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Upon Root Induction of *Eucalyptus marginata* donn EX SM. In  
Vitro**

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**EFFECT OF CARBOHYDRATE SOURCE, AUXIN AND ENDOGENOUS  
ETHYLENE UPON ROOT INDUCTION OF *EUCALYPTUS MARGINATA*  
DONN EX SM. *IN VITRO***

**BY**

**MEREDITH MARGARET FAIRBANKS**

**A Thesis Submitted in Partial Fulfilment of the  
Requirements for the Award of**

**Bachelor of Science (Biological Science) Honours**

**at the Faculty of Science, Technology and Engineering, Edith Cowan University**

**Date of Submission: 7th June, 1996.**

## USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

## ABSTRACT

The utilisation of eucalypts around the world is increasing, mainly through the development of plantations. Clonal propagation has become important in some countries for production of such plantation trees. Micropropagation has been utilised to produce clones of trees selected for specific characteristics such as disease resistance, salt tolerance and fast growth rates. However, a suitable micropropagation protocol for all eucalypts has not been produced. One component of the micropropagation protocol, in which there is considerable difficulty, is the induction of adventitious roots on micropropagated shoots. Of particular interest, is the development of these procedures for *Eucalyptus marginata* (jarrah) that have been selected for dieback (*Phytophthora cinnamomi* Rands.) resistance.

The effect of different sugar sources was examined on the rooting of jarrah shoots. Sucrose, glucose and fructose were all effective in promoting roots on jarrah *in vitro*. The effectiveness of each sugar varied between clones. In particular, three clones produced higher rooting on a medium containing fructose. For two of these clones the increase was as high as 30%.

Interactions between auxin, sugar and ethylene were examined. Optimum root induction was obtained when approximately 10  $\mu\text{M}$  of auxin (indole butyric acid) and 2% sugar (sucrose or fructose) was used in the medium. As auxin and sugar concentration in the medium increased, the amount of ethylene produced also increased. Similarly, this increase in auxin, sugar and ethylene reduced the chlorophyll

content of the shoots. The use of fructose appeared to produce lower amounts of ethylene than sucrose when used at the same concentration. The ethylene produced seemed to have no effect on the rooting response.

The increases in rooting provided in some clones may be applicable to other clones that are difficult to root. This may lead to more efficient micropropagation with more clones being able to be produced in large numbers. This will increase the genetic diversity of clones that is currently available for jarrah breeding programs.

## DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signature \_\_\_\_\_

Date 3/6/96

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# CHAPTER ONE

## INTRODUCTION

### 1.1 EUCALYPTS

The genus *Eucalyptus* dominates the native vegetation (mainly open woodland) of more than half of Australia, where over 500 species have been described. The forty-one million hectares of Australian eucalypt forest provides a great variety of wood products. Juvenile trees provide pulpwood, charcoal and fuelwood, poles, mining timber and fibreboard. Mature trees provide strong and durable wood used for high-quality sawn timber for furniture and joinery, construction and railway sleepers. Eucalypts of all ages and sizes are capable of producing volatile oils for pharmaceutical and industrial uses (Eldridge *et al.*, 1993).

Eucalypts are also the most widely planted hardwood trees in the world and can be grown in most tropical and temperate regions. There are over six million hectares of eucalypt plantations in these regions throughout the world (Eldridge *et al.*, 1993). In Australia there are just over 100,000 hectares of eucalypt plantations (Stephens *et al.*, 1993).



## 1.2 CLONING

The use of clonal propagation is important in the control of genetic variation and maintenance of desirable gene pools. Clonal propagation allows the gene pool to be maintained as the daughter plants are genetically identical to the mother plant (Mehra-Palta, 1982; Haissig *et al.*, 1992).

Eucalypts can be cloned by a variety of techniques including; cuttings, graftings, micropropagation, callus cultures, organ cultures, suspension cultures, protoplast cultures and somatic embryogenesis (Le Roux and van Staden, 1991). This enables desirable genotypes, such as high quality pulp, salt tolerance, frost tolerance, oil yield and disease resistance (for example, resistance to *Phytophthora cinnamomi*, jarrah dieback) (Le Roux and van Staden, 1991; McComb *et al.*, 1996) to be used in a range of purposes.

When using conventional cuttage, or tissue culture, one of the limiting factors of cloning is the inability to induce adventitious root formation (Haissig *et al.*, 1992). It is, therefore, essential to develop propagation methods that maximise adventitious root formation. This enables genetically elite material to be fully exploited. Micropropagation is a method of clonal propagation that has shown considerable promise for utilisation of small scale plantings such as in seed orchards.

### 1.3 MICROPROPAGATION

Plant tissue culture, in general terms, refers to the growth of plant tissues on a nutrient medium in which a range of plant developmental processes can occur (Kantha, 1982). Many methods of tissue culture are currently employed for plant propagation: (1) Regeneration from callus and/or protoplasts - this procedure uses hormonal combinations to generate the formation of growing shoots from unorganised masses of parenchymatous tissue (callus). Shoots are then treated with auxin to promote root growth (resulting in a clonal propagule or plantlet) and transferred to soil; (2) Organ cultures - of roots, lignotubers, anthers, microcuttings and shoot tips; (3) Suspension cultures - from callus with cytokinin, nodular aggregates formed from single cells in liquid culture, restabilised as friable callus cultures on agar medium; (4) Protoplast cultures - protoplasts are isolated and cultured onto solid medium which proliferates into callus. This technique can be used for the selection and propagation of frost resistant variants (Le Roux and van Staden, 1991); (5) Somatic embryogenesis - embryos arise from callus; and (6) Micropropagation - growing shoots are exposed to media that stimulates axillary bud growth, and consequently shoot multiplication (Le Roux and van Staden, 1991). The shoot is then transferred to a root-promoting medium, adventitious roots are produced and genetically identical plantlets result. Micropropagation is the preferred mode of production of clones as it is generally less likely to lead to genetic variants, often referred to as somaclones (Ahloowalia, 1986; Bajaj *et al.*, 1986).

### 1.3.1 Stages of Micropropagation

Micropropagation involves a number of stages; the isolation of the desirable plant characteristics, shoot multiplication, root induction, and acclimatisation (Biondi and Thorpe, 1981; Krikorian, 1995).

#### Shoot Multiplication

Stem tips and lateral buds are the most commonly used starting material. Bud production or precocious branching is increased by the application of hormones. This is generally achieved by increasing the level of applied cytokinins. Depending upon the type of shoot characteristics desired, pulsing - the application of cytokinin between subcultures, may be applied to modulate branching patterns. A carbon source and nutrients are also supplied. The carbon, usually in the form of sucrose, acts as an energy source (Krikorian, 1995).

Shoots are often required to remain on the shoot multiplication media to become stabilised, before rooting is possible. Stabilisation periods varies with the species and age of the plants, with older plant material often requiring longer stabilisation periods (Bennett *et al.*, 1994).

## Root Induction

Either single shoots or branched shoots, from stabilised shoot cultures, are placed on rooting media. The media is composed of auxins to stimulate roots, macro- and micronutrients supplied at lower levels than applied to shoot multiplication, and usually sucrose to act as an energy source (McComb and Bennett, 1986; Krikorian, 1995).

Factors affecting root induction include the composition of the media, species of the plantlet - whether it is referred to as a hard-to-root species, the age of the plantlet - juvenile plantlets tend to root better than older plant material, stabilisation periods, and clonal variation - within clones the ease of rooting varies and consequently the media composition may have to be altered (McComb and Bennett, 1986; Bell *et al.*, 1993).

## Acclimatisation

Acclimatisation refers to the transfer of the plantlet from tissue culture to soil. Plants are required to be established in the soil (ie- resume or start photosynthesis, and grow in the absence of supplied nutrients, vitamins, sucrose and hormones). Transplanting of plantlets raised *in vitro* typically requires prolonged regulation of both temperature and relative humidity to allow acclimatisation of plantlets to glasshouse conditions (Grout and Millam, 1985; Krikorian, 1995).

Major problems commonly experienced with transplanting are an inability of plantlets to regulate the transpiration stream and insufficient photosynthesis capability to achieve a positive carbon balance (Grout and Millam, 1985). This is due to poor *in vitro* plant development and poor photosynthetic performance. These problems may be overcome by manipulating the photosynthetic capacity of plants by decreasing medium carbohydrate concentration, or by increasing growth irradiance (Lees, 1994; Ziv, 1995).

### 1.3.2 Environmental Conditions Affecting Micropropagation

An advantage of *in vitro* culture is that both the physical and chemical environmental factors of the culture medium can be predetermined and altered throughout the culture growth cycle. The physical environmental conditions include headspace temperature, incident light at the culture surface (irradiance, spectra and photoperiod), and air movement. The chemical environmental factors of *in vitro* growth include pH and the composition of the medium (Kozai and Smith, 1995).

For successful micropropagation high light transmittance, isolation from water loss and contamination, satisfactory gas permeability, constant air temperature, and the provision of an adequate growing area are characteristics of a suitable culture vessel (Tanaka *et al.*, 1992; Kozai and Smith, 1995; Smith and Spomer, 1995).

Ventilation of tissue culture vessels is essential to ensure that poor aeration, which can be damaging to growth, does not occur. Air movement within the culture headspace can range from very nearly stagnant (tightly wrapped closures) to independently-circulating (vessels and closures with forced ventilation or supplementary introduced gases). The gaseous environment *in vitro*, and the quality of the micropropagated plants can be significantly affected by closure around vessel caps (Jackson *et al.*, 1991). Studies have shown, that limited air exchange favours the early initiation stages in culture. Secondary metabolite *in vitro* production, however, is decreased under conditions of low gas exchange (tightly-sealed caps) due to ethylene accumulation (Smith and Spomer, 1995). Vessels with high condensation, due to greater relative humidity (ie those sealed with parafilm, and therefore have a reduced gas exchange) can lead to flaccid and often hyperhydric shoots (McClelland and Smith, 1990). Hussey and Stacey (1984) reported that restriction of gas exchange between the culture vessel and the external atmosphere inhibits tuberizing and potato nodes develop poor shoots (small leaves), effects which appear to be due mainly to ethylene build up.

### 1.3.3 Uses of Micropropagation

Micropropagation is used to produce as many identical copies (clones) of plants as desired within a short period of time. It has several advantages over conventional cultivation of whole plants. These include; very rapid multiplication, all year round production, greater

physiological manipulation to improve responses and the multiplication of disease free material (Crocomo *et al.*, 1981; George, 1993).

Apart from commercial propagation for the production of large numbers of clones, micropropagation has been utilised in the following areas; seed orchards; progeny testing; conservation of rare, endangered or slow reproducing species, and the multiplication of desirable genotypes (such as pathogen resistant) (Biondi and Thorpe, 1981; George, 1993).

#### 1.4 SUGARS IN MICROPROPAGATION

Carbohydrates are essential for plant growth and are the primary energy-storage molecules in most living things. They serve as energy stores, fuels and metabolic intermediates and supply energy by the oxidation of their constituent elements (Candy, 1980; Walker, 1989).

Sugars, a group of carbohydrates, are separated into groups according to the number of carbon atoms and bond arrangements (Candy, 1980; Smith, 1993). Included are the monosaccharides such as glucose and fructose, the disaccharides such as sucrose, and the polysaccharides and sugar alcohols such as sorbitol.

#### 1.4.1 Sucrose *in situ*

Sucrose occurs in nearly all organs of plants and accumulates to high concentrations in a number of organs of some species (Hawker, 1985). It is the principal form of translocated carbon and it is the main storage sugar in plants (ap Rees, 1984).

Sucrose is synthesised by the transfer of glucose from uridine diphosphate glucose (UDP-glucose), an activated form of the sugar, to fructose 6-phosphate forming sucrose 6-phosphate which is then hydrolysed to sucrose (Smith, 1993).

#### 1.4.2 Sugars *in vitro*

Most sterile cultures are unable to support photosynthesis, so an exogenous carbohydrate is required (Wareing and Phillips, 1981; George, 1993). Findings have concluded that the best carbon source for plant growth *in vitro* is sucrose, followed by glucose, maltose and raffinose; with fructose, mannose and lactose less effective. Glucose in a few species results in better *in vitro* growth than sucrose. These findings may not necessarily apply to micropropagation of eucalypts, as plant species vary in their ability to utilise sugars (Damiano *et al.*, 1987; George, 1993).

Sugars (usually in the form of sucrose) are required for cellular metabolism and therefore regulate shoot and root growth and influence adventitious root formation providing energy



for these processes (Sacher, 1966; Jeffs and Northcote, 1967; Hyndman *et al.*, 1982; Capellades *et al.*, 1991). Root initiation requires a continuous supply of sugar from the medium for vigorous growth (ap Rees, 1984; Hawker, 1985; Cheng *et al.*, 1992; George, 1993).

#### Utilisation of Sucrose in Micropropagation

Sucrose is a non-reducing disaccharide which is either hydrolysed or taken up *in vitro* unaltered depending on plant species. When sucrose is hydrolysed it is broken down into glucose and fructose. In whole plants complete hydrolysis occurs 16 hours after a plant takes up sucrose, and thus carbohydrates are then in the form of glucose and fructose (Sacher, 1966).

Photosynthetic capability of micropropagated plantlets is influenced by the presence of sucrose in the culture medium (Hdider and Desjardins, 1994). Sucrose in micropropagation media has been found to inhibit chlorophyll formation and photosynthesis, making autotrophic growth less feasible (Rier and Chen, 1964; Edelman and Hanson, 1972). Hdider and Desjardins (1994) found that for *in vitro* strawberry plantlets, problems arose during acclimatisation, because of the presence of high concentrations of sucrose in the media which resulted in poor photosynthetic apparatus development.

### 1.4.3 Use of Sugars Other than Sucrose

While sucrose is the most common carbohydrate source used in plant tissue culture, some plants can metabolise other sugars more efficiently (Mac An tSaoir and Damvoglou, 1994). Sugars such as sorbitol, glucose and fructose have been investigated for their potential as effective growth promoters for a wide variety of species *in vitro* (Hew *et al.*, 1988; Marino *et al.*, 1993; Taji and Yuehua, 1994; Romano *et al.*, 1995). Romano *et al.* (1995) found that sorbitol (D-glucitol), a sugar alcohol, is the most effective carbon source for *in vitro* proliferation of some apple rootstocks. For apricot (*Prunus armeniaca* L.), sorbitol in combination with fructose, has been found to produce high proliferation rates and longer shoots than other carbohydrates including sucrose (Marino *et al.*, 1993).

### 1.5 HORMONES IN MICROPROPAGATION

Micropropagation most often requires the application of exogenous plant growth regulators that are essential for shoot multiplication and adventitious root formation. The main plant growth substances which are added to micropropagation media are auxins and cytokinins (George, 1993). The effects of these hormones upon plantlet development is variable, depending upon the concentration and the environmental conditions under which the cultures are maintained.

### 1.5.1 Auxin

Auxin occurs naturally as indole-3-acetic acid (IAA) but in micropropagation, artificial alternatives (such as naphthalene acetic acid (NAA), and indole butyric acid (IBA)) are used, as IAA tends to be readily denatured in culture media and is often less effective (George, 1993).

Auxins are incorporated into media to promote the growth of roots and elongate shoots (Krikorian, 1995). Different tissues respond differently to the same concentration and the same type of auxin. Even anatomically identical tissues give different responses depending upon their age and other physiological states (Marumo, 1986). When choosing the type and concentration of auxin several aspects have to be considered. These include; the growth or development required, the endogenous levels of auxin within the explant, the capacity of the cultured tissues to synthesise auxin naturally, and the interaction between applied synthetic auxin and the natural endogenous substances (George, 1993).

### Shoot Multiplication

Auxins are readily taken up by shoots from the medium (Kateava *et al.*, 1991). They are required to stimulate elongation of excised stem sections in micropropagation by promoting activity of the vascular cambium (Marumo, 1986) and are also necessary for vein growth (Wareing and Phillips, 1981). Shoots can be maintained on shoot

multiplication media indefinitely with frequent (every 4 weeks) subcultures. Auxins, supplied in multiplication media, prolong the life of the shoot by maintaining rooting ability, by preventing abscission and aging of the shoot (Krikorian, 1995).

### Root Induction

Adventitious root formation may be divided into three phases, namely dedifferentiation, induction and differentiation with high levels of auxin and low levels of cytokinin required during the induction phase (De Klerk *et al.*, 1995). The promoting effect of auxins varies among species and cultivars, making it difficult to understand the mechanism of their regulatory action (Baraldi *et al.*, 1995). Auxins supplied at a certain level are necessary for adventitious root production (Fabijan *et al.*, 1981; Nordstrom *et al.*, 1991). The level required for optimal root production will depend upon a number of factors, most importantly the species, type of auxin supplied, and the physiological condition of the shoot material (Fabijan *et al.*, 1981; Marumo, 1986; Baraldi *et al.*, 1995).

IAA and IBA are used to induce root formation in micropropagation, with IBA being the most commonly used auxin (Krikorian, 1995). Auxin stimulates root induction but inhibits root elongation (Wareing and Phillips, 1981). Therefore to encourage root elongation plantlets are often transferred to auxin free medium after root induction has taken place (McComb *et al.*, 1996).

### 1.5.2 Cytokinins

Cytokinins are necessary to promote shoot formation. They promote cell-division and differentiation, and auxiliary bud stimulation, thereby enabling shoot multiplication to be obtained (Koshimizu and Iwamura, 1986). Cytokinins are generally supplied in the media in the forms of synthetic regulators such as 6-Benzylaminopurine (BAP) and kinetin (George, 1993). Cytokinins influence the development and establishment of bud elongation. This is generally achieved by the increasing the cytokinin level. However it may be preferable to select a lower multiplication rate by using low levels of cytokinin, as too rapid an increase may lead to genetic variations (Krikorian, 1995).

Cytokinins generally inhibit root production and thus are removed from the media prior to rooting in difficult-to-root species (Krikorian, 1995).

### 1.5.3 Use of Other Hormone Groups in Micropropagation

Other hormones which may be included in micropropagation to promote growth include gibberellins, abscisic acid (ABA) and ethylene. The types of hormones and levels applied to the tissue culture medium depends upon the type of growth required, the age and species of the plant material and the amount of endogenous hormones contained in the plant material (George, 1993).

The addition of gibberellins to micropropagation media is minimal. Gibberellins may be employed where plantlets are preferred to have a uniform single stem, such as forest trees. In these cases gibberellins are applied to promote stem elongation (George, 1993; Krikorian, 1995).

ABA is not considered useful for micropropagation as it slows growth and moderates the effects of cytokinins and auxins (George, 1993; Krikorian, 1995).

Ethylene effects on plants *in vitro* is generally viewed as inhibitory and thus precautions are taken to reduce its concentration (Krikorian, 1995). Further implementations of ethylene in micropropagation is discussed in chapter four.

## 1.6 MICROPROPAGATION OF *EUCALYPTUS*

Micropropagation techniques have the potential for use in the genetic improvement of *Eucalyptus* (Le Roux and van Staden, 1991). Large scale micropropagation of eucalypts was first achieved in the 1980's (Bennett and McComb, 1982; Mehra-Palta, 1982; McComb and Bennett, 1986). Since then many eucalypt species have been successfully cloned (Le Roux and van Staden, 1991; McComb *et al.*, 1996). However, for the *in vitro* propagation of *Eucalyptus*, there are difficulties in the establishment of a good general protocol. This is due to the variability between eucalypt species, different clones, and other

physiological variations that can occur within a species (Damiano *et al.*, 1987; Le Roux and van Staden, 1991; McComb *et al.*, 1996).

### Shoot Multiplication

Multiplication rates of eucalypt shoot cultures depends upon a number of aspects including the explant, the age of the material, and the individual's genotype. Shoot multiplication media is largely developed and used for juvenile explants, but can be applied to mature explants (McComb and Bennett, 1986).

### Root Induction

Stabilisation of shoot cultures may be required before rooting is possible. This applies to both juvenile and mature explants but has been best documented from mature material (McCown and McCown, 1987; Bennett *et al.*, 1994; McComb *et al.*, 1996). Examples of this include three to four subcultures for *Solanum tuberosum* (Fussey and Stacey, 1984) and ten to twelve months for *E. marginata* (Bennett *et al.*, 1994).

Root induction is maximal on media with limited or no vitamins, and contains lower concentrations of nutrients than that used in shoot multiplication (Mehra-Pelta, 1982; McComb *et al.*, 1996). The induction of adventitious roots is difficult in many eucalypt species including *E. marginata* (Bennett *et al.*, 1994). Shoots from juvenile explants of

eucalypts usually root well, but for shoots obtained from mature trees, rooting is minimal upon media optimised for juvenile shoots (McComb *et al.*, 1996).

## 1.7 JARRAH

*E. marginata*, a member of the Myrtaceae family (Morley and Toelken, 1983), grows in the southwest of Western Australia with a production area of about one to two million hectares. Jarrah is one of the most important hardwoods in Australia and is known throughout the world for its toughness and durability. In Western Australia it represents about two thirds of the annual production of sawn timber (Boland *et al.*, 1987; Kelly *et al.*, 1989). Jarrah is suitable for many purposes including poles, sleepers, furniture, and flooring. It is easily worked with and is resistant to termites (Kelly *et al.*, 1989).

### 1.7.1 Jarrah Dieback

*Phytophthora cinnamomi* is a fungal pathogen affecting plants of south-west Australia. The fungus kills its host by destroying the roots and girdling the base of the stem, depriving the plant of access to nutrients and water. The primary symptoms of infection include; advancing fronts of necrosis (lesions) in the inner bark of roots and stems, and root rot (Shearer, 1994).



Of the 9000 plant species occurring in the south-west of Australia, as many as 2000 species may be susceptible to *P. cinnamomi* (Hardy *et al.*, 1994). Therefore, it is essential to develop strategies that reduce the damage, by cloning resistant species, and therefore conserve natural ecosystems. Included amongst these strategies is micropropagation, which can be adapted to any species (Shearer, 1994). Considerable variation in resistance to dieback can be found within the genus *Eucalyptus*. Members of the subgenera *Symphomyrtus* and *Corymbia* are tolerant, whilst members of the subgenus *Monocalyptus*, including jarrah, are susceptible to the pathogen (McComb and Bennett, 1986). The resistant and susceptible stocks available may provide the pedigreed stocks necessary for the detection of molecular markers for *P. cinnamomi* resistance in jarrah (Shearer, 1994).

## 1.8 AIMS

The aims of the study are to: (1) determine the effects of various carbon sources on root/plantlet production and ethylene production of jarrah cultures; and (2) determine the effect of auxins on root/plantlet production and ethylene production of jarrah cultures.

## 1.9 HYPOTHESES

The hypotheses that were tested include

(1) Carbon source concentration in the root induction medium influences the number of roots and the condition of the resulting plantlet of *Eucalyptus marginata*.

- (2) Sucrose concentration in root induction medium influences endogenous ethylene production of shoots.
- (3) Auxin in root induction medium containing sucrose influences plantlet production.
- (4) Auxin in root induction medium containing sucrose influences ethylene production.

## CHAPTER TWO

### MATERIALS AND METHODS

#### 1.1 PLANT MATERIAL

Clones of *Eucalyptus marginata* were obtained from research projects being conducted in the Department of Applied Science, Edith Cowan University. These cultures had been maintained for over two years *in vitro* and were thus stabilised. Clones which proved to have fast shoot multiplication rates included: 1 JN 30, 1 JN 98, 5 JN 336, 11 JN 50, 11 JN 379, 12 JN 35, 12 JN 96, and 91 JP 4 and therefore were used in numerous experiments. These clones have been identified as having varying degrees of resistance to *Phytophthora cinnamomi* (McComb *et al.*, 1994).

#### 1.2 STERILE TECHNIQUE

For handling of shoot cultures, and consequently the setting up of root induction experiments, a sterile technique was followed. All components necessary for subculturing and rooting experiments were autoclaved prior to use in a Labec autoclave (Laboratory Equipment, Australia) at 100kPa for 15 minutes. This included all media, instruments used for the transfer and cutting of shoots, plastic cutting plates, and bottles of 70% ethanol.

Subculturing took place in a laminar flow workstation (Email Westinghouse Pty Ltd, Australia). Prior to use, the workstation was exposed to ultra-violet lights for thirty minutes to reduce aerial and surface contaminants. The cabinet was then sprayed with 70% ethanol. During subculturing instruments were regularly resterilised in a sterilising unit (Sigma-Aldrich, Australia).

### 1.3 MEDIA PREPARATION

Shoot multiplication medium was modified from Bennett and McComb (1982) and contained full strength Murashige and Skoog (1962) (MS) minerals and vitamins (catalogue number 5519, Sigma, Australia) with added naphthalene acetic acid, benzyl amino purine, sucrose, agar (2.5g/L), and gelrite (2.5 g/L) (Table 1.1). All medium components were dissolved in milli Q water. The pH of the medium was adjusted to 5.8 prior to the addition of gelling agents. Fifty millilitres of medium was dispensed into 250ml polycarbonate containers (Disposable Products Pty.Ltd, Australia) prior to autoclaving.

Basal rooting medium consisted of, one quarter strength MS macronutrients, full strength MS micronutrients, 10 $\mu$ M indole butyric acid, carbon source as required, agar (2.5 g/l), and gelrite (2.5g/L) (Table 1.1). The pH of the medium was adjusted to 5.5 prior to the addition of gelling agents. Murashige and Skoog micronutrients stock solution was prepared from the method described by de Fossard (1976).

Table 1.1 Components of standard shoot multiplication and root induction media. Stocks of hormones and nutrients were maintained at 4 °C until required, with occasional checking to ensure compounds remained in solution.

<u>Compound</u>	<u>Shoot Multiplication Media</u>	<u>Root Induction Media</u>
	<u>Amount/L of Medium</u>	<u>Amount/L of Medium</u>
NH <sub>4</sub> NO <sub>3</sub>	20 mM	5 mM
KNO <sub>3</sub>	20 mM	5 mM
CaCl <sub>2</sub> .2H <sub>2</sub> O	3 mM	0.75 mM
MgSO <sub>4</sub> .7H <sub>2</sub> O	1.5 mM	0.375 mM
KH <sub>2</sub> PO <sub>4</sub>	1.5 mM	0.375 mM
H <sub>3</sub> BO <sub>3</sub>	100 μM	100 μM
MnSO <sub>4</sub> .H <sub>2</sub> O	100 μM	100 μM
ZnSO <sub>4</sub> .7H <sub>2</sub> O	30 μM	30 μM
KI	5 μM	5 μM
Na <sub>2</sub> MoO <sub>4</sub>	1 μM	1 μM
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.1 μM	0.1 μM
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.1 μM	0.1 μM
FeSO <sub>4</sub>	100 μM	
FeNa.EDTA	100 μM	25 μM
Naphthalene Acetic Acid (NAA)	1.25 μM	
Benzyl Amino Purine (BAP)	2.5 μM	

Indole Butyric Acid (IBA)		10 $\mu\text{M}$
Sucrose	20 g	20 g
agar	2.5 g	2.5 g
phytagel	2.5 g	2.5 g
pH adjusted	5.8	5.5

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#### 1.4 CULTURE CONDITIONS

Shoot cultures were maintained in a room under a photoperiod of 16 hours with a light irradiance of approximately  $36 \mu\text{mol.s}^{-1} \text{m}^{-2}$ . Shoots were cut into segments containing two to three nodes and placed on the multiplication medium, and transferred to fresh media every four weeks (subcultured) for continued growth.

After substantial growth on shoot multiplication media, shoot clumps were cut into approximately 1 to 2 cm single shoots and transferred randomly onto root induction media. Five or 10 shoots per container were placed in complete darkness for seven days at  $25^{\circ}\text{C}$  and then transferred to a germination incubator (Clayson Laboratory Apparatus Pty. Ltd, Australia) at a constant temperature of  $25^{\circ}\text{C}$  with a light irradiance of approximately  $24 \mu\text{mol.s}^{-1} \text{m}^{-2}$ .

Culture vessels were spaced at approximately 3cm, allowing for air flow around the entire vessel. Culture vessels were regularly rearranged on the shelving and checked for contamination.

Roots were counted on day 11, 12, 13, 14, 21 and 28. The average number of roots per shoot and percentage of rooted shoots per culture vessel were calculated each day the roots were scored.

### 1.5 CHLOROPHYLL DETERMINATION

Chlorophyll content was determined using a technique modified from Moran and Porath (1980). After 28 days on root induction media, 1 to 2 shoots were randomly chosen from each culture vessel, and the fresh weight determined. Shoots were placed in 5 ml of Dimethylformamide (BDH, Australia) and left in the dark overnight. Absorbency was measured at 647nm and 664nm on a U-1100 spectrophotometer (Hitachi, Ltd., Japan).

Chlorophyll was calculated using:

Total chlorophyll (chlorophyll a and b) =  $(ABS_{664} \times 7.04) + (ABS_{647} \times 20.27)$  in  $\mu\text{g/ml}$

Chlorophyll per grams of fresh weight was calculated by:

Chlorophyll content  $\mu\text{g/ml}$  per fresh weight = Total chlorophyll  $\times 5$  / fresh weight (g).

The average chlorophyll content was taken for each treatment from a sample size varying from 6 to 14.

## 1.6 QUANTIFICATION OF ETHYLENE

After 28 days gas samples were removed from culture containers using a 5ml gas-tight syringe and needle (Hamilton, Australia). Quantification of ethylene was accomplished using a gas chromatograph (GC) (Varian Australia Pty. Ltd) in combination with a flame ionisation detector (FID) and a packed column (stainless steel, Altech Association, Australia). Identification of ethylene was based on GC retention time and on elimination of the corresponding GC peak (Yoshii and Imaseki, 1982; Adkins, 1992; Hedden, 1993). As standard ethylene samples supplied were faulty and could not be replaced in time for the quantification of experiments, individual peak areas (area under the ethylene peak) were recorded and averages for each treatment calculated. All gases, Helium (carrier gas), Hydrogen and air, were supplied by BOC Gases, Australia.

## 1.7 DATA ANALYSIS

Results are expressed as the mean and standard error of the mean (SEM) for all the variables studied. For any results using percentages, the arcsin percentage transformation was used. Means were compared by two-way and one-way analysis of variance. Where differences were obtained due to experimental treatments, Duncan's new multiple range



test (Duncan, 1955) was applied with a significance level of 95%. Correlation coefficients and regressions were applied to parameters such as chlorophyll content, number of roots produced per shoot, ethylene concentration, auxin concentration and sucrose concentration.

## CHAPTER THREE

### EFFECTS OF CARBON ON ROOT INDUCTION

#### 1. INTRODUCTION

There are numerous reports on the effect of sugar, as a carbon source, upon shoot proliferation and root induction (Hildebrandt and Riker, 1953; Okonkwo, 1966; Hyndman *et al.*, 1982; Steffen *et al.*, 1988; Cheng *et al.*, 1992; Marino *et al.*, 1993; Taji and Yuehua, 1994; Romano *et al.*, 1995). Variations in these reports include; the type of sugar, concentration of sugar, and techniques used for the sterilisation of media.

Hildebrandt and Riker (1953) experimented with sucrose in callus tissue and found a concentration of 2% produced optimum growth (greatest increase in weight). Roots from callus grew progressively better on media through concentration from 0, 0.5, 1, 2, 3, and 4% sucrose while 5 and 6% produced no further increase in root length. These levels have been routinely incorporated into a large number of media formulations for different species. The optimum concentration for growth usually ranges from 2-5% (Okonkwo, 1966; Hyndman *et al.*, 1982; Cheng *et al.*, 1992; Hdider and Desjardins, 1994).

Stehsel and Caplin (1969) found that the best growth (fresh weight of carrot tissue) occurs on medium containing sucrose, with glucose second best and fructose was least effective.

They found that autoclaving the culture media produced positive and negative effects on both callus and organ growth. The magnitude of the effect was dependent on the type of sugar used.

Steffen *et al.* (1988), experimented with differing sugars and the effect of filter and autoclave sterilisation upon floret initiation of *Bougainvillea*. Their results differed from those of Stehnel and Caplin (1969), as floret initiation using different sugars was the same whether the media was filter sterilised or autoclaved. In addition sucrose and fructose were found to have similar effects on growth and development of young leaves.

An experiment on *Rosa* plantlets by Taji and Yuehua (1994), found that a concentration of 120 mM sucrose, glucose, or fructose, produced more roots per plantlet and higher number of roots compared with those of 6 mM and 60 mM. Higher concentrations resulted in better general appearance and growth and greater fresh weight. Higher sugar concentration, irrespective of type, enhanced root formation and survival of *Rosa* plantlets when transferred to soil.

Recent studies comparing sucrose with glucose and fructose have found that the most effective sugar varies with species and response required (Hew *et al.*, 1988; Romano *et al.*, 1995). Glucose is the most effective carbon source in relation to root promotion of cork oak (*Quercus suber*). Plantlets rooted on 4% glucose were found to have a significantly higher chlorophyll content and higher rooting (Romano *et al.*, 1995). Fructose has been

found to have various effects depending on plant species, and is the sugar most rapidly utilised by orchid meristem tissue (Hew *et al.*, 1988). Sucrose at a concentration of 3 percent was found to be the best carbon source during shoot proliferation and elongation of *Dendrobium* meristem tissue. At higher concentrations, these sugars limited root extension, but increased the number of roots produced.

Cheng *et al.* (1992), investigated the effects of different levels of sucrose and auxin to identify the optimal combinations for rooting and plantlet formation for *Eucalyptus sideroxylon*. Regardless of auxin type and explant type used, it was found that sucrose concentrations of 2-6% favoured root development. Higher concentrations of 8-10% lowered rooting percentage, inhibited callus formation and suppressed explant growth during culture.

The methods reported for the tissue culture of jarrah have all utilised sucrose as the sugar source (Bennett and McComb 1982; McComb and Bennett 1982; McComb *et al.*, 1996). As several studies have shown that sucrose is not always optimal, other sugars and their effects on plantlet production were investigated in this study.

## 2. MATERIALS AND METHODS

### 2.1 Experiment One: Root Induction Using Different Concentrations of Sucrose

Shoots of clones 5 JN 336 and 91 JP 4 were randomly placed on media with four sucrose concentrations, 0, 1, 2, or 4%. Treatments contained 5 shoots per culture container and were replicated 7 times. Mean rooting percentage and mean number of roots per shoot were scored progressively up to 28 days after the transfer of shoots to rooting media. Chlorophyll content was determined after day 28.

### 2.2 Experiment Two: Root Induction Using Different Sugars

Shoots of clones 1 JN 30 and 12 JN 96 were placed on root induction media containing treatments of 2% sucrose (CSR, Australia), 2% analytical grade sucrose (Ajax chemicals, Australia), 2% glucose (BDH chemicals, Australia), 2% fructose (Ajax chemicals, Australia) or 2% sorbitol (Sigma, Australia). Treatments contained five shoots per container with 7 replicates for clone 1 JN 30, and 8 replicates for clone 12 JN 96. Mean rooting percentage and mean number of roots per shoot were scored progressively up to 28 days after the transfer of shoots to rooting media. Chlorophyll content was determined after day 28.

### 2.3 Experiment Three: Root Induction Using Different Concentrations of Fructose

Shoots of clones 1 JN 30 and 91 JP 4 were placed on media containing concentrations of 0, 1, 2, or 4% fructose and compared with 2% sucrose. Treatments contained five shoots per container, with 6 to 9 replications. Mean rooting percentage and mean number of roots per shoot were scored progressively up to 28 days after the transfer of shoots to rooting media. Chlorophyll content was determined after day 28.

### 2.4 Experiment Four: Root Induction Using Different Combinations of Fructose and Glucose

Shoots of clone 5 JN 336 were placed on media containing 2% sucrose, 2% glucose, 2% fructose and varying ratios of glucose and fructose; 1glucose:1fructose (1% each), 3glucose:1fructose (1.5%:0.5%) and 1glucose:3fructose (0.5%:1.5%). There were eight replicates, 5 shoots per replicate, for each treatment. Mean rooting percentage and mean number of roots per shoot were scored progressively up to 28 days after the transfer of shoots to rooting media. Chlorophyll content was determined after day 28.

## 3. RESULTS

Roots generally appeared after 11 days on rooting medium. Rooting increased up to day 21 and generally remained at this level to day 28. In most cases the mean number of roots

per shoot were equivalent to the mean rooting percentage and therefore only the mean rooting percentage is presented. Mean rooting percentage was used as the number of roots per shoot has often been shown not to display a normal distribution when the non-rooted shoots are included (De Klerk *et al*, 1995).

### 3.1 Experiment One

The different levels of sucrose induced varying amounts of rooting with the absence of sucrose being significantly lower ( $P < 0.05$ ) than all other treatments (Figure 3.1 A). In addition, there was a difference in rooting between the two clones with 91 JP 4 producing significantly fewer roots ( $P < 0.05$ ).

For clone 5 JN 336 1, 2 and 4% produced the same percentage rooting, but for 91 JP 4, 2 and 4% sucrose was significantly higher ( $P < 0.05$ ) than both 0 and 1%. The chlorophyll content was not affected by the different media (Figure 3.1B).

### 3.2 Experiment Two

The different sources of sugar produced a variation in rooting, with fructose having at least 30% higher rooting ( $P < 0.05$ ) than any of the other treatments for clone 1 JN 30. There was a difference in rooting between the two clones with 12 JN 96 producing significantly fewer roots ( $P < 0.05$ ). No rooting was obtained for either clone on sorbitol medium and

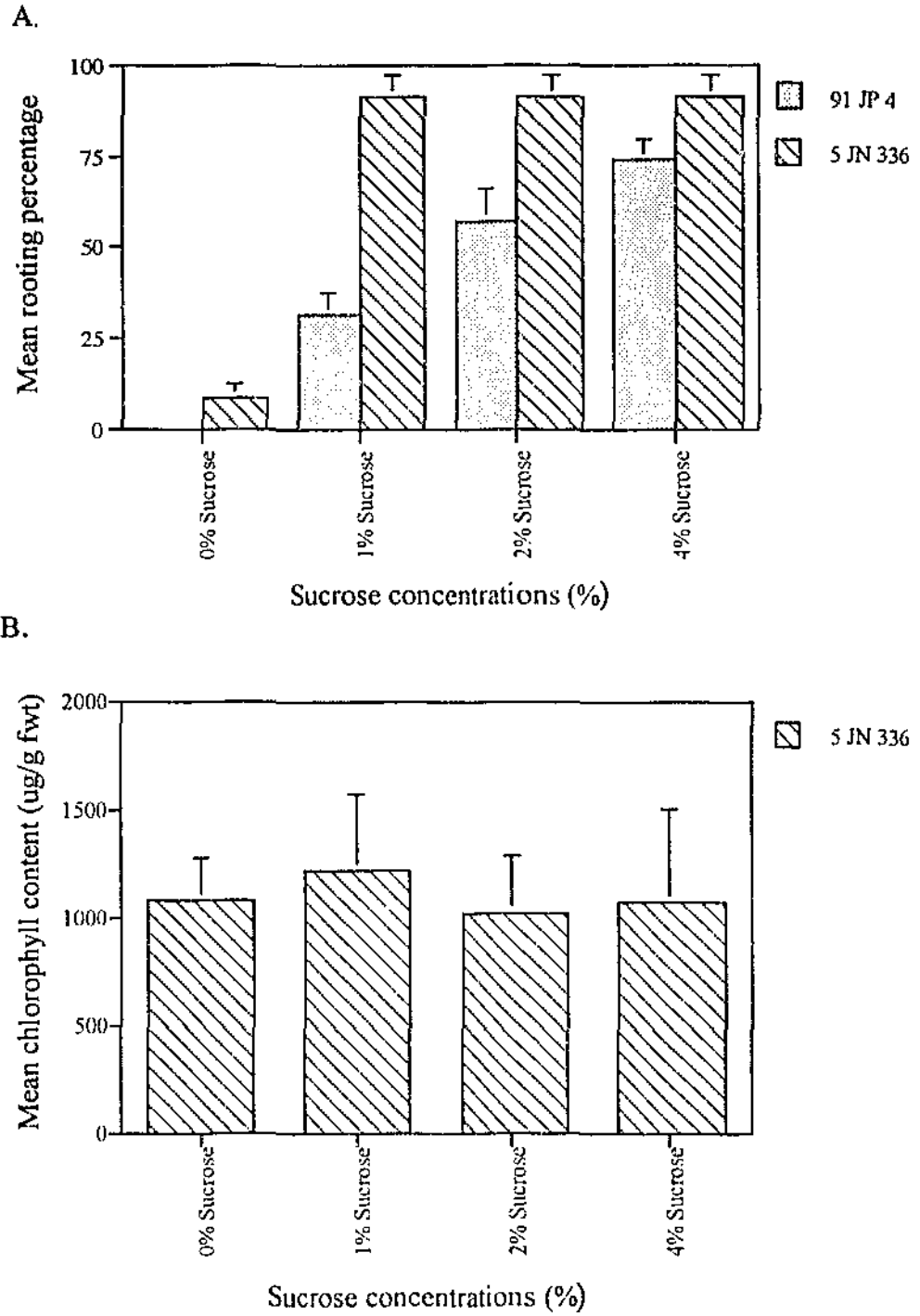


Figure 3.1 Rooting response of 91 JP 4 and 5 JN 336 to different sucrose concentrations. A. Mean rooting percentage (7 replicates) and, B. Chlorophyll content (14 replicates), after 28 days. Vertical bars denote SEM.



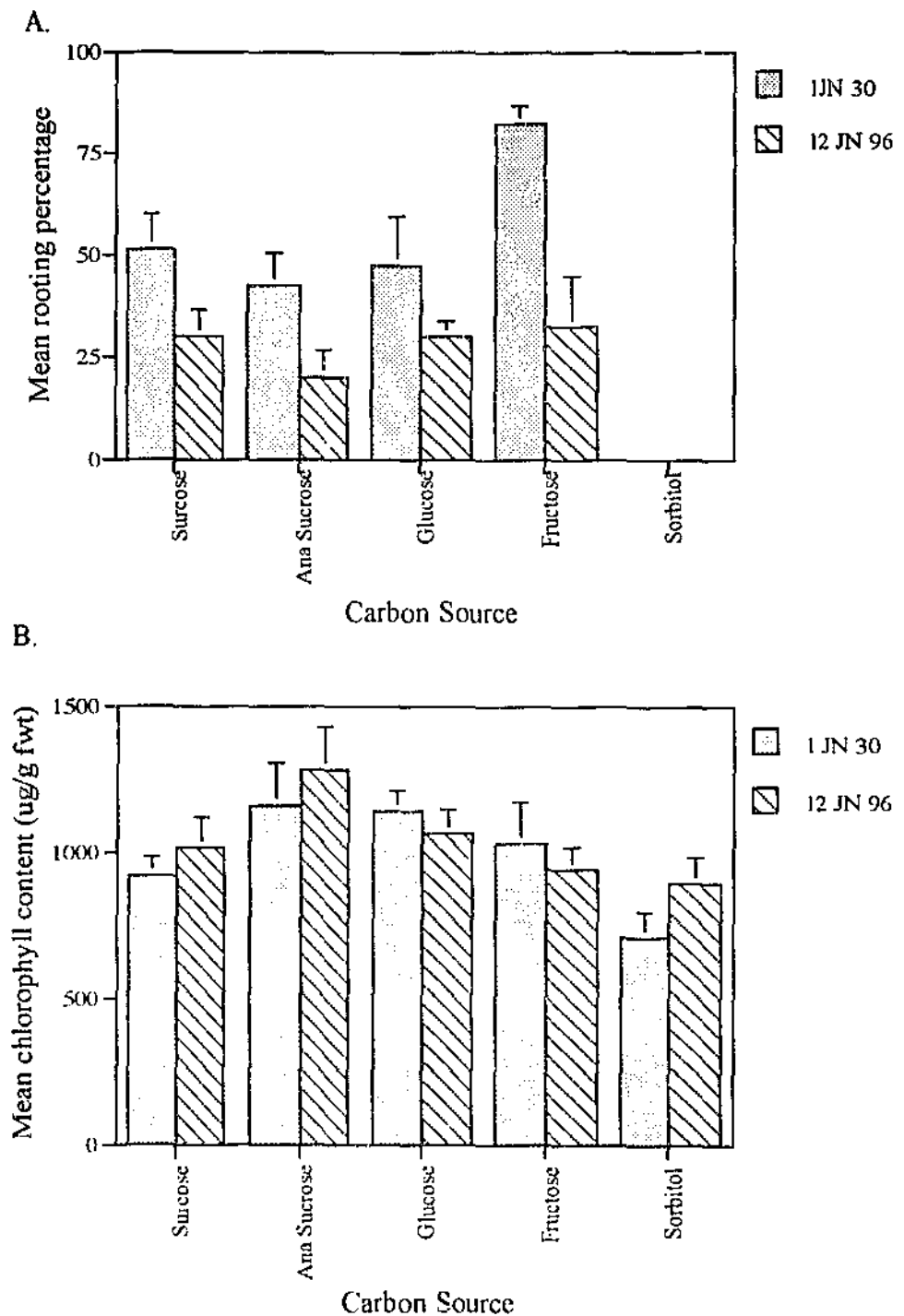


Figure 3.2 Rooting response of 1 JN 30 and 12 JN 96 to different carbon sources. A. Mean rooting percentage (7-8 replicates), and B. Chlorophyll content (7-8 replicates), after 28 days. Vertical bars denote SEM.

there was no difference in the other treatments for 12 JN 96 (Figure 3.2 A). The chlorophyll content was affected by the different media for clone 1 JN 30, with glucose and analytical sucrose being significantly higher than sorbitol ( $P < 0.05$ ), but not different from fructose or sucrose. The treatments had no effect on clone 12 JN 96 (Figure 3.2 B).

### 3.3 Experiment Three

Fructose was found to be necessary in the media to promote rooting, as zero fructose was found to give low rooting percentages for clone 1 JN 30 and no rooting for clone 91 JP 4 (Figure 3.3). One, 2 and 4% fructose compared to 2% sucrose was not significantly different at day 28. However, for clone 1 JN 30 there was earlier emergence of roots on 2% fructose compared to 2% sucrose ( $P < 0.05$ ), this trend was also evident (but not statistically significant) for clone 91 JP 4 (Figure 3.4).

Chlorophyll content for clone 1 JN 30, (Figure 3.3 B) was significantly less on 4% fructose than all other treatments except 0% fructose. Two percent sucrose was significantly greater than both 0 and 4% fructose. For clone 91 JP 4 (Figure 3.3 B) there was no difference between treatments.

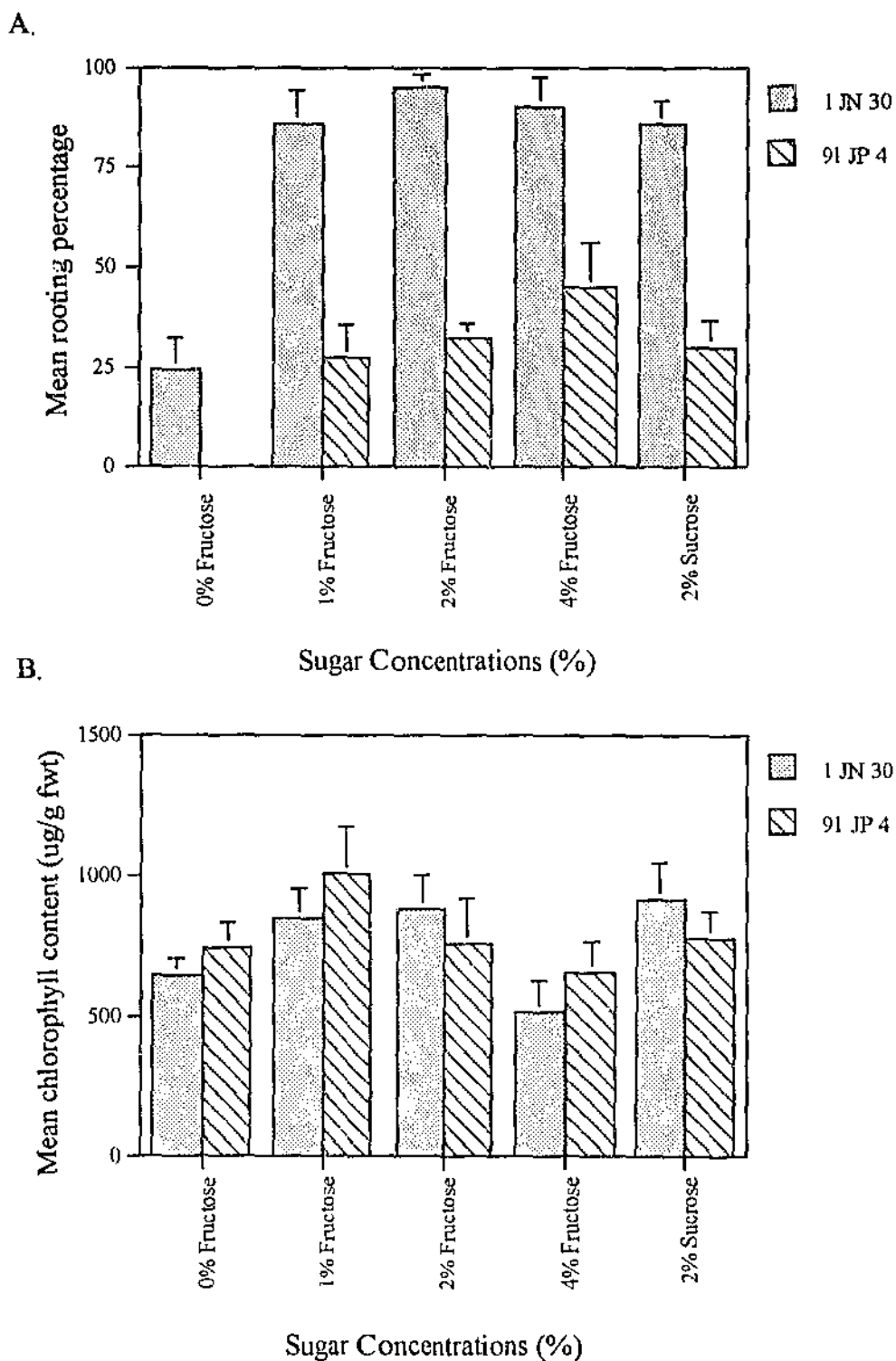


Figure 3.3 Rooting response of 1 JN 30 and 91 JP 4 to different fructose concentrations. A. Mean rooting percentage (6-9 replicates) and B. Chlorophyll content (9-7 replicates) after 28 days. Vertical bars denote SEM.

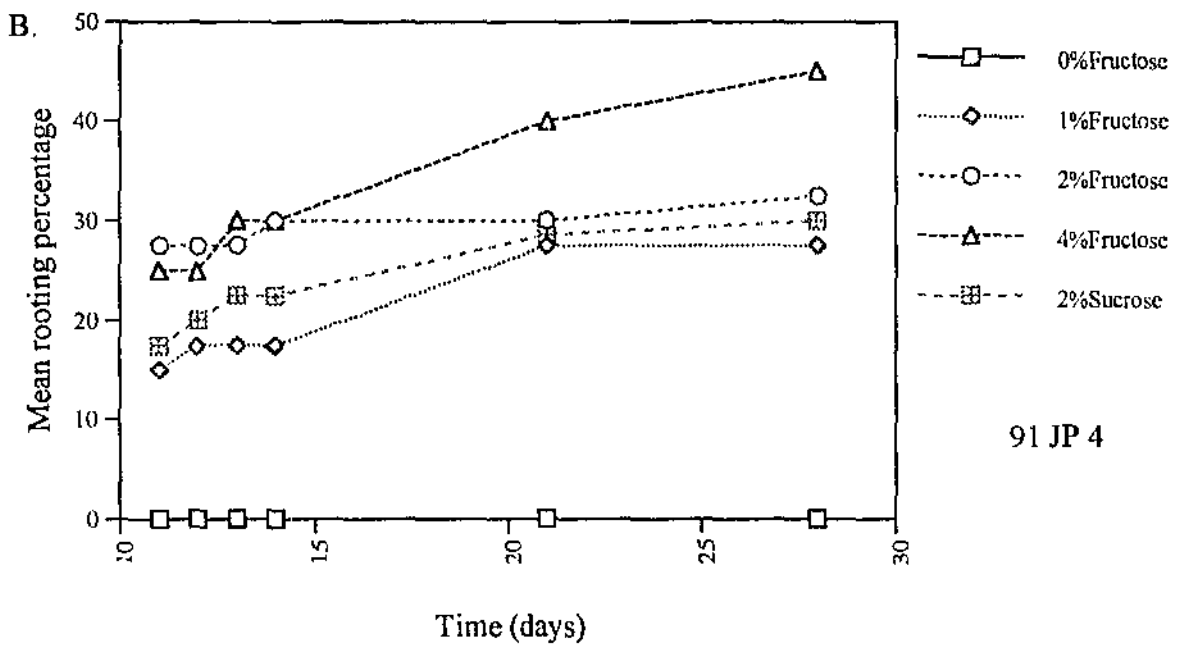
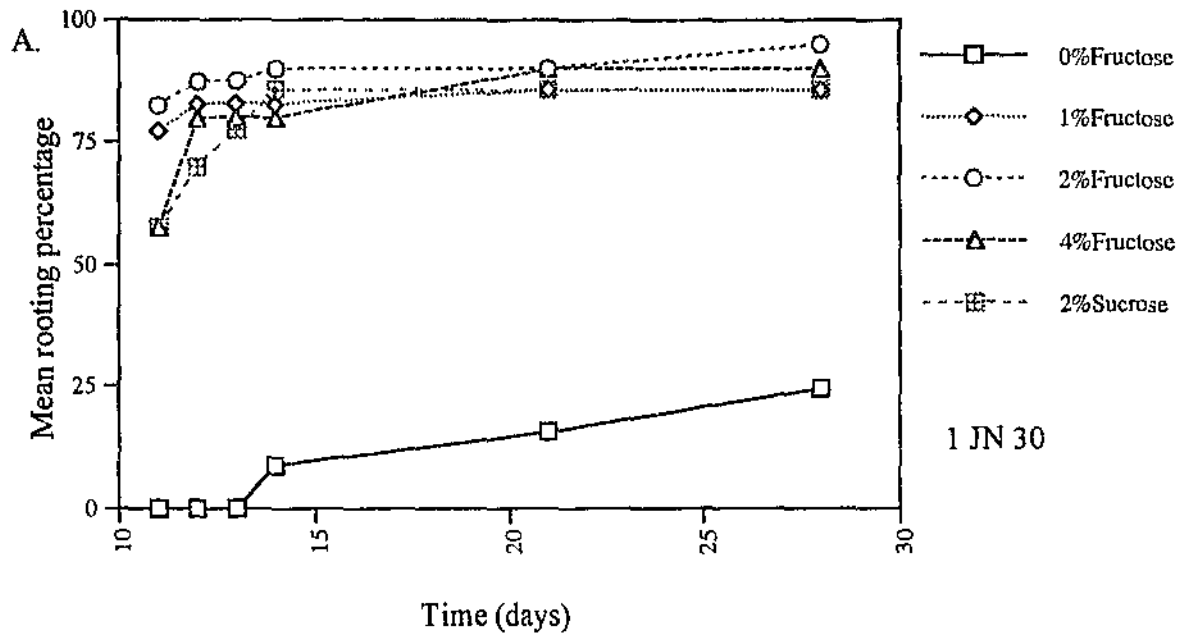


Figure 3.4 Rooting response of 1 JN 30 and 91 JP 4 to different fructose concentrations over time. A. Mean rooting percentage of shoots of clone 1 JN 30 (9-7 replicates) and B. clone 91 JP 4 (6-8 replicates) after 28 days.

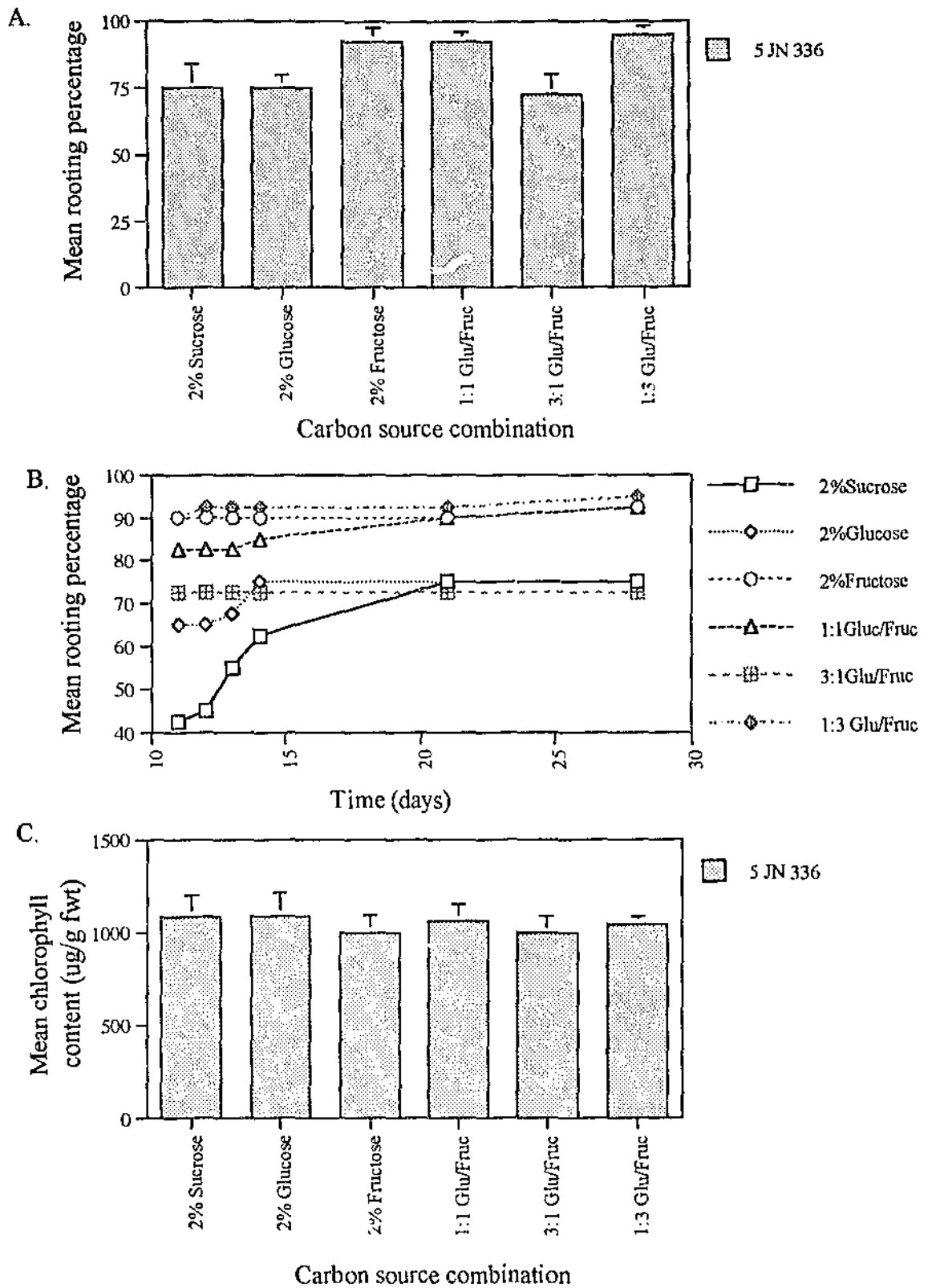


Figure 3.5 Rooting response of 5 JN 336 to different sugar combinations A. Mean rooting percentages of shoots (8 replicates), B. Mean rooting percentages over time (8 replicates) and C. Mean chlorophyll content (8 replicates) after 28 days. Vertical bars denote SEM.

### 3.4 Experiment Four

The variations in ratios of different sugars produced no difference in rooting after 28 days (Figure 3.5 A). However, all media containing fructose showed earlier emergence of roots ( $P < 0.05$ ) (Figure 3.5 B) with the media containing higher proportions of fructose giving the highest rooting percentages. For chlorophyll content the differing sugar combinations had no effect (Figure 3.5 C).

## 4. DISCUSSION

### 4.1 Experiment One

As rooting response was similar in medium of 2 and 4% sucrose for both clones, it can be concluded that further increases in sucrose will not increase rooting. This reflects the results of other reports that found a concentration between 2-5% to be optimum for various plant species (Hildebrandt and Riker, 1953; Okonkwo, 1966; Hyndman *et al.*, 1982; Hdider and Desjardins, 1994; Romano *et al.*, 1995). For studies on *Eucalyptus* species, sucrose concentrations between 2-6% (Cheng *et al.*, 1992) were found to favour root development, reflecting the results observed in this experiment.

Although it was expected that higher levels of sucrose would lower chlorophyll content (Rier and Chen, 1964; Edelman and Hanson, 1972), chlorophyll results indicate that

sucrose concentration does not effect plantlet condition (measured by chlorophyll content) for clone 5 JN 336.

#### 4.2 Experiment Two

From the five carbon sources, sorbitol was found to be the least effective carbon source for rooting. This reflects the results from other studies as Romano *et al.* (1995), found sorbitol to be completely ineffective on root induction of cork oak. Fructose for clone 1 JN 30, was found to increase rooting by 30%. This may be because it is more readily utilised (Smith, 1993). Fructose, can therefore be considered as the best carbon source for rooting for clone 1 JN 30. As this experiment produced unexpected results further experimentation of fructose took place in the following two experiments.

The difference in the response to the carbon sources from the two clones is an effect that has been reported previously for eucalypts. Often where one clone responds well to a medium variation other clones may not (McComb *et al.*, 1994).

#### 4.3 Experiment Three

Media with fructose were found to produce earlier emergence of roots as it was more readily utilised (Smith, 1993). This reflects the studies of Hew *et al.* (1988), who found that fructose was the sugar most rapidly utilised by *Dendrobium* meristem tissue. As the

hydrolysis of sucrose, the breakdown of sucrose into fructose and glucose, occurs in the medium and becomes readily available (ap Rees, 1984; Hawker, 1985; George, 1993), rooting rates increased. Therefore mean rooting percentages of 1, 2 and 4% fructose and 2% sucrose were not significantly different at the end of the 28 days.

It is recommended that the clones be grown on medium containing fructose for up to 14 days and then be transferred to stabilising medium so acclimatisation can occur. This process will limit the photosynthetic apparatus inhibition that results from sucrose in the medium, and thus would possibly increase chances of the plantlets survival when transferred to the soil (Hdider and Desjardins, 1994). Treatments were found to have a negative effect on chlorophyll content, as fructose concentration increased, chlorophyll content decreased for clone 1 JN 30.

#### 4.4 Experiment Four

Results indicate that up to day 14, all media containing fructose is a better carbon source for rooting of clone 5 JN 336 than those containing 2% sucrose. As mentioned before, it is suggested that these plantlets be taken off media at day 14, therefore reducing photosynthetic apparatus damage and enabling acclimatisation to occur two weeks earlier, as a substantial increase in root growth was not apparent after day 14.



#### 4.5 General Discussion

The results from these four experiments indicate that fructose can substantially increase rooting for particular clones. Fructose was also illustrated as generally appearing to be a better carbon source due to earlier emergence of roots. Plantlets grown on fructose media can be acclimatised at an earlier date, reducing problems associated with sucrose dependence.

The results indicated variations between clones with some statistically significantly increasing their rooting percentage on media with fructose, while others did not. The results of experiment three differed from the previous experiment in that for clone 1 JN 30, fructose was found to be no different from sucrose of the same concentration. In the previous experiment a difference was observed. This illustrates the variability that can occur within a clone, and how a clone may grow differently between subsequent trials and highlights the variation between trials. This has been reported in other species (Damiano *et al.*, 1987).

## CHAPTER FOUR

# INFLUENCE OF AUXIN AND SUCROSE ON ENDOGENOUS ETHYLENE PRODUCTION

## 1. INTRODUCTION

### 1.1 Ethylene *in vivo*

Ethylene synthesis is strongly influenced by the presence of auxin. Auxin regulates ethylene production by controlling the endogenous levels of 1-Aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene (Yoshii and Imaseki, 1982). Endogenous ethylene production is affected by conditions of stress, chemicals added to the culture media and senescence of plant material (George, 1993). Ethylene is involved in the regulation of several physiological processes that require, for their initiation, cell wall dissolution and cell lysis through an increased production or release of hydrolytic enzymes (Drew *et al.*, 1981). For the early stages of germination endogenous ethylene is inhibitory, for example, bud development in pea seedlings is retarded (Burg and Burg, 1968). At later stages of development endogenous ethylene is encouraging or essential, with root growth and inflorescence production being promoted in some species (Matthys *et al.*, 1995).

The effect of ethylene upon root initiation of cuttings *in vivo* is variable, depending upon the amount of ethylene and plant species (Fabijan *et al.*, 1981). Excess ethylene results in necrotic spots and some radial swelling of the hypocotyl and decreases the rate of root elongation (Fabijan *et al.*, 1981). The effect of ethylene upon growth *in vitro* is also variable.

## 1.2 Ethylene *in vitro*

The role of ethylene in micropropagation has received little attention, however, its effects on callus growth have been more thoroughly investigated. Ethylene has been found to suppress chlorophyll synthesis and chloroplast development, inhibit shoot formation if initially present in culture, and have both promotory and inhibitory effects on rooting *in vitro* (George, 1993; Biddington and Robinson, 1994). It was been reported that ethylene stimulates rooting, but can also inhibit or have no effect upon rooting depending on species. The varying effects on rooting depends upon the applied concentration and on the physiological condition of the explants (Bollmark and Eliasson, 1990; Buddendorf-Joosten and Woltering, 1994).

Ethylene has been known to decrease IAA levels in plant tissue (and therefore may have negative effects on rooting), stimulate the metabolism of IAA, and decrease levels of cytokinins in Norway spruce hypocotyl cuttings (therefore stimulating rooting) (Bollmark and Eliasson, 1990). Low concentrations of ethylene *in vitro* seem to be necessary for

organogenesis, higher concentrations result in negative effects on growth and development and generally induces senescence (Buddendorf-Joosten and Woltering, 1994).

### 1.3 1-Aminocyclopropane-1-carboxylate (ACC)

1-Aminocyclopropane-1-carboxylate (ACC) stimulates ethylene production (Yoshi and Imaseki, 1982; Adkins, 1992). It can be added to rooting media, to promote ethylene production in various plant tissues (and thereby engendering physiological effects) that normally produce little ethylene (Adams and Yang, 1979; George, 1993). Studies have found that the addition of high ACC concentrations (100 $\mu$ M) to standard MS medium induces necrosis and decreases rice callus growth by 15% (Adkins, 1992).

The production of ethylene via ACC, is enhanced by the provision of exogenous carbohydrates, light, auxin, cytokinins and carbon dioxide (Kumar *et al.*, 1987). For example, Garcia and Einset (1983) noted that the rate of ethylene production increased in cell suspension cultures when the cells were subjected to stress of some kind.

Although the effect of ethylene on various tissues is variable, it is generally expected that the build up of ethylene in the tissue culture systems of jarrah will be inhibitory to growth. This is based on the findings of McClelland and Smith (1990) and Jackson *et al.* (1991), who found that ventilation of the culture system is necessary to dissipate ethylene build up and promote healthy plantlet growth.

## 2. MATERIALS AND METHODS

### 2.1 Experiment One: Effect of Auxin on Ethylene Production

Clones 91 JP 4, 1 JN 30 and 11 JN 50 were placed on media containing 0, 0.1, 1, 10 or 100  $\mu$ M indole-butyric-acid (IBA). Ten shoots were randomly placed in each container to maximise ethylene production. Due to limited plant material sample size varied from 3, for clone 11 JN 50, to 5, for clones 91 JP 4 and 1 JN 30, replications per treatment. Containers were sealed with two strips of parafilm (Crown, Australia) 10 cm in length to prevent ethylene leakage. Mean rooting percentage, mean number of roots per shoot, ethylene area counts, and chlorophyll content were determined after day 28.

### 2.2 Experiment Two: Effect of Sucrose on Ethylene Production

Clones 91 JP 4, 12 JN 96 and 12 JN 35 were placed on media containing sucrose concentrations of 0, 1, 2, and 4% or 2% fructose. Replications of either 4 or 5 were used, depending upon the clone, with 10 shoots per container, which were sealed with parafilm. Mean rooting percentage, number of roots per shoot, ethylene area counts, and chlorophyll content were determined after day 28.

### 2.3 Experiment Three: Effect of Auxin on Ethylene Production with Applied ACC

Clones 5 JN 336 and 12 JN 96 were placed on media containing different amounts of auxin (0, 0.1, 1, 10 and 100  $\mu\text{M}$ ) with 25  $\mu\text{M}$  ACC. An additional treatment of 10 $\mu\text{M}$  IBA without ACC was also included. A 1mM stock of ACC was prepared in milli Q water and dispensed into the separate media. Ten shoots were placed in each container. Sample size of 4, for clone 12 JN 96, and 5, for clone 5 JN 336, was used per treatment and containers were sealed with parafilm. Mean rooting percentage, mean number of roots per shoot, ethylene area counts, and chlorophyll content were determined after day 28.

### 2.4 Experiment Four: Effect of Sucrose on Ethylene Production with Applied ACC

A combination of clones (due to limited plant material) 1 JN 30, 11 JN 50, 1 JN 98, 12 JN 35, and 11 JN 379 was placed over five treatments 0, 1, 2, and 4% sucrose with 25 $\mu\text{m}$  of ACC, or 2% sucrose without ACC. Two shoots of each clone were placed in each container (10 shoots in total) with five replicates per treatment and the containers were sealed with parafilm. Mean rooting percentage, mean number of roots per shoot, ethylene area counts, and chlorophyll content were determined after day 28.

### 3. RESULTS

Roots appeared at about day 11. Rooting increased up to day 21 and generally remained at this level to day 28. In most cases the mean number of roots per shoot reflected the results obtained using the mean rooting percentage and therefore only the mean rooting percentages are presented. Where there was an apparent difference between mean rooting percentage and mean number of roots per shoot, both graphs are included.

#### 3.1 Experiment One

The different levels of auxin induced varying amounts of rooting with the higher concentrations of auxin (10 and 100  $\mu\text{M}$ ) being significantly greater for all three clones ( $P < 0.01$ ) than the remaining treatments (Figure 4.1 A). In addition, there was a difference in rooting between the three clones with 91 JP 4 producing significantly fewer roots ( $P < 0.05$ ).

For clone 1 JN 30, 100 and 10  $\mu\text{M}$  IBA, rooting percentages were found to be significantly greater than all other treatments, and 1  $\mu\text{M}$  IBA significantly greater than 0  $\mu\text{M}$  IBA. For clone 11 JN 50, 100 and 10  $\mu\text{M}$  IBA, were also found to be significantly greater than all other treatments. For clone 91 JP 4, 0 and 0.1  $\mu\text{M}$  IBA, rooting percentages were significantly less than the other three treatments.

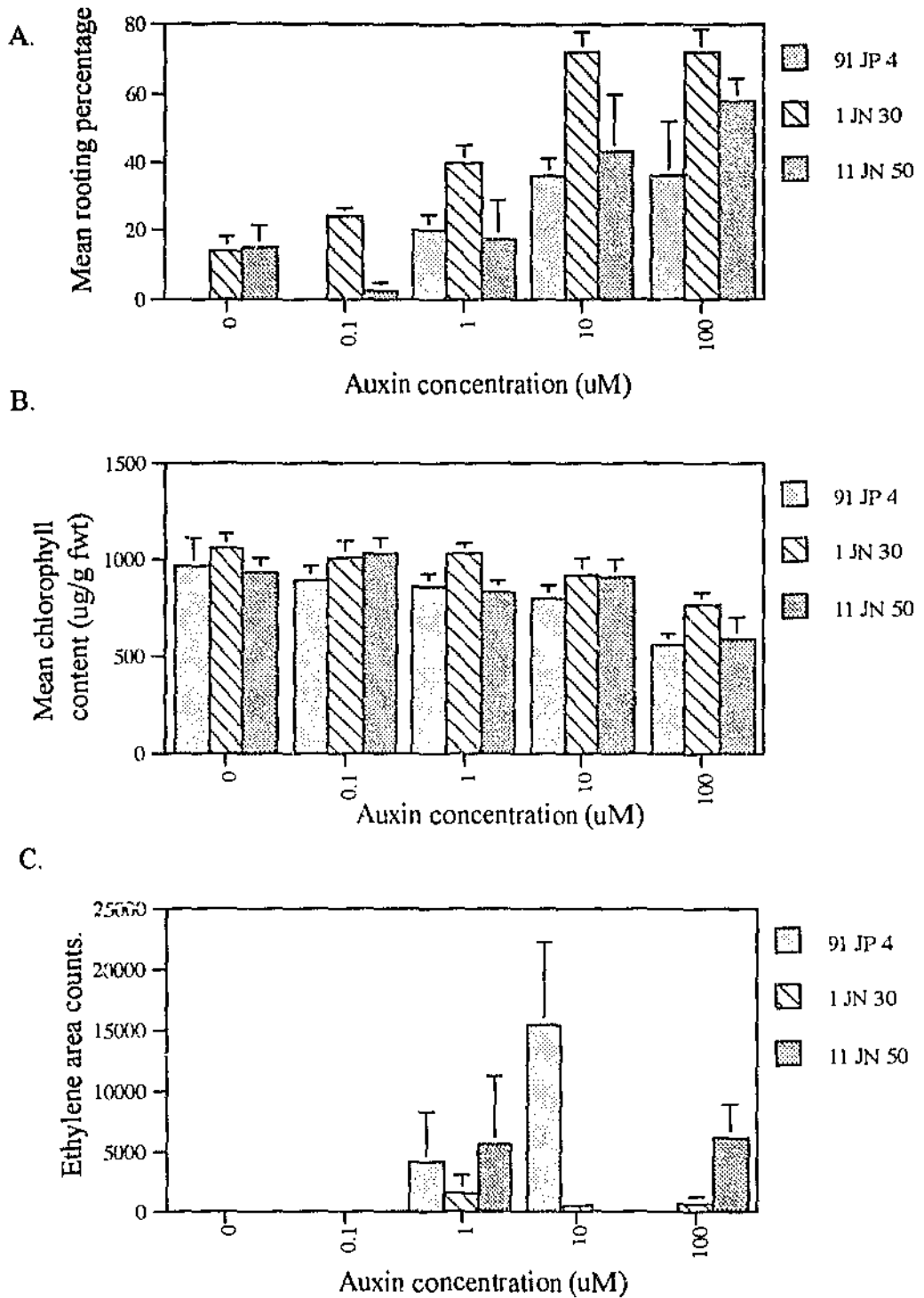


Figure 4.1 Rooting response of 91 JP 4, 1 JN 30, and 11 JN 50 to different auxin concentrations A. Mean rooting percentage, B. Mean chlorophyll content (6-10 replicates) and C. Mean ethylene area counts (3-5 replicates) after 28 days. Vertical bars denote SEM.



The different concentrations of auxin were found to have varying effects on the resulting chlorophyll content ( $P < 0.05$ ) with 100  $\mu\text{M}$  of auxin giving a significantly less chlorophyll content for all three clones tested (Figure 4.1 B).

The multiple range test indicated for clone 91 JP 4 that shoot chlorophyll content was greater on 0, 0.1, and 1  $\mu\text{M}$  IBA than on 100  $\mu\text{M}$  IBA. For clone 1 JN 30, 0 and 1  $\mu\text{M}$  IBA shoot chlorophyll content was significantly greater than 100  $\mu\text{M}$  IBA. For clone 11 JN 50, 0, 0.1 and 10  $\mu\text{M}$  IBA, chlorophyll content were found to be significantly greater than 100  $\mu\text{M}$  IBA.

The different clones were found to produce varying amounts of ethylene, with clone 1 JN 30 producing significantly less ethylene than 91 JP 4 at 10  $\mu\text{M}$  and 11 JN 50 at 100  $\mu\text{M}$  ( $P < 0.05$ ) (Figure 4.1 C).

For clone 91 JP 4, 10  $\mu\text{M}$  IBA produced significantly more ethylene than any other treatment. For clones 1 JN 30 and 11 JN 50, there was no difference found between the five auxin treatments and the ethylene produced. The correlations between mean rooting percentage and ethylene (Figure 4.2) ranged from positive weak to modest positive relationship. Indicating that as mean rooting percentage increased, ethylene increased.

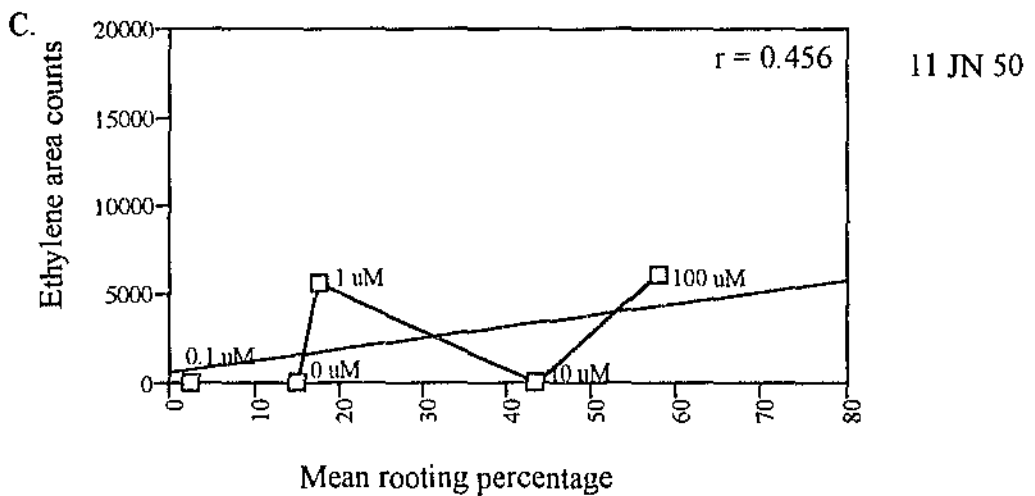
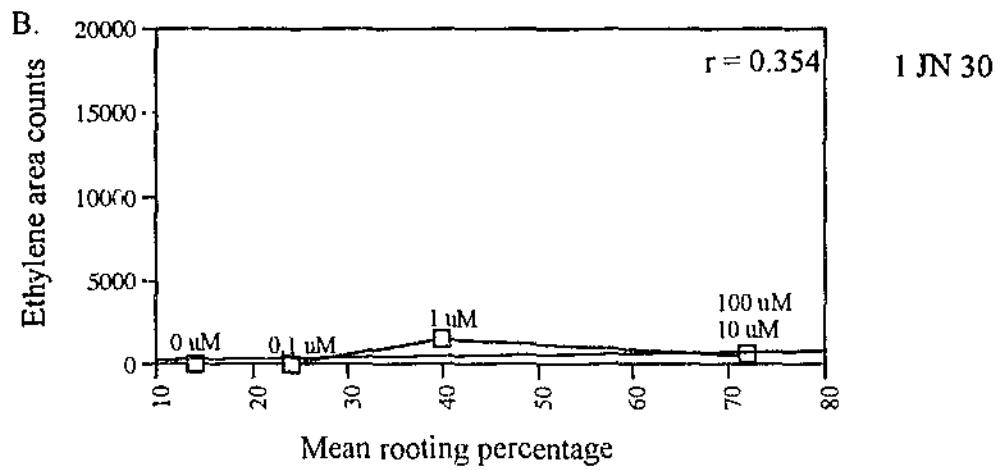
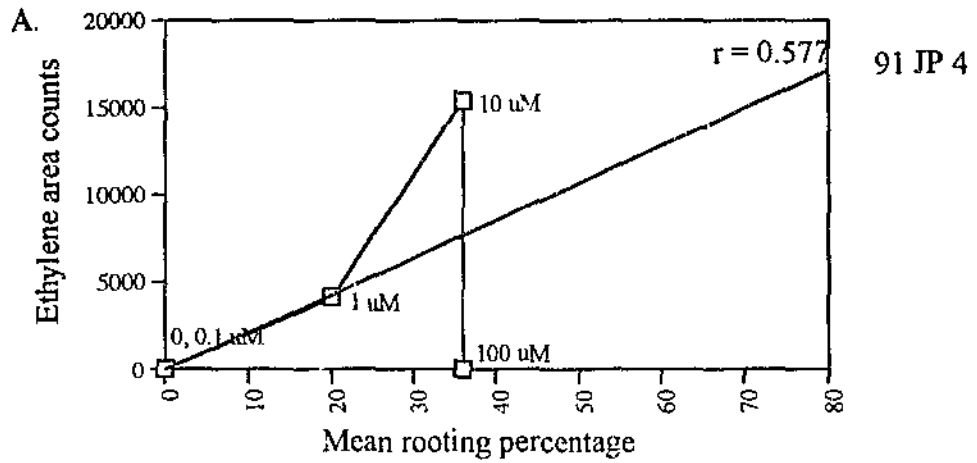


Figure 4.2 Correlation of mean rooting percentage and ethylene area counts of shoots after 28 days on media with differing auxin concentrations. A. Clone 91 JP 4 (5 replicates), B. Clone 1 JN 30 (5 replicates) and C. Clone 11 JN 50 (3-5 replicates).

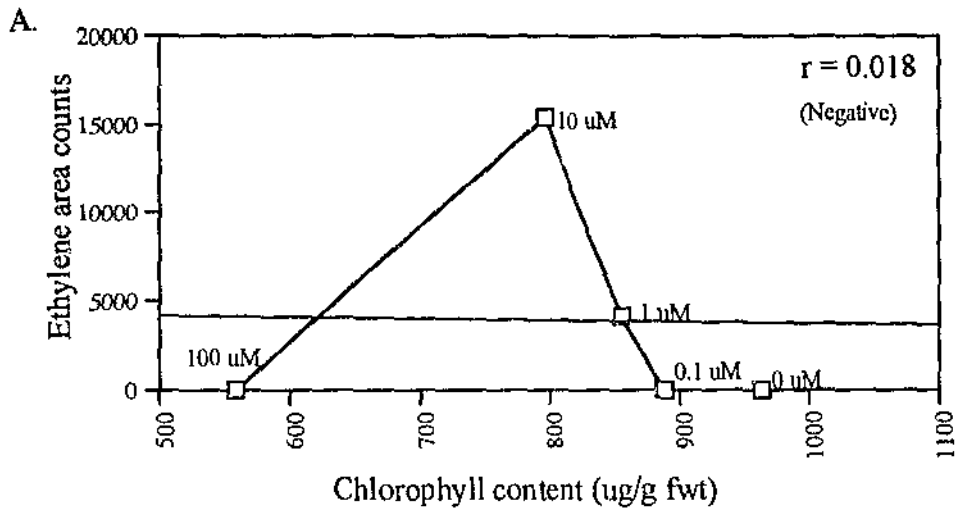
Correlations between chlorophyll content and ethylene range from very weak and weak negative correlations for clones 91 JP 4 and 1 JN 30 (Figures 4.3 A and B) and a strong negative correlation for clone 11 JN 50 (Figure 4.3 C).

The correlations between auxin concentration and ethylene production (Figure 4.4) ranged from very weak to a modest positive correlation.

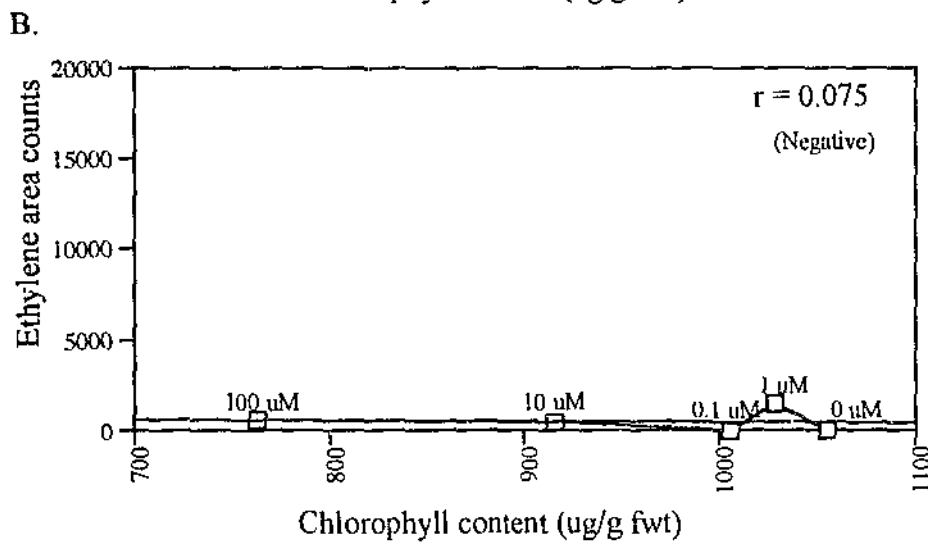
### 3.2 Experiment Two

The different levels of sucrose induced varying amounts of rooting with the absence of sucrose being significantly lower ( $P < 0.05$ ) than all other treatments (Figure 4.5 A). In addition, there was a difference in how each of the three clones responded to the treatments ( $P < 0.05$ ).

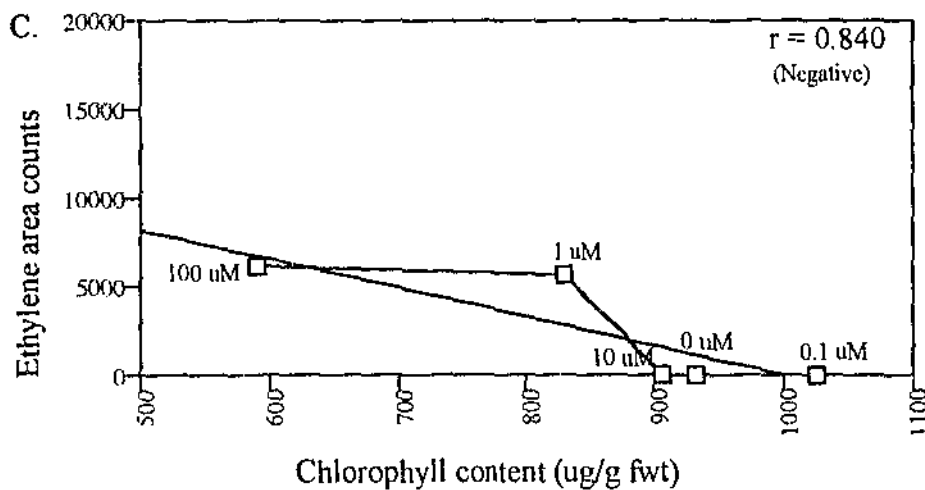
The multiple range test, for mean rooting percentage, indicated that for clone 91 JP 4, 0% sucrose was less than all other treatments, 2% fructose and 2% sucrose greater than 1% sucrose, and 4% sucrose greater than 1 and 2 % sucrose. For clone 12 JN 96, 1% and 2% sucrose were found to be greater than 0% sucrose, and 2% fructose was found to be greater than 0% and 4% sucrose. For clone 12 JN 35, 2% fructose was found to be greater than all other treatments; 2 and 4% sucrose were found to be greater than 0 and 1% sucrose and; 1% sucrose greater than 0% sucrose.



91 JP 4



1 JN 30



11 JN 50

Figure 4.3 Correlation of mean chlorophyll content and ethylene area counts of shoots after 28 days on media with differing auxin concentrations. A. Clone 91 JP 4. B. Clone 1 JN 30. C. Clone 11 JN 50. Replicates between 6 and 10.

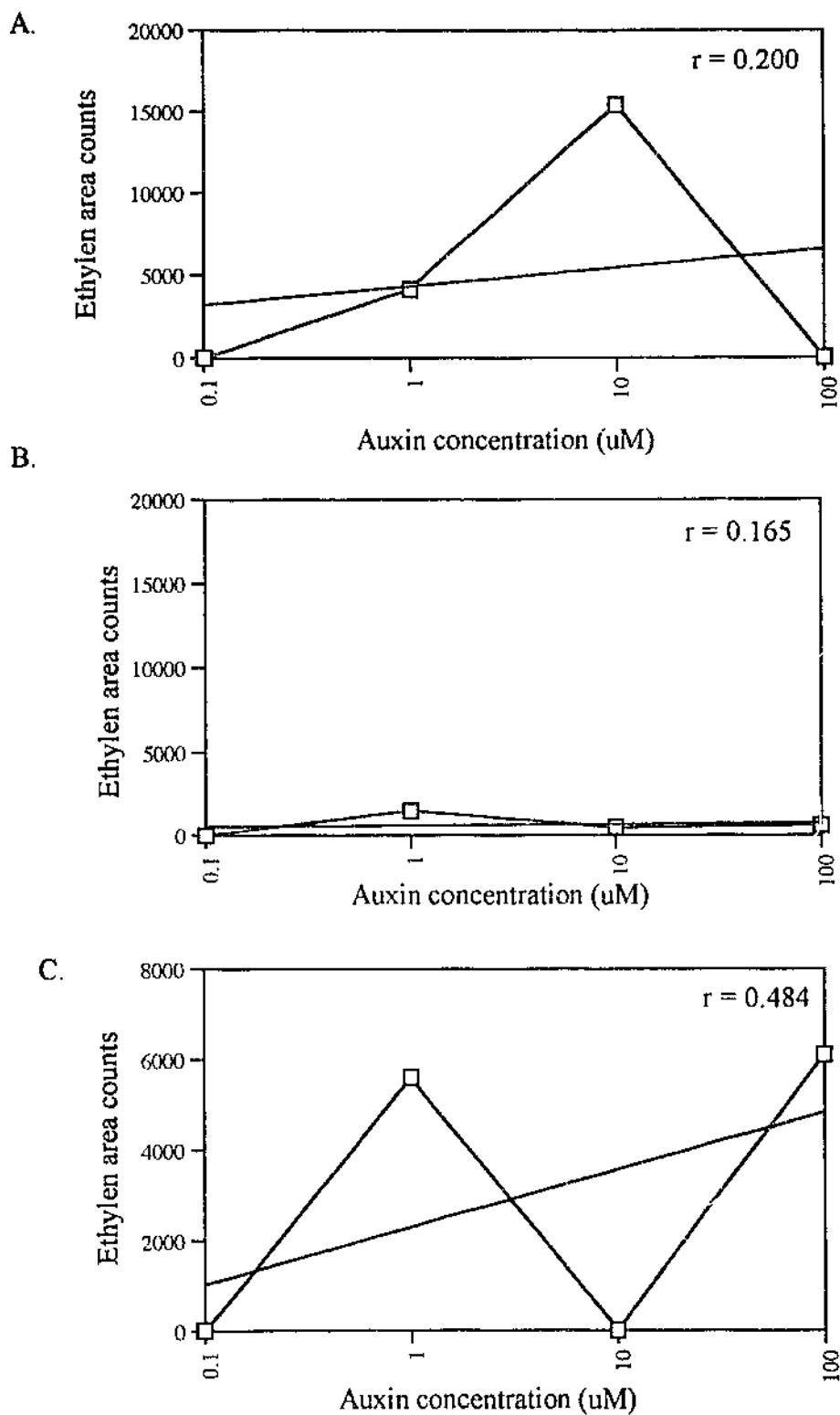


Figure 4.4 Correlation of auxin concentration and ethylene area counts of shoots after 28 days on media with differing auxin concentrations. A. Clone 91 JP 4. B. Clone 1 JN 30. C. Clone 11 JN 50. Replicates between 4 and 5.

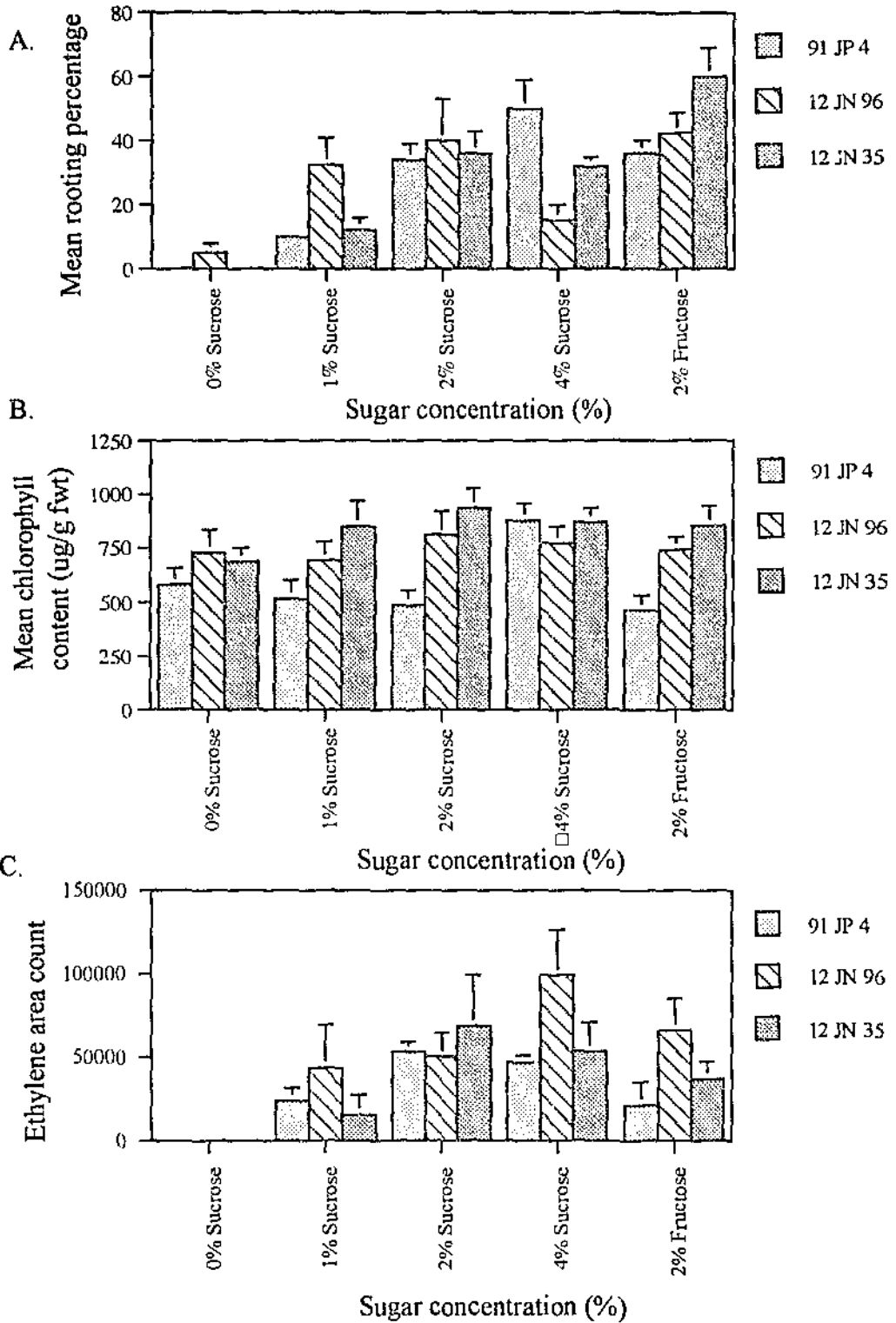


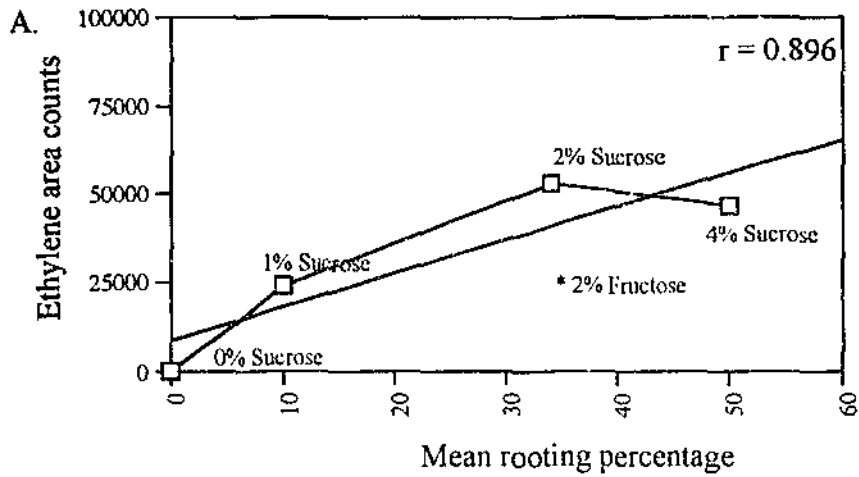
Figure 4.5 Rooting response of clones 91 JP 4, 12 JN 96 and 12 JN 35 to different sucrose concentrations A. Mean rooting percentage (4-5 replicates), B. Mean chlorophyll content (8-10 replicates) and, C. Mean ethylene area counts (4-5 replicates) after 28 days. Vertical bars denote SEM.

The differing sucrose concentrations were found to have differing effects on the corresponding chlorophyll contents ( $P < 0.05$ ) for clone 91 JP 4, with 4% sucrose having a greater chlorophyll content than any other treatments (Figure 4.5 B). For the two remaining clones, there was no significant difference found between the treatments.

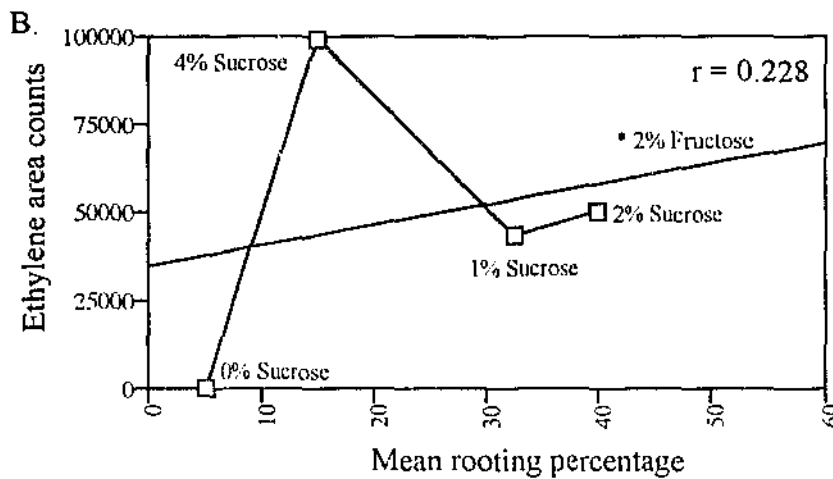
The different sucrose concentrations produced varying amounts of ethylene, with media without sucrose producing no measurable amounts of ethylene for all clones ( $P < 0.05$ ). In addition, the clones were found to produce differing quantities of ethylene under similar sucrose medium ( $P < 0.05$ ), with 12 JN 96 producing significantly more ethylene on 4% sucrose and 2% fructose than the other clones (Figure 4.5 C).

For clone 91 JP 4, 2 and 4% sucrose were found to have significantly greater ethylene area counts than 0, 1% sucrose and 2% fructose. For clone 12 JN 96, 4% sucrose had a significantly greater ethylene area count than 0% sucrose. For clone 12 JN 35, 2 and 4% sucrose were found to be significantly greater than 0% sucrose.

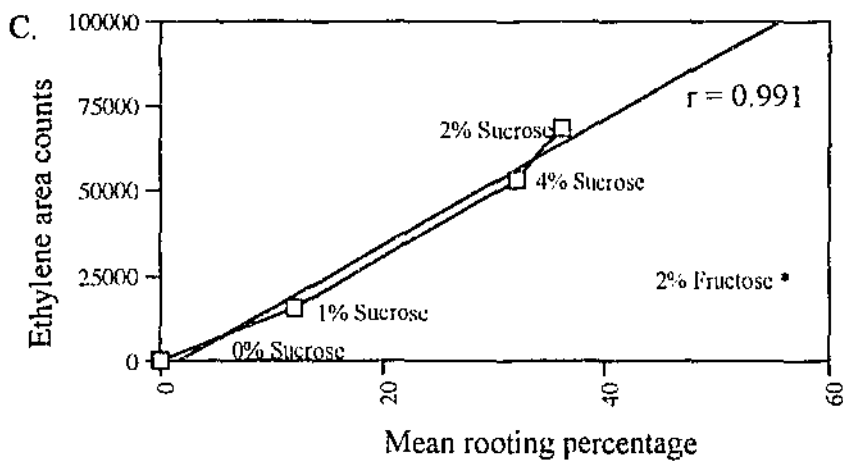
Correlations between mean rooting percentage and ethylene area counts (Figure 4.6) ranged from a weak to very strong positive correlation. Indicating that as rooting increased, ethylene production increased.



91 JP 4



12 JN 96



12 JN 35

Figure 4.6 Correlation of mean rooting percentage and ethylene area counts of shoots after 28 days on media with differing sucrose concentrations; A, Clone 91 JP 4 ; B, Clone 12 JN 96 ; and C, Clone 12 JN 35. Replicates between 4 and 5.



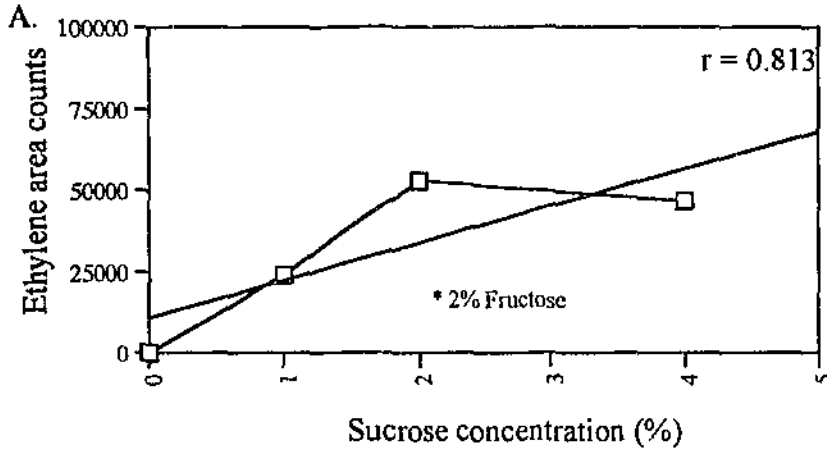
The correlation between sucrose concentration and ethylene area counts (Figure 4.7) ranged from strong to very strong positive correlation. Indicating that as sucrose concentration increased, ethylene production increased.

### 3.3 Experiment Three

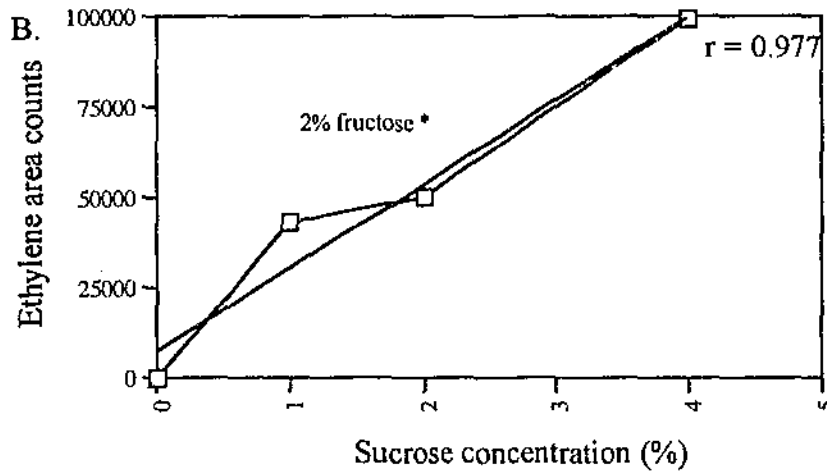
The differing auxin concentrations were found to influence the mean rooting percentage and mean number of roots per shoot (Figure 4.8 A and B), with 100  $\mu\text{M}$  plus ACC giving no root growth for both clones ( $P < 0.005$ ). There was no difference found between the two clones in relation to the effect of the treatments.

The multiple range test for mean rooting percentage indicated, for clone 5 JN 336, that 10  $\mu\text{M}$  - ACC was greater than all other treatments, 10 and 1  $\mu\text{M}$  IBA + ACC were greater than 0, 0.1 and 100  $\mu\text{M}$  IBA + ACC. For clone 12 JN 96, 1 and 10  $\mu\text{M}$  IBA + ACC, and 10  $\mu\text{M}$  IBA - ACC were found to be greater than 0, 0.1 and 100  $\mu\text{M}$  IBA.

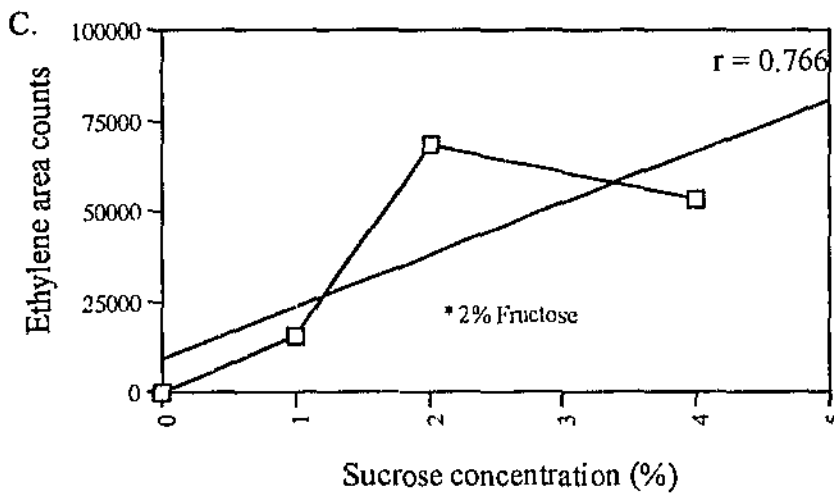
For mean number of roots per shoot (Figure 4.8 B), clone 5 JN 336, treatments of 10  $\mu\text{M}$  IBA with and without ACC were found to be greater than 0, 0.1, 1 and 100  $\mu\text{M}$  IBA. For clone 12 JN 96, 10  $\mu\text{M}$  IBA - ACC was found to be greater than all other treatments, and 1 and 10  $\mu\text{M}$  IBA + ACC greater than 0, 0.1 and 100  $\mu\text{M}$  IBA.



91 JP 4



12 JN 96



12 JN 35

Figure 4.7 Correlation of sucrose concentration and ethylene area counts of shoots after 28 days on media with differing sucrose concentrations; A. Clone 91 JP 4; B. Clone 12 JN 96 and; C. Clone 12 JN 35. Replicates between 4 and 5.

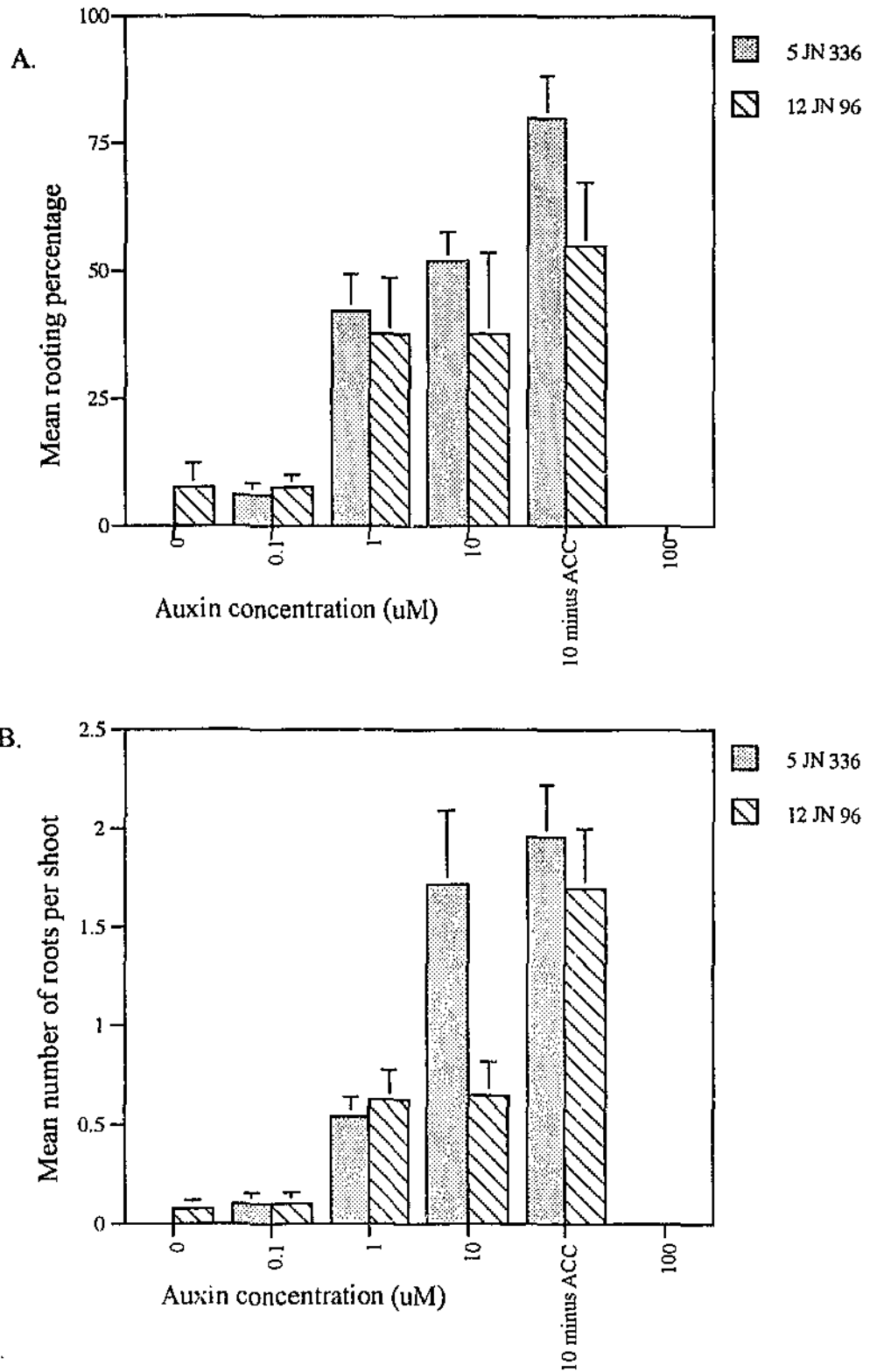


Figure 4.8 Rooting response of 5 JN 336 and 12 JN 96 to different auxin concentration plus ACC. A. Mean rooting percentage (4-5 replications) and B. Mean number of roots per shoot (40-50 replications) after 28 days. Vertical bars denote SEM.

The differing auxin concentrations were found to have varying effects on the resulting chlorophyll content ( $P < 0.05$ ) for clone 12 JN 96 with 10  $\mu\text{M}$  minus ACC being greater than all the other treatments. In addition, there was a difference between the two clones and their consequent chlorophyll content ( $P < 0.05$ ) (Figure 4.9 A).

The differing auxin concentrations were found to have varying effects upon the amount of ethylene produced for clone 5 JN 336 ( $P < 0.05$ ). For this clone, 10  $\mu\text{M}$  - ACC produced less ethylene than 10 and 100  $\mu\text{M}$  IBA + ACC. Ten  $\mu\text{M}$  IBA + ACC produced more ethylene than 0, 0.1, and 1  $\mu\text{M}$  IBA. One hundred  $\mu\text{M}$  IBA produced more ethylene than 0.1, 1  $\mu\text{M}$  IBA + ACC (Figure 4.9 B). For clone 12 JN 96 there was no difference found between any of the treatments.

Correlations between mean rooting percentage and ethylene area counts (Figure 4.10) ranged from a weak positive relationship for clone 5 JN 336, to modest negative for clone 12 JN 96. Indicating for clone 5 JN 336 ethylene increases as rooting increases, but for clone 12 JN 96, ethylene decreased as rooting increased. These correlations were similar for mean number of roots per shoot.

For chlorophyll content and ethylene area counts (Figure 4.11) correlations varied from a modest to a strong positive, indicating that as chlorophyll content increased, ethylene increased. Strong positive correlations were also found between ethylene area counts and auxin concentrations (Figure 4.12).

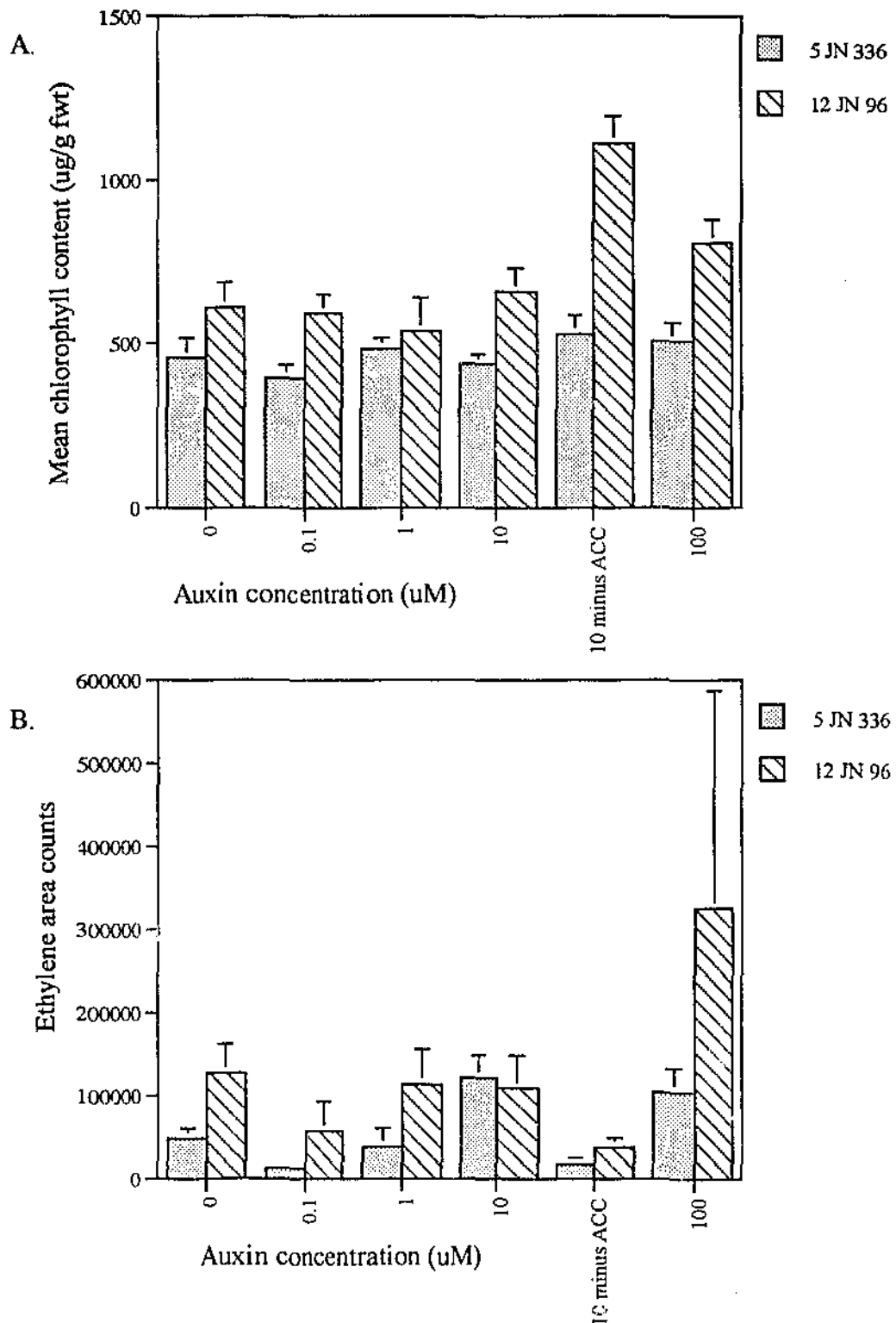
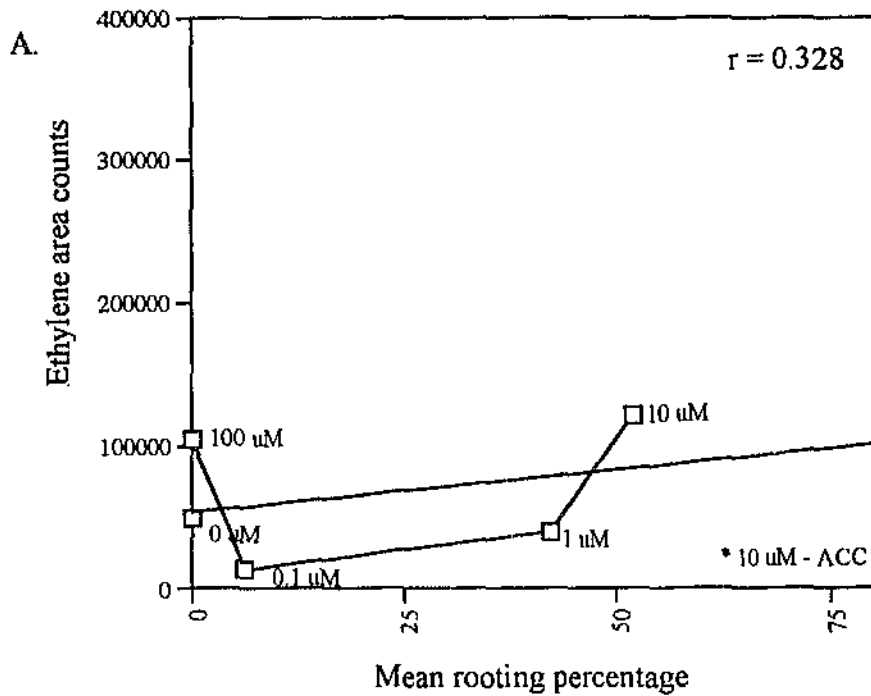
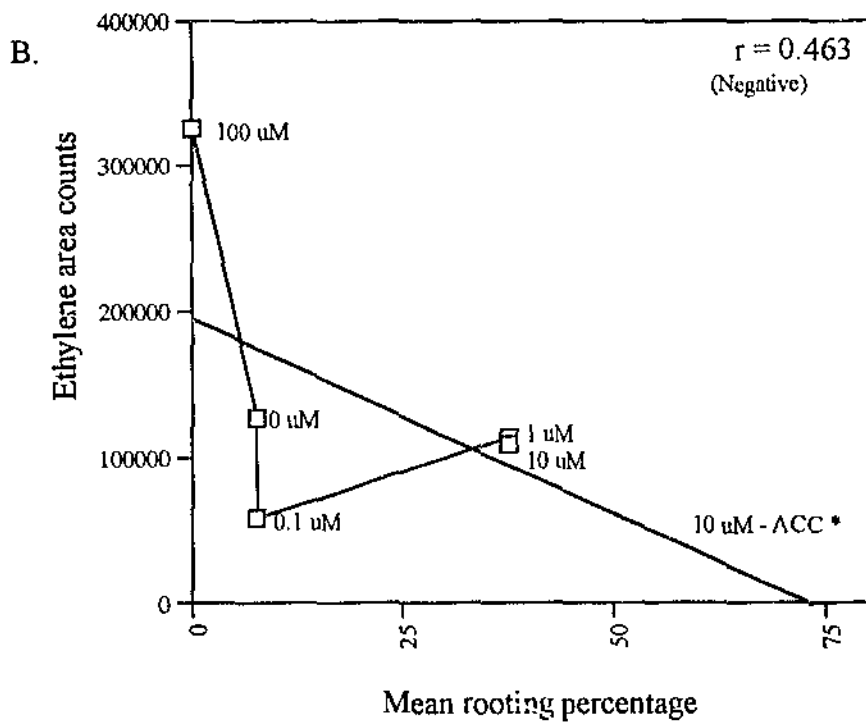


Figure 4.9 Rooting response of clones 5 JN 336 and 12 JN 96 to different auxin concentrations plus ACC. A. Chlorophyll content (8-10 replicates) and B Mean ethylene area counts (4-5 replicates) 28 days. Vertical bars denote SEM.

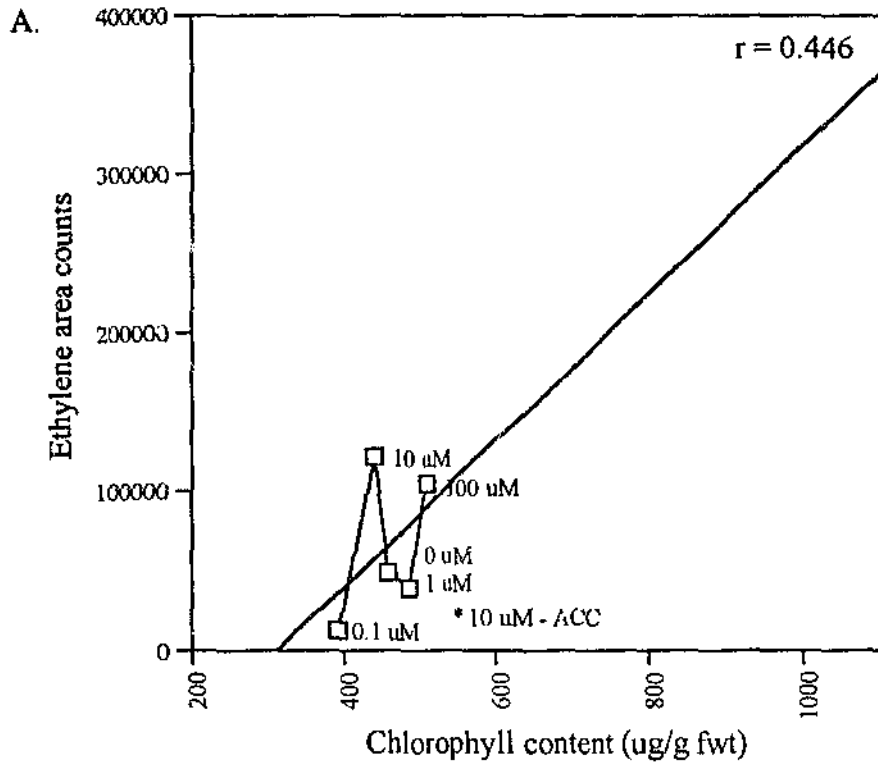


5 JN 336

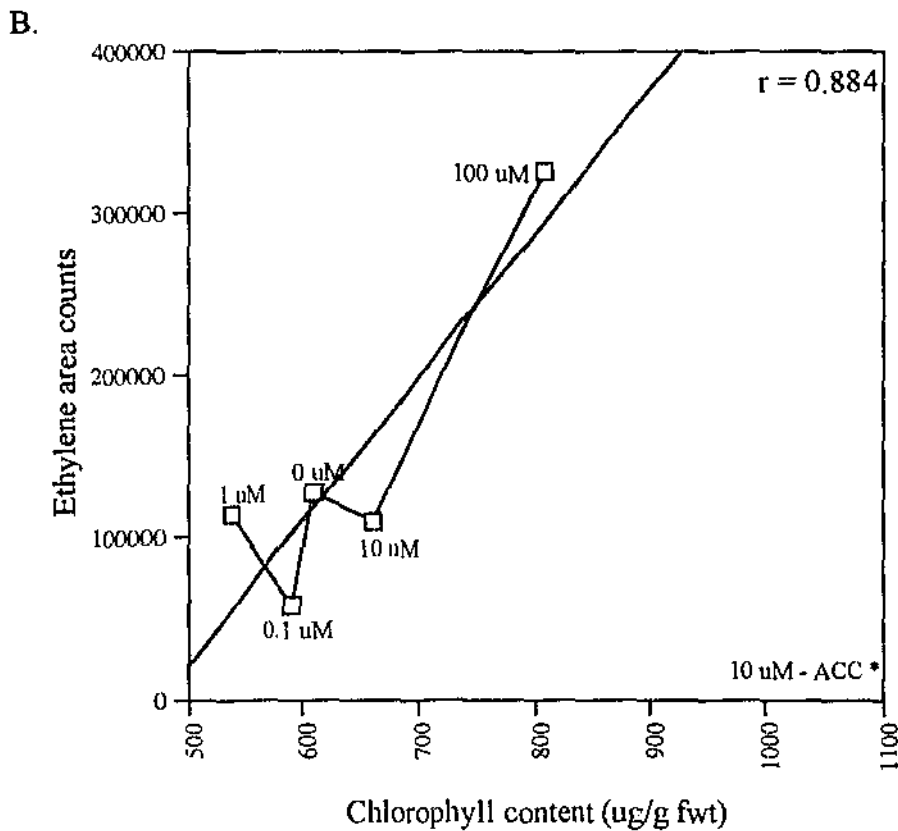


12 JN 96

Figure 4.10 Correlation of mean rooting percentages and ethylene area counts of shoots after 28 days on media with differing auxin concentrations with ACC. A. Clone 5 JN 336. B. Clone 12 JN 96. Replicates between 4 and 5.

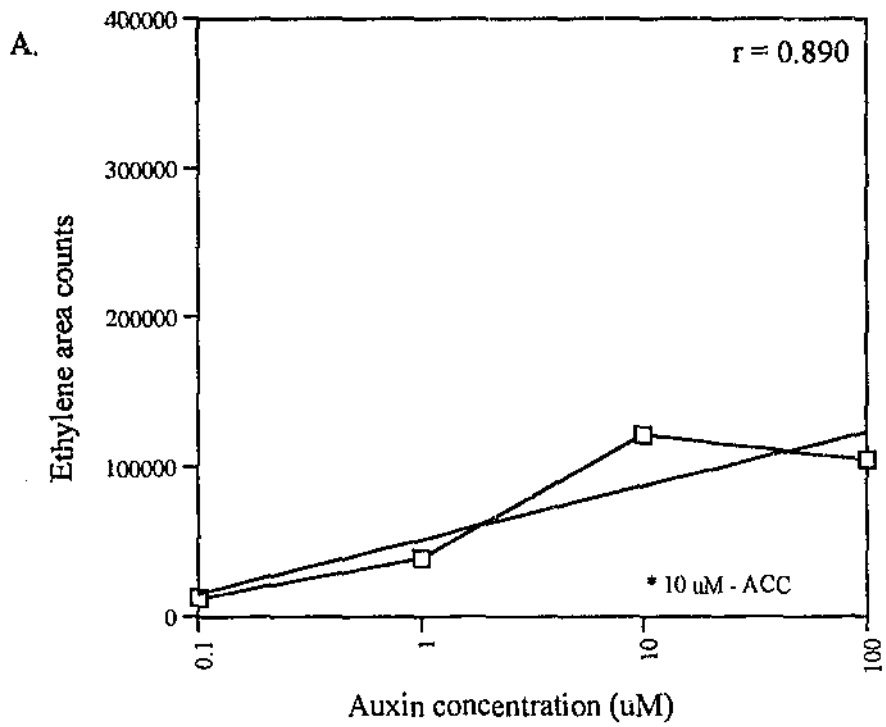


5 JN 336

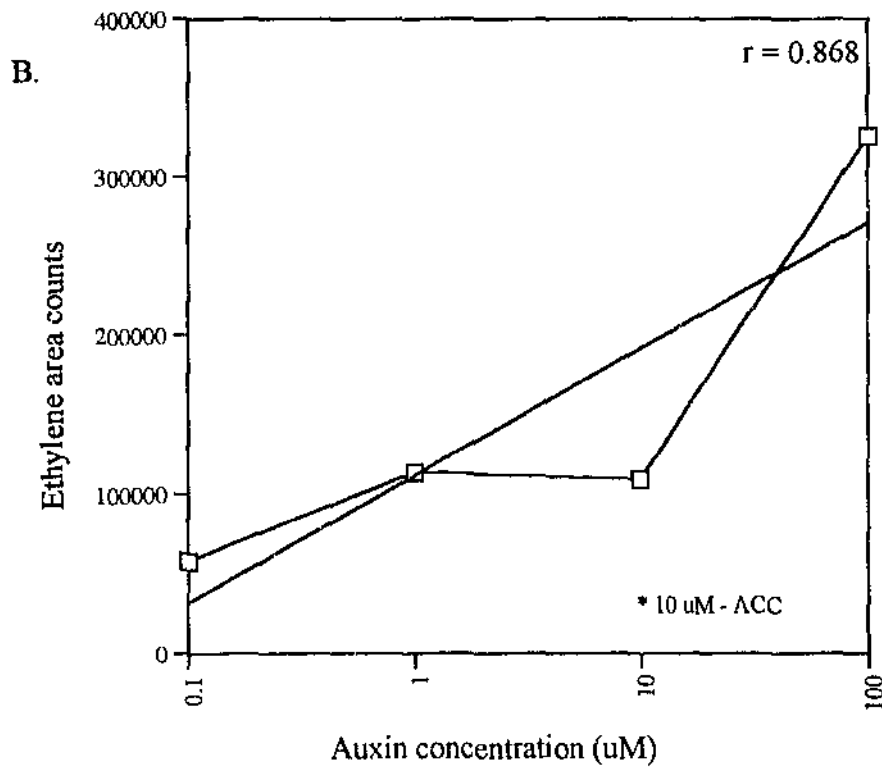


12 JN 96

Figure 4.11 Correlation of mean chlorophyll content and ethylene area counts of shoots after 28 days on media with differing auxin concentrations with ACC. A. Clone 5 JN 336. B. Clone 12 JN 96. Replicates between 8 and 10.



5 JN 336



12 JN 96

Figure 4.12 Correlation of auxin concentration and ethylene area counts of shoots after 28 days on media with differing auxin concentrations with ACC. A. Clone 5 JN 336. B. Clone 12 JN 96. Replicates between 4 and 5.



### 3.4 Experiment Four

The different levels of sucrose induced varying amounts of rooting ( $P < 0.05$ ) (Figure 4.13 A and B), with the difference being more pronounced in the mean number of roots per shoot. For mean rooting percentage 2% sucrose - ACC was greater than 0, and 1% sucrose and; 0% sucrose was less than all other treatments. For mean number of roots per shoot 2% sucrose - ACC was found to be greater than all other treatments and 0% sucrose + ACC was less than all other treatments.

The various sucrose concentrations were found to influence the resulting chlorophyll content ( $P < 0.05$ ) (Figure 4.14 A). Four percent sucrose was found to be greater than 0, 1, and 2% sucrose + ACC. However, 4% sucrose was not significantly different to 2% sucrose - ACC.

The treatments were found to vary the amount of ethylene produced ( $P < 0.05$ ) (Figure 4.14 B). One and 2% sucrose + ACC produced more ethylene than 0% sucrose + ACC and 2% sucrose - ACC.

Correlations for mean rooting percentage (Figure 4.15 A), and mean number of roots per shoot (Figure 4.15 B), ranged from modest to strong positive relationships. This indicates that as rooting increased, ethylene increased. For chlorophyll content, a negative weak correlation resulted.

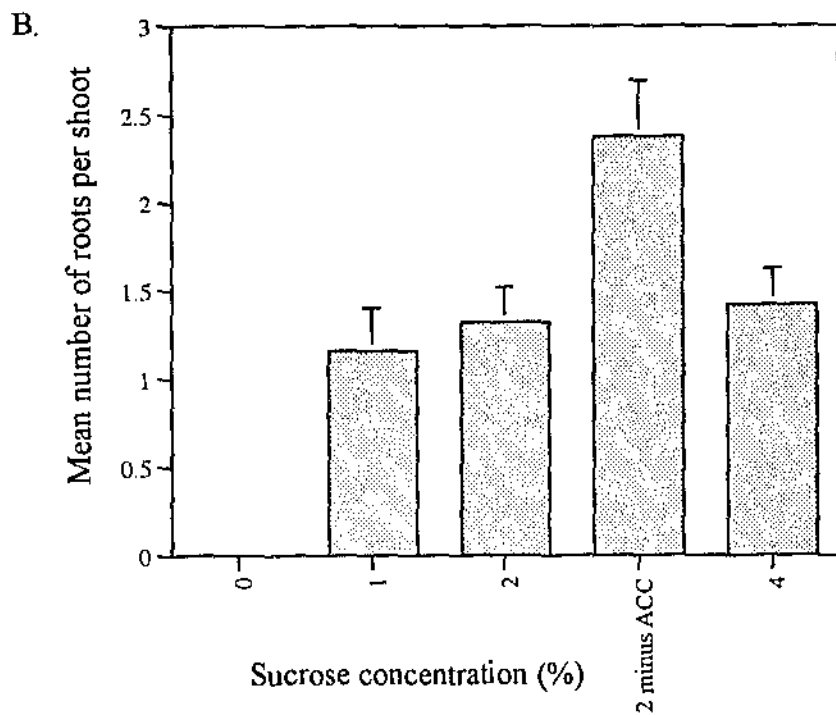
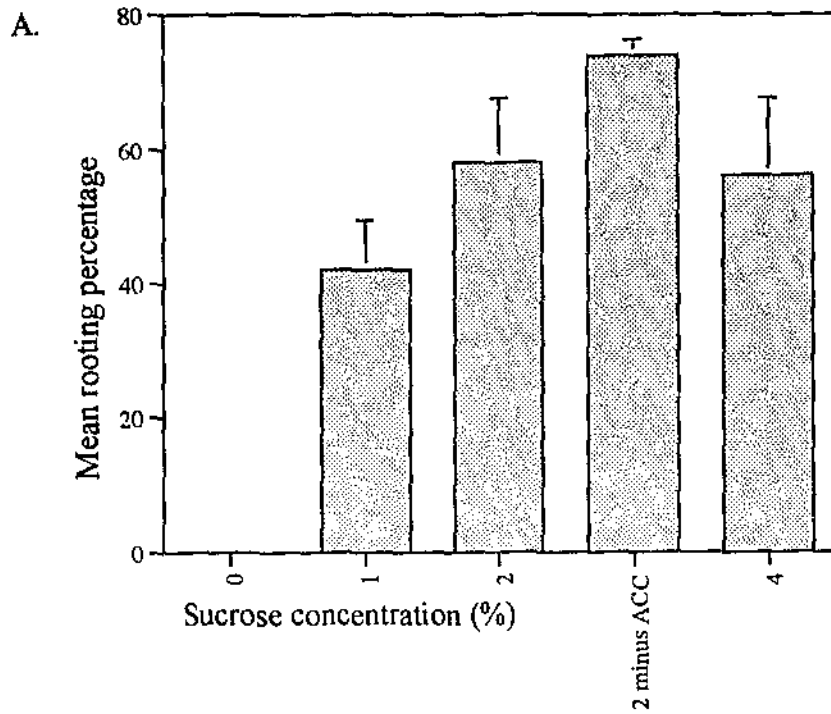


Figure 4.13 Rooting response of 5 clones to different sucrose concentration with applied ACC. A. Mean rooting percentage (5 replicates) and B. Mean number of roots per shoot (50 replicates) after 28 days. Vertical bars denote SEM.

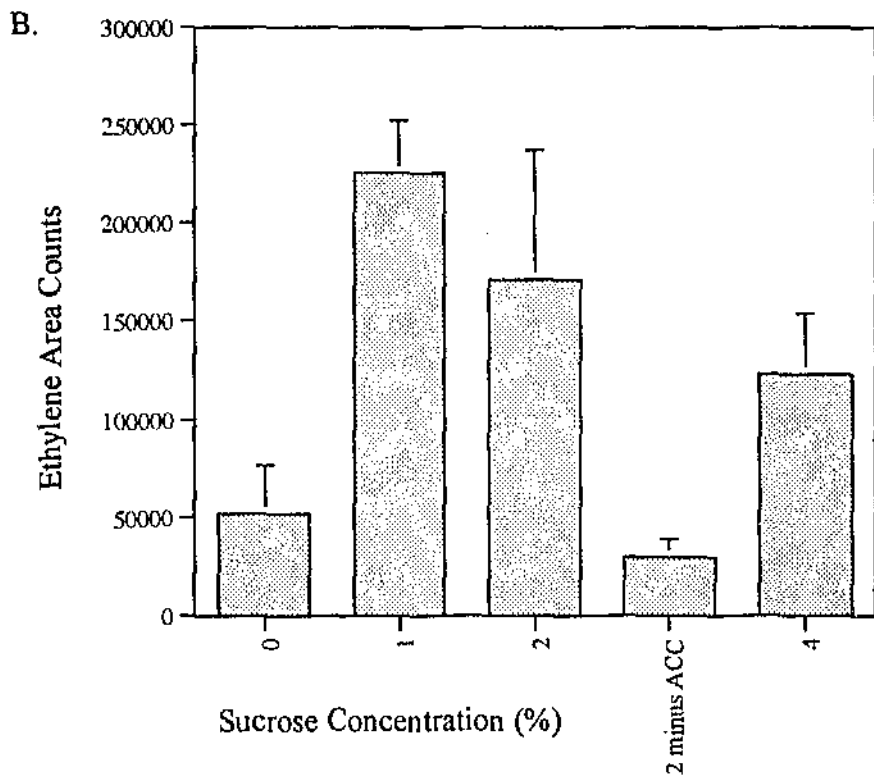
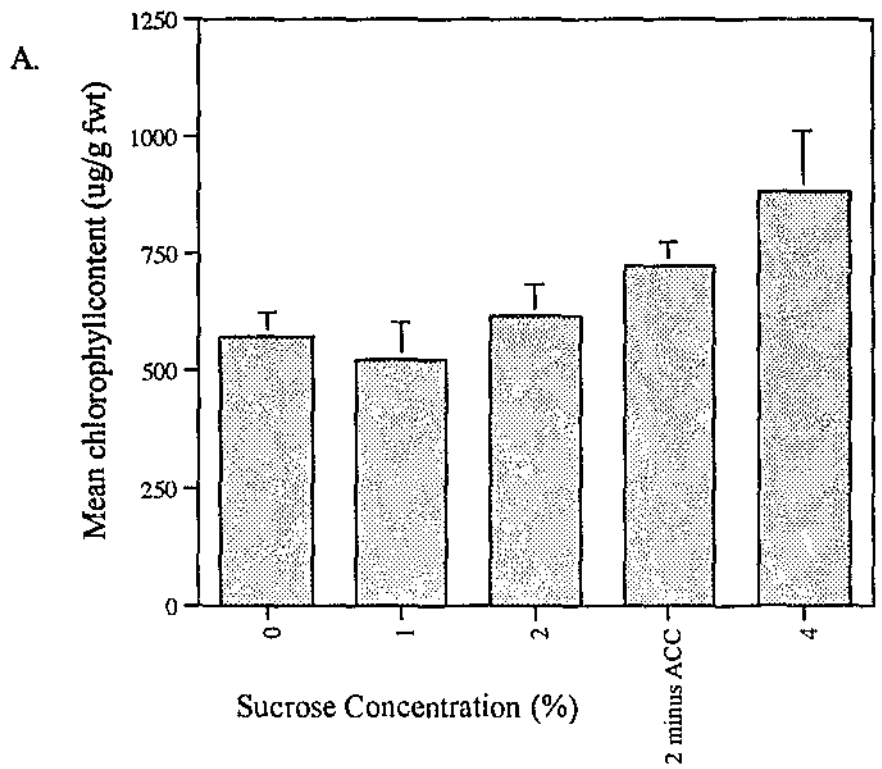


Figure 4.14 Rooting response of 5 clones to different sucrose concentrations with applied ACC. A. Mean chlorophyll content (10 replicates) and B. Mean ethylene area counts (5 replicates) after 28 days. Vertical bars denote SEM.

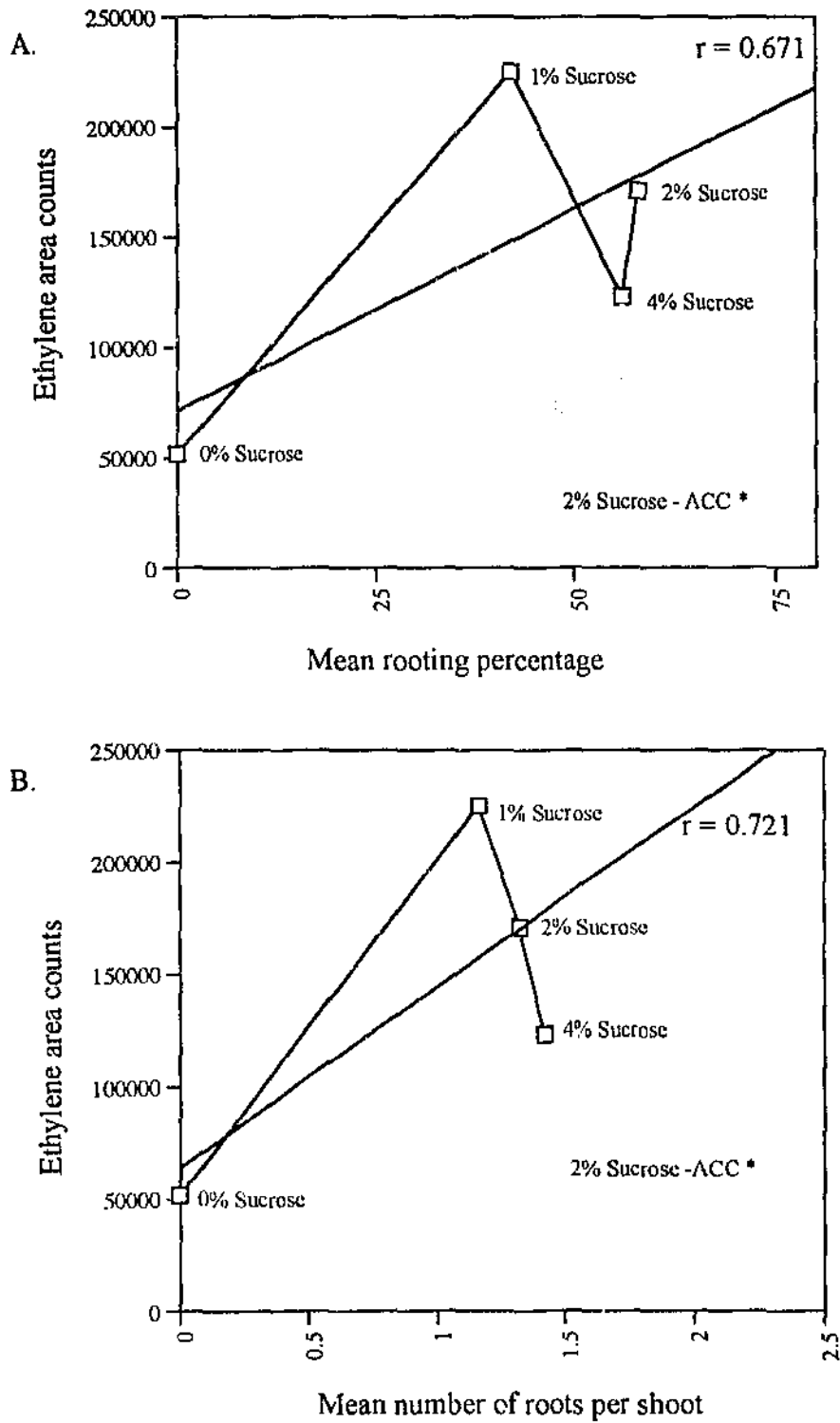


Figure 4.15 Correlation of A. Mean rooting percentage (5 replicates), and B. Mean number of roots per shoot (50 replicates) and ethylene area counts of shoots after 28 days on media with differing sucrose concentrations with ACC.

## 4. DISCUSSION

### 4.1 Experiment One

Auxin was indicated to be necessary for adventitious root production, as minimal rooting occurred at 0  $\mu\text{M}$  IBA for all clones tested. This is supported by the findings of other studies that experimented with levels of auxin on differing plant species and plant tissue (Jackson and Harney, 1970; Arteca *et al.*, 1988; Nordstron *et al.*, 1991; Pan and Zhao, 1994; Baraldi *et al.*, 1995). Auxin was also found to encourage endogenous ethylene production for only one clone, 91 JP 4. For this clone this concurs with the results from other studies (Burg and Burg, 1968; Batten and Mullins, 1978; Yoshii and Imaseki, 1982). In addition, for this clone, very high auxin was found to be associated with lower ethylene amounts, supporting the findings of Bollmark and Eliasson (1990). They found that once auxin reached a critical level, ethylene production decreased. It did not appear that the levels of ethylene reduced root production.

High auxin was found to be detrimental to shoot condition, as reflected in the chlorophyll content, with 100  $\mu\text{M}$  IBA significantly lowering the chlorophyll content for one of the three clones tested. High amounts of ethylene were found to be associated with low amounts of chlorophyll for one of the clones tested. Indicating that ethylene is inhibitory to shoot condition for this clone, and that the response of plantlets to ethylene varied between clones. These results are as expected as other studies have indicated that chloroplast

synthesis and chloroplast development *in vitro* is depressed by ethylene (Dalton and Street, 1976).

#### 4.2 Experiment Two

Sucrose was found to be necessary in the media for the production of adventitious roots. Two percent fructose was found to be the best carbon source for one out of the three clones tested. This supports the results found for experiment two in the previous chapter.

For one of the clones tested, 4% sucrose resulted in the highest chlorophyll content. This was unexpected as other studies have shown that high sucrose levels tend to limit chlorophyll apparatus (Rier and Chen, 1964; Edelman and Hanson, 1972).

The increase in ethylene associated with higher sucrose concentrations is similar to the findings of Kumar *et al.* (1987), who found that ethylene production is increased by the provision of exogenous carbohydrates. The ethylene produced did not significantly effect the resulting shoot condition (chlorophyll content) in any way. This maybe due to the limited amount of ethylene produced in the tissue culture system.

High rooting was found to be associated with high ethylene. This result suggests that the ethylene produced was not high enough to inhibit rooting.

### 4.3 Experiment Three

The presence of ACC in the media increased the production of ethylene substantially. This is supported by the findings of Adams and Yang (1979) who found that ACC increases ethylene production in various plant tissues which normally produce little ethylene.

Auxin concentration of 100  $\mu\text{M}$  IBA, combined with ACC, was found to inhibit root production for the two clones tested. It is suggested that the combination of high auxin and ethylene caused this effect. It is known that the regulation of ethylene is by a feedback mechanism of the level of endogenous auxin. High concentrations of auxin stimulate ethylene formation, but the ethylene then, in some way, causes a lowering of the auxin level in the tissue and therefore lowers rooting (Hayes, 1981; Wareing and Phillips, 1981).

For one of the clones tested, auxin minus ACC gave highest chlorophyll content suggesting that ethylene/ACC is inhibitory to shoot condition. This result is supported by Dalton and Street (1979) who stated that chlorophyll synthesis and chloroplast development *in vitro* is depressed by ethylene.

Ethylene production was found to be the highest at an auxin concentration of 10  $\mu\text{M}$  IBA for clone 5 JN 336. Further increases in ethylene production did not occur at higher concentrations (100  $\mu\text{M}$  IBA). This result is possibly due to the suggestion of Bollmark and Eliasson (1990), who state that as the auxin increases ethylene production, the

ethylene induces a decrease in the auxin production, which in turn decreases the ethylene production, until a stable equilibrium is reached.

#### 4.4 Experiment Four

Sucrose concentration of 2% without ACC was found to promote a higher rooting response than any other treatment. Thus ethylene/ACC presence was associated with lower rooting. This is supported by the results of Fabijian *et al.* (1981), who found endogenous ethylene to be inhibitory for root initiation of *Helianthus* hypocotyls. Also supported by the observations of George (1993) who states that endogenous ethylene is inhibitory to the early stages of root generation. Other studies which have recorded the inhibitory effects of ethylene on root induction include Adkins, (1992), Biddington and Robinson, (1994), Buddendorf-Joosten and Woltering, (1994) and Matthys *et al.* (1995).

Media with ACC was found to lower chlorophyll content, supporting the results of Dalton and Street (1976), who also found ethylene to be inhibitory to chlorophyll development. Chlorophyll content was found to increase as ethylene production decreased.

As sucrose concentration increased, ethylene increased. Media with ACC, except for 0 % sucrose, produced more ethylene than 2% sucrose without ACC. This indicates that adding exogenous ACC to plants *in vitro*, results in a substantial increase in ethylene production (George, 1993).



#### 4.5. General Discussion

These experiments indicate that when shoots are grown on abnormally high levels of auxin and sucrose, ethylene production increases. The quantity of ethylene production increased significantly when ACC was added to the media. Although ACC is not a component of normal root induction media, it enabled the observation and measurement of ethylene effects on the root production and shoot condition of jarrah *in vitro* to be determined.

The results indicate variations between clones and their ability to produce ethylene, with some clones producing substantial amounts of ethylene while others did not. This is often reported with regard to other aspects of micropropagation. Reported variations between clones include; their rooting rates, their multiplication rates, their optimum sugar concentration and optimum auxin concentration (McComb and Bennett, 1986; Damiano *et al.*, 1987; Bell *et al.*, 1993; Bennett *et al.*, 1994).

These results appear to be contrary to those of other studies (Dalton and Street 1976; Fabijan *et al.*, 1981; Adkins, 1992; George, 1993; Biddington and Robinson, 1994; Buddendorf-Joosten and Woltering, 1994; Matthys *et al.*, 1995) in that ethylene was associated with high chlorophyll and high rooting, indicating that it was not inhibitory to growth as expected. This is possibly due to the ethylene produced not being high enough to have significant effects. Direct comparisons between the ethylene produced in these

experiments and those of others could not be made, as the ethylene could not be quantified, and therefore was represented as mean peak areas and not actual values.

## CHAPTER FIVE

### GENERAL DISCUSSION

#### Effect of Sugar Source

When 2% sucrose was supplied in the medium, optimal rooting occurred for some of the clones tested. For the remaining clones, the most suitable carbon source was found to be fructose. These results concur with those of Damiano *et al.* (1987), who observed that each clone may have a different optimum medium, however, these authors did not use fructose. The results presented here suggest that particular clones prefer fructose over sucrose for *in vitro* adventitious root production. This may be related to the ease with which fructose is taken up by the shoots (Smith, 1993).

As there are no other studies currently available on the rooting response of *Eucalyptus* species to fructose, it is suggested that further investigations be undertaken as 2% fructose increased rooting by up to 30% in three clones (1 JN 30, 91 JP 4 and 12 JN 35). Not all clones were tested for their optimal carbon source, however, and it would be beneficial to test those currently hard-to-root clones, as the trend may follow other clones.

## Auxin, Sugar and Ethylene

Auxin was found to be required for adventitious root production. The standard concentration of 10 $\mu$ M from the current tissue culture protocol proved most effective. For the majority of clones this gave the best root production (for both mean rooting percentage and mean number of roots per shoot) out of the five auxin concentrations tested. Higher concentrations of 100 $\mu$ M, increased rooting percentage in a hard to root clone (11 JN 50) but, lowered the chlorophyll content significantly. The study showed that high amounts of ethylene were found to be associated with low amounts of chlorophyll for all clones tested. There is no clear association between high ethylene and low chlorophyll. This is surprising given the data presented by other studies (Dalton and Street, 1976; Chi *et al.*, 1990).

An increase in sucrose concentration up to 4% was found not to affect chlorophyll content and therefore resulting shoot condition of the plantlet. However, in another experiment, high sucrose concentration of 4%, with and without added ACC increased ethylene production.

Sucrose with ACC, and therefore ethylene, was found to lower rooting (mean number of roots per shoot), suggesting that ethylene is inhibitory to rooting. As fructose was found to produce considerably less ethylene, it may be a better carbon source for *in vitro* growth of jarrah.

High auxin concentration (100 $\mu$ M) in association with ACC, was found to inhibit root production for all clones tested. Chlorophyll content was also lower than the standard medium (10  $\mu$ M IBA, 2% sucrose, without ACC), suggesting that 100  $\mu$ M IBA, in combination with ACC, is detrimental to shoot condition. This may be due to the action of ethylene, ACC or high auxin. This supports the findings of George (1993), who suggested that some of the responses of plants to auxins are ultimately brought about by the ethylene produced in response to an auxin treatment.

The variability of clones, the limited amount of ethylene produced and the action of the sucrose and auxin treatments promoting ethylene production made it difficult to generalise about the effects of ethylene upon the rooting of jarrah. Ethylene was, however, implicated as being inhibitory to both shoot condition and root production when associated with high levels (100  $\mu$ M) of auxin plus ACC. These levels are not normally used in general micropropagation of *Eucalyptus*. Further research is warranted to examine both endogenous and exogenous ethylene, as some findings indicate that applied ethylene can promote root formation (Cummins and Fiorino, 1969; Krishnamoorthy, 1972).

## **Conclusions**

From these, and other people's findings, it is suggested that low amounts of auxin, enough to promote rooting, but to reduce ethylene production, be used. The balance between auxin and ethylene is hard to determine, but is an essential part of the plant tissue culture

environment, and may be critical for hard-to-root clones. Further investigations of auxin and ethylene relationships, would be appropriate to increase rooting and also increase survival of the plantlets when placed in soil.

Optimum concentrations of auxin for the clones tested were found to differ. It is very probable for those clones not tested, that their response to auxin concentrations also differs (Damiano *et al.*, 1987). As their tolerance levels of ethylene, produced by the auxin, varies, due to the variable characteristics of the clones (McComb and Bennett, 1986). It is therefore suggested that further investigations on auxin concentration for those important clones be performed. Time restrictions disallowed further findings to be determined in this study.

Although sealed tissue culture containers used for the ethylene experiments is not the normal protocol for micropropagation, quantities of ethylene built up under normal conditions (unsealed containers) from a preliminary experiment were found to be significant. From these results, ventilation of tissue culture systems is an issue that should be considered, as containers were shown to have quantities of ethylene that in some cases may have been related to reduced rooting response and poor shoot condition. Vented culture containers are commercially available for use in plant tissue culture. These containers allow the flow of gases such as ethylene, methane and carbon dioxide, out of the tissue culture environment. This significantly reduces gas build up, and in particular

ethylene accumulation, and therefore promotes the growth and development of healthy plantlets (Jackson *et al.*, 1991; Smith and Spomer, 1995).

The variability of clones was highlighted in these experiments. Not all clones were found to respond similarly to the applied treatments of sucrose and auxin. These results are supported by the observations of McComb and Bennett (1986), and Damiano *et al.* (1987), who did similar experiments with *Eucalyptus* clones.

It is known that ethylene, auxin and sucrose levels do influence chlorophyll content of plantlets by retarding chlorophyll formation and photosynthesis (Dalton and Street, 1976; Capellades *et al.*, 1991; George, 1993), and as fructose produces considerably less ethylene than sucrose of the same concentration, acclimatisation may well be more successful when fructose is used as the carbon source. It is therefore suggested that a concentration of 2% fructose in combination with an auxin concentration of 10 $\mu$ M IBA, be used for the *in vitro* adventitious root production of jarrah clones.

An increase in rooting of 30 % can increase the number of clones that can be utilised in micropropagation. Clones grown on sucrose with rooting rates of less than 30% are generally considered uneconomical to produce in large numbers. An increase as high as 30% would mean that many more clones could be readily produced. This could have a significant impact upon the way in which these clones could be used. In particular, it

would increase the genetic diversity that is now available for present jarrah breeding programs.



## CHAPTER SIX

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