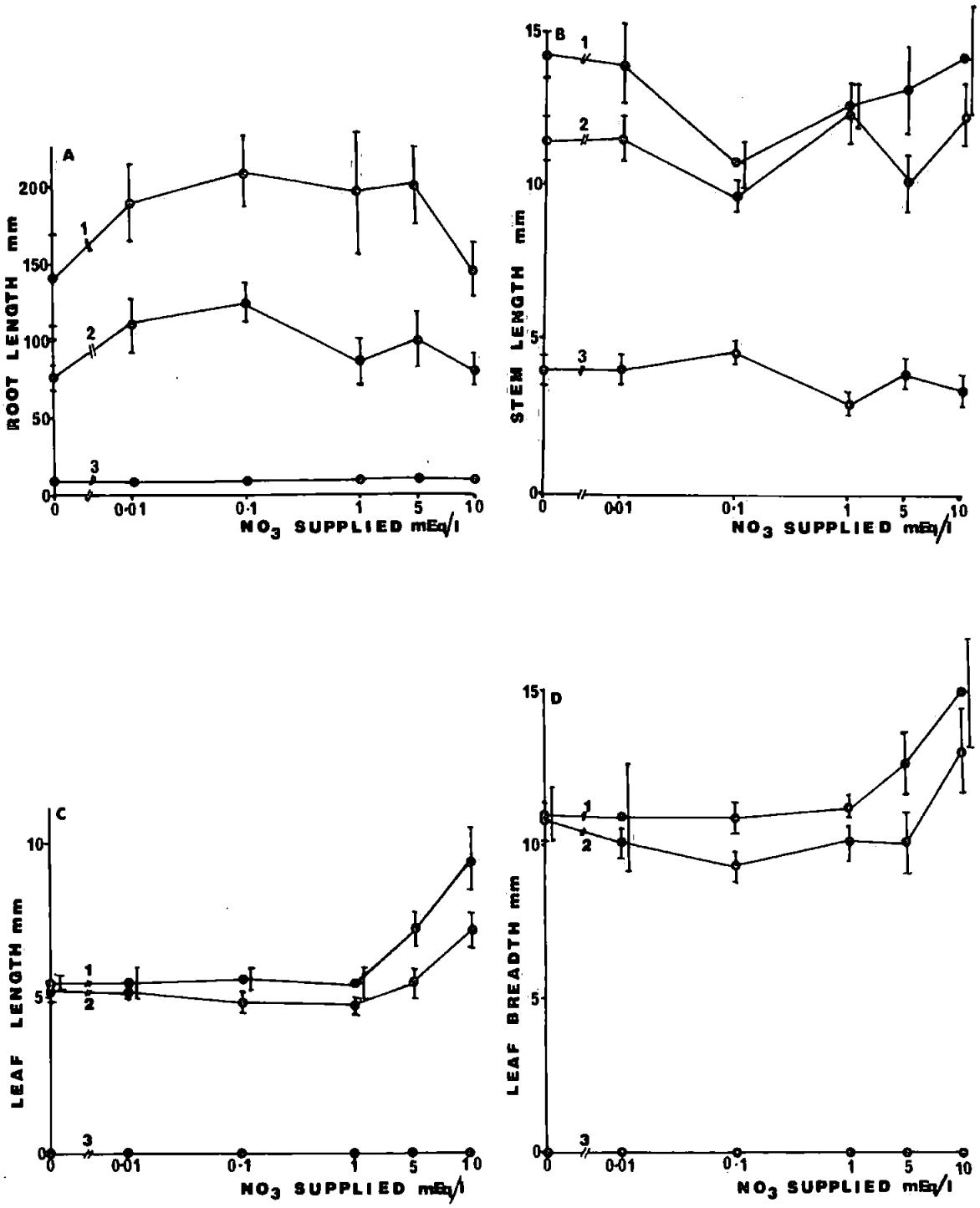

A
ROOT LENGTH mm
200
150
100
50
0
1
2
3
0
0·01
0·1
1
5
10
NO3 SUPPLIED mEq/l
B
STEM LENGTH mm
15
10
5
0
1
2
3
0
0·01
0·1
1
5
10
NO3 SUPPLIED mEq/l
C
LEAF LENGTH mm
10
5
0
1
2
3
0
0·01
0·1
1
5
10
NO3 SUPPLIED mEq/l
D
LEAF BREADTH mm
15
10
5
0
1
2
3
0
0·01
0·1
1
5
10
NO3 SUPPLIED mEq/l

Figure 3.2.3 Seedling growth in 1 mEq/l lead solutions of varying anion composition

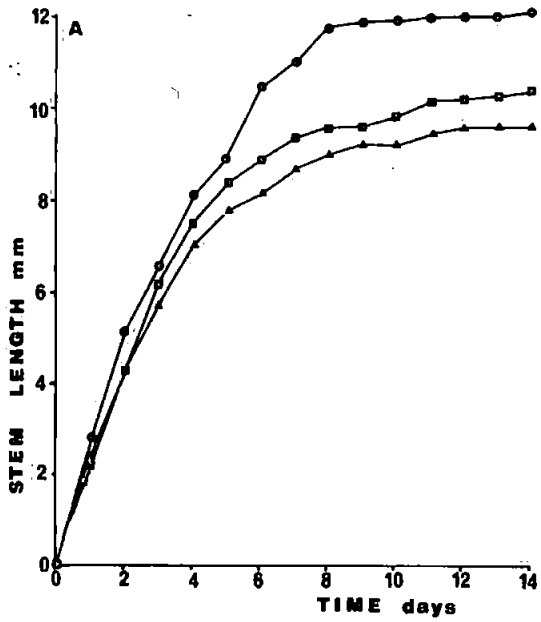
A. Stem length

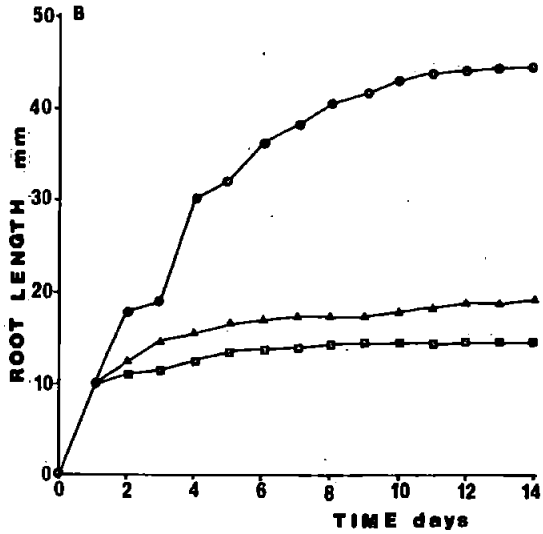
B. Root length

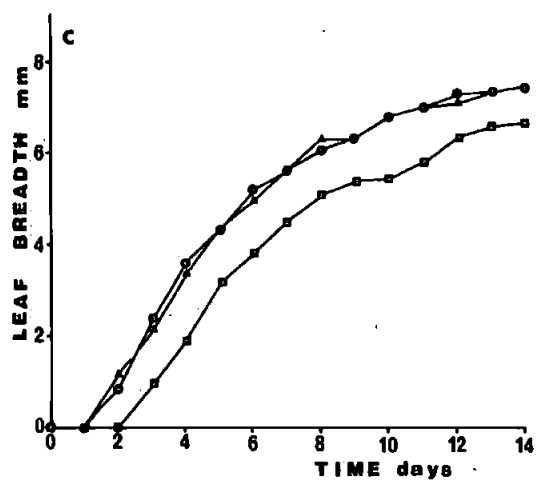
C. Cotyledon leaf breadth

D. Cotyledon leaf length

● - lead citrate; ■ - lead acetate; ▲ - lead nitrate


A
STEM LENGTH mm
TIME days
0
2
4
6
8
10
12
14


B
ROOT LENGTH mm
TIME days
0
10
20
30
40
50
2
4
6
8
10
12
14


C
LEAF BREADTH mm
TIME days
0
2
4
6
8
10
12
14

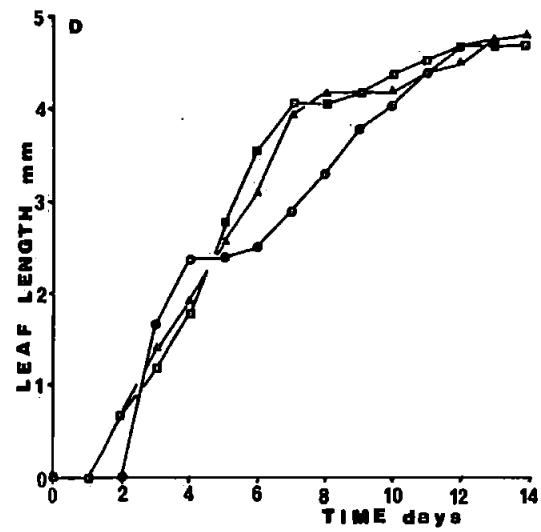
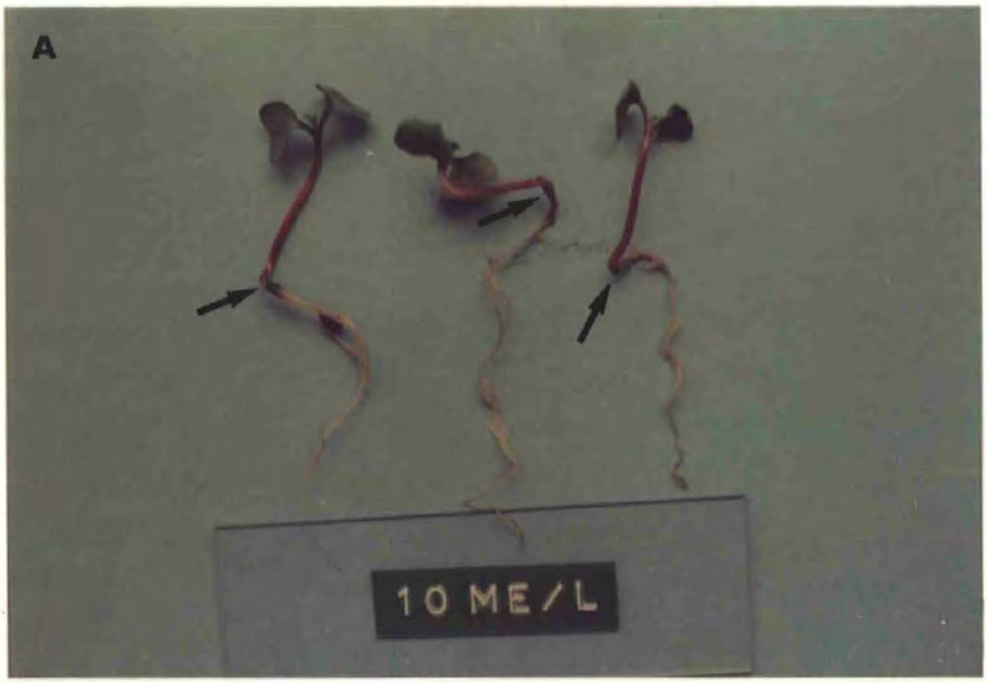

D
LEAF LENGTH mm
TIME days
0
1
2
3
4
5
6
8
10
12
14

Plate 3.2.4 Lead-induced discolouration in 7-day radish seedlings

A. 7-day old seedlings grown in 10 mEq/l lead nitrate solution for 4 days (approximately actual size). Note dark brown area at top of root (arrowed).

B. 7-day old seedling cotyledon leaves grown in 10 mEq/l lead nitrate solution (x6). Note general chlorosis and necrotic lesions (arrowed).


A
10 ME/L

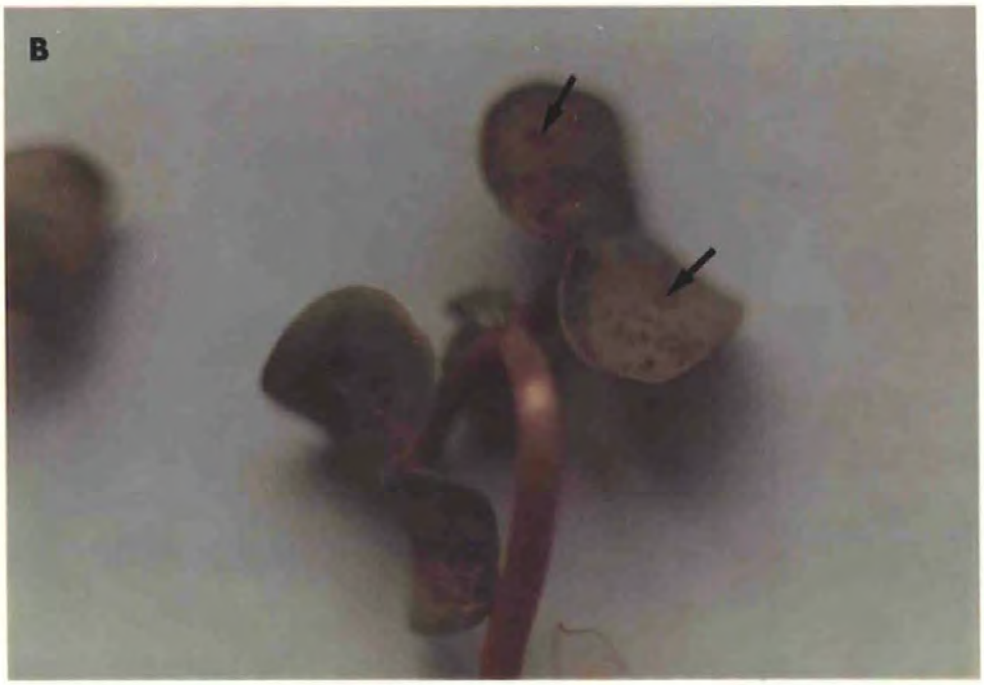

B

Figure 3.3.1 Fresh weights of hydroponically grown plants

A. 14 day old plants

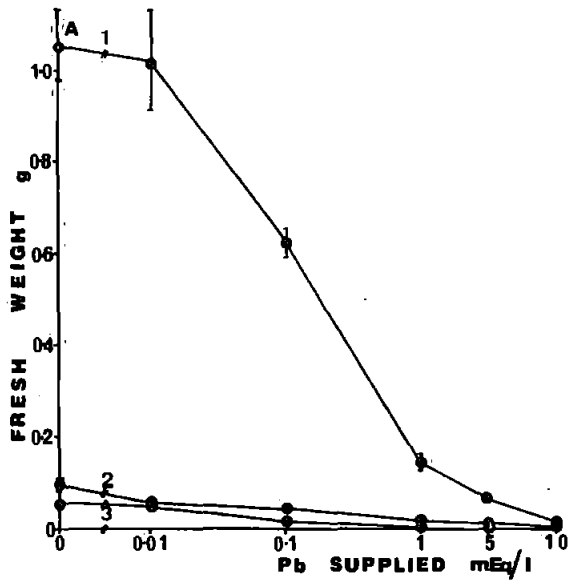
B. 21 day old plants

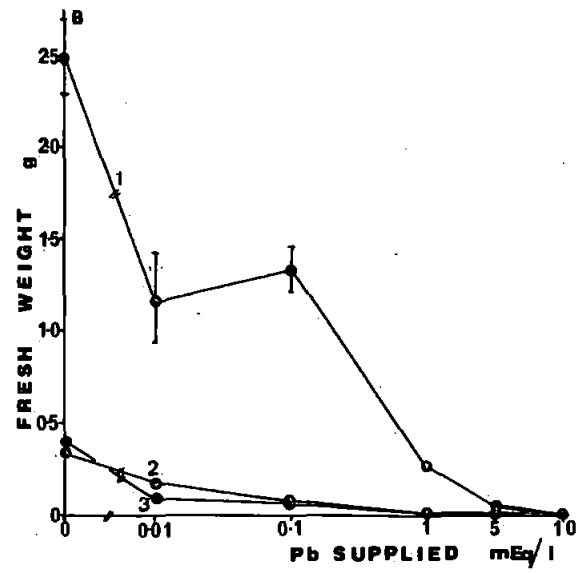
C. 28 day old plants

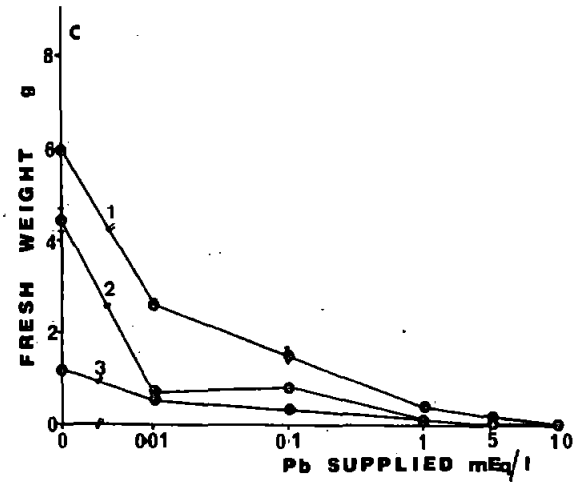
D. 35 day old plants

1 - leaves; 2 - stems; 3 - roots

Vertical line = $\pm$ standard error


A
FRESH WEIGHT g
1·0
0·8
0·6
0·4
0·2
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
FRESH WEIGHT g
2·5
2·0
1·5
1·0
0·5
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
FRESH WEIGHT g
8
6
4
2
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

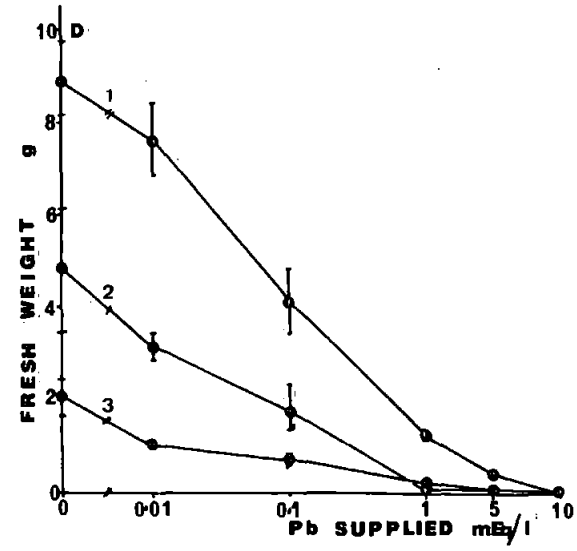

D
FRESH WEIGHT g
10
8
6
4
2
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 3.3.2 Dry weights of hydroponically grown plants

A. 14 day old plants

B. 21 day old plants

C. 28 day old plants

D. 35 day old plants

1 - leaves; 2 - stems; 3 - roots

Vertical line = $\pm$ standard error

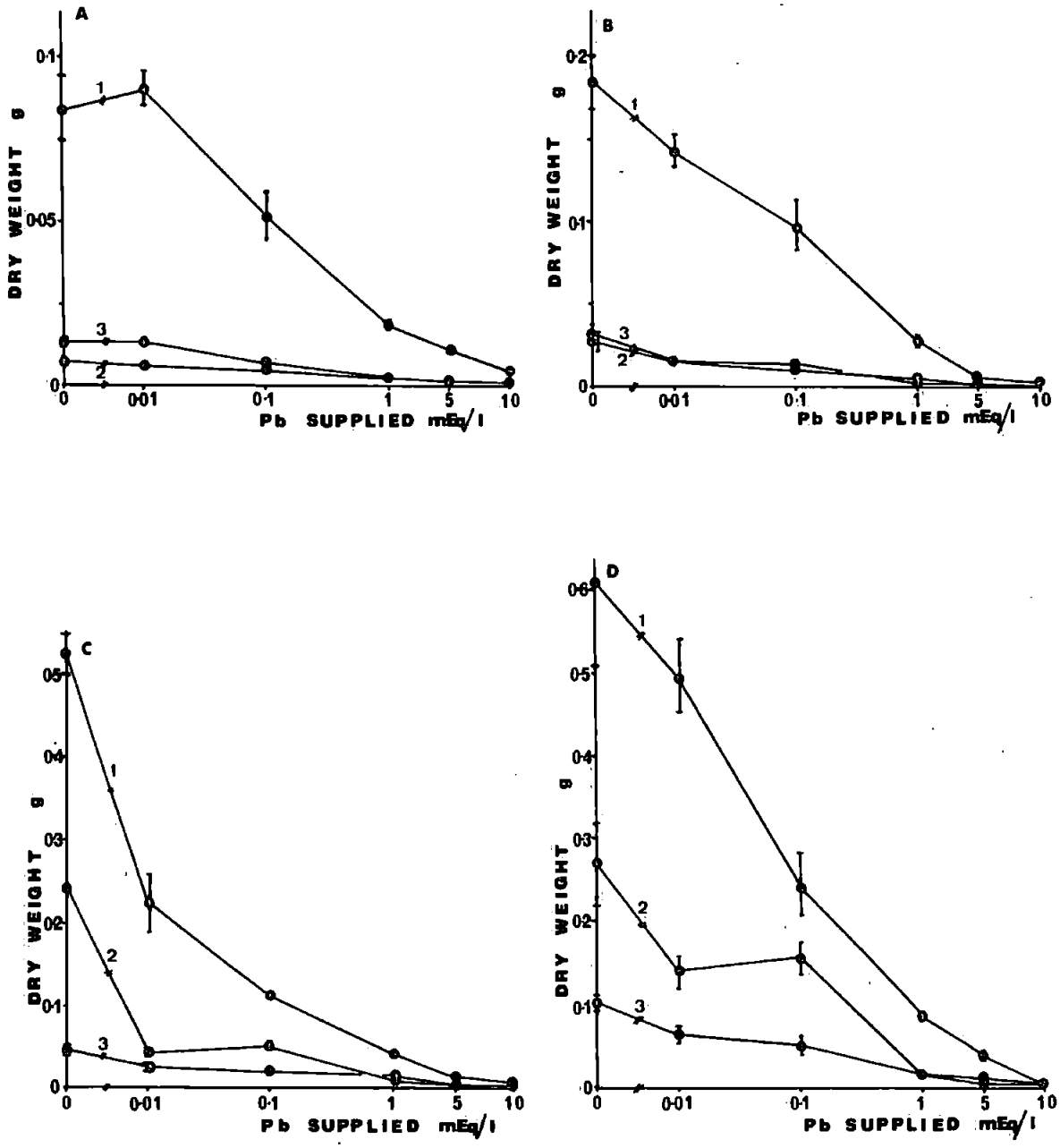

A
B
C
D
DRY WEIGHT g
Pb SUPPLIED mEq/l
0
0·01
0·1
1
5
10
1
2
3

Figure 3.3.3 Fresh weight/dry weight ratios of plants grown in hydroponic solution

A. 14 day old plants

B. 21 day old plants

C. 28 day old plants

D. 35 day old plants

1 - roots; 2 - stems; 3 - leaves

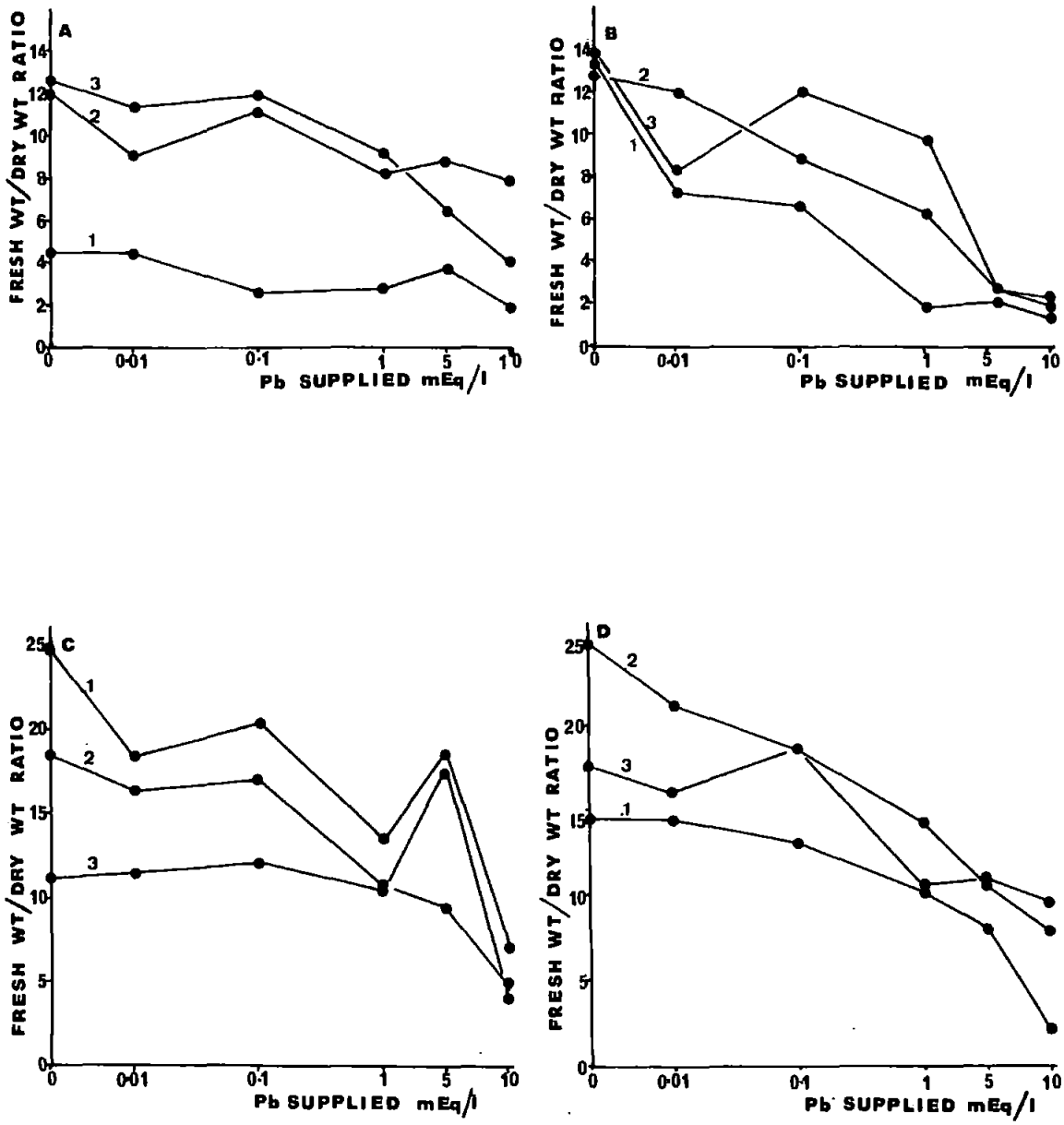

A
B
C
D
FRESH WT/DRY WT RATIO
Pb SUPPLIED mEq/l
0
0·01
0·1
1
5
10
1
2
3

Figure 3.3.4 Linear dimensions of roots and stems of hydroponically grown plants

A. 14 day old plants

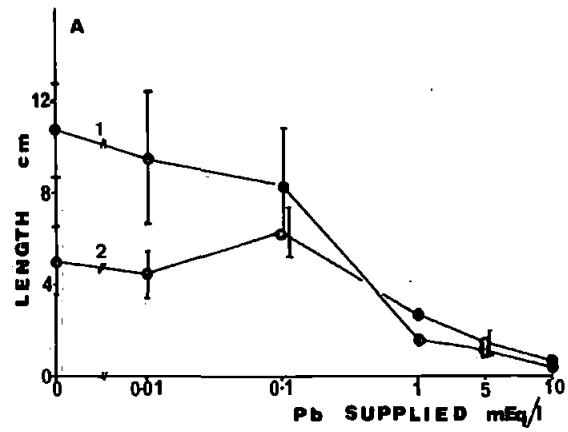
B. 21 day old plants

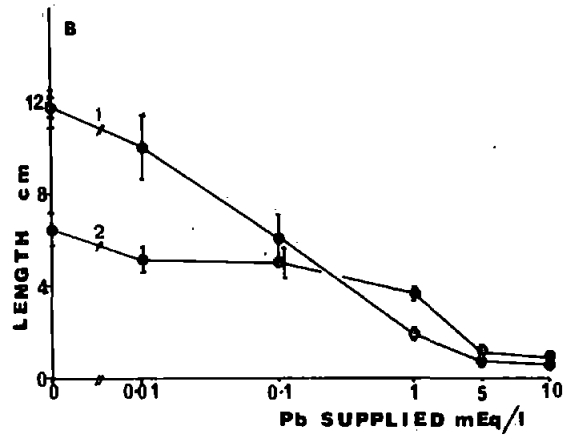
C. 28 day old plants

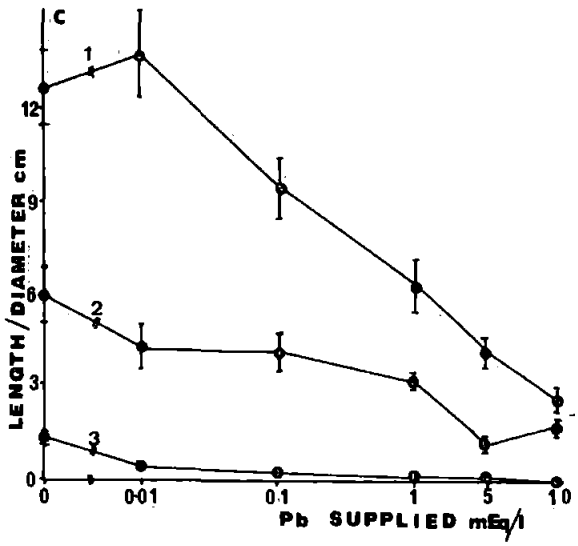
D. 35 day old plants

1 - root length; 2 - stem length; 3 - stem breadth

Vertical line = $\pm$ standard error


A
LENGTH cm
12
8
4
0
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
LENGTH cm
12
8
4
0
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
LENGTH/DIAMETER cm
12
9
6
3
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

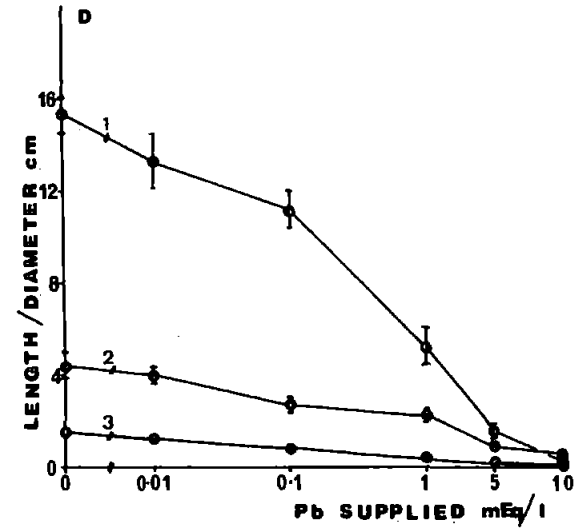

D
LENGTH/DIAMETER cm
16
12
8
4
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 3.3.5 Leaf areas of hydroponically grown plants

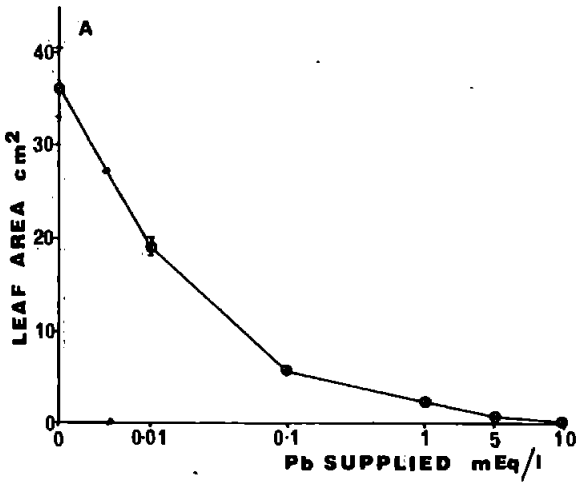
A. 14 day old plants

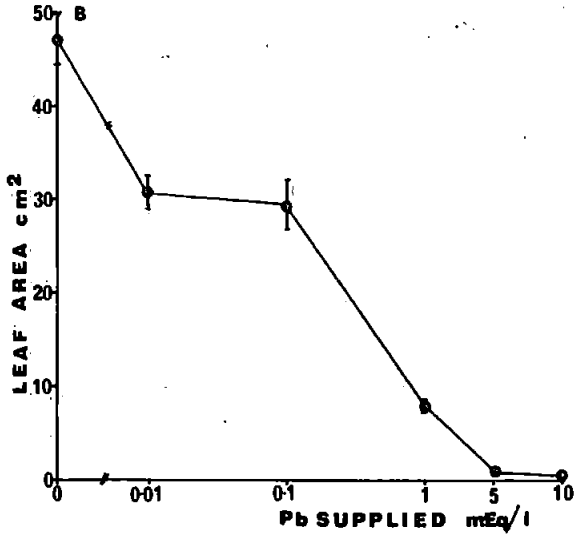
B. 21 day old plants

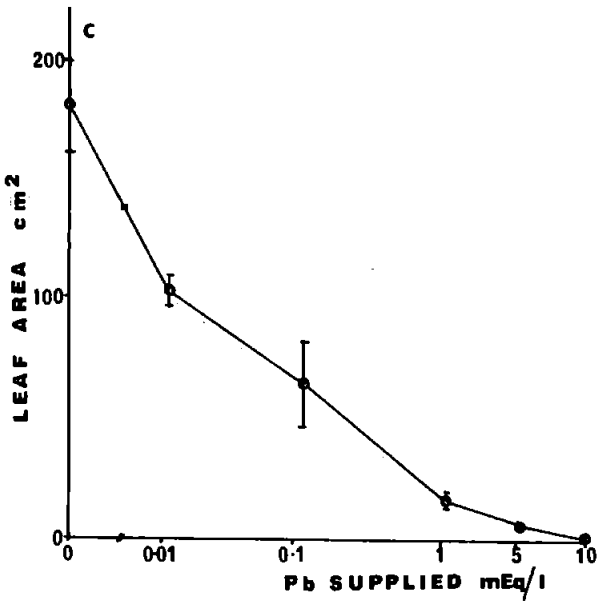
C. 28 day old plants

D. 35 day old plants

Vertical line = $\pm$ standard error


A
LEAF AREA cm2
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
LEAF AREA cm2
50
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
LEAF AREA cm2
200
100
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

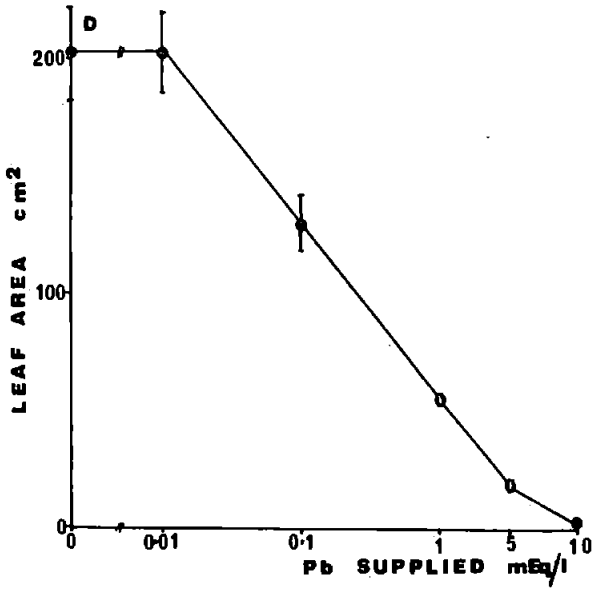

D
LEAF AREA cm2
200
100
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 3.3.6 Leaf development in hydroponically grown plants

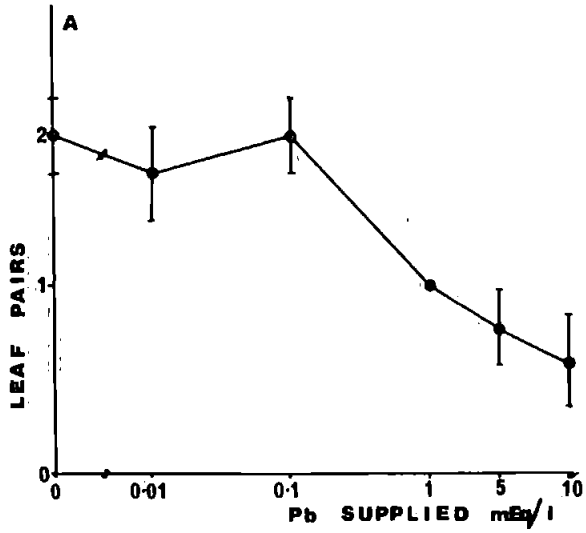
A. after 14 days

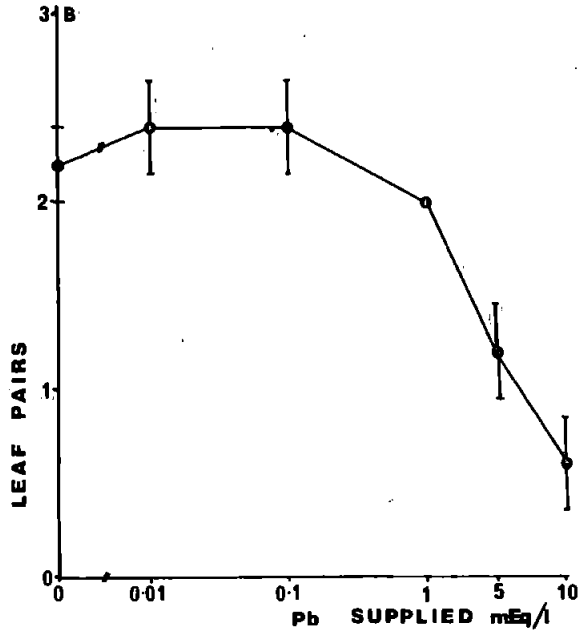
B. after 21 days

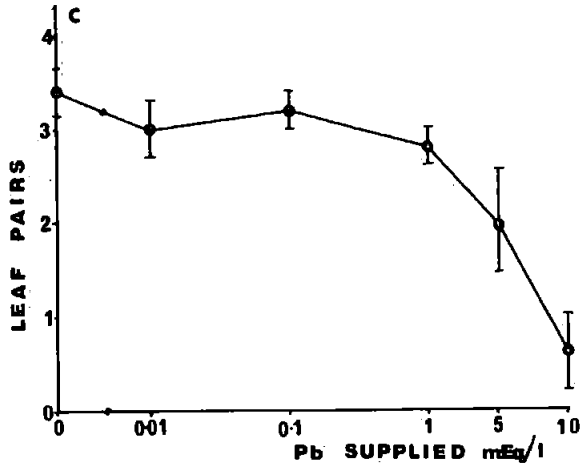
C. after 28 days

D. after 35 days

Vertical line = $\pm$ standard error


A
LEAF PAIRS
0
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
LEAF PAIRS
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
LEAF PAIRS
0
1
2
3
4
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

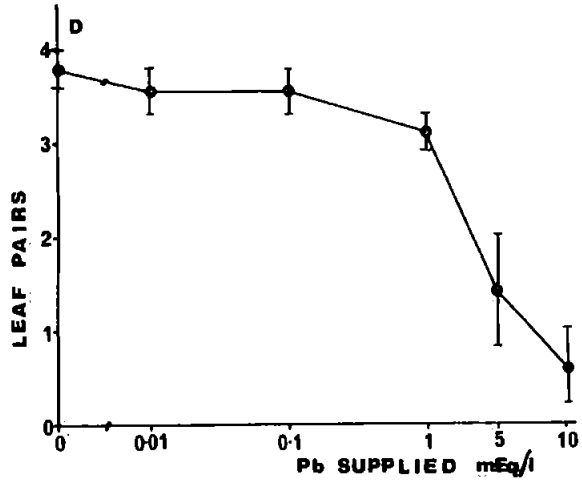

D
LEAF PAIRS
0
1
2
3
4
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 3.3.7 Dry weight/unit length for roots and stems of hydroponically grown plants

A. 14 day old plants

B. 21 day old plants

C. 28 day old plants

D. 35 day old plants

1 - stems; 2 - roots

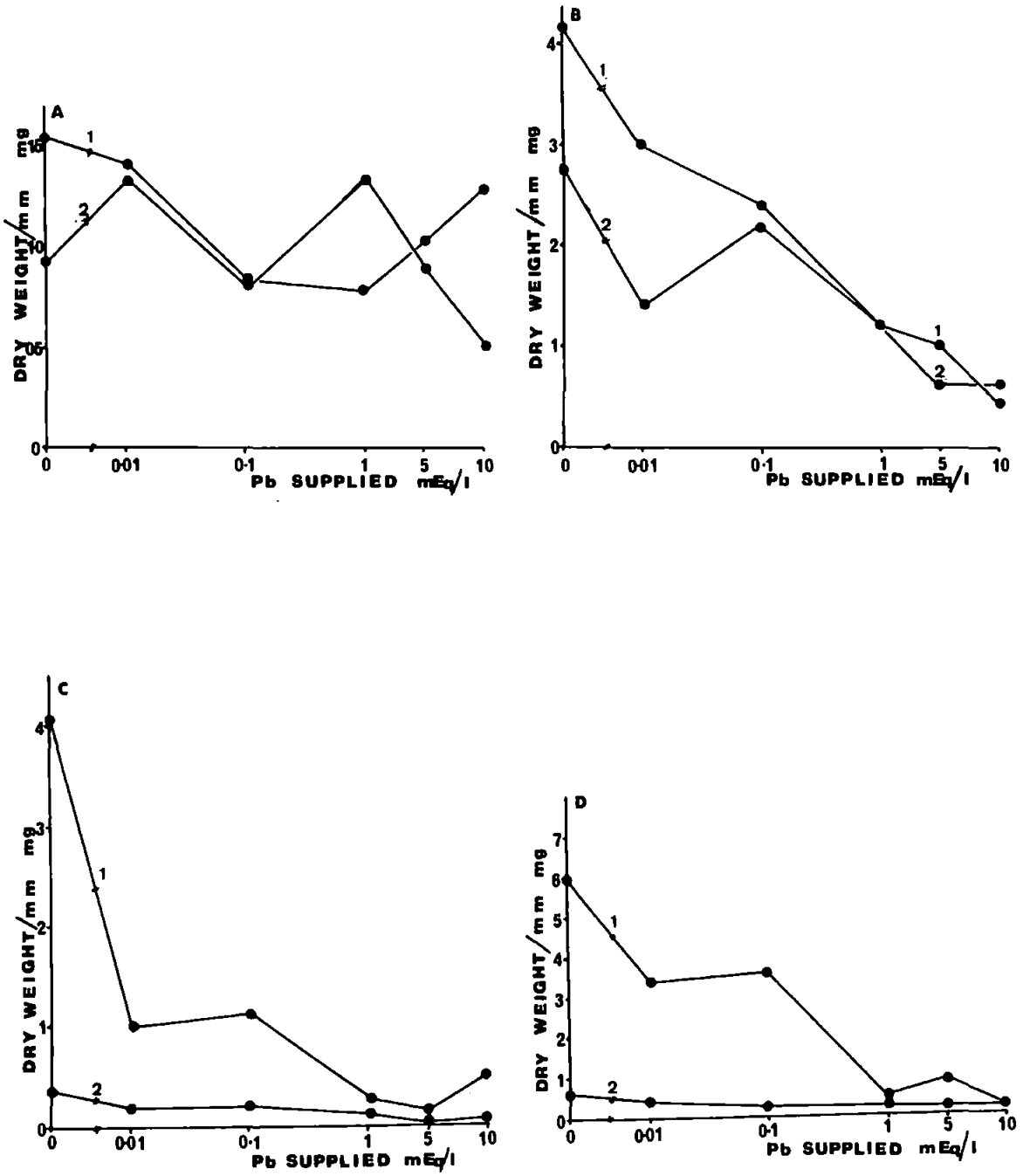

A
B
C
D
DRY WEIGHT/mm mg
Pb SUPPLIED mEq/l
0
0·01
0·1
1
5
10
1
2

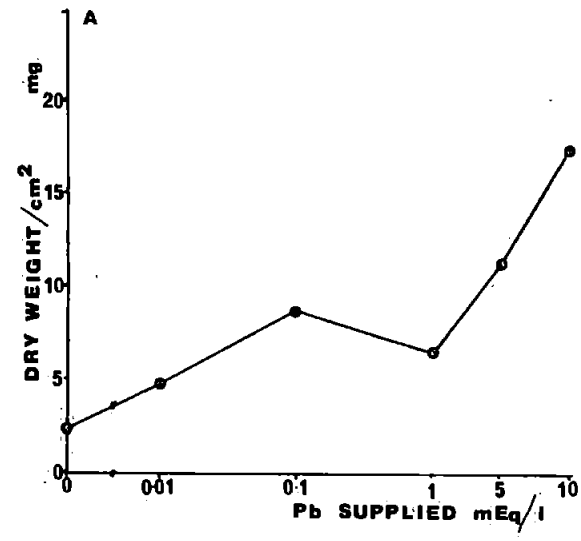
Figure 3.3.8 Dry weight/unit area for leaves of hydroponically grown plants

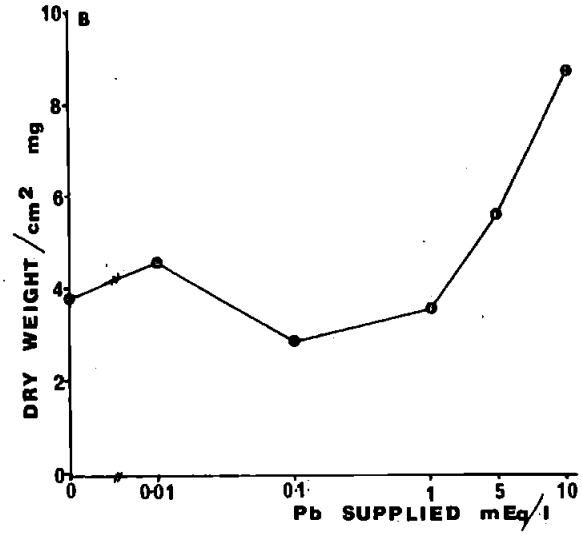
A. 14 day old plants

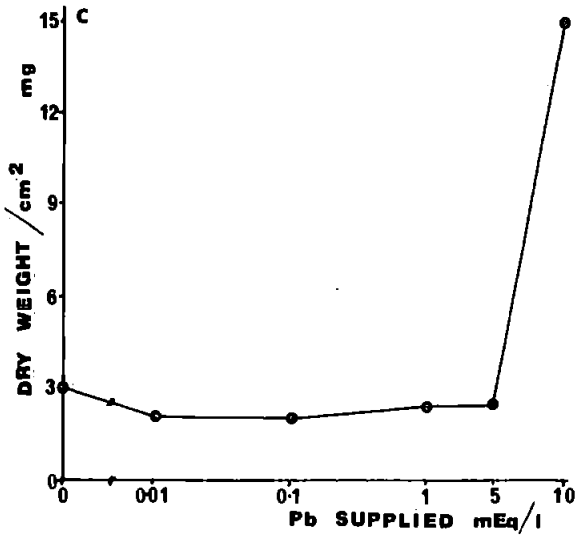
B. 21 day old plants

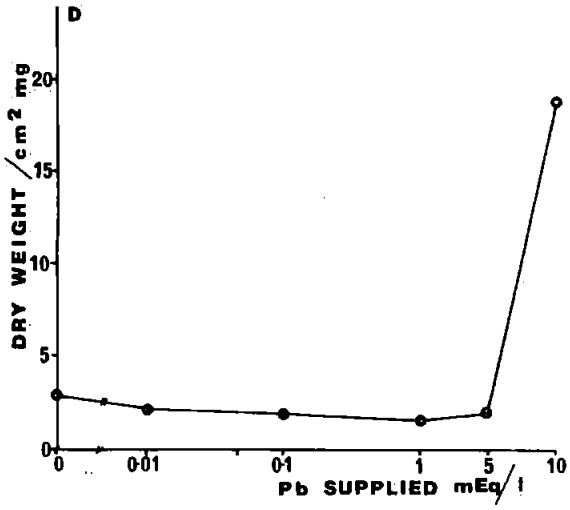
C. 28 day old plants

D. 35 day old plants


A
DRY WEIGHT /cm2 mg
0
5
10
15
20
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
DRY WEIGHT /cm2 mg
0
2
4
6
8
10
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
DRY WEIGHT /cm2 mg
0
3
6
9
12
15
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


D
DRY WEIGHT /cm2 mg
0
5
10
15
20
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

CHAPTER 4

THE UPTAKE OF ROOT-APPLIED LEAD BY *RAPHANUS SATIVUS*

The assimilation of lead by plants is a topic which has received much consideration by researchers in the past (e.g. Prat, 1927; Keaton, 1937; Miller and Koeppe, 1970; Baumhardt and Welch, 1972). Much of this interest has arisen because the uptake of lead by plants is the first step in the transmission of the metal along food chains which may eventually lead to man and his agricultural animals (Jones and Clement, 1971). It is, however, an area of research in which widely differing results have been obtained. Much of the work reported relates to lead levels in plants in the vicinity of lead pollution sources (e.g. roads, factories, smelters, spoil tips, etc.) and to a great extent the diversity observed must arise from the range of sources utilised in these studies (i.e. atmospheric, soil, solution) and in some cases because of the differences in the physical and chemical properties of the sources concerned (see Chapter 1). There also appears to be considerable variation in lead uptake between species (Hopkinson et al., 1972) uptake being greater, in general, in dicotyledonous than in monocotyledonous species (Suchodoller, 1967). A selection of the numerous reports concerning the uptake of root-applied lead by plants is shown in Table 4.0.1.

The present study was carried out in order to gain further information about the uptake and dissemination of lead and to evaluate the growth data presented in Chapter 3 in terms of the lead content of the parts of the plant concerned.

TABLE 4.0.1. SOME LEAD LEVELS IN PLANTS

Literature Source	Growth Medium	Lead Supply ppm	Plant Material	Plant Lead Concentration ppm		
				Roots	Stem	Leaves
Alloway & Davies 1971	Soil	11,200	Radish	499	136.1	
Brown & Bates 1972	Soil	100,000	*Grimmia doniana*	30,000		
Chisholm 1972	Soil	277	Beetroot	14.7	20.77	
Dedolph *et al* 1970	Soil	27	Radish	2	2.3	
John & van Laerhoven 1972	Soil	1000	Oats	58.6	35.7	
John & van Laerhoven 1972	Soil	1000	Lettuce	-	140.6	
Jones, Clement & Hopper 1973	Culture Solution	1.5	Perennial Ryegrass	215.4	11.1	
Jones, Jarvis & Cowling 1973	Soil	135	Perennial Ryegrass	36.8	6.1	
Lagerwerff* 1971	Soil	299	Radish	32.7	25.1	
Lagerwerff *et al* 1973	Soil	212	Corn	-	51.12	27.74
Marten & Hammond 1966	Soil	680	Bromegrass	-	34.5	
Motto *et al* 1970	Soil	164	Carrot	24	84.0	
Reilly & Reilly 1973	Soil	750	*Cynodon dactylon*	121.2	62.0	
Rolfe 1973	Soil	600	Various Trees	600	80	40
Rolfe 1973	Culture Solution	600	Various Trees	1000	500	400

*Exchangeable Lead only

4.1 *Lead Content of Seedlings*

Seedlings utilized in the 14-day growth studies were dried, ashed in a combination of dry and wet ashings (see Chapter 2) and analysed for lead content using an Orion lead ion specific electrode system (see Chapter 2). Prior to drying, however, one of three washing procedures was applied. In the first this consisted of no washing at all, but simply the removal of excess solution from the surface of the tissue by blotting with filter paper. The second treatment involved shaking the tissue in deionised water for a period of 30 minutes, and the remaining seedlings were agitated for 60 minutes in 0.01 mol/l calcium nitrate solution, then rinsed in deionised water. The last-mentioned treatment is thought to remove ions held in an exchangeable form within the plant (Goren-Suchodoller and Wanner, 1969; Kannan and Keppel, 1976). The material was subsequently divided into roots, stems and leaves and analysed. Because small amounts of tissue were involved it proved necessary to bulk some of the samples, and therefore in some cases only single point determinations were carried out.

It was clear, however, that in all cases there was a parabolic relationship between lead content and lead supplied, the lead content of the material, expressed on a dry weight basis, rising sharply at supply concentrations in excess of 1 mEq/l. Tissue concentrations of lead were higher in the unwashed than in the washed samples, and concentrations in the material washed in calcium nitrate solution were the lowest by far (see Figure 4.1.1).

The results indicated that lead was present in the plant in three phases. Firstly, a large proportion (about 50% in the roots) was in a soluble or free state, which could be removed by washing in deionised water. A second portion (up to about 47% in roots,

depending on supply concentration) was removable by washing in calcium nitrate solution, suggesting that it was present in an exchangeable form. The remaining portion was only released by the ashing processes, suggesting that it was in a more tightly bound form.

4.2 *Lead Content of Hydroponically Grown Plants*

Analyses of 2-, 3-, 4-, and 5-week old plants showed that a similar relationship to that seen in single salt culture existed between lead supply and the lead content of tissues of plants grown in hydroponic culture. In this case only a single washing procedure was employed, consisting of a short rinse in deionised water (5 minutes) to remove superficial contamination of the material. Subsequent analysis was carried out as described in section 4.1. Although the washing procedures were not comparable the values obtained showed that at the two week stage lead uptake was appreciably lower than had been observed in single salt cultures. In all parts of the plant levels increased with increasing exposure throughout the trial period. It was evident that lead was present at the highest concentrations in the roots, lower in the stems and least of all in the leaves, providing evidence for the existence of at least a partial barrier to the movement of lead through the plant. Movement of lead into the aerial parts did occur, however, and in 5 week old plants the leaves supplied with the highest concentrations of lead showed proportionally higher lead levels than those recorded at the lower supply concentrations (mean root : leaves lead ratio for 10 mEq/l treatment = 2.25 : 1. For 1 mEq/l treatment, mean ratio = 36.7 : 1). This was less evident in younger plants, and could be caused either by increased levels of lead passing into the leaves, or by the leaf acting as a sink for the pollutant, being

unable to dissipate its lead burden. In those experiments in which the analyses were replicated, the range of the results obtained, as indicated by the standard error, increased with increasing lead supply suggesting that the uptake of lead by the plant does vary from individual to individual (Figure 4.2.1).

4.3 *Comparison of Plant Lead Contents with Plant Dry Weight Yield*

Evaluation of the effect of tissue lead concentration on the development of radish plants in terms of their dry weight yields or linear dimensions gave results which were somewhat difficult to interpret, and which did not lend themselves to graphical presentation. It was obvious that the production of material by the plant, as evidenced by plant dry weight or plant dimension, decreased as the internal lead concentrations rose. Again a parabolic relationship between cause and effect was indicated.

But of greater interest was the disparity that existed in certain cases between tissue lead content and dry matter production. In the seedling studies anomalies were apparent in the results of the stem and cotyledon leaf measurements, where reductions in dimensions occurred but lead was not detectable in the tissue (Table 4.3.1). This was perhaps more strongly shown by the results of the hydroponic studies (Table 4.3.8), where stem and leaf dry weight were found to be reduced at sub-detectable internal lead concentrations.

Also leaf and stem tissue appeared to be more susceptible to low internal lead concentrations than the root. At the 5 week stage a lead content of 50 μg/g in the roots was reflected in a reduction in tissue dry weight over controls of about 50%; a similar concentration in the stem brought about an 88% reduction in dry weight, and in the leaves a 94% reduction was observed. Presumably, however, these figures are

biased because stem and leaf dry weights begin to drop before internal lead concentrations reach detectable levels.

4.4 *Discussion*

It is clear from the results that the uptake of lead by radish increases in a parabolic manner in relation to the logarithm of the lead concentration supplied. Although a number of authors (e.g. John and van Laerhoven, 1972; Hassett et al., 1976; Miller et al., 1977) support the finding that the lead content of tissue increases with lead supply, in the majority of these studies lead has been supplied at only two or three concentrations, and so it is not possible to derive any valid evaluation of the nature of the relationship between lead uptake and supply in these cases. However a similar parabolic relationship is apparent in the results of Hevesy (1923) who examined the lead content of Vicia faba plants exposed to a range of lead concentrations from 10^{-6} to 10^{-3} mol/l. In short-term (1 - 3 hour) narrow concentration range ($1 - 8 \times 10^{-4}$ mol/l) experiments, Goren and Wanner (1971) found a different relationship, the uptake of lead by Hordeum vulgare tending to achieve a plateau at a supply concentration of about 4×10^{-4} mol/l. While the two relationships are not mutually exclusive, it is difficult to render the short-term model compatible with the high tissue concentrations which have been observed in this study and by other researchers.

It is also clear from the present data that the concentration of lead within the plant increases with increasing period of exposure, but again conflicting evidence is presented in the literature. Jones, Clement and Hopper (1973) found that in ryegrass grown in hydroponic culture containing up to 1500 μg/l of lead, although the total lead

content of the tissue increased, the concentration decreased with time. This was thought to be because the dry weight of the tissue increased faster than the total lead levels. Goren and Wanner (1971) observed a reduction in the rate of uptake of lead from a 5×10^{-4} mol/l lead nitrate solution after about two hours. Kannan and Keppel (1976) reported a similar reduction in lead uptake by pea seedlings after about 90 minutes. In both cases, however, the internal concentration of lead continued to rise with time. In a more recent report John (1977) grew lettuces in hydroponic cultures containing lead at concentrations of up to 50 ppm. Samples were analysed for lead content at 2-, 4-, and 6-week intervals and the data obtained shows that lead concentration continues to increase with time, although the difference between the 2- and 4-week samples is more marked than that between those taken at 4- and 6-weeks.

Again the two models are not mutually exclusive, and the differences apparent between the various results are supportive of the theory that dicotyledonous species are more susceptible to lead than monocotyledonous species (vide supra) as, in this case at least, it is clear that the growth of the tissue does not continue at a sufficiently rapid rate to offset the uptake of the metal.

The data from the present study showed that lead is taken up more rapidly from single salt culture than from hydroponic culture solution, as the levels observed in two week old seedlings in single salt culture are far in excess of those found in plants of the same age in hydroponic culture. However, it must be borne in mind that the hydroponically-grown plants were far ahead of the single salt culture plants in their development at this stage, and the differences in internal concentrations may be heightened by the enhanced growth of

the hydroponically-grown specimens. It seems unlikely, however, that differences of such magnitude could be attributed solely to this. Although certain authors have reported the effect of changes of pH on lead uptake (John and van Laerhoven, 1972; Ardvik and Zimdahl, 1974; Zimdahl and Foster, 1976), pH differences cannot be the explanation here as all solutions were adjusted to pH 6 at the commencement of the trial periods (see Chapter 2). It is more likely that any differences which do exist are attributable to the presence of other ions in the hydroponic culture solution, as several researchers have reported the competitive effects of different anions and cations on lead uptake (Goren-Suchodoller and Wanner, 1969; Goren and Wanner, 1971; Jones, Jarvis and Cowling, 1973).

Consideration of the effect of different washing procedures on tissue lead content shows that the processes applied produce vast differences in the residual lead content of the tissue. From a theoretical standpoint it would be suspected that in the first case (unwashed tissue) a measure is obtained of the total lead in and on the tissue. In the second case (tissue washed in deionised water) the amount of lead removed provides an estimate of the lead in a soluble or free form in the tissue. In the third case washing with calcium nitrate solution would, based on the Law of Mass Action, result in the substitution of the more abundant calcium ions for lead in exchange sites in the plant. On this basis, the results of Figure 4.1.1 show that lead is present in radish in free, exchangeable, and in tightly-bound forms, but that the last form represents only a small proportion of the total lead present. Jones, Clement and Hopper (1973) also examined the removal of lead from the roots of ryegrass plants by washing with water or with calcium solutions, but found that lead was

not released in detectable amounts by the washing treatments. Kannan and Keppel (1976) also compared the effect of water and calcium washing treatments on the lead contents of pea seedlings, and found that about 10% of the total lead present was in the Water Free Space, i.e. was water-soluble, and about 40% was in the Donnan Free Space, i.e. could be removed by the calcium treatment. Such losses highlight the need for careful control of washing procedures in assays of this nature.

Comparison in terms of magnitude of the present results with those of other workers is difficult. As mentioned previously, much of the data concerning lead uptake by plants has been gained from soil experiments and so is not strictly comparable with the results of hydroponic experiments; in those cases where lead has been supplied in an aqueous form, results are diverse. For example, Rolfe (1973) reported levels between 600 and 4000 ppm of lead in the roots of various species of tree seedlings exposed to 600 ppm of lead, whereas Jones, Clement and Hopper (1973) reported that in ryegrass supply levels of 1500 μg/l of lead resulted in maximum levels of 24 ppm of lead in shoots and 321 ppm in roots. In the experiments of Hevesy (1923), exposure of Vicia faba to 10^{-1} Normal lead solution following growth in hydroponic culture resulted in a concentration of lead of 38% of ash in roots, and 12% of ash in leaves.

Such variability in lead concentration is difficult to explain. Doubtless it may be partly attributable to species difference, but it is interesting to note that in the experiments of Jones, Clement, and Hopper (1973) the mode of solution culture employed seemed to be of great importance. These authors found that uptake from flowing solution culture was about 10 times that observed in conventional culture, but do not discuss whether this difference arose merely from the difference

in the total lead supplied, or from differences in the culture systems. Comparison of results in the literature is also confused by the lack of full details of the growth systems employed; in some cases no mention is made of solution pH, aeration, or agitation; in others details of environmental conditions such as relative humidity, and airspeed are not supplied. In view of the range of factors which may affect lead uptake (see Chapter 1) such differences should be considered in the comparison of uptake data from differing sources. There is also considerable latitude in the washing procedures employed in the various experiments reported and in the subsequent ashing procedures. It is clear from the data shown in Figure 4.1.1 that the nature of the former procedure may be of great importance to the magnitude of the concentrations recorded, and similarly the data of Webber (1972) shows that the different ashing procedures which may be employed give rise to major differences in the recorded lead content of tissue. In general terms, however, it would appear that the results of this series of analyses are of a similar order of magnitude to those presented in the literature.

In the present study in all cases lead concentrations were higher in roots than in stems, and higher in stems than in leaves. Similar distributions have been reported by other researchers, both in soil and in hydroponic culture (e.g. Marten and Hammond, 1966; Alloway and Davies, 1971; Lagerwerff et al., 1973; Rolfe, 1973). This non-uniform distribution probably arises from the reduction in the concentration of freely-diffusible lead in the plant due to its removal to cell vacuoles or its binding to sites within the root as evidenced by the data in section 4.1. Hevesy (1923) also suggested that lead may be bound up in plant roots, thus reducing its movement to other

parts of the plant, and Jones, Clement and Hopper (1973) have suggested the presence of some form of partial barrier to lead mobility from the root. In radish this restriction of movement may, at least in part, be attributable to the accumulation of lead in the region at the top of the root which shows a dark staining in the presence of lead (see Chapter 3).

Perhaps one of the most pertinent conclusions which may be drawn from the comparisons of dry weight yields and lead contents lies in the differing reactions of plant parts to internal lead concentrations. From the results shown in Chapter 3, it might be suspected that a reliable assessment of lead uptake by the plant could be made on the basis of the inspection of its aerial parts, measuring, say, leaf area or stem length. The results in section 4.3 show that this is clearly not the case, as leaf dry weight drops noticeably before any detectable lead contamination of the leaf occurs. A similar, albeit less marked, situation is apparent in the stem. It may be concluded, therefore, that root measurements form the best estimate of lead uptake from a root-applied source. It would seem likely that the reductions in stem and leaf development at low lead supply concentrations result from reduced uptake of water and/or nutrients by the roots, either because of reduced surface area or reduced ability, or both.

One further point must be made. The Lead in Food Regulations (M.A.F.F. no. 1931, 1961) state that the maximum concentration of lead permissible in vegetables for human consumption is 2 ppm on a fresh weight basis. In this study it has been shown that the ratio of fresh to dry weight for radish stem tissue varies between 8:1 and 25:1 with varying lead supply concentration (see Chapter 3), which indicates that in radish the regulation maximum is equivalent to 16

to 50 ppm on a dry weight basis. It is of interest to note, therefore, that contamination of the portion of the radish grown for human consumption is not of direct relevance in these terms as in those cases where levels of lead in excess of the permitted maximum appear in this region, the stem fails to expand to marketable proportions (see Chapter 3). This does not, however, exclude lead from food chains where the primary consumer is not man, since other animals may not demonstrate the same selectivity in their choice of foodstuffs.

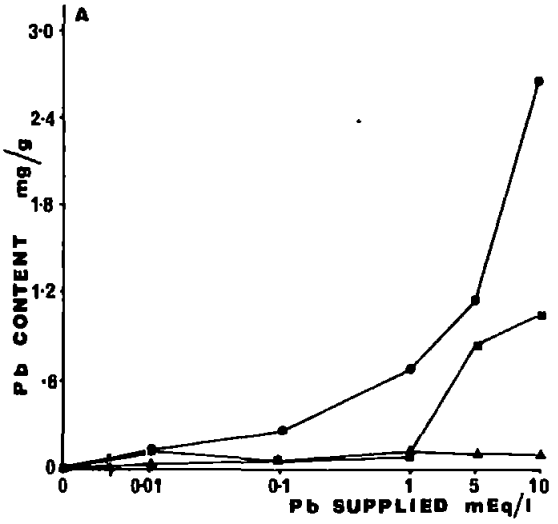
Figure 4.1.1 Lead content of 14-day old radish seedlings following various washing treatments

A. Unwashed tissue

B. Tissue washed in deionised water

C. Tissue washed in 0.01 mol/l calcium nitrate solution

● = roots; ▨ = stems; ▲ = cotyledon leaves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3·0
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1·8
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·6
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1
5
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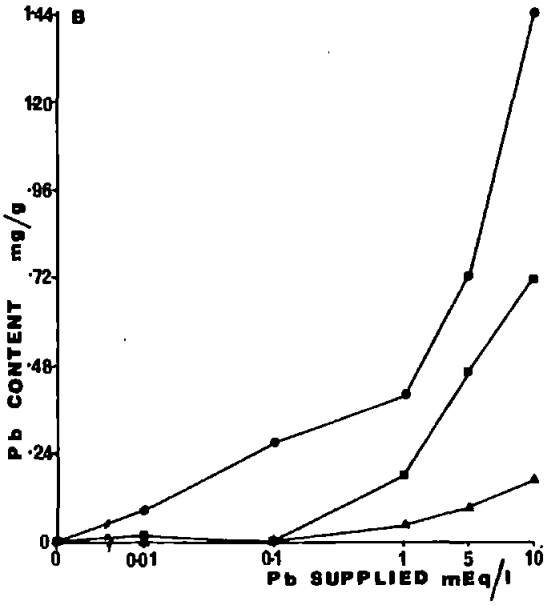
Pb SUPPLIED mEq/l


B
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Pb CONTENT mg/g
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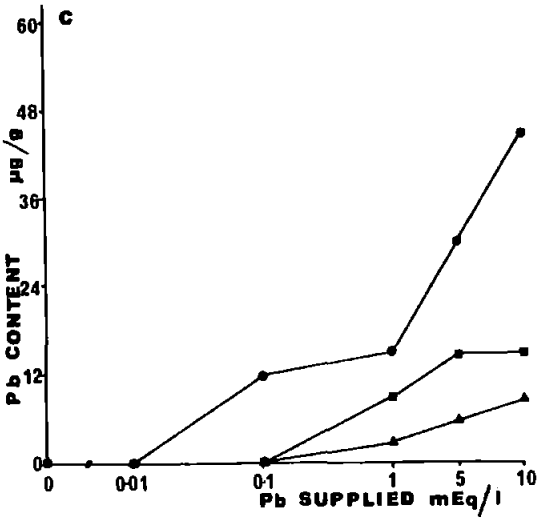
Pb SUPPLIED mEq/l


C
60
48
36
24
12
0
Pb CONTENT µg/g
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1
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Pb SUPPLIED mEq/l

Figure 4.2.1 Lead content of hydroponically grown plants

A. 2 week old plants

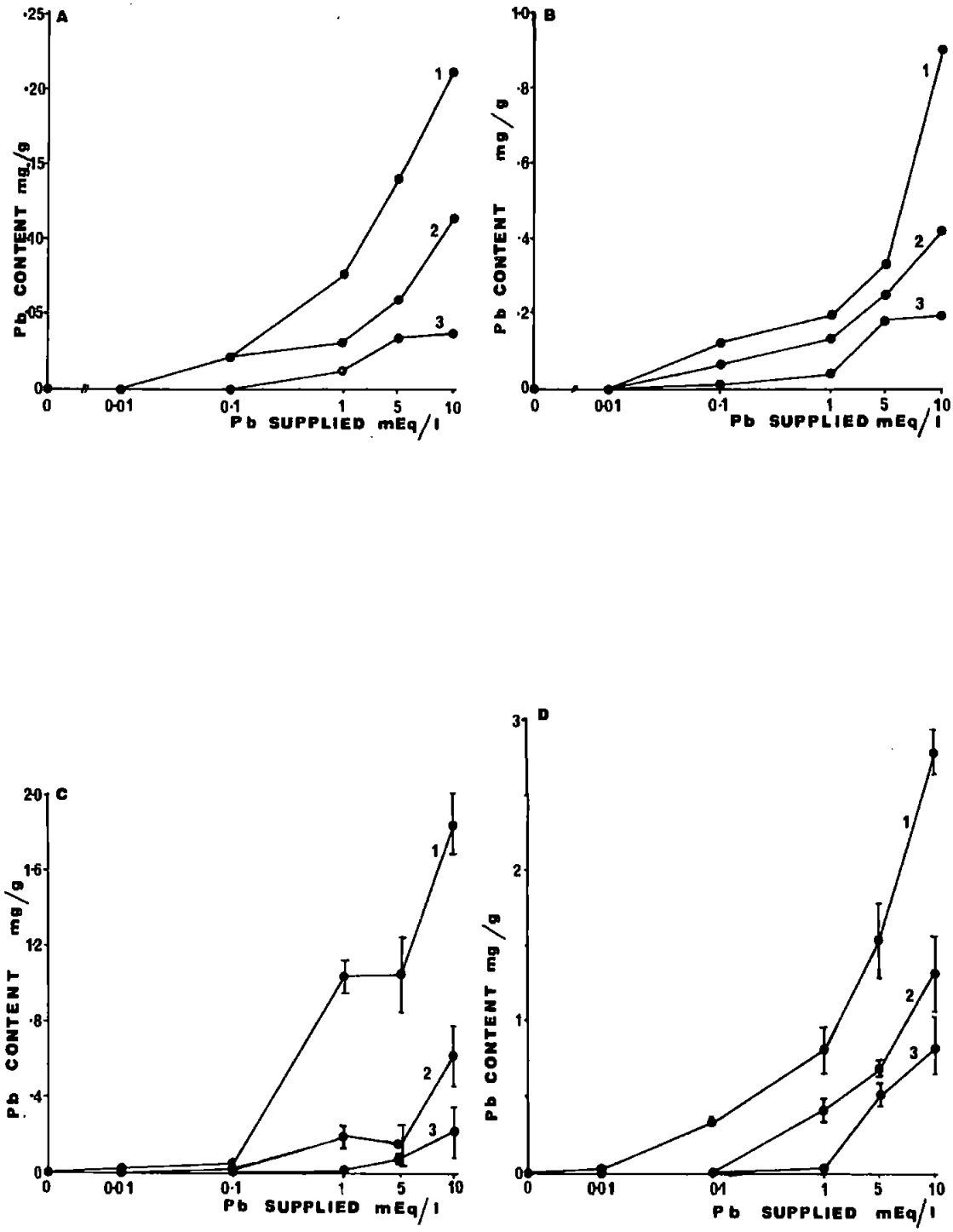
B. 3 week old plants

C. 4 week old plants

D. 5 week old plants

1 = roots; 2 = stems; 3 = leaves

Vertical line = $\pm$ standard error


A
B
C
D
Pb CONTENT mg/g
Pb SUPPLIED mEq/l
0
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1
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10
1
2
3

TABLE 4.3.1. COMPARISON OF DIMENSIONS OF SEEDLINGS GROWN IN SINGLE SALT CULTURE WITH TISSUE LEAD CONCENTRATIONS

(values represent mean ± standard errors where applicable)

A. Unwashed samples

Lead supplied mEq/l	Root lead conc. µg/g	Root length mm	Stem lead conc. µg/g	Stem length mm	Cotyledon leaf lead conc. µg/g	Cotyledon leaf breadth mm
0	0	121.8 ± 17.26	0	11.1 ± 2.99	0	8.0 ± 2.01
0.01	132	200.6 ± 17.84	168	10.0 ± 1.16	24	11.6 ± 2.05
0.1	243	120.2 ± 24.46	45	8.6 ± 2.05	75	7.0 ± 2.82
1	666	17.4 ± 2.86	111	8.2 ± 2.19	123	7.2 ± 1.21
5	1140	11.8 ± 0.71	825	6.4 ± 2.05	93	4.6 ± 1.88
10	2261	10.2 ± 0.17	1032	5.8 ± 1.12	140	4.0 ± 1.66

B. Samples washed in deionised water

Lead supplied mEq/l	Root lead conc. µg/g	Root length mm	Stem lead conc. ug/g	Stem length mm	Cotyledon leaf lead conc. µg/g	Cotyledon lead breadth mm
0	0	121.8 ± 17.26	0	11.1 ± 2.99	0	8.0 ± 2.01
0.01	87	200.6 ± 17.84	9	10.0 ± 1.16	0	11.6 ± 2.05
0.1	27	120.2 ± 24.46	45	8.6 ± 2.05	0	7.0 ± 2.82
1	396	17.4 ± 2.86	180	8.2 ± 2.19	45	7.2 ± 1.21
5	726	11.8 ± 0.71	465	6.4 ± 2.05	90	4.6 ± 1.88
10	1450	10.2 ± 0.17	711	5.8 ± 1.12	183	4.0 ± 1.66

C. Samples washed in 0.01 Molar calcium nitrate solution

Lead supplied mEq/l	Root lead conc. µg/g	Root length mm	Stem lead conc. µg/g	Stem length mm	Cotyledon leaf lead conc. µg/g	Cotyledon leaf breadth mm
0	0	121.8 ± 17.26	0	11.1 ± 2.99	0	8.0 ± 2.01
0.01	0	200.6 ± 17.84	0	10.0 ± 1.16	0	11.6 ± 2.05
0.1	4	120.2 ± 24.46	0	8.6 ± 2.05	0	7.0 ± 2.82
1	18	17.4 ± 2.86	9	8.2 ± 2.19	3	7.2 ± 1.21
5	30	11.8 ± 0.71	15	6.4 ± 2.05	6	4.6 ± 1.88
10	51	10.2 ± 0.17	15	5.8 ± 1.12	9	4.0 ± 1.66

A. 2 Week old plants

Lead supplied mEq/l	Root lead conc. µg/g	Root dry weight g	Stem lead content µg/g	Stem dry weight g	Leaf lead conc. µg/g	Leaf dry weight g
0	0	0.0126 ± 0.0013	0	0.0078 ± 0.0008	0	0.0834 ± 0.0096
0.01	0	0.0126 ± 0.0011	0	0.0064 ± 0.0010	0	0.0892 ± 0.0049
0.1	21	0.0066 ± 0.0012	21	0.0044 ± 0.0010	0	0.0518 ± 0.0064
1	76	0.0022 ± 0.0005	32	0.0022 ± 0.0007	1.3	0.0156 ± 0.0013
5	142	0.0010 ± 0	61	0.0014 ± 0.0002	35	0.0104 ± 0.0009
10	214	0.0002 ± 0	116	0.0008 ± 0.0002	38	0.0042 ± 0.011

B. 3 Week old plants

Lead supplied mEq/l	Root lead conc. µg/g	Root dry weight g	Stem lead content µg/g	Stem dry weight g	Leaf lead conc. µg/g	Leaf dry weight g
0	0	0.0318 ± 0.0062	0	0.0276 ± 0.0042	0	0.1834 ± 0.0143
0.01	5	0.0140 ± 0.0012	0	0.0154 ± 0.0028	0	0.1420 ± 0.0099
0.1	120	0.0128 ± 0.0025	64	0.0106 ± 0.0006	8	0.0986 ± 0.0153
1	192	0.0022 ± 0.0002	132	0.0046 ± 0.0006	36	0.0280 ± 0.0029
5	332	0.0004 ± 0	251	0.0012 ± 0.0002	180	0.0050 ± 0.0008
10	896	0.0004 ± 0	423	0.0004 ± 0	192	0.0026 ± 0.0011

Table 4.3.2 Comparison of dry weight yields of plants grown in hydroponic culture with tissue lead concentrations

(values represent mean ± standard error when applicable)

C. 4 week old plants

Lead supplied mEq/l	Root lead conc. µg/g	Root dry weight g	Stem lead conc. µg/g	Stem dry weight g	Leaf lead conc. µg/g	Leaf dry weight g
0	0	0.0490 ± 0.0076	0	0.2428 ± 0.0157	0	0.5348 ± 0.0254
0.01	29 ± 5.4	0.0284 ± 0.0047	0	0.0432 ± 0.0050	0	0.2248 ± 0.0344
0.1	38 ± 4.9	0.0188 ± 0.0036	10 ± 1.3	0.0496 ± 0.0036	0	0.1236 ± 0.0101
1	1040 ± 83.2	0.0096 ± 0.0021	192 ± 42.9	0.0092 ± 0.0026	19 ± 1.8	0.0406 ± 0.0091
5	1051 ± 189.1	0.0020 ± 0.0005	149 ± 64.4	0.0018 ± 0.0006	75 ± 28.6	0.0132 ± 0.0021
10	1842 ± 98.8	0.0018 ± 0.0004	629 ± 145.3	0.0020 ± 0.0008	215 ± 98.8	0.0030 ± 0.0008

D. 5 Week old plants

Lead supplied mEq/l	Root lead conc. µg/g	Root dry weight g	Stem lead conc. µg/g	Stem dry weight g	Leaf lead conc. µg/g	Leaf dry weight g
0	0	0.072 ± 0.0110	0	0.490 ± 0.0872	0	0.760 ± 0.1404
0.01	19 ± 6.3	0.062 ± 0.0085	0	0.604 ± 0.0233	0	0.778 ± 0.0997
0.1	340 ± 22.3	0.052 ± 0.0058	8 ± 1.4	0.176 ± 0.0590	8 ± 2.2	0.371 ± 0.1212
1	808 ± 126.6	0.026 ± 0.0107	408 ± 82.3	0.062 ± 0.0080	220 ± 4.9	0.143 ± 0.0429
5	1538 ± 202.5	0.004 ± 0.0009	686 ± 50.1	0.065 ± 0.0058	650 ± 155.2	0.027 ± 0.0049
10	2790 ± 136.8	0.003 ± 0.0009	1324 ± 22.0	0.0015 ± 0.0007	1240 ± 118.1	0.004 ± 0.0009

Table 4.3.2(continued) Comparison of dry weight yields of plants grown in hydroponic culture with tissue lead concentrations (values represent mean ± standard error)

CHAPTER 5

LIGHT MICROSCOPE STUDIES ON THE DISTRIBUTION OF LEAD IN *RAPHANUS SATIVUS*

As stated in Chapter 4, over the years a number of authors have reported a differential distribution of lead between various plant organs, such observations arising from chemical analysis of tissues. However, studies of the distribution of lead within the organs of the plant have been relatively scarce. A few authors have used lead chelates to investigate water pathways in plants. For example, Crowdy and Tanton (1970) applied the lead salt of ethylenediaminetetra-acetic acid to the roots of wheat plants and found that the salt moved relatively freely throughout the plant, even as far as the stomata, the periclinal epidermal walls, and the cuticle of the leaves. Similar movement was shown by Byott and Sheriff (1976) using excised leaves of Tradescantia virginiana. While such studies provide evidence concerning the pathways of solutions through plants, the information they contain cannot be interpreted in relation to the movement of lead in the plant, since in a chelated form the lead is unable to bind to anionic sites as it would in a free ionic form. However, the data does show that lead compounds are capable of movement throughout the plant, even when the metal is complexed in a large molecule. Hammett (1928a) reported that in Allium cepa, Phaseolus vulgaris, and Zea mays lead was concentrated in the roots and more specifically in the region of the meristem. In a later paper Hammett (1928d) suggested that lead was probably bound to organic sulphydryl groups. Malone et al. (1974), investigating lead uptake by corn plants,

found that lead was located mainly as a surface precipitate on the root and within the cell walls.

Other studies have tended to be at a subcellular level and in these the cell wall again features predominantly as a site of lead accumulation (e.g. Whitehead et al., 1971; Brown and Slingsby, 1972; Gullvåg et al., 1974).

The present study was carried out in order to determine the distribution and localisation of lead in the tissues of the radish. From the data of Chapter 3 it can be seen that lead exerts its greatest influence on growth in the early stages of development, and so in this study observations were mainly confined to the more juvenile stages of growth. Lead was applied to the plants as the nitrate salt and at three concentrations: 0.1, 1, and 10 mEq/l. Observations were also made on seed material which had been exposed to lead at 50 mEq/l and had failed to germinate. After treatment the tissue was washed in deionised water and treated with saturated hydrogen sulphide solution (see Chapter 2). The precipitate distributions observed were also verified by staining with sodium rhodizonate at pH 2.8 (see Chapter 2). Unless otherwise stated the photographs presented are of 10 mEq/l specimens treated with hydrogen sulphide solution. In weaker solutions of lead distribution tended to be analogous, but general contrast was much reduced.

5.1 *Lead Distribution in the Seed During Early Germination*

A dark colouring appeared in the testa of lead-treated seeds on exposure to hydrogen sulphide solution which was not present in controls. After 12 hours exposure to 10 mEq/l lead this took the form of a slight darkening of the entire area exposed to lead, in addition to which small, discrete, darker patches were visible. The areas

became more extensive with treatment time until after 36 hours virtually the entire exposed testa was black (Plate 5.1.1a).

In sections of testa four layers were visible. These consisted of an outer layer of cells, a layer of sclereid-like cells, a layer of coloured cells, and finally a layer of larger cuboid cells. In seeds exposed to lead for 12 hours lead was seen to be associated with the sclereid-like cells, and perhaps to a less obvious extent with the irregular outer layer (Plates 5.1.1b and c). This outer layer was identified histochemically using the methods of Chayen et al. (1969) and found to be rich in acid mucopolysaccharides. In addition discrete areas of lead, which were of an amorphous nature, were visible on the surface of this outer layer. In the testa of seeds which had been exposed to 10 mEq/l of lead for 36 hours or more, some staining was also evident in the other layers of cells. A three-dimensional diagrammatic representation of lead distribution in the testa at the 12 hour stage is presented in Figure 5.1.2. In seeds exposed to 50 mEq/l lead for 36 hours staining was intense throughout the testa.

The Radicle

While the testa was intact, staining of the embryo was not visible except in those seeds exposed to 50 mEq/l lead. In this case staining of the embryo was widespread, but tended to be less intense towards the centre of the seed. In specimens exposed to 1 and 10 mEq/l lead once the testa was ruptured the radicle took up lead quickly, as did the rest of the tissue. Twelve hours after radicle emergence lead was distributed throughout the developing root, but density of staining was variable and noticeably less dense in the meristematic region. The concentration in the meristem appeared to increase with time until at 36 hours after emergence the whole zone of division was highly

contaminated. Staining in the rest of the tissue appeared to be associated mainly with the cell walls, and was noticeably less dense in the root cap than in the rest of the tissue (See Plate 5.1.3, a-c).

The Hypocotyl

At this stage of development it was difficult to decide where the radicle stopped and the hypocotyl began, but in general the distribution of lead in the hypocotyl tended to be slightly more localised. At the top of the hypocotyl in the region of the stem apex there was a zone which was relatively slightly contaminated. The staining of this area did not increase during the 36 hour observation period, but instead the area low in lead tended to enlarge (Plate 5.1.4, a and b).

The Cotyledon

It was found that after 12 hours exposure to lead the cotyledons were heavily stained, the intensity being greatest in the region of the veins. Staining became less obvious with increasing time, becoming more localised and less intense in the vascular tissues (Plate 5.1.4, c and d). It was noted that during the period of exposure to lead the elongation which occurred in the hypocotyl tended to lift the cotyledons clear of the bathing solutions and so removed the cotyledons from direct contact with lead. It would seem, therefore, that the subsequent expansion of the cotyledons exceeded the increase in lead content arising from the transport of the metal into the cotyledons via the stem.

5.2 *Lead Distribution in Radish Seedlings*

The observations reported in Chapter 3 show that plants grown in solutions containing relatively high concentrations of lead tend to remain in the seedling stages of growth. For the purposes

of this study examinations were carried out on seedlings which had been germinated in deionised water and subsequently transferred to lead solutions for predetermined periods of time, or on seedlings germinated and grown in solutions containing lead.

It was noticed throughout the study that exposure of the plant to lead rendered the material difficult to section; the preparations were very susceptible to knife damage and in many cases this resulted in local damage to cell walls in the sections. This was less evident in resin preparations where the embedding medium provides more support for the structures.

The Root

The distribution of lead in the root tip of 14 day old seedlings exposed to 10 mEq/l of lead was similar to that seen in the radicle of the germinating seed after 36 hours, irrespective of the time of exposure to the metal. Further up the root staining was less dense and staining of the epidermis and peripheral region of the cortex occurred even after very short periods of exposure to lead. In these preparations it was clear that the lead distribution was highly localised, and most predominant in the intercellular spaces (Plate 5.2.1a). Although root hairs tended not to develop in solutions containing high concentrations of lead, examination of the root hairs of seedlings grown in deionised water and transferred to lead nitrate solutions showed that lead was taken up readily by the hairs, and was found not only in association with cell walls but also in the cytoplasm of the root hair cell (Plate 5.2.1b). Sections through the root in the region of root hair proliferation showed staining in the peripheral region of the cortex and heavy deposits in the endodermis. Staining of a less localised nature was also evident in the vascular tissue,

being most prominent where secondary thickening had occurred (Plate 5.2.1c). When the endodermis was penetrated by lateral root formation an accumulation of the stain was observed in the damaged area, suggesting that lead had moved in rapidly through the ruptured endodermis (Plate 5.2.1d). It is interesting to note that, even at this early stage of development of the lateral root, intracellular deposits of lead were apparent in the meristematic region. A similar distribution with respect to the endodermis was seen in longitudinal sections, and from these it was apparent that the accumulation of stain was more pronounced above the developing root than below it (Plate 5.2.2a), indicating that the lead was moving into the root and upwards.

In very young root material exposed to lead, accumulations of stain were visible in the phloem. Radish has an uncommon stelar structure in its juvenile stages in that both the protoxylem of the diarch xylem and the protophloem interrupt the pericycle and lie in juxtaposition to the endodermis. At this stage lead appeared to accumulate more quickly in the protophloem cells than in the protoxylem and in addition an accumulation of stain was visible in the inner cortex in the proximity of the phloem (Plate 5.2.2b). Examinations of this area under higher magnifications revealed that at this point black deposits were present on all walls of cells in the endodermis, indicating that in these few cells the Casparian strip may be absent (Plate 5.2.2c). In older tissue the pericycle was complete and the protoxylem and protophloem were divorced from the endodermis; also distribution in the cortex outside the endodermis was more uniform. However, in some preparations of older tissue localisations of stain were visible which suggested an accumulation in the phloem (Plate 5.2.2d). Investigations were also carried out to elucidate the nature of the

dark regions described previously as occurring at the top of the root on exposure to lead (see Chapter 3). Sections through this region showed that in the presence of lead a dark staining was visible in the cell walls even in specimens which had not been exposed to hydrogen sulphide solution. This would indicate that the lead is being localised as a dark compound of low solubility; this area may be rich in the sulphydryl groups which Hammett (1928) proposed as a binding site for lead (Plate 5.2.3a).

The Stem

In tissue in which any lateral expansion of the stem had occurred it was difficult to determine whether tissue was of root or stem origin, as there was no clear delineation between the two tissues, either internally or externally. For the purposes of this study the portion of the plant which would normally become swollen as a storage organ was taken to be stem tissue.

The major site of lead deposition appeared to be associated with the vascular tissue. In specimens treated with 10 mEq/l lead nitrate there was a distinct delineation in the stained portion of the tissue, the cortical region being only very lightly stained (Plate 5.2.3b). In tissue treated with 1 mEq/l lead nitrate there was little variation between vascular and cortical regions, although the major vessels still showed fairly heavy staining (Plate 5.2.3c). At concentrations below 1 mEq/l lead was not detectable. In these cases it was suspected that the tissues of the root were binding the lead and preventing its movement in bulk to the stem. At the higher treatment levels it was found that the intensity of staining became progressively less as the distance from the root increased. At leaf nodes lead was still predominantly associated with the vascular tissue, but there was

also evidence of an association of lead with developing leaf primordia and axilliary buds (Plate 5.2.3d). In both root and stem tissue staining in the vascular tissue was more pronounced where secondary thickening had occurred, and in the sections examined there was no evidence of lead remaining in the pores, indicating that the metal was bound to the lignified tissue rather than simply deposited on the inner surface of the elements (Plate 5.2.4a and b).

The Leaves

In the cotyledon leaves lead was deposited in two main sites, one in the basal and central portions of the cotyledon (Plate 5.2.5a) and the other in the form of more local concentrations in the distal parts of the cotyledon lobes (Plate 5.2.5b). These local concentrations possibly represent a build-up of lead in the terminal region of the vascular strands, or in some cases a residual deposit remaining after contact with the lead solution during germination or early growth. In addition in many leaves staining was evident in the region of the apical meristem of the cotyledon (Plate 5.2.5c). Consideration of the appearance of the stained cotyledon as a whole (Plate 5.2.5d) shows that the accumulations of lead present show a definite similarity to the chlorotic and necrotic areas mentioned previously (c.f. Chapter 3, Plate 3.2.1a). The association with necrotic regions was borne out by sections of lead-impregnated leaf tissue which showed that in the necrotic regions lead deposits were far more extensive than elsewhere (Plate 5.2.6a). In these sections it was also noted that, although staining of the vessel walls in the vascular tissue was apparent, the bulk of the stain associated with the vascular tissue was outside the major vessels and the lumina of the vessels were almost free of deposits (Plate 5.2.6b).

Even in plants where high levels of lead were supplied via the roots there was no real evidence for its deposition in the true leaves, unless lead solutions were supplied over prolonged periods. Alternatively placing the plant in lead solution at 10 mEq/l after excision of the roots at the base of the stem (deradication) resulted in contamination of the true leaves within 24 hours. This accumulation was almost entirely restricted to the vascular tissues, and there was no indication of the local accumulations observed in the cotyledon leaves. Distribution of stain was not uniform from leaf to leaf and in general contamination was more extensive in mature than in young leaves (Plate 5.2.7.a and b).

Control sections of all tissues exposed to solutions of 10 mEq/l lead nitrate and then washed in deionised water showed an even distribution of lead throughout the tissue. From this it was surmised that the distributions seen in the treated material were the result of dispersion patterns of lead, not of a variable ability to bind lead. However, an exception to this was observed in the xylem walls which were slightly darker in colour than the surrounding tissue when exposed to hydrogen sulphide solution.

5.3 *Discussion*

The findings of this study show that in the seed exposed to lead solutions at up to 10 mEq/l the testa prevents lead contamination of the seed until such time as it is ruptured by the developing radicle. This confirms previous data (see Chapter 3) that lead in the concentration range 0 - 10 mEq/l had no influence on germination. This protective feature of the testa would ensure that little contamination of the embryo can take place and could be of major significance under natural conditions.

Once the testa had ruptured lead was taken up very rapidly, notable exceptions occurring in the meristematic regions of the radicle and hypocotyl. There are several possible explanations for a reduced accumulation of lead in the meristematic tissue. The fact that in the root this effect disappears with time may indicate that the supply of lead to the meristem is attenuated by passage through the surrounding cells, or that movement of salts into this region is restricted or occluded during early development. This may relate to the pressure variations in the area surrounding the quiescent centre suggested by Clowes (1976) and may be analogous to the reduction in DNA precursors through the root tip region reported by Clowes (1970). The ability of plants to restrict the movement of toxic substances into the meristematic region may be an important mechanism of resistance to these substances, and may contribute to the considerable resistance of meristems to unfavourable environmental conditions, including variation in nutrient supply (Thompson and Miller, 1962).

The difference which is apparent between the distributions of stain observed in the radicle and hypocotyl apices is important, as such differences in meristem contamination may be at least partly responsible for the disparity observed in the growth of roots and stems exposed to lead. The reduced contamination of the apical meristem suggests that, in the meristem, cell division and expansion proceed at a sufficiently rapid rate to offset the permeation of lead into the region. This process would no doubt be facilitated by the lower concentrations of lead observed in the stem relative to the root (see Chapter 4).

In the seedling it is apparent that lead is capable of essentially unrestricted movement throughout the plant. The results indicate that

lead moves predominantly into the root apoplast and moves in a radial manner across the cortex and accumulates at the endodermis. The endodermis may therefore act as a partial barrier to lead movement between the root and shoot. The existence of such a function is implicit in the observation that damage to the endodermis by lateral root formation resulted in accumulations of lead in the stele adjacent to the damaged area. This would suggest that lead had moved in rapidly and in relatively large amounts through the damaged endodermis, and for this to occur lead must have passed through the cortex in relatively large amounts. Such a movement would be in agreement with an apoplastic movement of lead to the endodermis. As staining in the cortex was not particularly dense, it is possible that the lead outside the endodermis had been partly leached out by the embedding process. Nagahashi et al. (1974) also highlighted the efficiency of the endodermis as a barrier to apoplastic solute movement in experiments using lanthanum. The trivalent cation of lanthanum is unable to penetrate cell membranes, and in their study Nagashi and his co-workers found that the movement of the ion in corn roots was prevented entirely by the endodermis.

Such a function as a barrier to ions may in part account for the previous observation of higher accumulation of lead in roots than in stems and leaves (see Chapter 4) and may in addition explain the greater toxicity of lead to roots (see Chapter 3).

In the very early stages of root development an interesting distribution was evident in the accumulation of lead in the protophloem. The phloem forms a distribution system for many metabolites and assimilates in the plant (Crafts and Crisp, 1971) and so it is perhaps not surprising that lead might be deposited or precipitated in this

region; however, what is interesting is the existence of cells in the endodermis associated with these young protophloem cells which appear to lack a Casparian strip. Such an arrangement may be advantageous in the proximity of the root meristem in that it would facilitate the flow of metabolites from the phloem. The absence of a Casparian strip could also facilitate the movement of lead into the stele at this point. This is possibly reflected in an irregular distribution of the metal in the adjacent cortex. In older tissue an accumulation of lead in the phloem may be apparent, but it is impossible to tell if this is a residual deposit from an earlier phase of development or the result of movement of the metal through the phloem. Experiments by Hampp and Höll (1974) have shown, however, that lead may be transported in the phloem in the aerial parts of the plant, and that the lead content of the phloem tissue decreases quite rapidly with increasing distance from the point of application.

In the stem lead was mainly restricted to the vascular tissues, and more specifically to the xylem. Accumulations of lead were also observed in the upper part of the stem in developing leaf primordia. This localisation may be involved with the reduction in leaf formation observed previously (see Chapter 3).

Observations of lead distribution in the cotyledons showed that lead moved through the vascular tissue and tended to accumulate in discrete areas in the distal parts, in positions similar to those developing necrotic lesions in other cotyledon leaves (see Chapter 3). A similar accumulation has been reported by Roberts and Hutchinson (1975), although this resulted from an aerial source of lead and was thought to arise from increased retention of lead on the leaf surface. The application of lead to deradicated plants showed that lead is more

mobile when the restricting influence of the root is removed, and that lead taken up into the leaves is accumulated preferentially in the older leaves. This would suggest that the chlorosis, more pronounced in younger leaves, which was observed in the growth studies (see Chapter 3) may have been the result of an influence of lead on processes remote from the leaves which appeared chlorotic, rather than an effect on the metabolism of the relevant leaf itself.

In general the concentration of lead in the aerial parts of the plant decreased as the distance from the roots increased. This concurs with the analyses of lead-treated plants described previously (see Chapter 4). Examination of sections of treated material indicates that this may be because the lead was bound to the cell walls of the tissue through which it had passed. In addition, more binding seemed to occur in lignified than in non-lignified tissue.

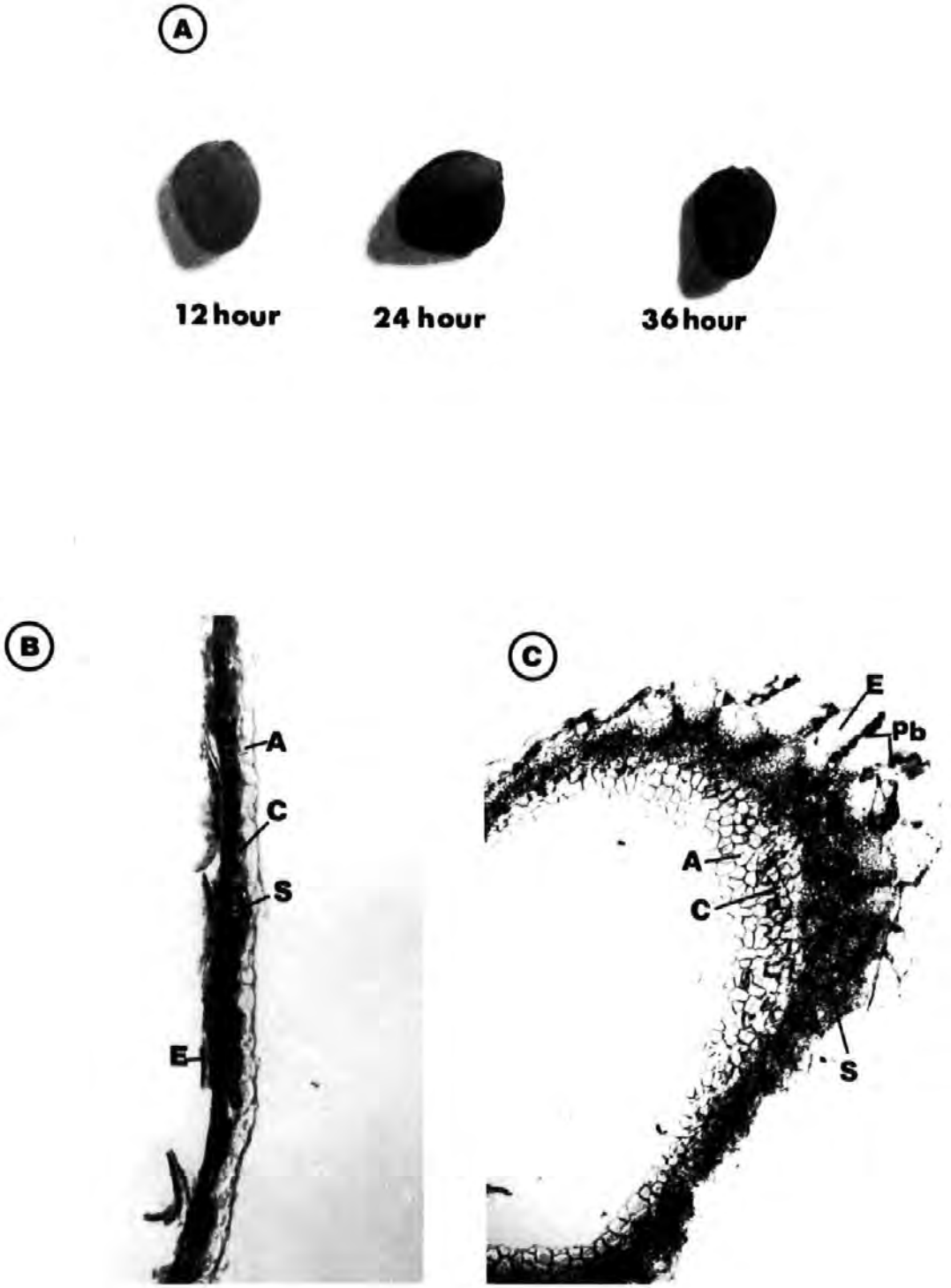
Plate 5.1.1 Light micrographs of lead distribution in germinating seeds

A. Colouration of the testa of radish seeds exposed to hydrogen sulphide after various periods of exposure to lead (x7).

B. Vertical section through testa of seed exposed to 10 mEq/l lead for 36 hours (x75). Note accumulation of stain in outer regions.

C. Tangential section of testa of seed exposed to 10 mEq/l lead for 36 hours (x75). Note superficial lead deposits.

A - aleurone layer; C - layer of pigmented cells; S - sclerenchyma layer; E - outer epidermis; Pb - lead deposits


A
12 hour
24 hour
36 hour
B
A
C
S
E
C
E
Pb
A
C
S

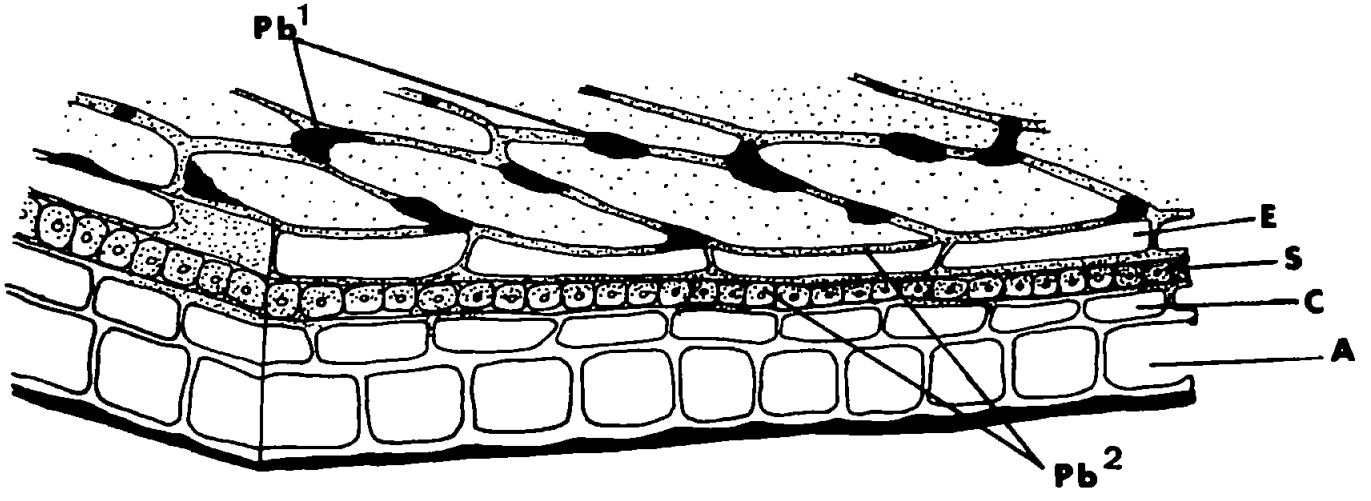


Figure 5.1.2. Distribution of lead in radish testa after 12 hours exposure to lead.

A - aleurone layer; C - layer of pigmented cells; S - sclerenchyma layer

E - outer epidermis; Pb^1 - amorphous superficial lead deposits; Pb^2 - small particle, internal lead deposits.

Plate 5.1.3 Light micrographs of lead distribution in developing radish radicles

A. Longitudinal section of radicle after 12 hours exposure to 10 mEq/l lead (x50). Note meristem is relatively free of lead.

B. Longitudinal section of radicle after 24 hours exposure to lead (x50). Note contamination of the meristem has increased.

C. Longitudinal section of radicle after 36 hours exposure to lead (x50). Note meristem is highly contaminated but the root cap remains relatively free of lead.

M - meristematic region; RC - root cap

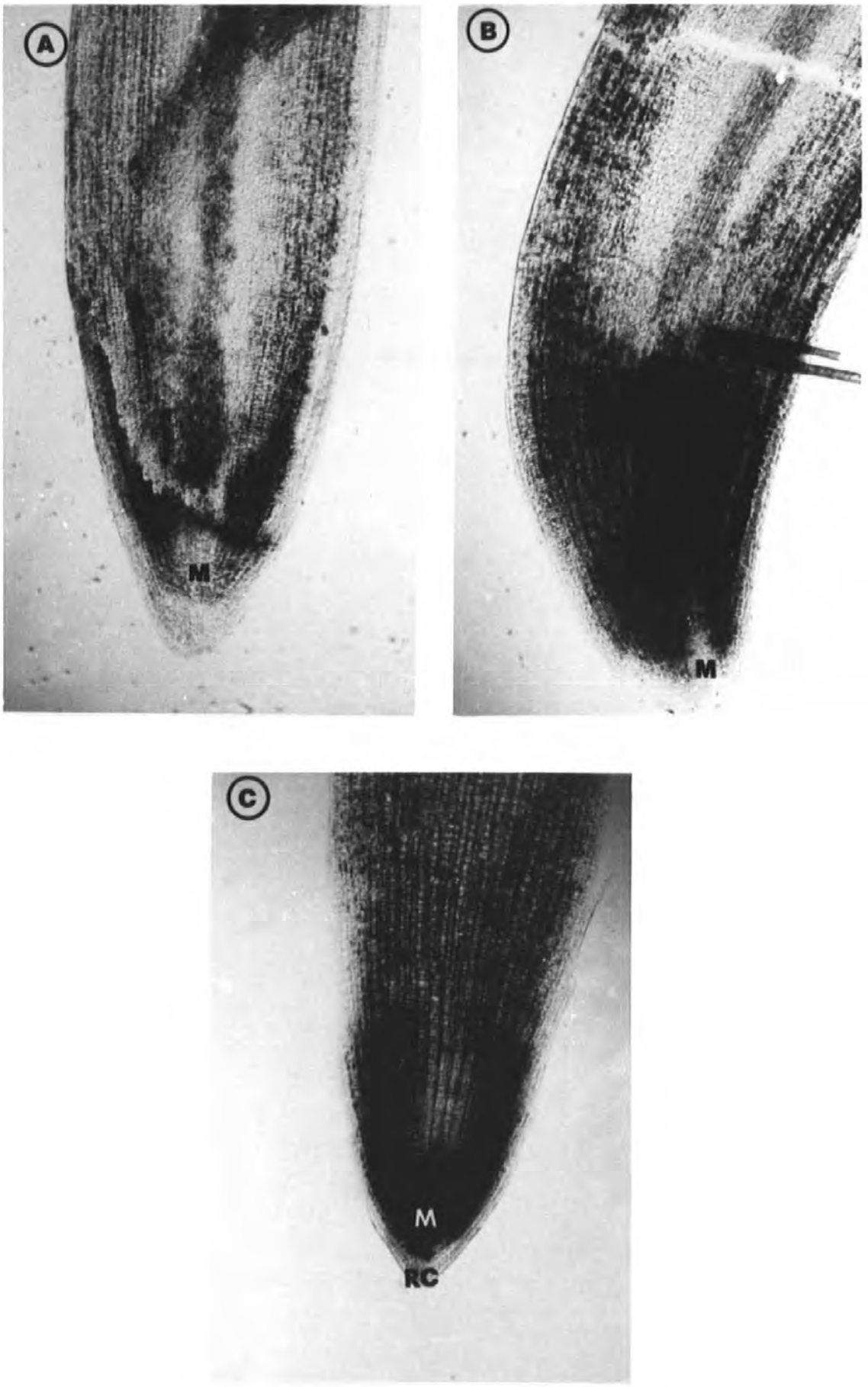

A
M
B
M
C
M
RC

Plate 5.1.4 Light micrographs of lead distribution in developing radish hypocotyls and cotyledons

A. Longitudinal section of apical meristem region of hypocotyl after 12 hours exposure to 10 mEq/l lead (x50). Note meristem is partially contaminated with lead.

B. Longitudinal section of apical meristem region of hypocotyl after 36 hours exposure to 10 mEq/l lead (x50). Note meristem is substantially free of lead.

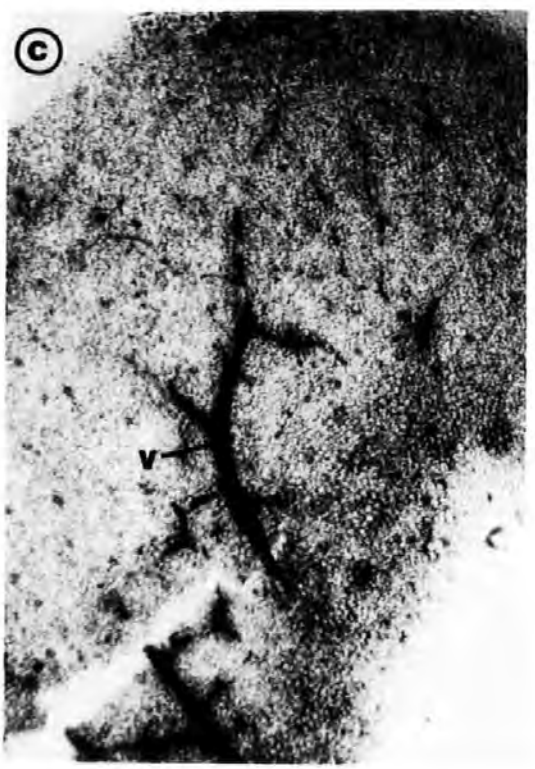
C. Horizontal section of seed cotyledon after 12 hours exposure to 10 mEq/l lead (x50). Note overall stain and intense staining of vascular tissue.

D. Horizontal section of seed cotyledon after 36 hours exposure to 10 mEq/l lead (x50). Note reduced staining, particularly in vascular tissue.

M - meristematic region; V - vascular tissue


A


B
M


C
V


D
V

Plate 5.2.1 Light micrographs of lead distribution in radish roots - 1

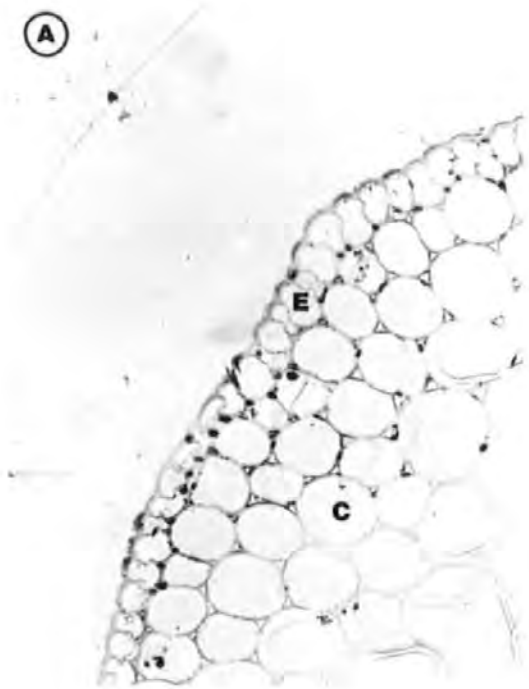
A. Transverse section of peripheral region of root behind root tip after exposure to 10 mEq/l lead for 1 hour (x400). Note intercellular deposition of lead in epidermis and outer cortex.

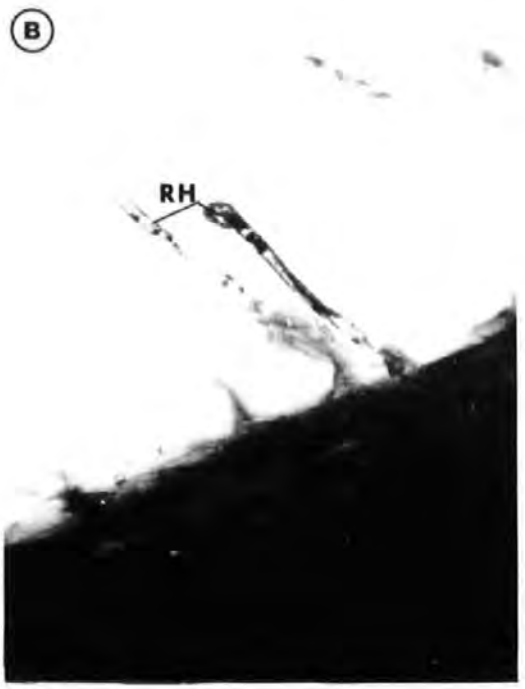
B. Whole mount preparation of radish root hairs after exposure to 10 mEq/l lead for 12 hours (x400). Note staining in cytoplasm.

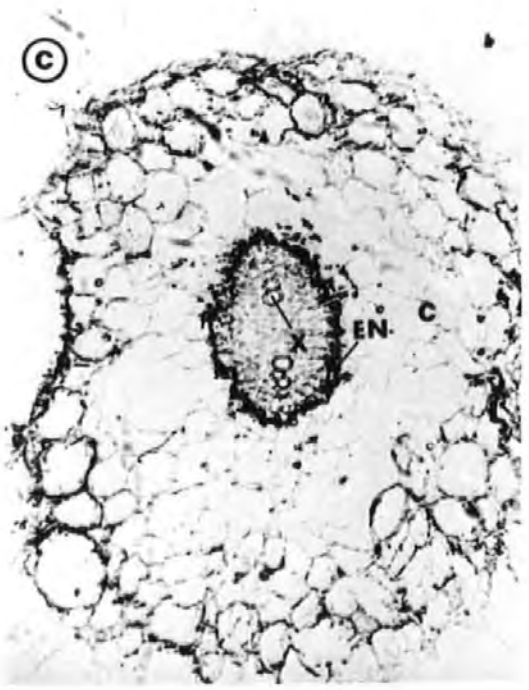
C. Transverse section of 14 day old root in region of root hairs after exposure to lead at 10 mEq/l for 24 hours (x50). Note heavy deposits in locality of endodermis.

D. Transverse section of 14 day old root in region of lateral root formation after exposure to 10 mEq/l lead for 24 hours (x50). Note heavy contamination of stele in area adjacent to damaged endodermis.

E - epidermis; C - cortex; RH - developing root hair;
EN - endodermis; X - xylem; R - developing lateral root.


A
E
C


B
RH


C
X
EN
C

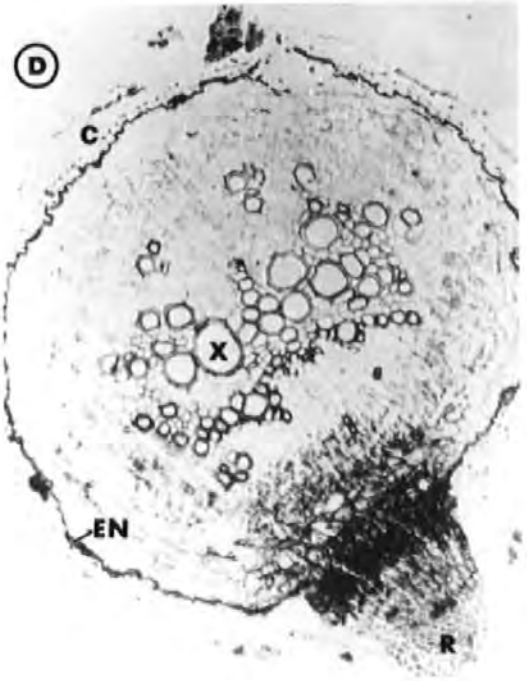

D
C
X
EN
R

Plate 5.2.2 Light micrographs of lead distribution in radish roots - 2

A. Longitudinal section of 14 day old root in region of lateral root formation after exposure to 10 mEq/1 lead for 24 hours (x50). Note intense staining in region above lateral root.

B. Transverse section of stele of 14 day old root c. 5 mm behind tip after exposure to 10 mEq/1 lead for 18 hours (x125). Note staining in phloem and corresponding inner cortex.

C. Transverse section of endodermis of 14 day root c. 5 mm behind tip after exposure to 10 mEq/1 lead for 18 hours (x350). Note presence of staining on all walls of endodermal cells adjacent to phloem.

D. Transverse section of stele of 14 day old root at base of region of root hair proliferation after exposure to 10 mEq/1 lead for 24 hours (x125). Note deposits in areas in region of phloem.

R - lateral root; X - xylem; P - phloem; E - endodermis; C - cortex.

Arrow indicates apical orientation

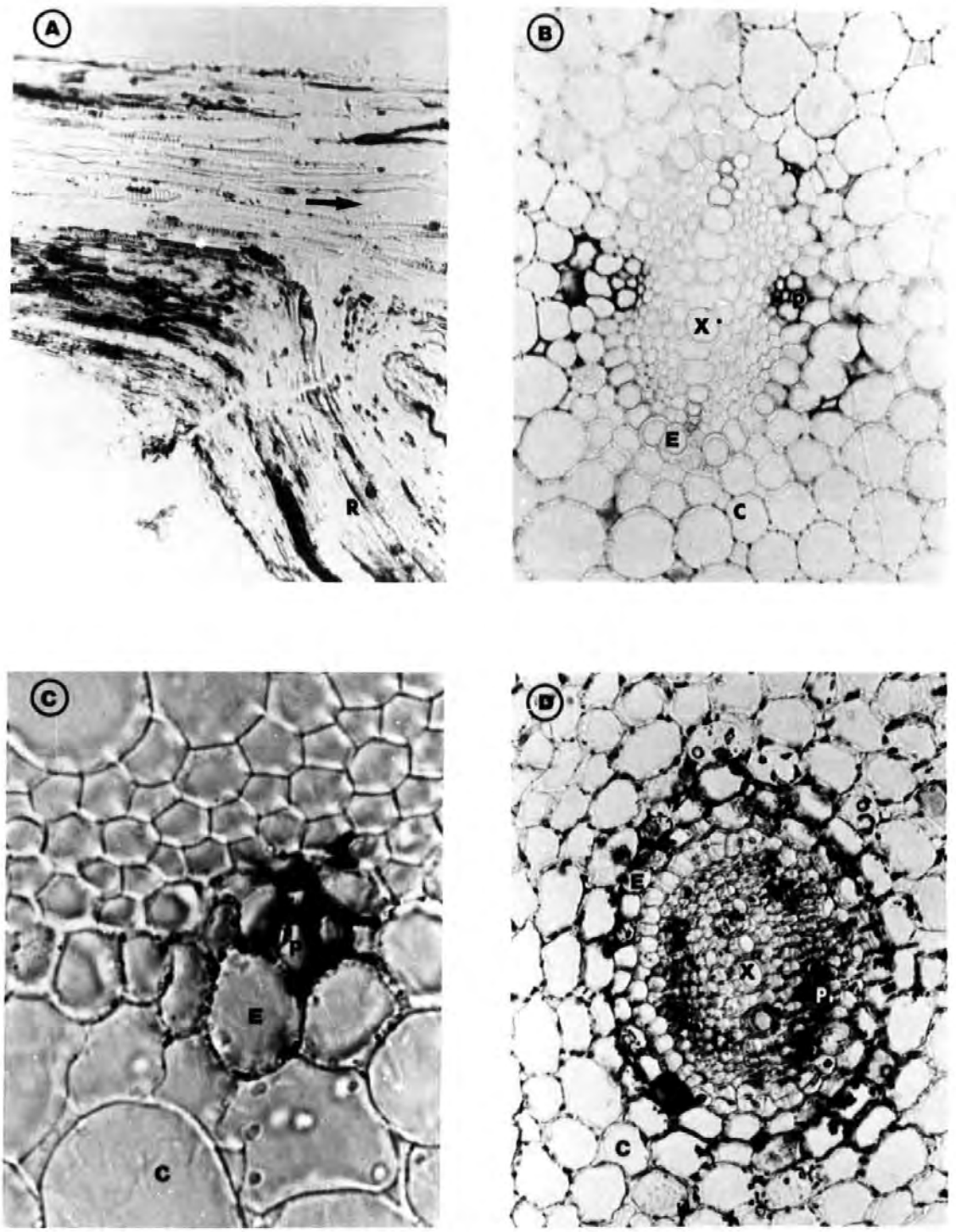

A
R
B
X
E
C
C
P
E
C
D
E
X
P
C

Plate 5.2.3 Light micrographs of lead distribution in radish stem tissue

A. Longitudinal section of coloured region at top of root of 14 day old seedling exposed to 10 mEq/l lead for 7 days (unstained) (x50).

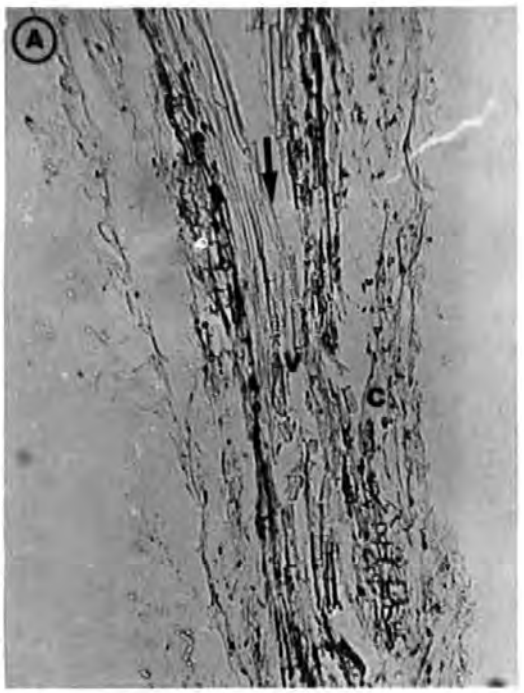
B. Transverse section of stem of 14 day old radish plant exposed to 10 mEq/l lead for 24 hours (x50). Note staining largely restricted to vascular region.

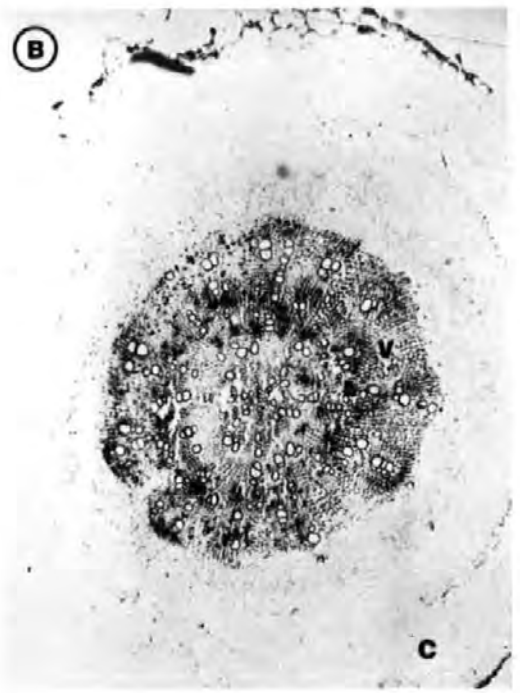
C. Transverse section of stem of 14 day old plant exposed to 1 mEq/l lead for 24 hours (x75). Note slight staining, restricted solely to xylem.

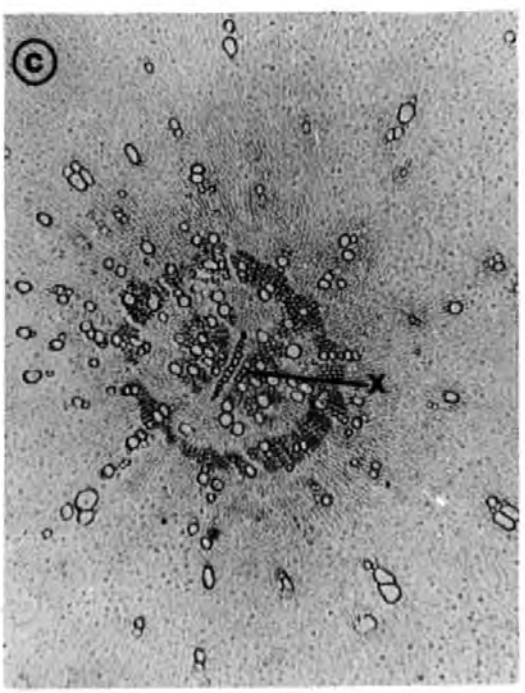
D. Longitudinal section of top of stem of 14 day old plant in region of lead initiation after 3 days exposure to lead at 10 mEq/l (x50). Note accumulation of stain in leaf primordia and axilliary bud.

V - vascular tissue; C - cortex; X - xylem; AB - axilliary bud; LP - leaf primordium.

Arrow indicates apical orientation


A
V
C


B
V
C


C
X

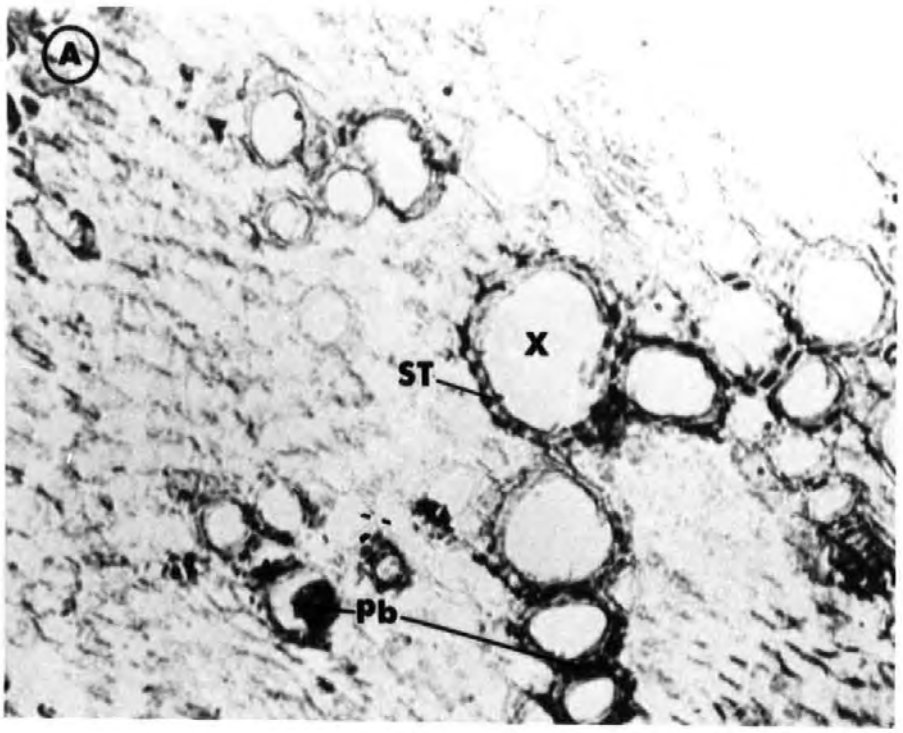

D
AB
LP
V

Plate 5.2.4 Light micrographs of lead deposits associated with secondary thickening of vascular tissues

A. Transverse section of 14 day old root after exposure to 10 mEq/l lead for 3 days (x400). Note heavy deposits in vessel walls.

B. Longitudinal section of 14 day old root after exposure to 10 mEq/l lead for 3 days (x400). Note absence of stain in pores of xylem secondary thickening.

X - xylem vessel; Pb - lead deposit; ST - wall with secondary thickening; P - pore.


A
X
ST
Pb

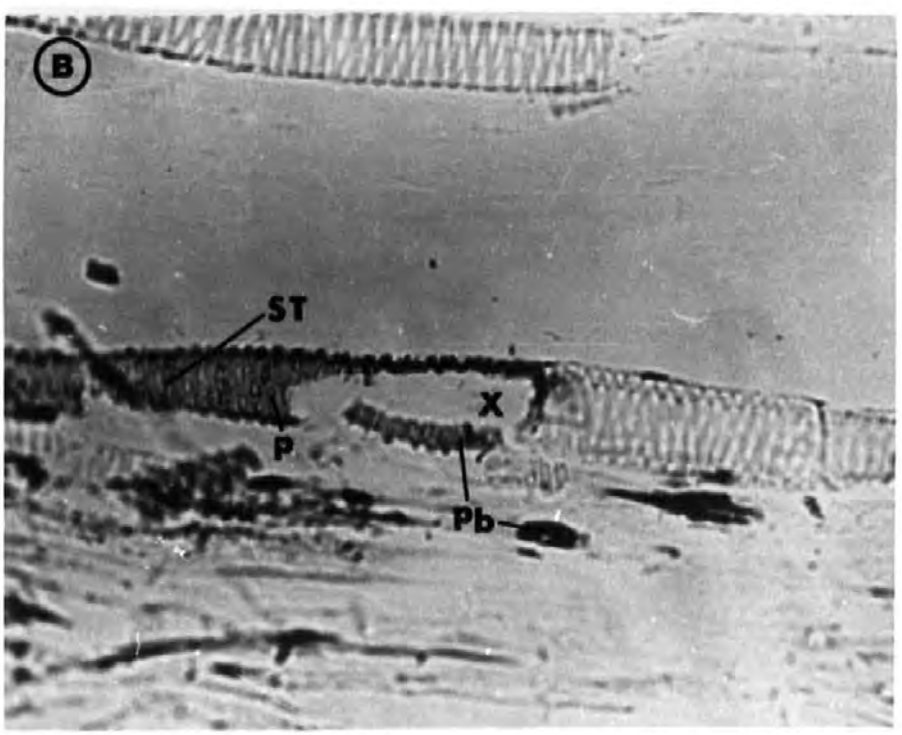

B
ST
X
P
Pb

Plate 5.2.5 Whole mount preparations of lead distribution in radish cotyledon leaves

A. Whole mount preparation of base of cotyledon/cotyledon petiole after exposure to 10 mEq/l lead for 7 days (x50). Note heavy staining in vascular regions.

B. Whole mount preparation of marginal region of cotyledon after exposure to 10 mEq/l lead for 7 days (x50). Note deposits around vascular tissue, and local accumulations.

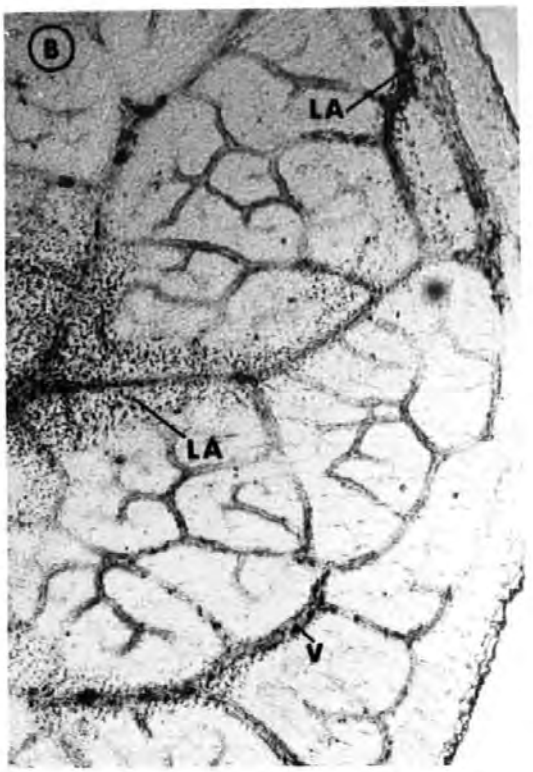
C. Whole mount of meristem region of cotyledon after exposure to 10 mEq/l lead for 7 days (x50). Note deposition in meristem region.

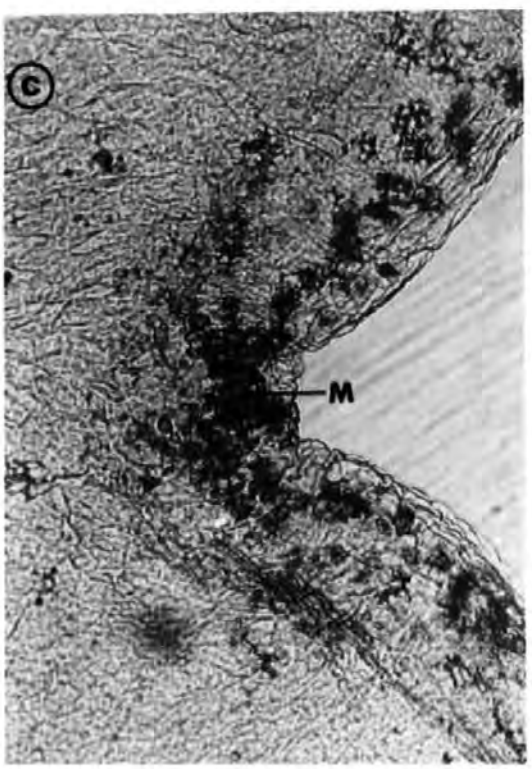
D. Whole mount of cotyledon exposed to 10 mEq/l lead for 7 days (x10). c.f. Plate 3.2.1a.

V - vascular tissue; LA - local accumulations of lead;

M - meristematic region.


A
V


B
LA
LA
V


C
M

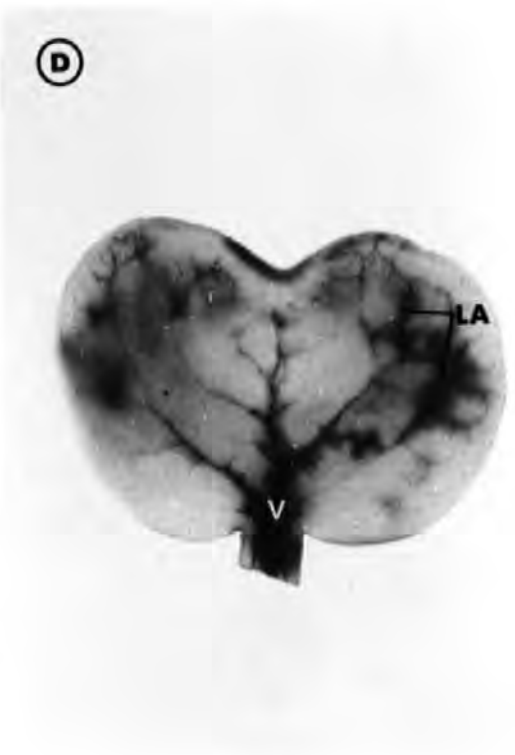
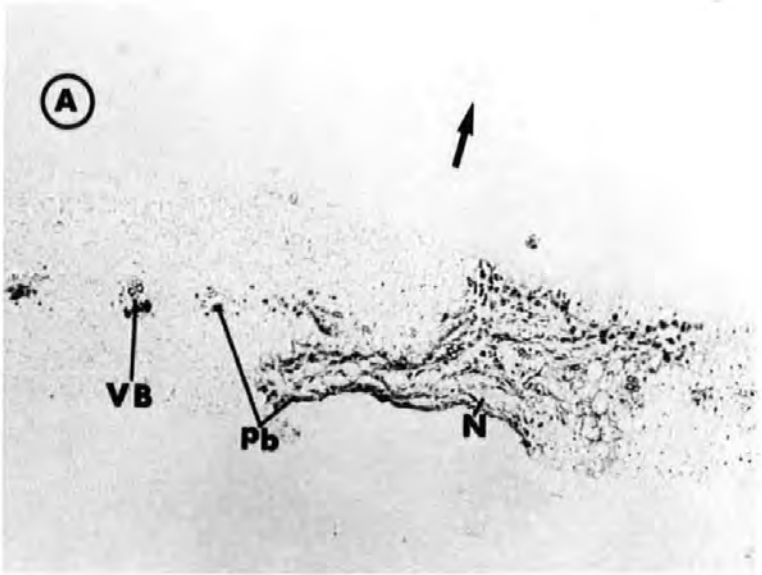

D
LA
V

Plate 5.2.6 Light micrographs of lead distribution in radish cotyledon leaf material

A. Transverse section of 14 day old cotyledon leaf exposed to 10 mEq/l lead for 7 days (x50). Note intense staining in region of damaged cells (necrotic region).

B. Transverse section of vascular strand in midrib of above leaf (x100). Note that the vessel lumina are largely clear of stain and that the bulk of the lead is in a position outside the major xylem elements.

VB - vascular bundle; N - necrotic area; Pb - lead deposits. Arrow indicates adaxial orientation


A
VB
Pb
N

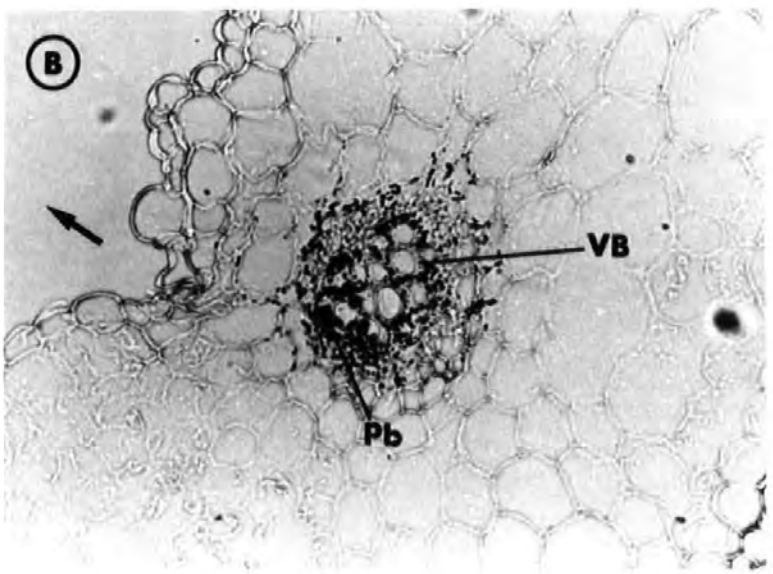
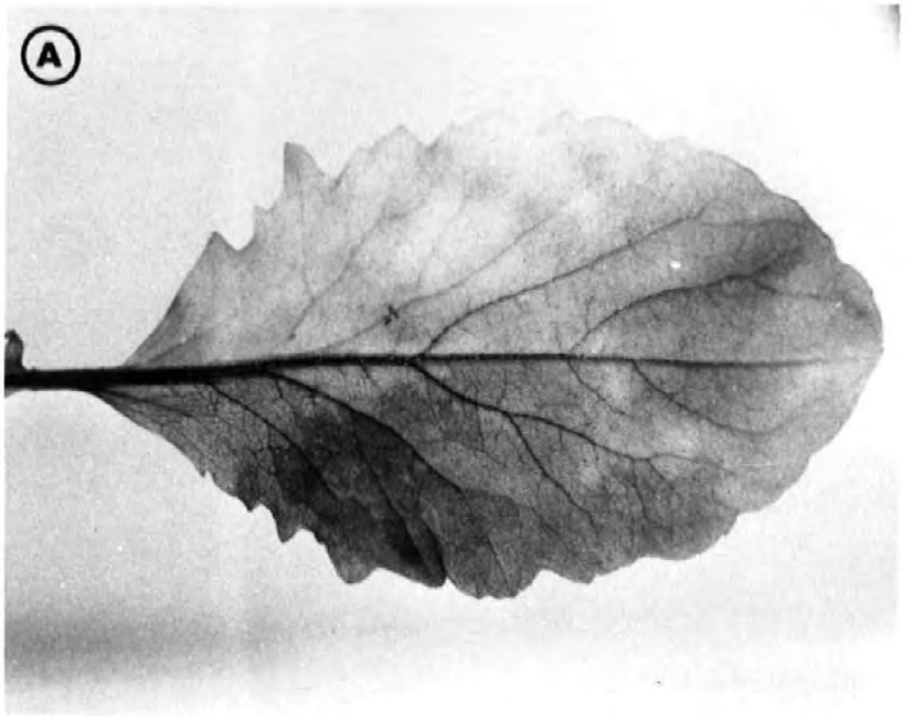
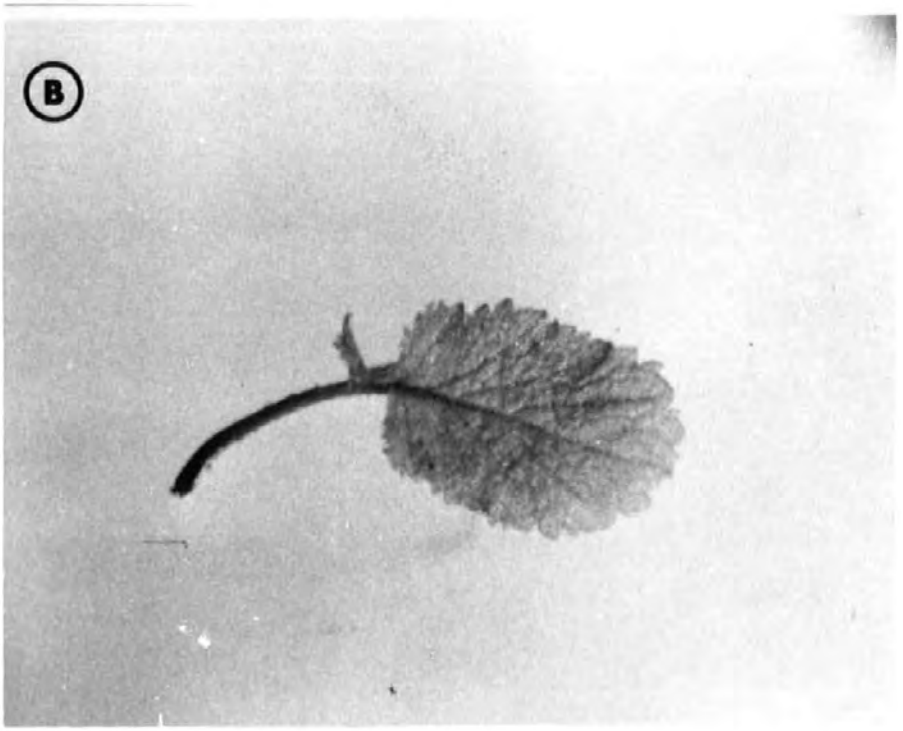

B
VB
Pb

Plate 5.2.7 Whole mount preparation of lead distribution in leaves of deradicated plants

A. Adaxial view of leaf of deradicated plant after immersion of cut end of stem in 10 mEq/l lead solution for 24 hours, followed by exposure to hydrogen sulphide solution and removal of natural pigmentation with 80% ethanol (x1.5). Note pervasion of lead throughout vascular tissue, even in fine reticulate network of veins.

B. Adaxial view of immature leaf from same plant as above, subjected to the same post treatment (x15). Note that lead is restricted to the petiole and the lower part of the midrib.


A


B

CHAPTER 6

SUBCELLULAR DISTRIBUTIONS OF LEAD IN *RAPHANUS SATIVUS*

The results presented in Chapter 5 revealed major accumulations of lead in the root, occurring predominantly in the vicinity of cell walls. For lead to exert an influence on the development of the plant, the metal must become closely involved with the plant at a cellular level. To elucidate this, transmission electron microscope (T.E.M.) studies were carried out to determine the distribution of lead at a subcellular level.

A number of studies on the uptake of lead and other heavy metals by cell organelles have been reported. These fall broadly into two groups: first, cell fraction separation studies, in which cells have been disintegrated and separated into a number of fractions by ultra-centrifugation, and second, electron microscopy studies. Experiments of the former type have been reported by several authors. In Agrostis tenuis zinc applied to cell organelle suspensions accumulated mainly in the cell wall/nucleus fraction (Turner and Gregory, 1966). When this fraction was absent, most of the zinc was found to be associated with the mitochondrial and ribosomal fractions. In a subsequent paper Turner (1970) highlighted the importance of the cell wall fraction in zinc tolerance. Rathore et al. (1972) carried out a similar study on zinc uptake in Phaseolus vulgaris and found little zinc in the cell wall, but more was apparent in the nucleus and mitochondria. Bittell et al. (1974) measured the uptake of lead by isolated mitochondria, and found that maximum uptake appeared to be about 10 times as great as that observed with other heavy metals. The difference was thought to be partly due to precipitation of the lead

in the bathing solution, but no mention of such a precipitate is made in the results presented. Skaar et al. (1973) in studies on the distribution of lead in the moss Rhytidiadelphus squarrosus used cell fractionation techniques and observed accumulations of lead in nuclei and mitochondria.

Experiments of this type are of limited value, since although they show which organelles have the ability to accumulate lead, only in certain cases is an indication of differential accumulation observed. Moreover three criticisms may be levelled against such studies. Firstly, the fractions used are aggregates of particles which sediment out after exposure to a certain gravitational force for a certain period of time; while examination of the pellets may show the resultant fraction to be composed primarily of a certain cell organelle, the pellets obtained are invariably impure and contaminated by other cell debris. Secondly, the separation of organelles and hence their removal from their natural situation may promote changes which are reflected in the organelle's accumulation of lead. The procedures involved in fractionation may, therefore, also cause redistribution of the metal. Thirdly, in some cases observations are made on material exposed to lead after fractionation; as a result the effect of barriers to lead movement within the plant are not taken into account.

Electron microscopic studies of lead distribution are now popular. Sharpe and Denny (1976) examined lead uptake in the hydrophyte Potamogeton pectinatus, and found that lead accumulated predominantly in the cell walls, and that the plasmalemma appeared to form a barrier to the movement of lead into the cytoplasm. Despite this lead appeared to move into the cell in pinocytic vesicles and was observed in the cytoplasm and in chloroplasts. Lead has also been reported in the cell

wall, the tonoplast, the nuclear membrane, in plasmodesmata, chloroplasts and in vesicles of the moss Rhytidiadelphus squarrosus (Skaar et al., 1973; Gullvåg et al., 1974; Ophus and Gullvåg, 1974). Malone et al. (1974) examined the subcellular distribution of lead in corn and found that lead deposits were restricted to the cell wall and dictyosome vesicles. Silverberg (1975) reported that the exposure of Stigeoclonium tenue to lead resulted in deposits of the metal in and on the surface of the cell wall and in vacuoles. Other authors have reported changes in the structure of various cell organelles in response to lead (Serkerka and Bobăk, 1974; Rebechini and Hanzely, 1974; Herich and Bobăk, 1974; Simola, 1977). These include swelling of mitochondria, breakdown of membranes, and disruption of the grana in chloroplasts. It is not made clear in these reports whether lead was detected within these structures or not.

The electron microscope is a useful instrument for the study of lead distribution in tissues since the metal is electron-dense, and its detection requires no specialised staining procedures. In fact the electron-opacity of lead is such that it is widely used as a general stain for electron microscopy, and improves the definition of membranes, glycogen and nuclear material in T.E.M. preparations. In view of this it is questionable whether it is suitable to use lead compounds (or any other electron-dense material) as a post-staining agent for T.E.M. preparations in which the distribution of lead is to be investigated. In spite of this a number of experimenters have used lead citrate-stained specimens in their examinations of lead deposition in plant cells (e.g. Herich and Bobăk, 1974; Malone et al., 1974; Silverberg, 1975).

In the present study young plants were subjected to root-applied lead at 10 mEq/l for various periods of time and sections prepared

from a range of parts of the plant. Sections were examined following single fixation in glutaraldehyde, double fixation in glutaraldehyde and osmium tetroxide, and on a few occasions, double fixation followed by a short period of staining in lead citrate (see Chapter 2). Observations were made on the basis of comparison of these preparations with control material as defined in Chapter 2.

6.1 *Glutaraldehyde-Fixed Specimens*

In an exploratory study sections of glutaraldehyde-fixed root specimens were examined. Electron-dense particles were visible throughout the sections, but were most numerous in the cell walls. These particles were of two types, crystalline and amorphous. There appeared to be no distinction between the distributions of the two types, and in both cases the size of the particle was highly variable. In the epidermal region of the root cell wall deposits were far denser in the inner regions of the wall (Plate 6.1.1a) but a more uniform distribution of the electron-dense particles across the cell wall was observed in the central region of the root. In this region of younger tissue prior to intercellular space formation intercellular junctions often show a heavier deposition than the remainder of the wall, suggesting a higher affinity for lead (Plate 6.1.1b). Although single fixation with glutaraldehyde does little to maintain membrane integrity and does not improve the electron contrast of the tissue, it was obvious that there existed local accumulations of electron-dense particles within the cell itself (Plates 6.1.1c and d). These were particularly notable in the regions of the cell which were in the proximity of prospective intercellular spaces (Plate 6.1.1d).

6.2 *Glutaraldehyde/Osmium Tetroxide-Fixed Preparations of Germinating Seed Material*

The Radicle

Cells of the radicle were found to be fairly uniform in their constitution, being typified by the presence within the cytoplasm of many osmophilic spherical bodies which tended to obscure other structures in the cytoplasm.

In many cases these bodies were seen to be limited by a single unit membrane and as the radicle developed they became more electron transparent, but retained their spherical appearance. In older material these structures were far less numerous and other cell organelles could be observed. These spheroids were thought to be food storage structures providing the reserves required for the radicle to develop (see Plate 6.2.1).

As in the glutaraldehyde preparations, a major site of lead accumulation appeared to be in the cell walls. This took the form of a finely diffuse deposit throughout the walls with heavy deposits in restricted areas (Plate 6.2.1a and b).

In the vicinity of these heavy deposits the cell wall appeared to be particularly fragile, for many fractures were observed (Plate 6.2.1b). Within the cytoplasm local aggregations of electron-dense particles were visible, which appeared to be most prolific in the region of those food storage structures which had become electron-translucent (Plate 6.2.1a). There was also evidence of binding of the particles to the plasmalemma, and of the formation of lead-containing pinocytic vesicles (Plate 6.2.1b) but there was no indication of lead accumulation within the vacuoles, or in mitochondria (Plate 6.2.1b and c). This was substantiated in older tissue, and in addition heavy deposits of electron-dense particles were apparent in the nuclei, with very

heavy deposits apparently associated with the nucleoli (Plate 6.2.1d).

The Hypocotyl

Examination of hypocotyl material supported the view that there was little structural difference between the tissues of the radicle and of the hypocotyl. In fact sections from this region appeared identical to the radicle sections in subcellular detail, except that in hypocotyl tissue there was perhaps greater vacuolation and intercellular space formation. As in the radicle, there was extensive contamination of the material with electron-dense deposits, these being most prominent in the cell wall. Again in the wall two types of deposit were visible, a fine diffuse deposit and a more localised heavy deposit which appeared to increase the susceptibility of the cell wall to damage during sectioning. Local accumulations were present in the cytoplasm, and again appeared to centre mainly on the translucent spheroid bodies (vide supra) (Plate 6.2.2a). Electron-dense accumulations were also found to be prominent in association with the plasmalemma, and with the middle lamella and the outer regions of the cell wall in the vicinity of intercellular spaces (Plate 6.2.2b).

The Seed Cotyledon

Examination of cotyledon preparations revealed, once again, fairly uniform cells which were packed with osmiophilic, approximately spherical bodies. However, while there was evidence of vacuolation these spheroids tended to be more closely packed and more irregular in size and shape. Lead contamination of the cell wall was minor, consisting only of very fine isolated deposits. There was no indication of local concentrations in the wall, as had been in evidence in the radicle and hypocotyl (vide supra). There was, however,

a heavy electron-dense deposit in the plasmalemma. Sparse deposits were present in the cytoplasm, even less dense than those observed in the radicle and hypocotyl. It appeared, however, that once again these were associated with electron translucent spherical bodies. There was also evidence of accumulation at the border of developing vacuoles in the cytoplasm (Plate 6.2.2c). At the epidermis of the cotyledon there was little indication of lead deposition in the cell wall, but again deposits were visible at and inside the plasmalemma (Plate 6.2.2d).

6.3 *Glutaraldehyde/Osmium Tetroxide-Fixed Preparations of Seedling Material*

The Root Cap

Examination of sections of root cap material revealed that cells could be divided broadly into two types. In the central region of the cap the cells were normal, approximating to typical plant cell ultrastructure (Plate 6.3.1a). However, in the peripheral regions of the cap the cells were different and typified by a whorled arrangement of endoplasmic reticulum and the presence of many vacuoles. Apart from this little cellular detail was discernible (Plate 6.3.1b). Similar observations on the cytology of root cap cells have been made with respect to Zea mays by Clowes and Juniper (1968). In cells from both the central and the peripheral regions osmiophilic spherical bodies were again present in the cytoplasm. This may have been because the material observed was still in an early stage of development.

Within the tissue lead was restricted almost entirely to an even particulate distribution throughout the cell walls. No local accumulations were evident, and there was no indication of cell wall

damage. Apart from the wall deposits electron-dense particles were observed in the vacuoles of the central cells, and in the peripheral cells in association with electron-translucent spherical bodies similar to those observed in section 6.2. There was no indication of lead accumulation at other sites (Plates 6.3.1a and b).

The Root Tip

Sections through the root tip region revealed that there was no sign of tissue differentiation, the cells being of a uniform nature. Even short exposures to lead resulted in the formation of heavy electron-dense deposits. In the peripheral region of the root tip there was little indication of contamination, but the subcellular integrity of the cells was disrupted and in many cases the cells appeared to be virtually empty. In more central cells very heavy electron-dense deposits were observed such that the cell walls were almost completely electron-opaque, but again the cellular matrix was disrupted. Those areas of cytoplasm which were visible appeared to be in poor condition, cellular detail being indiscernible. Also heavy electron-dense deposits were often present throughout the cytoplasm, making determination of cellular organisation impossible. In the central portion of the root tip deposits were less dense and cellular structure could be observed (Plate 6.3.2a). In these cells there was again evidence of a fine particulate deposit in the cell wall with additional local heavy deposits, particularly in the region of intercellular junctions. Damage was again more frequent in heavily contaminated portions of the wall (Plate 6.3.2b).

In addition, electron-dense deposits were observed in association with the plasmalemma and the tonoplast (Plate 6.3.2b), with the nuclear membrane and portions of endoplasmic reticulum (Plate 6.3.2c). In

several cases mitochondria were observed but there was no indication of specific accumulation of electron-dense particles in association with them, but both amorphous and crystalline electron-dense bodies were observed relatively frequently in vacuoles (Figure 6.3.2d). In virtually all cells there were signs of increased vesicle formation, heavy electron-dense deposits being associated with the vesicles.

The Differentiated Root

The exposure of plants to 10 mEq/l lead for prolonged periods (7 days) caused signs of heavy lead contamination. In the epidermal region the cellular structure of the cells was totally disrupted and the resulting cell lumen contained numerous electron dense particles. However, the cell walls themselves were only lightly contaminated. Plants exposed for short periods (12 hours) possessed heavy, vascular deposits of electron-dense material, which tended to be associated with amorphous osmiophilic debris. Such deposits as were present in the cytoplasm were of a disseminated nature, and no clear associations were apparent with the possible exception of a light, but regular, contamination of the plasmalemma. Cell wall deposits were also very light (Plate 6.3.3a). Further into the cortex even short exposures to 10 mEq/l lead brought about relatively heavy contamination of the cell walls, but cytoplasmic contamination was minimal (Plate 6.3.3b). Continued exposure to lead, however, resulted in contamination of the cell organelles in a manner similar to that described previously, electron-dense deposits being visible in association with the plasmalemma, the tonoplast, the endoplasmic reticulum and with vesicles. This was soon followed by dissolution of cellular structure (Plate 6.3.3c). In the inner cortex deposits in the cell walls became much heavier, even after periods of short exposure, and heavy cytoplasmic contamination

accompanied by disorganisation of the subcellular structure was observed (Plate 6.3.3d). Throughout the cortex irrespective of supply concentration or period of exposure electron-dense deposits appeared to be more abundant on radial cell walls than on tangential ones and heavy wall deposits were associated with cell wall fractures. At the endodermis, deposits of electron-dense material were visible on the radial walls which stopped abruptly about halfway across the cell (Plate 6.3.4a). Short exposures to lead (4 hours) resulted in a small accumulation of electron-dense material in this region (Plate 6.3.4b). This accumulation became larger with more prolonged exposure (12 hours) (Plate 6.3.4c). Also with more prolonged exposure electron-dense material appeared in the cytoplasm, and became associated with the plasmalemma, the nuclear membrane and with a profusion of vesicles (Plate 6.3.4c). In the pericycle prolonged exposure brought about a similar heavy contamination and dissolution of structure, but the cell walls remained fairly clear (Plate 6.3.4d). Inside the stele the general distribution of the electron-dense particles was different to that observed outside the endodermis, in that the majority of the deposits were intracellular. Exceptions to this occurred in the phloem and xylem. In the former region examination of juvenile glutaraldehyde-fixed material indicated a distribution very similar to that observed in light microscope studies (see Chapter 5), deposits being confined largely to the cell walls (Plate 6.3.5a). This was supported by the examination of glutaraldehyde/osmium tetroxide-fixed specimens, although in these preparations the deposits were less dense. Within the cytoplasm contamination was minor, being confined to slight contamination of the outer membrane of mitochondria and to occasional small aggregations of vesicles (Plate 6.3.5b).

In the xylem slight deposits were visible in vessel lumina, these tending to be of a disseminated nature, but heavy superficial deposits were evident on the walls and in those structures undergoing secondary thickening. Apart from this there were only light deposits within the cell walls (Plate 6.3.5c). Elsewhere in the stele exposure to lead resulted in a proliferation of vesicles which were associated with electron-dense deposits and which appeared to fuse into the vacuole. Prolonged exposure brought about general dissolution of cell structure and many cells were reduced to near-empty lumina (Plate 6.3.5d).

The Stem

Distributions of electron-dense particles in the stem epidermis were similar to those observed in the root epidermal cells. There was clear evidence of slight lead deposits in the cell wall, although they were widely disseminated. Deposits were also relatively abundant in the vacuole and in association with the nuclear membrane. Once again associations of electron-dense material with electron-translucent vesicles were visible in the cytoplasm and also occasionally with mitochondria. Cellular structure was generally normal (Plate 6.3.6a). In the central cortical region deposits in the cell walls were heavier, but again contamination of the cytoplasm was slight, being restricted to only minor deposits on the plasmalemma and the tonoplast. Heavier accumulations were visible in the vacuole, these being of an aggregate but amorphous nature (Plate 6.3.6b). In the vascular region deposits in the cell walls were much denser, and there was evidence of local accumulations and of wall fracture. Contamination of the cytoplasm was also much heavier, tending to lead to structural disorganisation. In cells which retained their

structural integrity heavy deposits were visible in the cytoplasm, associated with the endoplasmic reticulum and with vesicles. Sporadic deposits also appeared to be associated with the amyloplasts, although it was difficult to distinguish between these and the normally-occurring osmiophilic granules (Plate 6.3.6c). In the xylem a superficial deposit was observed on the vessel walls, similar to that seen in the vessels of the root.

The Cotyledon Leaves

In general cell structure was retained in the cotyledon leaves of lead-exposed seedlings to a greater extent than had been observed in roots or stems. Many cells demonstrated no definite lead deposits and appeared to be structurally unaffected by the lead. In the epidermis slight electron-dense deposits were observed in the walls of a few cells, and in these cases contamination appeared to be slightly denser in the anticlinal walls. Amorphous deposits were also noted in the cell vacuoles (Plate 6.3.7a). Developing stomatal complexes were observed in the epidermis, but these showed no particular affinity for the deposits. In a more central region of the cotyledon leaf deposits tended to be greater, but again there was much variation from area to area. Some cells were virtually free from contamination and the deposits that were present were confined to the vesicles and vacuoles. In some cases vesicles were fairly profuse although apparent contamination was only slight (Plate 6.3.7b).

Progressive vacuolation resulted in cells which were virtually completely vacuolated, and contained in their vacuoles, electron-dense material in association with cell debris. The chloroplasts were found to be virtually the last organelles to break down in the vacuolating cells, and these showed disruption of thykaloids so that

grana tended to be poorly developed (Plate 6.3.7c) although there was little evidence of lead contamination of the chloroplast itself. Closer examination of these cells revealed that the residual cytoplasm was still separated from the vacuole by a tonoplast, associated with which were relatively heavy electron-dense deposits (Plate 6.3.7d).

6.4 *Discussion*

As the electron-dense deposits reported throughout the series of studies of lead-contaminated plants were not apparent in control tissue it seems reasonable to assume that these deposits represent lead or complexed forms of lead in the cell. It also seems likely that the two distinct forms of lead deposit observed (crystalline and amorphous) might be representative of free and complexed forms of lead in the lead treated material.

It was clear from comparison of glutaraldehyde-fixed and glutaraldehyde/osmium-fixed preparations that the nature of the techniques involved caused definite changes in the amount of lead observed in the tissue. This was, in fact, observed in preliminary investigations when it was found that pretreatment with hydrogen sulphide solution improved retention. Even with this pretreatment a slight brown discolouration of the dehydrating solutions occurred and electron-dense particles were often observed in the embedding medium some distance from the plant material. In an attempt to overcome this problem, rapid procedures for dehydration and embedding were employed (see Chapter 2 for further details).

The origin of these changes in the amount of lead in the tissue probably arises from two factors. Firstly, the influx and efflux of solutions would tend to wash lead out of the section. This may even occur with the relatively insoluble lead sulphide, which has

a solubility coefficient of 0.0124 g/100 ml in water at 20°C (C.R.C. Handbook, 1977). Secondly the divalent cation osmium is presumably capable of substituting for lead in accordance with the Law of Mass Action. The observation of lead loss was further substantiated by the examination of unfixed lead-treated material which had been subjected to sulphide precipitation and rapid dehydration and embedded. In this tissue lead deposits were present in greater amounts than were observed with the other techniques, but the lack of preservation resulted in preparations which were useless except for the most superficial structural inspection.

It must be acknowledged therefore that lead ions can be lost from, and possibly relocated in, the material during preparation for electron microscope examination. The observations reported in this study were made on material in which the losses had been minimised by the preparatory techniques employed but the possibility of lead loss or migration cannot be ruled out.

In spite of this it is possible to draw certain conclusions about the distribution of lead within the cells of lead treated plants. From the glutaraldehyde preparations it is apparent that much of the lead present is found in the cell walls, and that in most areas intracellular deposits are much lighter. This was also confirmed in the double-fixed preparations.

Examination of glutaraldehyde/osmium tetroxide-fixed specimens tended to substantiate the observations made in light microscope studies - that in the germinating seed lead distribution was general, and that in the seedling lead deposition varied from area to area, becoming less dense with distance from the root. In the root cap region, deposits were sparse, but in the root tip they were very heavy;

in the root proper heavy deposits occurred in the region of the endodermis, and lead was associated with the xylem and phloem; in the stem lead was associated primarily with the vascular tissue; and in the cotyledon leaves there was an association with vascular tissue, superimposed on which were local accumulations.

The electron microscope studies offer further evidence for the mechanisms which may give rise to these non-uniform distributions. In the root cap/root tip region major anomalies occur in lead contamination of two closely associated groups of cells, the root tip being highly contaminated while the root cap is relatively free of lead. A major difference does exist in the internal organisation of these cells, in that massive vesicle formation is typical of the peripheral cap cells, even in control tissue. These vesicles have been shown to originate in the golgi apparatus and to be involved in the formation of mucilages in and around the root cap (Northcote and Pickett-Heaps, 1966). It is likely that mucilage production may be a major factor in restricting lead contamination of the root cap and may, in situations where contamination is slight, also protect the root tip from contamination.

Electron microscope studies of the accumulation of lead observed at the endodermis (see above, also Chapter 5) revealed that the heavy deposits in the inner cortex were mainly in the cell walls. There appeared to be a definite barrier about halfway along the radial wall of the endodermis through which lead did not pass. As exposure to lead solutions progressed, deposits on the cortical side of this area increased. Inside the endodermis cell wall deposits were slight in comparison with the cortical region, but some cytoplasmic contamination occurred. In fact the pericycle cells appeared to suffer

most in this respect, and in these cells cytoplasmic contamination was relatively heavy. Such distributions lend further support to the functioning of the endodermis as a partial barrier to lead movement as discussed in Chapter 5.

Notable exceptions to the increase in intracellular contamination occurred in the xylem and phloem. In the xylem deposits were observed mainly as a superficial particulate coating on the inner surface of the vessel walls. There are two possible reasons for this; either lead in the vessel walls is in a highly mobile form, easily leached out by the embedding processes, or the vessel wall is impervious to lead. In view of the particulate nature of the deposits observed on the inner surface of the wall, and the sharp delineation between contaminated and non-contaminated areas, the latter alternative appears more acceptable.

In the phloem the situation is more difficult to interpret, since lead is highly localised, and in these few cells cytoplasmic contamination is slight. The restriction of lead to the cell walls is indicative of some specialisation of the cell wall in this region, and may be linked to the electrical polarisation of the cell wall-cytoplasm unit required by the electroosmotic mechanism of phloem transport discussed by Spanner (1975), or to the presence of plasmalemma microvilli in the cell wall proposed by Spanner and Jones (1970).

Generally the studies of the stem and cotyledon leaves provided less new information. It is interesting to note, however, that contamination is evident in the epidermal cells of the root and cotyledon leaves, as this was not observable in the light microscope studies. The source of this contamination is debatable, but it probably arises as a surface contamination (either by surface wetting or as a residue

from an earlier stage of growth) as deposits tend to be slight, if any, in underlying tissue. Proliferation of the local areas of cells in the cotyledon leaves in which ultrastructural breakdown has occurred presumably causes the necrotic lesions in the leaves noted in the light microscope studies and the growth experiments (see Chapters 3 and 5).

Observations at the subcellular level are more interesting. Although visual examination gives no indication of the chemical nature of organelles, it would seem likely that the osmiophilic spheroids observed in the germinating seeds are at least similar to the spherosomes discussed by Clowes and Juniper (1968). As such it is likely that they do have a storage function, and that they surrender their contents progressively as the embryo develops. Assuming this role for the organelles, it is equally feasible that the release of nutrients vacates a number of ionic sites within the limiting membrane, which would explain the affinity these empty structures appear to possess for lead. Associated with the formation of these electron-translucent lead-contaminated vesicles was an increase in vacuolation, and in lead contamination of the vacuole.

This suggests that the vesicles may fuse into the vacuole and surrender their lead deposits into the vacuolar sap, rendering them more innocuous in the cell system. The existence of such a system would be of importance in the ability of the young plant to withstand subcellular contamination by toxic elements in general. In this respect it is worthwhile to bear in mind the suggestion of Bowes (1965) that spherosomes and vacuoles are organelles of the same kind which have diverged only in their late development.

The extensive production of vesicles, similar to those described above, was also clearly a characteristic feature of lead-contaminated

cells in the seedling stage of development, these tending to take the form of groups of electron-translucent vesicles associated with which were notably heavy lead deposits. The origin of these vesicles is debatable. Gullvåg et al. (1974) and Silverberg (1975) report the association of lead with vesicles which are pinocytic in origin, while Malone et al. (1974) and Zegers et al. (1976) report lead accumulation in dictyosomal vesicles. The difficulties of distinguishing between these two types of vesicles have been acknowledged by Clowes and Juniper (1968). In the present study there was little evidence of pinocytosis in the lead contaminated cells other than in the embryonic stages; in fact it was if anything less obvious than in control tissue. Also the vesicles observed were closely packed and highly localised, suggesting that they were at or near their site of production and that this site lay within the cytoplasm. If the vesicles are dictyosomal in origin, their profusion and close-packed nature would require a very rapid vesicle synthesis. A third possibility exists - that of endoplasmic vesicle formation. Such vesicles have been shown to arise in the egg cells of ferns (Bell and Mühlethaler, 1962) and Clowes and Juniper (1968) suggest that lysosome vesicles may arise from the endoplasmic reticulum. This concept is further enhanced by the association of lead in the root tip with the nuclear membrane, which is thought to be continuous with the endoplasmic reticulum (Clowes and Juniper, 1968); and by the formation of membrane-bounded vesicles from the nuclear membrane (Lafontaine and Chouinard, 1963). In addition it has been suggested that the spherosomes discussed earlier with respect to lead accumulation (vide supra) may arise from the endoplasmic reticulum (Clowes and Juniper, 1968). In view of this, at least some of the vesicles may

arise from the endoplasmic reticulum. Whatever the origin of the structures, they do accumulate lead. From observation of tissues exposed to heavier concentrations of lead and for greater periods of time it appears that the vesicles coalesce, rather in the fashion of collapsing soap bubbles, to form vacuole-like structures. The present evidence suggests that these structures may also fuse into the cell vacuole. Prolonged exposure results in a continuation of this process until the cell structure disappears completely.

The other important site of lead deposition, which appeared to be consistent throughout the study, was the cell wall and in many cells this appeared to be the major site of accumulation. Under heavy exposure two types of deposit were observed, a light, diffuse deposit and a heavy deposit which tended, in the cortex, to be localised to some extent in the radial walls. Associated with these heavy deposits was a tendency for the cell wall to fracture during sectioning. Such damage may arise as a result of impaired infusion of the embedding medium due to the presence of crystals (Reid, 1974), but since the damage to the cell wall was not always associated with scratches or tears in the section (which would be expected in the case of poor resin permeation) it seems likely that the damage arose prior to block polymerisation, and may have arisen from lead-induced structural changes in the cell wall.

The association of lead with other cell organelles appeared to be more variable. As stated previously, in root tips lead was seen to be in association with the nuclear membrane, and in a number of specimens lead accumulations at or in the plasmalemma were observed. An accumulation of lead at the nuclear membrane has been reported (Skaar _et al._, 1973) and there are reports of lead-induced damage to

the membrane (Gullvåg et al., 1974; Skaar et al., 1973). The paucity of these observations may be connected with the observation of Hammett and Justice (1928) that in Allium cepa and Zea mays cells in a mitotic region have an affinity for lead greater than that observed in non-mitotic regions.

In the embryonic stages of development there was also evidence of lead contamination of what was thought to be the nucleolus. Intranuclear lead deposition has been reported by Skaar et al. (1973) and by Gullvåg et al. (1974), and similar inclusions have been observed in the nuclei of lead treated rat kidney cells (Choie and Richter, 1972). The nucleolus is rich in ribonucleic acid (RNA) and if these structures are of a nucleolar nature their association with lead contamination may be linked to the observation that lead partially inactivates transfer RNA, and may depolymerise it (Farkas, 1968; Farkas et al., 1972). The lack of such deposits in older tissue suggests that the development of the cell organelle system in some way precludes lead to a certain extent from entering the nucleus.

Accumulation of lead at the plasmalemma has been reported by Sharpe and Denny (1976) who suggest that the plasmalemma may form a partial barrier to lead movement into the cytoplasm. Lead deposition at the tonoplast appeared to be more prevalent when lead deposits were observed in the vacuole and may therefore be of a secondary nature, occurring from within the vacuole when the vacuole is subject to a heavy lead burden. Vacuolar deposits of lead have been reported by Silverberg (1975) and the importance of the vacuole in heavy metal resistance, particularly in stem and leaf tissue, was discussed by Ernst (1974 and 1975).

Reports are available which describe lead deposition in association with chloroplasts and mitochondria (Skaar et al., 1973; Ophus and Gullvåg, 1974), and the uptake of lead by isolated mitochondria (Walton, 1973; Bittell et al., 1974). Other authors have failed to detect such accumulations (Malone et al., 1974; Silverberg, 1975). In the present study no consistent accumulation in these organelles was observed, and the occasional deposits which were observed may have been of a secondary nature, resulting from redistribution of lead during the dehydration and embedding procedures. However, it did appear that in the lead-treated plants granal stacks were poorly developed, although the chloroplasts themselves appeared to be the last organelles to break down in the vacuolating cells, retaining their structure when other organelles had disappeared. Similar alterations to chloroplast structure induced by exposure to lead have been reported by Rebechini and Hanzely (1974) and by Simola (1977). Such changes are perhaps surprising in view of the report by Murakami and Parker (1971) that divalent cations are more effective than monovalent ones at cementing together chloroplast thykaloids into grana. In view of this, and of the lack of specific lead accumulation in the chloroplasts it seems likely that the apparent changes in chloroplast structure arise through metabolic changes at a site removed from the chloroplast rather than through interactions of lead with the structure of the chloroplast itself.

Plate 6.1.1 Electron micrographs of lead distribution in glutaraldehyde-fixed radish root cells

A. Transverse section of glutaraldehyde-fixed root epidermal cells after 5 days exposure to 10 mEq/l lead nitrate solution (x6300). Note crystalline deposits in cell wall.

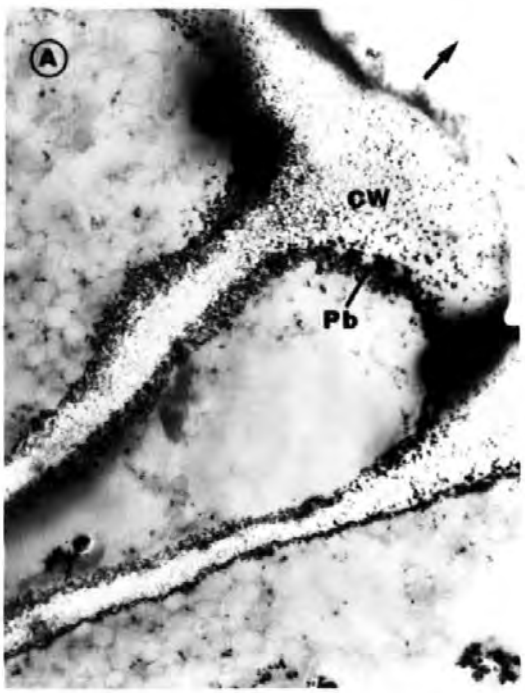
B. Transverse section of glutaraldehyde-fixed root cortical cell junction after 5 days exposure to 10 mEq/l lead nitrate solution (x24000). Note heavier deposits at junction and leading into middle lamella.

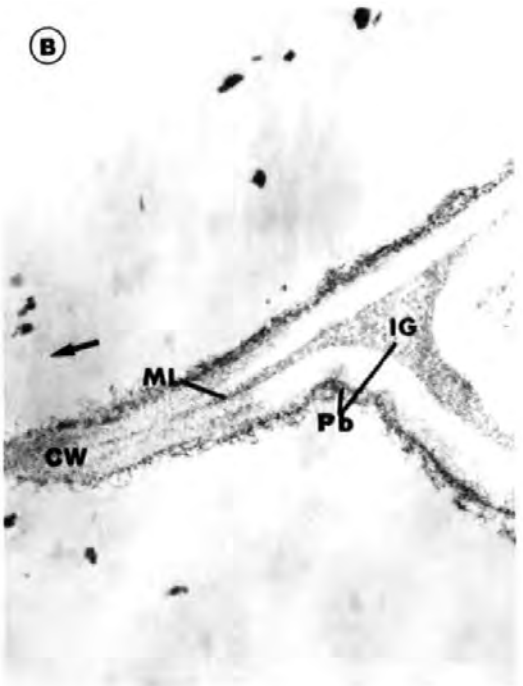
C. Transverse section of glutaraldehyde-fixed root cortical cell after 5 days exposure to 10 mEq/l lead nitrate solution (x13000). Note intracellular deposits.

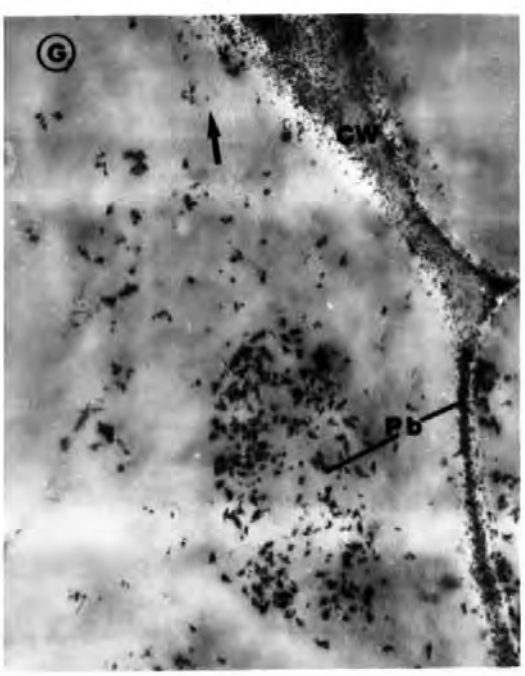
D. Transverse section of glutaraldehyde-fixed root cortical cell after 5 days exposure to 10 mEq/l lead nitrate solution (x13000). Note heavy deposits in cell in region of intercellular space.

CW - cell wall; Pb - lead deposits; IC - prospective intercellular space; ML - middle lamella.

Arrow indicates radial orientation


A
CW
Pb


B
IG
ML
Pb
CW


G
CW
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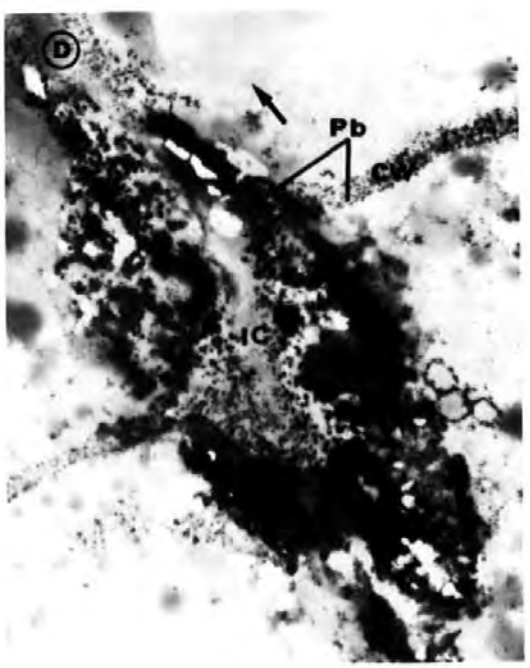

D
Pb
IC

Plate 6.2.1 Electron micrographs of lead distribution in radish radicle cells

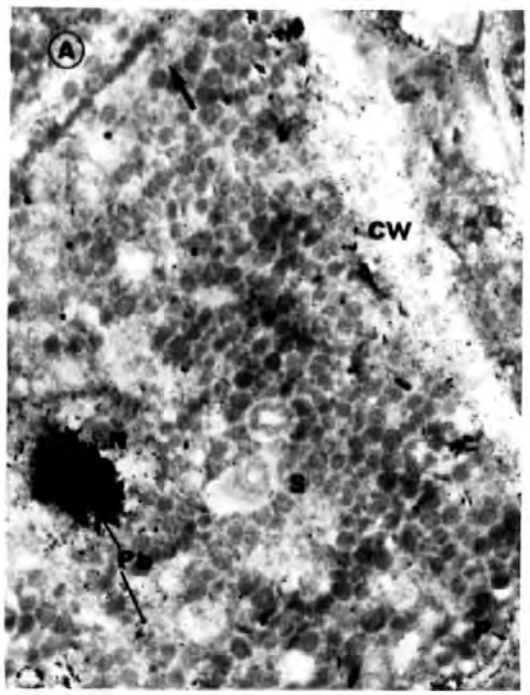
A. Transverse section of double-fixed cell of 12 hour old radicle germinated in 10 mEq/l lead nitrate solution (x7700). Note cell wall deposits, nuclear deposits, and local deposits amongst osmiophilic vesicles.

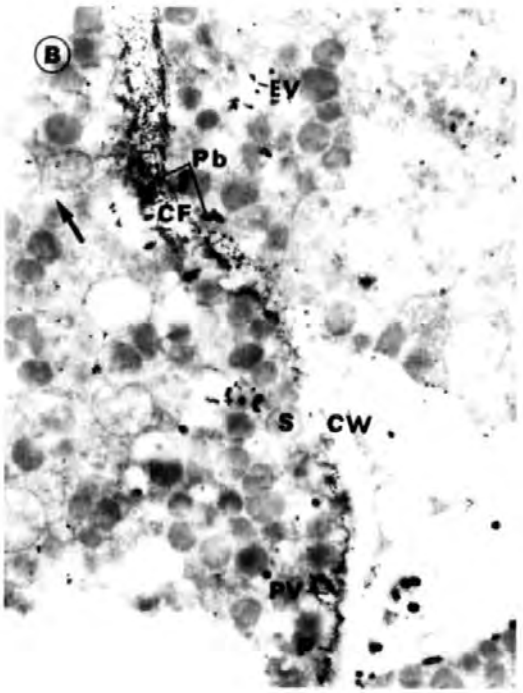
B. Transverse section of double-fixed cell of 12 hour old radicle germinated in 10 mEq/l lead nitrate solution (x13000). Note local deposits associated with cell wall fracture, pinocytic vesicles and plasmalemma.

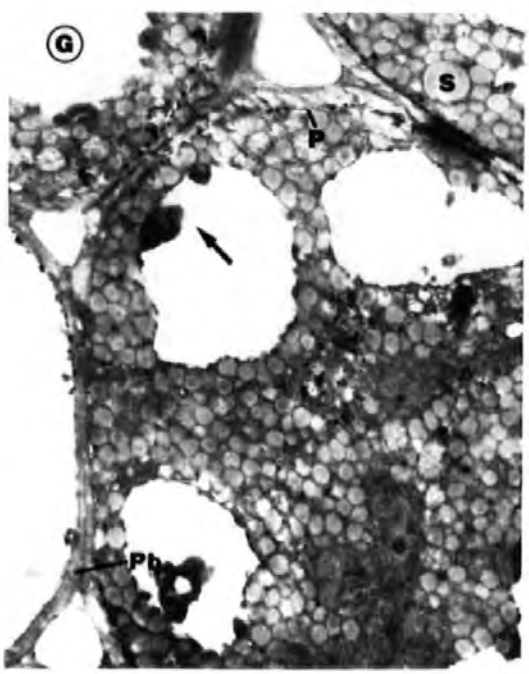
C. Transverse section of double-fixed cell of 12 hour old radicle germinated in 10 mEq/l lead nitrate solution (x7700). Note deposits in plasmalemma and in electron-translucent vesicles.

D. Transverse section of double-fixed cell of 12 hour old radicle germinated in 10 mEq/l lead nitrate solution (x17000). Note deposits in nucleus and heavy deposits in nucleolar region.

CW - cell wall; N - nucleus; P - plasmalemma; CF - cell wall fracture; V - vacuole; EV - electron-translucent vesicle; S - spherosome; Pb - lead deposits; PV - pinocytic vesicle. Arrow indicates radial orientation


A
CW
S
Pb


B
EV
Pb
CF
S
CW
PV


G
S
P
Pb

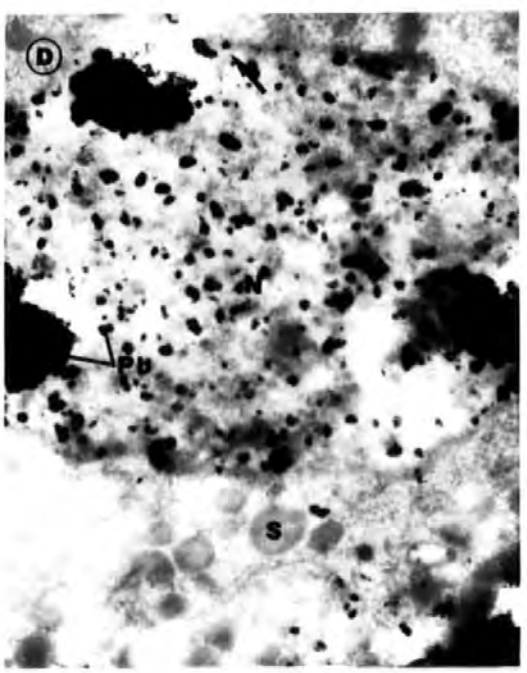

D
Pb
S

Plate 6.2.2 Electron micrographs of lead distribution in radish hypocotyl and seed cotyledon cells

A. Transverse section of double-fixed cell of 12 hour old hypocotyl germinated in 10 mEq/l lead nitrate solution (x7700). Note heavy deposits and fracture in cell wall, and local deposits in cytoplasm.

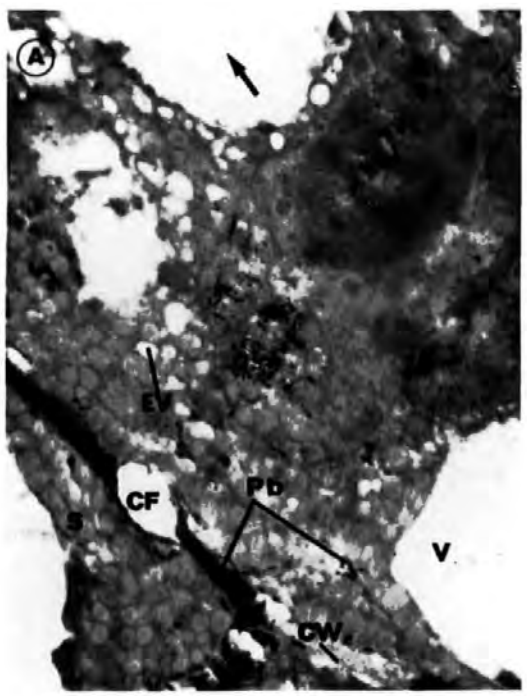
B. Transverse section of double-fixed cell of 12 hour old hypocotyl germinated in 10 mEq/l lead nitrate solution (x17000). Note deposits in middle lamella, outside of cell wall and at plasmalemma. Deposits are also visible associated with electron-translucent vesicles.

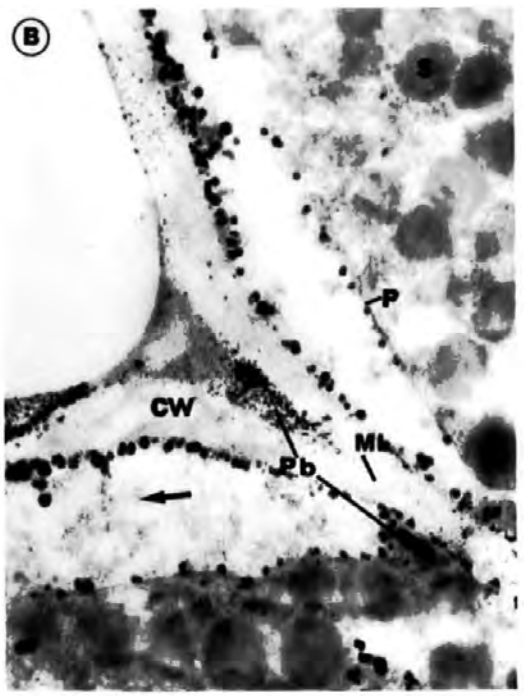
C. Transverse section of double-fixed cell of 12 hour old cotyledon germinated in 10 mEq/l lead nitrate solution (x5000). Note deposits in middle lamella, plasmalemma, vacuoles, and around translucent vesicles.

D. Transverse section of double-fixed abaxial epidermal cell of 12 hour old cotyledon germinated in 10 mEq/l lead nitrate solution (x10400). Note deposits around plasmalemma.

CW - cell wall; CF - cell wall fracture; ML - middle lamella; P - plasmalemma; V - vacuole; EV - electron-translucent vesicle; S - spherosome; Pb - lead deposits.

Arrow indicates radial orientation in A and B, abaxial orientation in C and D.


A
EV
CF
Pb
S
V
CW


B
S
P
CW
Mb
Pb


S
V

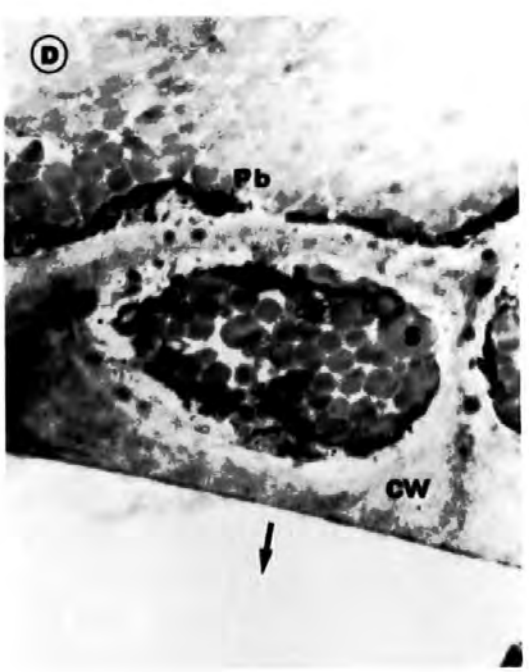

D
Pb
CW

Plate 6.3.1 Electron micrographs of lead distribution in radish root cap cells

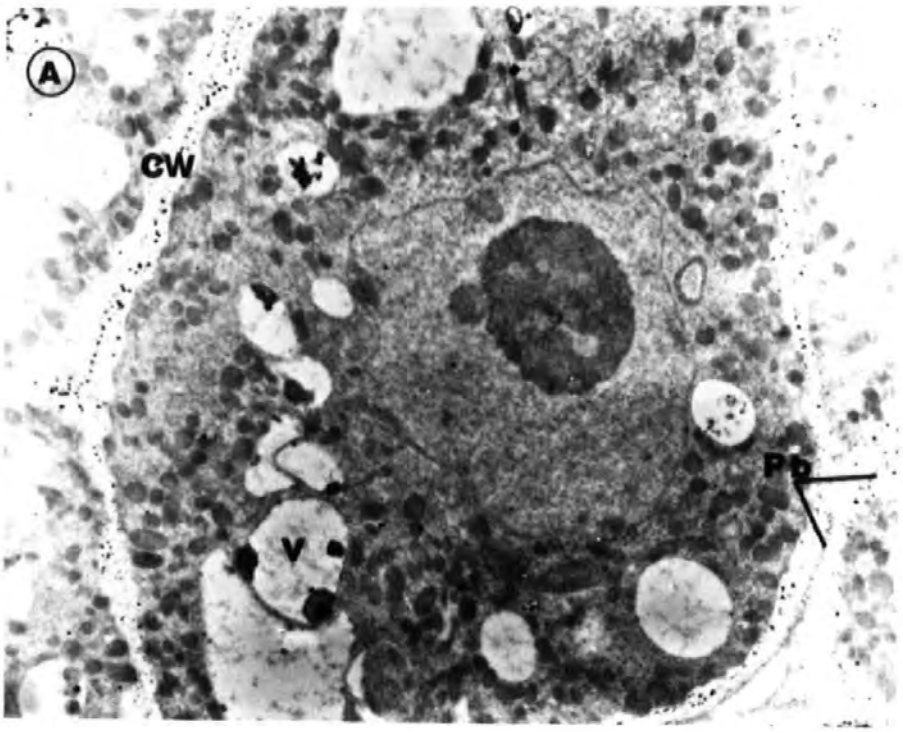
A. Transverse section of 7 day old double-fixed central root cap cell after 24 hours exposure to 10 mEq/l lead nitrate solution (x9000). Note deposits in cell wall and vacuoles.

B. Transverse section of 7 day old double-fixed peripheral root cap cell after 24 hours exposure to 10 mEq/l lead nitrate solution (x9000). Note deposits in cell walls and around vesicles.

CW - cell wall; EV - electron-translucent vesicle;

V - vacuole; Pb - lead deposits.

Arrow indicates radial orientation


A
CW
V
Pb

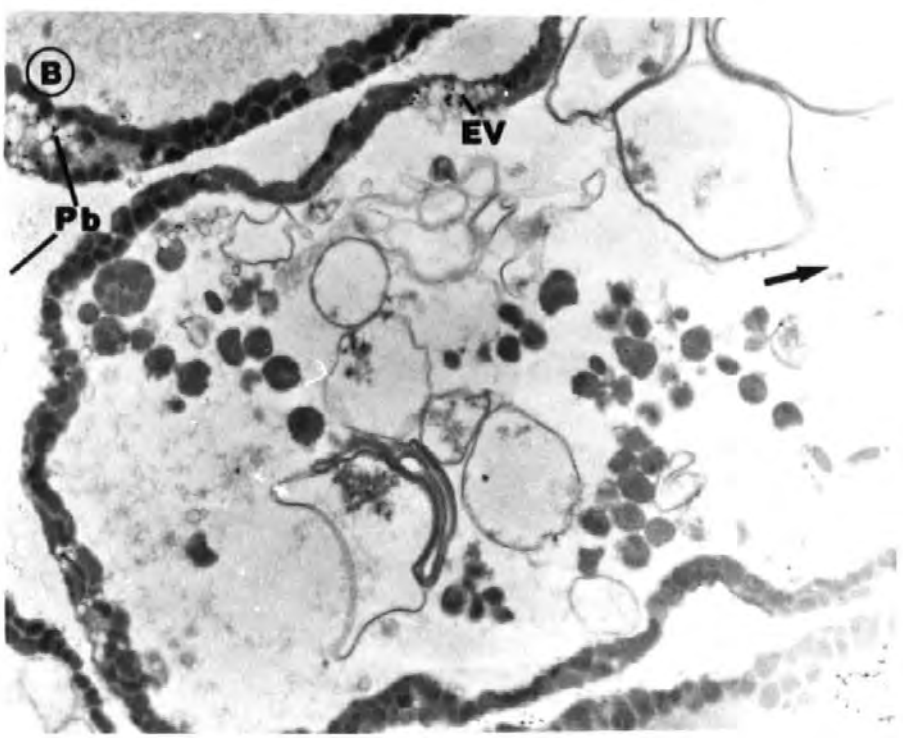

B
EV
Pb

Plate 6.3.2 Electron micrographs of lead distribution in radish root tip cells

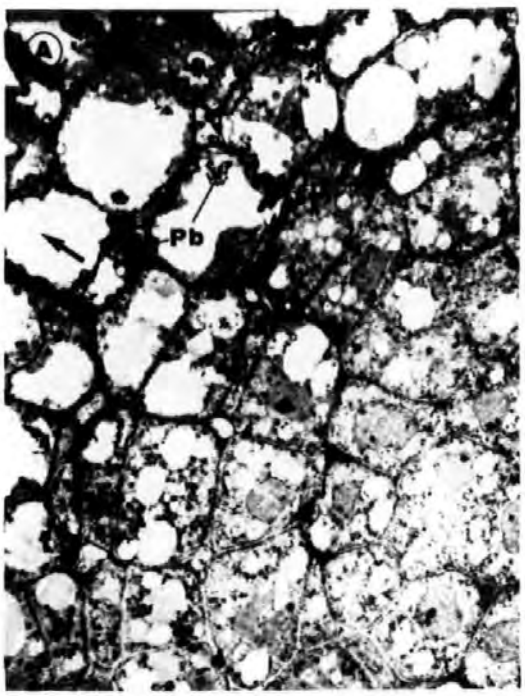
A. Transverse section of 7 day old double-fixed root tip after 24 hours exposure to 10 mEq/l lead nitrate solution (stained) (x1300). Note heavy wall deposits and internal breakdown in outer cells, lighter deposits in central region.

B. Transverse section of double-fixed cell from central region of 7 day old root tip after 24 hours exposure to 10 mEq/l lead nitrate solution (x5000). Note heavy deposits and fracture at intercellular space and deposits at plasmalemma, nucleus and vesicles.

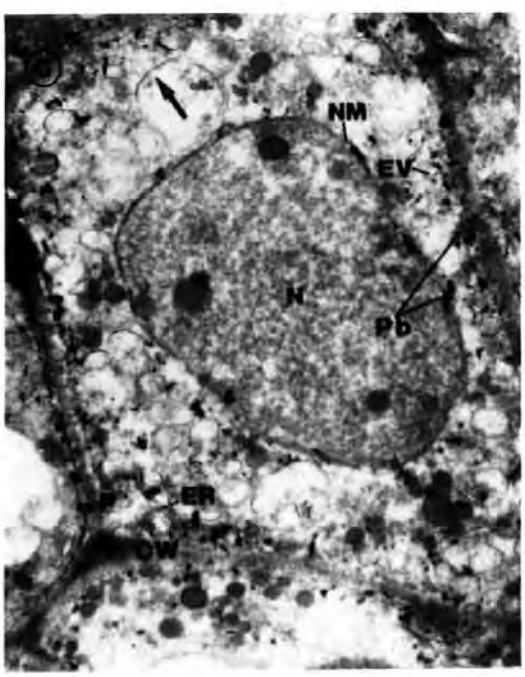
C. Transverse section of double-fixed cell from 7 day old root tip after 24 hours exposure to 10 mEq/l lead nitrate solution (stained) (x4000). Note deposits in cell wall, plasmalemma, vesicles, nuclear membrane and endoplasmic reticulum.

D. Transverse section of double-fixed cell from 7 day old root tip after 24 hours exposure to 10 mEq/l lead nitrate solution (stained) (x7700). As above, but note also vacuolar deposits.

CW - cell wall; N - nucleus; NM - nuclear membrane;
V - vacuole; EV - vesicle; ER - endoplasmic reticulum;
P - plasmalemma; Pb - lead deposits.
Arrow indicates radial orientation


A
Pb


B
CW
N
P
Pb
EV
V


NM
EV
N
Pb
ER
CW

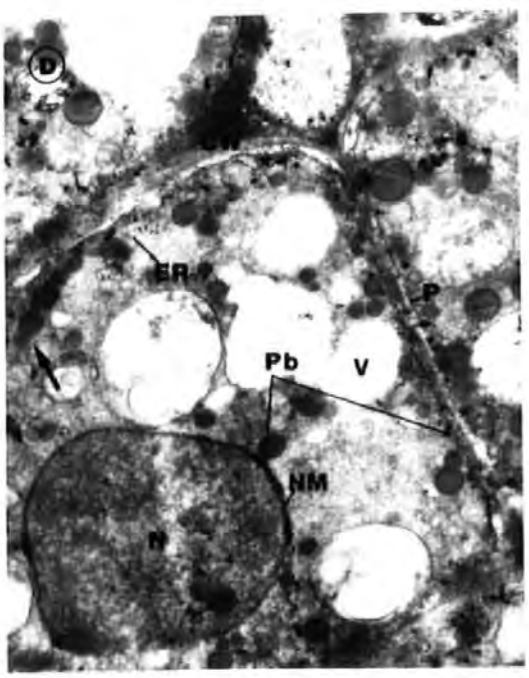

D
ER
P
Pb
V
NM
N

Plate 6.3.3 Electron micrographs of lead distribution in radish root cortical cells

A. Transverse section of 7 day old double fixed root epidermis after exposure to 10 mEq/l lead nitrate solution for 12 hours (x7700). Note light cell wall deposits and heavy deposits in vacuole associated with osmiophilic material.

B. Transverse section of 7 day old double-fixed root central cortex after 24 hours exposure to 10 mEq/l lead nitrate solution (x5000). Note relatively heavy wall deposits and fracture.

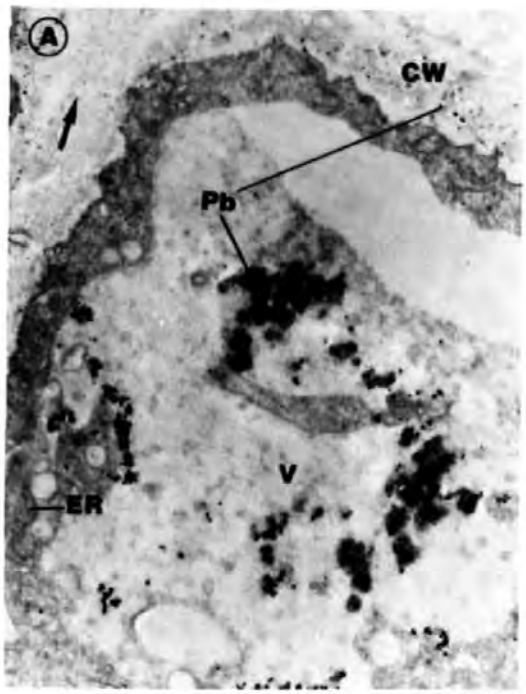
C. Transverse section of 7 day old double-fixed root central cortex after 3 days exposure to 10 mEq/l lead nitrate solution (x10400). Note heavy cell wall deposits and loss of cellular detail.

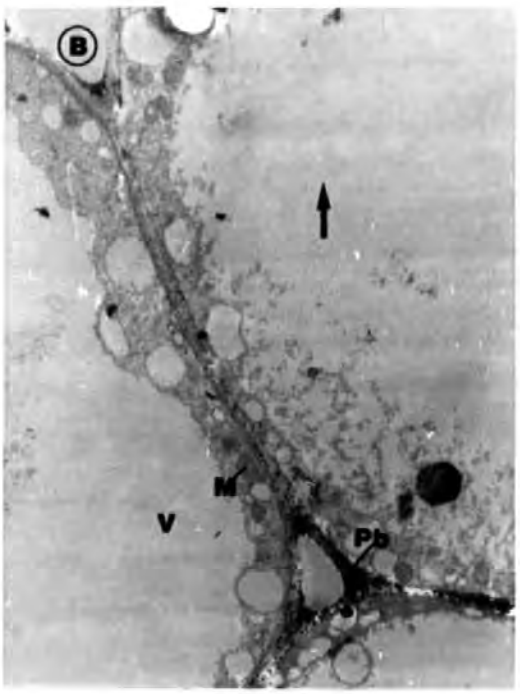
D. Transverse section of 7 day old double-fixed inner cortex after 24 hours exposure to 10 mEq/l lead nitrate solution (x3250). Note heavier wall deposits on inner walls and lack of cellular detail.

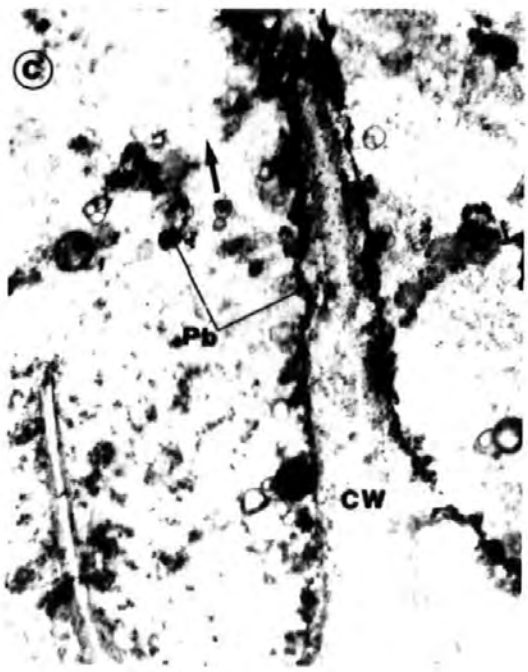
CW - cell wall; V - vacuole; Pb - lead deposits;

M - mitochondrion; ER - endoplasmic reticulum.

Arrow indicates radial orientation


A
CW
Pb
V
ER


B
M
V
Pb


C
Pb
CW

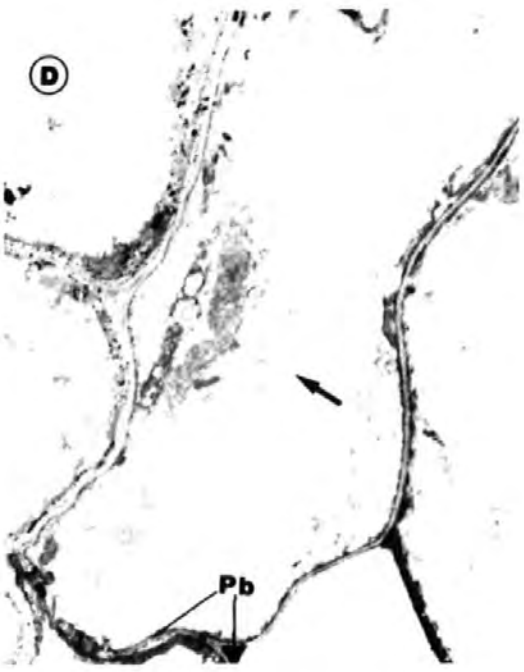

D
Pb

Plate 6.3.4 Electron micrographs of lead distribution in radish root endodermis and pericycle cells

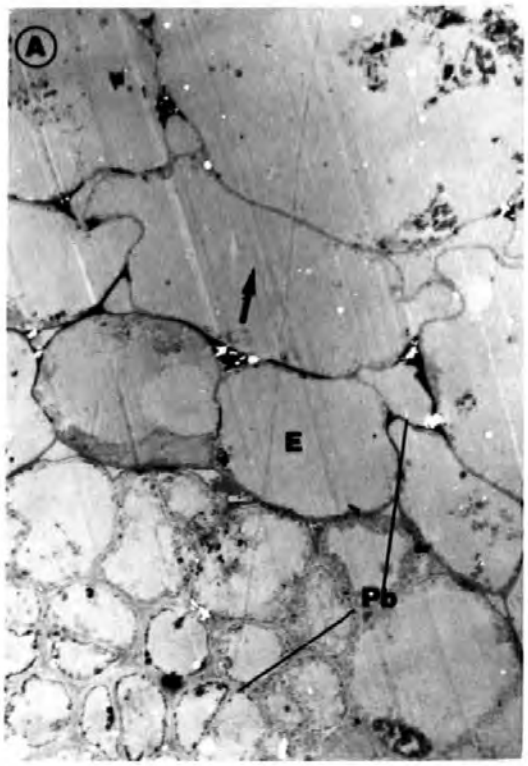
A. Transverse section of 7 day old glutaraldehyde-fixed root endodermis after 24 hours exposure to 10 mEq/l lead nitrate solution (x2670). Note lead deposits in radial endodermal walls which cease abruptly about halfway along wall. Also paucity of wall deposits inside endodermis.

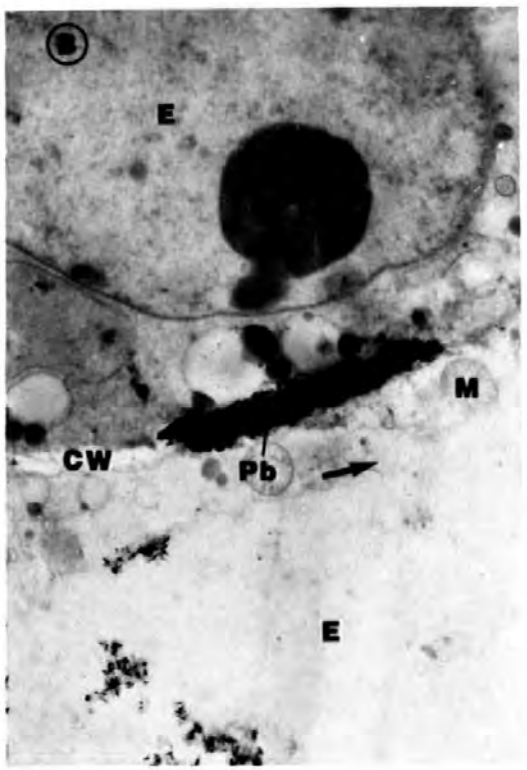
B. Transverse section of 7 day old double-fixed root endodermis after 4 hours exposure to 10 mEq/l lead nitrate solution (x10400). Note highly localised deposit in radial endodermal wall and vacuolar deposits.

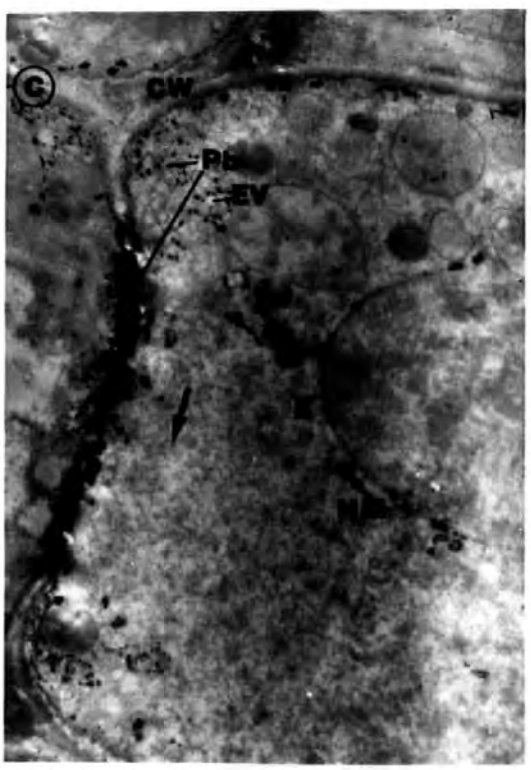
C. Transverse section of 7 day old double-fixed root endodermis after 12 hours exposure to 10 mEq/l lead nitrate solution (x7700). Note deposit in radial endodermal wall has become larger and deposits in the cytoplasm associated with vesicles and the nuclear membrane.

D. Transverse section of 7 day old double-fixed root pericycle after 3 days exposure to 10 mEq/l lead nitrate solution (x7700). Note lack of cell wall deposit, but heavy contamination of the plasmalemma and tonoplast and the association of deposits with the prolific vesicles. Vesicles also appear to be fusing into vacuole in right hand cell.

E - endodermis; CW - cell wall; V - vacuole; EV - vesicle; NM - nuclear membrane; Pb - lead deposits; M - mitochondrion. Arrow indicates radial orientation


A
E
Pb


B
E
M
CW
Pb
E


C
CW
Pb
EV

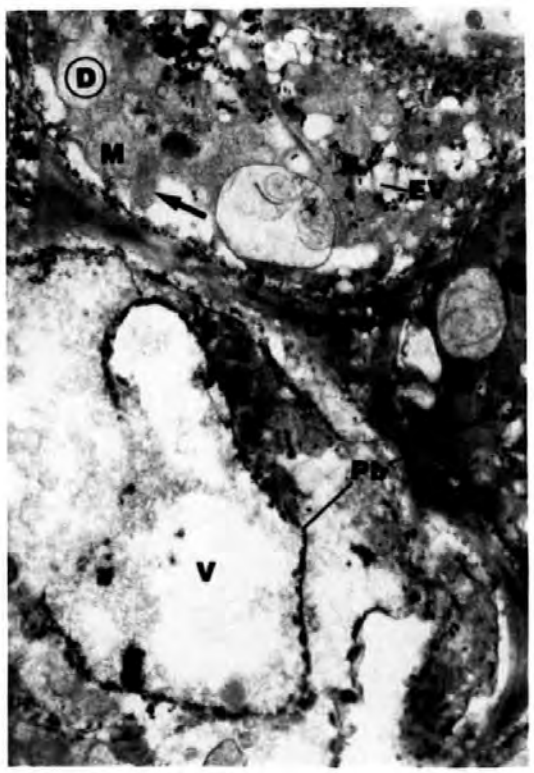

D
M
EV
Pb
V

Plate 6.3.5 Electron micrographs of lead distribution in radish root stelar tissues

A. Transverse section of 7 day old glutaraldehyde-fixed phloem group after 3 days exposure to 10 mEq/l lead nitrate solution (x2200). Note deposits are confined largely to cell walls.

B. Transverse section of 7 day old double-fixed phloem cells after 3 days exposure to 10 mEq/l lead nitrate solution (x5000). Note that the major deposits of lead are associated with the cell walls, although there are slight electron-dense deposits in the vacuole.

C. Transverse section of 7 day old glutaraldehyde-fixed xylem vessel after 3 days exposure to 10 mEq/l lead nitrate solution (x5000). Note that deposits lie principally on the surface of the secondary thickening. Slight deposits are also visible in the primary wall and in the lumen of the element.

D. Transverse section of 7 day old double-fixed stelar parenchyma cell after 3 days exposure to 10 mEq/l lead nitrate solution (x4000). Note that walls are free of deposits, but deposits are present inside the wall and cell organisation is disrupted. In the cell approximately in the centre of the field of view extensive vesicle formation has occurred and lead-carrying vesicles appear to be fusing into the vacuole.

CW - cell wall; ST - secondary thickening; V - vacuole; EV - vesicle; Pb - lead deposits.

Arrow indicates radial orientation

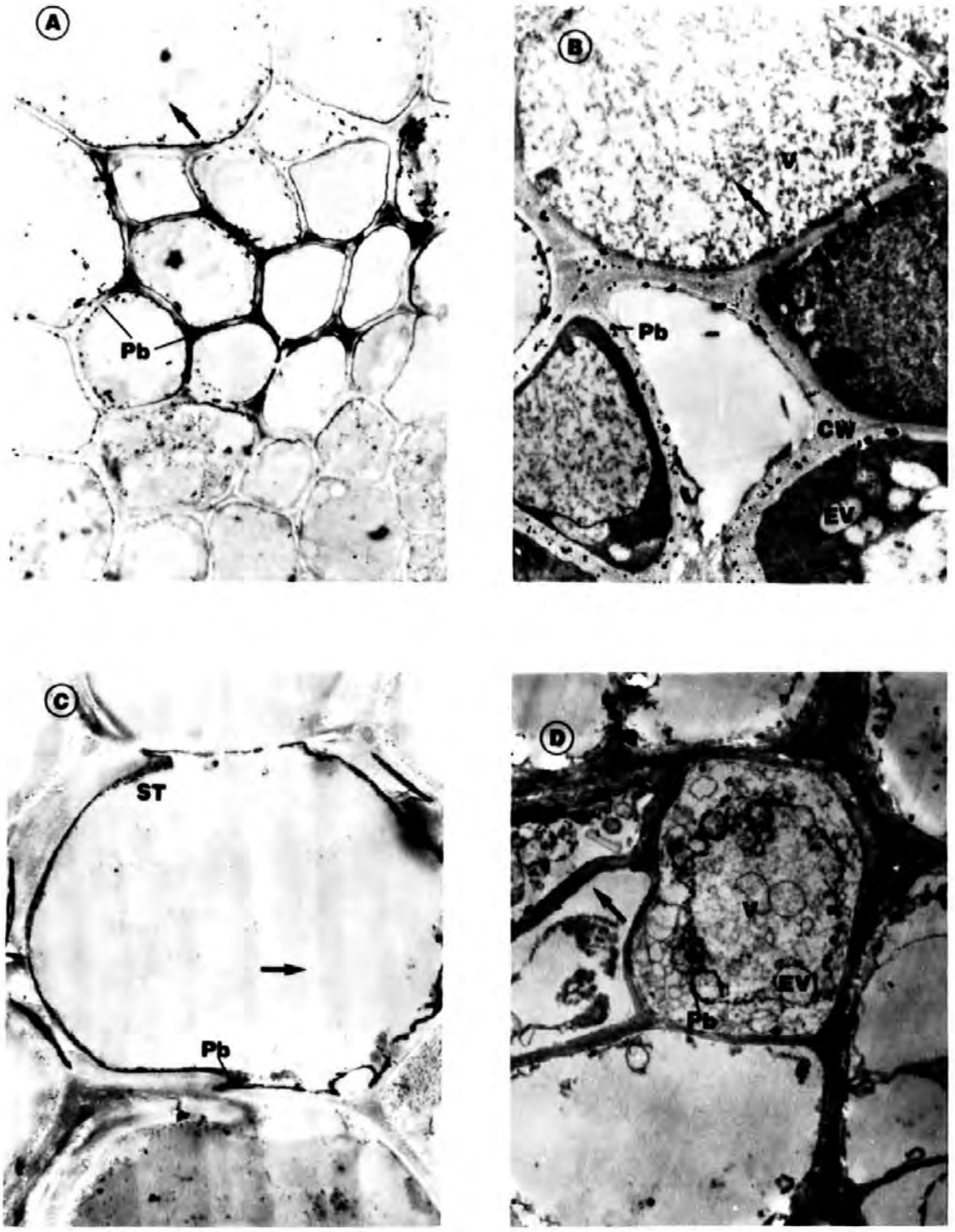

A
Pb
B
V
Pb
CW
EV
C
ST
Pb
D
V
EV
Pb

Plate 6.3.6 Electron micrographs of lead distribution in radish stem tissues

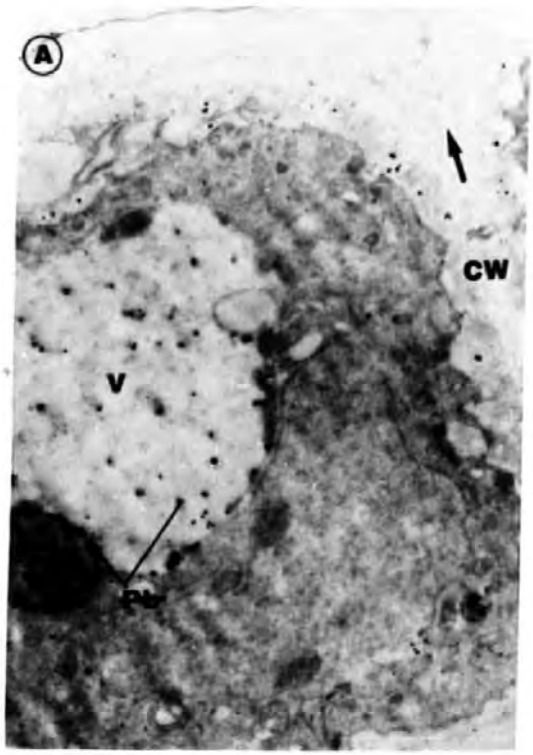
A. Transverse section of 7 day old stem epidermis of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x7700). Note light deposits in cell wall, and deposits in vacuole and at nuclear membrane.

B. Transverse section of 7 day old stem inner cortex of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x5000). Note that cell wall and vacuolar deposits are heavier and that vacuolar deposits are of a more amorphous nature. In addition there is slight contamination of the tonoplast.

C. Transverse section of 7 day old stem in region of vascular bundle of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x5000). Note heavy wall and intracellular deposits, and dissolution of cell structure.

CW - cell wall; V - vacuole; N - nucleus; M - mitochondrion; A - amyloplast; EV - vesicle; Pb - lead deposits.

Arrow indicates radial orientation


A
CW
V
Pb


B
CW
M
EV
Pb
V

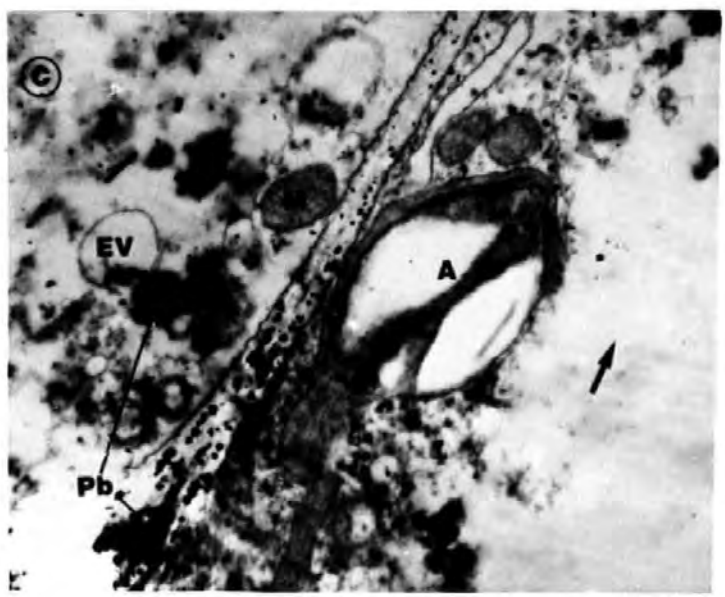

C
EV
A
Pb

Plate 6.3.7 Electron micrographs of lead distribution in radish cotyledon leaf tissues

A. Transverse section of 7 day old cotyledon through upper epidermis of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x5000). Note deposits in anticlinal cell walls and very slight deposits associated with osmiophilic material in the vacuole.

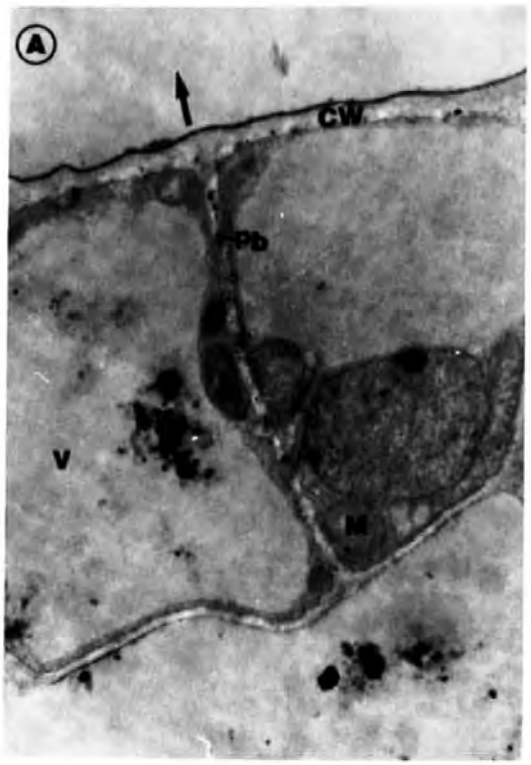
B. Transverse section of 7 day old cotyledon through region of high contamination in 'mesophyll' of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x10000). Note that deposits are slight, and are restricted to the vacuole, plasmalemma and vesicles.

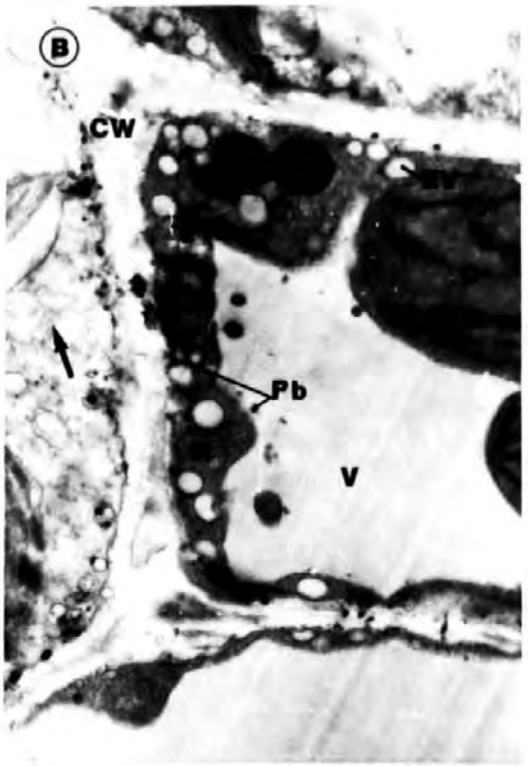
C. Transverse section of 7 day old cotyledon through region of high contamination in 'mesophyll' of plant exposed to root-applied 10 mEq/l lead nitrate solution for 3 days (x5000). Note deposits in plasmalemma and in association with vesicles, and in association with debris in vacuole.

D. Transverse section as above, showing deposits in the cell wall, tonoplast and plasmalemma (x10000).

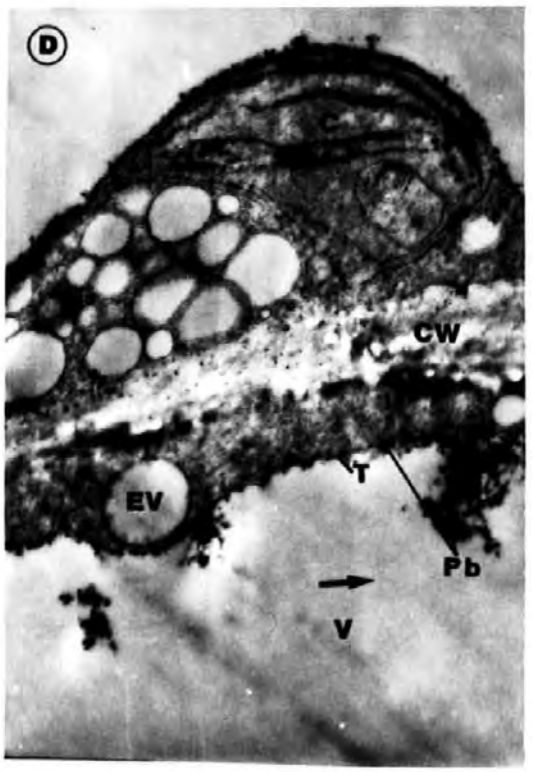
CW - cell wall; V - vacuole; EV - vesicle; C - chloroplast; M - mitochondrion; T - tonoplast; Pb - lead deposits.

Arrow indicates adaxial orientation


A
CW
Pb
V
M


B
CW
Pb
V


C
Pb
CW
V
EV


D
CW
T
EV
Pb
V

CHAPTER 7

ELECTRON MICROPROBE EXAMINATION OF LEAD-CONTAMINATED TISSUE

In the course of the electron microscope studies on lead distribution in radish cells reported in Chapter 6, electron-dense deposits were observed in lead-treated specimens which were absent in controls. On the basis of their absence in controls and the electron-scattering properties of lead, it was suspected that these electron-dense deposits represented lead deposits, in either an inorganic or organic form. In order to verify this suspicion, specimens were subjected to electron microprobe X-ray analysis. At present this facility is lacking in Plymouth, and so analyses were carried out in the Zoology laboratories at University College, Cardiff and at JEOL (U.K.) Ltd., London. Because of this the studies were of necessity of a fairly curtailed nature.

The electron microprobe is a relatively new tool in physiological studies. Previously Libianti and Tandler (1969) examined the distribution of phosphate ions in maize root tips with an electron microprobe. Heichel and Hankin (1972) have used the electron microprobe system to examine the elemental composition of lead-bearing particles on or in tree bark. More recently a similar system was used by Sangster (1976) for the analysis of silicon deposits in the roots of sorghum and by Stevens and Martin (1977) for the analysis of potassium in the guard cells of *Tradescantia pallidus*. More specifically two authors have reported the use of the microprobe system in the identification of deposits in studies on the localisation of lead in plant tissue - Ophus and Gullvåg (1974) working with *Rhytidiadelphus squarrosus*, and Silverberg (1975) working with

Stigeoclonium tenue.

In the present studies analyses were restricted principally to root tissue and were carried out in four phases. Initial studies were carried out using a Phillips EM300 microscope with an EDAX 505 X-ray analyser in the Zoology Department of University College, Cardiff. These consisted of small particle spot analyses, and analysis of bulk specimens for several cations simultaneously (see Chapter 2 for further details of the systems and techniques employed). Subsequently analysis on small areas was carried out at JEOL (U.K.) Ltd., London, using a JEOL 100CX analytical TEMSCAN in the Scanning Transmission (STEM) mode. The analytical principles of the above unit are the same as those of the Phillips unit, but the use of the STEM mode allows moving point analyses to be carried out for elemental mapping and also the use of thicker specimens. Studies were also carried out using the latter instrument at low magnification to investigate further the distribution of lead across the root. A problem exists in the microanalysis of lead in biological specimens in that the strongest, and hence the most useful, peaks of the lead spectrum are close to the major peaks for osmium and hence analysis for lead may be rendered inaccurate by the presence of osmium (T. Davies, personal communication). Therefore analyses were carried out on glutaraldehyde-fixed tissues.

7.1 *Small Spot Analysis*

Static small spot analysis was carried out on thin (gold) sections of 7-day old root material which had been exposed to 10 mEq/l lead nitrate solution for 3 days. These included a number of sites in the cortex, analyses being carried out on areas of the cell wall

contaminated by heavy electron-dense deposits, and on adjacent areas which appeared to be relatively deposit-free. It was clear from the resulting spectra that the heavy electron-dense deposits were rich in lead, and that this was by far the principal element present. However, even where deposits were visibly slight, measurable amounts of lead were detected (Plate 7.1.1). Analyses were also carried out in the stelar region, on the endodermis, the xylem and the phloem. In each case it was found that the electron-dense deposits observed coincided with high lead counts in the EDAX spectra. The highest peaks were recorded in the phloem region in juvenile root tissue (Plate 7.1.2).

In addition a series of spot analyses were carried out around the central region of the root to give an indication of the distribution of lead in relation to the endodermis. The lead counts were corrected for specimen thickness and density using the Bremsstrahlungen radiation counts (see Chapter 2) and are shown in Figure 7.1.3. While these results provided an indication of the distribution present, they were found to be very variable, presumably because of the small area under analysis on each occasion and the localised nature of the lead deposits.

7.2 *Small Area Analysis*

The small area analyses carried out at JEOL (U.K.) Ltd. were again on glutaraldehyde-fixed specimens of 7-day old root material which had been exposed to 10 mEq/l lead for 3 days. In this study analysis was carried out on a whole field of view basis. The study provided two sets of data. Firstly a line profile for lead was constructed across each field of view, and secondly a lead X-ray image of the area was produced. The areas examined were the same as those

examined in the initial study at Cardiff, but the scanning nature of the instrument allowed a far greater area to be analysed. Also the scanning mode, with its facility for subsequent electronic amplification of specimen contrast, allowed thicker sections (blue) to be used, so increasing the reliability of the measurement and reducing the problems of tissue distortion under the analysis beam.

Using this instrument the question of lead occurrence in the specimens appeared to be a little more complex. In general the correlation between electron-dense deposits and lead peaks was good, and lead containing deposits were identified throughout the sections, both in intercellular and intracellular locations (Plates 7.2.1 - 7.2.5). However, discrepancies were noticed. On occasion, areas of cell wall which appeared relatively free of deposits gave fairly strong lead signals. This was particularly evident in the cortical cell walls, and was best demonstrated by the lead X-ray images (Plate 7.2.2). Also it was found that within the stele intracellular deposits which from their extent in electron micrographs would appear to represent large lead deposits were in fact far less rich in lead than had been suspected. Again this was more clearly seen in the lead X-ray images (Plates 7.2.3 and 7.2.4).

7.3 *Low Magnification Analysis of the Root Cortex and Stele*

In order to verify, and to gain more information about, the role of the endodermis in lead distribution across the root the same technique as was employed in section 7.2 was used at the lowest magnification possible to examine lead distribution in transects across the endodermis, with particular reference to the area around the phloem groups of juvenile roots (see Chapters 5 and 6). The material

employed was similar to that examined in the study reported in section 7.2, and analyses were carried out on adjacent fields of view to derive a composite belt transect of the region. The resultant transects are presented in Plates 7.3.1 - 7.3.4. In each case although the traces themselves may be displaced, line profiles of lead distribution were constructed approximately across the centre of each field of view. The extent of each transect was limited by the mesh width of the grids even though the largest grid apertures available were employed, and the outer part of the root cortex could not be examined. The procedure was also rendered more difficult by the lack of any system for specimen rotation within the microscope, so that aligning the designated transect with the electron beam scan required the removal of the specimen from the microscope, manual rotation of the specimen within the holder and reinsertion of the holder into the microscope, this procedure being repeated until the desired line was achieved.

Four transects were compiled in this way, these running radially outwards from the stele into the cortex and traversing the endodermis at four locations: approximately opposite the protoxylem, opposite the protophloem, and at two locations between the protoxylem and protophloem groups.

It was clear from the lead X-ray images that, as suspected from the T.E.M. studies (see Chapter 6) lead existed outside the endodermis as a diffuse deposit and also as heavy localised deposits. Inside the endodermis a diffuse deposit of lead was again observable throughout the cells, and in many cases cell outlines could be clearly recognised in the stelar cells in the lead X-ray images. In addition on these traces the endodermis and the inner cortical

cells were visible in the form of cell outlines. The increased levels of lead in these cells did not appear to arise as a general increase in the concentration of the diffuse deposits so much as an increase in the frequency of the heavy deposits. However, the density in this region of the images recorded appeared to vary greatly, being particularly low in the phloem transect (Plates 7.3.1 - 7.3.4). This situation was made more explicit by the lead monitored through the centre of each transect. These showed clearly that in areas of the endodermis other than opposite the phloem heavy deposits of lead were to be found, these being greater in the outer than in the inner tangential endodermal walls, although appreciable amounts of lead were still registered associated with the inner walls. However, in the phloem transect the profile was completely different. The analysis showed that lead was present in the endodermis at this location, and that deposits were greater on the outer than on the inner tangential walls as before; but the magnitude of these deposits, when compared with those observed elsewhere in the endodermis, was far less. Also profile peaks were much lower in the cortical cells, although large peaks were recorded in the phloem region. It was also noticed that cortex peak heights were reduced in the xylem transect, although this was less obvious than had been observed associated with the phloem. The lead profiles were not particularly clear on the copies of the original 'Polaroid' traces, and so have been reproduced for comparison in Figure 7.3.5.

7.4 *Bulk Analysis of Lead-Treated Tissue using the Electron Microprobe*

In the last part of the microprobe study bulk analyses were carried out on seedlings which had been grown in lead-contaminated Hoagland's solution as employed in the growth experiments. The

material assayed was 7 days old, and had been exposed to lead nitrate at concentrations of 0, 1 and 10 mEq/l for a period of 4 days. The dried specimens were ashed at low temperature and solubilized in acid, and transferred to specimen grids as a fine spray. The grids were subsequently subjected to microprobe analysis. For further details of the techniques involved see Chapter 2. Preliminary analysis of culture solutions in the growth analysis experiments (see Appendix 4) had indicated that the addition of lead to the culture solution produced anomalies in calcium uptake, and therefore the relative concentration of this element in the tissues was of particular interest.

Analyses were carried out on root tissues alone, and on entire seedlings. The latter measurements produced highly variable results, and although it appeared that in general terms ion levels were lower in 10 mEq/l treated plants than in others, it was not possible to uphold this statistically. In the root tissue, however, the situation was clearer. Significant reductions in concentration with increasing lead supply were observed in analyses for sodium ($P < 0.001$), magnesium ($P < 0.01$), phosphorus ($P < 0.01$), potassium ($P < 0.001$) and calcium ($P < 0.05$) (Figure 7.4.1). Measurement was also made of sulphur content but these results were found to be inaccurate due to the proximity of the sulphur and lead peaks. The relationship for calcium was also verified by conventional total ion analyses of mature plant material grown in lead-contaminated culture solutions. These analyses also demonstrated a significant reduction in iron content, as had also been suggested by the preliminary studies (see Appendix 1.4). The results of these analyses for calcium and iron are expressed graphically in Figure 7.4.2.

7.5 *Discussion*

The electron microprobe analyses provided corroborative evidence for several proposals expressed earlier, and also provided an interesting insight on the effect of lead on the ionic status of the plant.

The analyses clearly showed that the electron-dense deposits viewed in the T.E.M. preparations contained lead and that the heavy deposits represented the predominant locations of lead within the tissue. However, it was also clear that, according to the lead X-ray images, areas existed where lead contamination was higher than would be suggested purely by visual interpretation, and in other areas, particularly in the stelar region, the lead content of some deposits was less than would be suggested by the extent of their electron-opacity. The deposits involved in the latter observation were, by comparison with the micrographs of Chapter 7, cytoplasmic in location and therefore probably organic in nature. It is likely that the discrepancy between lead content and electron-opacity arises from the organic nature of the deposits, because the inter-ionic spacing of the lead atoms in these larger molecules would be greater than in inorganic molecules. It may be concluded, therefore, that the interpretation of the electron-dense deposits observed in the electron micrographs presented in Chapter 6 as lead accumulations is valid, but that, apart from the comparisons between heavy and light deposits, visual estimation of the extent of electron opacity provides only an approximate indication of relative lead contamination.

The lead X-ray images and lead profiles of the root at low magnifications carried out at JEOL (U.K.) Ltd. upheld the proposal that the endodermis presented a partial barrier to radial lead movement

in the root. Although cortical lead contamination in the region of the endodermis was not as high as might be expected in several of the transects, the recurrent disparity between amounts of lead associated with the inner and outer tangential walls of the endodermis was supportive of a barrier function.

The electron microprobe analyses also demonstrated that, as had been suspected in the light and electron microscope studies of lead distribution in the plant, this barrier function was absent in the endodermal cells opposite the protophloem and that the phloem accepted a heavy lead burden, much of which was associated with the cell walls. The indication of relatively low lead contamination in the cortex as indicated by the lead profiles in several transects was, in all likelihood, attributable to the line taken by the profile across the cortex. In those cases where the deposits were low, the profiles lay predominantly across the tangential walls. The discrepancies between contamination of tangential and of radial cortical cell has been discussed in Chapter 6, and the difference is clearly demonstrated by the comparison of the lead profile in Plate 7.3.3, which was associated approximately with radial cell walls in the cortex, with the other profiles.

Perhaps the most interesting information, however, arises from the results of the bulk microprobe analyses of seedlings and the associated conventional analyses. They clearly show that the presence of lead in the young root tissue resulted in a reduction in the levels of other minerals present. However, whole seedling analyses were difficult to interpret. It is probable that the material subjected to analysis was still in a fairly early stage of development, and the food reserves associated primarily with the cotyledon would form a

significant contribution to the mineral status of the whole seedling and so confuse the results obtained. The analyses of whole mature plants by more conventional means showed that the presence of lead brought about a reduction in the levels of iron and calcium in the tissue, these reductions being most obvious at lead supply levels in excess of 1 mEq/l. The reductions observed in ion levels in juvenile root tissue are probably attributable in part to the ionic dilution effect brought about by the addition of large amounts of lead ion to the culture solutions, but as the changes in tissue content invoked by the various lead treatments were disproportionate from element to element it seems unlikely that this is the sole reason for the observed reductions in the ionic status of the tissue. The possibility must be considered, therefore, that lead causes a reduction in the roots' ability to take up these other elements.

Analyses of both culture solutions and mature lead-treated material supported the observation that lead contamination reduced the uptake of calcium into the entire plant, and reductions in iron uptake also occurred. However, there was no evidence that similar reductions occurred in the uptake of the other elements assayed in the microprobe studies. It is possible that this disparity arises partly through the nature of the tissues observed. In the microprobe studies the tissue under examination was root material alone, and much of the ion complement detected must have been associated with the apparent free space of the cortex, and therefore of similar proportional ion content to the bathing medium. In the whole plant studies this represents only a small proportion of the total nutrient complement of the tissue under analysis. It is possible that changes in the mineral complement of the whole plant occur in response to

lead supply, but with the exception of calcium and iron, these could not be detected by the techniques employed in the whole plant study.

Reports on the effect of lead on mineral uptake by plants are scarce. A number of authors (Hewitt, 1948a and b; Miller and Koeppe, 1970; Fiusello, 1973) have reported lead-induced chlorosis which could, in some cases, be rectified by the provision of additional iron but no mention is made of iron content of the tissue (see Chapter 3). Iron uptake as influenced by a number of cations (excluding lead) was, however, examined by Lingle et al. (1968) who reported that the interfering ions brought about a slight enhancement of iron uptake in iron-deficient soybeans at low interfering ion concentration, but that at higher concentrations uptake was severely reduced. An exception occurred in the case of zinc, when it was found that even low levels (0.5×10^{-6} mol/l) inhibited iron uptake. Rosen et al. (1977) also report an interaction between zinc and iron uptake in corn plants. A number of authors have reported reductions in iron uptake by plants in the presence of nickel, and also reduction in the availability of iron in the tissue (see Mishra and Kar, 1974).

The role of iron within the plant is multi-factorial. It is involved structurally in a number of enzymes and proteins (Hewitt and Smith, 1975) but perhaps one of the most important roles of iron lies in its structural involvement in the cytochrome and ferredoxin of electron transport chains (Salisbury and Ross, 1969; Epstein, 1972; Hewitt and Smith, 1975). Radical interference with these compounds will of necessity be reflected in a wide range of functions in the plant.

Prior observations of lead/calcium interactions are varied. Prat (1927) reported that calcium acted as an antagonistic agent in

lead uptake. Subsequently Broyer et al. (1972) reported that calcium did not significantly modify lead uptake. Johnston and Proctor (1977) found that plants growing on lead spoil tips rich in calcium accumulated large amounts of calcium, and suggested that this might enable the plant to withstand heavy metal toxicity. However, in these studies no examination has been made of the effect of lead on calcium uptake. This measurement has been made in the case of other potentially toxic metals, but again conflicting results have been obtained; Forster (1954) reported that cobalt contamination increased the calcium content of oat plant tops, while Ramani and Kannan (1976) found that manganese inhibited calcium uptake by excised rice roots.

The role of calcium in the plant is a particularly interesting one. Calcium is taken up by the plant in relatively large amounts (Beeson, 1941) and is generally described as a plant macronutrient. However, studies by Pharis, Barnes and Naylor (1964), and Wallace, Frölich and Lunt (1966) have shown that plants are able to develop normally in solutions containing very low concentrations of calcium as long as the concentration of other ions in the culture solutions are carefully controlled. Wyn Jones and Lunt (1967) conclude that the large amounts of calcium required by plants under natural conditions are required mainly to reduce the toxicity of other nutrients, and that in the absence of such toxic agents the role of calcium in the plant assumes a micronutrient status. In this respect it is of interest to note the observation made by a number of authors that calcium supply may counteract heavy metal toxicity (True, 1914; Prat, 1927; McBrien and Hassall, 1965; Chester, 1965). Gielink et al. (1966) demonstrated that the cell wall is the primary site of calcium accumulation in the plant cell, where it is thought to be

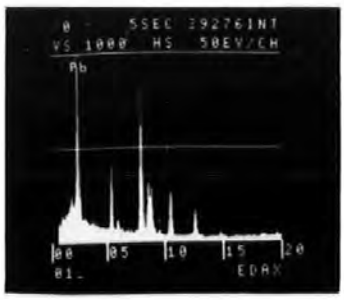
involved in structural elements of the wall (Siegel, 1962; Rogers and Perkins, 1968; Hewitt and Smith, 1975). Calcium has been shown to be involved in several processes within the cell, notably in the stability of cell membranes (van Stevenick, 1965; Morré and Bracker, 1976; Toprover and Glinka, 1976). It is clear that several sites exist in the plant cell where lead/calcium interactions may occur.

In the light of this it is clearly feasible that some of the visual symptoms of lead toxicity observed in the growth analysis experiments (chlorosis and necrosis at high lead supply levels, and a general stunting of growth - see Chapter 3) may at least in part be attributable to deficiencies in iron and calcium induced by the supply of lead to the plant.

Plate 7.1.1 Small spot electron microprobe analyses of glutaraldehyde-fixed specimens of 7 day old root material after 3 days exposure to 10 mEq/l lead nitrate in hydroponic solution.

The right hand rank of photographs show transverse sections of electron-dense cortical deposits. The sites arrowed and numbered show where analysis was carried out. The results of these analyses, photographed from a cathode ray display tube, are displayed in the left hand rank of photographs. These may be identified by the number in the bottom left hand corner of each trace, which corresponds with a site number in the right hand rank of photographs. In each case noticeable lead peaks (Pb) are present in the traces. The additional peaks in trace 1 reflect the wider range of KeV energies applied in this, a preliminary, run.

(Micrograph magnification x3000)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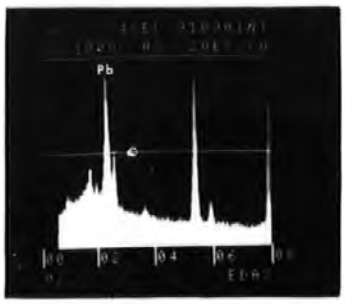
Pb
EDAX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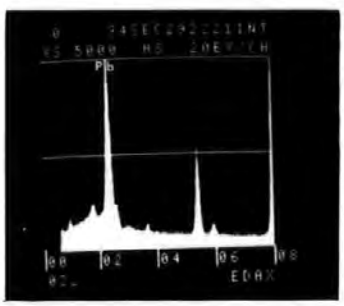
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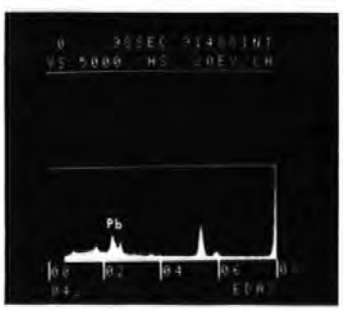
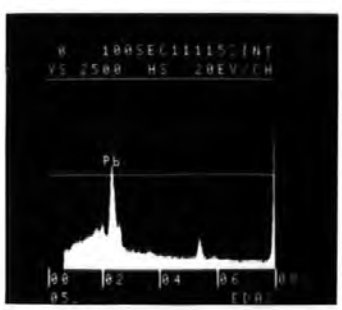
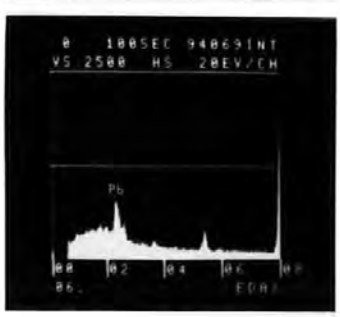

Pb


VS 2500 HS 20EV/CH
Pb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Pb


A
1
2


B
4
3

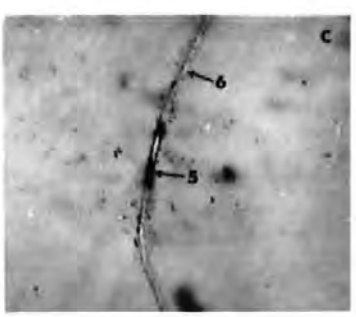

C
6
5

Plate 7.1.2 Small spot electron microprobe analyses of glutaraldehyde-fixed specimens of 7 day old root material after 3 days exposure to 10 mEq/l lead nitrate solution.

The right hand rank of photographs show transverse sections of electron-dense deposits in the stelar region:

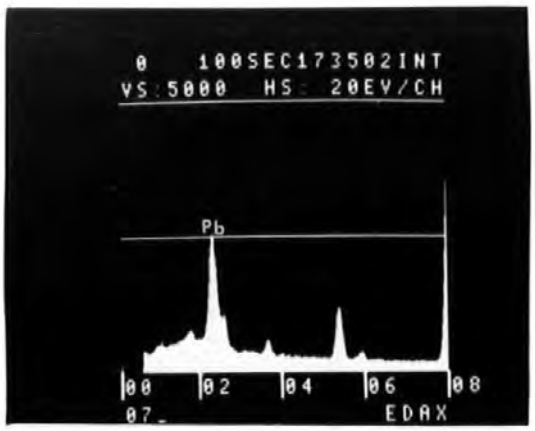
A. Endodermis outer tangential wall

B. Protophloem cells

C. Metaxylem vessel

Corresponding analyses are shown in the left hand rank of photographs and are numbered in the manner described in the legend for Plate 7.1.1.

(Micrograph magnification x4500)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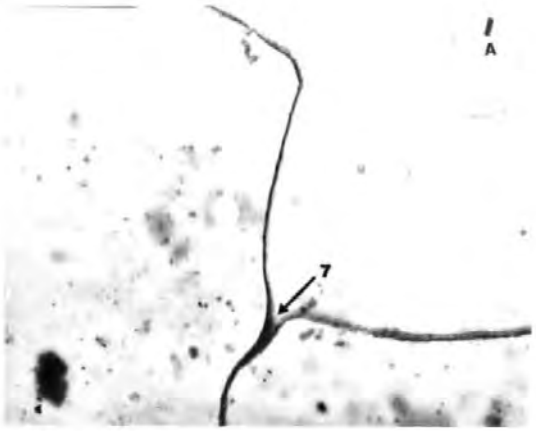
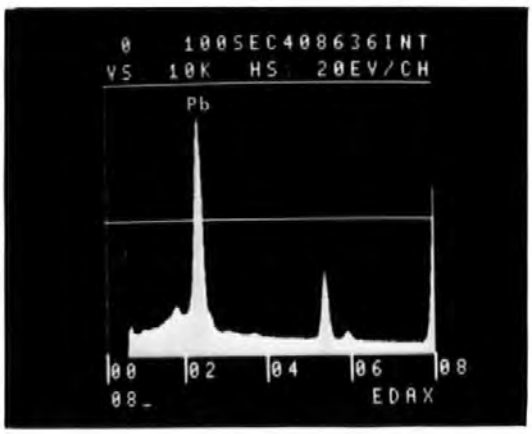
VS 5000 HS 20EV/CH
Pb
00 02 04 06 08
07_ EDAX


A
7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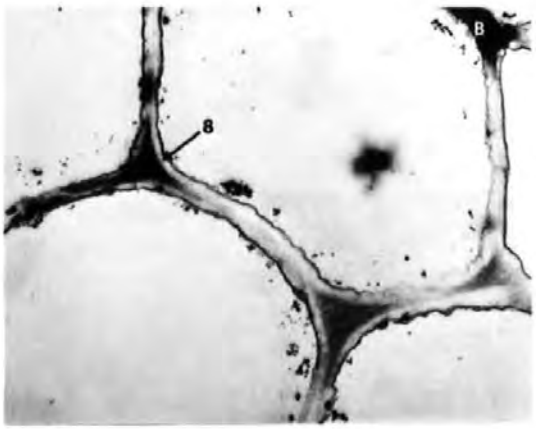
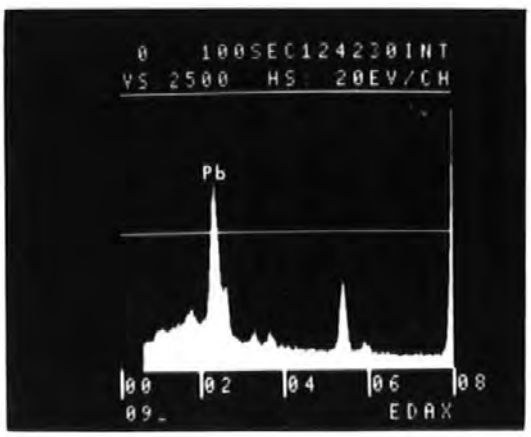
Pb
00 02 04 06 08
08_ EDAX

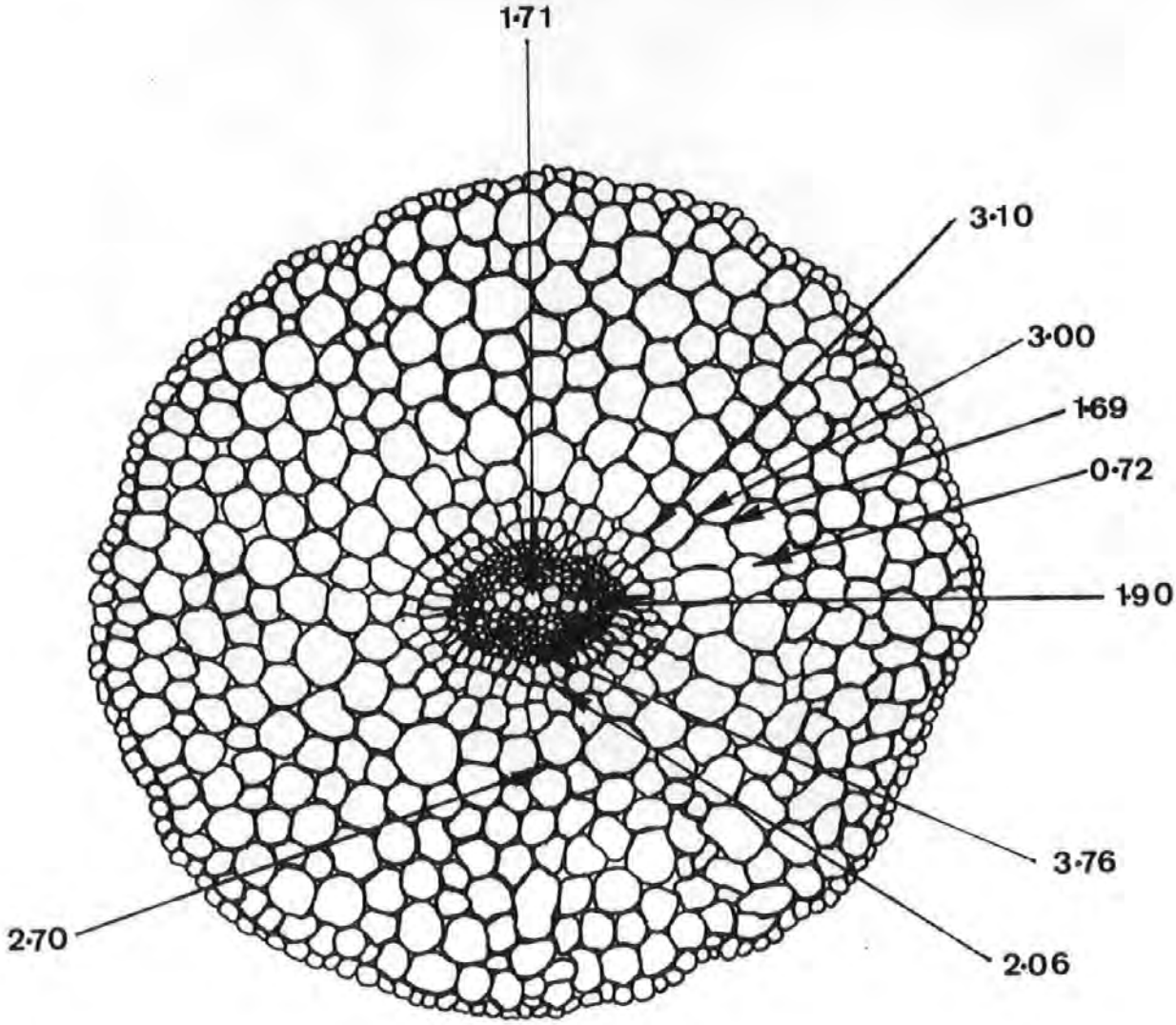

B
8


0 100SEC124230INT
VS 2500 HS 20EV/CH
Pb
00 02 04 06 08
09_ EDAX


C
9

Figure 7.1.3 Relative lead peak heights observed in small spot analyses around the inner cortex and stelar region of 7 day old radish roots exposed to 10 mEq/l lead nitrate solution for 3 days.

The figures are corrected for specimen thickness and density as measured by the Bremsstrahlungen or white radiation count.



Site Description	Lead Count	Bremsstrahlungen Count	Corrected Lead Count
Cortex cell wall	10604	3469	3.10
Cortex cell wall	7200	2369	3.00
Cortex cell wall	10811	3963	2.70
Cortex intercellular space	2316	1373	1.69
Cortex intracellular deposit	958	1338	0.72
Endodermis	4389	2135	2.06
Phloem cell wall	17824	4744	3.76
Protoxylem vessel	2853	1502	1.90
Metaxylem vessel	3389	1983	1.71

Plates 7.2.1 - 7.2.5 Small area STEM microprobe analyses of glutaraldehyde-fixed specimens of 7 day old root material exposed to 10 mEq/l lead nitrate solution for 3 days.

The upper photograph (A) in each plate shows a transverse section of the area under analysis, superimposed upon which is a profile of lead distribution across the specimens measured along the position arrowed. The lower photograph (B) shows a lead X-ray image of the same area at the same magnification.

Plate 7.2.1 Intercellular space in cortex (x6300)

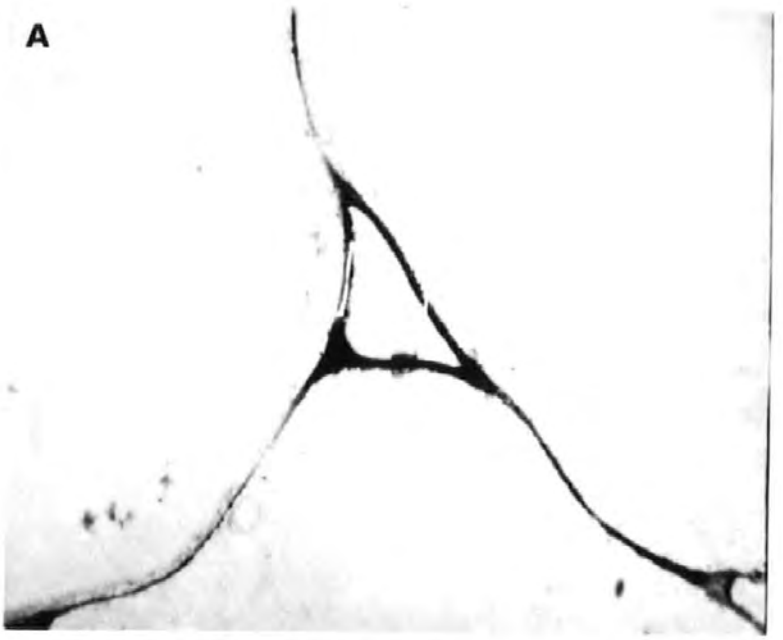
Plate 7.2.2 Intercellular space in cortex (x6300)

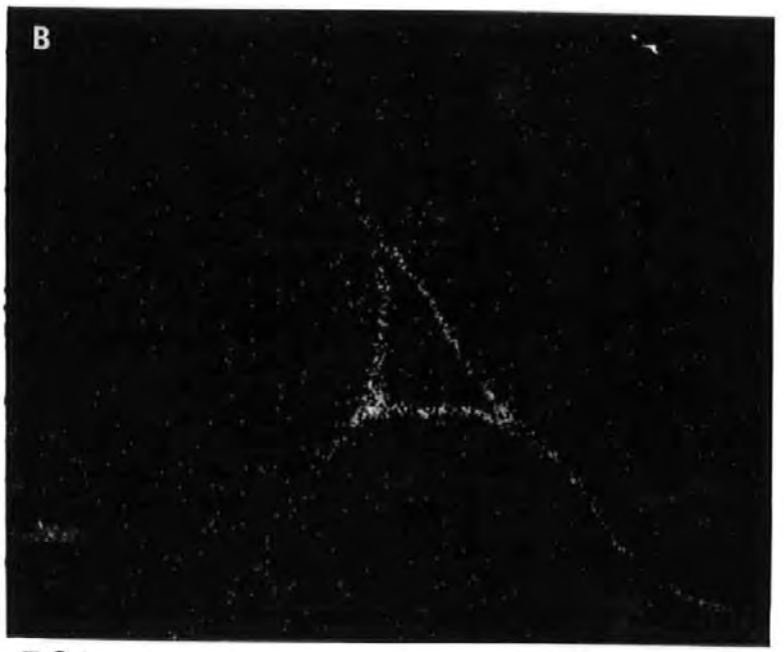
Plate 7.2.3 Parenchyma cell wall within stele (x7700)

Plate 7.2.4 Xylem vessel (x2600)

Plate 7.2.5 Phloem group (x3250)

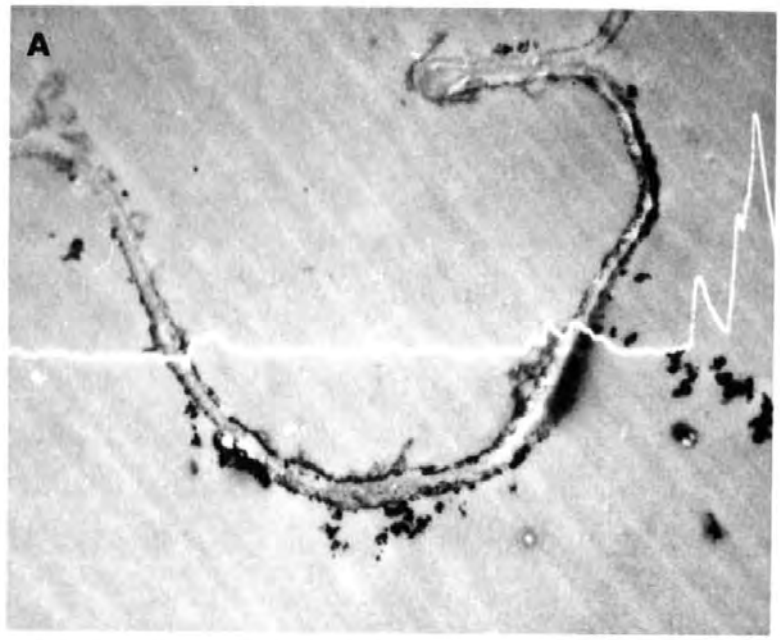
Certain of the random white signals observed in the X-ray images are attributable to electrical 'noise' in the instrument - notably 2 large white signals recurrent in the top right hand corner of a number of the X-ray images. Nevertheless it remains clear that on the whole the electron-dense deposits correlate with lead deposits, but that areas exist where the magnitude of the lead deposits differs from that suggested by visual inspection of the electron-dense deposits (see Plates 7.2.2, 7.2.3, 7.2.4).



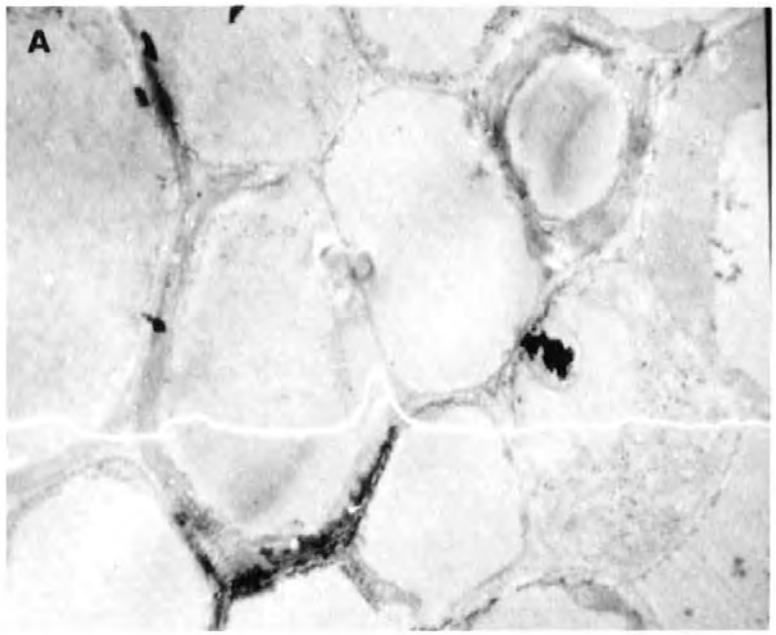


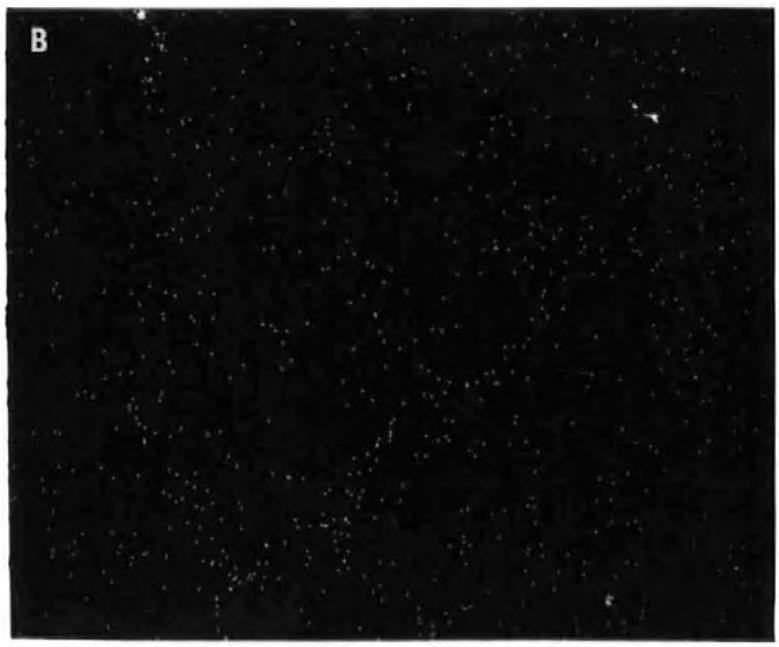
7.2.1

7.2.2

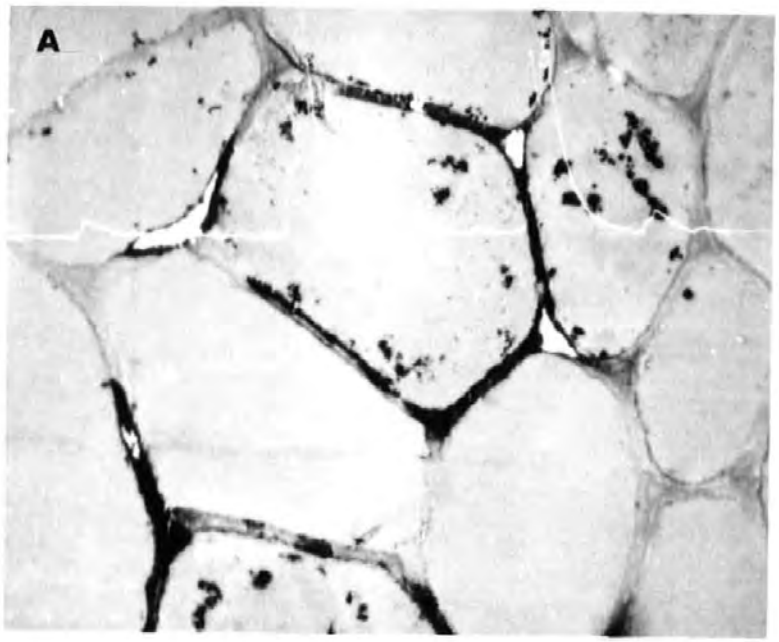


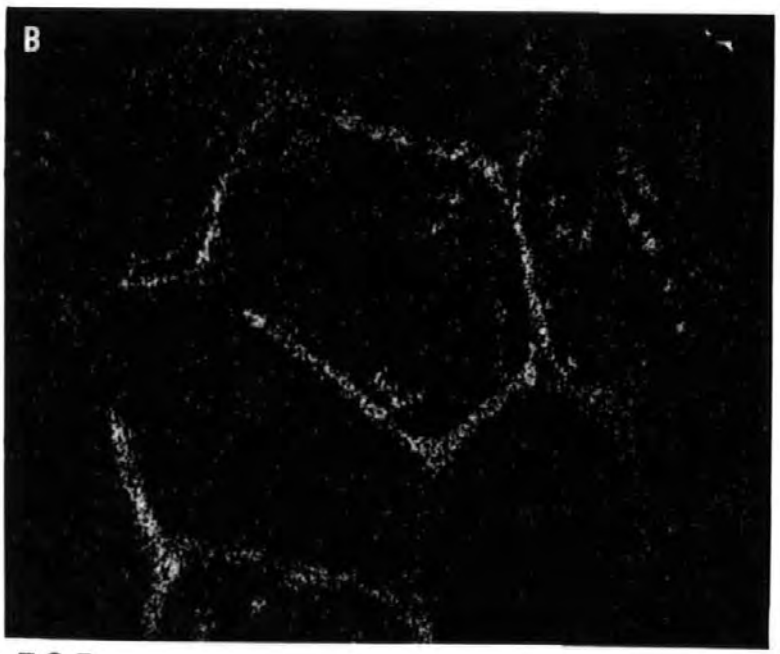
7.2.3





7.2.4





7.2.5

Plates 7.3.1 - 7.3.4 Low magnification transect analyses of glutaraldehyde-fixed specimens of 7 day old root material exposed to 10 mEq/l lead nitrate solution for 3 days.

Transects are constructed from several fields of view across the radius of the root, and again two sets of photographs are presented for each transect. In each case the photograph closer to the spine of the thesis shows a STEM micrograph of the specimen, with a lead profile measured across the centre of the field of view superimposed upon it. These are not particularly clear in the copies and have been reproduced in Figure 7.3.5. The photograph closer to the free edge of the page shows a lead X-ray image of the same area.

Plate 7.3.1 Transect through endodermis at phloem group (x2000)

Plate 7.3.2 Transect through endodermis close to protoxylem (x2000)

Plate 7.3.3 Transect through endodermis between phloem and xylem (x2000)

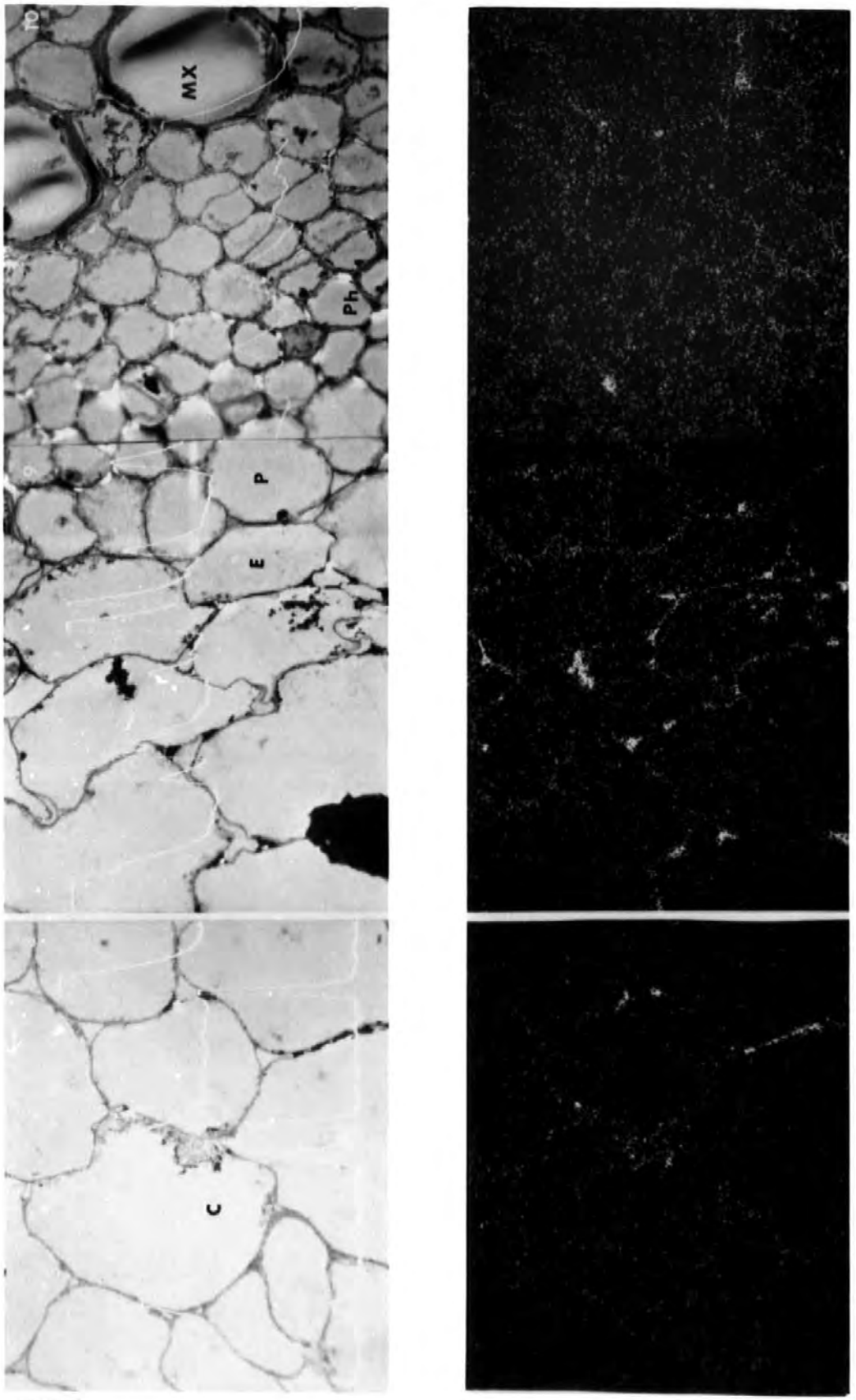
Plate 7.3.4 Transect through endodermis between phloem and xylem (x2000)

Note large endodermal accumulations of lead except opposite the protophloem; note also deposits tend to be heavier on outer than on inner walls of endodermis.

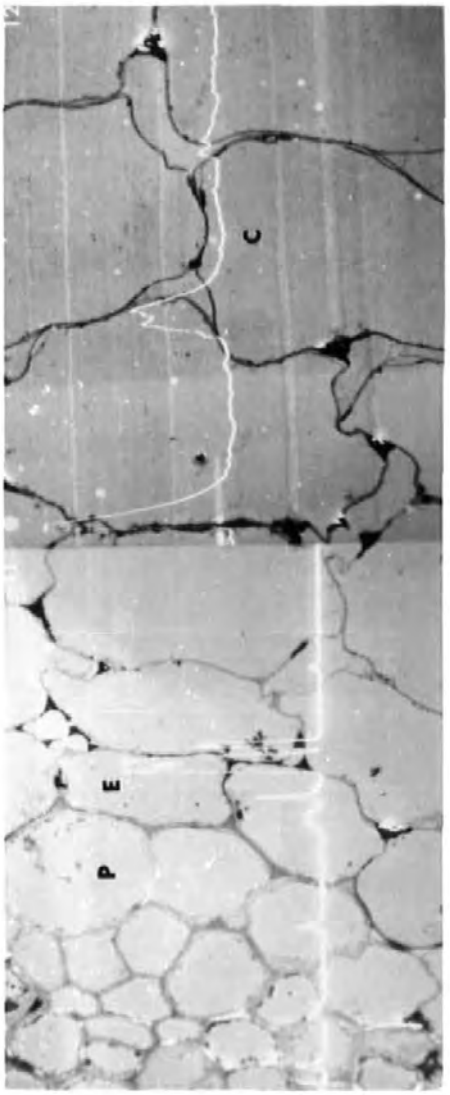
C - cortex; E - endodermis; P - pericycle; Ph - phloem; MX - metaxylem

7.3.1

7.3.2



7.3.3



7.3.4

Figure 7.3.5 Root lead profiles copied from Plates 7.3.1 - 7.3.4

The letters below the profiles indicate cell positions

A. Profile through endodermis at protophloem group (Plate 7.3.1)

B. Profile through endodermis close to protoxylem (Plate 7.3.2)

C. Profile through endodermis between phloem and xylem (Plate 7.3.3)

D. Profile through endodermis between phloem and xylem (Plate 7.3.4)

Note prominent lead peaks associated with endodermis tangential walls in B - D, but absent in A, where instead a large amount of lead is present in the protophloem.

C_1 - C_4 - cortical cell; E - endodermis; P - pericycle

PH - protophloem; MX - metaxylem

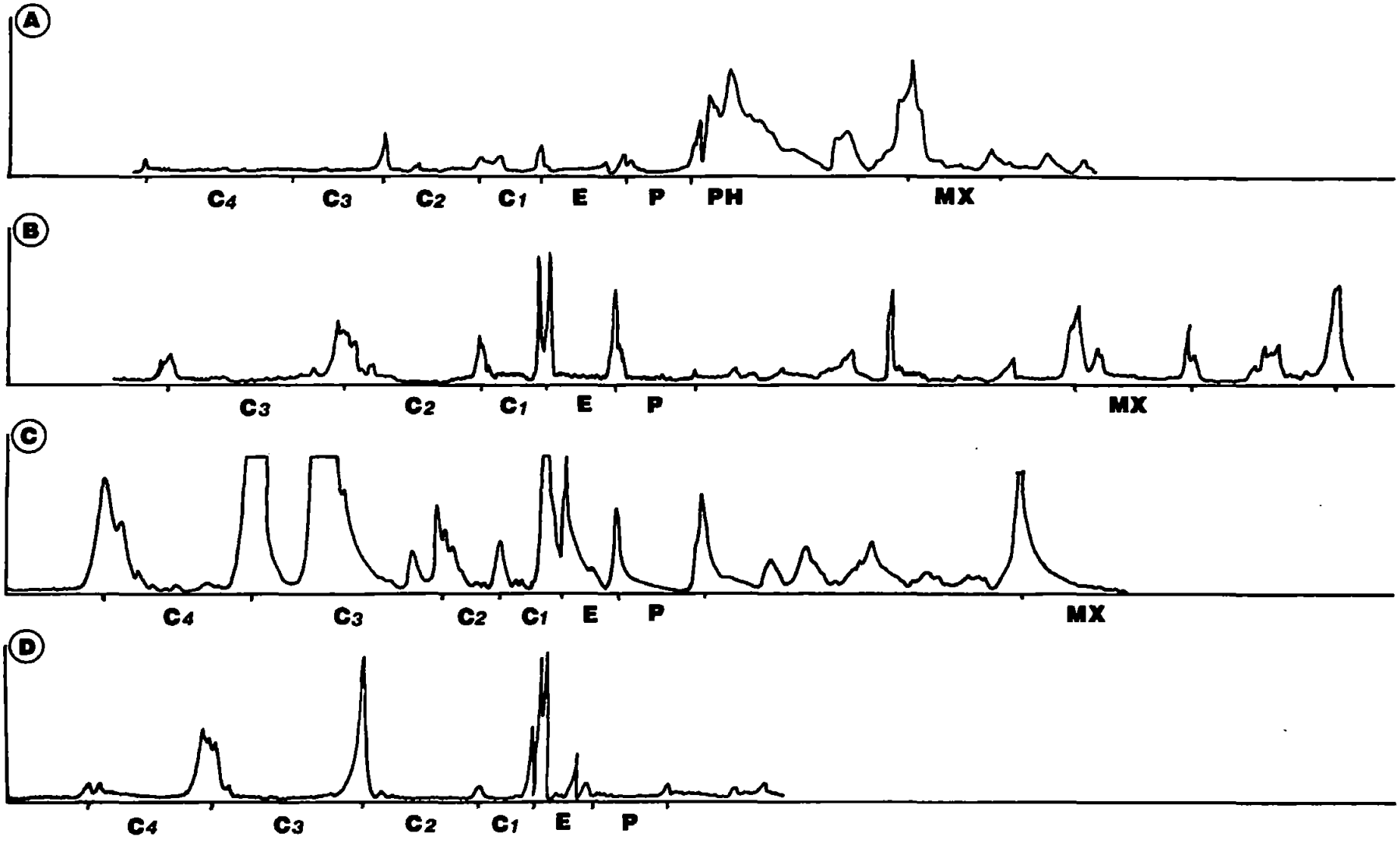
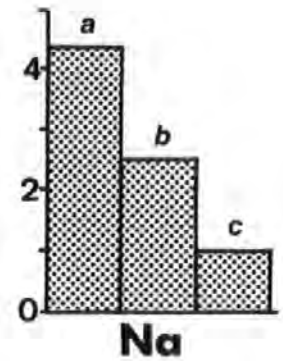
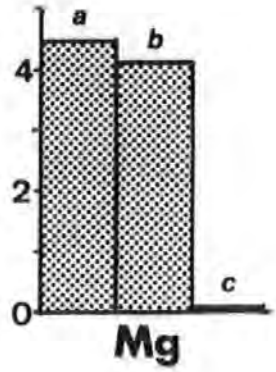

A
C4
C3
C2
C1
E
P
PH
MX
B
C3
C2
C1
E
P
MX
C
C4
C3
C2
C1
E
P
MX
D
C4
C3
C2
C1
E
P

Figure 7.4.1 Bulk analyses of root material for several elements

Analyses were carried out on 7 day old root material exposed to 0, 1, or 10 mEq/l lead nitrate for 4 days. Elements monitored included sodium, magnesium, phosphorus, potassium, calcium and lead. The vertical axes are again relative. Note that reductions occur in all cases except in lead with increasing lead supply but that the relative decrease is not constant from element to element.

a - 0 mEq/l; b - 1 mEq/l; c - 10 mEq/l


a
b
c
4
2
0
Na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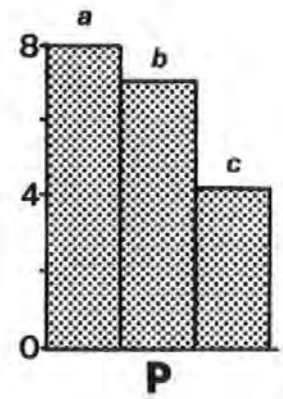
Mg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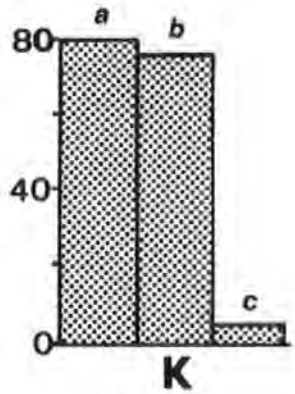
P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c
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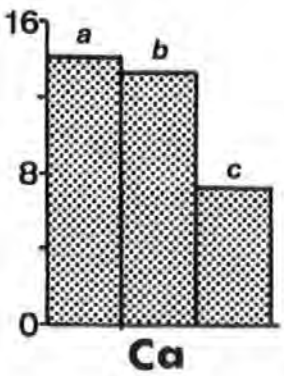
40
0
K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c
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8
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Ca

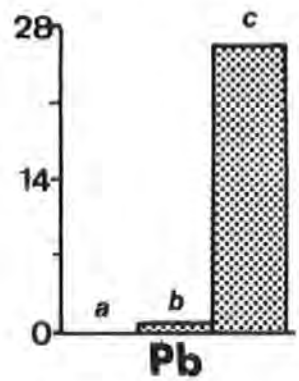
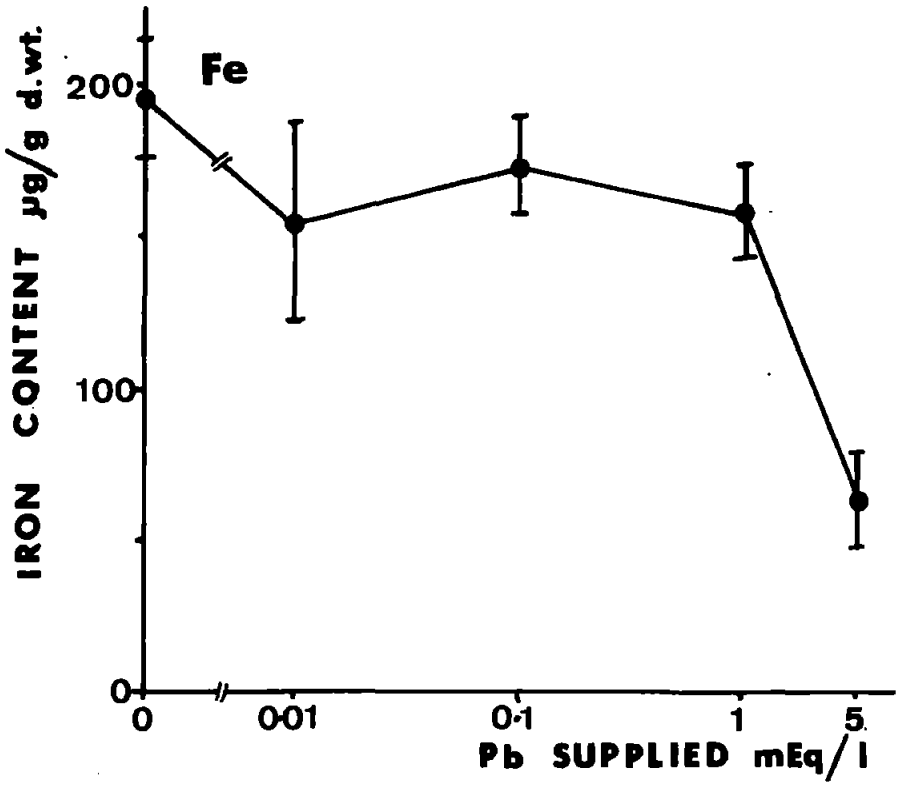
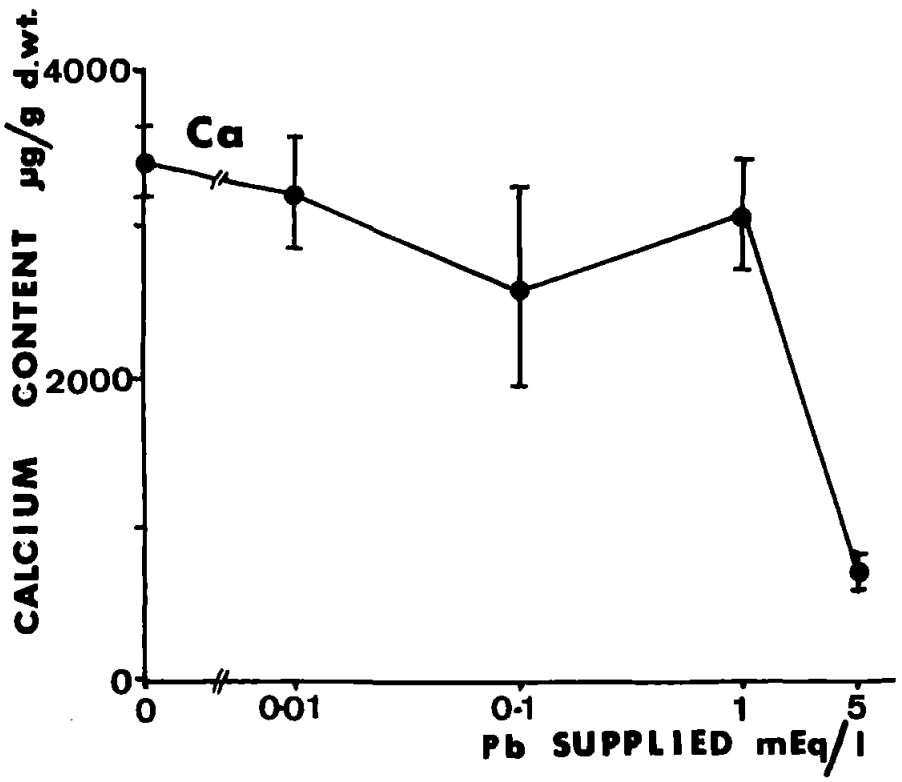

a
b
c
28
14
0
Pb

Figure 7.4.2 Conventional analysis of mature radish plants for iron and calcium content

The plants examined were 4 weeks old and had been hydroponically grown in lead-contaminated (0 - 10 mEq/l) Hoagland's solution.

Vertical line = ± standard error


Fe
IRON CONTENT µg/g d.wt.
200
100
0
0
0·01
0·1
1
5
Pb SUPPLIED mEq/l


Ca
CALCIUM CONTENT µg/g d.wt.
4000
2000
0
0
0·01
0·1
1
5
Pb SUPPLIED mEq/l

CHAPTER 8

EFFECTS OF LEAD ON THE HORMONAL REGULATION OF PLANT GROWTH

The results presented in Chapter 3 have clearly demonstrated that the reduction or even complete cessation of growth is one of the major effects of contamination of the plant by lead. This has also been shown by the reports of other researchers (e.g. Berry, 1924; Rasmussen and Henry, 1963). Such changes in plant development must be brought about either by changes in the rate of cell division and/or by the modification of cell expansion.

Previously a few researchers have examined the effect of lead on cell division. Hammett (1928c) and Levan (1945) reported that the accumulation of lead in meristematic regions might lead to reduction in the rate of cell division, and Deknudt et al. (1973) reported that chromosome aberrations were frequent in workers exposed occupationally to lead. However, the light microscope studies detailed in Chapter 5 have shown that although the root meristem becomes heavily contaminated with lead, the stem meristem remains substantially lead-free, and therefore alterations to the rate of cell division may not be the only cause of growth inhibition. This is supported by the abrupt manner in which root development ceases on exposure to high concentrations of lead, since some continuation of cell expansion would be anticipated if growth inhibition arose solely from an inhibition of cell division.

The process of cell expansion is thought to be mediated through the action of plant growth hormones such as indol-3ylacetic acid (IAA) and it has been proposed that the expansion process involves a number of steps. Firstly, the cell wall becomes less rigid due to the

breakage of bonds in the wall; secondly, water uptake occurs, enlarging the cell; and thirdly, new wall synthesis occurs, returning to the wall its former stability (Cleland, 1971).

The following experiments were carried out to examine the effect of lead on some of the processes involved in the growth of the plant. These experiments were involved mainly with the cell elongation process, and any cell wall changes were of particular interest in view of the extensive distribution of lead in the cell walls detected in the light and electron microscope studies (see Chapters 5 and 6).

8.1 Preliminary Experiments

Preliminary information was collected on the topics of lead-induced changes in cell division and cell expansion in radish seedlings, the experiments being carried out on 7-day old material.

The extent of cell division in radish root tips was examined in squashes of root tip material stained with Schiff's reagent (see Chapter 2). In this respect radish is not a particularly good experimental material. The chromosomes are small, and may only be viewed under a microscope magnification of x400. They are also fairly numerous (somatic number = 18) and the examination is rendered more difficult by a variable number of chromomeres, approximately of the same order of magnitude as the chromosomes, associated with the nucleus in its non-mitotic condition. Therefore it was only possible to distinguish cells in interphase, metaphase or telophase. The result of counts carried out after 24 hours exposure to lead at various concentrations between 0 and 10 mEq/l is shown in Figure 8.1.1a. It was quite apparent from these data that the percentage of cells in both metaphase and telophase declined with increasing lead supply.

Moreover at the higher levels of lead supply (5 and 10 mEq/l) extensive areas existed in the squashes where nuclear material was not differentially stained by the Schiff's reagent, but the whole of the cytoplasm adopted a red pigmentation suggesting that some disruption of the nuclear material had occurred. Despite this apparent breakdown of cellular integrity cells were still observed in which the nucleus was in metaphase or telophase, suggesting that some cell division had occurred.

The effect of lead on cell expansion was initially investigated using radish stem sections. The test material was prepared by removing a 10 mm segment from each stem 5 mm behind the stem apex. These segments were exposed to solutions containing IAA and lead nitrate simultaneously and the resultant increases in length measured after 3 days (see Chapter 2 for further details). It was found that although this procedure resulted in a reduction in elongation with increased lead supply the extent of this reduction was rather variable (Figure 8.1.1b). Experiments were also carried out to determine the effect of lead/IAA mixtures on developing radish seedlings, which were allowed to grow for 3 days on inclined sheets of filter paper impregnated with the test solutions. At the end of this period root development was measured (see Chapter 2). However these results proved somewhat inconclusive since all the lead treatments caused a drastic inhibition of root growth, although they did show that the provision of IAA failed to ameliorate the growth inhibition induced by lead contamination (Figure 8.1.1c).

8.2 Studies on Cell Elongation

Because of the erratic nature of the results of the preliminary cell expansion studies, subsequent observations were made using

coleoptile material of Triticum aestivum. Graminiferous coleoptiles have been adopted as classical experimental material for cell elongation studies by many researchers, from Went (1926) to Zegers et al. (1976).

In this study a series of modified wheat coleoptile bioassays were carried out using 10 mm segments of coleoptile, excised 3 mm from the tip of each coleoptile (see Chapter 2). Assays were carried out in the classical manner (see Chapter 2), but with the addition of various co- and pre-treatments.

The addition of lead nitrate to the wheat coleoptile bioassay system resulted in a strong inhibition of IAA-induced coleoptile elongation (Figure 8.2.1a). This effect was obvious at lead concentrations of 0.1 mEq/l and increased with increasing lead supply, but was absent in parallel experiments in which nitrate was supplied as the sodium salt. At lead concentrations of 10 mEq/l elongation was reduced to approximately one fifth of that observed in controls. In all treatments the provision of 1 μg/ml of IAA caused greater elongation than was evident in those tests in which lead was supplied alone (Figure 8.2.1a). The provision of IAA at levels in excess of that observed to produce optimum elongation in controls tended to correct the lead-induced inhibition. In the presence of 1 mEq/l of lead nitrate, an elongation response approximately equal to the maximum response in specimens treated with IAA alone was recorded at an IAA level of 10 μg/ml (Figure 8.2.1b).

In order to assess the relationship between IAA and lead in more detail a factorial experiment was carried out to investigate the effect of increasing levels of IAA on the inhibition of coleoptile elongation promoted by the supply of lead. IAA was supplied at five

concentrations up to a maximum of 20 μg/ml, while lead nitrate was supplied at 0, 1, 5, and 10 mEq/l. The results of this experiment confirmed that the inhibition promoted by lead could be only partly alleviated by IAA since at the higher supply levels of lead changes in IAA supply levels had little effect on coleoptile expansion (Figure 8.2.2).

To assess the general applicability of the IAA/lead interaction other auxin systems were investigated. To this end more coleoptile expansion bioassays were carried out, utilising a range of primarily synthetic auxins. These included phenylacetic acid (PAA), 1-naphthlylacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-(indol-3yl)butyric acid (IBA), and 4-chloro-2-oxobenzothiazolin-3ylacetic acid (Benazolin). The chemical structures of these substances are shown in Figure 8.2.3.

(i) Phenylacetic acid was first reported to possess auxin-like properties by Zimmerman et al. (1936) and although it was thought to be a purely synthetic auxin, it has subsequently been identified in plant material (Wightman, 1977). Also, two of its derivatives (phenylacetamide and p-hydroxybenzoic acid) have been extracted from plant material (Isogai et al., 1964; Vieitez et al., 1966). The effect of lead on PAA-induced elongation was found to be similar to that for IAA, namely a progressive reduction with increasing lead supply, but it was observed that increasing the supply levels of PAA did not alleviate the lead-induced elongation of inhibition (Figures 8.2.4a, 8.2.5a).

(ii) 1-Naphthylacetic acid was again first reported in the work of Zimmerman et al. (1936), and has achieved wide use in commercial auxin preparations. The response of NAA-induced coleoptile elongation

to lead was again found to be similar to that observed in the case of IAA-induced elongation. Experiments involving increasing NAA concentrations showed that no shift in optimum NAA concentration occurred in the presence of 1 mEq/l lead nitrate (Figures 8.2.4b, 8.2.5b).

(iii) The growth stimulatory abilities of 2,4-dichlorophenoxyacetic acid were recognised by Synerholm and Zimmerman (1947), although its primary commercial use today is at toxic concentrations as a selective herbicide. Its interaction with lead in the coleoptile elongation test was very similar to that observed in the IAA-induced elongation tests, in that increasing lead concentration caused an inhibition of elongation which could be partially corrected by increasing 2,4-D supply concentrations (Figures 8.2.4a, 8.2.5c).

(iv) 4-(Indol-3yl)butyric acid is very similar in chemical structure to IAA, differing only in the length of the side chain attached to the indole ring system. It was first reported as a substance with auxin-like properties by Zimmerman et al. (1936) but has subsequently been detected in plant extracts (Audus, 1972). Major commercial uses of IBA are in rooting compounds and in aids to fruit set and ripening. Once again the bioassays showed that exposure to lead reduced IBA-induced coleoptile elongation, but it was found that increasing levels of IBA had little effect on the lead induced inhibition (Figures 8.2.4d, 8.2.5d).

(v) 4-Chloro-2-oxobenzothiazolin-3-yl acetic acid was developed by the Boots Company Limited in the early 1960's as a selective herbicide. However, Ingram and Butcher (1972) found that low concentrations of benazolin were of value in the initiation and maintenance of plant tissue cultures, and also to a certain extent

in root initiation in plant tissue cultures. The compound has also been shown to be active in the coleoptile straight growth test (Brookes and Leafe, 1963). As with the other synthetic auxins tested, the addition of lead to this system brought about an inhibition of the elongation process which increased with increasing lead supply. However, once again this was not alleviated by increasing the benazolin supply (Figures 8.2.4e, 8.2.5e).

8.3 Lead-Induced Changes in the Physiology of IAA-Induced Cell Elongation

In the next phase of the study a series of experiments was carried out to provide further information on the subject of the lead-induced inhibition of auxin-directed cell expansion.

Initially the lead and IAA treatments employed were separated. The provision of lead as a one hour vacuum infiltration pretreatment made little difference to the response of the elongation process to lead, suggesting that the inhibition was not merely the result of lead nitrate-induced osmotic changes within the tissue (Figure 8.3.1a).

In subsequent experiments 1 μg/ml IAA was supplied as a 12 hour pretreatment in 0.5 mol/l mannitol solution. Such mannitol solutions have been used to prevent osmotically-directed water uptake (Burström, 1953; Ordin et al., 1956). The pretreatment was followed by exposure of the segments to aqueous lead nitrate solutions for a further 48 hours. It was obvious from these experiments that an effect on cell elongation still occurred, but this effect appeared to be relatively slight in comparison with previous experiments (Figure 8.3.1b).

Calcium has been found by previous researchers to exert an influence on coleoptile elongation and to be involved in IAA-induced

acidification (Cooil and Bonner, 1957; Nadler, 1976; Cohen and Nadler, 1976). Therefore the effect of the simultaneous addition of calcium nitrate and lead nitrate to the test system was next examined. This brought about a small but significant lessening of the inhibitory action of lead. This amelioration by calcium was optimal at calcium concentrations between 0.1 and 1.0 mEq/l (Figure 8.3.1c). Subsequent experiments suggested that the optimum calcium concentration was approximately 0.9 mEq/l, which was roughly equivalent to the level of lead supplied in these experiments (Figure 8.3.1d).

At this stage it was decided to look more closely at the basic processes involved in cell expansion. As stated previously (vide supra) the presence of cell expansion may be considered in two distinct, although concurrent, phases. Firstly changes occur in the cell wall rendering it more extensible, and secondly the cell takes up water causing it to expand. Experiments were therefore carried out on the effect of lead on these processes.

Wheat coleoptiles are not good material for water uptake studies because of the heterogeneity of the tissue (i.e. segments include both coleoptile and leaf tissue) and more importantly because their structure might render difficult the accurate and reproducible removal of surplus fluid. Other authors have examined water uptake in storage tissue and reported clear and reproducible results (e.g. Commoner et al., 1943; Masuda, 1965). Attempts were made to examine IAA-directed water uptake in radish tuber material, but these produced variable results, probably because of the extensive lignification of the tissue which occurs in enlarged radish tubers.

Hence estimates of IAA-induced water uptake in the presence of lead were made using freshly-harvested tuber tissue of Jerusalem

artichoke (Helianthus tuberosus) after the manner described by Masuda (1965) (see Chapter 2). These revealed an apparent effect of lead on water uptake in the presence of 1 μg/ml IAA (Figure 8.3.2a). Significant variations ($P < 0.001$) in percentage weight change were recorded at concentrations of 5 and 10 mEq/l, these changes taking the form of weight losses. It was noticeable that at these levels the artichoke discs became discoloured during the incubation period and at the time of the final weighing were of a rather spongy composition. In view of this change in the outward appearance of the discs the possibility must be considered that the weight losses arose, not from alterations in water uptake, but from the loss of a greater proportion of the water content of the tissue during blotting of the tissue. There was no variation in the diameter of the discs.

Measurements of cell wall elasticity/plasticity of lead/IAA treated segments were made using the system devised by Heyn (1931). In this technique segment deformation caused by an applied weight is measured in degrees (see Chapter 2). The results of these measurements showed that lead treatment brought about a reduction in the overall curvature of coleoptile segments placed under a constant inflexion stress. This reduction appeared to be the result of a decrease in both the plasticity and the elasticity of the cell walls (Figure 8.3.2b). However, the reduction in wall plasticity was not found to be significant when subjected to analysis of variance ($P > 0.05$). The relative contributions of the plastic and elastic responses to the total curvature of the tissue did not appear to change.

Because of this increase in cell wall rigidity in the presence of lead, and in consideration of the extensive contamination of the cell wall observed in both the light and electron microscope studies,

it was decided to enlarge this area of investigation and determine the ability of the different constituents of the cell wall to bind lead ions. Separation of cell wall fractions from coleoptile material was carried out using a combination of the techniques of Siegel (1962), Barratt and Northcote (1965) and Preston (1974). Aliquots of the resulting extracts were added to known volumes of lead nitrate solution, and changes in the concentration of available lead measured with an ion-selective electrode system (see Chapter 2). The results showed that, with the exception of lipids, lead absorption occurred in all the cell wall fractions prepared. This was most pronounced in the pectic substances and pectinic acid fractions, the amount of lead taken up by the pectinic acid fraction being approximately four times that taken up by the pectinic substances. Binding in other fractions was much lower, the next largest uptake occurring with hemicellulose A (Figure 8.3.3).

Ultraviolet absorbtion spectra of the compounds before and after exposure to lead also supported this observation, the addition of lead causing major alterations in absorbtion peaks in each fraction except lipids (Figure 8.3.4).

It became clear from these studies that an interaction existed between lead and auxin-induced cell elongation, which involved structural changes in the cell wall. The in vitro studies showed that normally supra-optimal levels of IAA could partially alleviate the effects of this interaction, and therefore experiments were carried out to determine whether any changes occur in endogenous auxin levels in response to lead contamination. A series of extractions were carried out (see Chapter 2) to obtain relatively pure samples of endogenous auxins and the amount of auxin present quantified by

bioassay and by colorimetric means (see Chapter 2). In both cases the concentration of auxin present in IAA-equivalents was found to increase with increasing lead supply (Figure 8.3.5).

8.4 Discussion

It was clear from the preliminary studies that lead exerted a strong influence on the processes of cell division, causing reductions in the percentage of cells undergoing mitosis. Lead-induced reductions in the rate of cell division have been reported in other studies (Hammett, 1928c; Levan, 1945) although Levan (op. cit.) suggests that the reduction is the result of an increase in colchicine mitoses rather than an increase in the percentage of cells in interphase. The observed reductions in cell division are possibly related to the association of lead with the root tip nuclei reported in the electron-microscope studies (see Chapter 6). It may be concluded that a reduction in cell division is a major cause of the reduced growth of root tissue observed in the presence of lead. However the light microscope studies indicate that this may not be so in the stem since heavy lead accumulations are absent from the stem meristem (see Chapter 5).

The present study showed that lead also exerted a considerable influence on the elongation process. From a theoretical standpoint, the effects seen in this series of bioassays might arise from a chemical reaction between lead and IAA in the bathing solutions, possibly arising from the formation of a complex with the carboxyl group of the IAA molecule similar to the copper/IAA complex reported by Perrin (1961). However, paper chromatography and nuclear magnetic resonance examinations of IAA and IAA/$Pb(NO_3)_2$ mixtures (see Appendix 1.5)

have indicated that no chemical interaction occurs. The inhibitory action of lead on cell elongation is not unique to the IAA system, as similar reactions were also observed when a range of synthetic auxins was used to induce cell elongation.

The observed inhibition of elongation by lead was found to be reduced by increasing the supply levels of IAA. In this respect it is of interest to note the observation of Mukherji and Maitra (1977) that exogenous supply of IAA could reduce the inhibition of growth produced in rice by lead. However, in the experiments involving synthetic auxins alleviation of lead inhibition was observed only in the bioassays involving 2-4,D. There are two possible interpretations for this apparent anomaly. Firstly, it is possible that the mechanisms by which lead-induced elongation is reduced are not susceptible to the other synthetic hormones. Secondly, differences may exist in the cellular responses of the experimental tissue to high levels of the various synthetic compounds and IAA. That such differences may exist is inherent in the observation of Audus (1972) that considerable variation occurs in the properties of these compounds (viz rate of penetration into cells and tissues, rate of transport and the rate of inactivation or destruction).

It would appear from the results of the analyses of lead-treated plant material that an increase in endogenous auxin occurs in response to lead exposure. The results presented in Figure 8.3.5 differ and this is probably attributable to the manner in which they were quantified. Colorimetric analysis is only of limited value since any indole groups are detected, not just auxins. Bioassays may be biased by any substances other than auxins (e.g. inhibitors, antiauxins) remaining in the extracts. However, in both cases significant

increases were observed with increasing lead supply. This indicates the existence of some feedback mechanism controlling the levels of endogenous auxin in the tissue. According to Mukherji and Maitra (1977) lead causes an increase in the activity of a number of enzymes, including both IAA oxidase and IAA synthase, the main enzymes associated with IAA degradation and synthesis. These authors believe that the growth inhibition induced by lead results from low endogenous auxin levels because of enhanced IAA oxidase activity. However in contrast the increase in endogenous auxin observed in this study must result from increased IAA synthesis. Influences of minerals on the IAA synthesis system have been reported previously. Al-Omary (1968) reported that in Zebrina pendula high calcium application coupled with adequate nitrogen supply caused a large increase in auxin content. Kutáček et al. (1966) demonstrated that the supply of zinc to barley grain restored the ability to synthesise tryptophan (and therefore auxin) which had been removed by grain irradiation. It is possible that lead influences the IAA synthesis system in a similar fashion.

Similar, albeit less marked, changes in cell elongation were found to be produced by the addition of calcium to the bathing solution. A calcium-induced inhibition of elongation has been reported previously (Cooil and Bonner, 1957; Ray and Baker, 1965; Pickard, 1970). Such inhibition may be a reflection of the findings of Cohen and Nadler (1976), Nadler (1976) and Rubinstein et al. (1977), who reported that calcium may cause acidification of the cell wall by cationic exchange with protons in the cell wall structure.

When calcium was added at the same time as lead there was a lessening of the lead induced inhibition which was most pronounced at concentrations of calcium approximately equivalent to the amount of

lead present. This would indicate a direct competition for uptake sites between the calcium and lead ions, and could perhaps partially explain the reduction in calcium uptake by radish plants which has been observed in the presence of lead (see Chapter 7).

Changes in percentage elongation of the type observed in the coleoptile straight-growth assays may result from differences in cell wall softening and/or water uptake. Cleland and Bonner (1956) showed that the cell wall plasticising action of auxins continued even in hypertonic mannitol solutions. In the present mannitol experiments all the test segments were pre-incubated in IAA in 0.5 mol/l mannitol solution; it would appear therefore that the small changes in elongation observed must arise from an effect of lead on the subsequent water uptake phase of elongation. It has been shown that prolonged exposure of plants to high concentrations of mannitol may, however, cause the uptake of mannitol into the test material (Burström, 1953; Groenwegen and Mills, 1960) and may therefore decrease the osmotic potential of the cell sap, or may elicit a toxic response (Strogonov and Lapina, 1964; Taylor, 1965). Such changes could explain the relatively low percentage elongation observed in these mannitol experiments, even in controls. An effect on IAA-induced water uptake appeared to be substantiated by the results of studies on water uptake, but this occurred only at supply levels of 5 and 10 mEq/l lead nitrate. However this may also reflect a direct effect of lead on membrane integrity rather than on interaction with IAA-directed water uptake, as permeability experiments have shown that treatment of plant tissue with high concentrations of lead causes a rapid release of ions from the cell which is indicative of major membrane disruption (see Appendix 1.6).

It is clear, however, that the presence of lead has a marked influence on the elasticity/plasticity of the cell wall, resulting in an overall increase in tissue wall rigidity which is only slightly reduced by IAA. Many hypotheses have been developed to explain the mechanism of auxin action (e.g. Burström, 1942; Commoner et al., 1943; Hackett and Thimann, 1952; Cleland, 1958; Penny, 1977; etc.). Almost all of these theories involve changes in the chemical status of the cell wall. Zegers et al. (1976) recently recorded a lead-induced alteration in the rate of elongation in IAA-treated coleoptile sections. Their electron microscope studies correlate this effect with a heavy deposition of lead in developing dictyosome vesicles, which may modify the elongation process via changes in the phosphate metabolism of the cell. However, according to Mühlethaler (1967) these vesicles are pectin-rich. Based on the present observation of binding to the pectic substances fraction of cell walls, it seems feasible that such lead accumulation may be solely the result of the pectin content of the vesicles. The ability of pectins to absorb lead is well-known, to the extent that in the past pectin has been recommended as a treatment for lead poisoning (Kertesz, 1951). This affinity for lead may also explain the adsorbtion of lead by hemicellulose A in the cell wall fraction studies, as hemicelluloses are thought to arise from the polymerisation of pectin groups (Buston, 1935).

A recent hypothesis for IAA-induced cell elongation put forward by Darvill et al. (1977) has suggested that auxin-induced changes in cell wall plasticity result from the breakage of cross-linkages between components of the cell wall matrix, and by a decrease in viscosity caused by the loss of side chains from the highly-branched molecules of the cell walls. It is thought that pectins and hemicelluloses

are involved in the formation of the side chains and cross-linkages contributing to cell wall viscosity (Bennett-Clark, 1956; Keegstra et al., 1973; Wilder and Albersheim, 1975; Albersheim, 1975). Therefore the effect of lead on IAA-induced cell extension may result mainly from the modification of cell wall cross-linkages, involving lead atoms, which give rise to a structure less susceptible to IAA-induced breakage. Such a hypothesis would also explain the effect of calcium on the cell elongation system as calcium treatment has been advocated for the 'firming' of many canned foodstuffs of plant origin (e.g. tomatoes) and its effectiveness is believed to be associated with the formation of calcium pectate within the material (Kertesz, 1951).

While lead may affect other parts of the system involved in the cell elongation process, a direct chemical effect would explain both the rapidity of the reaction, and the general increase in tissue rigidity which results on exposure of the tissue to lead. Such a chemical effect could also influence the activity of other plant hormones, and in this respect it is of interest to note that simple bioassays with gibberellic acid and kinetin have revealed that an interaction with lead may also exist in these systems (see Appendix 1.7).

Figure 8.1.1 Preliminary experiments

A. Changes in cell division in radish root material exposed to lead nitrate solutions for 24 hours

1 - Total percentage of cells undergoing division

2 - Percentage in telophase

3 - Percentage in metaphase

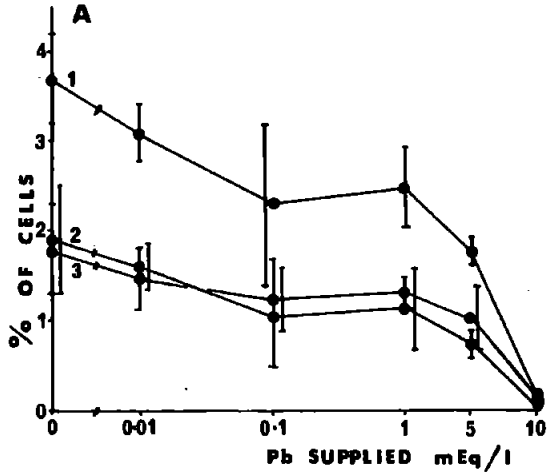
B. IAA-induced elongation of radish stem segments in the presence of lead nitrate

C. IAA-induced changes in radish root elongation in the presence and absence of lead nitrate

1 - 1 μg/ml IAA alone

2 - 1 μg/ml IAA and 1 mEq/l lead nitrate

Vertical line = ± standard error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% OF CELLS
Pb SUPPLIED mEq/l
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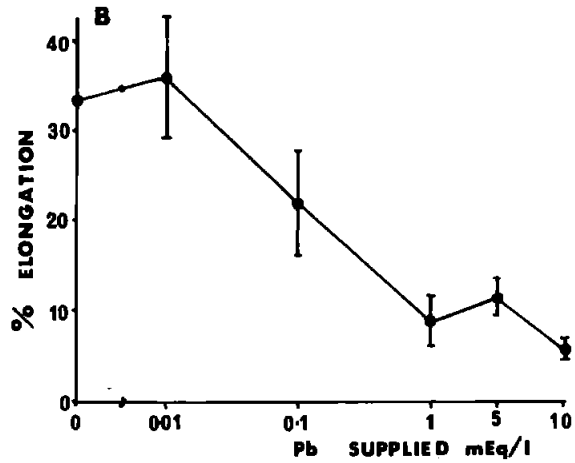
0·01
0·1
1
5
10
1
2
3


B
% ELONGATION
Pb SUPPLIED mEq/l
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0·01
0·1
1
5
10

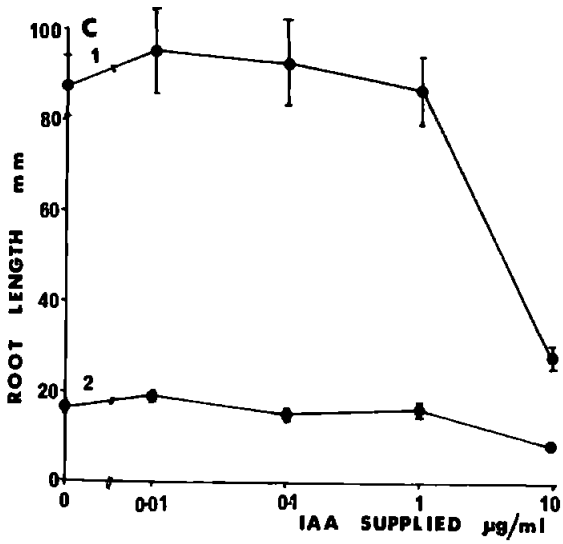

C
ROOT LENGTH mm
IAA SUPPLIED µg/ml
0
0·01
0·1
1
10
1
2

Figure 8.2.1 Effect of lead on IAA-induced cell elongation

A. The effect of increasing levels of lead nitrate on IAA-induced coleoptile elongation

1 - in the presence of 1 μg/ml IAA

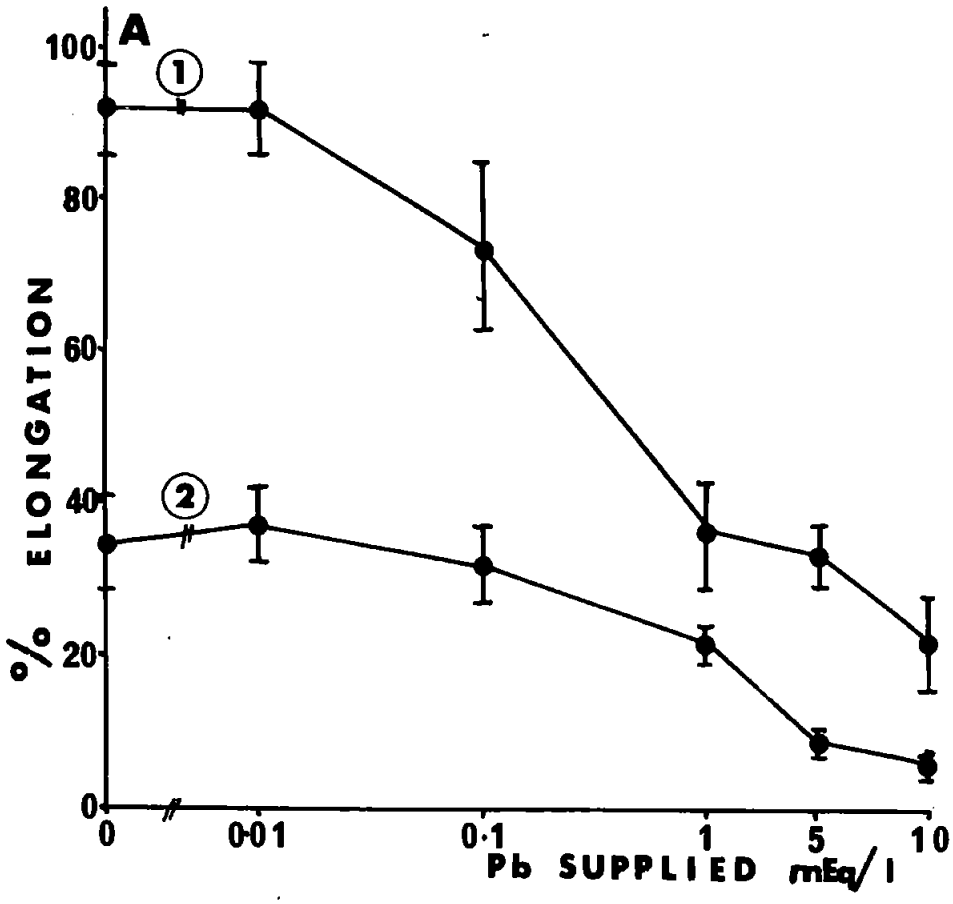
2 - without IAA

B. The effect of increasing levels of IAA on coleoptile elongation

1 - control

2 - in the presence of 1 mEq/l $Pb(NO_3)_2$

Vertical line = ± standard error


A
100
80
60
40
20
0
% ELONGATION
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

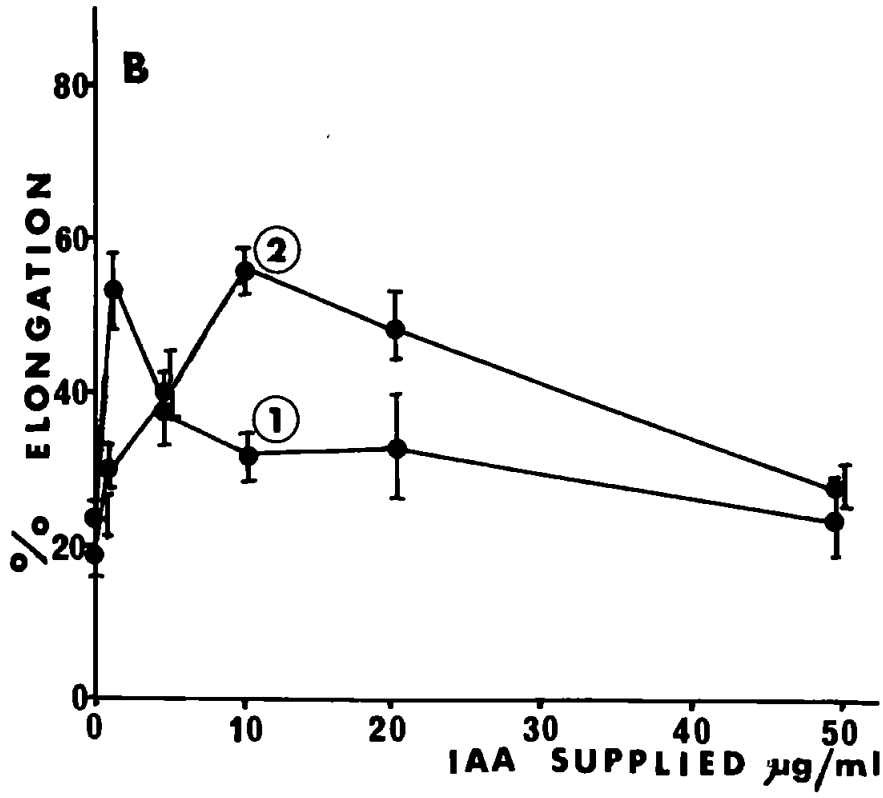
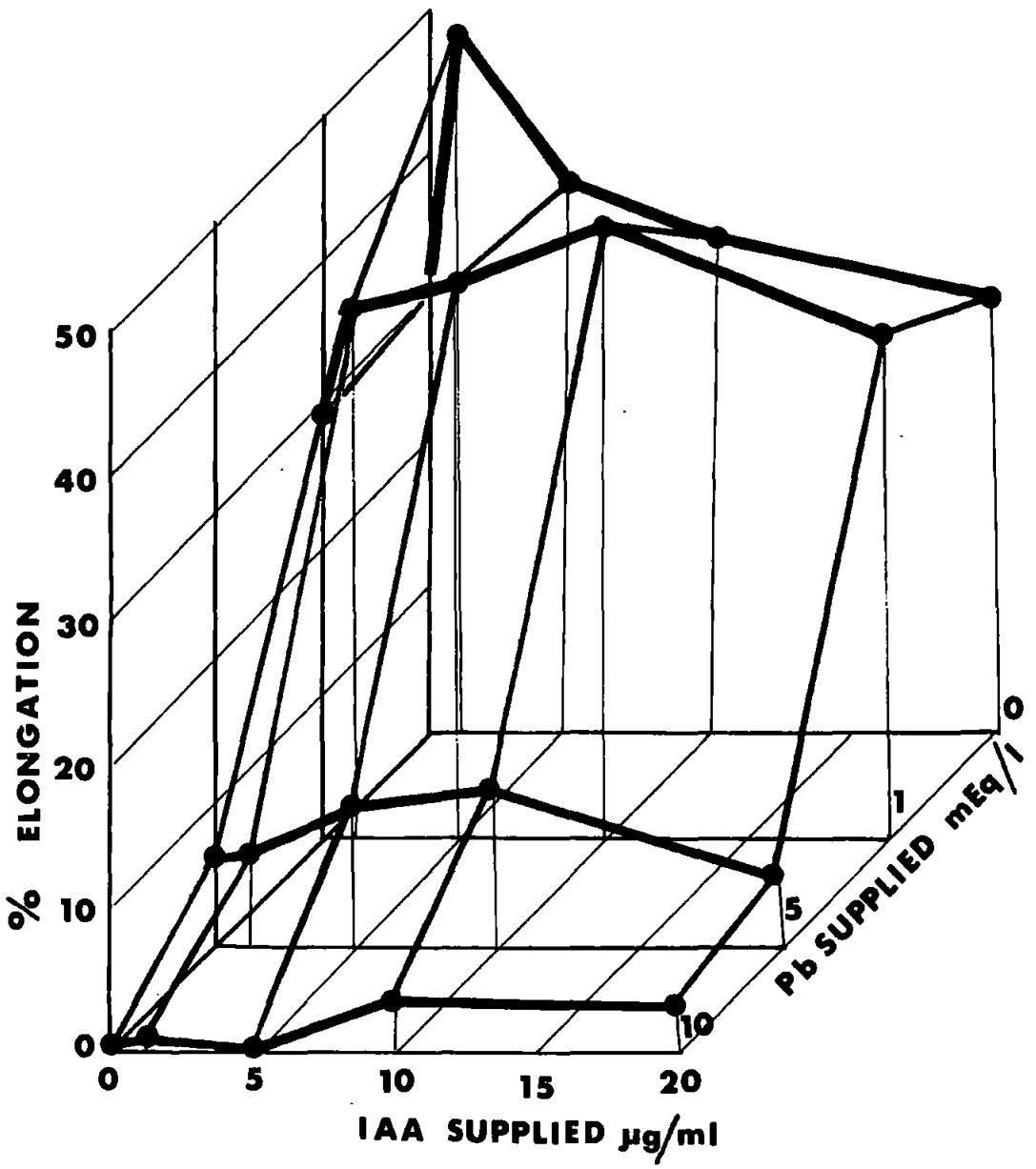

B
80
60
40
20
0
% ELONGATION
1
2
0
10
20
30
40
50
IAA SUPPLIED µg/ml

Figure 8.2.2 Factorial experiment on the effect of lead on IAA-induced cell elongation

Lead nitrate supplied at 0 - 10 mEq/l

IAA supplied at 0 - 20 μg/ml


% ELONGATION
50
40
30
20
10
0
0
5
10
15
20
IAA SUPPLIED µg/ml
0
1
5
10
Pb SUPPLIED mEq/l

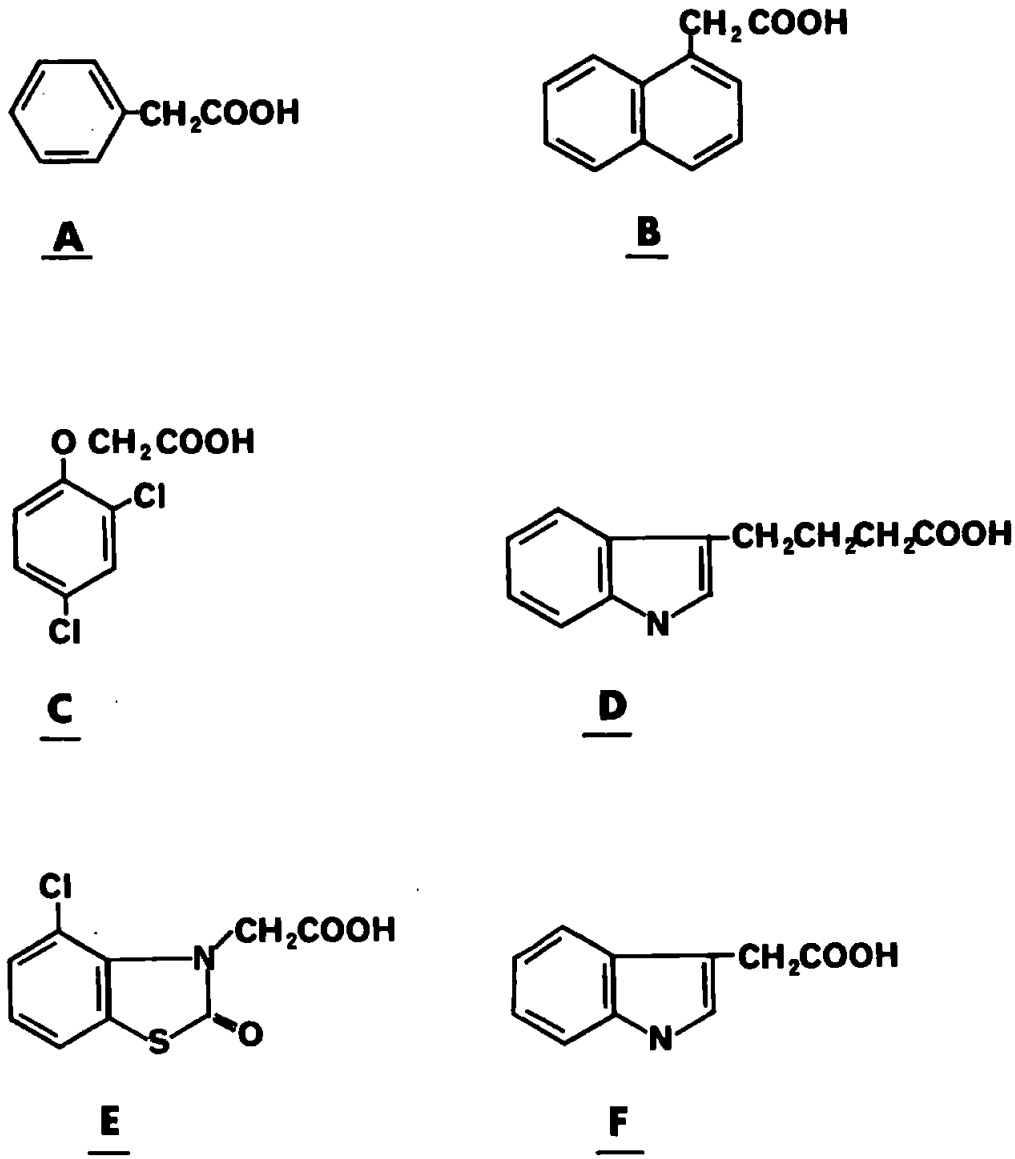


Figure 8.2.3

Chemical structures of auxins used in coleoptile elongation studies.

A. Phenyl acetic acid
B. Napth-1yl-acetic acid
C. 2,4-Dichlorophenoxyacetic acid
D. Indol-3yl-butyric acid
E. 4-Chloro-2-oxobenzothiazolin-3yl-acetic acid
F. Indol-3yl acetic acid

Figure 8.2.4 The effect of lead on coleoptile elongation induced by synthetic auxins

A. 1 μg/ml PAA

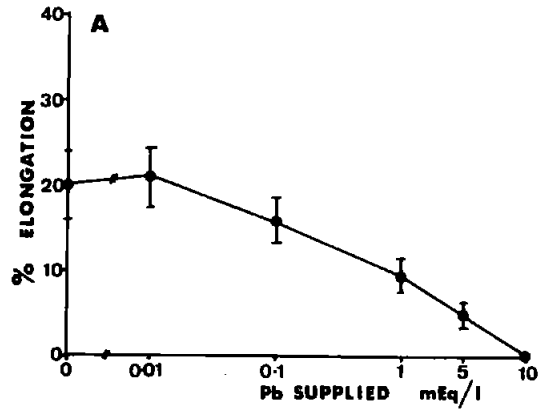
B. 1 μg/ml NAA

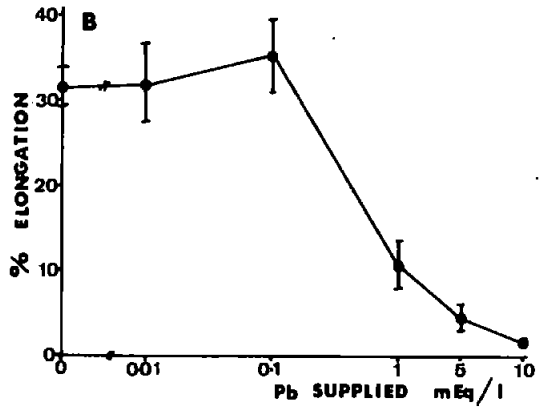
C. 2 μg/ml 2,4-D

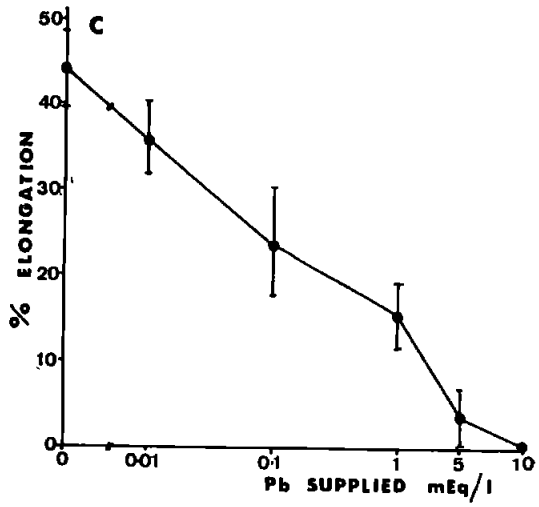
D. 1 μg/ml IBA

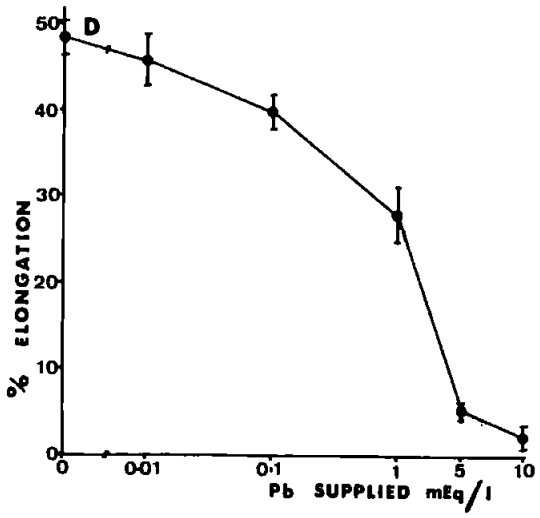
E. 5 μg/ml Benazolin

Vertical line = ± standard error


A
% ELONGATION
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
% ELONGATION
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
% ELONGATION
50
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


D
% ELONGATION
50
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

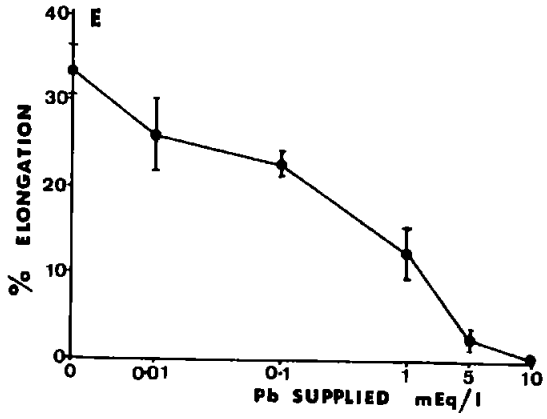

E
% ELONGATION
40
30
20
10
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 8.2.5 Effect of increasing levels of synthetic auxins on coleoptile elongation in the presence and absence of lead nitrate

A. PAA

B. NAA

C. 2,4-D

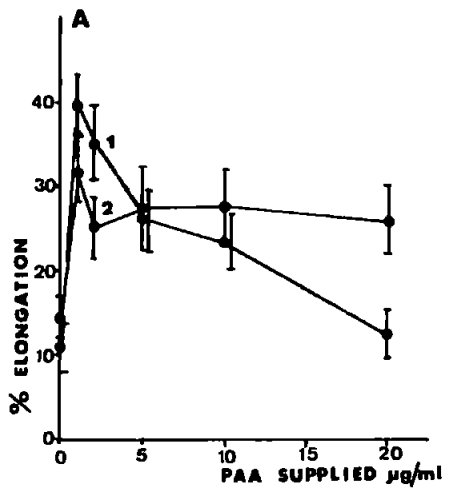
D. IBA

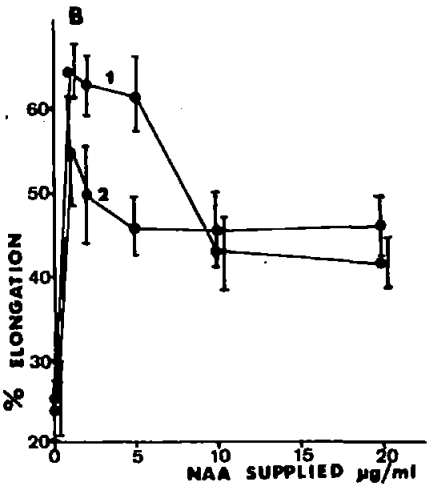
E. Benazolin

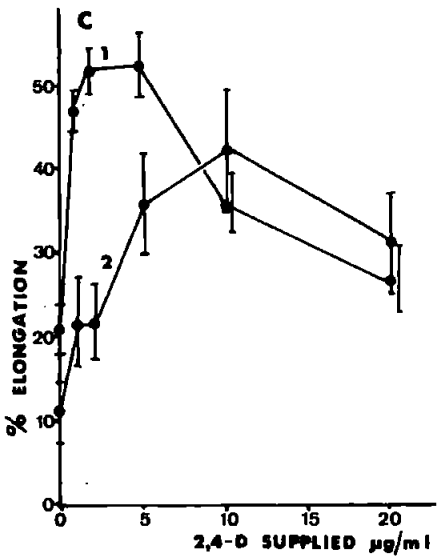
1. Control

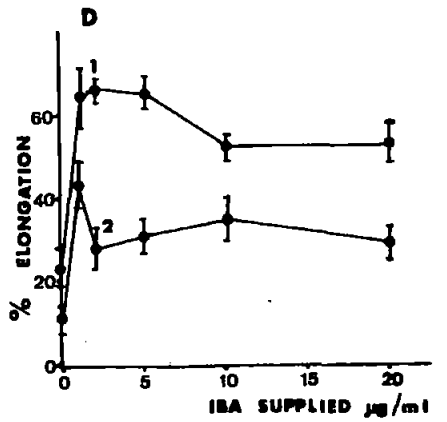
2. In the presence of 1 mEq/l $Pb(NO_3)_2$

Vertical line = $\pm$ standard error


A
% ELONGATION
PAA SUPPLIED µg/ml
1
2


B
% ELONGATION
NAA SUPPLIED µg/ml
1
2


C
% ELONGATION
2,4-D SUPPLIED µg/ml
1
2


D
% ELONGATION
IBA SUPPLIED µg/ml
1
2

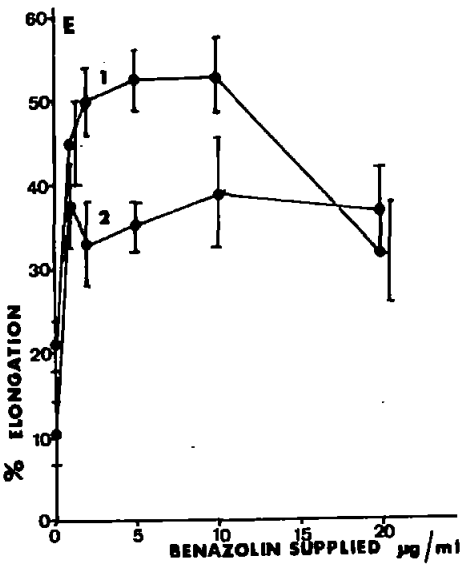

E
% ELONGATION
BENAZOLIN SUPPLIED µg/ml
1
2

Figure 8.3.1 The effect of pre- and co-treatments on the lead/IAA interaction

A. IAA-induced coleoptile elongation following vacuum infiltration with varying concentrations of lead for 1 hour.

B. Coleoptile segment elongation in lead nitrate solutions following 1 μg/ml IAA treatment in 0.5 mol/l mannitol solution.

C. Effect of log. range of calcium concentrations on IAA-induced coleoptile segment elongation

   1. In presence of calcium nitrate solutions only.

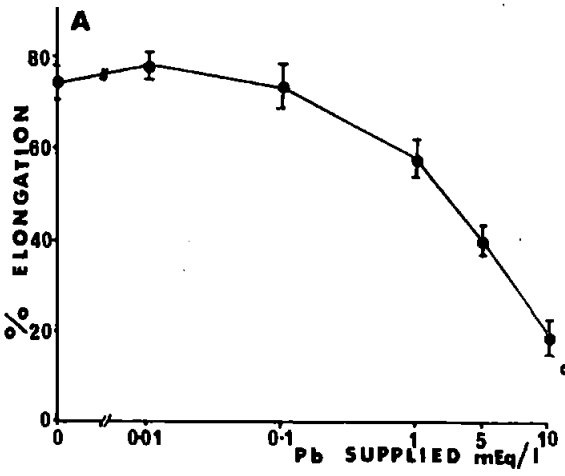
   2. In presence of calcium nitrate and 1 mEq/l lead nitrate.

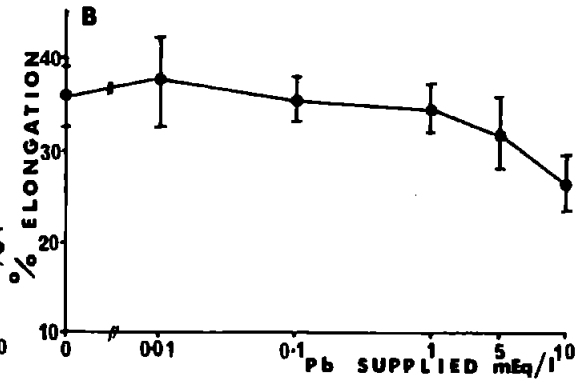
D. Effect of linear range of calcium concentrations on IAA-induced coleoptile segment elongation

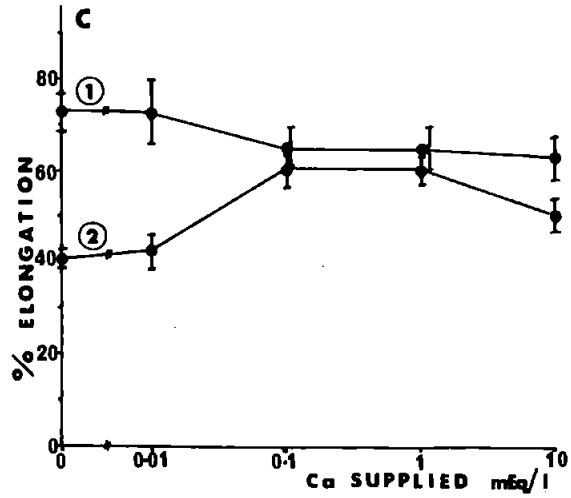
   1. In presence of calcium nitrate solutions only.

   2. In presence of calcium nitrate and 1 mEq/l lead nitrate.

Vertical line = ± standard error


A
% ELONGATION
80
60
40
20
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
% ELONGATION
40
30
20
10
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


C
% ELONGATION
80
60
40
20
0
1
2
0
0·01
0·1
1
10
Ca SUPPLIED mEq/l

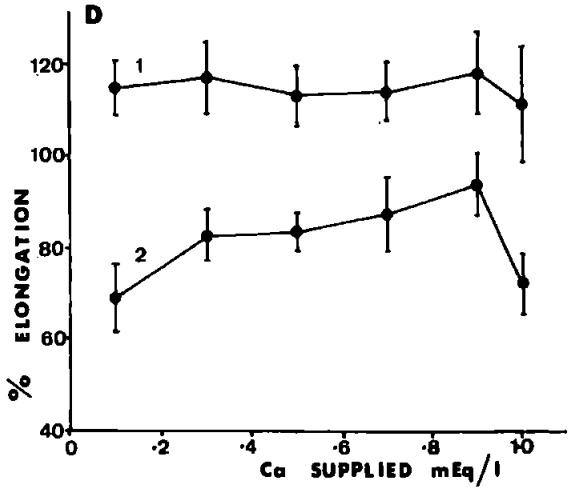

D
% ELONGATION
120
100
80
60
40
1
2
0
·2
·4
·6
·8
1·0
Ca SUPPLIED mEq/l

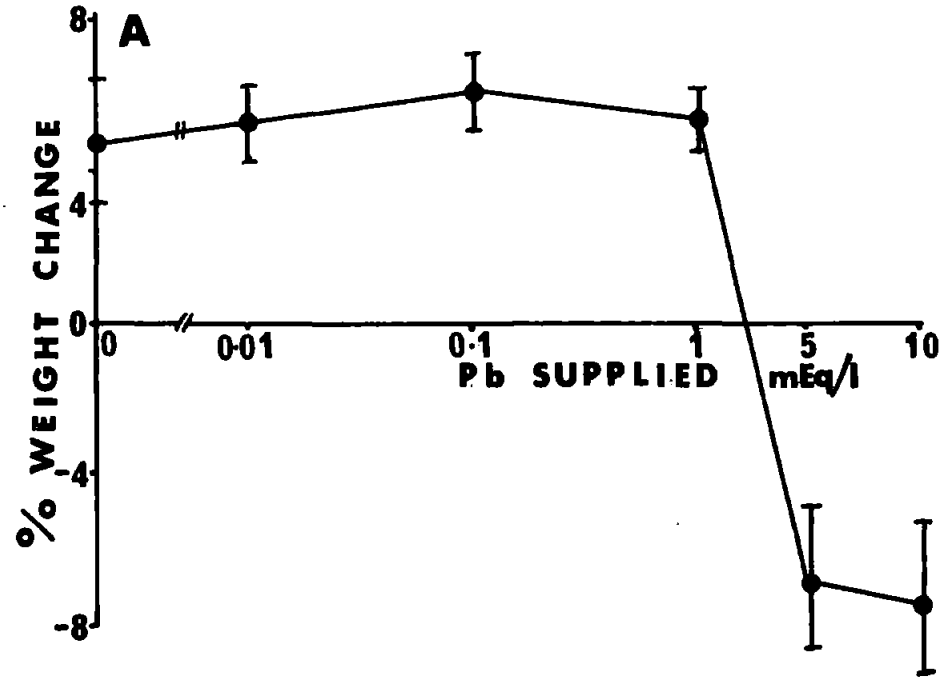
Figure 8.3.2 Effect of lead on IAA-induced water uptake and cell wall plasticity/elasticity

A. Effect of lead on IAA-induced water uptake in artichoke tuber discs (5 μg/ml IAA).

B. Curvature of coleoptile segments under constant inflexion load, following treatment with various concentrations of lead.

1. Total curvature
2. Elastic component
3. Plastic component

Vertical line = ± standard error


A
% WEIGHT CHANGE
8
4
0
-4
-8
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

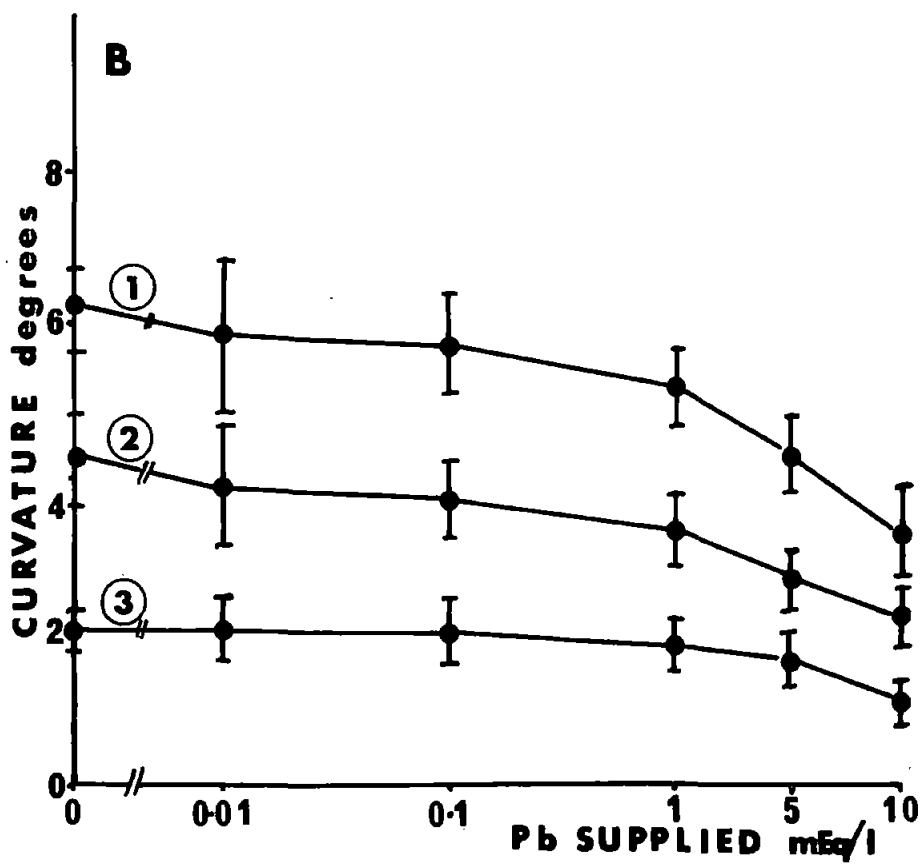

B
CURVATURE degrees
8
6
4
2
0
1
2
3
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

Figure 8.3.3 Absorbtion of lead by cell wall fractions

PS = pectic substances

PA = pectinic acid

L = lipids

HC/A = hemicellulose A

HC/B = hemicellulose B

HC/C = hemicellulose C

NCP = non-cellulosic polysaccharides

RC = residual cellulose

α-C = α-cellulose (commercial)

Vertical line = ± standard error

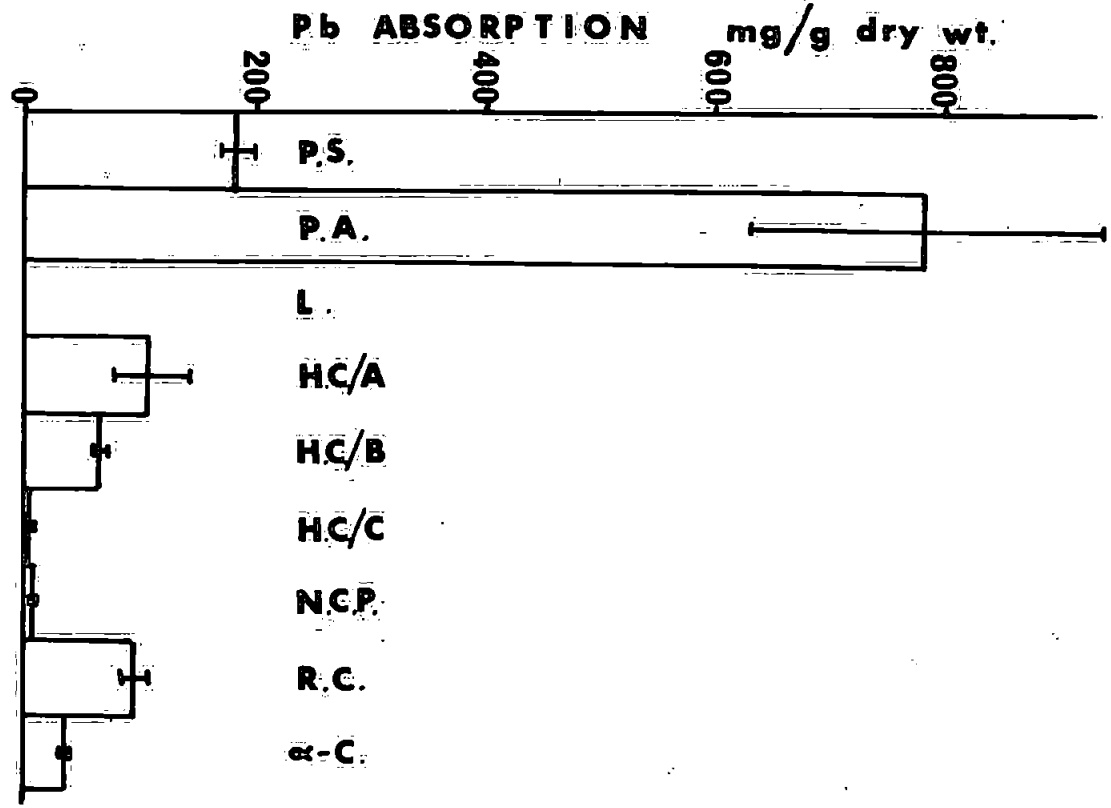

Pb ABSORPTION mg/g dry wt.
0
200
400
600
800
P.S.
P.A.
L.
HC/A
HC/B
HC/C
NCP.
R.C.
α-C.

Figure 8.3.4 Ultraviolet absorbtion spectra of cell wall fractions before and after addition of 10 mEq/l $Pb(NO_3)_2$

A. Pectic substances

B. Lipids

C. Non-cellulosic polysaccharides

D. Hemicelluloses

A_1-D_1 Uncontaminated samples

A_2-D_2 Samples containing same concentration of cell wall fraction after addition of lead nitrate solution.

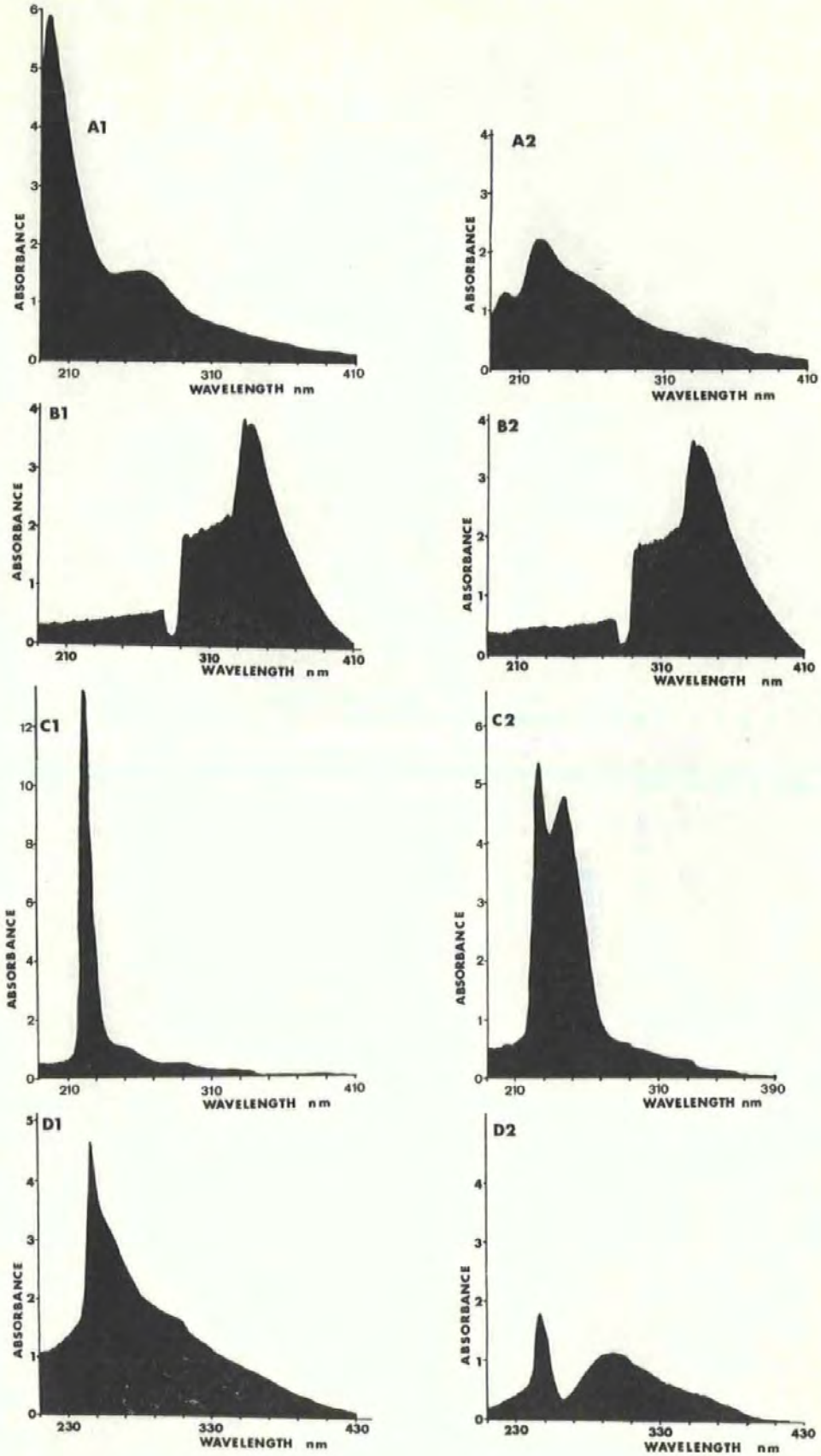
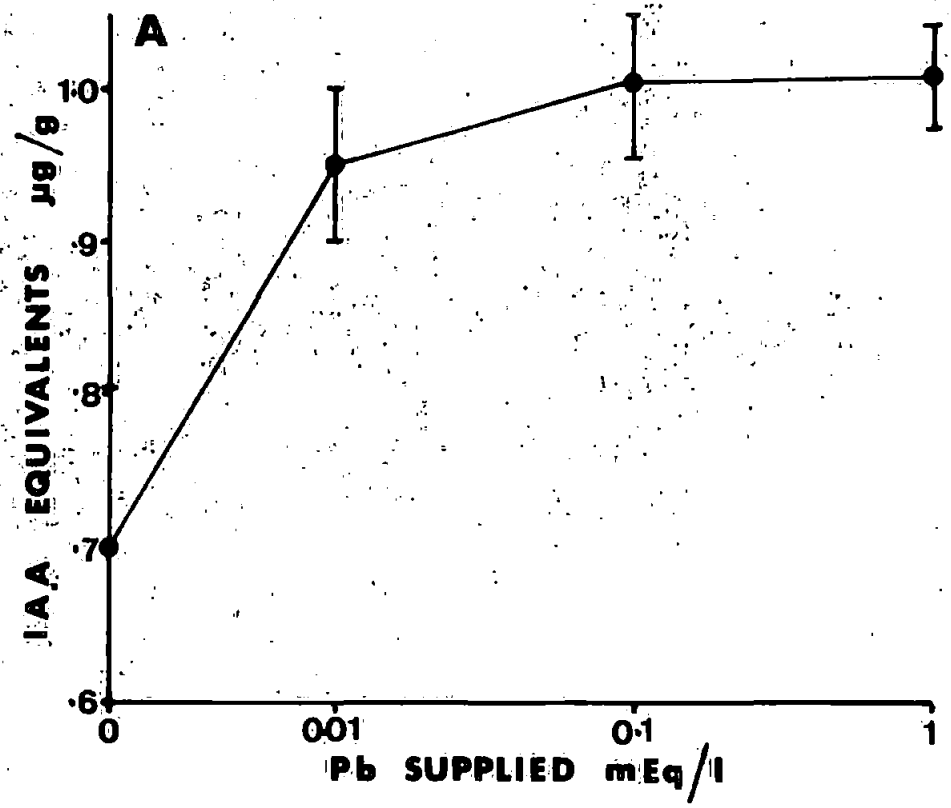

A1
A2
B1
B2
C1
C2
D1
D2
ABSORBANCE
WAVELENGTH nm
210
310
410
390
230
330
430

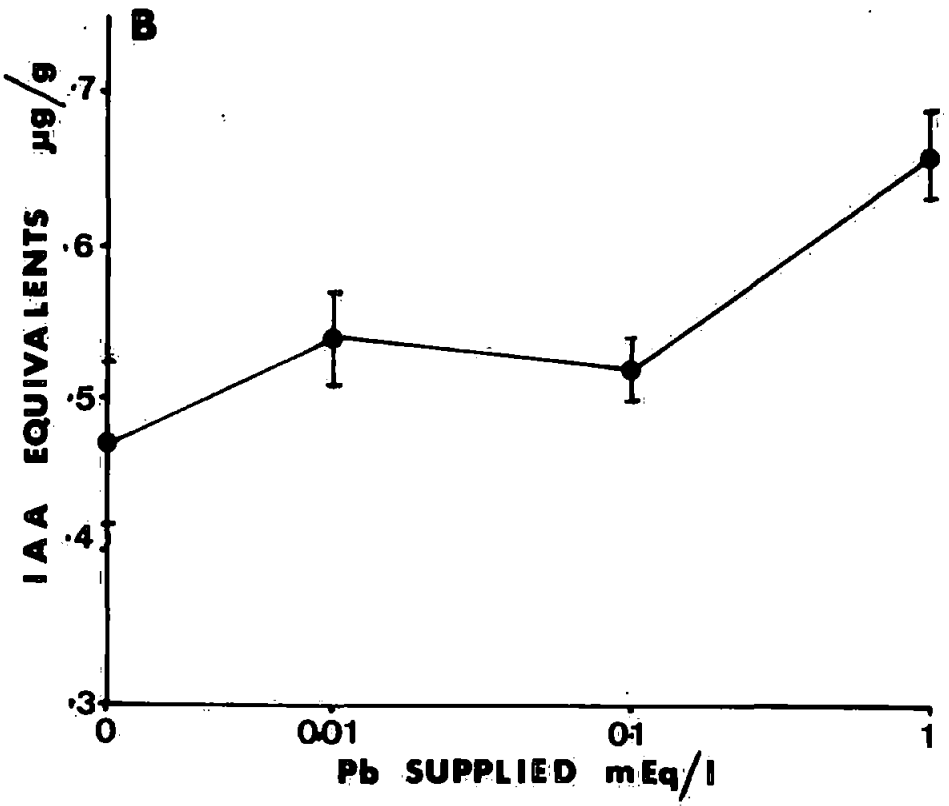
Figure 8.3.5 Effect of 3 days lead nitrate application on endogenous auxin levels in 7 day old radish seedlings

A. Colorimetric estimation using Ehrlich's reagent

B. Estimation using wheat coleoptile bioassays

Vertical line = $\pm$ standard error


A
IAA EQUIVALENTS µg/g
1·0
·9
·8
·7
·6
0
0·01
0·1
1
Pb SUPPLIED mEq/l


B
IAA EQUIVALENTS µg/g
·7
·6
·5
·4
·3
0
0·01
0·1
1
Pb SUPPLIED mEq/l

CHAPTER 9

CONCLUDING REMARKS

Lead is an element which has many important industrial applications, but its main interest from a biological standpoint lies in its potential toxicity to living systems. While much popular literature has been directed to the topic of lead poisoning in animals, including man (e.g. Bryce-Smith, 1971; Williams, 1971; Which?, April 1973; Adler, 1973) the study of lead in plants has been the source of less attention.

The experiments carried out during the course of this study have covered a number of subject areas and have entailed the use of a wide range of techniques. They have been intended to clarify the processes by which lead moves into and through the plant, and to provide information about the effect of lead on the plant at a physiological level.

The studies have shown that the seed exposed to lead is largely protected from metal contamination by the physical and chemical nature of the testa until such time as the testa is ruptured by the developing radicle. Once the embryo is exposed to the metal, however, contamination occurs which is both rapid and extensive. Very heavy contamination occurs in the root meristem region, but conversely the stem meristem remains relatively lead-free. A similar situation exists in radish seedlings exposed to root-applied lead.

In differentiated root tissue lead movement is primarily apoplastic, occurring predominantly along the radial cortical cell walls. Histochemical and X-ray microprobe studies have shown that the endodermis functions as a partial barrier to lead movement into the

stele, and as a result deposits of lead accumulate in the inner cortex. This function exists even when the endodermis is still in its primary condition, although in very young tissue the endodermis is penetrated by cells apparently lacking a Casparian strip and which, therefore, afford a means of entry of lead into the stele cells. In older tissue the endodermis may be physically disrupted by the emergence of lateral roots, allowing essentially unrestricted movement of lead into the stele at this point.

Broadly speaking, three possible pathways for ion migration across the cortex have been proposed: movement through the apoplast, movement through the symplast, and movement from vacuole to vacuole (see Bowling, 1976). Apoplastic transport is today almost universally accepted as the major mechanism of ion transport across the cortex, and the results of the present experiments are in agreement with such a pathway. Electron microscope studies have, however, shown that lead may enter the cell, and so it is feasible that limited symplastic transport may also occur. However, apoplastic contamination is such that any symplastic transport would be small in comparison to the total migration of lead in the cortex, although at the endodermis a different situation exists. Here apoplastic movement is prevented by the Casparian strip, and so the pathway must become 'symplastic'. This term may only be applied in a general fashion, as the present experiments have shown that lead contamination causes a sharp increase in membrane permeability, and eventually leads to membrane breakdown. Such an effect at the endodermis would render the endodermis 'leaky', and would have grave effects on the trans-root potential/endodermal resistor system of ion uptake discussed by Bowling (1973, 1976) as

a mechanism for ion movement into the stele. The nature of the changes which occur in the cell membranes in response to lead are unknown at present, but it is feasible that the metal could combine with phosphoryl, carboxyl, imidazole or sulphydryl groups within the membrane (Rothstein, 1959).

Within the stele cell wall lead deposits may still be observed, but these are far less dense than those present in the cortex. In general terms deposits throughout the stele are fairly uniform, with the notable exception of the vessel elements, where lead crystals are found in dense superficial deposits in association with secondary thickening, and of the protophloem elements during the early stages of development. Movement of lead into the stem occurs virtually exclusively in the vascular tissue, particularly the xylem, and migration of lead into the surrounding stem tissue is slight. In this fashion lead may be conducted into the leaves, and appears to accumulate predominantly in older tissues, presumably because of their greater surface area and hence greater water flux. In young seedlings extensive contamination may lead to the formation of local accumulations in the cotyledon leaves which may give rise to necrotic lesions, but in general the distribution of lead becomes more limited as the distance from the root increases. This reduction is reflected in the concentrations of lead measured in the roots, stems and leaves of plants grown in hydroponic culture.

At a subcellular level lead appears to be similarly pervasive. In contaminated tissue, deposits of lead are clearly visible within the cell wall, but the metal is capable of movement across the plasmalemma, although in the root cortex cytoplasmic deposits of lead are far less extensive than intramural deposits. Within the cytoplasm

the major reaction to lead appears to lie in membrane disruption which becomes more extensive as contamination increases. In the initial stages of contamination the most common site of lead deposition appears to be within the endoplasmic reticulum. This appears to produce vesicles with which the lead deposits are intimately associated, and which subsequently fuse into the vacuole. It is feasible that such a process could render innocuous small amounts of toxic contaminants of the cytoplasm. Heavy cytoplasmic contamination results in extensive vesicle formation, and eventually the membrane structure of the cell breaks down completely. Certain organelles are more susceptible to this process than others, and in the leaves the chloroplasts seem to be amongst the last structures to lose their structural integrity.

From a physiological standpoint lead is a potent and far-reaching modifier of plant development, and the effects of lead contamination may be seen in a number of processes including growth, chlorophyll synthesis, water and ion uptake and cell permeability.

It may be considered that such diverse changes in the physiology of the plant might be best explained by an effect of lead on some basal metabolic process such as respiration, particularly in view of the changes in iron uptake which have been recorded and the possible effects that might arise from disruption of iron metabolism. Indeed, a number of authors have reported heavy metal-induced alterations in respiration. In Aspergillus niger reductions in carbon dioxide evolution produced by copper, mercury and silver have been reported (Cook, 1926). Experiments using mitochondrial suspensions have shown that lead inhibits succinate oxidation (Koeppe and Miller, 1970), while Lee et al. (1976) found that contamination of soybean (Glycine max)

seedlings by cadmium increased respiration rate. In the course of the present studies examinations of the effect of lead nitrate on oxygen uptake were carried out. These have shown that in radish root material exposure to lead brings about a reduction in oxygen uptake which appears to arise at least in part in the Kreb's citric acid cycle/electron transport phases of respiration (See Appendix 1.8). However, even at the highest lead concentrations employed substantial oxygen uptake still occurs. Although the adverse effect of lead on respiration must be reflected in the plant's overall productivity, it seems likely that other factors must come into play.

Reductions in growth are perhaps the most obvious outward symptom of lead burden within the plant. Significant reductions occur in all the parameters of plant growth, and their magnitude appears to be related to the uptake of lead by the organ in question. Root growth seems to offer the best indication of lead toxicity, as the development of the upper parts of the plant may also be affected by other factors such as disturbances in water and mineral supply. Within the stem tissue a major effect of lead may be observed in the process of IAA-induced cell elongation, which appears to be connected with a partially reversible increase in cell wall rigidity, arising probably from a chemical reaction between lead and pectin and/or hemicellulose bridges in the wall structure.

In addition at high supply levels (5 and 10 mEq/l) lead exerts an influence on the process of IAA-induced water uptake. A similar influence may be observed on the water status of the whole plant, and it is likely that this is attributable to the changes in membrane permeability and integrity discussed earlier (vide supra).

In the root the situation is more complex. A reduction in cell division is apparent, and from the results of the growth experiments it would be suspected that an effect on root elongation might also exist. Considering the observations of reduced iron uptake in the presence of lead it is interesting to note that cessation of cell division has also been reported in iron-deficient plants (Brown and Possingham, 1957).

The mechanisms involved in root elongation have not been clearly defined at present. Auxins have been detected in the root in small amounts, principally in stelar tissue (Bowen et al., 1972; Bridges et al., 1973). Bowen et al. (1972) believed that the principal movement of auxin in roots occurred in an acropetal direction, but subsequent work by Davies et al. (1976) has demonstrated the production of IAA in the root cap and its subsequent migration into the growing zone, where they believe it acts as a growth inhibitor. An inhibitory effect of IAA in roots, this time mediated by ethylene synthesis, was also proposed by Chadwick and Burg (1967). A third alternative has been put forward by Greenwood and Yčas (1975) who proposed the theory that stelar IAA is not involved in root elongation at all, but that it in some way controls root cell differentiation. However, it has long been accepted that IAA at very low concentrations does exert a stimulatory influence on root elongation (see Audus, 1972). Whatever mechanisms are involved in the control of root elongation, the factors of cell wall rigidity and water uptake must influence the resultant increase in cell length.

It has been suggested that the cell wall forms a sink for lead within the plant system, and that the plasmalemma may function as a barrier preventing contamination of the cytoplasm (Sharpe and

Denny, 1976). While this may be the case, it is apparent that the heavy cell wall contamination observed in the course of the histochemical studies has itself a serious debilitating effect on the plant, which could presumably extend to any physiological process in which cell changes are involved. It would seem likely that some feedback mechanism exists in the induction of stem cell elongation by IAA, for in response to lead application endogenous auxin levels increase, as if to overcome the increased stability of the cell wall structure.

In addition to the gross effects observed in the growth of the plant, lead appears to promote a number of changes in its metabolism, including reduction in water and ion uptake, and in chlorophyll synthesis. Changes in the water and ion status of the plant must arise in part from the changes in cell membranes which have been observed in response to lead burden, and perhaps more specifically to such changes in the root, particularly in endodermal cells. However the possibility must be considered that it is changes in the mineral status of the plant which mediate the effect of lead on membranes, for it has been demonstrated that changes in the calcium content of plant material may influence membrane permeability (Epstein, 1961; van Stevenick, 1965; Toprover and Glinka, 1976; Morré and Bracker, 1976).

Chlorosis becomes apparent only at relatively high supply concentrations of lead. Chlorophyll synthesis may be affected by numerous factors, including genetic changes, lack of oxygen, mineral status, water stress, infection, etc. Hewitt (1963), in a discussion of heavy metal-induced iron deficiency, has discussed the possibility that the symptoms of chlorosis which may be observed in plants subjected to heavy metals could arise from the formation of

metallo-organic complexes, the metals competing with iron at a site in the chlorophyll synthesis pathway normally occupied by iron. While this is possible, particularly in view of the reduction observed in iron uptake, other possibilities may be considered. It may be that the chlorosis is not an effect of iron inbalances but is a reflection of other physiological abnormalities in the plant, such as water stress. Also, work carried out on haem synthesis in animals has suggested another possibility.

It is thought that the early stages of haem synthesis and chlorophyll synthesis, at least as far as Protoporphyrin IX, are identical (see Goodwin and Mercer, 1972). The pathway involved has been worked out mainly in animals, but most of the steps involved have subsequently been demonstrated in plants. The steps involved in the latter stages of the formation of Protoporphyrin IX, and the formation of its magnesium complex Magnesium-proporphyrin IX, are not fully understood but the possibility has been discussed that an iron-protoporphyrin IX complex might form an intermediate step, and that such an intermediate would be a possible site of heavy metal-iron interaction (Hewitt, 1963). An enzyme, iron chelatase, which incorporates iron into protoporphyrin IX has been derived from animal tissue and from photosynthetic bacteria, but has not been reported in higher plants (Goodwin and Mercer, 1972). However, an alternative site of action of lead exists earlier in the pathway. At this stage two molecules of δ-aminolaevulinic acid (ALA) are condensed by ALA dehydrase to form porphobilinogen, the immediate pyrrole precursor of the porphyrins (Gibson et al., 1955). This enzyme is activated by sulphydryl groups such as glutathione as cysteine (Granick, 1967) and the ability of lead to combine with sulphydryl groups is well known (e.g. Hammett, 1928d; Rothstein, 1959).

Moreover, more recent studies have demonstrated that lead exerts an inhibitory effect on glutathione synthesis in rat liver (Hsu and Anthony, 1975). Therefore the possibility of an interaction between lead and δ-ALA dehydrase cannot be ignored. Such an interaction would also be of importance in cytochrome synthesis.

A third possibility exists in that the observed chlorosis may be a reflection of the effect of lead on chloroplast structure. The instigation of chlorosis by inhibition of the structural development of the chloroplast has been discussed by Price (1970) and could be correlated with the alterations to thykaloid configurations noted in lead-treated leaf material in the electron microscope studies (see Chapter 6). It is possible that a number of factors contribute to the chlorosis observed in plants exposed to high levels of lead.

It is clear then that the toxic nature of lead in the plant system manifests itself in a number of processes intimately connected with the well-being of the plant. As these processes tend to be modified one by the other, it is impossible to be sure whether changes arise as a direct or indirect effect of lead contamination, although in all the cases described in the present study it is possible to extend theories which involve lead directly with the processes modified by its presence. In spite of this, two very definite and debilitating consequences of lead contamination may be recognised: firstly, an increase in the rigidity of the cell wall apparently caused by an alteration in the cross-linkages of the wall; and secondly, a disruption of cellular membranes resulting in changes in membrane permeability.

It is probable that further work on the topic of lead in plants will prove to be most productive in two areas of research. The most

obvious line of investigation entails the more accurate identification of the chemical changes which occur at a cellular level in response to lead pollution. In addition much information may be gained by the consideration of the effects of lead, in relation to the processes examined in the present study, on lead-tolerant plant species. More information must be obtained before a comprehensive picture of the effect of lead on plants can be produced; but it is hoped that the present study has described a frame within which the picture may be assembled.

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APPENDIX 1

SUPPLEMENTARY DATA

A range of data has been collected which, while not directly related to the main themes of this study, provide important correlative information to the main investigation.

1. Soil samples from preliminary site inspections.

Samples from these sites (Figure A.1.1) were collected from the surface 15 cm of the soil, sieved through a 2 mm soil sieve, and used in greenhouse growth experiments. Radish plants were germinated in the soil samples and grown on for a period of five weeks. At this time the plants were harvested, dried and weighed and analysed for lead content. Analysis of dried soil samples was also carried out.

For both soil and plant analysis the dry samples were ashed at 350°C for 3 hours in a Gallenkamp FSE 450 muffle furnace, then cooled and wet ashed in Normal nitric acid for one hour. Analysis was carried out using an 'Atomspek' atomic absorbtion spectrophotometer. Results of analyses and dry weight yields are represented in Figure A.1.2.

Figure A.1.1 Lead mine sites sampled in preliminary survey

a. O.S. map* showing location of Frankmill/Wheal Exmouth mine complex.

b. Plan of sample sites at Wheal Exmouth Mine.

c. Plan of sample sites at Frankmills Mine.

d. O.S. map* showing location of Callington United Mine complex.

e. Plan of sample sites at Holmbush Mine.

f. Plan of sample sites at Kelly Bray Mine.

g. Plan of sample sites at Redmoor Mine.

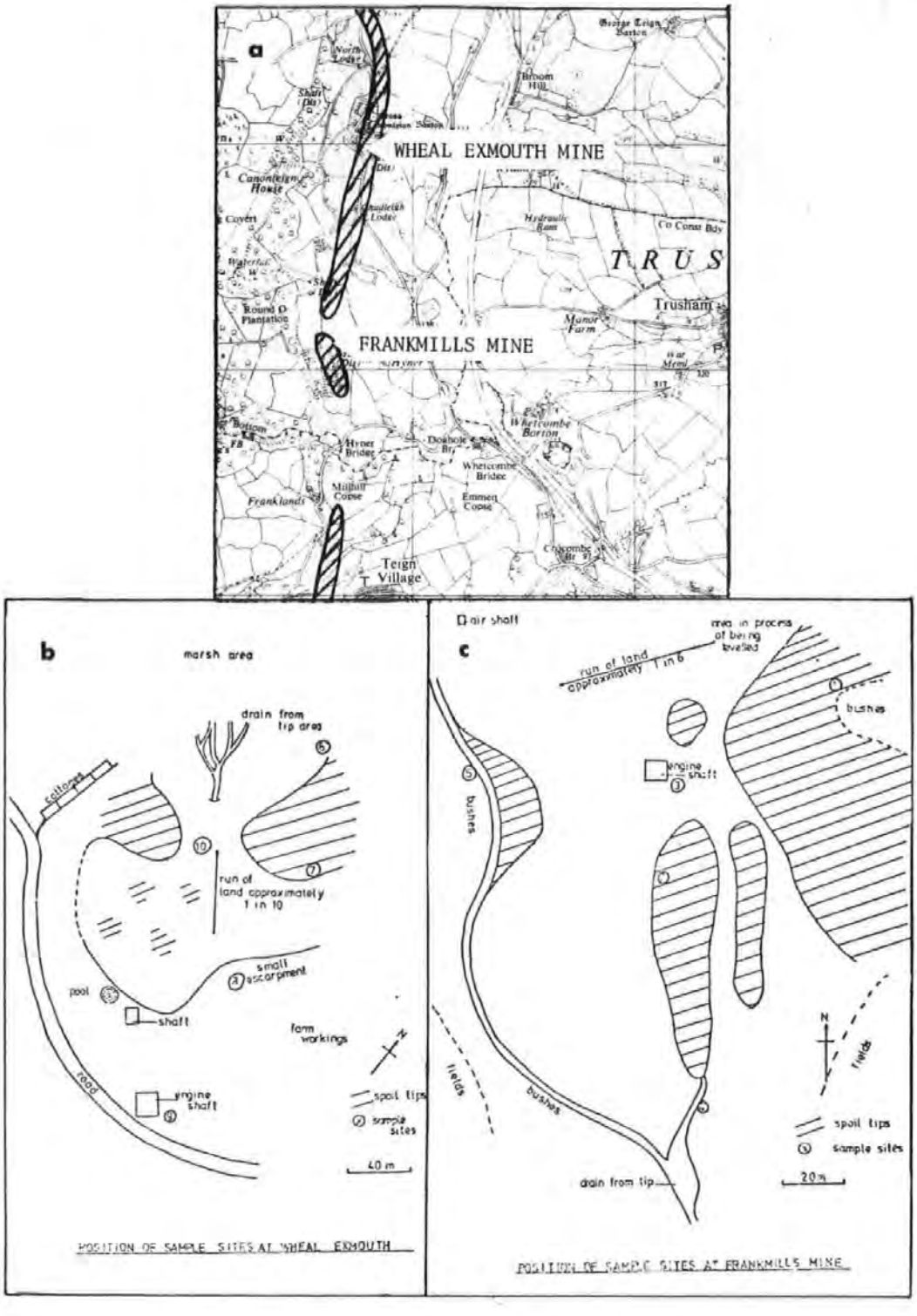
h. O.S. map* showing location of Wheal Betsy Mine complex.

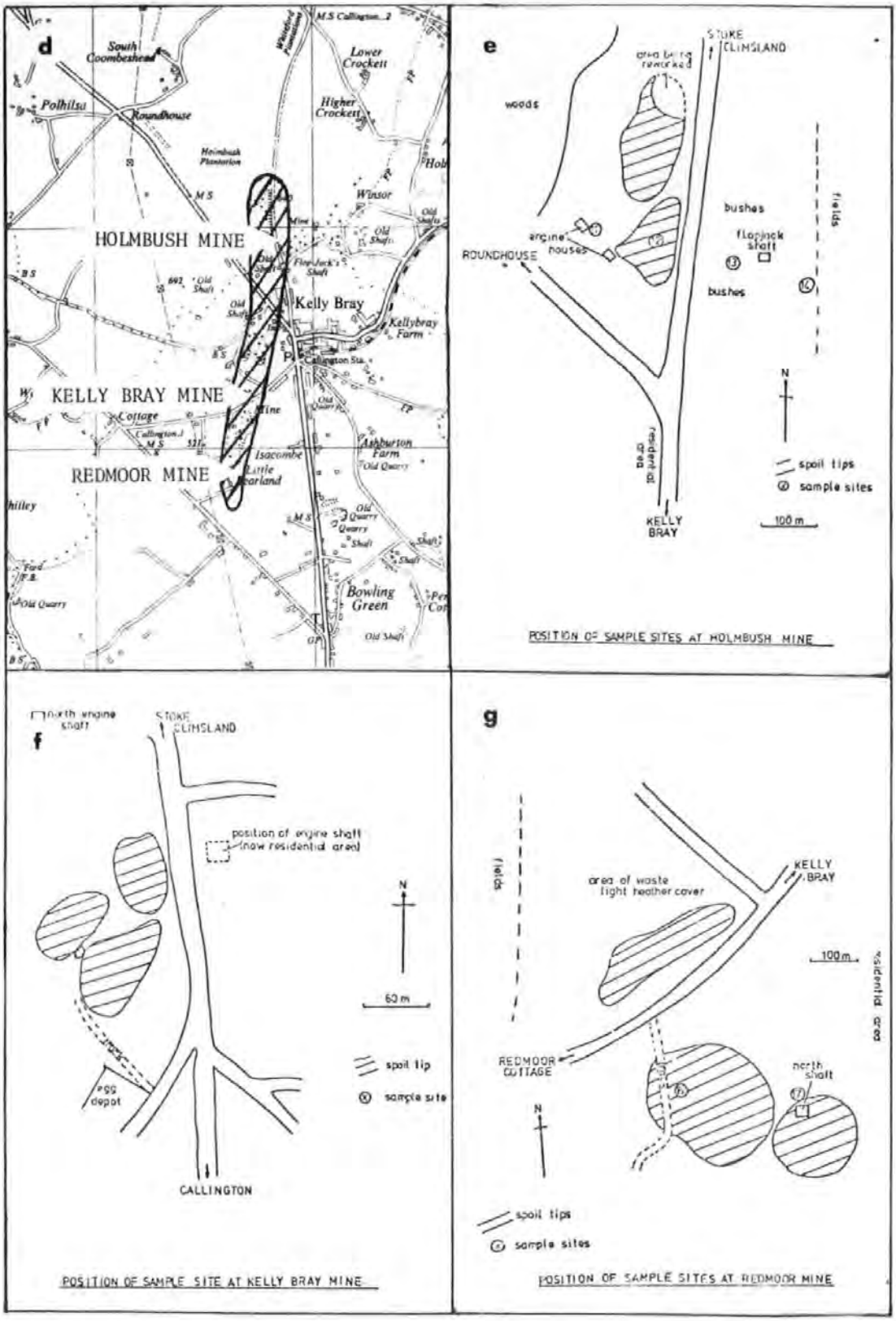
i. Plan of sample sites at Wheal Betsy Mine.

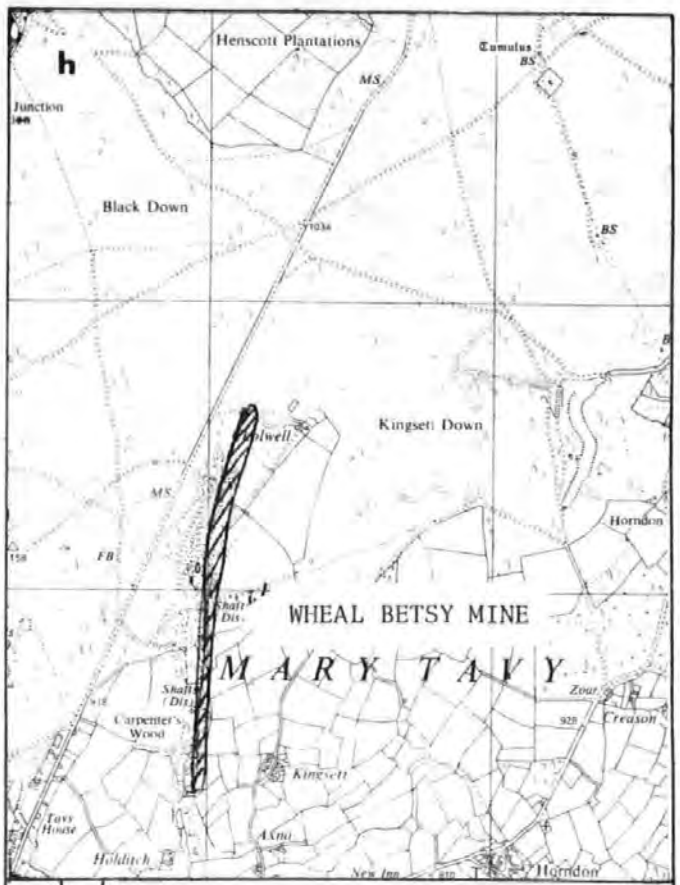
Shaded area represents position of lead lodes.

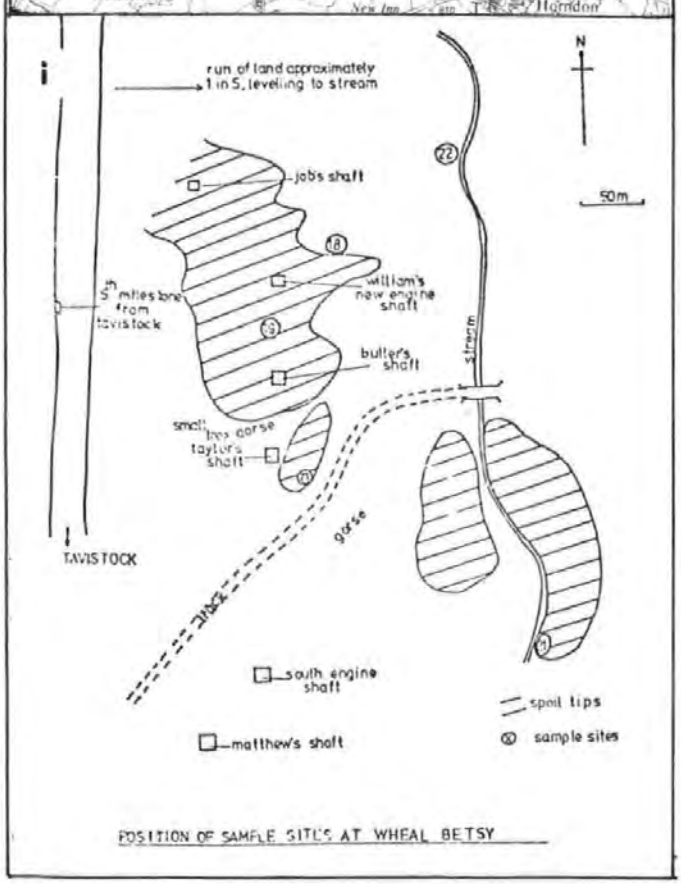
x represents location of sample sites.

*Based on Ordnance Survey maps S.X.58 (1969) S.X.37 (1964), S.X.88 (1968), 1:25000 series with the permission of the controller of Her Majesty's Stationery Office, Crown Copyright Reserved. Plymouth Polytechnic, Drake Circus, Plymouth, PL4 8AA.


a
WHEAL EXMOUTH MINE
FRANKMILLS MINE
TRUS
Trusham
Teign Village
Broom Hill
Canonteign House
Covent
Round O Plantation
Manor Farm
Whetcombe Barton
Hyner Bridge
Doshole Br
Whetcombe Bridge
Malpitt Copse
Emmett Copse
Franklands
Co Const Bdy
b
marsh area
drain from tip area
run of land approximately 1 in 10
small escarpment
pool
shaft
farm workings
engine shaft
road
spoil tips
sample sites
40 m
POSITION OF SAMPLE SITES AT WHEAL EXMOUTH
c
air shaft
area in process of being levelled
run of land approximately 1 in 6
bushes
engine shaft
fields
spoil tips
sample sites
drain from tip
20 m
POSITION OF SAMPLE SITES AT FRANKMILLS MINE


d
South Coombeshead
Polhilsa
Roundhouse
Lower Crockett
Higher Crockett
Hoimbush Plantation
Windsor
HOLMBUSH MINE
Flapjack's Shaft
Kelly Bray
Kellybray Farm
Callington Sta.
KELLY BRAY MINE
Cottage
Isacombe
Little Earland
Ashburton Farm
REDMOOR MINE
Bowling Green
Old Quarry
Old Shaft
e
STOKE CLIMSLAND
woods
area being remoded
bushes
fields
engine houses
flapjack shaft
ROUNDHOUSE
residential area
KELLY BRAY
N
spoil tips
sample sites
100 m
POSITION OF SAMPLE SITES AT HOLMBUSH MINE
f
north engine shaft
STOKE CLIMSLAND
position of engine shaft (now residential area)
N
60 m
spoil tip
sample site
POS depot
CALLINGTON
POSITION OF SAMPLE SITE AT KELLY BRAY MINE
g
fields
area of waste light heather cover
KELLY BRAY
100 m
residential area
REDMOOR COTTAGE
north shaft
N
spoil tips
sample sites
POSITION OF SAMPLE SITES AT REDMOOR MINE



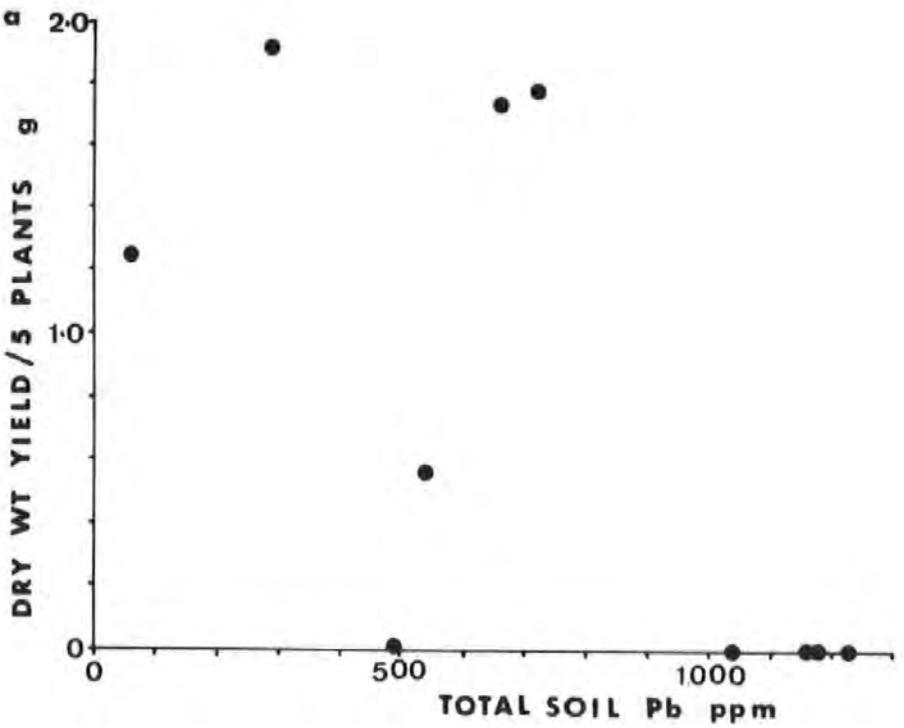


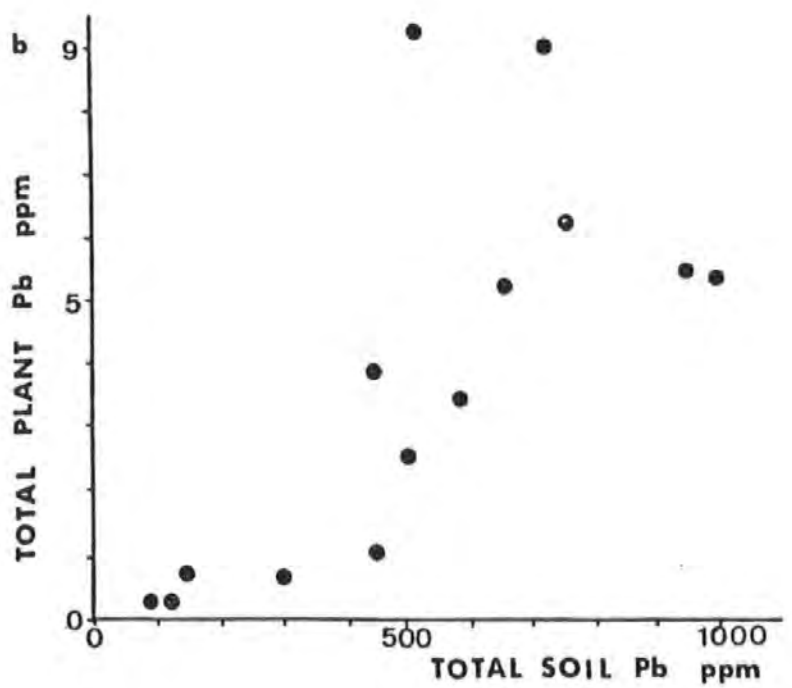
POSITION OF SAMPLE SITES AT WHEAL BETSY

Figure A.1.2 Analysis of plants grown on lead-contaminated soils

A. Dry weight yields of 5 week old radish plants grown on lead contaminated soils.

B. Total lead controls of 5 week old radish plants grown on lead contaminated soils.


a
2·0
1·0
0
DRY WT YIELD/5 PLANTS g
0
500
1000
TOTAL SOIL Pb ppm


b
9
5
0
TOTAL PLANT Pb ppm
0
500
1000
TOTAL SOIL Pb ppm

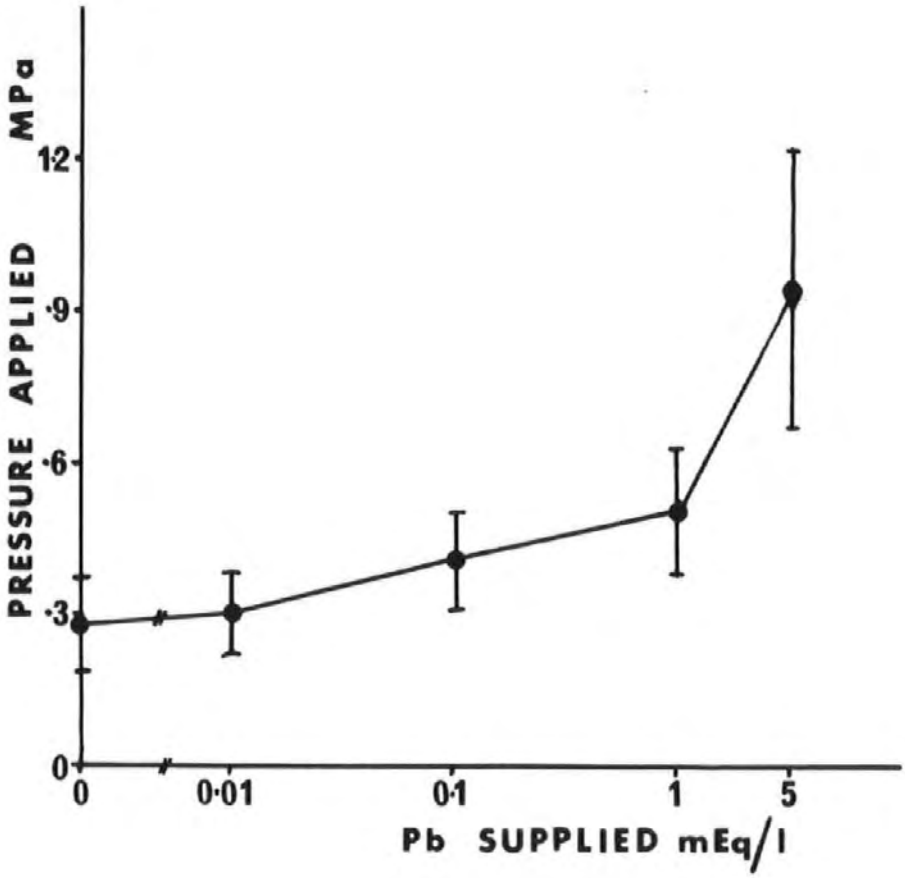
2. Leaf Water Potential of Hydroponically-grown Plants exposed to Lead.

Radish plants were grown hydroponically in Hoagland's solution as described previously (see Chapter 2). After a period of 5 weeks samples were taken for pressure-bomb determination of leaf water potential. Leaves were cut from each plant with a clean, sharp razor blade and transferred immediately to the pressure chamber of a PMS model 600 pressure bomb*. The chamber was pressurised with compressed air and the applied pressure at which sap began to appear on the cut petiole surface was recorded. At the 10 mEq/l treatment level it was found to be impossible to apply this treatment to the tissue, as the plants were too small; with 5 mEq/l treated specimens it was necessary to excise the plants at their junction with the root and use the entire aerial parts of the plant for water potential determination.

*PMS Instrument Company, 2750 N.W. Royal Oaks Drive, Cornvallis, Oregon, U.S.A.

Figure A.2.1 Leaf water potential of 5 week old radish plants grown in lead-contaminated hydroponic culture

Vertical line = $\pm$ standard error


PRESSURE APPLIED MPa
1·2
·9
·6
·3
0
0
0·01
0·1
1
5
Pb SUPPLIED mEq/l

3. Chlorophyll Synthesis in Lead-Treated Radish Seedlings

Seedlings were germinated on filter paper damped with deionised water and grown in the dark at 25°C for 7 days. At this time the seedlings were removed and the roots excised, illumination for this being provided by a green safelight (see Chapter 2). The stem and cotyledons were transferred to specimen bottles containing 10 ml of 0 - 10 mEq/l lead nitrate solution.

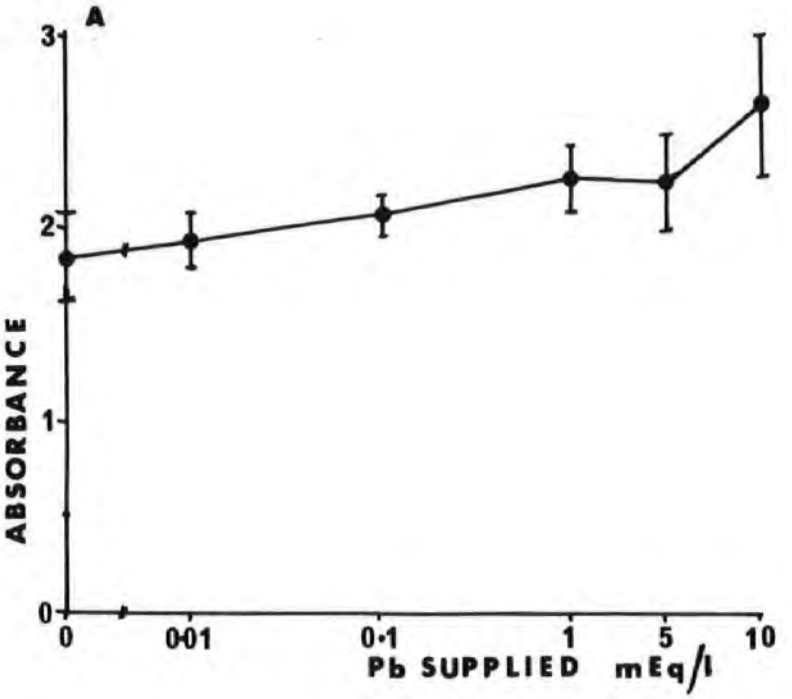
The seedlings were positioned 5 per bottle in such a manner that the cut ends were immersed in the test solution and the cotyledons did not overlap; the seedlings were held in place with a plug of non-absorbent cotton wool. The containers were returned to the dark incubators for a further 24 hours and subsequently placed under constant white illumination at 11,000 lux for a further 24 hours. The specimens were then removed and the cotyledons excised and weighed. These were then extracted in boiling refluxing 80% ethanol and chlorophyll content estimated by measuring the optical density of the extracts, cooled and made up to volume, at 660 ppm on a Cecil CE303 spectrophotometer. The values obtained were then corrected for tissue fresh weight (Figure A.3.1a). A parallel series was also set up in which the cotyledons were excised from the seedling at the junction with the stem and floated directly on 10 ml aliquots of lead solution in petri dishes. The cotyledons were subjected to light treatments and extraction as before (Figure A.3.1b).

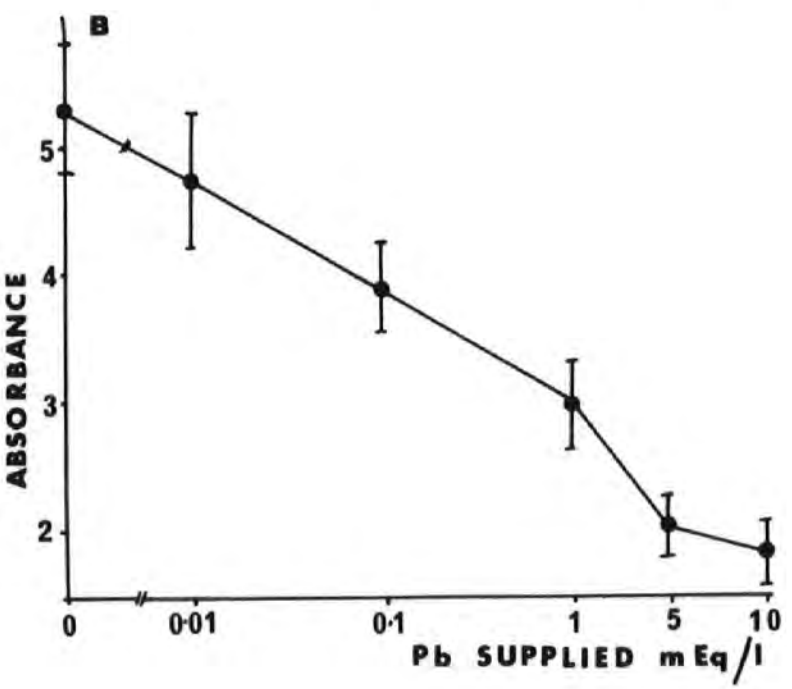
Figure A.3.1 Chlorophyll synthesis in lead-treated cotyledons

A. Chlorophyll synthesis in cotyledon/stem preparations. Absorbance of cotyledon extracts at 660 nm.

B. Chlorophyll synthesis in excised cotyledon preparations. Absorbance of cotyledon extracts at 660 nm.

Vertical line = $\pm$ standard error


A
ABSORBANCE
3
2
1
0
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
ABSORBANCE
5
4
3
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

4. Analysis of Culture Solutions.

During the course of measurement of nutrient levels in the Hoagland's solution employed in the growth analysis experiments, it was noticed that discrepancies existed in the figures recorded for lead-treated and control cultures at the end of each week run. Although analysis was carried out for calcium, copper, iron, magnesium, manganese, potassium and zinc (by atomic absorbtion spectroscopy or flame photometry as appropriate) consistent differences were observed only in the cases of calcium, iron, and possibly potassium. It was therefore decided to monitor residual calcium, iron, and potassium on a day to day basis during a plant growth period of seven days, using lead treatments of 0 and 1 mEq/l lead nitrate. The plants employed began the observation period at 14 days old, and 10 replicates of each treatment were analysed.

For the analysis 1 ml aliquots were withdrawn from the solutions under test and, after dilution as necessary, measured either with an EEL flame photometer (for calcium and potassium) or with an 'Atomspek' atomic absorbtion spectrophotometer (for iron). These analyses revealed significant differences in residual levels of calcium and iron ($P < 0.05$) but the differences observed were not found to be significant in the case of potassium. The results of the calcium and iron analyses are shown in Figure A.4.1.

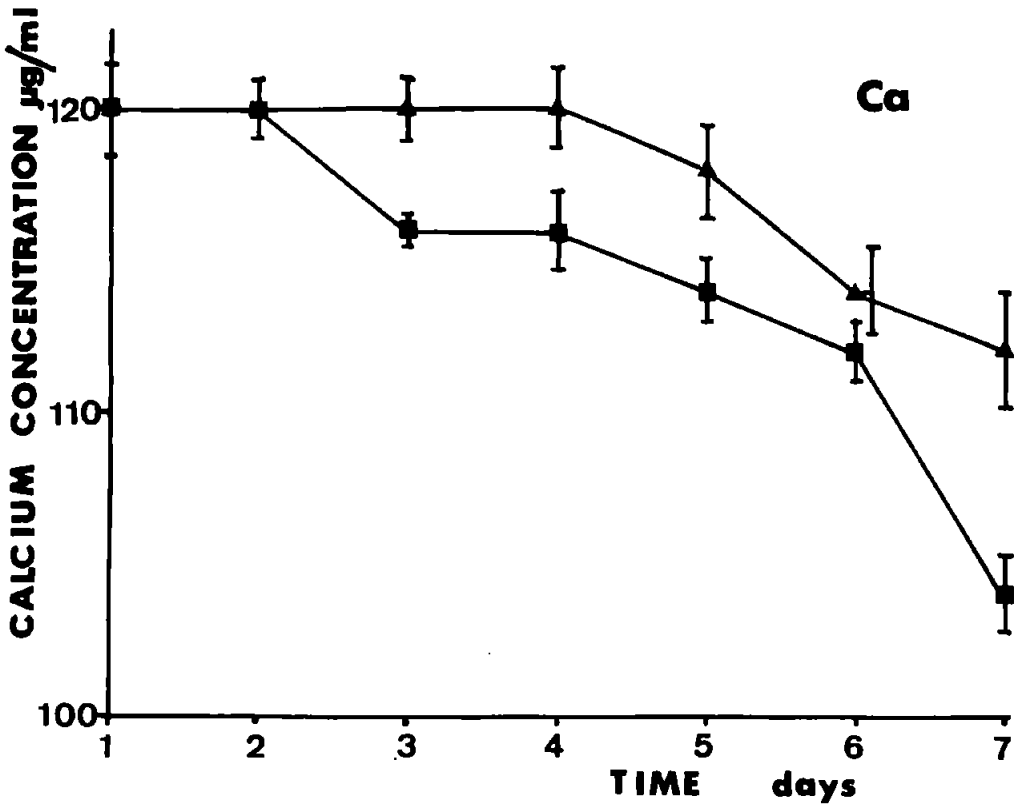
Figure A.4.1 Residual concentrations of calcium and iron in culture solution

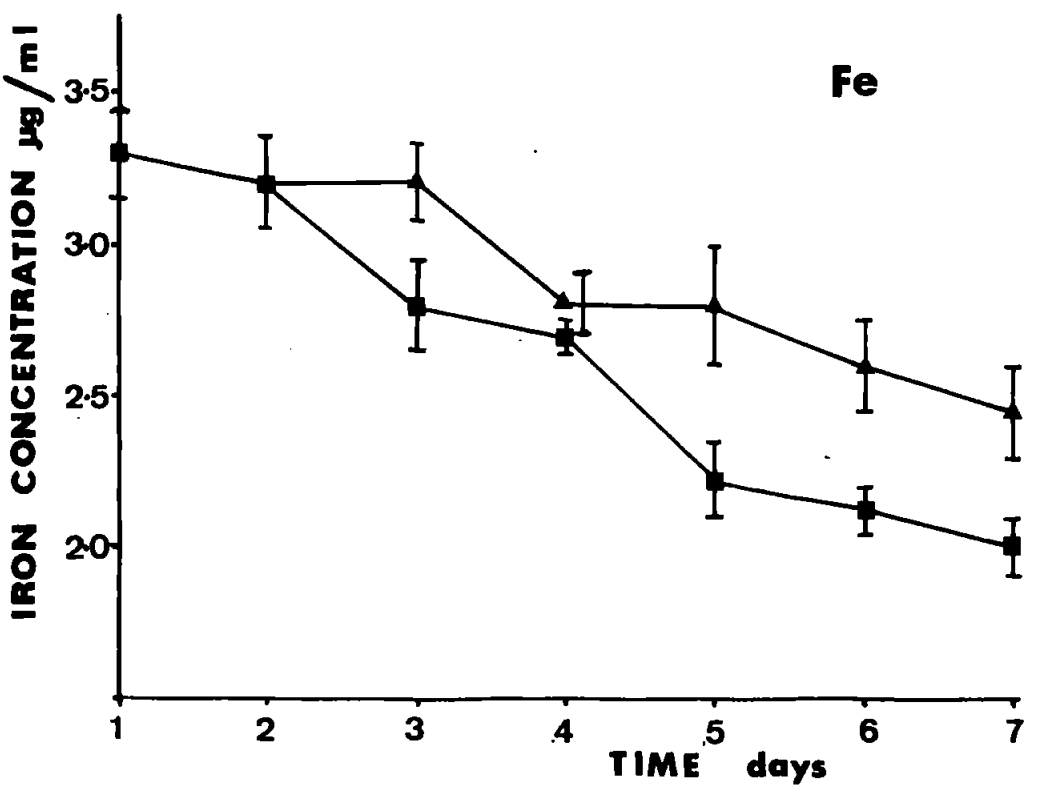
A. Residual calcium concentration during 7 day period in presence of 0 mEq/l and 1 mEq/l lead nitrate.

B. Residual iron concentration during 7 day period in presence of 0 mEq/l and 1 mEq/l lead nitrate.

■ - 0 mEq/l; ▲ - 1 mEq/l

Vertical line = ± standard deviation


Ca
CALCIUM CONCENTRATION µg/ml
120
110
100
1
2
3
4
5
6
7
TIME days


Fe
IRON CONCENTRATION µg/ml
3·5
3·0
2·5
2·0
1
2
3
4
5
6
7
TIME days

5. Examination of Chemical Interaction between Lead Nitrate and IAA

The coleoptile elongation experiments reported in Chapter 8 showed that the addition of lead to the IAA bathing solutions caused a reduction in elongation. It was considered possible that a direct chemical interaction between lead and IAA might occur and in order to examine this possibility IAA and lead nitrate/IAA mixtures were subjected to Nuclear Magnetic Resonance spectroscopy.

In this technique spectra are formed by the absorbtion of energy in the radio-frequency range by certain chemical nuclei exposed to a strong uniform magnetic field. The conditions under which such absorbtion occurs varies according to the chemical environment of the nucleus in question, and so provides a valuable research tool for the examination of chemical structures.

In the case of IAA two clear groups of peaks were observed, one corresponding to the indole ring structure and one to the acetic acid side chain. The addition of lead nitrate to the sample under test did not alter this spectrum, indicating that no substitution of atoms had occurred within the IAA molecule (Figure A.5.1).

That no direct chemical interaction occurred between IAA and lead nitrate was also indicated by paper chromatography of IAA and IAA/lead mixtures. Bands were identified with Ehrlich's reagent and with hydrogen sulphide solution, and although the Rf value for the IAA bond was reduced in presence of lead, there was no indication of lead presence in the IAA band, nor of indole presence in association with the heavy lead deposit remaining on the start line. A reduction in Rf value, as seen in the case of the IAA/lead mixture, is a frequent feature in chromatographs in which a large amount of substance is immobile in the solvent system employed (see Figure A.5.2).

Figure A.5.1 N.M.R. Analysis of lead nitrate/IAA mixtures

1. Saturated solution of $Pb(NO_3)_2$ in deuterated Dimethylsulphoxide (DMSO).
2. Saturated solution of IAA alone in deuterated Dimethylsulphoxide.

In each case chemical shift is measured relative to an internal standard of Tetramethylsilane.

Each trace shows a set of peaks corresponding to hydrogen atoms. The conglomeration of peaks at approximately 7 units represents the hydrogen atoms associated with the indole ring: the smaller peak at approximately 11 units represents the hydrogen atoms in the carboxyl group. The upper lines in each trace are peak integrations, and comparison of the amplitude of these lines gives an approximate indication of the number of hydrogen atoms represented in each peak or range of peaks.

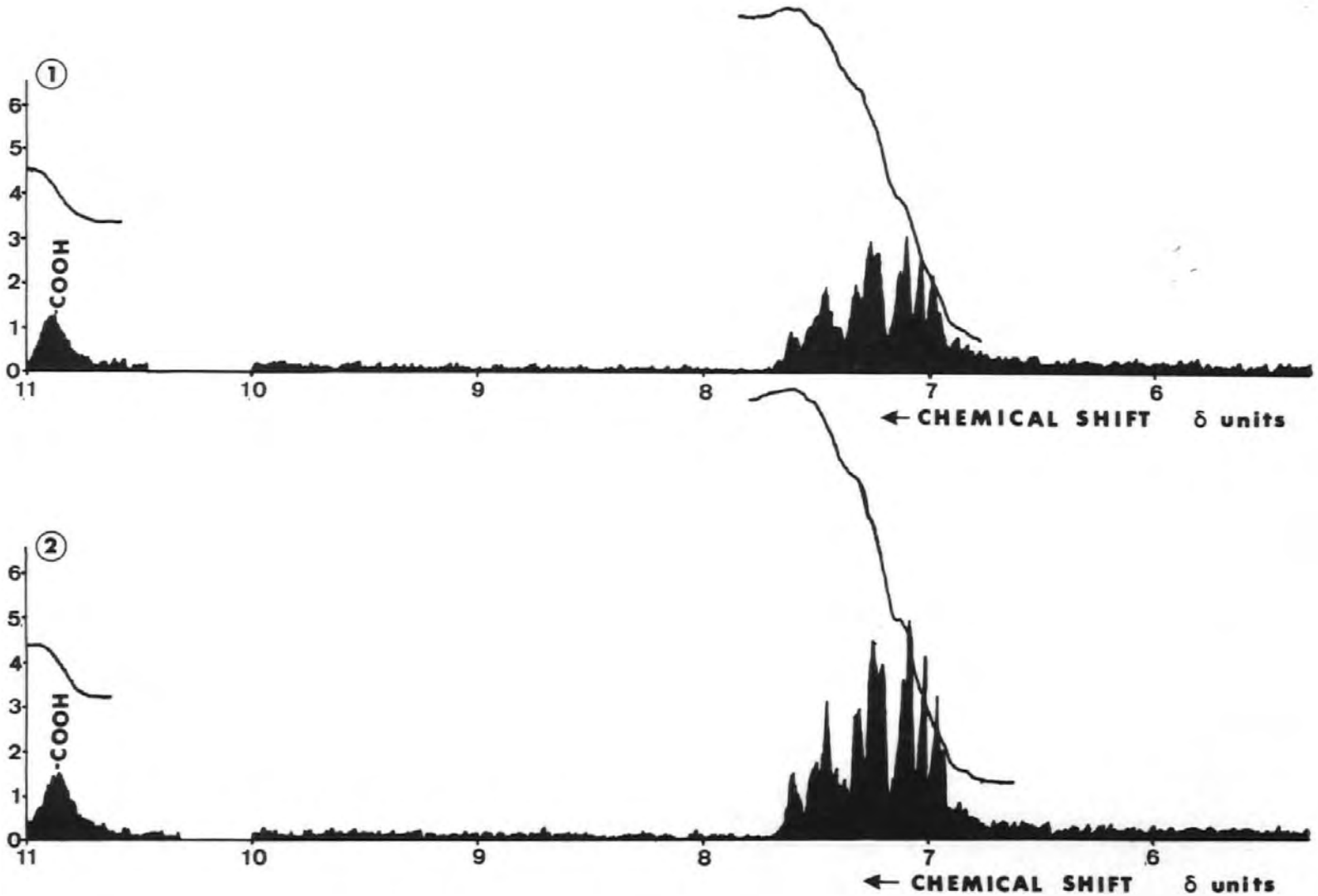
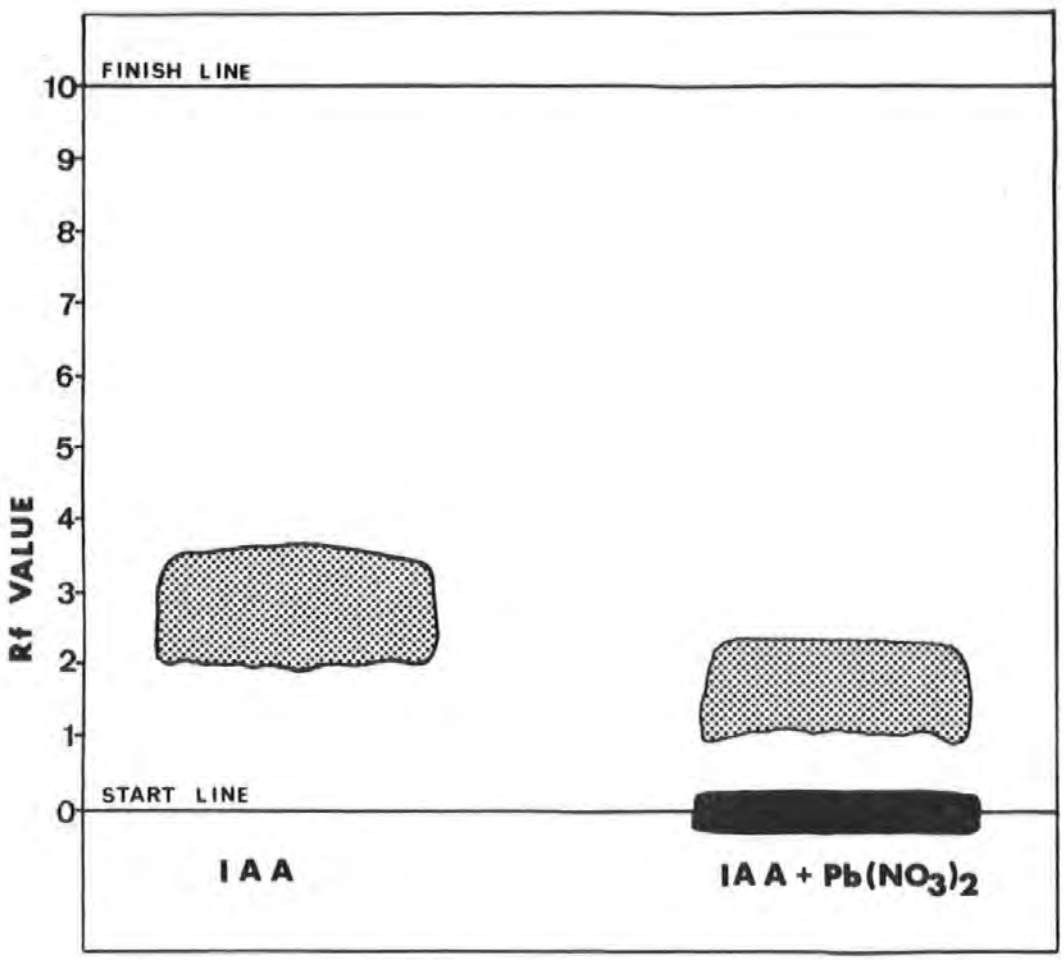

1
6
5
4
3
2
1
0
-COOH
11
10
9
8
7
6
← CHEMICAL SHIFT δ units
2
6
5
4
3
2
1
0
-COOH
11
10
9
8
7
6
← CHEMICAL SHIFT δ units

Figure A.5.2 Paper chromatography of IAA/lead nitrate mixtures

The left hand side of the start line was loaded with IAA alone; the right hand side with a mixture of IAA and lead nitrate. Chromatographs were developed in a 9:3:1 solution of isopropanol: water:ammonium hydroxide.

The shaded areas represent the IAA bands giving a positive reaction with Ehrlich's reagent. These bands also fluoresced under ultraviolet illumination (248 nm). The intense black area represents the only region of the chromatogram which gave a positive reaction with hydrogen sulphide solution.


FINISH LINE
10
9
8
7
6
5
4
3
2
1
0
Rf VALUE
START LINE
I A A
IA A + Pb(NO3)2

6. Estimation of the Effect of Lead on Membrane Permeability

Exosmosis of electrolytes was monitored after the manner described by Stiles and Stirk (1938).

Discs of radish leaf tissue, 7 mm in diameter, were cut and then washed in aerated deionised water for 24 hours. Discs were then placed in 50 ml samples of lead nitrate solutions. Ten discs were used in each replicate, and 5 replicates of each treatment were set up. The initial conductivity of each solution was measured using a Pye Unicam model E.7566/4 conductivity bridge with a Mullard E.7591/B conductivity cell. The samples were shaken at 20°C and 100 r.p.m. in a New Brunswick Psychrotherm Controlled Environment Shaker. Conductivity readings of the bathing solutions were taken at hourly intervals for 7 hours. Results were presented as the mean percentage increase in external solution conductivity. These showed that in deionised water a progressive release of ions occurred from the discs, while in 10 mEq/l lead nitrate solution rapid loss of ions occurred from the discs, suggesting a major reduction in membrane permeability in the presence of high concentrations of lead nitrate. This would be in agreement with the extensive membrane disruption observed in the electron microscope studies.

Figure A.6.1 Effect of lead on release of ions from radish leaf discs

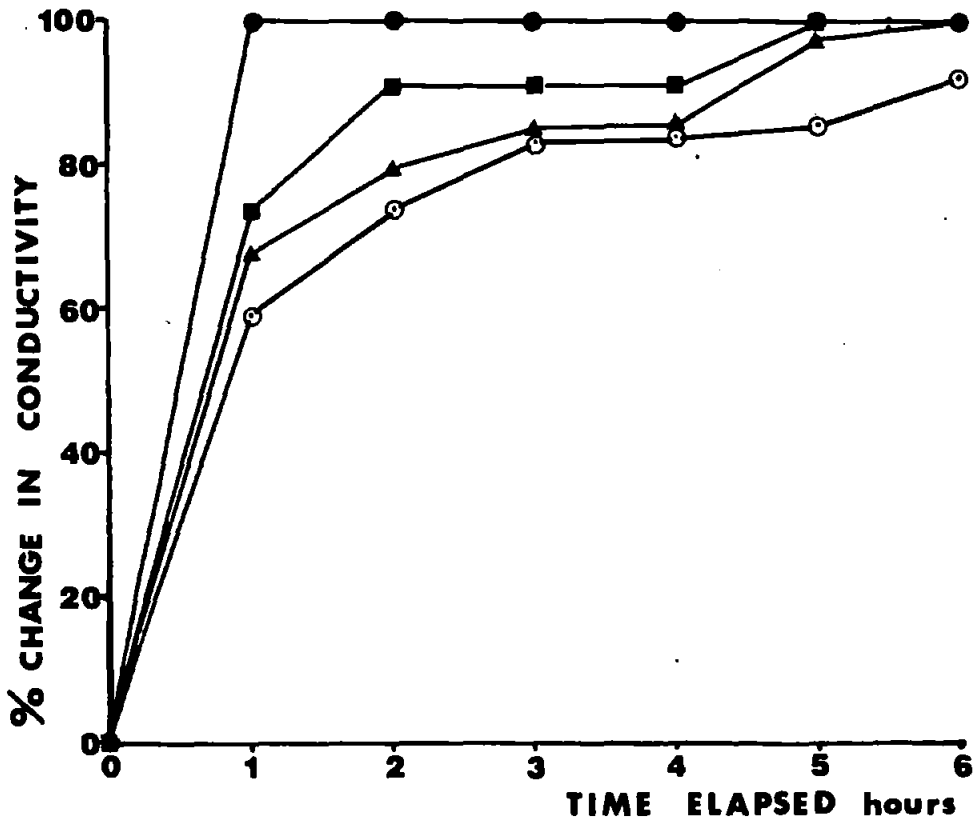
● — 10 mEq/l $Pb(NO_3)_2$

■ — 1 mEq/l $Pb(NO_3)_2$

▲ — 0.1 mEq/l $Pb(NO_3)_2$

⊙ — 0 mEq/l $Pb(NO_3)_2$

Individual points represent means of five replicates.


100
80
60
40
20
0
% CHANGE IN CONDUCTIVITY
0
1
2
3
4
5
6
TIME ELAPSED hours

7. The Effect of Lead Nitrate on Kinetin and Gibberellic Acid-Induced Cell Expansion

The reaction of gibberellic acid-induced elongation to lead contamination was examined using hypocotyl elongation bioassays.

Ten mm sections of hypocotyl were cut from 7 day old radish seedlings grown in the dark in deionised water. The sections, 10 at a time, were placed in solutions containing 1 μg/ml gibberellic acid and a range of concentrations of lead nitrate in petri dishes. Ten replicates of each treatment were set up. After three days incubation in the dark at 25°C the hypocotyl segments were re-measured with vernier calipers. The results showed that the provision of lead nitrate caused significant ($P < 0.001$) reduction in gibberellic acid-induced hypocotyl elongation; and are presented graphically in Figure A.7.1.

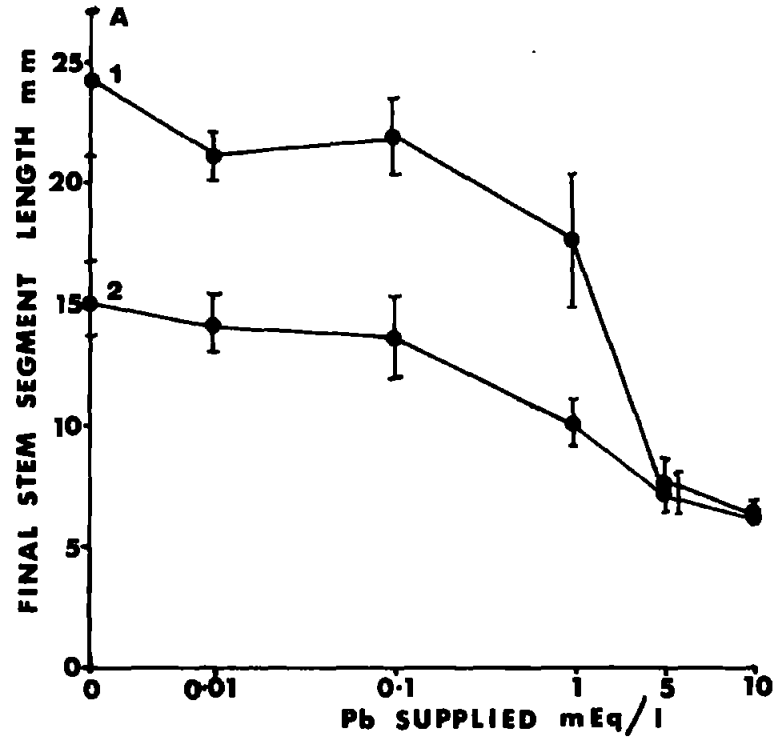
The reaction of kinetin-induced cell expansion to lead contamination was examined using coleoptile expansion bioassays. Cotyledons were excised from 30 hour old radish seedlings and transferred to petri dishes containing 1 μg/ml kinetin and a range of concentrations of lead nitrate. Ten cotyledons were placed in each dish, and 5 replicate dishes set up for each treatment. The dishes were placed under a constant illumination of 11,000 lux at 25°C, and after three days the cotyledons were removed, blotted dry and weighed. The results showed that lead nitrate caused a significant reduction in cotyledon expansion ($P < 0.001$) and are expressed graphically in Figure A.7.1.

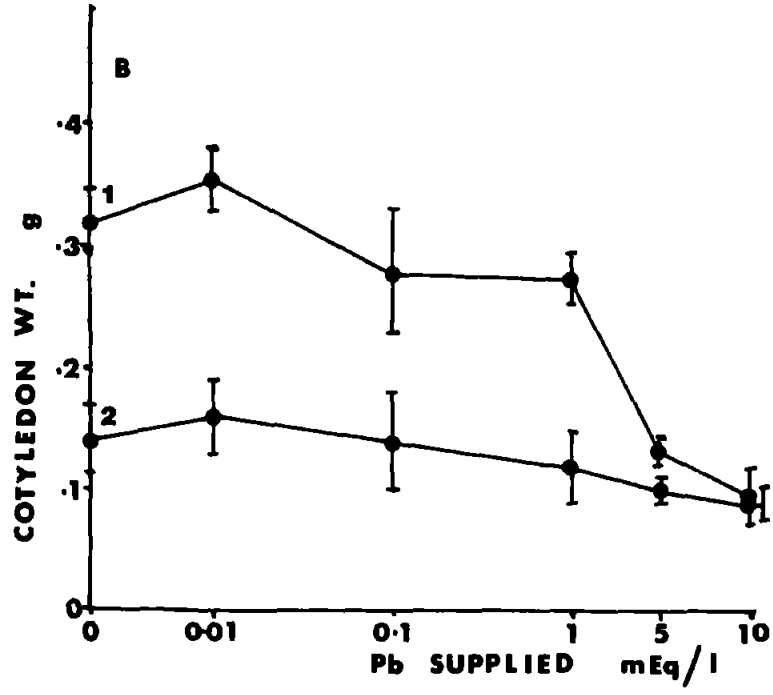
Figure A.7.1 Effects of lead on gibberellic acid- and kinetin-induced cell expansion.

A. Effect of lead nitrate on gibberellic acid-induced hypocotyl elongation.

B. Effect of lead nitrate on kinetin-induced cotyledon expansion.

Vertical lines = $\pm$ standard error


A
FINAL STEM SEGMENT LENGTH mm
25
20
15
10
5
0
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l


B
COTYLEDON WT. g
·4
·3
·2
·1
0
1
2
0
0·01
0·1
1
5
10
Pb SUPPLIED mEq/l

8. Effect of Lead Nitrate on Respiration

In order to investigate the effect of lead on tissue respiration measurements were taken of oxygen uptake by tissue immersed in lead solutions using a Gilson respirometer. Root samples were chosen as the experimental material because previous experiments had shown that the roots take up lead quickly, and in contaminated culture solutions show the highest accumulations of lead. Roots were excised from 7-day old radish seedlings and placed in a range of lead nitrate solutions in the reaction vessels of the respirometer. 0.4 ml of 50% potassium hydroxide solution was placed in the centre well with filter paper to absorb carbon dioxide. Measurements of oxygen uptake were taken at 15 minute intervals and corrected for temperature and atmospheric pressure using the formula supplied by the instrument's manufacturers:

$$K = \frac{(P_{at} - P_w)}{22.4 \times 760} \; \frac{273}{T_{lab}} + \frac{\alpha V_f}{V_g}$$

where K = correction factor

P_{at} = atmospheric pressure

P_w = partial pressure of water

T_{lab} = room temperature

α = solubility of oxygen at water bath temperature

V_g = volume of active flask

V_f = volume of liquid in active flask

At the conclusion of the experimental period the roots were washed and dried, and the oxygen uptake figures corrected accordingly.

The results showed that although oxygen uptake was reduced by increasing concentrations of lead, it continued at a substantial rate even in the presence of 10 mEq/l of lead (Figure A.8.1). In

subsequent experiments using only 0 and 10 mEq/l lead nitrate treatments oxygen uptake was monitored over an extended period (three days). The excised roots immersed in 10 mEq/l lead nitrate continued to respire for two days, while oxygen uptake in the control samples ceased during the following day (Figure A.8.2). The debilitating effect of lead on the root respiratory system could not be alleviated by the incubation of lead-treated roots in 0.01 mol/l sodium succinate solution, indicating that lead has an effect on Kreb's cycle or on the electron transport system. It does not, however, rule out the possibility of further effects in the earlier stages of the pathway prior to Kreb's cycle (Figure A.8.3).

Figure A.8.i Effect of lead nitrate on oxygen uptake by radish roots

Individual points represent means of 5 replicates

▲—▲ — 0 mEq/l

▲···▲ — 0.01 mEq/l

▲--△ — 0.1 mEq/l

●—○ — 1 mEq/l

●--● — 5 mEq/l

●····● — 10 mEq/l

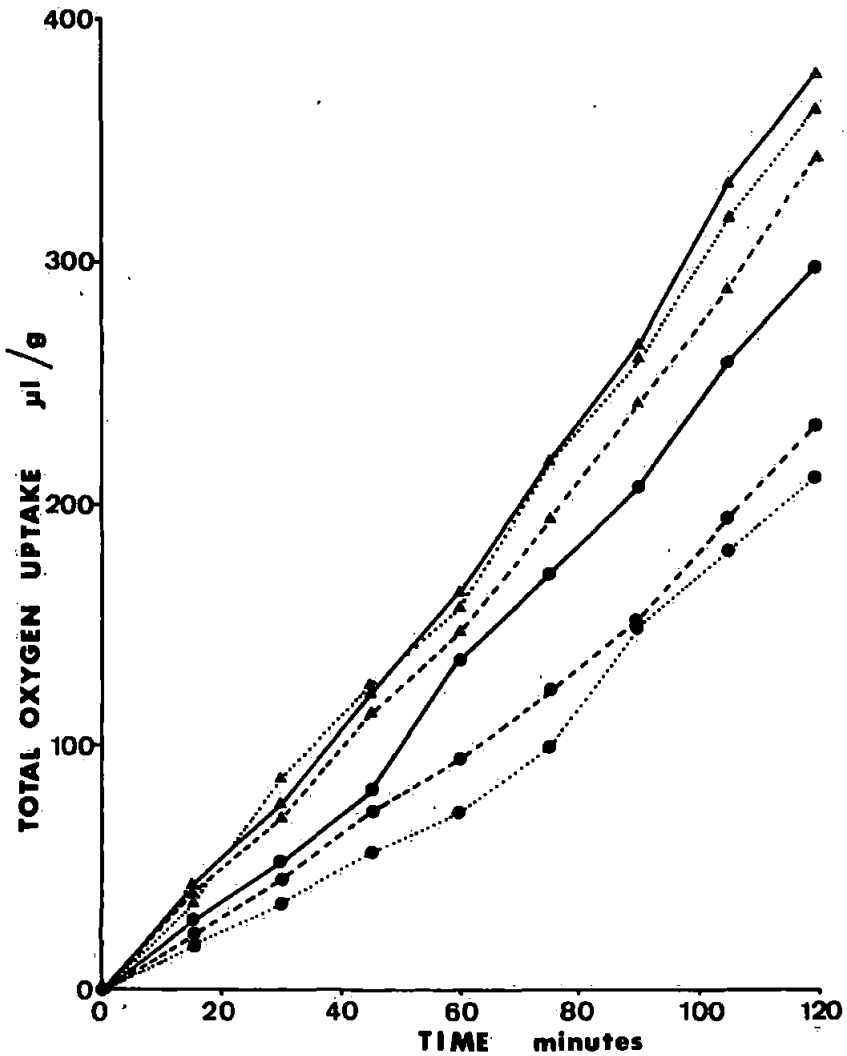

400
300
200
100
0
TOTAL OXYGEN UPTAKE µl /g
0
20
40
60
80
100
120
TIME minutes

Figure A.8.2 Effect of lead on oxygen uptake by radish roots over a period of three days

1. Control tissue
2. Tissue immersed in 10 mEq/l lead nitrate solution

Each point represents the mean of 4 replicates.

Vertical lines = $\pm$ standard deviation.

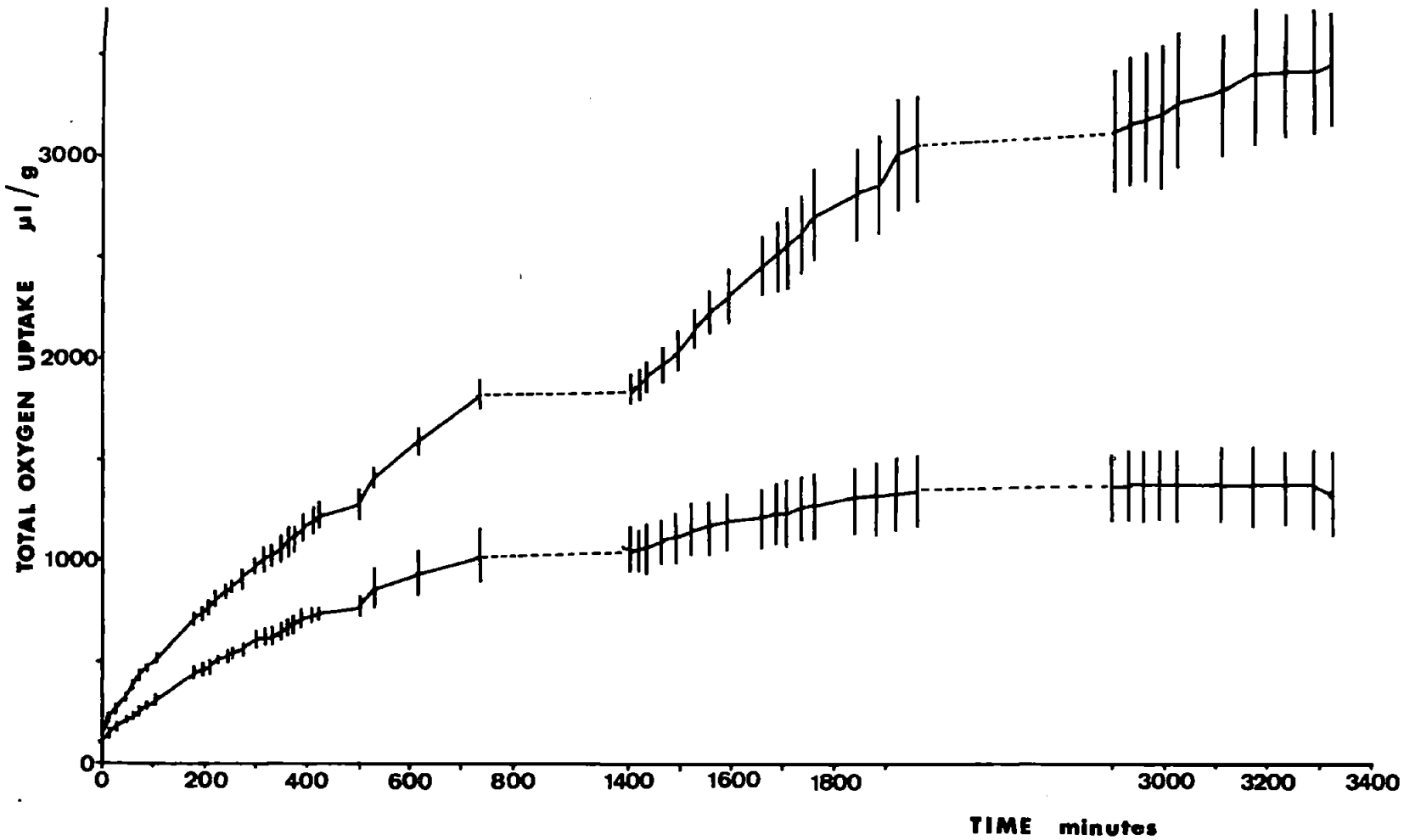

TOTAL OXYGEN UPTAKE µl/g
3000
2000
1000
0
0
200
400
600
800
1400
1600
1800
3000
3200
3400
TIME minutes

Figure A.8.3 Effect of 0.01 mol/l sodium succinate solution on lead-inhibited oxygen uptake in radish roots

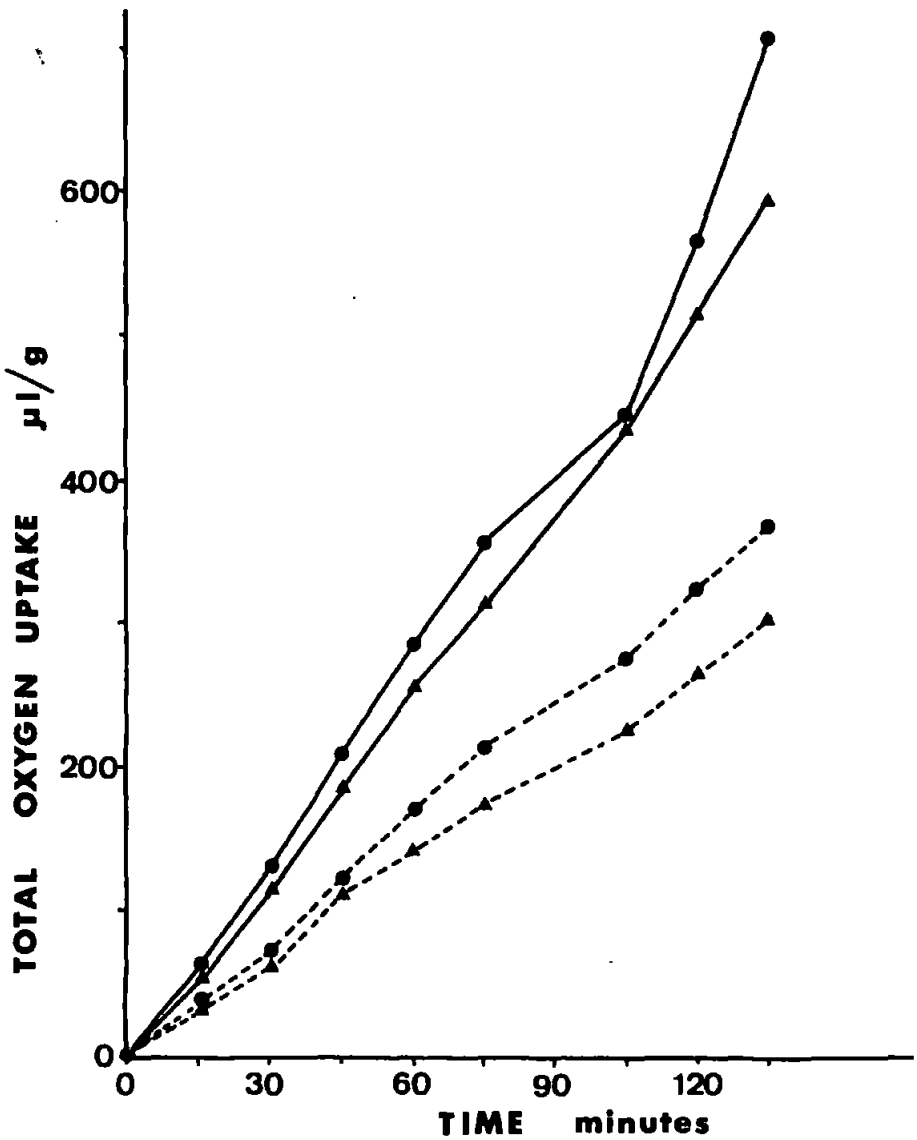
⊖—⊖ — Roots incubated for 2 hours in deionised water, then transferred to sodium succinate solution in the reaction vessels.

▲—▲ — Roots immersed for 2 hours in deionised water, then transferred to deionised water in the reaction vessels.

●--○ — Roots immersed for 2 hours in 10 mEq/l lead nitrate solution, then transferred to sodium succinate solution in the reaction vessels.

△--△ — Roots immersed for 2 hours in 10 mEq/l lead nitrate solution, then transferred to deionised water in the reaction vessels.

Each point represents the mean of 4 replicates.


TOTAL OXYGEN UPTAKE µl/g
600
400
200
0
0
30
60
90
120
TIME minutes

APPENDIX 2

REAGENTS AND PROCEDURES

1. Arnon and Hoagland's Solution

Plants cultured hydroponically were grown in Arnon and Hoagland's solution as described by Hewitt (1966), the constitution of which is as follows:

KNO_3	1.02 g/l	
$Ca(NO_3)_2$	0.492 g/l	
$NH_4H_2PO_4$	0.230 g/l	
$MgSO_4.7H_2O$	0.49 g/l	
$FeSO_4.7H_2O$	0.5%)	
	)	0.6 ml/l 3 x weekly
Tartaric Acid	0.4%)	
H_2BO_3	2.86 mg/l	
$MnCl_2.4H_2O$	1.81 mg/l	
$CuSO_4.5H_2O$	0.08 mg/l	
$ZnSO_4.7H_2O$	0.22 mg/l	
H_2MoO_4	0.09 mg/l	

This was supplemented by the addition of lead nitrate to the prepared solution as necessary.

2. Composition of Glass used in Milk Bottles used as basis for Culture Vessels

Silicon	73%
Sodium	13.7%
Calcium	10.9%
Aluminium	1.3%
Potassium	0.4%
Iron	0.4%
Sulphur	0.2%
Magnesium	0.1%

The above elements are present as oxides. Heavy metals, particularly lead and cadmium, are excluded.

(Courtesy of Rockware Glass Ltd., Rockware Avenue, Greenford.)

3. Tetrazolium Test for Seed Viability (Lakon, 1949)

Radish seeds were soaked in deionised water for six hours. At this time the seeds were bisected along the placental scar and transferred to a 0.5% aqueous solution of 2,3,5-triphenyl tetrazolium chloride. Seeds were incubated in the dark at 25°C for 24 hours, and then examined with a hand lens. Seed viability was indicated by the development of an orange pigmentation in the embryo, which arises by the reduction of the tetrazolium salt by dehydrogenases to form a red complex. In the case of radish seed this appears orange because of the inherent yellow colouration of the embryo. This test is, however, of limited use as it only indicates that one group of enzymes in the embryo is functional, and although a negative result indicates that the seed is not viable, a positive result does not necessarily indicate viability.

4. Procedure for Preparation of Plant Material for Light Microscopy

i Rinse fresh material briefly.

ii Cut into sections up to 3 mm x 3 mm.

iii Transfer to saturated aqueous hydrogen sulphide solution (20 minutes).

iv Wash in deionised water (2 minutes).

v Dehydrate in 70% alcohol (5 minutes).

vi Dehydrate in 90% alcohol (5 minutes).

vii Dehydrate in absolute alcohol (5 minutes).

viii Dehydrate in absolute alcohol (10 minutes).

ix Clear in xylol (30 minutes).

x Wax infiltrate (2 x 30 minutes).

5. In addition to the above procedure preparations were also stained with sodium rhodizonate, using the technique described by Glater and Hernandez (1972).

i Rinse material briefly in deionised water.

ii Cut into sections approximately 2 mm thick.

iii Freeze in Hamilton's freezing mixture (142.5 g sucrose, 2.8 g gum arabic and few drops formalin in 300 ml distilled water) and section*.

iv Rinse briefly in deionised water.

v Stain in fresh 0.2% aqueous sodium rhodizonate solution (30 minutes).

vi Add buffer solution (1.5 g tartaric acid + 1.9 g sodium bitartrate in 100 ml water) until pH stabilises at 2.8. Leave 10 minutes.

vii Rinse with deionised water to remove excess stain.

viii Mount in glycerol.

Specimens may also be dehydrated and mounted permanently, but such sections are of very limited value due to leaching during dehydration and embedding and rapid breakdown of the lead rhodizonate colour complex in storage.

6. Identification of Layers of Testa

A series of histochemical tests was carried out to obtain further information about the nature of the layers of the testa. Examinations were carried out on fresh sections cut as described for the sodium rhodizonate stain (vide supra).

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*Sections were cooled on an MSE 'Pelcool' freezing unit and cut using an MSE Base Sledge microtome set at 10 μm.

Initially simple staining was carried out using phloroglucinol and hydrochloric acid to identify lignified tissue. Next a number of preliminary stains were applied to localise any specific accumulations of chemical groups.

a. Stain in dinitrofluorobenzene. Positive reaction indicates protein.

b. Stain with toluidine blue at pH 6. Positive reaction indicates acidic components (e.g. nucleic acids).

c. Stain with Sudan black. Positive result indicates lipid.

d. Stain material in controlled periodic acid Schiff test. Positive result indicates carbohydrates.

Using these techniques a positive reaction was observed in each tissue layer for each stain; but a very heavy staining reaction was observed in the epidermal layer and in the aleurone layer of the testa in the test for carbohydrates. Accordingly further staining techniques were carried out to further identify this carbohydrate accumulation.

e. Stain with Best's carmine. Positive reaction indicates simple polysaccharides.

f. Stain in Lugol's iodine. Positive reaction indicates starch.

g. Stain with Alcian blue. Positive reaction indicates acidic mucopolysaccharides.

Other alternative mucoproteins and glycolipids may be ruled out as these groups would give positive staining reactions with dinitrofluorobenzene and Sudan black respectively.

Phloroglucinol/HCl Procedure

i Immerse in phloroglucinol (5 minutes).

ii Transfer to concentrated hydrochloric acid (1 minute).

iii Wash in distilled water.

iv Mount in glycerol

Dinitrofluorobenzene (DNFB) Procedure

i Immerse section in 1.5% DNFB in 65% ethanol saturated with sodium bicarbonate (2 hours).

ii Wash in 60% ethanol.

iii Immerse sections in 20% sodium dithionite at 37°C (10 minutes).

iv Wash in distilled water.

v Transfer to fresh 1% sodium nitrite in 0.05 N hydrochloric acid (5 minutes).

vi Wash in distilled water (1 minute).

vii Wash in distilled water (1 minute).

viii Wash in distilled water (1 minute).

ix Transfer to 0.5% 8-amino-1-naphthol-3-6-disulphonic acid in 1% sodium bicarbonate solution (15 minutes).

x Wash in distilled water.

xi Mount in glycerol.

Toluidine Blue Procedure

i Stain sections in 0.1% toluidine blue in acetate buffer (30 minutes). (1.36 g sodium acetate/100 ml water adjusted to pH 6 with acetic acid.)

ii Wash in acetate buffer.

iii Mount in glycerol.

Sudan Black Procedure

i Stain sections in Sudan black (30 minutes).

ii Rinse briefly in 70% ethanol to remove surplus stain.

iii Wash in 50% ethanol.

iv Wash in water.

v Mount in glycerol.

Periodic Acid - Schiff Procedure

i Fix sections (5 minutes) in picrate-formalin fixative (6% Formaldehyde, 3.5% ethanol, 0.18% sodium chloride and 0.15% Picric acid in aqueous solution).

ii Wash in 70% ethanol.

iii Immerse (5 minutes) in fresh periodate solution (400 mg periodic acid + 135 mg sodium acetate in 50 ml distilled water).

iv Wash in 70% ethanol.

v Wash in 5% potassium iodide/5% sodium thiosulphate solution (5 minutes).

vi Immerse in Schiffs reagent (Hopkins & Williams Ltd., Essex) (30 minutes) in the dark.

vii Rinse in 5% potassium iodide/5% sodium thiosulphate solution three times.

viii Mount in glycerol

Best's Carmine Procedure

i Fix sections in picrate-formalin solution (5 minutes).

ii Wash in 70% ethanol.

iii Immerse in periodate solution (5 minutes).

iv Rinse in 70% ethanol.

v Rinse in 5% potassium iodide/5% sodium thiosulphate solution (5 minutes).

vi Dehydrate and stain in Ehrlich's haematoxylin (15 minutes).

vii Wash in 5% sodium bicarbonate solution to 'blue'.

viii Wash in distilled water (5 minutes).

ix Stain with Best's carmine (2g carmine, 1g potassium carbonate, 5g potassium chloride in 60 ml distilled water).

x Wash in differentiating solution (20 ml ethanol + 10 ml methanol in 25 ml distilled water).

xi Mount in glycerol.

(For details of fixatives see Periodic acid - Schiff procedure)

Alcian Blue Procedure

i Fix sections in picrate-formalin fixative (vide supra).

ii Rehydrate and stain in 0.1% Alcian blue in 3% acetic acid (20 minutes).

iii Stain in 1% aqueous Alcian blue (1 minute).

iv Wash in tap water.

v Mount in glycerol.

7. Standard Procedure for Preparation of Plant Material for Transmission Electron Microscopy

i Rinse specimens and cut into small sections (1 mm x 1 mm)

ii Infiltrate with 6% glutaraldehyde in 0.2 M cacodylate buffer pH 7.2 (overnight at $0^{o}C$).

iii Wash in 0.02 M cacodylate buffer* (20 minutes).

iv Wash in 0.02 M cacodylate buffer (20 minutes).

v Infiltrate with 2% osmium tetroxide in 0.02 M cacodylate buffer (2 hours).

vi Wash in 0.02 M cacodylate buffer (10 minutes).

vii Wash in 0.02 M cacodylate buffer (10 minutes).

viii Dehydrate in 50% ethanol (15 minutes).

ix Dehydrate in 70% ethanol (15 minutes).

x Dehydrate in 90% ethanol (15 minutes).

xi Dehydrate in absolute ethanol (30 minutes).

---

*Cacodylate buffer - to 50 ml 0.2 M sodium cacodylate add 4.2 ml 0.2 M hydrochloric acid. Dilute to 500 ml with distilled water.

xii Dehydrate in absolute ethanol (1 hour).

xiii Infiltrate 1:3 mixture Spurr resin*:ethanol (1 hour).

xiv Infiltrate 3:1 mixture Spurr resin:ethanol (1 hour).

xv Infiltrate pure Spurr resin (1 hour).

xvi Infiltrate pure Spurr resin (overnight).

xvii Polymerise fresh Spurr resin for 8 hours at 70°C.

8. Rapid procedure for Preparation of Plant Material for Transmission Electron Microscopy, employing Sulphide Precipitation

i Rinse specimens and cut into small sections (1 mm x 1 mm).

ii Infiltrate with 0.02 M cacodylate buffer pH 7.2 containing a few drops of 5% ammonium sulphide solution (5 minutes).

iii Wash in 0.02 M cacodylate buffer pH 7.2 (5 minutes).

iv Infiltrate with 6% glutaraldehyde in 0.02 M cacodylate buffer (2 hours).

v Wash in 0.02 M cacodylate buffer (5 minutes).

vi Wash in 0.02 M cacodylate buffer (5 minutes).

vii Infiltrate with 2% osmium tetroxide in cacodylate buffer (1 hour).

viii Wash in 0.02 M cacodylate buffer (5 minutes).

ix Wash in 0.02 M cacodylate buffer (10 minutes).

x Dehydrate in 70% ethanol, agitating continuously (2 minutes).

xi Dehydrate in 90% ethanol, agitating continuously (2 minutes).

xii Dehydrate in absolute alcohol, agitating continuously (2 minutes).

---

*Spurr resin (Spurr 1969) Supplied by Taab Laboratories, Reading.

Epoxy resin	ERL 4206	10.0 g
additive	DER 736	6.0 g
hardener	NSA	26.0 g
accelerator	S-1	0.4 g

xiii Dehydrate in absolute alcohol, agitating continuously (5 minutes).

xiv Infiltrate with 1:1 mixture of Spurr resin:ethanol, agitating continuously (1 hour)

xv Infiltrate with pure Spurr resin overnight, agitating continuously.

xvi Polymerise in fresh Spurr resin for 8 hours at 70°C.

9. Glutaraldehyde Fixation Procedure

As above, but for step vii, read 'Wash in 0.02 M cacodylate buffer (1 hour)'.

10. Reynolds' lead citrate stain (Reynolds 1963)

i Prepare fresh lead citrate by dissolving 1.33 g of lead nitrate and 1.76 g of sodium citrate in 30 ml distilled water.

ii Shake for 1 minute; allow to stand for 30 minutes, shaking intermittently.

iii Add 8 ml of Normal sodium hydroxide solution and make up to 50 ml with distilled water.

iv Stain grid-mounted thin sections for 10 minutes in the presence of sodium hydroxide pellets in a petri dish.

v Rinse sections thoroughly in distilled water.

11. Formvar Coating of Grids for Microprobe Analysis

Microscope slides were cleaned and dried, and then dipped into a 0.5% solution of polyvinyl formal in chloroform. The slide was immediately withdrawn and excess fluid allowed to drain off one end. The slide was then dried under an infra-red lamp. The perimeter of one face of the slide was then scored with a needle and the film of formvar enclosed by the score line was floated off on to distilled water. Grids were placed on the formvar film and the film picked up with a piece of thin card of suitable dimensions. The formvar-coated

grids were then coated with carbon in an Edwards E306 Vacuum coating unit (Edwards High Vacuum, Crawley).

12. Staining of Chromosomes

i Remove apical 5 mm of roots and fix in acetic alcohol (3:1 absolute alcohol:glacial acetic acid) for 30 minutes.

ii Rinse in absolute alcohol and store in 70% alcohol until required for staining.

iii Hydrolyse root tips in normal hydrochloric acid at 60°C (6 minutes).

iv Transfer root tips to Schiff's reagent (Hopkins and Williams Ltd., Essex) in the dark (3 hours).

v Rinse in sulphurous acid (20 minutes).

vi Rinse in sulphurous acid (20 minutes).

vii Excise tip of root showing pink colouration and transfer to a slide in a drop of acetic alcohol. Separate cells by tapping gently with a blunt-ended rod.

viii Warm slide gently (c. 50°C) to spread and flatten cells.

ix Cover with cover slip and press gently, avoiding sideways movement.

x Invert slide in acetic alcohol in a smearing dish. Leave until cover slip drops off (up to 10 minutes).

xi Transfer slide and cover slip to absolute alcohol (1 minute).

xii Transfer to fresh absolute alcohol (1 minute).

xiii Mount in 'Euparal'.

Sulphurous Acid

Normal Hydrochloric acid	5 ml
10% Potassium metabisulphite	5 ml
Distilled water	90 ml

13. Colorimetric Estimation of Auxins (Anthony and Street 1970)

1 ml aliquots of the auxin extracts were added to 2 ml trichloracetic acid. After mixing 2 ml Ehrlich's reagent was added to each sample. After 60 minutes incubation at room temperature the colour complex was extracted into 5 ml chloroform and its absorbance measured at 580 nm using a CECIL CE303 grating spectrophotometer.

Ehrlich's Reagent

2 g p-dimethylaminobenzaldehyde (PDAB) dissolved in 100 ml 2.5 N hydrochloric acid.

Trichloracetic Acid

25% solution in deionised water.

APPENDIX 3

STATISTICAL TREATMENT OF DATA

In all cases where results were replicated, means, standard deviations and standard errors were calculated. Appropriate statistical analyses were applied to the data to determine the level of significance of the results. These analyses took the form of paired data t-tests, one- or two-way analyses of variance (Anovar), and correlation analysis. Unpaired data t-tests were also applied where necessary to determine the significance of differences between individual sets of replicates. Typical examples of the procedures employed are given in the following pages, together with significance figures for presented data.

1. Paired data t-test

NULL HYPOTHESIS: There is no significant difference between the mean growth increments in lead salts of differing anionic composition.

DATA

mean daily increment in root length mm				
lead acetate	lead citrate	lead nitrate	$d_{(n-a)}$*	$d_{(c-n)}$+
1.1	7.8	2.5	+1.4	+5.3
0.4	1.1	2.4	+2.0	-1.3
0.8	11.2	0.2	-0.6	+11.0
1.2	2.0	1.4	+0.2	+0.6
0.2	4.4	0.4	+0.2	+4.0
0.3	0.4	0.3	0	+0.1
0.5	2.1	0.2	-0.3	+1.9
0.1	1.7	0	-0.1	+1.7
0.1	2.3	0.6	+0.5	+1.7
0	0.6	0.3	+0.3	+0.3
0	0.3	0.5	-0.2	-0.2
0	0.1	0	+0.1	+0.1
0	0.6	0.3	+0.3	+0.3

$\bar{x}_a = 0.261 \quad \bar{x}_c = 2.662 \quad \bar{x}_n = 0.7$

$$\bar{d}_{(n-a)} = \bar{x}_a - \bar{x}_n = 0.339$$

$$\bar{d}_{(c-n)} = \bar{x}_c - \bar{x}_n = 1.962$$

$$S^2 = \frac{\Sigma d - \bar{d}^2}{N - 1}$$

Therefore, $S^2_{(n-a)} = 0.492$

$S^2_{(c-n)} = 10.56$

*difference between acetate and nitrate
+difference between citrate and nitrate

$$t = \frac{\bar{d}}{\sqrt{\frac{S^2}{N}}}$$

Therefore, for n-a,

$$t = \frac{0.339}{\sqrt{\frac{0.492}{13}}} = \underline{1.74}$$

for c-n,

$$t = \frac{1.962}{\sqrt{\frac{10.56}{13}}} = \underline{2.18}$$

By tables, $t_{.05,\ 12df} = 1.78$

$t_{.025,\ 12df} = 2.18$

Therefore, the null hypothesis is upheld for lead nitrate/lead acetate ($P > 0.05$) and rejected for lead nitrate/lead citrate - there are significant differences between the lead nitrate and lead citrate treatments ($P < 0.025$).

2. Unpaired data t-test

NULL HYPOTHESIS: There is no significant difference between dimension of seedlings treated with 0 and with 0.01 mEq/l lead nitrate.

DATA

	Root length mm		Cotyledon length mm		Cotyledon breadth mm	
	0 mEq/l	0.01 mEq/l	0 mEq/l	0.01 mEq/l	0 mEq/l	0.01 mEq/l
	145	195	6	5	10	8
	105	237	7	10	10	15
	157	188	7	10	10	12
	140	140	7	10	10	17
	62	145	0	4	0	6
Σx =	609	903	27	39	40	58
Σx^2=	80143	169587	183	341	400	758

$$t = \frac{\Sigma x_1/n_1 - x_2/n_2}{\sqrt{\left[\frac{\Sigma x_1^2 - (\Sigma x_1)^2/n_1 + \Sigma x_2^2 - (\Sigma x_2)^2/n_2}{n_1 + n_2 - 2}\right]\left[\frac{n_1 + n_2}{n_1 n_2}\right]}}$$

Therefore, for root length,

$$t = \frac{\frac{903}{5} - \frac{609}{5}}{\sqrt{\left[\frac{169587 - 163081.8 + 80143 - 741762}{8}\right] \times \frac{10}{25}}}$$

$$= \frac{180.6 - 121.8}{24.97} = \underline{2.35}$$

By tables, $t_{.025,\ 8df} = 2.31$

Therefore, the null hypothesis is rejected - the differences in root length are significant ($P < 0.025$).

For cotyledon length,

$$t = \frac{\frac{39}{5} - \frac{27}{5}}{\sqrt{\left[\frac{341 - 304.2 + 183 - 145.8}{8}\right] \times \frac{10}{25}}}$$

$$= \frac{7.8 - 54}{1.92} = \underline{1.25}$$

By tables $t_{.05,\ 8df} = 1.86$

Therefore, the null hypothesis is supported, the differences in cotyledon length are not significant $(P > 0.05)$.

For cotyledon breadth,

$$t = \frac{\frac{58}{5} - \frac{40}{5}}{\sqrt{\left[\frac{758 - 672.8 + 400 - 320}{8}\right] \times \frac{10}{25}}}$$

Therefore, $= \frac{11.6 - 8}{2.87} = \underline{1.25}$

By tables, $t_{.05,\ 8df} = 1.86$

Therefore, the null hypothesis is supported - the differences in cotyledon breadth are not significant $(P > 0.05)$.

3. One-way analysis of variance

NULL HYPOTHESIS: Lead treatment causes no significant change in coleoptile elongation.

DATA

Mean coleoptile length (mm)

Lead supplied (mEq/l)		0	0.01	0.1	1	5	10
Replicate	1	14.5	15.7	14.0	13.5	10	11.0
	2	14.3	15.5	13.4	12.0	10.6	11.0
	3	15.3	13.8	13.4	13.1	10.8	10.0
	4	14.7	15.5	14.2	14.3	10.5	10.0
	5	14.6	13.6	13.9	11.9	10.8	10.0
	6	16.0	14.5	13.3	12.5	10.0	10.0
	7	15.2	15.7	14.5	12.9	10.6	10.0
	8	15.6	13.6	14.0	14.1	11.0	10.0
	9	14.0	13.5	15.1	12.5	10.5	10.0
	10	14.5	14.4	14.0	11.1	10.0	10.0
	t =	148.7	146.0	139.8	127.9	104.8	102.0

$T = t_1 + t_2 \ldots + t_6 = 769.2$

$\Sigma x^2 = 10091.9$

$N = 60$

Correction term, $C = \frac{T^2}{N} = \frac{(769.2)^2}{60} = 9661.1$

Total sum of squares: $TSS = \Sigma x^2 - C = 10091.9 - 9661.1 = 430.8$

Total degrees of freedom: $Tdf = N - 1 = 59$

Pb sum of squares: $PbSS = \Sigma\frac{t^2}{n_t} - C,$

$$= \frac{148.7^2}{10} + \frac{146.0^2}{10} + \frac{139.8^2}{10} + \frac{127.9^2}{10} + \frac{104.8^2}{10} + \frac{102.0^2}{10}$$

$$- 9661.1$$

$$= 410.6$$

Pb degrees of freedom: $Pbdf = 6 - 1 = 5$

Within groups sum of squares: $WGSS = TSS - PbSS$

$$= 430.8 - 410.6 = 20.2$$

Within groups degrees of freedom: $WGdf = Tdf - Pbdf = 59 - 5 = 54$

Pb variance estimate $= \frac{PbSS}{Pbdf} = \frac{410.6}{5} = 82.1$

Within groups variance estimate $= \frac{WGSS}{WGdf} = \frac{20.2}{54} = 0.37$

$$F(Pb) \text{ ratio} = \frac{\text{Pb variance estimate}}{\text{within groups variance estimate}}$$

$$= \frac{82.1}{0.87} = 221.6$$

By tables $F_{.001}$, 5.54df = 4.9

Therefore, the null hypothesis is rejected - lead treatment does cause significant changes in coleoptile elongation ($P < 0.001$).

4. Two-way analysis of variance

NULL HYPOTHESIS: There is no interaction between lead and the effect on coleoptile elongation.

DATA

Pb supplied (mEq/l) ↓	IAA supplied (μg/ml) → 0	1	5	10	20
0	28, 25, 38, 32	43, 52, 52, 49	42, 32, 38, 41	32, 35, 38, 34	33, 34, 30, 25
1	1, 38, 38, 42	38, 39, 38, 37	38, 38, 39, 14	41, 38, 45, 47	35, 36, 37, 33
5	0, 8, 11, 5	6, 8, 9, 11	3, 0, 0, 0	2, 0, 1, 0	9, 8, 5, 4
10	0, 0, 0, 1	1, 4, 0, 0	5, 6, 5, 0	0, 0, 0, 0	3, 7, 5, 0

0 Pb total = 733

1 Pb total = 712

5 Pb total = 90

10 Pb total = 37

0 IAA total = 267

1 IAA total = 387

5 IAA total = 301

10 IAA total = 313

20 IAA total = 304

$N = 80 \quad T = 1572 \quad \Sigma x^2 = 56340$

Correction term: $C = \frac{T^2}{N} = 30889.8$

Total sum of squares: $TSS = \Sigma x^2 - C = 25450.2$

Total degrees of freedom: $Tdf = 80 - 1 = 79$

Total variance estimate: T var. est. = 3221.54

Pb sum of squares: $PbSS = \frac{\Sigma t^2}{n_t} - C = 21795.3$

Pb degrees of freedom: $Pbdf = 4 - 1 = 3$

Pb variance estimate: Pb var. est. = 7265.1

IAA sum of squares: $IAASS = \frac{\Sigma t^2}{n_t} - C = 487.95$

IAA degrees of freedom: IAAdf = 5.1 = 4

IAA variance estimate: IAA var. est. = 121.99

Pb x IAA interaction effect:

Totals

	0	1	5	10	20
0	123	196	153	139	122
1	119	152	129	171	141
5	24	34	3	3	26
10	1	5	16	0	15

$$Pb \times IAASS = \frac{\Sigma t^2}{n_t} - (PbSS + IAASS + C) = 54280 - 53173.05$$
$$= 1106.95$$

Pb x IAAdf = Pbdf x IAAdf = 3 x 4 = 12

Pb x IAA variance estimate: PbIAA var. est. = 92.25

Within cells sum of squares: WCSS = TSS - all other SS

= 25450.2 - (21795.3 + 487.95 + 1106.95)

= 2060

WCdf = Tdf - all other df

= 79 - (3 + 4 + 12)

= 60

Within cells variance estimate $= \frac{2060}{60} = 34.33$

F values:

for Pb: $F = \frac{7265.1}{34.33} = 211.63$ 3,60 df

for IAA: $F = \frac{121.99}{34.33} = 3.55$ 4,60 df

for IAA/Pb interaction: $F = \frac{92.25}{34.33} = 2.69$ 12.60 df

By tables, for 3,60 df $F_{.001} = 6.3$

for 4,60 df $F_{.025^2} = 3.1$

for 12,60 df $F_{.01} = 2.6$

Therefore, the null hypothesis is rejected - there are significant changes induced by both lead ($P < 0.001$) and IAA ($P < 0.025$). There is a significant interaction effect ($P < 0.01$).

5. Correlation coefficient

Root lead concentration, X µg/g	Root dry weight, Y g	$(X - \bar{X})$	$(Y - \bar{Y})$
0	0.0126	-75.5	+0.0067
0	0.0126	-75.5	+0.0067
21	0.0066	-54.5	+0.0007
76	0.0022	+ 0.5	-0.0037
142	0.0010	+66.5	-0.0049
214	0.0002	+138.5	-0.0057

$\bar{X}$ = 75.5 $\bar{Y}$ = 0.0059

$$R = \frac{\Sigma(X - \bar{X})(Y - \bar{Y})}{\sqrt{(\Sigma(X - \bar{X})^2 \Sigma(Y - \bar{Y})^2)}}$$

$$= \frac{-2.167}{\sqrt{37975.5 \times 0.00016}}$$

$$= \frac{-2.167}{2.469}$$

Therefore R = -0.879

By tables, $R_{.01}$, 5df = $\pm$ 0.875. Therefore, there is significant negative correlation between the two sets of data ($P < 0.01$).

6. Significance figures for presented data

KEY

Pb - lead; A/N - acetate:nitrate; C/N - citrate/nitrate; SL - stem length; SB - stem breadth; Na - sodium; Mg - magnesium; P - phosphorus; K - potassium; Ca - calcium; Fe - iron; TOT - total; TELO - telophase; META - metaphase; E - elastic; P - plastic; S - succinate; RL - root length

FIGURE	TEST APPLIED	RESULT			df	SIGNIFICANCE
3.1.1 a	2-way ANOVAR	F_{Pb}	=	1.58	5,798	$P > 0.05$
3.1.1 b	2-way ANOVAR	F_{Pb}	=	25.21	5,798	$P < 0.001$
3.1.1 c	2-way ANOVAR	F_{Pb}	=	2.48	5,798	$P > 0.05$
3.1.1 d	2-way ANOVAR	F_{Pb}	=	32.10	5,798	$P < 0.001$
3.2.1 a	2-way ANOVAR	F_{Pb}	=	28.47	5,336	$P < 0.001$
3.2.1 b	2-way ANOVAR	F_{Pb}	=	46.9	5,336	$P < 0.001$
3.2.1 c	2-way ANOVAR	F_{Pb}	=	40.10	5,336	$P < 0.001$
3.2.1 d	2-way ANOVAR	F_{Pb}	=	42.32	5,336	$P < 0.001$
3.2.2 a	2-way ANOVAR	F_{Pb}	=	27.35	5,336	$P < 0.001$
3.2.2 b	2-way ANOVAR	F_{Pb}	=	2.2	5,336	$P > 0.05$
3.2.2 c	2-way ANOVAR	F_{Pb}	=	45.01	5,336	$P < 0.001$
3.2.2 d	2-way ANOVAR	F_{Pb}	=	34.9	5,336	$P < 0.001$
3.2.3 a	2-way ANOVAR	F_{Pb}	=	42.8	2,169	$P < 0.002$
3.2.3 b	2-way ANOVAR	F_{Pb}	=	73.6	2,169	$P < 0.001$
3.2.3 c	2-way ANOVAR	F_{Pb}	=	57.1	2,169	$P < 0.001$
3.2.3 d	2-way ANOVAR	F_{Pb}	=	3.27	2,169	$P < 0.05$
3.2.3 a	Paired-data t-test	A/N	=	1.86	12	$P < 0.05$
		C/N	=	2.40	12	$P < 0.025$

FIGURE	TEST APPLIED	RESULT			df	SIGNIFICANCE
3.2.3 b	Paired-data t-test	A/N	=	1.7	12	$P > 0.05$
	Paired-data t-test	C/N	=	2.18	12	$P < 0.05$
3.2.3 c	Paired-data t-test	A/N	=	6.0	12	$P < 0.001$
	Paired-data t-test	C/N	=	0	12	$P > 0.05$
3.2.3 d	Paired-data t-test	A/N	=	0.78	12	$P > 0.05$
	Paired-data t-test	C/N	=	0	12	$P > 0.05$
3.3.1 a	1-way ANOVAR	F_{leaves}	=	60.28	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	22.18	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	44.38	5,24	$P < 0.001$
3.3.1 b	1-way ANOVAR	F_{leaves}	=	28.78	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	91.65	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	81.68	5,24	$P < 0.001$
3.3.1 c	1-way ANOVAR	F_{leaves}	=	16.89	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	157.75	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	115.55	5,24	$P < 0.001$
3.3.1 d	1-way ANOVAR	F_{leaves}	=	48.68	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	9.48	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	24.19	5,24	$P < 0.001$
3.3.2 a	1-way ANOVAR	F_{leaves}	=	43.13	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	14.33	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	38.4	5,24	$P < 0.001$
3.3.2 b	1-way ANOVAR	F_{leaves}	=	64.08	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	25.92	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	16.52	5,24	$P < 0.001$
3.3.2 c	1-way ANOVAR	F_{leaves}	=	123.8	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	=	147.4	5,24	$P < 0.001$
	1-way ANOVAR	F_{roots}	=	16.8	5,24	$P < 0.001$

FIGURE	TEST APPLIED	RESULT		df	SIGNIFICANCE
3.3.2 d	1-way ANOVAR	F_{leaves}	= 30.32	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	= 5.29	5,24	$P < 0.01$
	1-way ANOVAR	F_{roots}	= 91.20	5,24	$P < 0.001$
3.3.4 a	1-way ANOVAR	F_{RL}	= 28.76	5,24	$P < 0.001$
	1-way ANOVAR	F_{SL}	= 163.55	5,24	$P < 0.001$
3.3.4 b	1-way ANOVAR	F_{RL}	= 13.57	5,24	$P < 0.001$
	1-way ANOVAR	F_{SL}	= 20.90	5,24	$P < 0.001$
3.3.4 c	1-way ANOVAR	F_{RL}	= 12.63	5,24	$P < 0.001$
	1-way ANOVAR	F_{SL}	= 9.64	5,24	$P < 0.001$
	1-way ANOVAR	F_{SB}	= 41.2	5,24	$P < 0.001$
3.3.4 d	1-way ANOVAR	F_{RL}	= 283.07	5,24	$P < 0.001$
	1-way ANOVAR	F_{SL}	= 23.46	5,24	$P < 0.001$
	1-way ANOVAR	F_{SB}	= 23.08	5,24	$P < 0.001$
3.3.5 a	1-way ANOVAR	F	= 79.51	5,24	$P < 0.001$
3.3.5 b	1-way ANOVAR	F	= 122.76	5,24	$P < 0.001$
3.3.5 c	1-way ANOVAR	F	= 52.33	5,24	$P < 0.001$
3.3.5 d	1-way ANOVAR	F	= 49.71	5,24	$P < 0.001$
3.3.6 a	1-way ANOVAR	F	= 7.07	5,24	$P < 0.001$
3.3.6 b	1-way ANOVAR	F	= 9.07	5,24	$P < 0.001$
3.3.6 c	1-way ANOVAR	F	= 9.43	5,24	$P < 0.001$
3.3.6 d	1-way ANOVAR	F	= 13.43	5,24	$P < 0.001$
4.2.1 c	1-way ANOVAR	F_{roots}	= 69.71	5,24	$P < 0.01$
	1-way ANOVAR	F_{stems}	= 13.17	5,24	$P < 0.001$
	1-way ANOVAR	F_{leaves}	= 4.18	5,24	$P < 0.01$
4.2.1 d	1-way ANOVAR	F_{roots}	= 76.31	5,24	$P < 0.001$
	1-way ANOVAR	F_{stems}	= 29.39	5,24	$P < 0.001$
	1-way ANOVAR	F_{leaves}	= 27.06	5,24	$P < 0.001$

FIGURE	TEST APPLIED	RESULT	df	SIGNIFICANCE
4.3.1 a	Correlation coefficient	$R_{roots} = -0.749$	5	$P < 0.1$
	Correlation coefficient	$R_{stems} = -0.875$	5	$P < 0.01$
	Correlation coefficient	$R_{leaves} = -0.722$	5	$P < 0.1$
4.3.1 b	Correlation coefficient	$R_{roots} = -0.740$	5	$P < 0.1$
	Correlation coefficient	$R_{stems} = -0.918$	5	$P < 0.001$
	Correlation coefficient	$R_{leaves} = -0.782$	5	$P < 0.05$
4.3.1 c	Correlation coefficient	$R_{roots} = -0.820$	5	$P < 0.05$
	Correlation coefficient	$R_{stems} = -0.915$	5	$P < 0.01$
	Correlation coefficient	$R_{leaves} = -0.823$	5	$P < 0.05$
4.3.2 a	Correlation coefficient	$R_{roots} = -0.879$	5	$P < 0.01$
	Correlation coefficient	$R_{stems} = -0.853$	5	$P < 0.02$
	Correlation coefficient	$R_{leaves} = -0.737$	5	$P < 0.1$
4.3.2 b	Correlation coefficient	$R_{roots} = -0.650$	5	$P > 0.1$
	Correlation coefficient	$R_{stems} = -0.805$	5	$P < 0.05$
	Correlation coefficient	$R_{leaves} = -0.822$	5	$P < 0.05$
4.3.2 c	Correlation coefficient	$R_{roots} = -0.729$	5	$P < 0.1$
	Correlation coefficient	$R_{stems} = -0.464$	5	$P > 0.1$
	Correlation coefficient	$R_{leaves} = -0.535$	5	$P > 0.1$
4.3.2 d	Correlation coefficient	$R_{roots} = -0.904$	5	$P < 0.01$
	Correlation coefficient	$R_{stems} = -0.654$	5	$P > 0.1$
	Correlation coefficient	$R_{leaves} = -0.768$	5	$P < 0.05$
7.4.1	1-way ANOVAR	$F_{Na} = 12.47$	4,10	$P < 0.001$
	1-way ANOVAR	$F_{Mg} = 6.79$	4,10	$P < 0.01$
	1-way ANOVAR	$F_{P} = 7.62$	4,10	$P < 0.01$
	1-way ANOVAR	$F_{K} = 35.92$	4,10	$P < 0.001$
	1-way ANOVAR	$F_{Ca} = 3.54$	4,10	$P < 0.05$
	1-way ANOVAR	$F_{Pb} = 296.05$	4,10	$P < 0.001$

FIGURE	TEST APPLIED	RESULT		df	SIGNIFICANCE
7.4.2	1-way ANOVAR	F_{Fe}	= 6.24	4,20	$P < 0.01$
	1-way ANOVAR	F_{Ca}	= 12.89	4,20	$P < 0.001$
8.1.1 a	1-way ANOVAR	F_{TOT}	= 6.57	5,54	$P < 0.01$
	1-way ANOVAR	F_{TELO}	= 3.86	5,54	$P < 0.05$
	1-way ANOVAR	F_{META}	= 2.69	5,54	$P > 0.05$
8.1.1 b	1-way ANOVAR	F	= 8.17	5,54	$P < 0.01$
8.1.1 c	2-way ANOVAR	F	= 349.8	1,89	$P < 0.001$
8.2.1 a	1-way ANOVAR	F	= 20.91	5,54	$P < 0.001$
8.2.1 b	2-way ANOVAR	F	= 5.51	1,108	$P < 0.05$
8.2.2	2-way ANOVAR	F_{Pb}	= 211.63	3,60	$P < 0.001$
8.2.4 a	1-way ANOVAR	F	= 13.8	5,54	$P < 0.001$
8.2.4 b	1-way ANOVAR	F	= 25.8	5,54	$P < 0.001$
8.2.4 c	1-way ANOVAR	F	= 4.39	5,54	$P < 0.01$
8.2.4 d	1-way ANOVAR	F	= 221.6	5,54	$P < 0.001$
8.2.4 e	1-way ANOVAR	F	= 30	5,54	$P < 0.001$
8.2.5 a	2-way ANOVAR	F	= 17.2	1,108	$P < 0.001$
8.2.5 b	2-way ANOVAR	F	= 41.64	1,108	$P < 0.001$
8.2.5 c	2-way ANOVAR	F	= 0.59	1,108	$P > 0.05$
8.2.5 d	2-way ANOVAR	F	= 72.26	1,108	$P < 0.001$
8.2.5 e	2-way ANOVAR	F	= 13.87	1,108	$P < 0.001$
8.3.1 a	1-way ANOVAR	F	= 28.43	5,114	$P < 0.001$
8.3.1 b	1-way ANOVAR	F	= 3.45	5,114	$P < 0.01$
8.3.1 c	2-way ANOVAR	F	= 35.39	1,90	$P < 0.001$
8.3.1 d	2-way ANOVAR	F	= 59.65	1,108	$P < 0.001$
8.3.2 a	1-way ANOVAR	F	= 19.46	5,24	$P < 0.001$
8.3.2 b	1-way ANOVAR	F_{TOT}	= 6.23	5,54	$P < 0.001$

FIGURE	TEST APPLIED	RESULT			df	SIGNIFICANCE
8.3.2 b	1-way ANOVAR	F_E	=	5.15	5,54	$P < 0.001$
	1-way ANOVAR	F_P	=	2.38	5,54	$P > 0.05$
8.3.3	1-way ANOVAR	F	=	49.41	4,40	$P < 0.001$
8.3.5 a	1-way ANOVAR	F	=	6.16	4,15	$P < 0.01$
8.3.5 b	1-way ANOVAR	F	=	3.52	4,15	$P < 0.05$
A.1.2 a	Correlation coefficient	R	=	-0.374	22	$P > 0.1$
A.1.2 b	Correlation coefficient	R	=	0.813	14	$P < 0.001$
A.2.1	1-way ANOVAR	F	=	2.95	4,20	$P < 0.05$
A.3.1 a	1-way ANOVAR	F	=	3.17	5,24	$P < 0.025$
A.3.1 b	1-way ANOVAR	F	=	12.26	5,24	$P > 0.05$
A.4.1 a	2-way ANOVAR	F	=	20.74	1,126	$P < 0.001$
A.4.1 b	2-way ANOVAR	F	=	16.07	1,126	$P < 0.001$
A.6.1	2-way ANOVAR	F	=	7.76	5,120	$P < 0.001$
A.7.1 a	1-way ANOVAR	F	=	20.0	5,54	$P < 0.001$
A.7.1 b	1-way ANOVAR	F	=	15.82	5,24	$P < 0.001$
A.8.1	2-way ANOVAR	F	=	5.77	5,192	$P < 0.001$
A.8.2	2-way ANOVAR	F	=	462.6	1,324	$P < 0.001$
A.8.3	2-way ANOVAR	F_{Pb}	=	41.41	1,48	$P < 0.001$
	2-way ANOVAR	F_S	=	6.56	1,48	$P < 0.025$