IN VITRO SELECTION AND CHARACTERIZATION OF LEAD RESISTANT SOMACLONAL VARIANTS FROM DAUCUS CAROTA L.

Bateson, Janice Mary

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IN VITRO SELECTION AND CHARACTERIZATION OF LEAD RESISTANT SOMACLONAL VARIANTS FROM DAUCUS CAROTA L.

Janice Mary Bateson, BSc(Hons).

A thesis submitted in partial fulfilment of the requirements of the Council for National Academic Awards for the degree of Doctor of Philosophy.

JULY 1990

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"And suppose we solve all the problems it presents? What happens? We end up with more problems than we started with. Because that's the way problems propagate their species. A problem left to itself dries up or goes rotten. But fertilize a problem with a solution—you'll hatch out dozens!"

N.F. Simpson
'A Resounding Tinkle'
In vitro selection and characterization of lead resistant somaclonal variants from *Daucus carota* L.

Janice Mary Bateson

**ABSTRACT**

Lead was shown to inhibit both callus initiation and callus growth in cultures of *Daucus carota* L. subsp. *sativus* (Hoff. Thell.) cv. Nantes "Tiptop" and "Nanthya". Taproot explants of *Daucus carota* were stressed with lead. The callus cell lines which initiated under this stress were shown to exhibit resistance to the effects of lead ions. The growth of the selected and nonselected cell lines on non-lead containing media was comparable and the resistance possessed by the selected cell lines did not result in reduced growth rates in the presence of lead. The resistance characteristic was shown to be stable and to be successfully transmitted over mitotic and meiotic barriers.

Plants were regenerated from the selected cell lines and ion uptake studies were conducted on isolated cortical tissue from mature taproots. The uptake of lead into the cortical cell tissue from the selected lines was shown to be reduced and a greater proportion of the lead that did enter the tissue was present in the Apparent Free Space and did not enter the cells.

The regenerated plants were self-pollenated to produce an F1 generation. F1 plantlets were grown in hydroponic culture containing various concentrations of lead. The selected plants were seen to be resistant to the lead stress. The sites of lead accumulation in these roots were determined using x-ray microanalysis in a scanning electron microscope with a cryo-stage. The lead was found to be associated with the epidermal layer and cell walls.

The mechanism of the lead resistance is discussed along with the implications of selection for somaclonal variants from initiating callus cultures.
I wish to thank the many people at Polytechnic South West for their assistance in the completion of this thesis.

I would also like to thank Dr. Brian Grout for his help and guidance over the years.

My eternal thanks go to Robert, without whose unstinting support and assistance this thesis would not have been completed.
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CHAPTER ONE

INTRODUCTION

The fundamental basis of plant tissue and cell culture is the potential of all living cells in a plant body, irrespective of their ploidy level and form of specialisation, to potentially give rise to whole plants. This important theory, of cellular totipotency was first proposed by Haberlandt in his classic paper of 1902 (see Krikorian and Berquam, 1969 for translation). He clearly stated the desirability of culturing the isolated vegetative cells of higher plants "to provide insight into the properties and potentialities possessed by the cell as an elementary organism, and important information about the inter-relationships and complementary influences to which cells within the multicellular organism are exposed". To the present day, this is still a major concern of plant research, and there is an obvious move to bring greater precision to the cellular aspects of plant tissue and cell culture research, and to utilize, where possible, molecular approaches.

Many attempts to culture plant cells in vitro were reported following Haberlandt's paper but none resulted in cell division. Knotte (1922) and Robbins (1922) reported some progress towards the culture of excised root tips, but it was not until 1934 that the first real step was taken in producing actively growing plant cell cultures, with the establishment of a growing clone of tomato roots from excised root-tips (White, 1934). In the same year Gautheret obtained limited growth of Salix capraea (Common Willow)
root cambium using Knop's solution, glucose and cystine hydrochloride (Gautheret, 1934). Cell proliferation in culture was also being obtained by Nobecourt (1937, 1938) using explants from carrot roots.

The discovery of the importance of the B vitamins for growth of cultured roots (White, 1937) and the increasing recognition of auxin (indole-3yl-acetic acid - IAA) in the control of plant growth (Went and Thimann, 1937) led Gautheret to include these growth factors in his culture medium with the result that he obtained greatly enhanced, but still limited growth of cambium from Salix capraea (1937, 1938). Gautheret (1939) used the inorganic salt mixture of Nobecourt along with glucose, vitamin Bl, cystine hydrochloride and IAA to obtain continuous growth of small explants from carrot cambium and phloem. In the same year White (1939) and Nobecourt (1939) independently reported establishment of similar cultures. However, these cultures were all derived from meristematic cells and it was still not possible to induce divisions in isolated, mature and differentiated cells.

In the search to replace the need for meristematic tissue in the original tissue explant, Skoog and his colleagues, in the early 1950's, were interested in auxin stimulation of plant cells and tissue grown in culture. They found that cells in pith sections of tobacco stems divided much more rapidly if a piece of vascular tissue was placed on top of the pith (Miller and Skoog, 1953). They tried to identify the chemical factor from the vascular tissue, using growth of the tobacco pith cells as a bioassay system. These cells were cultured on agar
medium containing known sugars, mineral salts, vitamins, amino acids and IAA. IAA itself increased growth for a time by causing enormous cells to be formed, but these cells did not divide. Many of these cells were polyploids with several nuclei. In seeking substances that would promote cell division, they found an adenine-like compound in yeast extracts that was highly effective in promoting growth of tobacco pith cells (Jablonski and Skoog, 1954). It was the use of yeast in the culture medium that had enabled White in 1934 to establish the first continuously-growing root cultures. Further investigations by Miller and colleagues into the ability of DNA to promote cytokinesis led, in 1956, to the discovery of a very active compound formed by the breakdown of aged or autoclaved herring sperm DNA. This compound was named kinetin, after its ability to promote cytokinesis. This, and compounds which exhibit activity like that of kinetin, were given the group name cytokinins. Kinetin itself was not found in plants and is not the active substance found by Skoog in vascular tissue, however, related cytokinins are present in most plants. Steward and Caplin, also using tissue culture techniques in 1954, found several cytokinins in coconut milk which enhanced cell division in carrot root tissue. The most active of these were later shown to be compounds previously given the names of zeatin and zeatin riboside and found in the milky endosperm of corn (Zea mays) (Letham, 1964, 1974; Miller, 1965). Since then other cytokinins have been identified in numerous parts of seed plants (for reviews see George and Sherrington, 1984; Sailsbury and Ross, 1985;
The discovery of cytokinins has made possible the induction of division in cells of highly mature and differentiated tissue, such as mesophyll and endosperm of dried seeds (Bhojwani and Razdan, 1983). In a continuation of their earlier work, Skoog and Miller (1957) introduced the concept of hormonal control of organ formation. They demonstrated that the differentiation of roots and shoots in tobacco pith cultures could be controlled by the auxin/cytokinin ratio. Steward, in the early 1950's, had initiated work on cultured carrot explants which for the first time involved analysis of culture growth in quantitative terms (Steward et al., 1952). This was to lead to the wide use of coconut milk as a nutrient in culture media and, far more importantly, to the discovery of embryogenesis (Steward, 1958; Steward et al., 1958; Reinert, 1958; 1959). The identification of these two processes of organogenesis and embryogenesis resulted in the ability to regenerate plants from cultured tissues, however, to realize the goals of Haberlandt, stated over 50 years before, of culturing single vegetative cells, the techniques of single cell culture still had to be developed. Muir, in 1953, reported that callus fragments of tobacco, transferred to agitated liquid culture medium, formed a suspension of single cells and cell aggregates. It was shown that this suspension could be propagated by subculture. Similarly, Steward reported that the medium bathing carrot root explants became turbid with free-floating single cells and small cell aggregates. The suspension grew and could be serially subcultured (Steward
and Shantz, 1956). Hence, the goal set by Haberlandt was achieved.

It is now possible to establish cell cultures from many species and genera and it has been forecast that this number will reach over 3000 by 1989 (Cocking, 1986). It is also possible to establish many different kinds of culture (there are numerous very good reviews, including: Bhojwani and Razdan, 1983; Evans et al., 1982; George and Sherington, 1984; Vasil, 1984).

Plant or cell growth in vitro can be either classed as organised or unorganised growth. The former occurs when organised plant parts (or organs), such as the apical meristems of shoots or roots, are transferred to culture where they may continue to grow with their structure preserved. It, also, occurs when organised structures are formed afresh during the culture of previously unorganised tissues. This process of organ formation is called organogenesis or morphogenesis and, as described earlier, is under hormonal control. Unorganised growth occurs fairly frequently when tissue excised from whole plants is cultured in vitro. The tissue formed typically lacks any recognisable structure and is termed dedifferentiated tissue. It is possible to regenerate many species from their in vitro cultures via organogenesis (Flick et al., 1983) or somatic embryogenesis (Ammirato, 1983; Hu and Wang, 1986) and, also, to propagate plants from numerous explants, including leaf sections, anthers, meristems, isolated single cells and protoplasts (Bhojwani and Razdan, 1983; Gamborg and Shyluk, 1981; George and Sherrington,
Many tissue and cell culture techniques are of great importance in agriculture and horticulture, such as clonal propagation and rapid multiplication of specific genotypes (Bhojwani and Razdan, 1983), secondary product synthesis (Yamada and Fujita, 1983; Whitaker and Hashimoto, 1986) and virus elimination (Evans et al., 1983). It is also possible to culture plant cells without a cell wall, called protoplast cultures (Evans et al., 1983; Gamborg et al., 1981). These were first produced by Cocking in 1960 by digesting the cell walls of root tip cells using a fungal cellulase. When cultivated these cells reform their cell walls and divide. Anther and pollen culture are also of use in agriculture and horticulture as haploid plants can be induced (George and Sherington, 1984).

Tissue culture technology finds major applications in the agricultural and horticultural industries. The culture techniques play a major part in the improvement of existing plant varieties and in the development of new ones. One of the aspects of plant improvement is the use of meristem tip culture resulting in the elimination of virus infestations and hence healthier plants (Giles, 1985). The other aspect consists of gathering, or creating, many gene combinations, and then in selecting and fixing the most agronomically desirable ones and finally, in releasing them through commercial varieties after seed manipulation (Dore, 1987). Conventional plant breeding suffers from a number of drawbacks (Shaw, 1984), particularly in its reliance on the natural processes of fertilization to introduce modifications to the genetic make-up of a plant and the
restriction of the gene pool to the range of plants which are sexually compatible (Armstrong, 1985). Another major problem of conventional breeding is the time scale involved, for example in winter wheat it may take eight years to produce a true breeding line and it may be twelve years in all before the seed is on sale to the farmer (Day, 1985). Due to the nature of the tissue culture process the number of plant propagules that can be developed in a short time are very large. Hence, this allows the rapid introduction of new lines and varieties into the marketplace, for example this reduces the introduction time of new lily varieties from 15-16 years to 7-8 years (Giles, 1985).

One approach to the genetic modification of plants is by the selection of desirable mutant plant types. Genetic mutations can be induced with physical or chemical mutagens or, in tissue culture, they can occur spontaneously. This spontaneous genetic variation is termed somaclonal variation. An important factor in the use of plant tissue culture for plant breeding is the occurrence of this high genetic variability in cultures (Evans and Mizrahi, 1988; Seal et al., 1984). Clonal plants regenerated from cell cultures were seen to exhibit altered phenotypes. This was thought to be an annoying 'artifact' of tissue culture, arising from epigenetic events (Scowcroft and Larkin, 1984). However, in both sugar cane (Heinz, 1983) and Pelargonium (Skirvin and Janick, 1976) tissue generated variation was seen as circumventing the considerable constraints imposed on the use of conventional breeding
practice for improving asexually propagated species. Extensive agronomically useful variation was also observed in tobacco (Devreux and Laneri, 1974) and among protoplast derived plants of potato (Shepherd et al., 1981). The employment of tissue culture to induce useful genetic variation grew in credence. Chromosome instability in tissue culture appeared to be the rule rather than the exception (Bayliss, 1980; D'Amato, 1977) and this instability was recognised as an important event in the tissue culture cycle. It was termed somaclonal variation (Larkin and Scowcroft, 1981).

Many possible mechanisms giving rise to somaclonal variation have been suggested, such as ploidy changes, nuclear fragmentation, inter- and intra-chromosomal interchanges, somatic crossing over and sister chromatid exchanges, gene amplification and diminution and transposable genetic elements (Chaleff, 1981; Larkin and Scowcroft, 1981; Shepard, 1981; Skirvin, 1978). The phenomenon has been observed in cultures of many plant species (Scowcroft and Larkin, 1983) and much of the variation is demonstrably genetic (Scowcroft et al., 1983). It has been observed that the ploidy levels in cultures tend to increase with the cultures age (Mathews and Vasil, 1975), resulting in both polyploid (Bayliss, 1980) and aneuploid (Heinz et al., 1977) cells. As well as chromosome number changes, structural alterations also occur. These range from single base changes in the DNA (Brettell et al., 1986), to gross chromosomal aberrations (McCoy et al., 1982) such as, deletions, duplications, translocations and centric fusions, and, also, to gene amplification or
transposition of control elements (Jacobs et al., 1987). In tissue cultures somaclonal variation has been seen to be the result of four types of mutational events which occur at very high frequencies. These are single gene mutations; transposable element activation; quantitative trait variation and chromosome breakage. It has been suggested that these four seemingly different mutational events are directly or indirectly related to the modification of DNA, specifically through DNA hypo- or hyper-methylation (Phillips et al., 1990). This methylation may be involved in controlling the cell to cell inheritance of gene activity patterns with increased methylation usually correlating with loss of gene activity and decreased methylation with increased activity (Holliday, 1989).

The range of character variation produced by cell culture is large. Burk and Malzinger (1978) found as much variation in yield, grade index and flowering time among somaclonally derived double haploids, as among a segregating F2 population of two cultivars. The wide genetic variation induced by tissue culture could be an important tool in plant breeding for unique genotypes.

There are a number of factors which increase the amount of somaclonal variation which occurs. The choice of genotype (i.e. cultivar) for the establishment of cultures will greatly effect the occurrence of variation (McCoy et al., 1982; Sun et al., 1983), as will explant source (Dix, 1986). It was shown very early on that variants can be recovered from in vivo root and petiole cuttings but not from stem cuttings of geranium (Skirvin and Janick, 1976).
It is widely accepted that the length of the culture step is important (Ammirato, 1986) and that most long-term cultures are chromosomally variable (Evans and Sharp, 1986). The correlation between duration of culture and accumulation of chromosomal variants has been documented for many plant species, including *Daucus carota* (Smith and Street, 1984). It has also been known for several years that the composition of the culture medium (Bayliss, 1980) and the type of growth regulator used (Bayliss, 1975) can influence the frequency of karyotypic alterations in cultured cells. The growth regulator 2,4-dinitrophenoxyacetic acid (2,4-D) has been most frequently considered to promote chromosome variability (Evans and Sharp, 1986; McCoy, 1982). The type of culture also plays a major part in the induction of variation, with the callus stage being the most unstable (Karp, 1989), however, even in tissue culture systems traditionally viewed as being genetically stable, such as somatic embryos and meristems, somaclones have been detected (Evans and Sharp, 1986).

An understanding of the underlying genetic and/or molecular events of somaclonal variation not only has an academic value, but is also most important practically. The plant propagator, in seeking clonal uniformity, would wish to mitigate against such variation by judicious choice of cultivar, explant source and culture regime. The plant breeder, on the other hand, may wish to enhance or, if possible, induce specific types of genetic variation by manipulation of these factors. A knowledge of these events and their underlying causes is essential if we hope to
control and utilise somaclonal variation.

It should be noted that the definition of the terms 'mutant' and 'variant' differs, although the use is somewhat lax in some literature. A variant cell line is one which differs phenotypically from the normal population, whether it is by genetic or epigenetic changes. The term mutant, on the other hand, is used to denote a line in which the genetic basis for the variation has been confirmed.

The success of in vitro variant isolation depends, ultimately, upon the selection scheme used. This can take the form of either direct, indirect or counter selection.

Direct selections is used when the variant has a direct application, eg. herbicide resistance. This is the simplest form of selection to accomplish and, because of this, most mutants that have been isolated in vitro from higher plants have been by direct selection (Challeff, 1986; Sacristan, 1986).

Indirect methods of selection are used for certain phenotypes that are difficult, or impossible, to select for directly in cell culture. The trait desired in the whole plant may not be detectable at the cellular level. Therefore, a factor, expressed in the culture, must be identified which correlates to the desired trait in the whole plant. For example, when drought resistance is required, it has been suggested that selection takes place for over-production of proline, which is observed as a response to drought stress (Flick, 1983). It has also been shown that tobacco cultures selected for cadmium resistance
will also show resistance to temperature stress (Huang and Goldsbrough, 1988).

Counter, or negative, selection is used when there are no conditions in which the mutant-type has a selective advantage over the wild-type. In cases such as these counter-selective agents are used which, for example, are lethal only to dividing cells (Depicker et al., 1988; Dix, 1986).

The selection of agronomically important traits using plant cell culture usually necessitates the selection being conducted at the cellular level. The level of differentiation is often different from that at which the character is ultimately desired, i.e. the whole plant. Although the character may be expressed in the cell culture, this may not be the case in the whole plant (Chaleff, 1986). Conversely, in some instances the reverse is the case. For example, some herbicide action occurs only at the whole plant level and not in cell cultures (Hughes, 1983), also most agronomically important traits, such as root architecture, plant height, yield and maturation time, are multigenic and, hence, may not be altered qualitatively by mutation at a single locus. This is coupled with the problem that often the poor understanding of the molecular and cellular basis of a characteristic, such as plant height, prevents the identification of the correlative cellular functions for which an in vitro selection scheme might be devised.

The selection for somaclonal variants, in vitro, can be used to isolate plant cell lines resistant to many varied compounds present in toxic concentrations. Published
reports of resistance being exhibited, as a result of somaclonal variation in tissue culture, are very numerous. This has resulted in the isolation of cell lines resistant to metals, such as aluminium and manganese (Conner and Meredith, 1985; Meredith, 1978; Ojima and Ohira, 1983), zinc and copper (Wu and Autonovics, 1978), cadmium (Huang et al., 1989). Cell lines have also been isolated from a large range of species which show resistance to many varied substances and types of stress. There are many hundreds of reports of this type in the literature and a very small number of recent references are shown in Figure 1.1. These have been chosen to illustrate the wide range of variant characteristics which have been obtained, via somaclonal variation, from many different species by using various selection schemes and culture types.

As a result of research on somaclonal variants a number of commercially valuable breeding lines have been developed, for example, in sugarcane (Liu, 1981) and Pelargonium sp. (Skirvin and Janick, 1976). Many other mutants, whilst not of any direct economic importance, may be useful as selective markers in somatic hybridization. For example, amino acid analogue resistant cell lines are used in the selection of somatic hybrids of Daucus carota (Harms et al., 1981). Some selections result in cultures which express the desirable characteristics, often, however, the prolonged time in vitro and other factors not yet fully understood, result in a culture which cannot be regenerated (Reisch, 1984). The use of tissue culture as
Figure 1.1
Examples of recent research where the desired characteristic has been selected via somaclonal variation.

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<tr>
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<td>C &amp; S</td>
<td>Aluminium Tolerance</td>
<td>Ojima &amp; Chira, 1988</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>I</td>
<td>C</td>
<td>Resistance to Verticillium Wilt</td>
<td>Latunde-Dada &amp; Lucas, 1988</td>
</tr>
<tr>
<td>Nicotiana plumbaginifolia</td>
<td>C</td>
<td>C</td>
<td>Nitrate-reductase Deficiency</td>
<td>Pelsy et al., 1988</td>
</tr>
<tr>
<td>Nicotiana sp.</td>
<td>C</td>
<td>C</td>
<td>Expression of the T-DNA Gene 2</td>
<td>Depicker et al., 1988</td>
</tr>
<tr>
<td>Triticum aestivum</td>
<td>D</td>
<td>C</td>
<td>Aminoethyl-L-cysteine Resistance</td>
<td>Kumpaisal et al., 1988</td>
</tr>
<tr>
<td>Various species</td>
<td>D</td>
<td>Sh</td>
<td>Salinity Tolerance</td>
<td>Chandler et al., 1988</td>
</tr>
<tr>
<td>Citrus sinensis</td>
<td>D</td>
<td>C</td>
<td>Salt Tolerance</td>
<td>Ben-Hayyim &amp; Goffer, 1989</td>
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<td>D</td>
<td>C</td>
<td>Cadmium Tolerance</td>
<td>Domanlicka &amp; Opatrny, 1989</td>
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<td>C</td>
<td>Herbicide Tolerance</td>
<td>Ishida et al., 1989</td>
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<td>D</td>
<td>C</td>
<td>Copper Resistance</td>
<td>Tobita et al., 1989</td>
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<tr>
<td>Prunus avium</td>
<td>D &amp; I</td>
<td>C</td>
<td>Salt &amp; Drought Tolerance</td>
<td>Ochatt &amp; Power, 1989</td>
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<td>D</td>
<td>C</td>
<td>Regeneration Efficiency</td>
<td>Heszky et al., 1989</td>
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<td>D</td>
<td>C</td>
<td>Frost Resistance</td>
<td>Galiba &amp; Sutka, 1989</td>
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<td>D</td>
<td>Mi</td>
<td>Salt Tolerance</td>
<td>Shohet et al., 1990</td>
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<tr>
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<td>S</td>
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<td>Thiemann &amp; Barz, 1990</td>
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<td>E</td>
<td>Salt Tolerance</td>
<td>Piqueras &amp; Hellín, 1990</td>
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<tr>
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<td>E</td>
<td>Salt Tolerance</td>
<td>Ibrahim et al., 1990</td>
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<tr>
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<td>D</td>
<td>C</td>
<td>Aluminium Resistance</td>
<td>Ojima et al., 1990</td>
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<tr>
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<td>D</td>
<td>S</td>
<td>Glycophosphate Resistance</td>
<td>Shyr &amp; Wichola, 1990</td>
</tr>
<tr>
<td>Phoenix dactylifera</td>
<td>D</td>
<td>E</td>
<td>Salt Tolerance</td>
<td>Aboelsi-El, 1990</td>
</tr>
</tbody>
</table>

¹ - the type of selection scheme used. Direct = D. Indirect = I. Counter = C.

² - the culture stage during which the selection was made. Callus cultures = C. Microspores formation = Mi. Mesophyl cell cultures = Me. Regenerating cells = R. Suspension cultures = S. Shoot cultures = Sh. Embryo formation = E.
a breeding tool for selecting a desired characteristic is negated unless plant regeneration of the selected line is possible and that, most importantly, the character seen in vitro is expressed in vivo. It has been recognised that the potential applications for both directed cellular selection systems and somaclonal variation manipulations can only be realised when regeneration from cells can be easily accomplished in a wide range of species (E.E.C. Biotechnology Directorate, 1986).

A major difficulty often encountered with plant cell lines selected for resistance to growth inhibitors is the instability of the character when removed from the selective pressure. (Gonzales and Widholm, 1985). There are a number of possible reasons. The genetic lesion responsible may have a high reversion rate or the alteration may simply be a transient genetic or epigenetic adaptation. However, a major reason may be the selective advantage of any surviving wild-type cells when the selection pressure is removed. Hence, the selected lines should be maintained under the selective pressure until the stability has been determined. This may be achieved by culturing part of the selected line without the selective pressure. If, after a set period of time, the cells still show the resistance when placed under the selective pressure then the stability of the character can be presumed.

Resistant lines are normally cloned to ensure a homogeneous culture (Gonzales and Widholm, 1985) but a theoretical model has been developed and tested (Portnoy and Murphy, 1983) describing the distribution of mutants in
aggregates of cultured cells, which indicate that, with time, cell clumps break apart randomly with no reassociation. It is, therefore, possible to calculate the number of generations required to yield a high probability that all the cells, within a given aggregate, are descended from a single cell. In practice 75 generations are usually adequate for clumps of 13 cells or less to develop into clonal cell lines (Hauptman and Widholm, 1982).

In plant breeding secure genetic stocks are a fundamental requirement (Holden and Williams, 1984) and mutants produced in vitro need subsequently to be held in a stable form, preventing any further somaclonal variation (Gonzales and Widholm, 1985). For many years the standard method of maintaining stocks was continuous growth under more or less optimum conditions (Withers, 1985), but there were serious drawbacks, namely, cost, the risks of somaclonal variation, of contamination and of accidental loss. Therefore, for both practical and scientific reasons, the techniques of storage by cryopreservation were developed. Growth is suspended in cell cultures by freezing at ultra-low temperatures, after appropriate pre-treatment of the cells (Withers, 1985). Storage is usually at -196°C in liquid nitrogen.

Heavy metal toxicity and pollution are of increasing environmental concern. In the last few years many reports have shown that lead, even at very low levels, can cause important, irreversible, subclinical effects (Clarkson, 1987). Increasing concern is now being expressed by the
media, by pressure groups and in Parliament about the dangers presented by the lead in our environment (Auto Express, 1988; DHSS, 1980; MAFF, 1982; The Independent on Sunday, 1990). Agricultural practices, such as excessive use of phosphatic fertilizers (Friber et al., 1974; Varma and Katz, 1978) and sewage sludge (Council for Agricultural Science and Technology, 1980) have resulted in the heavy metal contamination of much agricultural land. Industrial activities, especially mining and smelting, have, also, resulted in the heavy contamination of land which might otherwise have been used for crop production. Houses may be built on this otherwise derelict land and this leads to the exposure of their inhabitants to dangerous levels of heavy metals through the consumption of home-grown vegetables (Gallancher et al., 1987). A major source of lead contamination of soils in both urban areas and agricultural land bordering major roads, is the airborne lead resulting from the combustion of lead-containing fuels by motor vehicles (Moore, 1986).

There is a long history of human exposure to abnormally elevated levels of lead in food and drink, due to practices such as cooking in lead-lined or lead-glazed pots and the supply of water through lead pipes (Grandjean, 1975) and, also, some communities living in areas of lead mineralisation are exposed to higher levels of lead than the general population (Harrison and Laxen, 1981). People are exposed to lead through breathing lead-polluted air (Moore, 1986), in the workplace (Araki et al., 1986; Brune et al., 1987) and through the ingestion of lead in food and drink (DHSS, 1980; Lessler, 1988). Recently, however,
concern has centred upon pollution arising from the use of lead additives in petrol, which cause emissions of lead aerosol when burned (Auto Express, 1988).

Lead serves no useful physiological function and for many years the human health effects have been recognised (Bernard and Becker, 1988; DHSS, 1980). The chronic effects of lead occur when levels of lead in the bloodstream exceed 70-80 ug Pb per 100ml of whole blood, compared to typical levels in the population of 10 to 30 ug Pb per 100ml of whole blood (Harrison and Laxen, 1981). Blood-lead concentrations at the highly elevated levels causing chronic effects are the result of long-term or very acute exposure to lead (Moore, 1986). A far more important problem is the recent realization that more subtle adverse effects to health occur at much lower levels of exposure. Concern has been directed particularly at the possible slight, but irreversible, damage to brain development in children (Lansdown and Yule, 1986).

Inorganic compounds of lead cause neurological and behavioural effects (Clarkson, 1987) and the alkyl derivatives of lead are major neurotoxins (Clarkson, 1988; Costa, 1988). Lead has a significant effect upon blood pressure (Victery, 1988), reproduction (Wiebe and Barr, 1988) and kidney function (Bernard and Becker, 1988). Exposure to lead, also, lowers both psychological performance and the motor and sensory conduction velocities of the median nerve (Araki et al., 1986). A number of hypotheses have been proposed for the mechanism of lead toxicity on the nervous system. Lead is a potent inhibitor
of heme synthesis and a reduction in heme-containing enzymes could compromise energy metabolism. Function of the brain may be affected by lead because of its interference with neurotransmitters such as gamma-amino-isobutyric acid (Clarkson, 1987) and there is also evidence to show that lead interferes with membrane transport and binding of calcium ions (Clarkson, 1987).

Archeological discoveries indicate the presence of lead objects and pigments during the early Bronze Age (Lessler, 1988). Extensive evidence of ancient lead mining exists and metallic lead was also produced as a by-product of silver mining (Harrison and Laxen, 1981; Lessler, 1988). Lead is believed to have been the first metal smelted by ancient man (Smith, 1986) and has been an important substance in human societies for many thousands of years, being put to uses as diverse as a construction material, a glaze on pottery and in sweetening wine (Harrison and Laxen, 1981). A sweet lead acetate syrup, called sapa, was added to food and wines during the Greco-Roman and Medieval times. This was considered to have caused the Roman Empire's downfall, due to lead poisoning of the ruling classes (Gilfillan, 1965).

There are three main sources of lead in soils. Firstly, the parent geological materials from which soils are derived are important natural sources of lead and, as a consequence of mineralisation, lead is found in three ore forms; Anglesite (PbSO₄), Cerussite (PbCO₃) and the most abundant of all, Galena (PbS) (US Geological Survey, 1976). In the South West of England these are found mainly in North-South lodes, occupying a tangential position relative
to granite intrusions into the area (Dines, 1956).

Secondly, lead occurs in soils due to pollution from ore-mining, the associated smelting activity and to industrial waste tipping. These forms of lead pollution appear to be regional problems, but often result in soils with the highest levels of contamination (Lee and Tallis, 1973).

Thirdly, atmospheric lead makes a significant contribution to lead in the environment (Chamberlain, 1983) and exhaust fumes from the internal combustion engine contribute approximately 80% of this (Harrison and Laxen, 1981). Cars in Britain emit 3000 tonnes of lead each year (DTI, 1987). This is in the form of inorganic salts of lead, such as lead halide, and unchanged tetra-alkyl-lead (Harrison and Laxen, 1981).

Lead has also been added to soils as an insecticide (Chisholm, 1972) or due to the application of contaminated fertilizers (Zimdahl and Ardvik, 1973) and sewage sludge (Dowdy and Larson, 1975).

In South West England spoil tips and tailings from old mine workings present a major source of lead in the environment. These produce tracts of land with poor rates of re-vegetation, even after a century or more (Bradshaw and McNeilly, 1981). In areas previously worked for mineral deposits, concentrations may reach 40,000 ppm, but more typically are in the range 100–2000 ppm (DOE, 1974) whereas levels for unpolluted soils are 2–200 ppm, with a mean level of 50 ppm (Bradshaw and McNeilly, 1981). Lead levels around roads as a result of exhaust emissions are lower, with the
highest being 3000ppm (Hemphill et al., 1974), but more usually 200-1000ppm (Davies and Holmes, 1972). Contaminated soils are defined as having lead-ion levels of 100μg Pb/g or greater, and highly contaminated soils as having lead-ion levels of 1000-10,000 μg Pb/g or higher (MAFF, 1982). Root vegetables grown on these can accumulate lead in excess of the present British legal limit for fresh food, which is 1μg/g fresh weight (MAFF, 1982). Lead contamination of the soils in mining areas can be very widespread. In mining areas in Wales levels in excess of 5000ppm were found in local gardens and 3000ppm in local fields (Davies and Roberts, 1975).

It is generally agreed that in the United Kingdom a major contribution to the lead in blood comes from food (Gallacher et al., 1984; Davies and White, 1981; MAFF 1982; DHSS 1980). Estimations attribute 0.5% of dietary lead to animal products (Smart et al., 1981) and because only a small proportion of lead absorbed by plant roots is transported to the foliage, approximately 13% of dietary lead has been attributed to root vegetables (Davies and White, 1981). Gallacher et al. (1984) demonstrated that blood-lead concentrations could be raised by 28% due to the ingestion of lead-contaminated vegetables. It has been recognised that this lead in vegetables presents a serious public health risk and that plants excluding this lead would be of significant importance, both agriculturally and academically (DHSS, 1980; Zurera et al., 1987).

Lead present in the soil may not always be available to plants, as pH, clay content, competing ions and organic matter content all affect the availability (Farrah and
Pickering, 1977; Zimdahl and Foster, 1976). Lead in culture media may, again, not always be available for uptake by the cells as other media components may act as competitors for the uptake sites or they may chemically complex with the lead so that it will not enter the cells (Cantoni et al., 1986).

The effects of lead upon animals have been more widely studied than on plants (Vallee and Ulmer, 1972 for review of animal literature; Koeppe, 1981 for review of plant literature). Numerous reports from the animal literature have shown that lead forms mercaptiles with the -SH group of cystine and less stable complexes with other amino-acid side chains. Lead has also been shown to bind and alter the activity of many membranous subcellular particles, including mitochondria, chloroplasts, nucleotides and nucleic acids and chromosomes. Much the same effects have been shown in plants, with changes in chloroplast fine structure, such as a reduction in granal stacks, reduction in the amount of stroma in relation to the lamellar system and an absence of starch grains. Lead has, also, been shown to cause chromosomal aberrations in plants and to become intimately associated with many different cellular and subcellular membranes. This association often results in the manifestation of toxic lead effects, such as reductions in the activity of phosphatase and enzyme-bound ATPase. The effect of high concentrations have been reported upon photosynthesis (via photosynthetic electron transport associated with photosystem II, but not photosystem I. Homer et al., 1981); respiration; energy relations and on an
array of selected enzymes. Inhibition effects are seen on yield (either fresh, dry weight or grain) and in pigment concentration changes (especially chlorosis of the leaves), which are often a visible sign of toxicity.

Plants may adapt to extreme environments, that is, environments in which a stress is exerted upon the plant. A stress may be any environmental factor which elicits from the plant a physiological or morphological change. This change may be either elastic (reversible) or plastic (permanent) (Tal, 1983). Plants resistant to the toxicity of a certain compound, such as high lead-ion levels in the soil, may not necessarily be tolerant to that toxicity.

There are four main mechanisms whereby resistance to toxicity, or stress, can be achieved (Levitt, 1980): (i) Phenological escape - where the stress is seasonal the plant may adjust its life-cycle so as to grow in the most favourable season. (ii) Exclusion - the plant may be able to recognise the toxic ion and prevent its uptake, so as not to experience the toxicity. (iii) Amelioration - the plant may absorb the ion but act upon it in such a way as to minimize its effects. Variously this may involve chelation, dilution, localization, or even excretion. Finally, (iv) True tolerance - the plant may have evolved a metabolic system which can function at potentially toxic concentrations, possibly by means of distinct enzyme molecules. Species most able to resist toxic ion are often found to employ more than one of these mechanisms (Fitter and Hay, 1981).

The increasing level of lead in soils warrants the production and use of plant varieties capable of either
lead exclusion or sequestration of the toxic metal in non-consumed plant parts and a logical solution to this problem would be a gene, or genes, coding for heavy metal binding proteins (Misra and Gedamu, 1989). Heavy metals in vertebrates and fungi are detoxified by low-molecular weight, cysteine-rich, heavy metal-binding proteins called the metallothioneins (Grill et al., 1985). For many years it has been proposed that higher plants contain metal-chelating proteins (Jowett, 1958) and recently a number of metal-binding, metallothionein-like proteins have been isolated from differentiated tissue and cell cultures of higher plants (Bennetzen and Adams, 1984; Casterline and Barnett, 1982; Grill et al., 1985; Grill et al., 1987; Jackson et al., 1987; Lolkema, 1984; Wagner, 1984). These small peptides, called phytochelatins, have been shown to sequester heavy metals (Grill et al., 1985; Jackson et al., 1987) and are functionally analogous to metallothioneins (Grill et al., 1987). Phytochelatins are linear polymers containing repetitive γ-glutamyl acid bonds (Grill et al., 1985) and it is because of these that they cannot be considered as primary gene products. Their synthesis is regulated at the transcriptional level in response to stress (Misra and Gedamu, 1989).

Physiological studies of the mechanisms of stress resistance can benefit by using tissue culture. It serves as an excellent model system in the study of mechanisms operating on the whole plant (Tal, 1983). Cell cultures offer many advantages for the isolation of variants in higher plants. Unlike the whole plant, a large number of
individuals (i.e. cells) can be screened at one time for the desired trait. The homogeneity of the cell population avoids any complications that may result from the morphological variability and the highly differentiated state characteristic of tissues in the whole plant. The cells are grown in a defined medium under uniform cultural conditions, thereby allowing a reproducible selection scheme to be employed. Experiments can be carried out throughout the year, independent of seasonal fluctuations and variations in the environment. It is also possible to use cell culture to study the response of isolated tissue or cells. This can then be related to their relative contributions to the whole plant, or to differentiate between mechanisms which operate only on the cellular level or those which depend on the organisation of the cells in the whole plant. Plant material at every level of organization may lend itself to selection of variants, and in some species there may be several different ploidy levels to choose from (Yeoman, 1986). Several features are most important when selecting the material to form the parental cell line. The ploidy level, that is, whether to use haploid, diploid or polyploid tissue, must be considered. Haploid tissue is essential to isolate any recessive mutations, such as auxotrophs. However, this must be balanced against the fact that haploid cultures are difficult to obtain from some species and, historically, they are chromosomally unstable in vitro. The use of haploid cell lines is unnecessary for the isolation of dominant mutants and stable mutations for resistance to many antimetabolites are possible using diploid cell lines.
The type of culture system used is also very important. Callus, cell suspensions and plated cells have all been successfully used for variant selection (Dix, 1986) and each have their different advantages and disadvantages which must be taken into account. Callus cultures have been considered a model system for selection studies (Dixon, 1982), but they have a number of important disadvantages. The growth rate is relatively slow, compared to that of suspension cultures, and the cells may not be uniformly exposed to a selective agent included in the agar solidified media. The cells themselves may not be uniform and their chemical and physiological status may alter through the callus mass. Gradients may well develop where some cells may be buffered by their neighbours and so escape the selective pressure, or the converse may occur where a resistant cell may encounter physical or chemical constraints from its neighbouring cells preventing the formation of a resistant callus mass. It is also possible to have cross-feeding within a callus mass resulting in phenotypic masking. Resistant cell lines may harbour sensitive cells which could out-grow the resistant cells under non-selective conditions, and sensitive or chimerical plants could be regenerated from such a line. However, this can be negated by a post-selection cloning step to purify the cell line. The disadvantages of using a callus cell culture system can be countered, and possibly outweighed, by the advantages. On a solid agar-solidified medium diffusion is reduced, compared to a liquid culture medium.
therefore the effects of dead cells and any toxic products released by them are reduced. More than one resistant colony can be selected per culture vessel because growth on solid media confines the dispersal of cells. The major advantage, however, is that the space and equipment needed are relatively small compared to those required when using a suspension culture system and the sub-culturing period is measured in weeks rather than days.

Suspension cultured cells growing rapidly can be manipulated in a similar way to micro-organisms. The major problem is the size of the cell aggregates and, although filtration of the suspension will remove the larger cell clumps, the small cell aggregates may present the same problems as encountered with callus. The surviving cells in a suspension system will be cultured in a medium enriched by dead cells and any toxic products they have released and the effects of cross-feeding will be greater in suspension cultures. Another consideration is that within any one culture vessel only one variant cell line can be isolated. Cell colonies, which may have originated from separate sources, cannot be kept separate. The advantages of both callus and suspension cultures can be exploited by plating out cells from suspension cultures onto agar-solidified media containing the selective agent. Hence, all the advantages of callus cultures may be obtained whilst ameliorating the disadvantages of the large cell masses. It was a system such as this which was to be used in this study, however, as will be seen later, variant lines were obtained before suspension cultures were initiated.
A carrot (*Daucus carota* L. subsp. *sativus* (Hoff.) Thell.) cell culture system is very amenable to tissue culture manipulations and has a good regenerative potential (Ammirato, 1983) and the responsiveness of carrot tissue to growth induction has made it a model system for studies on factors promoting cell division (Steward *et al.*, 1969). Haploid cultures of carrot are difficult to obtain (Ammirato, 1986). This is due to the problems in surface sterilizing the compound umbel, and to the difficulties in inducing flowering in a biennial plant.

A wide range of variant cell lines have been isolated from carrot cell cultures. These include those resistant to amino acid analogs (e.g., 5-methyltryptophan; Widholm, 1974), purine and pyrimidine analogs (e.g., 5-fluorouracil; Sung and Jacques, 1980), and antibiotics (e.g., cycloheximide; Sung *et al.*, 1981), as well as colour variants (Miller *et al.*, 1980) and developmental mutants (Breton and Sung, 1982). Carrot cell lines resistant to the metals aluminium and manganese have been isolated by selection, but no plants could be regenerated (Meredith, 1978; Ojima and Ohira, 1983).

Crop plants are major accumulators of lead from those environments where intolerably high levels are found, and plants that exclude such heavy metals would be of significant agricultural importance, especially in the South West. Most importantly, this study will provide valuable evidence in the broader biotechnological investigations into the feasibility of using spontaneous, or somaclonal, cell variants as unique breeding stock.
(via cell culture selection) for desirable agricultural characteristics. It will also provide evidence as to the validity of selecting for desired mutations from tissue in the process of initiating callus rather than from callus cultures.
Plant material.

Seeds of two subvarieties of *Daucus carota* L. cv. 'Nantes' were kindly donated by Breeders Seeds Ltd., Kent, England. The two subvarieties were:

1) an out-bred form with the trade name 'Tiptop'.

2) an F1 hybrid with the trade name 'Nanthya'.

Taproot tissue was obtained by growing seeds in John Innes No.1 compost in an unheated greenhouse. The taproots were used when they were approximately 6cm long.

Culture media.

Callus cell cultures of *Daucus carota* are most often initiated and maintained on Murashige and Skoog medium (Ammirato, 1986), but Gamborgs B5 medium may also be used (Reinhert, 1981). Therefore, lead was added to both these media formulations and the total and available lead ions were measured. The results of this can be seen in the Appendix.
The media compositions are as follows:-

Murashige and Skoog Medium (as Murashige and Skoog, 1962)

CaCl2 .2H2O .......................... 440.000mg/l
FeSO4 .7H2O .......................... 27.800mg/l
KH2PO4 .................................. 170.000mg/l
KNO3 ................................... 1900.000mg/l
MgSO4 .7H2O .......................... 370.000mg/l
Na2.EDTA ................................ 37.300mg/l
NH4NO3 .................................. 1650.000mg/l
CoCl2 .6H2O .......................... 0.025mg/l
CuSO4 .5H2O .......................... 0.025mg/l
H2BO3 ................................... 6.200mg/l
KI ....................................... 0.830mg/l
MnSO4 .4H2O .......................... 22.300mg/l
Na2MoO4 .2H2O ......................... 0.025mg/l
ZnSO4 .7H2O .......................... 8.600mg/l
Nicotinic Acid .......................... 0.500mg/l
Pyridoxine HCL .......................... 0.100mg/l
Thiamine HCL .......................... 0.100mg/l
Myo-Inositol .......................... 100.000mg/l
Glycine .................................. 3.000mg/l

Supplemented with - 2% w/v Sucrose
0.8% w/v Agar
0.05mg/l Dichlorophenoxyacetic acid
pH - 5.8

Gamborgs B5 Medium (as Gamborg and Wetter, 1975)

CaCl2 .2H2O .......................... 0.150mg/l
Na2HPO4 .H2O .......................... 150.000mg/l
(NH4)2SO4 .................................. 134.000mg/l
KNO3 ................................... 2500.000mg/l
MgSO4 .7H2O .......................... 250.000mg/l
Ferric EDTA ............................ 40.000mg/l
CoCl2 .6H2O .......................... 0.025mg/l
CuSO4 .5H2O .......................... 0.025mg/l
H2BO3 ................................... 3.000mg/l
KI ....................................... 0.750mg/l
MnSO4 .4H2O .......................... 10.000mg/l
Na2MoO4 .2H2O ......................... 0.250mg/l
ZnSO4 .7H2O .......................... 8.600mg/l
Nicotinic Acid .......................... 1.000mg/l
Pyridoxine HCL .......................... 1.000mg/l
Thiamine HCL .......................... 1.000mg/l
Myo-Inositol .......................... 100.000mg/l

Supplemented with - 2% w/v Sucrose
0.8% w/v Agar
0.1mg/l Dichlorophenoxyacetic Acid
pH - 5.6

The media was supplied in powder form by Imperial Laboratories Ltd., Salisbury, Wiltshire and was
reconstituted using distilled water. Sterilization was by autoclaving at 121°C (15lbs psi) for 15 minutes.

Gamborgs B5 medium was used in all initiation, maintenance and selection experiments, unless otherwise stated.

Conditioned media.

Conditioned media was used for maintaining the very small pieces of selected callus resulting in Chapter 4. The Gamborgs B5 media was conditioned by growing carrot callus on it for two weeks (method as Reinhert, 1983).

The small pieces of callus to be maintained were placed on the conditioned media. Once growth was seen and the callus pieces had reached a diameter of approximately 0.5cm they were transferred to conventional Gamborgs B5 media. Multiplication and transfer was then as standard.

Lead ions.

In all cases, lead ions were added as lead nitrate \((\text{Pb(NO}_3\text{)}_2)\) AnalR grade - minimum assay 95.5% supplied by BDH Chemicals Ltd., Poole, England.

Culture manipulations.

All transfers and manipulations were all carried out in a sterile air hood (Slee Ltd., London, England). Unless otherwise stated the transfer period was four weeks and the culture containers Geneco Biovessels supplied by Genetic Research Instrumentation Ltd, Dunmow, Essex, England.
Growth conditions of seedlings.

The seeds were placed in petri dishes (9cm diameter) containing filter paper (Whatmans No.1 Ashless, 7cm diameter) and the appropriate lead-ion solution (see Experimental design Chapter 3). These were then incubated at 25°C ±1°C, with a 16 hour photoperiod of 2200 LUX, provided by Thorn 30W 'Warmwhite' fluorescent tubes.

Plant regeneration.

Eight friable pieces of callus (0.5-1.0cm diameter) were removed from each of their selected cell lines and from their control cell lines (NAN/4 and TT/12). These were roughly macerated with forceps and two pieces per container were spread over the surface of media in Geneco biovessels. The media used was Murashige and Skoog (as detailed earlier) containing no hormones. The cultures were placed in the light (2200Lux) with a 16 hour photoperiod and a temperature of 25°C ±1°C.

After four weeks most of the cells were green in colour and definite roots and shoots could be seen. These were large enough (approximately 1cm long) to be transplanted to fresh media of the same composition.

Once the regenerated plants were approximately 5cm tall and the characteristic leaf form could be seen, they were removed from culture and the roots washed free of media with distilled water. They were planted in Fisons 'Jiffy' pots using a 1:1 mixture of peat and sand. They were then kept in a plastic propagator and misted twice daily for two
weeks. After this period they were planted into three inch pots without the removal of the fibre pot, again using a 1:1 mixture of sand and peat, and placed in a Fisons growth cabinet at 25°C, 8 hour photoperiod (2200 Lux) and 90% humidity. Over 6 weeks the temperature was slowly dropped to 10°C and the humidity to 40%.

Transplant to the field was in mid-April were they were allowed to grow and mature.

F1 seed production.

Plants were regenerated; 20 from the selected cell line TTL1 and 20 from the control cell line TT12. These were grown to maturity in a glasshouse and allowed to flower. Paper bags were placed over each flower-head and the flowers within brushed at regular intervals to induce self fertilisation. Seed formed and it was allowed to dry on the plant before collection. Seed was collected from each plant. It was sorted and any undeveloped (i.e. flat husks) or split seed were discarded. This sorting process occurs during the commercial production of seed (Breeders Seed Ltd., England. Personal communication).

Pollen germination and morphology.

The pollen was incubated in a solution containing 3% w/v sucrose and 0.01% w/v boric acid at 25°C for four hours. The pollen samples were then studied under a microscope and germination of the pollen tube could easily be seen.

Pollen morphology was observed under a microscope at x40.
Production and growth conditions of callus cell lines.

Full details and data of the production of the stock cell lines are shown in the Appendix. Briefly the method is as follows; 20 plants of each subvariety were grown with mature taproots of approximately 6cm x 1.5cm. 6 taproot explants from each plant were used to establish callus. After a period of eight weeks (two transfers) the fastest growing (i.e. largest) callus piece from each seed parent was transferred and multiplied to form 40 stock lines of callus tissue. The logistics of maintaining 40 stock lines resulted in the decision to reduce the numbers of cell lines. Therefore, after 4 subculture periods the 4 fastest growing cell lines were saved from each subvariety as well as the two cell lines from which the selected cell lines originated.

The ten control cell lines were designated NAN/2, NAN/3, NAN/4, NAN/5, NAN/6, TT/7, TT/10, TT/12, TT/13, TT/14 and the selected cell lines were designated TT/L1 (which originated from TT/12), NAN/L1 and NAN/L2 (which both originated from NAN/5).

Callus cultures were maintained on Gamborgs B5 media supplemented with the appropriate level of lead ions if necessary. The culture vessels were, in all cases, Geneco Biovessels (Genetic Research Instrumentation Ltd., Dunmow, Essex. UK.) and the cultures were maintained in the dark at 25°C, +/-1°C.
Seed Screen.

The optimum temperature and light levels for the germinating seed were as defined by Breeders Seeds Ltd.

The seeds were placed in petri dishes (9cm diameter) containing filter paper (Whatmans No.1 Ashless, 7cm diameter) and the appropriate lead-ion solution (see Experimental design Chapter 3). These were then incubated at 25°C +/-1°C, with a 16 hour photoperiod of 2200 LUX, provided by Thorn 30W 'Warmwhite' fluorescent tubes.

The following lead-ion concentrations were used; 0ppm, 0.1ppm, 1.0ppm, 10ppm, 50ppm, 100ppm, 150ppm and 200ppm. Two petri dishes per concentration, containing filter paper, were filled with 5mls of the appropriate lead-ion concentration. The lead ions were added in the form of lead nitrate. Each petri dish contained 20 seeds of Tiptop. This was the number which comfortably fitted in allowing each to be numbered. Hence, 40 seeds of Tiptop were used per concentration. It is a small sample size in terms of a whole population but large enough to obtain accurate means and standard errors (Haber and Runyon, 1973) for use in determining lead-ion concentrations to be used later in a larger screen. The same protocol was repeated with 320 seeds of Nanthya.

Measurements of the radicle from germinated seeds were taken daily for eight days, in mm, by laying the radicle along a clear plastic ruler.

Large seed screen.

The lead-ion concentration used was determined from the small-scale screen. This was the level at which
little or no germination took place (50ppm), and it was used as a screen.

Approximately 10,000 seeds (5000 of each sub-var) were placed 500 per petri dish which also contained filter paper and 10mls distilled water supplemented with 50ppm lead ions.

It was important to the validity of the results that the only stress the seeds received was that from the lead-ion, and so to prevent water stress (either from excess or lack) 5mls of distilled water was added to each dish every two weeks. This effectively reduced the lead-ion concentrations, but, more importantly, the total amount of lead-ions to which the seeds were exposed did not change.

**Sensitivity of carrot callus to lead ions.**

The lead-ion concentrations for this series of experiments were nominally determined using shop-bought carrots (Marks and Spencers - Variety unknown).

Tap-root explants were initiated and callus established for 4 transfer periods (16 weeks) after which small callus pieces (5-7mm diameter) were transferred to media containing one of the following lead-ion concentrations: 0ppm, 1.0ppm, 10ppm, 50ppm, and 100ppm.

Growth was assessed by measuring callus diameter. According to Fowler and Janick (1974) this can be taken to be an estimation of callus growth.

The callus diameters were measured after a four week period. Following the results obtained from above the sensitivity of callus, from the subvarieties Tiptop and Nanthya, to lead ions was assessed over eight weeks on
media containing the following lead-ion levels; 0ppm, 1.0ppm, 10ppm and 50ppm.

**Sensitivity of callus initiation to lead ions.**

Tap-root explants of the unknown variety were used to assess the sensitivity levels of callus initiation. Lead ion levels of 0ppm, 0.01ppm, 0.1ppm, 1.0ppm and 10ppm were used. 20 taproot explants (0.5cm diameter, approximately 1mm deep) were placed on media supplemented with each concentration of lead ions in petri dishes. 30mls of media and five explants per dish.

A number of variables were seen during callus initiation and production, such as explant expansion, number of sites of callus initiation on an explant and the size of these developing callus pieces. A simple positive or negative score of callus presence is not sufficient to quantify the changes taking place. A scale was developed on which the varied changes taking place on each explant could be assessed and then quantified for future comparison. A small amount of summarisation of the data was necessary to enable recordings to be taken, but this was kept to the barest minimum (see below).

The following scale was used to produce a three scores for each explant:

(a) Explant expansion = explant diameter in mm.

(b) Callus initiation sites - if n<5 then n=n
    if n>5 then n=5

(c) Callus diameter -if <2mm diameter then diameter= 1.5mm
    if >2mm and <4mm then diameter= 3.0mm
    if >4mm then diameter = 6.0mm

Callus areas were calculated using the summarised
diameters and totals per explant were obtained by the sum of the areas of the callus pieces present.

Six explants were removed from each of the taproots of the 40 plants (20 of each form) grown up from the seed. These were used to initiate callus. The remainder of the taproot from each plant was used to complete a large screen of callus initiation at a lead ion concentration of 10ppm. This was the lowest level at which no callus initiation took place using taproot explants of an unknown variety.

For the mass screen 250 explants from each taproot. There were 20 taproots from each of the two subvarieties, therefore, 5000 explants were used from each subvariety: 10,000 in total. These were placed in petri dishes containing media supplemented with 10ppm lead ions. The screen was continued until all the explants became necrotic. In effect this was 14 weeks.

Growth analysis of the cell lines.

Two filter paper squares per dish were placed on 30mls of media, with or without 10ppm lead ions, in petri dishes. The filter paper was then removed and weighed to obtain a wet weight. 20 callus pieces (approximately 1.0cm diameter) of each cell line were weighed and placed one per filter paper square. The filter paper being necessary due to the highly friable nature of the callus.

After four weeks the filter paper, plus callus, was re-weighed to obtain a fresh weight measurement. A dry weight measurement was obtained by placing the filter
paper plus callus in an incubator (60°C) on a plastic tray until no more water-loss was obtained (4 days). The filter paper and callus were removed from the incubator into a desiccator to cool for an hour and then re-weighed; together and then the filter paper separately. The final fresh weight was expressed as a percentage of the initial fresh weight to give a measurement of percentage biomass increase.

Flame atomic absorption spectrophotometry.

To prevent contamination of the sample during sampling or analysis all the apparatus was precleaned by leaching with 2M nitric acid for 24 hours. A valuable check upon contamination is provided by running standard blank solutions alongside those to be tested. These provide information as to lead in any of the reagents and indicate contamination if it occurs.

Plant material for analysis was dried in an oven at 105°C for 24 hours and then wetashed using the method of Allen and Parkinson (1969). 100mg-200mg of plant material was placed in a Kjeldahl flask to which was added, in order, 5ml of nitric acid, 1ml of perchloric acid (60%) and 0.5ml sulphuric acid. The flask was heated gently until all the brown fumes of nitric acid had been boiled off and then the heat increased until the dense white fumes of the sulphuric acid could be seen. The remaining liquid was cooled and made up to 25mls with distilled water.

Liquid samples for analysis were first acidified to 1% by nitric acid.

The atomic absorption spectrophotometer was a Varian
Model 901 (Varian Ltd., London, England) and was set on spectrophotometer programme 2 with a lamp current of 4 amps.

Establishment and maintenance of cell suspension cultures.

Cell suspensions were required for cryopreservation and these were produced from each cell line using the method of Gamborg and Wetter (1975).

Two friable callus pieces (approximately 0.5cm in diameter) were added to a 125ml flask, containing 50mls of liquid Gamborgs B5 media (as detailed before). These were placed on a shaking bed (125rpm, 4cm displacement) under the same conditions as for culture growth.

After two weeks 10mls of suspension was transferred to a 250mls flask containing 50mls of fresh media. This was repeated at 7 day intervals to produce a suspension of single cells and very small cell aggregates. Cell density was determined by cell counts using a haemocytometer.

Cryopreservation of cell lines.

The method for cryopreservation was as Nag and Street (1975). Portions (10mls) of the cell suspensions from each cell line were taken at day 6 of the 7 day transfer cycle. These were chilled in an ice bath for 15 minutes and then an equal volume of chilled medium containing 10% of the cryoprotectant Dimethylsulphoxide (AnalaR grade. Supplied by BDH Chemicals Ltd., Poole, England) was added in four equal aliquots over 60 minutes.

The resulting suspensions were then dispensed into
sterile plastic ampoules (1.4 x 4.5cm) in aliquots of 1.5mls.

The ampoules were stood in ice for 10 minutes before transfer to a programmable freezer (Kryo 10 series, Chamber model 10-16. Supplied by Planner Biomed Ltd., London, England) in which the solutions were cooled at 2°C/min down to -100°C. They were then transferred to storage in liquid nitrogen (Liquid nitrogen refrigerator, capacity 7.25 litres. Supplied by Union Carbide U.K. Ltd.)

Thawing of the samples was achieved by placing the desired ampoule in a water bath at 40°C for 30 minutes. The suspension (containing 5x10^-9 cells) was added to 100mls of media and then 0.1mls of the resulting suspension was placed onto the surface of media in a petri dish (9cm diameter). The media composition was Gamborgs B5 as stated earlier in this chapter and for the selected lines the media was supplemented with 10ppm lead ions. The culture conditions for the dishes were as those for seed germination.

After four weeks the resulting colonies were counted. Each viable cell can potentially form a colony and for 100% viability 500 colonies must be present. The percentage viability of each sample was counted.

Ion uptake by cortical tissue.

Ten mature taproots from selected plants and ten from nonselected plants were used for this experiment. The taproots were cut into slices approximately 2mm thick and then discs (5mm diameter) of cortical tissue were cut with
a cork borer. 250 discs were used from each taproot and the fresh weight of tissue from each taproot was in the range 2.26-2.29g.

Each tissue sample was put through the following series of events. The environmental conditions throughout were 25°C and 2000lux.

1) Tissue washed in aerated deionised water for 18 hours.
2) Incubated in 100mls of 10ppm lead solution in a conical flask (250mls) on a rotary shaker (100rpm, displacement 4cm) for 120 minutes. A sample (3mls) of the bathing solution was taken every hour.
3) The tissue was blotted of all surface moisture with a paper tissue and then placed in deionised water (100mls) in a conical flask (250mls) on a rotary shaker (100rpm, 4cm displacement) for 30 minutes, Samples (3mls) of the bathing solution were taken every 10 minutes.
4) The tissue was blotted of all surface water with a paper tissue and then placed in 100mls of 0.1M calcium nitrate solution in a conical flask (250mls) for 90 minutes on a rotary shaker (100rpm, 4cm displacement). Samples (3mls) were taken every 30 minutes.

All samples were acidified to 1% with full strength nitric acid and the concentration of lead ions determined using flame atomic absorption spectrophotometry.

**Hydroponics.**

The three hydroponic tanks (30cm long, 15 cm wide and 20cm high) were constructed from 5mm inert plastic and the joints were internally sealed with silicon sealer. Each tank had a lid of identical material containing 120 holes (
8mm diameter and 10mm apart). The tanks were coated externally with matt black paint to exclude light and reduce any algal growth. At the bottom of the tanks there was an air stone, fed from a conventional aquarium electric air pump, to ensure adequate aeration of the plant roots and to reduce precipitation of the lead added to the solutions.

Each tank held three litres of half strength Hoagland and Arnons solution (as described by Hewitt, 1974). The solution was replaced once a week. 150 seeds from each group were germinated and grown for two weeks on damp filter paper in petri dishes (20°C, 3000lux, 16 hour day). The most vigorous 90 from each group were selected and divided into three groups of 30. 60 plantlets (30 from each group) were "planted" in each of the three hydroponic tanks in a random pattern. They were supported in the holes with non-absorbant cotton wool and it was also used to plug the spare holes.

The plantlets were allowed to acclimatise in the tanks for one week. Lead ions were added at a level of either 0, 10 or 100ppm when the nutrient solutions were replaced. The plants were then allowed to grow under these conditions for two weeks. At the end of the first week the nutrient solutions, containing their requisite lead-ion concentrations, were replaced with fresh solutions. Throughout this time the iron citrate was added three times per week in the required concentration in order to prevent iron chlorosis developing (Hewitt, 1974).
Specimen freezing and cryomounting.

The specimens were carefully removed from the tanks and the surface of the roots well washed with deionised water before being frozen by using a plunge cooling device (Ryan and Purse, 1985). They were plunged, individually, 100mm into liquid ethane (93K) at approximately 2ms⁻¹. The method of cryomounting and the cryomounting device is as described by Ryan et al. (1988).

Cryo-scanning electron microscopy.

Specimens were examined at 3kV (uncoated) or 10kV (coated) in a JEOL JSM 35C fitted with a modified cryostage (Ryan et al., 1985). The microscope airlock also contained a cryostage, which cooled to 103K, on which frozen specimens were fractured with a cold scalpel and sputter-coated when required. The roots were freeze-fractured so that tissue approximately 1mm from the surface of the tissue block was exposed. Freeze-etching was done in the microscope at a specimen temperature of 193K where necessary. The specimens were coated with graphite in the cold stage, again, where necessary.

The specimens were exposed to the electron beam of the SEM for a considerable time before, during and after analysis.

This continued bombardment by electrons caused the specimen picture to break up. This is termed "charging" and is the reason why, unfortunately, the photographic quality is poor. The beam used during the small spot analysis to produce the plots was intense and holes can
be seen in the cells where the analysis took place.

Statistics.

The data was processed on a PRIME mainframe computer using the Minitab Statistical Package (Minitab Inc., 3081 Enterprise Drive, State College, PA 16801, USA).

Levels of significance of the data was obtained using the unpooled, two sample t-test. The computer prints the results of the t-test and the confidence interval to compare two independent samples. Further details can be found in Ryan et al., 1985. The computer will calculate the p-statistic to four decimal places. If the figure is less than this it will be considered as zero.

Correlations between two sets of data were also calculated on the Minitab system.
AIMS.
Within this project an attempt is made to select for, and study resistance to lead-ions resulting from somaclonal variation. The initial starting point is to define the concentration of lead-ions to be used as a primary screen for resistance by determining the resistance level in a seed population.

INTRODUCTION.
A population of cells or plants will generally show some resistance to an inhibitory chemical, although this resistance may be very slight. There will also be variations, within the population, of this resistance. The concentration of a chemical used as a screen for resistant cells or plants must be such that it inhibits growth of the entire unmodified population to some extent. The screen, therefore, will be constructed in such a way as to select only for truly mutant cells or plants. This enables such cells or plants to be distinguished from those exhibiting resistance at the top end of the variation occurring naturally within the population. In a review Bradshaw and McNeilly (1981) noted that the levels of available lead-ions (from lead nitrate – Pb(NO₃)₂) commonly used in whole plant resistance tests was 12ppm or 5.79x10⁻² mMdm⁻³, and so a range of lead-ion concentrations around this figure were used, from 0.1ppm to 200ppm (4.83 x 10⁻⁴ to 9.65 x 10⁻¹ mMdm⁻³). The
dissociation of lead nitrate occurs fully in distilled, deionised water and so it can be assumed that the total concentration of lead-ions approximates to the concentration of available lead ions. However, the total lead-ion concentration in cell culture media may not approximate to the available lead-ions as they may complex with the chemicals in the medium forming insoluble or non-dissociating compounds (Lane, 1978), hence, the lead may not be 'available' to exert an inhibitory action on the material in vitro (see Appendix).

The lead used is added to solutions as lead nitrate. This will fully dissociate and so for each lead ion present there will be two nitrate ions. Nitrogen is an essential mineral element, being a major constituent of proteins and amino acids (Marschner, 1986). In the form of nitrate it stimulates plant growth and is often added to crops as fertilizer (Sahrawat, 1980). During early stages of plant growth a very high nitrogen availability can enhance shoot elongation and inhibit root elongation (Klemm, 1966). It can also result in increased leaf area with decreased leaf thickness (Marschner, 1986). These effects of nitrate must be considered due to its presence in solution, however, at the comparatively low levels being used here it may stimulate the general growth without inhibiting the root growth.

The extent of natural resistance within the seed population can be determined in a number of ways, such as measurements of root expansion, leaf expansion or studies of the morphology. At this stage, however, the
simplest and quickest is to look at the germination rate and the subsequent radicle lengthening.

The seeds or plants used for any practical assessment of resistance to the effects of lead-ions will only, be a relatively small segment of the population, and as *Daucus carota* is less genetically uniform than most crops (Ammirato, 1986), the sample used will only contain a portion of the genetic variation present. One must assume that this is equally distributed over the Normal Distribution curve of the character under study. The greater the sample size used, the greater the confidence limits are for stating that the results are representative of the population as a whole (Haber and Runyon, 1973).

Resistance to the effects of specific heavy metals has a genetic base and is a continuously varying characteristic (Bradshaw and McNeilly, 1981), suggesting a number of genes involved with additive effects. Large numbers of seed, therefore, are required in an attempt to screen the whole genomic variability in the population.

The aim is to determine if, within the range of genotypes available in the population, any variation exists which confers a high level of resistance to lead toxicity. Having, therefore, examined the background levels of resistance and determined the concentration of lead at which no pre-existing variants are present, a ratio is obtained, below which no natural variants can be said to occur. For example, if when 5000 plants were stressed with a set concentration of lead, no resistant
variants were observed and if, when screening for resistant cell lines or plants, they occur more frequently than 1 in 5000 it may be concluded that they are very unlikely to have occurred due to natural variation and have probably resulted from mutations. In the case of cultured material, these mutations may result due to the genetic instability of the tissue, that is, due to somaclonal variation. A mass screen, therefore, must be carried out using, in this case, the lowest level of lead-ions at which it is thought no germination takes place.

The biochemistry and physiology prevalent in seed germination and the growth of in vitro cultures are widely different and this experiment is not necessarily ideal for determining the lead levels to be used for screening cells in vitro. The validity of using these results for tissue culture work is debatable, however, the range of lead-ion concentrations to be used must be determined somehow. It is known that as the complexity of cell structures decreases the sensitivity to stress increases (Street, 1974), so the toxic effectiveness of lead-ions to seed germination and to cell cultures is more likely to be of a similar magnitude than the levels used in whole plant toxicity studies.

A fact which also must be taken into account is that seed germination studies are a quick and effective way of assessing the background variation within the population, whilst only using a small amount of space.

A small scale screen was designed to find the toxicity of a range of lead solutions. The results from this were
then used to construct a large scale screen.

**EXPERIMENTAL DESIGN.**

Seeds were germinated in the following lead-ion concentrations; 0ppm, 0.1ppm, 1.0ppm, 10ppm, 50ppm, 100ppm, 150ppm and 200ppm. Two petri dishes per concentration, containing filter paper, were filled with 5mls of the appropriate lead-ion concentration.

Each petri dish contained 20 seeds. This was the number which comfortably fitted in allowing each to be numbered. Hence, 40 seeds of Tiptop were used per concentration. It is a small sample size in terms of a whole population but large enough to obtain accurate means and standard errors (Haber and Runyon, 1973) for use in determining lead-ion concentrations to be used later in a larger screen. The same protocol was repeated with 320 seeds of Nanthya.

Measurements of the radicle from germinated seeds were taken daily for eight days.

**Large seed screen.**

The lead-ion concentration used was determined from the small-scale screen. This was the level at which little or no germination took place (50ppm), and it was used as a screen.

Approximately 10,000 seeds (5000 of each sub-var) were placed 500 per petri dish which also contained filter paper and 10mls distilled water supplemented with 50ppm lead ions.

It was important to the validity of the results that the only stress the seeds received was that from the
lead-ions, and so to prevent water stress (either from excess or lack) 5mls of distilled water was added to each dish every two weeks. This effectively reduced the lead-ion concentrations, but, more importantly, the total amount of lead-ions to which the seeds were exposed did not change.
RESULTS.

Small seed screen.

The addition of lead ions to the bathing solution around the seeds had marked effects upon both the germination rate and on the subsequent radicle lengthening. The mean radicle lengths of the seeds over 8 days in different lead ion concentrations are shown in Figures 3.1 and 3.2. It is possibly easier to compare the effects of the lead ions by expressing the radicle length as a percentage of the control (i.e. radicle length in a solution of 0ppm lead ions) and these are shown along with their associated germination rates in Figure 3.3.

The mean radicle length of germinating Tiptop seeds with no lead ions was 26.9mm. This could be reduced to 8.71mm by the addition of 10ppm lead ions and to 1.31mm by the addition of 200ppm lead ions. The effect on germinating Nanthya seeds appeared less dramatic as the mean radicle length of the control was 9.4mm. This was reduced to 4.55mm by 10ppm and to 0.38mm by 200ppm. These effects appeared to be less than those on Tiptop seeds, however, when the radicle lengths were expressed as percentages of the control it can be seen that the effects upon the two subvarieties are similar.

For Tiptop seeds a lead-ion concentration of 0.1ppm had very little effect upon germination (85.0% as opposed to 87.5% for the control) and radicle length (97.4% of the control). A small effect upon both germination (77.5%) and radicle length (77.5%) was seen with 1.0ppm and at 10ppm the effect seen was greater still for both germination (75.0%) and radicle length (42.9%) (p =
0.0007). At 50ppm, however, the effect of the lead-ions were marked, with germination being 57.5% and radicle length only 7.6% of that of the control. Lead-ion concentrations of 100ppm, 150ppm and 200ppm showed germination respectively being 57.5%, 55.0% and 50.0%. Radicle length was respectively 5.9%, 5.8% and 3.6% of the control. These were all significantly different (p = 0 in all cases).

Nanthya seeds show much the same trend, with the lowest level at which major inhibition of radicle lengthening occurred being 50ppm lead-ions.

The only variation in the Nanthya seed results is at the 0.1ppm concentration, here the radicle lengthening after eight days is significantly (p = 0.25) greater than the control (146% as opposed to 100%). Germination is also slightly greater (85% as opposed to 75%). A possible explanation for this can be found in the presence of nitrate in the seed environment. This nitrate was present as the lead ions were added in the form of lead nitrate. This may, in the case of Tiptop, be counteracting the effects of the very small concentration of lead-ions present, and in the case of Nanthya, not only counteracting the effect of lead-ions but also stimulating the lengthening, so the result after eight days is greater than the control. In the case of both Tiptop and Nanthya, at 50ppm lead-ions germination was significantly inhibited by approximately 50% (p = 0 and p = 0.0022 respectively) and very little subsequent growth took place, with a zero growth rate by day eight. At 10ppm, although germination
was inhibited this did not occur to the same extent. Subsequent radicle lengthening occurred, but after eight days this was only approximately 45% that of the control and was significantly inhibited ($p = 0.0007$ for Tiptop and $p = 0.08$ for Nanthya).

To summarise, the germination rate is inhibited by 50% at 50ppm, or above, lead-ion concentrations, whilst 50% inhibition of radicle lengthening occurs at 10ppm, or above, concentrations.

At all lead-ion concentrations the growth of Nanthya was less than that of Tiptop. This was possibly to be expected because, although Nanthya is the F1 hybrid and may possess hybrid vigour, the outbred Tiptop has very strong foliage growth production which is higher than other Nantes-types (Breeders Seeds Ltd., 1985 Catalogue).

**Large seed screen.**

The dishes were checked weekly for germination and radicle growth.

Germination is completed when the radicle can be seen, and after ten weeks the germination rate for Tiptop was 40% and for Nanthya 35%, however, as the radicle lengths were less than 0.5mm they were not recorded. In all cases the exposed radicle became dark brown and shrivelled.
FIGURE 3.1.

Mean radicle length over time, in mm, of germinating *Daucus carota* seeds (TIPTOP) imbibed in a range of lead-ion concentrations.

<table>
<thead>
<tr>
<th>Pb $^{2+}$ conc.</th>
<th>Sample size</th>
<th>Mean radicle length (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day1</td>
<td>Day2</td>
</tr>
<tr>
<td>0.0</td>
<td>40</td>
<td>0.00</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
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FIGURE 3.2.
Mean radicle growth over time, in mm, of germinating *Daucus carota* seeds (Nanthya) imbibed in a range of lead-ion concentrations.

<table>
<thead>
<tr>
<th>Pb(^{2+}) conc.</th>
<th>Sample size</th>
<th>Mean radicle length (sd)</th>
<th>Day1</th>
<th>Day2</th>
<th>Day3</th>
<th>Day4</th>
<th>Day5</th>
<th>Day6</th>
<th>Day8</th>
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</tbody>
</table>

57
FIGURE 3.3.

Mean radicle lengths, expressed as a percentage of the control, and the associated germination rates after 8 days.

<table>
<thead>
<tr>
<th>Lead-ion conc.</th>
<th>Seed Sample</th>
<th>Radicle size</th>
<th>Radicle length</th>
<th>Germination rate</th>
</tr>
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<tbody>
<tr>
<td>0.0ppm</td>
<td>TT</td>
<td>40</td>
<td>100.0%</td>
<td>87.5%</td>
</tr>
<tr>
<td>0.1ppm</td>
<td>TT</td>
<td>40</td>
<td>97.4%</td>
<td>85.0%</td>
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<tr>
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<td>40</td>
<td>77.5%</td>
<td>77.5%</td>
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<td>40</td>
<td>42.9%</td>
<td>65.0%</td>
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<tr>
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<td>TT</td>
<td>40</td>
<td>7.6%</td>
<td>57.5%</td>
</tr>
<tr>
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<td>TT</td>
<td>40</td>
<td>5.9%</td>
<td>57.5%</td>
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<tr>
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<td>TT</td>
<td>40</td>
<td>5.8%</td>
<td>55.0%</td>
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<tr>
<td>200.0ppm</td>
<td>TT</td>
<td>40</td>
<td>3.6%</td>
<td>50.0%</td>
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<tr>
<td>0.0ppm</td>
<td>NAN</td>
<td>40</td>
<td>100.0%</td>
<td>75.0%</td>
</tr>
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<td>NAN</td>
<td>40</td>
<td>146.0%</td>
<td>85.0%</td>
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<tr>
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<td>NAN</td>
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<td>40.0%</td>
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<td>NAN</td>
<td>40</td>
<td>5.1%</td>
<td>40.0%</td>
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<td>NAN</td>
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<td>NAN</td>
<td>40</td>
<td>4.0%</td>
<td>30.0%</td>
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</table>
DISCUSSION.

As a prerequisite to germination the seed must imbibe water from its external environment and for any heavy metal ions present to inhibit this germination they must enter the seed to reach their site of effect.

Lead has been shown to cause chromosomal aberrations and become associated with many cellular and subcellular membranes. (Koeppe, 1981, for review). This often causes effects on many enzymes, such as reductions in the activity of phosphatase and enzyme bound ATPase. These effects are manifested in respiration, photosynthesis and yield (either fresh, dry or seed weight).

It can be assumed that the inhibition of germination, seen partially at 10ppm lead-ions and fully at concentrations above this, can be attributed to the effects of the lead-ions, present in the bathing water, entering the seed. In all cases the inhibition of germination is comparable to the inhibition in radicle lengthening, hence implying that the inhibitive effects of lead-ions are exerted upon both germination and radicle lengthening, although germination does not appear as sensitive as radicle lengthening. Lead has been found to inhibit the elongation of coleoptile segments and it was hypothesized that lead was interfering with a factor directly involved with cell elongation (Zeger et al., 1979). This suggests the specific involvement of enzymes in the cell wall and the ATPase associated with the plasmolemma. It has also been noted that due to the charge on, and chelating effects of, the lead ions the net electroosmotic flux into the cells is altered (Fensom,
1977) and this results in a general perturbation of the ionic transport systems across the cell wall and plasmamembrane (Pitman et al., 1977). These factors might explain why, at 10ppm lead-ions, when germination takes place the subsequent radicle lengthening is almost totally inhibited, implying that lead ions have an inhibiting effect upon either or both of cell division and cell elongation.

The addition of lead nitrate to a solution will alter the water potential and thoughts should be given to the possible osmotic effect of the ions, which, if significant, would cause water stress in the seeds or radicles. Many of the metabolic events associated with germination are able to proceed in osmotically stressed embryos, but it is the initiation of radicle elongation (the event that terminates the germination process) which is prevented by osmotic stress (Ross and Hegarty, 1979). Ross and Hegarty (1979) assessed germination and radicle growth in 13 vegetable crop species, including Daucus carota, over a wide range of water potentials and found that the process of radicle elongation can be inhibited by a water potential more negative than -8 bars. A lead nitrate solution of 50ppm when fully dissociated has a water potential of -2.6 bars and so an osmotic factor is an unlikely contributor to the date reported for these seed screens.

The LD50's of germination rate and radicle lengthening occur at 50ppm and 10ppm lead-ions respectively, therefore, the lead-ion concentration which will be used further in the selection studies lies in the area 10-50ppm
lead-ions.

It is expected that cell cultures will be more sensitive to the effects of lead-ions than seed germination, for as the complexity of cell structures decreases the sensitivity to stress increases (Street, 1974) and so the level at which most, but not all, germination is inhibited (i.e. 10ppm) will be used for future culture work.

Large seed screen.

Over a period of ten weeks exposure to a lead-ion concentration of 50ppm resulted in germination rates of 40% and 35% for Tiptop and Nanthya respectively, however, no radicle lengths were recorded as all were below 0.5mm and accurate measurement was impractical.

Although in some cases germination takes place, as no subsequent radicle lengthening occurs it may be assumed that there are no naturally occurring variants which show any resistance to the effects of a lead-ion concentration of 50ppm within the samples of the seed populations of both forms of Daucus carota cv. Nantes. It may be said, therefore, that if any natural genotypes occur which confer an absolute resistance to the lead toxicity exerted by 50ppm lead-ions then these exist at a level below 1 in 5000 seeds.

If resistant variants occur at a ratio greater than 1 in 5000, when screening for resistance to lead toxicity, then it would be unlikely that these were part of the natural genotypic variation within the population, and may be present due the effects of mutation or somaclonal
variation.

SUMMARY.

Germination and subsequent radicle lengthening were significantly effected by lead ion concentrations of 10ppm and above. A mass screen of seeds at 50ppm found none that were resistant to this level of stress.
CHAPTER 4

DETERMINATION OF THE LEAD-ION SENSITIVITY OF CARROT CALLUS GROWTH AND CALLUS INITIATION.

GENERAL AIMS.

This series of experiments defines the range of lead-ion concentrations over which detrimental effects can be observed upon both culture initiation and subsequent cell growth. The ultimate aim was two-fold; to determine a lead ion concentration suitable for use in future in vitro screening, whilst assessing the effect of lead-ions on the complex processes of callus initiation, with a view to selection of variants.

4.1 THE SENSITIVITY OF CARROT CALLUS TO LEAD ION CONCENTRATIONS IN THE CULTURE MEDIUM.

INTRODUCTION.

It was thought that callus cultures may be more sensitive to lead-ions than both germination and radicle lengthening due to the reduced tissue organisation in respect of both its morphological characteristics and its metabolic stage of development.

The results from Chapter 3 indicated that the lowest lead ion levels causing marked detrimental effects on radicle growth were in the region 10-50ppm lead ions. Using these results, therefore, as guide-lines, the basic effects of different lead ion levels upon the inhibition of callus growth were determined.

These experiments were firstly conducted using callus from Daucus carota of an unknown variety, thus allowing the maximum callus from the known varieties to be used once
the general levels for use were determined.

**EXPERIMENTAL DESIGN.**

The lead-ion concentrations for this series of experiments were nominally determined using shop-bought carrots (Marks and Spencers - Variety unknown).

Tap-root explants were initiated and callus established for 4 transfer periods (16 weeks) after which small callus pieces (5-7mm diameter) were transferred to media containing one of the following lead-ion concentrations: 0ppm, 1.0ppm, 10ppm, 50ppm, and 100ppm.

Growth was assessed by measuring callus diameter. According to Fowler and Janick (1974) this can be taken to be an estimation of callus growth.

The callus diameters were measured after a four week period. Following the results obtained from above the sensitivity of callus, from the subvarieties Tiptop and Nanthya, to lead ions was assessed over eight weeks on media containing the following lead-ion levels; 0ppm, 1.0ppm, 10ppm and 50ppm.
RESULTS.

It can be seen from Figure 4.2 that, after four weeks, media containing a lead-ion concentration of 10ppm inhibits practically all callus growth (measured as increase in callus diameter). Media containing 50ppm lead ions inhibits all callus growth and that containing 100ppm lead ions results in a net reduction in the callus diameters. It was therefore decided to use the same concentrations, except 100ppm, to determine the sensitivity of Tiptop and Nanthya callus growth over a longer period.

The lead-ion concentration of 100ppm resulted in the callus pieces shrivelling and turning from a yellow/cream colour to dark brown. This can be seen in the photograph Figure 4.1. As the aim of this experiment was to determine the lowest lead-ion concentration causing total inhibition, for later use as a screen for resistance, and a level 50ppm lead ions also resulted in no growth, it was decided not to continue with the 100ppm level.
FIGURE 4.1

Photograph showing the effects of 100ppm lead ions on the morphology of *Daucus carota* callus. The shrivelled brown callus pieces have been grown on media containing 100ppm lead ions. These can be readily compared with the large creamy/yellow pieces grown on media without lead ions.
FIGURE 4.2

Graph showing the relationship between the mean increase in callus diameter over four weeks, at different media lead ion concentrations, for callus tissue of Daucus carota (Variety Unknown).
FIGURE 4.3

Graph showing the relationship between the mean increase in callus diameter over eight weeks, at different media lead-ion concentrations, for callus tissue of Daucus carota (Nantes cv. Tiptop).
FIGURE 4.4

Graph showing the relationship between the mean increases in callus diameter over eight weeks, at different media lead-ion concentrations, for callus tissue of Daucus carota (Nantes cv. Nanthya).
DISCUSSION.

Over a period of eight weeks the lead ion level of 10 ppm resulted, for both Tiptop and Nanthya cell lines, in an almost total inhibition of the increase in callus diameter and hence growth. A net reduction in mean callus diameter was seen with 50 ppm lead ions.

The method for estimation of callus growth (Fowler and Janick, 1974) was found to be highly inadequate. The callus diameters were difficult to measure for, as growth took place, the callus pieces increased unevenly in size resulting in non-circular and uneven margins. The friable nature of the callus tissue resulted in small pieces breaking off the main mass, again rendering measurement of the diameter difficult.

Fowler and Janick assessed the effectiveness of their method on slow-growing, non-friable strawberry callus, the shape of which approximated to a hemisphere. The carrot callus was observed to differ, to some degree, on all three of these points. The method has a major flaw as to its accuracy, for although the correlation coefficient (r) between the cubed diameter of the callus and the callus weight was high (0.94) the variation in the results was also very large. Callus pieces weighing 57 mg and 295 mg both had the same cubed diameter of 725 mm$^3$, also two pieces of callus, both weighing 57 mg, had cubed diameters of 425 mm$^3$ and 725 mm$^3$. The large summarization of data inherent in this method means that a very large sample size must be used before any accurate conclusions can be drawn.

The factors mentioned all result in a conclusion
that this method for callus growth estimation is adequate for the determination of general trends, but inadequate, due to its high level of inaccuracy, for the direct quantification of those trends.

Tissue from an unknown variety of carrot was initially used to determine the lead ion levels to be used in this experiment. There can be varietal difference in response to stress, however, in D. carota these are not great (Ammirato, 1986). The strength, therefore, of this approach is that it allows the maximum amount of callus from the known varieties to be used in the final experiment. Valuable tissue is not sacrificed in determination of the general levels of stress to be used.
4.2 CALLUS INITIATION FROM TAPROOT EXPLANTS AND THE SENSITIVITY OF THIS PROCESS TO LEAD IONS IN THE CULTURE MEDIUM.

INTRODUCTION.

Callus initiation is the result of cellular and molecular processes which are very different to those resulting in undifferentiated callus growth (Hall, 1984; Street, 1977; Tranh Van and Trinh, 1986). These major changes, taking place within the cells as callus is initiated may enable a variant cell line, resistant to lead ion stress, to be isolated from this stage by subjecting the changing cells to the desired stress. This was attempted and the implications discussed.

Once the lowest concentration of lead-ions to cause 100% inhibition of callus initiation was determined, all the available taproot explants from the experimental plants of Tiptop and Nanthya were used for a mass screen at this concentration. The aim being to determine the upper limits of the naturally occurring resistance within the population.

The lowest lead-ion level which results in total inhibition of callus initiation was initially determined using taproot explants from *Daucus carota* of an unknown variety, hence, enabling all the available taproot explants from the stock plants to be used for the mass screen.

EXPERIMENTAL DESIGN.

Tap-root explants of the unknown variety were used to assess the sensitivity levels of callus initiation. Lead
ion levels of 0ppm, 0.01ppm, 0.1ppm, 1.0ppm and 10ppm were used. 20 taproot explants (0.5cm diameter, approximately 1mm deep) were placed on media supplemented with each concentration of lead ions in petri dishes. 30mls of media and five explants per dish.

A number of variables were seen during callus initiation and production, such as explant expansion, number of sites of callus initiation on an explant (i.e. discrete areas of callus formation, separated from each other by at least 1mm) and the size of these developing callus pieces. A simple positive or negative score of callus presence is not sufficient to quantify the changes taking place. A scale was developed on which the varied changes taking place on each explant could be assessed and then quantified for future comparison. A small amount of summarisation of the data was necessary to enable recordings to be taken, but this was kept to the barest minimum (see below).

The following scale was used to produce three scores for each explant:

(a) Explant expansion = explant diameter in mm.

(b) Callus initiation sites - if n<5 then n=n
    if n>5 then n=5

(c) Callus diameter - if <2mm diameter then diameter= 1.5mm
    if >2mm and <4mm then diameter= 3.0mm
    if >4mm then diameter = 6.0mm

Callus areas were calculated using the summarised diameters and totals per explant were obtained by the sum of the areas of the callus pieces present.

Six explants were removed from each of the taproots of
the 40 plants (20 of each form) grown up from the seed. These were used to initiate callus. The remainder of the taproot from each plant was used to complete a large screen of callus initiation at a lead ion concentration of 10ppm. This was the lowest level at which no callus initiation took place using taproot explants of an unknown variety.

For the mass screen 250 explants from each taproot. There were 20 taproots from each of the two subvarieties, therefore, 5000 explants were used from each subvariety: 10,000 in total. These were placed in petri dishes containing media supplemented with 10ppm lead ions. The screen was continued until all the explants became necrotic. In effect this was 14 weeks.
RESULTS.

A number of factors were assessed, namely:

a) Explant expansion (mm diameter).

b) Number of sites of callus initiation.

c) Total area of callus (mm²).

The interrelation of the three variables with the lead ion concentrations is shown in Figures 4.5, 4.6 and 4.7.

When no lead ions were present in the culture media the explant diameter increased over the ten week period to between 12mm and 16mm. The mean diameter increased to 12.9mm. The groups of explants subjected to 0.01ppm, 0.1ppm, 1.0ppm and 10ppm lead ions in the culture medium had respective mean diameter increases of 11.6mm, 14.5mm, 14.4mm and 14.2mm. None of these groups means, however, were significantly different (p=0.027, p=0.0026, p=0.0004 and p=0.0086 respectively) from that of the control (0ppm lead ions).

The number of sites of callus initiation, after ten weeks, on those explants placed on media containing no lead ions varied between 5 and 12, with the mean being 9.1. The presence of 0.01ppm lead ions in the culture media resulted in the mean number of callus initiation sites, after ten weeks, being reduced to 6.6. This is not significantly different from the control (p= 0.014). The presence of 0.1ppm and 1.0ppm lead ions in the media resulted in reductions in the mean number of callus initiation sites to, respectively, 5.0 and 3.8. These are both significantly different from the control (p=0 in both cases) but not from each other (p=0.11). Over the ten week experimental period
no sites of callus initiation were seen when the culture medium contained 10ppm lead ions.

The presence of 0.01ppm lead ions in the culture media significantly reduced \( \text{p}=0.0003 \) the mean area of callus formed. Significant reductions were also seen in the mean when the media contained 0.1ppm \( \text{p}=0 \) and 1.0ppm \( \text{p}=0 \). 10ppm lead ions resulted in no callus initiation and therefore no callus area.

Mass screen for callus initiation on taproot explants of known varieties.

Lead ions (10ppm) in the culture media resulted in no callus initiation over ten weeks, and so this level was used for the mass screen of all the available taproot explants from both Tiptop and Nanthya.

Callus initiation was scored on a presence or absence basis every week for fourteen weeks. At this stage all the explants were very soft, dark brown and necrotic. The results are shown in Figure 4.8.

Three small area of callus formed. This was removed from the explants and placed on conditioned media (see Chapter 2) for four weeks to multiply the tissue. It was subsequently subcultured and maintained on media containing 10ppm lead ions as cultures can show an eventual loss of resistance under non-selective conditions (Gonzales and Widholm, 1985). The three selected cell lines were designated NAN/L1, NAN/L2 and TT/L1.
FIGURE 4.5

Graphical representation of the relationship between mean taproot explant expansion and lead ion concentration over ten weeks using an unknown variety of Daucus carota.
FIGURE 4.6

Graphical representation of the relationship between mean number of callus initiation sites per explant and lead ion concentration over ten weeks, using an unknown variety of Daucus carota.
Graphical representation of the relationship between the mean area of callus (in mm²) per explant and lead ion concentration, over ten weeks using an unknown variety of Daucus carota.
FIGURE 4.8.

Table showing the number of sites of callus initiation on taproot explants from the Nantes subvarieties Tiptop and Nanthya when placed on culture media containing 10ppm lead ions.

The absence of callus is indicated by * for Tiptop and $ for Nanthya.

<table>
<thead>
<tr>
<th>Taproot sample number</th>
<th>Taproot size (mm)</th>
<th>Number of explants showing calli initiation.</th>
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</table>
DISCUSSION.

Lead ions, of up to 10ppm, in the culture media had no effect upon taproot explant expansion. The number of sites of callus initiation and the area of initiated callus were both adversely affected by lead ions in the media. Noticeable effects being seen at 0.1ppm, whilst at 10ppm callus initiation was completely inhibited. Lead has been shown to inhibit metabolic processes, such as respiration, and to inhibit cell division (see Chapter 1 for a review). It may be expected, therefore, that that explant expansion, if it was caused purely by cell expansion would continue whilst callus initiation was inhibited.

There is no correlation \((r=0.03)\) between expansion and callus initiation at any level, indicating that expansion and callus initiation are not linked.

The number of sites of callus initiation was positively correlated \((r=0.08)\) with the area of callus. This is the simple response that may have been expected. Established callus exhibited no competitive exclusion of newly initiating callus.

Two small area of callus originated on the same explant from the plant Nanthya/5. These were used to form the cell lines NAN/L1 and NAN/L2. It must be considered if these initiation events are connected or independent. If the acts of callus initiation are completely independent then the probability of them occurring on the same explant, or on separate explants, is identical. On the other hand, the events may be connected. for example, the taproot from which the explant originated may have an altered genotype,
with a mutagenic event occurring early in its seed history. If this were the case it would have been expected that other explants from the same taproot would show callus initiation, however, this was not the case.

An area of initiating callus may have originated from a single cell in which had occurred a mutation event. This may then have become determined for callus formation and had dedifferentiated. The resultant variant cell line may therefore be considered clonal. Alternatively a small area of cells may become determined and dedifferentiated to form a clump of callus. The resulting cell line may be a mixture of genotypes with possibly the truly variant cells protecting or ameliorating the effects of the stress on any nonvariant cells. The resulting variant cell line would therefore not be homogeneous and there may be a gradient of resistance exhibited by the cells, with some being fully resistant and some being only partially resistant. It can be considered that individual cells are not determined (Meins, 1987) but it is the cell-cell interactions and morphogenic fields that direct groups of cells to develop in specific ways. This may have implications if the genetic mutation conferring the characteristic under selection did occur only in one cell. It must be mentioned that at this point in the investigation nothing is known about the concentrations or gradients of lead ions within the explant and the actual stresses which these are placing on the individual cells. The lead ion resistance of any initiated callus can only be presumed at this stage. It must be demonstrated by further experiments.
Taproot explants placed on media containing 10ppm lead ions showed no callus initiation apart from the three small areas of presumed resistant callus. Established callus exhibited a somewhat lesser sensitivity to the effects of the lead ions in that growth did take place, even if it was at a reduced level.

The molecular, biochemical and physiological processes resulting in callus initiation, such as cellular dedifferentiation, are very different to those of callus growth, such as conventional cell division (Bornman, 1974). The implications of selecting for variants at the stage of callus initiation must be considered.

Cellular differentiation is a complex process and its understanding implies an understanding of the underlying cellular biochemistry and physiology, along with its genetic control. The present acceptance of the theory of totipotency may have lead to an oversimplified view of plant development (Henshaw et al., 1982). Cellular differentiation may be divided into a number of processes loosely termed determination, differentiation and development, the boundaries of which are vague. During determination the cellular state alters so that the external stimuli to which it may respond and the patterns of that response are resolved (Mutaftschiev et al., 1987). It is during this phase that both the genes to be expressed and the degree of their expression is determined. Differentiation is the actual differential expression of the cells genetic state when proteins are formed by the transcription and translation of the 'determined' genetic code. Callus forming from a differentiated tissue (such as
in this case taproot cortex) is termed dedifferentiated tissue (Ammirato, 1986). This is to distinguish its reversion to the undifferentiated state. Finally, development is, as it implies, the actual development of the tissue, organ or plant.

Genetic information contained in the DNA is utilized to make proteins in a two-stage process. The first stage, known as transcription, involves the synthesis of RNA from the DNA template. These DNA-prescribed RNA molecules serve as templates for the synthesis of polypeptides in a complex process called translation, whereby the RNA orders and polymerizes amino acids into polypeptide chains. Proteins consist of one or more of these chains (Bodley, 1987). Most transcripts must be processed before they are used and there exists a wide variety of enzymes which carry out post-transcriptional modifications to the RNA molecules (Rodriguez and Chamberlain, 1982). The polypeptide chains then fold into complex, three-dimensional structures to produce proteins with vastly differing properties and functions (see Zubay, 1987 for a full review). The DNA can be divided into transcription units which contain: i) a promoter region which informs the RNA polymerase where to bind, how tightly to bind and how frequently to initiate an RNA chain; ii) a central coding sequence which contains the genetic code required to produce the specific polypeptides and iii) a terminator region which causes the RNA polymerase to stop transcribing (Zubay, 1987). The strength of the promoters (defined in terms of the frequency of RNA initiation) differs greatly (Burgess et
Determination takes place at the level of the DNA and the processes controlling this stage are unclear (Barker-Burgess and Burgess, 1987; McClure, 1985). The major problem for research workers when trying to understand determination is that the cell appears to show no detectable signs that the determination has taken place (Knowland, 1984). Determination results from stable changes that persist even in the absence of the agent responsible for the change (Meins, 1987) and a tissue that normally forms a specific structure is said to be determined provided that it continues to form that structure when placed in a new environment. Plant regeneration experiments have shown that many cell types, even those in determined organs, are totipotent. This apparent paradox is resolved by assuming that determination in plants is essentially a supracellular phenomenon (Meins, 1987). Individual cells are not themselves determined. It is the cell-cell interactions and morphogenic fields that direct groups of cells to develop in specific ways and in principle determination could be a clonal process in which cells become committed and transmit this state to daughter cells when they divide (Jeffree et al., 1987). According to this view commitment is inherited by cells but is not permanent. Plant growth regulators play an important part in mediating plant development (Jeffree et al., 1987; Meins, 1987) and a major question is whether these hormones actually control the determination of the different cell types, or if they only stimulate the expression of the cells already determined developmental potential. The action of a hormone
governing the activity of a single gene, or group of functionally related genes is very important. These studies, however, do not start until after a particular type of differentiated cell characterised, if not defined, by the proteins it synthesizes in response to the hormones has appeared. Genes are not always controlled by hormones and hormones can have a profound effect upon a cell without necessarily affecting the activity of its genes (Baulieu et al., 1978). In so far as differentiation involves selective gene expression, hormones which control differentiation must act by regulating gene expression (Zubay and Palmiter, 1987).

The actual presence of a stress has been shown to stimulate altered gene expression. The processes of transcription and translation are redirected to the synthesis of different proteins, whilst pre-stress gene expression is inhibited (Sachs et al., 1980; Rowland and Stromer, 1986). Metal-complexing proteins have been found in both animals (Grill et al., 1987) and plants (Misra and Gedamu, 1989), however, the mechanisms responsible for the transcriptional and translational control during stress are not well defined (Russell and Sachs, 1989) and it is unclear if the responses are due to mutational changes or if they are the result of gene expression being induced by the presence of the metal stress. The resistance-effect need not necessarily be induced by the presence of lead itself. Compounds produced, accumulated or depleted by the presence of lead may be the active inducers. This subject is discussed further in Chapter 8.
Gene expression, however it may be caused, is complex and the exact complement of expression products is different in different organs, in cells at different stages of development and in plants grown under different conditions (Ooms, 1987). This demonstrates the complexity of gene expression and its control but it indicates very little as to the importance and biological function of the corresponding individual genes during determination and differentiation. The acts of determination and differentiation are times of high genetic activity within the cell (Knowland, 1984). Gene activity is a widely used phrase, but it is not particularly easy to define and may have different meanings in different contexts. It can be used to refer to the total amount of a single protein that is made in a certain time by a large number of cells, in which case it describes the total activity of a gene in a tissue at the level of protein synthesis. This can be described as the integrated activity of a gene producing a particular protein. It is far more useful to use the term gene activity to describe the activity of a single gene, either at transcription or translation. The absolute activity of a gene can then be described as the number of transcriptions produced per gene in a given time or as the number of protein molecules synthesized per gene in the same time (Palmiter, 1975; Zubay, 1987). The differential activity of a gene is a concept which refers to its own activity relative to that of other genes. It can be defined as the rate at which a gene is transcribed or translated relative to the total rate of transcription or translation. This is perhaps a more important concept than
the absolute activity of a gene. It is clearly difficult to measure as the total rates of translation or transcription are difficult to measure (Knowland, 1984). A large amount of gene activity, by whatever definition, must be taking place during cellular determination and callus initiation. To put it crudely, the genes initially being expressed dictate that, in this instance, the cells are those of taproot cortex. These genes must be 'switched off' and those allowing undifferentiated callus growth must be 'switched on'. The cells will now respond to hormonal (growth regulator) signals allowing their potential for totipotency to be fulfilled. A great many more potential genetic states are opened up to the cells, depending upon the external stimuli to which they are exposed. There is, also, greater movement of transposable elements (Shepherd, 1988) which are thought to be one of the causative agents of somaclonal variation (Jacobs et al., 1987).

A point to appreciate is that even a small mutation can have a significant effect. A mutation leading to an amino acid alteration is a sensitive function of the location and role of that amino acid in the fully formed protein. In some cases the change is so insignificant that it would ordinarily go unnoticed. In other cases it would produce highly visible changes in the functioning of the protein (Bodley, 1987). More importantly, if a mutation occurs within the promoter region of a gene the strength of this promoter may be increased resulting in gene amplification. The strength, however, may also be decreased (Bollum et al., 1987).
It would not be surprising to consider that the application of a selection pressure at the time of cellular determination for callus growth would result in that pressure being applied to a greater diversity of genetic states, and hence increasing the probability of a variant cell line being formed. Somaclonal variation is the result of spontaneous genetic mutation (see Chapter 1) and the probability of it occurring is very high when genetic activity is also high. As a result of placing the explant directly under the selection pressure, all the processes of cellular determination and differentiation are subjected to the stress. This lead stress in the media also removes all those mutations which may have occurred that are not resistant to lead. More normally, callus has been initiated and then the selection pressure has been applied (see Chapter 1) but in this case both have been accomplished together.

It can be considered that individual cells are not determined (Meins, 1987) but it is the cell-cell interactions and morphogenic fields that direct groups of cells to develop in specific ways. This may have implications if the genetic mutation conferring the characteristic under selection did occur only in one cell. An area of initiating callus may have originated from a single cell in which had occurred a mutation event. This may then have become determined for callus formation and had dedifferentiated. The resultant variant cell line may therefore be considered clonal. Alternatively a small area of cells may become determined and dedifferentiated to form a clump of callus. The resulting cell line may be a...
mixture of genotypes with possibly the truly variant cells protecting or ameliorating the effects of the stress on any nonvariant cells. The resulting variant cell line would therefore not be homogeneous and there may be a gradient of resistance exhibited by the cells, with some being fully resistant and some being only partially resistant. There is evidence to suggest, therefore, that a variant cell line may not be homogeneous.

A variant cell line selected as callus is initiated may have a different genetic basis compared to one isolated from fully formed callus cells. Somaclonal variation has been found to be the result of many different types of genetic mutation (Larkin and Scowcroft, 1984) from point mutations to gross chromosomal changes. During callus initiation there are a great many potential genetic states open to the cell and a great deal of genetic activity is taking place (Zubay, 1987), including a greater movement of transposable elements (Shepherd, 1988). The presence of a stress has been shown to stimulate altered gene expression (Rowland and Stromer, 1986) and therefore it would not be surprising for a genetic mutation or altered gene expression to occur. A translocation or inversion may be more likely than a point mutation but any conclusions as to the type of mutations in the resulting variant lines can only be drawn after extensive karyotypic or RFLP analysis.
SUMMARY.

A lead ion concentration of 10 ppm severely inhibits callus growth and is also greatly inhibitive of callus initiation. A mass screen of taproot explants at 10 ppm lead ions resulted in the initiation of three callus cell lines.
CHAPTER 5

CHARACTERIZATION OF THE LEAD ION RESISTANT CALLUS CELL LINES.

AIMS.

The aim of this series of experiments is to quantify the responses of the callus cell lines, both selected and unselected, to the presence of lead in the culture medium.

INTRODUCTION.

Growth analysis of the cell lines.

In order to characterize the growth responses of the cell lines to lead, a quantification of the growth is needed. This enables the resistance to lead exhibited by the selected lines to be directly compared to unselected, control, cell lines. A quantitative measure of resistance, if any, can, therefore, be demonstrated.

There is some difficulty when using such a loosely used term as 'growth'. It is commonly used to define a simple increase or rate of increase (or decrease in the case of negative growth) in such widely varying parameters as weight, height, size, etc. It is more properly used to indicate an irreversible change in biomass and it is in this context it is used here.

Fresh weight is a measure of the weight of the hydrated tissue and as such an increase by be caused by an increase in either the biomass or in the percentage water content. In some cases it is easier to monitor changes in biomass by dry weight measurements. This approach, however, has disadvantages and a number of workers have chosen to measure fresh weight (Rahman and Kaul, 1989; Reddy and
Vaidyanath, 1986). The most obvious disadvantage is the destruction of the tissue necessitated by the dry weight measurement, hence a piece of tissue may only be monitored once. The use of dry weights also presents a practical problem in that a comparatively large callus piece, of say 1g, may only have a dry weight of 10mg. This is difficult to weigh accurately, any errors will constitute a greater percentage of the whole and the variation in results will be reduced, as differences in fresh weights of up to 100mg will be undetectable when the dry weights are measured. Preserving variation is paramount. Large variations in growth response or a continuous variation in response are very important factors when considering the genetic basis of the resistance character. They may have implications as to the influence of multigene families (Zubay, 1987) or the presence of gene amplification (Bollum et al., 1987).

Callus growth was assessed by measurement of the fresh weight increase and was expressed as the percentage increase over the test period. The increase in fresh weight can only be said to be biomass production if the percentage water content remains constant. Differences seen in the fresh weight increases over the test period may simply be due to changes in the percentage water content of the tissue. The dry weights of the cell lines used and of all the experimental tissue were obtained, allowing the percentage water contents to be calculated.

The method for the estimation of callus growth used earlier (Fowler and Janik, 1974) was found to be unsatisfactory (see Chapter 4), mainly due to its
inaccuracy. It was therefore decided to monitor the increase in fresh weight by direct weighing under aseptic conditions. This has a number of disadvantages. The most obvious being the risk of contamination, however, this can be avoided by careful manipulation of the tissue. A more important problem, which can not readily be overcome, is that two pieces of callus with similar appearance may not have the same growth potential, so it is important to ensure that the tissue is as homogeneous as possible and that a large sample size is used. The disadvantages are, however, far outweighed by the major advantage of enabling accurate fresh weight increases of each callus piece to be recorded, hence allowing statistical methods to be applied to the results.

The selected lines (designated TT/L1, NAN/L1 and NAN/L2) were selected by, and subsequently maintained on media containing 10ppm lead ions. An assessment of the growth of these callus lines was undertaken on media containing 10ppm lead ions. This was compared to the biomass increase of control cell lines (initiated and subsequently maintained on non lead-containing media) when exposed to the same lead stress.

A flow chart of cell line origins and production can be seen in the Appendix.

When a stress factor is not present the whole plant, or cell culture, resistant to that stress often has reduced growth rates compared to their non-resistant forms (Bradshaw and McNeilly, 1981; Gonzales and Widholm, 1985). Callus growth for the selected and control cell lines was, therefore, also compared on lead free media.
Tissue lead concentrations.

Once it has been established that a cell line exhibits resistance, it is desirable to define the nature of this resistance. If the resistant cell line is also tolerant then the lethal chemical will enter the cell but have no obvious effect upon growth or development and the uptake can be either obligative (uncontrolled) or facultative (controlled) (Rhue, 1979). On the other hand, the resistance may be due to an exclusion mechanism, in which the tissue in some way diverts the chemical, or restricts it to a certain area, thus preventing it from reaching its site of effect. For example, the resistance to aluminium shown by selected carrot cell lines (Ojima and Ohira, 1985) is due to the chelating effect of citric acid which is abundantly released into the culture medium by the cells. This release of citric acid is stimulated by the presence of aluminium ions. The aluminium ions are prevented, therefore, from reaching their site of effect and the cellular aluminium concentration of these cells is not increased. The cell line appears to be resistant to aluminium, although once the mechanism is understood there is a case for considering this avoidance rather than resistance.

The concentrations of lead were determined, for both the control and selected cell lines, after the test period on growth media containing 10ppm lead ions. The lead concentrations were measured using atomic absorption spectrophotometry.
Stability of the resistance mechanism.

A major difficulty encountered with plant cell lines which have been selected to exhibit a resistance mechanism is the instability of this characteristic when the selection pressure is removed (Gonzalez and Widholm, 1985). The resistance mechanism exhibited by the selected lines may have a stable genetic basis, resulting from some form of genetic mutation. The resistance, however, would be unstable if it were due to a transient genetic or epigenetic adaptation, or if the genetic lesion responsible had a high reversion rate. It has been suggested by many workers (see Chapter 1 for a review) that one of the causes of somaclonal variation is the presence, in the cell genome, of transposable elements. These can become dislodged from their position and integrate at a new chromosomal location (Larkin et al., 1985; Saedler et al., 1983). A prominent property of these mutations, however, is that they can frequently revert to wild-type due to somatic instability. The resistance may, on the other hand, be a physiological response caused by the presence of lead ions. The genes for this response will already be present with the lead acting in some stimulative or inductive way. Cell lines may become adapted, to a greater or lesser extent, to a particular stress, such as water stress (Fallon and Phillips, 1989) or the presence of a stress may induce a synthesis pathway. In Soybean suspension cultures, for example, ferritin synthesis is induced by excess iron in the culture medium (Proudhon et al., 1989). In both cases the response is dependant upon the presence of the stress. It was, therefore, most desirable to determine if the
resistance characteristic was stable when the presence of lead ions were removed. The results of such an experiment allow conclusions to be drawn as to the probable causes of the cellular resistance.

Cryopreservation of the cell lines.

It is most important that a variant cell line can be stored without any loss of the variant characteristics. It has been discussed earlier (Chapter 1) that prolonged periods in culture can result in spontaneous genetic mutations with possibly the eventual loss of the desired characteristics. The genetic stability of the cultures can be increased by limiting the growth. This is obtained either by decreasing the temperature at which the cultures are maintained or by adding retardent chemicals to the media (Withers, 1984). These cultures, however, may still be lost or damaged due to a number of external factors, such as contamination, toxin build-up or human error (Bhojwani and Razdan, 1983). The only real protection against these eventualities is the cryostorage of some of the variant tissue, which can then be used to reinitiate cultures of the variant cell line if the original is lost. Cryostorage, or cryopreservation, is the storage of cells or tissues at very low temperatures, usually at -196°C in liquid nitrogen (Grout and Morris, 1987).

Freezing in biological materials is complex because of their heterogeneous nature and a major problem is cellular disruption caused by ice-crystal formation during freezing or recrystalization during thawing (Withers, 1979). Ice
crystals cause mechanical damage to the cells. Disruption also results from phase separation as water molecules migrate to form crystals during freezing, whilst the solutes and other cell constituents are concentrated in the matrix, or grain boundary, between crystals (Ryan et al., 1988). Ultrastructural deformation and disruption by ice crystals can be avoided, or reduced, by the use of a cryoprotectant. Low-molecular-weight cryoprotectants (such as glycerol and dimethylsulphoxide) are water soluble molecules able to penetrate cell membranes. They interact strongly with water, ions and bipolymers giving rise to metabolic and physiological changes (Franks, 1978). High-molecular-weight non-penetrating polymers (such as polyvinylpyrrolidone and hydroxyethyl starch) may also be used.

The cryopreserved cell lines formed the reference lines from which new cultures could be reinitiated if any undesirable genetic variation was noticed in the cell lines used for experimentation.

EXPERIMENTAL DESIGN.

Growth analysis of the cell lines.

Callus pieces (approximately 1.0cm diameter) from each of the cell lines were placed on culture media containing 10ppm lead ions for four weeks. The final fresh weight was expressed as a percentage of the initial fresh weight to give a measurement of the percentage biomass increase. The percentage water contents of the cell lines before the test period were obtained by measuring the fresh and dry weights
of representative tissue from each of the cell lines. The percentage water contents of the cell lines after the test period were obtained by measuring the dry weights of all the tissue used in the test.

The callus pieces were obtained by subdivision and subculture. The subdivision took place by carefully separating large, friable tissue pieces. The tissue was not cut. This reduced any unnecessary tissue damage. The resulting callus pieces were of roughly equal size and their weights ranged from 0.278g to 0.688g.

A photograph was taken illustrating the visual effects of lead-ions on callus growth. Four pieces of callus were taken from each of the cell lines TT/L1 and TT/12 after the test period (TT/12 originated from the same seed parent as TT/L1). These pieces were of comparable weight, covering the range of tissue weights at transfer. A direct comparison of weights could, therefore, be made for each pair.

**Tissue lead concentrations.**

It was not practicable, or possible, to analyse every callus piece resulting from the growth analysis, therefore, to minimise any unnecessary variation caused by the random selection of samples for analysis, the dried callus tissue was bulked into two groups; control lines and selected lines.

The two samples were wet-ashed (method in Chapter 2), made up to 25ml samples with distilled water and then fed into the flame atomic absorption spectrophotometer.
Stability of the lead resistance mechanism.

The selected lines had been established for five months prior to this experiment and were constantly maintained on media containing 10ppm lead ions.

20 callus pieces (0.5-1.0 cm diameter) from each of the selected lines were transferred to Gamborg's B5 media containing no lead ions. Transfer to fresh media (containing no lead ions) was every four weeks. Upon transfer the callus pieces were split, as in normal multiplication procedures, but only one piece from each original callus piece was retained and transferred, hence at the end of the experimental run there were ten callus pieces from each selected cell line (ie. each piece originating from one of the original pieces).

Transfer to non-lead containing media was for five months. The 60 callus pieces were then transferred to media containing lead ions and a four week period of growth analysis commenced.

Earlier in this chapter a growth analysis was undertaken on 20 callus pieces from the stock of each selected cell line (ie. they had been constantly maintained on lead-ion containing media). These results are repeated here and act as the control.

Cryopreservation of the cell lines.

The methods for cryopreservation and sample thawing are as Nag and Street (1975) and are fully described in Chapter 2. Ten samples from each of the cell lines were cryopreserved and after four weeks one sample from each cell line was thawed and the viability determined by
measurement of the plating efficiency.

RESULTS.

Growth analysis of the cell lines.

The percentage fresh weight increases of all the cell lines were monitored over the four week test period both on media containing 10ppm lead ions and on non lead-containing media. The results are shown in Figure 5.1.

The dry weights were obtained of a) a random tissue sample from all the cell lines before the test period and b) all the experimental callus pieces after the test period. The results are shown in Figure 5.2.

The differences in biomass increase of the cell lines in response to the lead stress may be either masked or apparently enhanced by changes in the percentage water content of the tissue. The water content of sample groups of tissue from each cell line were determined before the experimental period. The water content of the tissue from each cell line did not change to any marked degree over the test period and the water content of the cell lines were very similar to each other.

The summary of results (Figure 5.1) illustrates the effects of 10ppm lead ions added to the growth medium on the percentage fresh weight increases, over four weeks, of all the cell lines. This is in comparison to the fresh weight increases, over four weeks, of all the cell lines on non lead-containing medium. It can clearly be seen that the fresh weight increases for the control cell lines (TT/7, TT/10, TT/12, TT/13, TT/14, NAN/2, NAN/3, NAN/4, NAN/5, and NAN/6) are vastly inhibited by the presence of 10ppm lead.
ions in the growth media. In comparison, the fresh weight increases of the selected lines (TT/L1, NAN/L1 and NAN/L2) show no significant alterations when stressed with 10ppm lead ions.

The fresh weight increases of the selected cell lines, both on lead-containing growth media and non-lead media, are comparable to the fresh weight increases of the control cell lines on non lead-containing growth media. The mean performance of the selected lines on lead-containing media show no significant differences to those of the selected lines on non lead-containing media (P=0.003).

The growth rates (measured as percentage biomass) shown by Tiptop control cell lines are approximately twice those shown by the Nanthya control cell lines. The same observation can be made between the selected Tiptop cell line and the selected Nanthya cell lines. In the field, Nanthya plants are far more vigorous than those of Tiptop (Breeders Seeds, personal communication), however, this hybrid vigour does not appear to have an effect in vitro.

The initial weights of callus used in this series of experiments varied over the range 0.278g - 0.688g. Obviously, before any conclusions concerning the cell line growth rates are drawn it is important to determine that the initial callus weight had no effect upon those growth rates.

There are a number of ways to measure the association between two variables, which in this case are initial weight and subsequent percentage increase in fresh weight. The most common measure is the Pearson product moment...
correlation coefficient (usually designated by the letter $r$). It is defined as follows:

$$r = \frac{(x-x)(y-y)}{(x-x)^2 (y-y)}$$

The correlation coefficient is always between +1 and -1. There is almost no association between $x$ and $y$ if $r$ is near 0, and the closer $r$ is to +1 or -1 then the easier it is to predict $y$ from $x$. It is usually taken (Ryan et al., 1985) that an association between $x$ and $y$ is only implied if $r$ is less than -0.5 or greater than +0.5. There are certain cases where $r$ is near 0 but there is still a clear association between $x$ and $y$. This occurs when a graphical representation of $x$ versus $y$ shows an increase and the a comparable decrease (or vice versa), however, this is not the case in any of the correlations studied here.

To ensure that initial weight had no effect upon subsequent percentage fresh weight increase, correlation was carried out on the selected lines and their origin lines (i.e. TT/L1, TT/12, NAN/L1 and NAN/5) on lead-containing media. The correlation coefficient for initial weight vs subsequent percentage increase in weight are as follows; TT/L1 = -0.418, TT/12 = -0.288, NAN/L1 = -0.288 and NAN/5 = -0.154. None of these correlation coefficients were less than -0.5 or greater than 0.5 so it can be concluded that there is no association between initial callus weight and its subsequent fresh weight increase.

It was seen earlier that the variability of growth rates, expressed as increases in fresh weight, within the selected line TT/L1 was very large. This is illustrated by
the photograph (Figure 5.3) and can readily be shown by comparison of callus No.2 (initial weight = 0.384g) and callus No.4 (initial weight = 0.389g), although their initial weights are almost identical. No.2 increased in weight by 1.611g whereas No.4 increased by 0.578g; a difference in growth rate of 271% (i.e. 419% compared to 148%).
FIGURE 5.1

Chart illustrating the relationship between the percentage increase in fresh weight of all cell lines on both media containing 10 ppm lead ions and non-lead-containing media.
FIGURE 5.2

Percentage water content of a) sample tissue from the cell lines to be used in the experiments, b) all the tissue grown on media containing 10 ppm lead ions, and (c) all the tissue grown on non lead-containing media.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Sample size</th>
<th>%H₂O (a) mean</th>
<th>SD</th>
<th>%H₂O (b) mean</th>
<th>SD</th>
<th>%H₂O (c) mean</th>
<th>SD</th>
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<td>92.4</td>
<td>0.58</td>
<td>92.1</td>
<td>0.52</td>
</tr>
<tr>
<td>TT/12</td>
<td>20</td>
<td>93.1</td>
<td>0.53</td>
<td>92.8</td>
<td>0.59</td>
<td>91.9</td>
<td>0.58</td>
</tr>
<tr>
<td>NAN/2</td>
<td>20</td>
<td>92.6</td>
<td>0.59</td>
<td>92.5</td>
<td>0.56</td>
<td>92.5</td>
<td>0.55</td>
</tr>
<tr>
<td>NAN/3</td>
<td>20</td>
<td>92.2</td>
<td>0.54</td>
<td>92.8</td>
<td>0.52</td>
<td>92.3</td>
<td>0.59</td>
</tr>
<tr>
<td>NAN/4</td>
<td>20</td>
<td>92.8</td>
<td>0.58</td>
<td>93.0</td>
<td>0.61</td>
<td>92.3</td>
<td>0.55</td>
</tr>
<tr>
<td>NAN/5</td>
<td>20</td>
<td>92.8</td>
<td>0.59</td>
<td>92.5</td>
<td>0.59</td>
<td>92.2</td>
<td>0.58</td>
</tr>
<tr>
<td>NAN/6</td>
<td>20</td>
<td>92.0</td>
<td>0.61</td>
<td>91.7</td>
<td>0.62</td>
<td>92.1</td>
<td>0.50</td>
</tr>
</tbody>
</table>
FIGURE 5.3

Photograph showing the change in fresh weights of callus pieces from selected and control cell lines, over 4 weeks on lead containing media.

<table>
<thead>
<tr>
<th></th>
<th>Initial weight</th>
<th>Weight increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1 (control)</td>
<td>0.382g</td>
<td>0.059g</td>
</tr>
<tr>
<td>No.2 (selected)</td>
<td>0.384g</td>
<td>1.611g</td>
</tr>
<tr>
<td>No.3 (control)</td>
<td>0.381g</td>
<td>0.042g</td>
</tr>
<tr>
<td>No.4 (selected)</td>
<td>0.389g</td>
<td>0.578g</td>
</tr>
<tr>
<td>No.5 (control)</td>
<td>0.411g</td>
<td>0.018g</td>
</tr>
<tr>
<td>No.6 (selected)</td>
<td>0.424g</td>
<td>1.364g</td>
</tr>
<tr>
<td>No.7 (control)</td>
<td>0.561g</td>
<td>0.111g</td>
</tr>
<tr>
<td>No.8 (selected)</td>
<td>0.555g</td>
<td>1.085g</td>
</tr>
</tbody>
</table>

[Diagram showing callus pieces with numbers 1 to 8 and a scale of 1 cm]
Tissue lead concentrations.

The tissue lead concentrations were determined using a flame atomic absorption spectrophotometer. The results can be seen in Figure 5.4. The level of detection (called the Characteristic Concentration) of this machine is 0.0045 units and this is given by 0.11ug/ml of lead, so this is the minimum level of detection. Obviously, at lead levels near this the variability within the machine will constitute a greater percentage of the reading, hence the percentage error is greater.

The machine is calibrated (see Chapter 2) and then subsequent readings are given expressed as a concentration (ug/ml) for a 25ml sample. The two samples were made up from differing dry weights of tissue and so the following equation was used to convert the readings to whole tissue lead concentrations (ug lead per g of tissue):

\[
\text{Reading} \times \text{Volume} \\
\text{Weight of whole sample}
\]

Figure 5.4 Tissue lead concentrations of the selected and control tissue used in the growth analysis which took place on media containing 10ppm lead ions.

<table>
<thead>
<tr>
<th></th>
<th>Weight of callus</th>
<th>No. of Readings</th>
<th>Mean ugPb/g</th>
<th>(SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0.365g</td>
<td>10</td>
<td>42.81</td>
<td>(6.29)</td>
</tr>
<tr>
<td>SELECTED</td>
<td>1.171g</td>
<td>10</td>
<td>21.36</td>
<td>(1.74)</td>
</tr>
</tbody>
</table>
The tissue-lead concentration of the control cell lines is approximately twice that of the selected cell lines.

**Stability of the lead resistance mechanism.**

The three selected callus cell lines were grown on non-lead media for five months. After this time they were returned to media containing 10ppm lead ions and their increase in fresh weight monitored. This was compared to callus from the selected lines which had remained on lead-containing media for the five months. The results are shown in Figure 5.5. The percentage water contents of the cell lines are not significantly different (p=0.003), implying that the percentage increases in fresh weight are, in fact, percentage increases in biomass. The long term absence of lead ions has not significantly altered these increases (p=0.0012) and the selected lines show no loss of lead resistance after a period without lead ions.
FIGURE 5.5.

The increase in fresh weight, over a four week test period, of callus from selected cell lines which had previously been maintained on either lead-containing or non-lead media for five months.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Treatment</th>
<th>Sample number</th>
<th>% FW increase mean (sd)</th>
<th>%H₂O mean (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT/L1</td>
<td>No lead</td>
<td>20</td>
<td>291.6 (133.5)</td>
<td>92.6 (0.59)</td>
</tr>
<tr>
<td>TT/L1</td>
<td>Lead</td>
<td>20</td>
<td>322.4 (144.4)</td>
<td>92.3 (0.56)</td>
</tr>
<tr>
<td>NAN/L1</td>
<td>No lead</td>
<td>20</td>
<td>158.1 (45.9)</td>
<td>91.8 (0.50)</td>
</tr>
<tr>
<td>NAN/L1</td>
<td>Lead</td>
<td>20</td>
<td>167.5 (52.2)</td>
<td>92.1 (0.54)</td>
</tr>
<tr>
<td>NAN/L2</td>
<td>No lead</td>
<td>20</td>
<td>172.3 (55.7)</td>
<td>93.0 (0.58)</td>
</tr>
<tr>
<td>NAN/L2</td>
<td>Lead</td>
<td>20</td>
<td>188.7 (63.1)</td>
<td>92.6 (0.51)</td>
</tr>
</tbody>
</table>
DISCUSSION.

Growth analysis of the cell lines.

The percentage water content of all the cell lines, on both lead-containing media and normal media, before and after the test period, indicate that any changes in fresh weight were not due to changes in the percentage water content. This implies that the differences seen in the percentage fresh weight increases of the cell lines were due to differences in the rate of biomass production.

The presence of 10ppm lead-ions in the growth medium vastly inhibits biomass production in the control cell lines. The low level of growth exhibited by the unselected, control cell lines on lead-containing media may, or may not, be sustainable. The tissue may continue growth at this reduced level or, on the other hand, may be in the process of dying with the growth rate reducing with time. Therefore, it may be desirable to perform experiments relating cell viability to time whilst the cells were stressed with lead.

The mean performances of the selected lines on lead-containing medium are not significantly different from their performances on non lead containing medium, implying that 10ppm lead-ions have no inhibitory effects upon biomass production in these cell lines.

It has been noted that whole plants, or cell cultures, resistant to a stress will often have reduced growth rates, compared to their non-resistant forms, when the stress is not present (Gonzales and Widholm, 1985). For example, Plants growing successfully on heavy metal polluted mine tailing will not grow in unpolluted areas where they are
outcompeted by their nonresistant forms (Bradshaw and McNeill, 1981). This, however, is not true in the present case. The percentage fresh weight increases of the selected lines and their controls, on non lead-containing medium, are not significantly different. Therefore, in this case, the possession of a mechanism for resistance to lead-ion stress does not inhibit biomass production when those ions are not present.

It can be seen that the standard deviation, and hence the variation or dispersion of results, is very large for all three of the selected lines, compared to those of the control lines. This is especially pronounced in the case of TT/L1. Hence, the variability in biomass production in response to lead-ion stress is far greater in the selected lines than in the controls. It may be pertinent to undertake another selection step to attempt to isolate the high performing variants within the cell lines.

It is interesting to note that the selected lines originated from very small clumps of callus tissue and that, with time, these will develop into clonal cell lines (Hauptman and Widholm, 1982), however, these cell lines exhibit a wide variability in response to the lead-ion stress. It appears that in selecting for resistant cells, even though an approximation to clonal cell lines have developed, the variation of response inherent within the cell populations are very much larger than those of the control cell populations, even though the control cell lines are not clonal.
The range of growth responses show what is termed continuous variation (Zubay, 1987). The resistance mechanism could be due to gene amplification (Bollum et al., 1987) which is the increased transcription or translation of a gene, such as one for a metal-chelating protein (see Chapter 8). On the other hand, the resistance mechanism may be due to the control of a multigene family. This is typified by a continuous variation in response (Zubay, 1987). Rhue (1979) noted that in many plants a combination of tolerance mechanisms are operating simultaneously to produce the observed tolerance and that this results in the genetic control being complex with the genes involved being at many loci. An increase in visible variation was also noted by Johnson (1979) who stated that selection on polygenic variation changes the gene frequencies or maintains the gene frequencies in non-random associations. It is suggested, therefore, that the resistance to lead stress is controlled by a multigene family. Multigene families are very prevalent and even phenotypes for which genetic and gene product data was suggestive of single gene control are proving to be related to small multigene families (Larkin et al., 1985). This conclusion is commensurate with the theory that somaclonal variation is caused by transposable elements. Single base pair and point mutations, which have also been associated with somaclonal variation, would effect small portions of the genome (Zubay and Gottesman, 1987) whereas transposable elements often effect large portions of the chromosome (Shepherd, 1988)
Tissue lead concentrations.

The total tissue-lead concentrations of the selected cell lines is approximately half that found in the unselected calli. This may be indicative of an exclusion mechanism. There may be a chelating chemical which is excreted into the media, as in aluminium tolerance in carrot cells (Ojima and Ohira, 1985) or it may be due to the lack of, or a blockage in, the mechanism which transports the lead through the plasmalemma or tonoplast.

The observed results, however, may not necessarily indicate an exclusion mechanism for lead. The unselected calli is slowly dying and lead may be entering by diffusion into the dead tissue. This implies that lead toxicity has already occurred at a lower tissue-lead concentration, which may be found in the selected calli, indicating them to be truly tolerant.

We can see, therefore, that although this experiment has gone some small way in enlightening us as to the presence or absence of lead in the cells, we can draw no firm conclusions from it. The site of the lead accumulation must be investigated. An exclusion mechanism would result in a build-up of lead ions around the periphery of the callus piece or root. If a tolerance mechanism were present the lead ions would be seen distributed evenly about the root or cell.

The site of lead accumulation is examined by x-ray microanalysis in Chapter 7.

Stability of the lead-resistance mechanism.

The growth responses of the three selected callus cell
lines, which were maintained without lead for five months, were not significantly different from the same cell lines continuously grown on lead-containing media.

The results show that the tolerance exhibited by the selected cell lines is not induced by the presence of lead-ions and this implies that the underlying mechanism is of a stable genetic nature. That is, the resistance mechanism has resulted from some permanent genetic change.
AIMS.

In Chapter 5 it was seen that the selected cell lines exhibited an in vitro cellular resistance to lead ions and it is necessary to characterise this resistance in some way. Plants will be regenerated and the ion uptake in taproot cortical cells will be studied. The results will indicate if the resistance characteristic is present in the whole plant and any differences between the uptake in roots from selected and unselected plants. These will allow us to draw conclusions as to the stable transmission of the resistance characteristic over mitotic barriers, and as to the cellular mechanism of this resistance in so far as it relates to ion uptake.

INTRODUCTION.

Plant regeneration.

The potential for plant regeneration distinguishes plant tissue cultures of animal tissue. The regeneration of plants can occur via a number of pathways, such as adventitious embryogenesis, including somatic cell embryogenesis, androgenesis and parthenogenesis (Murashige, 1984). The theory of totipotency (see Chapter 1 for a review) implies that plant regeneration can be achieved from any cell. In practice, however, the expression of this totipotency is confined to cells called meristemoids.
(Torrey, 1966) which give rise to roots, shoots or embryos. Any cell can become a meristemoid but the stimuli for this type of cellular determination and the processes controlling it are poorly understood (Barker-Burgess and Burgess, 1987). Plant regeneration will be universally possible only when we are able to manipulate the transformation of non-meristemoid cells into meristemoids.

A genetic mechanism for resistance at the undifferentiated cellular stage is only of academic importance unless it alters the phenotype of the whole regenerated plant. The regenerative potential of cultures after selection is most important if the expression of the variant character is required at the level of the whole plant. The problem is two-fold. Many cell lines which show resistance have been found to be non-regenerative (Chaleff, 1986; Evans, 1984) and even if regeneration is possible the variant character may not be expressed in the highly differentiated state of the whole plant. It is widely accepted that most long-term cultures are chromosomally variable (Evans, 1984; Larkin et al., 1985; Reisch, 1983) and this may be a major cause of some of the problems. Apart from the genetic alterations that are the cause of the desired variation there may be gross changes in the genome. These other mutations may often affect the regenerative potential of the cell. Regeneration, therefore, is in itself a selection step in that many of the abnormal cells are screened out.
Ion uptake by cortical tissue.

Much can be understood about the control of lead toxicity, and the resistance to its effects, by studying the site of accumulation of the lead and the mechanisms by which the uptake occurs. The uptake and transport of ions in roots is complex and can be summarised as follows below.

The soil contains minerals, many of which are essential for plant growth. The mineral nutrient concentration of the soil often does not correspond with the requirements of the plant and there are many mineral ions present at high concentrations in the soil which are either not required by the plant or may be positively harmful. The uptake mechanism of the plant must, therefore, be selective (Epstein, 1972).

A transverse section through a root will show three distinct zones, an outer ring of cells containing the root hairs, which is called the rhizodermis; a wide band of highly vacuolated cells called the cortex; and a central core called the stele (Sailsbury and Ross, 1985). The stele contains the vascular tissue and is bounded by a thin layer of cells called the endodermis. Mineral ions, in solution, must pass through the rhizodermis and cortex before passing into the stele where they enter the xylem vessels for transportation to other areas of the plant. Low molecular weight solutes (e.g. ions, organic acids and amino acids) will move by diffusion through the rhizodermis into the cortex where the cell walls and water-filled intercellular spaces are accessible. The main barriers to this passive process are the plasmalemma of the individual cortical
cells and the endodermis. In the radial and transverse walls of the endodermis cells there are hydrophobic incrustations of suberin (called the Casparian strip) and these constitute an effective barrier against passive solute movement into the stele (Marschner, 1986; Sailsbury and Ross, 1985).

Primary cell walls consist of a network of cellulose, hemicellulose and glycoproteins. This network contains interfibrillar and intermicellar spaces, generally called pores. These do not constitute a barrier to mineral ions and low molecular weight solutes, however, high molecular weight solutes (e.g. metal chelates, fulvic acids and toxins) are severely restricted from entering the free space of root cells. Within this free space carboxylic groups act as cation exchangers and cations can accumulate in the free space where anions are repelled. The negative charge on the cell walls of the apoplast (the cell wall continuum in plant tissues) resulted in Hope and Stevens (1952) introducing the term apparent free space (AFS). This can be divided into the water free space (WFS), which is freely accessible to ions, and the Donnan free space (DFS), where cation exchangers and anion repulsion takes place. Ion distribution within the DFS is the typical Donnan distribution which occurs in soils at the surface of negatively charged clay particles. Plant species differ considerably in their cation exchange capacity (CEC). The exchange adsorption in the AFS of the apoplast is not essential for ion uptake or transport through the plasma membrane into the cytoplasm, however binding of cations
increases their concentration in the apoplast and hence also in the vicinity of the active uptake sites at the plasma membranes. A positive correlation can be observed between the CEC and the ratio of \( \text{Ca}^2+ \) to \( \text{K}^+ \) contents in different plant species (Crooke and Knight, 1962).

The main sites of selectivity in the uptake of cations, anions and solutes in general is the plasma membrane of individual cells. It is an effective barrier to diffusion into, or out of, the cytoplasm. The tonoplast (vascular membrane) is also an effective barrier to diffusion and in most fully differentiated cells, such as cortical cells, the vacuole constitutes more than 90% of the cell volume (Leigh et al., 1981) and it is the main compartment for ion accumulation. Ions may be transported over these membranes by active processes.

Plants were firstly regenerated from selected and control lines and the cortical tissue from their taproots was used for ion uptake experiments. The uptake of lead from a lead nitrate solution was monitored, followed by the release of lead from the Water Free Spaces and then the displacement of lead from the Donnan free spaces by calcium ions. The uptake and release of lead ions were monitored using flame atomic absorption spectrophotometry.

**EXPERIMENTAL DESIGN.**

Plants were regenerated to provide taproot tissue for use in this experiment. Ten mature taproots from selected plants and ten from nonselected plants were used for this experiment. The taproots were cut into slices approximately 2mm thick and then discs (5mm diameter) of cortical tissue
were cut with a cork borer. 250 discs were used from each taproot and the fresh weight of tissue from each taproot was in the range 2.26–2.29g.

Each tissue sample was put through the following series of events. The environmental conditions throughout were 25oC and 2000lux.

1) Tissue washed in aerated deionised water for 18 hours.
2) Incubated in 100mls of 10ppm lead solution in a conical flask (250mls) on a rotary shaker (100rpm, displacement 4cm) for 120 minutes. A sample (3mls) of the bathing solution was taken every hour.
3) The tissue was blotted of all surface moisture with a paper tissue and then placed in deionised water (100mls) in a conical flask (250mls) on a rotary shaker (100rpm, 4cm displacement) for 30 minutes, Samples (3mls) of the bathing solution were taken every 10 minutes.
4) The tissue was blotted of all surface water with a paper tissue and then placed in 100mls of 0.1M calcium nitrate solution in a conical flask (250mls) for 90 minutes on a rotary shaker (100rpm, 4cm displacement). Samples (3mls) were taken every 30 minutes.

All samples were acidified to 1% with full strength nitric acid and the concentration of lead ions determined using flame atomic absorption spectrophotometry.
RESULTS.

Cortical tissue from various selected and nonselected plants was incubated in a solution of 10ppm lead ions. The lead content of this solution was monitored over time and an uptake of lead by the tissue was indicated by a corresponding decrease in the lead content of the bathing solution. The results of this and the other parts of the experiment are shown in Figure 6.1 and 6.2. The lead contents of the bathing solutions were plotted against time and these show the shape of classic uptake/release curves. The rates as well as the final amounts reached by these curves differ. The rate was measured over the exponential part of the curve and the results can be seen in Figure 6.3. The mean rate of lead uptake by the selected cell lines is lower than that of the nonselected cell lines. The total amount of lead remaining in solution is greater for the selected lines, hence indicating that the total mean lead uptake was lower than that in the nonselected cell lines.

In the second part of the experiment the tissue was placed in water and the lead content of the bathing solution was measured over time. Lead present in the water free space will be leached in to the bathing solution. The rate of this leaching and the total amount of lead leached are shown in Figure 6.4. The mean rate of leaching from the selected lines is 14.05μg/min (sd = 6.77) whilst the mean rate of leaching from the nonselected cell lines is 6.31μg/min (sd = 3.82). The total amount of lead leached from the selected lines is much greater than that from the nonselected lines. It should be borne in mind that the
amount of lead initially in the nonselected tissue is greater than that of the selected tissue and so the total lead loss is expressed as a percentage of the initial lead content (calculated from Figure 6.3). These results are shown in Figure 6.4 and serve to emphasis the difference between the selected and nonselected lines. The selected lines show a mean percentage loss of 30.2% (sd = 7.1) whilst the nonselected lines have a mean percentage loss of 9.4% (sd = 5.1).

In the third part of the experiment the tissue was transferred to calcium nitrate solution. The calcium ions will preferentially exchange with the lead ions which are bound to the cation exchange sites in the Donnan free space. The subsequent increase of lead into the bathing solution was monitored over time. The rates and total lead losses are shown in Figure 6.5. Again the initial lead contents of the cell lines were different and so the lead loss to the bathing solution has been expressed as a percentage of the initial total content (calculated from Figures 6.3 and 6.4). The rate of leaching of lead ions into the bathing solution was much higher from the selected lines with the mean rate at 8.25μg/min (sd = 1.41) than the nonselected lines with their mean rate at 4.98μg/min (sd = 0.88). The mean total lead lost is again far higher in the selected lines and this observation is enhanced by the percentage figures with the selected lines having a mean percentage loss of 48.2% (sd = 8.8) whilst the nonselected lines have a mean percentage rate of 19.7% (sd = 2.4).
FIGURE 6.3

Table showing the initial rate of lead uptake from the bathing solution and the total amount of lead remaining in that solution after the experimental period.

<table>
<thead>
<tr>
<th>Selected cell lines</th>
<th>Rate 1</th>
<th>Total 1</th>
<th>Control cell lines</th>
<th>Rate 1</th>
<th>Total 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.40</td>
<td>96</td>
<td>1</td>
<td>13.28</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>13.75</td>
<td>25</td>
<td>2</td>
<td>12.98</td>
<td>110</td>
</tr>
<tr>
<td>3</td>
<td>6.55</td>
<td>274</td>
<td>3</td>
<td>13.25</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>9.18</td>
<td>166</td>
<td>4</td>
<td>13.53</td>
<td>83</td>
</tr>
<tr>
<td>5</td>
<td>10.72</td>
<td>111</td>
<td>5</td>
<td>14.07</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>9.83</td>
<td>195</td>
<td>6</td>
<td>13.17</td>
<td>82</td>
</tr>
<tr>
<td>7</td>
<td>12.45</td>
<td>69</td>
<td>7</td>
<td>14.02</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>3.97</td>
<td>559</td>
<td>8</td>
<td>12.60</td>
<td>141</td>
</tr>
<tr>
<td>9</td>
<td>8.85</td>
<td>334</td>
<td>9</td>
<td>12.47</td>
<td>174</td>
</tr>
<tr>
<td>10</td>
<td>12.02</td>
<td>178</td>
<td>10</td>
<td>11.97</td>
<td>158</td>
</tr>
</tbody>
</table>

|x       | 9.67   | 200     |                     | 13.13  | 100     |
sd      | 2.87   | 156     |                     | 0.66   | 44      |

Rate 1 is expressed in ug of lead per minute per tissue sample and is the rate at which lead ions are being lost from the bathing solution (i.e. taken up by the tissue) over the first 60 minutes of incubation.

Total 1 is expressed in ug of lead and is the amount of lead ions remaining in the bathing solution after 180 minutes. The total amount of lead in solution at the start of the experiment was 1000ug.

* The tissue samples are identical in volume and mass, namely, 250 cortical tissue discs (2mm thick, 5mm diameter) with a mass of 2.2g.
FIGURE 6.4.

Table showing the initial rate of lead release from the tissue when incubated in water. The total amount of lead lost is shown along with the initial amount of lead that the tissue held (calculated from Figure 6.3). The total lead loss is then expressed as a percentage of the initial tissue lead content.

<table>
<thead>
<tr>
<th></th>
<th>Rate 2</th>
<th>Total 2</th>
<th>Initial content</th>
<th>Percentage loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.60</td>
<td>295</td>
<td>904</td>
<td>32.6</td>
</tr>
<tr>
<td>2</td>
<td>19.70</td>
<td>272</td>
<td>975</td>
<td>27.9</td>
</tr>
<tr>
<td>3</td>
<td>15.10</td>
<td>232</td>
<td>726</td>
<td>32.0</td>
</tr>
<tr>
<td>4</td>
<td>20.30</td>
<td>345</td>
<td>834</td>
<td>41.4</td>
</tr>
<tr>
<td>5</td>
<td>11.20</td>
<td>176</td>
<td>889</td>
<td>19.8</td>
</tr>
<tr>
<td>6</td>
<td>9.20</td>
<td>227</td>
<td>805</td>
<td>28.2</td>
</tr>
<tr>
<td>7</td>
<td>16.70</td>
<td>276</td>
<td>931</td>
<td>29.6</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>83</td>
<td>441</td>
<td>18.8</td>
</tr>
<tr>
<td>9</td>
<td>9.70</td>
<td>236</td>
<td>666</td>
<td>35.4</td>
</tr>
<tr>
<td>10</td>
<td>13.00</td>
<td>301</td>
<td>822</td>
<td>36.6</td>
</tr>
<tr>
<td>x</td>
<td>14.05</td>
<td>244</td>
<td></td>
<td>30.2</td>
</tr>
<tr>
<td>sd</td>
<td>6.77</td>
<td>74</td>
<td></td>
<td>7.1</td>
</tr>
</tbody>
</table>

Control cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Rate 2</th>
<th>Total 2</th>
<th>Initial content</th>
<th>Percentage loss</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

Rate 2 is the rate, over the first 10 minutes, at which lead ions are being washed from the tissue by incubation in water. It is expressed in ug lead per minute per sample. Total 2 is the amount of lead ions which have been washed out of the tissue after 30 minutes incubation in water. It is expressed in ug lead per sample.
FIGURE 6.5.

Table showing the initial rate of lead release from the tissue when incubated in calcium nitrate solution. The total amount of lead lost is shown along with the initial amount of lead that the tissue retained (calculated from Figures 6.3 and 6.4). The total lead lost is then expressed as a percentage of the initial tissue lead content.

<table>
<thead>
<tr>
<th>Selected cell lines</th>
<th>Rate 3</th>
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<th>Initial content</th>
<th>Percentage loss</th>
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<td>6.43</td>
<td>201</td>
<td>494</td>
<td>40.7</td>
</tr>
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<td>489</td>
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<tr>
<td>sd</td>
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</table>

Control cell lines

<table>
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<th>Initial content</th>
<th>Percentage loss</th>
</tr>
</thead>
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<td>857</td>
<td>18.2</td>
</tr>
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</tr>
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<td>4.60</td>
<td>152</td>
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<td>x</td>
<td>4.98</td>
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<td>19.7</td>
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<tr>
<td>sd</td>
<td>0.88</td>
<td>26</td>
<td></td>
<td>2.4</td>
</tr>
</tbody>
</table>

Rate 3 is the rate, over the first 30 minutes, at which lead ions are being displaced from the tissue by calcium ions. It is expressed in ug of lead per minute per sample.

Total 3 is the amount of lead ions which are displaced from the tissue by calcium ions over 90 minutes. It is expressed in ug lead per sample.
DISCUSSION.

The selected material does not take up lead ions as readily as the nonselected lines and what lead they do take up is removed in greater proportions by water and calcium ions. A greater proportion of the lead entering the tissue of the selected plants is present in the apparent free space and hence can be removed by either water or calcium ions. The uptake of lead into the cells is less than in the nonselected lines. Suggesting that the cellular boundaries of the cell wall and plasmalemma are either less permeable to lead ions or that the ions may be bound into insoluble complexes in, or on, the cell walls. An indication as to which of these possible mechanisms is present can be seen by noting that the displacement of the bound lead from the Donnan Free Spaces was much greater in the selected cell lines than in the nonselected lines. This implies that the observed resistance may be due to an increase in number or efficiency of the cation binding sites and these may, or may not, be specific for lead ions. It would be very interesting to carry out more experiments to further elucidate these findings. This could lead to a series of experiments to look at the cation exchange capacity of the cells and the specificity of any increased cation binding potential. Kinetic experiments using competitive ions to block binding sites would help to determine if the actual number of sites has increased or if it is just an increase in efficiency.

Further experiments in Chapter 7 will attempt to determine the sites of lead accumulation in the roots of selected and nonselected plants.
CHAPTER 7

EXPRESSION OF THE RESISTANCE CHARACTER IN IMMATURE PLANTS OF THE F1 GENERATION AND ANALYSIS OF THE SITE OF LEAD ACCUMULATION IN THESE USING X-RAY MICRO ANALYSIS

AIMS.

Plants of the F1 generation will be stressed with lead and the effects on their growth observed in an attempt to determine if the resistance characteristic is passed over meiotic barriers. Roots from the stressed plants will be analysed to determine to sites of lead accumulation and to therefore draw some conclusions as to the mechanism of the resistance.

INTRODUCTION.

It is important that a resistance characteristic can stably pass over meiotic barriers and be fully expressed in an F1 generation before it is of any practical use to the plant breeder. A number of somaclonal variants express phenotypes that are not inherited (Karp, 1989) and, to determine accurately the extent of somaclonal variation, sexual progeny should therefore be examined (Evans and Sharp, 1988). Plants were regenerated from the cell lines TTL1 and TT12 and seed obtained (see Chapter 2). The flowers are produced in compound umbels borne terminally on the branches. The main axis forms the first order, or primary, umbel and from this main stalk several lateral branches arise producing secondary umbels (Gray and Steckel, 1980). The flowerheads develop and mature at different times and this is thought to account for the variation between umbel types in seed size, vigour and
germination rates (Gray and Steckel, 1980; Jacobsohn and Globerson, 1980). Care was, therefore, taken to only use seed originating from the secondary umbels, which was the most numerous umbel type.

Seed from secondary umbels were germinated (method in Chapter 2) and then placed in hydroponic tanks, where they were stressed with differing lead-ion concentrations. Hydroponics is the growth of plants without soil. The development of hydroponics has gone hand in hand with the increasing knowledge of plant physiology and the understanding of the requirements for plant growth. It was pioneered on a commercial scale by Gericke in the late 1930's with much of the work on the essential micronutrients required being carried out by Arnon and Hoagland (Arnon, 1938; Arnon and Hoagland, 1940). This was placed on a firm scientific base in a major reference text by Hewitt (1974). A full review of this subject was completed by Resh (1983). The use of hydroponics when studying the effects of lead ions on plant growth has been used by a number of workers (Jarvis et al., 1977; Lane, 1978; Miller and Koeppe, 1970) and has a number of distinct advantages over the use of soil. The addition of lead to soil can result in many complexes forming of both reversible and irreversible nature (Davies, 1981; Lane, 1978). These are very difficult to predict and vary greatly upon the type of soil used (John, 1972). The nutrient solution used in hydroponic culture is of defined composition and although some precipitation may be present, it is part of a dynamic system and as ions are removed from
The soluble fraction the loss is compensated for by the movement of ions back into solution from the precipitate. The precipitation does not reduce the availability of the ions (John, 1977).

The roots of the immature plants, grown at varying lead levels in the hydroponic tanks, were studied as frozen-fixed material using a x-ray probe microanalyser in a scanning electron microscope fitted with a cold stage. Within an electron microscope a stream of energetic electrons interact with the solid sample. This interaction yields a variety of different types of electrons and electromagnetic waves, of which x-rays are just one, which are the result of elastic and inelastic scattering events (Chapman, 1986). The x-rays generated are unique to the chemical elements present. These are detected by the microanalyser and presented on a screen as a series of peaks. The x-ray detector measures the counts (volts) at different energies (KeV). Each electron shell of an element radiates at a distinctive energy level when in collision with an electron. If there is a high concentration of a particular x-ray energy a peak will form. There will be a number of peaks for each element, one for each of the particular electron shells. It will be noted that on the plots the electron shell is indicated after the element type. X-ray spectrometry, however, can only be used to detect elements. The technique does not distinguish between chemical states (for detailed reviews see Moreton, 1981; Morgan, 1985). X-ray microanalysis has been used, for example, in detailed studies of the elemental composition of oil palm suspension.
cultures (Warley et al., 1985) and the physiology of salt stress in both tissue cultures (Dix et al., 1983) and whole plants (Hajibagher and Flowers, 1989).

Cold-stage scanning electron microscopy (cryo-SEM) enables biological specimens to be observed in a near-natural state after freezing, avoiding artifacts induced by other preparative methods (Morgan et al., 1978; Morgan, 1985; Sargent, 1986). Wet chemical techniques of sample preparation expose tissue specimens to a sequence of aqueous media, which introduces artifacts that invalidate attempts to localise diffusible constituents. These include the loss, or redistribution, of elements during the processes of fixation, dehydration, embedding and staining (Morgan, 1985). Elements can also be introduced into the specimen by these techniques.

Whole mounting of the sample and air or freeze drying is again not suitable due to the possibility of ion translocation during the slow and uncontrolled drying phase. There would also be a major problem caused by contributions to the analysis from cytoplasmic or extracellular elements underlying and overlaying the area of interest (for reviews see Hutchinson, 1979; Moreton, 1981; Morgan, 1985; Plattner and Bachmann, 1982). Cryofixation of specimens provides the only reliable means of permitting physiological studies of diffusible ions. It instantly arrests all physiological activity and hence the movement of the diffusible constituents, whilst retaining a specimen with good structural information.

Freezing in biological materials is complex because of
their heterogeneous nature and a problem with cryofixation
can be the disruptive artifact of ice-crystal damage. This
results from phase separation as water molecules migrate
to form crystals during freezing, whilst the solutes and
other cell constituents are concentrated in the matrix,
or grain boundary, between crystals (Ryan et al., 1988).
Ultrastructural deformation by ice crystals can be avoided,
or reduced, by the use of a cryoprotectant. Low-molecular-weight cryoprotectants (such as glycerol and
dimethylsulphoxide) are water soluble molecules variously
permeable to cell membranes. They interact strongly with
water, ions and biopolymers giving rise to metabolic and
physiological changes which render them unsuitable for
x-ray microprobe analytical studies (Franks, 1978).

High-molecular-weight non-penetrating polymers (such
as polyvinylpyrrolidone and hydroxyethyl starch) can be
used, but it appears that they do actually penetrate the
cells (Barnard, 1980) and disturb ion distribution, to some
extent, in intercellular and extracellular compartments
(Kuijpers and Roomans, 1983). The only way, therefore, of
reliably controlling disruptive ice-crystal formation,
without creating artefacts by redistribution of ions, is
by careful consideration and manipulation of the specimen
freezing protocol.

In this study ice-crystal damage and its prevention was
studied in rapidly frozen, cryomounted specimens of carrot
cortical tissue and compared to that found in specimens of
marine arrow worms (*Sagitta elegans*) prepared in a
comparable manner. Cryomounting and low temperature
cementing techniques were specially developed for this
x-ray study. These results have been published (Ryan et al., 1989) and are presented in the Appendix section.

X-ray microanalysis may be used as a quantitative assay but there are a number of problems associated with this approach. There is a major difficulty in the preparation of accurate standards (Warley et al., 1985) and the matrix used for calibration standards must be one which has the same interaction qualities, with regards to x-rays and electrons, as the tissue to be analysed (Spurr, 1975). Even when rigorous technique is followed, the quantification of the observed x-ray spectra is difficult and somewhat unreliable (Zierold and Schafer, 1978). In the current situation it is a relative comparison of two different plant types which is important, not the quantification of the elements present. X-ray microanalysis was, therefore, carried out on a quantitative basis to locate and map sites of lead accumulation within the tissues.

EXPERIMENTAL DESIGN.

Seed Production.

Plants were regenerated by the method described in Chapter 6; 20 from the selected cell line TTL1 and 20 from the control cell line TTL2. These were grown to maturity in a glasshouse and allowed to flower. Paper bags were placed over each flower-head and the flowers within brushed at regular intervals to induce self fertilisation. Seed formed and it was allowed to dry on the plant before collection.
Random samples of 1000 pollen grains were collected from secondary umbels on both the selected and control plants.

Viability and germination rates of the two pollen samples were determined (method as Weatherhead et al., 1978) and the pollen morphology was observed using a light microscope (x40 magnification).

Seed was collected from the secondary umbels. The seed from each individual plant was not kept separate but bulked to form two groups; that from the selected plants and that from the control plants. It was sorted and any undeveloped (i.e. flat husks) or split seeds were discarded. This sorting process occurs during the commercial production of seed (Breeders Seeds Ltd., England, personal communication).

Hydroponics.

The design and construction of the hydroponic tanks is fully described in Chapter 2.

Each tank held three litres of half strength Hoagland and Arnons solution (as described by Hewitt, 1974). The solution was replaced once a week. 150 seeds from each group were germinated and grown for two weeks on damp filter paper in petri dishes (20°C, 3000lux, 16 hour day). The most vigourous 90 from each group were selected and divided into three groups of 30. 90 plantlets (30 from each group) were "planted" in each of the three hydroponic tanks in a random pattern. They were supported in the holes with non-absorbent cotton wool which was also used to plug the spare holes.
The plantlets were allowed to acclimatise in the tanks for one week. Lead ions were added at a level of either 0, 10 or 100 ppm when the nutrient solutions were first replaced. The plants were allowed to grow under these conditions for two weeks. At the end of the first week the nutrient solutions, containing their requisite lead-ion concentrations, were replaced with fresh solutions. Iron citrate was added three times per week in the required concentration in order to prevent iron chlorosis developing (Hewitt, 1974).

The roots from the plants were used for the subsequent x-ray microanalysis. The leaves were bulked into two samples; those from the selected plants and those from the nonselected plants. They were dried and then, after sample preparation as detailed in Chapter 2, the lead concentration was measured.

**Specimen freezing and cryomounting.**

The specimens were carefully removed from the tanks and the surface of the roots well washed with deionised water before being frozen by using a plunge cooling device (Ryan and Purse, 1985). They were plunged, individually, 100 mm into liquid ethane (93K) at approximately 2 ms⁻¹. The method of cryomounting and the cryomounting device is as described by Ryan et al. (1988).

**Cryo-scanning electron microscopy.**

Specimens were examined at 3 kV (uncoated) or 10 kV (coated) in a JEOL JSM 35C fitted with a modified cryostage (Ryan et al., 1985). The microscope airlock also contained a cryostage, which cooled to 103K, on which
frozen specimens were fractured with a cold scalpel and sputter-coated when required. The roots were freeze-fractured so that tissue approximately 1mm from the surface of the tissue block was exposed. Freeze-etching was done in the microscope at a specimen temperature of 193K where necessary. The specimens were coated with graphite in the cold stage, again, where necessary.

The specimens were exposed to the electron beam of the SEM for a considerable time before, during and after analysis.

This continued bombardment by electrons caused the specimen picture to break up. This is termed "charging" and is the reason why, unfortunately, the photographic quality is poor. The beam used during the small spot analysis to produce the plots was intense and damaged areas can be seen in the cells where the analysis took place.

RESULTS.

Seed production, pollen germination and morphology.

The pollen germinated readily and both the control and selected plants had germination rates of 100% in samples of pollen collected at random from secondary umbels. The pollen morphology appeared to be normal and no abnormally large pollen grains were seen.

The regenerated plants appeared to possess normal phenotypes. No abnormalities in leaf type, flower type or general morphology were seen.
Plant growth in hydroponic tanks.

The results of the plant growth can be seen in Figure 7.1. Exposure of the unselected plants to lead ions resulted in reductions in leaf area, leaf fresh weight, leaf dry weight, root extension, root fresh weight and root dry weight. It also resulted in an increased mortality of the plants. In marked contrast, the selected plants exposed to the same levels of lead ions exhibited no deleterious effects.

The unselected (control) plants were observed to have a distinctive morphology. They showed the classic symptoms of lead toxicity (chlorotic leaves - Koeppe, 1981) and of plant stress (red stems - Bradshaw and McNeilly, 1981). The plants which died wilted, turned brown and then shrivelled. No measurements, such as leaf area, could be obtained and their mass was too small for a determination of their lead content using either atomic absorption spectrophotometry or x-ray microanalysis.

Carrot plantlets grown in the hydroponic tanks for three weeks, without the addition of lead ions, had roots which were approximately 1-2mm in diameter, creamy white in colour and had many lateral branches of 20-30mm. In contrast, roots which had been exposed to 100ppm lead ions were typically very thin (less than 0.5mm), medium to dark brown in colour and the lateral branches were short (2-4mm). The roots exposed to 10ppm lead ions exhibited a morphology which was intermediate between these two extremes.

The lead content of the leaves was measured by atomic
absorption spectrophotometry. The leaves from both the selected and control plants were found to have lead levels which were below the limits of detection for the machine (i.e. 0.11ug).

Cryo-scanning electron microscopy.

The cryo-mounted carrot cells showed good three-dimensional information. However, the surface cells of the carrot specimens exhibited ice-crystal damage when frozen in liquid nitrogen. Cells at the surface of specimens frozen in liquid ethane showed little obvious crystal damage and no matrix of salts or other constituents were seen during freeze-etching.

In all carrot specimens deep seated cells (i.e. approximately 1mm from the surface) showed similar freezing damage and the same range of ice crystal size was found in carrot specimens frozen in quenchants of greatly differing efficiencies. No difference could be seen in specimen quality when they were either frozen on metal supports or frozen on foil supports and then cryomounted.

X-ray microanalysis and x-ray maps.

Analysis was performed on different cellular compartments and structures to ascertain whether there were any differences in elemental concentrations, especially those of lead. Plots were recorded on a semiquantitative/qualitative basis and to ensure that this was the case once the detector recorded a total of 8000 counts in the area between 4.5KeV and 5.0KeV the analysis was terminated. There were no major chemical peaks in this area and the counts recorded here were due to background
radiation. Hence, the analysis runs were very similar.

The process of x-ray microanalysis on the cryo-SEM was very time consuming and expensive. Only one specimen could be processed and studied per day. It was, therefore, expedient to initially determine the major areas of lead accumulation, thus allowing the limited resources available to be concentrated on these areas.

Figures 7.2 and 7.3 show x-ray maps of the freeze-fractured roots from unselected (control) and selected roots, respectively. These were grown in hydroponic tanks containing 100 ppm lead ions in the nutrient solution. The x-ray maps were produced by a 5 minute scan of the area at 10549 eV. This is the energy which is characteristic of the lead (Lα) peak. This was used as it is unequivocally that of lead and is not shadowed or distorted by a peak from any other element. The x-ray maps were unfiltered and so up to 50% of the readings may be due to background radiation, however, this occurs evenly over the whole scan area and does not mask the results.

It can be seen that there is a concentration of lead around the outer portion of both the roots. This is far more obvious in the root from the selected plant. A close-up of the root surface and epidermal layer was also mapped (Figure 7.4) and a scanning electron micrograph taken of the area. The concentration of lead in the epidermal layer is evident. The x-ray maps indicate the main area of lead accumulation to be around the external portion of the roots. It was on these areas, therefore,
that further work was concentrated.

Analysis was carried out on roots mainly from plants stressed with 100ppm lead. Plots were obtained from many cells in differing root areas and various roots. The results presented here are representative of these findings.

In the walls of epidermal cells from unselected roots there are noticeable concentrations of lead (Figure 7.6). The other two main elements indicated can be discounted. The copper is most probably a contaminant from the specimen chamber of the cryo-SEM and the iron from the hydroponic solution. Higher iron concentrations can be found in plants which are grown in this way (Hewitt, 1974). The lead, however, can only be due to absorption of that present in the hydroponic nutrient solution. The plot of the epidermal cell wall from a selected root (Figure 7.7) is vastly different, with the lead ($\mu$) peak exceeding the scale on the y axis. Further analysis with a y axis of up to 4KeV, as opposed to 2KeV, indicates that this peak is 2800eV high.

In the centre of the same epidermal cells as used for Figures 7.6 and 7.7 the situation is dramatically different. In both plots (unselected root - figure 7.8; selected - figure 7.9) there is no indication of any lead present. It can be said that no contribution is made by lead to the conglomeration of peaks at 1-4KeV as no indication of the other lead peaks (Pb L$\alpha$ and Pb L$\beta$) can be seen further down the plots. It is this observation which results in the deduction that the peak indicated on figure 7.9 is solely due to sulphur (k$\alpha$) and not to lead. The
small copper peak can, again, be put down to contamination from the cryo-SEM chamber.

The centre of the epidermal cell from the selected root contains large amounts of potassium. The external surface of the roots, which were thoroughly washed, show dramatic differences between selected and unselected plants. The unselected root surface possesses no large accumulations of any particular element. That of the selected root, however, has large concentrations of iron, calcium and particularly lead. This was bound to the root surface and could not be washed away.

Figures 7.12 and 7.13 show analyses of the centre of epidermal cells from selected and unselected roots, respectively, which were stressed with 10ppm lead. These results reinforce the findings shown in Figures 7.8 and 7.9. The cell from the unselected root does, in comparison to that of Figure 7.8, contain a concentration of potassium. That contained in the cell from the selected root is, however, dramatically larger with the peak exceeding the scale. Further analysis indicated that this peak reached approximately 3000eV.
FIGURE 7.1.
Selected and control plants grown in hydroponic culture with the addition of various concentrations of lead ions.

<table>
<thead>
<tr>
<th>Lead-ion Concentration</th>
<th>Sample Mortality</th>
<th>Results of surviving plants only</th>
<th>Results of all plants¹</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>Mean leaf area, mm²</td>
<td>Mean leaf length, mm</td>
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<td>Unselected Plants</td>
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</tr>
<tr>
<td>Oppm 30</td>
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<td>206 (114)</td>
<td>50 (23)</td>
</tr>
<tr>
<td>10ppm 28</td>
<td>17</td>
<td>118 (65)</td>
<td>30 (15)</td>
</tr>
<tr>
<td>100ppm 29</td>
<td>20</td>
<td>182 (46)</td>
<td>39 (12)</td>
</tr>
<tr>
<td>Selected Plants</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oppm 30</td>
<td>0</td>
<td>221 (83)</td>
<td>47 (18)</td>
</tr>
<tr>
<td>10ppm 28</td>
<td>2</td>
<td>293 (100)</td>
<td>57 (20)</td>
</tr>
<tr>
<td>100ppm 29</td>
<td>2</td>
<td>285 (94)</td>
<td>53 (20)</td>
</tr>
</tbody>
</table>

All means are shown along with their standard deviations in brackets.

¹ The data form those plants dying during the experiment were taken as zero and used as such in the calculations of the means.
² The leaf areas were measured on an area quantifier in mm².
³ The fresh weights were measured in mg
⁴ The root extentions were measured as the length of the main taproot in mm.
FIGURE 7.2

X-ray map of a freeze-fractured root from an unselected (control) plant, grown with 100ppm lead ions.
FIGURE 7.3

X-ray map of a freeze-fractured root from a selected plant, grown with 100ppm lead ions.
FIGURE 7.4

X-ray map of the epidermal portion of the area shown in figure 7.3.
FIGURE 7.5

Scanning electron micrograph of the area mapped in figure 7.4.
Small spot analysis of the epidermal cell wall in a root from an unselected (control) plant, grown with 100 ppm lead.
Small spot analysis of the epidermal cell wall in a root from a selected plant grown with 100 ppm lead.

Figure 7.7
100 ppm lead ions from a root of an unselected (control) plant, grown with small spot analysis in the center of an epidermal cell. FIGURE 7.8
Small spot analysis in the centre of an epidermal cell from a root of a selected plant, grown with 100ppm lead ions.

FIGURE 7.9

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Small spot analysis of the external surface of a root from an unselected (control) plant grown with 100ppm lead ions.

Figure 7.10
Figure 7.11: Small spot analysis of the external surface of a root from a selected plant grown at 100 ppm lead ions.
10 ppm lead ions from a root of an unselected (control) plant, grown with small spot analysis in the centre of an epidermal cell.
Small spot analysis in the centre of an epidermal cell from a root of a selected plant, grown with 10 ppm lead.

Figure 7.13
DISCUSSION.

Lead was shown to have an inhibitory effect upon both the growth and mortality of the unselected plants. The addition of lead ions to the hydroponic tanks resulted in a large increase in the mortality of the unselected plants. Lead ions, both at 10ppm and 100ppm, had some inhibitory effect upon the mean leaf area of those plants surviving, although it must be noted that this effect is not significant (p=0.013). If, however, the results are expressed as the mean leaf area for all the plants in the group (the results of those plants that died being taken as zero) then a vast reduction in mean leaf area can be seen. Similar effects can be seen at 10ppm, 100ppm and for the other parameters. Lead ions had very little effect upon the growth of the selected plants and they exhibited no stress symptoms, i.e., chlorotic leaves and reddened stems, suggesting that the variant characteristic had successfully crossed meiotic barriers.

Leaves from both the control and selected plants were found to contain lead at undetectable levels. Any lead entering the roots was not transported to the leaves.

The gross effects of lead were seen to occur at 10ppm and this is a relatively low level for whole plants (Bradshaw and McNeilly, 1981). They were, however, very young and hence less resistant to stress of any kind (Sailsbury and Ross, 1985).

Ion toxicity is the result if the cytoplasmic ion concentration exceeds that which is compatible with normal enzymic activity (Levitt, 1980) and x-ray microanalysis is of great importance in the study of
this most profound of physiological problems. The tolerance of the metabolism of halophytes to a variety of salts has been investigated over the years, most latterly by Hajibagheri and Flower (1989) using x-ray microanalysis. The conclusion reached is that compartmentation of ions is a key process in this tolerance. This would appear to consistent with the case here. Lead has been shown to profoundly disrupt many subcellular activities, such as photosynthesis, respiration, energy relations and an array of enzymic functions (for a review see Chapter 1). These are mainly brought about due to the intimate association of lead with many different cellular and subcellular membranes (Koeppe, 1981). It becomes obvious, therefore, that a mechanism by which lead ions are prevented from entering the cell, and hence reaching the site of their effect, would confer a degree of tolerance to lead. This type of action is not in fact true tolerance (as described by Levitt, 1980), it is in fact termed amelioration. This is were the plant may absorb the potentially toxic ion but act upon it in such a way as to prevent it reaching its site of effect. Variously, this may involve chelation, dilution, localization or even excretion.

This process of avoidance is the means by which plants resistant to lead are able to cope with the stress and, in general, the lead is excluded from its site of effect by precipitation in, or on, the cell wall (Levitt, 1980). It should be noted, however, that genotypical differences exist among plant species, in respect to the
strategies adopted for tolerance/avoidance. These are well documented in tomato (Rush and Epstein, 1981) and lupins (Van Steveninck et al., 1982), and they may also occur within a species among genotypes (Marschner et al., 1981).

The specific mechanisms of lead-ion uptake have been shown to differ within the plants regenerated from the same cell line (see Chapter 6).

It has been shown that roots from selected plants accumulate lead in the cell walls of their epidermal cells and especially on the root surface. The lead is prevented from entering more than the epidermal layer of the roots. Roots from unselected plants had some accumulation of lead in the epidermal cell walls but little or none on the root surface. It must be concluded that, in these plants, the entry of lead into their roots is virtually unrestricted and that the lead is transported to the site of its effect.

The information presented in the plots from the central vacuoles of the epidermal cells is very interesting. The epidermal cells of unselected roots grown at 10 ppm contain a small amount of potassium, whilst those from unselected roots grown at 100 ppm contain virtually none. On the other hand, the epidermal cells from selected roots, grown at both 10 ppm and 100 ppm, contain vast quantities by comparison. Potassium is a univalent cation and its uptake is highly selective and closely coupled to metabolic activity (Marschner, 1986).

Potassium acts as a charge carrier of high mobility that forms only weak complexes in which it is readily exchangeable (Wyn-Jones et al., 1979). It is very important
in enzyme activation (Suelter, 1970), is the dominant counter-ion to light induced H+ flux across thylakoid membranes (Lauchli and Pfluger, 1978) and plays a key role in cell extension, stomatal movement and turgor related movements (Marschner, 1986). It is, also, well established that potassium is required for protein synthesis (Evans and Wildes, 1971; Koch and Mengel, 1974; Marschner, 1986; Wyn-Jones et al., 1979). High potassium levels in cells indicate that large amounts of cell extension, protein synthesis and anion transport are taking place. This can be related to the cells of the selected roots and indicates that they are growing and functioning well. In contrast, the epidermal cells from unselected roots grown at 10ppm contain reduced amounts of potassium and those from unselected roots grown at 100ppm contain only small amounts. This would suggest that the roots from unselected plants have reduced growth, and this is borne out by visual observations. It must be borne in mind that the unselected plants used for the x-ray microanalysis were not representative of their group as a whole. The plants available for analysis from the unselected group were unusual in the fact that they were alive. They were showing greatly retarded growth and may have been in the process of succumbing to the lead stress.

The roots from selected plants have been shown to resist the effects of lead by its accumulation in the epidermal cell walls and on the root surface. The plasma membrane of the root epidermal cells must possess binding sites with an increased affinity for lead ions. Many
lead ions, also, appear to be excluded by the root, thus causing them to accumulate at the root surface.
GENERAL DISCUSSION

It has been seen in Chapter 3 that both germination and radicle lengthening of carrot seeds (*Daucus carota* cv. Nantes, subvarieties Tiptop and Nanthya) can be partially inhibited by 10ppm lead ions and fully inhibited by concentrations above this. It was assumed that the lead was interfering with a factor directly involved with cell elongation, suggesting the specific involvement of enzymes in the cell wall and the ATPase associated with the plasmalemma (Koeppe, 1981). A large screen of seeds (5000 from each subvariety) at 50ppm lead was carried out. In some cases germination occurred but there was no subsequent radicle lengthening. It was therefore assumed unlikely that there were any naturally occurring gene combinations within this commercially available population which conferred resistance to the lead toxicity exerted by 50ppm lead ions.

The processes of callus initiation and growth did not necessarily have the same sensitivity to lead ions as did seed germination and radicle lengthening, however, the results from Chapter 3 indicated a guide to the level which could be used in subsequent experiments.

The growth of carrot callus was seen to be very sensitive to lead ions and a concentration of 10ppm in the culture medium resulted in vast inhibition of the increase in callus diameter. Callus initiation was also assessed and 10ppm lead ions in the culture medium was shown to totally inhibit callus formation. The explant expansion, however, was not affected, but this may have been caused by
cell expansion rather than cell division. A large screen of
taproot explants for callus initiation on medium containing
10ppm lead ions was undertaken. Three small areas of
callus were formed and these were the basis of the three
selected lines. At this stage the resistance of the callus
could only be presumed.

The biomass increases of the nonselected lines growing
on normal medium were comparable to those of the selected
lines. However, on lead containing media the biomass
increases of the nonselected lines were severely inhibited
whilst those of the selected lines were unaltered. The
nonselected cell lines at this stage were found to contain
approximately twice as much lead as the selected lines. The
selected lines, therefore, possessed resistance to lead and
the possession of this mechanism for resistance did not
inhibit biomass production when lead was not present.

The biomass productions of the selected lines were
unaltered after a period of growth on normal media and the
cell lines could be successfully cryopreserved without any
loss of the resistance characteristic. This implied that
the mechanism for lead resistance had a stable genetic
basis resulting from some form of genetic mutation or
altered gene expression occurring as a result of somaclonal
variation (Zubay and Gottesman, 1987).

Further experiments attempted to elucidate the nature of
the resistance mechanism. Plants were successfully
regenerated and cortical tissue was used from mature
taproots for ion uptake experiments. It was found that the
tissue from the selected plants took up less lead than
tissue from the nonselected plants. A greater percentage

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of the lead in the selected tissues, compared to that in the nonselected tissues, could be removed by water. This was from the Water Free Space. Of the remaining lead in the tissue some was in the Donnan Free Space, and hence removable by calcium ions, and the rest was not removable, i.e. the lead may have chemically complexed in the cell walls or have passed over in plasmalemma into the vacuole were, again, it may have formed chemical complexes (Marschner, 1986). A greater percentage of the remaining lead in the selected tissue was removed by calcium ion, compared to the percentage removed from the nonselected tissue. Basically, therefore, the selected lines have a reduced uptake for lead and a greater proportion of this lead is present extracellularly, suggesting that the resistance mechanism is in part due to a barrier to lead at the cell wall.

The selected and nonselected plants were regenerated from what were assumed to be clonal cell lines, however, it can be seen, for taproot tissue from both selected and nonselected plants, that the variations in response to lead are continuous as opposed to being discrete (see Chapter 5). This suggests that the resistance character is caused by a multigene family and that the expression of this character may be caused by a gene amplification (Zubay and Palmiter, 1987).

It is important that a resistance characteristic can stably pass over meiotic barriers and be fully expressed in the F1 generation before it is of any use to the plant breeder (Ammirato, 1986). A number of somaclonal variants
express phenotypes that are not inherited and, to determine accurately the extent of somaclonal variation, sexual progeny should therefore be examined. Consequently the regenerated plants were allowed to flower and seed was collected. There appeared to be no morphological abnormalities in either the plants or the pollen. The seeds were germinated and grown in hydroponic tanks where they were subjected to either 0ppm, 10ppm or 100ppm lead ions. In the absence of lead ions the growth of the selected seeds was comparable to that of the nonselected seeds. When subjected to either 10ppm or 100ppm lead ions, however, the growth of the nonselected F1 plants was greatly inhibited and they exhibited the classic signs of lead stress, i.e. chlorotic leaves and red stems. The selected F1 plants exposed to the same levels of lead ions exhibited no deleterious effects. The resistance characteristic was obviously expressed by the F1 progeny of the selected plants, thus demonstrating the stable transmission of the resistance character over meiotic barriers. It was difficult to say if any Mendelian inheritance of the resistance character could be seen. There appeared to be a graduation in intensity of the resistance exhibited by the plants, rather than the 3:1 ratio expected from Simple Mendelian inheritance. The results tend to indicate that the resistance character is under the control of multiple genes or a multigene family rather than being due to a single point mutation.

Various roots from the F1 plants grown in the hydroponic tanks were cryo-mounted and then freeze-fractured on a cryo-stage in the scanning electron microscope. X-ray maps
of selected and nonselected roots grown at 100ppm show that there is a concentration of lead around the outer portion of both roots. This is greater in the selected root. A close-up of the root surface and epidermal layer from the selected root was also mapped. The concentration of lead in the epidermal layer is evident. Further x-ray microanalysis demonstrated that the epidermal cell walls from selected roots contained far greater concentrations of lead than those found in nonselected roots. X-ray plots from the centre of the same epidermal cells show no indication of any lead being present. The external surfaces of the roots were thoroughly washed prior to the experiment and whilst the surface of the selected roots showed an accumulation of lead and other minerals, these were not seen on the surface of the nonselected roots. It can be seen, therefore, that the roots from selected plants accumulate lead in the cell walls of their epidermal cells and especially on their root surfaces. The lead is prevented from entering more than the epidermal layer of the roots. This resistance is, in fact, not tolerance at all but amelioration and this is where the plant absorbs a potentially toxic ion but acts upon it in such a way as to prevent it reaching its site of effect (definitions of resistance and tolerance can be found in Chapter 1). Roots of from the nonselected plants showed some accumulation of lead in the epidermal cell walls but little or non on the root surface. It must be concluded that, in these plants, the entry of lead into their roots is virtually unrestricted and that the lead is transported to the site of its effect. Preliminary investigations on
the cortical cells from these roots indicated very large concentrations of lead. A great problem existed when studying the nonselected plants in that the typical reaction of the plant when stressed with lead was to die. The plants available for study, therefore, were atypical of the group as a whole and even these were very difficult to handle, showing extreme signs of stress and in the process of dying. It would be most informative to study the differences between these two groups of plants further.

To summarise, *Daucus carota* taproot explants were exposed to lead stress and callus was initiated. The variant cell lines had the following properties:

i) They exhibited resistance to lead.

ii) The resistance was not deleterious to the growth of the cell lines when the lead stress was not present.

iii) The resistance characteristic was stable in the absence of lead and was transmitted over meiotic barriers to the F1 generation.

iv) The resistance was found to result in a reduced uptake of lead into isolated cortical cells, and of the lead taken in a greater proportion was in the Water and Donnan Free Spaces.

v) The F1 generation plants from the variant line were shown to concentrate lead on the external surface and in the epidermal layers of their roots.

It is very interesting that the variant cell lines were successfully selected for at the stage of callus initiation. Callus cultures are known to be genetically unstable and it is because of this that they are used so often as the culture type from which attempts are made to
select for a desired variant. In the callus stage the cells can be induced to become determined and subsequently differentiate into wide range of specific organs or types of cells. To put it crudely, in the callus state more of the cell's genome is 'available' to receive stimuli causing it to be expressed. When a carrot taproot cell is determined and becomes differentiated it loses its natural capacity to form other types of cells, in effect, the relevant genes are inactivated. It is still totipotent, but is no longer meristematic. When it dedifferentiates, however, to form callus the genes must be activated. A great deal of the inactive portion of the genome must become active again and the portion of the genome which directs this activity will also be active, therefore the genetic activity in dedifferentiating cells is likely to be greater than in normally growing callus cells. With a greater genetic activity there is an increased chance of genetic instability, or error, resulting in mutation of the genome. The increased genetic activity may also result in a larger number of genetic states on which a stress may be exerted to cause an altered gene expression.

A number of different hypotheses may be proposed. The relatively large amount of gene activity, and hence greater genetic instability, may have resulted in a mutation conferring the desired characteristic. On the other hand, the genetic basis for the desired resistance may already be present in the genome and the presence of the lead stress at this time of increased genetic activity may have resulted in the expression of the desired gene or genes. A
third possibility may arise where the genetic basis for the desired characteristic is, again, present but that the genome or gene expression may be altered in such a way as to negate or remove an inhibitory factor, hence allowing the potential of the cell to be fully expressed. Finally, gene amplification may be taking place. There may not actually have been any changes in the genome but the number of times the desired gene is transcribed and translated may have increased. There is evidence to suggest that other workers are beginning to consider the method of selecting variants from differentiating cells to be a valid. Variants have been selected from cells undergoing embryogenesis (AboEl-Nil, 1990), microspore formation (Shohet et al., 1990) and regeneration (Ibrahim et al., 1990).

It has been shown that a variant which exhibits a stable resistance mechanism is the result of selection at the stage of callus initiation. It may, therefore, be valid to use this method to produce other types of variants from carrot or from other species. If we can produce stable variants in the comparatively simple culture system of carrot and define the genes controlling the variant characteristic then it could be possible to transfer the desired genes to other plant species which are more difficult to manipulate in culture. An example of this is the transfer of heavy metal tolerance from Nicotiana tabacum to Brassica napus (Misra and Gedamu, 1989). Resistance to lead could be transferred from carrot cultures to cultures of various species of grass, such as Agrostis. The resulting lead resistant grass could then be used plant polluted land for amenity use.
The resistant carrot plants produced in this study are unsuitable for use as crop plants as they accumulate lead in the outer portions of their roots. They do not, however, accumulate the lead to such an extent that they could be used to remove lead from contaminated soils in the same way as fungi is used to remove heavy metals from effluent waste (Lewis and Kiff, 1988).

It would be very interesting to follow a number of lines of further enquiry.

i) Secondary selections could be made to further improve the resistance. It was noted that the selected callus cell lines exhibited a wide variation in biomass increase on lead-containing media and further improvements of the cell lines could be undertaken by selecting for the high performance callus pieces or by stressing the callus with progressively higher concentrations of lead ions.

ii) Further investigations into the ion uptake mechanism could be done to look at the cation uptake and the specificity of the cation exchange sites, or at the kinetics of the uptake, using such methods as competitive ions to block various sites.

iii) The ion uptake experiments in Chapter 6 used isolated cortical tissue. It would be desirable to carry out ion uptake and ion kinetic experiments on whole plants with mature taproots using, possibly, radioisotopes of lead. Mature taproots could also be used to study any differences in the uptake in different portions of the root.

iv) The F1 generation plants exhibited interesting
differences in their lead contents and the partitioning of this lead. Further experiments would be desirable looking in more detail at the sites of lead accumulation and at the tissue succumbing to lead stress. It would possibly be better to grow the plants at a level of lead ions causing intermediate effects on the control plants, rather than the level used which caused widespread deaths. These experiments could possibly be done using radioisotopes or by utilizing the electron dense property of lead rather than using cryo-SEM x-ray microanalysis which is expensive, time consuming and restricted by specimen preparation problems.

v) A greater understanding of the genetic basis and the type of genetic change resulting in the resistance mechanism could be obtained by a number of directions of study: karyotypic analysis; RFLP analysis; back-crossing with parent plants; crossing two resistant plants and study of the resulting generation for signs of Mendelian segregation and recession or dominance of the characteristic.

The results of this study may make us think a little more about the way we select for variant cell lines. Have we been doing it in the most efficient way and have we sufficiently thought about the genetic and metabolic states of the cells which we are using in our selection schemes? Once a variant has been selected all manner of sophisticated techniques are used to elucidate its nature and possibly less effort is given to understanding the means by which we originally obtain the selection.

The aims of this project have been fulfilled and the
initial questions we asked have been answered, however, many, many more questions have become apparent resulting in a number of interesting lines of study which could be followed.
APPENDIX 1

TOTAL AND AVAILABLE LEAD IN DIFFERENT CULTURE MEDIA

Lead ions will form stable complexes with many chemicals (see Phipps, 1981 for review) and once these have formed the lead ions will no longer be available to exert a stress on a biological system. For example, the total lead concentration of a soil may be very high, but, due to the complexing action of the soil components, the available lead is very low, and no toxic effects are seen in plants grown on it (Nriagu, 1978).

The above mentioned factors make it prudent to determine both the total and available lead-ion concentrations in media used.

Callus cell cultures of *Daucus carota* are most often initiated and maintained upon Murashige and Skoog media (Ammirato, 1983) but Gamborgs B5 media may also be used (Reinhert, 1981). Hence, both these media were assessed to determine their suitability for use. The media compositions can be seen in Chapter 2.

Varying concentration of lead ions (added as lead nitrate) were added to these medias and the total available lead was determined using a lead solid-state electrode (Model 94-82, Orion Electrodes, Cambridge, Massachusetts, USA) and a double junction reference electrode, filled with 10% KNO₃ in the outer chamber. These measured the activity (concentration) of the free lead ions in solution. The meter was calibrated in ppm.

In Gamborgs B5 medium the total lead ions were the same
as the available lead ions for concentrations up to 150 ppm. In Murashige and Skoog medium, however, the available lead ions were the same as the total lead ions for concentrations up to 30 ppm. At total lead concentrations above this the available lead ion concentration did not increase.

In Murashige and Skoog medium at total lead ion levels higher than 30 ppm a white precipitate was seen. This was thought to be lead chloride.

Murashige and Skoog medium contains 440 mg/l of calcium chloride, whereas Gamborg's B5 contains only 0.15 mg/l. It is thought that when the calcium chloride dissociates in solution the free chloride ions associate with the lead ions, forming lead chloride.

It is due to these results that Gamborg's B5 medium is used throughout the study.
APPENDIX 2

PRODUCTION OF THE CELL LINES

Selection, in vitro, for a given characteristic firstly requires a large number of cells or cultures from which this selection is to take place. Production of this stock for experimentation itself constitutes a form of selection as only a certain number of individual plants can be used to produce the cell lines. These may not necessarily be representative of the variability present in the whole plant population, however, it is assumed that the variation within the sample selected is the same as that in the population as a whole.

The genetic stability in cell or tissue cultures is often low (Handro, 1981) and, although this can be useful when selecting for variants, its presence in control cell lines is most undesirable. Hence, the fastest growing callus piece from each seed parent was rapidly multiplied to form the stock lines, which were subsequently cryopreserved, hence forming reference lines. These would be reinitiated in the event of any noticeable genetic variation occurring in the control lines.

The cell lines formed were not strictly clonal, ie. originating from one cell, but instead originated from single explants from separate seeds. However, Portnoy and Wilson (1981) developed a theoretical model which shows that cell clumps, with time, tend towards becoming pure types, and so we can assume that the cell lines produced are, to all practical purposes, uniform and distinct.

There are virtues in studying the slower growing callus
pieces, but as time was at a premium this line of investigation was not followed.

PROCEDURE

20 plants of each sub-var were produced with tap-roots of approximately 5cmx1.5cm. 6 tap-root explants from each plant were used to establish callus.

After a period of eight weeks (two transfers) the fastest growing (ie. largest) callus piece from each seed parent was transferred and multiplied to form 40 stock lines of callus tissue.

After 4 subculture periods the 4 fastest growing cell lines were saved from each sub-variety.

The two cell lines from which the selected callus originated were also saved.
RESULTS.

Resultant diameters of both Tiptop (TT) and Nanthya (NAN) callus after four weeks. The * indicated those callus pieces from which the cell lines were formed.

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<td>4 - 14mm</td>
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<td>TT</td>
<td>1 - 15mm</td>
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<td>2 - 18mm*</td>
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<td>3 - 17mm*</td>
<td>TT</td>
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<tr>
<td>TT</td>
<td>1 - 18mm*</td>
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<td>TT</td>
<td>5 - 19mm*</td>
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<td>6 - 16mm</td>
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<td>8 - 16mm</td>
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<td>8 - 17mm*</td>
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<td>7 - 18mm*</td>
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<td>8 - 15mm</td>
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<td>5 - 16mm</td>
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<td>TT</td>
<td>5 - 16mm</td>
<td>TT</td>
<td>6 - 18mm*</td>
<td>TT</td>
<td>7 - 16mm</td>
<td>TT</td>
<td>8 - 15mm</td>
</tr>
</tbody>
</table>

A 177
The fastest growing callus (marked *) from each seed parent was saved and multiplied to form 40 stock lines.

The logistics of maintaining 40 stock lines resulted in the decision to only maintain the eight most vigorous lines, as well as the two from which the selected lines originated. After four sub-culture periods the number of callus pieces (approximately 0.5-1.0 cm diameter) originating from each initial callus was noted:

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of calli</th>
<th>Cell line</th>
<th>No. of calli</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAN 1</td>
<td>19</td>
<td>TT 1</td>
<td>12</td>
</tr>
<tr>
<td>NAN 2</td>
<td>38 *</td>
<td>TT 2</td>
<td>18</td>
</tr>
<tr>
<td>NAN 3</td>
<td>44 *</td>
<td>TT 3</td>
<td>28</td>
</tr>
<tr>
<td>NAN 4</td>
<td>36 *</td>
<td>TT 4</td>
<td>18</td>
</tr>
<tr>
<td>NAN 5</td>
<td>34 *</td>
<td>TT 5</td>
<td>24</td>
</tr>
<tr>
<td>NAN 6</td>
<td>34 *</td>
<td>TT 6</td>
<td>18</td>
</tr>
<tr>
<td>NAN 7</td>
<td>24</td>
<td>TT 7</td>
<td>32 *</td>
</tr>
<tr>
<td>NAN 8</td>
<td>28</td>
<td>TT 8</td>
<td>26</td>
</tr>
<tr>
<td>NAN 9</td>
<td>26</td>
<td>TT 9</td>
<td>16</td>
</tr>
<tr>
<td>NAN 10</td>
<td>32</td>
<td>TT 10</td>
<td>36 *</td>
</tr>
<tr>
<td>NAN 11</td>
<td>22</td>
<td>TT 11</td>
<td>24</td>
</tr>
<tr>
<td>NAN 12</td>
<td>32</td>
<td>TT 12</td>
<td>36 *</td>
</tr>
<tr>
<td>NAN 13</td>
<td>28</td>
<td>TT 13</td>
<td>40 *</td>
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<tr>
<td>NAN 14</td>
<td>24</td>
<td>TT 14</td>
<td>32 *</td>
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<tr>
<td>NAN 15</td>
<td>16</td>
<td>TT 15</td>
<td>24</td>
</tr>
<tr>
<td>NAN 16</td>
<td>30</td>
<td>TT 16</td>
<td>28</td>
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<td>NAN 17</td>
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<td>TT 17</td>
<td>16</td>
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<tr>
<td>NAN 18</td>
<td>24</td>
<td>TT 18</td>
<td>22</td>
</tr>
<tr>
<td>NAN 19</td>
<td>22</td>
<td>TT 19</td>
<td>30</td>
</tr>
<tr>
<td>NAN 20</td>
<td>24</td>
<td>TT 20</td>
<td>26</td>
</tr>
</tbody>
</table>
The fastest growing callus pieces (*) were used to form the following eight experimental control cell lines:

from Nanthya - NAN 2, 3, 4, 5, 6
from Tiptop - TT 7, 10, 12, 13, 14

Selected lines NAN/L1 and NAN/L2 originated from NAM/5.
Selected line TT/L1 originated from TT/12.
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ABSTRACT

Somaclonal variation, occurring during callus initiation on taproot explants in vitro has given rise to a number of variant cell lines, selected using lead stress. These variants grow vigorously in the presence of lead, and their resistance is not diminished by prolonged growth on lead-free medium or following recovery from cryopreservation. Plants regenerated from these cell lines retain the lead-resistance characteristic in hydroponic conditions.

This present study describes the growth of the variant cell lines and looks at uptake/release and localisation of lead in the regenerated plants.

INTRODUCTION

It is widely recognised that lead in vegetables will present a serious public health risk (e.g. DHSS 1980) and that plants that exclude lead would be of significant agricultural importance, especially in areas where
contaminated soils are evident.

In South West England, for example, spoil tips and tailings from old mine-workings present a potentially hazardous source of lead in the environment. This produces tracts of land with poor rates of revegetation, even after a centuary or more, and many of these are still rich in heavy metals, often reported as soil levels of over 40,000 ppm lead (Bradshaw and McNeilly 1981).

Contaminated soils are defined as having lead-ion levels of 100 μg Pb/g or greater, and highly contaminated soils as having lead-ion levels of 1000 to 10,000 μg Pb/g or higher (MAFF, 1982). Root vegetables grown on these soils can accumulate lead in excess of the present British legal limits for fresh food, which is 1 μg/g fresh weight (MAFF, 1982).

A major contribution to the lead in blood in the United Kingdom comes from food (DHSS 1980; MAFF 1982). Estimations attribute 0.5% of dietary lead to animal products (Smart et al, 1981) and 13% to root vegetables (Davies and White 1981). The ingestion of lead-contaminated vegetables can raise blood-lead concentrations by 28% (Gallencher et al 1984).

Crop plants are major accumulators of lead from these environments where high levels are found, and plants that exclude such heavy metals would obviously be of significant agricultural importance.

Lead has many varied effects upon both plant and animal biological systems (Vallee and Ulmer (1972) for review of
animal literature; Koeppe (1981) for review of plant literature). Numerous reports from the animal literature have shown that lead forms mercaptiles with the -SH group of cystine and less stable complexes with other amino-acid side chains. Lead has also been shown to bind and alter the activity of membranous subcellular particles in plants and animals, including mitochondria, chloroplasts, nucleotides, nucleic acids and chromosomes. Effects of high lead concentrations have also been reported on photosynthesis; chloroplast fine structure; respiration; energy relations; enzyme activity; yield (either fresh, dry weight or grain); pigment concentration changes (often a visible sign of lead toxicity) and on chromosomes, causing aberrations. Lead also becomes intimately associated with many cellular and subcellular membranes. The aim of this study was to investigate the possibility of generating somaclonal variants (Scowcroft and Larkin, 1981) from a vegetable cell culture and to screen them for apparent resistance to high external lead levels. Successful selection might then be used to derive new plant lines with the acquired resistance characteristic. Carrot (Daucus carota L. subsp. Sativus (Hoffm.)) was chosen as an amenable culture system in vitro (Ammirato 1983) with a high regenerative potential.

This study will also provide valuable evidence in the broader, biotechnological investigations into the feasibility of using spontaneous, or somaclonal, cell variants as unique breeding stock (via cell culture selection) for desirable agricultural characteristics.
MATERIALS AND METHODS

Lead stress on taproots in vitro.

Taproot explants (10 x 2 mm) were surface sterilized and incubated in vitro on Gamborg's B5 medium (Gamborg, O.L. 1975) supplemented with 0.1mg l⁻¹ 2.4-D, 2% w/v sucrose and 0.8% w/v agar at 25°C +/-1°C in the dark.

Lead, as lead nitrate, was incorporated into the media at 5, 10, 50, 100, 200ppm. Explants were examined regularly for signs of callus development.

Growth of selected cell lines on -Pb and +Pb medium.

Following initiation on lead-containing medium, selected callus lines were multiplied and then transferred to fresh 10ppm +Pb media for fresh weight analysis over a four week growth cycle. Comparative samples were also transferred to -Pb media for parallel assay.

Plant regeneration.

Callus cultures were transferred to Gamborg's B5 media supplemented with sucrose (2% w/v) and agar (0.8% w/v) only. They were placed under Thorn EM 'Warmwhite' lights at 25°C +/-1°C. After four to six weeks small embryos had formed and these were transferred to fresh media, where they formed roots and leaves.

Resistance of regenerated plants to lead stress.

Regenerated plants, derived the resistant cell line TT/L1 and the non-resistant cell line from which TT/L1 originated, were established in a simple hydroponic growth system, with the roots unsupported in aerated half strength Arnon and
Hoaglands solution (see Hewitt 1966 for details). Lead, as lead nitrate, was included in experimental solutions at 10ppm and 100ppm. After two weeks growth cycle survival was recorded, together with leaf area, and both leaf and root total lead content. This latter was obtained by wet acid digestion of the tissue and then by use of a flame atomic absorption spectrophotometer.

Uptake/release of lead from taproot tissues.

Regenerated plants of the selected line TT/L1 and the non-selected line from which it originated were grown to taproot maturity under conventional horticultural conditions. Subsequently, 100 discs of cortical tissue (5mm diameter, 2mm depth) were taken from each of the twenty plants (ten selected and ten non-selected) forming twenty experimental groups. These were washed in aerated, deionised water for 20 hours. They were then placed in 100mls of deionised water containing 10ppm lead as lead nitrate, in a 250ml flask, for three hours, 0.5ml samples of the bathing solution were removed every hour. The tissue was removed, blotted dry and placed in 100mls of deionised water in a 250ml flask for thirty minutes, 0.5ml samples of the bathing solution were removed every ten minutes. After this the tissue was again blotted dry and placed in a 250ml flask containing 100mls of 0.01M calcium nitrate solution. This was shaken for 90 minutes and 0.5ml samples of the bathing solution were removed every 30 minutes. The tissue samples were then digested by a wet ashing technique and the lead content determined, along with the bathing solution samples,
by flame atomic absorption spectrophotometry (Model - Varian 99A).

It should be noted plants regenerated from selected and non-selected callus both came from clonal cell lines.

RESULTS

Lead stress on taproots in vitro.

In a preliminary in vitro screen using a limited number of taproot explants results indicate that 10ppm (0.05mM) prevented both callogenesis and cell proliferation completely. Subsequently, a larger screen was carried out using two cultivars, Tiptop and Nanthya, with a stress of 10ppm lead ions and involving 5000 taproot explants from 20 plants. After 14 weeks, this resulted in one site of callus initiation on a single explant of Tiptop and two sites on a further explant of Nanthya. These calli, designated TT/L1, NAN/L1 and NAN/L2, were fast growing and presumed to be lead resistant. They were bulked up to provide populations for subsequent investigations and maintained on lead-containing media to prevent possible loss of resistance under non-selective conditions. Samples of each line were cryopreserved at this stage.

Growth of selected lines on -Pb and +Pb medium.

The variant lines are shown to grow vigorously, both in the presence and absence of lead (Fig 1) and the possession of the mechanism of tolerance does not appear to have reduced growth rates, compared to non-resistant lines, when
both are grown on lead-free media (Fig 1).

Examination of the standard deviation associated with data from such experiments indicates greater variation of performance within selected populations than controls (Fig 2).

Resistance of regenerated plantlets to lead stress.

The advantage of selection can be seen from the survival data presented in Table 1. Surviving plants from selected lines were normal and healthy, whereas survivors from the non-selected lines were characterised by chlorosis, red stems and malformed leaves.

The tissue lead appeared restricted to root tissue, and had not been translocated to the foliage in either group. Leaf areas were, however, significantly affected.

Selected plantlets showed a reduced root tissue lead content at an external level of 10 ppm, but no significant difference from non-selected material at external levels of 100 ppm.

Uptake/release of lead from taproot tissues.

The data presented in Fig 3 indicates that variation in lead uptake, from a 10 ppm external solution, was relatively low. Water and calcium exchanged release was also low with little variability within the population of samples.

By contrast the selected lines showed great variation in uptake, despite the fact that the tissue samples were from clonal material. Similarly, the water and calcium exchange was significantly greater than in non-selected material and again showed a wide range of variation.
FIGURE 1. Fresh weight increase (%) of selected and non-selected carrot callus lines grown on +Pb (10ppm) and -Pb medium. (n = 20)
Shaded bars represent performance on +Pb medium, whilst unshaded bars indicate +Pb growth levels. The favourable selections are indicated by *.

FIGURE 2. Percentage fresh weight increase of replicate samples of selected (*) and non-selected callus lines on +Pb (10ppm) medium. Bars indicate standard deviations (n = 20) and show differences between selected and non-selected material.

N = Nanthya, T = Tip Top.
TABLE 1. Survival, leaf area and tissue lead content of plants regenerated from selected and non-selected cell lines. The plants were challenged with lead under hydroponic conditions for two weeks.

<table>
<thead>
<tr>
<th>Population Type</th>
<th>No</th>
<th>% Survival</th>
<th>Lead (ppm)</th>
<th>Leaf area (cm +/-sd)</th>
<th>Leaf lead (g/g dry wt)</th>
<th>Root lead (g/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selected 25</td>
<td>96</td>
<td>0</td>
<td>6.32(1.5)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Non-select. 25</td>
<td>96</td>
<td>0</td>
<td>6.87(1.8)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Selected 25</td>
<td>92</td>
<td>10</td>
<td>2.93(1.0)</td>
<td>nd</td>
<td>63(sd=15)</td>
<td>110(sd=20)</td>
</tr>
<tr>
<td>Non-select. 25</td>
<td>32</td>
<td>10</td>
<td>1.26(0.5)</td>
<td>nd</td>
<td>110(sd=20)</td>
<td>140(sd=30)</td>
</tr>
<tr>
<td>Selected 25</td>
<td>76</td>
<td>100</td>
<td>1.66(0.6)</td>
<td>nd</td>
<td>190(sd=25)</td>
<td>190(sd=25)</td>
</tr>
<tr>
<td>Non-select. 25</td>
<td>36</td>
<td>100</td>
<td>0.93(0.3)</td>
<td>nd</td>
<td>140(sd=30)</td>
<td>140(sd=30)</td>
</tr>
</tbody>
</table>

nd = not detectable within constraints of the assay.

FIGURE 3. Uptake of lead (a) from a 10ppm lead solution into isolated taproot tissues of both lead resistant and lead susceptible plants. Lead release following water (b) and calcium (c) exchange are also indicated.
DISCUSSION

Lead stress on taproots in vitro.

The selection resulting in the three variant lines, occurred at a very low frequency and only at the initiation of callusing and not from growing suspensions. The selection has, therefore, taken place during the major changes occurring with the dedifferentiation of organised tissue when gene activity might be expected to be high.

Growth of selected lines on -Pb and +Pb medium.

Growth of the selected callus lines on +Pb media is significantly greater than that of the non-selected callus lines, but the variation within these lines is large compared to that of the non-selected material. This suggests that there may be a wide range of effectiveness, or possibly expression, of the lead-resistance character associated with the genetic event that gave rise to each line.

When a stress is absent a whole plant, or cell culture, resistant to that stress often has reduced growth rates compared to the non-resistant forms (Bradshaw and McNeilly 1981; Gonzales and Widholm 1985). However, in this case it can be seen that the selected lines have fresh weight increases which are comparable with the non-selected lines on +Pb medium (Fig 1). Moreover, the fresh weight increases of the selected lines both on -Pb and +Pb media are not significantly different, whereas, plants resistant to a
stress often have lower growth rates than their non-resistant counterparts growing under non-stressed conditions. Therefore, the possession of the newly acquired mechanism for lead tolerance has no disadvantageous effects on fresh weight increase and appears stable in the absence of lead.

Resistence of regenerated plantlets to lead stress.

Regeneration is important in determining transmission of the resistance character over mitotic barriers and through patterns of differentiation. The genetic mechanism for resistance at the undifferentiated cellular stage is probably only of academic importance unless it alters the phenotype of the whole regenerated plant. Further, many selected cell lines have been found to be non-regenerative (Challeff 1986) or chromosomally variable (Reisch 1983), and so regeneration studies are valuable in this respect also.

The evidence presented in Table 1 indicates clearly that the growth of plantlets from control populations is significantly inhibited in lead-containing media. The selected plants show a reduction in leaf area with lead, but this was not as large as that of the control plantlets, and the survival rate was good. It should be noted that when stressed with lead, survival of the non-selected plants was 30-36%. Those surviving were not, therefore, representative of the total population.

The results indicate that the resistance mechanism, however based, is active and effective at the whole plant level.
In media containing 10ppm lead ions the selected plants have a root lead level of just over half that found in the non-selected plants. This suggests some form of exclusion or inhibited/depressed uptake mechanism. On the other hand, at 100ppm lead ions, the root-lead concentrations are not significantly different. However, whilst the selected plants are healthy and green the controls are not. This may suggest that once the lead level is too great for an uptake mechanism of resistance to be effective, the selected plants also possess a cellular tolerance/hardiness mechanism that the control population does not. This cellular hardiness of selected lines has been noted by other workers and possibly results in heavy metal tolerant lines also exhibiting tolerance to cold stress (Huang and Goldsbrough 1988).

The leaves of the plants from the lead-susceptible (control) populations exhibited the classic symptoms of lead toxicity (Peterson 1978). Their leaves, however, along with those from the lead-resistant population, contained lead levels which were undetectable by this assay. The lead, present in the roots was not transported to the leaves. This agrees with other work (Koeppe 1981 for review) suggesting that lead is complexed within, as well as being adsorbed onto, the root.

**Uptake/release of lead from taproot tissues.**

In this study the taproot tissue from the lead resistant plants exhibits its resistance character in three ways:

a) the mean (n=10) rate of uptake of lead from solution is slower and the final concentration is also less, when
compared to control populations.

b) The mean water exchangeable fraction of the tissue lead from resistant plants is greater than that in the susceptible plants.

c) the mean chemically exchangeable fraction of tissue lead is again greater for resistant plants.

The lead resistant tissue exhibits a lead exclusion mechanism and a greater proportion of the tissue lead is held in an exchangeable form. Two phases of lead uptake by roots have been identified (Jarvis, Jones and Clement 1977) an initial rapid phase which is probably an exchange phenomenon, and a slow sustained phase which may be under metabolic control. The mean uptake of the plants from the selected populations show a depression of this initial uptake; however, the range of response can be seen to be large with plants exhibiting no change or very great change.

The variability of response exhibited by lead-resistant tissue is far greater than the lead-susceptible, even though the tissue of both types were regenerated from single cell lines and should, therefore, exhibit clonal properties (Dix 1986).

CONCLUSION

The production of carrot cell lines resistant to lead stress by isolation of variants, demonstrates that a genetic basis for lead tolerance may exist, which can, also, be expressed in whole plants regenerated from the cell lines. This may be important for developing crops to be grown for
use in agriculture or for their amenity value. Hence, derelict land polluted by lead can be exploited.

The transmission of the resistance character across meiotic barriers is being investigated in seed populations derived from the plants described in this study.

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THE INFLUENCE OF CONTAINER DIMENSIONS ON THE MULTIPLICATION RATE OF REGENERATING PLANT CELL CULTURES

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SUMMARY

The growth of regenerating *Daucus carota* callus tissue depends upon the distance from the tissue to the seal of the container. Growth increases as the distance decreases.

The correct choice of container design may be necessary, therefore, to optimise early multiplication rates in Stages 1 and 2 of a micropropagation system.

KEYWORDS

Containers, gas-exchange, multiplication.

INTRODUCTION

When attempting to optimise growth of regenerating cultures the influence of the gaseous environment is commonly ignored. The evolution of ethylene into such an environment is likely to affect growth in vitro as is the accumulation of carbon dioxide (Belcher et al., 1987; Deproff et al., 1985), together with other morphogenically active compounds (Gould and Murashige, 1985).

Other, physical factors will also moderate any biological effects of components of the gaseous atmosphere. The most significant will be the total volume of gas-space above the culture and the diffusion distance from the cultured tissue to the point of gas exchange with the external atmosphere, ie, the seal of the vessel.

In this study two types of cylindrical culture vessel were constructed. The first maintained a constant volume but altered the distance for gas exchange, whereas the second maintained this distance as constant, but varied vessel volume. The significance of volume and diffusion distance can then be evaluated in terms of the growth performance of a simple culture system, in this instance a regenerating callus culture of *Daucus carota*.

MATERIALS AND METHODS

A single piece of callus tissue (0.15-0.35 g fresh wt) was placed in each culture vessel, containing Gamborg B5 medium supplemented...
with 2% w/v agar and 0.1 mg l\(^{-1}\) 24 D.

Cylindrical vessels were used with a gaseous volume of 350 ml and diffusion distances of 50, 100 and 130 mm. The second set of vessels maintained a diffusion distance of 100 mm and gaseous volumes of 100, 340 and 500 ml. Cultures using each vessel type were contained in large perapex boxes to maintain a greater uniformity of external environment.

Growth was monitored by sterile fresh weight measurement at each sample point and expressed as a percentage of the initial fresh weight.

RESULTS

The relationship between fresh weight increase of the regenerating callus cultures and gaseous volume in vitro, with constant diffusion distance, is expressed in Figure 1. The effect of variable diffusion distance and constant gaseous volume is expressed in Figure 2.

![Figure 1](image1.png)

**Figure 1.** The growth of regenerating callus cultures of Daucus carota with differing gaseous volumes in vitro and a 100 mm diffusion distance.

![Figure 2](image2.png)

**Figure 2.** The growth of regenerating callus cultures of Daucus carota with differing diffusion distances and a 350 ml gaseous volume in vitro.
DISCUSSION

The growth rates displayed in Figure 1 are not significantly different, and are not reduced by nutrient depletion over the time course of the experiment. Most importantly, it is also evident that the growth rates are not affected by a variable gaseous volume in vitro, when the gas diffusion distance from the cultured tissues to the seal of the vessel is kept constant.

On the other hand, when gaseous volume remains constant and the diffusion distance is altered (Figure 2), the increases in fresh weight are markedly changed. It is apparent that growth rate increases as diffusion distance decreases, with a doubling of fresh weight being possible in identical gaseous volumes (in cylindrical vessels) if the diffusion distances are adjusted appropriately. This may be due, at least in part, to an effective reduction in gaseous concentration of compounds such as carbon dioxide and ethylene where the diffusion distance is reduced. Critical analysis of compounds such as these is the obvious next step in this investigation.

A further implication of these observations may be apparent when the growth of an in vitro culture is deliberately to be restricted, e.g. in plant germplasm storage. The growth rate of stored cultures might simply be diminished by choice of a culture vessel with an appropriately large diffusion distance.

REFERENCES


THE INFLUENCE OF CONTAINER DIMENSIONS ON THE MULTIPLICATION RATE OF REGENERATING PLANT CELL CULTURES.

RÉSUMÉ

Le développement du cal tissu régénéré de Daucus carota compte sur la distance du tissu au plomb du récipient. Le développement augmente pendant que la distance diminue.

La choix juste du dessein de récipient peut être nécessaire, donc, à optimiser des taux de multiplication dans les phases 1 et 2 du système de micropropagation.
CRYO-SCANNING ELECTRON MICROSCOPY OF ICE CRYSTAL DAMAGE IN RAPIDLY FROZEN, CRYOMOUNTED SPECIMENS

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Summary

Thin arrow-worm specimens showed good cryofixation with no ice crystals being observable within 25 μm of the surface, after freezing in liquid ethane and mounting with low temperature cements. Carrot specimens, regardless of cryopreparation method, showed severe damage 1 mm from the surface. This suggests that there is no advantage in using special coolants for cryo-scanning electron microscopy, unless small specimens are to be frozen and fractured for internal examination.

Keywords
Cryofixation, cryo-SEM, freeze-fracture, freeze-etching, freeze-drying.

Introduction

Cold-stage scanning electron microscopy (cryo-SEM) enables biological specimens to be observed in a near-natural state after freezing, or cryo-fixation, avoiding artefacts induced by other preparative methods (1-3)
A major problem of cryofixation is the disruptive artefact of ice crystal damage. This results from phase separation as water molecules migrate to form crystals during freezing, while the solutes and other constituents are concentrated in the matrix, or grain boundary, between the crystals.

Specimens destined for cryo-SEM are normally frozen after mounting on the support required for insertion into the microscope. The method of mounting already-frozen specimens described here was developed to examine specimens which were plunge-frozen free from contact with any thermal mass, in an attempt to reduce ice crystal damage (4). It has also been used to prepare arrow-worm specimens with large fluid-filled cavities, containing about 97% water, for X-ray microanalysis (5).

This report describes results from specimens frozen in two coolants which differ greatly in their cooling efficiency (6-7). It also describes a cryomounting technique and considers whether or not it alters ice crystal damage in specimens which were already frozen.

Materials and Methods

Specimens: Arrow-worms (Sagitta elegans: Chaetogntha) 15 mm long and 1 mm diameter were caught off Plymouth and frozen within 2 hours of coming into the laboratory. Blocks of cortical parenchyma (6 x 4 x 2 mm) were cut, with a razor blade, from the large storage tap roots of carrots (Daucus carotta: Umbelliferae).

Freezing methods: specimens were frozen by two methods using a plunge-cooling device (8). Some were plunged 100 mm into liquid ethane (93 K) at about 2 ms⁻¹ and others were dropped into liquid nitrogen 100 mm deep. Arrow-worms were plunged while suspended across the ends of a V-wire glued onto a Reichert FC4 cryoultramicrotome specimen pin; the trunk of the specimen was not in contact with any thermal mass and was cooled very efficiently. Immersed control animals were held so that they protruded from a hole containing seawater in a standard specimen support, they could then be fractured after freezing. Carrot specimens were placed on one end of a strip of aluminium foil, and the other end then attached to the plunger using Blu-Tack. Control carrot specimens were frozen by mounting them on standard specimen supports, on a thin layer of silver paint, and dropping them into liquid nitrogen or into liquid ethane in the plunger device.

The cryomounting device: this is a polystyrene Igloo box containing a brass plate on a metal block. A tin box (150 x 90 x 55 mm deep) containing a polystyrene layer (10 mm thick) rests on the plate and contains the low-temperature "hot-plate", or cryomounting block. This is made of brass (50 x 30 x 25 mm high) with a cartridge heater (110V/100W) inserted from one end. A thermocouple near the heater connects to a set-point temperature controller. The top of the block is milled to form a well
(11 mm diameter x 4 mm deep) to house specimen supports. A clamp holds a second thermocouple onto the specimen support, for direct temperature measurement. Liquid nitrogen covers the plate to provide cooling in the system.

**Modified specimen supports:** these were made by cutting two slots, 2 mm wide and 2 mm deep, in standard 5 x 10 mm diameter SEM specimen supports.

**Electroconductive paints:** Dotite Type D-550 (supplied by J.E.O.L. for mounting dried SEM specimens) and Leit-C Conductive Carbon Cement (from Agar Scientific, Stansted, Essex) were used. The latter is useful for X-ray microanalysis because it contributes little to spectra. The silver paint solvent is iso-butyl methyl ketone (m.p. 189 K), while that for Leit-C is a commercial product (m.p. 150 K) containing xylene. At temperatures near the solvent melting points the paints do not "wet", or glue, specimens readily; this is also a function of their solvent content which should not be too high.

**Cryomounting:** The carrot specimens were mounted in silver paint, at 213 K, by filling the grooves in the modified specimen supports at room temperature and then placing them on the cooled mounting block. The temperature of the paint was monitored directly and controlled by the set-point controller. Pre-frozen specimens, stored in liquid nitrogen, required about 10 minutes on the cryoblock to equilibrate thermally before they could be wetted satisfactorily. After mounting, the stubs were placed in a small container of liquid nitrogen brought into the tin box. Plunged arrow-worms were snapped off the V-wires and cryomounted using carbon cement. It should be noted that, for a few minutes, freeze-substitution can occur at this temperature involving possible ionic redistribution in the surface layer of the specimen. This would affect X-ray microanalysis results. Our observations were made 1 mm from the paint/specimen interface.

**Cryo-scanning electron microscopy:** specimens were examined at 3 kV (uncoated) or 10 kV (coated) in a JEOL JSM 35C fitted with a modified cryo-stage (9). The microscope airlock also contained a cryostage, which cooled to 103 K, on which frozen specimens could be fractured with a cold scalpel and sputter-coated when required. Arrow-worms were fractured across the trunk. Carrot specimens were freeze-fractured so that tissue approximately 1 mm from the surface of the tissue block was exposed. Freeze-etching was done in the microscope at a specimen temperature of 193 K, and freeze-drying at 213 K after the exposed cells were dried at 193 K (10).

**Crystal measurement:** data given are cavity widths, as measured with a graticule on micrographs. No allowance was made regarding the angle of crystals to the fracture plane, or for the precise depth of fracture within a specimen or individual cell. Crystal lengths were large and indeterminate; in many cases they appeared to be almost equal to the cell diameter.
Results

The cryomounted arrow-worms showed small crystal domains 0.4-0.9 μm in width, except for the outer 25 μm which showed no discernible damage (fig. 1). The animals frozen on supports in liquid nitrogen showed damage that rendered them almost unrecognizable (fig. 2); the outer 50 μm (corresponding to the body wall) contained crystals 0.54-5.40 μm across, and the fluid-filled inner spaces contained larger ones up to 8.8 μm in width.

Uncoated, freeze-etched body wall of arrow-worm specimens:

Figure 1. Specimen plunged 100 mm into liquid ethane, cryomounted, and freeze-fractured, with outer surface at far left. The fluid-filled body cavity shows uniformly small diameter crystals. The outer 25 μm deep layer of the body wall appears free of crystal artefact. Scale bar 25 μm.

Figure 2. Specimen frozen by immersion in liquid nitrogen while protruding from a seawater-filled well in a solid support, with the outer surface at the bottom (arrow). Scale bar 25 μm.

The surface cells of carrot specimens showed crystal damage when frozen in liquid nitrogen; a freeze-etching specimen mounted on a solid support showed crystal widths of 1.3-1.6 μm (fig. 3). The same cells, freeze-dried, can be seen in fig. 4. Surface cells were normally devoid of contents after freeze-drying, leaving only an empty shell except for a few residual organelles or pieces of cellular debris. The matrix of constituents deposited between the ice crystals in surface cells, where the crystals are smaller, appears to be very fine and friable; during freeze-etching and freeze-drying it tends to crumble and sublime in the microscope (the coarse matrix of fractured, deeper cells also disappeared when specimens were removed from the microscope for sputter coating). Cells at the surface of specimens frozen in liquid ethane showed little obvious crystal damage and no matrix of salts and other constituents was seen during freeze-etching (fig. 5), although the surface of residual amyloplasts or mitochondria showed markings which may reflect fine-order damage (fig. 6).
Deep-seated cells in all carrot specimens, i.e. approximately 1 mm from the specimen surface and exposed by freeze-fracturing, showed similar freezing damage. Figs. 7-10 show crystal damage and the resulting residual matrix in freeze-dried cells from the four preparation methods. The crystals ranged in width from 0.15 to 2.25 μm in all specimens, and can be seen to branch (fig. 10).
Freeze-fractured and freeze-dried, deep-seated carrot cells:

Figure 7. Specimen plunged 100 mm into liquid ethane at 2 ms\(^{-1}\) on a foil support and then cryomounted. Scale bar 10 \(\mu\)m.

Figure 8. Specimen dropped into liquid ethane 100 mm deep on a solid support. Scale bar 10 \(\mu\)m.

Figure 9. Specimen plunged 100 mm into liquid nitrogen at 2 ms\(^{-1}\) on a foil support and then cryomounted. Scale bar 10 \(\mu\)m.

Figure 10. Specimen immersed in liquid nitrogen on a solid support. Note the branching of the crystal cavity (arrow). Scale bar 10 \(\mu\)m.

Discussion

Carrot parenchyma and arrow-worms are sensitive indicators of freezing damage, consisting largely of cavities containing dilute solutions of salts and organic solutes. The cryomounted arrow-worms showed no ice crystals in a 25 \(\mu\)m deep surface layer, which indicates good cryofixation. They also demonstrate that 10 min at 213 K does not induce ice crystal damage, supporting Steinbrecht (11) who found that cryofixed sensory hairs needed 45 min at 230 K to induce fine-order deterioration.

The cryomounting technique was developed following attempts at clamping, which shattered already-frozen specimens. Similar low temperature cementing techniques have been described previously for cryoultramicrotomy and freeze-fracture purposes, using butyl benzene, m.p. 185 K (12); toluene, m.p. 178 K (13); and heptane, m.p. 182 K (14).
The arrow-worm results also highlight the limitation of operating the SEM at low kV using a large probe diameter, resulting in poor resolution, because cryosubstituted, resin sectioned arrow-worms showed at best only a 15 µm deep surface layer free of crystals (unpublished results). The operating conditions did not appear to damage specimens while they were hydrated and at low temperatures; but after freeze-drying they were noticeably beam sensitive.

The freeze-dried carrot cells showed good three-dimensional information, although they may deform slightly while drying in the microscope. It is possible that "ante-melting" occurred deep inside the specimen resulting in a small amount of distortion, or freeze-drying collapse (this probably explains the apparent peripheral contraction of cytoplasm in figs. 7-10). "Ante-melting" describes the melting of ice or a eutectic mixture during slow rewarming of rapidly frozen specimens when crystal metastability is lost. It precedes further freezing resulting in ice crystal growth prior to final melting (15).

No problems were encountered while freeze-etching and freeze-drying at 193-213 K, above the solvent melting points, due to the efficient cold-trap, although if the temperature was raised rapidly to about 223 K then the paint would appear to liquefy, well up, and envelop the specimen.

Crystal cavities were frequently seen to branch in a dendritic fashion, and were similar in form to those considered by Bald (16) and shown in polyvinylpyrrolidone solutions (17-18).

Crystal size was clearly smaller close to the cell wall than in the centre of cells; this was a position effect within each cell and does not imply that the smaller-sized crystal area was closer to the specimen surface. It reflects the water content of the peripheral cytoplasmic area of the cell compared to that of the central vacuolar area.

The same range of ice crystal size was found in carrot specimens frozen in quenchants of greatly differing efficiencies. It was also found in specimens frozen on metal supports and in specimens frozen efficiently on foil supports and subsequently cryomounted.

Conclusions
The arrow-worm results show that good cryofixation does not deteriorate with cryomounting, i.e. the technique does not induce crystal growth which is observable by cryo-SEM. Cryofixation in the surface layers of specimens has been found previously to depend on the chosen coolant (19) and, because this is seen here, it can be inferred that the uniform results seen deeper within specimens do not reflect coolant efficiency. They imply that heat transfer to the surface of the specimen is limited, presumably by specimen thermal diffusivity. This demonstrates that, while not
necessarily being a limitation for X-ray microanalysis purposes (20), good cryofixation of untreated, deep-seated fresh tissue is unattainable with the rapid freezing methods used in this report.

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