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NOVEL SCREENING METHODS FOR PLANT MICROPROPAGATION

By

Neil Armitage BSc (hons)

To be submitted as a thesis
for the degree of Doctor of Philosophy
at the University of Durham
(Dept. Biological Science)
September 1991

18 AUG 1992

Declaration

No part of this thesis has previously been submitted for a degree at this or any other University.

I hereby declare that the work presented here is entirely my own.

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Novel Screening Methods for Plant Micropropagation   Neil Armitage

Abstract

The techniques of plant micropropagation have not been successfully applied to all species. This study was carried out with the objectives of developing new techniques for rapidly assessing the relative merits of cultural treatments and identifying fundamental and genotype-specific problems associated with micropropagation.

Anatomical characteristics of micropropagated *Hosta spp.* and *Paeonia lactiflora* were investigated. A root exodermis was present and apoplastic tracer studies indicated it was functionally and anatomically the same as *ex vitro* root exodermes in the literature. Specialised cells rather than simple wound tissue were present at the plantlet /medium interface An endodermis was present in the shoot base of *Hosta* plantlets. It is suggested that the basal zone of the shoot is functionally a specialised "root".

It is hypothesised that carbohydrate status (or solute potential) of vascular cambia and/or root initial cell is important in the induction of adventitious root formation.

Growth medium GA_3, and possibly raised inorganic phosphate, resulted in increased shoot "health" but inhibited rooting in *P. lactiflora* cultures, possibly through changes in assimilate partitioning and sucrose uptake. A low mobility esterase isoenzyme was specific to these changes. Water relations are identified as a critical factor in the micropropagation of *P. lactiflora*.

Cold storage of *Hosta spp.* led to sequential leaf senescence, abscission and changes in isoenzyme patterns. No true dormancy was identified in culture, although it was demonstrated after weaning if a requirement for cold storage was not met. *In vitro*, "dormancy" was expressed as a reduction in the rate of new leaf production. Removal of this growth inhibition was correlated with the appearance of a highly mobile esterase isoenzyme. The possibility of using this isoenzyme to predict subsequent *in vitro* growth inhibition and *ex vitro* dormancy is discussed.

The objectives of this study were fulfilled, and the direction of future research is discussed.

September 1991

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Special thanks must also be extended to the company and staff of NeoPlants Ltd. of Preston, who not only helped to finance this studentship, but donated extra materials and time for my benefit. I think particularly of Julian Nicholas, who was extremely patient with me and provided valuable guidance, Carlo Leifert who was also very helpful and generously provided accommodation in Preston, where I was made to feel at home, and Robert Lees and Anges Harbour with whom I also had useful discussions.

I extend my gratitude to the Science and Engineering Research Council, who partly funded the project in collaboration with NeoPlants.

I thank my family (especially Mum and John) for their support, both moral and financial, without which I would still be gurgling in a cot waiting for someone to feed me! All my friends deserve thanks, but particularly Kathryn who went through all this at the same time.

Hoping that nobody will read this bit, thinking I'm soft in the head, I also thank Dylan Thomas for writing these lines, which have kept me going through a few rough patches!

The force that through the green fuse drives the flower

Drives my green age; that blasts the roots of trees

Is my destroyer.

And I am dumb to tell the crooked rose

My youth is bent by that same wintry fever.

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List of Abbreviations

AAT	Aspartate aminotransferase
ABA	Abscisic acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BAP	Benzyl aminopurine
ANS	8-Anilino-1-naphthalene sulphonic acid
cAAT	Cytoplasmic aspartate aminotransferase
CR	Choisya rooting medium
CX	Choisya multiplication medium
DNA	Deoxyribonucleic acid
EnActS	Enzyme activity staining
Est	Esterase
GA	Gibberellins
GA_3	Gibberellic acid
HR	Hosta rooting medium
HX	Hosta multiplication medium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IMS	Industrial methylated spirit
LAP	Leucine aminopeptidase
LDH	Lactate dehydrogenase
L-DOPA	3,3-Dihydroxy-L-phenylalanine
mAAT	Mitochondrial aspartate aminotransferase
MPHC	Multiple plantlet humidity control apparatus
MS	Murashige and Skoog medium
MSMO	Murashige and Skoog medium with minimal organics

NAA	Naphthaleneacetic acid
PAGE	Polyacrylamide gel electrophoresis
PAR	Photosynthetically active radiation
PEG	Polyethylene glycol
Pi	Inorganic orthophosphate
PhR	Photinia rooting medium
PPi	Inorganic pyrophosphate
PPO	Polyphenol oxidase
PR	Paeony rooting medium
PVP-40	Polyvinyl-pyrrolidone
PX	Paeony multiplication medium
Rh	Relative humidity
Rf	Relative front (relative mobility)
RMM	Relative molecular mass
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-7	SDS-PAGE molecular weight markers
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPHC	Single plantlet humidity control apparatus
SPS	Sucrose-phosphate synthase
SSS	Saturated salt solution
TEMED	N, N, N',N'-Tetramethylethylenediamine
WPM	Woody plant medium

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1. Introduction

The techniques of micropropagation, although one of the first commercial successes of plant biotechnology, are more closely related to the craft-based skills of horticulture than the science of botany. This study was initiated with a view to investigating some of the fundamental principles and problems of micropropagation, and developing new techniques for rapidly assessing the relative merits of different chemical treatments and environmental conditions.

In the pursuit of these goals, it was decided to base the study around the micropropagation of two herbaceous perennial plants, only including other species when necessary to confirm any findings. One of these plants (*Hosta spp.*) is relatively easily micropropagated, with well established techniques in use by the commercial micropropagation company Neo Plants Ltd. The other (*Paeonia lactiflora*) is intransigent to micropropagation, with no successful system developed to date. It was hoped that by investigating these two systems, one could be contrasted with the other to help understand why their responses to culture are so different, and identify the specific problems associated with the micropropagation of each plant.

1.1. Micropropagation

1.1.1. History of Micropropagation

As long ago as 1878, the German botanist Vöchting predicted that small fragments of plant material, under appropriate external conditions, could be regenerated into whole plants. Twenty four years later Haberlandt (1902) attempted, but failed, to grow plant cells in aseptic culture; he was able to keep them alive for nearly a month, and envisaged a day when, "one could successfully cultivate artificial

embryos from vegetative cells". It was not for another 32 years, when White (1934) showed that sterile tomato (*Lycopersicon esculentum*) roots could be grown and maintained *in vitro* when supplied with yeast extract, that any further progress was made. In the late 1930's and early 1940's, Gautheret and others demonstrated that isolated storage tissue from plants such as carrot (*Daucus carota*) and potato (*Solanum tuberosum*) could be grown aseptically as a mass of undifferentiated cells, or callus (Wareing and Phillips, 1981).

The use of plant growth regulators in tissue culture dates from 1937, when Gautheret and Nobecourt independently used IAA to promote cell growth in carrot (*D. carota*) cultures. It was not until much later that Miller *et al*, (1955) discovered that kinetin (a cytokinin) promoted cell division. The roles of auxins and cytokinins in shoot and root induction in tobacco (*Nicotiana tabacum*) callus cultures were recognised by Skoog and Miller (1957), and this allowed some control of morphogenesis *in vitro*. In 1962, Murashige and Skoog published their 'Revised medium for rapid growth and bio-assays with tobacco cultures'. It proved to be a medium on which many plants could be grown successfully in culture, and has since become almost a standard medium, others only being resorted to if success is not first achieved on 'MS' medium.

The techniques outlined above, along with others, are collectively termed "tissue culture", and have been used as a research tool by geneticists, botanists and plant physiologists for some time. Their application to the commercial mass production of plants is a relatively recent development. Morel (1960), whilst attempting to produce virus-free orchids, discovered that shoot tip cultures produced many protocorms, which could be separated and placed back into culture to

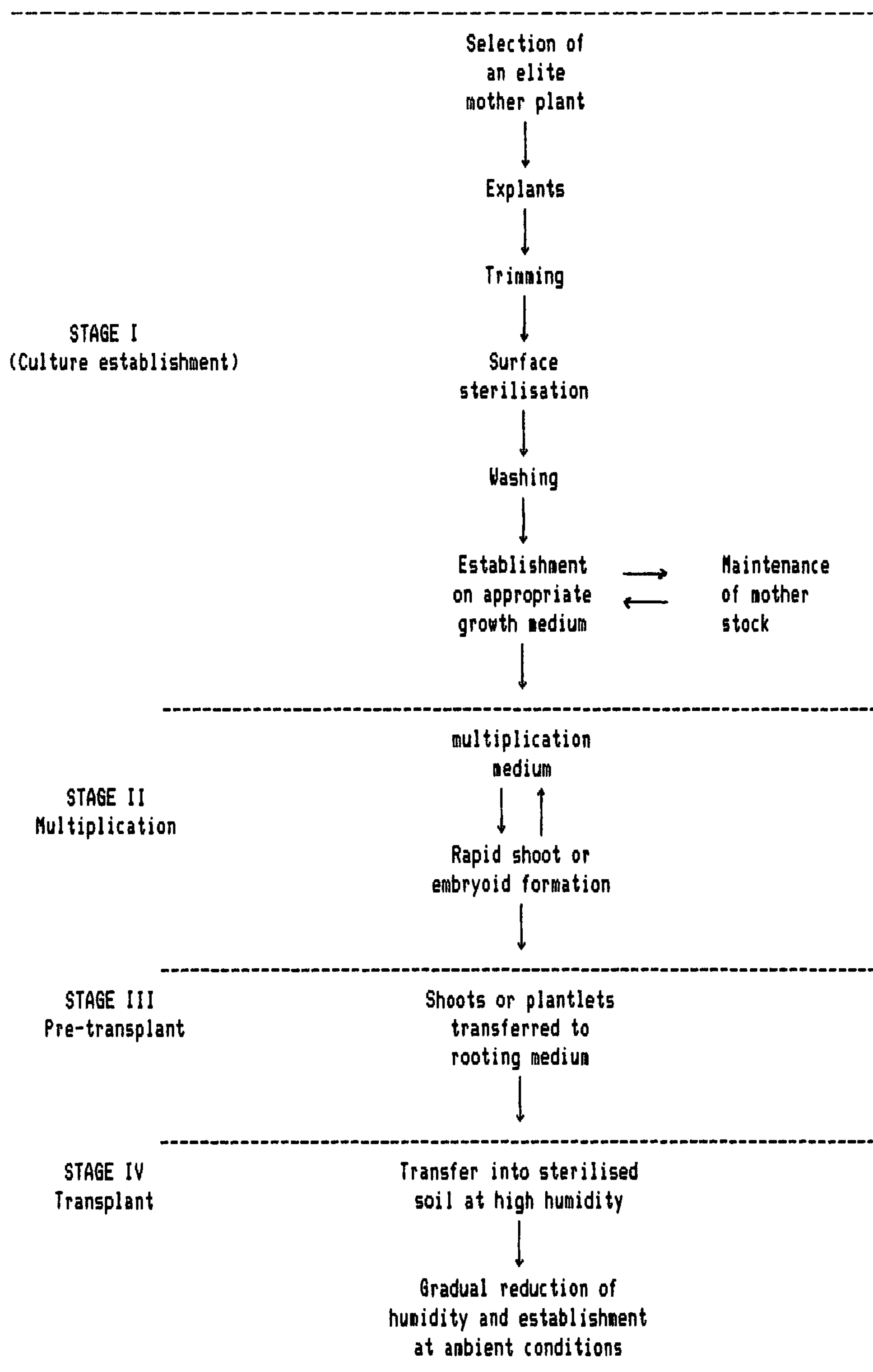
produce new plants. Traditional methods for the propagation of orchids were very slow, and the new technique greatly speeded up production (Morel 1964), and made orchids available to the general public. Similar procedures have now been applied to many other plants, and micropropagation has become one of the first commercial successes of the new science of plant biotechnology.

Micropropagation has now been effectively used to proliferate hundreds of vascular plants, and new species are being propagated in this way all the time. In addition to the direct commercial benefits of being able to produce very large numbers of genetically identical plants, a carefully defined micropropagation regime is becoming increasingly important in other areas of research. The disciplines of crop breeding and selection require the availability of a reliable true-to-type method of propagation. This must be able to maintain the germplasm sources on which any breeding programme relies, and to rapidly regenerate and proliferate any newly-generated genetic lines, so they may be quickly used in further breeding or utilisation in the field. The emerging technologies of plant genetic engineering (recombinant DNA), also have these requirements, and micropropagation is well suited to their needs.

1.1.2. Micropropagation Techniques

The basis of micropropagation is the rapid clonal propagation of plants under sterile conditions from small segments of plant tissue. These are usually meristematic areas such as shoot or root tips, axiliary nodes or adventitious nodes, which are grown under controlled conditions on defined growth media in containers capable of excluding microbial infection. After a period of growth, (usually 3-4 weeks), the

Fig. 1 General scheme for micropropagation



miniature plants (plantlets) are removed from their growth containers and dissected in order to recover any newly generated nodes, with which the process is repeated. In this way, very large numbers of clones of the original plant can be produced in a relatively short time.

Murashige (1974) divided micropropagation into 3 stages, and later added a fourth stage. These are outlined in Figure 1.

1.1.2.1. Culture Establishment

The first step in any micropropagation system is the selection of source material. The genotype and physiological status of the mother plant can have a great influence on subsequent micropropagation. Explants taken from plants exhibiting vigorous growth have often been shown to be more successful in culture (e.g. Mantell *et al*, 1978 and Litz & Conover, 1981). Although this seems to be invariably the case for woody plants, the situation is less clear with herbaceous plants, for example; Wright & Anderson (1980) showed that flower stem explants from tulips only give rise to shoots when taken in the dormant (dry storage) stage, and once stem elongation has commenced, regenerative capacity is lost. However, most herbaceous plants can be regenerated from actively growing plants.

It appears that familiarity with a plant's natural propagation is often the best indicator of the best source plant, (Mantell *et al*, 1985). This would also seem to be true for the choice of tissue or organ from which the explant is taken. The plant part chosen should contain a high proportion of meristematic tissue, or cells which are still able to express totipotency. Successful cultures are rarely obtained from senescing tissues (Mantell *et al*, 1985). It has been suggested that regardless of source within the same plant, if cultures are kept in

identical conditions, they will eventually, (12 subcultures for wheat), become indistinguishable (Barker, 1969). This may be morphologically correct, but it is not yet certain if some of the mother plant cells' determinism is retained in long-term cultures.

With the mother plant and excision area determined, the resultant explants then need to be de-contaminated. This is usually accomplished by surface sterilisation with sodium hypochlorite or mercuric chloride, (Street, 1977). The explants are then ready for establishment in sterile culture. During this stage explants need to adapt physiologically from what is normally a photosynthetically active state on the mother plant, to a mainly heterotrophic mode of nutrition in the artificial conditions *in vitro* (Lamb *et al* 1985). The regular transferral of explants on to fresh growth media is often necessary, due to the exudation of harmful endogenous substances from the explants' wounded tissue (see Section 1.1.4.3.). The inclusion of absorbent material (e.g. charcoal), or antioxidants (e.g. ascorbic acid and sodium selenite) in media has also been used to combat this with some success, (Mantell *et al*, 1985; Elmore *et al*, 1989). At this point, any infected tissue needs to be removed, and the most vigorously growing plantlets selected for the second stage of micropropagation defined by Murashige (1974).

1.1.2.2. Multiplication

During this stage of shoot proliferation, the conditions for optimum growth and multiplication of plantlets should, as far as possible, be elucidated and maintained. This can be a very laborious procedure, for although cultural conditions are controlled by the experimenter, there are many variables involved. These include media constituents and environmental factors, both of which can be relatively

easily defined, but when it is considered that each media component or environmental factor might be tested individually for its optimum level in a particular species, and often for each variety within the species, it is obviously a gigantic task. In addition to this, many of the factors are likely to affect each other, making the number of combinations almost unimaginable. For this reason, most researchers use MS media, or slight variations from it, in their investigations. Standard growth conditions are also usually utilised (often 25-6°C, 16 hour day or continuous light of a relatively low intensity).

The factor most investigated, because of the large potential benefits, is growth media hormone levels. The relative concentrations of auxins and cytokinins are known to be very important in determining the morphology of plants in culture. Plantlets grown on a basal medium containing no plant growth hormones, tend to resemble seedling-like shoots with strong apical dominance. This only gives a limited potential for shoot multiplication. Cytokinins are often used to suppress apical dominance and produce large quantities of axillary shoots, which are ideal for this purpose. Optimum levels for axillary shoot formation are usually in the range of 0.5-10 mg/l (Mantell *et al*, 1985). Auxins are sometimes included at low concentrations to promote cell growth, but high concentrations are likely to cause the proliferation of callus tissue. As a general guide, if the production of axillary shoots is desired, the ratio of cytokinin to auxin should be high (e.g. 100:1). If adventitious shoot formation is required, the non-meristematic tissue is exposed to low, approximately equal levels of both auxin and cytokinin. (Hartman & Kester, 1983).

Other plant growth regulators have been used to influence the morphology and physiology of plants in culture. Gibberellins (usually

GA_3) are the most commonly used, either to act synergistically with auxins to promote cell extension, or, more controversially, to increase cell division in a similar way to cytokinins (Jones & MacMillan, 1984). In addition to this, gibberellins are known to influence dormancy, and have been used pre-culturally to remove any residual dormancy and achieve "normal" growth (e.g. Howard & Oehl, 1981). It is possible that some of the increased growth observed by the inclusion of gibberellins in culture media is due to the removal of any residual "dormancy" in plantlets, (see Section 1.2.). This, however, has not been fully investigated to date.

It should be noted that the responses of plants to hormones *in vitro* are gross effects, and not directly comparable with the subtle relationships and functions of endogenous hormones. They artificially "force" morphogenesis, are often man-made and not found naturally (e.g. the cytokinin BAP), or are used at concentrations seldom found in plants.

With species that have proved very difficult to micropropagate, it has sometimes been found that the addition of crude plant extract allows successful culture. Yam *et al* (1991), attempting to micropropagate Taro (*Colocasia* sp.) species, found that the addition of a crude extract of tubers from the same species enabled them to grow healthy Taro plantlets in culture. The use of extracts from banana (*Musa* sp.) fruit also improved micropropagation, but not to the same extent as Taro tubers. This indicates that some essential ingredient(s) are missing, or not present in high enough concentration in the growth medium. It is possible that this particular requirement is unique to Taro, and that other species also have a unique requirement, but, more interestingly, it is also possible that easily-propagated plants simply have a

tolerance to the absence or low levels of these "essential" ingredients, and would perform better in their presence. Consequently, the empirical use of plant extracts is not a satisfactory answer, and is, in fact, resorting to the methods used in the 1930's. They may work in a commercial sense, but these compounds need to be isolated and scientifically investigated so their full potential can be utilised.

Inorganic nutrients have been shown to affect growth and organogenesis *in vitro*. An important factor seems to be the type of nitrogen source used, (e.g. Mantell *et al*, 1985; Selby & Harvey, 1990).

The environmental requirements of plants multiplying in culture have also received little attention. Plants are finely-adapted to the conditions they find in their natural habitat. Their physiology and development is delicately tuned to diurnal and seasonal variations in temperature, light quantity and quality. The changes in water regime, soil chemistry, and atmospheric conditions may also be important (see section 1.1.4.). So the application of knowledge about a plant's ecology could be very useful, and should be thoroughly investigated.

Optimum photoperiod requirements for plantlet growth can vary considerably between species. Murashige & Nakano (1968) found that a 16 hour day was best for tobacco (*Nicotiana tabacum*), whilst Maragara (1969) found a 9-hour day to be more effective with cauliflower (*Brassica oleraceae*).

Although micropropagated plants have a low dependency on photosynthesis, light, apparently absorbed by photosynthetic pigments, plays an important role in morphogenesis (Mantell *et al*, 1985). For example, *Asparagus* spear production is increased in the light (Hasegawa *et al*, 1973). Light has been shown to be morphologically active *in vitro*

at both the red and blue ends of the spectrum. Seibert (1973) found that blue light induced adventitious shoots in tobacco (*N. tabacum*) callus, and Kadkade & Seibert (1977) showed that adventitious shoot formation in lettuce (*Lactuca sativa*) was much increased by very small doses of red light, and is probably regulated by phytochrome. For a number of cultured plants, the appropriate red/far-red response signature of phytochrome has been demonstrated, with red light stimulating shooting and far-red stimulating rooting (Mantell *et al*, 1985).

Temperature requirements can also vary from the somewhat arbitrary 25°C frequently used. For example, the optimum temperature for shooting in *Begonia* cultures is 18°C (Fonnesbech, 1974), whereas *Asparagus* cultures have been shown to grow best at 27°C (Hasegawa *et al*, 1973).

The varying responses to hormonal levels observed in different species, or even varieties within a species, testifies to the potential benefits of research into these other factors for each genotype, if time was available.

1.1.2.3. Pre-Transplant

Murashige's stage III of micropropagation involves the rooting and weaning of the plantlets produced in stage II. It is normally necessary to transfer plantlets on to a "rooting" medium, on which the plantlets are encouraged to initiate, and sometimes extend roots. During this stage plantlets increase in size rather than proliferating, leading to the production of single plants when weaned. These morphological changes are, again, usually brought about by changing the hormonal composition of the media. The exclusion of all hormones is sometimes sufficient to allow rooting (e.g. *Hosta spp.*), but the inclusion of an auxin will often promote the initiation of roots, although it can also inhibit root

extension, so plantlets may have to be removed from this medium after roots have been initiated or begun to appear.

The effect of gibberellin (GA_3) on rooting in micropropagules has been widely investigated, often with contradictory results. Evidence does, however, point to a role in root elongation, rather than initiation (Waring & Phillips, 1981, Carvalho *et al*, 1989). Whether or not GA_3 has a beneficial effect on *in vitro* rooting in a particular species is likely to depend on the complex interrelation of anatomical and physiological factors, (see Section 1.1.3.2.).

Rooting is also thought to be inhibited by high inorganic salt concentrations (e.g. Sriskandarajal & Mullins, 1981, Lamb *et al* 1985), and is often carried out on 1/2MS, 1/4MS or other low ionic strength media such as Lloyd & McCown's (1981) Woody Plant Media. This could be an osmotic effect, or due to one or more of the constituents of MS being inhibitory to rooting at the concentration found in full strength MS.

The influence of light regime on rooting is also thought to be important. For example, Wainwright & Flegmann (1984) found that light intensity during multiplication of blackcurrant (*Ribes nigrum*) influenced the speed of subsequent rooting. The effect of light during rooting culture, although dramatic, seems to vary between species (Hartman & Kester, 1983; Nicholas, 1985)

1.1.2.4. <u>Transplant</u>

After rooting, it is nearly always necessary to acclimatise plantlets before they can withstand the stresses of being transferred to their natural environment. There are many factors which may cause stress to plants during acclimation. Water stress is the most obvious of these, when it is considered that plants are being transferred from the "water-

logged" environment *in vitro* (see section 1.1.4.1.1.), to the often harsh water stresses naturally placed on plants. For this reason, plants are often left for a time in conditions of high humidity (eg. a mist room) for a period of adjustment.

The causes of this water relations incompetence are thought to be both physiological and anatomical. Defective stomatal formation and function are thought to play a part (Brainerd & Fuchigami, 1982; Brainerd *et al*, 1981), as is excessive cuticular transpiration (Wardle *et al* 1983), and a lack of functional vascular tissue (P. Gates pers. com.) or effective hydraulic connection between shoot and root (Grout & Aston, 1977), (see section 1.1.3.3.). Another factor, which is increasingly acknowledged to be important is photosynthetic competence (Conner & Thomas, 1982; Evans & Lees, 1987). The transition from mainly heterotrophic to autotrophic nutrition does not seem to be easy, and this could be the cause of many plants dying in the nursery.

1.1.2.5. <u>Summary</u>

It is evident that much basic research is needed if some of the more difficult species are to be proliferated through micropropagation. The present systems are adequate for the propagation of "easy" species, but even they might benefit from further work. In the following section I will discuss the problems of micropropagation in more detail, and look at some of the ways in which they can be approached.

In the light of all this, it seems sensible to re-examine the basis of the technique of micropropagation, looking at environmental factors, such as water relations, light (quality and quantity), and temperature, as well as nutrition and hormonal environment in culture. A number of important questions need to be asked; "Is it surprising that some plants

don't do well in water-logged conditions?", " Can micropropagation be made environmentally more 'natural' for the plant?", "How important are toxin build up and allelopathy in such a closed system?", "To what extent is a carbohydrate source necessary, and when it is present; why is the growth rate so slow in comparison to heterotrophic plant cell or bacterial culture?", "Does wounding during sub-culture affect growth rate?".

Such questions are numerous, and it is only now that they are beginning to be investigated, after the first flush of success with present knowledge. The rewards of having a more detailed understanding of basic biology and problems of plants in culture could be enormous.

1.1.3. Anatomy and Physiology of Micropropagated Plants

1.1.3.1. Shoots

The health and uniformity of shoots is a vital factor for subsequent rooting and weaning in commercial micropropagation (J.R. Nicholas, pers. com.). Anatomical and physiological investigation can give an indication of any possible problems, and suggest ameliorative measures.

1.1.3.1.1. Vitrification

It is commonly reported that culture conditions can result in "vitreous" leaf development (e.g. Phan & Hegedus, 1986). The leaves in vitreous plants contain larger (X3 length and width), often morphologically disrupted mesophyll cells and much larger air spaces between the cells (Von Arnold & Eriksson, 1984). This gives a "bloated", translucent and glass-like (hence "*vitre*"ous) appearance to the shoot. Evans & Lees (1987) reported that in the chloroplasts of vitreous plantlets, only a low level of photosynthetic coupling is present. Some

of these observations have also been made in plantlets which are not obviously vitreous (Davis, 1987), and it is possible that vitreous plants are just extreme cases of problems due to conditions in culture.

Vitrification is thought to be due to modifications in cell wall elasticity. Davis (1987) reported that cell wall plasticity was 9 times higher in vitreous leaves. He also reported that vitrification could be reduced by increasing cytokinin (BA), and including $CaCl_2$ in the medium. Other media factors have also been reported to alleviate vitrification. These include the brand and concentration of agar (Von Arnold & Eriksson, 1984; Jones *et al*, 1985), type of carbon source, NH_4^+ concentration and the addition of phloridzin (Jones, 1987). Decreasing the medium sucrose concentration increased vitrification in rose (*Rosa* sp.) cultures (Lanford & Wainwright, 1987), and Debergh *et al* (1981) suggested that the osmotic differential between shoot and medium may be a contributing factor. Ziv *et al* (1983) found that the high humidity found inside culture vessels appeared to encourage vitrescent shoots, and that reducing it helped to alleviate the problem.

It seems that vitrification is a stress response to the alien water relations and nutrition experienced in culture. The phenomena is complicated and will require extensive research to identify the mechanisms involved.

1.1.3.1.2. <u>Chlorosis</u>

The "yellowing" of leaves has been associated with inorganic nutrient levels. Hewitt (1952) described how additions of ferric phosphate alleviated chlorosis in hydroponically-grown plants. He also suggested that the form of nitrogen source could be important. It is difficult to attribute chlorosis to the deficiency or toxicity of any

individual mineral due to the complex inter-relationships of uptake (see Clarkson, 1984).

Leaf chlorosis also occurs when plants are kept in the dark (etiolation), although this seems to be a separate phenomenon, which acts through the delayed development of chloroplasts (Danks *et al*, 1983).

1.1.3.1.3. Stomata and Cuticle

Abnormal development and structure of *in vitro* stomata has been reported (eg. Brainerd & Fuchigami, 1982). In *Photinia*, Harbour (1987) showed that the mean stomatal pore size was much greater *in vitro* than in fully acclimated plants. Short *et al* (1984) demonstrated that the stomata of micropropagated African Violet (*Saintpaulia* sp.) were unable to close properly, and suggested that this could be due to an anatomical abnormality. Indeed, Brainerd & Fuchigami (1982) reported such a phenomena.

Reduced leaf epicuticular wax levels have been reported (Wardle *et al*, 1983), which is thought to allow excessive cuticular transpiration during weaning, (Short *et al*, 1984).

1.1.3.1.4. Leaf Senescence and Abscission

The synchronous senescence of leaves on a seasonal basis, such as autumnal leaf loss in deciduous woody plants, or when the shoots of some herbaceous perennials die back leaving only their underground parts, is a natural response to avoid an unfavourable season (Noodén, 1988). Leaf senescence and abscission are also stress symptoms (Noodén & Leopold, 1988; Sexton & Roberts, 1982). In micropropagated plants, container ethylene accumulation can be a severe problem (Jackson *et al*, 1991), and ethylene is also a crucial hormone in the process of both senescence and

abscission (Sexton & Roberts, 1982) and stress responses (Beyer *et al*, 1984). It is possible that both these stimuli operate in culture.

Senescence and abscission are tightly controlled and highly ordered on a cellular and metabolic level (Sexton & Woolhouse, 1984). A number of external factors affect the rate of senescence, including drought, mineral nutrition, light intensity, daylength, disease (Wareing & Phillips, 1981) and light quality (Guiamet *et al*, 1989). Although senescence and abscission are not rigidly linked, they often take place sequentially, with the withdrawal of nutrients from the organ being followed by abscission of that organ. When they occur separately, this is usually the result of extreme stress (Sexton & Woolhouse, 1984).

When senescence is initiated, nutrients are removed from leaves and stored in other organs. Vascular connection via sieve tubes may remain functional very late, even after leaf yellowing (Benner & Noodén, 1984). The first stages of abscission are initiated early on in this process (Noodén, 1988).

Abscission usually takes place along a layer of cells 1-5 deep in a flat uninterrupted plane across the structure, such as near the base of the petiole in leaves (Sexton & Roberts, 1982). This area is called the separation layer, which together with other adjacent specialised cells compromises the abscission zone. The cells of the abscission zone can often be recognised by being smaller, more densely protoplasmic, having smaller intercellular spaces, containing large deposits of starch and having more highly branched plasmadesmata than adjacent cells (Sexton & Roberts, 1982). These cells can also be distinguished histochemically by their intense staining for respiratory enzymes (Poovaiah & Rasmussen, 1974), conspicuous nuclei and nucleoli (Sexton & Woolhouse, 1984) and

substantial deposits of what most workers believe is lignin (Sexton & Roberts, 1982).

The reduction in separation layer break-strength has been attributed, at least in part, to the action of a specific cellulase isoenzyme (Durbin *et al* 1981). Other enzymes thought to be involved include pectinase and hemicellulase (Sexton & Woolhouse, 1984). Most plant growth hormones seem to have an effect on abscission, with auxins, cytokinins and gibberellins having an inhibitory effect, and ABA and ethylene an accelerating effect. For a good review of this area see Sexton & Roberts (1982) and Noodén & Leopold (1988).

1.1.3.2. <u>Roots</u>

The formation, or at least initiation, of roots in micropropagated plantlets is essential for subsequent weaning. Difficulties have been experienced in the rooting of many species (see Hartman & Kester, 1983; Jones, 1983; Wainwright, 1987). An understanding of the anatomical and physiological processes leading up to rooting is essential if any progress is to be made in this area.

Roots that arise spontaneously at locations other than the primary root or embryonic axis are termed "adventitious". It is these roots that are important in relation to plantlet rooting.

Irrespective of species or material, the origins of adventitious roots can almost always be traced back to vascular tissue (Lovell & White, 1986). In stems, the initial events and cell divisions are usually close to vascular bundles or in the interfasicular region (e.g. Mitsuhashi-Kato *et al*, 1978; Cline & Neely, 1983). The formation of primordium initials (and all subsequent root development) sometimes takes place close to this cambium (eg. hypocotyl cuttings in *Griselina*

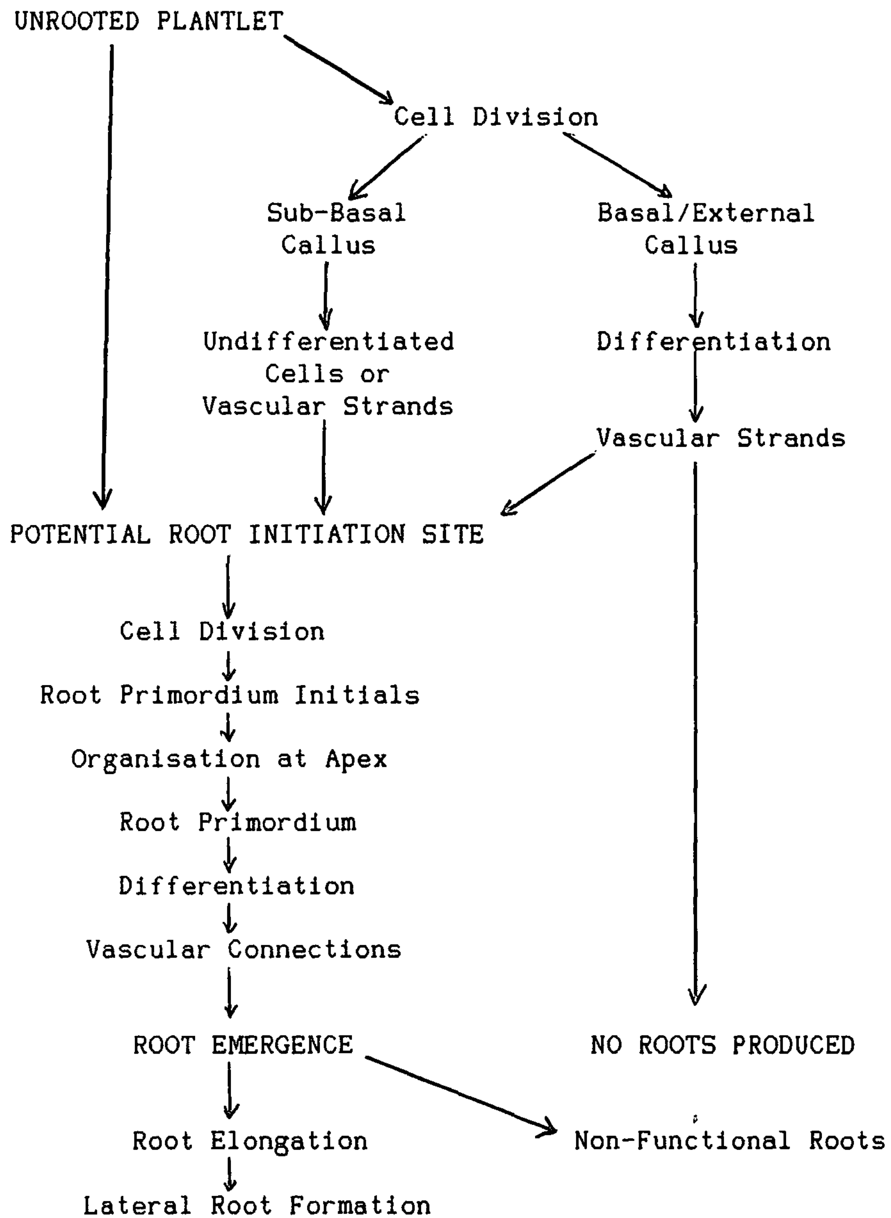
littoralis), but continued division of the cambium can take place, forming an internal callus, from which rows of cells push outward into the cortex, where root primordia are formed.

When basal or sub-basal callus is produced the situation is a little more complex. The somatic induction of vascular tissue in the callus (Brutsch *et al*, 1977), or the induction of xylem and phloem differentiation in the interfascicular region, which then move downwards and outwards into the callus as strands of vascular tissue takes place. Root primordia then appear to form near, but not at the end of these vascular strands (White & Lovell, 1984c; Lovell & White, 1986). Vascularisation of callus may be an essential pre-requisite for primordia formation, but is an unreliable indicator of subsequent root formation as massive vascularisation of basal regions can take place without the formation of root primordia, also possibly preventing their formation (White & Lovell, 1984b; Lovell & White, 1986). Whilst bearing this in mind, it is interesting to note that auxins are well known to stimulate both vascular development (e.g. Sorokin & Thimann, 1964; Wareing & Phillips, 1981) and root initiation (e.g. Hartman & Kester, 1983).

When the root primordium has grown to about 150 cells, the first signs of organisation can be observed (Lovell & White, 1986). By the time the root is ready for emergence, it resembles a seedling root tip. At this stage, it is reported that a vascular connection has not been made, but vascular tissue develops from the primordium to bridge the gap to the nearby vascular strand at about the same time as root emergence (Lovell & White, 1986). There have been reports of this process being

Fig. 2. Schematic Representation of Rooting

(Adapted from Lovell & White, 1986)



delayed in micropropagated plants (Grout & Ashton, 1977; P. Gates pers com.).

Physical barriers to root emergence have been reported in both cuttings (Strangler, 1956; Beakbane, 1969) and micropropagated plants (Nicholas, 1985). These barriers may take the form of a compact mass of dense callus, sclerenchyma bands or large volumes of induced vascular tissue (Lovell & White, 1986).

Photoperiod has also been shown to have an effect on rooting. Vince-Prue (1975) reported that the number of roots and the proportion of rooting in woody plant cuttings was increased in long days for most species, but decreased in some. This is consistent with the variation in rooting response shown in the dark and light in micropropagated plants (Nicholas, 1985).

The deleterious effect of prolonged exposure to auxins has been well documented (e.g. Zimmerman & Fordham, 1985; Nicholas *et al*, 1986). Welander (1983) attributed the low weaning survival of such plantlets to excessive callusing and poor root extension.

1.1.3.3. Vascular Tissue

There is some doubt as to whether the roots of some plantlets in culture have a vascular connection to the main vascular system of the plantlet (see Section 1.1.3.2.). This could be due to the alien water and nutrition regime being imposed on plants *in vitro*, and its demands on the vascular system. The lack of a significant gradient of water potential through the plantlet, due to the aerial parts being exposed to near 100% relative humidity in culture vessels, could affect the differentiation of xylem vessels. Reducing the transpiration rate was reported by Saks *et al* (1984) to diminish the number of newly formed

secondary xylem fibres in the stem of *Helianthus*, but this was markedly reversed in the presence of the cytokinin, Kinetin, suggesting that low transpiration rates reduce the root originated cytokinin supply. Reverse transpiration could also be an important process in water relations *in vitro*.

Auxin is thought to be the major hormone controlling vascular differentiation, but its effects seem to be mediated by other hormones, such as gibberellin, cytokinins and ethylene (Aloni, 1987).

Exogenously applied GA_3 has little effect on vascular differentiation when applied alone, but acts synergistically with IAA, and the combination seems to be able to effectively replace leaves in stimulating vascularisation in *Coleus* (Aloni, 1979). In *Populus robusta*, GA_3 has been shown to promote cambial division, but the cells remain undifferentiated until IAA is applied, when normal development continues, (Wareing & Phillips, 1981). A low ratio of auxin to gibberellin is thought to promote phloem rather than xylem development (Wareing *et al*, 1964).

Cytokinins can also promote vascular development, but only in the presence of auxin and GA_3; ethylene can also have a stimulative effect (see Aloni, 1987).

Exogenous sucrose has been shown to affect xylem differentiation, but reports vary as to whether this is due to a simple plant growth promotion (Wright & Northcote, 1972), or to a role in differentiation (Wetmore & Rier, 1963). Aloni (1980) found that increasing medium sucrose caused a decrease in callose deposition on sieve plates, and suggested that this is the effect being observed.

The mainly heterotrophic rather than photosynthetic nutrition means that any functional phloem should be loaded in the roots, or near the base of the explant, with sugars from the media rather than with photosynthetic assimilates in the leaves. Thus transport is required acropetally rather than basipetally as is normally the case in mature tissues. Indeed the small size of the plantlet may mean that normal symplastic transport from cell to cell may be sufficient for the plantlet's needs. It seems likely that vascular differentiation will be affected in such a situation, where it is either not needed, or the resultant tissue is required to perform the reverse of its normal function.

1.1.3.4. Fluorescence Microscopy

Anatomical investigation of plants in culture could yield interesting results, as anatomy is the visible result of morphogenesis. The technique of fluorescence microscopy has been shown to be useful in this respect (Nicholas *et al*, 1986; Gates, 1991).

Thick sections of cultured plant material can be stained with an appropriate fluorochrome, and cells can be viewed remarkably clearly with very little preparation (Gates, 1991). This could be a very useful tool when looking at rooting of explants, as meristematic areas can be identified long before roots are visible from the outside of the plantlet (Nicholas *et al*, 1986). A selection of available fluorochromes includes Calcofluor M2R for cellulose staining (Hughes & McCully, 1975), Auramine O (Considine & Knox, 1979) for the visualisation of lignin, cutin and endomembrane activity (Harris & Gates, 1984), Acridine Orange to reveal nuclei (Pearse, 1980), and 8-anilo-1-naphthalene sulphonic acid (ANS) as a general stain, fluorescing strongly in organic solvents, or when bound to hydrophobic sites (Stryer, 1968), or used as a vascular

tracer (Gates & Oparka, 1982). Staining of a section with two or more fluorochromes is possible. Sampling regimes could be worked out to determine the critical stages of root formation under different cultural conditions.

Not only rooting may be examined using this technique. Many of the problems of micropropagation could be investigated, such as the leaf anatomy during vitrification, the development of vascular tissue, cutinisation, and numerous other phenomena.

The technique is especially powerful when used in combination with fluorescent dye tracers, which can be used to follow water (Peterson *et al*, 1981) or chemical movements within the plant and between the plant and its environment.

1.1.4. Stress

Various potential sources of stress exist in culture, and they may be important factors limiting the successful micropropagation of intransigent species. Such stresses include:

1. Water/osmotic stress
2. Oxygen stress
3. Nutrient stress
4. Wounding at sub-culture
5. Media toxin accumulation

1.1.4.1. Water Stress

1.1.4.1.1. General

The first striking feature on the observation of a plant tissue culture vessel is often condensed water on the inside surfaces. Most plants are unlikely to meet these conditions in their natural habitat,

and many will be unadapted and unable to cope in this alien environment. Water-logging of plants outside culture has a number of effects; transitory wilting of leaves, drop in internode extension growth rate, excessive stem thickening, epinasty of petioles, and premature senescence and abscission of leaves, (Wareing & Phillips, 1981). All of these effects are observable *in vitro* (Jackson *et al*, 1991). This does not necessarily reflect a disturbed water balance, but may be due to hormonal imbalances produced by imperfect root function or ethylene accumulation in the culture vessel.

Water stress has also been shown to affect carbohydrate partitioning (Dancer *et al*, 1990). It has been argued that this is only a consequence of the inhibition of photosynthesis (Vassey & Sharkey, 1989), but increased sucrose and reduced starch in response to water stress has also been shown in non-photosynthetic tissue (Meyer & Boyer, 1981; Oparka & Wright, 1988; Dancer *et al*, 1990). This could be relevant in starch accumulating plants (such as Paeony) grown in culture, where starch may be involved with osmoregulatory responses.

The effect of media osmotic potential on plantlets is poorly understood. Full strength MS media has an initial osmotic potential of approximately -5 bars, whereas rooting media are frequently higher than this (eg Paeony rooting, -1.6 bar). It is not known how this varies through a sub-culture, or what consequences it has for the plantlets.

Various methods have been used in attempts to create a more normal transpiration stream and water balance in plants grown *in vitro*. Vanderschaeghe & Debergh (1987) looked at the bottom cooling of culture vessels. Reduced humidity in the culture vessel is achieved by means of water condensing on the cooled growth media from the warmer gaseous

phase. Kozai *et al* (1986) used foam plugs to increase gas exchange in and out of the culture vessel. Jackson *et al* (1991) used loosened lids and different sized containers. Wardle *et al* (1983) suspended bags of silica gel in the culture vessel and used an over-layer of lanolin to prevent media dehydration. Using cauliflower, they demonstrated that the reduced humidity caused an increase in leaf epicuticular wax and a reduction in stomatal aperture, resulting in a greater resistance to water loss. However, plantlet mortality due to dehydration in culture negated any advantages of using this system.

All of these authors suggested that some of the morphological abnormalities and problems with acclimation could be overcome using this kind of technique, but failed to produce any convincing results from weaning trials in confirmation. Indeed, each methodology may have specific problems associated with it. Increased necrosis is reported in *Sorghum* somatic embryos when condensation water covers the media (Wernicke *et al*, 1982), root:shoot ratio has been shown to be reduced by low media temperature in relation to atmospheric temperature (Larigauderie *et al*, 1991) and low air humidity (<96% Rh) has been reported to be detrimental to rooting in pear (*Pyrus communis*) shoots (Lane, 1982). One of the main difficulties encountered when trying to assess the role of humidity gradients by increasing water vapour diffusion, is the associated increase in ethylene diffusion. Ethylene levels in the culture vessel have been shown to be very important, although some species are considerably more tolerant than others (e.g. Jackson *et al*, 1987). Jackson *et al* (1991) suggested that ethylene accumulation was solely responsible for the anatomical abnormalities that occurred in tightly sealed potato cultures, which did not occur in loosely sealed containers.

Slavík (1974) found that saturated salt solutions are the best way of controlling air humidity in closed containers. This is an approach which could lessen some of the problems discussed above. In addition, a constant humidity is maintained by the "buffering" of excess salt crystals in a supersaturated solution. Slavík warns, however, that solutions must be regularly stirred for a constant humidity to be achieved.

1.1.4.1.2 Water Potential Measurements

Measurement of the water status of plants is a complicated and involved subject. Numerous techniques have been developed, each with its own set of advantages and disadvantages. There are basically three types of measurement: ① Compensation methods, ② Direct methods, and ③ Pressure chamber methods. A very good discussion of these can be found in Slavík (1974). Compensation methods will be used exclusively in this study, due to their relative simplicity and lack of requirement for complex equipment.

The water potential (Ψ_w) of tissues is the factor used to express overall water status. Ψ_w is made up of several components; the osmotic or solute potential (Ψ_s); the pressure potential or turgor (Ψ_P) and the matric potential (Ψ_M). Values of all these factors are less than zero or zero, the more negative value often erroneously being considered a "higher" pressure.

1.1.4.2. Oxygen Stress

The possibility of anaerobis in micropropagated plants is a subject of increasing concern. Jackson *et al*, (1991) found that oxygen levels in tightly sealed micropropagation vessels dropped from 20.5% to approximately 4% over 28 days, which is approaching the 3% often found

in flooded soil (Jackson, 1985). Even if oxygen levels in the gaseous envelope are high enough to support aerobic respiration, localised anaerobis seems likely within the plantlet at such sites as the plantlet base, where oxygen penetration is impeded by the static agar-based medium (Jackson *et al*, 1987). Barrett-Lennard & Dracup (1987) showed that semi-solid agar can inhibit root elongation by two thirds, in association with a demonstrable lack of oxygen. This was alleviated by "crumbling" the agar. The approximately 10^4 fold decrease in the diffusivity of oxygen in water compared to air at 20° (Jackson *et al*, 1987), could go some way to explaining this, especially if the agar is covered with a layer of condensed water.

When it is considered that the precise areas (shoot base and roots) most likely to be affected by anaerobis are those which are morphologically and physiologically very important to plantlets, it is surprising that so little attention has been paid to the potential problems.

Little is known about the effects of anaerobis on nutrition, water uptake and root initiation, but it is not difficult to imagine that it would be detrimental. Erdmann *et al* (1988) found that externally imposed oxygen shortage slowed the cell cycle in wheat root meristems. Specialised "gas transporting" tissue, or aerenchyma, consisting of loosely packed cells with large intercellular spaces, have been shown to form in response to root oxygen stress in a number of species (Justin & Armstrong, 1987). The tolerance of plant roots to low oxygen concentrations also depends on root diameter, root porosity and the respiratory activity of root cells (Armstrong & Beckett, 1985; Laan *et al*, 1991), with thin, highly porous roots with a low root respiration rate having a higher tolerance. Laan *et al* (1991) showed that

temperature was important in oxygen uptake in *Rumex* spp. subjected to oxygen stress. At temperatures equivalent to most growth rooms, diffusion pathways were critical, but at lower temperatures uptake was much more dependant on the activity of cytochrome oxidase.

1.1.4.3. Wounding at Sub-Culture

When explants are excised or multiplied, they are subject to severe physical damage under the scalpel blade. Some plants are likely to be less tolerant to this than others. Woody plants, for example, do not often reproduce by mechanical disruption, and so would not be expected to be very tolerant.

Plants respond in a number of ways to wounding. They increase the deposition of lignin, suberin, cutin and callose around the cut surface, which may greatly affect the uptake of metabolites (Tran Thanh Van, 1981), hormones and water. The deposition of polyphenols and oxidation products is a problem in the establishment of many plants *in vitro* (Mantell *et al*, 1985)

Wounding can also directly affect the rooting of cuttings, probably by the removal of mechanical resistance to root emergence (Lovell & White, 1986; section 1.1.3.2).

1.1.4.4. Media Toxin Accumulation

The substances released into the media in the wounding response (see above) are often very toxic to plants, and if allowed to remain in the media are certain to affect plant health. The inclusion of absorbents such as charcoal to absorb any secreted toxins, or antioxidants to render them harmless, can to some extent ameliorate this situation (Mantell *et al*, 1985). The regular replacement of media is often helpful, especially with newly excised explants, but does not seem

to be a commercially practical solution in the large scale multiplication of plantlets. The wounding response in established cultures is usually less severe, but can be a problem is some species (Jones, 1983).

Also to be taken into account are any excretory products of the plant such as protons pumped out in the uptake of ions from the media, or pH balance of the cytoplasm. Indeed, the pH of media can drop significantly over a subculture in some species, even when uncontaminated plants are used (C. Leifert *pers. com.*).

Another factor that could be important is the concentration of media constituents reaching toxic levels as the media dehydrates due to plantlet water uptake and water vapour loss from culture vessels.

1.1.4.5. Stress Metabolites

The accumulation of the imino acid proline has been associated with stress, and in particular water stress, (eg. Singh *et al*, 1973, Handa *et al*, 1986). Handa and his co-workers showed that proline accumulation in cultured plant cells is most clearly related to cell Ψ_s. However, attempts to use proline as a quantitive expression of drought tolerance have not been successful (Ahmad & Hellebust, 1988). The levels of proline accumulated have been shown to be insufficient to account for the increased tolerance of cultured plant cells exposed to the osmoticum polyethylene glycol, (Handa *et al*, 1983), but many other organic solutes such as glycinebetaine and sorbitol can also be accumulated in addition to, or in replacement of proline (Ahmad & Hellebust, 1988), and proline may be at least partly compartmentalised in the cytoplasm (Handa *et al*, 1983). Factors other than Ψ_s, such as drought avoidance and physiological mechanisms for reducing water loss may also account for

why proline accumulation does not correlate well with drought tolerance. Amberger-Ochsenbauer & Obendorfer (1988) showed that the choice of sample material within the plant is very important, with factors such as plant age, leaf age and leaf region giving large differences in proline content.

The mechanism for proline accumulation induction is still a mystery. Gottlieb & Bray (1991) showed that endogenous ABA levels rise along with proline in *Arabidopsis* subjected to drought, and that exogenous ABA increased proline accumulation. However, Chou *et al* (1991) showed that ABA was not the direct signal. LaRosa *et al* (1991) ruled out regulation at the level of proline's immediate precursor, and suggested that regulation might take place much earlier in proline biosynthesis.

1.2. Dormancy

One of the mechanisms whereby plants increase their chance of survival during periods of unfavourable conditions is to cease growth and protect vulnerable tissue. When this lack of growth is accompanied by an inability to grow, even if conditions become favourable, a plant is said to be "dormant". When the absence of growth is due entirely to current environmental conditions, it does not possess true dormancy, but is "quiescent", (this has also been called imposed or enforced dormancy).

Most work on the dormancy of vegetative plants (as opposed to seeds), has been concerned with woody plant bud dormancy. However, Villiers (1975) reported that young, undeveloped leaves remain appressed around the tip of the shoot during the period of dormancy in over-wintering non-woody plants.

Dormancy has been studied extensively in both seeds and adult woody plants, but no published research has been found concerning dormancy in culture. This creates some difficulties as it is possible that dormancy in plantlets has some characteristics from both seed and adult stages.

Although the details vary between seed, plant and species, a number of general observations can be made on the mechanisms of dormancy. It is always initiated and broken by specific environmental factors, and follows a strictly defined pattern between these two points. The initiation of dormancy is often brought about by light related factors, although in some cases is caused by stress. Loss of dormancy is usually temperature related (Berrie, 1984; Wareing & Phillips, 1981).

A reduction or increase in photoperiod is generally accepted to be the factor that initiates bud dormancy, and exit from dormancy is thought to be controlled by extended periods at chilling temperatures (0-10°C), (Noodén & Weber, 1978). The dormancy of some seeds (eg *Lactuca sativa*) can be broken by a short exposure to red light (Wareing & Phillips, 1981) , but this does not seem to be the case in buds.

The exogenous application of GA_3 has been shown to at least partially overcome dormancy in many seeds and woody plant buds (eg. Wareing & Phillips, 1981; Berrie, 1984; Armitage, 1987). In some cases, GA_3 may only be able to hasten germination or bud-break after the chilling requirement of dormancy has been met, but there are many authenticated reports of GA_3 effectively replacing any chilling requirement (Wareing & Phillips, 1981).

Endogenous levels of GA's have been shown to change during seed stratification and bud cold treatment, reaching a peak when germination becomes possible, and similar changes have been observed in cytokinin

levels, whereas endogenous ABA levels decline over stratification (Wareing & Phillips, 1981; Berrie, 1984). It is thought that the balance between GA_3 or cytokinins and ABA is responsible for the maintenance of dormancy.

The mechanisms of dormancy in temperate herbaceous perennials is poorly understood, but they exhibit similar behaviour to woody species (Berrie, 1984). Dormancy in Hostas grown in the field is exhibited in the form of foliar die-back in the Autumn months, leaving only the below ground plant parts. The apical meristem is enclosed in a "sheath" of dead leaves and the root system remains intact. Nothing has been found in the literature concerning dormancy in Hostas.

Some comparisons can be made between seed dormancy and that in micropropagated plantlets. Villiers (1975) reported that excised dormant seed embryos will germinate, but subsequent growth may be slow or abnormal. J. R. Nicholas (*pers. com.*) reported a similar effect in cold stored Hostas. Villiers also showed that the speed of germination of excised apple embryos increases with the length of cold store, rather than there being a "trigger-like" removal of dormancy.

The growth of Plum (*Prunus insititia*) plantlets after potting has been shown to be much improved by *in vitro* cold storage at 0°C for two months or post-transplant application of GA_3 (Howard & Oehl, 1981). This would seem to indicate that some form of dormancy was operating in culture. *Hosta* and other species also exhibit this phenomena (J. R. Nicholas *pers. com.*). It is not known if this "dormancy" *in vitro* is the same as *in vivo* dormancy. Indeed, it is not known if true dormancy even exists in culture, although it seems likely that it does, considering the wide variety of possible stress factors discussed earlier. The

mechanism for the inception of "dormancy" is difficult to understand in any other terms than stress in the supposedly constant conditions of culture. Dormancy may indeed be a relatively unconsidered factor in the failure of many plant species to micropropagate effectively.

1.3. Isoenzymes

1.3.1. Background

In 1895, Emil Fischer recognised that enzymes catalysing the same reaction might differ in other properties, and he advised that the species and organ or tissue of origin could be important, and should be stated when publishing results. The discovery that various coenzymes (eg. coenzyme A and NAD) have a uniform structure independent of origin, probably led to the mistaken conception that apoenzymes and enzymes also have common structures (Wieland and Pfleiderer 1962). This was not seriously questioned until 1943 when Warburg and Christian showed that aldose from yeast differed from that of animal tissues. In the wake of this discovery, many other workers found that enzyme properties differed between, and even within species. Differences were also observed between organs and tissues of the same individual. It is now thought that at least half of known enzymes exist in different forms within organisms (Rider and Taylor 1980). This revolutionised many areas of biology, and thinking in fields as diverse as metabolic regulation, genetics and evolution had to be drastically revised. It is now known that enzyme heterogeneity can occur even during the ontogeny of an organ or tissue (eg. Adams *et al*, 1981; Farnham *et al*, 1990). It is this aspect of enzyme heterogeneity that could prove to be most useful in this study, giving insights into the way environmental changes are reflected in the

metabolism of the plant, which in turn could produce biochemical markers for physiological and developmental changes of plants in culture.

1.3.2. Definitions

The term 'isoenzyme' (synonymous with the term 'isozyme') was coined by Markert and Møller (1959) to indicate enzymes with multiple forms, but the same substrates. They excluded enzymes with wide, and not always overlapping substrate specificity, such as esterase, alkaline phosphatase and peroxidase, which they considered to consist of separate, discrete types of enzymes. More recently, it has become possible to gather isoenzymes into different groups, according to the source of heterogeneity. However, this does not help much in making the distinction between closely related enzymes and disparate isoenzymes, which in any case may be arbitrary.

Markert (1968) proposed that isoenzymes be separated into allelic, nonallelic, homomultimeric, heteromultimeric, conformational, hybrid, conjugated etc... This assumes that enough is known about the enzyme, and it must be remembered that different isoenzymes of the same enzyme may be included in more than one of these categories.

Shaw (1969) classified isoenzymes into two broader groups: (a) isoenzymes with genetic causes, and (b) non-genetic isoenzymes. He identified the second group with the possibility of artefactual production, and ruled them out of studies of a species genetics. This does not necessarily apply here, as these may indeed be the most interesting isoenzymes, although care should be taken to rule out isoenzymes produced during experimental handling or storage.

The current definition recommended for an isoenzyme incudes only enzyme multiplicity due to genetic causes, (IUPAC-IUB, 1976). In

practise this is difficult to follow, as a full scale investigation in to each enzyme would be required. This would be very time consuming, so a better working definition would include all multiplicity not produced by experimental handling.

1.3.3. Separation and Identification

1.3.3.1. Electrophoresis

The study of isoenzymes was made possible by the development of a technique known as electrophoresis, and to some extent chromatography and later isoelectric focussing. These techniques separate crude enzyme extracts by virtue of physical properties (eg. molecular charge, size or shape).

The technique most commonly utilized to investigate enzyme heterogeneity was first used by Hunter & Markert (1957), and coupled the use of starch (and later polyacrylamide) gel electrophoresis with subsequent histochemical staining of the gel.

The medium proposed for electrophoretic separation of isoenzymes in this study is polyacrylamide, due to the good resolution attainable over a wide range of chemical and physical conditions with relatively short migration distances. Although many enzyme activity staining (EnActS) methodologies were formulated using starch gels, most can be quite easily adapted to polyacrylamide gels. One disadvantage is the high toxicity of acrylamide. Vertical slab gels will be used because of a need for the rapid comparison of large numbers of samples.

1.3.3.2. Enzyme Activity Staining (EnActS)

The technique of EnActS aims to produce a non-diffusible coloured precipitate at the site of enzyme activity on the gel. Newer

methodologies, however, sometimes do not require a precipitate, although the principle of low or negligible diffusion still applies (Vallejos, 1983). The pH of the medium is ideally the optimum of the enzyme being studied, but it is sometimes necessary to compromise with the optima of any "linking" reactions when the reaction product is not visible, or pre-incubate gels in a solution of substrate at the optimum enzyme pH.

1.3.4. Sources of Enzyme Heterogeneity

1.3.4.1. Genetic Causes

The most obvious source of isoenzyme generation is the occurrence of different alleles within a population coding for the same enzyme. This could be relatively easily explained by mutations between the parental and sibling loci in the course of evolution, producing a pool of slightly different, but functional isoenzymes. In monoploid plants, this would only allow for the expression of one (if homozygous) or two (if heterozygous) enzyme variants. This does not explain the occurrence of more than 15 forms of the same enzyme that have been found in the same individual (see Scandalios, 1974).

Most functional enzymes are formed from two or more sub-units, and this allows for much greater possible variation. These are called multimeric enzymes. An enzyme system where only one, genetically uniform sub-unit is involved is termed homomultimeric. When an enzyme consists of different allelic variants, it is called heteromultimeric. The resultant number of heteromultimeric isoenzymes will depend on the number of sub-units making up the completed enzyme. With random association of subunits a dimeric enzyme (two subunits) has three possibilities, a trimeric has four, a tetrameric has five etc. In cases such as these, where the genetic locus is active, both alleles will be

expressed, and the isoenzyme profile should remain constant throughout the organism, although the total activity will vary (Rider and Taylor 1980). If the situation were this simple, individuals homozygous for that gene would still only exhibit a single iso-form of the enzyme. It would also rule out any biochemical role for isoenzymes (as opposed to enzymes), and interest in them would be confined to genetics.

Another common genetic mechanism which is known to produce isoenzymes is gene duplication in conjuction with subsequent mutations at the two (or more) loci. Taken in conjuction with multiple sub-unit composition, this vastly increases the number of possibilities. For example, a trimeric enzyme with one locus has 4 possibilities, with two loci has 20 possibilities, and with three loci has 52 possibilities. A similar effect is seen if the plant is polyploid.

These different alleles, which may, or may not be expressed are in part responsible for isoenzyme profile variation between tissues, organs and developmental stages.

1.3.4.2. Non-Genetic causes

Multiple enzymes can also be a result of chemical and physical factors. Isoenzymes may arise post-translationally by the binding of varied numbers of co-enzyme molecules (Jacobson 1968), divalent metals, sialic acid, AMP etc., as well as the deletion or addition of groups of amino acids from the polypeptide chain (Scandalios, 1974). Differences in the tertiary or quaternary structures of the enzyme has also shown to be significant (Hotchkiss, 1964). These isoenzymes could be very sensitive to ontological and environmental changes, making them very useful in this study.

Enzymes such as peroxidase and LDH have been shown to produce isoenzyme variants during preparation and storage (Scandalios, 1974). This makes it vitally important to check that 'isoenzyme' bands on gels are not in fact artifacts.

1.3.5. Enzyme Properties and Functions

1.3.5.1. α Esterase (E.C. 3.1.1.2.)

This enzyme catalyses the hydrolysis of α-ester bonds in uncharged carboxylic esters, although Kahler & Allard (1970) reported that certain α esterases have some β-esterase activity. Esterases have acidic pH optima in plants and their action is generally limited to short chain fatty acids (Krisch, 1971).

There is some controversy about whether the esterase group of enzymes are actually isoenzymes or separate enzymes. These enzymes are frequently termed non-specific esterases, due to the wide substrate specificities often found.

Esterase is a ubiquitous enzyme in plants, and polymorphism has been observed in many species (e.g. Gates & Boulter, 1979 a,b, 1980; Tanksley & Rick, 1980; Haufer, 1985). Atkinson *et al* (1986) reported that at least one isoenzyme of esterase was monomeric in Cacao.

1.3.5.2. Aspartate Aminotransferase (E.C. 2.6.1.1.)

Aspartate aminotransferase (AAT, *syn.* Glutamate Oxaloacetate Transaminase) catalyses the transfer of nitrogen between glutamate and aspartate.

There are two distinct forms of AAT. One group of isoenzymes is thought to be localized in the mitochondria (m-AAT), and the other in the cytoplasm (c-AAT). They differ in physical and chemical properties

(Christen & Metzler, 1985). Gracia *et al* (1987) showed that they could be distinguished using their pH optima; total AAT activity being shown when incubated at pH 7.4, and mainly m-AAT being revealed at pH. 6.2.

Multiple forms have been demonstrated in many species (eg. Gottlieb, 1973; Farnham *et al*, 1990).

1.3.5.3. Polyphenol Oxidase (E.C 1.10.3.1)

Polyphenol oxidase (PPO, *syn.* catechol oxidase, phenolase, and o-diphenol oxygen oxidoreductase), catalyses two distinct reactions; the oxidation of o-diphenols to o-diquinones, and the o-hydroxylation of monophenols (Mayer and Harvel 1979). Although other enzymes oxidise phenols, PPO is specific for these reactions and in any case is the most prevalent enzyme of this sort in plants (Vaughn and Duke 1984). In most cases diphenol oxidase activity is much greater than monophenol hydroxylase activity.

Little is known about the function of PPO in higher plants, (Vaughn & Duke, 1984), although it has been studied for many years. Histochemical studies have indicated that nearly all the PPO in green tissues is localized in plastids, (Vaughn & Duke, 1984), although it does appear in the cytoplasm of degenerating or senescent tissues, (Flurkey & Jen, 1978). PPO has been isolated with thylakoid material, and Lax & Vaughn (1991) showed that PPO is at least peripherally associated with the photosystem II. Several roles have been suggested, such as mediator in photosynthetic electron transport and the Mehler reaction, and host defence mechanisms, but no conclusive evidence has been presented (Vaughn *et al*, 1988).

Boulillenne & Boulillenne-Walrand (1955) proposed that PPO located in the pericycle, phloem and cambium is involved with auxin in the

formation of a complex implicated with the promotion of root initiation. More recent work has tended to discount this hypothesis, but PPO is likely to be involved as phenols often show auxin-like activities (Mantell *et al*, 1985).

PPO does not seem to be activated until it crosses the plastid membrane, and most phenolic compounds are isolated from the enzyme in the vacuole (Vaughn & Duke, 1984). There are at least three forms of inactive PPO. These are, (1) a form synthesised in the cytoplasm and transported to the chloroplast, (2) PPO present in the chloroplast, and (3) active enzyme which has then become deactivated. All these forms are antigenically distinguishable, (Lanker *et al*, 1987). Enzyme latency can be removed in crude extracts by a variety of treatments, for example; pH change, fatty acids, detergents, proteases and SDS, (King & Flurkey, 1987). Little is known about any *in vivo* activation mechanism, apart from what can be concluded by these results.

1.3.5.4. <u>α Amylase</u>

The enzyme α amylase is involved with the hydrolysis of starch. Specifically, it catalyses the breakdown of α-(1,4) glucosidic bonds in linear and branched carbohydrates. The metabolic role of α amylase in plant growth and in particular its role in seed germination, has been the object of much study for many years. However, the physiological role of amylase in vegetative plant tissues is still poorly understood.

It is known that α amylase is associated with the mobilisation of stored starch in germinating cereal seeds, and that this is regulated by endogenous GA_3 levels (Callis & Ho, 1983; Al-Helal, 1985). GA_3 produced in the embryo diffuses to the aleurone layer of barley (*Hordeum vulgare*) seeds and induces the transcription of α amylase genes (Bernal-Lugo *et*

al, 1981). α-Amylase has also been shown to increase in association with starch mobilisation in germinated pea (*Pisum sativum*) cotyledons, but GA_3 had no effect on amylase production, although the presence of the embryo is still required (Sprent, 1968).

Parys *et al* (1983) suggested that end-product (here, endogenous reducing sugar levels) regulation of amylase in pea (*P. sativum*) might be important, but Gepstain & Ilan (1981) found this not to be the case in dwarf french bean (*Phaseolus vulgaris*).

Most amylase in vegetative tissues seems to be located away from the site of starch synthesis and degradation (Saeed & Duke, 1990a,b). 70-90% of amylase activity has been found to be extrachloroplastic in several species (Okita *et al*, 1979, Kak funda *et al*, 1986, and Robinson & Preiss, 1987). Recent research has shown that as much as 87% of total α amylase activity is located in the apoplast (Beers & Duke, 1990, Saeed & Duke, 1990a). No satisfactory explanation can be found for this, and no suitable substrates have been found at this location (Beers & Duke, 1990), although Saeed & Duke (1990b) suggest that it could be linked to plastid function and senescence.

The existence of multiple forms of the enzyme has been confirmed in the seeds of many plants, (e.g. pea (*P. sativum*): Yomo & Varner, 1973, Al-Helal (1985) Beers & Duke (1990); barley (*H. vulgare*): Callis & Ho, 1983, Jacobson *et al*, 1986; and soyabean (*Glycine max*): *Adams et al*, 1981).

1.3.6. Isoenzymes as Biochemical Markers

The analysis of isoenzymes in studies of gene expression during development has been practised since the mid-1970's. Scandalios (1974) suggested that isoenzymes could provide sensitive markers for metabolic

processes and differentiation. He also pointed out that a change in isoenzyme pattern does not necessarily reflect a change in gene transcription, but could be due to such things as translational and post-translation changes, or regulatory action at any number of control points. It is also possible for isoenzymes already present to be activated, as demonstrated by Lanker *et al*, (1987).

The expression of esterase isoenzymes has been shown to vary with light and temperature in *Dianthus* callus cultures (McCown *et al*, 1970), developmental state in leaves in *Xanthium* (Chen *et al*, 1970), senescing leaves of *Festuca pratensis* (Thomas & Bingham, 1977) and the nitrogen regime of *Vicia faba* (Gates & Boulter, 1979b).

One of two aspartate aminotransferase isoenzymes has been shown to be induced during rhizobial infection of legume roots in association with the onset of nitrogen fixation (Boland *et al*, 1982; Jones *et al*, 1990; Farnham *et al*, 1990). In pumpkin cotyledons, AAT expression has been shown to be linked to developmental state (Splittstoesser & Stewart, 1970).

Adams *et al* (1981) and Al-Helal (1985) showed, respectively in soyabean and pea, that starch hydrolysing isoenzyme patterns varied through seed germination and subsequent development. Leah & Mundy (1989) showed that the application of ABA (which inhibits germination and development) to germinating barley seeds, promotes the accumulation of a protein which inhibits germination-specific α amylase isoenzymes.

Peroxidase isoenzymes have been shown to be specific to organogenesis of cultured *Nicotiana tabacum* (Kay & Basile, 1987). Jain *et al* (1990) also found this to be the case in callus cultures of *Datura innoxia*.

Other isoenzyme systems known to change during seed development include many of the starch hydrolysing enzymes and leucine-aminopeptidase (LAP), (see Al-Helal, 1985; Collier & Murray, 1977). The enhanced expression of the gene for one isoform of α amylase was found when barley plants were exposed to water stress, (Jacobson *et al*, 1986).

1.4. Polypeptides as Biochemical Markers

The use of protein profiles in attempts to provide markers for various biological processes has recently become of great interest. Leshem & Sussex (1990) found that a group of polypeptides of molecular weight 20-25 kDa could be used to predict organogenisis in cultured melon (*Cucumis melo*) cotyledons. When these polypeptides were present for more than 3 days from the start of culture, roots were developed; when they were present for 3 days or less, shoots were regenerated. Stafstrom & Sussex (1988) used similar markers to delineate stages in axillary bud development in peas (*Pisum sativum*) , Villalobos *et al* (1984) predicted the time of bud regeneration in detached pine (*Pinus* spp.) cotyledons using the appearance of particular polypeptides. Rosenberg & Rinne (1989) were able to follow soybean (*Glycine max*) seed maturation, hydration, germination and seedling growth using protein profiles. Storage proteins have also been used as markers for somatic embryogenesis in *Trifolium* (McGee *et al*, 1989).

Hahn & Walbot (1989) showed that the cold treatment of expanding rice seedling leaves resulted in the synthesis of several polypeptides. Meza-Basso *et al* (1986) showed a similar phenomena in rape seed (*Brassica napus*) seedlings. Proteins specific to cold acclimation have also been observed (Guy *et al*, 1985).

1.5. Gene Expression and Plant Development

With the rapid growth of techniques for studying molecular genetics, attention is now beginning to be focussed at the gene level of morphological and physiological processes. Cold acclimation specific genes have been isolated in barley (Dunn *et al*, 1990), wheat (Sutka, 1989) and alfalfa (Mohapatra *et al*, 1989).

Skriver & Mundy (1990) give a good review of the growing number of genes that have been found to be induced by ABA and osmotic stress.

Hughes & Galau (1991) investigated the developmental and environmental induction of mRNAs in *Gossiypium hirsutum* embryo cultures, and found mRNA changes associated with the GA-stimulated embryo germination and GA-mediated delayed abscission.

Watillon *et al* (1991) found that the accumulation of two gene transcripts was specific to the presence or absence of cytokinin in the growth medium of micropropagated apple (*Malus domestica*). They suggested that these mRNAs may be associated with the physiological events associated with cytokinin induced shoot proliferation.

This kind of approach to identifying markers for specific physiological and morphological events is preferable to using the end products of gene activation or inactivation, such as proteins and isoenzymes, which are subject to modification after translation. However, post-translational changes may be important, and this kind of work is still very much in its infancy, so examination of isoenzyme and proteins is still an important tool for furthering understanding about the biochemistry behind physiological phenomena.

2. Materials and Methods

2.1. Micropropagation

2.1.1. Plant Material

Plant material was generously donated by Neoplants Ltd of Preston in the form of aseptically cultured mother stocks. These stocks were shown to be free from microbial contamination by Neoplants, using confidential techniques. The species and varieties supplied were as follows:

Hosta fortunei var. Francie

H. sieboldiana elegans var. Gold Edger

H. tardiana (*H. sieboldiana* x *H. tardiflora*) var. Blue wedgwood

Hosta hybrids (origins unknown): var. August Moon
var. Golden Prayers
var. Midas Touch

Paeonia lactiflora var. Bowl of Beauty

Choisya ternata var. Sundance

Daphne blagayana

D. odora

Photina x fraseri var. Birmingham

2.1.2. Tissue Sterilisation

In cases where new plant material was needed, or contaminated plantlets rescued, the following procedure was followed:

Meristematic nodes were excised from the shoots of the source plant, leaving some excess tissue around the meristems. They were then immersed in 70 % industrial methylated spirit (IMS) for 1 minute to aid the penetration of the sterilant. Rescued culture material was then immersed for 3 minutes in a 10 % solution of sodium hypochlorite, and

other material was immersed for 10 minutes. Four washes in sterile distilled water in sterile conditions were employed to remove any residual sterilant from the explants, which were then transferred to sterile culture.

2.1.3. Media Preparation

Two different media salt combinations were used: Murashige and Skoog (MS), minimal organics salts, (Murashige and Skoog, 1962) and Woody Plant Media (WPM) salts, (Lloyd and McCown, 1981). Both of these were obtained commercially in a ready to use form from Sigma.

Plant growth regulators used in the growth media were the auxins Indole Acetic Acid (IAA) and Indole Butyric Acid (IBA), the cytokinin 6, Benzylaminopurine (BAP), and the gibberellin Gibberellic Acid (GA_3). The auxins and cytokinins were dissolved in water and autoclaved with the rest of the media constituents. GA_3 was dissolved in methanol (1 mg/ml) and filter sterilised, as the high temperatures experienced during autoclaving affects the hormone (see Section 3.1.2.).

All media contained 1 % agar unless otherwise stated.

Media were autoclaved in 1 litre batches, and unless otherwise stated, poured aseptically in 50 ml aliquots into pre-sterilised plastic culture vessels, with 9 cm diameter and 4.5 cm height (supplied by Neoplants Ltd). The agar was allowed to solidify on a level surface, and the containers could then be stored for up to 1 month at 2°C before use.

Hosta multiplying media (HX)	Full strength MS salts
	1 mg/l BAP
	30 g/l Sucrose

Paeony multiplying media (PX)	Full strength MS salts 1.5 mg/l IAA 0.8 mg/l BAP 30 g/l Sucrose
Choisya multiplication media (CM)	Full strength MS salts 0.5 mg/l BAP 30 g/l Sucrose
Hosta rooting media (HR)	Half strength MS salts 15 g/l Sucrose
Paeony rooting media (PR)	Half strength WPM salts 0.5 mg/l IBA 20 g/l Sucrose 2 % Agar
Choisya rooting media (CR)	Half strength WPM salts 0.5 mg/l IBA 20 g/l Sucrose
Photinia rooting media (PhR)	Half strength WPM salts 1.0 mg/l NAA 20 g/l Sucrose

2.1.4. Plantlet Sub-Culturing

All work was carried out in a laminar flow cabinet, using equipment that was autoclaved and regularly flamed with ethanol. Plantlets were taken out of stock tubs and placed on a 14 cm sterile Petri-dish. Meristematic areas (apical and axiliary buds), were carefully excised and placed on growth media. This was done so that only a small amount of the plant tissue was in contact with the medium but enough to insure the plantlet remains in place. Tubs were then closed and placed in a growth room at 25°C, continuous light with a photon flux of 380-450 µEi $m^{-2}s^{-1}$ PAR until the next sub-culture (3-4 weeks). It was sometimes necessary to carry out rooting in the dark, and for much shorter periods of time (4-6 days), before transferring to the cold store or weaning facilities. In

these cases it was ensured that plantlets were larger in size when sub-cultured.

After the rooting, plantlets were transferred to a cold-room at 4°C with a low photon flux, (<40 μEi $m^{-2}s^{-1}$ PAR) for 2-5 months before weaning.

Plantlets were transferred into small plant pots containing perlite. Weaning was then carried out in a glasshouse mist-room at 25°C.

2.1.5. Maintenance of Mother Stock

A mother stock of all species and varieties was maintained by multiplying every 4 weeks on the appropriate media, and transferring half the multiplied plantlets to the cold room. This ensured that no accident or growth/cold room malfunction could kill all the samples of one variety. When plantlets were required for an experiment, a proportion of the mother stock was multiplied up separately until the required number had been achieved.

2.1.6. Scoring

A record of multiplication rates was kept to detect any problems with mother stocks or multiplication runs.

A number of non-destructive indices were used to assess plantlet health and vigour: Leaf number, tiller number, leaf colour, leaf type, and the presence/number of young leaves. Where it was possible to use destructive indices, multiplication rate, dry weight accumulation and leaf area were utilised.

Rooting was assessed non-destructively by scoring the number of rooted plantlets, number of roots per plantlet, and in some cases root length. Root type was also recorded, eg. auxiliary, storage, aerial, photosynthetic (green), and fibrous.

Scoring was carried out every 5-14 days depending on the time-scale of the experiment.

2.2. Humidity Control

2.2.1. Saturated Salt Solutions

The control of relative humidity inside culture vessels was attained by holding the growth medium and plantlets above saturated salt solutions (SSS). The data provided by Winston and Beres (1960), was used to determine which salts were used, and a summary of the relative humidity above a range of salts at 25°C are included in Appendix 1, (Winston and Beres' data ranges from 2-50°C).

To ensure salt saturation, distilled water was brought to the boil, and enough salt added to saturate at that temperature, (being careful not to over-saturate). After the solution had cooled slightly, a small amount of salt was added, and then the solution was allowed to cool to room temperature. At this stage an excess of salt was added, and the solution was allowed to equilibrate for about a week. Those solutions producing very low humidities, (below about 50% Rh.), were used as a sludge of liquid and crystalline salt.

The volume of container used did not exceed one litre, as a device would be required above this volume to keep the air in motion (Slavik, 1974). The surface area of salt solution was maximised, to aid diffusion of water vapour. Containers were then either hermetically sealed, or left with a small gap around the lid to allow some gas exchange.

2.2.2. Single Plantlet Humidity Control (SPHC)

Glass vials (20 ml) were glued with Araldite Rapid into the bases of glass jars (see figure 3a). After autoclaving, 200 ml of the

Fig. 3a Single Plantlet Humidity Control Apparatus

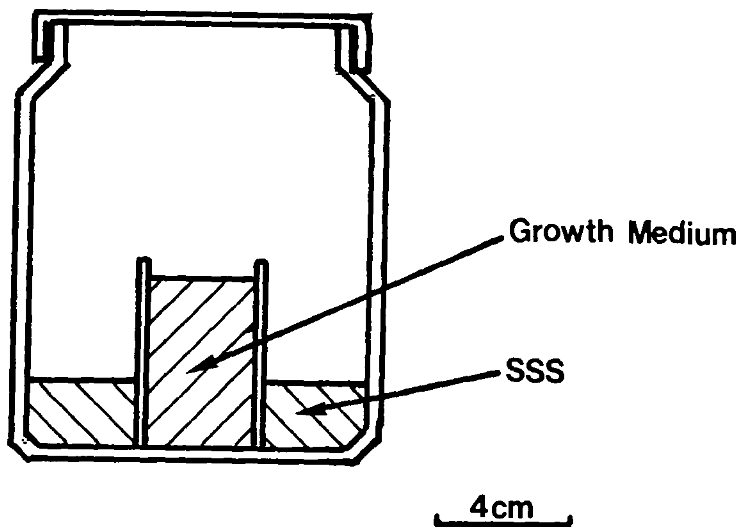
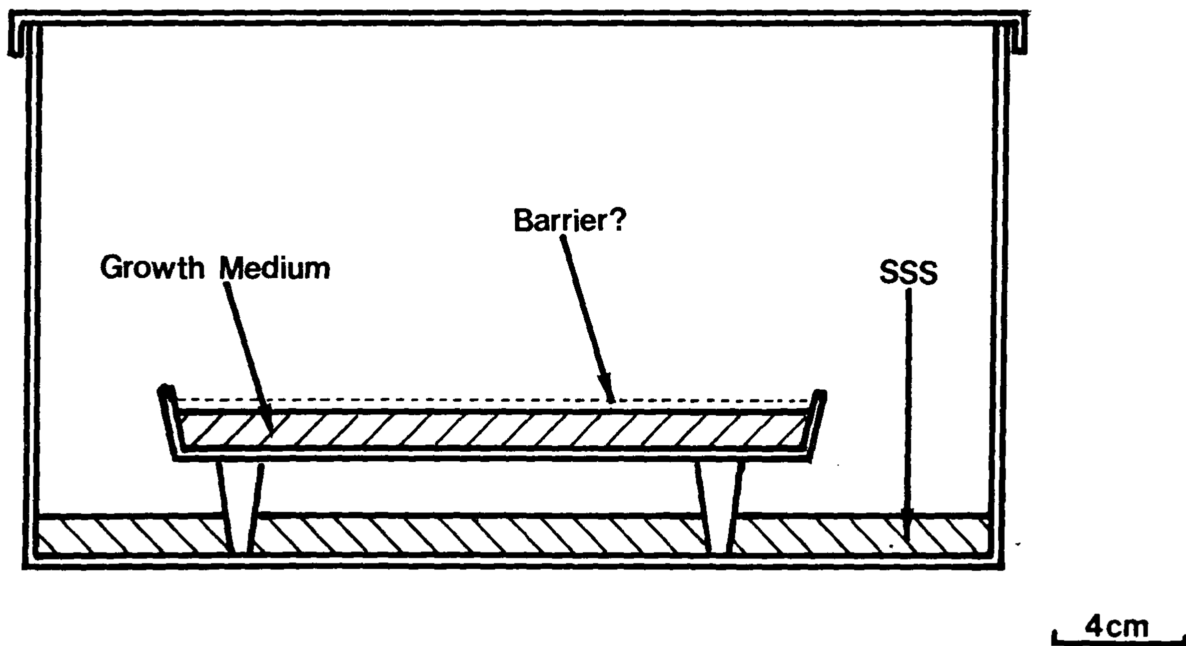


Fig. 3b Multiple Plantlet Humidity Control Apparatus



appropriate SSS was transferred aseptically into the outside compartment of the apparatus, making sure that crystals of salt are present to 'buffer' the solution. 20 ml of the growth medium was then pipetted into the glued glass vials. A single plantlet was then transferred aseptically on to the growth medium in each jar. The apparatus was swirled gently each week to stop a layer of unsaturated salt solution forming on the salt solution/air interface.

2.2.3. Multiple Plantlet Humidity Control (MPHC)

Culture humidity control was obtained for more than one plantlet in 14 cm diameter, 7 cm height evaporation dishes, containing a 9 cm glass petri-dish lifted up 2 cm from the base of the dish by glueing 4 small plastic tubes (modified Ependorfs) to the base, (see figure 3b). The apparatus was autoclaved with aluminium foil covering the top, which was replaced with the top of a disposable sterile 14 cm petri dish after 50 ml of growth medium had been poured in the glass petri dish, and 100 ml of the appropriate SSS poured into the bottom of the evaporation dish. Ten plantlets were then inserted and the apparatus sealed with Nescofilm. In some cases, small slits in the Nescofilm allowed gas exchange.

2.3. Water Potential Measurements

2.3.1. Smear Method

The 'smear' method of water potential determination (Shardakov, 1938; Slavik 1974) was employed. Intact plantlets were immersed in small volumes of a range of sucrose solutions, until one was found to be isotonic with the tissue. This was determined by adding a small amount of the dye bromophenol blue to each test solution after 2 hours immersion. Then, with a pasteur pipette, introducing a sample of this

solution into the middle of a test tube containing a sucrose solution identical to that in which the plantlet was first immersed; if the drop (visualised by the dye) does not rise or fall, the solution is judged to be isotonic. A table of water potentials of different sucrose solutions is provided in appendix 3. A correction factor of 1.54 was used to compensate for the 0.6-0.7 reflection coefficient of sucrose, which causes a reduction in the observed water potential (more negative).

2.3.2. Gravimetric Method: Gaseous Phase

This method was developed from Arcichovskij & Arcichovskaja (1931). Accurately weighed whole plantlets were placed in closed vessels, each of which had its humidity controlled by a solution of NaCl, (see appendix 2) The vessel consisted of a SPHC with the glass vial glued upside down, so that the tissue sample could be placed on top. The apparatus was then placed in an accurate water-bath, set at 20°C inside the cold room for 24 hours. Plantlets were then re-weighed. Compensations for respiratory weight loss were made by measuring the wet weight of a series of plantlets, and killing them immediately in boiling water, and then their dry weight was determined. The experimental samples had their dry weights measured after the experiment and a correction factor was calculated.

The ratio of final and initial weights were plotted against the water potentials of the series of relative humidities. The water potential of the plantlets was inferred from the point where the ratio was 1.0.

2.3.3. Gravimetric Method: Liquid Phase

Accurately weighed plantlets were immersed in 50 ml of serial NaCl solutions for 3 hours at 20°C (as above). Plantlets were then re-

weighed, and the percentage weight loss or gain plotted against the osmotic potential of the solutions (see appendix). The point where no weight loss was observed corresponded to the water potential of the sample. Serial solutions were equilibrated over-night at 20°C before use.

2.3.4. Pressure Chamber Method

A pressure chamber (see Slavik, 1974) was utilised to assess the water potential of micropropagated plantlets. The pressure was gradually increased until sap could be observed exuding from the cut stem. This pressure was taken to be equivalent of the plantlet water potential.

2.4. Proline Assay

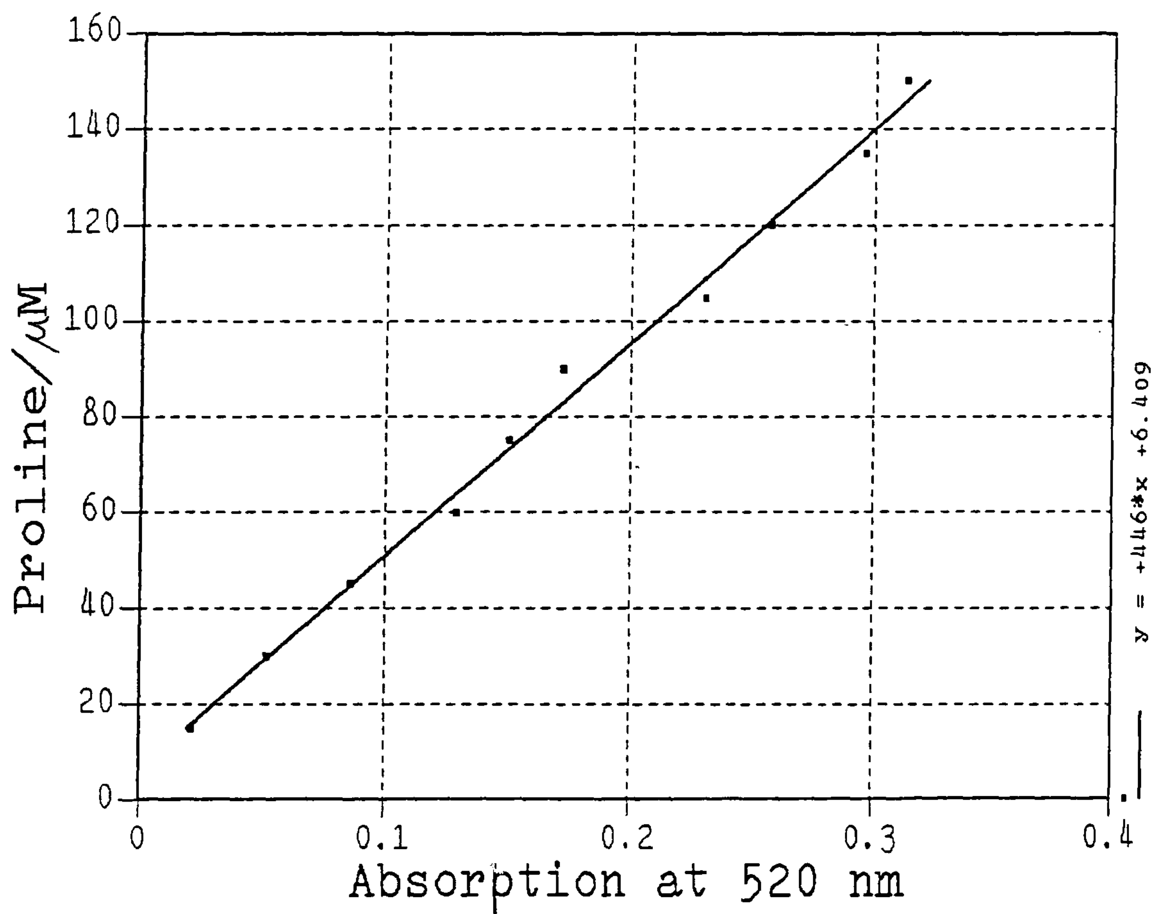
This assay was adapted from (Singh *et al* 1972).

An Acid Ninhydrin solution was made up:

1.25 g Ninhydrin
30 ml Glacial Acetic Acid
20 ml 6 M Phosphoric Acid
Warm to dissolve

This solution was used on the same day as it is stable for only 24 hours even if refrigerated. Approximately 0.5 g of accurately weighed plant material was homogenised in 10 ml of 3 % Sulphosalicylic acid, and centrifuged for 2 minutes at 13,000 revs/min. To 2 ml of supernatant, 2 ml Acid Ninhydrin and 2 ml Glacial Acetic Acid were added and the mixture was incubated at 80°C for one hour. After the mixture was cooled, 4 ml of Toluene was then added and the solution was mixed using a whirly mixer for 20 seconds. The red coloured toluene was decanted off the top of the sample, and its absorption at 520 nm measured, using distilled water incubated with toluene as a control. A calibration curve was constructed using $0-10^{-3}$ M L-proline (Figure 3c).

Figure 4. Proline Calibration



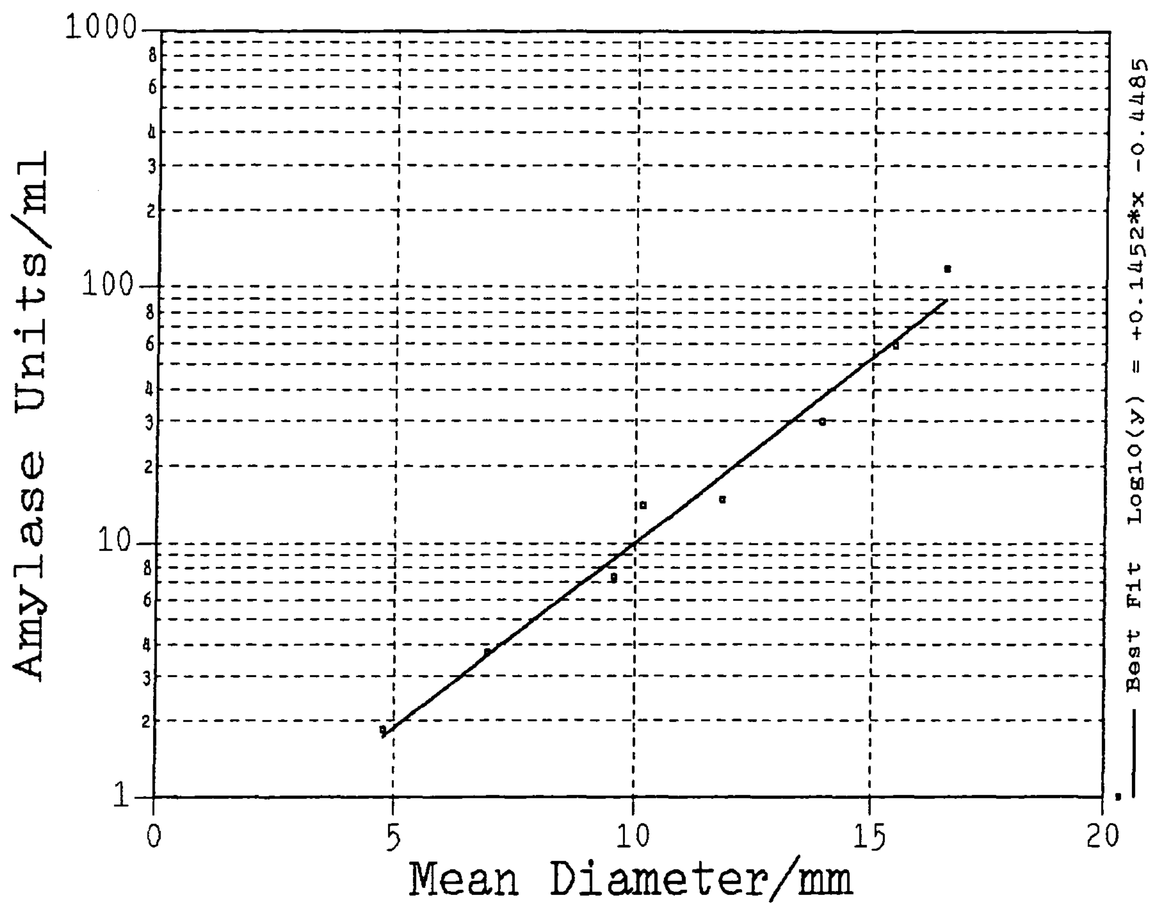
2.5. Amylase Assay

A semi-quantitative assay for amylase activity was adapted from Al-Helal (1985). The following solutions were made up:

Incubation medium	2.0 g Agarose 1.0 g Soluble starch 180 ml Distilled water Boil the mixture and as the starch and agarose dissolve, add 20 ml of 0.5 M Sodium acetate buffer, pH 6.9. Bring volume back up to 200 ml.
Staining solution	0.1 g I_2 1.0 g KI 100 ml of 0.1 M acetic acid. Stir the solution for 20 minutes in a fume cupboard.

15 ml aliquots of incubation medium were transferred using a warmed pipette into 9 cm plastic Petri dishes. These were then left on a level surface while the agarose set. They were then placed in a hermetically-sealed container with damp tissues to stop evaporation increasing the gel concentration, and allowed to equilibrate at 2°C for at least 48 hours. Eight 50 µl holes were punched in the gel, and then numbered on the bases of the Petri dishes. Crude plant extracts were prepared by grinding plant material in a cooled mortar and pestle for 5 minutes, with a ratio of 100 mg of plant tissue to 1 ml TRIS-Chloride buffer, pH 7.8. Extracts were then spun in a bench microcentrifuge at 13,000 revs/min for 4 mins. A 50 µl sample containing amylase was then pipetted into the holes and the enzyme allowed to diffuse into the gel for 24 hours at 25°C. At the end of this period, amylase activity was visualised by washing the staining solution over the gel. The diameter of the hydrolysed area was measured using a

Figure 5 Amylase Assay Calibration



compass and ruler. Each sample was replicated 5 times and a mean value calculated.

A new calibration was performed for each experiment using Amylase from the fungi *Aspergillus oryzae*, with an activity of 90 units/mg (supplied by Sigma). One unit being sufficient to hydrolyse 1 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C.

2.6. Polyacrylamide Gel Electrophoresis (PAGE)

2.6.1. Native Gels (N-PAGE)

The methods used are adapted from those used by Davis (1964). An anodic discontinuous buffer system was utilised to improve band separation.

2.6.1.1. Enzyme Extraction

All materials and equipment were cooled on ice before use to ensure minimum enzyme activity loss. Prepared samples were either frozen at -20°C for future use, or left on ice for use the same day. Care was necessary when freezing samples, as some enzymes de-nature quickly when frozen, and this is especially important if some isoenzymes become inactive more rapidly than others in the same sample. Immediate electrophoresis was performed where no tests had been carried out proving the efficacy of sub-zero storage.

2.6.1.1.1. α Esterase (EC 3.1.1.-)

The following solutions were made up:

Tris-Chloride buffer, pH 7.8	50 ml of 0.2 M TRIS 70 ml of 0.1 M HCl Adjust pH to 7.8 using pH meter

Esterase extraction media	1.9 g $CaCl_2$ 3.8 ml Triton X-100 0.95 ml 2-Mercaptoethanol 95 ml TRIS-Chloride Buffer, pH 7.8 Make up to 100 ml in a volumetric flask.

200 mg of plant material was ground with 2.0 ml of extraction media and a pinch of acid washed sand in a mortar and pestle for 5 minutes. The resultant green paste was transferred to two 2 ml Ependorf tubes and spun in a bench microcentrifuge at 13,000 revs/min for four minutes. The supernatants were then transferred into clean Ependorfs with a Pasteur pipette and stored on ice.

2.6.1.1.2. Aspartate Aminotransferase (EC 2.6.1.1)

TRIS-Chloride Buffer, pH 7.8 (see Section 2.6.1.1.1.) was used as an extraction media. The procedure used was the same as for α Esterase.

2.6.1.1.3. Polyphenol Oxidase (EC 1.10.3.1)

Extraction 1

After Thomas *et al* (1978)

Extraction media	10 ml of 0.2 M Potassium buffer, pH 7.0 1.0 g Polyvinylpyrrolidone (PVP-40 10 ml of 0.1 M Ascorbic acid 1.0 g Triton X-100 Adjust to pH 7.0 and make up to 100 ml in volumetric flask.

1 g of plant material was homogenised in 2 ml of extraction buffer, in a cooled pestle and mortar for 10 mins.

This crude extract was then centrifuged for 5 mins. at 13,000 revs/min in a bench centrifuge, and the supernatant was kept on ice. The pellet was then re-suspended in 1 ml of extraction buffer for 5 mins, and centrifuged again. 1.6 volumes of cooled acetone was added to 1 volume of the pooled supernatants, and the precipitate was centrifuged down. The pellet was then suspended in a small volume of buffer. This was then re-precipitated with 1.6 volumes of acetone and centrifuged. The pellet was then homogenised in a small volume of buffer, and centrifuged. The clear brownish supernatant was the extract.

Extraction 2

After Martinez-Cayuela *et al* (1988) with *Cherimoya sp.* The following solutions were made up:

Extraction medium 1	250 ml of 0.1 M Sodium phosphate buffer, pH 6.5. 0.25 g Cysteine.
Extraction medium 2	125 ml of 0.1 M Sodium phosphate buffer, pH 6.5. 125 ml Distilled water
Extraction medium 3	62.5 ml of 0.1 M Sodium phos-phate buffer, pH 6.5. 187.5 ml Distilled water

20 g plant material was homogenised in a Sorval Omnimixer for 5 mins at high speed (position 8), with 200 ml of cold acetone (-20°C). This was then passed through filter paper. The insoluble material was successively washed with 200 ml of cold acetone (-20°C) and 200 ml of cold diethyl ether (-20°C). The residue was dried and the powder was re-suspended in 250 ml of Extraction medium 1, homogenised again at medium speed for 5 mins. This was

then centrifuged for 15 mins at 10,000 X g. The supernatant was fractionated with ammonium sulphate and the 40-75% fraction was re-suspended in 9-15 ml of Extraction medium 2. This was then dialysed for 24 hrs at 4°C against Extraction medium 3.

Extraction 3

After Flurkey (1986), SDS and freezing in liquid nitrogen was employed to activate any latent enzymes. 0.5 g of tissue was frozen in liquid nitrogen and then homogenised in a pestle and mortar for 3 minutes, with 1 ml of 0.25 M Tris/0.2% SDS Buffer, pH 6.8 (see Section 2.6.2.2.), and a pinch of sand. This was centrifuged for 3 mins. @ 13000 revs/min. in bench microcentrifuge. The supernatant was the extract and could be stored on ice for use on same day, or frozen in liquid nitrogen and kept at -20°C for long term storage.

2.6.1.1.4. α-Amylase (EC 3.2.1.1.)

TRIS-Chloride Buffer, pH 7.8 (see Section 2.6.1.1.1.) was used as an extraction media. The same procedure as α-Esterase was used.

2.6.1.2. Gel Preparation

The gel constituents were developed from Davis, (1964), and LKB 2001 gel moulding apparatus was used, following instructions from the manual. A stacking gel of 4.4% and a resolving gel 10% acrylamide concentration were used in all cases. A 20 % sucrose solution, was added to all samples in a ratio of 1:5, and then mixed thoroughly. 100 μl of sample was added to each small well, or 150 μl to each large well. This was achieved with a 200 μl Gilson pipette with capillary tips. Different, clean tips were used for the loading of each sample.

The modification suggested by Lanker *et al* (1987) of adding 0.01 % SDS to the gel was used in an attempt to activate latent polyphenol oxidase isoenzymes (see Section 1.4.5.3.).

2.6.1.3. Gel Running

A 10 mA constant current per gel was applied for 15 minutes, and then 30 mA per gel for the remaining running time. The initial low current setting allowed isotachophoretic stacking in the sample.

The power was switched off when the bromophenol blue tracking dye reached the bottom of the gel. Running time was usually 3-3½ hours. Gels were then immediately removed from the equipment. The plastic spacers were eased out from between the glass plates using a soft plastic implement. The plates were then levered apart and the gel dropped into the appropriate staining solution (prepared during running time, see below). It is important to take a small slice out of one corner of the gel with a razor blade, to enable the different sides of the gel to be distinguished after it has been dropped into the staining solution.

2.6.1.4. Gel Staining

If more than one stain was used for a single gel, the gel was cut carefully with a razor blade, with each strip or running track being exposed to different staining regimes. This was especially useful when evaluating and adjusting different methods. Controls were always run when using a new stain or new plant material to detect any artefactual bands. This was normally achieved by following the same method, but excluding the substrate(s) of the enzyme. As an additional control enzymes were denatured before electrophoresis by boiling for 5 minutes.

2.6.1.4.1. α Esterase (EC 3.1.1.-)

Method 1

This method was adapted from Rudolph and Stahman (1966). Gels were incubated in the following solution for 2-3 hours at room temperature, or 30 minutes at 37°C.

Staining solution	100 ml Tris-chloride buffer, pH 7.4 (see below) 2 ml of 1 % α naphthyl acetic acid in acetone, (may need heating to dissolve). 25 mg Fast Blue RR.

The solution was made up as freshly as possible, as it rapidly becomes ineffectual with exposure to light.

Method 2

The following solutions were made up:

0.2 M Sodium Acetate buffer, pH 5

Solution A	12.0 g HAc. Make up to 1000 ml with distilled water
Solution B	27.2 g $C_2H_3O_2Na \cdot 3H_2O$ Make up to 1000 ml with distilled water

296 ml of solution A was mixed with 704 ml of solution B, and the pH of the mixture was adjusted to pH 5.0

Staining solution	100 ml of Sodium Acetate buffer, pH 5.0 2 ml of 1 % α naphthyl acetic acid in acetone, (may need heating to dissolve).

300 mg Fast Garnet GBG
200 mg Fast Blue RR

The solution was made up as rapidly as possible, as it rapidly becomes ineffectual.

2.6.1.4.2. Aspartate Aminotransferase (EC 2.6.1.1.)

Method 1

This method was adapted from Decker and Rau (1963). The following solutions were made up:

Solution (A)	100 ml of 0.1 M Tris, pH 8.5 100 mg α-Ketoglutarate 200 mg Aspartic acid
Reagents (B)	10 mg Pyroxidal-5-Phosphate 150 mg Fast Blue BB

Solution (A) was mixed at least 15 minutes in advance as the acids are not readily soluble. Solution (A) was then poured into a flask containing the reagents (B) just before use. The solution should be bright yellow. Then the gel was then placed in the mixture and incubated in the dark for 2-4 hours, or until blue bands appear.

Method 2

Gels were incubated gel at room temperature for 15 mins in the following solution:

Incubation solution	100 ml of 0.1 M Tris-chloride buffer, pH 8.0 200 mg L-Aspartic acid 100 mg α Keto-glutaric acid 5.0 mg Pyroxidal-5-Phosphate Bring pH back to 8.0 using a concentrated solution of NaOH.

The gels were then washed and stained for 15 minutes in the following solution:

Staining solution	100 ml of 0.5 M NaOH-citric buffer, pH 3.5 300 mg Fast Garnet GBG Prepare just before use and ensure the Fast Garnet is fully dissolved before immersing the gel.

Method 3

The appropriate amounts of substrates for detecting mitochondrial (mAAT), or mitochondrial and cytoplasmic (mAAT+cAAT) aspartate aminotransferase were used according to the following table.

Table 1. Substrate Concentrations

	L-ASPARTIC ACID	KETO-GLUTARIC ACID	pH
mAAT	10mM (133.1mg/100ml)	10mM (146.1mg/100ml)	6.2
mAAT+cAAT	20mM (266.2mg/100ml)	4mM (58.4mg/100ml)	7.4

The substrates were added to 100 ml of the appropriate buffer (see below), and stirred vigorously for 15 minutes. As the substrates are strongly acidic, the pH of the staining solution was brought back to either pH 6.2 or 7.4 with 1 M NaOH as specified.

Table 2. Buffers

	0.2M Na_2HPO_4	0.1M Citric Acid
pH 6.2	66.1ml	33.9ml
pH 7.4	181.7ml	18.3ml

In a dark room, 10mg of Pyroxidal-5-Phoshate (co-enzyme), and 200mg of Fast Blue B Salt were added. The stain was used immediately in the dark room.

2.6.1.4.3. <u>Polyphenol Oxidase (EC 1.10.3.1)</u>

<u>Double Paper Print Technique</u>

Two pieces of filter paper (Whatman 3MM), just larger than the gel, were cut, soaked in 0.1 M potassium phosphate buffer pH 7.0, and allowed to dry. The filter paper for monophenolase staining was then soaked in a 1% solution of tyramine-HCl in methanol, and the diphenolase filter paper in a 1% solution of D-catechin as recommended by Thomas *et al* (1978). The filter papers were allowed to dry before placing the gel in a "sandwich" between them. They were left in contact for 1-5 mins. and then incubated at 40°C for a further 5-10 mins.

<u>Direct Staining Technique</u>

This method was adapted from Lanker *et al* (1987), to detect the presence of any latent enzyme in the samples. Gels were pre-incubated in 1 % Acetic acid for five minutes. They were then transferred to the following staining solution:

Staining solution	200 ml Potassium phosphate buffer, pH 7.0
	78.9 mg L-DOPA (2mM)
	5.8 mg (+) Catechin (0.1 mM)
	3.5 mg Tyramine-HCl (0.1 mM)
	20 mg SDS (0.01% w/v)
	200 µl Catalase

If monophenolases only were required, catechin and 3,3-Dihydroxy-L-phenylalanine (L-Dopa) were excluded from the staining solution. If only diphenolases were required, tyramine and L-Dopa were excluded.

2.6.1.4.4. α Amylase (EC 3.2.1.1)

The method was adapted from Brewbaker *et al* (1968). The gels were incubated for 2 hours at room temperature in 150 ml of freshly prepared 1% soluble starch solution.

1% starch solution	150 ml of 0.2 M Sodium Acetate buffer, pH 5.0 (see above). 1.5 g Soluble starch Add starch slowly to near boiling buffer, whilst stirring continuously. Allow to cool and bring back to 150 ml using buffer.

Gels were then washed twice in 0.1 M acetic acid and stained for 20 minutes in the following solution:

Staining solution	100 ml of 0.1 M Acetic Acid 0.1 g KI 20 mg I_2 Stir solution with magnetic stirrer in a fume cupboard for 15 minutes.

If gels lost colour during storage, an exposure to the staining solution would visualise their bands.

2.6.1.5. Gel Shrinking

All gels were immersed for 48 hours in a 50 % solution of methanol in a hermetically sealed container. This concentrated the isoenzyme bands, and allowed them to be identified more easily. It also has the advantage of making the gels more robust and easier to handle. Measurements of the distance between the origin and tracking dye (bromophenol blue) were always taken prior to gel shrinking. Gels can be

stored in this solution until gel drying, provided that the 50 % methanol solution is replaced regularly.

2.6.1.6. Gel Viewing and Recording

Shrunk gels were laid on pieces of transparent plastic sheet, and then placed on a light-box. Gels were then photographed using a single lens reflex camera, tripod on a light-box with 100 ASA black and white film. Films were processed according to the manufacturers instructions.

Gels were then scanned using a laser gel scanner, which allowed the identification of very faint bands.

2.6.1.7. Gel Drying

It was important to photograph before gel drying as this can be a difficult procedure, and gels may be damaged or destroyed.

Two pieces of gas permeable cellophane were cut to size and immersed in 50 % methanol for 5 minutes. The gel was then sandwiched between the cellophane and transferred to a Pharmacia Vacuum Gel Drier. The gel drier was then sealed and the vacuum applied. A glass plate and lead weights were placed over the gel drier to ensure an air-tight seal.

The gel was then left for 24 hours, but longer periods were required for gels with a high acrylamide content, or if the vacuum applied was less than 700 mm Hg.

2.6.2. SDS Polyacrylamide Gel Electrophoresis

The methods used were adapted from Laemmli (1979), and utilise a discontinuous buffer system to improve band resolution.

2.6.2.1. Protein Extraction

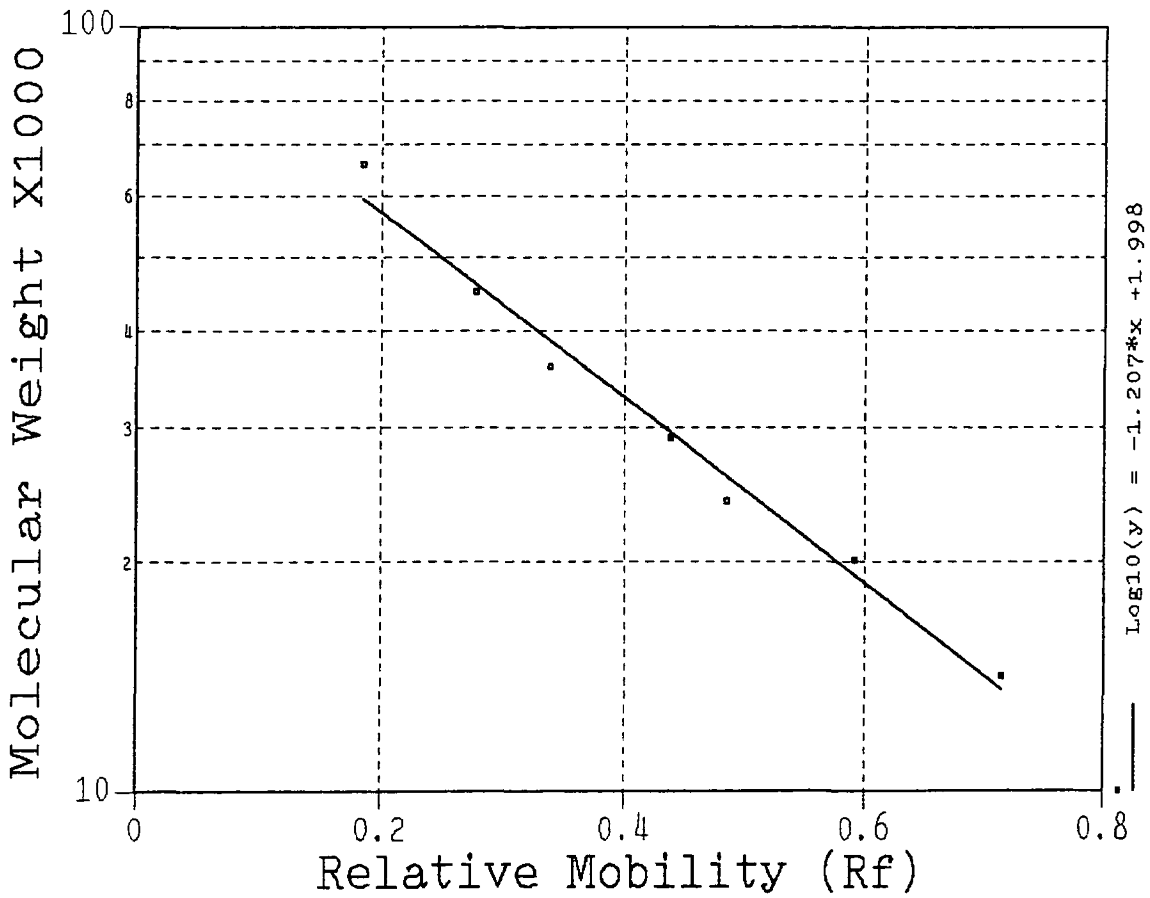
The following solutions were made up:

Sample buffer	25 ml 0.25 M Tris-SDS buffer, pH 6.8 2 g Sodium dodecyl sulphate (SDS) 10 ml Glycerol 5 ml 2-Mercaptoethanol 0.1 ml of 1 % Bromophenol blue Make up to 100 ml in a volumetric flask, working in a fume cupboard. Store in a tightly sealed bottle.
Dialysing solution	250 ml of 0.01 M Sodium phosphate buffer, pH 7.4 (see above) 0.25 g SDS (0.1 %)

Tissue samples of 500 mg were ground up with 5 ml of sample buffer and a pinch of acid-washed sand in a mortar and pestle for 5 minutes. The resulting slurry was transferred into 2 ml Ependorf tubes and spun in a bench microcentrifuge at 13,000 revs/min for four minutes. The supernatants were decanted into Ependorfs with no lid. Visking tubing was then secured over the top of the Ependorfs with elastic bands, ensuring that a water-tight seal was made. The samples were then dialysed against the dialysing solution for 24 hours at room temperature with a slowly rotating magnetic flea. Samples were then transferred to labelled 2 ml Ependorfs and stored at -20°C until use.

Relative molecular mass (RMM) marker sets (Sigma SDS-7) were prepared alongside the samples. See Figure 4 for marker details.

Figure 6. Example of a Calibration of RMM Markers for 10% Gel*



* Note that each gel requires a separate calibration

2.6.2.2. Gel Preparation

The gel constituents were developed from Laemmli (1979), and LKB 2001 gel moulding apparatus was used, following the instructions from the manual. 3% stacking and 10% resolving gel acrylamide concentrations were used

2.6.2.3. Gel Running

Gels were initially set running at 10 mA/gel constant current for the first 15 minutes, and the changed to 40 mA/gel for the rest of the running time, which usually lasted between 3½ and 4 hours.

Relative molecular weight markers (Sigma: SDS-7) were always run alongside samples, so that the molecular weight could be estimated, and individual proteins could be compared from different gels.

2.6.2.4. Gel Staining

Gels were stained for total protein content according to Reisner *et al* (1975). The following solutions were made up:

Staining solution	25 mg Coomassie brilliant blue G250 perchloric acid solution 50 ml Methanol 7 ml Acetic acid Make up to 100 ml with distilled water in a volumetric flask
De-staining solution	25 ml Methanol 7 ml Acetic acid Make up to 100 ml with distilled water in a volumetric flask.

Gels were left over-night in the staining solution at room temperature, then de-stained for 12 hours, with regular replacements of the de-staining solution. Gels could be stored temporally in the de-

staining solution until photographing and gel drying (see Section 2.6.1.6-7).

2.7. Fluorescence Microscopy

2.7.1. Sectioning

The material for sectioning was removed aseptically from the micropropagation tubs, or from mature plants immediately prior to use. Plantlets were sectioned with a single, steady stroke of a new, double edged razor blade. In each sample, 3 plantlets were sectioned in the horizontal plane, and 3 in the vertical.

2.7.2. Staining

Sections were stained in either 0.01% w/v Calcofluor M2R (Hughes and McCully, 1975) for 1 minute to visualise cellulose, or in 0.001% w/v Auramine O (Considine and Knox, 1979) for 5 minutes to detect lignin, cutin and endomembrane activity (Harris and Gates, 1984). Alternatively, sections were stained sequentially in both fluorochromes (double stain), with thorough rinsing with distilled water between stains to prevent any precipitation. Stained sections were then mounted in a dilute iodine solution (0.001% w/v).

Sections were also stained and mounted in a 0.001% w/v 8-anilino-1-naphthalene sulphonic acid (ANS) solution as a general stain (Gates and Oparka, 1982), as it fluoresces strongly in organic solvents or when bound to hydrophobic sites on proteins (Weber and Laurence, 1954; Stryer, 1968).

Acridine orange was also used in a 0.00001% w/v solution for 3 minutes, to visualise the cell nuclei in samples.

2.7.3. Viewing and Recording

Samples were examined using a Nikon Diaphot inverted microscope, equipped with an epi-fluorescence attachment. An ultraviolet excitation filter was used for calcoflor stained material, giving a blue fluorescence from cellulose cell walls, a blue excitation filter was used to induce yellow fluorescence in Auramine stained material. With double stained samples, a blue-violet or violet filter could be used to excite the sample. A violet filter was used with ANS, and a blue filter for Acridine Orange. Sections were then photographed with either a front or side mounted camera, using a variety of film types, which were processed according to the manufacturers instructions.

2.8. Statistical Methods

Standard errors (S.E.) were calculated from the standard deviation (S.D.) of samples using the following formula:

$$S.E. = S.D./ \text{ Sample size}$$

95% confidence levels were calculated simply by doubling the S.E., since 95% of samples will fall within two S.E.'s of the mean if the distribution is Normal. 99% confidence levels were calculated by multiplying the S.E. by three, using similar logic.

3. Experimental Methods and Results

3.1. *Paeonia lactiflora* Micropropagation

3.1.1. Anatomical Survey of *P. lactiflora*

Materials and Methods

A wide range of material was sectioned and stained using different fluorochromes under various settings of the fluorescence microscope. Anatomical observations were made of plantlets from both multiplication and rooting media at different stages throughout sub-culture, and post weaning. Particular attention was focussed on root anatomy, the plant/media interface, vascular tissue, and root and shoot initials.

Results

Examples of photographs taken during this survey have been included, (Plates 1-5).

Plate 1: Bud Formation in Multiplying Cultures of *Paeonia lactiflora*

A. Longitudinal section through the base of a plantlet after 3 days on multiplying medium, showing the first few divisions of a shoot meristem (m), accumulation of starch (s) in surrounding tissues and the base of a leaf petiole (p). Also note the thickened walls (t) of cells to the right of the meristem and the intercellular spaces (is) at the bottom right of the picture. The section was double stained with Auramine and calcofluor and viewed using a BV filter. Scale bar = 0.04 mm

B. As A., but transverse section and after 10 days on medium. Also note the vascular connection (v) to the shoot meristem (m). Scale bar = 0.04 mm

C. As A., but after 14 days on medium. Scale bar = 0.10 mm

D. As A., but after 21 days on medium. Also note the vascular connection (v) to the shoot meristem (m) and the apparent absence of a cuticle. Scale bar = 0.10 mm

Planes of section through plantlets

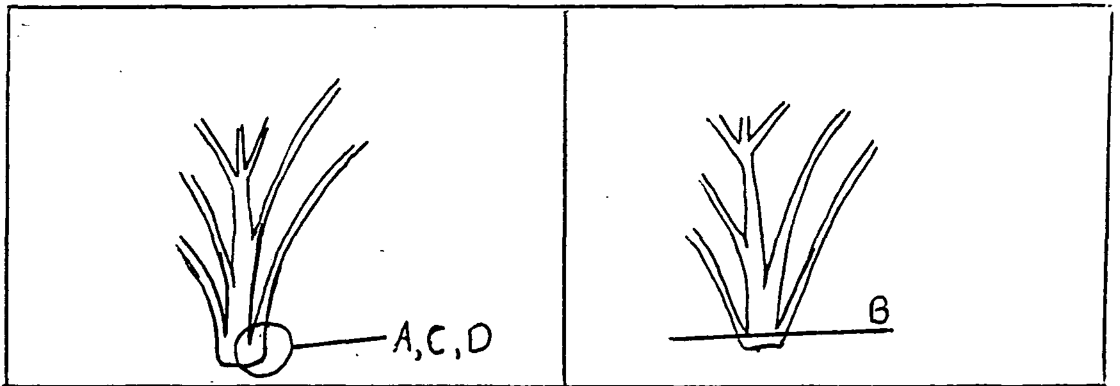


Plate 1: Bud Formation in Multiplying Cultures of *Paeony lactiflora*

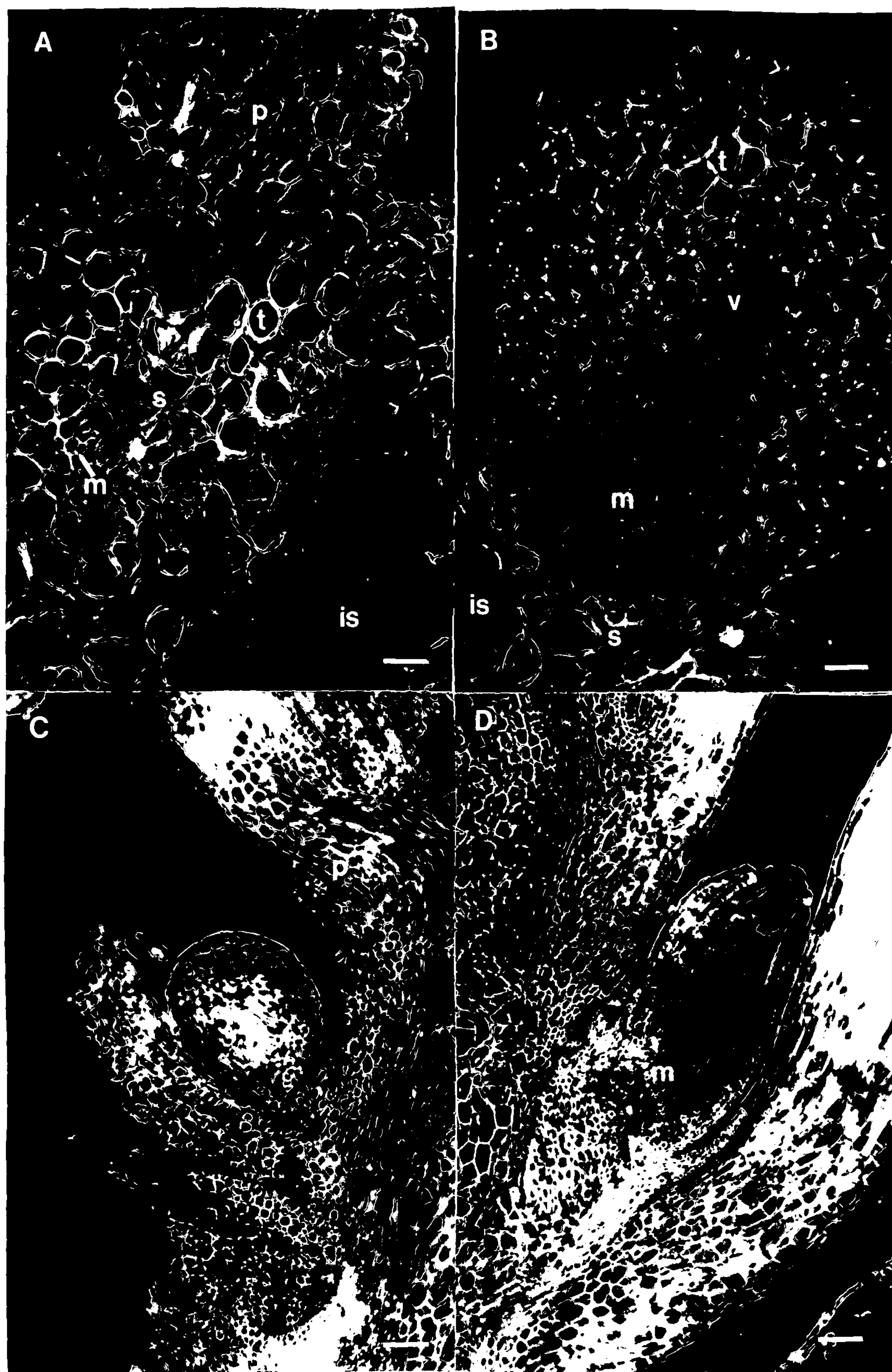


Plate 2: *Paeonia lactiflora* on PX Medium

A. Transverse section of plantlet after 7 days on medium, showing starch accumulation (s) in cells in the parenchyma cells outside the vascularised area. Also note the disorganised xylem (x) development in different planes. The section was double stained with Auramine and calcofluor and viewed using a BV filter. Scale bar = 0.1 mm

B. Transverse section near the base of plantlet after 7 days on medium, showing starch accumulation (s) in cells near the growth medium, and shoot meristems (m). The section was double stained with Auramine and calcofluor and viewed using a BV filter. Scale bar = 0.1 mm

C. Longitudinal section of plantlet, showing secondary thickening in cell walls (w) close to the medium (M). Stained with Auramine and viewed using the B filter. Scale bar = 0.02 mm

Planes of section through plantlets

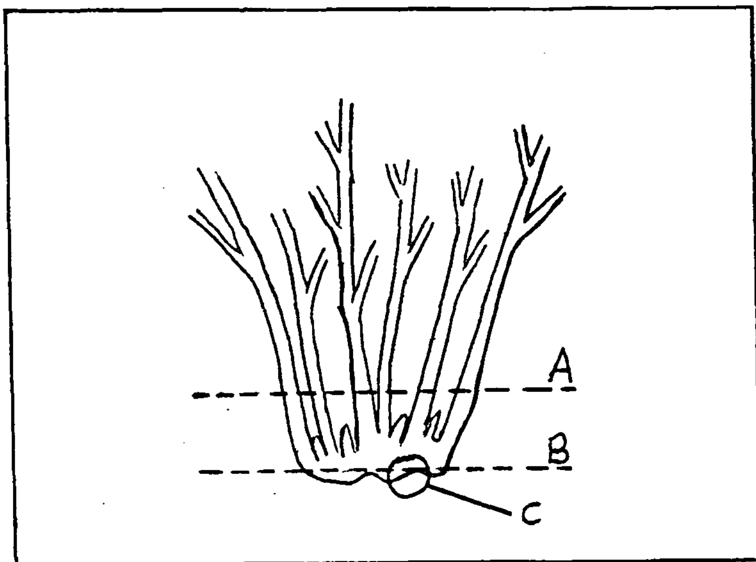


Plate 2: *Paeony lactiflora* on PX Medium

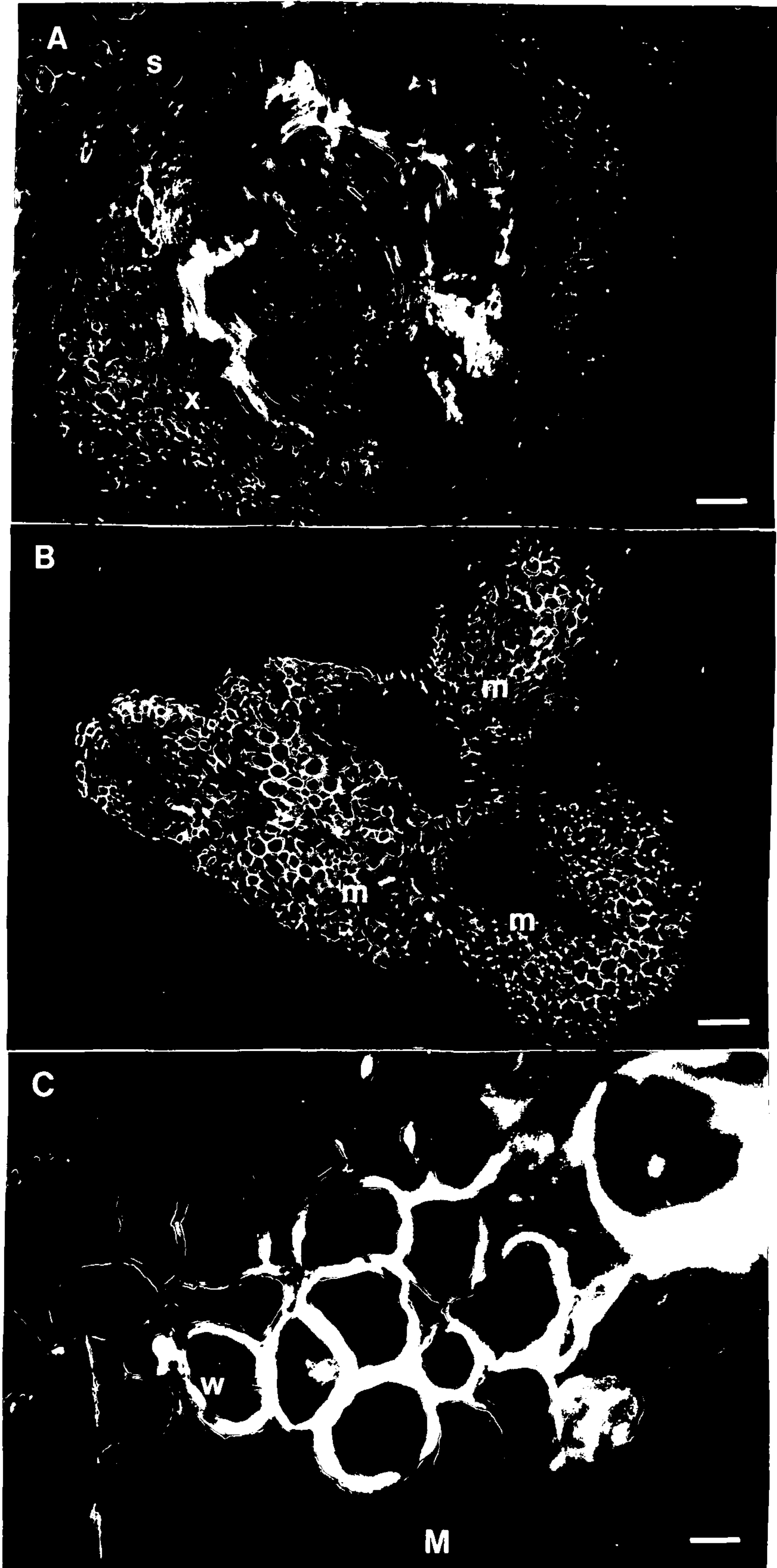


Plate 3: Root Primordia in *Paeonia lactiflora* on PR Medium

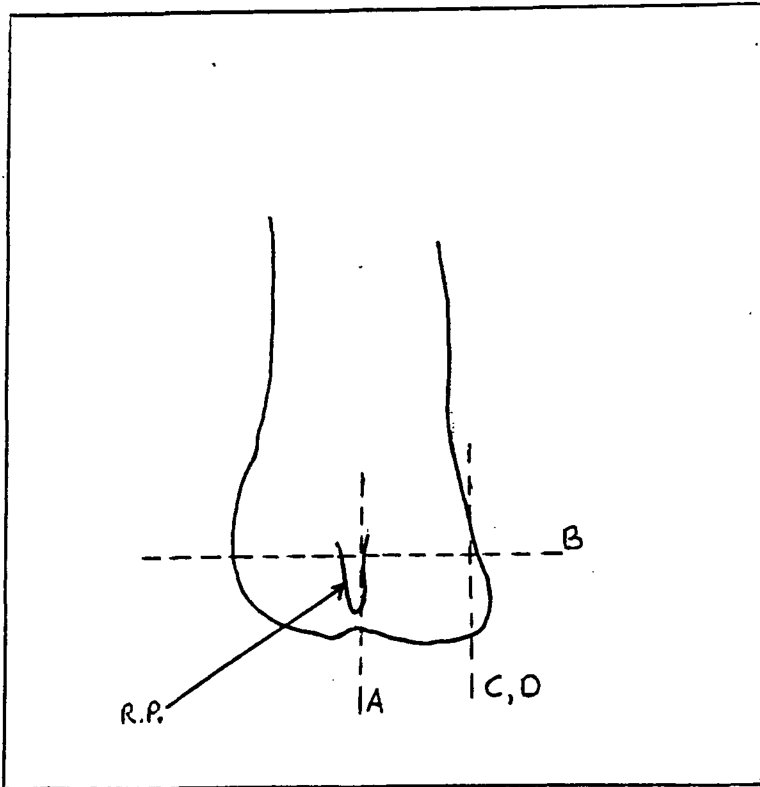
A. Longitudinal section of a plantlet showing the formation of a root primordium (rp). Note that the section is slightly out of the plane that would show the meristematic cells themselves and the starch granules (s) in cells surrounding the primordium. Calcoflor staining. Scale bar = 0.04 mm

B. Transverse section, as A. Scale bar = 0.04 mm.

C. Longitudinal section, showing an earlier stage of primordium development and xylem elements (xe) apparently leading towards the root primordium (rp). Calcofluor and Auramine staining. Scale bar = 0.04 mm.

D. As C, showing xylem elements (xe) in detail. Scale bar = 0.01 mm.

Planes of section through plantlets



RP = Root Primordium

Plate 3: Root Primordia in *Paeony lactiflora* on PR Medium

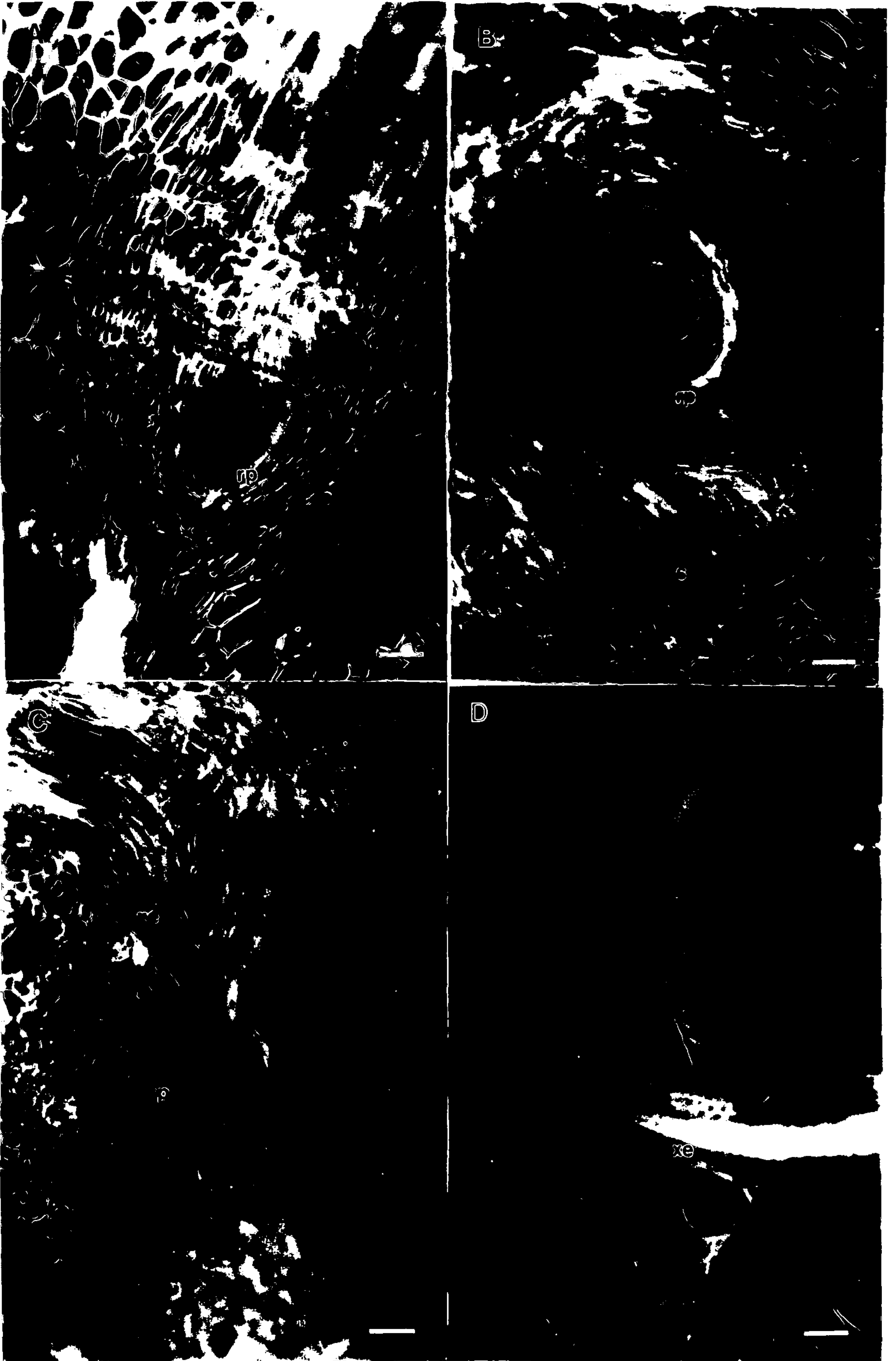


Plate 4: *Paeonia lactiflora* Root Anatomy in Growth Room

A. Transverse section of root, showing starch granules (s) and cortical intercellular space (is). Auramine and calcofluor staining. Scale bar = 0.04 mm.

B. As A., showing details of the stele/cortex boundary and large cellulose(?) thickened cell (c), (see D.). Scale bar = 0.02 mm.

C. As B., showing the thickened anticlinal cell walls of the endodermis (en). Calcofluor staining. Scale bar = 0.01 mm.

D. As C., showing the abnormal cellulose (?) thickening (c) in a cell bordering the cortex. Calcofluor staining. Scale bar = 0.01 mm.

E. As C., but in a younger (smaller) root, showing similar abnormally cellulose (?) thickened cells (c) inside the endodermis (en). Calcofluor staining. Scale bar = 0.01 mm.

F. Transverse section of root showing suberin (?) deposition in the epidermal cells, forming an exodermis (ex). M is the growth medium and note the lack of root hairs. Auramine staining. Scale bar = 0.01 mm.

Plate 4: *Paeony lactiflora* Root Anatomy in Growth Room

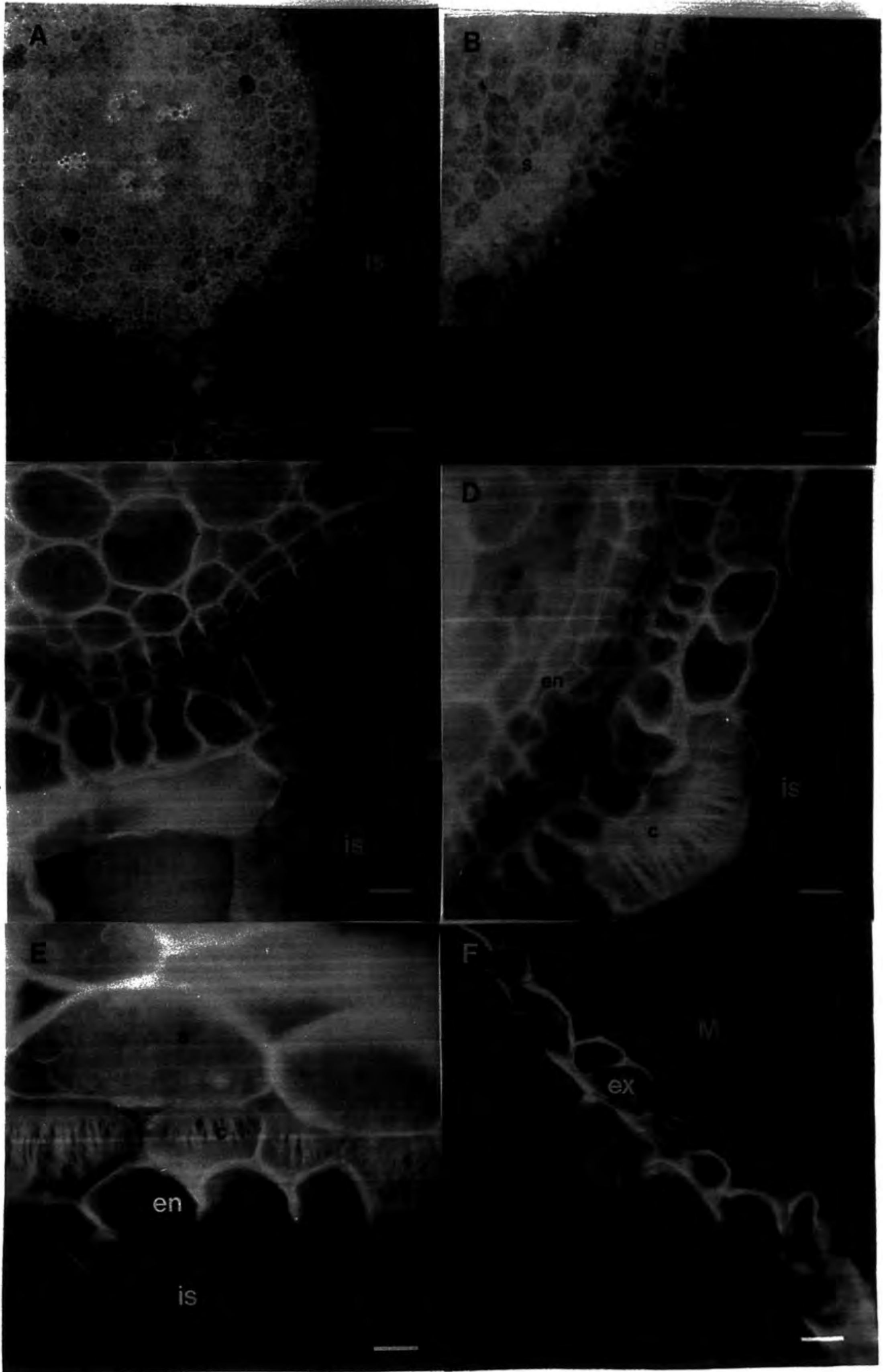


Plate 5: *Paeonia lactiflora* Root Anatomy after 6 Months in Cold Storage

A. Transverse section of young (small) root, showing large amount of black phenolic compound (p) deposition in cortical cells. Auramine staining. Viewed using transmission phase contrast. Scale bar = 0.10 mm.

B. As A., without phase contrast. Note the sparse xylem (x) development. Auramine staining. Scale bar = 0.10 mm.

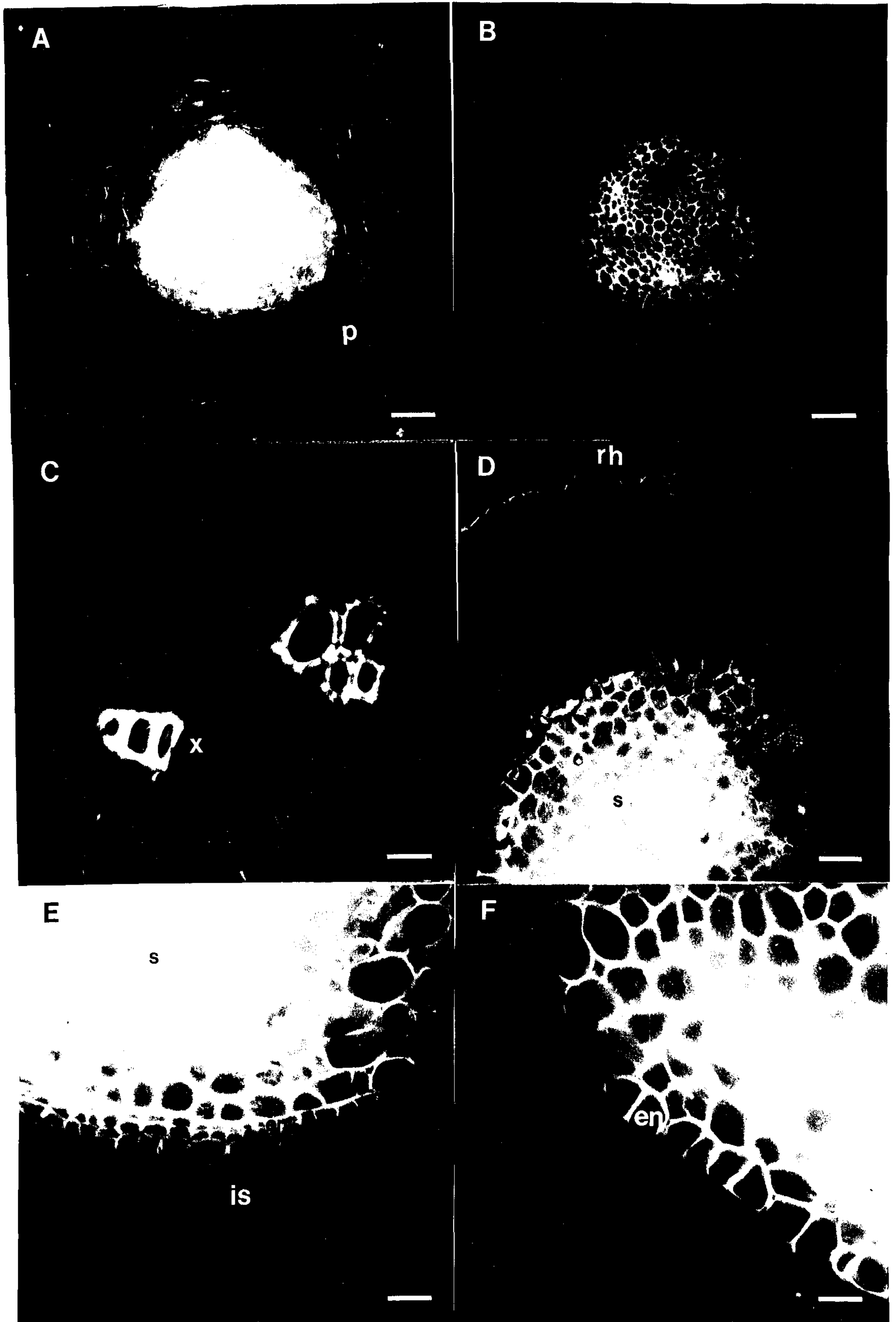
C. As B., showing detail of xylem (x). Auramine staining. Scale bar = 0.01 mm.

D. As B., showing partially differentiated root hairs (rh) and starch granules (s) in some of the cells in the stele. Also note the lack of cortical intercellular spaces. Auramine staining. Scale bar = 0.02 mm.

E. Transverse section of older (larger) root, showing starch granules (s) in the stele, endodermis, and the persistence of cortical intercellular spaces (is). Auramine staining. Scale bar = 0.02 mm.

F. Transverse section of a young (small) root, showing the formation of at least a partial endodermis (en), by anticlinal cell wall suberisation (?). Auramine staining. Scale bar = 0.01 mm.

Plate [illegible]. *Paeonia lactiflora* Root Anatomy after 6 Months in Cold Storage



3.1.2. Autoclaved and Filter-Sterilised GA_3

Materials and Methods

10 plantlets were transplanted on to each of 10 tubs of PX with autoclaved GA_3 (2 mg/1) and 10 tubs of PX with filter sterilised GA_3 (2 mg/1), and then placed into the growth room.

Results

Table 3. Comparison of autoclaved and filter sterilised GA_3

	% Alive	% Petiole Exten.	% Chlorotic	Mult. Rate
F.S'd	100	70.0±12.0*	9.6±3.9	2.89±0.13
Auto'd	100	66.0±12.6	67.0±19.1	1.77±0.12

* 95% Confidence level

No significant difference ($p>0.05$) in petiole extension between autoclaved and filter sterilised GA_3 was found. However, chlorosis was significantly ($p<0.05$) lower in the filter sterilised treatment, and multiplication was significantly ($p<0.05$) higher. Filter sterilised GA_3 was used in all subsequent work.

3.1.3. Paeony GA_3 Multiplication Experiments

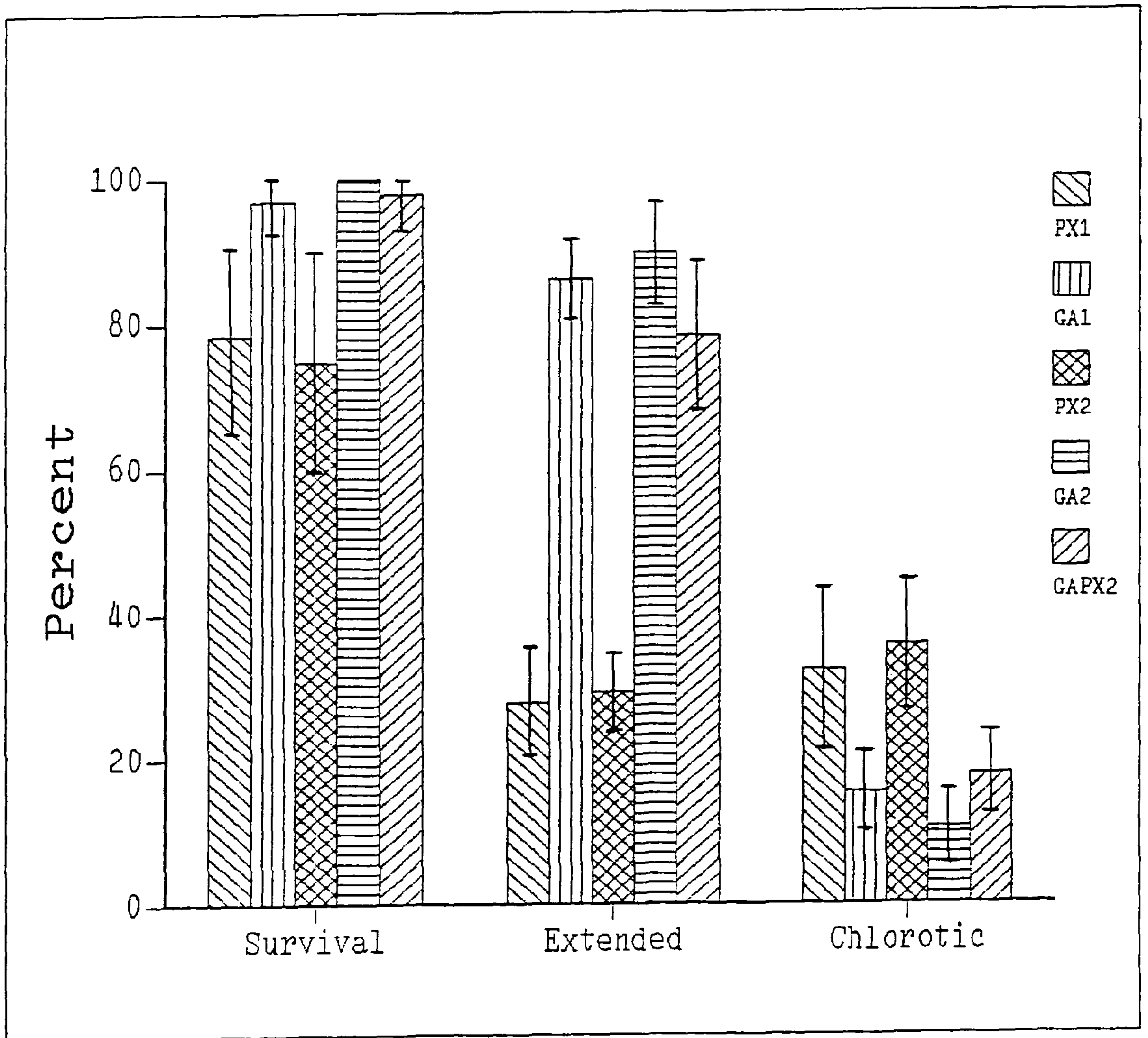
3.1.3.1. Preliminary Experiment

Materials and Methods

As a preliminary investigation, 5 tubs of Paeony (13 plantlets /tub), were subcultured on to PX medium including 2 mg/1 GA_3. A control of 5 tubs without the GA_3 was used.

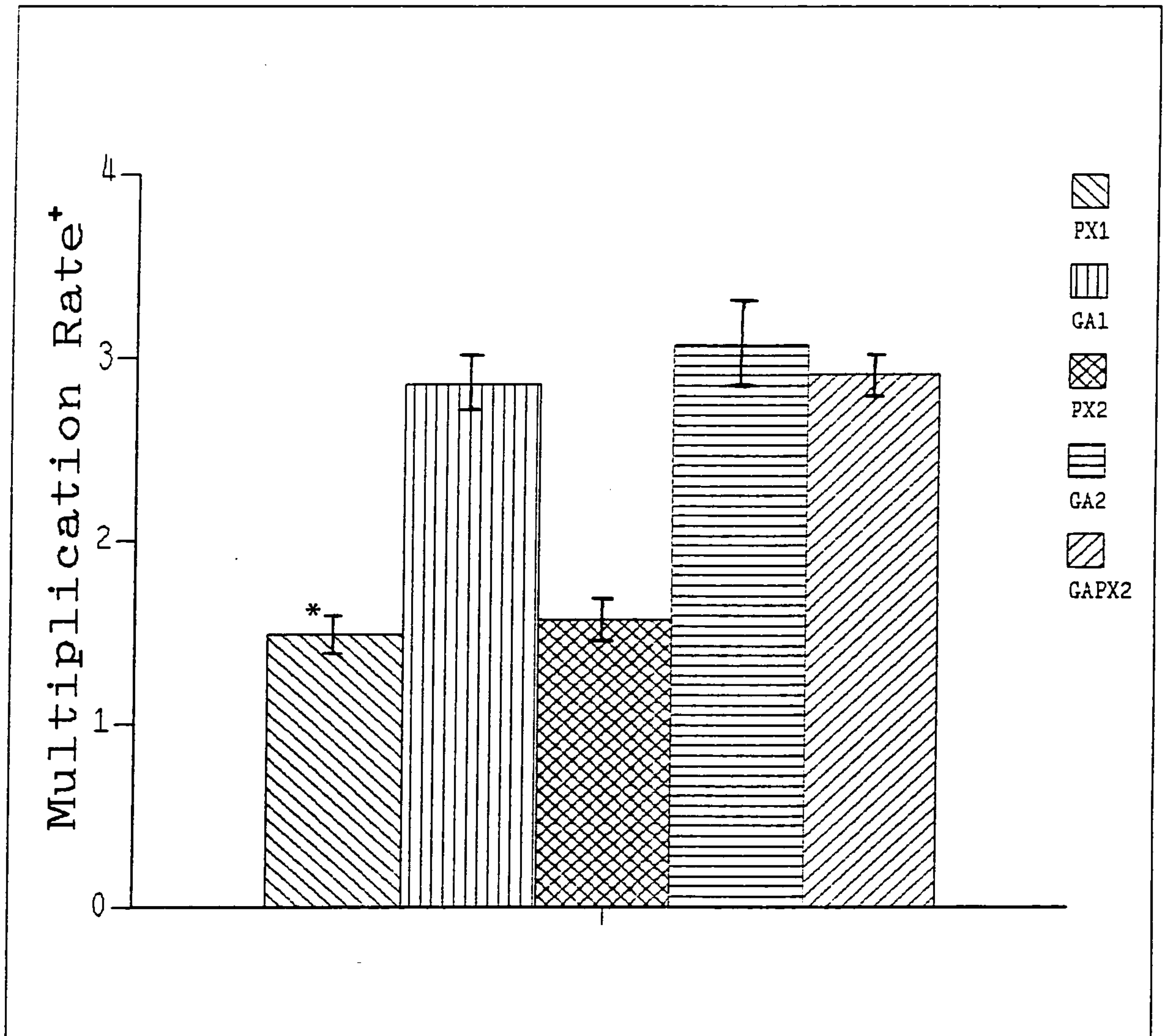
Leaf growth and appearance were recorded weekly, and an assessment of multiplication rate was made after 4 weeks in the growth room. One tub of each treatment was subcultured back on to the same medium, to determine whether any observed effect is maintained.

Figure 7. Plantlet Survival, Leaf Petiole Extension and Chlorosis in Relation to medium GA_3 and Subsequent Transfer on to Medium Without GA_3



* 95% Confidence level

Figure 8. Effect of GA_3 on *P. lactiflora* Multiplication Cultures and Maintenance of the Effect After Transfer to Medium Without GA_3



* 95% Confidence level, † Increase in dividable node number after 1 subculture (4 weeks)

Results

The addition of GA_3 to Paeony multiplication media resulted in a significant ($p \leq 0.05$) increase in the proliferation of plantlets (see figure 6). GA_3 also increased plantlet survival ($p \leq 0.05$) and petiole extension ($p \leq 0.05$) and reduced leaf chlorosis ($p \leq 0.05$), (see figure 7). These effects, with the possible exception of leaf chlorosis, were maintained even after GA_3 treated plantlets were returned to media without GA_3 at the next subculture ($p \leq 0.05$).

3.1.3.2. Effect of Different Medium GA_3 Levels on Paeony Multiplication Cultures

Materials and Methods

4 replicates (15 plantlets per tub), of the following treatments were placed in the growth room:

① PX media (control)
② PX + 0.5 mg/l GA_3
③ PX + 1.0 mg/l GA_3
④ PX + 1.5 mg/l GA_3
⑤ PX + 2.0 mg/l GA_3
⑥ PX + 3.0 mg/l GA_3
⑦ PX + 4.0 mg/l GA_3

GA_3 was filter sterilised in a solution (10 mg/l), and added aseptically to media before being poured into the tubs. Plantlets were scored every 5 days for the number of leaves, petiole extension and chlorosis, and at 4 weeks the multiplication rate was assessed.

Esterase and AAT gels were run on the samples after 21 days in the growth room, and the gels scanned with the laser scanner.

Results

Multiplication rate was increased with increasing medium GA_3 and reached an optimum at 1.5 mg/l (see figure 9). Petiole extension increased with GA_3 and continued to increase to a maximum at 3 mg/l, but at 4 mg/l it was slightly lower (see figure 10). Leaf chlorosis was

reduced by concentrations of up to 2 mg/l, where it was not observed, but higher concentrations of GA_3 led to chlorosis increasing again until at 4 mg/l it was more prevalent than when GA_3 was absent (see figure 11).

Esterase and AAT gel scans of treatments 1 and 5 are shown in figures 13 & 14). Using data provided by the gel scanner (area under absorbance trace which is a measure of total activity on the gel), the following enzyme activities were calculated from multiple scanning runs on two independently extracted and run gels.

Table 4. Total Esterase and AAT activities

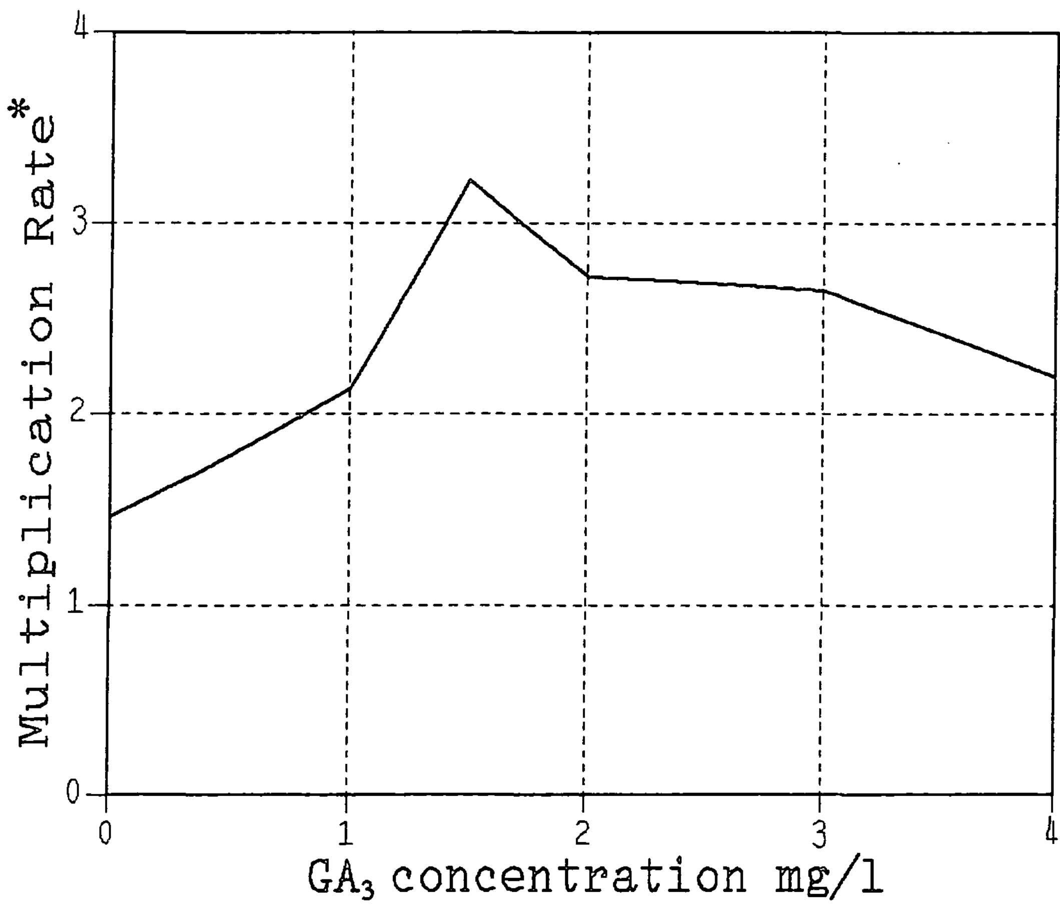
Media	Esterase Activity /Absorption Units	AAT Activity /Absorption Units
PX	4.44±0.24*	8.33±0.14
PX + 0.5mg/l GA_3	5.08±1.04	8.41±0.20
PX + 1.5mg/l GA_3	5.94±0.67	8.24±0.89
PX + 2mg/l GA_3	5.77±0.55	8.34±0.18
PX + 3mg/l GA_3	3.87±1.95⁺	8.09±1.09⁺
PX + 4mg/l GA_3	4.85±1.17	8.40±0.11

* 95% confidence level, ⁺ Possible sampling error

Total esterase activity was significantly ($p \leq 0.05$) increased over the control by treatment with 1.5 and 2.0 mg/l GA_3. Est-5 contributed approximately 40% of the observed increase in esterase activity at 1.5 mg/l GA_3. Est-2 and Est-10 disappeared in all GA_3 treated plantlets.

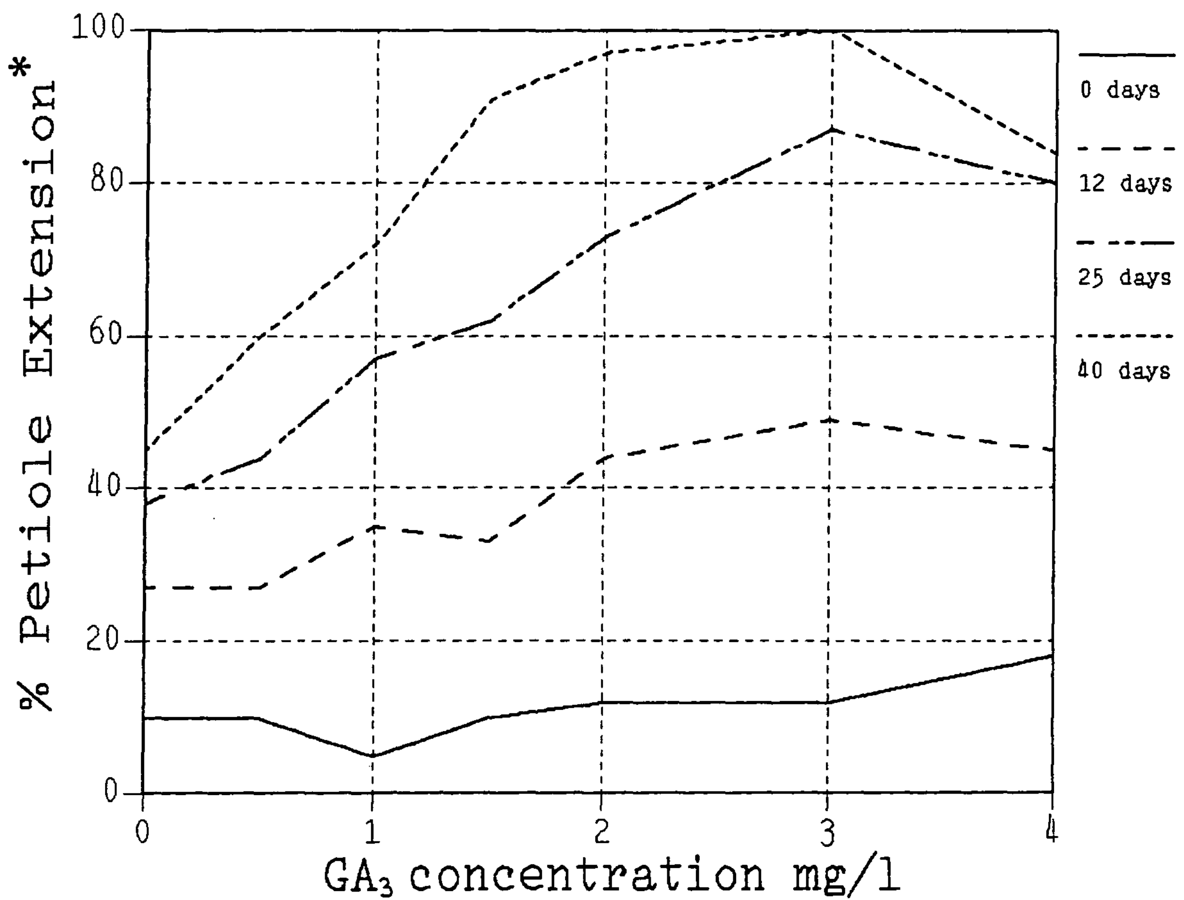
An error in one of the extractions probably led to the low enzyme activities and large confidence level in the 3 mg/l GA_3 treatment, so that extraction was excluded from figure 12. The broken line in figure 10 shows the data with this extraction included.

Figure 9. Relationship Between Medium GA_3 Content and *P. lactiflora* Multiplication Rate



* Increase in dividable node number after 1 subculture (4 weeks)

Figure 10. Relationship Between Medium GA_3 Content and *P. lactiflora* Leaf Petiole Extension



* Percentage of plantlets *with* extended leaf petioles present.

Figure 11. Relationship Between Medium GA_3 Content and *P. lactiflora* Leaf Chlorosis

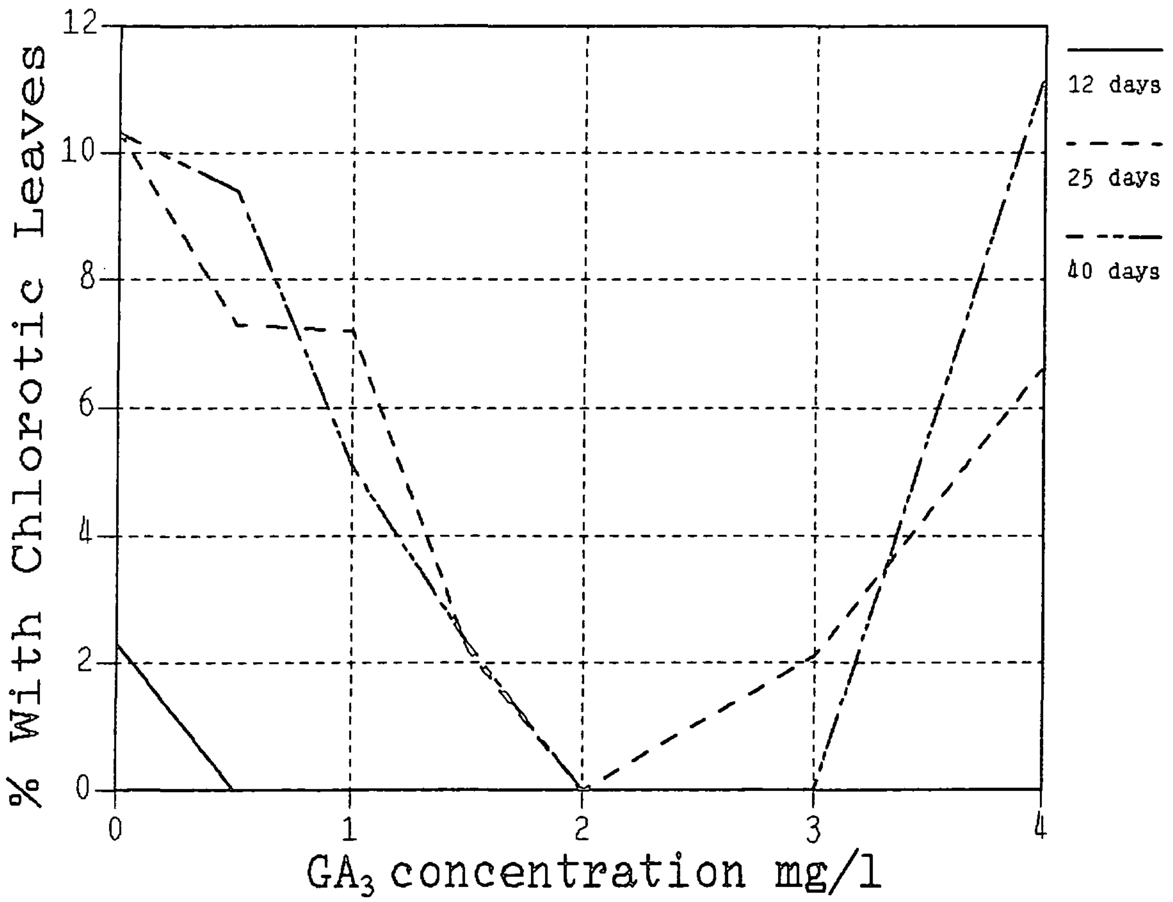
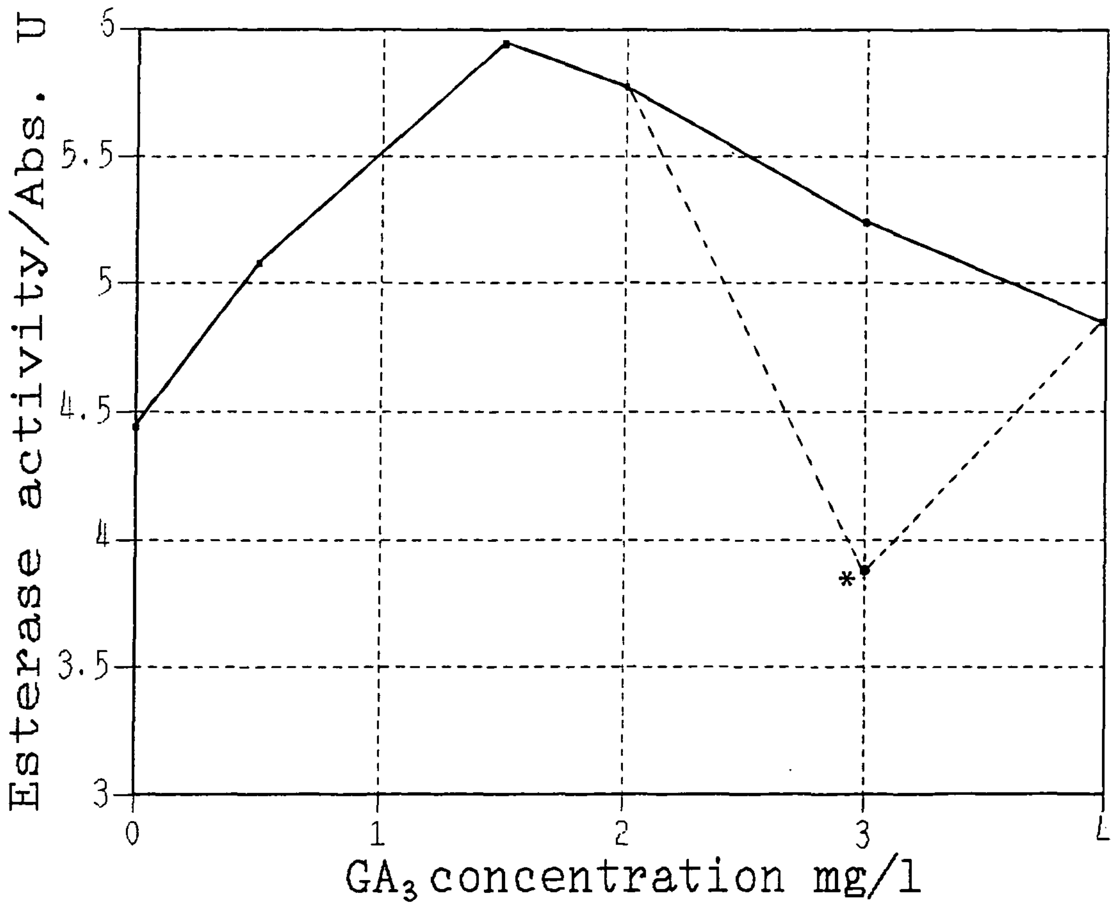
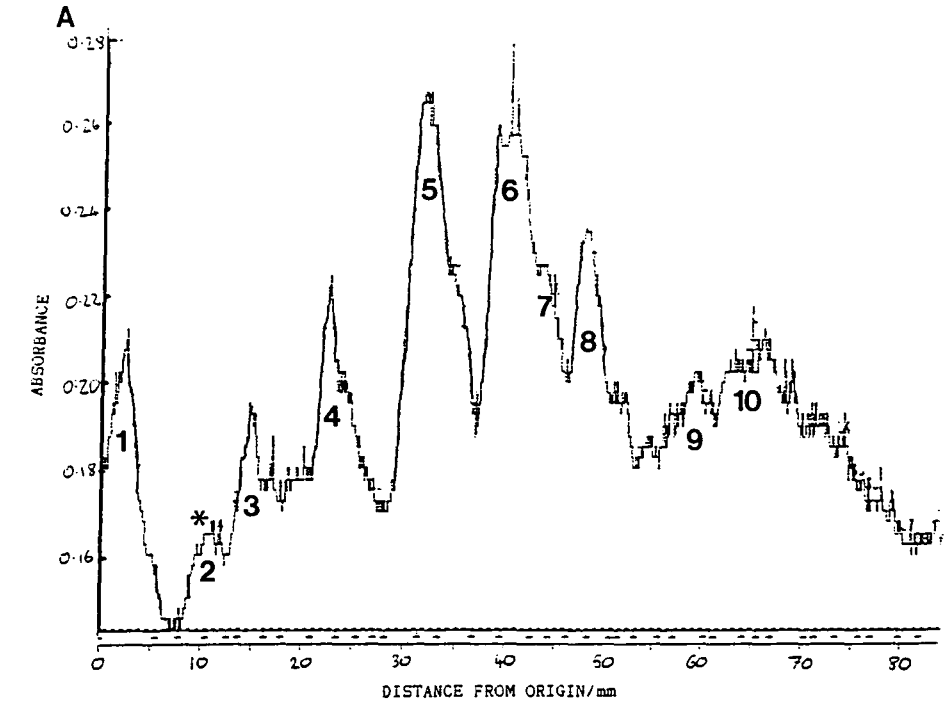


Figure 12. *P. lactiflora* Esterase Activity in Relation to Medium GA_3



* Probable sampling error: see text.

Figure 13. Differences in Esterase Isoenzyme Profiles Between *P. lactiflora* Multiplying Plantlets in the Absence (A) and Presence (B) of Medium GA_3



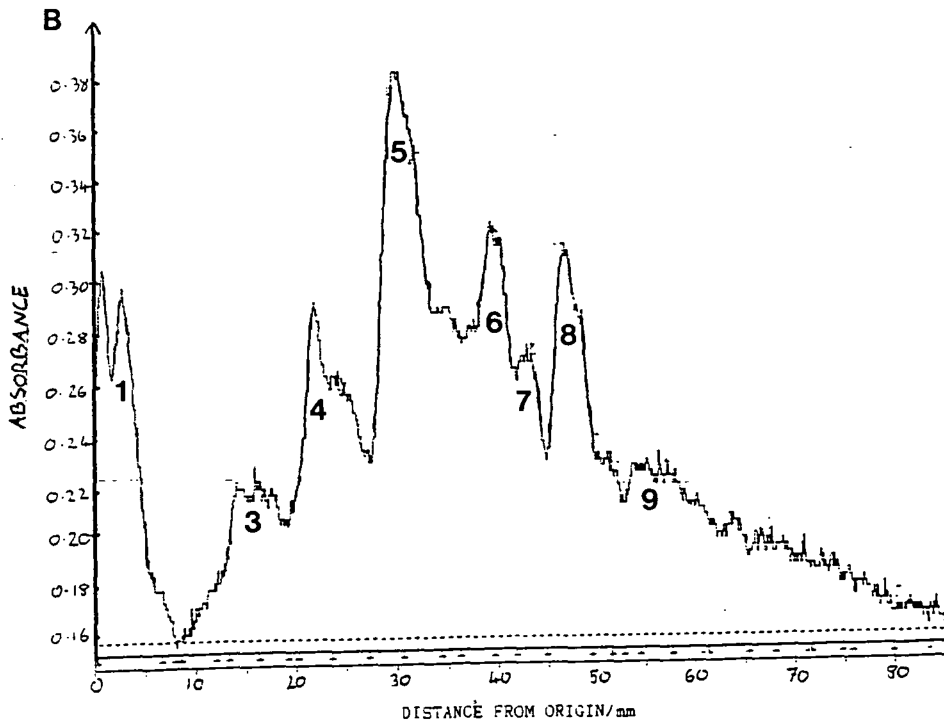
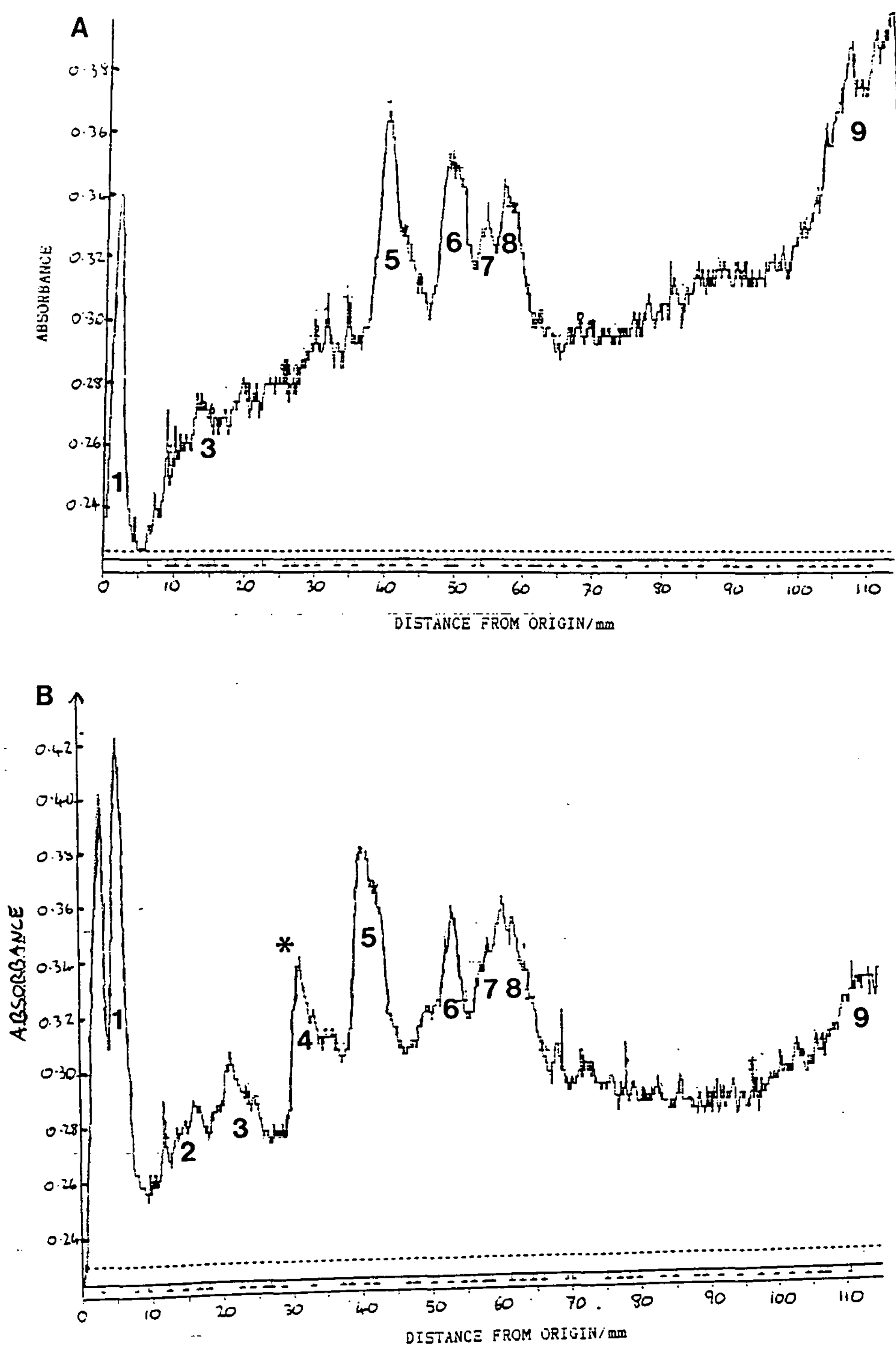


Figure 14. Differences in AAT Isoenzyme Profiles Between *P. lactiflora* Multiplying Plantlets in the Absence (A) and Presence (B) of Medium GA_3



3.1.4. Effect of Repeated Subculture on Paeony

Materials and Methods

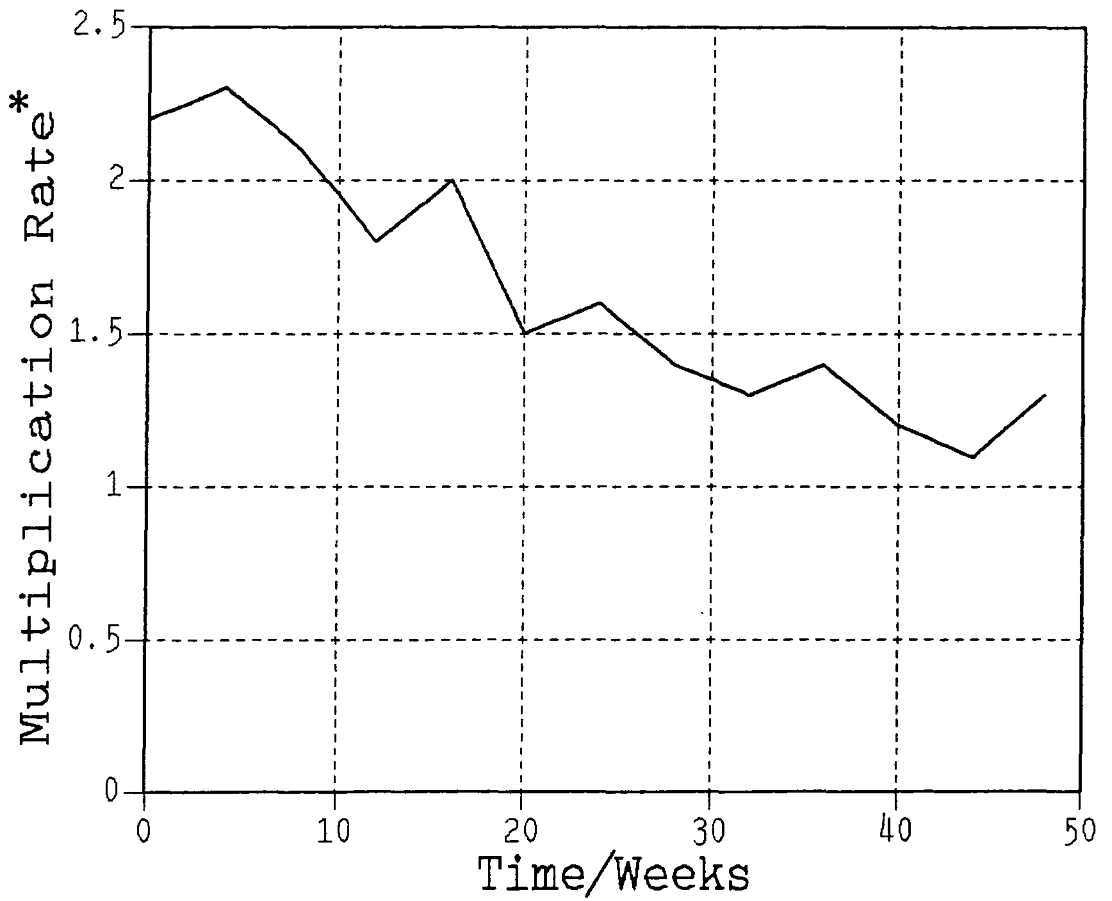
Data from the multiplication of stock cultures were compiled. At week 48, half the stock cultures were placed in the cold store (4°C). 2 tubs were taken monthly from the cold store and subcultured onto fresh medium. These were then placed in the growth room for 30 days and their multiplication rates were assessed.

Results

The mean multiplication rate declined by about 11% per month, and after 50 weeks had fallen from X2.3 per subculture to just above X1 (see figure 15). At 20 weeks onward, plantlets started to become chlorotic, stunted and produced leaves that were larger and thicker in cross-section, than the leaves before that time. These plantlets may have been vitreous.

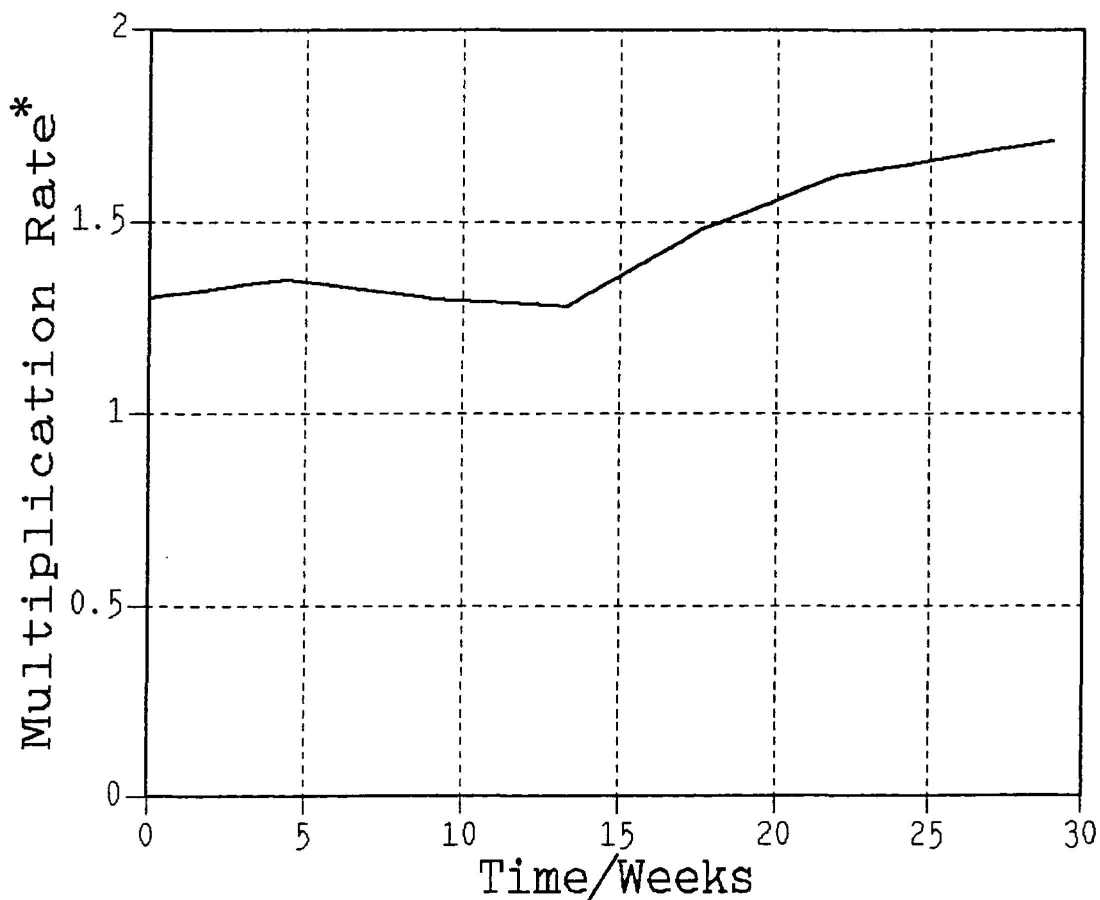
After 15 weeks at 4°C the multiplication rate of cultures started to rise slightly, reaching X1.75 after 29 weeks (see figure 16).

Figure 15. Decrease in *P. lactiflora* Multiplication Rate Over Numerous Subcultures



* Increase in dividable node number after 1 subculture (4 weeks)

Figure 16. Effect of Cold (4°C) on Slowly Multiplying *P. lactiflora* Cultures



* Increase in dividable node number after 1 subculture (4 weeks)

3.1.5. GA$_3$ and Amylase in Paeony Rooting Cultures

Materials and Methods

5 replicates, (10 plantlets per tub), of the following treatments were set up:

① PR media (control)
② PR + 0.5 mg/l GA$_3$
③ PR + 1.0 mg/l GA$_3$
④ PR + 3.0 mg/l GA$_3$

Plantlets were scored for number of rooted plantlets, and number of roots per plantlet, every 5 days for 4 weeks in the growth room, and were then transferred to the cold room (2°C) for 3 months and scored every 2 weeks.

Samples were taken for amylase radial diffusion assays, and microscopical observation before cold storage, and after 2 months in the cold.

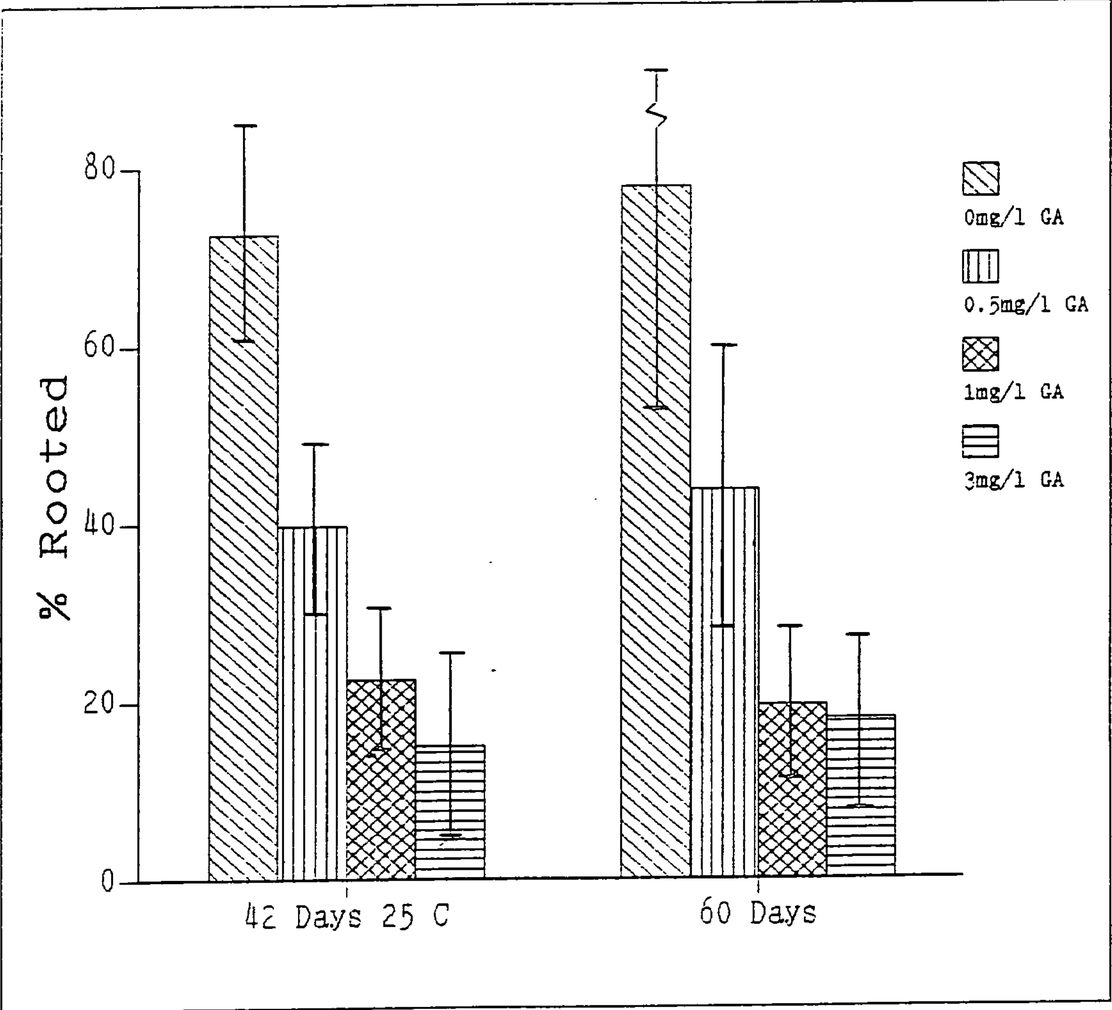
10 tubs with 10 plantlets/tub were rooted in the normal way. Plantlets shoots and roots were observed under the fluorescence microscope, and assayed for amylase at weekly intervals in the growth room and every 2 weeks in the cold room.

Results

The inclusion of GA$_3$ in rooting media significantly ($p \leq 0.05$) reduced rooting in Paeony plantlets. This inhibition applied to the proportion of plantlets rooting (see figure 17) and the subsequent amount of roots produced by plantlets that managed to root (see figure 16). A small amount of rooting did continue during cold storage, but this was only significant ($p \leq 0.05$) in the number of roots produced per rooted plantlet from the 1 mg/l GA$_3$ treatment (see figure 18).

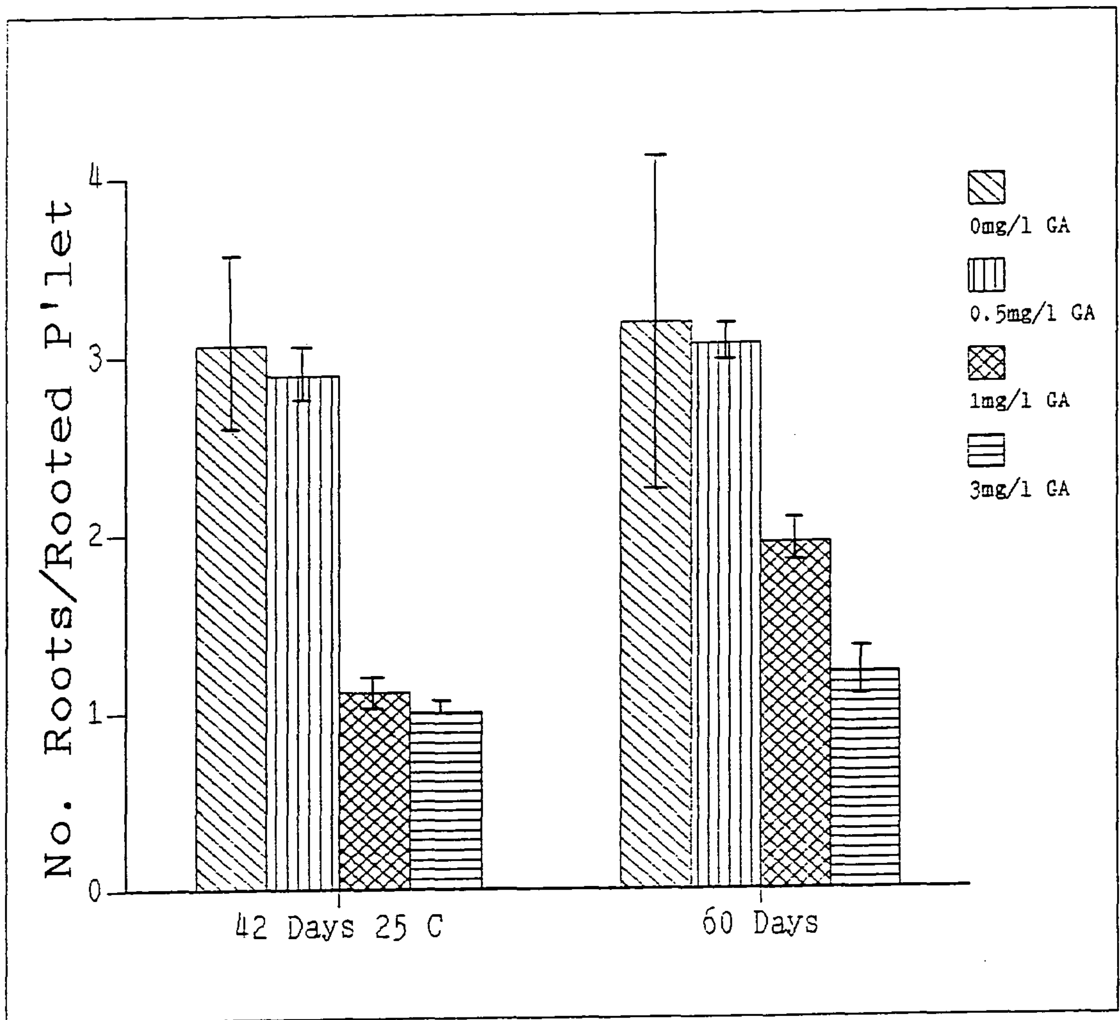
The amount of amylase activity in a sample used in radial diffusion could be calculated using the following equation:

Figure 17. Effect of Medium GA_3 on the Proportion of *P. lactiflora* Plantlets Rooting



* 95% Confidence level

Figure 18. Effect of Medium GA_3 on *P. lactiflora* Root Proliferation



* 95% Confidence level

Figure 19. Amylase Levels in *P. lactiflora* Through Rooting Culture

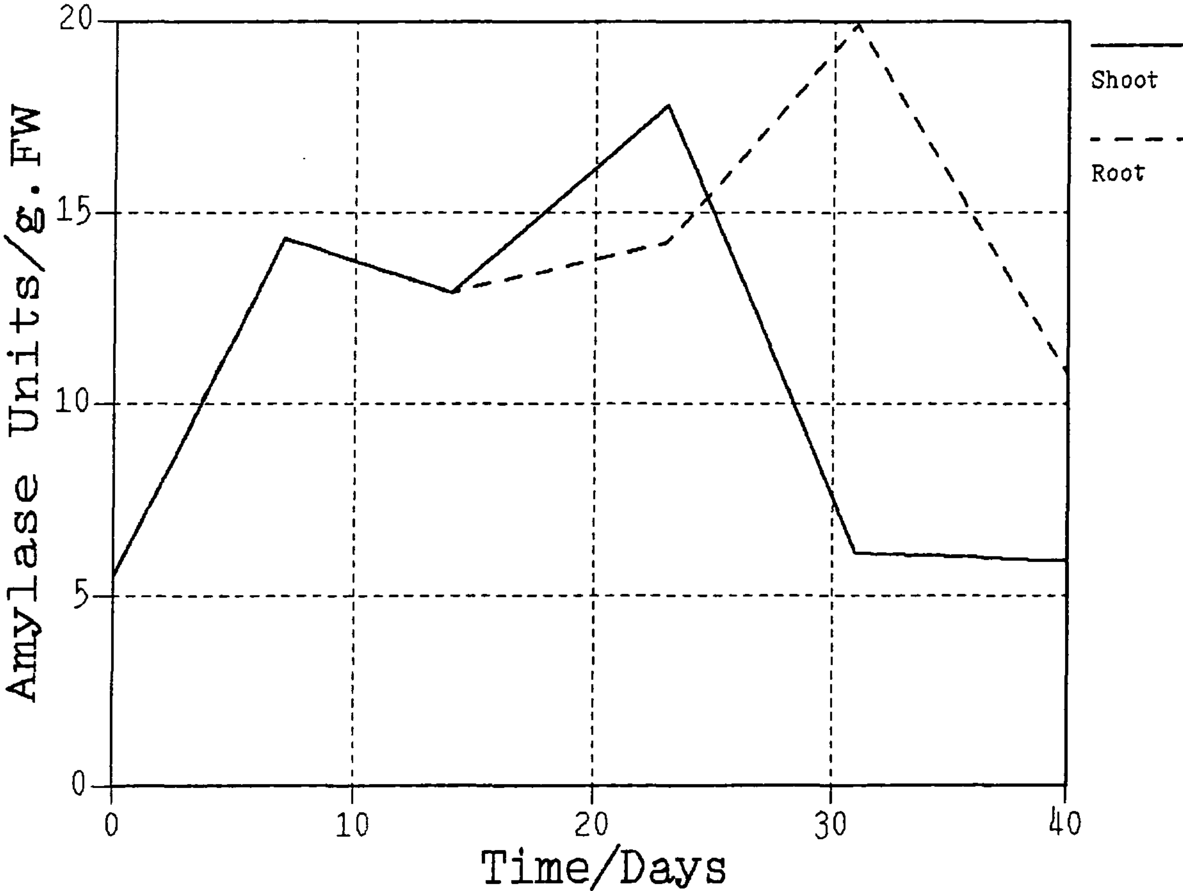
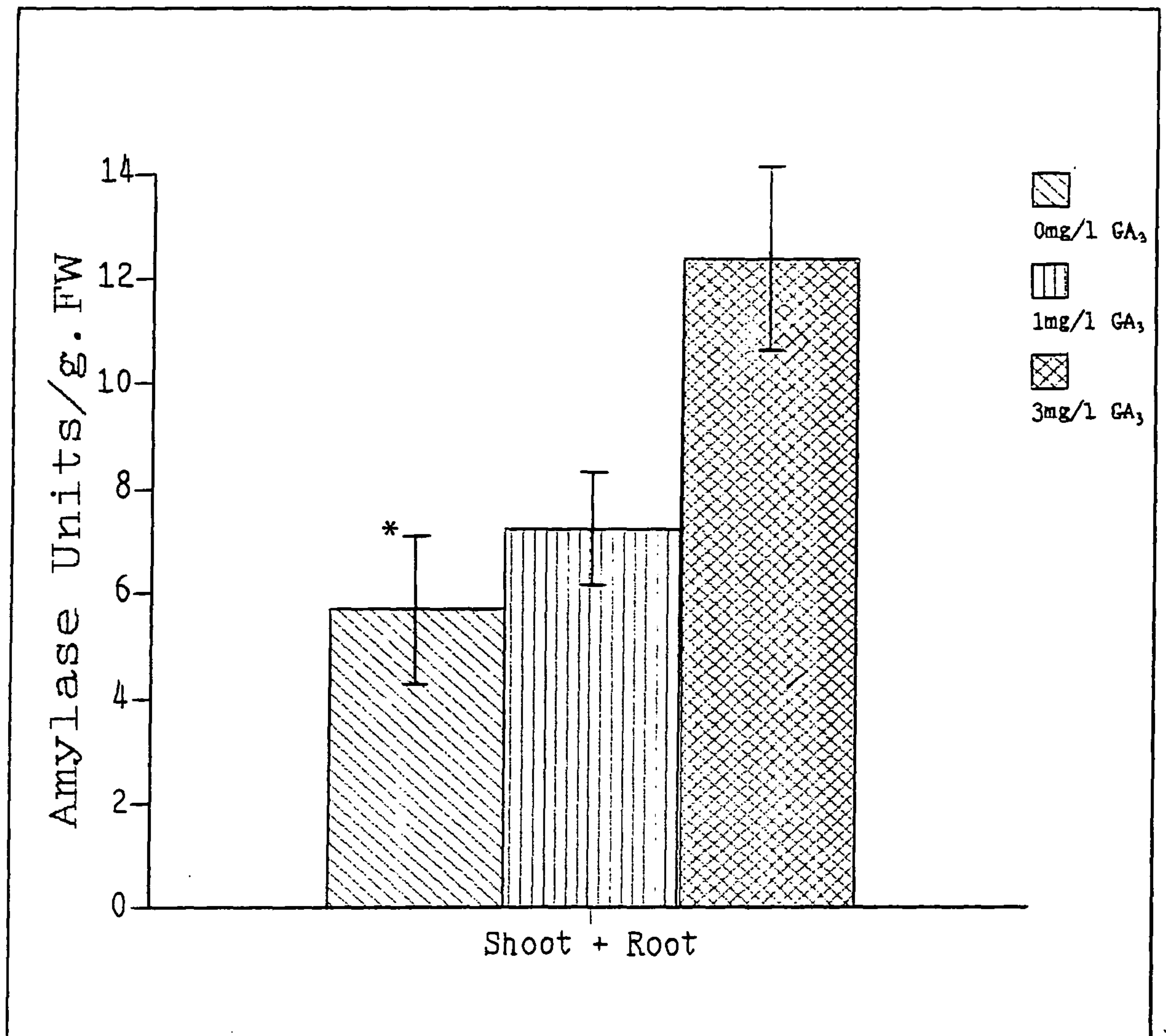


Figure 20. Effect of GA_3 on Amylase Levels in Rooting *P. lactiflora* Cultures



* 95% Confidence level

Figure 21. Effect of GA_3 on Amylase Levels on Rooting *P. lactiflora* Plantlets Shoots and Roots

* 95% Confidence level

$$\log A = 0.145d - 0.4485$$

Where A = Amylase activity units and d = Diameter of hydrolysed area in millimetres. This equation was produced from a best fit line of figure 13. Alternatively, the figure 5. could be used directly.

Plantlet amylase levels rose until day 23, when plantlets had produced roots. After this point the shoot amylase level declined to a level comparable with the start of the subculture, but root amylase levels continued to rise until day 31, after which it declined (figure 19).

Plantlet amylase levels were significantly ($p \leq 0.05$) increased by 3 mg GA_3 after 40 days in the growth room on rooting media (see figure 20). Amylase levels in shoots were significantly ($p \leq 0.05$) increased by at least 2.7 times when GA_3 was included in rooting medium, but root amylase content was depressed slightly by GA_3 ($p \leq 0.05$) (see figure 21).

3.1.6. Effect of Phosphate on Paeony Rooting

Materials and Methods

5 replicates with 10 plantlets in each were used for each of the following treatments:

① PR media only (0.625 mM Pi)
② PR + 100 mg/l KH_2PO_4 (1.36 mM Pi)
③ PR + 400 mg/l KH_2PO_4 (3.56 mM Pi)
④ PR + 800 mg/l KH_2PO_4 (6.50 mM Pi)

Tubs were placed in the growth room for 4 weeks, and then transferred to the cold room (4°C) for 3 months. Rooting was scored every 5 days in the growth room and every 2 weeks in the cold room. Two plantlets were removed for examination under the fluorescence microscope at each sample. On day 21, Esterase and AAT gels were run, using 2 plantlets (from different tubs), from each treatment.

Results

The application of 400 mg/l additional phosphate to rooting cultures appeared to increase leaf production (figure 22) and increase the proportion of plantlets rooting (figure 23), but not at a significant levels ($p > 0.10$ and $p > 0.20$ respectively). However, the trends of this experiment were repeated in a later experiment (results not presented), although a shortage of plant material also made these results inconclusive. The proportion of plantlets growing had decreased by day 8 in low Pi media (PR & PR+100), but increased steadily until day 14 in high Pi cultures (PR+400 & PR+800) and then declined. In low Pi cultures, a peak of activity was reached after 21 days, after which it declined (figure 24). Further work is required to determine if this effect is due to chance or is significant. The level of replication in future experiments needs to be much higher due to the high level of variation of plant health observed within treatments. Gel scans of treatment ① and ③ are shown in figures 25 & 26. Using data provided by the gel scanner (area under absorbance trace which is a measure of total activity on the gel), the following enzyme activities were calculated from multiple scanning runs on two independently extracted and run gels.

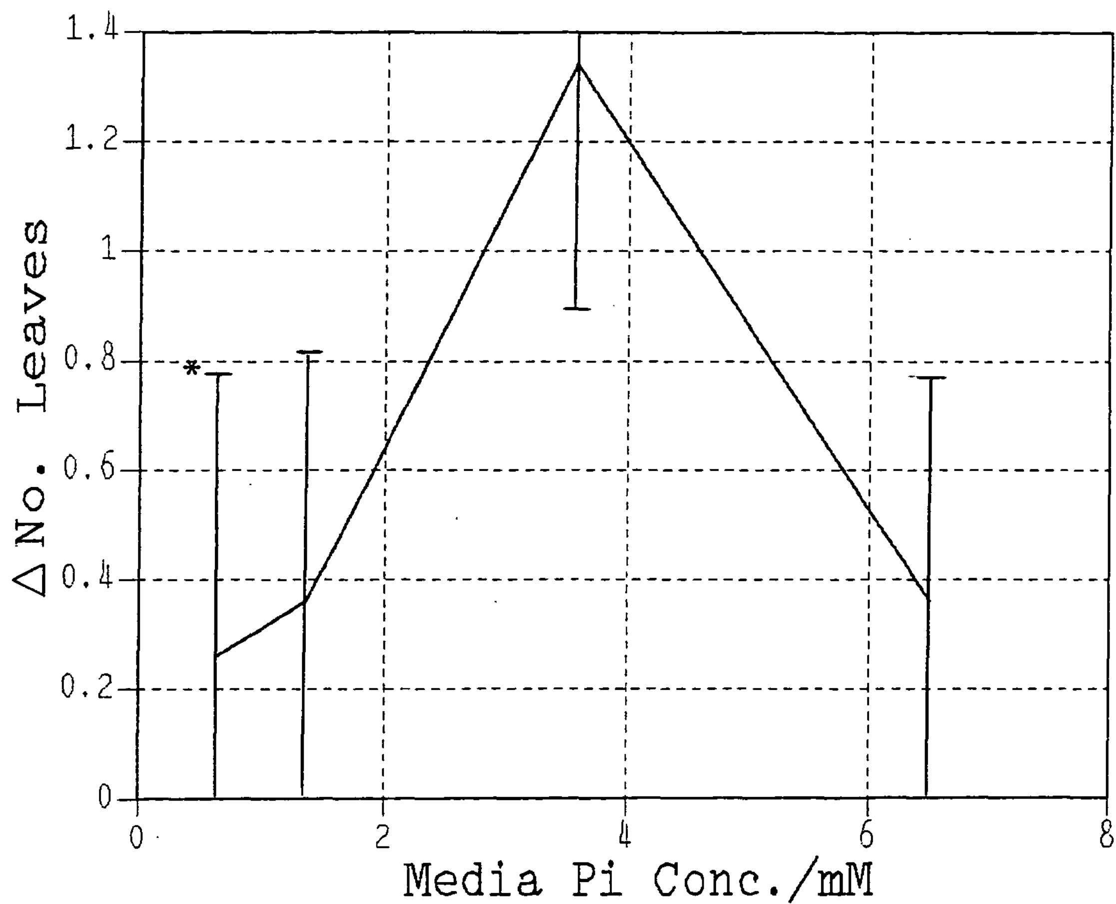
Table 5. Total Esterase and AAT activities

Media	Esterase Activity /Absorption Units	AAT Activity /Absorption Units
PR	3.34±0.73*	7.83±0.37
PR + 100mg/l PO_4	3.41±0.84	7.42±0.23
PR + 400mg/l PO_4	3.39±1.04	7.76±2.97
PR + 800mg/l PO_4	3.46±0.63	7.89±0.89

* 95% confidence level

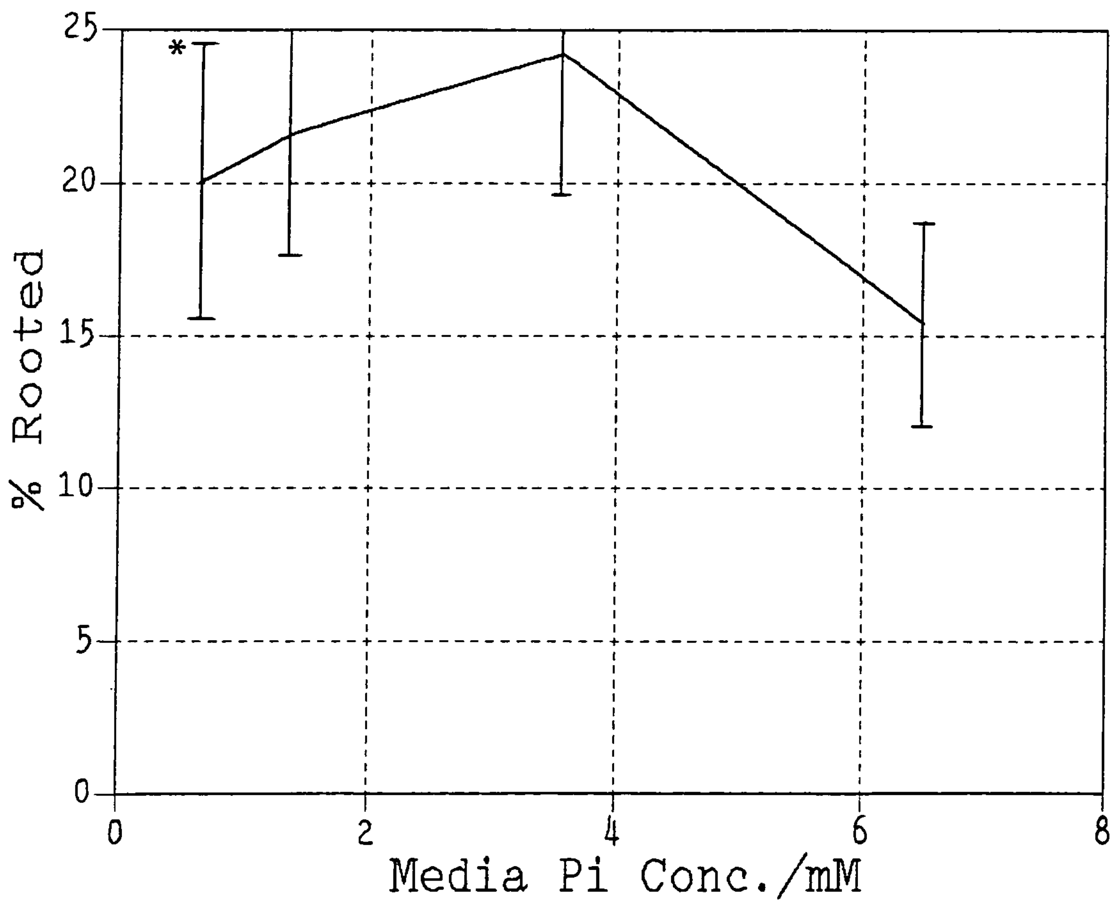
No significant difference was found in esterase or AAT activity in response to additional media phosphate. However, both esterase and AAT

Figure 22. Effect of Medium Phosphate on *P. lactiflora* Leaf Proliferation Between Days 14 and 35 of Rooting Culture



* 90% Confidence level

Figure 23. Effect of Medium Phosphate on the Proportion of *P. lactiflora* Plantlets Rooting After 35 Days



* 80% Confidence level

Figure 24. Effect of Medium Phosphate on the Proportion of *P. lactiflora* Plantlets With Newly Emerged Leaves

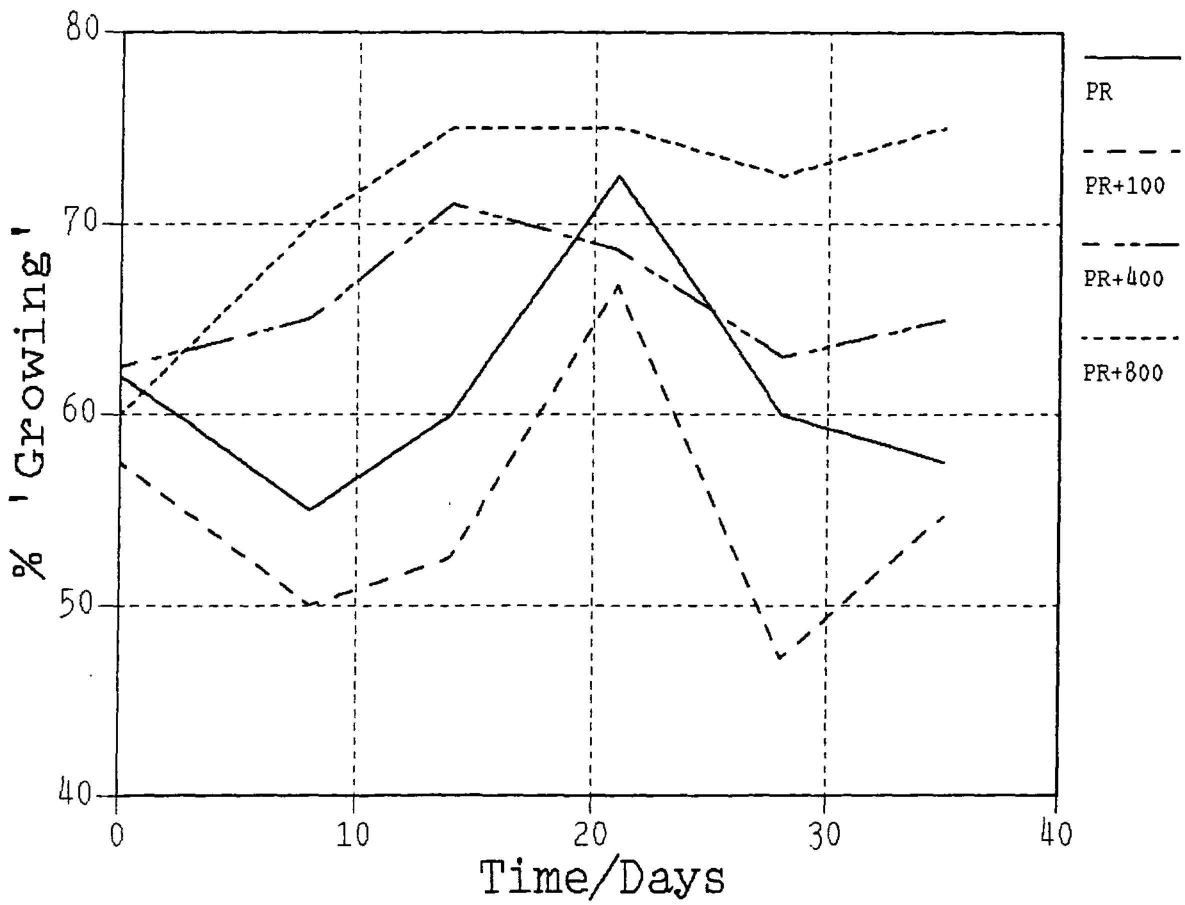
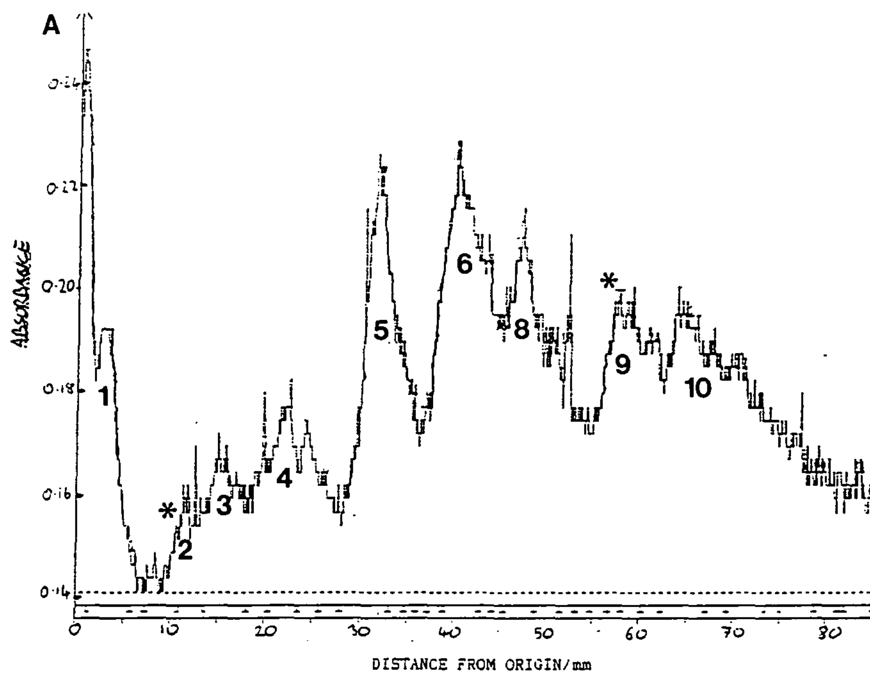


Figure 25. Differences in Esterase Isoenzyme Profiles Between *P. lactiflora* Rooting Plantlets with 0.625 mM (A) and 3.56 mM (B) Medium Phosphate After 14 Days



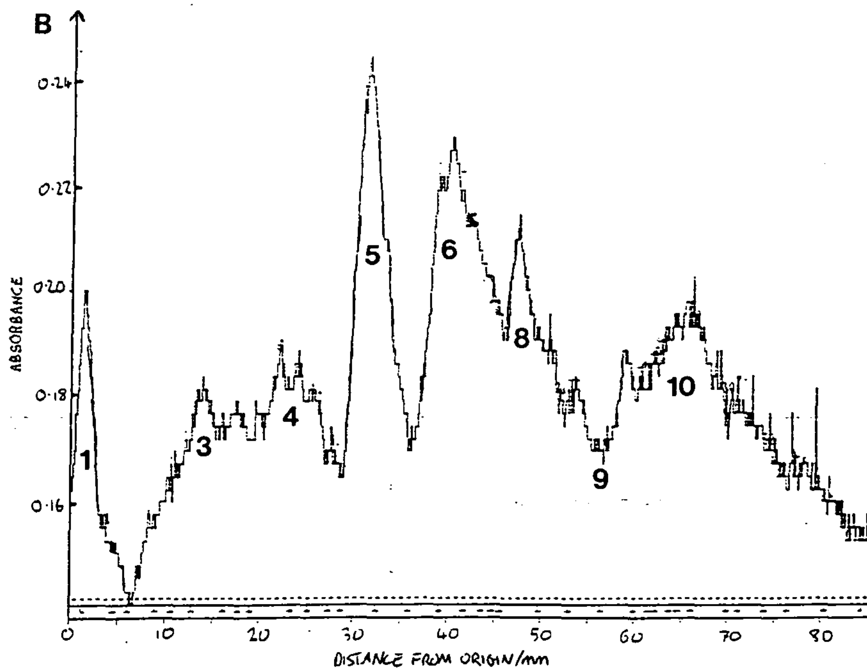
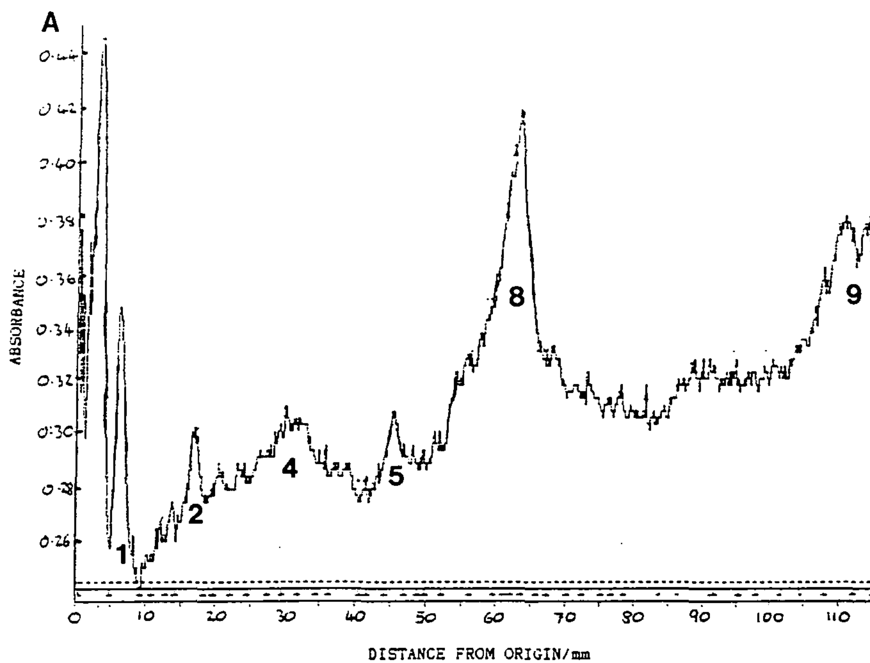
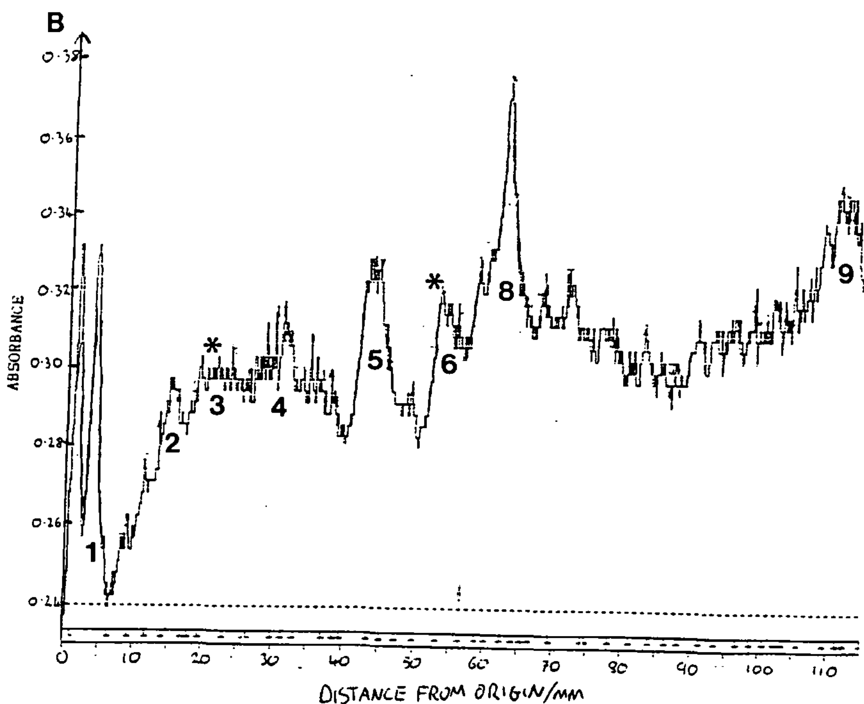


Figure 26. Differences in AAT Isoenzyme Profiles Between *P. lactiflora* Rooting Plantlets with 0.625 mM (A) and 3.56 mM (B) Medium Phosphate After 14 Days





activity were significantly ($p \lesssim 0.05$) reduced on PR in comparison to PX (see section 3.1.3.2.)

The contribution of Est-5 increased slightly, in the same way, but to a lesser extent than GA_3 treatments. Est-2 disappeared with all Pi treatments, as was the case with GA_3 treatment; however, Est-10 remained. Est-9b was not present in Pi treatments (figures 25 & 13).

3.1.7. Paeony Osmotica Experiment

Materials and Methods

The osmotic potential of mannitol solutions were calculated from data on freezing point depression at various concentrations found in the CRC Handbook of Physics and Chemistry (1968). The salt nutrient component (½WPM) was calculated from osmolality data supplied by Sigma, and the sucrose component from the tables in Slavík (1974). This gave a figure of -1.59 bars for PR media (not including hormones).

Treatments were replicated 4 times, with 10 plantlets per tub.

① PR : -1.59 bar
② PR + 0.110 M mannitol : -4.42 bar
③ PR + 0.251 M mannitol : -8.06 bar
④ PR + 0.568 M mannitol : -16.97 bar

A similar experiment was carried out using polyethylene glycol (R.M.W. 200) (PEG-200).

Results

All of the plantlets on medium including mannitol and PEG-200 died within a week. Meristems died first, followed by necrosis of the whole plantlet.

3.1.8. Culture Storage and Media Dehydration

Materials and Methods

18 tubs of Paeony (10/tub) on multiplication medium were stored in the growth room, of which 3 were sampled and transferred to rooting medium at intervals over 5 months. Multiplication tubs and media were

Figure 27. Effect of Multiplication Culture Storage on the Subsequent Proportion of *P. lactiflora* Plantlets Rooting

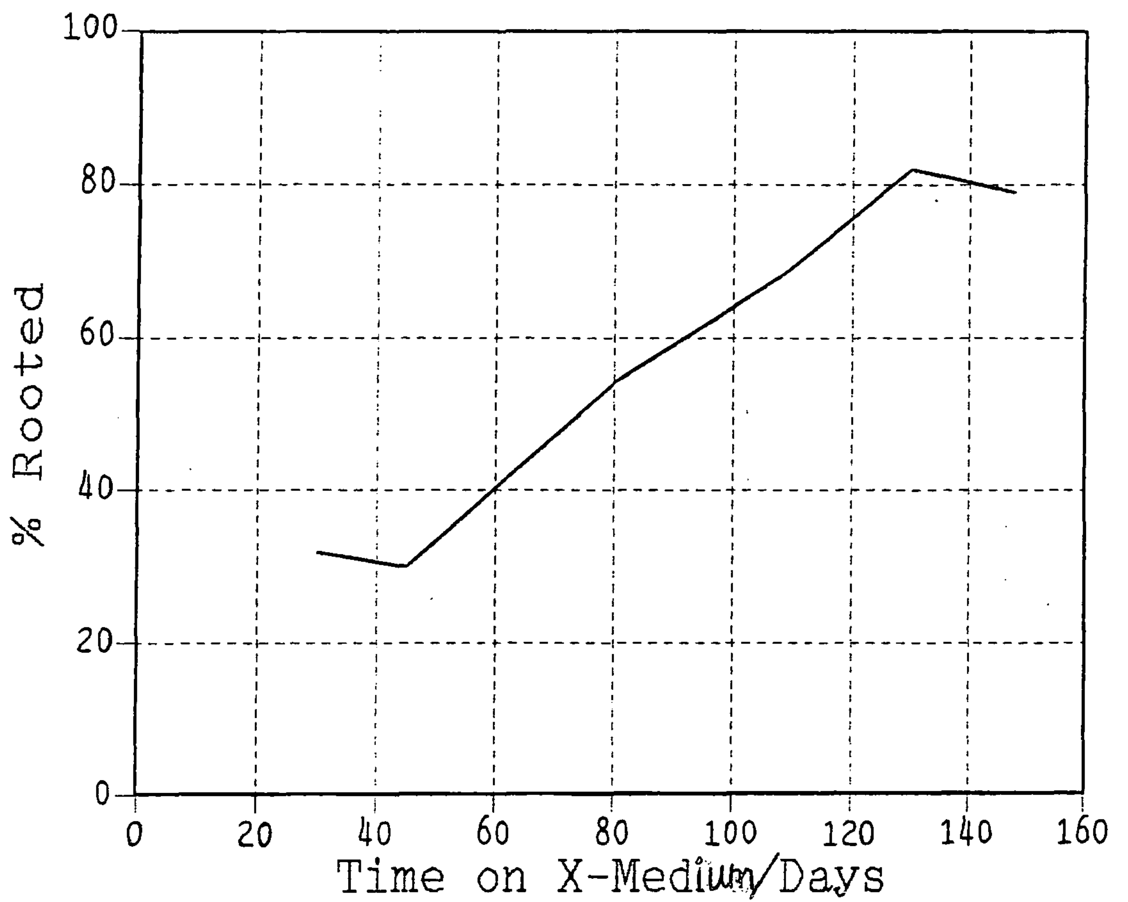
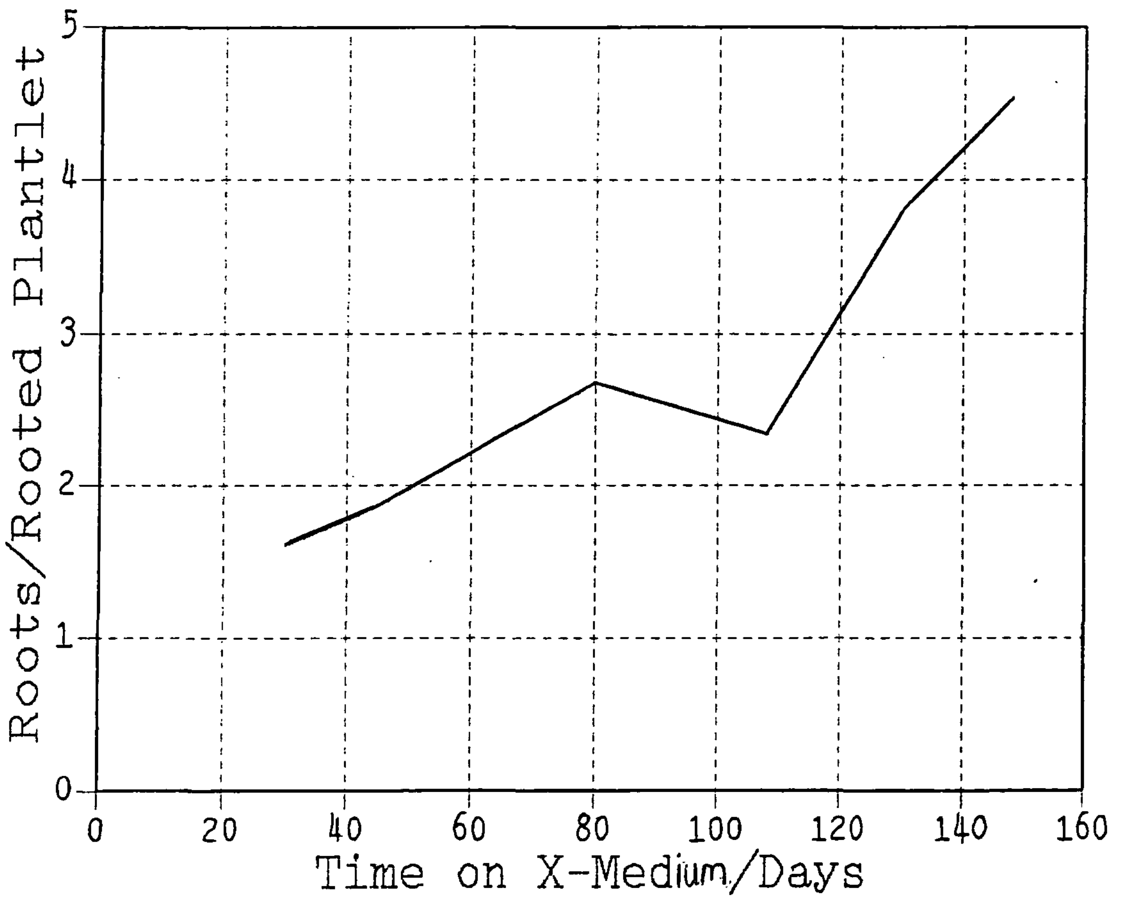


Figure 28. Effect of Multiplication Culture Storage on the Subsequent Proliferation of *P. lactiflora* Plantlet Roots



accurately weighed before and after plantlets were added. Cultures were weighed weekly in the first month, and then every 2 weeks thereafter.

Results

The proportion of plantlets rooted increased by a factor of 2.6 when the time on multiplication medium was increased from 30 days to 148 days (see figure 27). The mean number of roots per rooted plantlet increased by a factor of 2.9 over the same period (see figure 28).

Culture media without plantlets lost 13% of water content by day 30 and 38% by day 160; when plantlets were on the media, the water loss was 24% and 66% respectively (see figure 29). However, the plantlets remained green, and consisted of a high number of small shootlets.

3.1.9. Paeony Pre-Rooting Stress Experiment

Materials and Methods

Treatments prior to rooting:

① PX; 50 ml + 1% Agar
② 130 days on PX
③ PX; 10 ml + 5% Agar
④ PX x5; 10 ml + 5% Agar
⑤ Distilled water; 50 ml + 1% Agar
⑥ Distilled water; 10 ml + 5% Agar

8 tubs per treatment and 10 plantlets per tub were used and treatments were scored every 5 days for plant health (number of leaves, leaf colour), and rooting quantity and quality. They were then transferred to the cold-store after 4 weeks, and scored every 2 weeks.

Results

Low levels of survival and rooting were observed in all the artificially-induced stress treatments (see figure 30).

Figure 29. Medium Dehydration in *P. lactiflora* Multiplication Cultures in the Presence and Absence of Plantlets

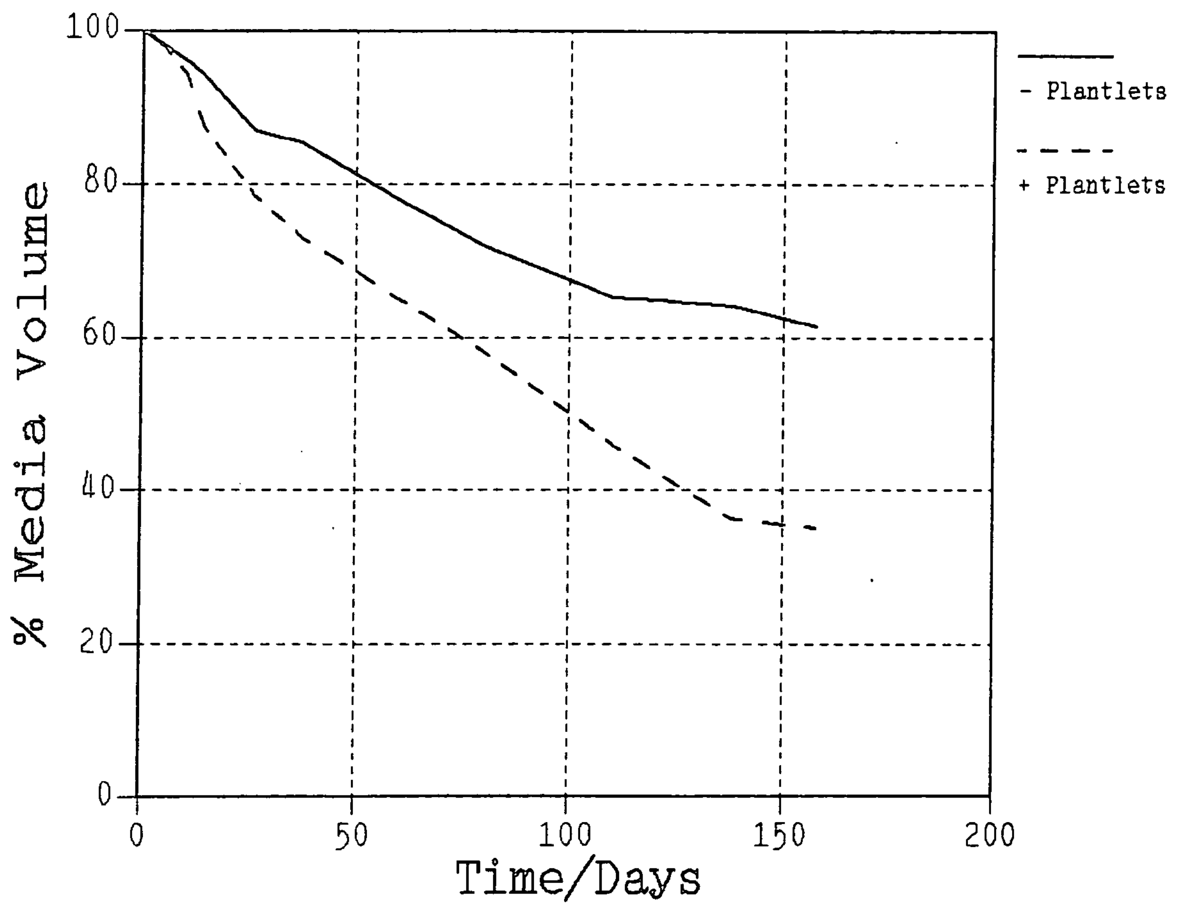
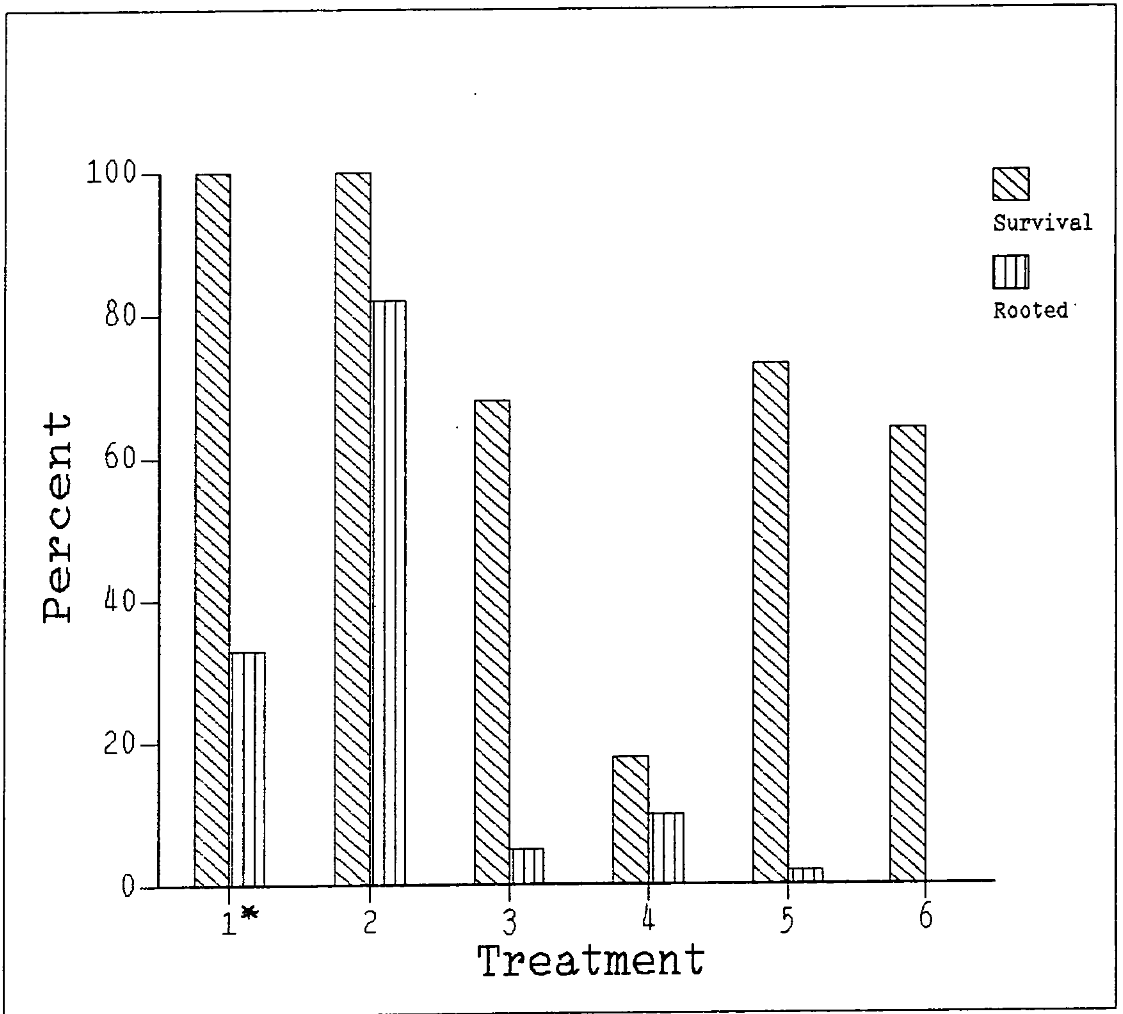


Figure 30. Survival of Pre-Stress Treatments and Subsequent Proportion of Remaining *P. lactiflora* Plantlets Rooting



* 1= PX, 50 ml + 1% agar; 2= 130 days on PX; 3= PX, 10 ml + 5% agar; 4= PX (x5 conc.), 10 ml + 5% agar; 5= Distilled water, 50 ml + 1% agar; 6= Distilled water, 10 ml + 5% agar.

Death of the plantlet was defined as necrosis of the meristem, as many detached leaves remained green on the media, but were obviously not viable plantlets.

3.1.10. PPO Extractions

Materials and Methods

In an attempt to investigate the role of PPO in *in vitro* rooting, several PPO Extractions (see Section 2.6.1.1.3.) were performed using plantlets from PX, fresh and stored growth room PR and cold stored PR.

Results

PPO levels appeared to be similar, and confined to a single isoenzyme. However, activities and resolution were low in all the extractions examined (Plate 11A).

3.1.11. Paeony Water Relations

3.1.11.1. Plantlet Dehydration Experiment

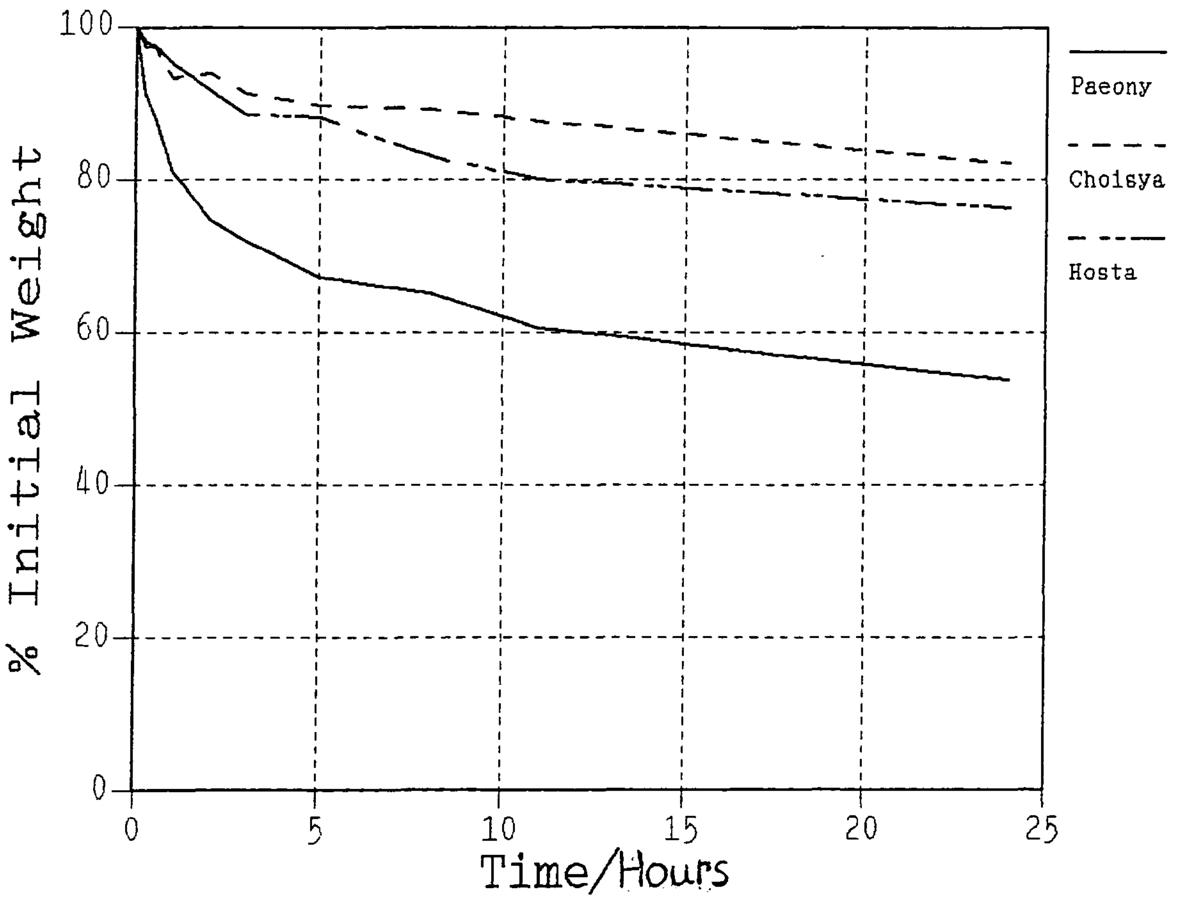
Materials and Methods

10 plantlets of each of Paeony (21 days, PR), Hosta (25 days, HR) and Choisya (24 days, CR) were placed in ambient conditions (room temperature of approximately 26°C, 50-70 %Rh) on a bench, and allowed to dehydrate. Plantlets were weighed accurately at regular intervals.

Results

The dehydration curves for the three different species are shown in figure 30. Paeony plantlets were visibly wilted after 10 minutes, whereas *Choisya* and *Hosta* plantlets appeared only slightly wilted after 24 hours. Paeony plantlets experienced a much more rapid water loss than plantlets of the other species (see figure 31).

Figure 31. Plantlet Dehydration Under Ambient Laboratory Conditions



3.1.11.2. Water Potential of Plantlets on Different Media

Materials and Methods

The 'smear' method of water potential determination (Section 2.3.1.) was employed. A table of water potentials of different sucrose solutions is provided in Appendix 3. The water potentials of a selection of plantlets were also assessed in a pressure chamber (Section 3.3.4.).

The initial water potentials of selected media were calculated using data supplied by Sigma and Appendix 3.

Results

Table 6. Water potential of selected plantlets

Sample	Water potential/bars
Hosta on multiplication media after 3 weeks	-3.82
Hosta on rooting media after 3½ weeks	-5.73
Paeony on multiplication media after 3 weeks	-3.13
Paeony on rooting media after 4 weeks	-1.61

Table 7. Water potential of selected media

Media	Water potential/bars
Hosta multiplication	-6.44
Hosta rooting	-3.22
Paeony multiplication	-6.44
Paeony rooting	-2.22

No sap exudate was observed at either cut or uncut shoot bases of any plantlet in the pressure ranges tested (0-50 bars) in the pressure chamber.

3.2. *Hosta* Micropropagation

3.2.1. Rooting and Apoplastic Tracer Experiments at Reduced Culture Humidity

3.2.1.1. Experiment 1

Materials and Methods

0.01% Calcofluor was incorporated into the HR media of 10 tubs (10 plantlets/tub) of Hosta 'August Moon'. 5 tubs without calcofluor were used as a control. Every 5 days, 3 plantlets were removed aseptically from different tubs and examined under the fluorescence microscope using the UV filter, and the number of rooted plantlets were scored.

Results

No uptake of calcofluor was observed during the course of the experiment. The calcofluor fluorescence remained on the surface of root hairs, roots and basal callus, (see Plate 8).

There was no significant difference in the level of rooting with or without calcofluor, (see figures 32 & 33).

3.2.1.2. Experiment 2

Materials and Methods

The above experiment was repeated using Choisya. In addition to this, 6 Rooted Choisya plantlets with a single root were chosen, laid on microscope slides and gently secured with sellotape. The slides were then pushed into CR media containing 0.01% Calcofluor, ensuring that the tip of the root was pushed into the growth medium. The tubs were left open to ambient laboratory conditions (approximately 25°C/50-80% RH). Plantlets were sampled every hour for six hours, and the root examined under the fluorescence microscope using the UV filter.

Plate 6: *Hosta* Shoot and Root Anatomy on HX, and on HR at Reduced Culture Humidity

A. Transverse section of *Hosta* plantlet after one week on HX at the centre of the plantlet base/medium (M) interface, showing plasmadesmata pit fields (pf), cell walls thickened (t) with suberin (?) and periclinally dividing cells (pc). Calcofluor and Auramine staining. Scale bar = 0.02 mm.

B. As A, but showing altered cell differentiation away from the exact base of the plantlet (but still in contact with the medium, M). The cells are still thickened (t) with an Auramine positive compound (suberin?), but the periclinal division derived cells are replaced by large (up to 0.1 mm diameter), thin walled cells (l). Note also the apparent absence of pit fields. Calcofluor and Auramine staining. Scale bar = 0.04 mm.

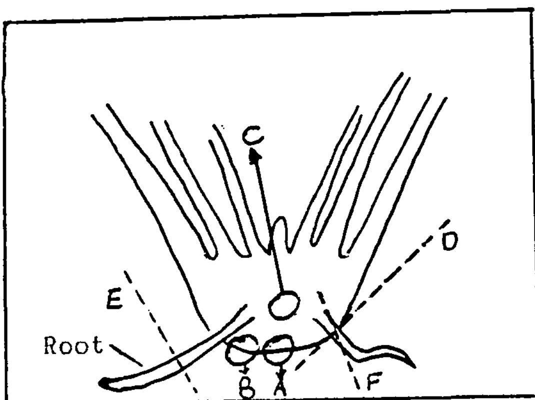
C. Transverse section of *Hosta* plantlet on HR, showing disorganised vascular development (v) and the presence of an "endodermis" (en) in the shoot basal callus. Auramine staining. Scale bar = 0.04 mm.

D. Longitudinal section of plantlet, showing transverse section of a root at the point in emerges from the basal callus. Note the presence of an endodermis (en) and an exodermis (ex). Auramine staining. Scale bar = 0.04 mm.

E. Transverse section of a root formed during 2 weeks at 85% RH. Note the absence of an endodermis (*), but the presence of an exodermis (ex). Auramine staining. Scale bar = 0.04 mm.

F. Longitudinal section of an emerging root of a *Hosta* from cold storage (4°C), showing the lack of an endodermis (*), the presence of an exodermis (ex) and a vascular connection with the shoot. Auramine staining. Scale bar = 0.04 mm

Planes of section through plantlets



Hosta Shoot and Root Anatomy on HX, and on HR at Reduced Culture Humidity

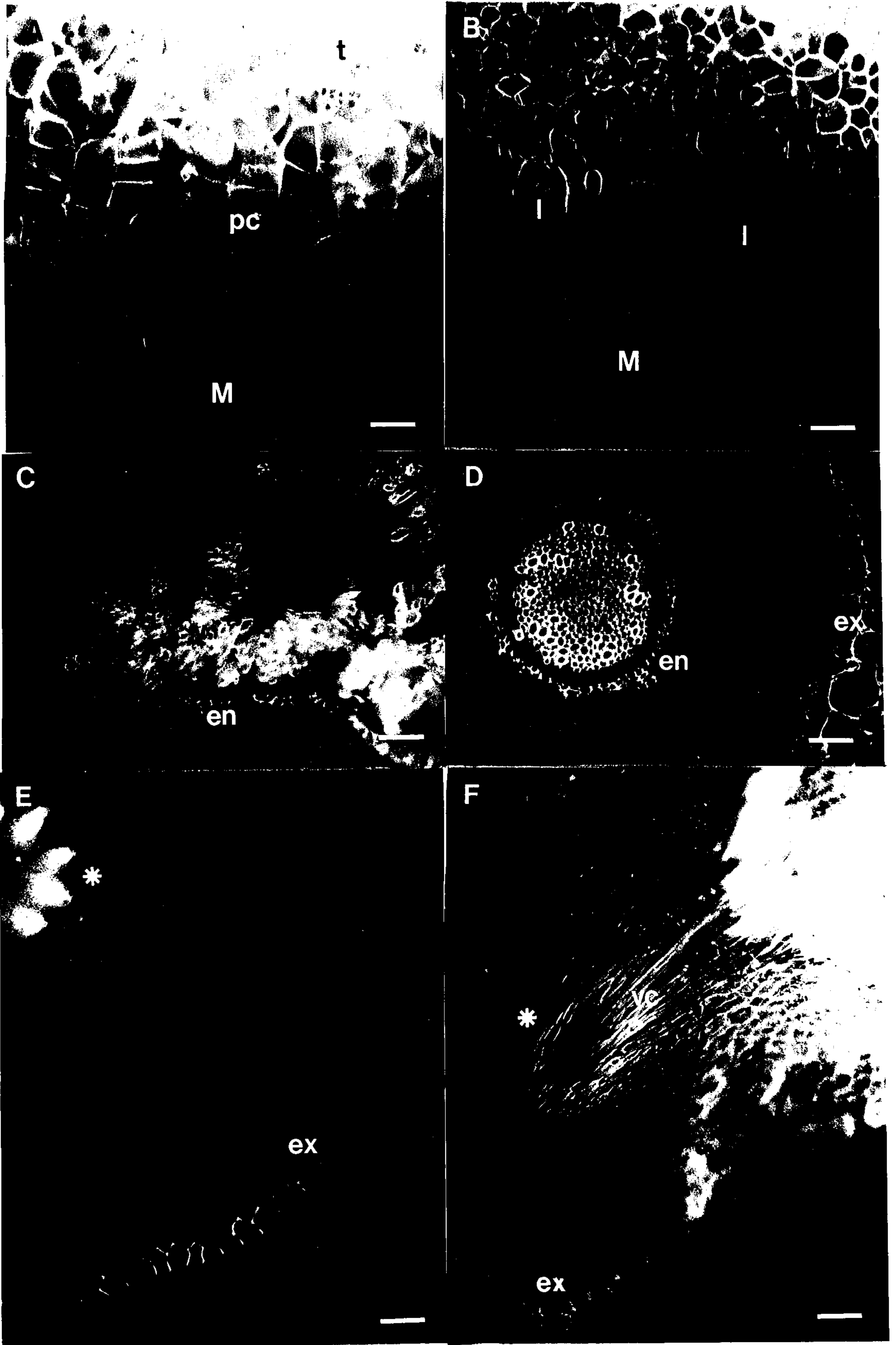


Plate 7: *Hosta* Shoots on HX Medium and on HR Medium in Cold Storage and Reduced Humidity

A. Longitudinal section of *Hosta* plantlet from multiplication media (3 weeks) showing apical meristem (am) and leaf primordia (lp). Calcofluor staining. Scale bar = 0.10 mm.

B. Longitudinal section of a *Hosta* plantlet after 6 weeks at 4°C, showing sequential leaf abscission (in the direction of the arrow), abscission layers (a), disorganised vascular tissue (v), shoot base endodermis (en) and an emerging root (r). Also note the vascular connection of the abscising leaf nearest to the apical meristem and the brighter fluorescing bottom cell layer in the abscission zone. Auramine staining. Scale bar = 0.10 mm.

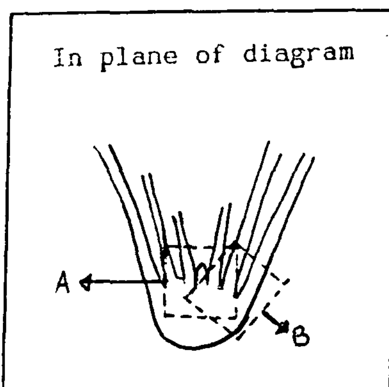
C. Surface view of cultured *Hosta* leaf after 2 weeks at 100% RH, showing closed stomata. Auramine staining. Scale bar = 0.02 mm.

D. Surface view of cultured *Hosta* leaf after 2 weeks at 85%% RH, showing apparently open stomata. Auramine staining. Scale bar = 0.04 mm.

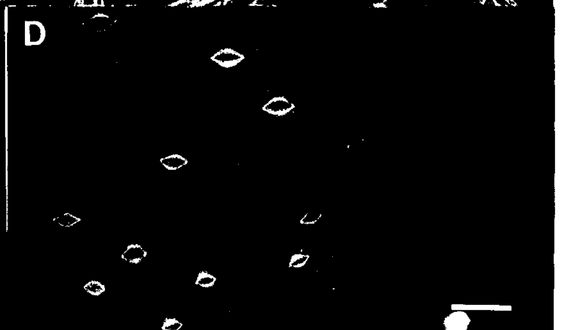
E. Transverse section of cultured *Hosta* leaf after 2 weeks at 100% RH, showing closed stomata, and secondary thickening on both the inside and outside surfaces of guard cells, but not in the external anticlinal walls (*). Also note the presence of a leaf cuticle (c), at least on the top surface of the leaf. Auramine staining. Scale bar = 0.02 mm.

F. Transverse section of a cultured *Hosta* leaf after 2 weeks at 32.5% RH, showing a secondarily thickened area (lenticel?) on the under side of the leaf. Note the air space (a) and the nearby xylem (x) and phloem (p). Auramine staining. Scale bar = 0.04 mm.

Planes of section through plantlets



Hosta Shoots on HX Medium and on HR Medium in Cold Storage and Reduced Humidity



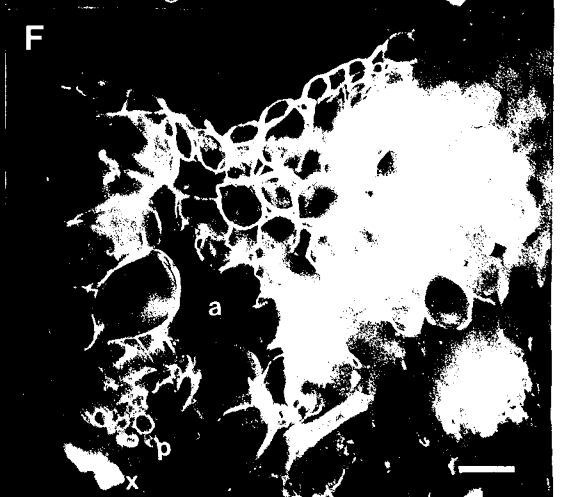


Plate 8: Tracer Experiments

A. Longitudinal section of a rooted *Choisya ternata* plantlet, showing abnormal vascular development (v), suberised (?) intercellular spaces (i) and exodermis (e), and TS of emergent roots (r). Auramine staining. Scale bar = 0.10 mm.

B. Longitudinal section of unrooted *C. ternata* plantlet placed on medium containing calcofluor apoplastic tracer for one week, showing lack of tracer penetration. No staining, viewed using UV filter. Scale bar = 0.10 mm.

C. Transverse section of rooted *C. ternata* plantlet rooted in medium containing calcofluor, showing calcofluor uptake (c) restricted to the vascular system of newly formed roots and possible calcofluor uptake through breaks in the shoot "exodermis" (*). No staining, viewed using UV filter. Scale bar = 0.04 mm.

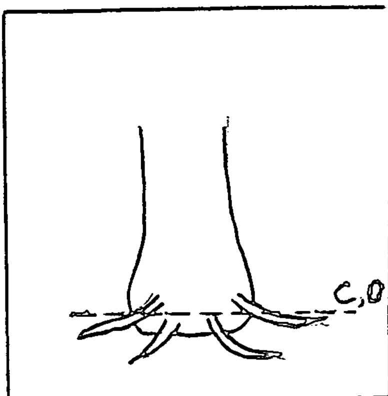
D. As C.

E. Transverse section of *Hosta* plantlet root kept on medium containing calcofluor at 85% RH for 1 week, showing tracer penetration only up to the exodermis (ex). Also note the apparently incomplete formation of the endodermis (en). No staining, viewed using UV filter. Scale bar = 0.10 mm.

F. Longitudinal section, as E. Scale bar = 0.04 mm.

G. Longitudinal section of *Hosta* plantlet shoot base, showing tracer penetration only into the large cells (l) in contact with the medium. Also note the mucilage (m) secretion into the medium. No staining, viewed using UV filter. Scale bar = 0.04 mm.

Planes of section through plantlets



A
r
r
v
r
r
B
C
*
c
*
D
*
E
en
ex
F
ex
G
m

Figure 32. Effect of Calcofluor (0.01%) on Proportion of Rooted *Hosta* Plantlets

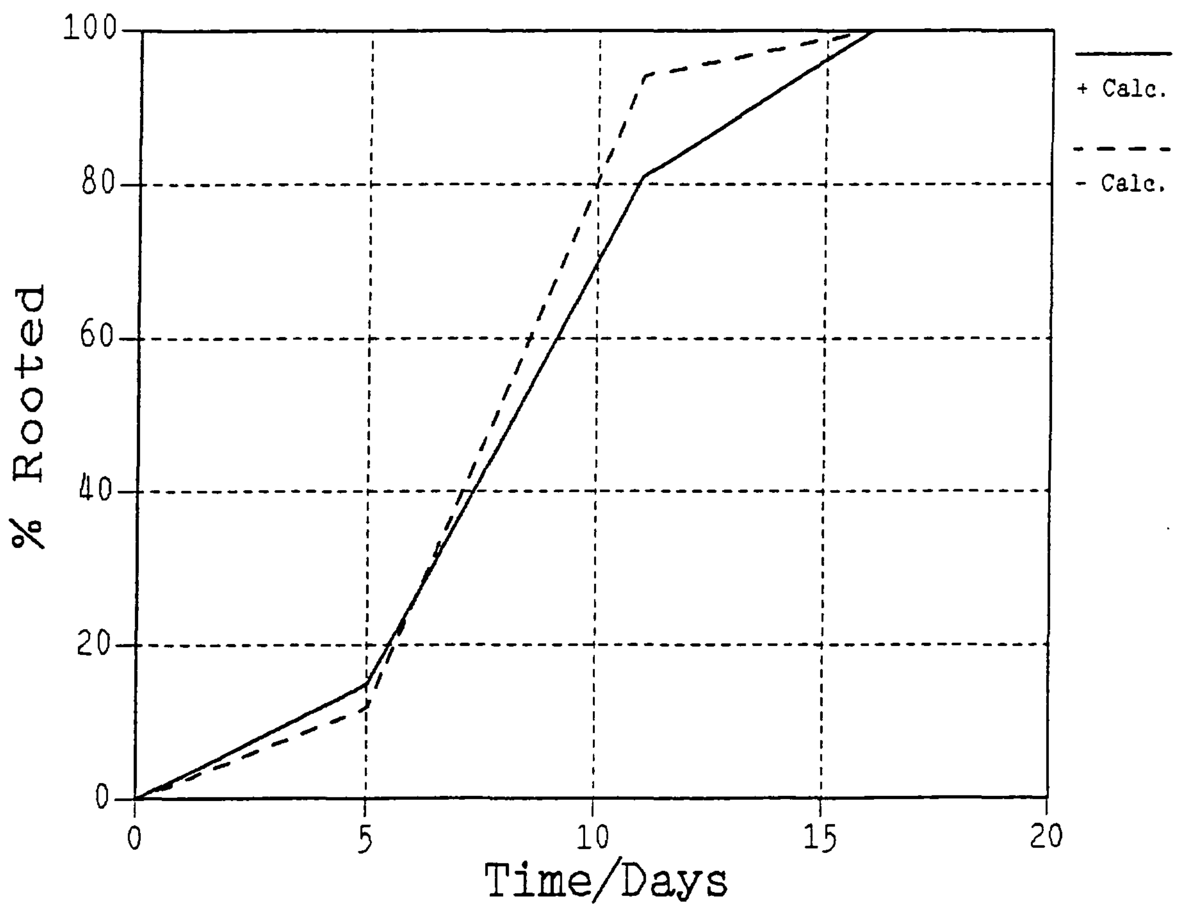
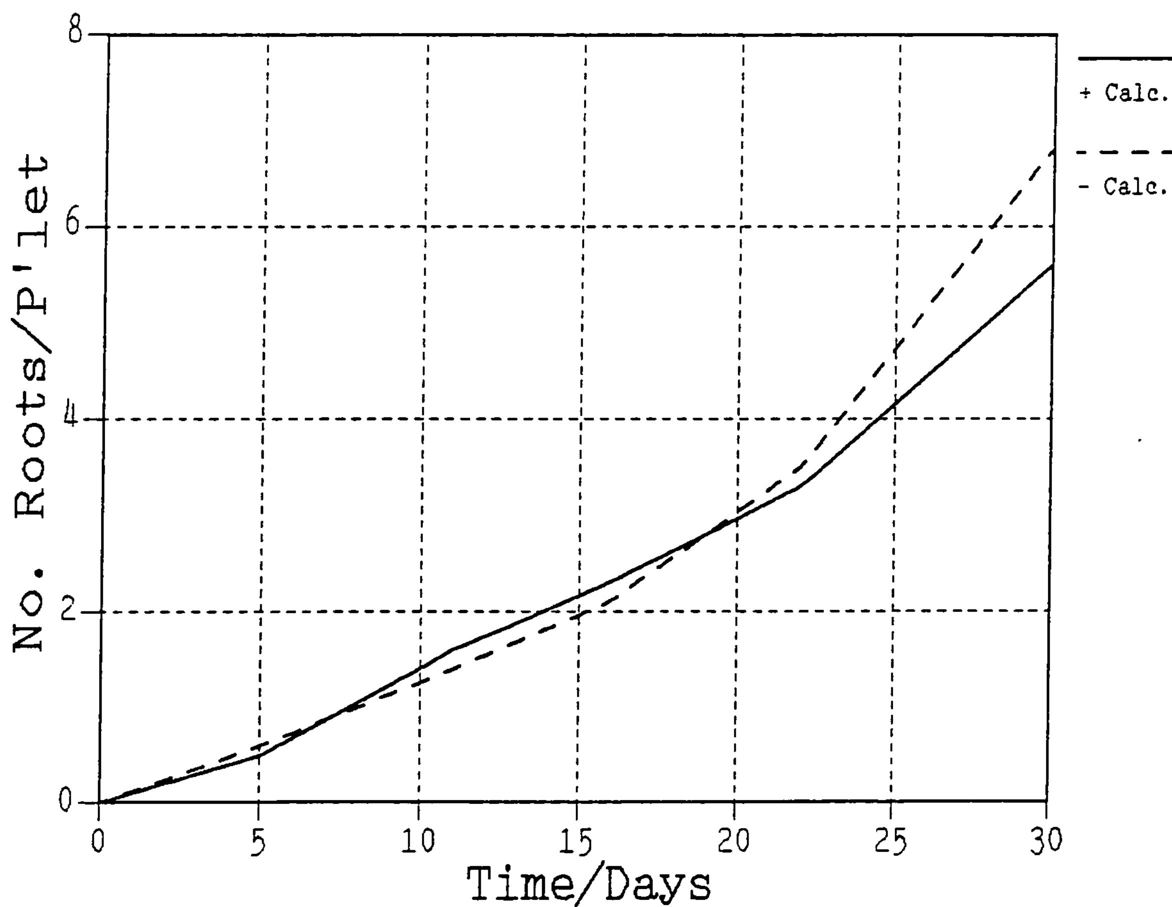


Figure 33. Effect of Calcofluor (0.01%) on *Hosta* Plantlet Root Proliferation



Results

No calcofluor penetrated more than a few cells into the base of the plantlets (Plate 8B), but calcofluor uptake was observed into the vascular tissue near newly emerged roots (Plates 8C/D). No uptake was observed by "adult" roots. No effects on rooting were detected with the presence of calcofluor in the growth media.

3.2.1.3. Experiment 3

Materials and Methods

12 SPHC vessels, containing 200 ml of the saturated solutions; KH_2PO_4 (96% RH), KNO_3 (95.5% Rh), and sucrose (85% Rh) in the outer chamber, and HR medium, with 0.01% calcofluor and without calcofluor in the inner chamber were set up. All glassware and solutions were autoclaved. After the media had cooled and set, one *Hosta* 'August Moon' plantlet directly from multiplying media was placed on the media on the inner compartment of each of the vessels. Three samples for each treatment were used.

Cultures were incubated at 25°C with a 16 hour day, and samples taken weekly. Sections of the plantlets were observed under the fluorescence microscope with the ultra violet filter, to follow the movement of the fluorescent tracer dye calcofluor. Staining with auramine enabled identification of vascular tissue in the sections (using the blue filter). Both stains were visualised together using either a violet or blue-violet filter. Surfaces of leaf material were stained with auramine, and viewed sandwiched between a glass slide and cover slip under the fluorescence microscope (blue filter) to observe any stomata present.

Figure 34. *Hosta* Plantlet Root Proliferation at Different Culture Humidities

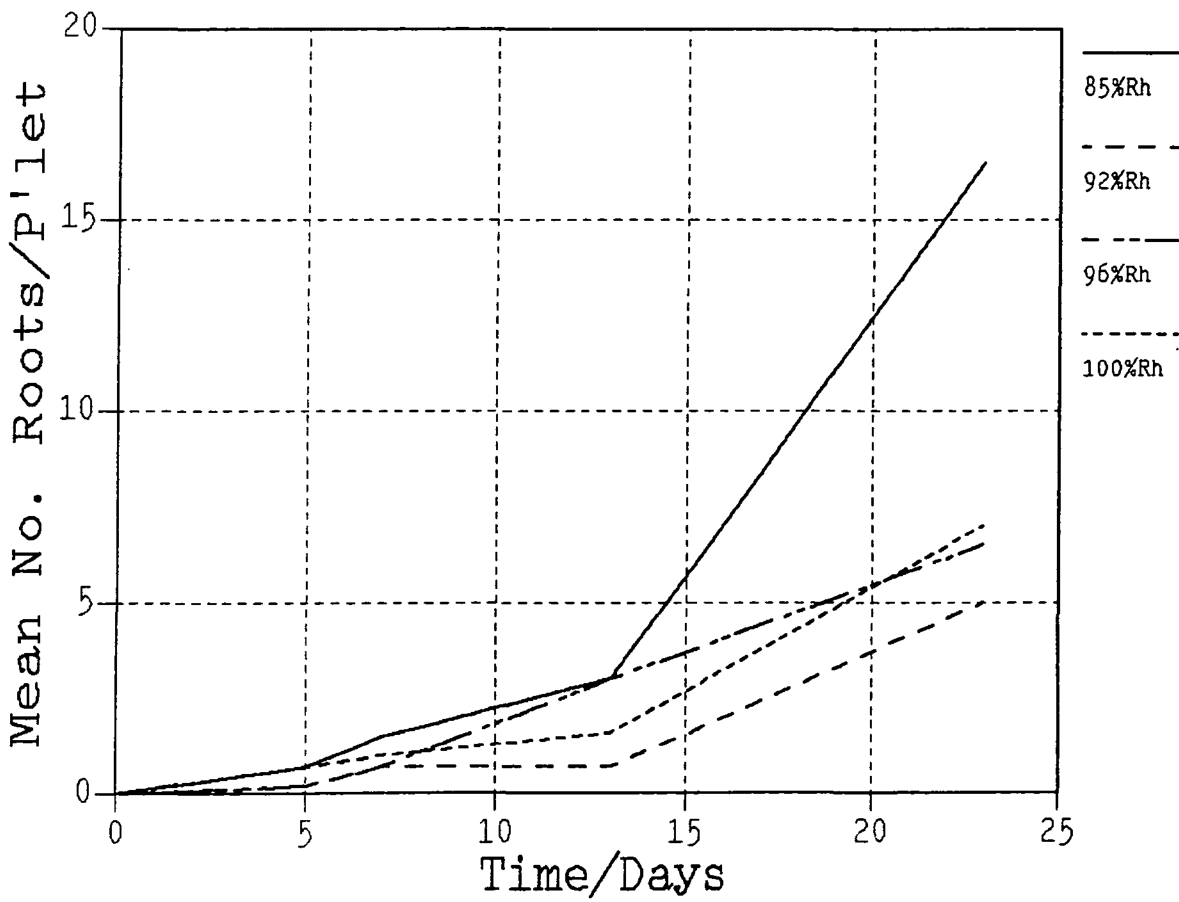
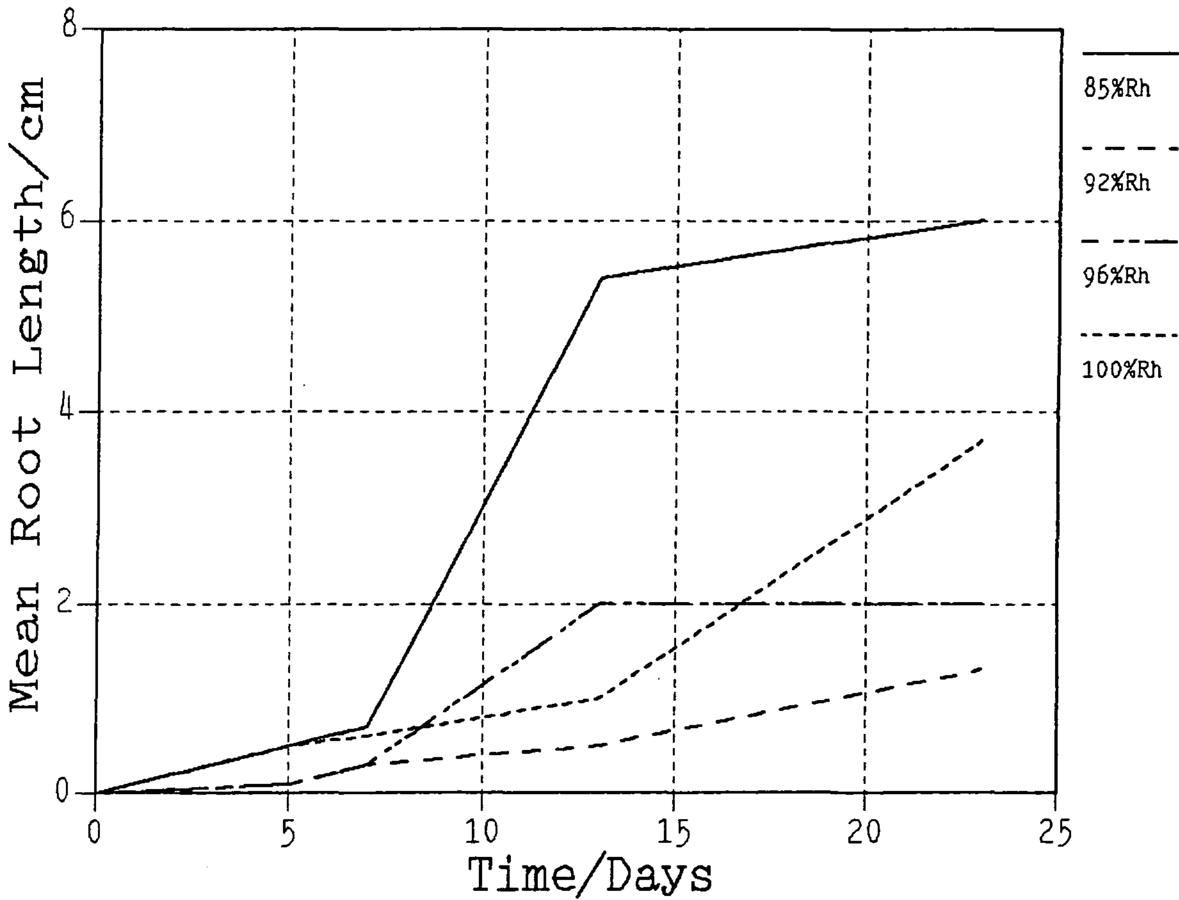


Figure 35. *Hosta* Plantlet Root Elongation at Different Culture Humidities



Results

No uptake of calcofluor was observed into the vascular tissues of the plantlets after three weeks in the range of humidities investigated in this experiment. However, some uptake was observed close to the boundary between the media (containing the calcofluor) and the plantlet. The epidermal cells invariably fluoresced with the characteristic bright blue of calcofluor, and the intensity dropped sharply with distance from the boundary. The epidermal cells in contact with the media were observed to possess what seemed to be a highly developed cuticle, which fluoresced strongly when stained with auramine. In addition to this, the walls of cells two to three cells within the epidermis fluoresced with the yellow of auramine. This phenomenon was also observable in the roots after they had emerged. Cells in contact with the media possessed other distinct characteristics. The cells immediately in contact with the media tended to be greatly enlarged, having up to five times the diameter of the callus/cortical cells (Plate 8G). Behind and in between these there are sometimes columns of small periclinally dividing cells. These cells were much more common at the exact centre of the shoot basal area.

Two forms of root were formed in the media. The earliest roots to form were usually green and sometimes stunted. Later roots are yellowish and possibly longer and thinner.

Closed stomata were observed in 100% RH cultures (Plates 7C & E), but stomata were apparently open in 85% RH cultures (Plate 7D).

3.2.1.4. Experiment 4

Materials and Methods

The same methods as experiment 3 were used with the saturated salt solutions in Table 8. Three samples of each treatment were used, but no control without calcofluor as 0.01% calcofluor had no adverse affects in experiments 1-4.

Table 8. Relative humidity of selected saturated salt solutions

Saturated Salt Solutions	% Relative Humidity
Distilled Water*	100.0
KH_2PO_4	96.0
KNO_3*	92.5
$ZnSO_4$	88.5
KCl*	85.0
$(NH_4)_2SO_4$	80.0
NaCl*	75.0
NaCl + KCl	71.5
$CaCl_2$	67.0
NaCl + Sucrose*	63.0
Glucose	55.0
$Ca(NO_3)_2$*	50.5
$MgCl_2$*	35.2

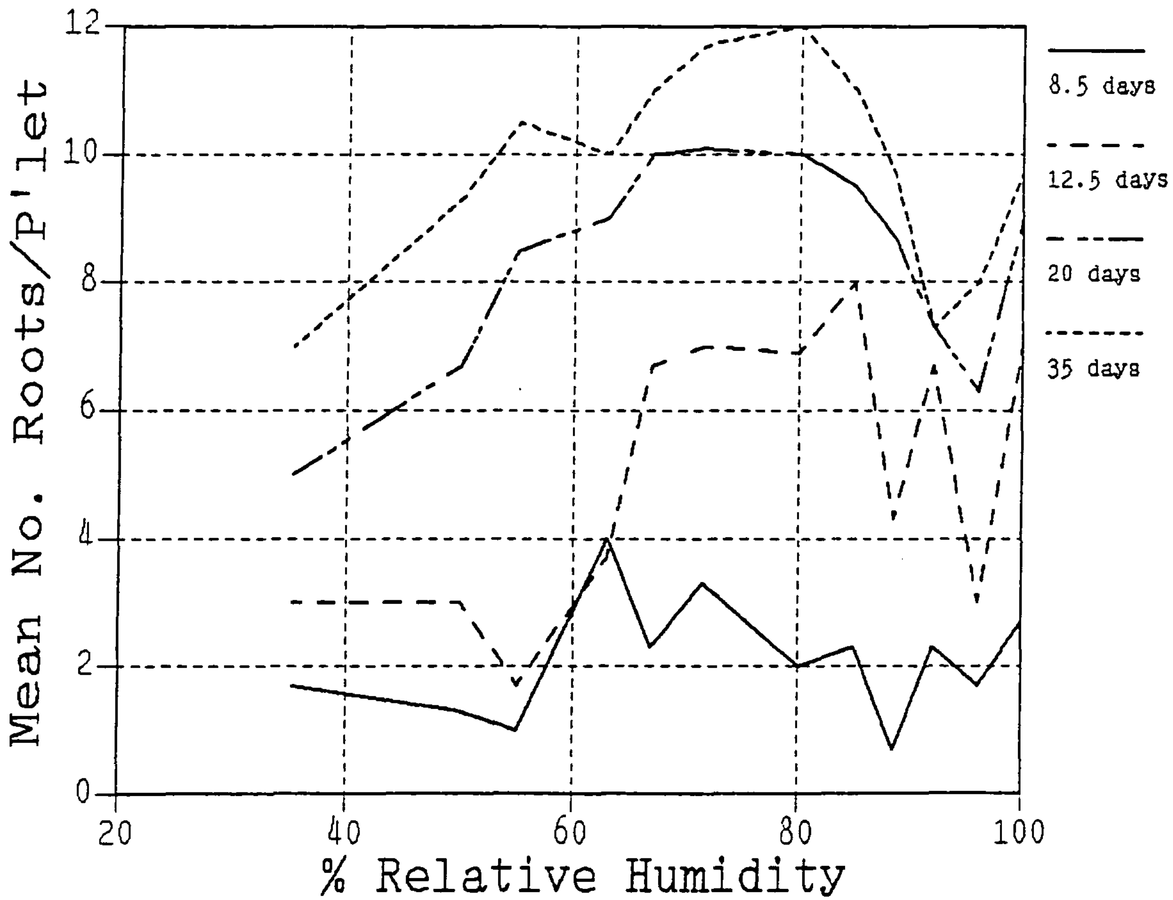
* Treatments for Experiment 5.

Results

No calcofluor was detected in vascular tissue and the only penetration observed was a few cells from the plantlet base as in plate 8G, and the epidermis of roots (Plate 8E). What seemed to be lenticels were observed on the under-side of leaves in very low humidity samples (Plate 7F).

Root proliferation was inhibited by a reduction in culture humidity from 100% to 95%, below which rooting was promoted up to an optimum at 80% RH, and then inhibited again at humidities below 80% RH (figure 36).

Figure 36. Effect of a Wide Range of Culture Humidities on Root Proliferation in *Hosta* Plantlets



3.2.1.5. Experiment 5

Materials and Methods

Experiment 5 was repeated with the treatments asterisked in table 8). Six SPHC apparata were utilised for each treatment, half of which were used to assess plantlet water potential with the "smear" method (section 2.3.1.) on day 6, and the other half for proline assays (section 2.4.) on day 9.

Results

An inverse relationship between culture RH and plantlet water potential was observed (figure 37). Optimum root proliferation was coincident with plantlet water potentials of -10 to -15 bars. An inhibition of rooting was experienced between -4 and -5 bars and below -15 bars (figure 38). Proline was accumulated (or concentrated by plantlet dehydration) with decreasing plantlet water potential (figure 39). In fact a logarithmic relationship appears to exist, with proline increasing significantly below -15 bars (figure 40)

3.2.1.6. Experiments 6

Materials and Methods

A 2 mm layer of the basal callus of 10 unrooted Hosta plantlets was removed using a new double edged razor blade. These were then immediately inserted into rooting medium containing 0.01% calcofluor, in SPHC's containing a saturated solution of NaCl (75% Rh). Two plantlets were examined after 15 minutes, and 1, 3, 6, 24, and 48 hours under the fluorescence microscope using the UV filter.

Results

After 15 minutes, the calcofluor had penetrated a few cells deep, and after 3 hours had permeated the whole of the basal callus.

Figure 37. Relationship Between Culture "Humidity" and *Hosta* Plantlet Water Potential on Day 10 of Subculture

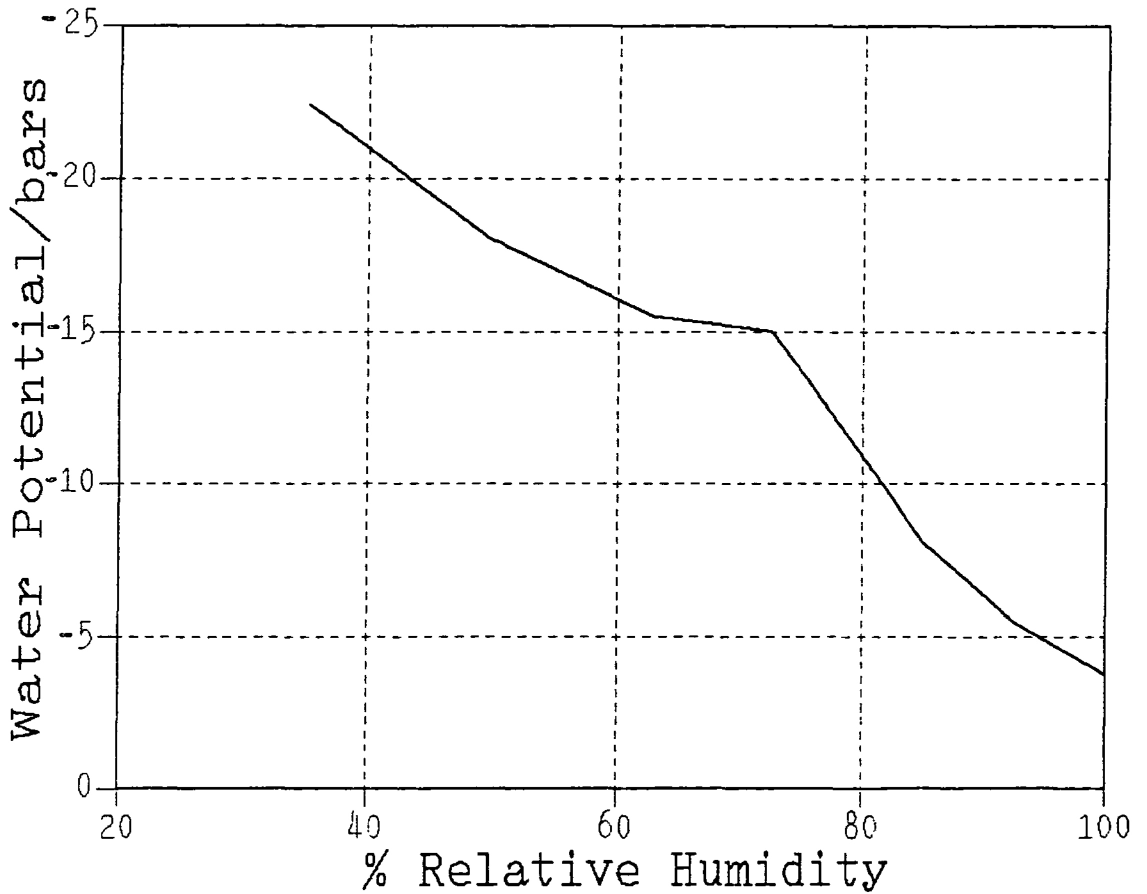


Figure 38. *Hosta* Plantlet Water Potential on Day 10 and Subsequent Number of Roots Produced on Day 35

Figure 39. Relationship Between *Hosta* Plantlet Water Potential on Day 10 and Proline Content on Day 9

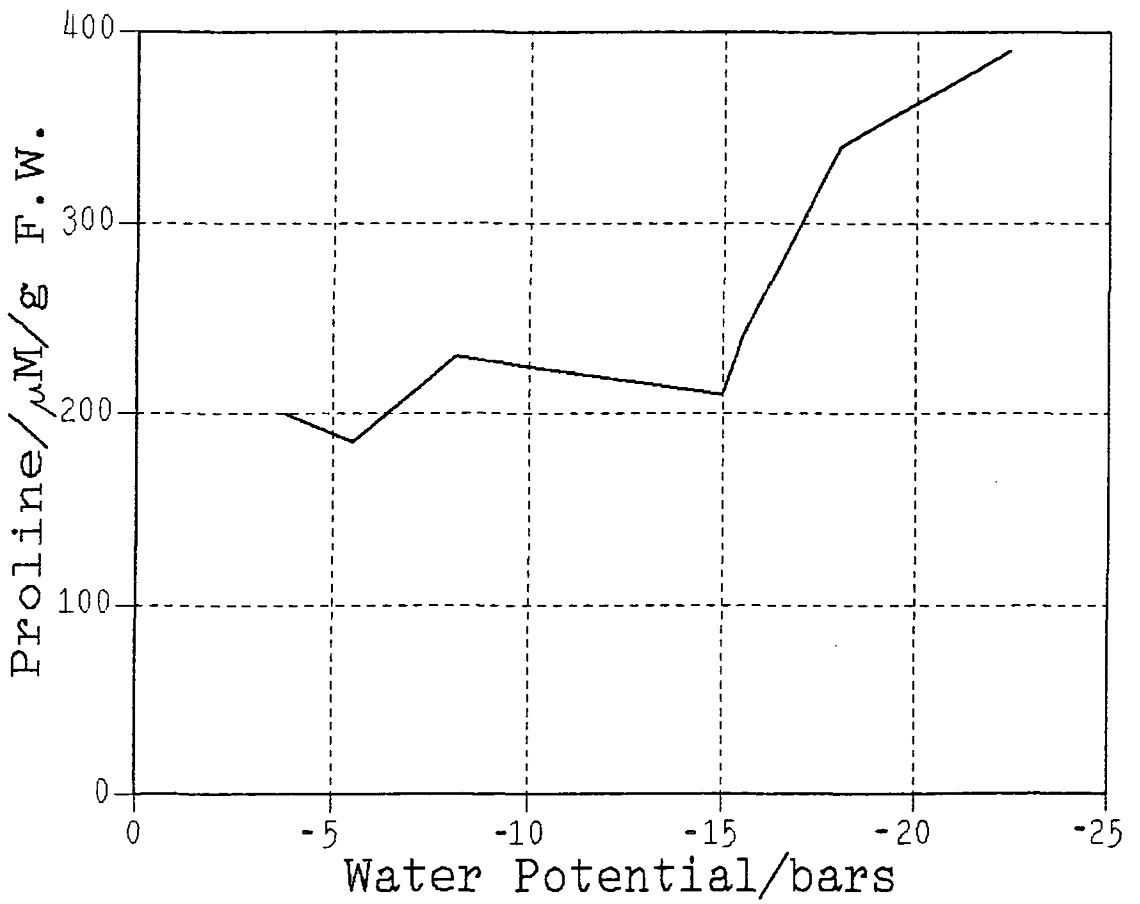
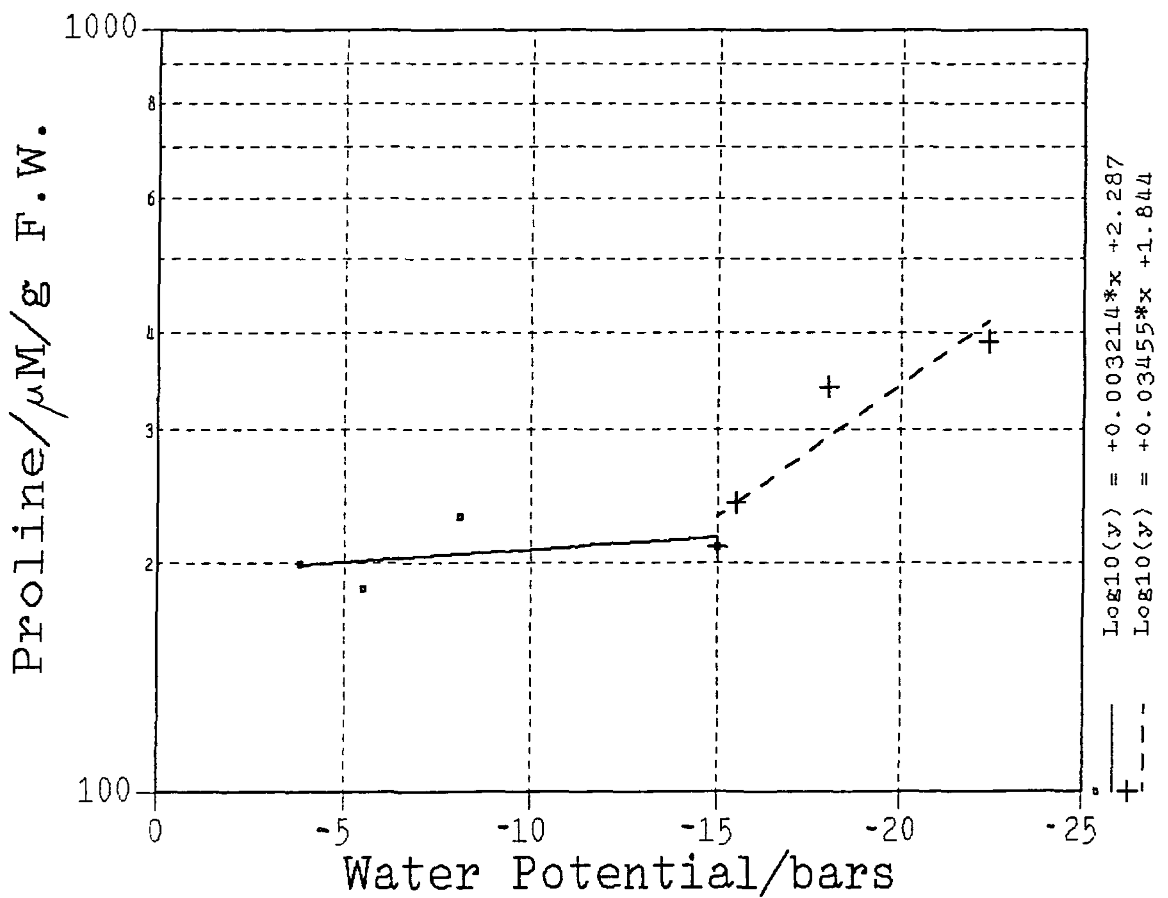


Figure 40. Logarithmic Relationship Between *Hosta* Plantlet Water Potential on Day 10 and Proline Content on Day 9, Showing Proline Accumulation Below -15 Bars



3.2.1.7. Large Scale Culture Of Hosta at Reduced Humidity

Materials and Methods

MPHC apparatuses were used to culture larger amounts of Hosta under reduced humidity. A saturated solution of sucrose was used to obtain 85% R.H., and distilled water to obtain 100% R.H. Both were autoclaved along with the HR media, and poured under sterile conditions in a laminar flow cabinet into the bottom of the evaporation dish until the level was just below the bottom of the petri dish. 5 replicates of 25 explants of Hosta from multiplication media were placed in each petri dish. They were then incubated at 25°C on a 16 hour day. A control of 5 tubs of "normally" (not in SPHC or MPHC) cultured plantlets was used.

Results

No significant difference in rooting was observed between the controls and the 100% Rh treatment (data not shown). The growth media in the 85% Rh treatment dried out and plantlets died after 10 days.

3.2.1.8. Prevention of Media Dehydration

Methods

5 ml of either paraffin wax or Rape seed oil were autoclaved and poured on top of solidified growth media in 6 MPHC's using saturated NaCl (75% Rh). With the paraffin wax, small holes in the set wax were cut using a scalpel, and unrooted Hosta plantlets were then pushed into the media below. Rape seed oil was poured onto the growth medium after the plantlets had been placed on the media.

Results

The Rape seed oil treatment plantlets wilted and died within 5 days. The paraffin wax treatment survived 20 days before the media dried out.

3.2.2 Hosta Dormancy

3.2.2.1. Hosta Leaf Abscission

Materials and Methods

5 tubs of Hosta 'Francie', and 'Golden Prayer' on rooting media, were placed in the cold store (2°C), and sampled initially after a week, and then every 2 weeks. Plantlets were examined using epi-fluorescence microscopy, utilising double staining with Auramine and Calcofluor. A photographic record of any anatomical changes was kept.

Results

Auramine proved to be effective in visualising the abscission layer at the base of senescing Hosta leaves, (see Plate 7B). Abscission layers became visible in the outer, older leaves, before they appeared to be senescing, and their appearance advanced inwards until reaching the innermost, youngest leaves, in which no abscission layers were observed either in this experiment, or in the period of study (Plate 73).

Visible senescence (Plate 9A), in the form of leaf yellowing, closely followed the formation of an abscission layer in a particular leaf.

3.2.2.2. Cold Induced 'Dormancy' and AAT Pattern

Materials and Methods

The AAT isoenzyme profiles of 7 varieties of cold stored *Hosta* were analysed.

Two tubs of rooted *Hosta* plantlets of 3 different varieties (148; 'August Moon', 259; 'Blue Wedgewood' and 250, 'Francie') were placed in the cold (4°C) from the growth room. AAT isoenzymes were analysed before placing in the cold and after 1 or 2 weeks in the cold.

10 tubs of rooted *Hosta* (var. '325') plantlets were placed in cold store (4°C in the dark), from the growth room, (25°C, continuous light). Plantlets were then moved back to the growth room after 3 days and

Plate 9: *Hosta* Dormancy in Culture and the Nursery

A. *Hosta* plantlets after 1 week at 11/16°C. Note the senescing leaves.

B. *Hosta* plantlets before going into cold storage. Note the greener leaves in comparison to A.

C. Weaned *Hosta* plantlets in the nursery. The plants nearest to the camera have been cold stored (11/16°C) for 2 weeks, whereas the further plantlets have been cold stored for 21 weeks. The "1 week" plants have been in the nursery for 28 weeks, and the "20 week" plants for 9 weeks.

D. As C., in detail.

E. weaned 1 week cold stored plantlets taken out of their containers. Note the storage roots emerging from the compost (cf. F).

F. Weaned 20 week cold stored plantlets taken out of their containers. Note the lack of thick storage roots and the taller, greener leaves in comparison to E.

Hosta Dormancy in Culture and the Nursery

returned to the cold room after a further 3 days. AAT isoenzymes were analysed every day.

Results

Two groups of AAT isoenzymes were identified. The first, low mobility isoenzymes were only expressed when plantlets had been in cold storage for more than 3 days, and disappeared after 2-3 days in the growth room (see figure 42a/b; plate 10). This group of isoenzymes were designated AAT-A's (1-7), and the more mobile isoenzymes, which were present in all samples were designated AAT-B's (1-4). Although not tested, it is assumed that the same phenomenon occurs in the three other varieties, which also possessed AAT-A and AAT-B isoenzymes.

Both sets of isoenzymes are assumed to be mitochondrial in origin (see section 1.3.5.2. & 2.6.1.4.2.- method 3).

3.2.2.3. Hosta 'Dormancy' and α Esterase Patterns

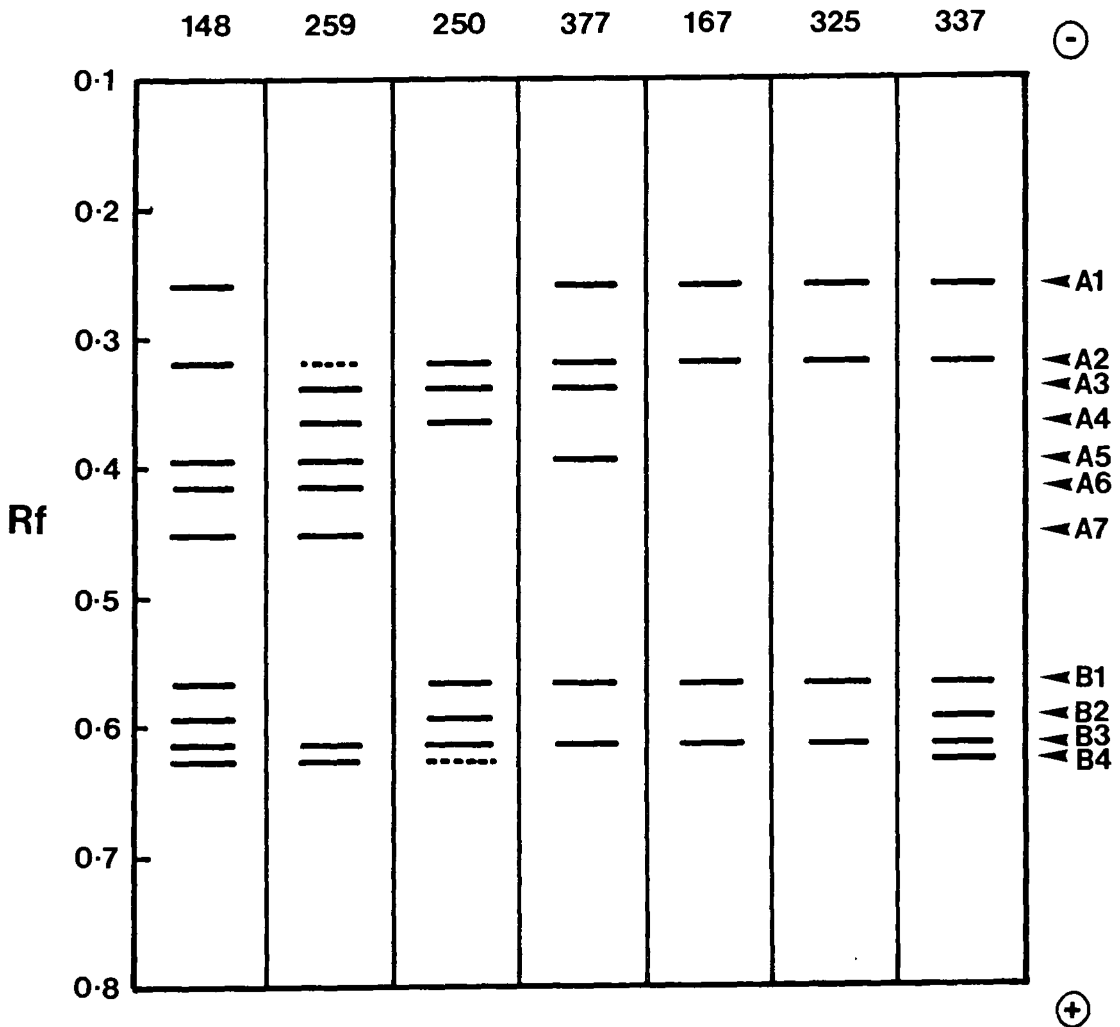
Materials and Methods

5 tubs of Hosta 'Golden Prayer' were placed in the cold store, and sampled every week for AAT and Esterase isoenzymes. After 5 weeks, the remaining tubs (2) were placed in the growth room for 1 week, sampled and then returned to the cold store, and sampled a further two times.

Results

After 3 weeks in the cold store, the three highest Rf esterase isoenzymes disappeared and did not re-appear during the course of the experiment (data not shown). The AAT isoenzymes followed the same pattern as Section 3.2.2.2..

Figure 41. AAT Profiles of 7 Varieties* of Rooted, Cold Stored *Hosta* Plantlets



* 148 = 'August Moon', 259 = Blue Wedgewood, 250 = 'Francie', 377 = 'Golden Prayer'. Other varieties are only known by their code numbers by the author.

Figure 42a AAT Profiles of 3 Varieties* in the Warm (25°C) and Cold (4°C)

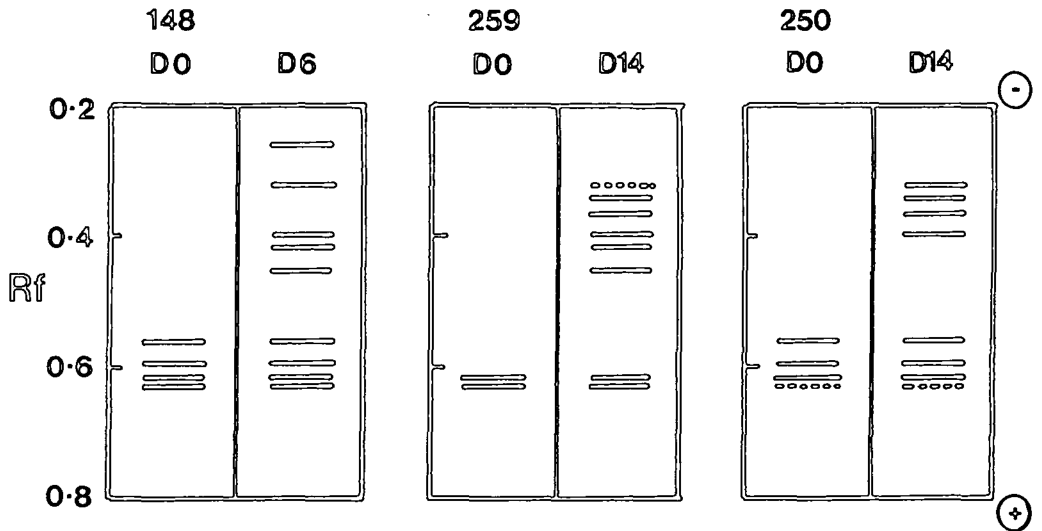
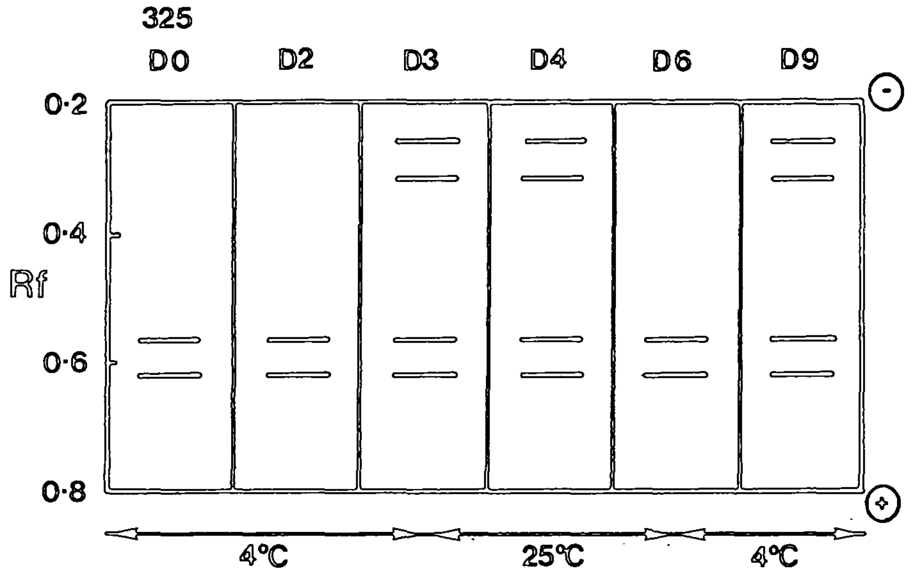


Figure 42b AAT Profiles of *Hosta* '325' During Warm/Cold Transfers



* See figure 41

3.2.2.4. Major Experiment 1

Materials and Methods

Two varieties of Hosta were used: Blue wedgewood
Francie (variegated)

400 plantlets on rooting media (10 per tub) were placed in the cold room at Neoplants (11°C/16°C), after 3 weeks in the growth room. An initial sample of 10 plantlets was prepared as a standard sample for electrophoresis. Samples of 4 tubs were taken back up to Durham every 2 weeks for 18 weeks.

These were analysed in the following way:

2 plantlets were removed under sterile conditions from each tub, (8 plants) for the electrophoresis experiments: a) Esterase, b) Total protein (coomassie blue), c) Aspartate aminotransferase. (see section 2.6.)

One plantlet from each tub was examined under the fluorescence microscope, and any changes in anatomy noted. The remaining plantlets (30), were placed in the growth room, and their ability to re-grow assessed by counting fully green leaf number at 5 day intervals.

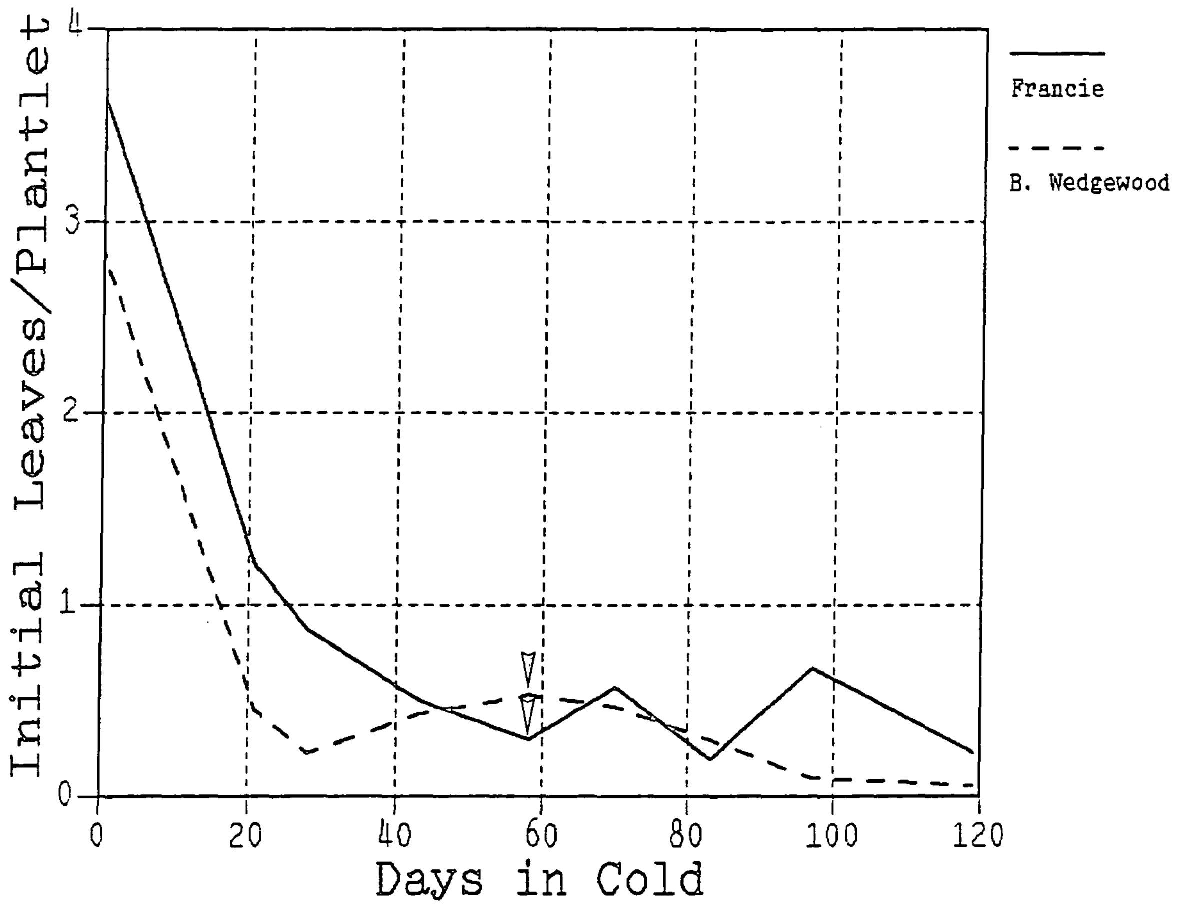
Results

3.2.2.4.1.. Dormancy Acquisition and Release

The leaf number at the point of removal from the cold store (and transfer to the growth room, 25°C), was plotted against the length of time the samples had been in the cold room, (Figure 43).

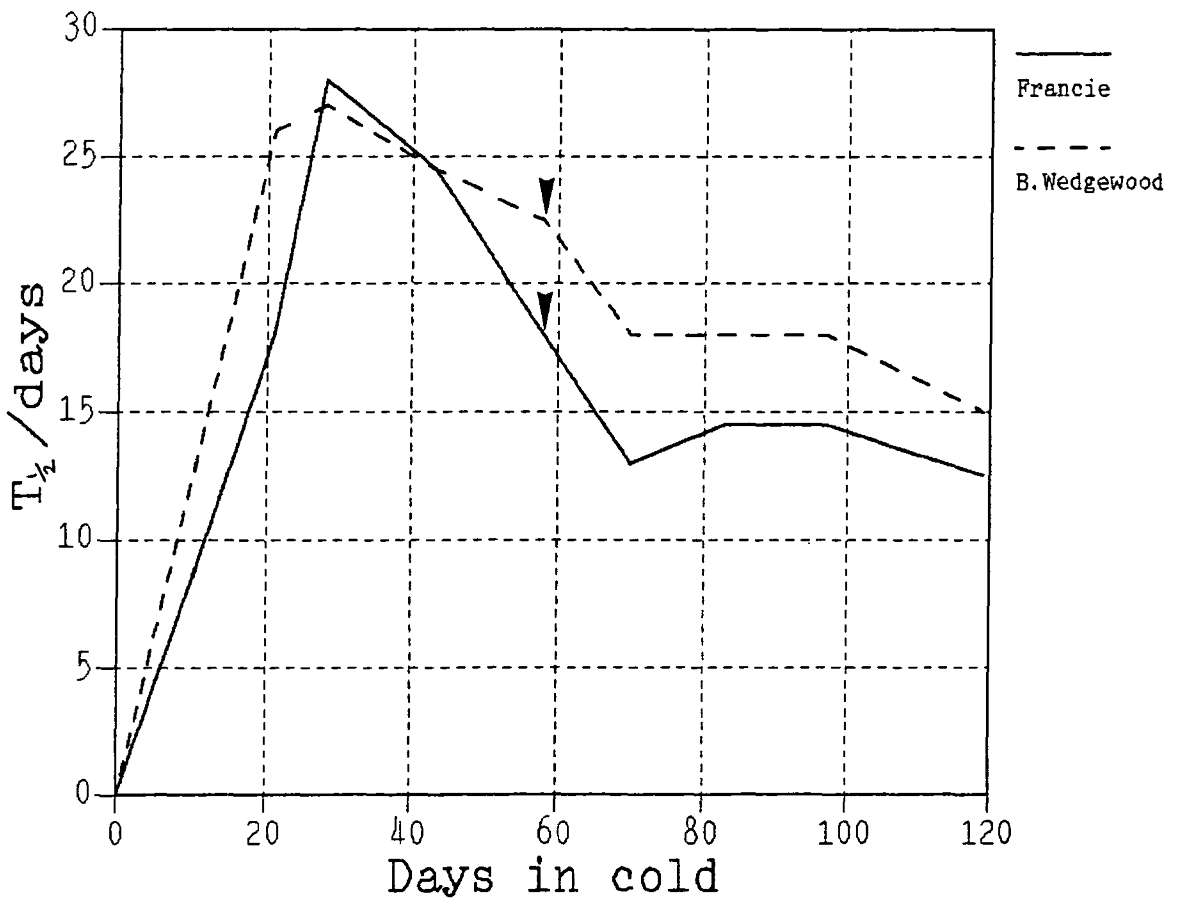
Leaves re-grown in the growth room after cold storage appeared to be longer and thinner than the original leaves, but this is a subjective observation, and was not quantified.

Figure 43. *Hosta* Leaf Senescence in Culture at 11/16°C



Arrows indicate re-appearance high Rf Esterase isoenzymes

Figure 44. *Hosta* Culture "Dormancy" at 11/16°C



Arrows indicate re-appearance high Rf Esterase isoenzymes

To obtain a graphical representation of the rate of the re-growth of leaves in the growth room after differing cold treatments, the initial number of leaves (Li; figure 43), was subtracted from the maximum leaf number attained (Lm). Leaf re-growth data was then adjusted by this figure, and plotted (not shown). The time to reach half the maximum leaf number ($T_{½}$) was then determined using these graphs. The $T_{½}$ of each sample was then plotted against time in the cold (figure 44). It should be noted that the higher the value of $T_{½}$, the slower the rate of growth, and the deeper the level of "dormancy".

Table 9. Dormancy data for Hosta var. 'Francie' (variegated leaves)

Days in cold	Lm*	Li+	Lm-Li	½(Lm-Li)	$T_{½}$°
0	3.65×	3.65	0	0	0
21	3.64	1.20	2.44	1.22	18.0
28	4.03	0.87	3.16	1.58	28.0
43	3.95	0.50	3.45	1.73	24.5
58	3.73	0.30	3.43	1.72	18.0
70	3.10	0.57	2.53	1.27	13.0
83	3.20	0.20	3.00	1.50	14.5
97	3.43	0.67	2.76	1.38	14.5
119	2.97	0.23	2.74	1.37	12.5

* Lm = Maximum leaf number achieved in growth room
+ Li = Initial leaf number when placed in growth room
° $T_{½}$ = Time taken to reach half the overall gain in leaf number.
× All figures are leaf number per plantlet (mean of 30)

Table 10. Dormancy data for Hosta Var. 'Blue Wedgwood'

Days in cold	Lm*	Li+	Lm-Li	½(Lm-Li)	$T_{½}$°
0	2.85	2.85	0	0	0
21	2.20	0.45	1.75	0.88	26.0
28	2.60	0.23	2.37	1.18	27.0
43	2.80	0.43	2.37	1.18	24.5
58	2.47	0.53	1.94	0.97	22.5
70	2.37	0.47	1.90	0.95	18.0
83	2.37	0.30	2.07	1.04	18.0
97	2.70	0.10	2.60	1.30	18.0
119	2.33	0.06	2.26	1.13	15.0

* See Table 8

The initial leaf number (L_i) declined rapidly in the first two weeks of cold store, and then continued to decline more slowly throughout the period of cold store (see fig. 43). The centermost leaves, and the shoot meristem remained green throughout the experiment. The basal callus tissue also avoided senescence, and was seen to contain some dividing cells, indicating that either slow growth was continuing in the cold, or that development was simply arrested almost immediately after entry into the cold, ready for continued growth when conditions are conducive to growth. Roots of all varieties grew in the cold room. This was confirmed by marking root tip positions on the base of tubs.

The rate of leaf re-growth reached its lowest level after 30 days in the cold for both varieties, after which it increased until day 70, when it appeared to stabilise (figure 44).

3.2.2.4.2. Dormancy 'Markers'

In both varieties, the 3 high Rf ('Francie': 0.81, 0.85, 0.88, and 'Blue Wedgewood': 0.80, 0.83, 0.86), isoenzymes disappeared by day 28, (see fig. 47, which shows a similar effect at 4°C), and the isoenzyme with the lowest Rf, (0.81 and 0.80 respectively) of the group re-appeared by day 58. The disappearance of the bands coincided with the peak of 'dormancy', and the re-appearance with a decline in 'dormancy' (arrows in figure 44).

The changes in AAT patterns in both cases were very similar to the previous results at 4°C, with the AAT-A isoenzymes appearing soon after the plantlets entered the cold (data not shown).

Total protein changes through the course of cold store were very complex and difficult to interpret. In both cases there was a peak in the intensity of a protein (approximately 50,000 rmm) when plantlets were first

placed in the cold (refer to Plate 11B). No other trends could be deduced from the gels.

3.2.2.5. Major Experiment 2

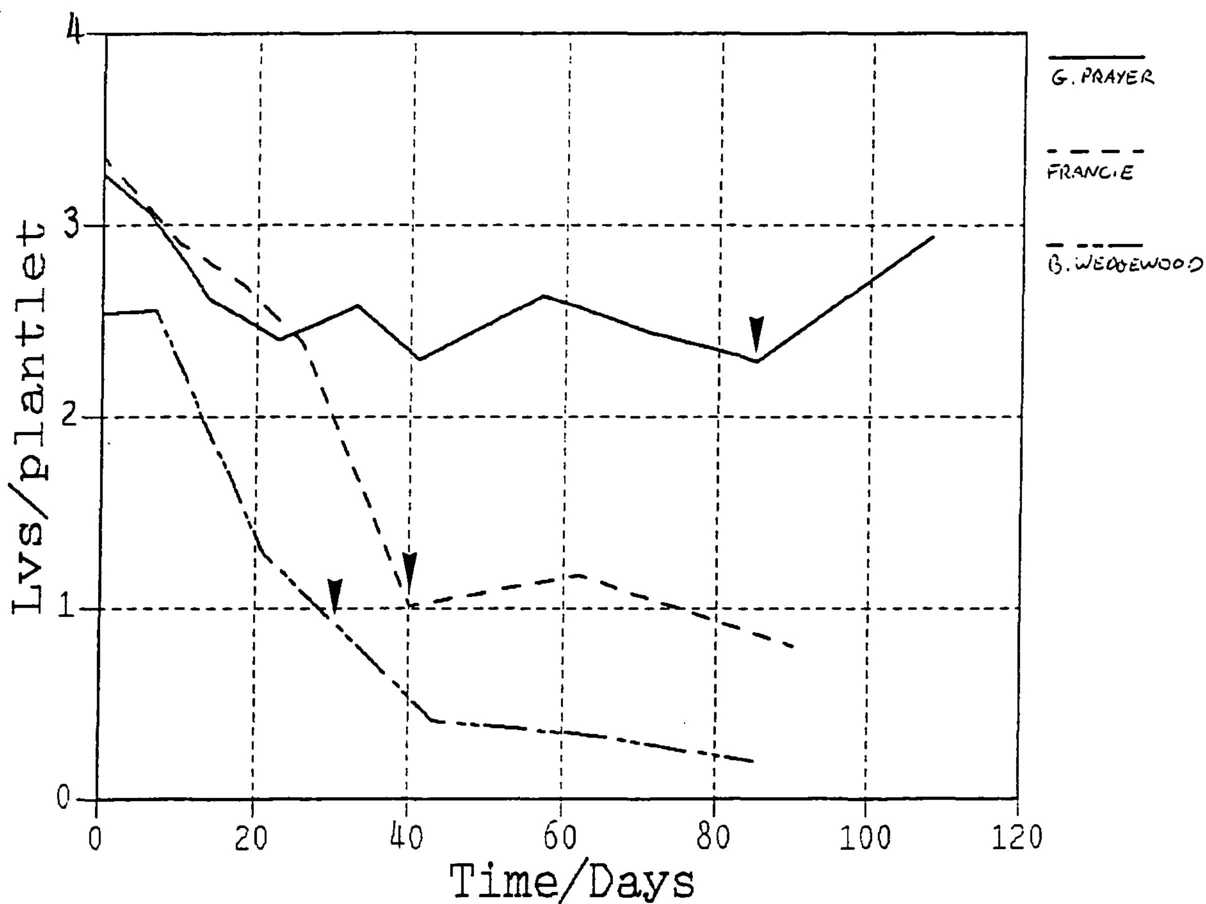
Materials and Methods

This experiment was performed entirely at Durham, to test the results of the first experiment. Three varieties were used: Francie, Golden Prayer and Blue Wedgewood. Experimental details were similar to the first, except in the following ways. Cold store temperature was 4°C, as opposed to 11/16 °C. Plantlets were placed into the cold store after only one week on rooting media, as opposed to 3 weeks. This reflected the changed practises of Neoplants, and allowed the plantlets to be subcultured onto new rooting media when they were removed from the cold store. This was due to a minimal root growth and elongation, and ruled out the possibility that growth media nutrient depletion was responsible for the results observed in expt 1.

Results

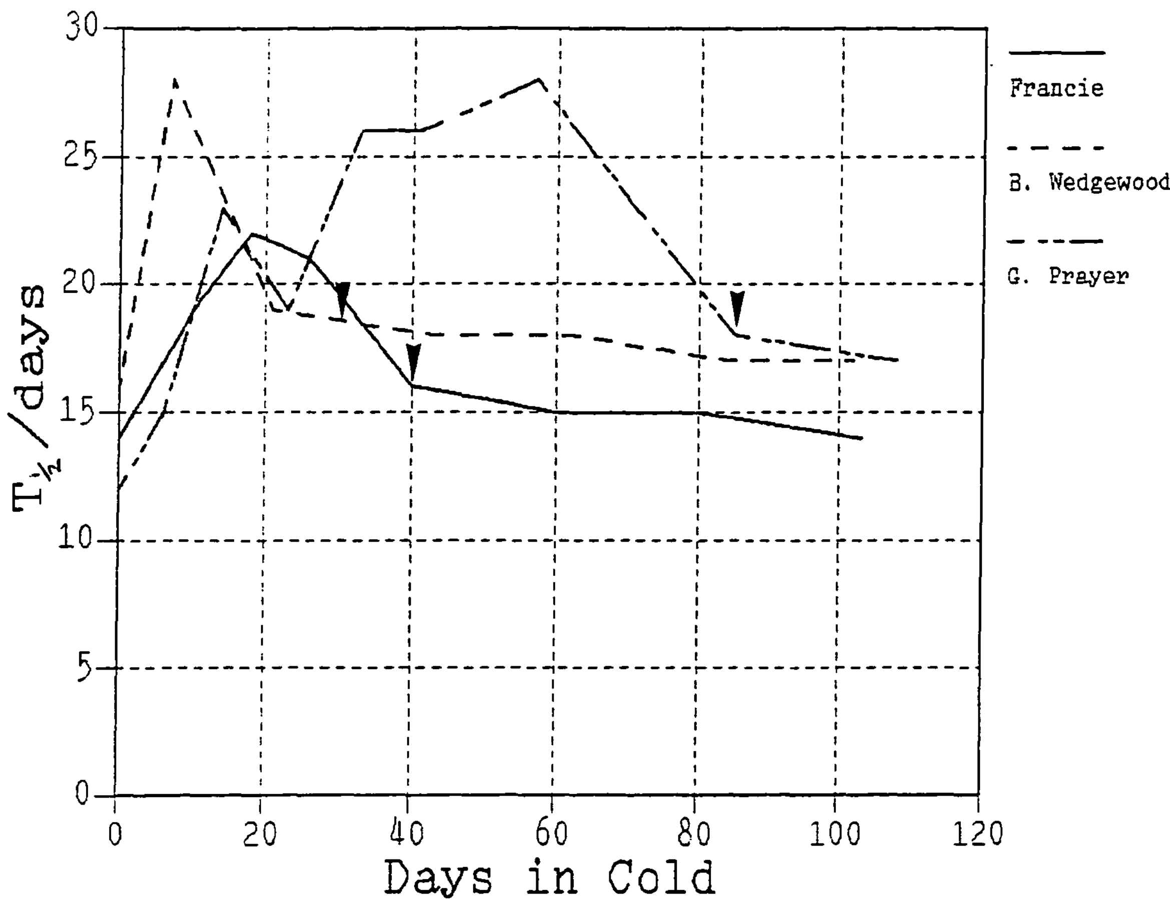
Growth data was analysed in the same way as Experiment 1, and presented in graphical form in figures 45 & 46. As in Experiment 1, high Rf esterase isoenzymes appeared to be coincident with "dormancy" loss (figure 46). The leaf number in cold store decreased rapidly in the first 40 days with 'Francie' and 'Blue Wedgewood', and continued to decrease more slowly until the end of the experiment. The leaf number with 'Golden Prayer' decreased initially, then levelled off at a level significantly higher than the other two varieties. By day 100, there appeared to be some re-growth of 'Golden Prayer' leaves at 4°C (figure 45).

Figure 45. *Hosta* Leaf Senescence in Culture at 4°C



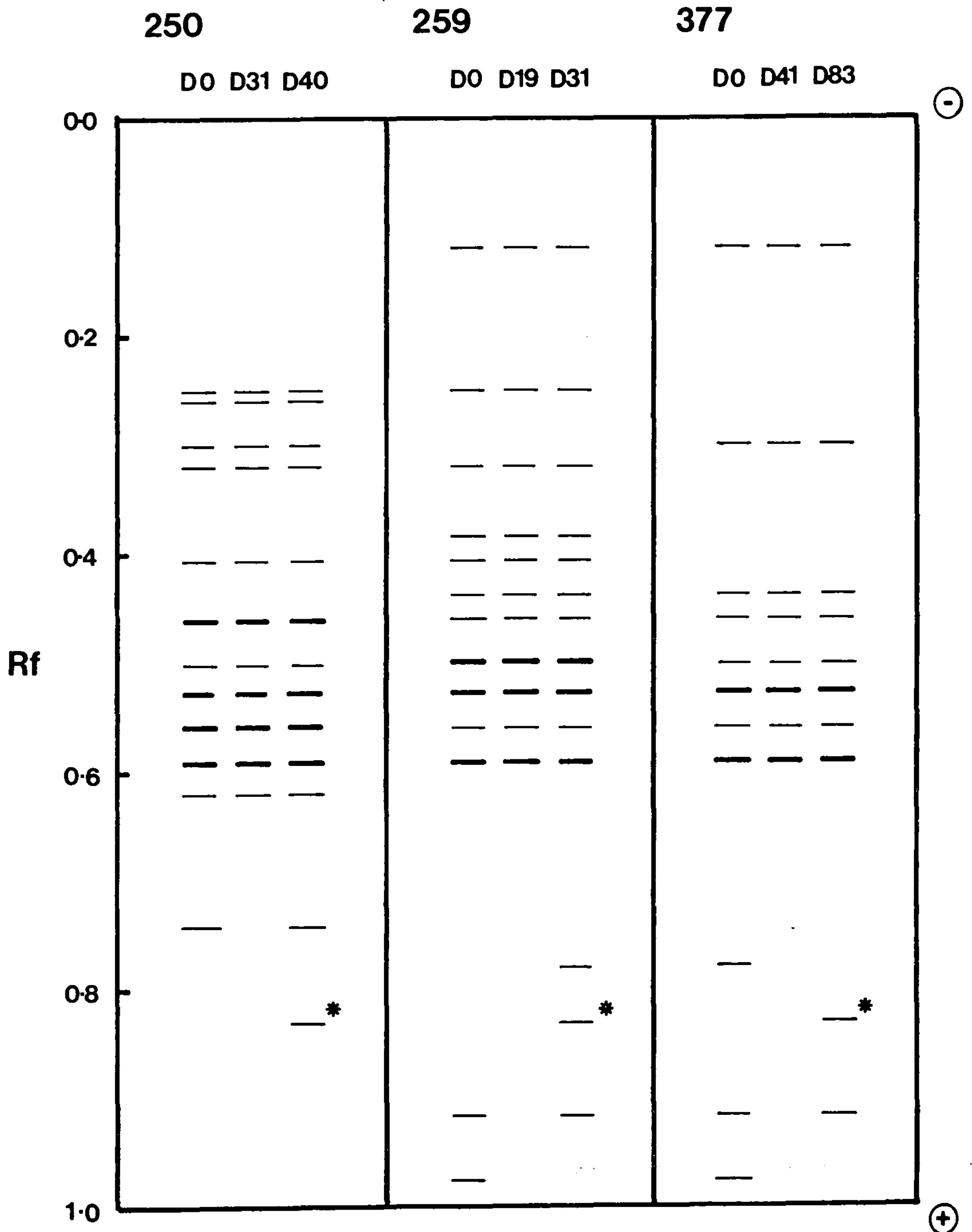
Arrows indicate re-appearance high Rf Esterase isoenzymes

Figure 46. *Hosta* Culture "Dormancy" at 4ºC



Arrows indicate re-appearance high Rf Esterase isoenzymes

Figure 47. Changes in Esterase Patterns Though Cold (4°C) Storage for 3 Varieties* of *Hosta*



* 250 = 'Francie', 259 = 'Blue Wedgewood' and 377 = 'Golden Prayer'

Plate 10. Isoenzyme Gels

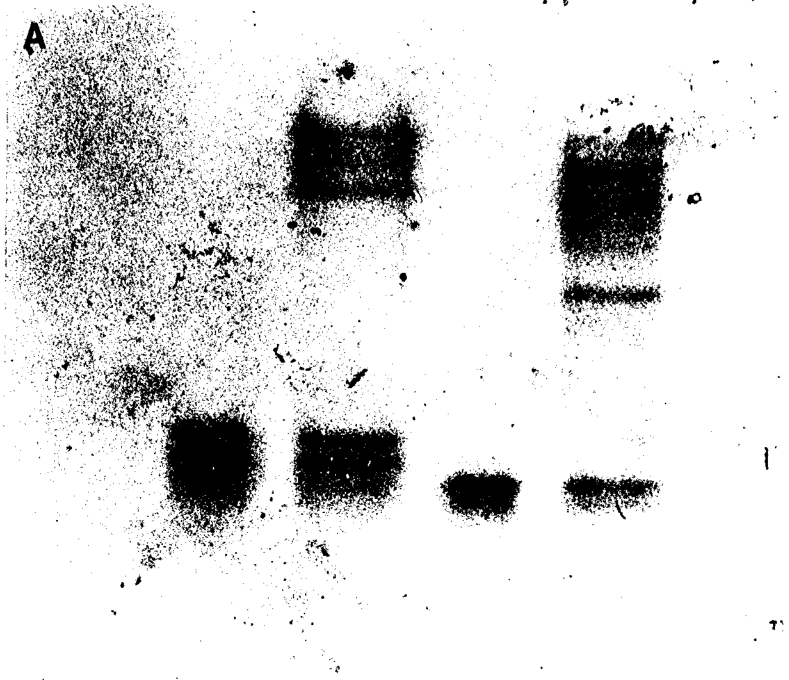
A. AAT isoenzymes in *Hosta* 'Francie' (250) and 'Blue Wedgewood' (259) rooting cultures from the growth room at 25°C (tracks 1 & 3 from the left respectively) and after 14 days in the cold at 4°C (tracks 2 & 4 from the left).

B. Esterase isoenzymes in *Hosta* 'Francie' (250) through a period of cold storage (4°C). Arrows indicate position of "marker" isoenzyme. From the left, track 1: 0 days in the cold, 2: 9 days, 3: 19 days, 4: 23 days, 5: 31 days, 6: 40 days

C. As B, but 'Blue Wedgewood' (259). From the left, track 1: 0 days in the cold, 2: 9 days, 3: 23 days, 4: 31 days, 5: 40 days, 6: 60 days, 7: 83 days.

D. As B, but 'Golden Prayer' (377). From the left, track 1: 0 days in the cold, 2: 9 days, 3: 23 days, 4: 60 days, 5: 83 days.

Plate 10: Isoenzyme Gels



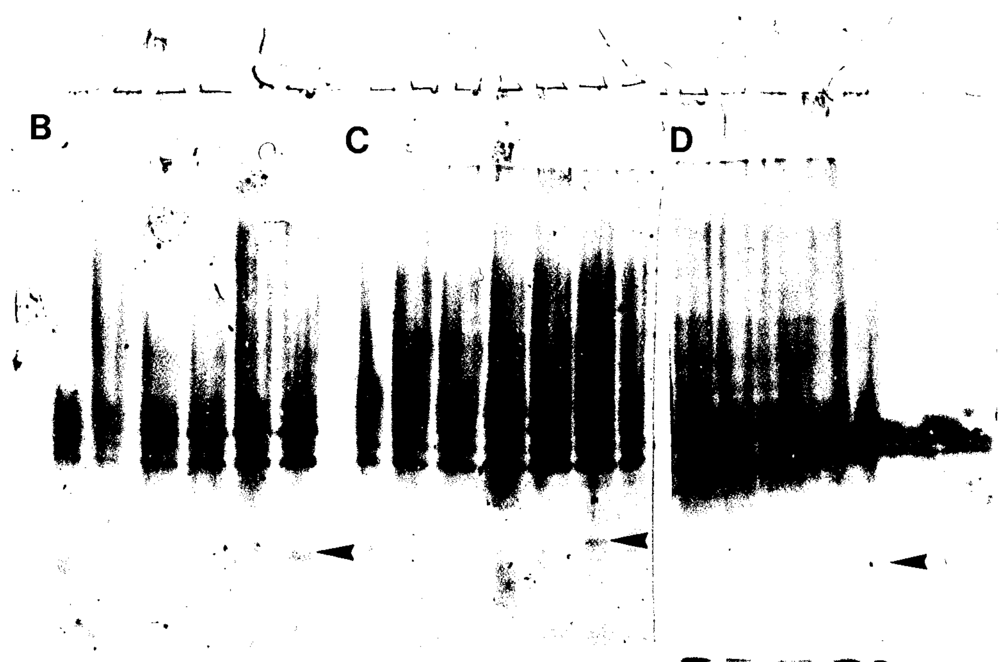


Plate 11. PPO Extraction and Total Protein Gels

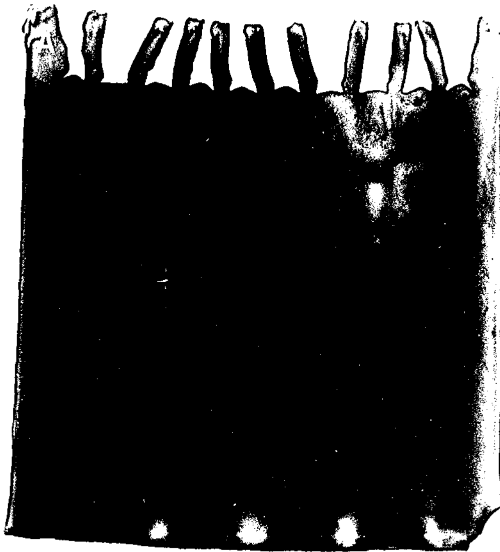
A. *Paeonia lactiflora* PPO extraction procedures 1, 2 & 3: tracks 2, 3, & 4 respectively,(see section 2.6.1.1.3). Tracks 5 and 6 are Hosta Francie PPO extraction 3, on PX and PR respectively. All other tracks were not loaded.

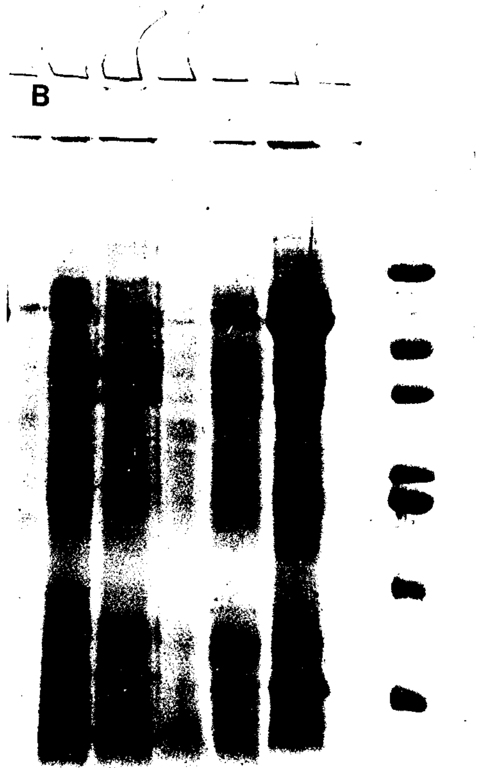
B. SDS-PAGE gel stained for protein (see section 2.6.2.4.), showing *Hosta* 'Francie' (250) polypeptides through a period of cold storage. From the RIGHT, Track 1: SDS-7, 2: 0 days in cold, 3: 9 days, 4: 23 days, 5: 31 days, 6: 40 days, 7: 60 days.

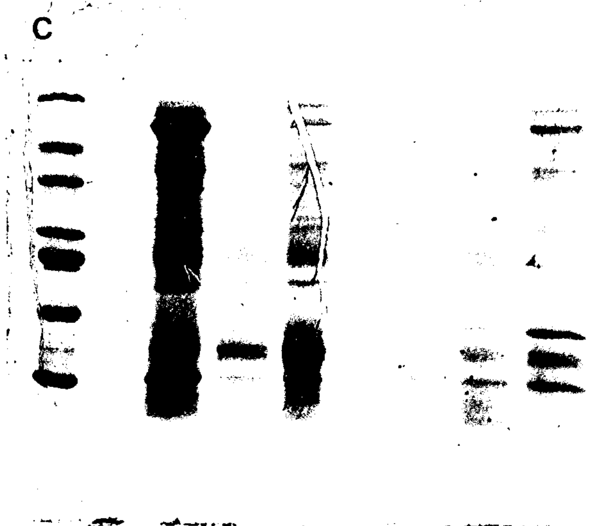
C. As B, but 'Blue Wedgewood' (259). From the left, Track 1: SDS-7, 2: 0 days in cold, 3: 5 days, 4: 9 days, 5: 14 days, 6: 23 days, 7: 31 days 8: 40 days, 9: 60 days.

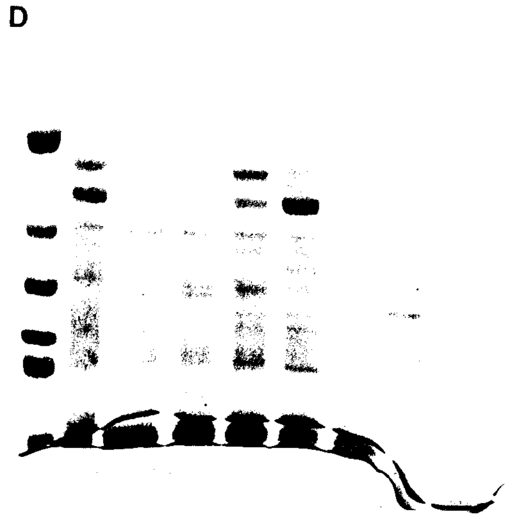
D. As B, but 'Golden Prayer' (377). From the left, Track 1: SDS-7, 2: 0 days in cold, 3: 9 days, 4: 23 days, 5: 31 days, 6: 40 days, 7: 60 days, 8: 83 days, 9, 109 days.

Plate 11: PPO Extraction and Protein Gels









3.2.2.6. Multiplication of *Hosta* Plantlets After Cold-Storage

Materials and Methods

10 tubs of *Hosta* 'Francie' from the previous experiment (40 days cold storage) were transferred to multiplication medium after being allowed to re-grow in the growth room. 10 tubs of non-cold stored 'Francie' were multiplied alongside these cultures for 3 subcultures.

Results

No significant difference was detected between cold stored and non-cold stored plantlets (results not shown).

3.2.2.7. Hosta Dormancy Out of Culture

Materials and Methods

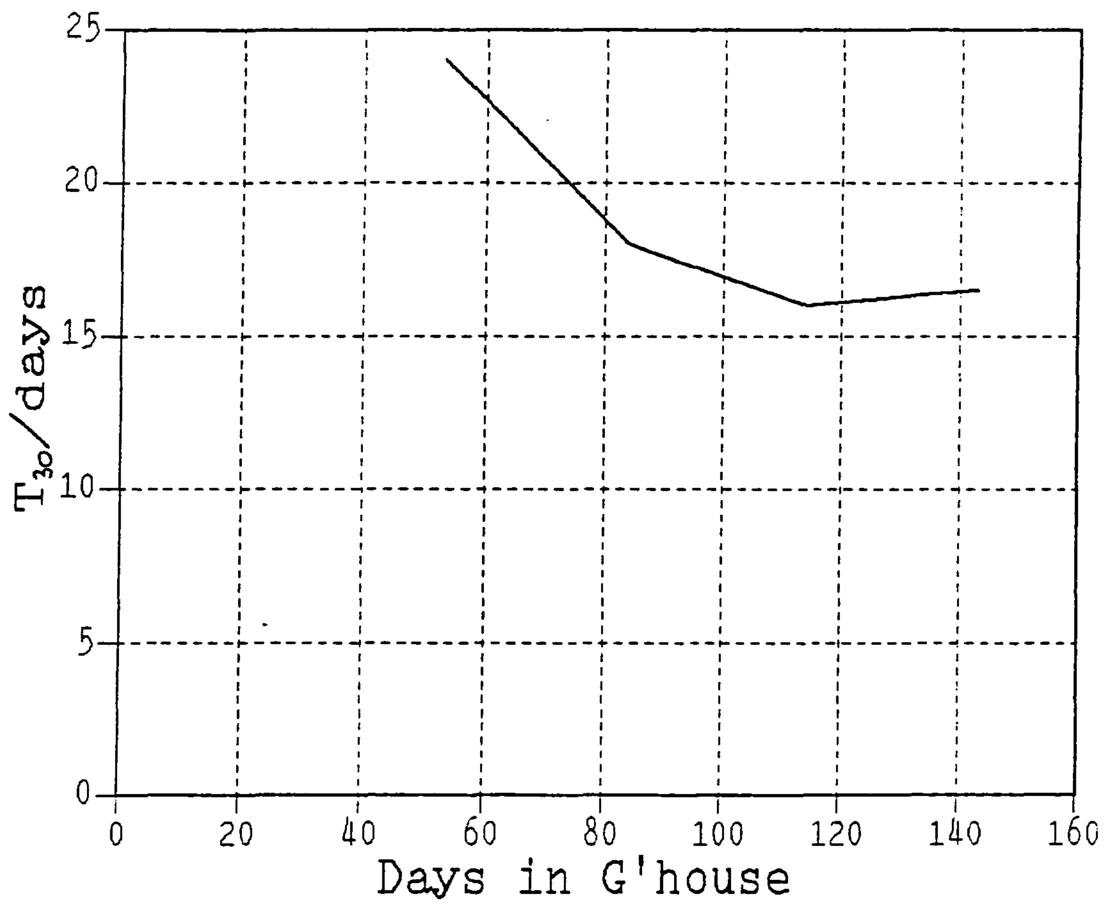
25 plants of the variety 'Blue Wedgewood' were weaned and transferred to the unheated section of the glass-house on 23rd November 1989. These were sampled every 3 weeks, by placing 5 plants in a growth cabinet at 25°C under continuous lighting. The plants were kept well watered, and scored every 5 days for leaf number.

Results

Data were analysed in a similar way to experiments 1 and 2, except the time to reach half the number of leaves on the 30th day at 25°C ($T30_{½}$ was used instead of the time to reach half the maximum leaf number ($T_{½}$). This was because the plants have no fixed maximum leaf number within the time-scale of this experiment, and 30 days is approximately the time taken for cultured plants to reach their maximum leaf number.

"Dormancy" declined after what was presumably a peak at 53 days, and reached a steady level after 100 days, (figure 48)

Figure 48. Dormancy in Weaned *Hosta* 'Blue Wedgewood'



3.2.2.8. Radioactive Labelling

Materials and Methods

50 µCi of ^{35}S-methionine was added to each of 4 cold-stored, rooted Hosta 'August Moon' tubs containing 15 plantets each. These were then left in the cold room (4°C) for 1, 3, 7 and 14 days, and the samples were subjected to SDS-PAGE. The gels were then dried, and exposed to x-ray photographic film for 2 days and then 2 weeks at -20°C.

Results

The only radioactivity detected was at the solvent front, and at the bottom of the sample wells.

4. Discussion

4.1. *Paeonia lactiflora* Micropropagation

P. lactiflora is a herbaceous perennial that exhibits slow growth and low levels of rooting when the techniques of micropropagation are applied. Hay (1987) advised that gardeners "should avoid disturbing [*P. lactiflora* plants] unless absolutely necessary.", and a "large number" of cuttings should be taken as only a small percentage will root. These two factors could help to explain why *P. lactiflora* is difficult to micropropagate, as subculturing can be regarded as excessive disturbance and wounding, and adventitious rooting is necessary to any micropropagation system.

4.1.1. Anatomy of *P. lactiflora* Plantlets

4.1.1.1. Multiplication Stage

In order to gain a better understanding of why *P. lactiflora* is so difficult to propagate using tissue culture, whilst other species such as *Hosta spp.* and *Choisya ternata* are relatively easily micropropagated, a survey of plantlet anatomy was conducted. This revealed a great deal of information about the cellular organisation of plantlets, which was often difficult to interpret in terms of the physiology of the whole plantlet. "Abnormal" differentiation and development were common features in diverse cell populations within the plantlets of *P. lactiflora* and other species. This is not necessarily indicative of cellular disorganisation, but perhaps a reflection of the "plasticity" of cellular development in response to cultural conditions.

The formation and initial stages of the growth of *P. lactiflora* auxiliary shoots is followed in Plate 1. An understanding of axiliary bud formation is important because it is the major factor contributing

to the multiplication rate. Evidence of the first few cell divisions which eventually lead to the shoot meristem (m: Plate 1) can be seen in Plate 1A. These most frequently form between leaf petioles (p: Plate 1) near the base of the plantlet. *P. lactiflora* plantlets did not produce much callus tissue at the shoot base on multiplication medium, so shoot meristems were often formed only a few cells away from the growth medium. This may account for the large amounts of accumulated starch (s: Plate 1) in adjacent cells. This starch is visible as a lightly coloured granular material, which often spills out of the cells when they are sectioned. Starch accumulation may also be related to a suppression of *in vivo* reducing sugars or solute potential, which may be necessary for meristematic activity to be initiated (see section 4.2.10.).

The secondary thickening of cell walls (t: plates 1A & 1B) in cells near the meristematic site may also be important. However, these cells do not seem to be vascular tissue as one might expect, because they do not appear to lead anywhere. This secondary thickening may be structural. Dubois *et al* (1990) suggested that the production of cell wall callose and "isolation" of a cell, may be necessary before a new metabolic program can be initiated. This speculation was made because every stimulus for callose deposition in isolated cells they investigated also resulted in an increase in somatic embryogenesis in roots of *Cichorium*. It could be significant that the stimulation of callose synthesis by Ca^{2+} levels has been reported (Kauss & Jeblick, 1986), and root primordia have also been shown to accumulate high levels of Ca^{2+} (Ferguson, 1979).

The bright specks of calcofluor fluorescence in cell walls at the top of Plate 1B are probably plasmadesmata pit fields, which often occur in abundance near the medium surface (see later). Large amounts of

cellulose (calcofluor positive staining) is often deposited around the boundary and neck of plasmadesmatal pits (P. Gates *pers com.*). A large density of pit fields in cells near the medium was also observed in *Hosta* (Plate 7A).

The large intercellular spaces (is: Plate 1) in the parenchymatous tissue surrounding the meristematic area, and indeed most of the plantlet base during the earlier stages of subculture, may be a response to adverse cultural conditions.

Plates 1C & 1D show the later stages of shoot meristem development. Little or no starch is visible in the immediate area of the shoot, and a vascular connection (v: Plates 1B & 1D) with the basal tissue is demonstrated.

Plate 2 shows disorganised vascular development in the stem, which suggests that the tissue is non-functional with respect to translocation. This sort of vascularisation was frequently observed in a wide range of micropropagated plants, and is likely to be a reflection of a reduced transpiration rate and altered phloem function, where most of the plantlet is a carbohydrate sink to the medium derived sugar (see section 1.1.3.3.).

Another phenomenon, that was found to be universal in all the plantlets studied, was the secondary thickening of cells at the plantlet/medium interface. This is shown in *P. lactiflora* in plate 2C, in *Hosta* in plates 7A/B and *Choisya* in plate 9A. This is discussed in detail later (section 4.2.2.)

4.1.1.2. Rooting Stage

Starch accumulation (s: Plates 3-5) is also evident in *P. lactiflora* rooting cultures (Plates 3A/B/C). As was the case for shoot

meristems, there appears to be little or no starch present in the actual root initial or root primordia (rp), perhaps indicating a high metabolic activity and growth rate.

In *P. lactiflora*, root initials form adventitiously in the sub-basal callus (Plate 3A/B/C), rather than directly from vascular cambia. However, vascular strands are visible near the newly formed root initial in plate 3C, and this was observed on several occasions during this study. This is consistent with the hypothesis that nearby vascular differentiation is a pre-requisite for root initiation (see section 1.1.3.2.).

After root emergence, a massive amount of starch accumulation was observed (s: Plates 4A-E). This is somewhat reduced, and perhaps confined to the stele after cold storage (Plate 5), which could be due to the replacement of cortical cells in the cold by periclinal divisions of cells just outside the endodermis (en: Plates 4C-D, 5D-F), (see below). These meristematic cells appear not to be the peridermis, which is usually located just inside the endodermis, and is the origin of lateral roots.

All *P. lactiflora* roots, before cold storage, possessed large cortical intercellular spaces (is: Plates 4A/B). In many sections these spaces were continuous around the cortex. It is not known if these spaces are of a gaseous or fluid nature, but if they are gaseous, they may be aerenchyma formed in response to root anoxia (see Section 1.1.4.3.). The large diameter of these roots may be a contributory factor to anaerobis. This may also explain why cold stored roots have no "aerenchyma" tissue, as Laan *et al* (1991) showed that diffusion pathways were important at growth room temperatures, but less so at lower

temperatures, when active oxygen uptake was much more important. In this case, a symplastic connection would be important and the filling of the cortex with cells derived from the outer stele could be a response to this need in the cold.

Other features worth noting are the relatively sparse xylem differentiation, which suggests that these are storage, rather than functional roots, and the strangely differentiated cells (c: Plates 4D/E), found both inside (Plate 4D) and outside (Plate 4C) the endodermis, which possess what is probably extensive cellulose thickening. These cells are similar to those found in imbibing rice seeds and may be associated with water uptake (P Gates, *pers. com.*). These roots also possess an exodermis, the significance of which is discussed in section 4.3.2.. It is also worth noting that very few root hairs were observed in these "storage" roots, or tubers. When they were present (rh: Plate 5D), they were only partially developed. The few lateral roots that were formed possessed more normal root hairs (results not presented).

4.1.2. Effect of GA_3 on Multiplication Cultures

It was found to be necessary to filter-sterilise GA_3 (see table 3), presumably because of the breakdown of the structure of GA_3 in the high temperature and pressure of the autoclave. This is an interesting result as it has been noted that some workers autoclave GA_3 (eg Jones *et al*, 1977; Howard & Oehl, 1981), and some filter-sterilise (eg. Kononowicz & Janick, 1984) for use *in vitro*, and this makes comparisons difficult. It is therefore recommended that GA_3 is filter-sterilised for all applications *in vitro*, making it easier to compare with *in vivo* results, where no sterilisation is required.

The optimum concentration of GA_3 in media varied depending on the factor investigated. Maximum multiplication was obtained at 1.5 mg/l (see figure 9), petiole extension at 3.0 mg/l (see figure 10), and chlorosis was only fully avoided at 2.0 mg/l (see figure 11). As petiole extension is not necessarily a desirable trait, it is suggested that a compromise between the other two factors is found, perhaps using 1.75 mg/l, or 2 mg/l as the multiplication rate at this level is satisfactory and leaf chlorosis can be totally avoided.

The application of gibberellins is well known to promote internode extension (eg. Jones & MacMillan, 1984), but in this case, only petiole, rather than general internode extension was observed. The reason for this is not understood by the author, but petiole extension probably contributes to the observed increase in multiplication rate by making the excision of nodes easier. This, however, is not the only factor causing increased multiplication, as the action optimum for GA_3 promoted petiole extension is much higher than that for multiplication, (see above). A possible explanation for the increased adventitious shoot development in response to GA_3 is the reported synergistic action with auxins on plant growth, or a cytokinin-like action on cell division and suppression of apical dominance (see section 1.1.2.2.).

GA_3 is also known to retard chlorophyll loss and increase chlorophyll content in many plants (Sabater, 1984). However, these effects have been associated with delaying the onset of senescence, and not the alleviation of chlorosis. A higher chlorophyll content has also been linked with an improved ionic balance resulting from GA_3 treatment of rice (*Oryza sativa*) leaves (Prakash & Prathapasenan, 1990). Since chlorosis is often a symptom of inorganic nutrient deficiency (see

section 1.1.3.1.2.), a possible mode of action of GA_3 is enhanced ionic uptake through increased membrane permeability (Pauls *et al*, 1982).

GA_3 has also been shown to affect the organic nutrition of plants. Patrick & Mulligan (1989) found that ^{14}C sucrose uptake was stimulated by 150% with the addition of GA_3 to the medium of *in vitro* cultured *Phaseolus vulgaris* stem tissues. This is also relevant for GA_3 in Paeony rooting cultures (section 4.2.3.).

The persistence of these effects even after plantlets are subcultured on to media containing no GA_3 (see figures 7 & 8) suggests that some form of "dormancy" has been overcome, or that the plantlets have been induced to switch to a more "juvenile" growth form. It has been established that GA_3 has an important role in release from dormancy (see section 1.2.), and Thomas & Vince-Prue (1984) reported that GA_3 application causes rejuvenation in many plants (also see section 4.2.2.), so such a speculation is not groundless.

The addition of GA_3 cause a significant increase in the total extracted esterase activity, which peaked at 1mg/l GA_3, and was significantly ($p<0.05$) higher than the control in all treatments except 3mg/l, which is explained in section 3.2.1.2. (see figure 12). No change was observed in total AAT activity (see table 4). The reason for the increased esterase activity is not known, except that it may reflect an increase in metabolic activity. It may be significant that a high proportion (at least 36%) of the increased esterase activity comes from a single isoform, suggesting a change in particular metabolic pathway. It is interesting to note here that in almost any gibberellin "feeding" experiment, GA conjugates *in vivo*, and commonly forms GA glucosyl esters, which are physiologically inert (Graebe, 1987). When they are

hydrolysed, an active GA molecule is released along with a reducing sugar. It has been suggested that these may be storage molecules for GA's (Graebe, 1987).

Esterases are known to be induced by the application GA_3 to cereal aleurone layers (Jacobsen & Knox, 1972), although their role is unknown. Perhaps they are involved with GA conjugation.

The method used to measure total enzyme activity is not entirely satisfactory, because, although plant material was weighed, and a standard amount of sample buffer added per gram (see section 2.6.1.1.), these measurements were not as accurate as desirable because a quantitative assay was not envisaged at the time. In addition to this, a high background interference was experienced, making it difficult to assess the relative activities of different isoenzymes. The increase in esterase activity was, however, observed in two totally separate extractions and four electrophoresis runs, so some credence must be given to these results.. To confirm this phenomena, it is suggested that a more accurate assay be utilised in any future work.

GA_3 in media also resulted in changes to the isoenzyme patterns of both AAT and esterase. In esterase, EST-2 (* in figure 13), a low Rf (0.12) isoenzyme and Est-10, a high Rf (0.75) isoenzyme disappear with the addition of GA_3 to growth media. EST-2 is expressed in response to both GA_3 and Pi, and may be useful as a marker for the phenomena associated with the use of chemical additions to the medium; i.e. increased sucrose uptake from the medium, changes in assimilate partitioning and a reduction in rooting. The contribution of Est-5 (Rf 0.34), is much increased with the addition of GA_3 to the medium (see above). An AAT isoenzyme, AAT-2 (Rf 0.24) also seems to be exclusive to

the addition of GA_3 to the growth medium (* in figure 14). The contribution of AAT-4 is much increased by GA_3. There also seems to be a reduction in the staining intensity of the high Rf AAT isoenzyme(s), AAT-9 (Rf 0.86-0.95) when GA_3 is added.

Gibberellin, then, seems to improve the "health" of Paeonys in culture and make them more amenable to micropropagation. It is suggested that an isoenzyme marker(s) could be identified for this, which could be of help with the choice of other culture variables.

4.1.3. Effect of Repeated Subculture

The observed reduction in plantlet multiplication over time to an unacceptable level of under X1.5 per subculture after 25 weeks (6-7 subcultures), (see figure 15) is difficult to understand. Plantlets became chlorotic, vitrified and grew very slowly, if at all, after this period of time, and the necrosis of shoot nodes was common.

This phenomenon has not been observed during the culture of Paeony at Neoplants (J. R. Nicholas *pers com.*), but they use plantlets from cold stored mother stocks and multiply the required amount of plantlets from this source, which means that so many subcultures are rarely necessary. In addition, every laboratory uses slightly different techniques and growth room facilities, however much the standardisation of conditions is attempted. These differences could be responsible for the observed effect.

The partial alleviation of this problem by long-term storage at low temperature (4°C) (see figure 16), could help explain this, suggesting that some form of dormancy is involved. However, unlike the situation with "healthy" cultures (see section 4.2.1.) the addition of GA_3 to culture media did not improve the situation, either before or after the

cold treatment (results not presented). This does not rule out the possibility that this phenomenon is a dormancy effect, as gibberellin does not always break dormancy (see section 1.2), but it certainly makes it less likely, and suggests that this phenomenon is distinct from the GA_3 response of Paeony multiplication cultures (figure 9).

A transition from a "juvenile" growth phase to an "adult" one could be triggered by a particular set of conditions found in the Durham growth room, and this could be the cause of the problem, but the differences in growth phase are not as distinct in herbaceous plants as in woody plants (Thomas & Vince-Prue, 1984), and again, a response might be expected to medium GA_3 addition, as Rogler & Hackett (1975) were able to induce juvenile phase characteristics with GA_3 in ivy (*Hedera helix*).

Another possibility is that accumulated stress factors caused the plantlets to stop growing (see section 1.1.4.). Reports that mature Paeony plants are very susceptible to physical wounding when split for propagation purposes (eg. Hay, 1987), add some weight to this hypothesis, but no healthy plantlets were available for a comparison of stress metabolites, such as proline or glycinebetaine, at the time of study.

It is evident that more work is required to understand, and hopefully avoid this problem in the micropropagation of Paeonys. Until that time it is advised that procedures similar to those used at Neoplants (see above) are used.

4.1.4. GA_3 and Amylase in Paeony Rooting Cultures

GA_3 has been shown to inhibit (eg. Coleman & Greyson, 1976; Wightman *et al*, 1980; Jarvis & Yasmin, 1987; Carvalho *et al*, 1989) or promote (eg. Nanda *et al*, 1972; Hansen, 1976) adventitious root

formation. Smith & Thorpe (1975) reported that exogenously applied GA_3 could be either inhibitory or promotive to rooting in *Pinus radiata* cuttings, depending on the time of application. Inhibition occurred between 0 and 4 days, but application after that time resulted in an increase in the formation of root primordia. However, Carvalho *et al* (1989) found, using *Pereskia grandiflora* petioles, that a treatment with GA_3 during the first 24 hours, although being inhibitory, was not *histochemically* distinguishable from the control until 12 days later.

It is possible that a treatment with GA_3 early in rooting interferes with the very early stages of rooting, perhaps with the vascular differentiation which appears to be a pre-requisite for root initiation (Aloni, 1987). Wareing & Phillips (1981) reported that if GA_3 is applied in the absence of IAA in *Poplus robusta*, division of the vascular cambia occurs, but the xylem remains undifferentiated and retains its protoplasmic contents. Such xylem does not undergo secondary thickening (Aloni, 1979). It may be that secondary thickening and protoplasmic disintegration of the cambial or somatic xylem is the pre-requisite for root initiation near the end of the vascular tissue. The presence of vascular strands leading to a *P. lactiflora* root initial (Plate 3C), seems to support this. Perhaps the transport of some hormonal "trigger" for the first root initial divisions to take place is required.

GA_3 could also interfere with the secondary thickening of the initial meristematic cell, which Dubois *et al* (1990) suggested may be a pre-requisite for a change in cellular metabolic program. They pointed out that the deposition of callose and other compounds which is often observed in somatic embryogenesis (and in root initial cells: Aloni, 1987) may serve to "isolate" the cell from its neighbours whilst

critical metabolic changes occur. Dubois *et al* (1990) do not make clear whether they envisage either apoplastic or symplastic isolation or both.

The cases of GA_3 promotion of rooting may be explained by comparison with seed germination. Karssen *et al*, (1989) found that a gibberellin-like substance diffused from the embryo to the endosperm of tomato seeds where it induced the hydrolysis of cell walls that are part of the mechanical resistance against the protrusion of the radical. Groot *et al* (1988) confirmed this using exogenously applied GA_3. Perhaps a similar mechanism occurs in GA_3-promoted rooting. Indeed, physical barriers to rooting have been reported in cuttings (Strangler, 1956; Beakbane, 1969), and micropropagated plants (Nicholas, 1985).

The inhibition of rooting observed in this study (see figures 17 & 18), is consistent with these results, in that GA_3 was applied at the beginning of the rooting culture. Very few root initials were formed when GA_3 was included in the medium, although no quantitative assessment of this was made.

Endogenous levels GA_3 are known to regulate the transcription of α amylase genes which in turn leads to the mobilisation of starch reserves in barley (*Hordeum vulgare*) seeds (see section 1.3.5.4.). The increase in Paeony shoot amylase levels in response to exogenously applied GA_3 (figure 20) may also result in increased starch hydrolysis, leading to higher cellular reducing sugar levels, but this can not be stated conclusively in Paeony without assessing *in vivo* sugars. Carvalho & Dietrich (1986) showed that exogenously applied GA_3 resulted in an increase in reducing sugar (mostly glucose and fructose) levels in *Peresbia grandiflora* leaf petioles, and suggested that a large pool of these carbohydrates could be involved in the inhibition of root

differentiation. This in fact had been shown previously by other workers (Lovell *et al*, 1972, 1973; Veierskov *et al*, 1976). Endogenous reducing sugar levels are also thought to regulate amylase (Parys *et al*, 1983) and other starch degradative enzymes (Beck & Ziegler, 1989).

The increase in amylase activity through a rooting subculture (figure 19) probably reflects an increase in growth rate and demand for carbohydrates for glycolysis as plantlets become established in culture rather than *in vivo* increases in sugars or starch hydrolysis. Indeed plates 3 & 4 demonstrate that extensive starch accumulation takes place throughout this period, especially in roots, where amylase activity actually decreased in response to GA_3 (figure 21).

Patrick & Mulligan (1989), found that GA_3 stimulates the *in vitro* ^{14}C sucrose uptake by *Phaseolus vulgaris* stem tissues. A similar effect was reported by Miyamoto & Kamisaka (1988), who found that GA_3 stimulated the transport of sucrose from *Pisum sativum* cotyledons to the growing point. This could be the mechanism for the inhibition of root initial formation by GA_3, and it may be that either secondary vascular thickening of vascular tissue requires a low cell reducing sugar levels, or that in order to lower cell reducing sugar levels, secondary deposition on the initial meristematic cell wall is required.

Starch accumulation has been shown to occur in the cells surrounding a potential meristem (but not in the meristematic cells themselves) just prior to the formation of meristematic centres. This starch then disappears in later stages of meristem development (Patel & Thorpe, 1984; Von Arnold, 1987). This was shown to be the case for Paeony root initials and shoot meristems in this study (see Plates 3 & 1). Von Arnold (1987) suggested that the role of this starch could be to

provide a readily available source of energy for organogenesis. This may well be the case, but it also seems likely, considering the above discussion, that the starch accumulation also serves to lower reducing sugar levels around the meristematic zone, allowing the initial stages of primordial formation to go ahead uninhibited by high cytoplasmic sugar levels. More circumstantial evidence for this was given by Thorpe *et al*, (1986), who showed that starch was synthesised directly from media sucrose in tobacco (*Nicotiana tabacum*) calli, and Von Arnold (1987) finding that bud formation on *Picea abies* embryos could take place on media without sucrose, but sucrose was required for further development.

It may be that it is not the reducing sugar levels that are important *per se*, but the cell osmotic potential. Loach (1977) found that leaf water potential, which may reflect the water potential at the rooting site, below -10 bars inhibited rooting in *Rhododendron* cuttings. This would also explain why low ionic content rooting media (eg. WPM, ½MS) are so much more successful at rooting *in vitro*.

It is interesting that exogenous inorganic phosphate (Pi) levels also have the effect of increasing starch breakdown (into reducing sugars) (Steup *et al*, 1976), and increasing sugar uptake from growth media, (Li & Ashihara, 1989), and in this study reduced Paeony rooting at high levels of Pi (see section 4.1.5.)

It is easy to imagine that a high reducing sugar level after root initiation has taken place would be beneficial. With the resultant increase in available energy, root differentiation and growth would be stimulated. This may be the reason that carbohydrate is often stored in

adjacent cells as starch, which contributes nothing to cell solute potential, but is a readily available source of energy.

A shifting of the metabolic balance away from the accumulation of starch reserves and towards reducing sugars by GA_3 (Thorpe et al, 1986; Graebe, 1987; Beck & Ziegler, 1989) may be responsible for the faster shoot growth observed in GA_3 treated plantlets.

An increase in osmotic competence, associated with an increase in readily available low molecular weight molecules could also play a part in increasing shoot health (see section 4.1.7.).

4.1.5. Effect of Phosphate on Paeony Rooting Cultures

In this study, a six fold increase in medium Pi levels appeared to cause an increase (X5) in leaf production between days 14 and 35 of rooting culture (figure 22), and a slight increase in rooting (figure 23) of Paeony plantlets. With a 10 fold increase in medium Pi, these effects seemed to be reversed. These results were not statistically significant, but the results of a repicate experiment showed similar trends. Insufficient plant material was available to achieve a statistically significant result.

First impressions would seem to indicate that these are deficiency-toxicity responses. Pi deficiency is known to rapidly reduce plant growth, decreasing photosynthesis rates (Dietz, 1989), change assimilate partitioning (Sicher & Kremer, 1988), result in bluish, bronzed or purple leaves and poor root growth (Tootill, 1984). The application of exogenous Pi has been shown to reduce the root/shoot ratio in hydroponically grown *Chionochloa spp.* (Chapin *et al*, 1982), and in *Agave lechuguilla* (Nobel *et al*, 1989). However, it is not certain that either deficiency or toxicity is taking place in Paeony rooting cultures within the Pi concentration range utilised.

Marschner (1986) estimated that plants need one molecule of Pi for every 500 carbon molecules assimilated during photosynthesis if they are to maintain *adequate* growth. Assuming that there is a similar requirement for heterotrophically acquired sucrose, and that no photosynthesis is taking place *in vitro*, then the Paeonies on PR media should not exhibit Pi deficiency before the media carbohydrate source is depleted (the ratio of Pi:carbon in PR is 1:400, as calculated from data supplied by Sigma). The assumption that no photosynthesis is taking place is not necessarily valid, but photosynthesis would have to account for more than 20% of carbon acquisition before Pi deficiency could be envisaged as a problem. Mantell *et al* (1985) and the work of Evans & Lees (1987) suggests that this level of photosynthesis does not occur in micropropagated plants. These results, then, do not appear to be due to a relief from phosphate deficiency. Dalton *et al* (1983) found that as much as 13% of Pi in MS medium could precipitate in agar after 7 days, but this would not appear to be a significant amount (see above).

Phosphate toxicity also seems unlikely as many workers (eg. Mohabir & John, 1988; Mimura *et al*, 1990) have used concentrations of Pi well in excess of those used here without any toxicity being reported.

Pi is known to be a key factor in starch metabolism, and Paeony roots are starch storage organs (see plate 4A), so it seems possible that changing medium Pi levels might affect the synthesis and/or the degradation of starch in Paeonies, perhaps leading to higher reducing sugar levels that might explain the increased shoot growth (figure 22) and reduced rooting (see figure 23 and section 4.1.4).

Some starches, especially those from roots and tubers, contain a measurable phosphate content (Banks & Greenwood, 1975). Although this is

only 1 Pi group to 2400 glucose residues in amylose, it is thought to be much higher in the amylopectin component of root or tuber starch (Banks & Greenwood, 1975). This suggests that Pi might be more important in the starch metabolism of roots than, say, cereal seeds on which most of the research seems to be concentrated. Most work on the regulation of starch metabolism has been performed using chloroplasts, but there are indications that amyloplasts use similar, if not identical pathways (eg. MacDonald & ap-Rees, 1983; Mohabir & John, 1988). This is in spite of the fact that chloroplasts contain mostly "transient" starch which varies on a diurnal basis, although they are used for starch storage to varying degrees (Banks & Greenwood, 1975).

Starch synthesis in cell-free potato (*Solanum tuberosum*) tuber amyloplasts was shown to be reduced by the addition of 10mM Pi to the suspending medium by Mohabir & John (1988), who attributed this to an inhibition of triose-phosphate uptake. Conversely, starch breakdown is thought to be stimulated by high Pi (Steup *et al*, 1976; Heldt *et al*, 1977; Goyal *et al*, 1987).

The regulation of starch synthesis is thought to be almost entirely centred around the enzyme ADPG-pyrophosphorylase (Beck & Ziegler, 1989) (which catalyses the reaction Glucose-1-phospate + ATP $\leftrightarrow$ Adenosine-5'-(α-D-glucopyranosyl pyrophosphate) [ADPG] + pyrophosphate). The equilibrium position of this reaction is highly dependant on the ratio of Glucose-1-phospate to pyrophosphate (Banks & Greenwood, 1975). High *in vivo* Pi levels inhibit the enzyme (Beck & Ziegler, 1989), and Schneider *et al* (1981) suggested that phosphorylase might play a mediatory role between starch synthesis and degradation in potato (*S. tuberosum*).

There are, however, difficulties in proposing that growth media Pi additions directly influence the partitioning of acquired carbohydrates. It is well established that plant cells compartmentalise and store Pi in the vacuole, and that there is only slow exchange between the metabolically active Pi found in the plastids and cytoplasm, and the "inactive" Pi in the vacuole (Bieleski, 1973; Foyer & Spenser, 1986). Mimura *et al* (1990) found, using barley (*Hordeum vulgare*), that increasing the nutrient medium Pi levels caused very little change in cytosolic Pi levels, but did increase Pi in the xylem sap and mesophyll cell vacuoles. It is suggested that under normal conditions, the total pool of both chloroplastic and cytosolic Pi and organic phosphate esters remains constant (Heber & Heldt, 1981; Mimura *et al*, 1990), presumably with the vacuolar Pi being made available to replenish any losses. Therefore, only Pi deficiency can explain any effect *in the leaves and stem* of the plant. Mimura *et al* (1990) showed that barley roots function as the main barrier to phosphate uptake from high Pi rooting medium, but do not specify a mechanism. Presumably the root endodermis plays an important role, and this being so, Pi could still be important in the regulation of starch metabolism in Paeony roots, as starch is deposited outside the endodermis, as well as the stele (see plate 4A).

The addition of inorganic phosphate (Pi) to growth media has been shown to result in increased sucrose uptake and metabolism in suspension-cultured *Catharanthus roseus* cells, (Li & Ashihara, 1989). Examination of figure 24 shows that leaf growth started earlier in high Pi cultures. In fact, below 3.56 mM medium Pi, the proportion of plantlets producing new leaves declined from the initial value until day 8, and only reached a level comparable with the high Pi treatments at day 21. This result could well be due to increased sucrose uptake, which

Li & Ashihara found to be 1.5-3.0 times higher in the presence of 1.25 mM Pi. This is a similar effect as observed with GA_3 addition to the medium (see section 4.2.3.), and it may be that a similar mechanism for root inhibition is taking place in both cases. It is worth noting that although the proportion of plantlets producing new leaves is highest in the 6.5 mM Pi treatment, the leaf number per plantlet in this treatment declined from its optimum at 3.56 mM Pi. This suggests that it is not a case of simple sucrose uptake stimulating plantlet growth.

Reinhert & Bajaj (1977) reported that Pi could suppress the root promoting effect of auxins, and this may be the case, but it seems more likely that the mechanisms discussed above are in operation.

Total *in vivo* esterase and AAT levels remained constant at different concentrations of media Pi (see table 5). However, the isoenzyme profiles of both enzymes changed in response to Pi (see figures 25 & 26). Est-2 disappeared when high levels (above 1.36 mM) of Pi were utilised, which was also observed with the addition of GA_3 (see figure 13), and Est-9b also disappeared. It is interesting that Pi and GA_3 should both cause the disappearance of Est-2, when it is considered that the effects of the addition of these two chemicals is remarkably similar. This isoenzyme may prove to be a good "marker" for one of these phenomena (increased growth and media sucrose uptake and inhibited rooting). AAT-6 appeared in the high Pi treatments, and an increased level of AAT-5 and AAT-9 compensated for a drop in the activity of AAT-8 (figure 26).

In summary, several hypotheses have been put forward to explain the results obtained. The most promising of which seem to be increased sucrose uptake from the rooting medium and, possibly some regulatory

role for Pi in starch metabolism and carbon partitioning. More work is required to establish what is actually happening. I suggest that examining *in vivo* and *in vitro* reducing sugar levels, and the roles of phosphorylase isoenzymes would be a productive starting point to understanding both the increased growth rate and effects on adventitious rooting of media additions of Pi and GA_3.

4.1.6. Effect of Osmotica on Paeony Rooting Cultures

Both mannitol and PEG appeared to be toxic to Paeony plantlets and therefore unsuitable as osmotica in this species. Although Paeony plantlets have been shown to have water relations problems (see section 4.2.9.), it seems unlikely that osmotic factors alone caused their death, as the water potential of PR with the lowest concentration of mannitol added was less negative (-4.42 bar) than PX media (-6.44), which did not kill the plantlets. There have been previous reports of mannitol toxicity, and it has been shown to be absorbed by many plants (e.g. Groenewegen & Mills, 1960; Slavík, 1974), and PEG has also been shown to be toxic to some plants (Leshem, 1966; Slavík, 1974).

Further work is required to identify an osmoticum that is not toxic. This would be a very useful tool for investigating many of the areas covered in this study of Paeony. Unfortunately, insufficient plant material was available to screen a large range of osmotica, due to the problems discussed in section 4.2.2. Promising osmotica include the various higher molecular weight (1000-20,000) PEG's, although these have been reported to reduce water fluxes in roots (Slavík, 1974). The criteria that must be met are, first of all non-toxicity, but also impermeability to the plasmalemma and preferably metabolic inertness.

4.1.7. Culture Aging and Medium Dehydration

The increase in rooting levels (figures 27 & 28) and increased shoot "vigour" and subsequent multiplication rates (results not presented), shown after pre-treatment with extended periods on multiplication medium could have a number of causes. It is possible that it is a stress response to dehydration, nutrient deficiency or a response to the accumulation of excreted compound, such as phenolics, in the growth medium.

During the preparation of this manuscript, Harris & Mantell (1991) published a paper, reporting that in the related species *Paeony suffructicosa*, "the length of the final subculture used prior to rooting treatment was critical for determining the subsequent rooting responses of microshoots." They found that increasing the final multiplication culture from 4 to 5 weeks gave a higher level of level of rooting. The results presented here show further increases in rooting by up to 20 weeks of multiplication stage storage (figures 27 & 28). *P. suffructicosa*, unlike *P. lactiflora*, is a woody plant ("tree paeony"), so this effect seems to be related to genotype rather than growth habit. It is not known if this effect occurs in other species within the genus *Paeonia*, or indeed in other genera. It is certainly an effect that needs to be investigated in other difficult to root species.

Figure 29. shows that considerable medium dehydration occurs in long-term stored multiplication cultures (see section 4.1.9).

The leaf water status of stem cuttings has been shown to influence rooting (Loach, 1977, 1988), with low water potentials inhibiting rooting. Perhaps the transition from the water "stressed" environment of

the dehydrated multiplication cultures to the relatively "unstressed" conditions on the rooting media in some way stimulated rooting.

Increasing exogenous sucrose levels have also been implicated in increased rooting outside any osmotic effect (eg. Lovell *et al*, 1972; Nanda & Jain, 1972), but in a series of papers, sucrose content was shown not to be consistently associated with rooting in pea (*Pisum sativum*) (Veierskov & Andersen, 1982; Veieskov, *et al*, 1982 a,b)

von Arnold (1987) found that in *Picea abies* adventitious bud formation, the initial stages of bud development could take place on media containing no sucrose, but further development of the meristem required sucrose in the media. If the mechanisms are similar, it is possible that root initials were "allowed" to be formed in the stored multiplication cultures by low endogenous sugar levels, and later, in the rooting medium developed to form roots. This is supported to some extent by the observation that high endogenous sugar levels inhibit callose deposition in xylem tracheids (Aloni, 1980), and root initiation seems to require vascular differentiation (Aloni, 1987). This could also explain the high level of multiplication observed when plantlets were returned to multiplication media (results not presented). Unfortunately, this was not investigated using the fluorescence microscope, but any future work should certainly include this.

The inclusion of phenolic compounds in media has been shown to increase root proliferation in a range of woody plants (see Jones, 1983), but nothing was found in the literature concerning this phenomena in herbaceous plants. In apple (*Malus* spp.), the effect is thought to be dependant on the physiological state of the plantlet. It may be due to "rejuvenation" of tissues (Jones, 1983). Phenolic compounds can

demonstrate auxin-like activity, or decrease levels of auxin oxidation (Bandurski & Nonhebel, 1984) which may stimulate root initiation.

The activity of polyphenol oxidase (PPO) in P. lactiflora cultures was found to be low (section 3.1.10.). This may be due to enzyme latency (section 1.3.5.3.), which was not removed as expected by extractions freezing in liquid nitrogen or treating with SDS (Plate 11). Alternatively it could suggest that phenols are accumulating due to low oxidation rates, although enzymes such as peroxidase are also capable of phenol oxidation. More work is required to establish if phenols are responsible for the effect described here.

4.1.8. Effect of Pre-Rooting Stress

In this experiment (section 3.1.8), an attempt was made to mimic the effects of culture "ageing" (section 4.1.7.). This was done by reducing the water content of cultures at different nutrient levels (treatments 3, 4 & 6), and by excluding nutrients altogether (treatment 5). All of these treatments appeared to be too stressful for the plantlets, which experienced a high mortality rate (figure 30). All of these treatments reduced rooting, even when this mortality was taken into account (figure 30).

This indicates that plantlets require an extended period to adapt to these conditions, perhaps becoming more photoautotrophic, as found by Champion (1982), who showed that *Cinchona ledgeriana* shoots became increasingly dependant on photosynthesis after 21 days of culture.

4.1.9. Paeony Culture Water Relations

Paeony plantlets lost water much more rapidly than other plantlets (figure 31). The water potential of rooted Paeonies was less negative

than other plantlets (table 6), and this may go some way to explaining why they lost so much water.

Paeony leaves appeared to have a thinner cuticle and larger cells, with more intercellular air spaces than *Choisya* or *Hosta* (results not presented, but compare plates 1D & 7E). This is one of the symptoms of vitrification (see section 1.1.3.1.1.).

If Paeony plantlets are osmotically "incompetent", in the same sense as vitrified leaves, then this may explain the high mortality observed when they are stressed (section 4.1.8.)

Considerable water loss is experienced by cultures, (15-30% over 4 weeks, 40-65% over 23 weeks; figure 29). The increase in media weight loss when plantlets were present has two possible causes. Firstly, plantlet respiration is likely to exceed photosynthesis (see section 1.1.2.4.) so a proportion of the sucrose will be metabolised and diffuse out of the container as carbon dioxide. Secondly, any transpiration by the plantlets (which is almost inevitable in the light due to temperature differential between the leaf and culture vessel atmosphere) would raise the relative humidity near the vessel lid and increase the diffusion gradient. It is possible that a "canopy" effect also operates, which would reduce water loss from the vessel, or at least directly from the medium surface.

The failure of the pressure chamber method to produce any results (section 3.1.11.2.) indicates that vascular tissue is non-functional, or perhaps that stomata are closed, as found in *Hosta* (Plate 7C).

4.1.10. <u>Proposed Mechanisms for the Control of Rooting</u>

A summary of possible mechanisms and factors affecting whether or not a plantlet will produce roots is presented in figure 49. There

seem to be two stages that are crucial to the success of adventitious rooting. The first being the formation of a potential initiation site, which is thought to be dependant on previous vascular differentiation and development (eg. Aloni, 1987), and the second being the growth and emergence of the root (eg. Nicholas, 1985; Lovell & White, 1986).

The "primary" control factors at both stages seem to be the endogenous levels of auxin, GA_3 and reducing sugars. According to this model, root initiation (0-4 days) should be increased by high rooting medium IAA, due to a stimulation of vascular differentiation. At this time, a low concentration of cytokinin would have its effect by inducing vascular cambia. Although GA_3 can stimulate IAA levels (eg. Prakash & Prathapassan, 1990), it can also be antagonistic (Sexton & Woolhouse, 1984). GA also promotes the elongation of pre-vascular cells (Aloni, 1987), but it inhibits secondary thickening of these cells (Aloni, 1979), as do high cytosolic reducing sugar levels (Aloni, 1980; Langford & Wainwright, 1987). It is therefore proposed that the secondary thickening of these cells is a prerequisite for the formation of root initials in the immediate area. Perhaps the vascular tissue has to be functional to transport some signal for root initiation.

Dubois *et al* (1990) suggest that meristem initial cell isolation by secondary cell wall thickening may be important. Control factors for this seem likely to be similar to those for vascular tissue secondary thickening.

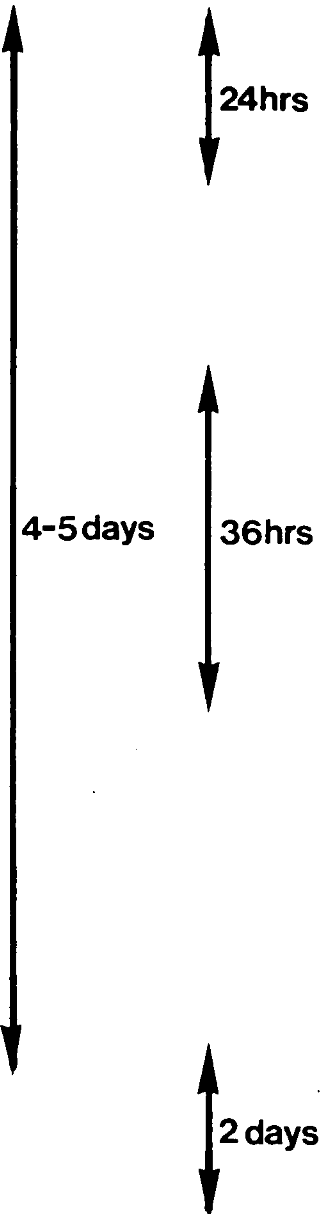
Increased rooting due to etiolation, could exert its effect by the consequent reduction in photosynthesis (by being kept in the dark) resulting in reduced sugar content, promoting vascular differentiation. Exogenous phosphate and high media ionic content have the effect of

increasing *in vivo* reducing sugars, and thus inhibit root initiation. The frequently observed starch accumulation around meristematic sites would reduce sugar content and promote root initiation..

According to the model, after 4 or 5 days, auxin should be inhibitory to root development (excessive callus production and vascularisation), and GA_3 should promote root development and elongation. High endogenous sugar levels would be metabolically advantageous and a low cell water potential would promote expansive growth.

This model would go some way to explaining the results of Smith & Thorpe (1975), who found that GA_3 inhibited rooting when applied to *Pinus* cuttings between 0-4 days, but promoted rooting thereafter. It would also explain why the reports of the effects of GA_3 on rooting are so contradictory. In addition to this it would help to explain why a low medium ionic content can promote rooting (eg. Hartman & Kester, 1983).

The model predicts, therefore, that the ideal rooting conditions would be: 1. Culture for 2-4 days in the dark, on media containing high concentrations of IAA and perhaps a cytokinin, but with low sucrose and low ionic content. 2. Transfer on to new medium containing GA_3, high sucrose, high mineral nutrients and in the light until the roots have emerged.



Endogenous

Exogenous

3, 27

Unrooted Plantlet

Cytokinin

Cambium

Basal/Sub-Basal Callus

13

3

IAA

Vascular Differentiation

1,2,33

26

3

Elongation

3

GA3

12

Etiolation

Secondary Thickening

1

6

2,15

Potential Root Initiation Site

20,24,34

20

Root Initial

32

ATP

30,31

Reducing Sugars

4,12,29

Root Primordium

13

IAA

8

Ψs

16

26

Emerged Root

33

GA3

Photosynthesis

9

10,14

Phytochrome

21

IAA/GA/Cytokinin Control or Substitution?

28

4,11,14

Starch

7,23

4,10,11

18

Pi

5

22

Cytokinin

IAA

GA3

Intensity

Light

Photoperiod /Quality

N Source /Level

Phenolics

21

23,25

Sucrose

18

Pi

Ψw

19

Notes for Figure 49

Stars on arrows indicate a negative effect.

* Effects of reducing sugars may be attributable to cell solute potential.

† Possible cellular "isolation" by secondary wall thickening (Dubois *et al*, 1990)

1. Aloni (1979)
2. ——— (1980)
3. ——— (1987)
4. Beck & Ziegler (1989)
5. Bieleski (1973)
6. Carvalho *et al* (1989)
7. Dancer *et al* (1990)
8. Darbyshire (1971)
9. Davis & Potter (1989)
10. Foyer & Spencer (1986)
11. Goyal *et al* (1987)
12. Graebe (1987)
13. Hartman & Kester (1983)
14. Heldt *et al* (1977)
15. Ingold *et al* (1988)
16. Karrssen *et al* (1989)
17. Langford & Wainwright (1987)
18. Li & Ashihara (1989)
19. Loach (1977)
20. Lovell & White (1986)
21. Mantell (1985)
22. Mimura *et al* (1990)
23. Miyamoto & Kamisaka (1988)
24. Nicholas (1985)
25. Patrick & Mulligan (1989)
26. Prakash & Prathapasan (1990)
27. Saks *et al* (1984)
28. Selby & Harvey (1990)
29. Thorpe *et al* (1986)
30. Veireskov *et al* (1982a)
31. Veireskov *et al* (1982b)
32. von Arnold (1987)
33. Wareing & Phillips (1981)
34. Zimmerman & Fordham (1985)

4.2. Hosta Micropropagation

Hosta species, like *Paeony lactiflora*, are herbaceous perennials, but unlike *P. lactiflora* they have proved to be amenable to micropropagation techniques. Thomas (1982) reports that hostas are easy to divide and transplant at any time of year. High multiplication rates are attainable on MS medium in the presence of only a low level of cytokinin (section 2.1.3.). The only problem encountered with *Hosta* multiplication in this study was with high light intensities (photon flux > $500 \mu Ei \cdot m^{-2} s^{-1}$), which led to leaf epinasty and reduced growth (results not presented).

A high level of rooting was achieved on ½MS, with no growth hormones. This makes *Hosta* an ideal model plant for studies into the rooting and water relations of plants *in vitro*.

Transplanting was successful in mist room conditions (section 1.1.2.4.), but was found to be improved by cold storage for a number of months, which also allows commercial propagators to adjust plant production to seasonal market requirements (J.R. Nicholas *pers. com.*). However, if plantlets were prematurely removed from the cold store, they exhibited what appears to be a form of dormancy (J.R. Nicholas *pers. com.*; see plate 6). One of the aims of this project was to investigate this phenomenon and develop a technique whereby propagators can predict if *Hosta* plantlets are "dormant", or would grow normally when transplanted. This could not be ascertained by casual observation of plantlets, and simply placing plantlets in the growth room was not satisfactory for two reasons. Firstly, a period of a few weeks could be too long to be of use to the commercial propagator (J.R. Nicholas *pers. com.*), and secondly, even "dormant" plantlets, when placed in the growth

room, exhibit new leaf growth, only ceasing to grow when transplanted (see plate 9).

4.2.1. Hosta Rooting at Reduced Culture Humidities

The reduction of culture vessel relative humidity (RH) from approximately 100% to 85% resulted in an increase in the number of seminal roots produced per plantlet (figures 34 & 36), number of lateral roots produced (results not presented), and the mean root length (figure 34). Below this level of humidity rooting was inhibited (figure 36). The optimum culture vessel RH for the number of roots produced was 80% (figure 36).

Attempts to scale up the number of plantlets maintained at reduced humidity failed due to media dehydration. Even when medium anti-evaporative barriers were employed, medium dehydration still occurred (section 3.2.1.6.). This suggests that a substantial transpiration stream is present in lowered humidity cultures, which is consistent with the observation that stomata are open in low humidity cultures (Plate 7C; see below).

Plantlet water potentials, as measured by the "smear" method (section 2.3.1.), across a range of humidities are presented in figure 36. It seems unlikely that culture vessel humidity approached the desired %RH near the leaves of the plantlet, especially at the lower humidities, as the difference between the equivalent water "potential" of air between 100% and 85% RH is -220 bars, and at 35% RH is a massive -1575 bars (Slavík, 1974). The difference between the plantlet water potentials and these theoretical levels (which are never found in plants), can be accounted for in a number of ways. Firstly, even micropropagated plants have some control over their water status other

than by the components that make up their water potential. They possess some form of leaf cuticle, stomata (if functional), and leaf diffusive resistance. Secondly, in the still air of a culture vessel, a gradient of humidity will inevitably form between leaves/growth medium and the surface of the saturated salt solution (SSS). In addition to this, unless the SSS is stirred continuously, a layer of . unsaturated solution will inevitably form between stirrings at the SSS surface. The water stress, then, is not as severe as would at first appear, although plantlets exposed to "humidities" lower than "50% RH" did visibly wilt in the first 5 days of culture (but then regained turgidity).

The stomata of *Hosta* plantlets cultured at 100 %RH were closed (plates 7C & 7E), but the stomata observed in "85 %RH" cultures appeared to be open. Other workers have found that stomata are open in "normal" (100% RH) cultures (see section 1.1.3.1.3.), but the technique used here was very rapid and does not kill the leaf tissues. Indeed, it was possible to observe the leaf whilst still attached to the plantlet. It will be necessary to confirm these findings with other species. The open stomata in reduced humidity cultures, again, indicates that a transpiration stream is present, and perhaps that an increased rate of photosynthesis is taking place in reduced humidity cultures. It has been suggested that increased photosynthesis stimulates the rooting of cuttings by increasing the available supply of carbohydrate (eg. Davis & Potter, 1989), but this seems less likely in culture as an adequate supply of sucrose is available from the growth medium. However, increased IAA supply from the leaves may stimulate rooting.

Loach (1977) found that leaf water potentials below -10 bars linearly inhibited the rooting of cuttings in several species. In addition to this, Darbyshire (1971) showed that IAA oxidase activity was

promoted, and endogenous IAA levels decreased by lowering leaf water potential from -2 bar to -14.5 bar. The proposed model for rooting (figure 49) predicts that root initiation will be inhibited by low cell solute potential.

Figure 37 shows that, at plantlet water potentials below -10 to -15 bars (RH >70-80%) inhibition of rooting in an approximately linear fashion is experienced. An increase in IAA oxidase activity could be responsible for this phenomena, although Darbyshire measured leaf IAA oxidase activity, which may not be a reflection of activity in the basal regions where rooting takes place. IAA has long been known to be readily absorbed by intact roots and transported upwards in vascular tissue (Skoog, 1938). So *in vitro*, the media auxin content is likely to be at least as important as leaf derived auxin, especially in the rooting regions. At this point it is interesting to note that IAA transport appears to take place not only in the the phloem, but also in the vascular cambium (Bandurski & Nonhebel, 1984), which is the site of the first events which finally lead to root initiation (see section 1.1.3.2.).

Handa *et al* (1983) showed that cellular proline accumulation was most closely correlated with cell solute potential in cultured plant cells. They found that proline appeared to be only accumulated below -10 to -15 bars water potential, and this was also found to be the case in this study with micropropagated *Hosta* (figure 39). If proline accumulation is an indicator of increased solute potential in micropropagated *Hosta*, it would suggest that plantlet water potential changes up to -15 bars are due to changes in matrix or pressure

potential. This would correlate the inhibition of root proliferation with osmotic adaptation by solute accumulation.

Handa *et al* (1986) also showed that a logarithmic relationship existed between solute potential and proline accumulation. In *Hosta* below -15 bars, a logarithmic relationship seems to exist (figure 40). The slope of this relationship is very close to that found by Handa and his colleagues. However, below this water potential there seems to be a discontinuity and the slope is much lower. This may be due to the fact that water content adjustments were not made in this study.

The reduction in root proliferation at 50 and 32.5% RH could be due to other factors, such as the transitory wilting mentioned earlier, which occurred at the time when root initiation usually takes place.

The increase in root number between -3 and -12 bars plantlet water potential appears not to be consistent with the model proposed for rooting control (figure 49). The lowering of plantlet water potential from -3.3 to -5.5 bars did seem to inhibit root proliferation, in agreement with the model, but a further decrease to -15 bars appeared to stimulate rooting (figure 38).

This may be at least partially explained by changes in sucrose-phosphate synthase (SPS) activity. SPS plays a central role in the regulation of sucrose synthesis (Quick *et al*, 1989). Dancer *et al* (1990), (their figure 5) showed that in heterotrophic cell cultures of *Chenopodium rubrum*, endogenous SPS activity increased by approximately 40% when growth medium water potential was decreased from -4 to -6 bars. SPS activity then declined to the original level (-4 bars), when the media water potential was reduced to approximately -10 bars, after which the SPS activity continued to fall. Although Dancer *et al* (1990) found

no peak in cell sucrose levels at -6 bars, the increase in SPS activity was concomitant with an increase in protoplast volume and fresh weight. This indicates that solute accumulation occurred at this water potential.

If this solute accumulation occurs in *Hosta* plantlets, it could explain the inhibition of root proliferation, and would fit these results into the model.

Increased root elongation between 100% and 85% RH (-3.8 and -7 bars) was observed (figure 35). This is difficult to explain, but it may be related to an increase in available sucrose through increased SPS activity (see above), which could contribute to the maintenance of higher root pressure potentials.

In summary, although the inhibition of root proliferation between -3.8 and -10 bars seems to conflict with the proposed mechanisms for the control of rooting (figure 49), this may not be so. Further work into the role of SPS activity and metabolite accumulation is necessary.

4.2.2. <u>Uptake of Apoplastic Tracers from Growth Medium</u>

The fluorochrome Calcofluor, along with many other large molecules, is thought to be excluded from the symplasm, which should make it an effective apoplastic tracer (Peterson *et al*, 1981; Section 1.1.3.4.). In this study, calcofluor uptake was only observed in the first few layers of cells next to the medium containing calcofluor (Plates 8B, 8E-G). An exception to this was uptake into the vascular tissue near newly emerged roots of *Choisya* (see below).

The cell walls of all cells in contact with, and a few cells away from the culture medium showed positive staining with Auramine (Plates 2C, 5D, 6B, 6D-F, 7B, and 9A). Auramine stains positive for lignin,

cutin and suberin. These compounds are hydrophobic, and it is likely that one or more of them is responsible for the lack of calcofluor penetration.

Wilms *et al* (1990) found that suberin was deposited in Tobacco (*Nicotiana tabacum*) plantlet cells at cut surfaces in contact with the medium after 4-7 days. They suggested that this forms a barrier to water and hormone uptake. The results presented here indicate that a similar phenomenon occurs in *Hosta* and *Choisya* plantlets. Wilms *et al* also reported that this suberisation could be reduced by higher agar concentrations, but this was not tested in this study. If that is the case, differential hormone and nutrient uptake could explain why agar concentration can be so important in plantlet morphology. Wilms' group also found what they called callus cells between the suberised cells, suggesting that they may be a route of uptake. These may be equivalent to the large cells (up to 0.5 mm diameter) with little or no secondary thickening (l: Plate 6B), which were distributed throughout the basal surface, but concentrated away from the central basal area (see below). No apoplastic route through these cells was discovered using calcofluor tracer in this study, but calcofluor was taken up into these cells (Plate 8G), and also when the basal surface was removed or wounded (section 3.5.1.5). As calcofluor is too large to pass through the plasmalemma (Peterson *et al*, 1981), it must be assumed that vesicle budding off the plasmalemma is responsible. The lack of further progress into the cytoplasm of other, adjacent cells may be due to the molecule being too large to pass through the plasmadesmata (PD), which occur in large quantities near the plantlet/medium interface. Another possible function for these large cells is mucilgel excretion, which is consistent with the speculation about a high level of vesicle production

in these cells. Careful observation of Plate 8G, shows the presence of what appears to be mucilage (m) near one of these cells. This substance was observed frequently during the course of this study. This may be a root-like response to the aqueous environment. Another interesting root-like characteristic of shoot bases, at least those of *Hosta* species, is the presence of an endodermis (Plate 7B). Endodermises are rare in plant stems (Mauseth, 1988). This is believed to be the first such report of an endodermis is such a location. The endodermis does not form a complete ring around the disorganised vascular tissue formed in the shoot base, but only appears to form in areas adjacent to cells in contact with the medium.

The presence of large amounts of PD and these "mucilage" cells, indicates that the tissues of the shoot/medium interface are not merely a wound response, as suggested by Wilms *et al* (1990), but a group of cells specialised in uptake of nutrients, and possibly the symplastic uptake of water through membrane budding. This tissue could be considered a highly adapted "root" involved not only with the uptake of water and mineral nutrients, but also of carbohydrate. An investigation of the shoot/medium interface with transmission electron microscopy should be able to confirm these findings, and is suggested as a next step in investigating this area.

So far I have only discussed the role of the shoot base, but what of the root system? Similar suberinisation or cutinization occurred the epidermal area of all the roots studied (Plates 5D, 6D-F). Calcofluor did not penetrate further than this layer (Plates 8E-G). The region two or three cells immediately beneath the epidermis is known as the hypodermis (Toothill, 1984; Mauseth, 1988). Until very recently, the role of the hypodermis was thought to be solely structural, consisting

of sclerenchyma cells. However, Perumalla (1986) found that 88% out of 213 angiosperm species studied had a root hypodermis with a Casparian band. Peterson (1988) proposed that the term "exodermis" be used to denote a hypodermis with a Casparian band. Observation of the hypodermal regions of *Paeony* (Plate 5D) and *Hosta* (Plates 6D-F) roots, reveals no evidence of a Casparian band. However, Peterson (1988) could only visualise exodermal Casparian bands by using an alkali pre-treatment, which removed the superficial suberin lamellae that is deposited soon after the Casparian band. This could explain why root exodermises have not been reported earlier, as Mauseth (1988) reported that exodermal suberin lamellae are deposited immediately, whereas in the endodermis, their formation can be some time after Casparian Band deposition. This pre-treatment was not used in this study. The suberin lamellae do not block apoplastic water movement (Clarkson *et al*, 1987; Peterson, 1988), so the pre-treatment is essential in the histochemical identification of an exodermis. However, the presence of an exodermis was deduced from the lack of calcofluor penetration further than the hypodermis (Plates 8E/F). Indeed, careful scrutiny of Plate 8E reveals that the anticlinal walls of the hypodermis do appear thicker than the periclinal walls, as visualised by the cellulose staining of the cell walls by the tracer.

Roots formed in reduced humidity and cold storage often possessed incomplete endodermises, (Plates 8E & 6F). Occasionally, no endodermis was present at reduced humidities (Plate 6E). Endodermises have been reported to be almost universally present in roots, with only three species known not to possess one (von Guttenberg, 1968). As endodermises were observed in "normally" cultured plantlets and weaned plants, it is suggested that the laying down of an endodermis is somehow delayed or interfered with in these particular sets of conditions.

The results obtained here for tracer uptake by micropropagated *Hosta* and *Choisya* roots are the same as those reported by Peterson *et al* (1981) who used *Zea mays* and *Vicia faba* seedlings grown in vermiculite. This indicates that difficulties encountered when weaning plantlets are not related to non-functional roots, but to stomatal functioning, thin cuticles or photosynthetic incompetence (see section 1.1.4.1.1.), except perhaps a lack of vascular connection with the shoot. However, vascular connections have been demonstrated for *Hosta* (Plate 6F) and *Choisya* (Plates 8C/D, see below). The proposal that vascular connections are a problem with emerged roots in culture may stem from the difficulty of obtaining the correct plane and position of a longitudinal section reveal a long enough portion of vascular tissue to establish if a vascular continuity exists. However, this may be a problem in some species under certain conditions, as a vascular connection has been shown not to occur until the later stages of primordia development, and occasionally has been shown to be concurrent with root emergence (see Section 1.1.3.2.).

The presence of calcofluor in the vascular tissue leading from newly emerged roots of *Choisya* (Plates 8C/D), not only demonstrates a vascular connection with the shoot, but apoplastic uptake from the medium. Peterson *et al* (1981) found that a fluorescent tracer similar to calcofluor (Tinopal CBS) only gained access to the cortex and stele of *Zea mays* and *Vicia faba* roots where a secondary root which had just emerged was present. They found that the point of entry was when the secondary root broke through the "epidermis" (more properly the exodermis). In Plates 8C and 8D, the asterisks indicate this area in the shoot exodermis where the roots emerged, and the tracer does seem to have penetrated to a greater depth. Perhaps the tracer entered the stele

out of the plane of section. However, if this is the only point of entry for the tracer, it indicates that water transport is occurring both acro and basipetally. As the same result was obtained with plantlets cultured at lower humidities (results not presented), where a transpiration stream would be expected (see above), this hypothesis must be treated with some caution.

There have been some reports of apoplastic uptake by the root tip (see Robards & Jackson, 1976). The exodermal Casparian band is thought to mature 30-120 mm from the root tip, but is thought to be mature within 5 mm of the root tip in slowly elongating roots. (Peterson & Perumalla, 1984; Perumalla & Peterson, 1986). There is some evidence that the *epidermal* walls near the root tip are impermeable to apoplastic dyes (Peterson & Perumalla, 1986), and it may be that the connection between the exodermis and these modified epidermal cells is not yet established in newly emerged *Choisya* roots. Differences in the balance between the development of these two barriers might explain why calcofluor uptake was not observed in newly emerged *Hosta* roots.

It appears, then, that in most cases, both in cultured and non-cultured plants, apoplastic water and nutrient uptake from the media or soil is confined to epidermal cells. Uptake can then proceed either apoplastically or symplastically to the endodermis, where it must once again enter the symplasm to gain entry to the stele.

4.2.3. Dormancy in *Hosta*

4.2.3.1. Physiology of Dormancy

Leaf senescence and abscission occurred to some extent in all cold stored *Hosta* cultures (figures 43 & 45) and also in plants out of culture in autumn, where aerial plant parts die back leaving only the

root system and apical bud. This indicates that a similar phenomenon is taking place *in vitro*.

Unexpectedly, the rate of leaf abscission at 11/16°C in the Neoplants cold room was greater than at 4°C at Durham (compare figures 43 & 45). This is a strong indication that leaf senescence and abscission is not a temperature stress response, but a process controlled by some other factor that varied between the two cold room environments. The alternating temperatures (11/16°C) at Neoplants could conceivably cause an increased rate of senescence, but this seems unlikely as the temperature differential is not great and any controlled environment will vary by a few degrees either side of the mean temperature. In addition to this, there is no evidence that temperature is involved with the regulation of senescence and abscission.

Abscission in cultured *Hosta* occurred progressively (Plate 7B), also indicating that this is not a stress response. The effects of light quality and quantity are quite well documented. Guiamet *et al* (1989) found that progressive leaf senescence in *Glycine max* was strongly influenced by the ratio of incident red:far red light, with red light delaying senescence and far red having the opposite effect. The type of fluorescent light tubes used in the different cold rooms may have influenced the rate of *Hosta* senescence. Light intensity has also been reported to affect senescence and abscission (Wareing & Phillips, 1981), but this may be due the preferential absorbance of red light by any canopy, or other changes in R:FR ratios due to changes in the light source.

The variety 'Golden Prayer' was much less sensitive to these changes, only exhibiting a low level of abscission and actually showing

some leaf growth at 4°C. As this variety is a hybrid of unknown origin (section 2.1.1.), it is not possible to postulate that this is due to adaptation to a cold environment, but this may indeed be the case. The increased chilling requirement adds weight to this assumption (see later). Roots were formed above the growth media *in vitro* in 'Golden Prayer', and were frequently green. It is not known if this bears any significance to the results obtained.

Hosta in culture can not be said to become truly dormant, as all cultures were able to grow when removed from cold storage. However, plantlet transferred to the nursery did become truly dormant if removed from the cold too early whilst still in culture. When dormant in the nursery, some leaves grew back and then appeared to at least partially senesce, but little abscission was observed (Plate 7B). This phenomenon was not observed in culture, and the possibility that nutrient depletion masked this effect was ruled out by the fact that the multiplication rate of culturally "dormant" plantlets when re-introduced to multiplication culture was no different to that of non-cold stored plantlets (section 3.6.5.2.). However, it can not be ruled out that some other factor is responsible for the lack of true dormancy in culture. In the nursery, dormancy occurred when plantlets had been cold stored for as little as one week, and was only overcome by several months cold storage (Plate 9).

The time taken to reach half the maximum leaf number after the removal of cultures from the cold ($T_{½}$) increased during the first few weeks of cold storage, and then declined again after a few months (figures 44 & 46). Unlike the rate of abscission, this phenomenon was speeded up at a lower temperature (compare figures 44 & 46 for varieties 'Francie' and 'Blue Wedgewood'). A lowering of temperature has often

been reported to decrease the time required to break dormancy by cold treatment, as is the case here.

It is evident that chilling requirement differs among *Hosta* species, with *H. tardiana* ('Blue Wedgewood') having the least requirement, then *H. fortunei* ('Francie'), with the hybrid 'Golden Prayer' needing a considerably longer period of cold storage to remove its "dormancy".

It is worth noting that the conditions that appear to initiate "dormancy", also seem to be responsible for release from dormancy. Dormancy is normally initiated by reducing daylength in temperate plants (eg. Wareing & Phillips, 1981), but in this case daylength is actually increased in the cold room. However, other factors such as nutrition, water status, temperature and light intensity can modify the time of dormancy inception (Berrie, 1984).

Dormancy in the nursery seems to be similar to culture "dormancy" (see section 3.5.6.; Figure 48), but was not followed from the inception of dormancy through to release. Isoenzyme profiles were similar to those found *in vitro* (results not presented), but were not followed through dormancy.

More work is required to prove conclusively that the "dormancy" found *in vitro* leads directly to the true dormancy observed in the nursery, but it is a reasonable assumption that this is the case, as both phenomena follow a very similar time-scale and a large degree of varietal variation in cold store requirements have been observed (J. R. Nicholas *pers. com.*). Indeed, this variation was the major reason for the initiation of this study.

4.2.3.2. Biochemical Markers for Dormancy

4.2.3.2.1. AAT Isoenzymes

A total of 11 AAT isoenzymes were identified from the *Hosta* species studied (figure 41). Using staining procedures at different substrate concentrations and pH, artefactual bands were identified and it was established that all of the isoenzymes found were mitochondrial in origin (see section 1.3.5.2.; Gracia *et al*, 1987)

AAT isoenzymes can be categorised into two distinct groups. The first, low mobility isoenzymes (AAT-A1-7) and a second anodic (high mobility) set of isoenzymes (AAT-B1-4). AAT-B isoenzymes were expressed in all samples studied, but AAT-A isoenzymes were only found in cold stored cultures (figure 42; plate 10). AAT-A's appeared after 3 days in the cold (4°C), and disappeared after 3 days when transferred back to growth room conditions (figure 42b). This effect was repeatable throughout cold storage and for up to six three day alternations between cold and warm condition. Thus, AAT isoenzyme patterns were found not to provide a suitable system for "dormancy" markers.

Splittstoesser & Stewart (1970) found that a low mobility group of AAT isoenzymes disappeared first during the germination of pumpkin seeds, so it is possible that AAT-A's are associated with quiescence or slow growth rather than any dormancy effect.

Farnham *et al* (1990) found that these two groups of AAT's existed over a wide range of species, and suggested that AAT-A's (equivalent to their AAT-1's) were the predominant in roots, whilst AAT-B's (AAT-2's) were the major forms in leaves and legume root nodules. The results presented here do not appear to support this hypothesis, as AAT-B's are

expressed even when leaves have abscissed; however, AAT-A's were unique to cold stored roots.

AAT is an important enzyme in the carbon and nitrogen metabolism of all plants, and it appears that AAT-B's are the isoenzymes associated with this general metabolism in *Hostas*, as they were found in all samples. The appearance of AAT-A's in cold stored roots could be associated with more specific pathways such as the production of cold acclimation specific proteins (as found by Mohapatra *et al*, 1989; Dunn *et al*, 1990).

4.2.3.2.2. Esterase Isoenzymes

The major esterase isoenzyme bands showed no qualitative and only small quantitative changes when transferred into cold conditions and in subsequent cold storage. However, the relatively faint, highly mobile bands did show changes (figure 47, Plates 10B-D). The faintness of these bands is not necessarily indicative of low *in vivo* activities, but may reflect low low affinities for the substrate used (α naphyl acetate), which is an artificial substrate. When plantlets were transferred into the cold, all this group of bands disappeared in the three varieties and two temperature regimes tested (figure 47, Plates 10B-D, Sections 3.2.2.4. & 3.2.2.5.). Some of these bands, or new ones, then appeared after a period of cold storage. One band (*: figure 47) was only detected under these circumstances, and occurred in all varieties and conditions tested.

The appearance of this band correlates well with the removal of culture "dormancy" (arrows: figures 44 & 46). The possible significance and use of this isoenzyme as a biochemical marker for culture "dormancy" is discussed below.

Chen *et al* (1970) showed that the number and mobility of esterase isoenzymes increased with *Xanthium* leaf age. The expression of new high mobility isoenzymes has also been reported with increasing leaf age and senescence in *Festuca pratensis* (Thomas & Bingham, 1977). In this study, the number of *Hosta* high mobility esterases actually *decreased* during the period of leaf senescence (compare Plates 10B-D with figures 43 & 45). This indicates either that the isoenzyme changes in *Hosta* cultures are not related to leaf senescence in cold storage, or that cold induced *Hosta* leaf senescence and abscission is a separate phenomenon to leaf ageing and subsequent senescence. It seems likely that both of these hypotheses are correct, as band changes do not seem to correlate with leaf senescence, occurring well after leaf senescence has halted at 11/16°C, but while senescence is still in progress at 4°C (arrows: Figures 43 & 45). In addition to this, unlike the cases reported above, no increase in total esterase activity was observed in conjunction with leaf senescence.

McCown *et al* (1970) found that esterase isoenzyme patterns in *Dianthus* callus cultures changed in response to light and temperature. When they placed cultures in the cold the number of expressed isoenzymes was reduced, with 5 or 6 bands (in different varieties) only being expressed in the warm. *Dianthus* also had a group of highly mobile isoenzymes, some of which were expressed in the cold. However, as the callus cultures were maintained in warm or cold conditions for over a year, their results do not conflict with those presented here.

There are many problems associated with assigning specific roles to alterations in isoenzyme profiles to specific metabolic or physiological events (Section 1.3.6.). This is even more true when dealing with esterases, which have very diverse substrate specificities. The band

which correlates with dormancy loss may be involved with the hydrolysis of ABA, or some other substance which controls the maintenance of dormancy, but this is mere speculation without an intensive study of the biochemistry and distribution of this isoenzyme. However, this isoenzyme has been shown to correlate well with "dormancy" loss in two species and one unknown hybrid of *Hosta*, with different cold storage requirements under two temperature regimes.

4.2.3.2.3. Proteins

Large quantitative and qualitative changes in total extractable protein were observed when *Hosta* plantlets were transferred into the cold and in subsequent cold storage (Plate 11), and careful analysis of the gels has yielded no protein that might be useful as a marker for dormancy.

A large increase in the amount of extracted protein was observed in the first few days of cold storage. This could be an artifact caused by some compound being produced by the plantlets in the warm that interferes with the extraction. The majority of the proteins extracted with the relatively simple extraction procedure used in this study will be globulins.

A protein of approximately 50,000 rmm was induced in large quantities upon entry into cold conditions in varieties 'Francie' and 'Blue Wedgewood', but not in 'Golden Prayers' (see plate 11). After approximately 2 weeks in the cold, this band is much reduced in intensity, reaching levels equivalent to 'Golden Prayers'. 'Golden Prayers' appears to be more cold tolerant than the other varieties tested (see Section 4.2.3.1.). This protein, then, seems likely to be a "cold stress" protein. Such proteins have been reported previously in

many species and are thought to be the result of *de novo* synthesis (Hahn & Walbot, 1989). Other induced proteins have been shown to be specific to cold acclimation (Dunn *et al*, 1990).

The attempt to identify what proteins are synthesised *de novo* using radioactive labelling (section 3.2.2.7.) failed, probably due to very low levels of uptake of the radioactive amino acid from the culture medium in the cold. This line of investigation was dropped at this stage as the time required for radioactive tracer uptake, combined with the long exposures of gels to photographic plate means that results could be obtained just as rapidly by simply placing the cultures in the growth room and assessing the growth rate. However, more useful techniques might be developed using isotopes of mineral nutrients, such as nitrate and phosphate, which should be readily taken up by the plantlets.

4.3. Conclusions

This study has investigated various aspects of plant micropropagation and attempted to use indices other than simple growth data to interpret and predict physiological problems. Using fluorescence microscopy, features such as root and shoot initiation could be used as very early predictive indices for multiplication and rooting culture success. In addition to this, a low mobility esterase isoenzyme was identified that is correlated with increased shoot health and inhibited rooting. Although this work is at an early stage, it may prove to be crucial for the screening of large numbers of cultural treatments, the results from which could be obtained in a few days rather than weeks.

Fluorescence microscopy work also led to the suggestion that water relations are a critical factor for *P. lactiflora* in culture, having large, almost vitreous cells and a thin leaf cuticle. Investigations of

in vitro root anatomy and function using fluorescence microscopy coupled with the apoplastic fluorescent dye tracer calcofluor indicates that water uptake in roots in culture is the same as in roots of most weaned plants (ie, symplastic after the epidermis). In addition to this, vascular connections were observed, suggesting that the requirement for high humidity weaning is not a result of abnormal or non-functional roots. *Hosta* shoot bases can be regarded as "roots", perhaps specialising in carbohydrate uptake. Evidence also pointed to this being the case for *P. lactiflora* and *Choisya ternata* plantlets.

Using data reported in the literature, investigations of reduced humidity culture of *Hosta*, the inclusion of GA_3 and elevated inorganic phosphate in the growth medium of *P. lactiflora*, possible mechanisms for the control of rooting were discussed and summarised in figure 49. It was hypothesised that in addition to hormonal factors, *in vivo* reducing sugars and/or cell solute potential could play an important role in determining whether adventitious rooting occurs. This may explain why rooting is so unpredictable in some species, as it would depend on subtle balances in plantlet respiration, photosynthesis, sucrose uptake and translocation. It may also explain why low ionic strength media are often required for rooting.

The work on *P. lactiflora* had to be abandoned due to problems with the multiplication and growth of cultures. This could have been due to a growth room malfunction or some other unidentified problem in the micropropagation system in Durham. Work continued with *Hosta*, and the problem of *in vitro* "dormancy" was addressed.

No true dormancy was identified in culture, but rather an inhibition of leaf re-growth after senescence and abscission in cold

storage. This contrasted to dormancy in the nursery where growth ceased completely in conditions conducive to growth if a requirement for a period of cold store was not fulfilled. It is suggested that these effects are the same, as dormant plants in the nursery do exhibit some leaf re-growth before they become fully dormant. *In vitro*, the situation could be that medium nutrients are exhausted before plantlets become dormant. However, all cold stored plantlets appeared to grow normally when re-introduced to multiplication culture.

A highly mobile esterase isoenzyme seems a likely candidate for a biochemical marker of this cultural "dormancy", and may prove to be very useful in predicting when plantlets have had their cold requirement fulfilled. However, the connection between culture "dormancy" and true dormancy, and the effectiveness of this isoenzyme as a marker for dormancy in other systems both in and out of culture needs to be further investigated.

In summary, much information has been gathered about the behaviour of plants in culture, but this is a very large and diverse field which requires more attention than can be given in the course of a single PhD. Tentative hypotheses have been put forward in the hope that they will be more deeply investigated in the future. The results presented here increase the understanding of plants in culture, but there remain many areas that require investigation before the techniques of micropropagation can be utilised with many "difficult" species, and to their full potential.

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Appendix 1

After Slavik (1974)

Relative humidity values over saturated solutions at 25°C

Compound	%Rh	Compound	%Rh	Compound	%Rh	Compound	%Rh
P_2O_2	0,0	$CoBr_2$	41,5	KBR+sucrose	63,0	$Na_2CO_3 \cdot 10H_2O$	87,0
NaOH	7,0	$CrCl_3$	42,5	NaCl+sucrose	63,0	$ZnSO_4 \cdot 7H_2O$	88,5
$LiBr \cdot 2H_2O$	7,0	$CeCl_3$ (tech.)	45,5	$NaNO_2$	64,0	$MgSO_4 \cdot 7H_2O$	89,0
KOH	8,0	KCNS[4]	46,5	$K_2S_2O_3$	66,0	$BaCl_2$	90,0
$ZnBr_2$	8,5	$LaCl_3 \cdot 7H_2O$	47,0	$CuCl_2 \cdot 2H_2O$	67,0	$MgSF_2$	91,5
H_3PO_4	9,0	$LiNO_3 \cdot 3H_2O$	47,0	$NaCl+NaNO_3$	69,0	Na tartrate	92,0
$LiCl \cdot H_2O$[1]	12,0	$Mg(CNS)_2$	47,5	Na methane		KNO_3[8]	92,5
Ethanol amine		$K_4P_2O_7 \cdot 3H_2O$	49,5	sulphonate	72,5	NH_4HPO_4	93,0
sulphate	12,5	$NH_4NO_3+NaNO_3$	50,0	NaCl+KCl	71,5	Pyrocatechol	93,5
$CaAc_2 \cdot H_2O$ +		$Ca(NO_3)_2 \cdot 4H_2O$	50,5	$NaAc \cdot 3H_2O$	73,0	$Pb(NO_3)_2$[9]	95,5
Sucrose	13,0	KBr+urea	51,0	$NaNO_2$	74,0	$CaH_4(PO_4)_2 \cdot H_2O$	96,0
$CaBr_2$	16,5	$Zn(MnO_4)_2 \cdot 6H_2O$	51,0	$NaCl+Na_2SO_4 \cdot 7H_2O$	74,0	KH_2PO_4	96,0
$CaAc_2 \cdot H_2O$	17,0	Sucrose+urea	52,0	$BaBr_2$	74,5	$CaHPO_4 \cdot 2H_2O$	97,0
$Ca(CNS)_2 \cdot 3H_2O$	17,5	$Na_2Cr_2O_7 \cdot H_2O$[5]	53,0	NH_4Br_2	75,0	K_2SO_4	97,5
$LiI \cdot 3H_2O$	18,0	$Mg(NO_3)_2 \cdot 6H_2O$	53,0	K tartrate[7]	75,0	$K_2Cr_2O_7$	98,0
$CaZnCl_2$[2]	20,0	$NiCl_2 \cdot 6H_2O$	53,0	NaCl	75,5	$KClO_3$	98,0
$KHCO_2$ (formate)	21,5	$Ba(CNS)_2 \cdot 2H_2O$	54,5	Urea	76,0	Distilled H_2O	100,0
KAc	22,5	Glucose	55,0	NH_4Cl	78,0		
$NiBr_2 \cdot 3H_2O$	27,0	$Pb(NO_3)_2+NH_4NO_3$	55,0	$AgNO_3+Pb(NO_3)_2$	78,5		
$CaCl_2$[3]	29,5	$MnCl_2 \cdot 4H_2O$	56,0	$(NH_4)_2SO_4$	80,0		
$KF \cdot 2H_2O$	30,5	NaAc+sucrose	57,0	KBr	80,0		
$MgBr_2 \cdot 6H_2O$	31,5	$NaBr \cdot 2H_2O$[6]	57,5	$Zn(CNS)_2$	80,5		
$Sr(CNS)_2 \cdot 3H_2O$	31,5	$SrBr_2 \cdot 6H_2O$	58,5	NaH_2PO_4	81,0		
$MgCl_2 \cdot 6H_2O$	32,5	$FeCl_2 \cdot 4H_2O$	60,0	$AgNO_3$	82,0		
$MnBr_2 \cdot 6H_2O$	34,5	$NaMnO_4 \cdot 3H_2O$	61,5	KCl	85,0		
$Ca(MnO_4)_2 \cdot 4H_2O$	37,5	$NH_4NO_3+AgNO_3$	61,5	Sucrose	85,0		
NaI	38,0	$CuBr_2$	62,0	Resorcinol	85,0		
$FeBr_2 \cdot 6H_2O$	39,0	NH_4NO_3	62,5	$KCl+KClO_3$	85,0		

[1] $LiCl \cdot XH_2O \rightarrow LiCl \cdot H_2O$ at 12,5°C
[2] Prepare by mixing $Ca(OH)_2 + ZnO + 4HCl$.
[3] May be unstable around 20°C.
[4] Very toxic!
[5] Very good; small temperature coefficient.
[6] Uncertain around 20°C; large temperature coefficient.
[7] Potassium tartrate is excellent.
[8] Large temperature coefficient.
[9] Solution slightly acid due to hydrolysis, but stable.

Appendix 2

Humidity and Osmotic Potential above NaCl Solutions at 25°C

Molarity	Osmotic Potential/Bars	%Rh
0.05	-2.34	99.83
0.10	-4.39	99.68
0.15	-6.44	99.53
0.20	-8.50	99.37
0.25	-10.57	99.22
0.30	-12.65	99.07
0.35	-14.77	98.92
0.40	-16.89	98.77
0.45	-19.05	98.61
0.50	-21.22	98.44
0.55	-23.41	98.27
0.60	-25.60	98.10
0.65	-27.82	97.94
0.70	-30.04	97.79
0.75	-32.30	97.63
0.80	-34.56	97.47
0.85	-36.86	97.31
0.90	-39.16	97.14
0.95	-41.48	96.97
1.0	-43.80	96.80
2.0	-97.7	93.0
3.0	-168	88.3
4.0	-252	83.0
5.0	-327	78.4
Saturated solution	-374	75.8

*Taken from Slavic (1974)

Appendix 3

Osmotic Potential of Sucrose Solutions at 20°C

M	bar	M	bar	M	bar
0.000	-0.00	0.200	-5.36	0.400	-11.25
05	13	05	50	05	42
10	26	10	64	10	58
15	40	15	79	15	74
20	54	20	94	20	89
25	67	25	-6.08	25	-12.05
30	80	30	22	30	22
35	94	35	36	35	38
40	-1.07	40	50	40	53
45	21	45	65	45	69
50	34	50	79	50	85
55	47	55	93	55	-13.02
60	61	60	-7.07	60	18
65	74	65	22	65	34
70	87	70	36	70	50
75	-2.01	75	51	75	68
80	14	80	65	80	84
85	27	85	80	85	-14.01
90	41	90	94	90	17
95	54	95	-8.09	95	33
0.100	67	0.300	24	0.500	50
05	81	05	38	05	67
10	95	10	53	10	83
15	-3.08	15	68	15	99
20	21	20	82	20	-15.15
25	34	25	97	25	33
30	47	30	-9.12	30	49
35	61	35	26	35	67
40	75	40	41	40	84
45	88	45	56	45	-16.03
50	-4.01	50	70	50	20
55	14	55	86	55	38
60	27	60	-10.00	60	56
65	41	65	15	65	74
70	54	70	30	70	93
75	67	75	46	75	-17.11
80	81	80	62	80	28
85	94	85	78	85	46
90	-5.08	90	94	90	65
95	22	95	-11.10	95	83

M	bar	M	bar	M	bar
0.600	-18.00	0.800	-25.87	1.000	-35.05
05	18	05	-26.08	05	30
10	37	10	30	10	56
15	56	15	51	15	86
20	74	20	72	20	-36.16
25	93	25	94	25	42
30	-19.12	30	-27.15	30	67
35	31	35	35	35	92
40	49	40	55	40	-37.18
45	68	45	76	45	43
50	86	50	96	50	68
55	-20.06	55	-28.16	55	94
60	24	60	36	60	-38.19
65	43	65	57	65	44
70	61	70	77	70	70
75	81	75	97	75	-39.00
80	99	80	-29.17	80	30
85	-21.18	85	43	85	56
90	37	90	68	90	81
95	58	95	88	95	-40.06
0.700	77	0.900	-30.09	1.100	32
05	97	05	34	10	93
10	-22.16	10	59	20	-41.43
15	37	15	85	30	-42.04
20	56	20	-31.10	40	55
25	76	25	30	50	-43.05
30	95	30	50	60	66
35	-23.16	35	76	70	-44.27
40	35	40	-32.01	80	77
45	55	45	26	90	-45.38
50	74	50	52	1.200	99
55	95	55	77	10	-46.60
60	-24.15	60	-33.02	20	-47.21
65	37	65	28	30	81
70	59	70	53	40	-48.42
75	80	75	78	50	-49.03
80	-25.01	80	-34.04	60	64
85	22	85	29	70	-50.24
90	44	90	54	80	95
95	66	95	80	90	-51.56
				1.300	-52.27

Taken from data summarised by Slavik (1974)