Acclimatization physiology in tissue cultured plants

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ABSTRACT

Physiological and morphological aspects of acclimatization were studied in cultured tomato (*Lycopersicon esculentum* Mill.), banana (*Musa accuminata* L.) and date palm (*Phoenix dactylifera*). The nutrient availability from agar solidified culture medium was determined to establish the nutrient status of the cultured plantlets before transfer to *ex vitro* conditions. Analysis of the plant tissues demonstrated decreasing tissue concentrations of the major elements nitrogen, phosphorus and potassium with decreasing concentration of basal salts in the medium. The effects of agar and increasing sodium concentration in the culture medium was studied in cultured banana plants. Plantlets grown on agar solidified medium with increased levels of sodium, exhibited reduced growth and stomatal movement. The use of agar as a solidifying agent was shown to reduce root growth, development and stomatal functioning in these plants.

The efficiency of ion and water uptake, and translocation in *in vitro* and acclimatized tomato plants was assessed using [^{32}P]-orthophosphate and [^{3}H]-tritiated water. The functional capacity of the root system formed *in vitro* was established, and assessed following acclimatization treatments at 40% and 80% relative humidity. Comparative studies with tomato seedlings demonstrated reduced efficiency of ion translocation to the shoot in plantlets growing *in vitro*. However, transport to the shoot improved during acclimatization. Ion absorption studies on *in vitro* and acclimatized palm plants demonstrated phosphate uptake and translocation in both plant types.

A detailed examination of the tissue structure through the root/shoot junction and roots of cultured, acclimatized and seedling tomato plants illustrated differences in the vascular development between the three plant types. However, no major abnormalities were observed which could have accounted for the reduced translocation efficiency in the cultured plants. Increased vascularization present in the root/shoot junction of the cultured plants may increase resistance to the transpiration flow through the region. The type of root system produced *in vitro* and the root/shoot ratio was manipulated using varying IAA and sucrose treatments. Improved root development and plantlet survival rates were achieved by reduced exposure to IAA during the root initiation phase followed by root elongation on IAA free medium supplemented with sucrose. Acclimatization at low relative humidity (40%) was achieved by producing plantlets with balanced root/shoot ratios and a well developed root system.

ABA	abscisic acid
ARC	Agricultural Research Council solution
BA	benzyladenine
BAP	benzylaminopurine
Ci	curies
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
GA_3	gibberellic acid
IAA	indole-3-acetic acid
IBA	indole-3-buytric acid
mRNA	messenger RNA
MES	morpholino-ethane-sulphonic acid
MS	Murashige and Skoog
NAA	naphthaleneacetic acid
PBS	phosphate buffered saline
PGR	plant growth regulator
RNA	ribonucleic acid
RWC	relative water content
SEM	scanning electron microscope
tRNA	transfer RNA
Tween	polyoxyethyelensorbitan (mono-oleate)
VTZ	vascular transition zone
v/v	volume per volume
w/v	weight per volume

Contents

Chapter 1: Introduction

1.1. General introduction

In recent years the production of plants using tissue culture techniques has become increasingly important in horticulture, agriculture, plant breeding and in the production of pharmaceutical products from plant tissues. One of the techniques which has become particularly important in horticulture is micropropagation. This technique involves *in vitro* vegetative multiplication of plantlets from a variety of starting tissues. These may be axillary or adventitious meristems, leaf, stem or root tissue. The genetic stability of the plantlets produced will vary according to the tissue and culture procedure chosen (Pierik, 1988). Using controlled environmental conditions and growth media containing essential minerals, carbohydrates, vitamins and optimum levels of plant growth regulators, prolific production of embryoids and shoots can be obtained *in vitro*. Micropropagation enables large scale production of high value crops or new varieties in a relatively short space of time. Plantlet multiplication rates of 1.5 - 2 times per month can be achieved *in vitro* with many species. The technique also allows production of disease free stock (George and Sherrington, 1984).

Stages involved in micropropagation are:

1. Selection of parent material and initiation of cultures.
2. Multiplication of plant tissue *in vitro*.
3. Division and root initiation on individual shoots.
4. Acclimatization and establishment of plants *ex vitro*.

Considerable potential exists for the propagation of plants for horticulture, agriculture and forestry using this technique. However, the widespread use of micropropagation is presently hindered by a number of problems. Those mentioned by Pierik (1988), are as follows; uncontrolled somaclonal variation, particularly in systems using adventitious bud proliferation and callus mediated plantlet production. A lack of basic knowledge concerning the physiological and environmental factors affecting organ and somatic embryo formation. The recalcitrant nature of many woody species, particularly lack of rejuvenation and root induction in culture, and the presence of latent contaminating micro-organisms has meant that considerable potential still remains for mass propagation within this group of plants. Mass propagation *in vitro* is labour intensive and production costs

can be high, hence the current techniques are not always economically viable for commercial use. The acclimatization of plantlets (transfer from culture to *ex vitro* conditions of lower relative humidity and growth in a conventional peat based substrate), often proves to be one of the most problematic stages of culture. This final point forms the basis for the work presented and discussed in this thesis.

Problems associated with acclimatization of cultured plants

It has been realized for some time that micropropagation systems have the potential to yield thousands of plants, cheaply and quickly with successful adaptation of *in vitro* plantlets to normal field conditions, following a short acclimatization period (Boxus, 1974). Transplanting of *in vitro* raised plantlets requires careful regulation of both temperature and humidity, to allow gradual adaptation to *ex vitro* conditions. Many species require a prolonged period of gradual exposure to reduced levels of humidity (Short, Warburton and Roberts, 1987). This often involves use of costly fogging units, which maintain high glasshouse humidity, with pulsed spraying of pressurised water (500 psi), to produce fine water droplets, ranging in size between 10 and 30 microns. Spraying times can be varied to produce the required humidity (Scott, 1986). Although these units are expensive, they regulate water availability much more precisely than mist units, which often result in conditions which are too wet. Excess water and inadequate air movement can lead to loss of plantlets through rotting disorders and fungal diseases. Substantial losses of plants may occur during this period (Conner and Thomas, 1982). Losses as high as 70% have been reported with some species e.g. *Prunus cerasus* L. (plum) (Marin and Gella, 1988), *Phoenix dactylifera* date palm (D. Blakesley, personal communication).

Anderson (1981) reports acclimatization periods of six weeks for many woody species, but suggests that this time may be reduced if plantlets are transferred from culture with a partially developed root system. On a commercial scale many of these procedures are uneconomic and at present cannot compete with conventional methods of propagation in low value crops, such as soft fruit, vegetables and some ornamentals. Tissue culture production costs are reported to be 3 - 4 times higher than in some nursery crops (Swartz *et al.*, 1981). A greater understanding of the physiological problems associated with this stage of culture may lead to more successful acclimatization in problem species.

Some of the problems associated with acclimatization have been identified, and over the past few years an attempt to understand some of the physiological causes behind them has been made. The main areas which have been recognised are:

1. Plantlets have little control over water loss, resulting in severe wilting and dehydration leading to irreparable damage to tissues.
2. The shoot/root connective region appears to be poorly developed, and may prove to be inefficient in nutrient and water translocation.
3. Plantlets have very limited photosynthetic capacity due to the heterotrophic mode of nutrition formed in culture. Leaves formed *in vitro* seem to be incapable of photosynthesising.
4. The root system formed *in vitro* appears to have limitations in more conventional growth media and may not function efficiently.

The reasons for many of these problems may lie with the tissue culture conditions in which the plants have been grown.

Epicuticular wax development

Within the culture vessel the relative humidity is 100%. It has been proposed that this could be the reason for the poor development of structured epicuticular waxes often observed in cultured plants (Grout and Aston, 1977 ; Wardle *et al.*, 1979; Sutter, 1985; Sutter *et al.*, 1988). Studies of epicuticular wax formation have been carried out on a number of species (e.g. *Brassica oleracea* (cauliflower), Grout 1975; *Dianthus caryophyllus* (carnation), Sutter and Langhans 1982; and *Liquidambar stryaciflua* (sweetgum), Wetzstein and Sommer, 1983), using contact angle methods (Fogg, 1947), scanning electron microscopy and extraction procedures (Jeffree *et al.*, 1975). Sutter (1985) compared a wide variety of cultured and glasshouse grown plants using scanning electron microscopy and gas liquid chromatography. In only *Brassica* species was the structured appearance, composition and amount of wax considerably reduced in the cultured plants. Differences did occur in other species, but they were variable and not consistent between *in vitro* and glasshouse grown plants. Differences between the chemical composition of waxes among plants grown *in vitro* was consistent, but relevance of such differences to the survival of these plants *ex vitro* could not be determined. Sutter (1985) points out that reliance on only one of the above mentioned techniques may lead to inaccurate estimates of plant survival

capabilities. In an attempt to quantify cuticular water loss Sutter *et al.* (1988) reported that the removal of wax from *Brassica* seedlings increased water loss by 2.5 times. However this value may vary with species and culture conditions. In acclimatized plum plants, wax deposits were shown to be thicker on the adaxial leaf surface (Fuchigami, Cheng and Soeldner, 1981), which would suggest that the major water loss is occurring via the abaxial surface. Substantial wax deposits were observed on leaves of *Fragaria ananassa* Duch. (strawberry), and *Maranta leuconeura* (maranta) formed *in vitro* (Sutter, Novello and Shackel, 1988). These studies suggest that the morphology of wax development may be determined by other factors apart from humidity e.g. temperature or light, or may be under genetic control. In addition the relationship between amount of wax and water loss is not consistent (Hunt and Baker, 1982) and as formation *in vitro* is variable, water loss must be influenced by other factors such as boundary layer and stomatal functioning.

Stomatal structure and functioning

Poor stomatal functioning has been reported in a number of species cultured *in vitro* (Brainerd and Fuchigami, 1981; Short *et al.*, 1984; Donnelly and Vidaver 1984a; Marin, Gella and Herrero, 1988). Both anatomical (Wetzstein and Sommer 1982; 1983) and physiological (Donnelly and Vidaver, 1984b; Wardle *et al.*, 1979) differences exist between stomata of cultured and glasshouse grown plants. Ziv *et al.* (1987) proposed that changes in guard cell wall structure and elasticity prevent normal closure of *in vitro* stomates. Variations in stomatal shape, guard cell wall thickness and starch distribution have been reported by Marin *et al.* (1988).

It has been known for some time that stomatal functioning is associated with movement of potassium ions into guard cells from subsidiary cells, resulting in changes in guard cell turgor. This increases or decreases stomatal aperture. The movement of potassium ions may be under the influence of the synergistic effects of abscisic acid and calcium ions (DeSilva, Hetherington and Mansfield, 1985). Jarvis and Mansfield (1980) found that although NaCl and KCl were equally effective in promoting stomatal opening, closure mechanisms seemed to be dependent on K^+ ions. Thus the high sodium levels found in guard cells of cultured plants (Wardle *et al.*, 1979) could be inhibitory to normal stomatal functioning. Brainerd and Fuchigami (1982) looked at stomatal functioning in

Malus domestica Borkh. (apple) and concluded that *in vitro* raised plants lacked the ability to close stomates. Stomates do not respond to changes in light regime, abscisic acid (ABA), increased levels of mannitol or carbon dioxide ($C0_2$). However the mechanism was acquired during acclimatization at lower relative humidity. Other workers have also found that changes in stomatal function occur following exposure to low humidity (Brainerd and Fuchigami, 1982; Marin, Gella and Herrero, 1988).

The frequency and distribution of stomata may also contribute to increases in water loss. Donnelly and Vidaver (1984a) report that in cultured red raspberry, stomata are slightly raised and present on both abaxial and adaxial surfaces and many are found on the periphery of the leaf. This anatomy persisted in leaves during transfer to soil, but had disappeared in leaves formed two months after transfer. In cultured plum, stomata are only located on the abaxial surface and water loss is predominantly from this surface of the leaf (Fuchigami *et al.*, 1981). Studies on conventionally grown plants suggest that light irradiance and humidity affect stomatal distribution (Gay and Hurd, 1975; Cutter, 1978).

Attempts have been made to reduce humidity within the culture vessel in an effort to improve wax formation and induce stomatal function. Increasing the agar concentration of the medium or using different rooting substrates, has in some cases slightly improved cuticular wax development and stomatal functioning (Wardle, Dobbs and Short, 1983; Ziv *et al.*, 1987; Short, Warburton and Roberts, 1987; Kramer, 1988). However increasing the agar content of the medium resulted in retarded shoot growth and poor root development. Additional elements, such as sodium are introduced into culture media from agar. The increased levels of such contaminants are often detrimental to plantlet growth and metabolism (Debergh, 1983; Singha *et al.*, 1985).

Alternatively, reduction in humidity prior to acclimatization has been obtained by removing the vessel lid for varying periods of time (Brainerd and Fuchigami, 1981) or by using a variety of vessel closures (Short *et al.*, 1987). Based on observations from these studies, high water loss can be attributed to a combination of cuticular loss, higher stomatal frequency and larger stomatal apertures.

Use of antitranspirants such as DC 200, Wiltpruf and Protec to prevent water loss during the acclimatization stage has only been marginally successful. Sutter and Hutzell (1984) report reduction in water loss in cultured plants treated with various antitranspirants, but experienced severe phytotoxic effects on delicate tissues. Wardle *et al.* (1979) reported some reduction in transpiration and increase in survival of plantlets treated with polyvinyl resin, and the use of abscisic acid (ABA) as a naturally occurring antitranspirant was successful in *Brassica* sp.

Photosynthesis *in vitro*

Modified leaf anatomy has been observed in a number of cultured species e.g. cauliflower, *Rubus idaeus* L. (raspberry), sweetgum and plum, and may be a contributory factor in increased water loss. Epidermal cells were frequently shown to be distorted or collapsed. In all cases, smaller cells were present that were less densely packed, with large intercellular spaces and substomatal cavities. The palisade layer was much reduced (Donnelly and Vidaver, 1984a; Donnelly, Vidaver and Lee, 1985; Marin and Gella, 1988). Leaves tended to be much thinner than those of conventionally raised plants. These structural modifications correlate with those of mesophytic leaf types, usually found in plants which have developed under conditions of high humidity or shade (Esau, 1965). Low photosynthetic activity is often found in cultured plants and has been associated with the low light levels and carbohydrate supplied during the culture period (Grout and Aston, 1978; Grout and Donkin, 1987; Short *et al.*, 1987).

Differences in chloroplast structure have been observed (Wetzstein and Sommer, 1982) and low $C0_2$ assimilation rates have been recorded in a number of studies on cultured plants (Grout and Aston, 1978; Grout and Crisp, 1977; Donnelly and Vidaver,1984b; Fujiwara, Kozai and Watanabe, 1988). This may be explained by low levels of chlorophyll and ribulose biphosphate carboxylase (rubisco) activity detected (Grout and Donkin, 1987). In some cases this can be improved by increasing light intensity and $C0_2$ levels during culture. A number of species appear to improve and adapt during acclimatization to lower relative humidity and higher light intensity (Grout and Millam, 1985; Smith *et al.*, 1986; Fujiwara *et al.*, 1988).

On transfer to *ex vitro* conditions, plantlets undergo a switch from heterotrophic to phototrophic nutrition. Increasing photosynthetic flux and CO_2 availability help to induce this (Kozai *et al.*, 1988; Desjardins *et al.*, 1988). Lowering or removing sucrose from the culture media stimulates limited photosynthesis in some cases, but this is not sufficient to compensate for the resultant reduction in growth (Langford and Wainwright, 1987; Rahman and Blake, 1988b).

Wardle *et al.* (1979) have suggested that leaves formed *in vitro* act only as storage organs, and that subsequent leaves produced *ex vitro* function photosynthetically. Studies by Thorpe *et al.* (1986) identified the accumulation of starch reserves in *in vitro* formed leaves with low photosynthetic activity. The accumulation of labelled rubidium in *in vitro* leaves during culture, followed by redistribution of the isotope into the new growth of *Chrysanthemum morifolium* Ram. (chrysanthemum), during acclimatization supports this theory (Wardle *et al.*, 1983). These workers propose that leaves produced *in vitro* essentially act as cotyledons. Plantlets produced in culture often experience severe water stress on transfer to *ex vitro* conditions. The effects of water stress in nutrient transport experiments may need to be taken into account.

Root formation, function and transport

Water stress may be imposed on cultured plants by the apparently poor vascular junction formed between the shoot and the root (Grout and Aston, 1977 ; Marin and Gella, 1987). In many culture systems shoots or microcuttings form roots *in vitro* via a basal callus region, callus being formed as part of the wounding response by the severed tissues (Yeoman, 1984; Jeffree and Yeoman, 1982). The wounding response has been reported in a number of species including tomato (Yeoman *et al.*, 1978), cauliflower (Grout and Aston, 1977) and cherry (Marin and Gella, 1987; Gebhardt and Goldbach, 1988; Borkowaska and Michalzuk, 1989). Stress occurs where proliferating shoots are detached from the explant and rooted independently. Alternative methods of plantlet production, such as embryogenesis would not be expected to produce these problems; however not all species respond to this method.

Uninterrupted continuation of vascular strands into the root is more likely to occur if roots can be initiated directly from vascular tissue within the stem. Robertsee

and McCully (1979) observed vascular tissue regeneration in pea roots around a wounded area and found that the regenerated area was functional if the strands were continuous with the vascular tissue of the stem. The influence of auxin type, concentration and exposure time used during the rooting stage is important if direct root initiation is required. Direct rooting of *Pinus contorta* Dougl. ex Loud.(pine) from hypocotyl cuttings has been achieved using indole-3-butyric acid (IBA) treatments (Gronroos and Von Arnold, 1987). Auxin and sucrose concentration has been shown to be important in determining vascular development and root formation (Aloni, 1987). Thus adequate sucrose supply during the culture period may be a prerequisite to the production of well developed plantlets.

It has also been suggested that roots formed *in vitro* may not function effectively in more conventional growth media, or that these roots collapse and are lost altogether on transfer to soil (Debergh and Maene, 1981; Maene and Debergh, 1983). Root initiation is usually obtained in culture by exogenous application of auxin to the shoot base. This often leads to problems with formation of basal callus, and root elongation and lateral root development may be impeded (Nicholas *et al.*, 1986). Excessive thickening of root tissue has been observed in some species. Auxins are usually supplied at fairly high concentrations and often via one prolonged application. This may not be the most effective regime for obtaining an extensive and functioning root system. Studies have been carried out on the effects of various treatments on rooting cultured shoots as follows: effect of auxin type and concentration on rooting in raspberry and strawberry species (James, 1979) and *Malus sylvestris* Mill. (apple) (Hutchinson, 1984); effect of temperature and light on rooting in *Malus domestica* (Zimmerman, 1984); and effect of sucrose and nitrogen on rooting in *Rosa* sp. (rose) (Hyndman *et al.*, 1982), *Philodendron erubescens* K. Koch & Aug. (philodendron) and *Cordyline terminalis* L. (cordyline), (Maene and Debergh, 1985). In a recent study, Wainwright and Scrace (1989) looked at the effect of carbohydrate source and concentration, supplied at the rooting stage, on plantlet acclimatization. In the majority of studies measurements of rooting were based on root numbers and root length, while little reference was made to the effect of culture conditions on root structure or function. Subsequently, limited observations have been made on *in vitro* root structure (Donnelly, Vidaver and Lee, 1985) and function (Marin and Gella, 1987; Borkowaska and Michalczuk, 1989). The study of root physiology

and anatomy in cultured plants is very limited. It is likely that poor water and ion absorbtion by the root compounds problems of water stress during the acclimatization stage.

Aims of thesis

The work described in this thesis aims to study the development of the root system formed *in vitro* through the first stages of acclimatization, in order to establish if the root is functional and if it is able to satisfy shoot demands. The majority of the experiments have been carried out on tomato plantlets. Tomato belongs to the Solanaceae, an economically important family. Past studies have demonstrated that tomato responds well to culture (Padmanabhan *et al.*, 1974), and seedling material is readily available for comparisons to be drawn.

The initial work in this study examines the availability of three major nutrient elements in varying concentrations of culture media. This is followed by analysis of the plant material, to establish if tissue concentrations are adequate. Conclusions are then drawn, concerning the relative availability of these elements during the culture period. Once effective supply of nutrients from the culture medium had been demonstrated, the uptake and transport efficiency of cultured plantlets was determined. The experiments described in chapters 5 and 8 used radiotracers (tritiated water, $^{3}H_2O$ and orthophosphate, ^{32}P) to study water and ion uptake in cultured plantlets.

Studies on the effects of auxin and sucrose concentration on root initiation, development and *ex vitro* establishment are presented in chapter 6. Further information was sought regarding the ability of the plantlets to maintain a favourable water balance during acclimatization. Work on the anatomy and development of the root vascular system was designed to demonstrate any structural problems which may impede water conductance through the plantlets. The control of water loss from the shoot was assessed by examining stomatal function and tissue water status. The capability of the *in vitro* formed root system to adapt to new environmental conditions and its importance in acclimatization is discussed in the concluding chapter of this thesis. In addition, banana plantlets have been used for studies on stomatal function and cuticle development and brief observations have been made on date palm, a high value crop which is

particularly difficult to acclimatize.

2.1 Materials

All chemicals used were obtained from BDH limited, Atherstone, Warwickshire and were of Analar grade unless otherwise stated and all biochemicals used were obtained from Sigma Chemical Company, Poole, Dorset unless otherwise stated. The plant growth regulators used were all of tissue culture quality, and were dissolved in absolute ethanol and sterile distilled water before use.

Murashige and Skoog (1962) basal salts (see appendix II), were supplied by Flow Laboratories Limited., Irvine. Difco-Bacto agar was obtained from Difco Laboratories, East Molesey, U.K. Spurrs resin for microscopy was obtained from Taab Laboratories, Tadley, Reading.

Radioisotopes [^{32}P]-Orthophosphate in (Phosphoric acid) (PBS11, specific activity 8000 μCi/mMole) and tritiated water [^{3}H]-H_2O (TRS8, specific activity <5 mCi/ml) were supplied by Amersham International Plc, Amersham, U.K. Soluene-350 solubilizer and Hi-ionic fluor liquid scintillant, for preparation of tissue for scintillation counting were obtained from Packard Chemicals Limited, Reading. Materials used for photography and autoradiograpy were as follows; Ilford Pan F and Technical Pan, Fuji Chrome 400. Ilford Microphen and Ilfotec HC developer with Ilford Hypam fixer. Kodak Omat SX X-ray film, LX24 developer and FX41 fixer.

Lycopersicon esculentum Mill. cv Sonatine F1 hybrid seeds were obtained from Pine Tree Vandenberg Limited, Surrey.

Distilled/deionised water was used for all aqueous solutions which, when known to be stable, were stored at 4°C or otherwise frozen at -20°C.

2.2 Methods

2.2.1 Test species and culture conditions

2.2.1a Tomato culture

All culture procedures were carried out in a laminar flow hood (Intermed, microflow) using aseptic technique.

Lycopersicon esculentum Mill.cv. Sonatine was chosen for the initial nutrient and ion absorbtion experiments. Cultures were initiated from leaf explants taken from 28 day old seedling plants which had been grown under sterile conditions on 80 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), with 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ Difco-Bacto agar. The pH was adjusted to 5.7 ±0.1 prior to autoclaving. The molten medium was then poured into sterile 9 cm square polyethylene boxes (Flow Laboratories) and allowed to solidify. Seeds were surface sterilized in 10% (v/v) sodium hypochlorite solution, with Tween 80 added as a wetting agent, for 15 minutes and were then rinsed twice in sterile distilled water. Twenty five seeds were sown in each vessel. Seedlings were grown at 25 ±2°C and 110 $\mu E.\ m.^{-2}s.^{-1}$, provided by cool white fluorescent tubes (Thorn. EMI), with a 16 hour photoperiod. After 28 days growth, the third true leaf was removed and a 10 mm diameter disc was removed from the lamina. The discs were collected in sterile distilled water, prior to inoculation onto shoot initiation media. A series of initial experiments were carried out using varying concentrations of auxin and cytokinin to establish the optimum combination for shoot proliferation. In these experiments 0, 0.5, 1.0, 2.0 and 5.0 $mg.l^{-1}$ of the cytokinins, benzyladenine (BA) or kinetin, were used in combination with 0, 0.5, 1.0, 2.0 and 5.0 $mg.l^{-1}$ of the auxin, indoleacetic acid (IAA).

For some studies, tissues from glasshouse seedlings were used. Seeds were germinated in trays of a peat based compost (Levington seed and cutting compost) and were grown for 32 days under natural daylight, with the glasshouse temperature set at 20°C. After this time the third true leaf was removed and surface sterilized in 2% (v/v) sodium hypochlorite solution for 10 minutes. The tissue was then rinsed twice in sterile distilled water. A 10 mm diameter disc was then cut from the central region of the leaf lamina.

The leaf explants were inoculated abaxial surface down onto the surface of the media as described by Padmanabhan *et al.* (1974),. Shoot initiation media contained Murashige and Skoog basal salts (Murashige and Skoog, 1962), 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ Difco-Bacto agar, with one of the combinations of growth regulators. The media were adjusted to pH 5.7 ±0.1 prior to autoclaving at 15 psi/121°C for 15 minutes, and were then poured into 50 mm petri dishes. After inoculation the dishes were sealed with Parafilm. Cultures were maintained at 25 ±2°C with 110 $\mu E. m.^{-2}s.^{-1}$ light intensity provided by cool white fluorescent tubes, and a 16 hour photoperiod. Ten replicates of each treatment were set up. After 14 days the treatments were assessed for callus growth and shoot/root initiation. Shoots produced were cut at the base and transferred to 10 ml of liquid MS media with 30 $g.l^{-1}$ sucrose and 0.5 $mg.l^{-1}$ IAA in 60 ml screw top vessels. Shoots were supported on filter paper bridges or perlite during the root elongation stage. Roots were initiated after 5-7 days and were allowed to elongate for a further 12-15 days, under the growth conditions described previously.

2.2.1b Tomato seedling production for whole plant studies

Tomato seedlings were germinated in plastic stubs packed with Perlite. Before sowing, the stubs were inserted into trays of moist perlite. Seeds were sown in a layer of perlite placed on top of a Netlon gauze disc (Netlon Ltd, Blackburn) inside each stub. Seedlings were germinated in a controlled environment cabinet (Fisons series II model 140G2) at a temperature of 25 ±2°C, with 110 μE. $m.^{-2}s.^{-1}$ and a 16 hour photoperiod. Seeds were kept moist with nutrient solution (modified ARC, Appendix II).

2.2.1c Banana culture

Proliferating cultures of *Musa accuminata* cv Gran Canaria were obtained from Twyford Plant Laboratories. The plantlets had been initiated from dormant basal buds of mature plants, and cultures were maintained on MS media with 30 $g.l^{-1}$ sucrose and 1.5 $mg.l^{-1}$ benzyladenine, solidified with 8 $g.l^{-1}$ Difco-Bacto agar (Basic medium see Appendix II). Cultures were grown in 250 ml screw top vessels and the growth conditions were the same as those described for Tomato cultures. After 28 days growth, large well developed plants were removed for rooting. The remaining plantlets were prepared, by trimming off excess shoot and root tissue, before transfer to fresh media. Root elongation was obtained on solidified media without any cytokinin. After a further 20 days on rooting media the plantlets were at a suitable growth stage to undergo acclimatization. Cultures were routinely transferred to fresh media every 28 days.

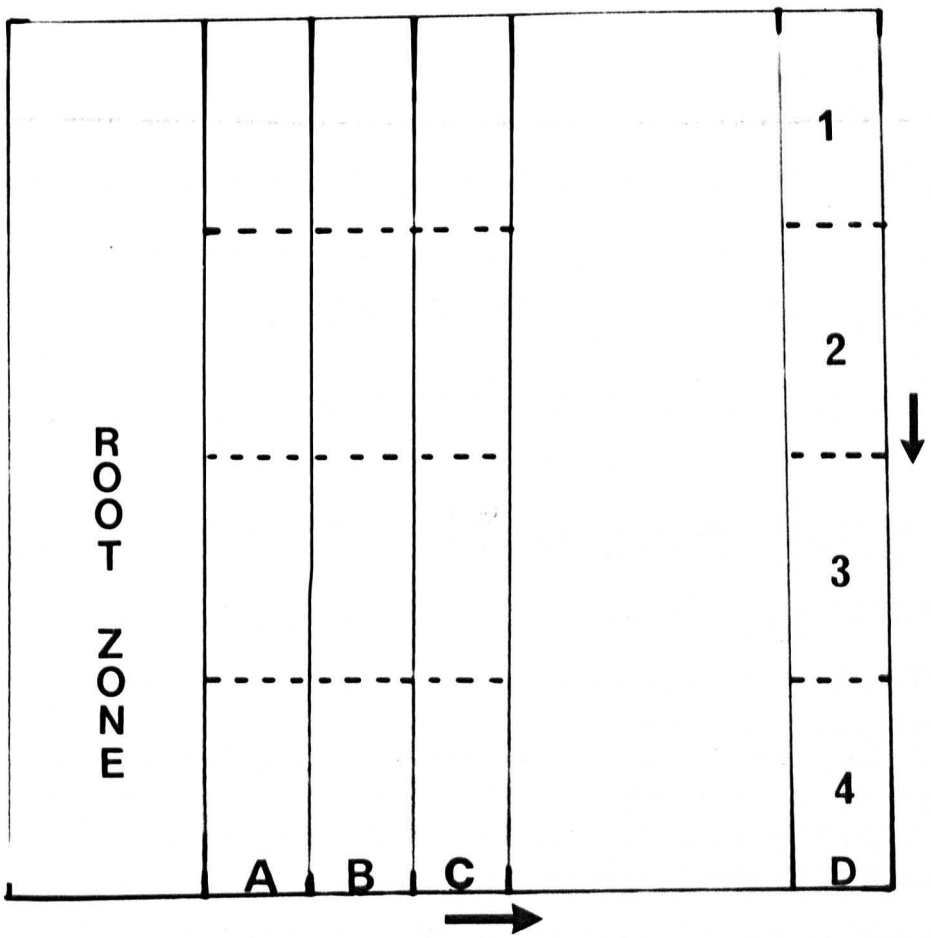
2.2.1d Date Palm Culture

Rooted male plants of *Phoenix dactylifera* cv. Jarvis 1 were obtained from Twyford Plant Laboratories. The plants were supplied in sealed 100 ml tubes, on root elongation media. The plants were considered to be at a suitable growth stage to undergo acclimatization. A further twelve plants were supplied following a period of acclimatization. These plants were potted into perlite, and were grown in a closed propagator at 30 ±2°C, with a relative humidity of 85-90% for 4 weeks. Natural daylight was supplemented with fluorescent tubes, providing an increased irradiance of 150 $\mu Em.^{-2}s.^{-1}$. The plants were watered daily and root fed twice weekly with a balanced liquid fertilizer. Both *in vitro* and acclimatized plants were used in ^{32}P uptake studies (chapter 5).

2.2.2 Media and plant tissue analysis.

Basic MS salts with 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ Difco-Bacto agar were prepared as described previously in section 2.2.1. The medium was then dispensed in 80 ml aliquots into 9 cm square polythene vessels. A filter paper barrier was incorporated into the vessel as shown in Figure 2.1, to restrict root growth to a small area of the vessel. Seeds of *L. esculentum* cv. Sonatine were surface sterilized as described previously and were sown as shown in figure 2.1. The filter paper barrier confined the seedling roots, but did not impede nutrient flow. The MS medium was used at full, half and one tenth concentration. The vessels were sealed with Parafilm and the seedlings grown at 25 ±2°C, with a light intensity of 130 $\mu E.\ m.^{-2}s.^{-1}$ and a 16 hour photoperiod for 28 days.

Figure 2.1: Sampling method for culture medium taken after 28 days growth. Three sample cores (0.5 mm diameter) were taken from the region adjacent to the root zone (A-C), and one from the opposite area of the vessel (D) 9 cm from the root zone. Each core was then sub-divided into four 1.5 cm sections (1-4) prior to analysis.


ROOT ZONE
A
B
C
1
2
3
4
D

After this time the plants were removed from the medium, and roots were rinsed in distilled water before drying at 70°C. Fresh weights of plant tissues were recorded. The medium was sampled by taking three 5 mm diameter cores from the area adjacent to the filter paper (A-C) and one core from the area at the edge of the vessel (D). Each core was divided into four 1.5 cm sections (Figure 2.1). Each section was melted and the volume made up to 10 ml with distilled/deionsed water. The samples were analysed for orthophosphate using the molybdenum blue method (Murphey and Riley, 1962) using ammonium molybdate and stannous chloride reagents. Potassium was estimated by tri-acid digestion (Sahrawat, 1980) and atomic absorption spectroscopy (Baird model: Alpha 1) and organic nitrogen was determined using the Kjeldahl method (Allen, 1974).

The plant tissues were dried for 48 hours at 70°C in a forced draught oven, dry weights were recorded and the tissues ground. A maximum sub-sample from each treatment of 0.1 g was digested in 6.5 mls of tri-acid mixture (1 ml Perchloric acid: 5 ml Conc. Nitric acid: 0.5 ml Conc. Sulphuric acid). After dilution (10 times for phosphate assay and 25 times for potassium determination) with distilled/deionised water the samples were analysed for phosphate using the molybdenum blue method and for potassium by atomic absorption spectroscopy. Further sub-samples of tissue (0.1 g) were digested in 3 mls Conc. Sulphuric acid with a selenium Kjeldahl tablet as catalyst. Organic nitrogen was determined after steam distillation of the Kjeldahl digest using a Markham apparatus (Allen, 1974). The Organic nitrogen content was only determined for the plant tissue. Fresh standard dilutions and blank digests were prepared for each determination. For phosphate and potassium standard curves see Appendix III.

2.2.3 Ion uptake studies

Tomato seedlings and plantlets were transferred to a balanced nutrient solution (modified ARC solution see appendix II) in 2.5 litre polythene vessels with four plants per vessel. Plants were supported in plastic stubs containing a shallow layer of perlite. The solutions were continuously aerated throughout the experimental period. Plants were grown in a controlled environment cabinet at 25 ±2°C, with a light intensity of 110 μE. $m.^{-2}s.^{-1}$ and a 16 hour photoperiod. Two levels of relative humidity at 40% and 80% were established as experimental treatments. Plants were grown under the test conditions for 7 or 14 days, before assessment of ion uptake using [^{32}P]-orthophosphate.

One set of cultured plants was exposed to the isotope in the culture vessel. After the 20 day rooting period, plantlets were transferred for a 24 hour standardization period to fresh medium which was then replaced with medium labelled with [^{32}P]-orthophosphate, 5 μCi/100mls. (Sp. Act. 8000 μCi/mMole). Plantlets were exposed to the isotope for 4 hours, and then treated as described below. Before the isotope uptake, individual acclimatized plants were transferred to 100 mls of fresh nutrient solution for 24 hours. Following this standardization period, the solution was replaced with 100 mls of solution containing 5 μCi of [^{32}P]-orthophosphate (as above). Plants were exposed to the isotope for 4 hours, the roots were then rinsed twice in unlabelled solution and blotted dry with filter paper. The plants were partitioned into root, stem, leaf, and 1 cm of the root/shoot junction (which was designated as the vascular transition zone, VTZ). Fresh weights, shoot height and leaf number were recorded, leaf area and root length being measured using image analysis (Seescan Image Analyser). Plant fractions were then dried in a force

draught oven at 70°C for 48 hours. A 10 mg sample of each fraction was wetted with 0.5 ml of distilled water for 30 minutes and then 1 ml of Soluene 350 tissue solubilizer (Packard Chemicals) was added and the tissue solubilized for 24 hours at 40°C in a dark incubator. Following solubilization, 10 ml of Hi-ionic fluor liquid scintillant (Packard Chemicals) was added and the samples were counted on a Canberra Packard scintillation counter (model: Tri-Carb, at 99% counting efficiency). Each treatment was replicated 10 times. Data was analysed using analysis of variance statistical analysis system package (SAS Institute limited).

2.2.3a Autoradiography

Separate plants from each treatment were prepared for autoradiography. Whole plants were placed between sheets of filter paper and pressed in a photographic press at 70°C for 15 minutes to arrest xylem movement. The plants were then dried for 8 hours at 65°C, before covering with Saran wrap and exposing to X-ray film (Kodak; Omat SX) in a cassette with an intensifying screen at -70°C for 8 hours. The autoradiographs were developed at room temperature for 3 minutes (Kodak; LX24 developer) and fixed for 3 minutes (Kodak; FX41 fixer). Photographs of autoradiographs were taken with Ilford Technical Pan film (25 ASA). Photographic films were developed at room temperature for 15 minutes with Ilford Ilfotec HC developer, and fixed for 1 minute in Ilford Hypam fixer.

2.2.4 Morphological development

2.2.4a Root and vascular transition zone anatomy

Plant material from *in vitro* plants and from both cultured plantlets and seedlings, after each acclimatization treatment, was taken and prepared for light microscopy using aldehyde/osmium fixation and resin embedding according to the following schedule:

1) Tissue was cut transversely into 2.5 mm deep sections through the shoot/root junction, thus dividing each junction into four parts. Root samples were taken from the region adjacent to the base, and further root samples were taken 1 cm behind the root tip.

2) The tissue was fixed for 4 hours in 3% (w/v) glutaraldehyde (Sigma) in 0.025 M phosphate buffered-saline (PBS), pH 6.8 at 4°C, followed by extensive rinsing in three changes of 0.025 M PBS, over 1 hour at 4°C.

3) Post-fixation was carried out in a 1% aqueous solution of osmium tetroxide for 4 hours at room temperature, followed by rinsing in three changes of distilled water at 4°C overnight.

4) The tissue was then dehydrated through the following alcohol:water series, 2.5, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 and 100% alcohol, followed by two changes in dessicated 100% ethanol, with a maximum of 20 minutes in each solution.

5) The ethanol was then replaced with Spurr's resin through the following series; resin:alcohol v/v, 1:3, 1:2, 1:1, 3:1, allowing 1 hour for each change and bringing the tissue into pure resin for 1 hour. Infiltration in pure resin was continued for a further 5 days, changing the resin twice in every 24 hours. Polymerisation was at 70°C for 9 hours.

Semi-thin sections (1 μm) were cut with a glass knife on a Sorvall MT500 ultramicrotome. Sections were transferred with the aid of an eyelash, to drops of analar water on glass microscope slides and allowed to dry on a hot plate at 50°C for 5 minutes. The sections were then stained in 5% methylene blue in borax for 1-2 minutes, heated gently and then rinsed in distilled water and allowed to dry.

The sections were viewed on a Nikon (Diaphot TMD) microscope and photographed using a Nikon (F-301) camera with Ilford Pan F film (5O ASA). Films were developed at room temperature for 6 minutes in Ilford Microphen developer and fixed using Ilford Hypam fixer.

2.2.4b Stomatal function and anatomy

Leaflets of *L. esculentum* produced *in vitro* were collected from 20 day old rooted plantlets, and fresh abaxial strips were taken and collected into 0.45 mM potassium chloride in 10 mM morpholino-ethane-sulphonic acid (MES) buffer, pH 6.8, until sufficient had been accumulated. The strips were then aerated for 30 minutes at 25 ±2°C and a light intensity of 100 μE. $m.^{-2}s.^{-1}$. Following this pre-treatment, the stomata were either induced to close by incubating the epidermal strips in darkness for 1 hour, or further opening was stimulated by incubation at 150 μE. $m.^{-2}s.^{-1}$ for 1 hour.

The strips were mounted on a glass slide and stained with 0.01% Calcofluor (Optical brightner, Sigma) for 15 minutes (Hughes and McCully, 1975). The degree of stomatal opening and guard cell wall thickening was assessed using a Zeiss fluorescent microscope with a UV light source and blue excitation filter.

The preparations were photographed using an Olympus OM4i camera and Fujichrome film (400 ASA).

A total of five fields of view were photographed from a minimum of three epidermal strips per treatment. The arrangement of chloroplasts was noted and the relative thickness of guard cell walls estimated.

Stomatal aperture

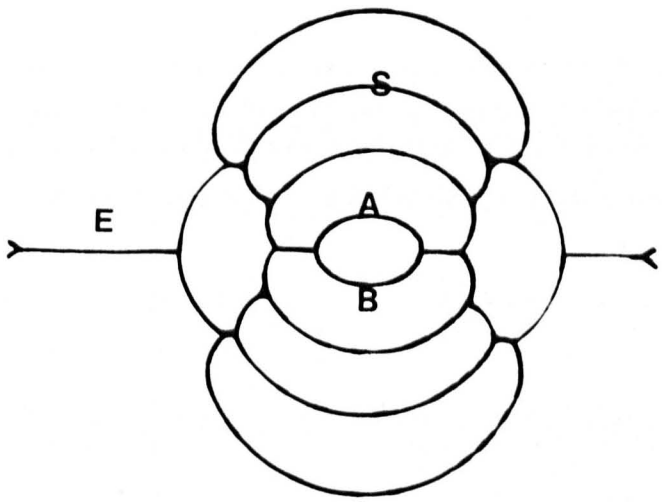
Leaflets were removed from *in vitro* plants, and from plants undergoing acclimatization treatments as described in section 2.2.3. Abaxial epidermal strips were taken from leaflets and immediately assessed for stomatal aperture or were assessed after a 5 minute stress period at 30% relative humidity, 25 ±2°C.The strips were mounted in distilled water and apertures were measured using an eyepiece graticule, each aperture being measured at its widest point, as illustrated in Figure 2.2. A total of 100 stomata were measured from five fields of view, chosen at random, from each treatment. Stomata with apertures of less than 2 μm were considered to be closed (Sutter and Langhans, 1982). Means and standard errors were calculated using the Microtab statistical package (BBC microcomputers).

2.2.4c Leaf surface morphology

Leaflets were removed from *in vitro* plantlets and both *in vitro* and *ex vitro* leaflets (formed following acclimatization) were removed from plantlets undergoing

Figure 2.2: Diagrammatic representation of a stomatal complex, measurements of stomatal aperture were taken between points A and B.

E = epidermal cell, S = subsidiary cells.


S
E
A
B

the acclimatization treatments previously described in section 2.3.

Small pieces of the leaves were frozen in liquid nitrogen for 30 seconds, and were then freeze dried at -40°C and 10^{-6} bar (Edwards freeze drier), for 8 hours.

Sections were mounted either abaxial or adaxial surface uppermost on aluminium stubs before coating with gold (Biorad E5100 series sputter coater). After, coating samples were stored in a dessicator. The leaf tissues were examined on a Cambridge Stereoscan 150 scanning electron microscope (SEM) and were photographed with Ilford Technical Pan film (25 ASA). Films were developed as described in section 2.2.3.

2.2.5 Auxin and sucrose manipulation of root development

Tomato shoots were removed from leaf disc explants, and roots were initiated on liquid media containing a combination of IAA and sucrose from one of the following, 0, 0.25, and 5.0 $mg.l^{-1}$ IAA and 10, 15 and 30 $g.l^{-1}$ sucrose. The shoots were exposed to the root initiation media for either 5 or 10 days, under normal growth conditions described in section 2.2.1. Following this initial phase plantlets were assessed for root number,length, shoot height, leaf number and basal callus development, which was scored on a scale of 0 - 10 (0= no callus, 10 = total cover of the shoot base).

Following the initial assessment, shoots were transferred to root elongation medium containing sucrose at 30 $g.l^{-1}$ or MS salts only, for a period of 15 or 10 days respectively giving a total root development period of 20 days. After this period each plant was acclimatized as described in section 2.2.3, at 40% relative

humidity. Plantlets were then assessed for root number, length, shoot height, leaf number and percentage plantlet survival after 2 and 7 days at the reduced humidity. After the 7 day period plants were partitioned into root, stem, leaf and shoot/root junction. Fresh weights were recorded and the tissues dried for 48 hours at 70°C, before dry weights were recorded. Results were analysed using the analysis of variance statistical package SAS.

2.2.6 Plant Water Relations

2.2.6a Measurement of water movement in the cultured plant

Rooted plantlets were produced as described in section 2.2.1. The ability of the plantlets' root system to absorb and transport tritiated water was assessed following 1, 3, 5 and 7 days acclimatization under the regime previously described, at 40% relative humidity. A comparison with 7 day old seedlings was also carried out.

Plants were exposed to 15 ml of [^{3}H] tritium (specific activity, < 5 mCi/ml) labelled nutrient solution (0.5 μCi/ml solution) for either 30 or 60 minutes. Following the uptake time the roots were rinsed twice in distilled water and then blotted dry. The plants were then divided into 1-2 cm sections as far as the 1st leaf and the fresh weights recorded. The remainder of the shoot was weighed.

The plant fractions were then macerated and solubilized using 1 ml soluene 350 with 0.5 ml isopropanol, for 8 hours at room temperature. A 5 ml aliquot of Hi-ionic fluor liquid scintillant was added to each sample before scintillation counting in polythene minivials, using a Canberra Packard scintillation counter (at 70% counting efficiency).

2.2.6b Determination of leaf relative water content and water loss

Assessments of leaf relative water content and percentage water loss were made using the method described by Brainerd and Fuchigami (1981). Measurements were made on leaf tissue formed *in vitro*, and during acclimatization at 40 and 80% relative humidity, for both seedlings and cultured plants.

Five leaflets were removed from seedlings and 10 leaflets from cultured plants (5 formed *in vitro* & 5 formed *ex vitro*) on day 6 and day 13 of the 7 and 14 day experiments respectively. Two 10 mm diameter leaf discs were removed from the central area of each leaflet. The discs were placed abaxial surface up on a petri dish and the weight was recorded every 2 minutes, for the first 30 minutes and then finally after 60 minutes. The discs were then soaked in a petri dish with distilled water for 4 hours, blotted dry and reweighed. Dry weights were recorded after drying for 48 hours at 70°C.

Relative water content was calculated and compared using the method of Barrs and Weatherley (1962), taken from Kramer (1983). The equation given in the paper by Brainerd and Fuchigami (1981) was not used as this gives a measurement which is the water deficit value.

2.2.7 Measurement of ion uptake and transport in Date palm

Individually rooted palm plants were obtained from Twyford Plant Laboratories as decribed in section 2.2.1. The *in vitro* plants were exposed to 10ml of ^{32}P-orthophosphate labelled (0.5 µCi/ml nutrient solution) nutrient solution in the culture vessel, following a 24 hour standardization period in fresh medium. Acclimatized plants were supplied by Twyfords Plant Laboratories, growing in

perlite. The perlite was rinsed from the roots and the plants were standardized and then exposed to labelled nutrient solution for 4 hours as previously described.

Following the exposure period the roots were rinsed in unlabelled nutrient solution and then blotted dry. The plants were partitioned into root, root/shoot junction and leaf. Fresh weights were recorded before assessment of root length and leaf area by image analysis. The plants were then dried at 70°C for 48 hours and then prepared for scintillation counting according to the method described in section 2.2.3. Two plants from each treatment were prepared for autoradiography as described in section 2.2.3.

Chapter 3: Test species and culture conditions

3.1 Introduction

The genus *Lycopersicon* includes species which contain a large number of genotypes, several of which are economically important e.g. Campbell 37, Saladette, Marglobe. These two factors make tomato an ideal experimental system for studies of *in vitro* regeneration and induced genetic modification.

Earlier studies on tomato have reported plantlet regeneration from a variety of explants including leaf (Padmanabhan *et al.*, 1974; Rafique Uddin *et al.*, 1988; Monacelli *et al.*, 1988), stem internodes (Ancorra & Sree Ramulu, 1981; DeLanghe & DeBrunjne, 1976) hypocotyl and cotyledon (Gunay & Rao, 1980).

Tomato has frequently been used for nutrient studies both in conventional growth media and liquid culture systems (Friis-Nielsen, 1973; Maynard *et al.*, 1980; Halbrooks and Wilcox, 1980). The accumulated information on tomato nutrient requirements, ion uptake and transport (Clarkson, 1980) and water relations (Bingham 1985) provide a useful basis for comparison with work on *in vitro* and acclimatized plantlets. In addition, seedlings of *L. esculentum* can be grown under controlled environment conditions and acclimatized under the same regimes as the tissue cultured plantlets. The seedlings can then be used in experiments to compare efficiency of nutrient uptake, aspects of water relations and anatomy of vascular continuity, in the two plant types. The variety Sonatine is a commercially important F1 hybrid providing fast growing and uniform seedling material as a source of culture explants.

Initiation of cultures from leaf discs or stem internodes of seedling tissue provides shoots which can be rooted independently. Shoots and roots develop from single cells or groups of cells within the explant, which become sites of cell division. Manipulation with plant growth regulators (PGR's) will lead to the formation of embryos, shoots or roots. Organogenesis can occur directly or indirectly from the explant. Organogenesis and the degree of callus formation is influenced by the morphogenic potential of the explant, genotype and type and concentration of PGR's in the culture medium.

Media and PGR combinations which promote rapid cell proliferation and callus formation do not usually give rise to morphogenetic meristems. Leaf explants and stem internodes form shoots directly from the explant. This pathway of regeneration is desirable, as propagation rates can be kept high from readily available explants. Explants from leaf bases consistently form proliferating cultures. The area of the responsive zone depends on the developmental leaf age and the age of the parent plant. Changes occur in regions of the leaf which determine its proliferative capacity (Meins and Wenzler, 1986). This may partly be linked to the presence of various cytokinin metabolites, which have been identified in leaf lamina and petioles, where they appear to accumulate (Blakesley and Lenton, 1987). Plantlets produced directly from the explant should be genetically identical to the parent material. Some somaclonal variation may occur within the cultured plantlets, but this can be kept to a minimum by good culture technique and short culture periods (George and Sherrington, 1984).

Successful shoot and root regeneration have been achieved in the genus *Lycopersicon* using the cytokinins benzylaminopurine (BAP), a very active

synthetic cytokinin and the cytokinin 6-Furfurylaminopurine (kinetin) in combination with the auxins IAA or IBA. The initial work of Skoog and Miller (1957) demonstrated that shoots could be induced from tobacco explants, by using high cytokinin:auxin ratios in the culture media. However the type of morphogenesis produced varied according to plant species, culture conditions and growth regulators used. Cells vary in their potential to form roots and shoots and can become determined to express these responses through various endogenous and exogenous stimuli. Isolated explants are initially unable to synthesize sufficient growth regulators to sustain growth. These are initially supplied exogenously in the culture medium. However some tissues respond at a critical threshold exposure to the growth regulator (concentrations of 5×10^{-7} M for kinetin) and synthesis within the tissue is induced (Meins and Lutz, 1980). This mechanism has been termed habituation and appears to result from epigenetic changes, which then direct changes in cell phenotypes. The cells lose the requirement for exogenous growth regulators and a positive feedback system is set up which controls PGR synthesis and degradation. Cells from leaf and cortex of tobacco appear to inherit different states of cytokinin requirement, and plantlets arising from these cell types exhibit a similar state, the shift from cytokinin requiring to cytokinin autotrophic being inducible and reversible in culture (Meins and Wenzler, 1986).

In tissue culture systems cytokinins are used to promote synchronous cell division and induce shoot production, to some extent mimicking their role in whole plants where they are produced in root tissue and move acropetally in the xylem (Letham and Palni, 1983). Cytokinins occur endogenously both as free compounds, e.g. ribosides and N-glucosides, and as component nucleotides in tRNA. The

principle metabolites formed following exogenous application of cytokinins are the cytokinin riboside 5'-phoshates. The cytokinins present in tRNA are thought to be involved in mRNA translation, whilst the free cytokinins appear to have specific role in the regulation of plant development (Roberts and Hooley, 1988). For example cytokinin was shown to promote formation of compact morphogenic callus in culture (Kirkham and Holder, 1981).

The primary effect of naturally occurring auxins, such as IAA are now known to be at the molecular level (Key *et al.*, 1986). Gene activation ultimately results in cell division and cell elongation, leading to the morphological alterations observed. Auxin is required for cell wall regeneration and induction of mitosis. It is possible that a twin system operates; an early phase in which auxin stimulates proton excretion, as proposed in the acid growth theory (Rayle and Cleland, 1970), followed by a later phase mediated by the primary action of auxin on gene expression (Theologis, 1986). Auxins have the capacity to alter biosynthesis of particular proteins in various plant systems. It has been known for a number of years that application of auxin induces specific changes in protein synthesis in elongating hypocotyl tissue (Zurfluh and Guilfoyle, 1980; Bates and Cleland, 1980). Auxin induced polypeptides have been detected within 30 minutes of application (Meyer *et al.*, 1984) and changes in mRNA have been detected within 10 - 20 minutes. More recently mRNA sequences have been isolated from 2,4-D treated hypocotyl segments and have been used to produce cDNA clones, enabling a more detailed characterization of the hormonal response (Walker and Key, 1982, Hagen *et al.*, 1984). It has been proposed (Theologis, 1986) that the rapidly induced mRNA's code for polypeptides which mediate proton secretion and cell elongation, whilst the mRNA's indirectly regulated by auxin may code for systems

conjugating cellulases and other hydrolases required for cell division, differentiation and adventitious meristem initiation, all of which are important in cell and tissue culture.

In whole plant studies it has been recognized for sometime that IAA is a controlling factor in both xylem and phloem regeneration (Jacobs, 1952). Auxin can limit vascularization in both callus and whole plant tissue. In callus tissue the concentration of auxin determines the site and type of differentiation (Jeffs and Northcote, 1967). Lower concentrations promote phloem differentiation and higher concentrations both induce xylem and phloem (Aloni, 1980). Vascular tissue is often formed in association with cambial or vascular tissue present in the explant. This has been demonstrated in a number of species, including tomato leaf explants (Monacelli *et al.,* 1988). Vessel size and arrangement is thought to be determined by concentration gradients formed within the tissue (Sachs, 1981); this is discussed in more detail in chapters 6 and 7. In tissue culture systems the ratio of auxin to cytokinin used in the growth medium is an important factor in determining the type of differentiation achieved. Generally high auxin to cytokinin ratio results in embryo and root production, whilst high cytokinin to auxin ratio results in shoot production. Modification of the induction ratios is usually required in the second stage of culture, to allow for shoot proliferation and elongation (George and Sherrington, 1984).

Aims and related studies on date palm and banana plants

The date palm was identified by J.Reid and D.Blakesley of Twyford Plant Laboratories as exhibiting particular problems during the early acclimatization stage. Plantlet losses were reportedly as high as 70% in some cultivars. The loss of plants appeared to be associated with a breakdown within the root/shoot

junction. Nutrient and water transport to the shoot may therefore be impeded, resulting in shoot dehydration and poor growth. The root system of these plants is fragile and brittle and requires careful handling during the potting stage to avoid damage. The brief study on date palm aimed to identify if any inherent weakness in the structural anatomy of the root or root/shoot tissues, reduced the nutrient absorbtion capacity of these plants.

Cultivars of *Musa* are a major staple food in parts of the world. Most commercial cultivars are triploids and therefore seed-sterile. It is only feasible to propagate these cultivars by vegetative methods (Banerjee *et al.*, 1985). Recently *in vitro* techniques have been developed for clonal propagation (Srinivasa Rao & Chacko, 1983). The effect of varying gelling agent type and sodium content of the culture media on stomatal functioning of *in vitro* and acclimatized banana plants was also studied; brief observations were made on cuticle formation *in vitro* and following acclimatization, this work is presented in appendix I.

The aim of the initial work presented in this chapter was to produce a reliable and rapid culture system for the production of uniform adventitious shoots of *Lycopersicon esculentum* cv. Sonatine, which could then be rooted *in vitro,* acclimatized and used in comparative studies with seedling plants.

3.2 Results and Discussion

Selection of parent material

In order to determine the most reliable method for the production of the parent material from which cultures were to be initiated. Tissue explants (leaf and nodal explants) were taken from glasshouse and aseptically raised tomato plants at the

same stage of development.

The leaf tissue and nodal explants produced from glasshouse grown seedlings was frequently contaminated in culture and results are not presented. Contamination of glasshouse grown seedlings by bacteria and fungi proved to be a major problem. Some of the bacterial contaminants appeared to be endogenous in the tissue and therefore surface sterilization of tissue failed to produce clean cultures. Increasing the duration and concentration of surface sterilization resulted in the elimination of fungal contaminants, but was detrimental to the tissue. The use of antibiotics in the growth medium could have suppressed bacterial growth, but as their effect on explant differentiation would have had to be evaluated, and alternative clean tissue was available, they were not included in this study. The aseptically produced tomato leaf explants were free from contaminants and proved to be the most successful tissue to use for culture initiation.

Effect of light intensity on explant regeneration

A brief initial study was carried out to determine the effect of high and moderate light intensity (60 and 110 $\mu E.\ m.^{-2}s.^{-1}$) on organogenesis and culture growth, with varying concentrations of cytokinin and auxin. At the higher light intensity (160 $\mu E.\ m.^{-2}s.^{-1}$), the leaf tissue produced high levels of anthocyanins. Distinct purple colouration of the leaf lamina was observed at all PGR concentrations. This was followed by tissue browning and necrosis. Callus production and shoot initials were not observed in any of the treatments. Inhibition of callus growth and early shoot bud initiation by high light intensities has been reported in jerusalem artichoke, *Helianthus tuberosus* L. (Fraser *et al.*, 1967). One explanation for this light inhibited growth may be that the initial cell divisions are suppressed by light,

through the action of vitamin B12, resulting in a reduction in DNA replication (Yeoman and Davidson, 1971). Additionally, photodegredation of IAA has been implicated by Fridborg and Eriksson (1975), who suggest that the resulting reduced action would affect its role in differentiation and the initiation of shoot bud meristems. However higher light irradiance has reportedly enhanced shoot proliferation rates in begonia, *Begonia x hiemalis* (Welander, 1978) and root elongation in *Dracaena fragans* (Vint-halter *et al.*, 1990). Survival of plantlets during acclimatization may also be improved by a higher photon fluxes during the final stages of culture (Kozai *et al.*, 1990). Leaf discs grown at the lower light intensity (110 $\mu E.\ m.^{-1}s.^{-1}$), produced most prolific shoot regeneration.

Effect of growth regulators on explant regeneration

The highest numbers of shoots were produced by the combinations of IAA and kinetin shown in Table 3.1. In the IAA and BAP treatments, reduced growth was observed at all concentrations. Limited organogenesis was observed in some of the treatments, root initials were produced directly from the leaf tissue without an intermediate callus stage, at concentrations of 0.5 and 5 $mg.l^{-1}$ IAA each in combination with 0.5 $mg.l^{-1}$ BAP. However only low numbers of shoot initials were produced with 0, 0.5 and 2.0 $mg.l^{-1}$ IAA in combination with 2 $mg.l^{-1}$ BAP. The response on media containing IAA and BAP was much lower than that achieved by using IAA and kinetin and none of these combinations were used subsequently for plantlet production.

In the majority of explants which exhibited regeneration the following series of events was observed. Callus was almost always produced first from the cut edge of the mid-rib at the basal end of the leaflet, as observed in previous work

(Monacelli *et al.*, 1988). Slight callusing occurred on the other cut edges, but showed no signs of differentiation. After a period of 20 days in culture the majority of explants which showed no growth response, had turned brown. Optimum shoot production was obtained on medium containing 0.5 $mg.l^{-1}$ IAA with 0.5 $mg.l^{-1}$ kinetin, or 0.5 $mg.l^{-1}$ IAA with 5 $mg.l^{-1}$ kinetin. Each of these cultures produced more than five shoot intials.

Shoot elongation and subsequent development of initiated shoot buds was superior on medium containing 0.5 $mg.l^{-1}$ IAA with 0.5 $mg.l^{-1}$ kinetin. At the higher concentration of kinetin shoots became stunted. Therefore subsequent sub-cultures were made onto the lower kinetin concentration and were also moved into larger vessels to allow for shoot proliferation and elongation. Cultures were sub-cultured a maximum of four times, to reduce variation in the shoots produced by repeated culturing. New cultures were initiated every four or six weeks. Lowering the cytokinin to auxin ratio during the second stage of culture allowed the initiated shoots to elongate. This may have been due to the stimulation of cell extension by the increased ratio of IAA to kinetin in the medium (Mutaftschiev *et al.*, 1987).

Shoots were rooted in liquid MS medium with 0.5 $mg.l^{-1}$ IAA, which was the optimum concentration for root development (Table 3.1). Plantlets were rooted for 20 days in liquid medium, using filter paper bridges to support the shoot (Figure 3.1). Liquid medium prevented damage to the root system during transfer from culture, and promoted root hair growth which is usually suppressed in agar solidified media (Yie and Liaw, 1977), probably as a result of poor aeration. In addition to the abundant root hairs, the roots were much more branched in liquid medium.

Vitrification is a condition of cultured plants described by Debergh *et al.* (1981) in which the stem and leaves appear translucent and water soaked. This problem is often associated with plantlets grown on liquid medium (Ziv *et al.*, 1987). Only a very few plants became vitrified during the course of this study and these were not used in any experiments.

The results described in this chapter permitted optimisation of the PGR content of the medium and culture time to achieve optimum tomato plantlet production. All subsequent cultures of tomato were initiated from aseptically raised seedling leaf explants, on MS medium with 0.5 $mg.l^{-1}$ kinetin with 0.5 $mg.l^{-1}$ IAA, for 20 - 28 days. Followed by 20 days rooting in liquid MS with 0.5 $mg.l^{-1}$ IAA. Cultures were maintained at 25 ±2°C, with 110 $\mu E.\ m.^{-2}s.^{-1}$ light intensity and a 16 hour photoperiod. Plantlets were then judged to be at a suitable growth stage to undergo acclimatization studies.

Maintenance and acclimatization of banana plants

Cultures of banana were maintained and rooted using the growth conditions recommended by Twyford Plant Laboratories and these are presented in appendix II. No modifications were made to the growth media and problems with plantlet proliferation or rooting were not encountered. Plantlets were routinely subcultured as described in section 2.2.1c. Individual plantlets were potted into a peat based compost and acclimatized in the glasshouse at 60% relative humidity, with the temperature set at 20°C for 14 days, after which time the plants were grown on the open bench (Figure 3.2). These plants were used in comparative studies with *in vitro* plantlets on stomatal function, described in appendix I.

Table 3.1 Effects of varying IAA and Kinetin concentrations on growth and organogenesis in tomato leaf explants.

IAA Conc. mgl^{-1}		Kinetin Conc.mgl^{-1}									
		0		0.5		1.0		2.0		5.0	
		L	H	L	H	L	H	L	H	L	H
0	S	-	-	-	-	-	-	-	-	*	-
	C	-	-	**	*	-	-	*	*	*	-
	R	-	*	-	-	-	-	-	-	-	-
0.5	S	-	-	***	-	-	-	-	-	***	-
	C	-	-	***	*	-	-	*	-	**	-
	R	***	***	**	*	-	-	**	*	-	-
2.0	S	-	-	-	-	-	-	-	-	***	-
	C	-	-	**	-	-	-	**	**	**	**
	R	***	***	*	**	-	-	***	-	-	-
5.0	S	-	-	-	-	-	-	-	-	-	-
	C	*	-	**	-	-	-	*	-	**	*
	R	**	**	*	-	-	-	*	-	-	*

10 replicates per treatment
L= low light intensity
H= high light intensity

S=Shoot
C=Callus
R=Root

Degree of response:
Callus production interpreted as an increase in tissue volume of: x2 *, x4 **, x8 ***
Shoot and root numbers: 0-5 *, 5-10 **, 10+ ***

Figure 3.1: Cultured tomato plantlet, rooted on liquid MS medium containing 0.5 mg l^{-1} IAA for 20 days, prior to acclimatization experiments

Figure 3.2: Banana plants following glasshouse acclimatization and 3 months growth.


3·1


3·2

Chapter 4: Media and plant tissue analysis

4.1 Introduction

The development of plant tissue culture media commonly in use today has taken place since the late 1800's and early 1900's, from salt-based liquid nutrient solutions e.g. Knops, Pfeffers and Hoagland's solutions. Early media, which were used for culture of isolated roots, callus and embryo culture contained relatively low concentrations of inorganic ions e.g. potassium and nitrate (White, 1943; Knudson, 1946; Schenk and Hildebrandt, 1972). Many of these early media contained undefined organic additions such as coconut milk and fruit pulp.

Improvements in the composition of these media in recent years, by the inclusion of higher concentrations of inorganic ions, nitrogen sources (particularly ammonium), vitamins and a carbohydrate source, has facilitated diverse cultures of isolated explants. The most widely used plant tissue culture medium is that of Murashige and Skoog (1962). This medium was originally developed for the culture of tobacco, but is now used for the culture of many plant species and is usually considered to be a high salt medium. Modifications to the original MS formula have since been made to fulfil the specialised requirements of some plant species or cultures e.g. Durzan *et al.* (1973), Linsmaier and Skoog (1965), Nitsch and Nitsch (1969), Anderson (1984).

Plant tissue culture media are often used in a solid or semi-solid form with agar almost always used as the gelling agent. This is probably because of its traditional use in bacterial culture and its being fairly economical when compared to alternative compounds such as agarose. However, the adsorptive properties and

impurity content of many brands of agar raise doubts about its use as a gelling agent.

The nutrient concentration in standard MS medium could be considered to be supraoptimal when compared with conventional solution culture media e.g. Hoagland's or Long Ashton solution. However, when the nutrient solution was solidified with agar, the brand and concentration of product used has been shown to have an effect on *in vitro* plant growth (Debergh, 1983; Von Arnold & Eriksson, 1984; Singha *et al.*, 1985). Debergh found that increasing agar concentration as a means of overcoming vitrification in cultures of jerusalem artichoke, had the undesired effect of lowering shoot and root production. This was attributed to either differential availability of calcium, potassium and sodium within the gel profile or the introduction of impurities from the agar. Alterations in the elemental composition of the basal medium by the additions of three brands of agar at varying concentrations (Singha *et al.*, 1985) were shown to result in decreases in the levels of potassium, calcium, magnesium and manganese found in apple and pear explants, with increasing agar concentration, but increases in explant concentrations of phosphorus, iron, zinc and aluminium increased with increasing agar concentration.

Romberger and Tabor (1971) found that, in spruce (*Picea abies* Karst.), shoot explants, increased dry matter yield resulted from reducing agar concentration. However, culture vigour declined. A ten percent reduction in agar concentration to 1% (w/v), resulted in a 54% increase in growth. This response was partly attributed to restricted diffusion of macromolecules within the gel matrix. Restricted diffusion would reduce the volume of medium which could effectively

contribute to the growth of the culture, thus agreeing with Debergh's findings of a vertical distribution gradient of Ca^{2+}, K^{+}, and Na^{+} within the culture medium. To be an effective mode of translocation concentration gradients need to be high. Romberger and Tabor (1971) propose that this is probably only the case for large molecular weight molecules. One major macromolecule considered is invertase, which would facilitate the uptake of glucose and fructose, from an initial carbohydrate source of sucrose. However, sucrose is partially hydrolysed on autoclaving (Skirvin *et al.*, 1986). Reduced diffusion of invertase may not be entirely responsible for the depressed growth observed.

Large tissue/medium volume ratios may also have an effect on mineral ion uptake and availability through efflux of ions, resulting in pH changes in the medium surrounding the explant or plantlet root. Neither agar or agarose can be regarded as chemically inert and the gel matrix will almost certainly have physical effects on nutrient movement.

Agar solidified media may also induce water stress in cultured plantlets (Singha, 1982). Brown *et al.* (1979) proposed that the carbohydrate source in the medium also acts as an osmoticum. Therefore if sucrose is replaced or supplemented with mannitol any osmotic requirement should be satisfied. Difco-Bacto agar also has the effect of lowering the water potential of the medium (Brown *et al.*, 1979), and badly affects shoot regeneration above a concentration of 1.7% (w/v). This effect was further compounded by reducing the sucrose concentration.

High concentrations of sucrose and agar inhibit turgor driven growth and cell expansion (Zimmerman, 1977; 1978). This type of inhibition would affect both callus and shoot formation. Agar cannot act as an osmoticum but does reduce the

water potential of the medium. Agar is responsible for the matrix potential of the medium, whilst the carbohydrate source seems to be associated with the osmotic potential (Debergh *et al.* 1981).

Root growth in agar solidified media is often inhibited, with only limited branching and a complete lack of root hair development (Lee *et al.,* 1986). This is probably due to poor aeration within the medium. More mobile elements such as potassium and nitrogen are present in basal MS salts in supraoptimal concentrations, and should therefore not be limiting to growth. Less mobile elements such as phosphorus may be exhausted more quickly, and as absorbtion is mainly via the root hairs (Clarkson, 1985) reduced uptake may become growth limiting.

The aim of this part of the investigation was to assess the availability of three major nutrients; nitrogen, potassium and phosphorus from differing concentrations of agar solidified MS medium, and to analyse the levels of these nutrients in the plant tissue. The concentrations of MS basal salts used represented two concentrations normally employed in tissues culture systems for shoot induction, proliferation and rooting (100 and 50% MS, the latter concentration is often used at the rooting stage). The 10% concentration was used to provide a low initial nutrient supply, which would become limiting to plant growth within the experimental period. Thus it was intended to study the availabilty of these nutrients from the agar solidified media and to determin if N, P or K became limiting during the culture period. Any reduction in plant growth or vigour may result in survival problems during the acclimatization stage.

The results presented in this chapter represent an assessment of nutrient availability from agar solidified MS media at varying concentrations. Values for P and K content of medium taken from serial samples across the vessel(see section 2.2.2, Figure 2.1) when used in conjunction with data from plant tissue analysis for N, P and K, should give an indication of the availability of these elements from the medium, and this is discussed in the following section.

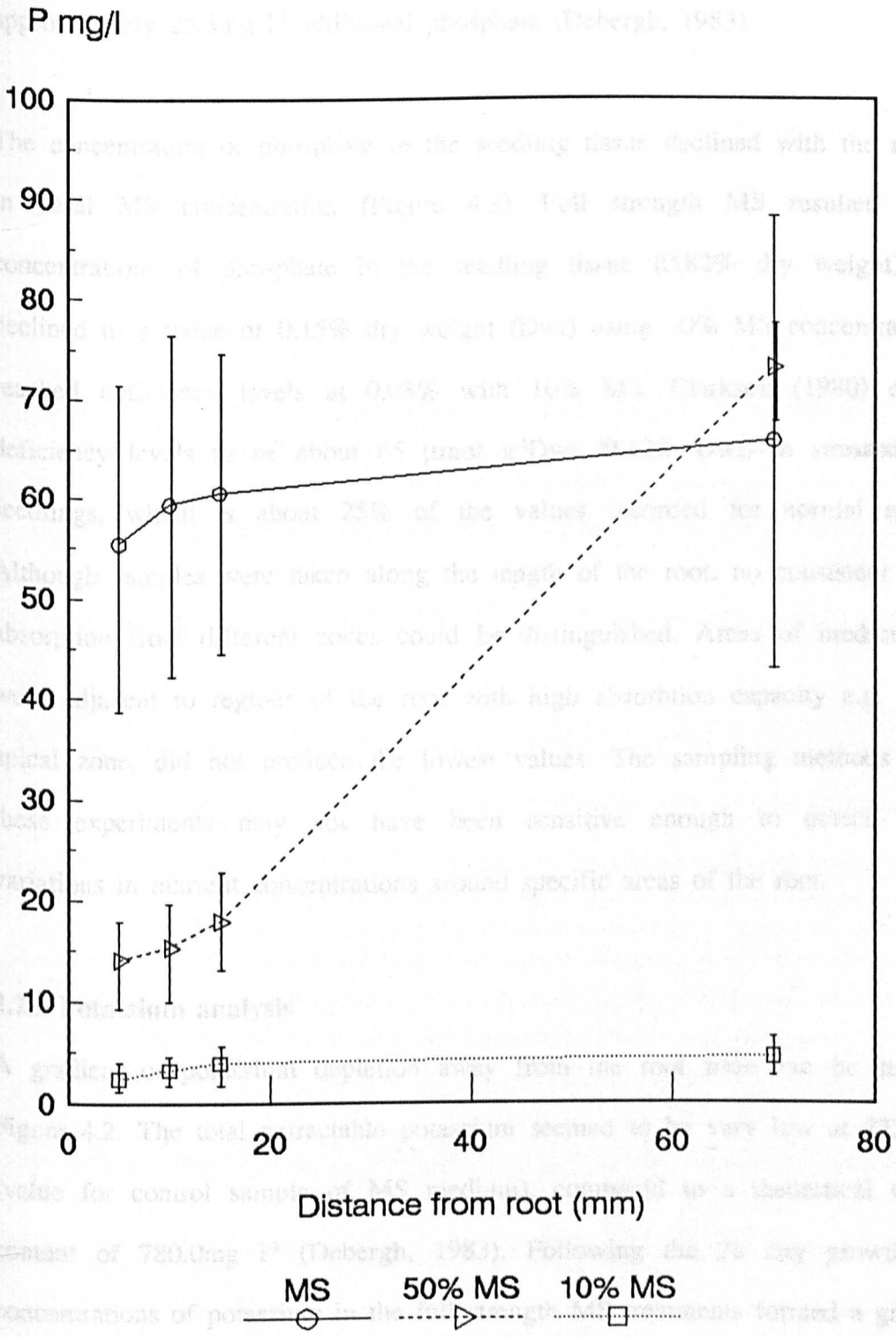
4.2 Results and Discussion

In order to study nutrient availability in the culture media samples were taken from regions of the culture vessel adjacent to the root zone. Three 5 mm diameter cores of medium were taken consecutively, moving away from the root zone (section 2.2.2, figure 2.1) with the final core taken from the side of the vessel furthest away from the roots. Each core was divided into 1.5 cm sections, which corresponded to different regions along the root length in an attempt to detect any differential uptake occuring in different regions of the root. Each sample was analysed for P and K as previously described (Methods section 2.2.2) using 100%, 50% or 10% concentrations of MS medium.

4.2.1 Phosphate analysis

The values obtained for analysis of phosphate concentrations in the media are shown in Figure 4.1. There appeared to be a gradient in the depletion of phosphorus across the vessel, particularly in the 50% MS treatment. The values recorded closest to the root zone are consistently lower in all concentrations of media, but was more pronounced in the 50% MS treatment. As the total concentration of MS basal salts was reduced the extractable P also fell after 28 days of seedling growth. The control samples of MS and 50% MS, produced

Figure 4.1: Concentration of phosphate, in varying strength MS media (100%, 50% and 10%) sampled from different vessel locations and analysed after 28 days seedling growth. Values represent means of 10 replicates, ± SEM


P mg/l
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Distance from root (mm)
MS
50% MS
10% MS

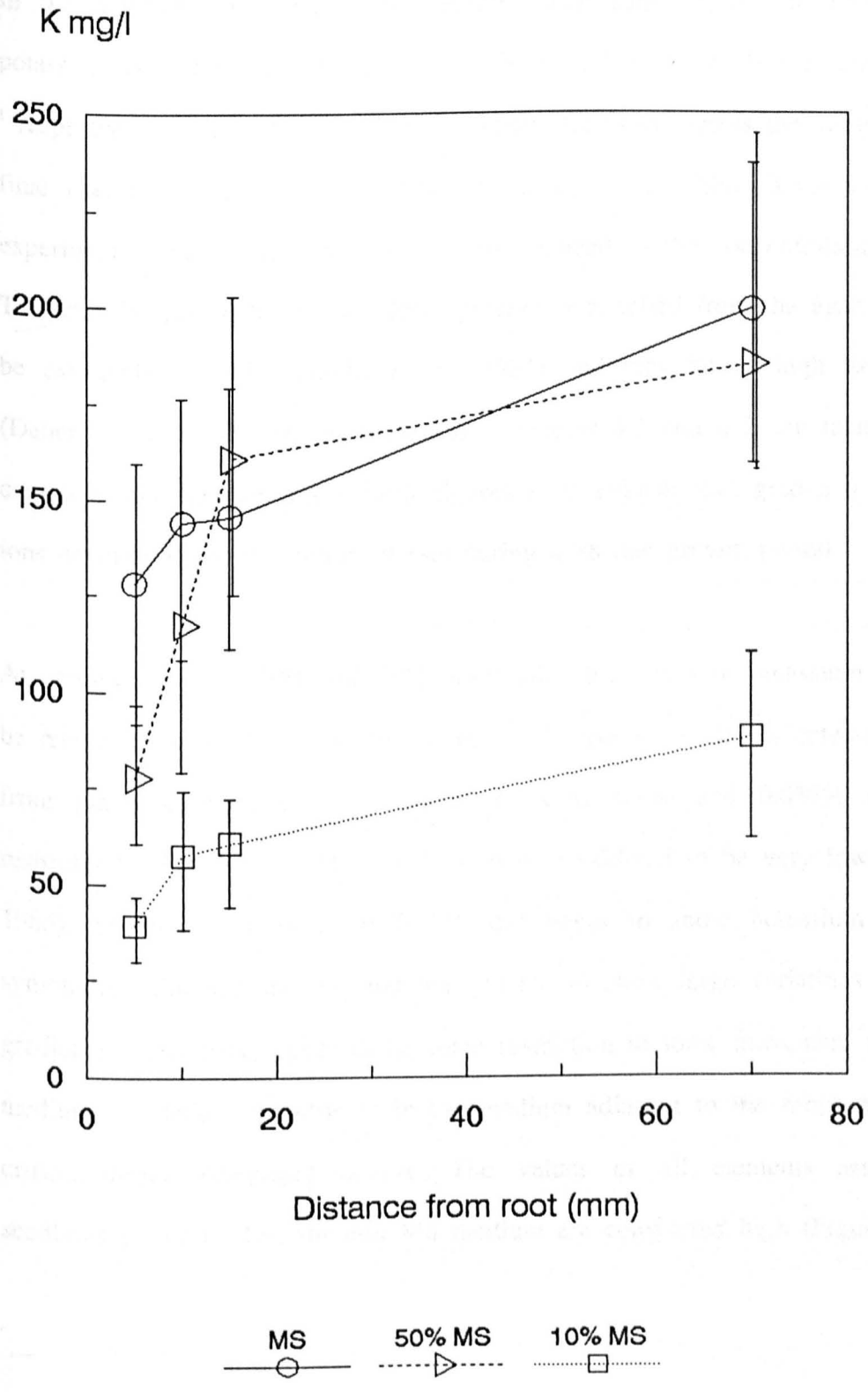
values of 83.3 and 37.5mg l^{-1} respectively, both higher than the medium taken following seedling growth. In full strength MS, the total P measured was higher than that supplied in the MS salts. The additional P was probably introduced from the Difco-Bacto agar, which when added at a concentration of 8g l^{-1} supplied approximately 25.3mg l^{-1} additional phosphate (Debergh, 1983).

The concentration of phosphate in the seedling tissue declined with the reduction in basal MS concentration (Figure 4.3). Full strength MS resulted in high concentrations of phosphate in the seedling tissue (0.82% dry weight), which declined to a value of 0.15% dry weight (Dwt) using 50% MS concentration and reached deficiency levels at 0.03% with 10% MS. Clarkson (1980) estimated deficiency levels to be about 65 µmol g^{-1}Dwt (0.12% Dwt) in stressed tomato seedlings, which is about 25% of the values recorded for normal seedlings. Although samples were taken along the length of the root, no consistent trend in absorption from different zones could be distinguished. Areas of medium which were adjacent to regions of the root with high absorbtion capacity e.g. the sub-apical zone, did not produce the lowest values. The sampling methods used in these experiments may not have been sensitive enough to detect localised variations in nutrient concentrations around specific areas of the root.

2.2.2 Potassium analysis

A gradient of potassium depletion away from the root zone can be seen from Figure 4.2. The total extractable potassium seemed to be very low at 273.1mg l^{-1} (value for control sample of MS medium), compared to a theoretical total MS content of 780.0mg l^{-1} (Debergh, 1983). Following the 28 day growth period concentrations of potassium in the full strength MS treatments formed a gradient

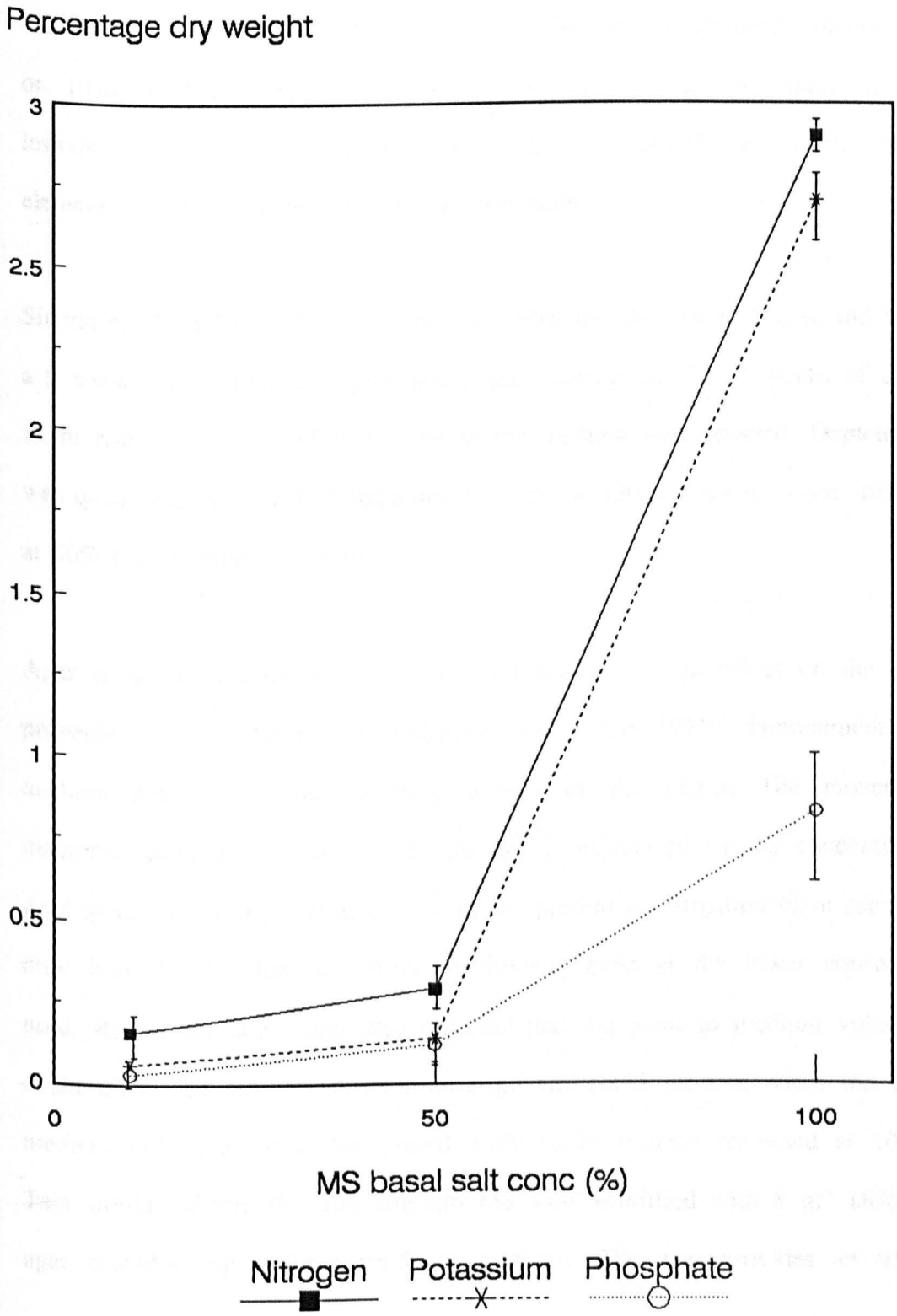
Figure 4.2: Concentration of potassium in varying strength MS media (100%, 50% and 10%) sampled from different vessel locations and analysed after 28 days seedling growth. Values represent means of 4 replicates, ± SEM


K mg/l
250
200
150
100
50
0
0
20
40
60
80
Distance from root (mm)
MS
50% MS
10% MS

across the vessel ranging from 199.0mg l^{-1} in the region furthest away from the roots (D) to 124.2mg l^{-1} in the region adjacent to the root zone (A),(Figure 4.2). In the experimental media with reduced basal salts (50% and 10% MS) the potassium concentrations in region D of the vessel were 183.4mg l^{-1} and 86.0mg l^{-1} respectively. These concentrations gradually decreased across the vessel, reaching final values of 77.5mg l^{-1} and 40.1mg l^{-1} in region A. Although the values in the experimental media are low, they are not reduced to the concentrations expected. This may be partly due to additional potassium supplied from the agar, which can be extremely variable (Singha *et al.,* 1985) and may be as high as 317mg l^{-1} (Debergh, 1983). The values presented in figures 4.1 and 4.2, are taken from the complete core sample. From both figures it is evident that gradients of nutrient ions develop across the culture vessel during a 28 day growth period.

At concentrations of 50% and 10% basal salts, the levels of potassium should not be restrictive to seedling growth. However, the potassium levels detected in tissue from plants cultured in these conditions were 0.4% and 0.057% dry weight respectively (Figure 4.3). These values were considered to be very low (Bingham, 1985). Seedlings grown at 10% MS did begin to show potassium deficiency symptoms. Although this method was unable to show large variations in nutrient gradients, there does appear to be some restriction to ionic movement through the medium. Depletion of nutrients in the medium adjacent to the roots may become critical during prolonged culture. The values of all elements assayed from seedlings grown on full strength MS medium are considered high (Figure 4.3).

Figure 4.3: Tissue concentrations of N, P and K recorded in tomato seedlings following 28 days growth on varying strengths of MS medium. Values represent means of 4 replicates, ± SEM


Percentage dry weight
3
2.5
2
1.5
1
0.5
0
0
50
100
MS basal salt conc (%)
Nitrogen
Potassium
Phosphate

2.2.3 Nitrogen analysis

In seedlings grown on full strength MS medium the soluble nitrogen content of the tissue was recorded at 2.9% Dwt. Nitrogen concentrations in seedlings grown on 50% and 10% MS were reduced to 0.3% and 0.15% Dwt respectively (Figure 4.3). Both values being considered adequate for growth. However, plants growing on 10% MS were much smaller and chlorotic, and were beginning to develop lesions on the leaf lamina, these symptoms demonstrated the low availability of all elements at this much reduced MS concentration.

Singha et al. (1987) observed a general media depletion of P, Ca, K and Mg over a 9 week culture period of pear and apple. During the first 2 weeks of culture a slight rise in the level of K present in the medium was detected. Depletion of P was quite severe, with 50% depletion by week 6. Other nutrients were still present at 50% concentration by week 9.

Agar is not chemically inert and the gel matrix has an effect on the physical properties of the medium (Romberger and Tabor 1971). Pretreatment of the medium also affects the resulting growth of the plants. The movement of macromolecules and water through the gel is influenced by the concentration of agar used. At the concentration used in the present investigation there seems to be only limited inhibition of mineral movement, even at the lower concentrations used. Romberger and Tabor also reported that the plant to medium volume ratio could affect the rate at which culture growth could proceed. Here the plant to medium ratio was large, but growth only really became restricted at 10% MS. This would indicate that full strength MS salts solidified with 8 gl^{-1} Difco-Bacto agar provides supraoptimal levels of nutrients. The agar provides an additional

source of nutrients, but also introduces high concentrations of sodium into the medium, which can be detrimental to plantlet metabolism and growth (Appendix III). Debergh (1983) found that phosphate and some micro elements were absorbed onto the gel and were only extractable using acid. In this study phosphate was recovered from MS medium in high concentrations and correspondingly high levels were found in plant tissues.

The full and half strength concentrations of MS media used in these studies were not found to be limiting to plant growth over the 28 day growth period. Gradients of P and K were seen to develop across the vessel and a depletion zone began to form in the area adjacent to the roots. Nitrogen was not assayed for in the medium, but this would have provided further useful information on nutrient supply from the medium. As it is a more soluble nutrient, nitrogen would not be expected to become limiting at either highest MS concentrations. None of the tissues assayed in these experiments exhibited low concentrations of soluble nitrogen. When an extremely low concentration of MS was tested, gradients of nutrient supply across the culture vessel became more pronounced and the plants showed signs of nutrient stress. These results may indicate that prolonged culture on agar solidified medium with reduced strength MS salts may lead to decreased plant growth rates and this may be an additional cause of poor survival *ex vitro*.

Chapter 5: Assessment of Phosphate uptake and translocation in cultured plants

5.1 Introduction

The relationship between growth and metabolism of root and shoot systems must be based primarily on mutual dependence for substrates, the shoot supplying the root with photosynthates and the root supplying the shoot with mineral ions and water (Brouwer, 1983). Normal growth is dependent upon the integrated functioning of roots and shoots. In plants cultured *in vitro*, abnormal physiological development may result in reduced growth rates, high percentages of leaf abscission or whole plant losses at the acclimatization stage. It has been suggested that discontinuity in vascular tissue between shoot and root, observed in cultured cauliflower plants (Grout and Aston, 1977) could result in reduced transport of ions and water to the shoot. Poor functioning of *in vitro* formed roots in conventional potting mixes (Maene and Debergh, 1983) would also result in slow growth rates and low percentages of plantlet survival. Root systems produced in culture, particularly in agar solidified media, tend to be unbranched and lack root hairs. This type of root system may have reduced absorbing power for mineral ions (Gardner *et al.*, 1981; Itoh and Barber, 1983), especially those considered to be less available such as phosphorus. Slow diffusion of phosphate through soil may sometimes limit uptake as the rhizosphere ion concentration becomes depleted (Clarkson, 1985).

In the experiments described in this chapter radiolabelled orthophosphate was used to elucidate the efficiency of the root system formed *in vitro* to absorb and transport phosphate from solution culture. Efficacy of xylem translocation from the

root to the shoot following varying acclimatization treatments was also assessed. A solution culture system was used, as it provided an ideal intermediate system between *in vitro* conditions and soil culture. Roots could be tested for ion absorption with little damage being inflicted on the root system, which would result in leakage of ions back into solution. Optimum growth rates could also be achieved by more accurate control of nutrient supply and root temperature. Plantlet and seedling growth measurements are presented and discussed.

Radial movement of nutrient ions and water across the root to the xylem are either via an apoplastic or symplastic pathway (Drew, 1987, Nye and Tinker, 1977) (Figure 5.1). Apoplastic transport of ions occurs passively in the cortical cell wall spaces of the root by diffusion or mass flow and are then actively absorbed for transport through the endodermis. Symplastic movement involves active absorbtion into the cell, at or near the root surface. The ions then move between cortical cells and endodermis via the cytoplasm and plasmodesmata. Although both pathways operate, ions tend to move predominantly by one process or the other (Clarkson, 1974).

Phosphorus is predominantly absorbed as the phosphate anion $H_2PO_4^-$ below pH 7. Most of this is converted to organic phosphate on entry to the root. It tends to be quickly absorbed and incorporated into the cytoplasm in the form of sugar phosphates and nucleoside phosphates and other organic complexes (Loughman, 1987). Following dephosphorylation, inorganic phosphate is transported via the xylem to the shoot where it forms an essential part of many organic compounds, such as sugar phosphates, phospholipids, DNA and RNA (Nye and Tinker, 1977).

Figure 5.1: A schematic diagram of the interaction of symplastic and apoplastic pathways in the root cortex and stele (from Nye and Tinker, 1977)

Factors affecting phosphate uptake include plant age, degree of root development, nutrient status of the plant and nutrient concentration of the growing solution (Clarkson, 1985). Root development and growth rate affects the amount of phosphate absorbed, which is in turn dependent on the demand created by shoot growth and the rate of nutrient supply to the root. Plant genotype has also been indicated as a determinant factor in phosphate absorption and distribution efficiency within species (Furlani, *et al.*, 1987).

In solution culture, circulation of media is important to prevent the ion concentration in the root boundary layer from becoming limiting (Cress and *et al.*, 1979) and maintenance of ionic concentration of the nutrient solution throughout the culture period promotes steady uptake and higher growth rates (Clarkson, 1985). Romer *et al.*, (1988) concluded that higher phosphate supply to the root results in higher uptake rates per root unit, whilst at low phosphate supply the available root absorbing surface becomes increasingly important.

Changes in external ion concentration can produce modulations in ion uptake via changes in internal cytoplasm concentrations, usually following periods of phosphate deprivation (Drew and Sakar, 1984). This may result in periods of increased loading, release or sequestration of ions by the vacuole. Long term changes in uptake probably result from modulations of carrier activity (Clarkson, 1985).

Drew and Sakar (1984) conclude however that the quantity of nutrients entering the root is not only determined by the efficiency of uptake (influx per unit of root length or weight) but also by the efflux rate into the xylem. The availability of elements important in maintaining membrane integrity, such as zinc, boron and

calcium also affect influx and efflux rates (Loughman, 1987). With phosphates the main influx is directed towards xylem transport to the shoot (Clarkson *et al.*, 1978). Controlled efflux into the xylem is maintained by regulation of cellular concentrations of nutrients. Nitrate status of the tissue has been shown to affect both root uptake of orthophosphate and secretion into the xylem (Lamaze *et al.*, 1987), both are thought to be mediated by NO_3^- depression of phosphate uptake into the vacuole. Transport through the xylem is determined by root pressure and transpirational demand for water from the shoot. Hsiao (1973) concluded that transpirational flow had more effect when solution concentration was high. This assumes that at high concentrations cell membranes become more permeable. Rapid transpiration moves ions in the xylem, keeping concentration in the root stele low and maintaining efflux rates into the xylem. In contrast Silberbush and Barber (1983) found that in soybeans transpiration had no or little effect on phosphate uptake and root morphology and the solution concentration had most influence on uptake. Compensation in uptake rates allows an early response to nutrient stress by roots. More long term responses include changes in growth rate and root/shoot ratios (Clarkson, 1985).

The size of the root system per unit shoot weight is influenced by nutrient availability, root temperature and translocation efficiency to the shoot (Barber, 1980). Altered distribution of N, P and K between root and shoot has led to suggestions that growth may be limited by restricted nutrient translocation at low root temperatures (Rufty *et al.*, 1981). Root development and morphology mainly affects uptake of less mobile ions such as phosphate, although this may be less important in an aerated solution culture system, it may be more critical in agar culture.

Long slender branched roots with well developed root hairs are predicted to have maximum absorbing efficiency (Reid, 1981). The main absorbtion is in the sub-apical zone of the root and in the zone where the root hairs begin to emerge, several millimetres behind the apex. Factors affecting root morphology and development such as endogenous growth regulator supply, substrate aeration and humidity are discussed in the following chapter.

In these experiments the ionic concentration of the nutrient solution was kept constant, aiming to produce "high ion status" plants, with steady state concentrations of nutrients in the roots and shoots, producing high growth rates and minimum variation in root/shoot ratios (Clarkson, 1985). Strong uptake of phosphate can continue for some time following growth in low concentration solutions (Katz *et al.*, 1986). Standardization periods in renewed solutions prior to uptake experiments help to eliminate these effects.

Aims of the study on ^{32}P uptake in cultured and seedling tomato plants

The initial part of this study aimed to investigate the effect of acclimatization regime on relative growth rates in the two plant types. The isotope uptake studies were designed firstly; to determine if the root formed *in vitro* was functional, and secondly; to assess the relative efficiency of ion absorbtion and translocation in the *in vitro* and acclimatized tomato plantlets when compared to seedlings at similar stages of growth.

5.2 Results

Phosphate uptake and translocation was compared in cultured and seedling tomato plants following 7 or 14 days acclimatization at either 40% or 80% relative humidity (RH). Micropropagated and seedling plants were selected for this comparative study on the basis of shoot height similarity at the beginning of *ex vitro* treatments. After 7 or 14 days, there was still no significant difference between cultured plants and seedlings, or between the two humidity treatments, as shown by Figures 5.2 and 5.3.

Figure 5.4 shows the numbers of leaves produced *in vitro* and *ex vitro* by the cultured plants, compared with seedlings over the same period. Seedling leaf production was unaffected by the ambient humidity whereas the micropropagated plantlets initially abscised some of their *in vitro* formed leaves but then produced more leaves, particularly evident after 14 days at 80% humidity. Complete abscission of older leaves was evident when plants were transferred to the higher of the two humidities. When recorded as fresh weight (Table 5.1), the leaf tissue per cultivated plantlet was similar between treatments after 14 days. However, seedling leaf weight was significantly greater after 14 days at the lower humidity ($P<0.001$). Maintaining a lower shoot ambient humidity promoted root proliferation in solution and this was particularly noticeable in seedlings where the root mass at 40% humidity was very low at 7 days, but increased rapidly towards 14 days, when the mass was double that recorded at 80% humidity. Generally roots were well branched and fine with root hairs present in both types of plants.

Root morphology would not have limited mineral uptake in the cultured plantlets at either 7 or 14 days.

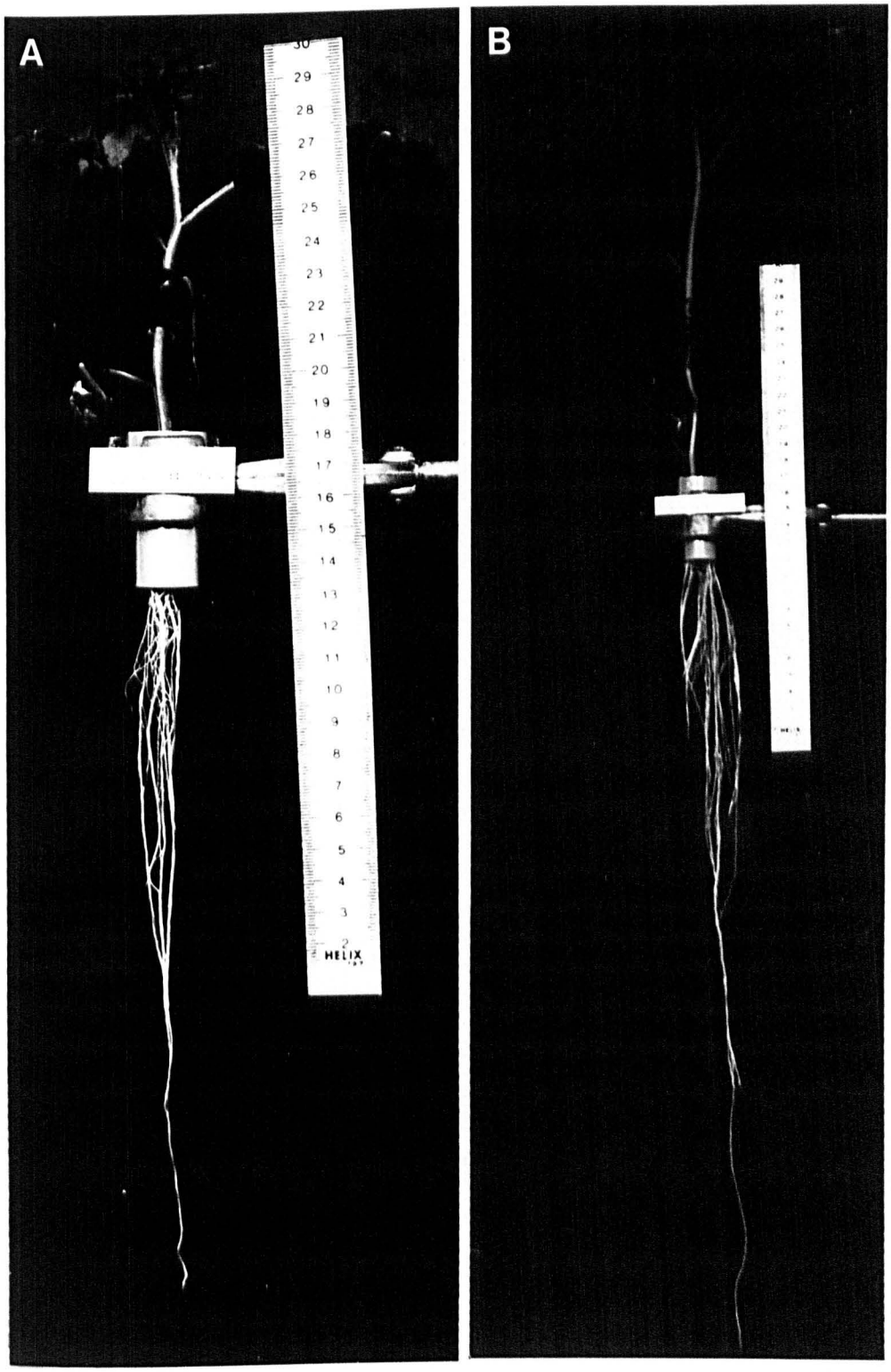
Table 5.1 Fresh (F) and dry (D) weights of micropropagated (M) and seedling (S) plants acclimatised at different humidities

Weights(g)		M 100%R.H.	80%R.H. 7 Days		80%R.H. 14 Days		40%R.H. 7 Days		40%R.H. 14 Days	
			S	M	S	M	S	M	S	M
Root	F	0.13	0.21	0.55	0.51	0.85	0.15	0.48	1.06	1.25
	D	0.007	0.01	0.03	0.04	0.07	0.01	0.02	0.07	0.09
Stem	F	0.24	0.47	0.73	1.26	2.50	0.28	0.54	1.27	1.76
	D	0.012	0.03	0.05	0.09	0.19	0.02	0.04	0.10	0.14
Leaf	F	0.47	0.96	1.61	1.90	3.80	0.54	1.56	3.09	3.91
	D	0.027	0.11	0.18	0.28	0.44	0.06	0.18	0.42	0.66
Shoot/ Root ratio	F	6.4	6.8	4.3	6.2	7.4	5.5	4.6	4.3	4.7
	D	7.0	13.8	8.3	10.0	9.5	11.2	11.0	8.0	9.6

Summary of analysis of variance (F values. Significance: *P<0.05 **P<0.01 ***P<0.001 n = 10)

Variate		Main Effects			Interactions		
		Time	Humidity	Plant type	Time Humidity	Time Plant type	Humidity Plant type
Root	F	44.6***	8.9**	9.3**	11.3***	0.3	0.0
	D	65.8***	3.1	10.2**	6.8**	0.5	0.1
Stem	F	46.5***	1.5	8.9**	0.0	1.8	0.4
	D	65.1***	1.1	13.4***	0.1	3.5	0.1
Leaf	F	52.5***	1.9	15.6***	4.9*	0.9	0.0
	D	58.7***	5.0*	11.0***	7.9**	1.2	1.1
Shoot/Root Ratio	F	2.9	20.2***	6.2**	4.9*	8.2**	5.0*
	D	7.8**	0.1	4.7*	0.4	6.5**	10.3**

Figure 5.2: Micropropagated tomato plantlets after 7 (A) and 14 (B) days growth in solution culture at 40% and 80% relative humidity respectively, and 25 ± 2°C, prior to use in isotope uptake experiments


A
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11
10
9
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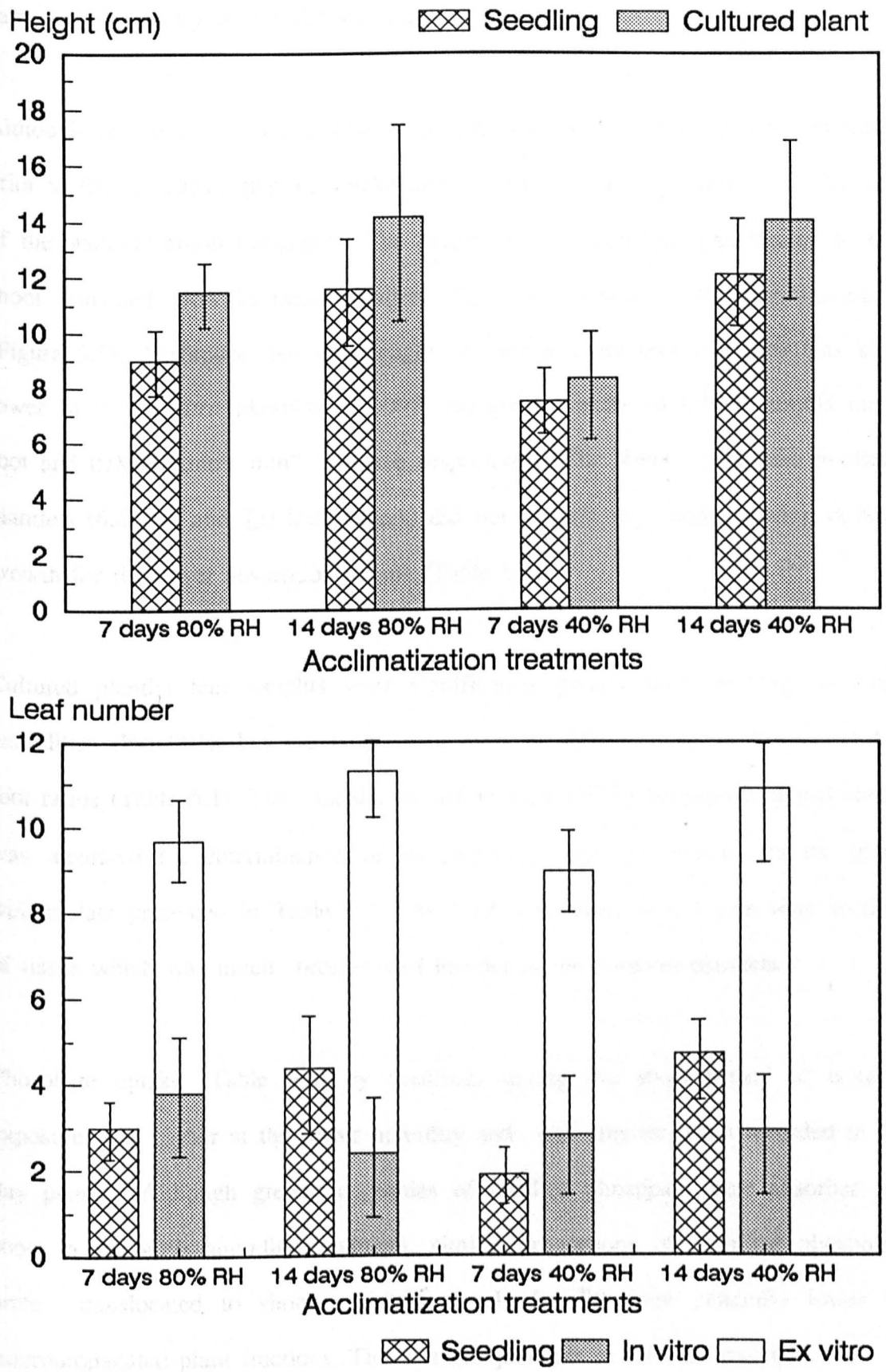
7
6
5
4
3
2
HELIX

Figure 5.3: Mean shoot heights of seedlings and micropropagated plantlets measured following treatments at 80% and 40% relative humidity, for 7 and 14 days. Values represent means of 10 replicates, ± SEM

Figure 5.4: Leaf numbers on seedlings and micropropagated plantlets measured after 7 and 14 days growth at each humidity of 40% and 80%. Values represent leaves formed at 100% (*in vitro*) and after acclimatization (*ex vitro*), to give total leaf numbers after 7 and 14 days growth. n = 10 ± SEM


Height (cm)
Seedling
Cultured plant
20
18
16
14
12
10
8
6
4
2
0
7 days 80% RH
14 days 80% RH
7 days 40% RH
14 days 40% RH
Acclimatization treatments
Leaf number
12
10
8
6
4
2
0
7 days 80% RH
14 days 80% RH
7 days 40% RH
14 days 40% RH
Acclimatization treatments
Seedling
In vitro
Ex vitro

However, uptake of ions by *in vitro* plantlets may be impeded by lack of root hairs and the poorly branched root system.

Although the same standardization treatment was given to the *in vitro* plantlets prior to the ^{32}P uptake period, uptake into the root was much lower than after any of the acclimatization treatments. The percentage of phosphate translocated to the shoot remained low in these plantlets. This can be seen in the autoradiograph (Figure 5.5). Absorption per unit length of root and per unit leaf area was also lower in the *in vitro* plantlets at 100% rh, giving values of 0.0007 μmols mm^{-1} root and 0.0043 μmols mm^{-2} leaf area respectively. The shoot : root ratio in these plantlets (6.4 Fwt and 7.0 Dwt values) did not suggest any compensation in root growth for the lower absorption values (Table 5.2).

Cultured plantlet leaf weights were significantly greater than seedlings at both humidities ($P<0.001$), but the larger root mass at 40% resulted in lower shoot : root ratios (Table 5.1). The vascular transition zone (VTZ) between root and shoot was separated for determination of radioactivity, but not included in the plant weight data presented in Table 5.1. The VTZ was taken as a 5 mm long section of tissue which was much broader and heavier in the cultured plantlets.

Phosphate uptake (Table 5.2) by seedlings during the short period of isotope exposure, was higher at the lower humidity and was greatest when recorded in 14 day plants. Although greater quantities of labelled phosphate were absorbed by roots in the 40% humidity treatment, similar proportions of absorbed phosphate were translocated to shoots. Activity levels for ^{32}P were generally lower in micropropagated plant fractions. The cultured plantlets at 80% rh absorbed less

Table 5.2 Phosphate absorption (Abs) and distribution in micropropagated (M) and seedling (S) plant fractions at different humidities

Weights(g)	M 100%R.H.	80%R.H. 7 Days		80%R.H. 14 Days		40%R.H. 7 Days		40%R.H. 14 Days	
		S	M	S	M	S	M	S	M
Root (µmol g^{-1}Dwt)	32.0	482.0	206.0	217.0	70.8	582.0	385.0	1107.0	179.0
Stem (µmol g^{-1}Dwt)	6.0	42.5	17.1	21.2	27.4	44.5	34.7	98.6	13.9
Leaf (µmol g^{-1}Dwt)	3.1	39.8	16.3	14.6	66.4	251.0	32.0	370.7	7.8
VTZ (µmol g^{-1}Dwt)	4.0	54.5	48.0	30.2	31.5	42.6	69.7	74.3	15.7
% Translocated to shoot	30.1	48.8	36.4	37.2	74.7	43.5	44.3	44.5	32.0
Abs.per unit root length (µmol g^{-1}Dwt)	0.0007	0.004	0.002	0.004	0.012	0.004	0.006	0.004	0.003
Abs.per unit dry Wt root (µmol g^{-1}Dwt)	0.064	1.0	0.36	0.5	0.63	1.1	0.76	2.6	0.27
Abs.per unit leaf area (µmol g^{-2}Dwt)	0.0043	0.024	0.014	0.009	0.0014	0.031	0.02	0.025	0.017

Summary of analysis of variance (F values. Significance: *P<0.05 **P<0.01 ***P<0.001 n = 10)

Variate	Main Effects			Interactions		
	Time	Humidity	Plant type	Time Humidity	Time Plant type	Humidity Plant type
% Translocation to shoot	1.49	6.59**	1.11	8.9**	8.19**	8.32**
Uptake (µmolmm^{-1}root length)	0.99	0.68	0.85	4.65*	0.79	0.77
Uptake (µmolmg^{-1} root Dwt)	2.87	23.4***	50.1***	7.81**	7.15**	22.45***
Uptake (µmolmm^{-2} leaf area)	16.2***	24.4***	18.6***	4.88*	0.39	0.02

Figure 5.5: Autoradiograph of micropropagated tomato plantlet after exposure to ^{32}P-orthophosphate *in vitro*, showing ^{32}P accumulation in the root. The shoot tip is indicated by the arrow.

when estimated after 14 days than after 7 days, but the proportion of absorbed phosphate transported to the shoots was markedly greater after 14 days growth (Figure 5.7&8).The reverse trend was apparent in seedlings, a decline in the percentage of phosphate translocated to the shoot from 49% at 7 days (Figure 5.7) to 37% at 14 days was observed (Figure 5.8 and Table 5.2). At the lower humidity the phosphate translocated to the shoot was low in both plant types at 7 days. After 14 days values had declined to 32% in the cultured plants but had remained the same in the seedlings.

fig 5.6

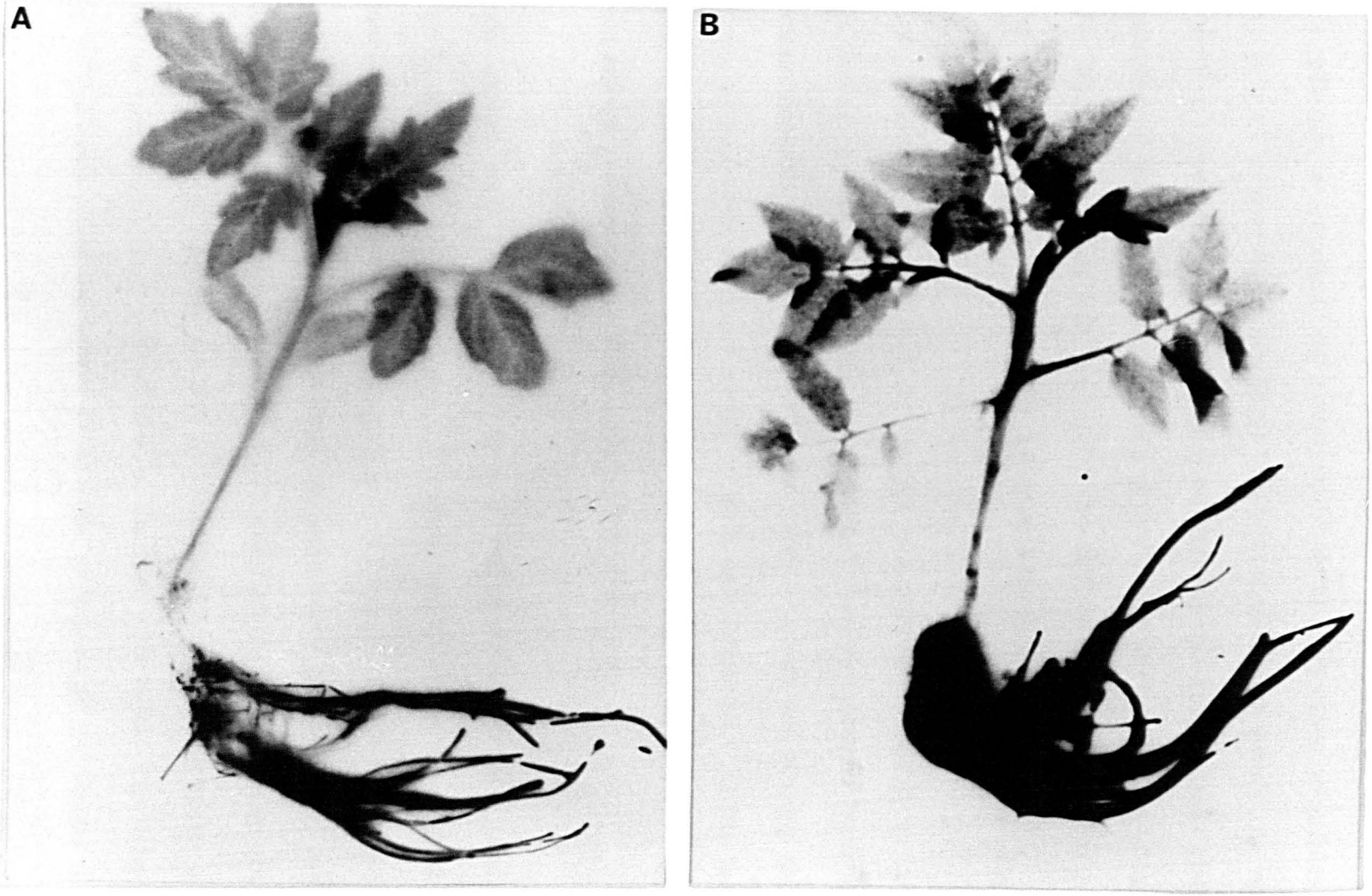
A lower quantity of phosphate was absorbed and translocated in the cultured plants at lower humidity, despite there being a greater root mass than in seedlings and more shoot tissue to create a stronger metabolic demand. With 40% rh treatments the seedling, leaf and stem tissues received high levels of P with effective translocation across the vascular transition zone. This is demonstrated in Figure 5.6. The overall water potential gradient created at this low humidity would be expected to enhance transpiration rates and movement of P through the apoplastic continuum would be promoted. Lower transpiration flow at higher humidity may account for the reduced total phosphate uptake, but when shoot metabolic demand is high, as in the cultured plantlets with good leaf expansion, the percentage of the absorbed phosphate translocated to the shoots is high (table 5.2 and Figure 5.8). This order of phosphate transport may be accounted for by symplastic movement, and will therefore be less dependent on total vascular continuity.

Lower values recorded for phosphate concentration in shoot tissues and percentage translocation to the shoot for *in vitro* plantlets at 100% rh may also be as a result

Figure 5.6: Autoradiographs of micropropagated (B) and seedling (A) tomato plants, labelled with ^{32}P, following 7 days growth at 40% relative humidity, showing a lack of translocation of isotope to the shoot in both plant types, but greater accumulation in the seedling root and shoot tissue.


A
B

Figure 5.7: Autoradiographs of micropropagated (B) and seedling (A) tomato plants, labelled with ^{32}P following 7 days growth at 80% relative humidity.

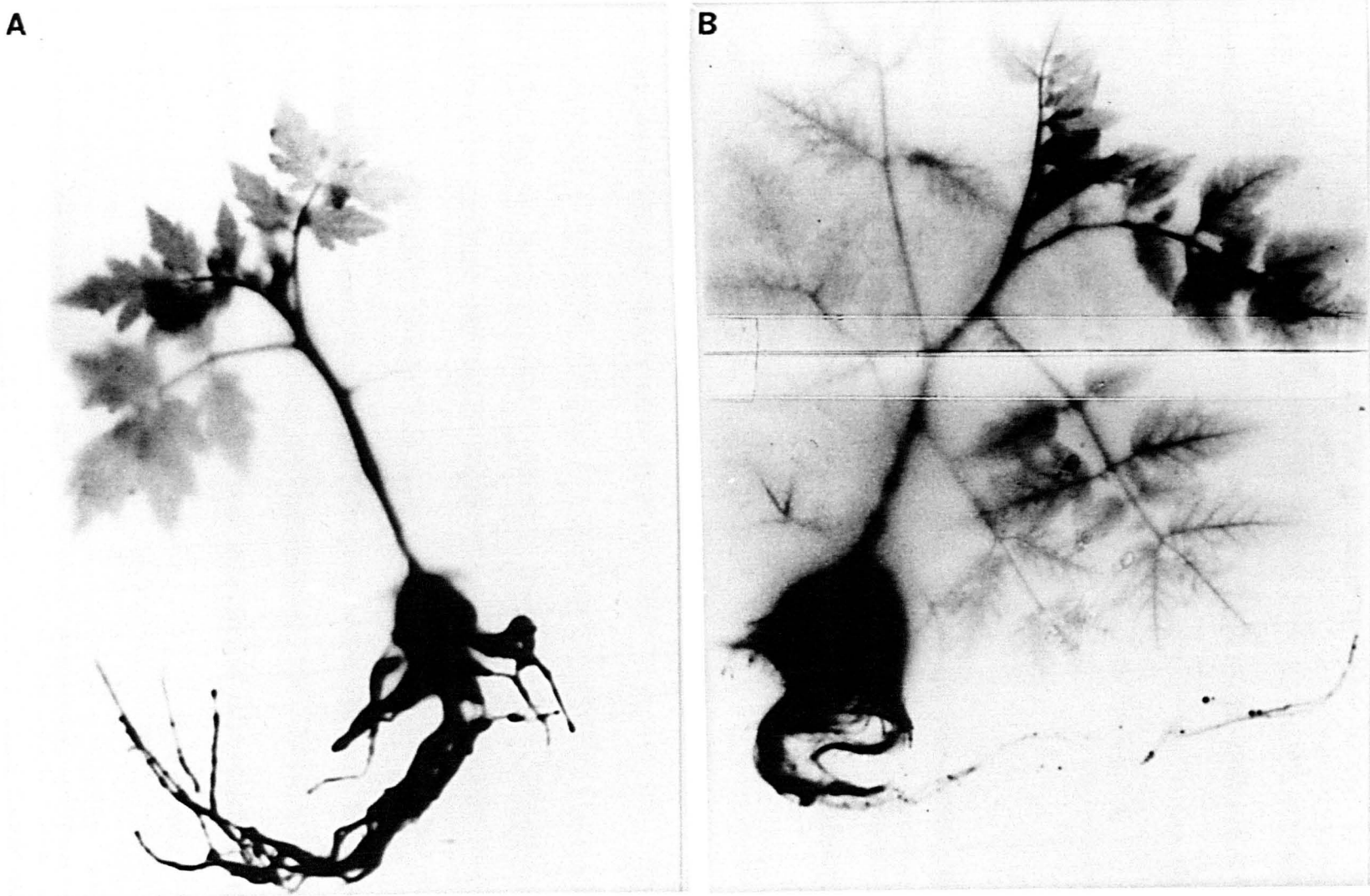

A
B

of symplastic movement. The value for absorption per unit leaf area of 0.0043 µmols mm^{-2} leaf is similar for those recorded for both plant types after 14 days at 80% rh, this is also demonstrated in the autoradiograph (Figure 5.8). This may suggest a reduction in movement via transpiration flow at these higher humidities. This may be more evident in the seedlings, where accumulation of isotope can be seen in the root (Figure 5.8). The degree of absorption per unit leaf area may give some indication of the extent of transpiration flow through the plants. The lower values for absorption per unit leaf area, could also indicate some development in control of water loss or may be the result of reduced shoot demand.

Uptake of phosphate per unit root dry weight was consistently higher in the seedlings ($P<0.001$). The root systems of the cultured plantlets appear to improve in absorbtion capacity over the first 7 days in solution culture and develop the same degree of absorption efficiency as the seedling roots. Absorption figures per unit length of root remain high throughout all acclimatization treatments, although the values are not as consistent as those recorded for seedlings.

^{32}P uptake studies were carried out on *in vitro* and acclimatized palm plants following the method described in section 2.2.7. Phosphate uptake into these plants was reasonably high with values of 4.8 µmol mg^{-1} dry weight of root for *in vitro* plants and 0.25 µmol mg^{-1} dry weight root for acclimatized plants. Final tissue concentrations of phosphate were recorded at 680 µmol g^{-1} dry weight for *in vitro* plants and 44 µmol g^{-1} dry weight for acclimatized plants. Concentrations of phosphate in root tissues were very similar in the two plant types. The values recorded in the palm plants are of the same order of magnitude for figures on tomato seedlings, presented in this chapter and by Clarkson (1980). However the

Figure 5.8: Autoradiographs of micropropagated (B) and seedling (A) tomato plants labelled with ^{32}P, following 14 days growth at 80% relative humidity, translocation to the shoot is greater in the cultured plantlets, accumulation of the isotope is evident in the seedling root.


A
B

values recorded for the acclimatized palm plants are much lower, and may well be considered to be approaching deficiency levels.

The percentage of phosphate translocated to the shoot is higher in the *in vitro* plants. As root mass and length is similar in the two plant types, and absorption values into the root are similar, the difference in final tissue concentration must be accounted for by the lack of transport to the shoot tissue in the acclimatized plants. The possible reasons for this are discussed at the end of section 5.3.

5.3 Discussion

The overall picture of phosphate absorption and transport in the two tomato plant types studied in these experiments seems to indicate that root concentrations of phosphate are the highest of all tissues, for each of the acclimatization treatments, and are in agreement with the figures given by Clarkson (1980). The seedling shoot values recorded at the lower of the two humidities are considered to be at an adequate level and the translocation value of 45% is average for tomato seedlings. However at the higher humidity the concentrations recorded in the root are acceptable, but the levels recorded in the shoot tissues could be considered to be low. Clarkson indicates that values below 65 μmol g^{-1} dry weight causes phosphate stress symptoms in tomato. This lower value may be accounted for by a decrease in transpiration flow. The *in vitro* plantlets exhibit very low phosphate concentrations in all tissue types and poor translocation. This improves during acclimatization, but the efficiency of uptake and translocation is only comparable to that recorded for seedlings at the highest humidity after 14 days growth. The cultured plants appear to be under most stress after 14 days at 40% relative humidity. Although the absolute values obtained in these experiments appear to be

quite low the relative comparisons of uptake and transport between the two plant types should still be valid. The low values obtained may be due to experimental error.

The conditions experienced by plantlets *in vitro* are reported to induce physiological differences in both the root and shoot of cultured plantlets. These differences are responsible for problems during the acclimatization stage. Transition from heterotrophic to autotrophic growth (Desjardins *et al.*, 1988), reduction in the ambient humidity and transfer to conventional growth substrates all require modifications in the plantlets physiology, in order that high survival rates are achieved.

It has been proposed that *in vitro* formed leaves act as storage organs, to allow for the development of adapted leaves during acclimatization (Grout and Aston, 1978; Wardle *et al.*, 1983b). This cannot be the case where micropropagated plantlets initially abscised some of the *in vitro* formed leaves on transfer to lower humidities, but then produced more leaves *ex vitro*. Although some of the tomato leaves became desiccated and abscised *ex vitro*, some survived and were clearly capable of adapting to the reduced humidity. The complete abscission of older leaves at the 80% humidity level may be explained by a model for adaptive developments which requires a threshold of reduced water potential to be experienced at an early stage, before the onset of physiological changes.

It has been reported that the poor functioning of cultured root systems may result from cellular damage incurred during transplantation from agar gel media (Aldrufeu, 1987). Modifications in structure and morphology may arise from

exposure to exogenous growth regulators e.g. kinetin for prolonged periods, and poor media aeration. The cultured plants used in these experiments were rooted in solution culture and then transferred to modified solutions well before isotope exposure periods to avoid root damage. Work on the anatomy of the *in vitro* and acclimatized roots is presented and discussed in Chapter 7. The lack of development of the root system, particularly the absence of root hairs in these roots probably contributed to the low absorbing capacity. Alternatively the low values may indicate low carrier activity development during the culture period. Although the pre-treatment period may allow short term changes in uptake in terms of loading and efflux into vacuole and xylem, the 24 hour period would not allow for more long term necessary modulations in carrier activity.

It has been demonstrated (Loughman, 1987) that mannose sequesters phosphate in the root as mannose-6-phosphate and prevents normal xylem loading and transport to the shoot. It is possible that a similar sequestration of phosphate may occur as other sugar phosphates in roots growing *in vitro*, where high levels of sucrose are present in the medium. On transfer to sucrose free nutrient solution slight improvements in translocation are observed.

From work with cuttings, it is apparent that reduced ambient humidity and partial desiccation of shoots stimulates rooting. This is a consequence of abscisic acid accumulation rather than a direct effect of drying (Orton, 1979; Rasmusen and Anderson, 1980). This may in part account for the increased root growth at the lower humidity. In cultured plants the increased root growth may be in response to the low rates of P absorption (Romer *et al.*, 1988). In these plants the extension in root growth was accompanied by root hair development. Despite absorption

limitations experienced by cultured roots growing in agar, some compensation in uptake may be achieved by increased levels of mineralization. This has been observed in lupin, which has a proteoid root system (Gardner *et al.*, 1981). Higher uptake in seedlings may be accounted for by increased branching and extension observed in the seedling root system. This agrees with the observations of Reid (1981) on the effect of root morphology on absorbtion capacity.

Poor vascular connections in the cultured plantlets could explain the restricted P translocation. Studies on root primordia initiation in cuttings show that they can develop in the vicinity of tracheids found within the callus. Vascular tissue may not link with the vascular bundles of the shoot (Brutsch *et al.*, 1977). A lack of continuity in the vascular tissues has been observed from serial sections through the vascular transition zone in cultured tomato (Chapter 7) and strawberry (Millam, pers. comm.). The development of an effective root/shoot transport system appears to be dependent on a degree of water stress being imposed on the shoot. Maintenance of a free transpiration stream develops ionic and growth regulator gradients within the stele leading to differentiation and development of normal vascular arrangements (Aloni, 1988). These processes must be impeded under the high humidity levels and low transpiration flow experienced by plants in culture.

Transport to the shoot in cultured plants *in vitro* and during early acclimatization may be predominantly by symplastic movement, and would therefore be less dependent on total vascular continuity. As the plantlets become acclimatized, development of more efficient vascular connections would be predicted through improvements in root growth and function, and changes in transpiration flow.

The low values recorded in the acclimatized palm plants appears to be due to variation in translocation efficiency to the shoot. The external morphology of the roots were similar in both plant types and no visible signs of damage could be seen. The roots also had similar lengths and masses, actual concentrations of phosphate in the two root types were almost identical, but the acclimatized root seems to retain a greater part of this. This may occur if the plants have experienced phosphate stress during the acclimatization period. The 24 hour standardization treatment may not have fully alleviated any deficits. The uptake rate into the shoot is about 20 times greater in the *in vitro* plants. The shoot/root junction was very fragile in many of the acclimatized palms. This region tends to be susceptible to pathogens and breaks down during the acclimatization period (D. Blakesley, pers. comm.). This may provide one explanation for the poor transport demonstrated by these plants.

Chapter 6: The effect of manipulating auxin and sucrose concentration on root development and morphology

6.1 Introduction

A well developed root system is necessary for efficient uptake and transport of nutrient ions and water to the shoot in cultured plants during their establishment *ex vitro*. Production of a well balanced plantlet with a functioning root system is essential for nutrient assimilation and growth, and results in higher plantlet survival during acclimatization.

The poor development of root morphology observed in many *in vitro* grown plants, particularly those produced in agar, (Lee *et al.*, 1986) has led to the proposal that the root system developed *in vitro* is non functional in a normal substrate (Maene and Debergh, 1983; Roberts *et al.*, 1990). Maene and Debergh later proposed that a system of rooting microcuttings under controlled conditions of high humidity in conventional substrates would produce a more effective root system (Maene and Debergh, 1987). Other workers have used radiotracers to demonstrate that roots formed *in vitro* are functional, although they are not as efficient during early stages of acclimatization or in culture as acclimatized or seedling roots (Borkowska and Michalczuk, 1989; Section 5.2; Marlow and Cumbus, in press).

Ex vitro rooting of microcuttings requires more costly and exactly controlled facilities to achieve higher survival percentages of plantlets, than those obtained with *in vitro* rooted plantlets.

Rooting of cuttings can be divided into the following stages (Moncousin, 1988).

1) Initiation of periclinal divisions in the cambium

2) Development of morphogenetical areas (root meristems)

3) Emergence of the root

The first two stages are examined and discussed in Chapter 7 of this thesis.

Root initiation, differentiation and elongation are regulated by both endogenous and exogenous chemicals and environmental stimuli (Aloni, 1987). In both conventional and microcuttings the root elongation stage is important for the development of lateral roots and root hairs. The extensiveness of the root system formed determines the effectivness of the root as an absorbing organ for essential nutrients and water.

Root formation *in vitro*

Production of *in vitro* rooted plantlets with well developed functioning roots should be possible by manipulation of the culture environment. The rooting phase of culture involves the separation of proliferating shoots into individual stems and their inoculation onto medium containing half strength basal salts, reduced or no cytokinin and a modified level of auxin (Hutchinson, 1984; Zimmerman, 1984).

In some recalcitrant species such as *Malus* and *Rubus* additions of activated charcoal or phloroglucinol to rooting media enhance the rooting response (James, 1979; James and Thurbon, 1981).

The auxins most widely used to induce rooting are IAA, NAA and IBA at concentrations ranging from 0.1 - 1.0 $mg.l^{-1}$. Exposure to auxin during the rooting phase is usually for a prolonged period prior to acclimatization. This may not be

the most effective method of producing an extensive root system (George and Sherrington, 1984). In some species this prolonged exposure inhibits lateral root formation and root extension. Exogenous application of IAA following an initiation phase did not enhance development of lateral roots in *Zea* (Hurren *et al.*, 1988). Application of exogenous auxin to stem bases or internodes for prolonged periods has also been shown to impede vascular formation above the site of application (Aloni, 1988). Chronic application of auxin (short period, high concentration) incorporated into rooting medium or in the form of a dip has, however enhanced rooting in some species (Moncousin, 1988).

Factors affecting root formation in shoots and cuttings

In many tissue culture systems little account seems to have been taken of the effect of endogenous auxin produced in the developing shoot (Jacobs and Short, 1986; Maldiney *et al.*, 1986; Label *et al.*, 1988). The root initials act as a sink, with vascular differentiation occurring via gradients of growth regulators formed in the procambium, cambium and differentiating elements (Aloni, 1988). Predictable patterns of vascular tissue are formed in this way (Aloni, 1987b). In tissue culture the formation of these patterns is modified at both the cellular and tissue level by prolonged stimuli from exogenous growth regulators, and in cell cultures from lack of consistent orientation of cell aggregates (Aloni, 1988).

In cuttings and shoot explants the utilization of endogenous auxin in the basal region may be influenced by the exogenous auxin supply (Jarvis and Shaheed, 1986; Mato and Vieitez, 1986). Reduction in the concentration of basal salts in rooting media has the effect of lowering total nitrogen concentration, which improves root formation in some species e.g. *Rosa* (Hyndman *et al.*, 1982), papaya

(Drew, 1987).

Effect of sucrose supply *in vitro*

The concentration of sucrose in culture media affects both root initiation and development (George and Sherrington, 1984). Interaction between sucrose and other media components such as nitrogen and auxin has been shown to affect root number (Hyndman *et al.*, 1982). The carbohydrate supply in rooting media may influence the acclimatization success of the plantlets (Zimmerman, 1983; Wainwright and Scrace, 1989). A number of studies have reported that optimum levels of carbohydrate are required for root initiation and shoot growth *in vitro*, resulting in higher survival percentages *ex vitro* (Zimmerman, 1983; Grout and Donkin, 1987; Langford and Wainwright, 1987; Rahman and Blake, 1988).

An alternative approach to improving plantlet acclimatization percentages has been to try and induce photosynthesis and stomatal function in cultured shoots by lowering media sucrose concentration (Short, Warburton and Roberts, 1987; Langford and Wainwright, 1987). Although some growth by photoautotrophy was stimulated, lowering sucrose concentration in the culture medium below about $10g.l^{-1}$, reduced plantlet growth and lead to lower survival percentages *ex vitro*. Growth of plantlets under conditions of high photosynthetic flux and CO_2 enrichment appear to be unaffected by lowering sucrose concentration (Kozai, Koyama and Watanabe, 1988; Desjardins *et al.*, 1988). Grout (1988) proposed that the addition of high levels of sucrose to media affects RuBPcarboxylase activity in the cultured leaves, preventing adaptation during acclimatization. Synergistic effects of light and sucrose have been observed in some studies (Lovell *et al.*, 1972; Rahman and Blake, 1988).

In the work described in this chapter, cultured tomato shoots were exposed to varying concentrations of sucrose and IAA during a root initiation phase of either 5 or 10 days. After this they were transferred to auxin free medium, with sucrose at 0 or 30mg.l^{-1}. The effect of these treatments on root growth and development, and plantlet survival *ex vitro* are presented in the following section.

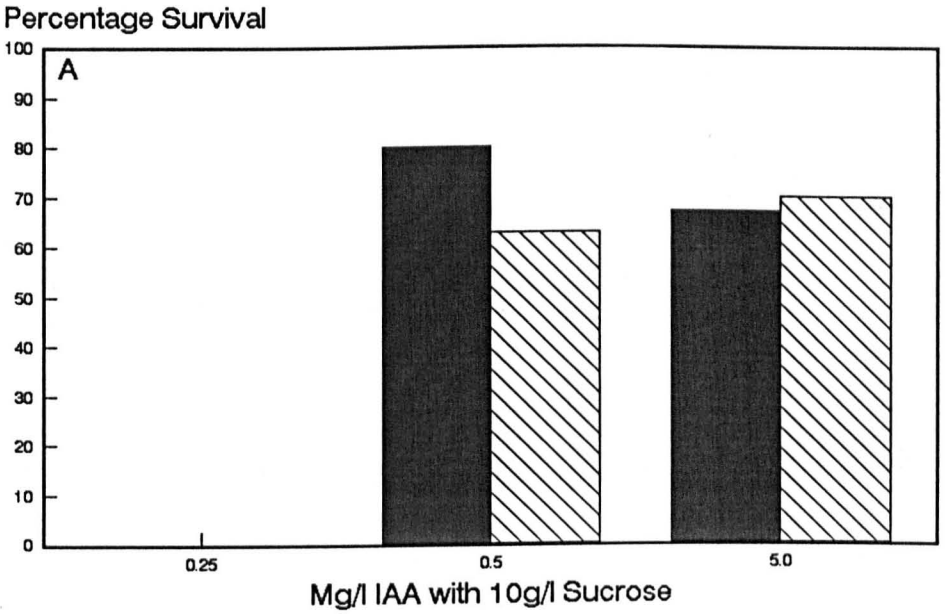
6.2 Results

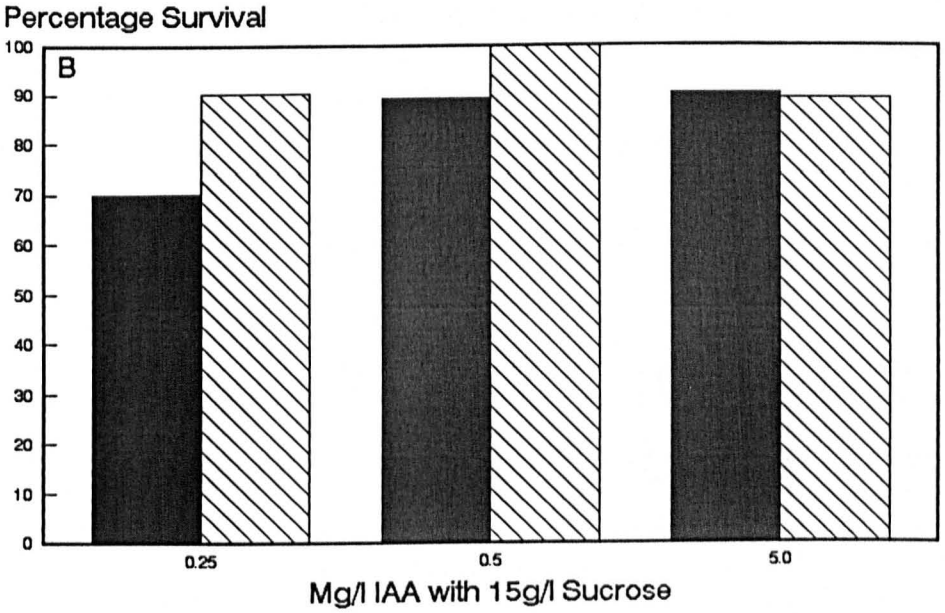
Effect of sucrose supply during culture on plantlet acclimatization

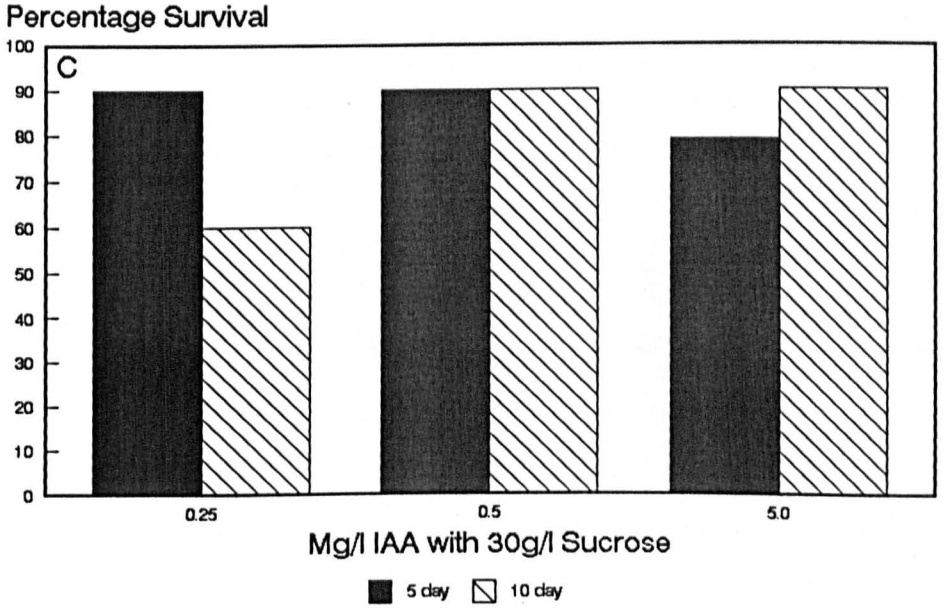
There are consistently higher survival percentages where sucrose is present in the elongation medium (Figures 6.1a-c). Survival of plantlets is improved in treatments where low sucrose in the initiation medium is supplemented by providing sucrose in the elongation medium (Figures 6.1a, b, 6.2a & b). Varying auxin concentration, and exposure time to the treatment have a significant effect, ANOVA ($P<0.05$) on the numbers of roots initiated. In treatments with low auxin and short exposure time, reduced plantlet growth and survival figures are improved by the addition of sucrose to the elongation medium. However when both auxin and sucrose concentration are low (Figure 6.1a) no improvement from the later supply of sucrose is evident. In the majority of treatments where sucrose is added to the root elongation medium, plantlet fresh weights are significantly ($P<0.001$) increased (Table 6.1), and a well branched root system is developed (Figure 6.3 and 6.6).

An increase in root growth produces more balanced root/shoot ratios (Figure 6.3 a-c and Table 6.1). The majority of treatments produce balanced plants with good root/shoot ratios. In two treatments, IAA 0.25 mg.l^{-1} with sucrose at 30g.l^{-1} (Figure

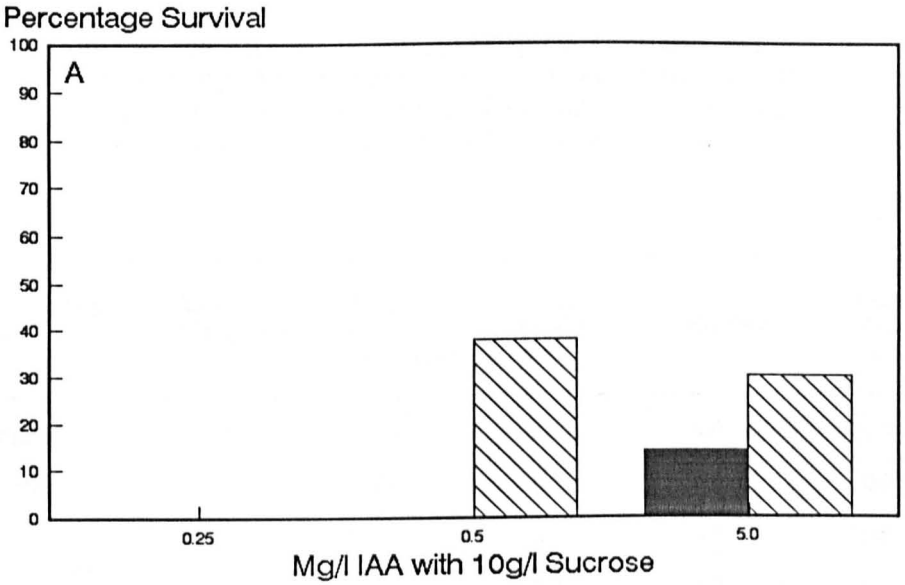
Figures 6.1 a-c: Percentage survival of micropropagated tomato plantlets after 7 days acclimatization at 40% r.h., after either 5 or 10 days culture with varying concentrations of IAA and sucrose at 10 (A), 15 (B), or 30 (C) $g.l^{-1}$, followed by root elongation on sucrose supplemented medium.

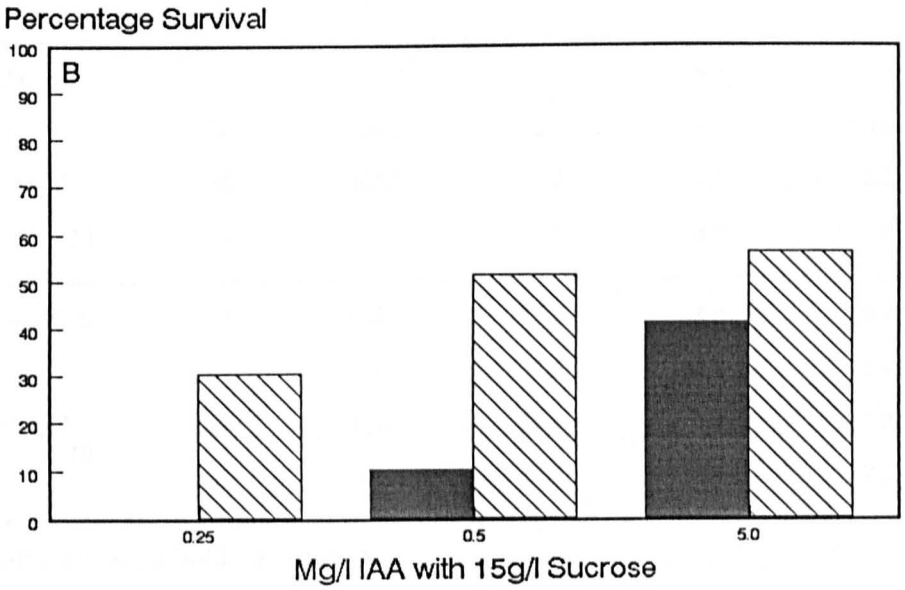

Percentage Survival
A
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 10g/l Sucrose


Percentage Survival
B
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 15g/l Sucrose


Percentage Survival
C
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 30g/l Sucrose
5 day
10 day

Figures 6.2 a-c: Percentage survival of micropropagated tomato plantlets after 7 days acclimatization at 40% r. h., after either 5 or 10 days culture with varying concentrations of IAA and sucrose at 10 (A), 15 (B) or 30 (C) $g.l^{-1}$, followed by root elongation on sucrose free medium.


Percentage Survival
A
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 10g/l Sucrose


Percentage Survival
B
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 15g/l Sucrose

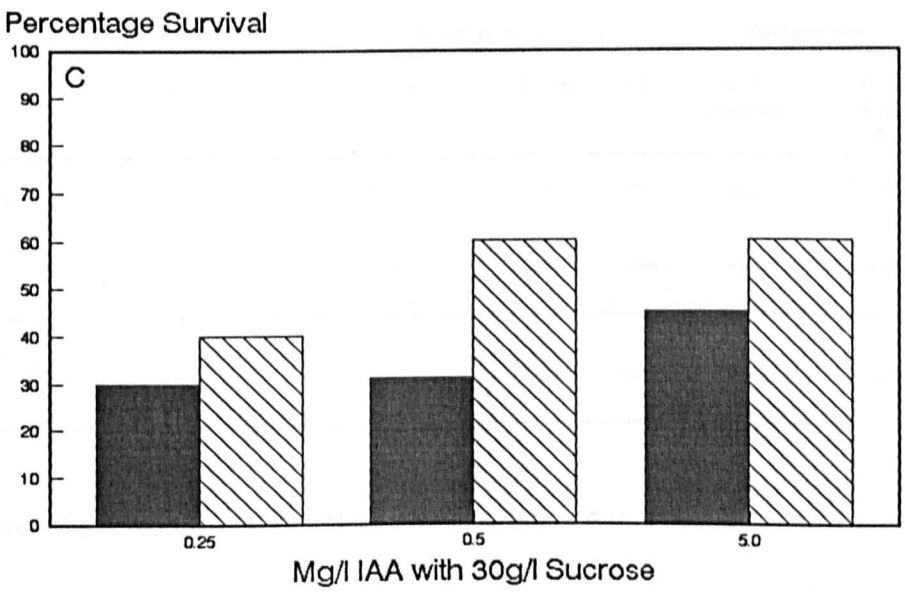

Percentage Survival
C
100
90
80
70
60
50
40
30
20
10
0
0.25
0.5
5.0
Mg/l IAA with 30g/l Sucrose
5 day
10 day

Table 6.1 Growth and survival of tomato plantlets after root initiation with varying concentrations of auxin and root elongation on medium with(+S) or without (-S) Sucrose. Followed by acclimatization for 7 days at 40% relative humidity.

Root Initiation Media	Days	Root Elongation Media	Root Fresh Weight(mg)	Shoot Height (cm)	Leaf Number	Shoot/ Root Ratio	% Plantlet Survival
1AA 0.5mgl^{-1}	5	+S	156.4	5.1	4.0	5.7	80.0
Sucrose 10gl^{-1}		-S	0.0	0.0	0.0	0.0	0.0
	10	+S	111.0	8.2	6.0	10.9	64.0
		-S	69.9	5.2	5.0	9.7	36.0
1AA O.5mgl^{-1}	5	+S	118.2	8.6	6.0	10.1	89.0
		-S	38.2	3.1	3.0	9.6	50.0
Sucrose 15gl^{-1}		+S	164.3	8.5	6.0	5.5	100.0
	10	-S	0.0	3.5	4.0	0.0	10.0
1AA 0.5mgl^{-1}	5	+S	306.7	15.2	8.0	9.9	90.0
		-S	69.7	4.6	3.0	6.9	30.0
Sucrose 30gl^{-1}		+S	317.2	12.0	8.0	7.9	90.0
	10	-S	162.6	9.5	5.0	7.2	60.0

SUMMARY OF ANALYSIS OF VARIANCE

Variate	Main Effects				Interactions		
	Auxin	Sucrose	Time	±S	Auxin Sucrose ±S	Auxin Time ±S	Time Sucrose ±S
Leaf Number	*	*	**	**	NS	NS	***
Root Fresh Weight	***	***	***	***	***	**	*
Shoot/Root Ratio	***	***	NS	***	*	NS	*

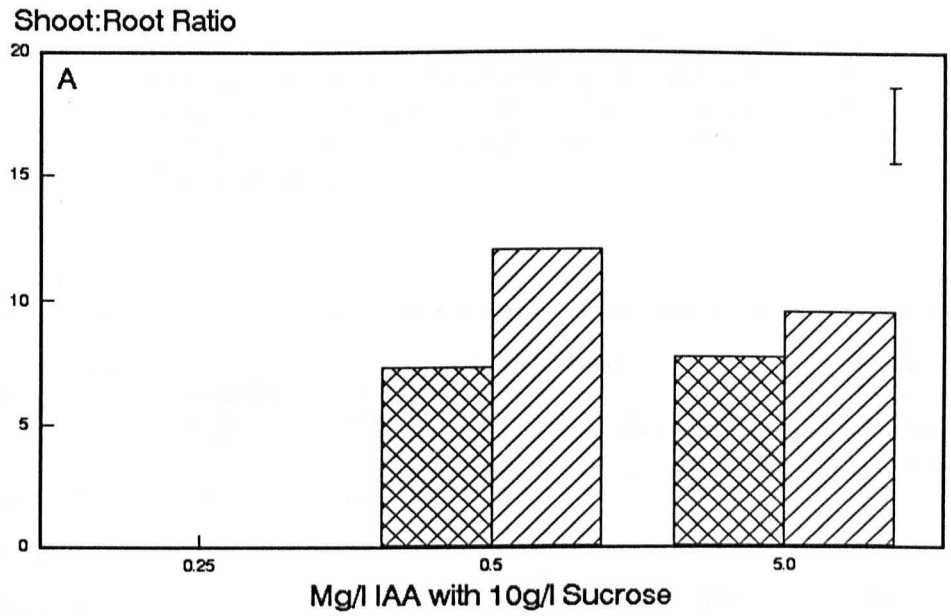
F Values Significance: * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ n=10

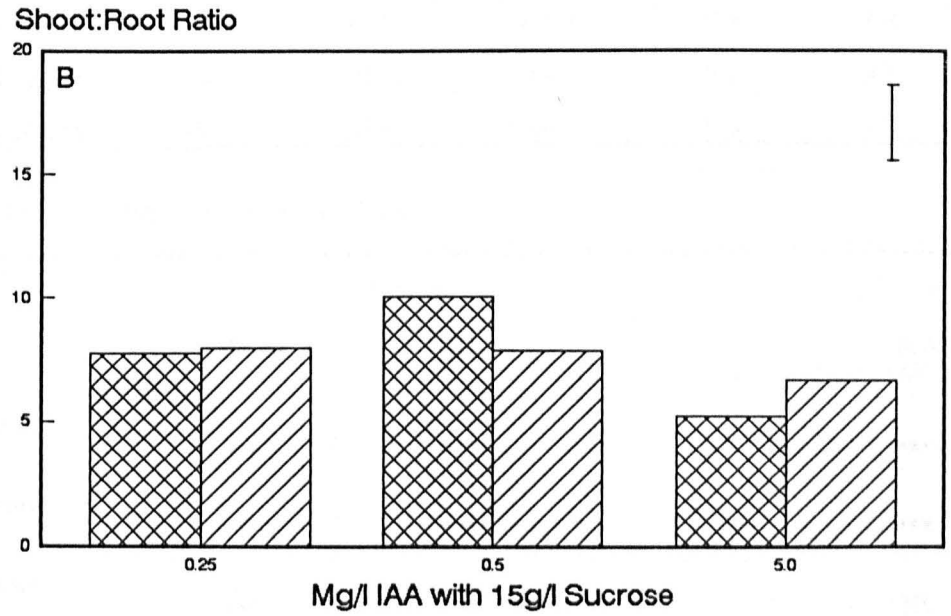
6.3c) and IAA 0.5 $mg.l^{-1}$ with sucrose at 10 $g.l^{-1}$ (Figure 6.1a), an increase in shoot/root ratio appears to result in lower survival figures.

Effects of varying the IAA concentration or exposure time to the initiation medium, on plantlet growth and survival seems to be compensated for if sucrose is then supplied during the root elongation stage (Figures 6.3 a-c and 6.4 a-c).

In treatments containing standard MS with 30 $g.l^{-1}$ sucrose, higher auxin concentration increases the number of root initials formed (Figure 6.5a). Longer exposure time only increases the number of initials formed at the two higher concentrations, but this is not reflected by corresponding increases in fresh weights (Figure 6.5b). The difference in root number between the 0.5 and 5.0 $mg.l^{-1}$ treatments does not appear to be significant (Figure 6.5a). Root and shoot fresh weights are consistently higher where sucrose is provided in the elongation medium (Table 6.2). Optimum shoot growth occurs with 0.5 $mg.l^{-1}$ IAA and a 5 day exposure time while increasing exposure time to 10 days results in significantly ($P<0.05$) lower shoot fresh weights (Figure 6.5c). In treatments where sucrose was not provided in the elongation medium, limited improvement in plantlet fresh weight occurred with a 10 day exposure time. Generally treatments with low sucrose and low auxin in the initiation media (Figures 6.4 a & b) produced plants with a less developed root system and a high shoot/root ratio and this appears to result in plants less able to withstand acclimatization. Low auxin and sucrose (IAA 0.25 $mg.l^{-1}$ with sucrose 10 $g.l^{-1}$ and IAA 0.25 $mg.l^{-1}$ with sucrose 15 $g.l^{-1}$, Figures 6.3 a & b) produced no viable plants.

Figures 6.3 a-c: Shoot/root ratios of micropropagated tomato plantlets acclimatized at 40% r. h. for 7 days, after culture with varying concentrations of IAA, and sucrose at 10 (A), 15 (B) or 30 (C) $g.l^{-1}$ for 5 or 10 days, followed by root elongation on medium containing sucrose. n = 10, vertical bar indicates LSD at 5%


Shoot:Root Ratio
A
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 10g/l Sucrose


Shoot:Root Ratio
B
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 15g/l Sucrose

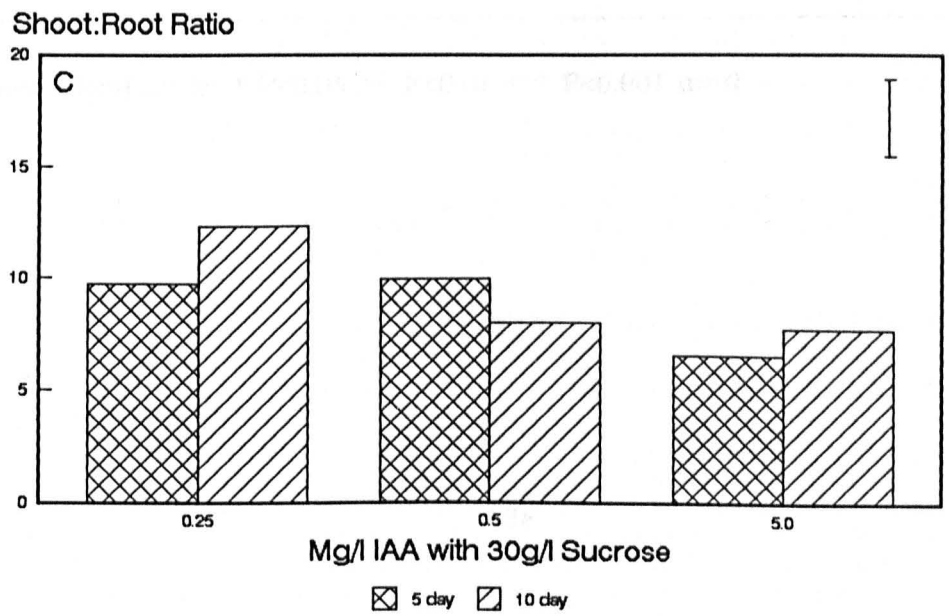

Shoot:Root Ratio
C
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 30g/l Sucrose
5 day
10 day

Table 6.2 Growth and survival of tomato plantlets after root initiation on varying concentrations of auxin and root elongation on medium with (+S) or without (-S) sucrose. Followed by 7 days acclimatization at 40% relative humidity.

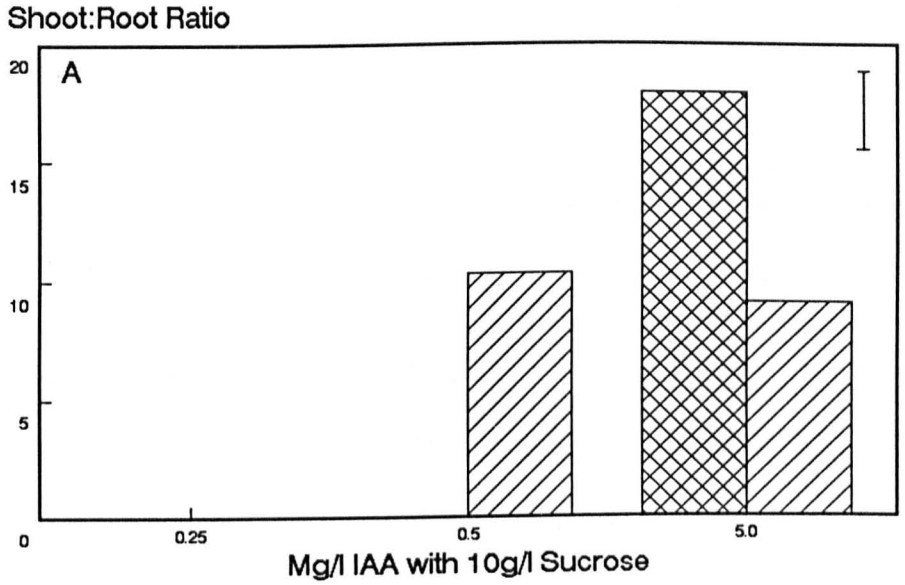
Root Initiation Media	Days	Root Elongation Media	Fresh Weights Mg Root	Stem	Leaf	Shoot/ Root Ratio	%Plantlet Survival
IAA 5.0mgl^{-1}	5	+S	203.2	437.5	947.1	7.7	90.0
Sucrose 30gl^{-1}	5	-S	59.5	193.6	230.4	8.4	20.0
IAA 0.5mgl^{-1}	5	+S	306.7	1068.3	1980.6	9.9	90.0
Sucrose 30gl^{-1}	5	-S	69.7	115.5	363.8	6.9	30.0
IAA 0.25mgl^{-1}	5	+S	102.5	364.4	637.0	9.7	90.0
Sucrose 30gl^{-1}	5	-S	27.4	64.4	77.5	7.0	30.0

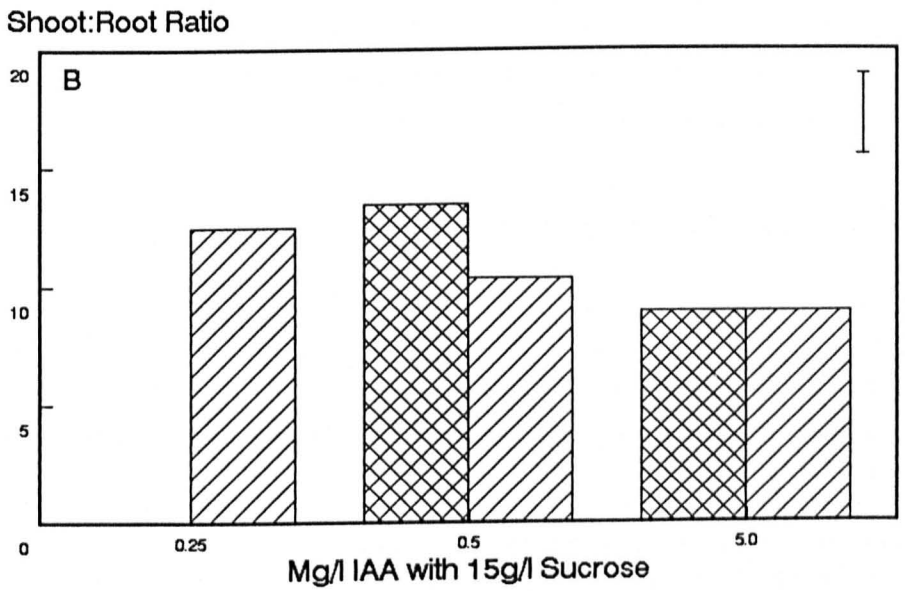
SUMMARY OF ANALYSIS OF VARIANCE

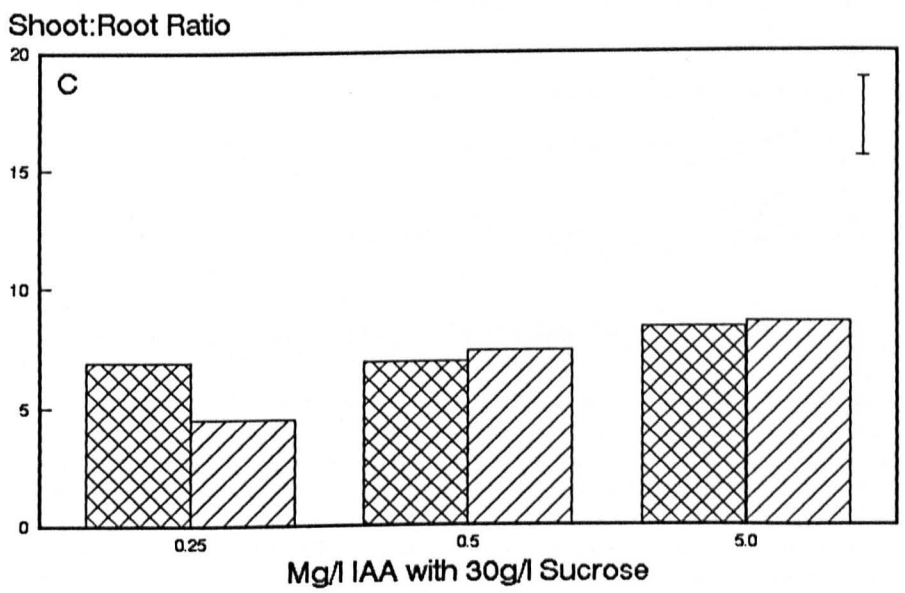
Variate	Main Effects		Interactions
	Auxin	±S	Auxin ±S
Root Fresh Weight	***	***	***
Shoot Fresh Weight	***	***	***
Shoot/Root Ratio	***	***	NS

F Values. Significance: * P<0.05 ** P<0.01 *** P<0.001 n=10

Figures 6.4 a-c: Shoot/root ratios of micropropagated tomato plantlets acclimatized at 40% r. h. for 7 days, after culture with varying concentrations of IAA, and sucrose at 10 (A), 15 (B) and 30 $g.l^{-1}$ (C) for 5 or 10 days, before root elongation on medium without sucrose. n = 10, vertical bar indicates LSD at 5%


Shoot:Root Ratio
A
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 10g/l Sucrose


Shoot:Root Ratio
B
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 15g/l Sucrose


Shoot:Root Ratio
C
20
15
10
5
0
0.25
0.5
5.0
Mg/l IAA with 30g/l Sucrose
5 day
10 day

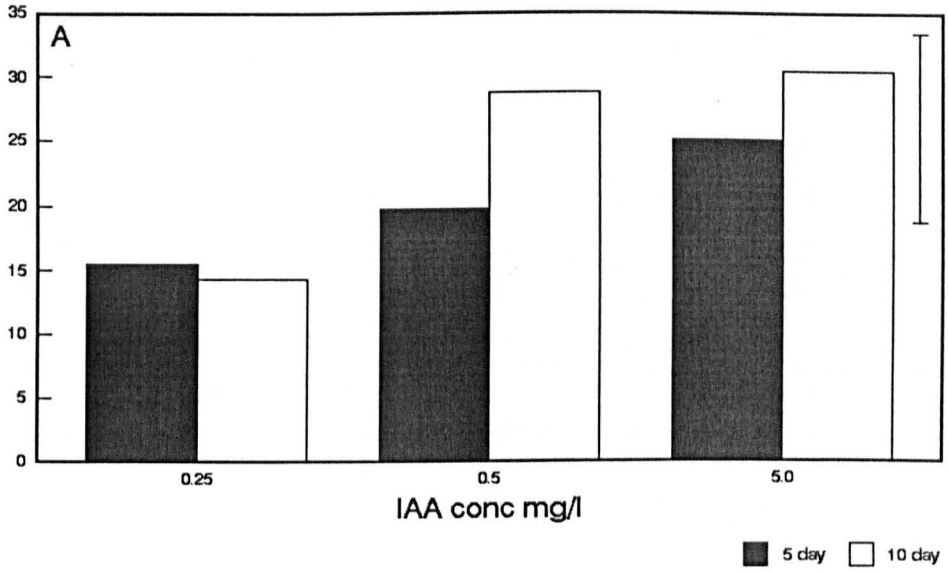
Figures 6.5 a-c: Growth of plantlets produced on standard MS medium with varying IAA concentration for either 5 or 10 days, followed by root elongation on medium with or without sucrose. n = 10, vertical bar indicates LSD at 5%

A) Number of root initials

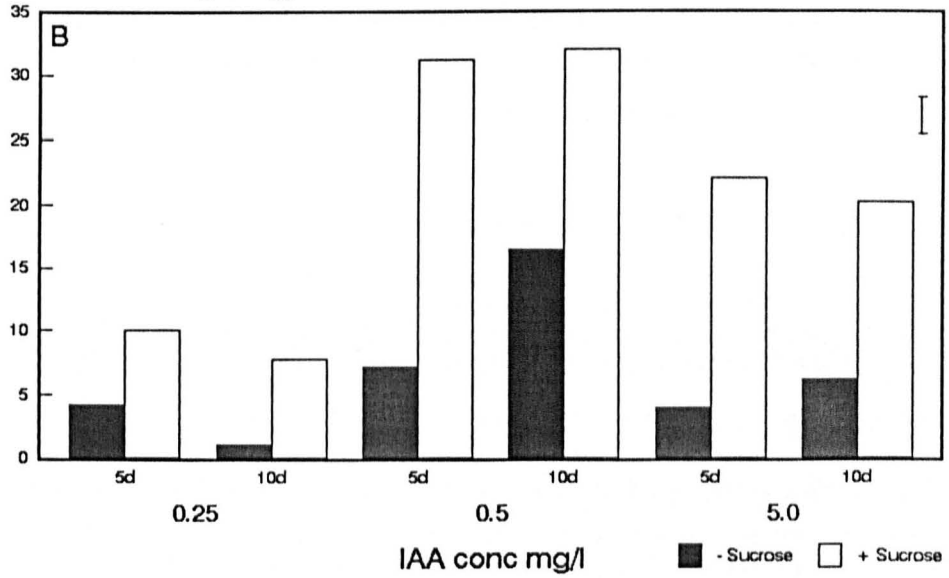
B) Root fresh weight ($g.l^{-1}$)

C) Shoot fresh weight ($g.l^{-1}$)

Root Number



Root fresh weights (g)



Shoot fresh weight (g)

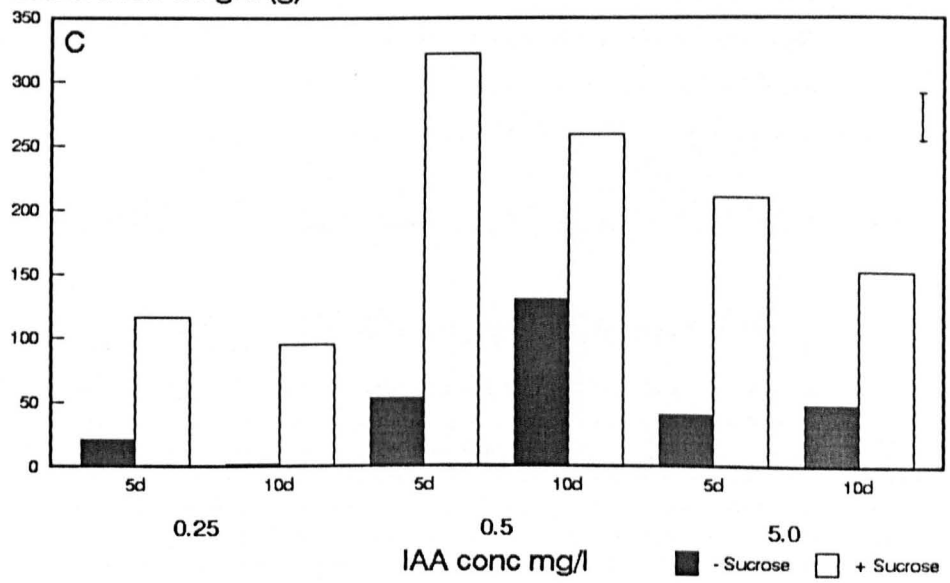
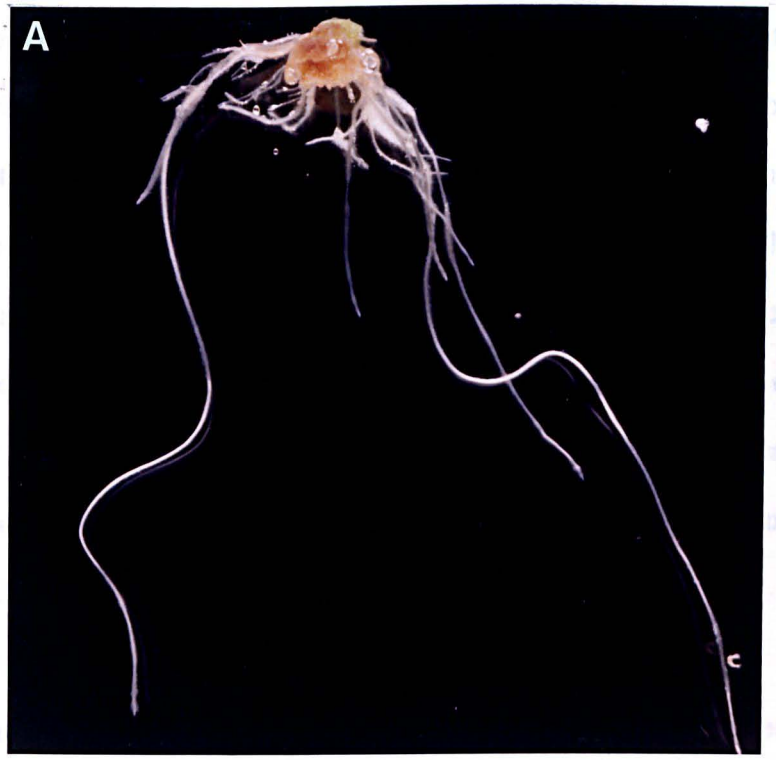


Figure 6.6 a&b: Root development in micropropagated *L. esculentum* following culture on root elongation medium without (A), or with (B) sucrose. The root system developed in the presence of sucrose is more extensive with increased lateral root development. Mag x 4.4


A


B

In treatments where sucrose was not provided in the elongation medium the effect of varying auxin concentration and exposure time to the initiation medium was significantly increased. Survival of plantlets is improved by increasing auxin concentration (Figures 6.2 a-c), with the exception of the 10 $g.l^{-1}$ sucrose treatments. Similar increases in root numbers and shoot growth result from longer exposure time to the initiation medium, at each auxin concentration ($P < 0.05$) (Figures 6.2 a-c). The optimum auxin concentration based on plantlet survival figures is 0.5 $mg.l^{-1}$. Survival during acclimatization is further improved with 30 $g.l^{-1}$ sucrose. Where a high shoot/root ratio is produced (Figures 6.4 a & b), the survival percentage of plantlets is reduced to 30% and less.

In treatments resulting in the lowest survival rates (Figures 6.2 a & b) the majority of growth occurred in the shoot, resulting in higher shoot weights and larger shoot/root ratios (Figures 6.4 a & b), there being only limited root growth and subsequent root development during the elongation stage being reduced. This may result in lower capacity for ion and water uptake through the acclimatization stage. Generally, higher sucrose levels at either stage of development produced plants with higher root weights which significantly ($P < 0.001$) improved the root/shoot ratio. A comparison of the effect of varying auxin concentration on root initiation suggests that increasing auxin concentration does induce the formation of more root primordia (Figure 6.5a). However, an increase from 0.5 to 5.0 $mg.l^{-1}$, did not produce significantly different primordia numbers. The variation in subsequent root growth and development results from interactions between auxin/sucrose concentration and exposure time. Optimum root weights (Figure 6.5b) and a well developed root system are produced by longer exposure to lower concentration of auxin. From general observations on root morphology, it appears

that longer exposure to higher auxin concentration increases root number but produces thick, short unbranched roots.

6.3 Discussion

In treatments where MS with a standard concentration of sucrose added to the elongation medium was used, significantly ($P< 0.001$) higher plantlet fresh weights and improved root/shoot ratios resulted. Increased plantlet survival percentages were achieved during acclimatization to lower relative humidities.

There appears to be no consistent trend to indicate that increasing auxin concentration results in improved survival due to increased root growth or development. There seems to be little effect over the range of auxin used in these studies, of either varying IAA concentration from 0.25 - 5.0 $mg.l^{-1}$, or from increasing the time for which shoots are exposed to the medium. Any initial variation in growth or survival can be compensated for if sucrose is provided at 30 $g.l^{-1}$ during the elongation stage of rooting. If sucrose is not provided at this stage, then the pre-treatment becomes more important and plantlet growth and survival improves as the sucrose concentration is increased from 10 - 30 $g.l^{-1}$. Additionally increased exposure time to the initiation medium produces beneficial responses, which result in higher survival percentages *ex vitro*.

Sucrose appears to be a critical factor in producing plantlets which are able to undergo successful acclimatization. Prolonged low sucrose supply produces very few viable plantlets. These results do not agree with other studies which have shown that lower sucrose concentrations in the medium stimulate improved rates of photosynthesis which enables plantlets to acclimatize more successfully (Short

et al., 1987; Grout, 1988). In the present study, it appears that sucrose concentration is important in producing a well balanced plant, with an extensive root system, which can then undergo successful transfer to conditions of lower relative humidity. Sucrose seems to have a greater effect on root/shoot ratio and root morphology, although in some studies its importance in root initiation has been demonstrated (Lane, 1978; Zimmerman, 1983). Zimmerman (1983) reports lower survival percentages in apple plantlets rooted on low sucrose concentrations. Lovell *et al.* (1972) indicate that endogenous carbohydrates levels may affect root initiation, and if this is the case then sucrose supplied to *in vitro* shoots will be important since alternative sources of endogenous carbohydrate are probably reduced.

However, it has been reported that the chlorophyll content of *in vitro* leaves is affected by the concentration of carbohydrate supplied during culture (Pamplin and Chapman, 1975; Grout and Aston, 1978). Subsequent studies (Grout and Donkin, 1987; Grout, 1988) have shown that high concentrations of sucrose during culture result in reduced chlorophyll levels and low RuBPcarboxylase activity. In these studies the leaves formed *in vitro* senesce rapidly on transfer to *ex vitro* conditions and contribute little to the carbon balance. The concentrations at which sucrose becomes limiting in this way can range from 30 to 80 $g.l^{-1}$, depending on the species. It is possible that both sucrose and light supplied during the culture period subsequently affect photosynthetic activity (Rahman and Blake, 1988), and survival percentages during acclimatization. Achieving an optimum balance of these components seems to be important in ensuring adequate root and shoot growth *in vitro*, whilst not impeding subsequent growth through inhibition of photosynthesis. In tomato 30 $g.l^{-1}$ sucrose supplied at either the root initiation or

elongation stage does not appear to be detrimental to plantlet establishment and growth over the first 7 days following acclimatization. However it should be pointed out that no measurements of photosynthetic activity were taken in these experiments. Plant species may vary in their sensitivity to sucrose, and the effect on photosynthetic enzymes.

Alternatively, if the *in vitro* leaves act as storage organs, as proposed by Wardle *et al.* (1983) and redistribution of metabolites occurs following transfer, then plantlets with increased growth rates in culture should give higher survival percentages *ex vitro*. This appears to be the case in the present study, as plantlets with higher leaf numbers and increased growth in culture adapted more successfully to reduced humidity, although some *in vitro* leaves were lost. Basal application of synthetic IAA on cuttings of *Coleus* has been shown to be inhibitory to vascular differentiation in an acropetal direction (Aloni, 1976). To some extent this imitates the case in the whole plant where auxin produced in young leaves induces vascular differentiation basipetally, but reduces vessel formation acropetally. IAA is known to have inhibitory effects on vascular differentiation in the shoot. Leaf primordia or alternative auxin sources affect the formation of continuous vascular strands by preventing newly induced vessels from joining up with strands already supplied with auxin. In rooting cuttings, auxin from the developing shoot has a promotery effect on the newly forming root system. In rooting microcuttings this is seldom taken into account and prolonged exposure to exogenous IAA seems to be inhibitory to extensive root development, and may also have detrimental effect on shoot vascular development, leading to reduced growth rates. The short, unbranched roots produced by long exposure to higher IAA concentrations may well be due to acropetal inhibition. The

mechanism of this inhibition may operate as proposed by Aloni (1976) through interruption of the basipetal action or reduced production by the shoot due to supply from the basal application.

The results of this work suggests that the production of plantlets with balanced root/shoot ratios produce highest survival rates through transfer to *ex vitro* conditions. This can be achieved with a regime incorporating an initial exposure to a high auxin concentration, followed by culture on auxin free medium. Media supplemented with sucrose in the final stages of culture improves plantlet growth and survival.

Chapter :7 Assessment of vascular development

7.1 Introduction

The initiation of roots on cultured shoots under *in vitro* conditions seems to be the most effective and cost efficient method of producing plantlets which are capable of undergoing acclimatization. This procedure is used by the majority of workers in plant tissue culture, and many commercial producers. However problems do exist with this method of rooting and *ex vitro* rooting has been proposed as an alternative (Maene and Debergh, 1983).

The low acclimatization success achieved with many micropropagated plant species, has partly been attributed to discontinuity in the shoot/root vascular junction (Grout and Aston, 1977 ; Grout and Millam, 1985), and the development of abnormal root structure (Maene and Debergh, 1983; Donnelly *et al.*, 1984). It has been reported that root formed under culture conditions (Maene and Debergh, 1983; 1987) resemble those normally formed under conditions of poor aeration, with large areas of aerenchymatous tissue developing in the root cortex. It has been suggested that this tissue breaks down during acclimatization, leaving the shoot with a non-functional root system. This would increase the water stress on the shoot, which is already under stress from a reduced control over water loss from the leaves (Sutter, 1985; Sutter *et al.*, 1988, Chapter 8 of this thesis). This evidence appears to indicate a general inadequacy in the vascular system formed *in vitro*, where shoots are often rooted through a basal callus, which inevitably develops following a prolonged rooting period, or where inappropriate concentrations of growth regulators are used. Callus tissue tends to be composed of large thin walled parenchymatous cells, which are loosely packed with an

unpredictable pattern or organisation (Dodds and Roberts, 1985).

The initiation of vascular tissue from callus cells has been extensively studied in graft unions (Jeffree *et al.,* 1987; Yeoman, 1984; Jeffree and Yeoman, 1983; Barnett and Weatherhead, 1988) and conventional cuttings (Jackson, 1986), providing a useful comparison for studies on root initiation *in vitro.*

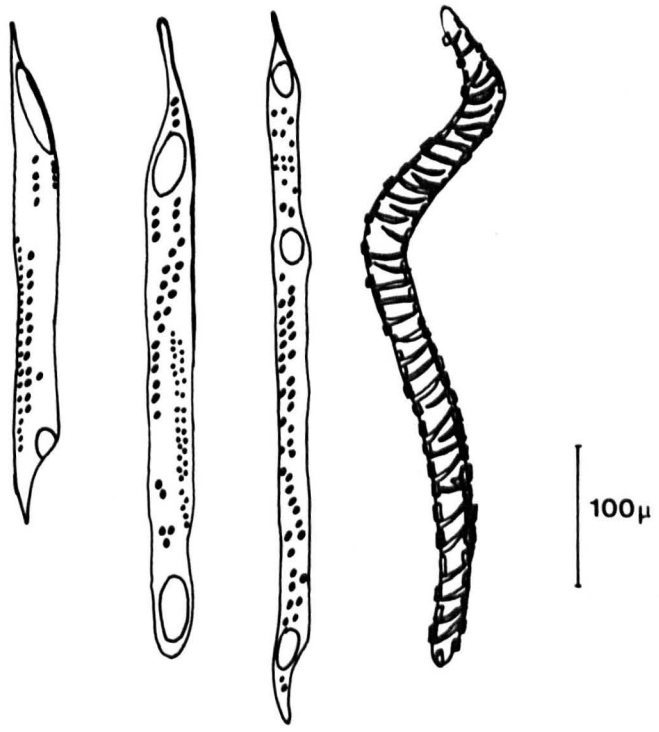
Vascular differentiation

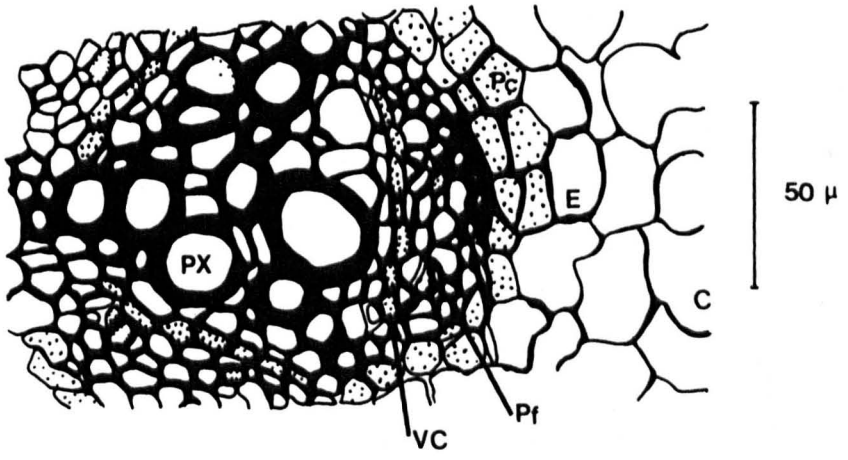
Vascular differentiation is a complex morphological process. The vascular system is formed continuously as the plant grows, from apical and lateral meristems. It is made up of two types of conducting tissue and several cell types; the xylem conducts water and soil absorbed nutrients flowing in an acropetal direction, and the phloem, which is composed of sieve tubes connected by a series of sieve plates, forms a network of phloem anastomoses (Aloni and Sachs, 1973). The phloem transports photosynthates and other organic compounds in a predominantly basipetal direction. The xylem is made up of tracheary cells which form the major conducting elements. The cells have secondary thickening and are of two types (Figure 7.1); tracheids, long non-perforated plates cells with bordered pits; and vessels, long continuous tubes made up of many vessel elements joined end to end by perforated plates (Esau, 1960). Although phloem is nearly always differentiated first, xylem is easily induced in tissue culture, and readily identifiable by simple staining and light microscopy (Figure 7.1).

In tissue culture systems xylem formation can be induced with growth regulators, auxin being the most important of these (Minocha and Halperin 1974; Bruck and Paolillo, 1984), but cytokinins, gibberellins and a carbohydrate source are also

Figure 7.1: Details of structure and development of primary and secondary xylem, cell types (A-D) and transverse section through alfalfa (*Medicago sativa*) root (taken from Esau 1960)

A-D = Vessel and tracheid cell types.
C= cortex, E= endodermis, Pc= pericycle, Pf= primary phloem, Vc= vascular cambium, Px= primary xylem.


100µ
A
B
C
D


Pc
E
PX
C
50 µ
VC
Pf

required (Aloni, 1980). Comer and Lenardo (1981) found that *Coleus* differentiated sieve elements in response to IAA and sucrose, but increased sucrose supply enhanced phloem formation. The interaction between exogenously supplied chemicals and the endogenous ones is not really understood at this stage, although it is certain that interaction do occur (Aloni, 1988). Polar auxin flow from the developing shoot stimulates vascular differentiation along auxin gradients which form between cells (Sachs, 1986), thus a continuous vascular system develops. In culture this may be disrupted by exogenously supplied auxin. Prolonged supply of auxin to the shoot base, or basal callus cells may have a different effect from the basipetal system in whole plants. Only a few studies have been made on the uptake, translocation and subsequent action of exogenously applied growth regulators on callus or shoot cultures *in vitro* (Nordstrom and Eliasson, 1986; Biondi, Canciani and Bagni, 1984). Both these studies looked at cytokinin uptake, and concluded that BAP is taken up throughout the culture period, but tends to accumulate in the basal part of apple shoots, whereas BA was rapidly absorbed and translocated to the upper part of elm shoots where it was degraded. In both cases it was suggested that formation of adventitious buds was stimulated. The formation of new roots has been studied in conventional cuttings, and this forms the main comparison for the work on *in vitro* microcuttings presented in this chapter.

It would appear that roots are not essential to vascular differentiation in tissue culture systems, however they normally perform two main functions; firstly they orientate the direction and pattern of vascular differentiation from stem base to root tip, by acting as a strong sink for auxin flow. Secondly the root apices are centres of cytokinin production (Aloni, 1987). Removal of roots in *Helianthus* sp. was shown to decrease secondary xylem formation (Sachs, 1984).

Pattern differentiation

The pattern of vascular formation, and in particular xylem is determined by fluxes of growth regulators moving through the tissue, usually from the cambial regions outward (Sachs, 1981). Vascular tissues are organised in longitudinal strands and form a predictable pattern for each species (Aloni, 1987). The xylem and phloem are differentiated to either side of the vascular cambium, which forms a band between the two tissue types. The number of centres of differentiation will determine the type of pattern which forms e.g. diarch, triarch or tetrarch. Tomato root has a diarch vascular arrangement (Byrne *et al.*, 1982). In whole plants the pattern is closely correlated with leaf development, and arises from basipetal flow of auxin, followed by horizontal diffusion through the tissue.

Studies on wound tissue have shown that once vascular strands have been formed, they provide the fastest route for IAA transport (Jacobs and Short, 1986). The elements then enlarge and auxin diffuses out from these elements. Vascular regeneration in cuttings and excised organs will be affected by the inherent polarity of the parent material, and may develop in association with it.

Severing vascular continuity disrupts internal growth regulator flow and leakage occurs around the wound area. Wounding induces regenerative short and narrow vessels. The repair vessels are initiated from wounded tissue via growth regulator and sucrose gradients, and are usually formed through intermediate parenchyma cells (callus). Although parenchyma cells appear to be uniform, the presence of structural, physiological and biochemical gradients has been demonstrated in *Nicotiana*, these gradients have varying potential for cytodifferentiation (Jacobs, 1984). Schulz (1987) looked at regeneration of sieve elements after the complete

severance of roots, and found effective transport resumed after 3 days. However all cells that resemble tracheary elements in tissue culture may not be functional in water conduction. The resumption of complete function may occur over a gradual period following wounding. Aloni and Jacobs (1977 a & b), report the initial formation of sieve tubes and vessels 3 days after wounding which, increased over 4 days. The final size of primary phloem fibres was determined by the ratio of IAA:gibberellic acid (GA_3). Higher concentrations of IAA produced short fibres with thick walls, a primary wounding response, whilst high GA_3 produced the opposite effect. Auxin has been shown to induce phloem in the absence of a root system. Any regulatory effect of cytokinin from the root apices is obviously absent in cuttings and cultured shoots. Formation of vessels and elements in a complete ring arrangement illustrate auxin flux in a circular direction and possible accumulation in parenchyma tissue (Aloni, 1988).

The activity of exogenously applied auxin will also influence the differentiation of vascular elements. Variations in IAA and IAA-oxidase levels have been detected in the basal part of chestnut (*Castanea sativa* Mill.) stems during the first days of root initiation (Mato and Vieitez, 1986). An increase in basal IAA was complemented by a decrease in the IAA-oxidase level during rooting of cultured shoots, pre-treated with IBA. The results indicated that the exogenous IBA influenced the endogenous IAA concentration. Similar results have been reported in tomato stem cuttings (Aung *et al.*, 1975), where quantitative changes in root promoting substances were recorded over a 5 day period. Root primordia were identified in 10 day old stem cuttings. The root promoting substances described by Aung *et al.*, may be IAA conjugates. These amide and ester conjugates seem to protect IAA from degradation by peroxidases, and may provide a slow release

source of IAA for culture (Pence and Caruso, 1984). As work on vascular formation in cultured shoots is limited, studies on callus cultures have provided excellent information on early events in xylem formation.

Callus xylogenesis

Xylogenesis *in vitro* has been identified under the following stages; cell enlargement, secondary wall deposition and lignification, wall lysis and cell autolysis (Roberts, 1976). Secondary wall deposition and lignification are early features of xylem formation, followed by primary wall and protoplast hydrolysis.

Primary xylem differentiates from procambial tissue in shoots and whole plants (Esau 1960). Cambial cells have the ability to differentiate and are switched into vascular differentiation by auxins, cytokinins, gibberellins and other secondary substances including calcium, which is important in cell wall metabolism and the biosynthesis of cellulose and callose (Delmer *et al.*, 1984), polyamines and ethylene. In stem cuttings these substances are supplied via the xylem or phloem and diffuse laterally to the cambium. It has also been proposed (Goldsworthy and Rathorr, 1985) that this movement may be associated with small electrical potentials, which form gradients across the cells and tissues. It has already been stated that exogenous application of growth regulators in culture may disrupt endogenous activity. In callus cultures vascular tissue is often formed in close proximity to the culture medium. Externally supplied IAA and kinetin promoted surface formation of large numbers of xylem elements in lettuce callus (Miller and Roberts, 1984). Work on callus cultures (Dodds and Roberts, 1985) and graft unions (Jeffree *et al.*, 1987; Jeffree and Yeoman, 1983; Barnett and Weatherhead, 1988) indicate that vessel elements form via parenchyma cells. Dodds and Roberts (1985) studied

xylogenesis in cultured material which had been maintained *in vitro* for sometime, and had no original parent material to differentiate from, and hence no inherent polarity. Differentiation occurred as a series of randomly arranged bundles and strands of tracheary elements.

In more recent work, tracheary element formation has been shown to be related to the cell cycle. Initiation may be early in the cell cycle, as is the case in *Zinnia elegans* cultures (Fukuda and Komamine, 1982), or as in *Helianthus* cultures (Dodds, 1981) after a number of cell cycles.

As briefly mentioned in Chapter 3 of this thesis, the primary effect of auxin is on gene expression. Messenger RNA's encoding proteins specifically involved in secondary wall formation have been used to produce cDNA libraries, in an early attempt to identify the genes involved in the complex process of xylem formation. The use of biochemical markers has also helped to elucidate some aspects of xylogenesis. A number of specific gene products have now been identified during xylem differentiation. Bolwell (1985) describes changes in various enzymes involved in primary and secondary wall synthesis and formation. These auxin stimulated changes alter the ratios of cell wall constituents e.g. arabinose and xylose, which are important in lignification.

The aim of the work presented in the following chapter was to identify the vascular arrangement of tissue at the shoot/root junction and the root structure developed by tomato shoots rooted *in vitro*, using serial sections and light microscopy. The majority of the study focused on xylem formation as it was easy to identify any abnormality or discontinuity in the tissue formed in culture. The

vascular structure and arrangement of the *in vitro* plant was then used as a starting point to follow the development of this tissue through the various acclimatization regimes described in previous chapters. Changes observed in the structure are then discussed in relation to acclimatization responses and plantlet survival.

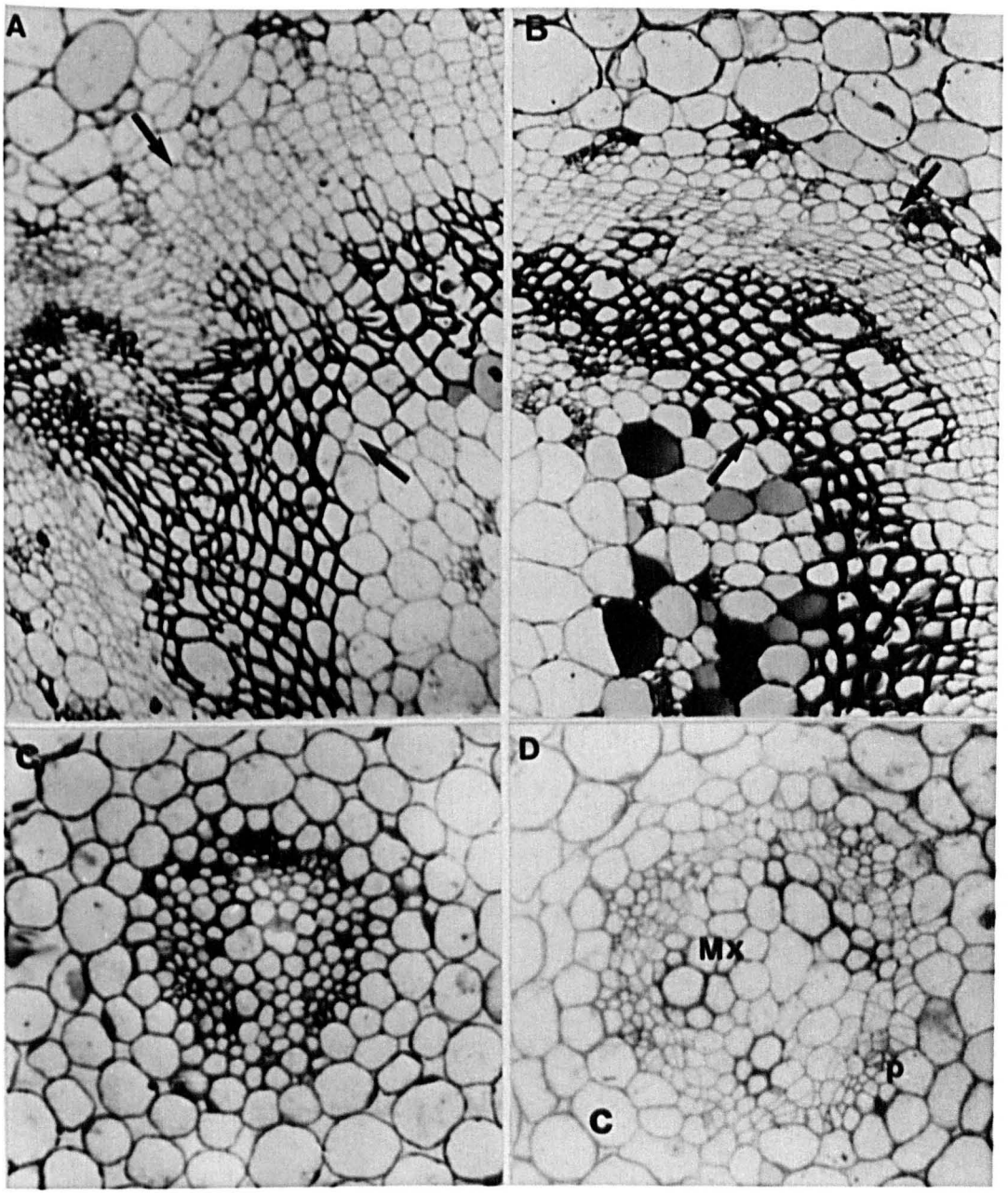
7.2 Results

Anatomy of the root/shoot junction in cultured and seedling *Lycopersicon esculentum*

The photomicrographs presented in the following section were taken from the shoot/root junction and root of both tomato seedlings and cultured plantlets. The vascular transition zone was divided transversly into four 2.5 mm portions. Following fixation and resin embedding, serial transverse sections were cut at 1 µm intervals through each 2.5 mm portion. The sections were then stained with a general stain (Aniline blue) to demonstrate xylem and other vascular structures, illustrated in Figure 7.1. The vascular tissue is easily distinguished in these sections. Further sections were taken from the root base and from the area 1 cm behind the root tip. Following staining the sections were mounted in distilled water and viewed under a light microscope (Nikon: Diaphot-TMD).

Cultured plantlets sampled following 20 days rooting *in vitro*, show extensive xylem development in the shoot/root junction (Figure 7.2 a & b). The vascular tissue is easily distinguished in both sections, and is indicated between the arrows in Figure 7.2. The development of a root primordium is seen in figure 7.2 a, this appears to originate in the cambial region. The xylem elements are well

Figure 7.2 A-D: Transverse serial sections taken through the shoot/root junction of *in vitro* tomato plantlets, rooted at 100% RH for 20 days. The section shows primary and secondary xylem (Mx) development from the stem base through to the root. A root primordium is visible in section A. Mag x 100
p= phloem, C= cortex.


A
B
C
D
Mx
C
p

developed and exhibit secondary sclariform and reticulate thickening. They form a continuous layer around the stem base. Phloem bundles are present on the outside of the cambium, indicated by heavier staining of the small closely packed cells, than the surrounding pericycle. The root base shows late metaxylem elements in the stele (Figure 7.2c) and phloem

fibre development in the adjacent area on the outside of the cambium. The endodermis is not clearly visible, as may have been expected in a section taken from this area of the root. The cortical cells appear to be irregular in shape and loosely packed, this may indicate callus parenchyma. In the section taken from the sub-apical zone (Figure 7.2d) the cells show no signs of distortion or collapse. Although these cells are also loosely packed the intercellular spaces are not comparable to those seen in arenchymatous development.

Vascular development in cultured plants following acclimatization

Following a seven day period of acclimatization at 80% relative humidity, the vascular development at the base of the stem, approximately 2.5 - 10 mm into the junction shows a well developed cambium differentiating phloem fibers (p) (Figure 7.3a). Extensive xylogenesis is evident forming continuous bands around the stem base; both late metaxylem (MX) and primary xylem can be identified. The band of xylem elements converge to form a central stele over a relatively short distance (the next 2.5mm) of the shoot/root junction. The structure in Figure 7.3 (b-d), show the cetral stele with primary and secondary xylem elements, with scalariform thickening (indicated by the arrows), the xylem elements appear to be

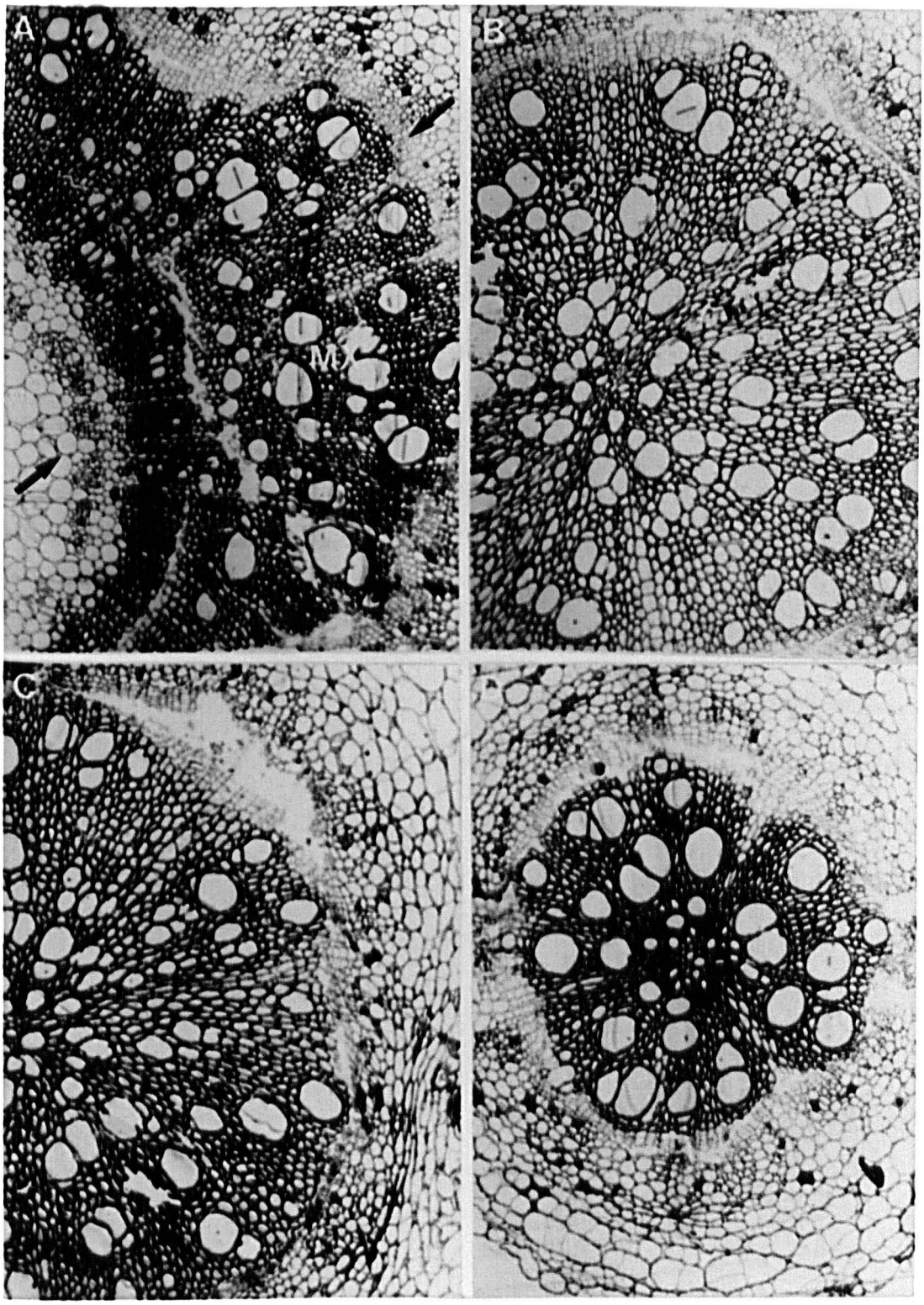
to the vascular tissue at the base of the stem. Metaxylem radiates out from the centre of the section. The central stele is surrounded by a well defined cambium. This can be identified from the small closely packed cells, undergoing periclinal

Figure 7.3 A-D: Transverse serial sections taken through the shoot/root junction of cultured tomato plantlets following acclimatization at 40% RH for 7 days.

A) Stem base showing interfascicular vascular development with primary and secondary (MX) xylem development

B & C) Gradual transition through the shoot/root junction showing development of the central stele

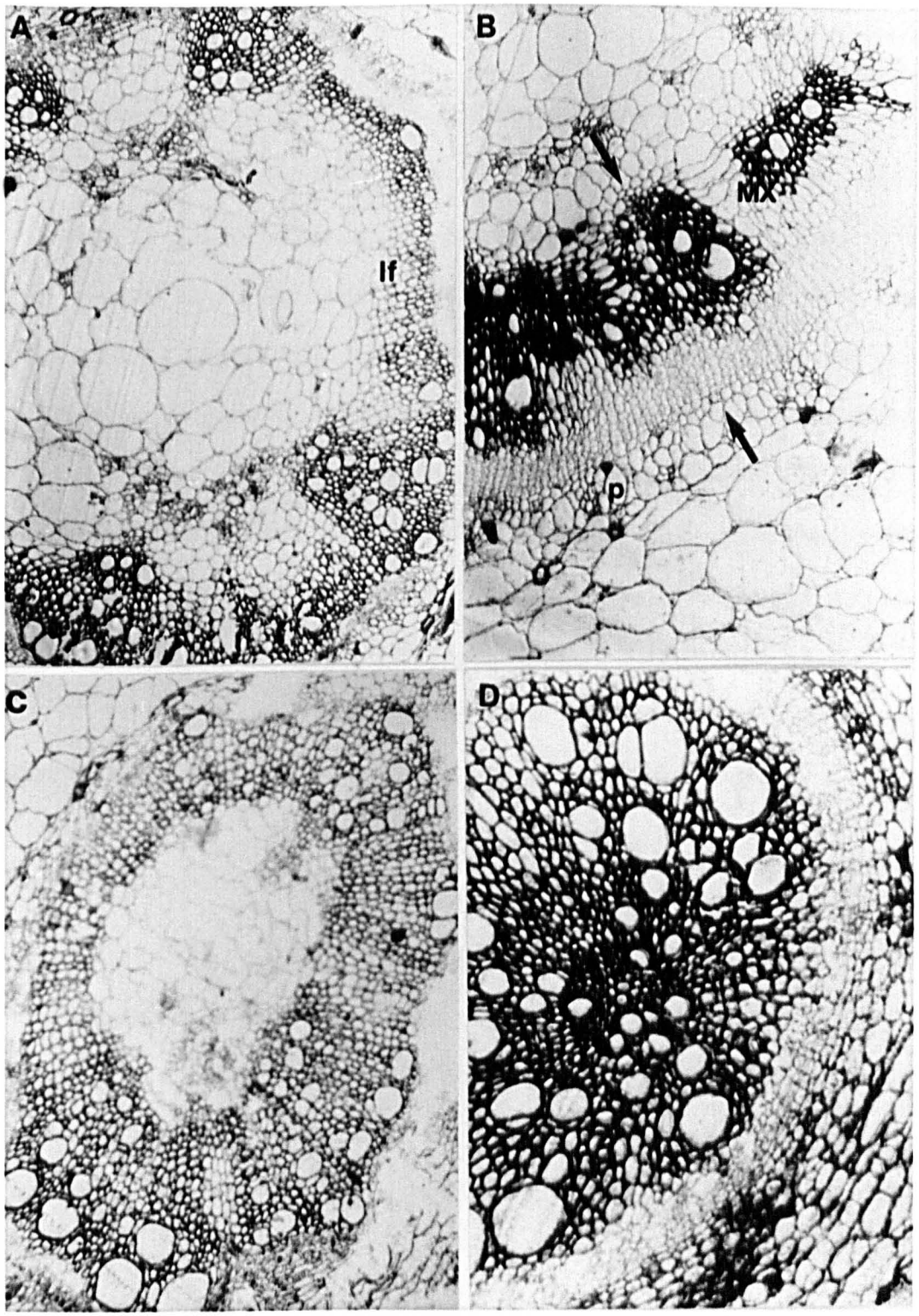
D) Section taken from the basal part of the shoot/root junction, showing the central stele surrounded by vascular cambium. Mag x 100


A
M
B
C

divisions. The sections taken from the basal region of the shoot/root junction (Figure 7.3c) are begining to exhibit some of the normal characteristics associated with normal seedling development. The cortical cells in the root base appear to dilate, and flare out as the tissue expands (Esau 1960).

The pattern of xylem and phloem formation in the cultured plantlets following 14 days acclimatization (Figure 7.4a-d), shows increased development from the structure observed in *in vitro* plants. Late phase metaxylem is present and the phloem is much more easily identified, as it is more differentiated. The outer pericycle shows a much more compact cell structure, and some signs of cell compression may be indicated, in certain areas. This can be caused by cambial derivatives which push the phloem fibers outwards and sideways causing damage to adjacent cells. Following a 14 day acclimatization period (Figure 7.4 a-d) at the lower humidity (40%), the vascular arrangement is similar to the previously described acclimatized plantlets. However there appears to be some pattern development in the early sections, and this may be coming from existing vascular tissue in the stem (Figure 7.4a). The pattern is not persistant and interfascicular vascular formation is demonstrated in the subsequent sections. This is similar to that seen in Figure 7.3. This interfascicular arrangement persists for a greater distance and the development of a central stele only begins at the root base (Figure 7.4d). The differentiation from the cambial tissues can be seen in Figure 7.4b, with proto and metaxylem differentiated toward the inside and phloem fibers on the outer side. The xylem development is easily identified in Figure 7.4c, but the vascular cambium and areas of phloem have been disrupted in preparation of the section.

Figure 7.4 A-D: Transverse sections taken through the shoot/root junction of cultured tomato planlets following acclimatization at 80% RH for 14 days. Showing interfascicular cambium development (if), phloem (p) and metaxylem (MX). Mag x 100


A
lf
B
MX
p
C
D

The basal part of the shoot/root junction (Figure 7.4d) shows a well developed central stele, surrounded by the cambial layer, undergoing cell division. The pericycle is beginning to differentiate a uniform layer of phellogen along the outer rim (Esau 1960).

Vascular development in *Lycopersicon esculentum* seedlings

In contrast the vascular development in the seedling plants exhibits the normal pattern found in *Lycopersicon* sp., as described by Esau (1960). This typical seedling structure is demonstrated in figures 7.5 and 7.6. Sections taken from seedlings exhibit a typical tetrarch arrangement of vascular tissue in the hypocotyl base (Figure 7.6a); gradually this changes into the diarch arrangement associated with the root. The photomicrographs in Figure 7.5 show half of the prepared section, the diarch structure is present at an early stage in the shoot/root junction in these sections. Late and early metaxylem elements can be seen in all these sections, and the cambium is well defined. Phloem has been produced towards the outside of the cambium. The shoot/root base (Figure 7.5d), shows heavy metaxylem formation, vascular cambium and phloem surrounded by a loosely packed cortex.

A complete picture of the normal transitional structure of the vascular tissue, from shoot to root is demonstrated by the series of photomicrographs in Figure 7.6. The transition from a multiarch vascular arrangement in the hypocotyl base, through to the diarch arrangement in the root can clearly be seen. All the cell types previously described can be identified, but the pattern formed is typical of *Lycopersicon* sp.

Figure 7.5 A-D: Transverse sections taken at 2.5 mm intervals through the shoot/root junction of tomato seedlings grown at 40% RH for 7 days. The gradual transition from the tetrarch vascular arrangement in the hypocotyl base (A) to the diarch arrangement in the root base (D) is demonstrated. The vascular cambium (VC) is clearly visible. Mag x 100

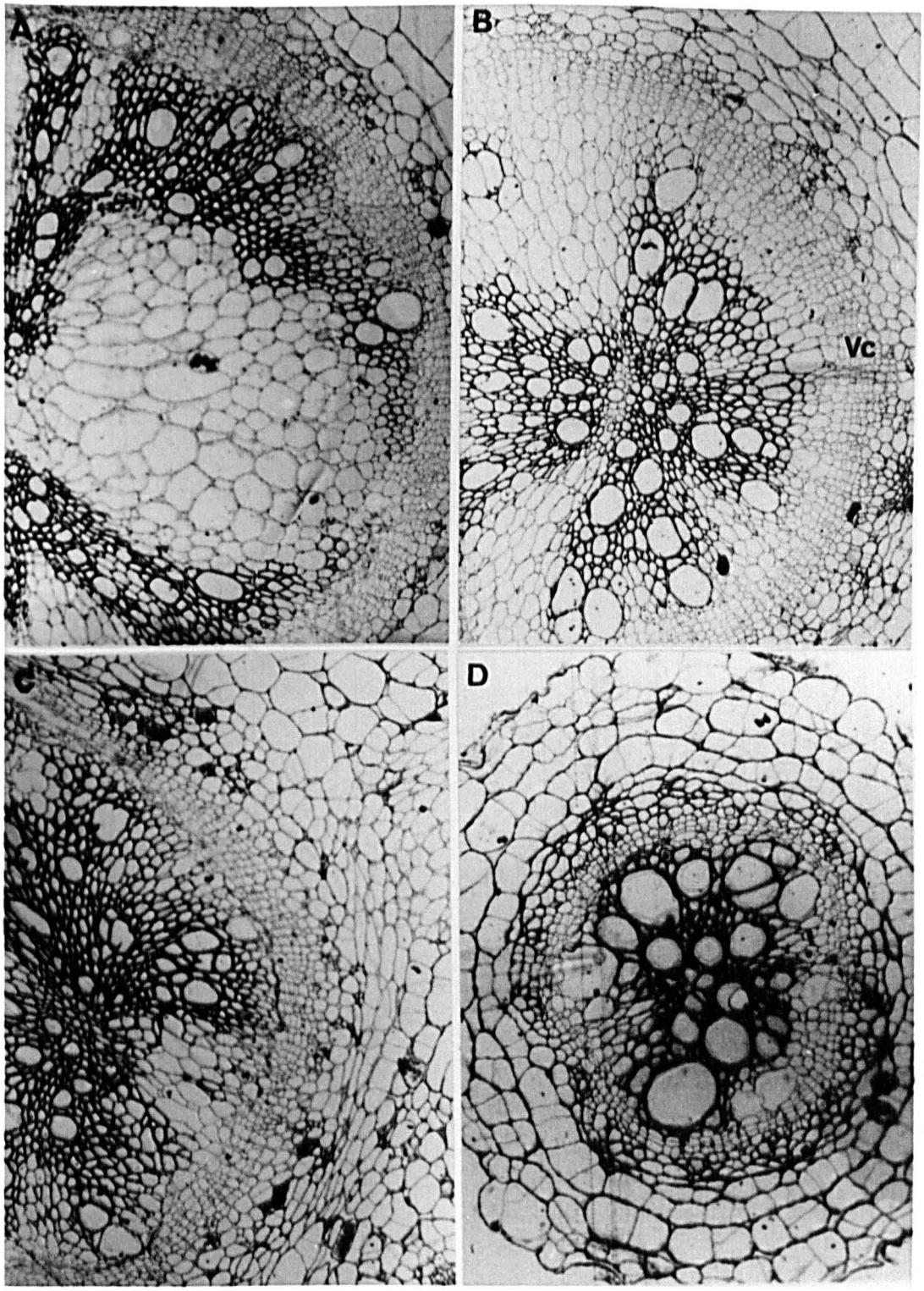
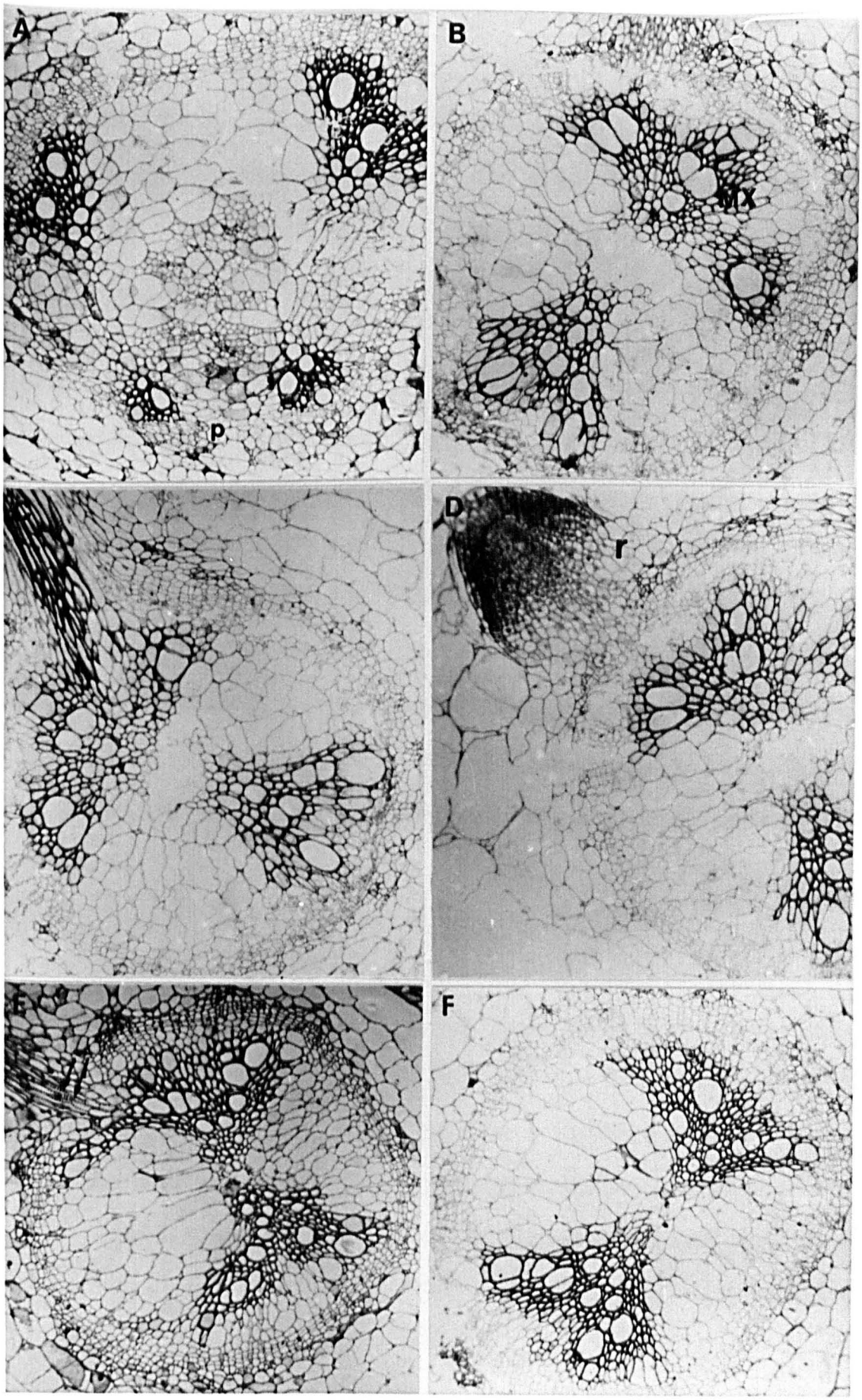

A
B
Vc
C
D

Figure 7.6 A-F: Transverse serial sections taken at 2.5 mm intervals through the shoot/root junction of tomato seedlings. Normal transitional development for *Lycopersicon esculentum* is demonstrated. Mag x 100
P=Phloem , r = root.


A
p
B
MX
D
r
E
F

In Figure 7.6d, a well developed root primordium (rp) is present, pushing through the cortex. The apex of the primordium is organized and vascular differentiation is occuring sub-apically, these new elements will eventually join up with xylem in the main trace. Some disruption of the cortex can be seen on either side of the emerging primordium where cells beside the root appear compacted. This can also be seen in the subsequent section (Figure 7.6e). Slight disruption of some of the tissues has ocurred during preparation of the section.

Sections taken from the root of seedlings and cultured plantlets exhibit the same basic structure (Figure 7.7). The root base of the cultured plant appears to have only limited late metaxylem development compared to the seedling. The sub-apical anatomy is very similar in both plant types (Figure 7.7 c&d). The cortical cells in the cultured plantlet are much more irregular in shape and appear to be slightly disrupted or collapsed. This may have happened during the preparation, or may be an indication that the cells are more fragile. This is not evident in the previous sections taken directly from *in vitro* plantlets. The cell packing is similar in both the seedling and acclimatized plant sections and there is no evidence of aerenchyma development.

7.3 Discussion

The transverse serial sections presented in this section failed to show up a callus block of cells betwen the shoot and the root, and from this evidence it would appear that the vascular strands between the shoot and root are continuous in the plants used is these studies and any discontinuities which may have been present were not apparent from these observations. If longitudinal sections were taken serially through the shoot/root juction, a more detailed picture of the entire region

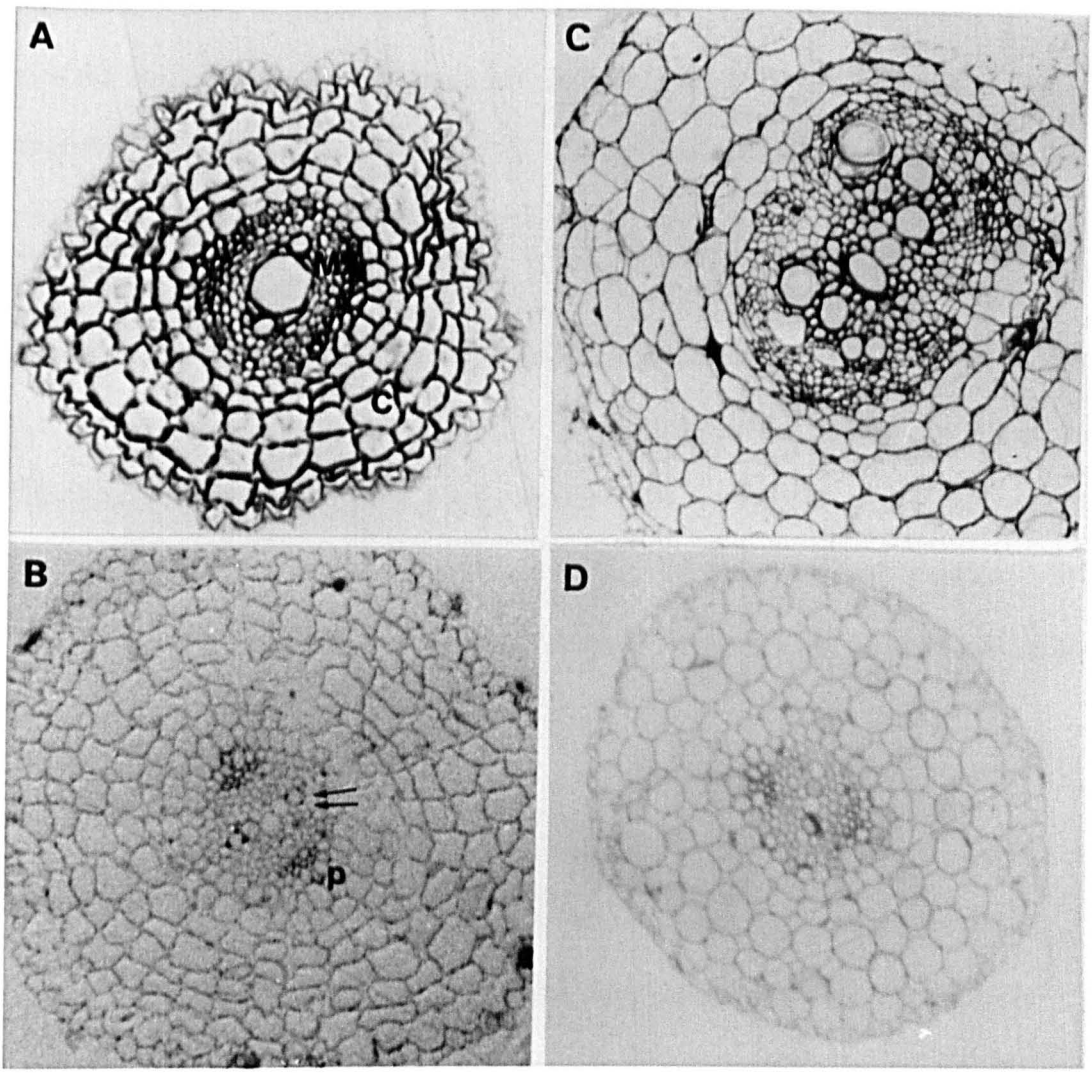
Figure 7.7 A-D: Transverse serial sections of root taken from seedling and cultured tomato plants.

A) Cultured plant root base, showing central stele showing metaxylem (MX) and cortical (C) cells

B) Cultured plant root tip, showing primary phloem (p)

C) Seedling root base

D) seedling root tip, all Mag. x 400


A
C
B
D
C
p

could be compiled. An attempt at longitudinally sectioned preparations was made, but the tissue proved diffecult to embed and cut. The limited availability of the tissue meant that this approach was not persued.

The differentiation of callus cells from the cut surface was evident when the microcuttings were examined 5 - 7 days after transfer to the rooting medium. However this was not excessive and examination of the internal structure indicates that the production of callus cells from the wounded tissue did not form a permanent barrier between the vascular tissue of the shoot and that differentiated subsequently. Callus cells are present in areas of the sections around the bases of the cuttings. The vascular tissue appears to develop in association with the vascular tissue of the shoot, although the normal vascular pattern is not developed. The development of extensive interfascicular cambium and xylem seen in these plants is consistent with that developed in cuttings which have had exogenous applications of kinetin and IAA (Saks *et al.*, 1984). Although the shoots used in these experiments were not exposed to kinetin in the rooting medium, it is possible that a latent effect is present in the tissue from the previous culture phase.

The xylem elements initiated from the newly derived cambium appear to be composed of both primary and secondary structures, as demonstrated in the structure of the *in vitro* plants taken directly from culture. The vessel diameter and pattern are influenced by auxin concentration, and the proximity of the tissue to the auxin. Work on *Phaseolus vulgaris* (Aloni and Zimmerman, 1983) has shown that there is an increase in vessel diameter and decrease in vessel density with increasing distance from the auxin source. This results in changes in the vascular

pattern developed. Increased amounts of secondary xylem were induced at close proximity to the auxin source, and this would be consistent with what may be expected *in vitro*, where the application is directly at the stem base and for a prolonged period.

There is extensive formation of secondary xylem in the sections taken from the cultured plants. It is not entirely clear what induces differentiation of the new cambium; Warren-Wilson (1978) suggests that an auxin/sucrose gradient acting basipetally may be involved. In conventional cuttings photosynthates and auxin would be supplied via the phloem. This is probably modified in cultured shoots, where photosynthesis is reduced and these factors are supplied exogenously. If the leaves formed *in vitro* act as storage organs as proposed by Wardle *et al.*, (1983), then any accumulated starch or carbohydrate may be utilised during the acclimatization period either for shoot growth or new root formation. However the pattern of vascular development observed in tomato plants from these treatments is not consistent with that developed by internal gradients. Early observations on the basal part of these stems, using SEM, revealed callus cells heavily packed with starch granules. These may provided an additional source of carbohydrate for the developing tissue during acclimatization.

Exogenous supplies of IAA and cytokinin are required for the initiation and differentiation of new vascular tissue and lateral root development (Sachs and Cohen, 1982). Studies on *in vitro* lettuce cells (Warren-Wilson *et al.*, 1982), showed that IAA induced callus, whilst a combination of IAA and zeatin promoted xylogenesis and high concetrations of both were found to be inhibitory.

The xylem structure in sections taken from plantlets acclimatized at 40% relative humidity (Figure 7.4), is very similar to that observed in plantlets from the 80& r.h. treatment. However there seems to be slightly more pattern development at the stem base in the reduced humidity treatment. There is reduced interfascicular xylogenesis and reduced late xylem formation. It should be noted that there is bound to be some variation in the position from which the sections were taken and only one plant from each treatment may not be fully representative. It is tentatively proposed that the increased transpiration flow resulting from the reduced humidity increases flow through the vascular tissues and leads to a more efficient and direct vascularization in the microcuttings.

The initiation of root primordia has been studied extensively in cuttings of *Griselina* sp. and *Agathis* sp. (White and Lovell, 1984a; b; c), and more briefly for tomato hypocotyl cuttings (Aung *et al.*, 1975). Aung *et al.*, report the presence of root primordia in 10 day old tomato hypocotyl cuttings. Examination of a 1 cm basal portion of the hypocotyl showed 4 -6 primordia. This 1 cm zone was apparently taken from the extreme base of the hypocotyl, since the arrangement of the vascular tissue resembles the pattern associated with root sections. The authors suggest that the root primordia were initiated acropetally in various positions in relation to the phloem and xylem. In cuttings of *Griselina* sp., root primordia developed in the cambial regions associated with recently derived phloem fibers (White and Lovell, 1984c). The cells closest to the cambium enlarge, but do not divide until a later stage. The outer cells continue to divide, and these cells initiate the root primordia. The cells nearest to the xylem differentiate at a much later stage and then form connections with the main stele. Vascular connections differentiate continuously between the shoot and root. By the time the root

primordium begins to push through the cortex, it is fully organized at the apex (as demonstrated in Figure 7.6d). In older *G. littoralis* cuttings the cambium is activated about 5 mm from the cut base (White and Lovell, 1984c). Areas of maximum cell proliferation are associated with the leaf traces. The activity is on the phloem side of the cambium as described previously, and the root initial pushes outwards through the cortex.

In order for the root to be functional, it is essential that vascular connections are established between the developing root and the vascular tissue of the stem. The final connections are made as the newly formed root emerges from the cortex.
In woody species these primordia may be associated with induced vascular tissue, either in the stem or basal callus. This may have obvious parallels with with cultured shoots. The basal callus is produced from proliferating cambium, phloem and xylem parenchyma cells. In some cases the xylem strands extend from the stem tissues into the callus. The interfascicular differentiation of tracheids and phloem seen in the microcuttings correspond with observations on conventional cutings of *Agathis* sp. (White and Lovell, 1984 a&b), and *Helianthus* sp. (Saks *et al.*, 1984).

The structure of the root bases in both seedlings and cultured plants appears to be very similar, with only minor variations in the structure of the cortex. The cortical cells in the cultured plants are irregular in shape and appear compressed in some areas. This may be due to disruption caused by the emergence of numerous root primordia. The suggestion that roots formed *in vitro* develop aerenchyma, and that this causes subsequent collapse of the root on transfer to *ex vitro* conditions (Maene and Debergh, 1983; 1987), is not apparent in the plants used in these

studies. The variations in root structure decribed by other authors (Marin and Gella, 1987; Donnelly *et al.*, 1985), are more consistent with the findings of this work.

Chapter 8: Plant water relations

8.1 Introduction

From a review of the current literature it seems that many cultured plants are unable to maintain a favourable water balance on transfer to the conditions of lower humidity experienced during acclimatization. There are a number of reports of low survival rates due to desiccation (Ziv, 1985; Sutter, 1985; Short *et al.*, 1987; Brainerd *et al.*, 1981).

The uncontrolled loss of water from *in vitro* formed leaves seems to result from a combination of poor stomatal functioning (Brainerd and Fuchigami, 1981; Short *et al.*, 1984; Marin, Gella and Herrero, 1988) and inadequate development of the cuticular wax layers (Grout and Aston, 1977; Sutter, 1985; Sutter *et al.*, 1988). The severity of these abnormalities appears to vary between plant species. It is generally recognized that under *in vitro* conditions the stomata of cultured plants remain open (Sutter *et al.*, 1988). During acclimatization, when plantlets are transferred to a lower relative humidity the stomata formed *in vitro* often fail to close or require a long period to respond to closure stimuli. A number of studies have compared closure responses between stomata of *in vitro* and glasshouse grown plants.

Stomatal functioning

Brainerd and Fuchigami (1981 & 1982) found that stomata formed *in vitro* failed to respond to closure stimuli. In apple plants, treatment with darkness, mannitol, ABA and carbon dioxide induced normal closure responses in glasshouse grown plants, but stomata of *in vitro* produced plantlets failed to respond. These plantlets

acquired a more effective closure mechanism during acclimatization.

Other workers have found similar results with other species. Sutter *et al.* (1988) report limited stomatal functioning in apple, cherry and sweetgum plantlets. However under water stress the stomatal conductance alone does not account for the degree of water loss. Cuticular transpiration forms a significant part of the water loss. They also found that under a gradual acclimatization regime stomatal function improves and conductance is reduced.

Loss of cellular integrity occurs under severe water stress and irreversible damage results in tissue death. The response of stomata of *in vitro* cherry plantlets improved during acclimatization (Marin and Gella, 1988). In this study cuticular development was similar in *in vitro* and in acclimatized plants and effective water transport through the root/shoot junction was demonstrated. These authors concluded that water loss and desiccation were not the sole cause of plantlet death. Stomata formed *in vitro* were rounder in shape and the guard cell walls were thinner, but no other structural differences in stomatal or cuticular development could be found. In a later study, Marin , Gella and Herrero (1988) observed variation in stomatal shape and distribution of starch bodies and chloroplasts in guard cells of both *in vitro* and acclimatized plants. Stomatal function improved during acclimatization and was comparable with glasshouse grown plants. Anatomical differences in leaf structure of raspberry also improved during acclimatization (Donnelly *et al.,* 1985). It appears that *in vitro* stomata have the ability to adapt during a controlled acclimatization stage.

Abnormality in stomatal function is related to structural (Wetzstein and Sommer, 1982; 1983; Ziv *et al.,* 1987) and physiological variations (Donnelly and Vidaver,

1984a; Wardle *et al.*, 1979). Stomata formed in culture appear to be rounder, this abnormal shape of the guard cells may prevent complete closure of the stomatal aperture. Some authors (Ziv *et al.*, 1981; 1987; Marin *et al.*, 1988) have reported callose deposits in guard cell walls. This may result in the gradual loss of cell wall elasticity and ability to close effectively.

Physiological aspects of stomatal movement

There are two distinct series of events connected with stomatal movement: hydroactive and hydropassive. Hydroactive processes involve changes in cellular ionic and organic content, are dependent on physiological and metabolic conditions and result in alteration of osmotic gradients. Hydropassive processes include resulting water fluxes, changes in turgor pressure and mechanical responses of the guard cell walls which result in changes in stomatal aperture.

Potassium is the main cation transported in guard cells during stomatal movement (Hsaio, 1976; Raschke, 1979). During stomatal opening, potassium ions move into guard cells. Proton efflux generates an electro-chemical potential, which is further stimulated by light (Bowling and Edwards, 1984; Inoue and Katoh, 1987). Potassium ions follow the electro-chemical gradient and accumulate in the guard cell vacuoles. The resulting increase in guard cell turgor and stomatal opening are due to uptake of water. Stomatal closure is thought to be brought about by cessation of potassium ion uptake and active efflux (MacRobbie, 1983; Edwards *et al.*, 1988). Calcium is known to be involved in the control of potassium ion fluxes. Calcium ions inhibit proton efflux and hence ion stimulated opening (DeSilva *et al.*, 1985; Inoue and Katoh, 1987).

Although potassium is the primary cation involved in stomatal movement under normal conditions, other cations can promote opening on detached epidermis. Willmer and Mansfield (1970) found that sodium ions could promote opening equally effectively. They suggest that sodium accumulates in the guard cells. Jarvis and Mansfield (1980) confirmed this. When comparing sodium and potassium chloride they found each to be equally effective in promoting increased guard cell turgor, but mechanisms permitting closure seemed to be absent with sodium chloride. They suggest that the efflux mechanism may be potassium specific. If this is the case, then the sodium content of the culture media may affect stomatal movement in the cultured plants. Increasing the agar content of the culture medium as a means of lowering humidity *in vitro* and improving acclimatization success (Ziv, 1985; Debergh *et al.*, 1981), may compound this problem as sodium is a major contaminant of agar (see Chapter 4; Debergh, 1983).

The effect of varying sodium concentration in the culture media , on potassium flux mechanism in stomata of cultured banana (*M. accuminata* cv Dwarf Cavendish), is presented in Appendix III. The effect of varying gelling agent, concentration and sodium content in the culture media on stomatal function is investigated using histochemical staining and UV fluorescence microscopy.

Plant water conductance

The development of an adequate root system is essential to vigorous shoot growth following culture. Shoot growth can become limited by root mediated cytokinin production, while leaf number and shoot growth can be related to root development (Richards, 1982). Adaptation to water deficits involves alteration in

plant water status, brought about by the growth regulator ABA. A range of physiological characters affect plant water status including stomatal conductance (Neill and Horgan, 1985), root hydraulic resistance (Bradford, 1983; Jones *et al.*, 1987) and decreased plant size (Bradford, 1983). The relative importance of the root and shoot in controlling stomatal behaviour using wilty mutants of tomato (Jones *et al.*, 1987), has shown that the primary control over leaf conductance is by the shoot. However Kramer (1988) stressed the importance of the root system in the control of water loss from the shoot and the influence on stomatal conductance via the plant growth regulators ABA and various cytokinins. Development of an extensive root system is important as water and nutrient ion uptake rates vary along the length of the root (Sanderson, 1983; Marschner *et al.*, 1987). Absorption and transport capacity is related to root vascular development and structure.

Water stress may occur from a decrease in root conductivity. This usually results in decreased growth, reduced foliar area and lower dry matter production. Increasing humidity around the shoot reduces transpiration and vapour pressure gradient, leading to a reduction in the rate of leaf extension (Bingham, 1985). The hydraulic conductance of the whole root system gives some indication of the ability of the root to supply the shoot with water. Under conditions of constant transpiration, changes in root conductance will lead to corresponding changes in leaf water status. The relative water content of the leaf tissue can be used as an index of plant water status (Kramer, 1988). Although this measurement does not take soil water potential into account, this is not a limiting factor with solution culture experiments.

The development of the epicuticular waxes varies between plant species (Sutter, 1985; Sutter *et al.,* 1988). Generally species native to conditions of high humidity have different structural wax development than in those which need to conserve water. It is the wax and cutin content of the cuticle which determine its permeability, rather than its thickness. The cuticle becomes permeable when wet and at high temperatures (Kolattukudy, 1981). During culture elevated temperature and humidity within the culture vessel will almost certainly affect cuticle development. Plants such as *Brassica*, which have a well developed cuticle under normal conditions show poor development *in vitro* (Grout and Aston, 1977; Sutter, 1985). However this does improve on exposure to lower relative humidity, as the cuticle dries and becomes less conductive.

Others factors which will affect plantlet water conservation and transpiration, include leaf number, area, age and structure. The structure of leaves formed *in vitro* have been described as mesomorphic. The single layer of palisade cells, reduced mesophyll layers and thin cuticle give a low internal to external ratio and this should result in lower transpiration rates. Although there is contradictory evidence, it is generally assumed that the major source of water loss from the *in vitro* formed leaf is via the stomata.

The experiments in the following section were designed to assess the importance of root conductance and translocation efficiency in cultured tomato plantlets during acclimatization. The effects of this on plant water stress acclimatization are discussed. Measurements on leaf relative water content and waterloss, together with observations on stomatal function and leaf surface morphology are aimed at assessing the relative importance of these factors on plantlet survival.

8.2 Results

Assessment of stomatal function

Stomatal apertures were measured on leaflets taken from leaf tissue formed *in vitro* and during acclimatization. The acclimatization regimes used were the same as those described in Chapter 5 of this thesis. The apertures were either measured immediately after removal from the plant, or following a stress treatment of 5 minutes at low humidity (30-35 % rh). This treatment was used to induce closure in the stomata and to give some indication of the degree of control over movement which the stomata had developed during the acclimatization treatments.

The stomata of cultured plantlets had consistently smaller apertures at the lower humidity (40 % rh), stomata on leaves formed *ex vitro* appeared to have a wider range of movement than those formed during culture (Table 8.1). The only exception to this was the lowest humidity treatment over the 14 day period, where stomata showed a very similar aperture size before and after the post-harvest stress treatment. The leaves formed *in vitro* may have undergone sufficient stress at 40 % rh over the 14 day acclimatization period to develop a higher degree of control over stomatal movement.

The stomata of cultured plants formed during acclimatization gave comparable values to those recorded for seedlings and the range of movement appeared to be of the same magnitude. Following transfer from culture, stomata formed *in vitro* had wider apertures than those formed *ex vitro*. By the seventh day of acclimatization the *in vitro* stomata exhibited limited closure when stressed, while the stomata formed *ex vitro* closed more effectively, as indicated by the smaller aperture sizes (Figure 8.1). These results seem to indicate that the stomata

Table 8.1 Stomatal aperture responses recorded in acclimatized and seedling *Lycopersicon esculentum*, measured directly on removal from the plant or following stress treatment at 30%rh, after growth under different humidity regimes.

Acclimatization Treatment			Stomatal Aperture (μm)	
			Unstressed	Stressed
Micropagated	40% rh	7 days		
		In vitro	3.1 ± 0.11	1.8 ± 0.14
		Ex vitro	4.0 ± 0.14	1.4 ± 0.12
	40% rh	14 days		
		In vitro	3.8 ± 0.16	1.9 ± 0.20
		Ex vitro	3.9 ± 0.15	1.9 ± 0.21
	80% rh	7 days		
		In vitro	3.8 ± 1.8	2.8 ± 0.12
		Ex vitro	5.1 ± 0.17	2.4 ± 0.11
	80% rh	14 days		
		In vitro	4.9 ± 0.14	3.0 ± 0.13
		Ex vitro	5.9 ± 0.16	2.2 ± 0.12
Seedling	40% rh	7 days	5.1 ± 0.23	2.6 ± 0.10
	40% rh	14 days	5.6 ± 0.20	2.0 ± 0.11
	80% rh	7 days	4.9 ± 0.14	1.6 ± 0.13
	80% rh	14 days	5.1 ± 0.18	2.5 ± 0.11

Each value represents the mean of 100 measurements ± standard error.

formed during culture have a smaller range of movement than those formed following acclimatization, the latter group are comparable with seedlings for corresponding treatments. The values for apertures taken from the lower humidity treatments were not consistently smaller, as might have been expected. In cultured plantlets the lower humidity treatments did result in smaller apertures, however the reverse was the case in the seedlings, where smaller apertures were recorded at the higher humidity (Figure 8.2a). Measurements taken from cultured plants acclimatized at 40% humidity, which had subsequently been stressed following excision may indicate that the stomates are capable of attaining smaller apertures at lower humidity during the first 7 days acclimatization. Stomata formed following acclimatization at 40% rh, showed a more effective closure response than those formed *in vitro* (Figure 8.2a). Acclimatization at 80% rh did not appear to induce such an effective closure mechanism. This is also demonstrated by the values recorded for water loss (Figure 8.2b). However it should be pointed out that none of the stomatal responses recorded in these experiments achieved complete closure values of 0 μm. The initial and closed apertures recorded for the seedlings (Figure 8.2a) showed no significant variation at the different humidities used in these experiments.

Cuticle development

Qualitative observations of micrographs of the leaf surface of non-acclimatized and acclimatized plantlets showed a slight improvement in cuticle formation on the tissue formed *ex vitro* (Figures 8.3a & b). The leaf surface appeared to be more ridged, this may be due to increased wax deposition. Detail of this ridged wax development is shown in Figure 8.3c. The surface of the *in vitro* tissue appears

Figure 8.1 A&B: Stomatal aperture measurements for cultured plants acclimatized for 14 days at 40% relative humidity, values for *in vitro* and *ex vitro* formed leaf tissue, unstressed (A) and after stress (B). Means of 100 measurements, ± SEM

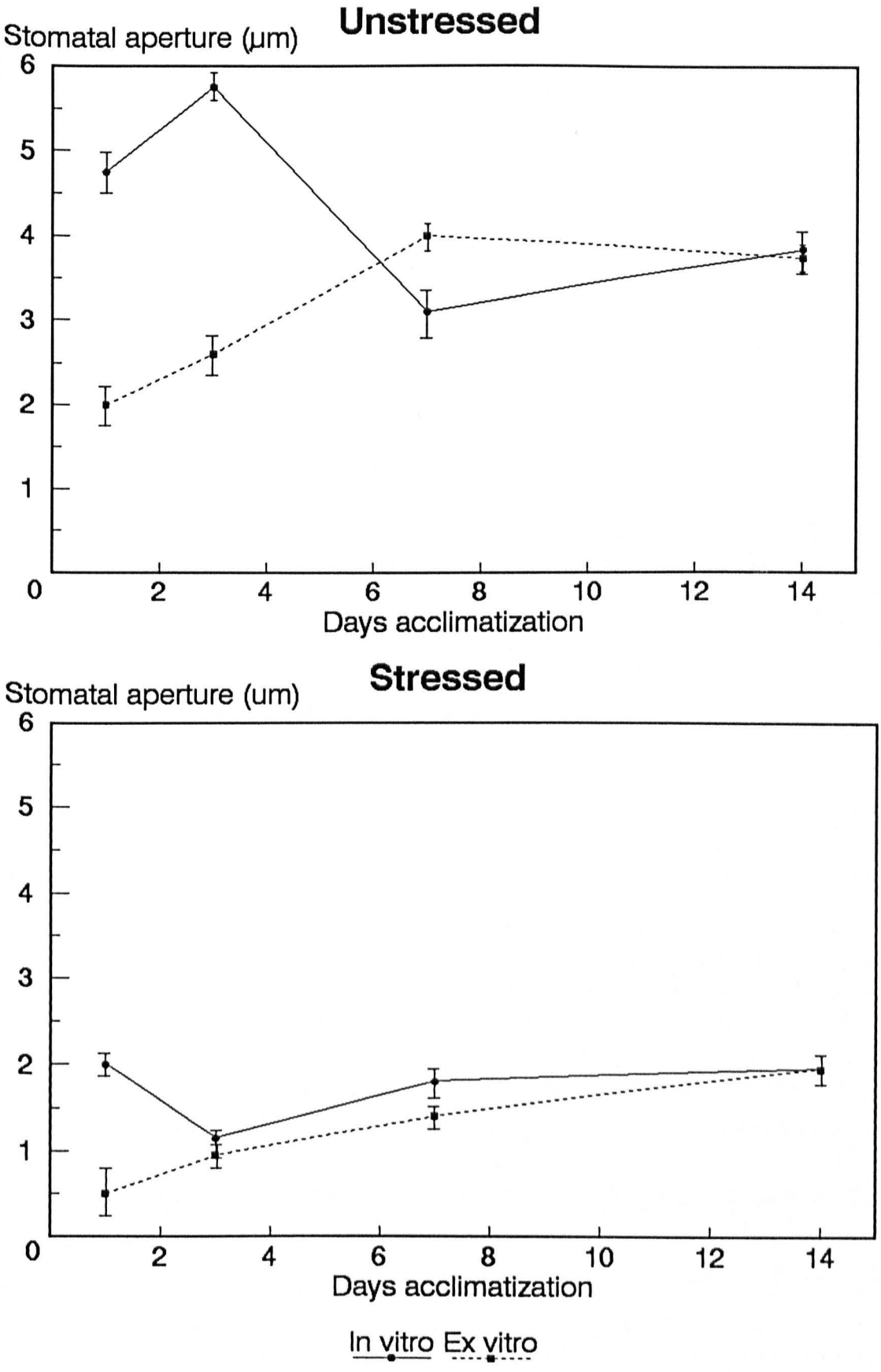
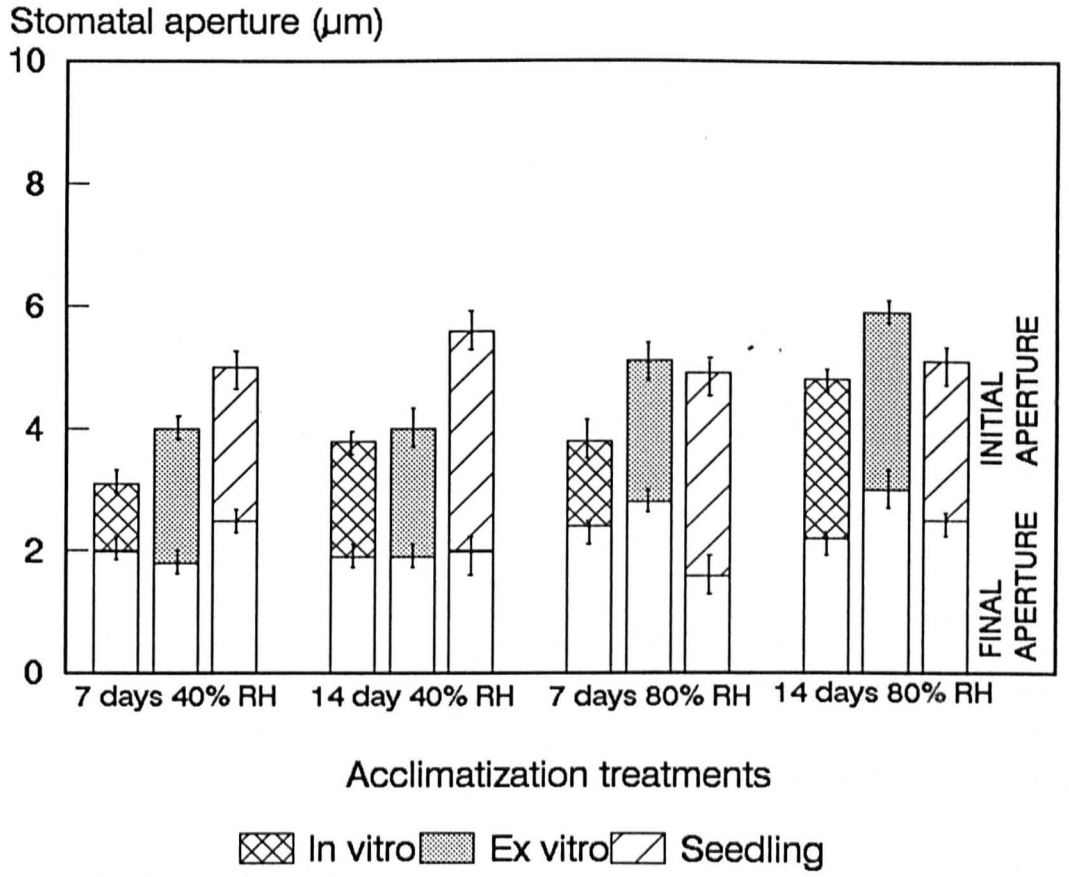

Unstressed
Stomatal aperture (µm)
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Days acclimatization
Stressed
Stomatal aperture (um)
6
5
4
3
2
1
0
2
4
6
8
10
12
14
Days acclimatization
In vitro
Ex vitro

Figure 8.2 A&B: Stomatal closure measurements and percentage leaf water loss for plantlets acclimatized for 7 or 14 days at either 40% or 80% relative humidity. Aperture values represent means of 100 measurements, ±SEM water loss figures represent means of 10 values,


Stomatal aperture (µm)
10
8
6
4
2
0
FINAL APERTURE
INITIAL APERTURE
7 days 40% RH
14 day 40% RH
7 days 80% RH
14 days 80% RH
Acclimatization treatments
In vitro
Ex vitro
Seedling

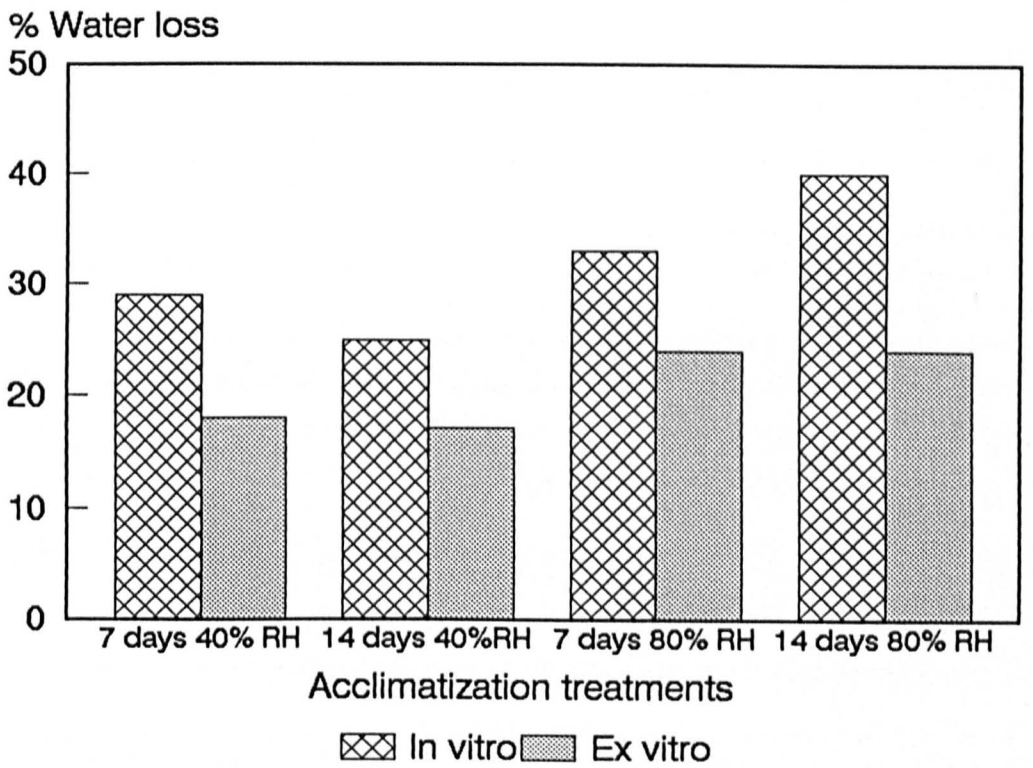

% Water loss
50
40
30
20
10
0
7 days 40% RH
14 days 40%RH
7 days 80% RH
14 days 80% RH
Acclimatization treatments
In vitro
Ex vitro

Figure 8.3 A-E: Scanning electron micrographs showing leaf surface morphology and cuticle development in cultured and seedling *Lycopersicon esculentum.*

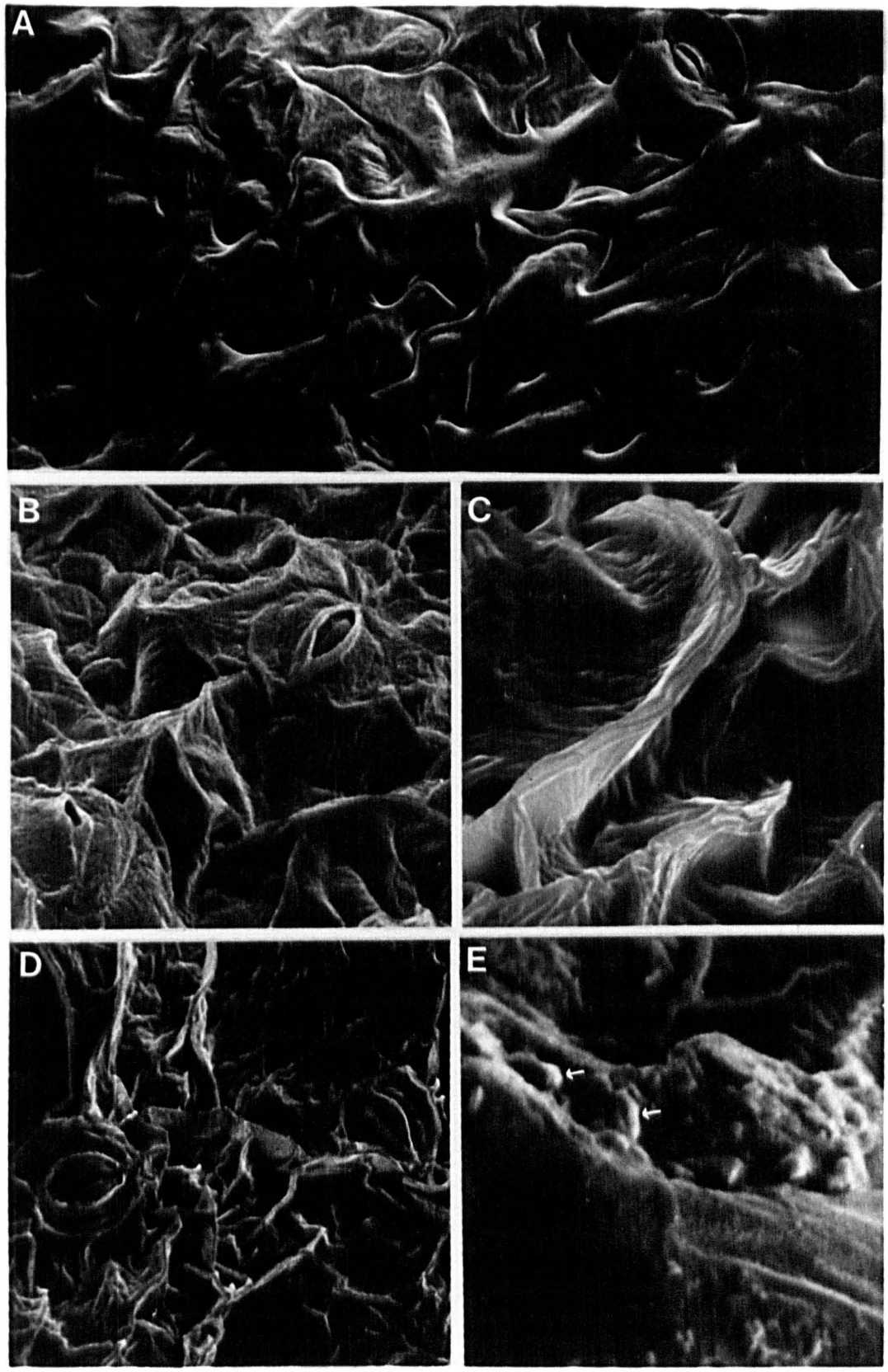
A) Abaxial leaf surface development after 20 days *in vitro.* Mag x 1000

B) Abaxial leaf surface development after acclimatization at 40% relative humidity. Mag x 1000

C) Acclimatized leaf tissue showing ridged development of the cuticle. Mag x 2000

D) Abaxial surface of seedling leaf. Mag x 1000

E) Detail of cuticle from seedling leaf showing ridged cuticle development and wax deposits. Mag x 2000


A
B
C
D
E

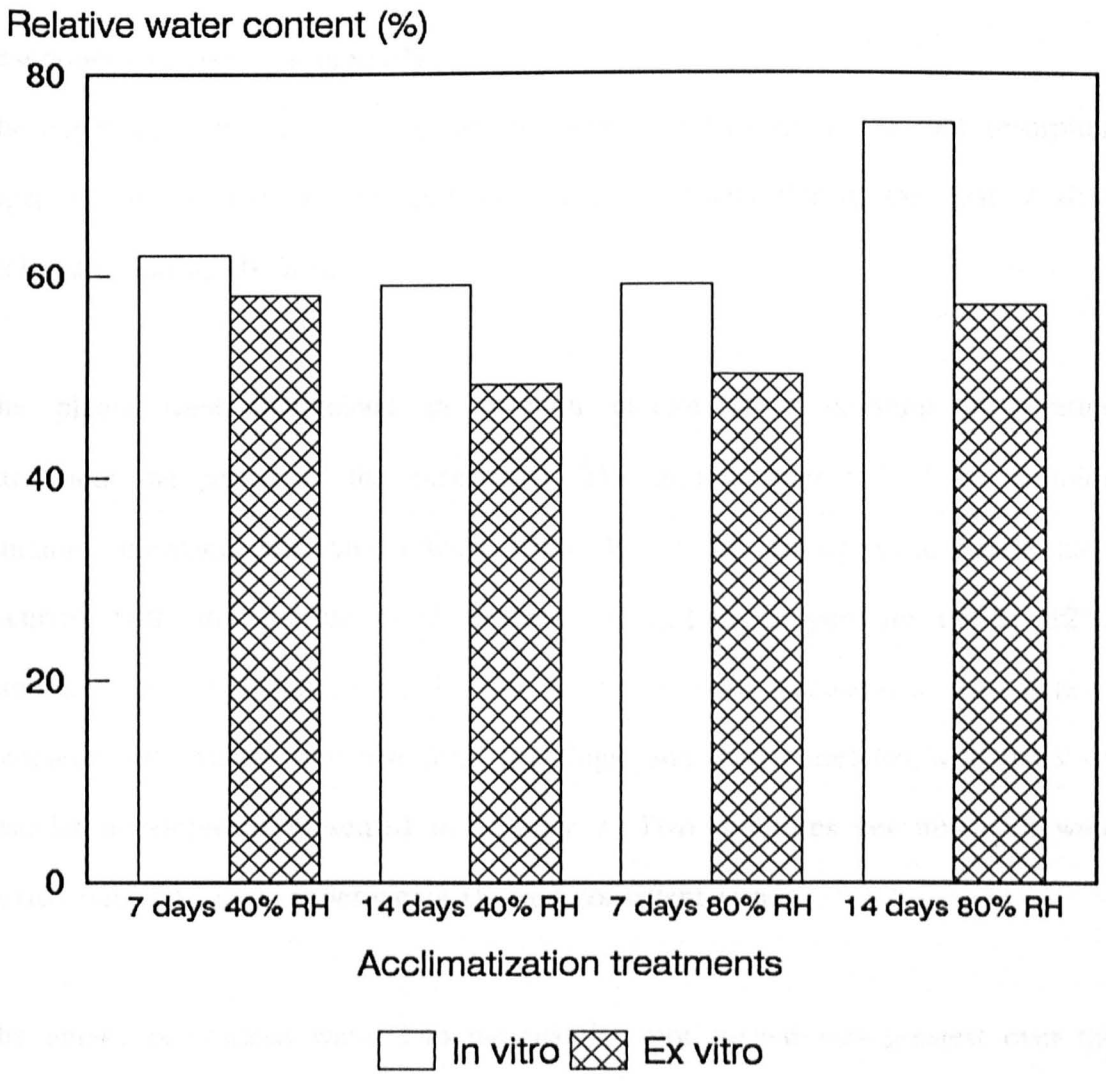
much smoother (Figure 8.3a). Figures 8.3 d and e show leaf surface development of seedlings taken after growth at 40% rh. The ridged cuticle is very similar to that seen in the acclimatized leaf tissue. Improved development of the cuticle in cultured plants during acclimatization at 40% r.h. is reflected in the decrease in figures rcorded for water loss (figure 8.2b). The surface morphology of the *in vitro* leaf may reflect the mesophytic nature of the leaves. Mesophytes and shade leaves tend to have a thin cuticle (Krammer, 1983).

Plant water status

Leaf relative water content (RWC) was used as an index of plant water status. A major limitation to the RWC technique is the accuracy with which the saturation point can be determined. Variation may occur through infiltration of water into intracellular spaces, changes in dry weight during water uptake or the continued uptake of water after the saturation point has apparently been reached (Krammer, 1983). Since most infiltration takes place through cut edges, use of standard size leaf discs incubated for a short time period (5 hours) should minimise this, whilst allowing an initial rapid uptake to satisfy the water deficit (Barrs and Weatherley, 1962). All discs were taken from leaves of the same age for each acclimatization treatment. Data for water loss was recorded over the first hour following excision from the plant. This data gives an indication of the extent of cuticular water loss when used in conjunction with the figures on stomatal aperture.

Leaf relative water content was consistantly lower in the acclimatized tissue (Figure 8.4). The highest water deficit in these tissues occured following 14 days at 40% rh. The RWC of the tissue formed *in vitro* remained constant over the 14 day period at 40% rh, but had increased following the same period at 80% r. h.

Figure 8.4: Leaf relative water content of *in vitro* and *ex vitro* tomato after acclimatization at either 40% or 80% relative humidity for 7 or 14 days. Means of 10 values,


Relative water content (%)
80
60
40
20
0
7 days 40% RH
14 days 40% RH
7 days 80% RH
14 days 80% RH
Acclimatization treatments
In vitro
Ex vitro

The RWC of leaf tissue formed *in vitro* ranged from 58 - 75%. The percentage water loss from *in vitro* and *ex vitro* leaf tissue indicated that under all acclimatization treatments used, the water loss from *in vitro* tissue was greatest (Figure 8.2b). In both *in vitro* and *ex vitro* tissue the highest water loss figure was recorded at 80% rh. There appeared to be very little improvement in water conservation following the longer period of acclimatization, at either humidity.

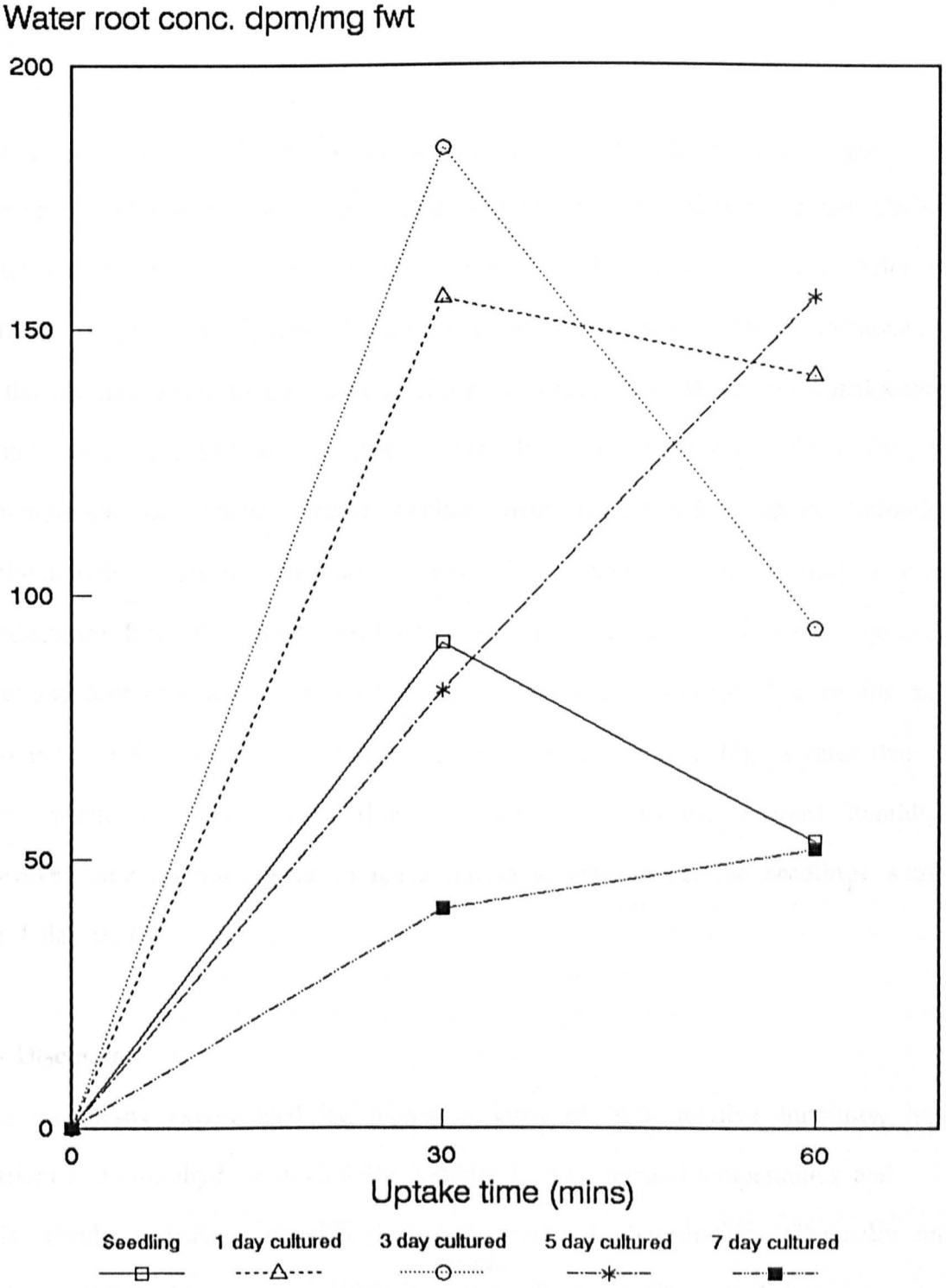
Assessment of root conductivity

The experiments in the following section were aimed to assess the root absorption capacity and conductivity of cultured tomato plantlets during the first 7 days acclimatization at 40 % rh.

The plants were maintained in solution culture at a constant temperature throughout the period of the experiment. The osmotic potential of the solution remained constant, and any effects from differential drying which may have occurred with soil culture were avoided. A culture temperature of 25 ±2°C, minimised any variation in uptake due to temperature fluctuations. The data are compared with values obtained from seedlings, and then correlated with work on vascular development presented in Chapter 7. Two replicates per treatment were carried out, but repeat experiments showed consistent trends.

The uptake of tritiated water into the plantlet root system was greatest over the first 5 days following transfer from culture (Figure 8.5). The absorbtion rates were similar over the first 30 minutes of exposure, but in the youngest plantlets and seedlings these values declined over the subsequent 30 minutes. After 5 and 7 days acclimatization the absorption rate continued to increase throughout the 60

Figure 8.5: Uptake of tritiated water into root tissue of seedling and acclimatized tomato plants after growth at 40% relative humidity, measured following 30 and 60 minutes exposure to the isotope. Each point represents the mean of 4 values taken from two experiments.


Water root conc. dpm/mg fwt
200
150
100
50
0
0
30
60
Uptake time (mins)
Seedling
1 day cultured
3 day cultured
5 day cultured
7 day cultured

minute exposure time, although the actual values were much lower in the 7 day old plantlets. The increased absorption recorded for plantlets at 1 and 3 days may be due to the development of a water deficit during the culture period, or alternatively an increase in the transpiration flow on transfer to the lower humidity.

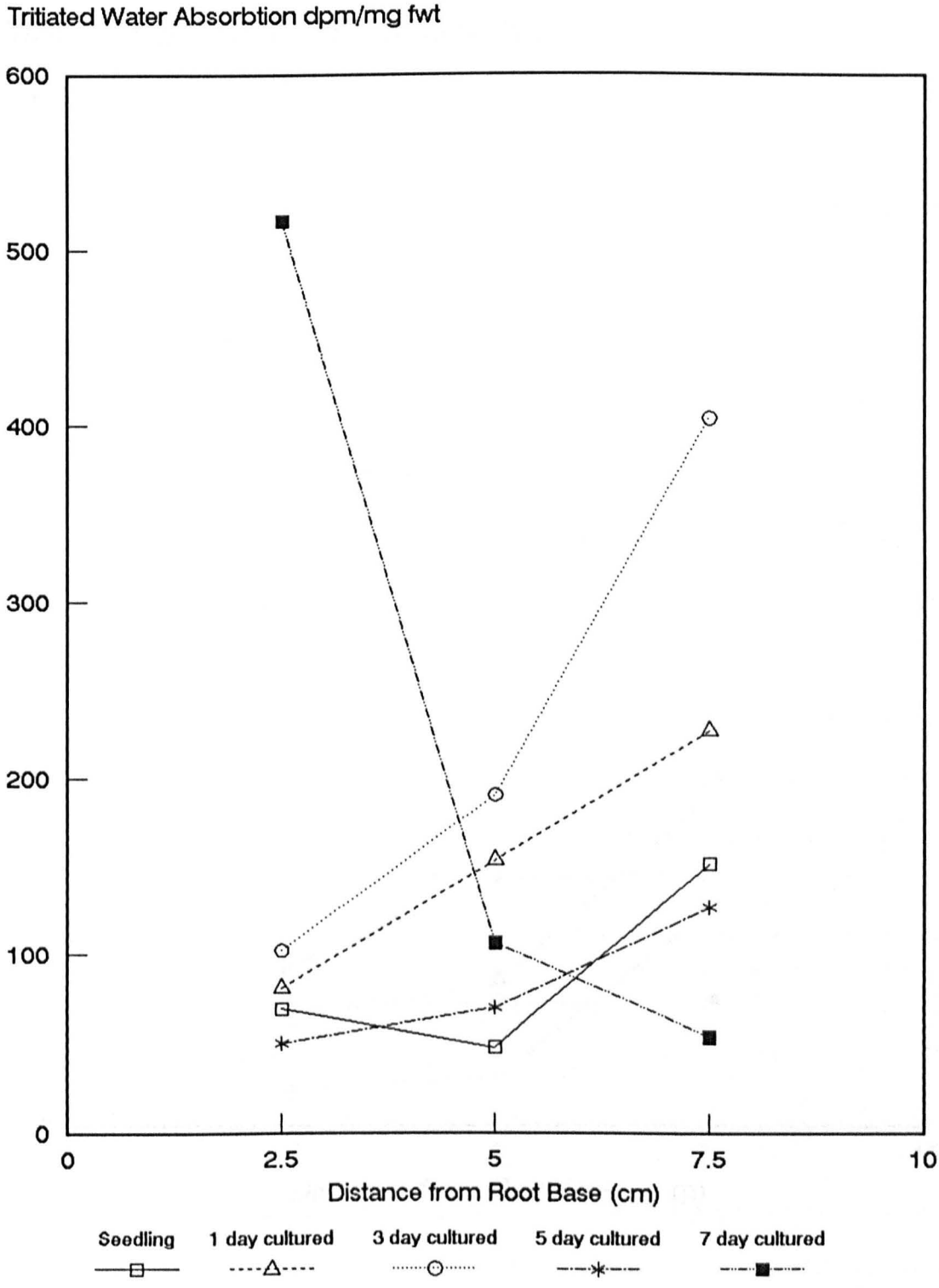
The distribution of tritiated water within the root is presented in Figure 8.6. During the 30 minute absorption period (Figure 8.6a) the majority of the labelled water was located in the area behind the root tip in the 7 day plants. After 60 minutes, (Figure 8.6b) increased water was located in the root base. Translocation of the tritiated water to the shoot is shown in Figure 8.7. Maximum translocation values were recorded in seedling plants. In cultured plants, values for the accumulation of labelled water decline over the initial 7 days following acclimatization. This may be due to a gradual development in the control of water conductance from the shoot, resulting in a gradual reduction in the transpiration flow and root conductance over the initial 7 days acclimatization. The results may also indicate the capacity for cultured plantlets to function at higher rates than *in vitro*, where the transpiration flow is reduced due to the extreme humidity. However they do not appear to reach the same capacity as the seedlings within the 7 day period.

8.3 Discussion

The conditions experienced by plants *in vitro* of high relative humidity, high nutrient and carbohydrate availabilty combined with elevated temperatures and light, result in various physiological and structural abnormalities (Donnelly and Vidaver, 1984b; Marin *et al.*, 1988; Ziv, 1987; Sutter, 1985), which become

Figure 8.6 A&B: Distribution of tritiated water in the roots of seedling and acclimatized tomato plants after growth at 40% relative humidity, measured following 30 (A) and 60 (B) minutes exposure to the isotope. Each point represents the mean of 4 values taken from two experiments.

A


Tritiated Water Absorbtion dpm/mg fwt
600
500
400
300
200
100
0
0
2.5
5
7.5
10
Distance from Root Base (cm)
Seedling
1 day cultured
3 day cultured
5 day cultured
7 day cultured

B

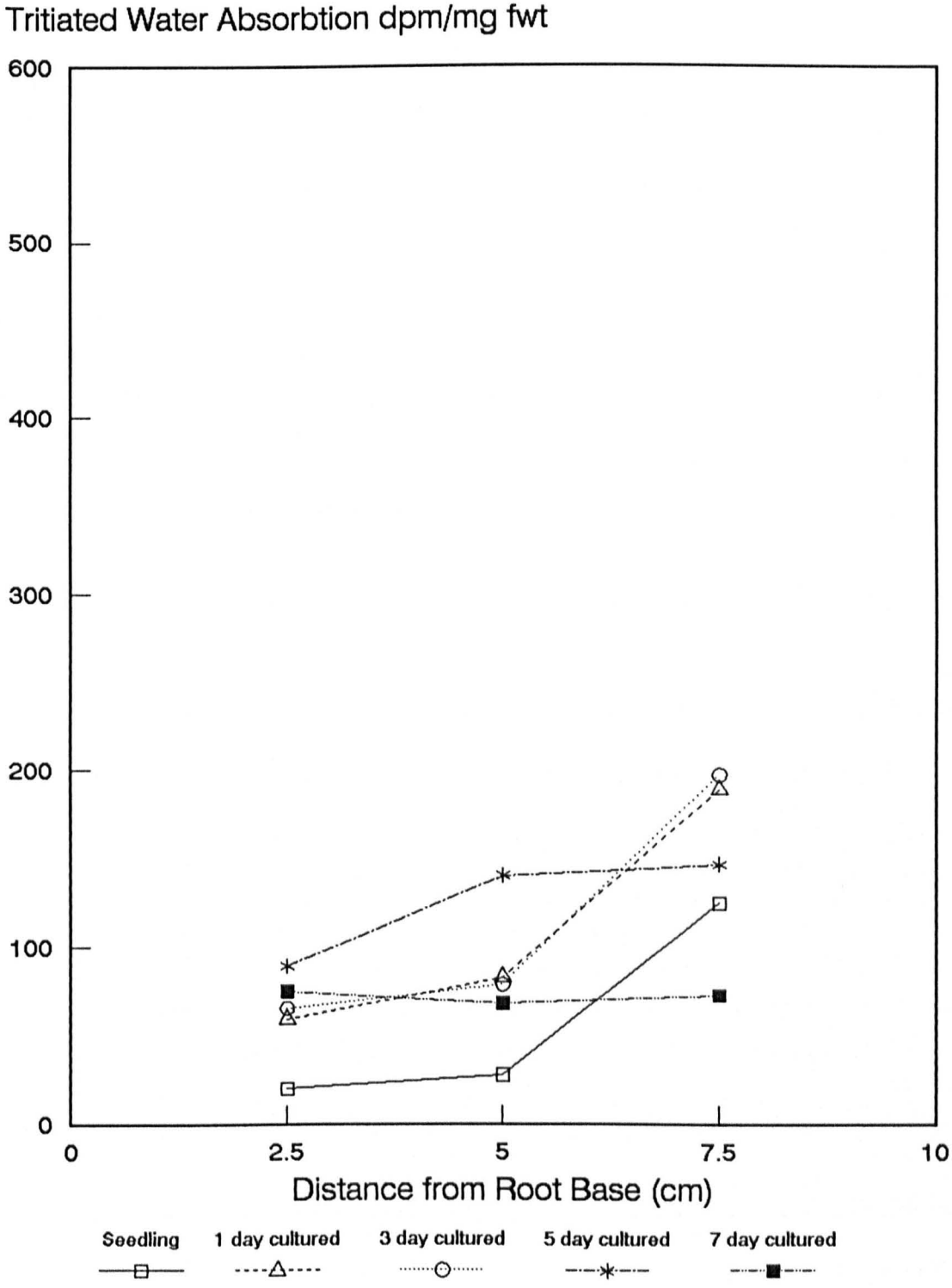
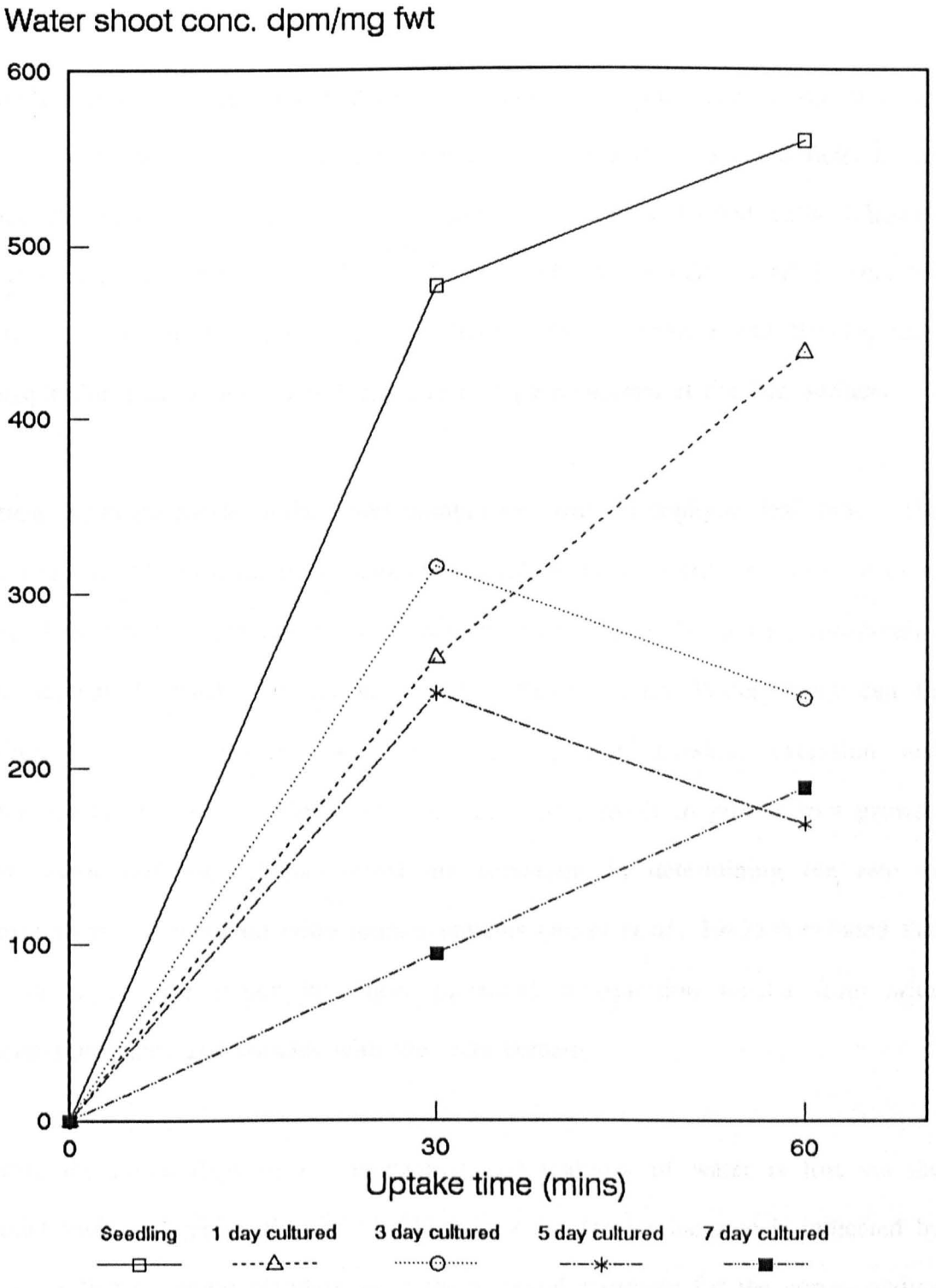

Tritiated Water Absorbtion dpm/mg fwt
600
500
400
300
200
100
0
0
2.5
5
7.5
10
Distance from Root Base (cm)
Seedling
1 day cultured
3 day cultured
5 day cultured
7 day cultured

Figure 8.7: Translocation of tritiated water into shoots of seedling and acclimatized tomato plants following growth at 40% relative humidity, measured after 30 and 60 minutes exposure to the isotope. Each point represents the mean of 4 values taken from two experiments.


Water shoot conc. dpm/mg fwt
600
500
400
300
200
100
0
0
30
60
Uptake time (mins)
Seedling
1 day cultured
3 day cultured
5 day cultured
7 day cultured

problematic during acclimatization. Plantlets often become dehydrated on transfer to a lower relative humidity and a compost based substrate. This can be attributed to excess water loss from the shoot and a reduction in water absorbtion through the root.

Several authors have reported malfunctioning stomata as the major source of water loss from the *in vitro* leaf. Additional cuticular loss results in water deficits in the shoot. Prolonged dehydration results in permanent damage to leaf cells, followed by leaf abscission and plantlet death. The high relative humidity (100%) generated *in vitro* is one of the main factors affecting plantlet growth and development. Transpiration flow is greatly reduced due to high resistance at the leaf surface.

Cuticle development is limited and comparable with mesophytic leaf types. The plantlets are able to maintain a favourable water balance in culture, but transfer to lower humidities, where resistance at the leaf surface is reduced and transpiration flow increased, usually results in water stressed plants. Water stress can be limiting to shoot growth and size, affecting cell division, extension and differentiation (Kramer, 1983). This can ultimately result in greater root growth. Both shoot and root characteristics are important in determining the rate of transpiration, but work on wilty tomato mutants (Jones *et al.,* 1987) concluded that the shoot has the major influence. Increased transpiration results from wide stomatal apertures and cuticles with low wax content.

During the initial days of acclimatization, the majority of water is lost via the abaxial surface (Fuchigami *et al.,* 1981), high cuticular conductance is indicated by Figure 8.2b for tomato plantlets since the stomatal apertures for the corresponding

plantlets are small. As the plantlets develop new leaves, at the lower humidity the ratio of the tissue formed in culture decreases. Tissues formed *ex vitro* exhibit greater stomatal control and increased cuticle development, leading to a balance in plant water status. Stomatal conductance is the principal componant of transpiration accounting for 80 - 90% of water loss, the degree of stomatal control varies with tissue age, but can be expected to be efficient in newly formed leaves. Rates of stomatal closure can also vary between species. Values for stomatal aperture taken from the abaxial surface of the leaf should give the best indication of stomatal conductance, since the stomata on the adaxial surface have a higher resistance and are less dense than on the abaxial surface. The incomplete closure of the stomata, recorded in all plant types may be attributed to the solution culture regime.

The increased water loss recorded at 80% rh may result from saturation of the cuticle, leading to increased permeability and therefore higher water conductance (Figure 8.2b). This is also reflected in the higher values for RWC (Figure 8.4) following the longer growth time (14 days). Typical figures for tissue water content normally vary between 80 - 85 % fresh weight. The values obtained of 49 - 62 % could be considered to be low, showing a water deficit. Although the stomata formed *ex vitro* have consistently wider apertures initially than those formed *in vitro*, the closure response is more efficient. Despite wider initial aperture values for the *ex vitro* tissue, the percentage water loss recorded is lower than for the *in vitro* tissue. This may be due to increased cuticle development at the reduced humidity, resulting in lower cuticular conductance or alternatively may be due to a faster stomatal response. Any physiological or structural abnormalities arising due to the culture environment, would not be expected in tissues formed

following transfer. If the stomatal malfunction is due to high sodium accumulation in guard cells this may be a transitory condition which will improve on transfer to low sodium containing substrate. However structural abnormalities in the guard cell walls, as proposed by Ziv *et al.*, (1987) could be expected to be more permanent.

Although the RWC of both tissue types (*in vitro* and *ex vitro*) is low, a greater water deficit develops in the tissue formed during acclimatization. This may be due to a greater demand from the *in vitro* tissue, via increased transpiration flow or due to greater control over water loss in the *ex vitro* tissue. Changes in RWC can occur diurnally; therefore tissue samples were collected at the same time of day for each set of assessments. Extreme water deficits were not recorded in any of the experiments, indicating adequate supply by the root system formed *in vitro*. Poor root conductance would almost certainly result in water stress in the shoot. Damage to the roots during transfer from culture, especially from high agar content gels could also lower root conductance and reduce the water absorbtion capacity of the root.

The importance of a well developed, branching root system is often underestimated when plantlets are transferred to glasshouse conditions. A large leafy shoot will soon produce a water deficit, if it is not supported by an adequate root system. Leaf conductance is mainly influenced by the shoot, and in conditions where water uptake into the root is not limited by soil water potential, it should be possible to gain maximum values for root and shoot conductance. Any structural resistance within the root may result in reduced conductance. Use of high agar content gels in culture is detrimental to root development, and ultimately results in decreased absorption capacity. Absorption of tritiated water by the *in*

vitro root system, shows a gradual decline in absorption over the first 7 days of acclimatization. A similar trend is observed for translocation to the shoot, with the exception of the seedling values, where translocation is more efficient. The decreasing rate of absorption into the root and transport to the shoot, with increasing number of days acclimatization may be explained by a gradual decrease in transpiration flow, due to increased control of leaf conductance, as the ratio of *ex vitro* formed tissue increases. However it is evident that the root system formed *in vitro* is functional and continues to function during acclimatization.

The reduced translocation efficiency of the cultured plants compared to the seedlings, is most probably due to internal structural resistance, from incomplete vascular development in the root/shoot junction (Grout and Aston, 1977). The absorption pattern of the root is similar to those of primary root systems. Water uptake varies along the length of the root (Sanderson,1983; Marschner *et al.,* 1987; Haussling *et al.,* 1988). The highest absorption is initially recorded in the zone behind the root tip and in the area of lateral root formation, subsequently increasing water enters the xylem vessels at the root base and becomes more evenly distributed throughout the root (Figures 8.3 a&b). At the lower humidity (40%rh) the increased absorption rate of the cultured plants may be due to higher leaf conductance.

Provided that the substrate is well aerated and roots are not damaged on transfer from culture, the root system formed *in vitro* is functional and able to supply shoot demand. Formation of an extensive root system is valuable since water and mineral ion uptake varies along the root length and is related to the extent of xylem and endodermis formation. A well branched root system provides maximum surface area. The apoplastic componant of radial transport will be important during

the first days following acclimatization, when the transpirational demand is high. As root conductance improves solute flow through the root increases and the vessel connections at the root/shoot junction should develop.

Removal of some *in vitro* leaves prior to acclimatization may aid as a temporary suppression of transpiration flow, through leaves with least control over water loss. Although antitranspirants may be useful initially (Sutter, 1985; Wardle *et al.*, 1979), they seem to impede stomatal function and prolonged use in cultured plants will not enhance root development and or improve vascular development in the root/shoot junction.

Chapter 9: General discussion

The studies presented in this thesis were designed to investigate the suitability of tomato plantlets grown *in vitro* to undergo successful acclimatization. A review of the general literature indicated key areas which could affect plantlet survival *ex vitro*. This study focused on root development and function in cultured plantlets.

Effect of culture medium on plantlet growth and acclimatization

The standard culture media used in plant tissue culture, such as MS appears to have more than adequate concentrations of the major growth requiring elements to support plantlet growth during the culture period. However analysis of media and plant tissue (Chapter 4) indicated that in culture systems which use reduced concentrations of basal media or prolonged culture periods, plantlets may become deficient in certain elements. This problem maybe aggravated further in culture regimes which use increased concentrations of solidifying agents such as agar to reduce humidity in the culture vessel. Movement of the elements N, P and K appeared to be restricted in the culture vessel and zones of depletion developed in the areas adjacent to the cultured tissue. Deficiency levels of nutrient supply will obviously occur sooner in cultures where reduced media concentrations are used. The use of liquid medium for rooting may help to alleviate this problem.

More precise control of rooting conditions is suggested by the work on auxin and sucrose interaction (Chapter 6). Prolonged exposure to high concentrations of auxin during the rooting phase seemed to be detrimental to root development. Although high concentrations of auxin produced large numbers of root initials. This may lead to problems with the mass formation of vascular connections to the

shoot, resulting in increased internal resistance to solute flow through the root/shoot junction. In addition, prolonged exposure to high auxin concentration appeared to promote the formation of short, thick unbranched roots. This type of root will obviously be less efficient in nutrient and water absorption. The promotion of a well developed, branched root system is possible by controlled application of auxin in conjunction with adequate sucrose supply. Sucrose is also important to vascular differentiation in the newly forming vascular tissue. Reduction of the endogenous supply from the shoot due to limited production of photosynthates means that the exogenous supply becomes more critical. The omission of sucrose from the culture medium in an attempt to induce early photosynthesis and improved acclimatization resulted in reduced plantlet growth and survival *ex vitro*. Stimulation of photosynthesis *in vitro* maybe achieved by combining increased light and $C0_2$ levels with reduced sucrose supply (Kozai *et al.,* 1990). A limited exposure time to sucrose during the later stages of root development may be sufficient to ensure optimum plantlet growth and survival during acclimatization, whilst having minimum effect on the photosynthetic mechanism. This approach needs to be studied in a wider range of cultured species.

Root development

The vascular development in the root and root/shoot junction in cultured plantlets is very similar to the structure found in conventionally rooted cuttings. The root induction regimes used in these studies promoted the development of vascular tissue which was continuous between the shoot and the root. There was no indication that callus tissue formed a persistent barrier to transport through this region. The root structure observed in the cultured plants was comparable with

that found in the seedling and there was no evidence to suggest that the roots collapsed or failed to function on transfer from culture, as has been suggested in other studies (Maene and Debergh, 1983; Smith *et al.,* 1990). The studies in this thesis were limited to roots produced in liquid culture and acclimatized in solution culture, thus avoiding any damage which may occur when roots are removed from agar solidified medium. Increased root development in liquid rooted plantlets, and subsequent improvement in acclimatization has also been demonstrated in other species (Lee *et al.,* 1986; Roberts and Smith, 1990).

The tomato plants produced for these experiments were as uniform as possible, and roots were initiated quickly and allowed to elongate before transfer to the acclimatization regime. Although many micropropagation systems use this method of rooting individual shoots, it is possible that other routes of plantlet production may not experience the same problems with vascular continuity between the shoot and the root. Plantlets produced by embryogenesis for example have a integral root primordium within the embryo which elongates and develops as in a normal seedling. Date palm is one example where plantlets are produced from embryoid structures. However, problems with acclimatization still occur. Although the root is functional structural weaknesses still appear in the shoot/root junction. Whether these weaknesses are inherent in the tissue or result from conditions experienced during acclimatization is an area for further investigation. A series of sections taken through the transition zone from *in vitro* and acclimatized palms would help to identify any structural abnormalities. More extensive studies on the uptake and translocation of phosphate in these plants would be aided by more careful monitoring of the nutrient regime under which the plants are grown during culture and acclimatization.

The isotope uptake studies in this thesis provide strong evidence to suggest that the root formed *in vitro* is capable of functioning in culture and following various acclimatization treatments. The problem of ion transport seems to be associated with the efficiency of translocation between the root and the shoot. There are no obvious structural abnormalities which could account for this, although some sequestration of phosphate may have occurred in the root, due to the conditions experienced in culture.

Control of water loss from the shoot develops during acclimatization at low humidity. This is illustrated by development of the cuticle and gradual improvement in stomatal function, demonstrated by the results in Chapter 8 and Appendix I. Interactions from high sodium contamination in the medium was not a problem in the tomato plants, where the roots were formed in liquid culture. The study on stomatal function in cultured banana (Appendix I) agrees with published work, demonstrating the inhibitory effect of sodium on stomatal functioning. In many plant tissue culture systems this may result from high media concentrations of sodium. The improved control over water loss acquired during acclimatization and resultant reduction in transpiration flow obviously has implications for phosphate transport to the leaves. Adequate supplies of essential nutrients to the shoot must be maintained via root supply to produce plants which are able to survive acclimatization. Removal of some of the *in vitro* formed leaves may alleviate water loss. The production of well developed plantlets with balanced shoot/root ratios is a prerequisite to successful plantlet acclimatization.

The work described in this thesis suggests that in order to maximise plantlet survival it is necessary to optimise concentration and exposure time of cultured tissues to major nutrients, PGR's and sucrose. By obtaining a balanced shoot/root ratio survival rates can be improved. The root produced in culture is functional and there is no evidence to suggest that there are major abnormalities in the vascular system. Subsequently it is important to provide appropriate conditions for growth. Transfer to low humidity was possible if good shoot/root ratios had already been achieved, but higher humidity was necessary for plants with poor root development.

Although these studies were carried out with tomato, the results may be applicable to other species.

Future work

One of the immediate aims of any future research would be to continue and repeat these studies with alternative species. In addition a more detailed study on nutrient movement through the culture medium over longer culture periods would give more information on the nutrient status of the plantlets during culture and early acclimatization. The supply of many of the microelements may also be severely restricted in solidified media. More extensive studies on the effect of sucrose during the root elongation stage of culture and any subsequent effects on photosynthetic activity are required to establish optimum concentrations and exposure periods for different species. The observation of starch reserves in basal callus and leaves formed *in vitro* raises questions about the possible utilization of these reserves during acclimatization. Determination of optimal PGR concentrations for shoot and root induction needs to take into account the effect of environmental

conditions on media factors. In particular photodegredation of auxin and the effect of temperature needs to be studied to permit more precise control of organ differentiation.

Further studies on the structure of the root and shoot/root junction from plantlets grown in agar culture and acclimatized in different substrates e.g. compost, sorbarods, rockwool etc, would provide valuable information on the ability of these roots to function under varying conditions. An extended study of the effect of auxin/sucrose concentration on vascular development in culture would provide information which could be used to improve root initiation and development. It may be useful to utilise some of the studies which have been carried out on conventional cuttings to determine optimum leaf area for successful transfer from culture. A balance between adequate photosynthate supply and minimum water loss needs to be achieved to optimise plantlet survival.

Appendix I

The effect of gelling agent and sodium concentration in the culture medium on stomatal functioning in *in vitro* and acclimatized *Musa accuminata*

The work presented in the following appendix was carried out in collaboration with Ms Kate Trapnell, as a final year undergraduate project under my supervision. The aim of the project was to determine if stomatal functioning could be affected by the concentration of sodium in the culture medium.

Materials and Methods

Materials and methods are the same as those presented in section 2.2, with the exception of the following; the experimental culture media (Appendix II) used contained sodium chloride at one of the following concentrations, 0, 10, 50 or 100 mM and used either agar (Difco-Bacto) at 8g l^{-1} , or agarose (Sigma Type II medium EEO) at 7g l^{-1}, as the gelling agent.

The plantlets were trimmed as described previously, inoculated onto the test medium and grown at 25 ± 2°C, at 100 μE $m^{-2}s^{-1}$ with a 16 hour photoperiod, for 49 days prior to assessment of stomatal function.

Assessment of stomatal function

Light microscopy

Abaxial strips of epidermis were taken from fully expanded leaves of cultured plantlets which had been grown under glasshouse conditions for 1 or 2 years, and from *in vitro* treatments. The strips were removed from the plants at the same

time on each occasion, when the stomata would normally have been in the open phase of the circadian rhythm. The strips were taken by scoring through the epidermis with a sharp blade and tearing the leaf lamina at an angle.

The epidermal strips were collected into beakers of distilled water and then transferred to petri dishes containing 0.45 mM potassium chloride in 10 mM morpholino-ethane-sulphonic acid (MES) buffer, adjusted with potassium hydroxide to pH 6.2. The buffer solution provides a background source of potassium ions.

Sodium cobaltinitrite histochemical test

The epidermal strips were rinsed for one minute in ice cold distilled water to remove external potassium ions before transfer to iced sodium cobaltinitrite (4g cobalt nitrate + 7g sodium nitrite dissolved in 15 mls glacial acetic acid). the epidermal strips were incubated in this solution for 15 minutes on ice and then rinsed in iced distilled water for a total time of 2 minutes. After washing the tissue was incubated in ammonium sulphide for 2 minutes. Excess ammonium sulphide was washed off with distilled water. The strips were then mounted in a drop of water and examined under the light microscope. Black precipitates showed the potassium distribution in the subsidiary and guard cells. Means and standard errors were calculated for the apertures. Measurements of stomatal apertures were carried out as described in section 2.7. The means of 100 apertures are presented. Photographs were taken using Ilford Pan F film (ASA 50) and Fujichrome 400.

Incubation treatments

Strips of abaxial epidermis were removed from plantlets grown on each treatment medium and collected into potassium chloride /MES buffer (10 mM, pH 6.2), and then incubated in MES buffer at 25°C for 30 minutes, under one of the following

treatments for 1 hour; darkness, light 60 μE $m^{-2}s^{-1}$ or 150 μE $m^{-2}s^{-1}$. The strips were then stained using sodium cobaltinitrite and apertures measured or alternatively, assessed using UV fluorescence microscopy. The fluorescence microscopy techniques are described in section 2.2.5 , sections were stained with .001% calcofluor brightner (Sigma chemicals). Any evidence of thickening in the guard cell walls and around the aperture was noted.

Estimations of sodium and potassium content of the culture media and plant tissue was carried out as described in section 2.2.3, using a tri-acid digest and atomic absorbtion spectroscopy, the settings used and calibration curves are presented in appendix III. The values represent means of four replicates.

Acclimatization

Plants were potted into a peat based compost (Fisons levington), and acclimatized in the glasshouse with the temperature set at 20°C, in a closed polyethene tent, giving increased humidity of 60-70% RH for 2 weeks. The plants were then transferred to normal bench conditions, and potted on every 4-6 months. The plants were watered 2-3 times per week, and root fed once every two weeks with Fisons Growmore liquid fertilizer.

Scanning electron microscopy

Leaf tissue was taken from *in vitro* plants, and from plants which had been growing under glasshouse conditions for 1 month or 12 months. The tissue was prepared for SEM as described in section 2.2.5 , photographs were taken using Ilford technical pan (ASA 25), and processed as in section 2.2.

Varying the sodium chloride content of the culture medium produced differences in plantlet size, vigour and development. Normal growth medium with no additional sodium chloride produced well developed plants with green leaves and well formed roots. This was the case with both gelling agents. Maximum growth was observed in plants growing on medium with 10 mM NaCl, well developed shoots and roots. There was no variation between the different gelling agents, and there was a positive stimulation of growth with this concentration of NaCl. However any further increase in NaCl concentration, resulted in stunted growth of both shoot and root tissue. The highest NaCl concentration (100 mM), produced plants with virtually no shoot growth and extremely stunted roots. The leaves were brown and necrotic. Again there was no added effect from either gelling agent.

Comparison of stomatal apertures and leaf surface morphology in glasshouse acclimatized and non-acclimatized banana.

The stomatal apertures in 1 and 2 year old glasshouse acclimatized plants were 2.3 + 0.23 and 1.91 + 0.19, respectively, compared with measurements of 1.24 + 0.15 from *in vitro* leaves. Significant differences were found between stomatal apertures in the acclimatized plants and *in vitro* plantlets (P 0.05). Brainerd and Fuchigami (1981) suggest that the stomata from *in vitro* leaves develop the ability to close during acclimatization. This was shown both in apple and *Brassica* (Sutter and Langhans, 1982) plantlets, where stomatal functioning improved after 4-5 days acclimatization, at low relative humidity. Marin and Gella (1988) found that non-functioning stomata in cherry plantlets could be induced to close by gradual acclimatization. However other workers (Wardle *et al.*, 1983) report that only

leaves formed following acclimatization had fully functional stomata and that non-functional stomata remained so. The majority of evidence seems to suggest that non-functioning stomata improve during gradual acclimatization.

The results of this investigation may have been affected by using detached epidermal strips, the apertures providing a useful comparison for the results from the experimental treatments.

The leaf surfaces from these treatments were prepared for SEM as described previously. Observations on *in vitro* and acclimatized leaves (Figure 1), show improved cuticle formation in the acclimatized plants. Globular deposits of wax can be identified on the leaf surface (Figure 1c). In contrast, the leaf surface developed *in vitro* appears to be very smooth and the outline of the underlying cells is clearly seen in Figure 1a.

Stomatal functioning in banana cultured at varying sodium chloride concentration and with different gelling agents.

Although some studies have shown increased stomatal opening in response to higher irradiation (Fischer, 1968; Humble and Hsaio, 1970; Travis and Mansfield, 1981), this is not the case in cultured banana. The response of stomata from banana plants cultured on varying concentrations of NaCl is illustrated in Figure 2a-c. A similar reduced response to light has also been reported in epidermal strips of *Commelina communis* L. which had been incubated in medium containing 50 mM NaCl (Jarvis and Mansfield, 1980). Normal dark stimulated stomatal closure was only evident in stomata from 10 mM NaCl agarose treatments (Figure 3). Stomata from tissues cultured at higher NaCl concentrations (50 and 100 mM)

Figure 1: Scanning electron micrograph showing the leaf surface morphology of *in vitro* and acclimatized banana leaves.

A) leaf surface following *in vitro* culture at 100% relative humidity. Mag x 1000

B) leaf surface following 21 days acclimatization under glasshouse conditions, with approximately 60% r.h. Mag x 1000

C) Area X from micrograph B, showing globular wax deposits on the leaf surface. Mag x 2000

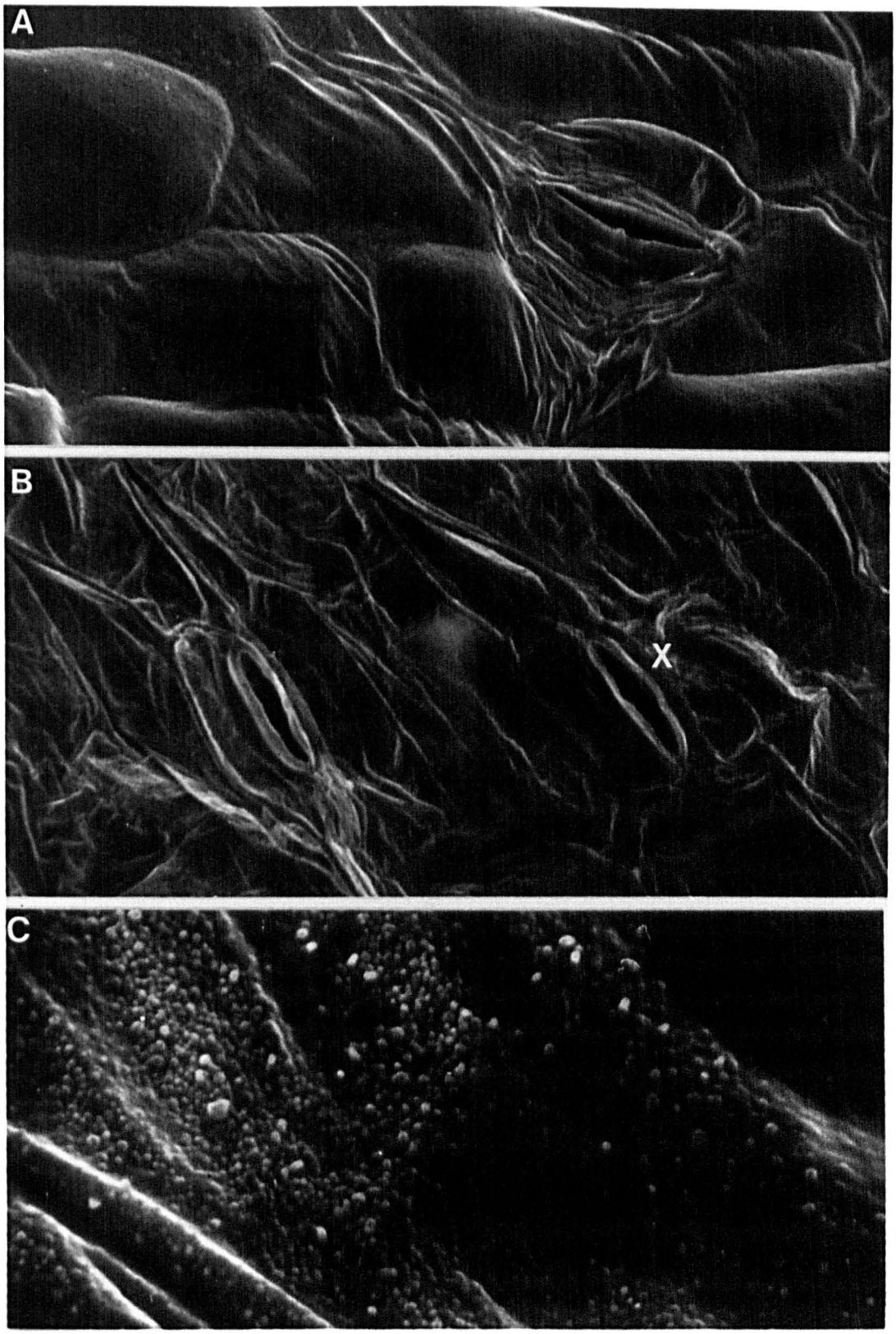
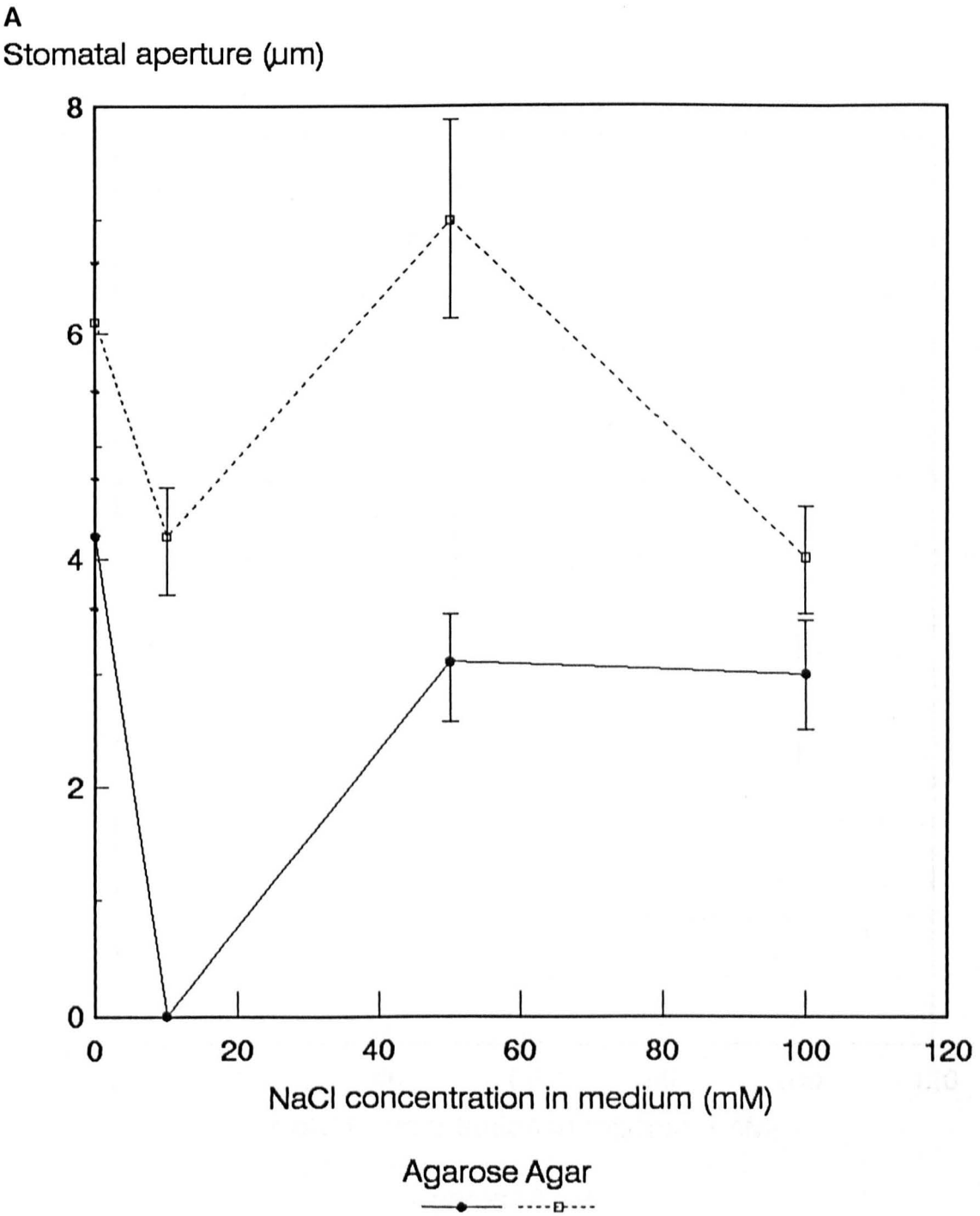

A
B
X
C

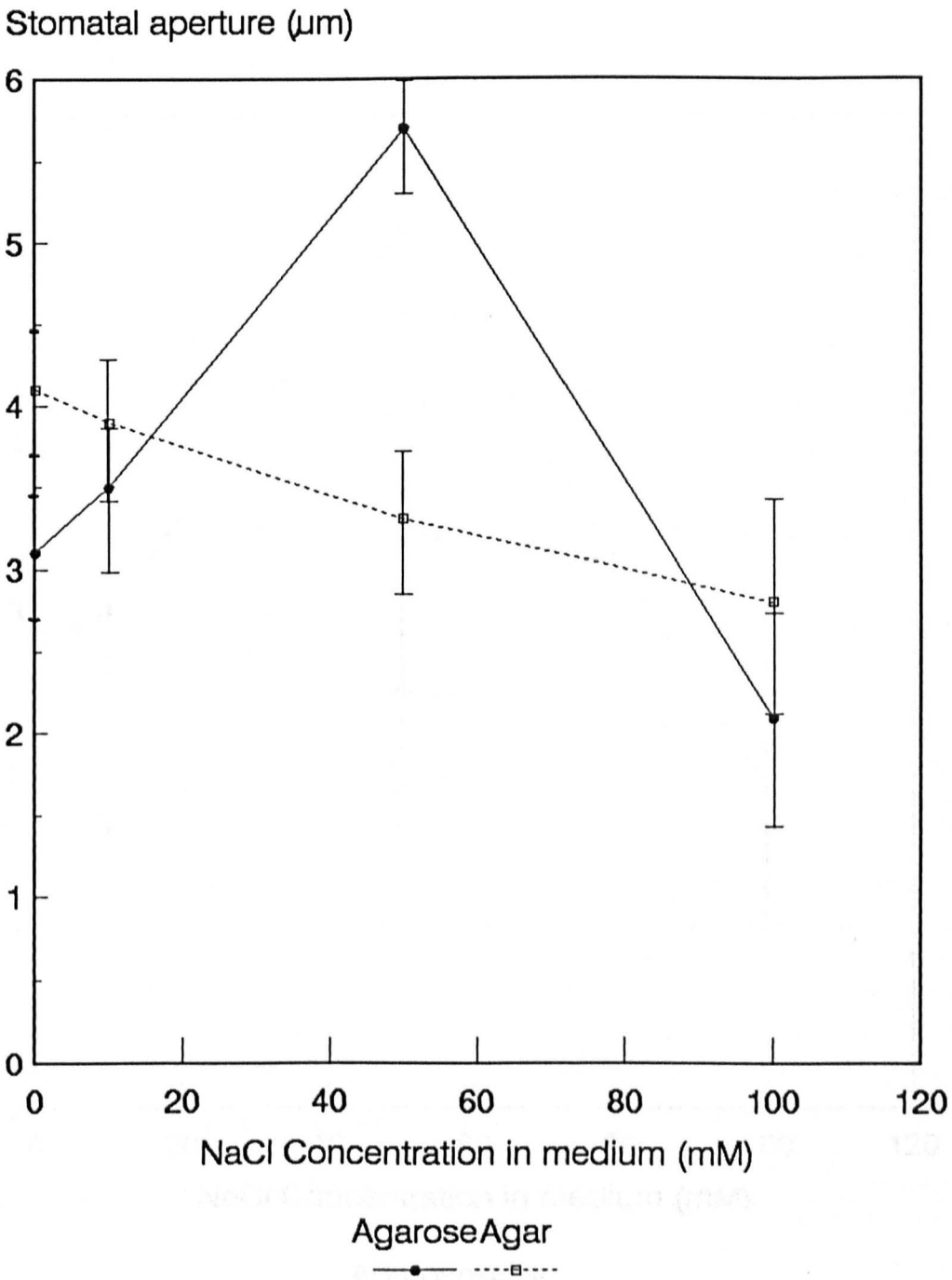
Figure 2 A-C: Stomatal aperture responses in *Musa accuminata* cultured on media solidified with agar or agarose containing 0 mM, 10 mM, 50 mM or 100 mM concentrations of sodium chloride, followed by different light treatments.

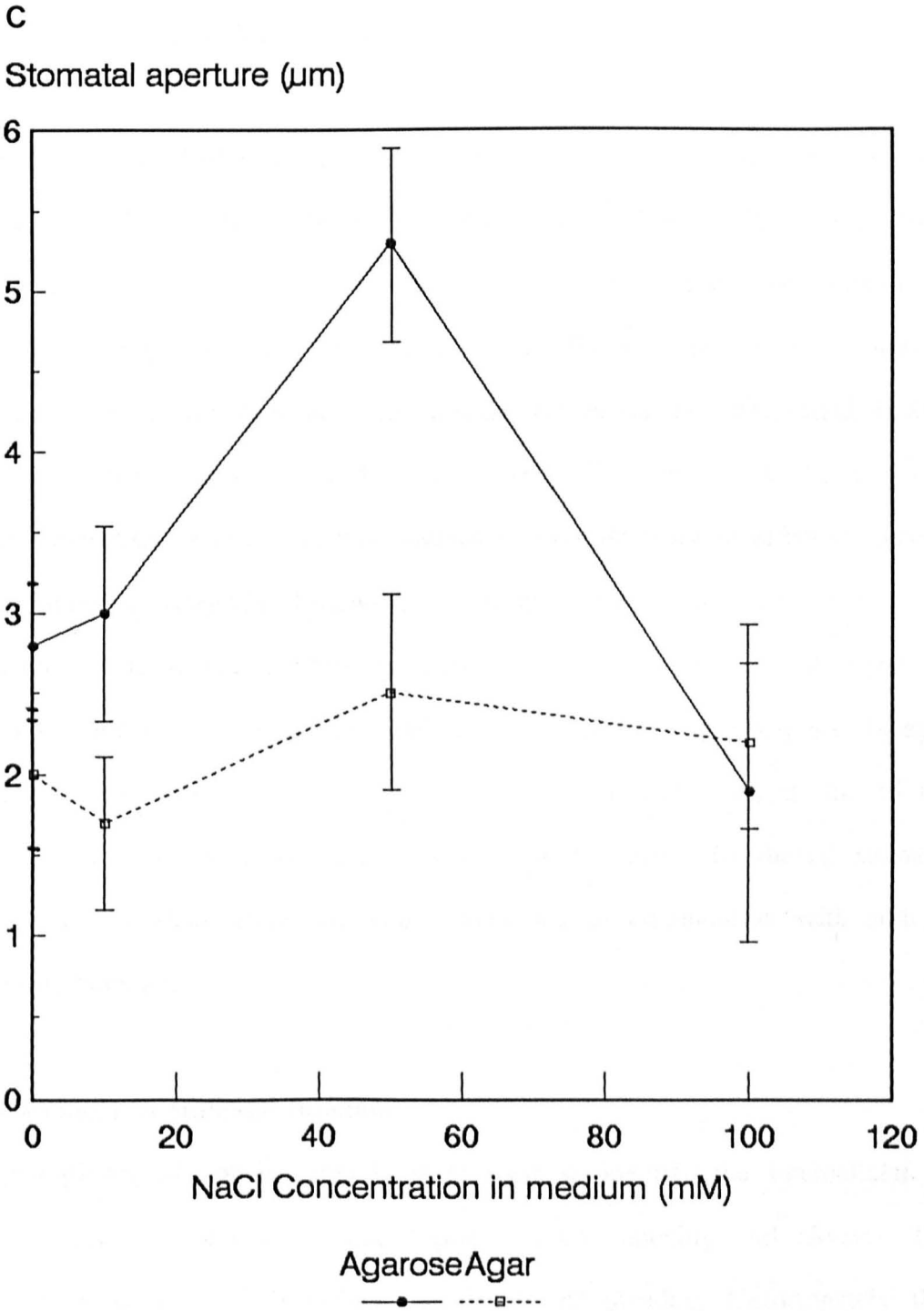
A) Dark incubation

B) Following incubation at 60 μE $m^{-1}s^{-1}$ irradiance

C) Following incubation at 150 μE $m^{-2}s^{-1}$ irradiance


A
Stomatal aperture (μm)
8
6
4
2
0
0
20
40
60
80
100
120
NaCl concentration in medium (mM)
Agarose Agar


Stomatal aperture (µm)
6
5
4
3
2
1
0
0
20
40
60
80
100
120
NaCl Concentration in medium (mM)
Agarose
Agar


C
Stomatal aperture (µm)
6
5
4
3
2
1
0
0
20
40
60
80
100
120
NaCl Concentration in medium (mM)
Agarose
Agar

were always slightly open (Figure 3). In some of the 50 mM NaCl agar treatment stomata were wide open. Maximum opening was obtained with 10 mM NaCl treatments (Figure 3). The normal potassium stimulated closure is probably ineffective in sodium loaded cells, the stimulated efflux does not occur, and the guard cells remain open (MacRobbie, 1983).

Movement may be further impeded by a lack of cell wall elasticity and the prescence of callose deposits along the inner guard cell wall (Ziv *et al.,* 1987, Marin *et al.,* 1988). Fluorescent staining demonstrated callose deposits around the stomatal aperture (Figure 3). The gelling agents did not produce any additional effects on stomatal function in these treatments. Jarvis and Mansfield (1980) suggest that concentrations exceeding 25 mM NaCl will affect the stomatal response mechanism. The results with banana show a decrease in effective closure i.e. the aperture obtained following light treatment, with increasing NaCl concentration. The results confirm previous work on the detrimental effect of sodium on stomatal functioning. The dark treatment invoked less response in agar cultured plantlets than the agarose ones, suggesting a further effect due to the agar. Potassium movement between subsidiary and guard cells during stomatal movement was studied using the same treatments in combination with sodium cobaltinitrite staining.

Ionic movement in stomatal function

Black precipitates of cobalt sulphide were used to identify the intercellular of potassium ions and their movement during stomatal opening and closure. The amount of potassium should reflect the degree of opening. Unfortunately this staining method disrupts normal stomatal function and previously open stomata

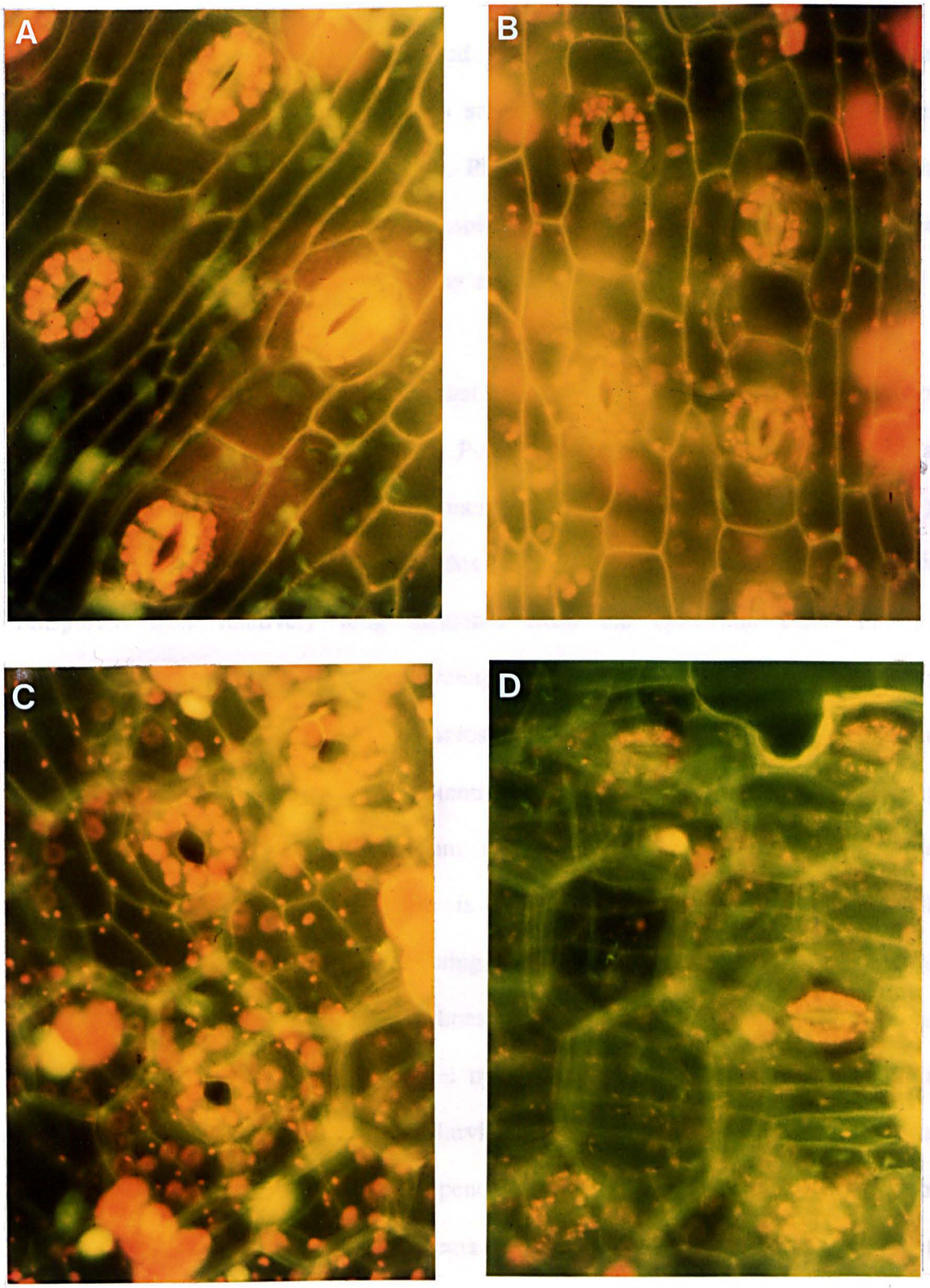
Figure 3: Photomicrographs showing varying closure responses in *Musa accuminata* cultured on media containing 10 mM, 50 mM or 100 mM concentrations of sodium chloride, followed by different light incubations

A) Slightly open stomata (50 mM NaCl)

B) Slightly open stomata (100 mM NaCl)

C) Wide open stomata (50 mM NaCl)

D) Closed stomata (10 mM NaCl) Mag. X 400


A
B
C
D

will close (Willmer *et al.*, 1987). Hence the aperture measurements were taken from unstained epidermis.

A summary of the results show closed stomata from 100 mM NaCl and agar treatment, following light incubation. A small accumulation of potassium ions was observed at the ends of the guard cells. Plantlets grown on agar solidified medium with no added NaCl remained open despite a dark incubation treatment, and did not accumulate potassium, this indicating a lack of normal function.

It has been recognized for some time that potassium plays a direct role as a major osmotic agent in stomatal functioning. Potassium ions move into the guard cells, with chloride acting as one of the balancing anions (Humble and Raschke, 1971). The use of potassium microelectrodes has demonstrated that potassium is transported over relatively long distances from the epidermal cells, through subsidiary cells to the guard cells (Penny and Bowling, 1974). Several workers have demonstrated a gradient of pH across the stomatal complex, from the guard cell outward in open stomata. The potential difference created by proton pumping in the plasmalemma allows potassium ions to move down the gradient and accumulate in the guard cell, this is known as the chemi-osmotic model. MacRobbie (1983), proposes that opening is maintained by control of ion efflux, rather than influx. Incubation in darkness results in stomatal opening, whereas potassium ions induce closure. Stomatal opening is also dependent on the calcium ion concentration in the apoplast. Jarvis and Mansfield (1980), showed that potassium and sodium selectivity depended on calcium presence. The role of calcium as a secondary messenger seems likely (DeSilva *et al.*, 1985), regulating ionic fluxes in the guard cells. Inoue and Katoh, (1987) suggest that calcium is

inhibitory to proton activity which transfers ATPase out of the guard cells, in which case opening would be impeded.

Interactions between potassium and sodium in stomatal movement.

Sodium appears to inhibit potassium uptake. This is evident in root absorbtion, where these two ions compete for carrier sites on the root membranes. It is probable that sodium has a similar disruptive effect in the chemi-osmotic mechanism discussed earlier in this section.

The effect of sodium potassium interaction on plant growth and stomatal movement is discussed taking into account the values obtained for sodium and potassium content of plant tissues. Results on plant growth show that plant weights decrease with increasing sodium content above 10 mM. There is a slight stimulation of shoot growth with 10 mM NaCl, at 50 and 100 mM growth is significantly reduced. Root growth was severely impeded at the highest concentration. Stimulation of shoot growth in rice by low concentration sodium has been correlated with increases in proline activity (Kavi Kishor, 1988). Sodium ions have a detrimental effect on many metabolic processes. In many plants sodium is mainly accumulated in the roots, however at high concentrations some transport to the shoot is inevitable, and damage to chloroplasts and interference in stomatal function results, leading to reduced plant growth and development.

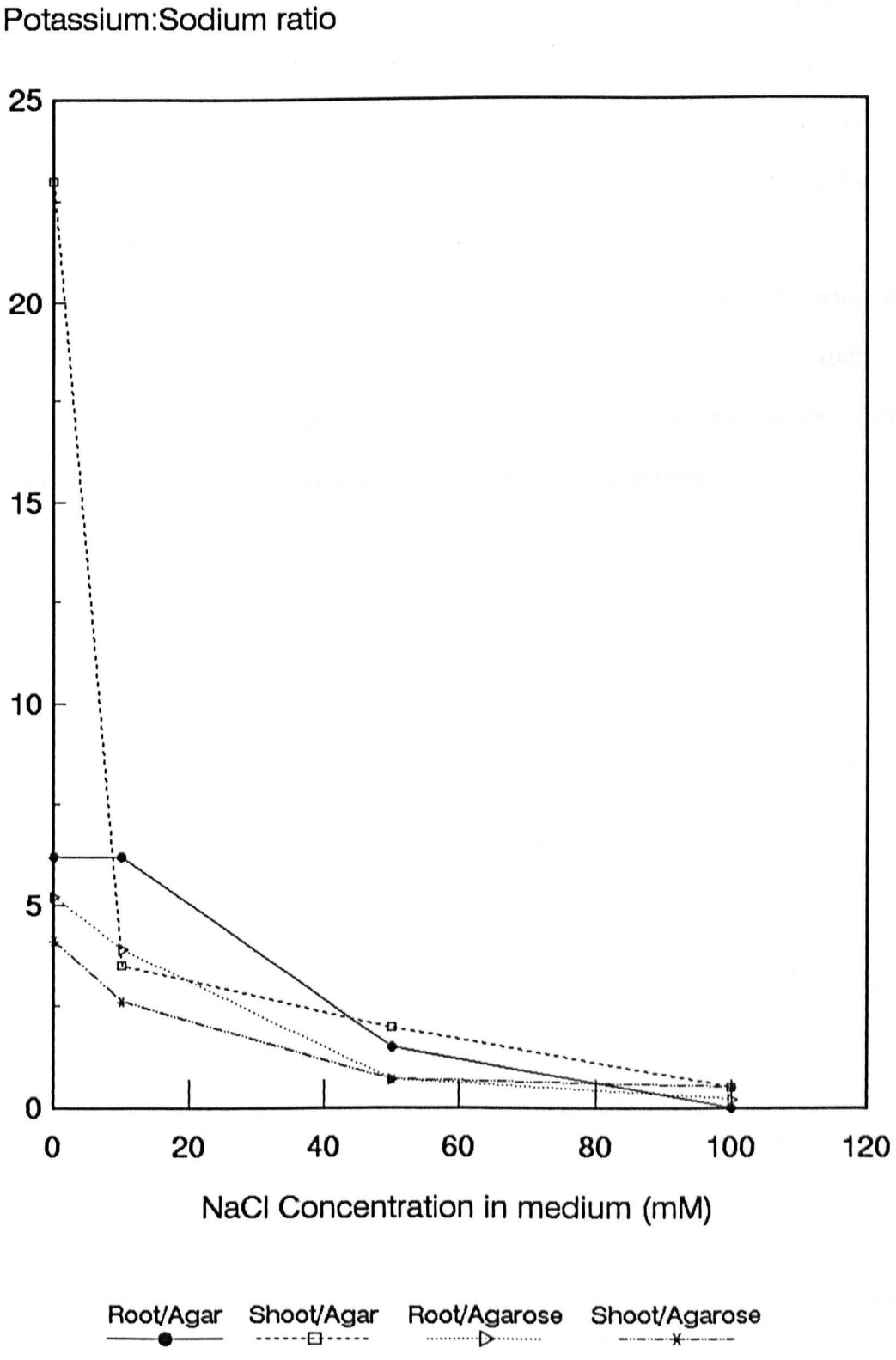
High concentrations of sodium and potassium were detected in the culture media, higher than in basal MS medium. The increased levels are most probably from the gelling agents. Difco-bacto agar proved to a major source of sodium , accounting for a 90-100 mM increase in sodium concentration. Debergh (1983), found an

increased level of potassium (1-2 %), and sodium (25 %) in media solidified with agar. These results show that agar is not the best gelling agent to use in tissue culture, although it may be the most economical.

The potassium content of shoot tissue grown on agarose solidified medium was stable between 16 and 21mg K g^{-1} Dwt (normally values represent 2-4% Dwt). Higher potassium levels were recorded in tissue from agar cultures, but a decrease in potassium content was observed as the NaCl content of the medium increased. The concentration of potassium in root tissue was also fairly stable ranging from 1.92 - 3.54 % Dwt. Much greater variation was observed in the sodium content of the shoot tissues, showing increased accumulation as the medium concentration was increased. Initially a slight rise, but followed by a pronounced increase at the higher NaCl treatments (50 and 100 mM). Root concentration of sodium follow a similar trend, and are higher in the agar cultured roots.

The decreasing K^+/Na^+ ratio, with increasing NaCl concentration (Figure 4), has also been reported in *Brassica* seedlings (Wardle *et al.,* 1981), and in apple and pear plants (Singha *et al.,* 1985), grown *in vitro*. This study on *Musa*, has demonstrated that although higher levels of sodium are found in roots, limited sodium retention by the roots may occur from competition for transport and binding sites in pathways to and from the stele (Jacoby, 1964, reported in Gauch, 1972). The concentrations of sodium detected in the *Musa* shoots, may cause the disruption in stomatal function reported.

Figure 4: Potassium:Sodium ratio in root and shoot tissue from *Musa accuminata* plants following culture on agar or agarose solidified medium with 0 mM, 10 mM, 50 mM or 100 mM NaCl


Potassium:Sodium ratio
25
20
15
10
5
0
0
20
40
60
80
100
120
NaCl Concentration in medium (mM)
Root/Agar
Shoot/Agar
Root/Agarose
Shoot/Agarose

Future work

Further work should be pursued to evaluate fully the effect of high calcium concentrations, present in many agar brands on inhibition of stomatal opening. A more detailed study of the structure of the guard cell walls in these plants, would improve our understanding of how abnormal formation contributes to stomatal malfunction. This study was limited by comparison of only two gelling agents, the plantgar (Flowlabs) was not used as it failed to set following autoclaving.

Epidermal strips may not give the most accurate assessment of potassium movement, as the mechanical damage to surrounding cells may induce production of metabolic inhibitors in respect to guard cell function (Willmer *et al.,* 1987). More accurate results may be achived using alternative techniques.

Appendix II

MURASHIGE AND SKOOG MEDIUM

	Concentration (mgl^{-1})
$CaCl_2.2H_2O$	440.0
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNa EDTA	36.70
H_3BO_3	6.20
KH_2PO_4	170.0
KI	0.83
KNO_3	1900.0
$MgSO_4.7H_2O$	370.0
$MnSO_4.4H_2O$	22.3
$Na_2MoO_4.2H_2O$	0.25
NH_4NO_3	1650.0
$ZnSO_4.7H_2O$	8.60
Inositol	100.0
Nicotinic Acid	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine	2.00

Appendix II

BASIC GROWTH MEDIUM (BM)

	Concentration
M & S	4.7 gl^{-1}
6-Benzylamino purine	1.5 μgl^{-1}
Sucrose	30.0 gl^{-1}

Appendix II

EXPERIMENTAL GROWTH MEDIA

500 mls BM + 0 mM NaCl + Difco Bacto Agar 8 gl^{-1}

500 mls BM + 0 mM NaCl + Agarose 7 gl^{-1}

500 mls BM + 10 mM NaCl + Difco Bacto Agar 8 gl^{-1}

500 mls BM + 10 mM NaCl + Agarose 7 gl^{-1}

500 mls BM + 50 mM NaCl + Difco Bacto Agar 8 gl^{-1}

500 mls BM + 50 mM NaCl + Agarose 7 gl^{-1}

500 mls BM + 100 mM NaCl + Difco Bacto Agar 8 gl^{-1}

500 mls BM + 100 mM NaCl + Agarose 7 gl^{-1}

Appendix II

Modified ARC Solution

	concentration (mg l^{-1})
$Ca(No_3)_2\ 4H_2O$	1771.1
$MgSo_4\ 7H_2O$	493.0
$NaH_2PO_4\ 2H_2O$	156.0
Fe EDTA	31.0
K_2SO_4	17.4
HBO_3	2.8
$MnCl_2$	1.8
$CuCl_2$	0.05
$ZnCl_2$	0.11
Na_2MoO_4	0.03

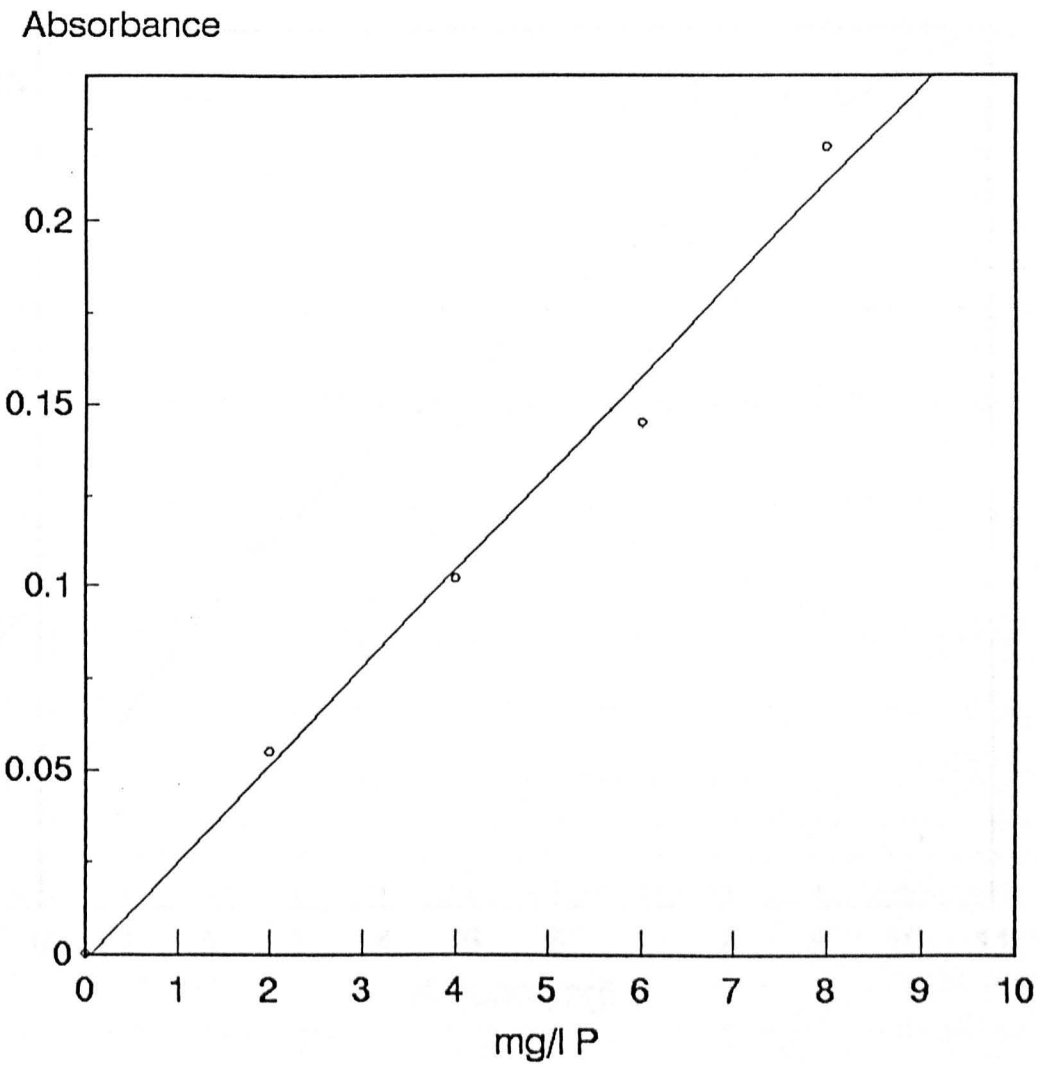
pH adjusted to 5.6

Appendix III

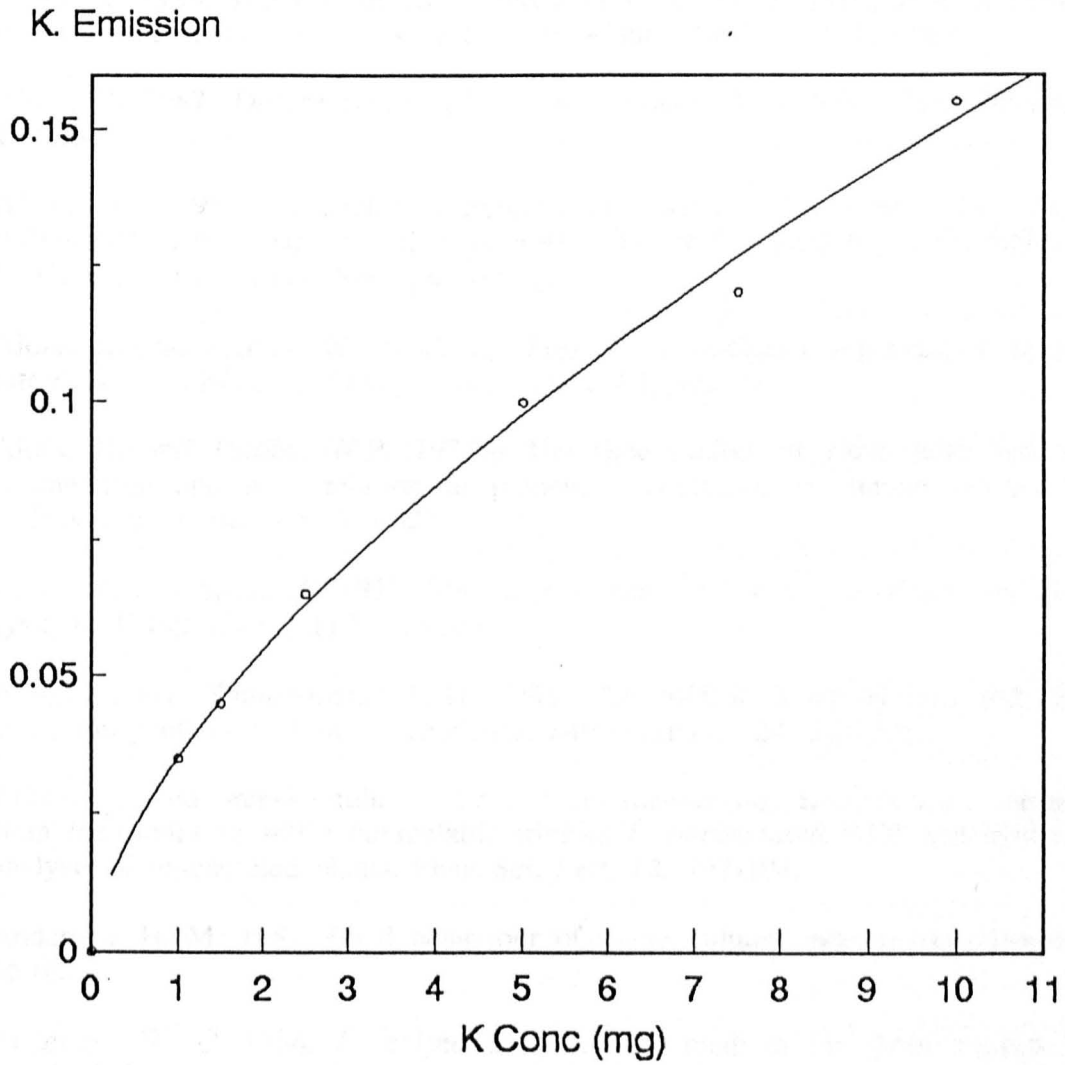
ATOMIC ABSORPTION SPECTROPHOTOMETER SETTINGS

a)	Potassium:	λ = 767
		Gain = 7
		Slit width = 4
b)	Sodium	λ = 589
		Gain = 8
		Slit width = 3

Phosphate calibration curve



Potassium standard curve



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