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The optimisation of nitrogen content for micropropagation of eucalyptus marginata

Andrew James Woodward
Edith Cowan University

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THE OPTIMISATION OF NITROGEN CONTENT FOR MICROPROPAGATION OF *EUCALYPTUS MARGINATA*

ANDREW JAMES WOODWARD

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ABSTRACT

The use of eucalypts for plantation timber and pulp is becoming increasingly important, as are eucalypts that are resistant to disease and insect herbivory. Where clones with desirable traits have been selected, it is important that these genotypes are preserved. Micropropagation of some eucalypts, and *Eucalyptus marginata* in particular, can be difficult possibly due to the currently used basal medium of Murashige and Skoog (M&S)(1962). By examining the nitrogen utilisation and the effects of medium pH of this species, it may be possible to improve general micropropagation protocol.

Six clones of the species *E. marginata* were obtained as shoot cultures. The pH of M&S medium was successfully buffered using 2-(N-morpholino)ethanesulfonic acid (MES) for both shoot multiplication and root induction. This did not result in any growth increase. Increased growth was achieved when shoots were grown on buffered medium that contained less nitrogen than M&S (20 mM and 40 mM) and the nitrogen was supplied solely as nitrate.

Shoots grown on buffered (10 mM MES) root induction medium produced more roots than those grown on unbuffered medium. Root induction medium containing nitrate as the sole source produced more roots than did media with ammonium and nitrates or solely ammonium. These results suggest that *E.*

marginata prefers nitrate to ammonium as a nitrogen source, and that the current level of total nitrogen may be too high.

The pH of culture medium for both shoot multiplication and root induction remained constant with nitrate as the sole nitrogen source but fell significantly when ammonium was the sole source. This occurred even when MES was supplied at 10 mM. Increasing the level of MES to 20 mM in the medium resulted in less shoot growth.

When different clones were subjected to the same treatments, significant differences in shoot growth and percentage of rooting between clones were observed, highlighting the genetic variability within the species.

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.

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1. INTRODUCTION

1.1 IMPORTANCE OF EUCALYPTS

1.1.1 Clonal Forestry

The development of plantations for timber and wood pulp production is becoming increasingly important around the world. This is because of an increase in world demand for timber and paper and an ever diminishing source of timber from the world's forests. In an effort to increase the efficiency and productivity of plantations clonal propagation is increasingly used to produce trees. This allows for rapid exploitation of genetic gains obtained from tree breeding programs, and produces a consistent product that is more readily harvestable and requires less processing. Currently, extensive clonal plantations have been established by producing plants from vegetative cuttings. Other clonal techniques are restricted to the production of high value plants for special plantings such as seed orchards.

Clonal propagation through micropropagation is seldom used for the production of trees for plantations. This is because of the high cost of production and the lack of a suitable protocol. However, where the plants are of particularly high value, or where there is a need to build up stock plants rapidly, micropropagation has been important (Eldridge *et al.*, 1993). The containment of tissue in aseptic culture has also helped in the exchange of genetic material around the world, there being less need for long term quarantine.

1.1.2 Plantation Eucalypts

Eucalypts are now the world's most widely planted hardwood trees and they form the major component of plantations in many temperate and tropical countries (Eldridge *et al.*, 1993). The development of clonal plantations has been most extensive in countries such as Brazil and The Congo and some more temperate countries such as Portugal (Eldridge *et al.*, 1993). The environmental conditions and low labour costs in countries such as Brazil are more conducive to propagation through cuttings, but there is still substantial loss of genetic material because some genotypes cannot be produced using this approach.

Micropropagation of eucalypts has been developed for most of the commercially important species with varying degrees of success (Le Roux and van Staden, 1991). In Australia micropropagation has been developed for eucalypts with high salt tolerance (Bell *et al.*, 1994) disease and insect resistance (Bennett *et al.*, 1992; McComb *et al.*, 1995), and oil and pulp production (de Little *et al.*, 1992). However, there needs to be significant advances in the tissue culture protocol before these important trees can be produced efficiently and economically.

1.1.3 Dieback resistant jarrah

A breeding program has been developed over the past 15 years to produce *Eucalyptus marginata* Donn ex Sm. (jarrah) plants that are resistant to the dieback fungus (*Phytophthora cinnamomi* Rands).

This research effort is being expanded to increase the number of clones available, establish seed orchards for revegetation schemes and establishment of dieback resistant plants on different sites.

This work involves the development of appropriate culture media and conditions for growth of shoots and roots. Of major concern is that the response of the plants to different media and culture conditions is dependent upon the individual plant; some clones of jarrah are easy to produce in large numbers while others are impossible. Using the present techniques, clones from about 30-40% of the seedlings that survive the screening trials can be readily produced. This means that 60-70% are excluded from large plantings. Unfortunately, this large percentage represents a waste of the time and resources required for the screening trials, and sometimes, the loss of potentially useful breeding material.

1.2 NITROGEN CONTENT AND TISSUE CULTURE MEDIA

1.2.1 Nitrogen requirements of whole plants

Nitrogen is one of the most important macroelements required for plant growth. It is taken up by the roots as inorganic nitrogen in the form of ammonium (NH_4^+) or nitrate (NO_3^-). Here it is combined with carbon by amination to form simple organic compounds, including simple amino acids and glutamic acid, before ascending the stem. In a few species, under conditions of high nitrogen availability, inorganic nitrogen may be transported

to the shoots for assimilation. Although inorganic nitrogen is available in both the reduced and oxidised forms, it is only the reduced form that can be used by the plant to form organic molecules. Therefore any nitrates taken up by the plant must first be reduced to ammonium, an energy requiring process. These basic organic compounds are then converted to other more complex molecules, through the process of transamination, for use in the plant's metabolism (Salisbury & Ross, 1992).

Plants are capable of producing all of the amino acids they require for normal growth from inorganic nitrogen taken from the soil. They also manufacture the nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP), which are energy transferring compounds used in photosynthesis. Nitrogen is also used in the construction of chlorophyll molecules, nucleic acids (DNA, RNA) and in many vitamins (Raven et al., 1992).

1.2.2 Effects of Ammonium and Nitrate on *in vitro* Plant Growth

Plants grown in tissue culture must be supplied with nitrogen as ammonium and as nitrate because plants can only metabolise nitrogen in the ammonium form. However, it is toxic in large amounts, and so nitrates must also be supplied (George, 1993). The level at which ammonium can be supplied before it becomes toxic and, further, what is the optimum level for each

species is not known and must be determined experimentally (Behrend & Mateles, 1976).

There are two effects of ammonium as a nitrogen source that need to be addressed in micropropagation of a particular species. First, are its effects on shoot growth and, secondly, its effects on root induction. With regard to both aspects, the problem of a change in pH is encountered.

Most work on root induction has centred on the levels and types of hormones required for this process (Bennett, McComb, Tonkin & McDavid 1994; Fukunaga, King & Child, 1978; Williams, Taji & Bolton, 1985). Little research has been conducted into the level of ammonium present, and at what level it inhibits root production. Ammonium is important for shoot growth, but at the same time it is known to be inhibitory to root induction (Behrend & Mateles, 1976; Sriskandarajah et al., 1990; Veliky & Rose, 1973).

Sriskandarajah et al. (1990) conducted an experiment into the effect of ammonium on rooting in three apple cultivars. It was found that a reduction in ammonium lead to an increase in two of the cultivars, but not in a third. A further reduction to zero ammonium reduced rooting in one that had increased, and not the other, but lead to an increase in the cultivar that had shown no increase previously.

Differences in number of roots were found in *Senecio x hybridus, Beta vulgaris, Pelargonium x hortorum* and *Anthurium scherzerianum* when levels of nitrogen in the medium were varied (Gertsson, 1988).

Root induction can be achieved with ammonium as the sole nitrogen source if the medium is buffered. Plants grown solely on ammonium exhibit four major toxicity symptoms. Firstly, a decreased level of mineral cations in tissue is noted. Secondly, there is a reduction in the level of organic anions in the plant. Thirdly, there is an increased accumulation of free amino acids. Fourthly, free sugars and starch accumulate in the shoots. However, most of these occur as a result of pH variations in the medium (Chaillou et al., 1991).

It has already been suggested that eucalypts prefer ammonium to nitrates as a nitrogen source in whole plants (Shedley et al., 1993), but ammonium toxicity has prevented an increase in the level of ammonium in eucalypt media *in vitro*. However, buffering of the media may relieve the toxicity symptoms and promote increased root induction and growth (Chaillou et al., 1991). These findings suggested an investigation into the effects on root induction of maintaining pH and altering the level of ammonium is appropriate.

1.2.4 Maintenance of pH

One of the major problems associated with growing plants *in vitro* is that of pH changes. As the plant utilises nutrients in the external medium, ions are released, causing a change in the acid balance of the medium. A change in the

pH can affect the types and amount of nutrients able to be taken up by the plant (Williams, 1993).

The main nutrient that affects this pH change is nitrogen. Inorganic nitrogen is supplied in two forms within plant media: reduced and oxidised. The reduced form of nitrogen is ammonium (NH_4^+), with reduction in this case referring to the removal of oxygen and the replacement by hydrogen. Plants utilise this reduced form of nitrogen in their metabolism, with reduced nitrogen being almost the sole form of nitrogen found in the plant. Most media contain more nitrate (the oxidised form, NO_3^-) than ammonium ions, but this is due to most media being poorly, if at all, buffered. This ratio of nitrate to ammonium is likely to be present only as a method of pH control (George & Sherrington, 1984).

As plants *in vitro* utilise nitrate, anions are released, causing a rise in the pH of the medium. This is balanced by hydrogen ions being released as the plant takes up and utilises ammonium. This causes the pH to fall again, and thus, the plant is able to take up more nitrate (George, 1993).

With *in vitro* eucalypt cultures pH falls, indicating ammonium utilisation, but it is not seen to rise again. This means that the plant is not taking up and using nitrates, as pH would again rise. As pH is now lower, the further problem of the plant not being able to take up any more ammonium is encountered. This

means that the plant is not taking up nitrogen of any form, and further growth is restricted.

Current evidence suggests that eucalypts prefer reduced nitrogen to the oxidised form (Shedley et al., 1993). A problem presented however, is how to supply more ammonium when standard media (eg Murashige and Skoog (1962)) cause a substantial fall in pH. One way to prevent this may be the use of buffers.

1.2.4.1 Use of Buffers for Plant Tissue Culture

A buffer is a chemical that has the ability to maintain a constant pH by forming complexes with hydrogen (H^+) and hydroxide (OH^-) ions as they are released into a system. In the case of plant media, this means that as ammonium is utilised by the plant, no major fall in pH will result, allowing for continued uptake of that nutrient. There are several buffers available for pH control in plant media. Some of these compounds are organic acids, which work by substituting for other organic molecules in the Kreb's cycle. There are also several inorganic buffer systems, including phosphate and sodium. However, it has been found that the phosphate buffer is needed in such high levels that growth can be inhibited rather than promoted (Street & Henshaw, 1966). The sodium buffers are ineffective because their buffering range is outside the pH required for optimum growth of plant tissue.

The final category of buffers contains those known as biological buffers. Unlike organic acids, these chemical compounds are not metabolised. Most of these substances were designed for use with tissue culture in animals, or other microorganisms, and as such have a buffering range outside of the pH required for growth of plants. These include, tris (hydroxymethyl)aminomethane (TRIS), N-tris(hydroxymethyl)methylglycine (Tricine), 4-(2-hydroxyethyl)-1-piperazine(2-ethanesulfonic acid) (HEPES) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS). However, there is one buffer which has been used effectively in controlling pH in plant tissue culture. This is 2-(N-morpholino)ethanesulfonic acid (MES). It is a commercially available product that acts in the range of pH 5-6. It has a low capacity to react with micronutrients, and is only toxic to a few species, or when used at very high levels (George, 1993).

MES has been used at 50mM to keep pH stable in culture of *Nicotiana* cells for 28 days, where unbuffered media had a significant pH change over the same time span (Tiburcio et al., 1989). In another experiment, it was found that a level of 10mM MES was effective as a buffer in media for several species of plants (Parfitt et al., 1988). The media used was for propagation of tobacco, carrot, peach, tomato and carnation, and while buffering stabilised pH, it did not result in an increase in growth. A large increase in the level of the buffer caused damage to the plants. Rooting media used for propagation of jackfruit (*Artocarpus heterophyllus*) was successfully buffered using a 20mM concentration of MES (Sathyanarayana & Blake, 1994). This allowed for

ammonium to be supplied as the sole source of nitrogen and actually lead to a high percentage of rooting.

The effect of buffers, their ability to control pH and to allow raising the level of ammonium to increase rooting and shooting, was examined. Based on the above examples, several levels of MES were investigated, as little work had been done in this area with eucalypts. The levels examined were 0, 10 and 20mM concentrations of the buffer compound.

1.3 AIMS

To determine:

1. the optimum nitrogen content in tissue culture media for the micropropagation of jarrah;

2. the effect of different NH_4^+:NO_3^- ratios on the growth of shoots and roots in jarrah cultures;

3. the relationship between nitrogen and changes in pH in jarrah cultures;

4. the effects of stabilising pH using buffers on the growth of jarrah cultures;

5. the most appropriate source of N for growth of jarrah

1.4 HYPOTHESES

1. Growth of jarrah shoots and root induction *in vitro* varies with total nitrogen concentration

2. Nitrogen source has a significant effect on medium pH in jarrah cultures

3. The pH of jarrah culture medium can be stabilised by the addition of buffers

4. Jarrah shoot growth and root induction *in vitro* can be improved by supplying appropriate nitrogen sources

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

Six clones of *Eucalyptus marginata* with varying resistance to *Phytophthora cinamomi* were used in the experiments. These were obtained from stocks at Edith Cowan University that had been established at least 2 years previously. This material was used for shoot multiplication as well as rooting experiments.

2.2 STERILE TECHNIQUE

Tissue culture involves growing plants under a defined set of conditions and in a sterile environment. Any materials for handling, growing and storing these cultured plants must first be sterilised.

Sterile conditions were achieved by autoclaving any materials to be used on or coming into contact with the plants or media containers (eg. instruments, plastic cutting plates, media containers, media, ethanol, rinsing water) at 121°C for 15 minutes.

Plant material was handled aseptically in a laminar flow cabinet which had been exposed to ultra-violet radiation for approximately 20 minutes, and then swabbed with 70% ethanol. Instruments were regularly re-sterilised using a Bacticinerator sterilising unit (Sigma-Aldrich, Castle Hill NSW).

pH measurement was conducted in the laminar flow unit, using an Intermediate Junction electrode (Ionode Pty Ltd, Tennyson QLD) that had been surface sterilised in autoclaved 70% ethanol for approximately 20 minutes. The culture vessels were opened only in

the laminar flow unit and the probe was re-sterilised by rinsing in 70% ethanol, then 2 rinses in sterile ultra-pure water, after measurement of each tub.

2.3 SHOOT CULTURE MAINTENANCE

Cultures were grown in 250 mL screw top polycarbonate containers containing 50 mL of solid medium. Shoots of selected clones were subcultured onto standard media every four to six weeks, depending on growth. Cultures were grown either in a culture room at 25°C ±4°C or a growth cabinet at 25°C ±1°C, both with a 16h photoperiod. Light was provided by cool white fluorescent tubes, and irradiance at the culture surface was approximately 24μmol.s^{-1}.m^{-2} in growth cabinets and 36μmol.s^{-1}.m^{-2} in the culture room.

2.3.1 Shoot Culture - experimental

For experiments on shoot growth, shoots were transferred to the experimental media, and allowed to grow for a period of four weeks in a growth cabinet with a photoperiod of 16 h light / 8 h dark. In some cases, the shoots were subcultured onto the same type of media for another four week period. For experimental procedures, shoots of approximately 1 - 2 cm in length and containing 2 - 3 leaves, were cut and placed upright, 5 per vessel, into culture containers. Distribution of shoots was random with material cut from one culture being placed into culture vessels with different treatments.

2.3.2 Root Induction

For rooting experiments, larger shoots (2 - 3 cm in length, with 4 -6 leaves) were cut from selected clones and placed upright, 5 per vessel, into culture vessels containing the different media, maintained for 7

days in total darkness and then transferred to either a growth cabinet or the culture room with a photoperiod of 16 h light / 8 h dark.

2.4 CULTURE MEDIA

2.4.1 Stock Solutions

Stock solutions of the plant growth substances IBA, NAA and BAP were used in media preparation. These were prepared by dissolving powdered auxins and cytokinins (Sigma-Aldrich, Castle Hill NSW) in analytical grade ethanol or 1M NaOH respectively, and made up to the required volume with ultra-pure water (ion-exchange filtered to 15 mΩ electrical resistance). Stock solutions were stored at 4°C, with auxins being kept in dark bottles to reduce deactivation by light.

For nitrogen experiments stock solutions of macro- and micronutrients were prepared using ultra-pure water and analytical grade reagents (BDH, Kilsyth VIC).

2.4.2 Media Composition

Culture media were prepared using Murashige and Skoog (M&S) Basal Medium Powder (Sigma Aldrich, Castle Hill NSW; Product number M5519), containing macro- and micronutrients, vitamins and organics. Agar (High Purity Agar, Coast Biochemicals, Auckland NZ), gellan gum (Phytagel™, Sigma-Aldrich, Castle Hill NSW), plant growth substances and sucrose (CSR Ltd, North Sydney, NSW) were also added.

For pH experiments, the buffer 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma-Aldrich, Castle Hill NSW; Product Number M8250)

was added in relevant concentrations. For nitrogen experiments, appropriate volumes of stock solutions were added.

2.4.3 Media Preparation

Media were prepared using analytical grade reagents and ultra-pure water. Glassware and culture vessels were washed in phosphate-free detergent and hot water, rinsed twice in tap water and given two rinses in deionised water before being oven dried at 60°C.

Media components were weighed and dissolved in ultra-pure water. Stock solutions of hormones, and in some cases macro- and micronutrients, vitamins and buffer, were added to the medium, solution made up to final volume, and pH adjusted to 5.8 with 1M KOH or 1M HCl. Powdered gelling agents were added to the media and dissolved by heating in a microwave oven on high at 10 min/L. Media was dispensed into culture containers while hot, then autoclaved. Media was stored at 4°C and in the dark until used.

TABLE 2.1: Composition of Murashige and Skoog (1962) Media

COMPONENTS ($mg.L^{-1}$)	CONCENTRATION
Macronutrients:	
Ammonium Nitrate	1 650.0
Potassium Nitrate	1 900.0
Calcium Chloride.$2H_20$	440.0
Magnesium Sulphate.$7H_20$	370.0
Potassium DiHydrogen Orthophosphate	170.0
EDTA-Iron(III) Sodium Salt.H_20	36.7

Micronutrients	
Boric Acid	6.2
Manganese Sulphate.$4H_20$	22.3
Zinc Sulphate.$7H_20$	8.6
Potassium Iodide	0.830
Sodium Molybdate.$2H_20$	0.250
Cupric Sulphate.$5H_20$	0.0250
Cobalt Chloride.$6H_20$	0.0250
Vitamins	
Nicotinic Acid (free acid)	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine (free base)	2.0
Organics	
Inositol	100.0
Sucrose	20 000.0
Hormones:	(μM)
Benzyl Amino Purine (BAP)	2.5
Naphthalene Acetic Acid (NAA)	1.25
Indole-3-Butyric Acid (IBA)	10.0
Gelling Agents:	($g.L^{-1}$)
Agar	2.5
Gelrite	2.5

2.5 ASSESSMENT AND ANALYSIS

2.5.1 Assessment of Shoot Growth

The relative amount of shoot growth, for both shoot multiplication and root induction experiments was determined by weighing each

shoot and measuring the amount of chlorophyll present in the shoot (Moran & Porath, 1980).

2.5.2 Assessment of Rooting

Number of roots was measured without having to remove or compromise sterility of the plant material. Number of roots per shoot in each tub was observed and recorded on a regular basis.

2.5.3 Chlorophyll Determination

Amount of total chlorophyll was determined by measuring the quantities of chlorophyll's a and b in each shoot (non-maceration method; Moran & Porath, 1980). This involved leaving the shoot in 5 mL of N,N-dimethyl formamide (DMF) overnight then reading in a spectrophotometer at the wavelengths of 647 and 664 nm. Using the fresh weight and chlorophyll values, the micrograms of chlorophyll per gram of fresh weight was calculated.

2.5.4 pH Measurement

The pH of each shoot in the media was measured by inserting a surface sterilised intermediate junction probe into the media at the base of the shoot to a depth of approximately 1 cm. Measurement was conducted aseptically so as not to compromise the sterility of the shoots. This process was used for both shoot growth and root induction experiments.

2.5.5 Statistical Analysis

The experiments were designed so that either a one way or two way analysis of variance (ANOVA) could be used. This was achieved by having equal numbers of replicates for each treatment at the start of

the experiment. A two way ANOVA was conducted on the data, and where a difference was found, a one way ANOVA was then performed. Where appropriate a multiple range test was also performed to determine differences between means.

3. EFFECTS OF NITROGEN SOURCE ON SHOOT MULTIPLICATION

3.1 INTRODUCTION

Several clones of *Eucalyptus marginata* were used in a series of experiments that investigated the effect of different nitrogen sources on shoot multiplication. A preliminary trial determined the level of buffer most effective for maintaining pH, with subsequent investigations varying total nitrogen and nitrogen source. The effects of varying the nitrogen source on shoot multiplication within and between clones was examined, as was the effect of stabilising pH.

3.2 MATERIALS AND METHODS

3.2.1 Experiment 1

The effect of different levels of the buffer 2-(N-morpholino)ethanesulfonic acid (MES) on shoot growth was investigated. The levels tested were 10 mM and 20 mM, with a level of 0 mM used as a control. Two clones were used, 1 JN 98 and 5 JN 56, with twenty shoots of each clone used for each treatment. The medium consisted of Murashige and Skoog basal medium (M&S) with the required level of buffer, shoot multiplication hormones and sucrose added. Shoots were grown for a period of 4 weeks, with fresh weight and chlorophyll determined after this time. The pH was measured daily for the first week, then weekly.

3.2.2 Experiment 2

Shoots of 4 clones (11 JN 379, 11 JN 50, 1 JN 30 and 5 JN 56) were subcultured onto media containing different total nitrogen levels and nitrogen source and the buffer MES supplied at a concentration of 10 mM (Table 3.1). At the nitrogen level contained in M&S (60 mM), a treatment containing the same nutrients, but without buffer, was used. Also at this nitrogen level was a treatment that substituted $CaNO_3$ for KNO_3, with the same substitution used at 120 mM total nitrogen. There were ten replicates used for each treatment for each of the four clones. The experiment was conducted over 4 weeks with fresh weight and chlorophyll measured at completion. The pH was measured daily for one week, then weekly.

3.2.3 Experiment 3

The effects of varying the nitrogen source and quantity on shoot growth and pH were measured using 4 clones (11 JN 379, 1 JN 30, 11 JN 50, 5 JN 56). Ten levels of ammonium or nitrate were used, with treatments containing no nitrogen, and standard M&S also included (Table 3.2). All media except for an additional standard M&S treatment contained 10 mM MES. Where ammonium was the sole source of nitrogen, $(NH_4)_2SO_4$ was used as a substitute for NH_4NO_3, and K_2CO_3 was used to supply the potassium normally obtained from KNO_3. No changes were needed in media with only nitrates, as NH_4NO_3 was the only macronutrient removed. Potassium carbonate was used

as a source of potassium for the treatment containing no nitrogen. There were 15 replicates for each treatment per clone. The shoots were grown for eight weeks, with a subculture onto the same media at 4 weeks. Fresh weight was recorded at the time of the subculture. The pH was measured weekly, with fresh weight and chlorophyll determined at completion.

Table 3.1: The nitrogen treatments used for Experiment 2, the effect of total nitrogen level on shoot growth (Total N = 2 NO_3^- : 1 NH_4^+)

TREATMENT NUMBER	TREATMENT
1	00 mM N
2	15 mM N
3	30 mM N
4	60 mM N
5	60 mM N (-MES)
6	60 mM N ($Ca(NO_3)_2$)
7	90 mM N
8	120 mM N
9	120 mM N ($Ca(NO_3)_2$)
10	240 mM N
11	480 mM N

Table 3.2: Nitrogen treatments used for Experiment 3, the effect of nitrogen source on shoot growth

NITRATE (mM)	AMMONIUM (mM)	M&S	00
20	20	60 mM N	00 mM N
40	40		
50	50		
60	60		
60 (-MES)	60 (-MES)		
70	70		
100	100		
120	120		
140	140		

3.3 RESULTS

3.3.1 Experiment 1

There was a significant difference (at the 95% confidence level) between clones, but no significant difference between the three levels of buffer (0, 10, 20 mM MES) for fresh weights. Clone 1 JN 98 shoots were significantly heavier than those of 5 JN 56 (Figure 3.1). For chlorophyll content, there was a significant difference between treatments but not between clones, with the highest chlorophyll content in those shoots that had no buffer in the medium (Figure 3.2). The most significant result was the effect of the buffer on pH, with a significant difference existing between treatments. The buffer was effective in keeping pH, (Figures 3.3 and 3.4) relatively stable at the levels 10 mM and 20 mM while the pH in the treatment without buffer had fallen

Figure 3.1: The effect of different concentrations of the buffer MES on fresh weight in clones 1 JN 98 and 5 JN 56. Means from 10 replicates. Vertical bars represent standard errors.

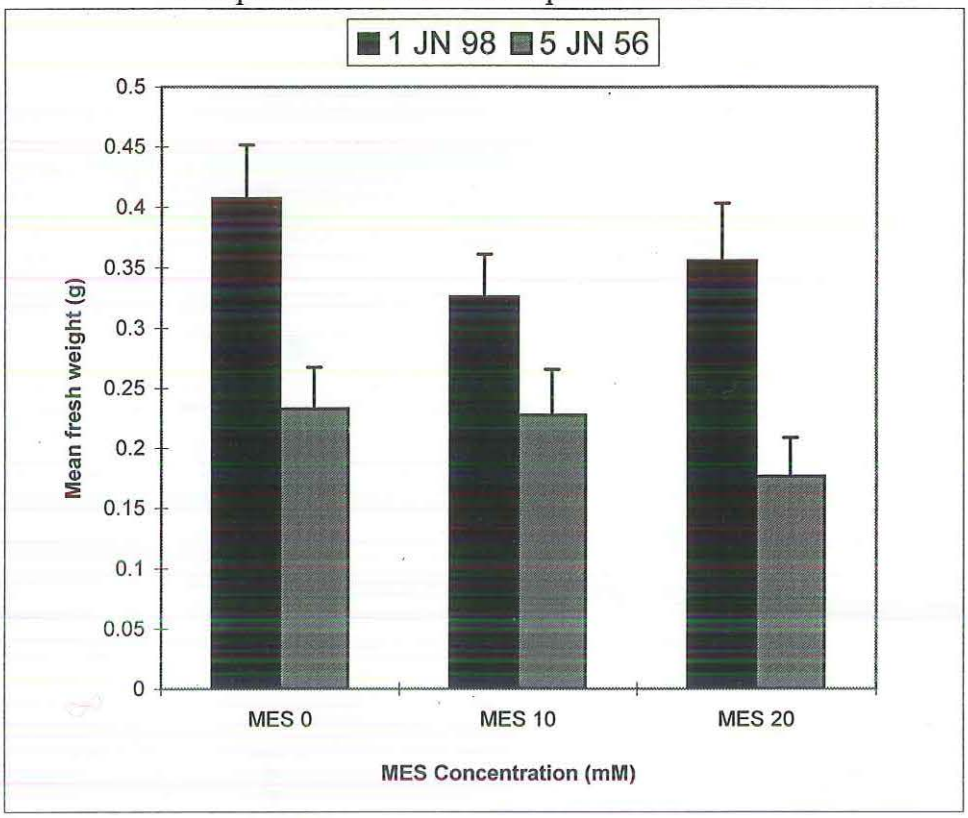


Figure 3.2: The effect of different concentrations of the buffer MES on chlorophyll content in clones 1 JN 98 and 5 JN 56. Means from 10 replicates. Vertical bars represent standard errors.

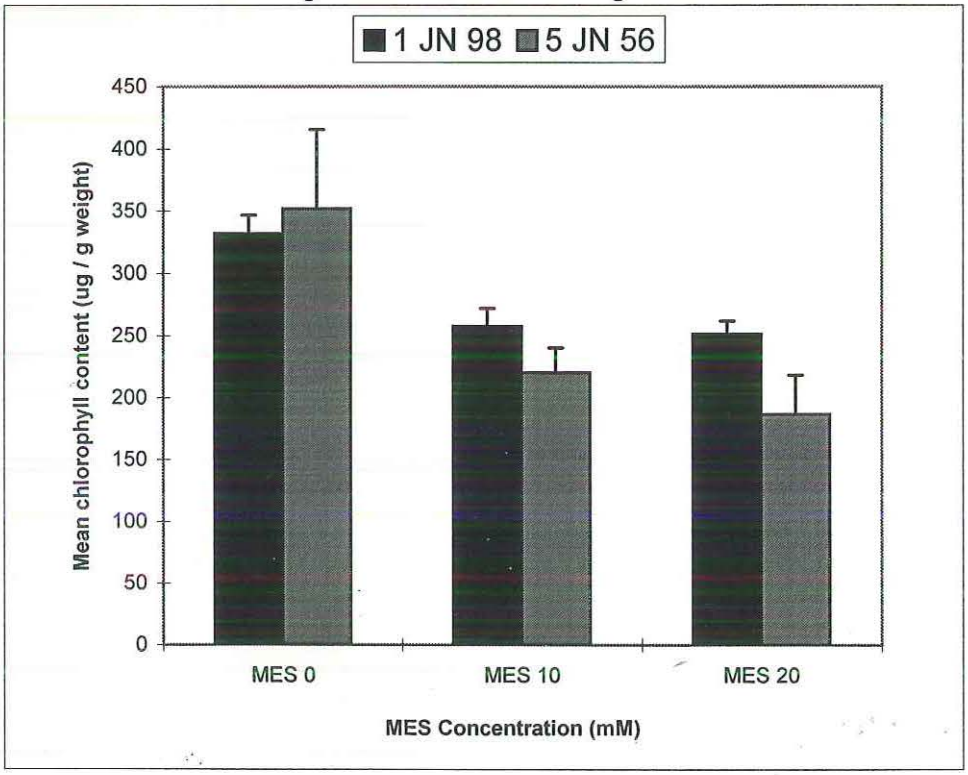


Figure 3.3: The effect of different concentrations of the buffer MES on pH for clone 1 JN 98 over 4 weeks

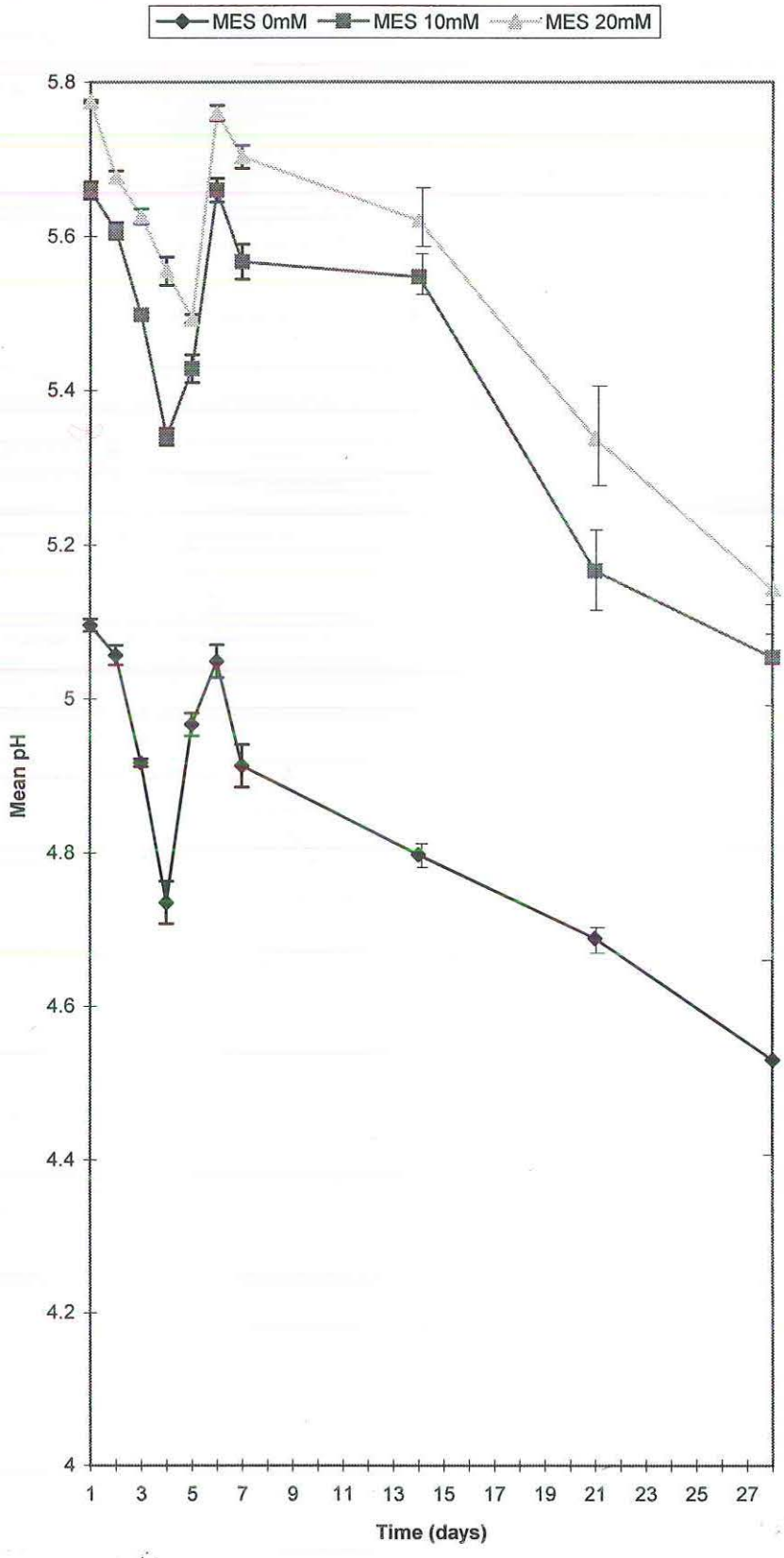
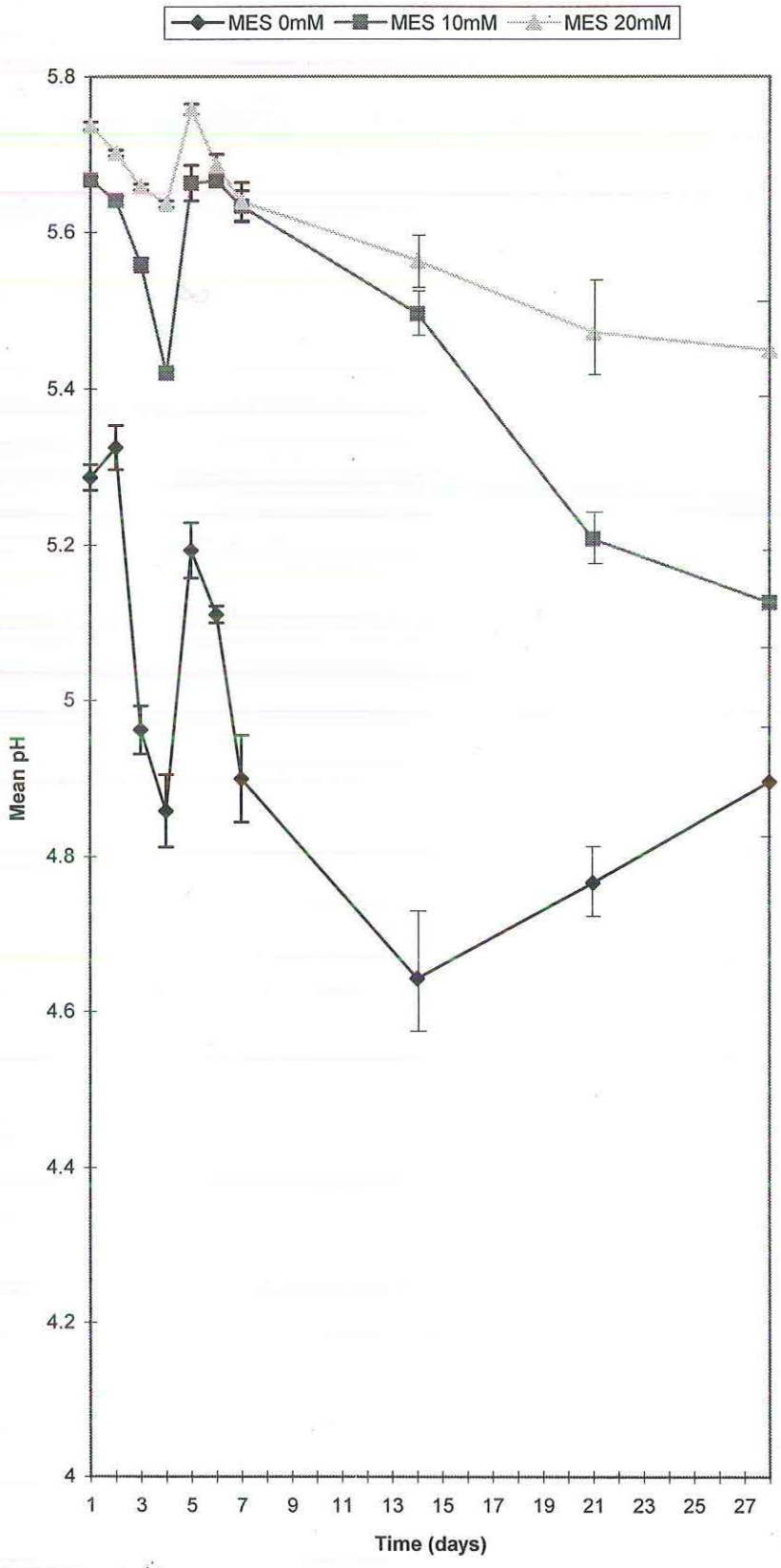


Figure 3.4: The effect of different concentrations of the buffer MES on pH for clone 5 JN 56 over 4 weeks



significantly. There was also a difference between clones for pH, with 5 JN 56 having higher pH at all treatments than 1 JN 98.

3.3.2 Experiment 2

Lower levels of nitrogen produced heavier shoots that contained more chlorophyll per unit mass than did those at higher levels of nitrogen. A significant difference existed between clones and between treatments within all four clones for both chlorophyll content (Figure 3.5) and for fresh weight (Figure 3.6). The total nitrogen levels of 15, 30, 60 and 60 mM $Ca(NO_3)_2$ produced the greater fresh weight for all clones, with some clones responding differently at different treatments. Clone 11 JN 379 had heavier shoots at 120 mM $Ca(NO_3)_2$, 5 JN 56 had significant higher fresh weight at treatments 15 through 120 mM total nitrogen, and 11 JN 50 had the treatments 15, 60 (-MES) and 90 mM total nitrogen producing highest fresh weights.

The chlorophyll content differed between clones and between treatments, with the highest chlorophyll contents at the levels of 0, 15, 30 mM total nitrogen and 60 mM $Ca(NO_3)_2$. In addition, clone 1 JN 30 had high chlorophyll contents at 60 mM, 60 mM (-MES) and 120 mM $Ca(NO_3)_2$. The treatment without buffer had a significantly higher chlorophyll content for 11 JN 379.

A significant difference in pH was found between clones and between treatments for each clone (Figure 3.7). pH was seen to decrease as nitrogen

Figure 3.5: Effect of different nitrogen concentrations on chlorophyll content in four clones. Standard errors were in the range 21 - 103.

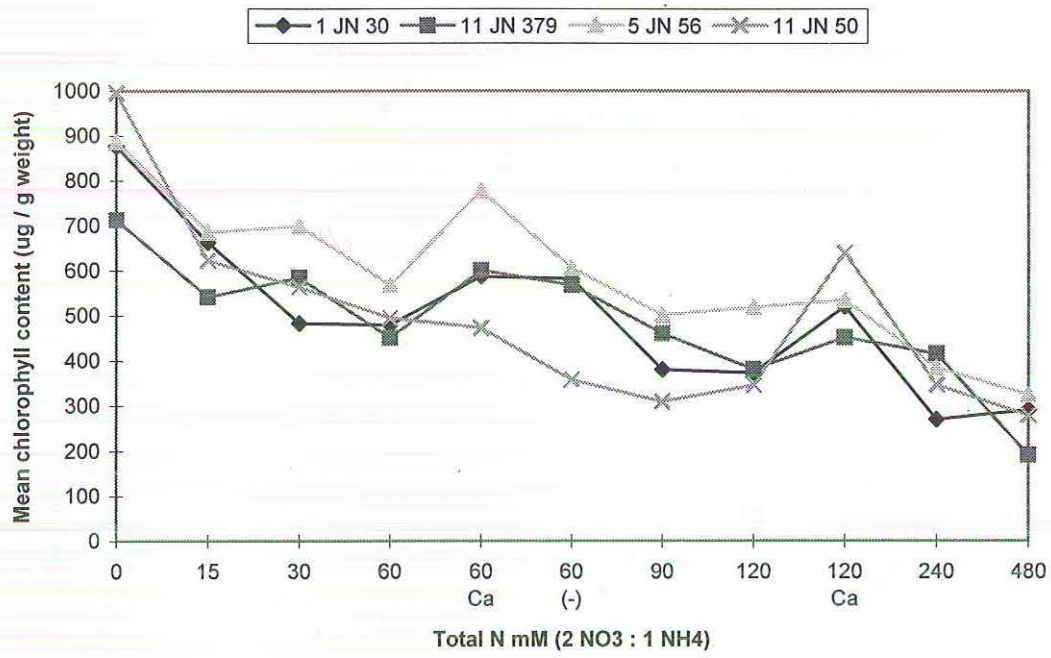


Figure 3.6: Effect of different nitrogen concentrations on fresh weight in four clones. Standard errors were in the range 0.0023 0.046

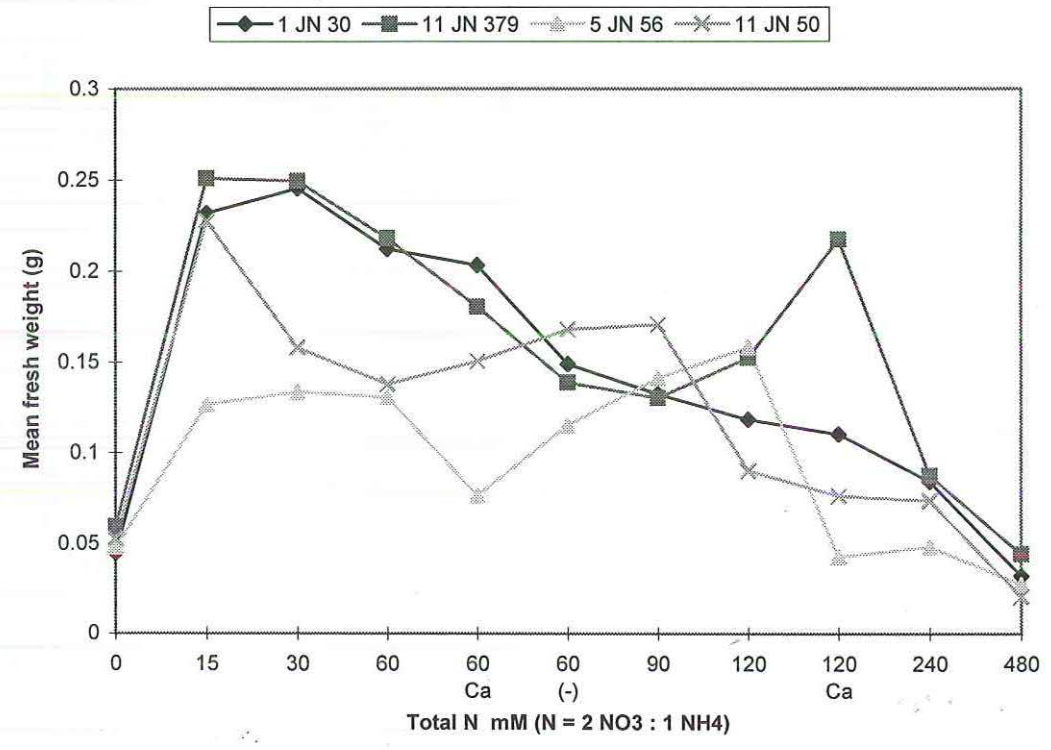
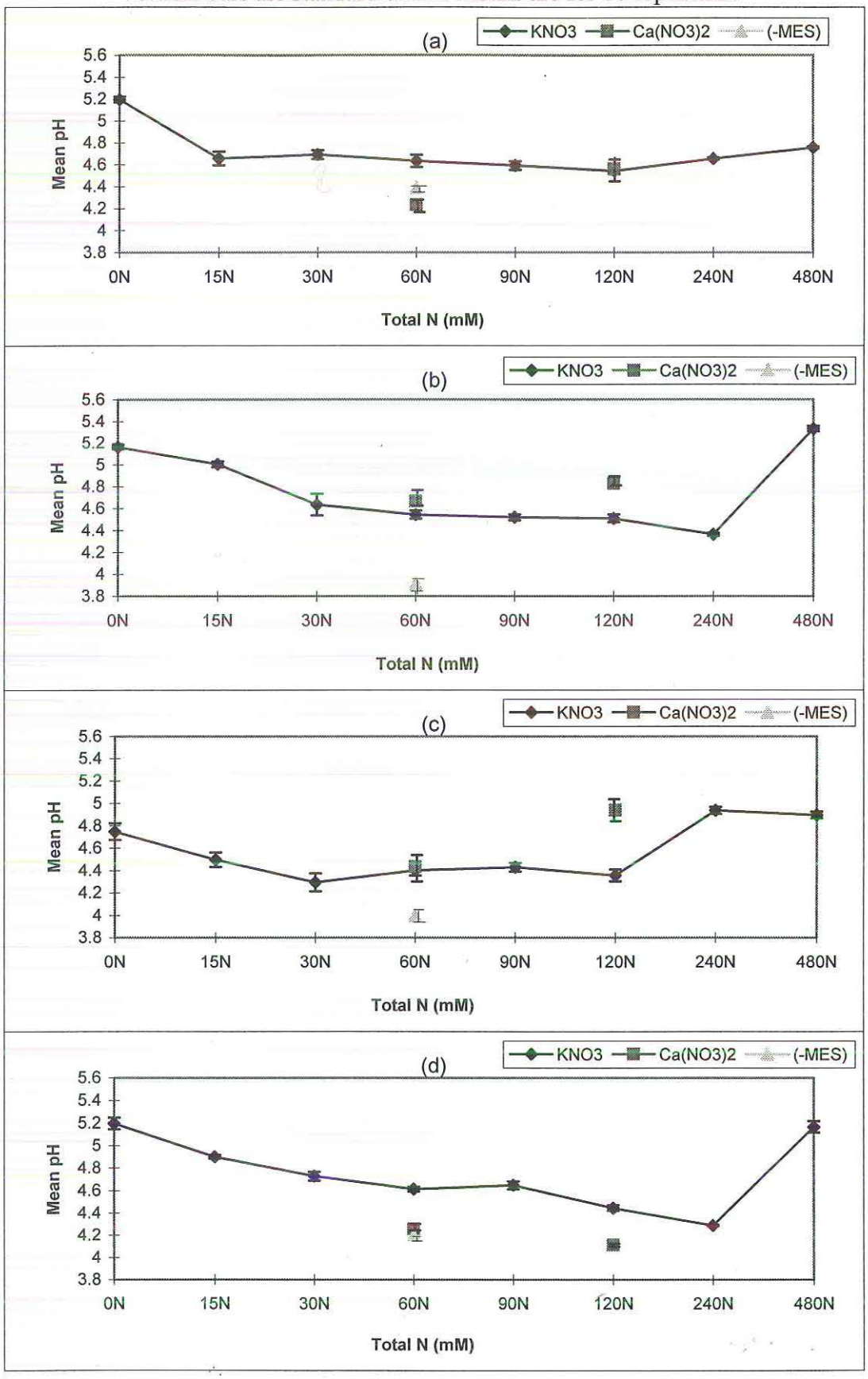


Figure 3.7: The effect of different nitrogen concentrations on pH in four clones: (a) 1 JN 30, (b) 11 JN 379, (c) 5 JN 56 and (d) 11 JN 50
Vertical bars are standard errors. Means are for 10 replicates.



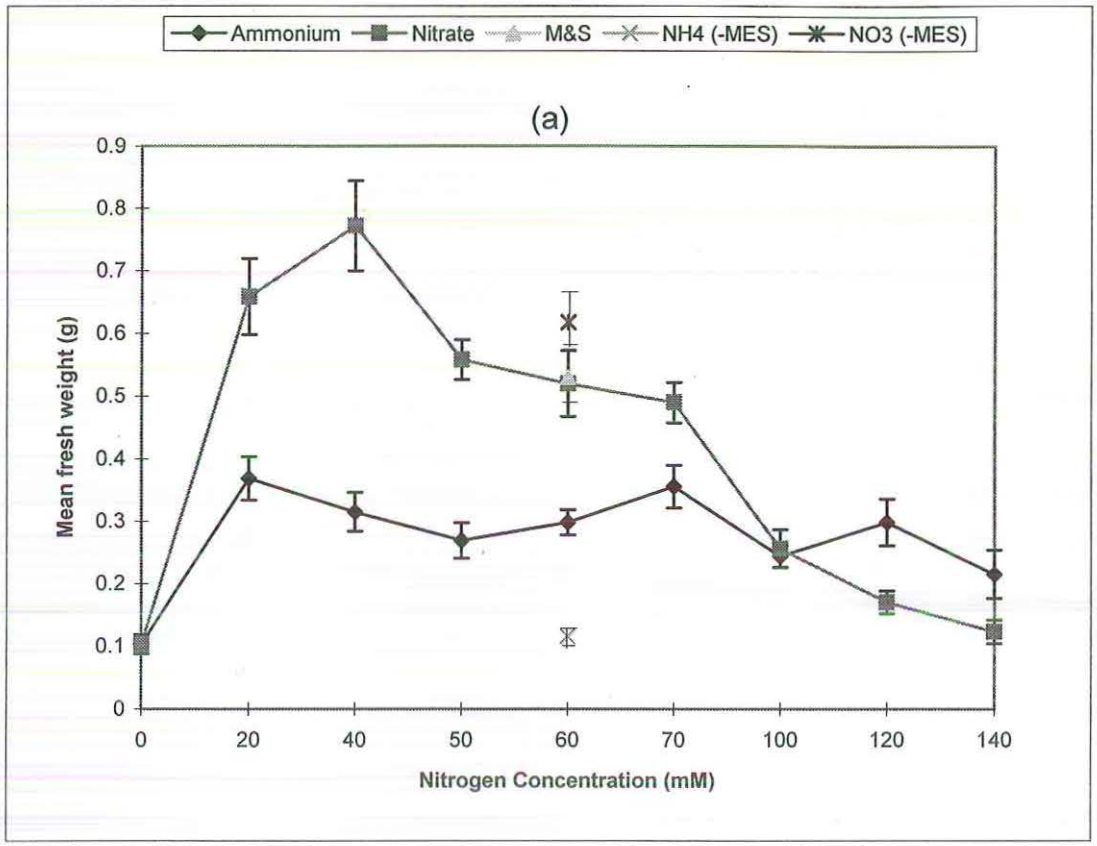
level increased, with a sharp decrease at the treatment without buffer, until the level of 120 mM nitrogen after which pH increased.

3.3.3 Experiment 3

The fresh weights were different between clones and between treatments for all clones (Figures 3.8a, 3.8b, 3.8c and 3.8d). The treatments that were significantly different varied between clones, but those producing highest fresh weight were 20 mM nitrate and 40 mM nitrate. The levels of 50 nitrate and 60 nitrate (-MES) also produced significantly heavier shoots for 11 JN 379 and 1 JN 30. Clone 5 JN 56 had significantly higher rooting with the Murashige and Skoog (M&S) basal nutrients (containing both nitrate and ammonium).

The highest chlorophyll values (Figures 3.9a, 3.9b, 3.9c and 3.9d) were obtained with 20 mM nitrate, 20 and 40 mM ammonium, 0 mM nitrogen and M&S. The significantly highest chlorophyll value for clone 11 JN 379 was found at 0 mM nitrogen, for 11 JN 50 at 20 mM ammonium, M&S and 40 mM ammonium. Clone 5 JN 56 had greatest chlorophyll content at 20 nitrate, 0 nitrogen, M&S and 20 ammonium. There was a significant difference in pH values between clones and between treatments for all clones (Figures 3.10a and 3.10b). For all clones, the treatments containing only nitrate had significantly higher pH than those with only ammonium.

Figure 3.8: The effect of nitrogen source on fresh weight for four clones: (a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are standard errors.



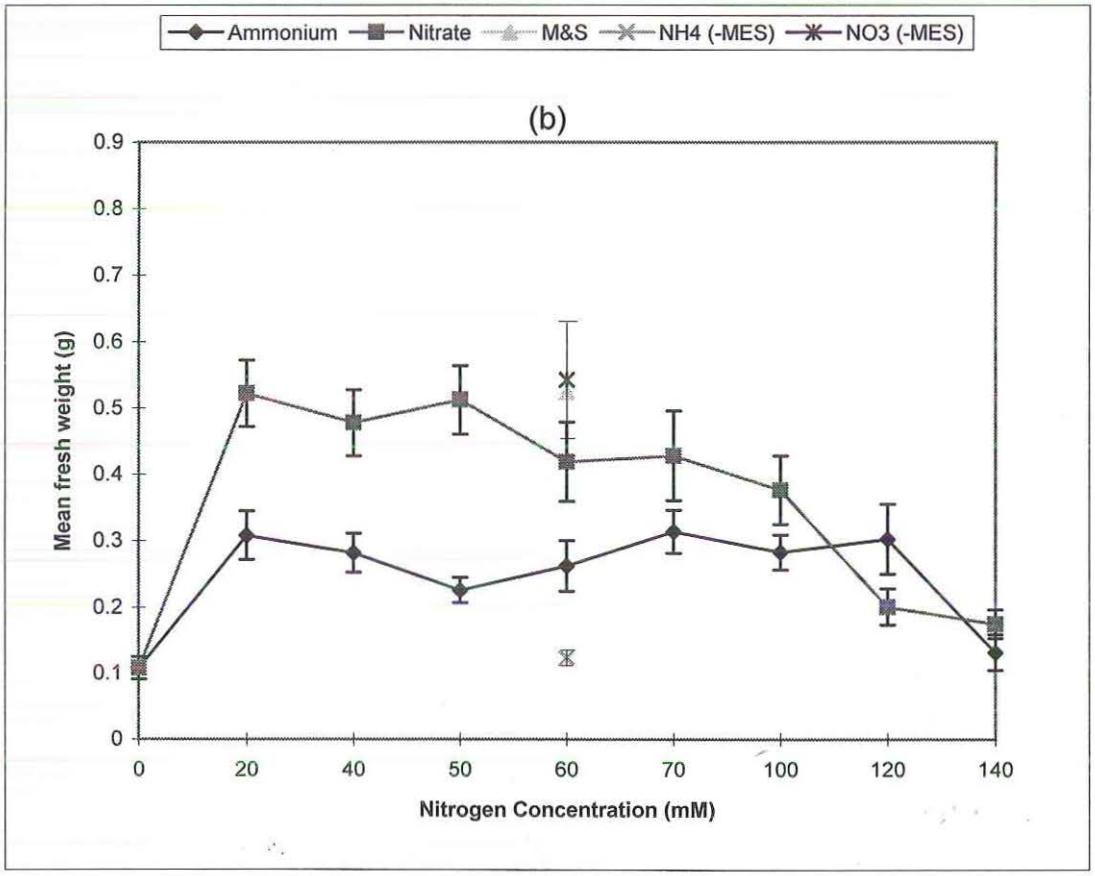
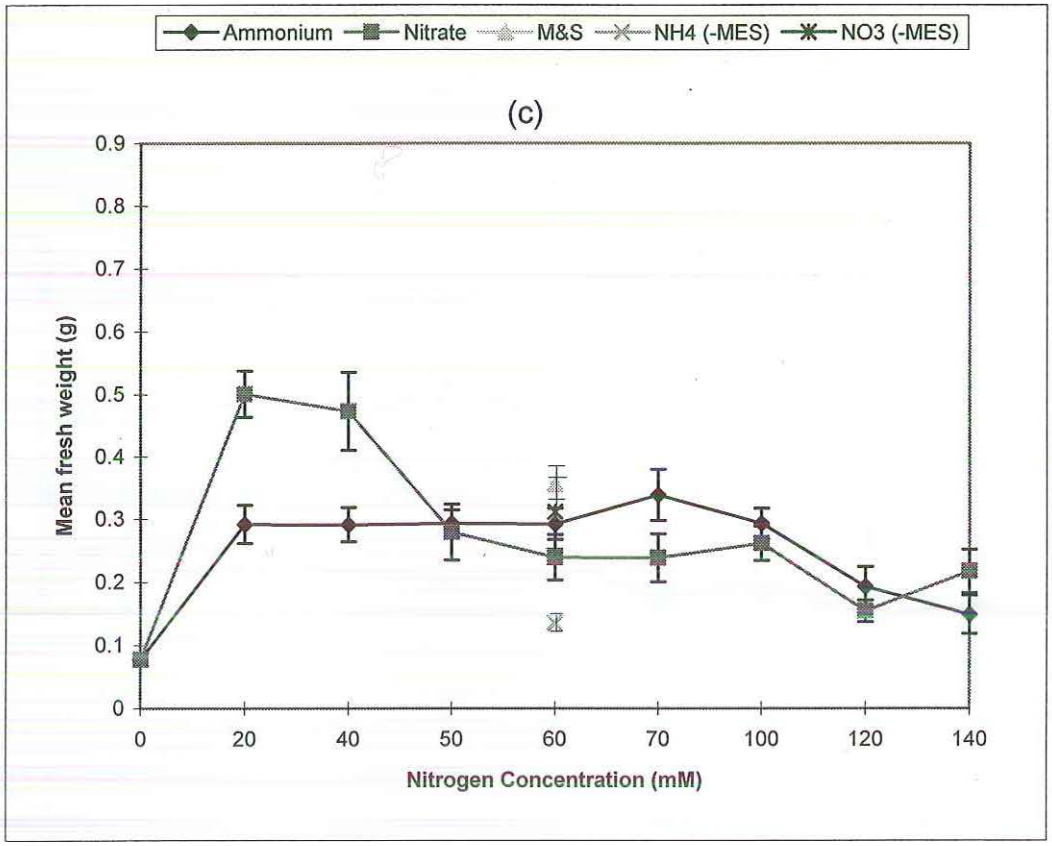


Figure 3.8 (cont'd): The effect of nitrogen source on fresh weight for four clones: (a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are standard errors.



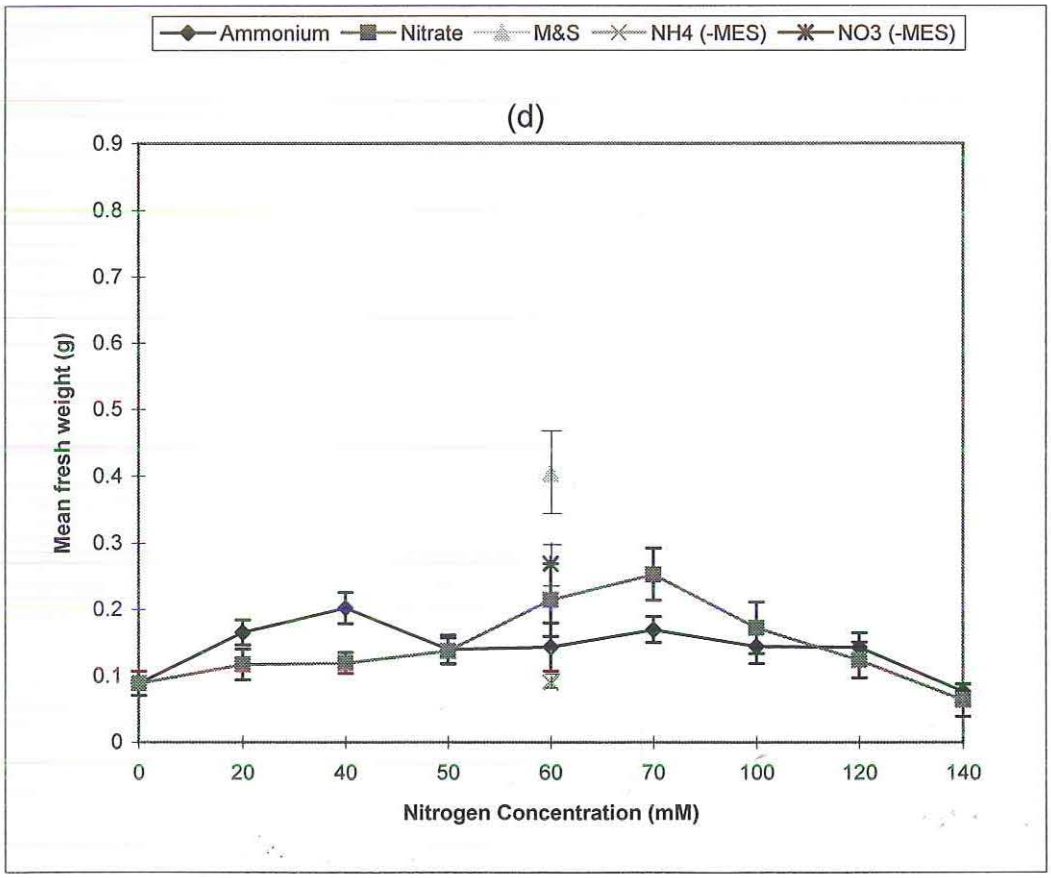
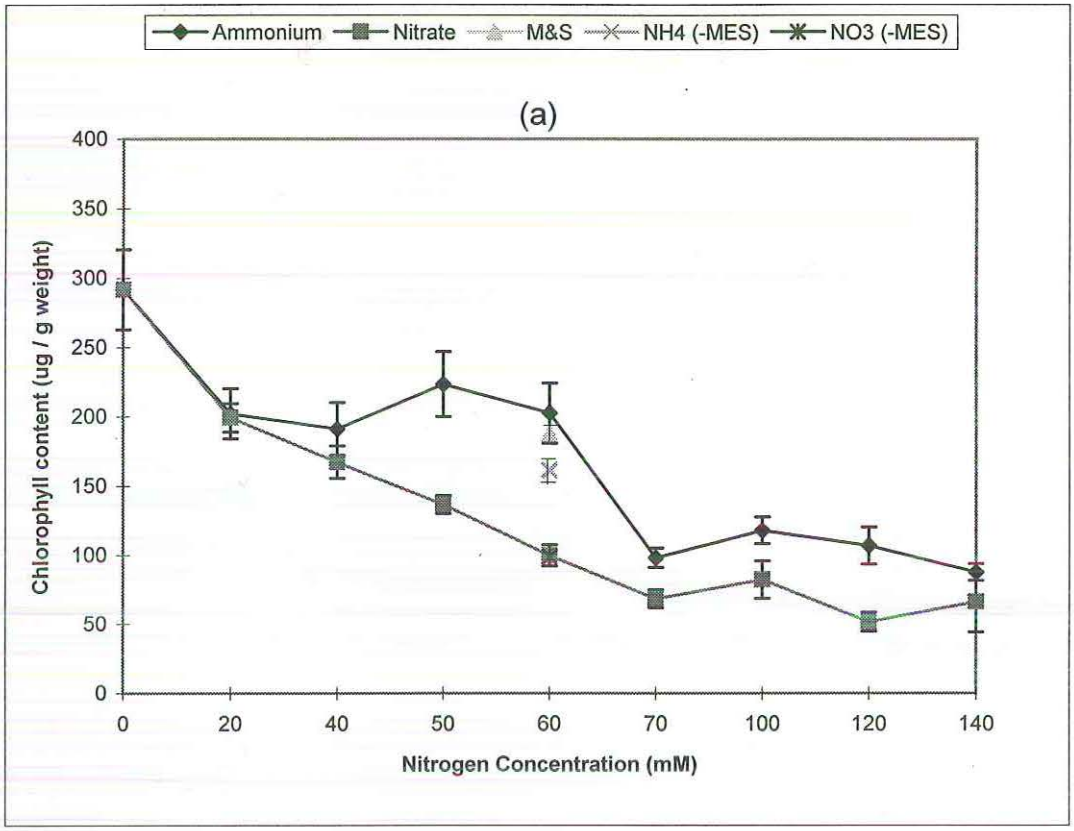


Figure 3.9: The effect of nitrogen source on chlorophyll content for four clones: (a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are standard errors.



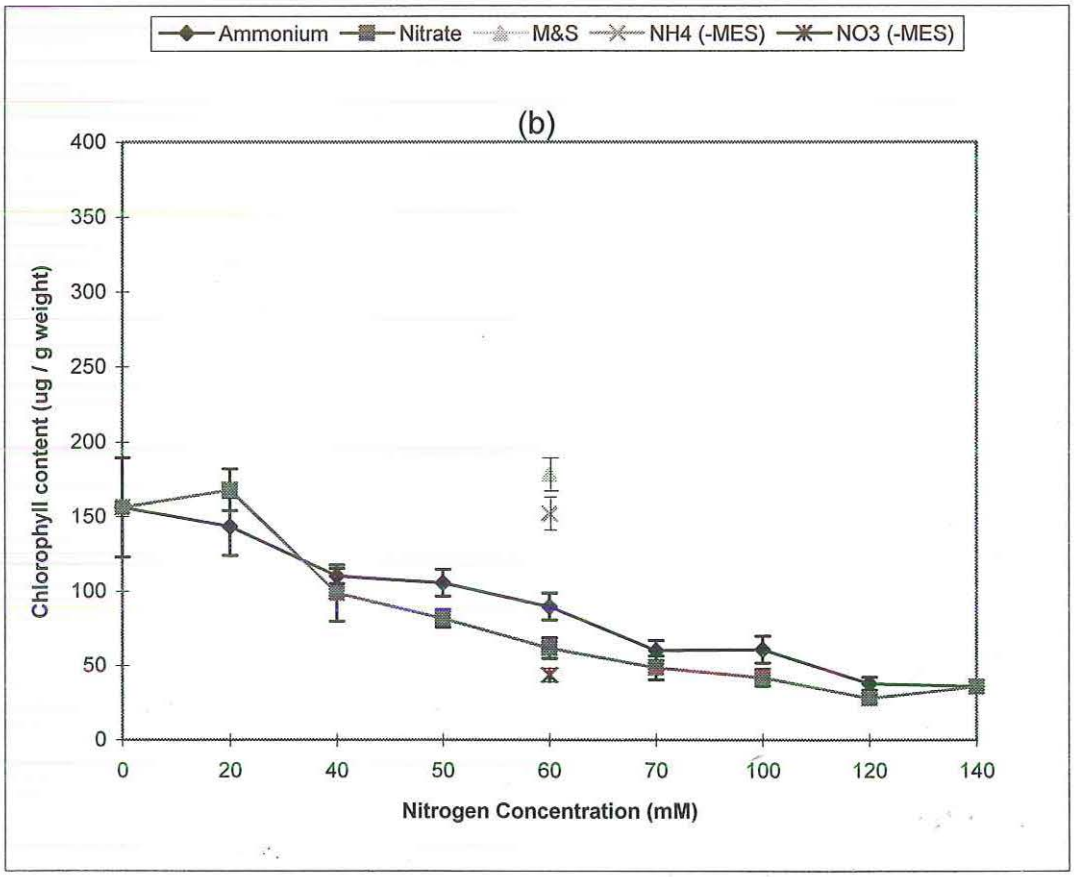
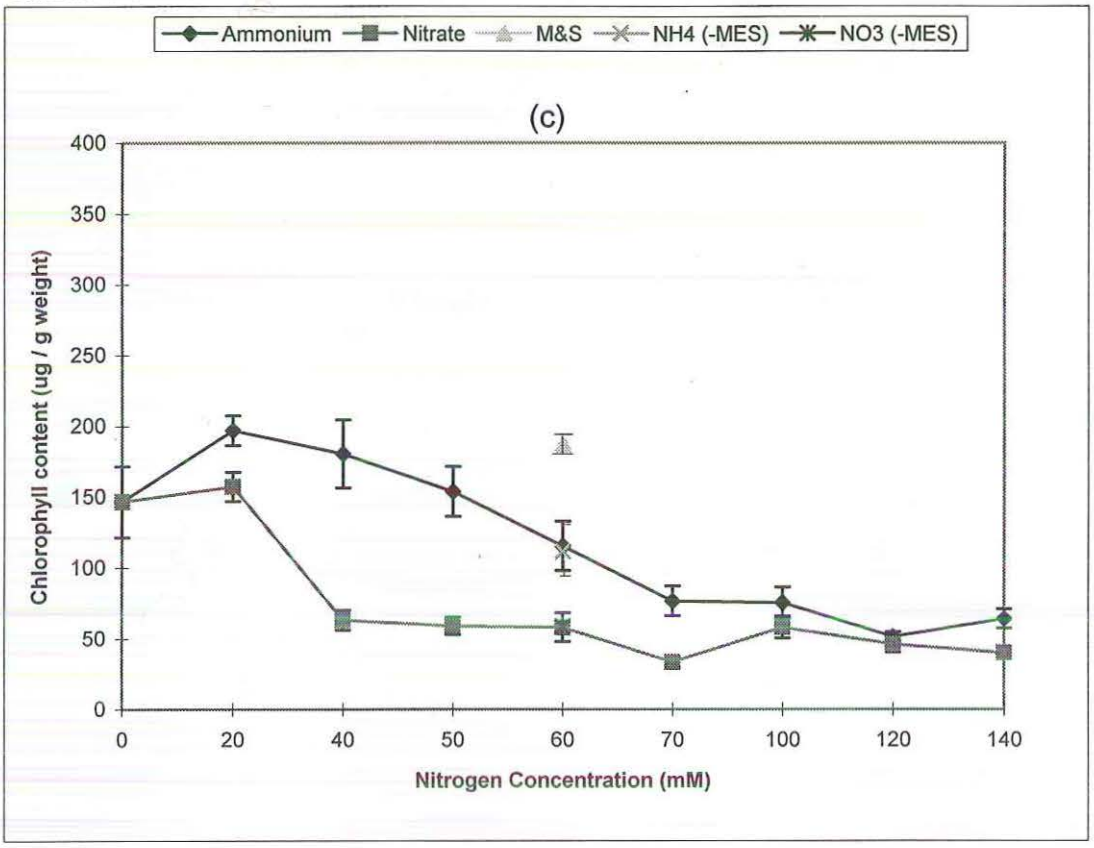


Figure 3.9 (cont'd): The effect of nitrogen source on chlorophyll content for four clo
(a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are
standard errors.



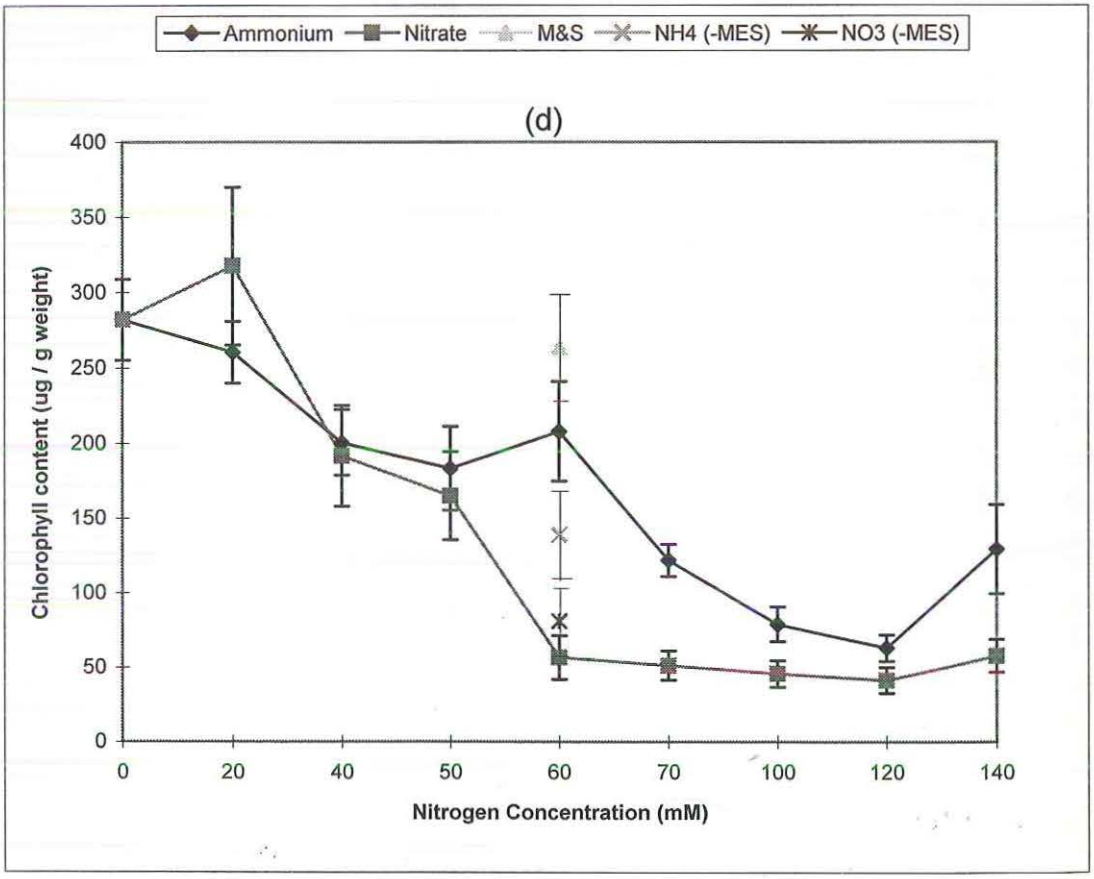
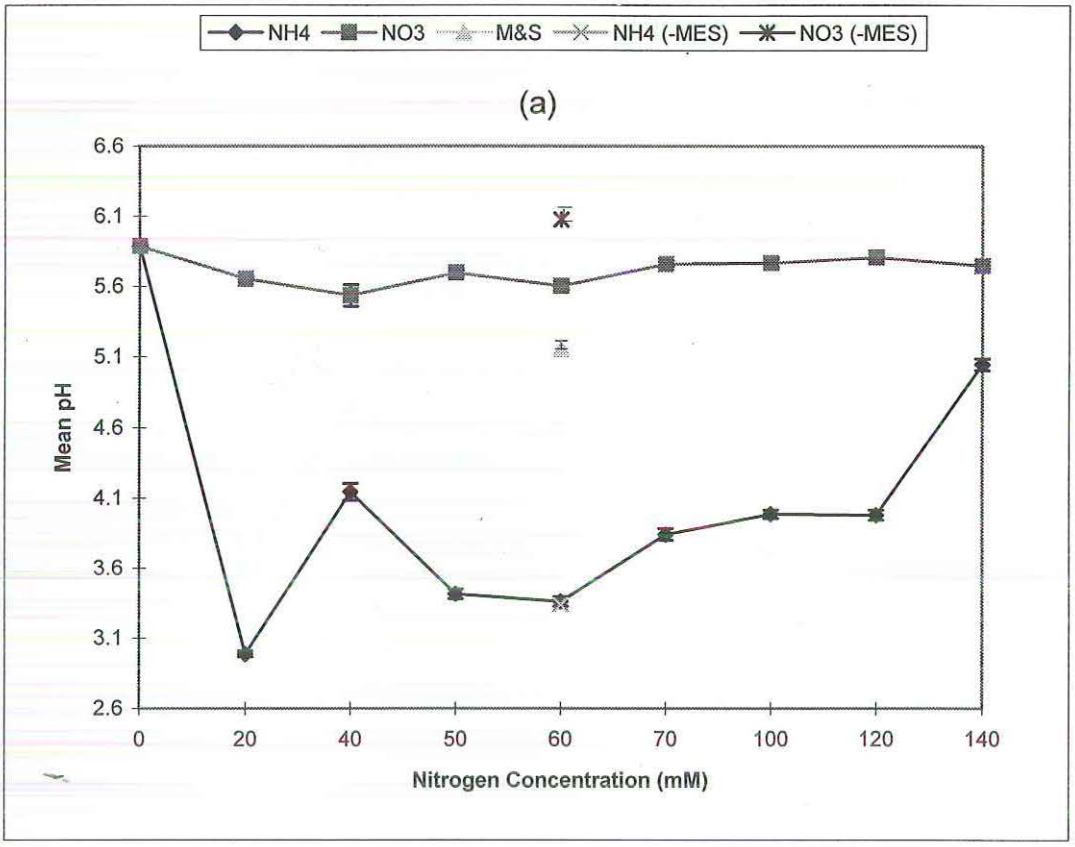


Figure 3.10: The effect of nitrogen source on pH for four clones:
(a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are standard errors. Values are means for 10 replicates



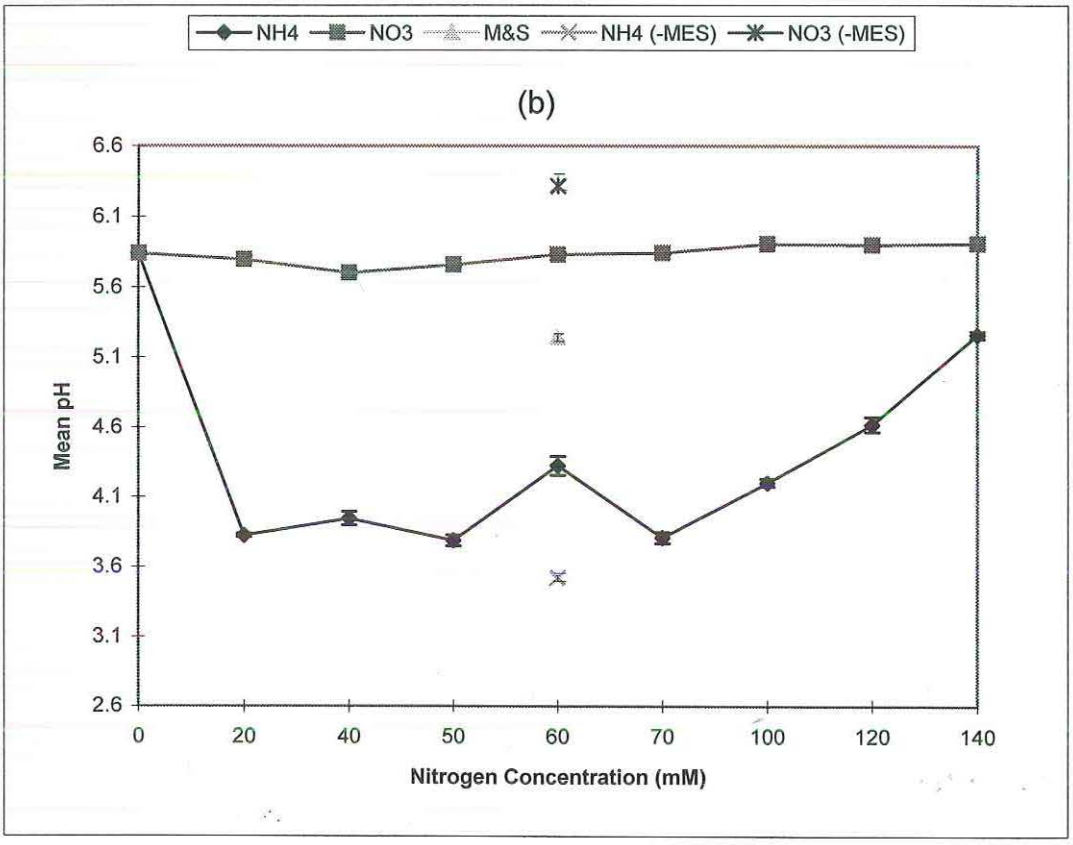
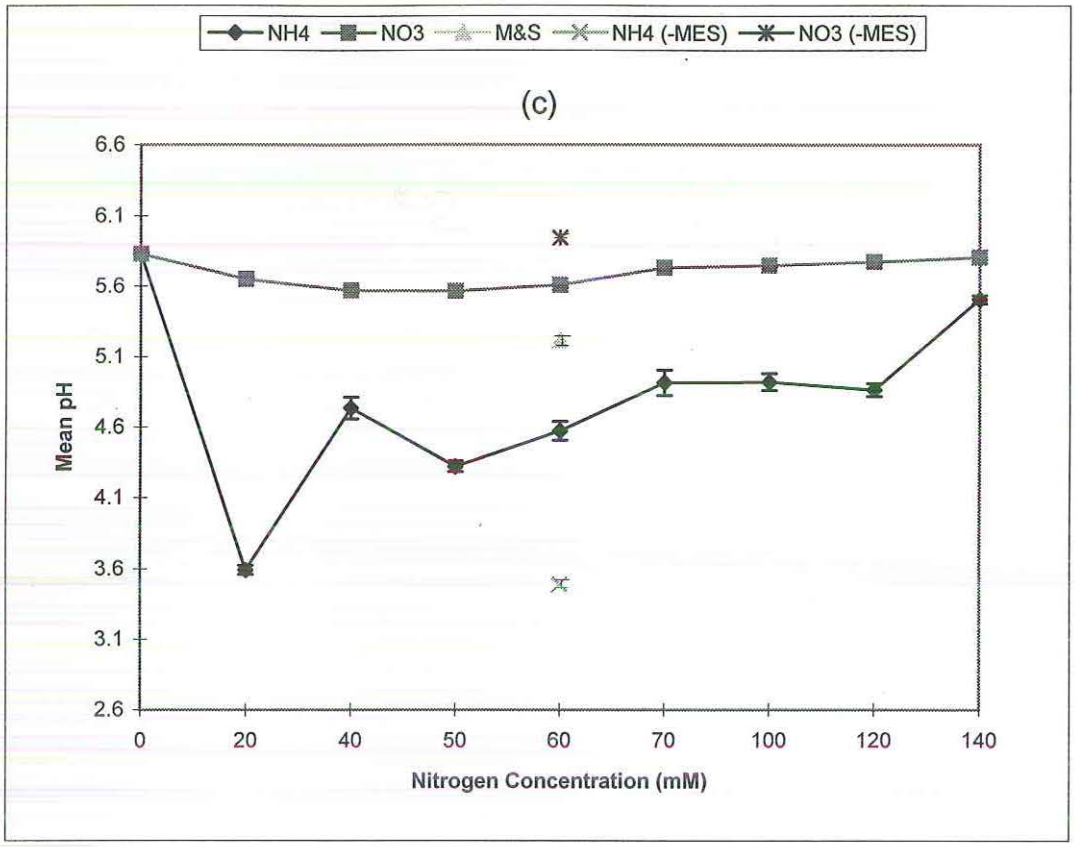
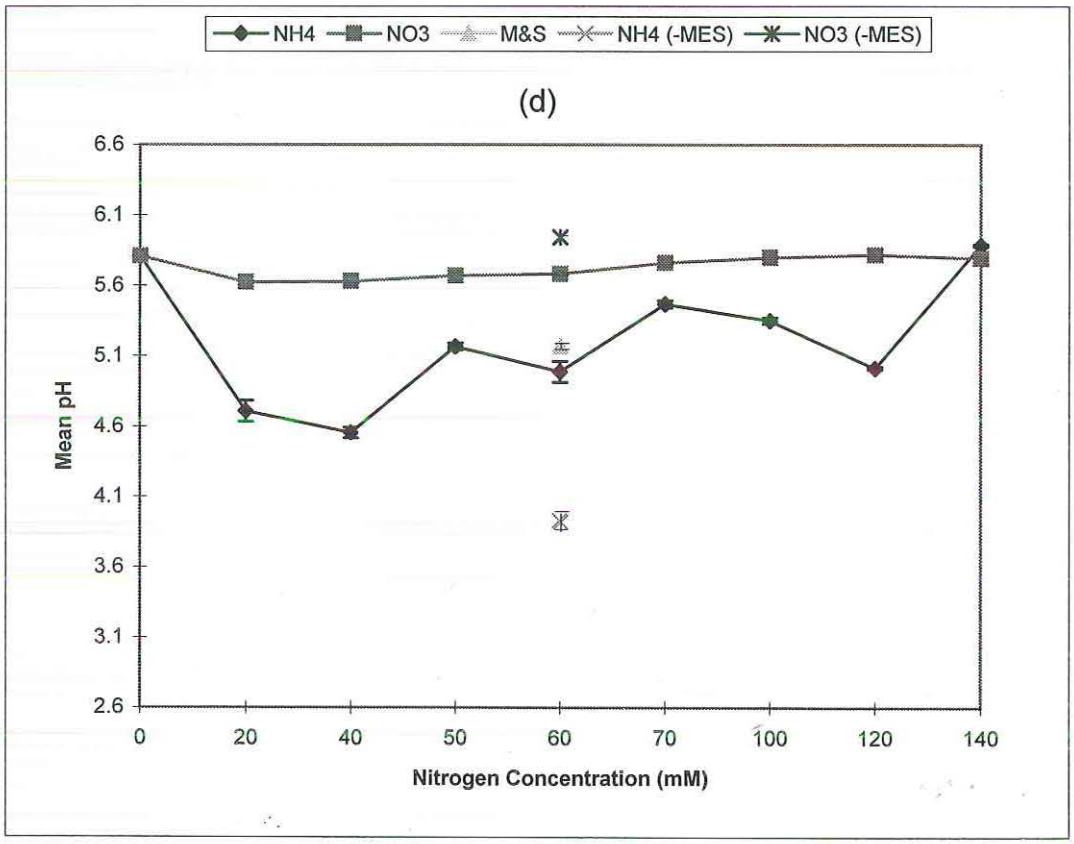


Figure 3.10 (cont'd): The effect of nitrogen source on pH for four clones: (a) 11 JN 379, (b) 1 JN 30, (c) 11 JN 50 and (d) 5 JN 56. Vertical bars are standard errors. Values are means for 10 replicates





3.4 DISCUSSION

3.4.1 Experiment 1

The buffer MES was effective in maintaining a stable pH in the medium, but had an adverse affect on fresh weight. The buffer caused a lowering of chlorophyll content in the medium, with shoots grown in unbuffered medium containing significantly higher chlorophyll content. As the same nitrogen source and concentration was used for all treatments, it is unclear as to whether the lowering of chlorophyll in the buffered shoots was due to the pH change or the buffer itself. Parfitt *et al* (1988) reported similar findings in experiments with several plant species, where buffering the medium kept pH constant, but lead to a decrease in shoot growth.

This lower chlorophyll content in shoots grown on buffered medium could be due to the buffer complexing with micronutrients and organics, or, an effect of stabilising the pH altering the availability of nutrients (Williams *et al*, 1990). Nitrogen is a major constituent of chlorophyll, and a decrease in available nitrogen could cause a lowering of chlorophyll content in shoots. At a higher pH, the plant would be able to take up more ammonium but less nitrate. If it requires more nitrate than ammonium, then a possible outcome could be decreased nitrogen uptake and thus less growth. If this is so it might suggest that *E. marginata* prefers nitrate to ammonium.

The 10 mM concentration of buffer was used in preference to the 20 mM concentration in subsequent experiments that varied nitrogen as it stabilised

pH almost as well, and resulted in less adverse shoot growth and chlorophyll content.

3.4.2 Experiment 2

Shoots grown on medium containing little nitrogen (less than or equal to that found in M&S) were significantly heavier and had higher chlorophyll content than on medium containing higher nitrogen concentration. There was a difference between clones in terms of the nitrogen concentration that resulted in greatest fresh weight. Three of the four clones tested had greatest fresh weight at levels of nitrogen lower (by 25 or 50 %) than that used in M&S, with only one clone preferring higher nitrogen (5 JN 56). This suggests that the current level of nitrogen (60 mM) used in M&S may be too high for growth of most *E. marginata* clones in tissue culture.

While the shoots grown on nitrogen free medium had the highest chlorophyll content, they had the lowest fresh weight. This suggests that chlorophyll content may not have been an adequate indicator of shoot growth, in terms of both fresh weight and shoot condition, in this experiment. Different clones preferred different nitrogen concentrations, with some preferring higher and others lower levels than that found in M&S. But the general trend was for increased growth on lower nitrogen concentrations. The pH result for each treatment was as expected, with a fall in pH with increased nitrogen.

This experiment showed the difference in nutrient requirements between clones, and supports the need for further investigation into the nutrient requirements of *E. marginata in vitro*.

3.4.3 Experiment 3

Significantly higher fresh weight was achieved in those shoots grown on nitrates, while highest chlorophyll content was found at low levels of ammonium and nitrates. This suggests that *E. marginata* shoots prefer nitrate as a source of nitrogen, but also require ammonium for normal growth. Again, there was a general trend for improved growth at the lower levels of nitrogen with two of the clones (11 JN 379 and 11 JN 50) preferring nitrate at 20 and 40 mM, and the other two clones (5 JN 56 and 1 JN 30) having higher fresh weights at higher levels (mM) with nitrate as the sole source. This highlights the difference between clones within a species, and suggests that nitrate is preferred to ammonium as a nitrogen source in *E. marginata*.

The pH changes were as expected with a fall in ammonium medium and little change in nitrate medium. The fall in pH in the ammonium medium indicates that *E. marginata* are taking up and seem capable of growing on ammonium as the sole nitrogen source. However, growth was not as great as that of shoots grown on nitrate as the sole nitrogen source. In 11 JN 379, the treatment of 20 mM ammonium (buffered) had a pH lower than 60 mM ammonium (unbuffered). This could mean that more ammonium was being taken up and used at lower concentrations, or that a level of 60 mM ammonium was too

toxic for normal growth, suggesting that *E. marginata* prefers low levels of ammonium.

In all clones at 60 mM nitrogen there was a fall in fresh weight when in ammonium and a rise in fresh weight when in nitrates, when the medium was unbuffered. This supports the suggestion from Experiment 1 that lower shoot growth on buffered medium was a result of the buffer affecting uptake of nitrogen. It also seems likely that this is a result of pH change and not a reaction between the buffer and micronutrient or organic components of the media. If the buffer does reduce the uptake of nitrates, and *E. marginata* does prefer nitrates, then this would account for a decrease in growth on M&S when the media was buffered.

4. EFFECTS OF NITROGEN SOURCE ON ROOT INDUCTION

4.1 Introduction

Several clones of *Eucalyptus marginata* were used in a series of experiments that investigated the effect of different nitrogen sources on root induction. A preliminary trial determined the level of buffer most effective for maintaining pH, with subsequent investigations varying total nitrogen and nitrogen source. The effects of varying the nitrogen source on root induction within and between clones was examined, as was the effect of stabilising pH. Varying the nitrogen source was performed to determine whether *E. marginata* prefers nitrate or ammonium for root induction. Based on the results of the previous root induction experiments, and using those treatments which provided greatest rooting, an investigation into the best nitrogen source and level was conducted.

4.2 Materials and methods

4.2.1 Experiment 1

The most effective level of buffer for stabilising pH for root induction was examined using two levels, 10 mM and 20 mM, of the buffer MES, with a level of 0 mM used as a control. Two clones were used, 1 JN 98 and 5 JN 336, with 10 replicates for each treatment (150 shoots per clone). The pH was recorded daily for one week, then weekly. Number of roots was counted daily for one week after initiation, then every 3 days. After 4 weeks chlorophyll content of the shoots was determined.

4.2.2 Experiment 2

Two clones, 1 JN 98 and 5 JN 336, were subcultured onto media containing either ammonium or nitrate as the sole nitrogen source. There were nine treatments used for each clone (Table 4.1) with a level of 10 mM MES used in all treatments. Each treatment was replicated 10 times for each clone (50 shoots for each treatment and for each clone). The number of roots per shoot was recorded every three days after root initiation, with pH recorded weekly. After four weeks chlorophyll content was determined as a measure of shoot condition.

Table 4.1: Nitrogen source and concentration for Experiment 2, the effect of nitrogen source on root induction

Nitrate	Ammonium
00 mM N	
7.5 mM	7.5 mM
15 mM	15 mM
30 mM	30 mM
60 mM	60 mM

4.2.3 Experiment 3

Two clones (1 JN 98 and 5 JN 336) were subcultured onto four treatments: M&S (10 mM MES), M&S (minus buffer), 7.5 mM nitrate (10 mM MES) and 7.5 mM ammonium (10 mM MES). Each treatment was replicated 10 times (5

shoots per tub) for each treatment and for both clones, and the experiment was conducted over 4 weeks. Number of roots per shoot and pH were measured at the end of four weeks, as were fresh weight and chlorophyll.

4.3 RESULTS

4.3.1 Experiment 1

There was no significant difference between clones for chlorophyll values (Figure 4.1), but a difference was found between treatments for clone 1 JN 98, with chlorophyll content being significantly highest in the control treatment. Clone 5 JN 336 had a significantly greater percentage of rooting than did clone 1 JN 98, but there was no difference due to treatment for either clone (Figure 4.2). There was no significant difference between clones for pH (Figure 4.3), but a large difference between treatments within each clone. The pH was kept stable when the media was buffered using 10 and 20 mM concentrations of MES, while pH fell in the unbuffered media.

4.3.2 Experiment 2

Chlorophyll content (Figure 4.4) differed between clones, and between treatments for clone 1 JN 98, but not 5 JN 336. Clone 1 JN 98 shoots grown in 15 mM and 30 mM nitrate media had the highest chlorophyll content.

There was a difference in roots per shoot (Figures 4.5a and 4.5b) between clones, and both clones had a difference in number of roots between treatments. The highest rooting for 5 JN 336 occurred when shoots were

Figure 4.1: The effect of different concentrations of the buffer MES on chlorophyll content for clones 1 JN 98 and 5 JN 336. Values are means for 10 replicates. Vertical bars are standard errors.

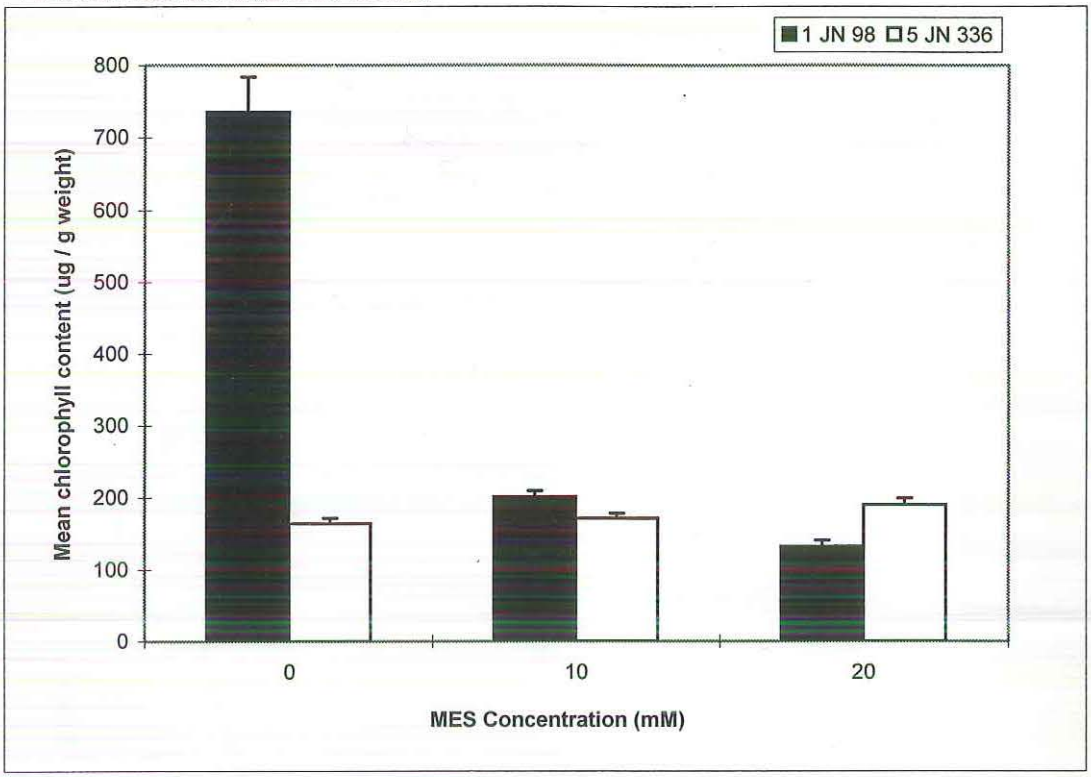


Figure 4.2: The effect of different concentrations of the buffer MES on root induction for clones 1 JN 98 and 5 JN 336. Values are means for 10 replicates. Vertical bars are standard errors.

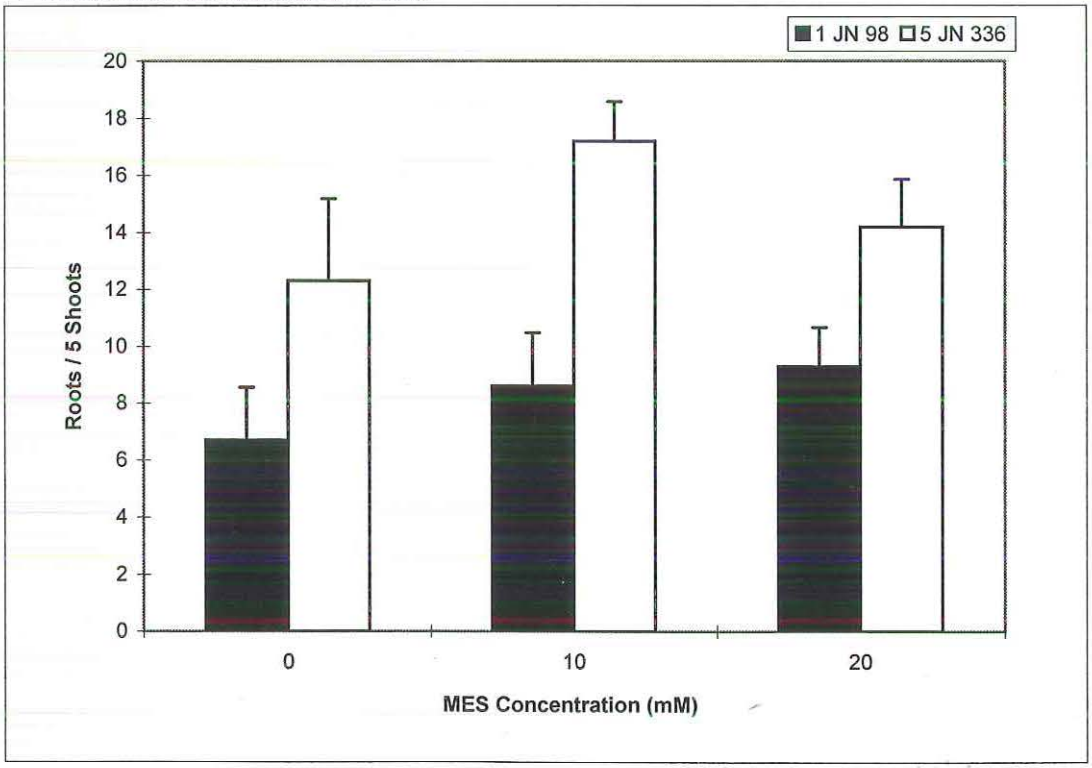


Figure 4.3a: The effect of different concentrations of the buffer MES on pH for clone 1 JN 98. Values are means for 10 replicates. Vertical bars are standard errors.

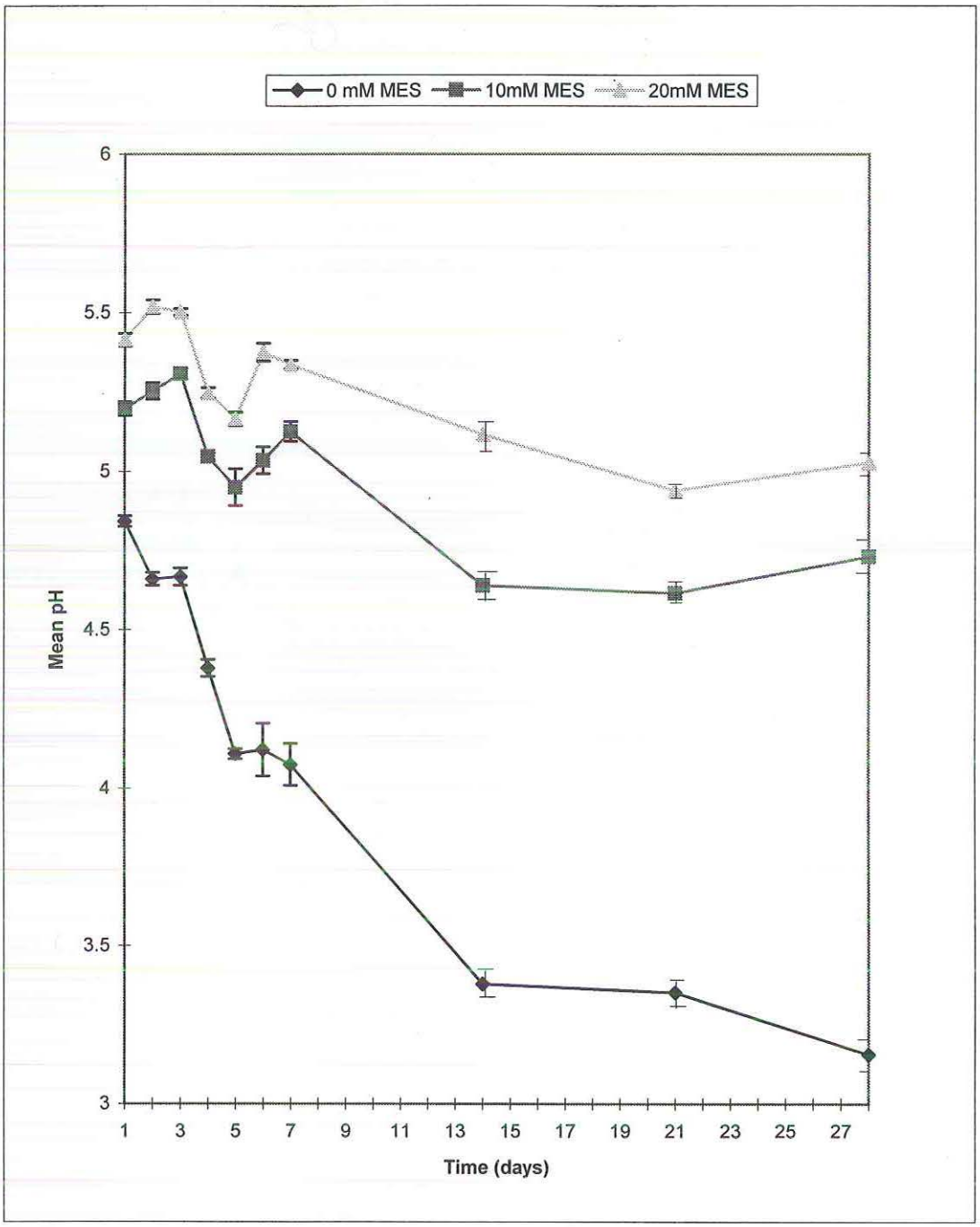


Figure 4.3b: The effect of different concentrations of the buffer MES on pH for clone 5 JN 336. Values are means for 10 replicates. Vertical bars are standard errors

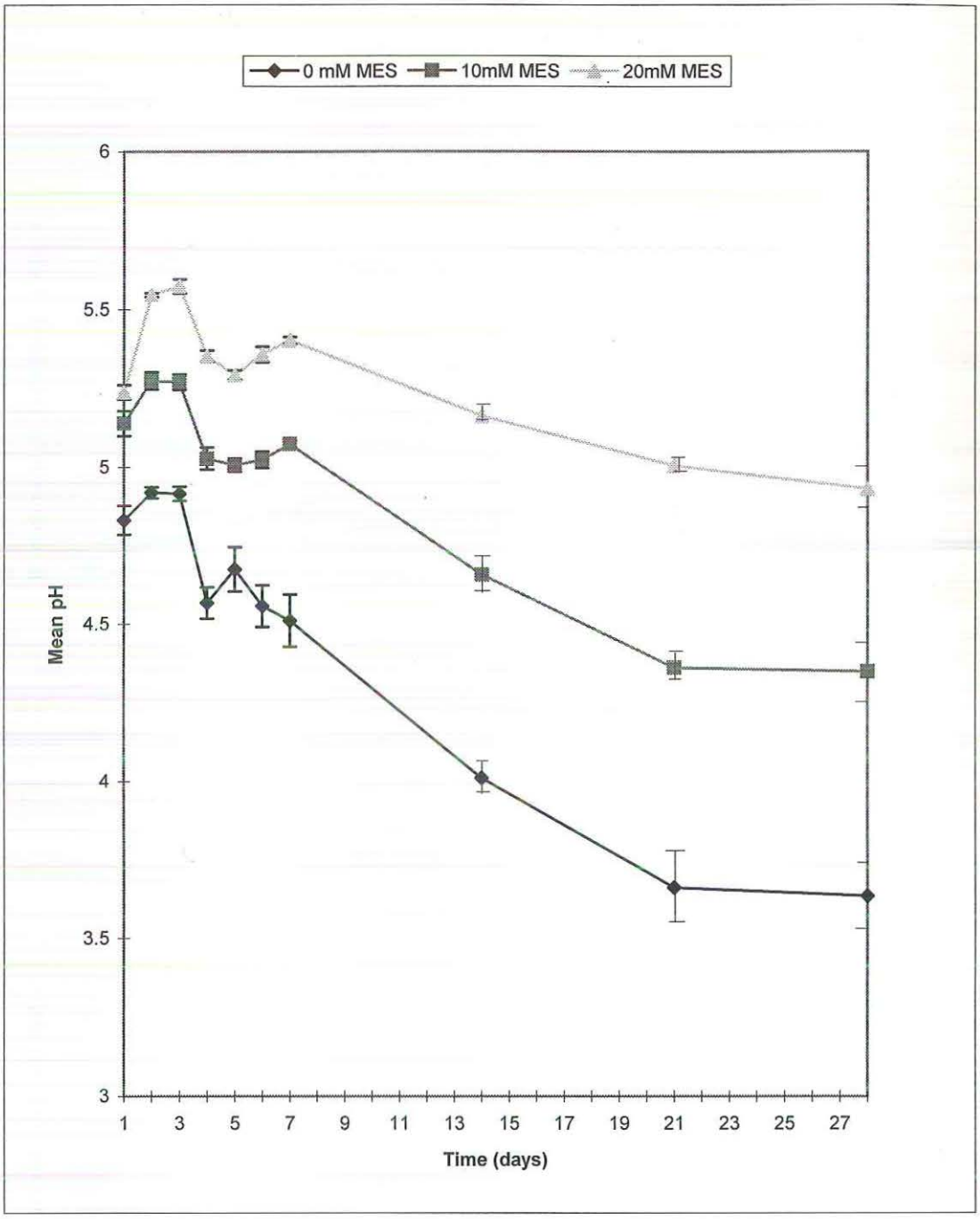
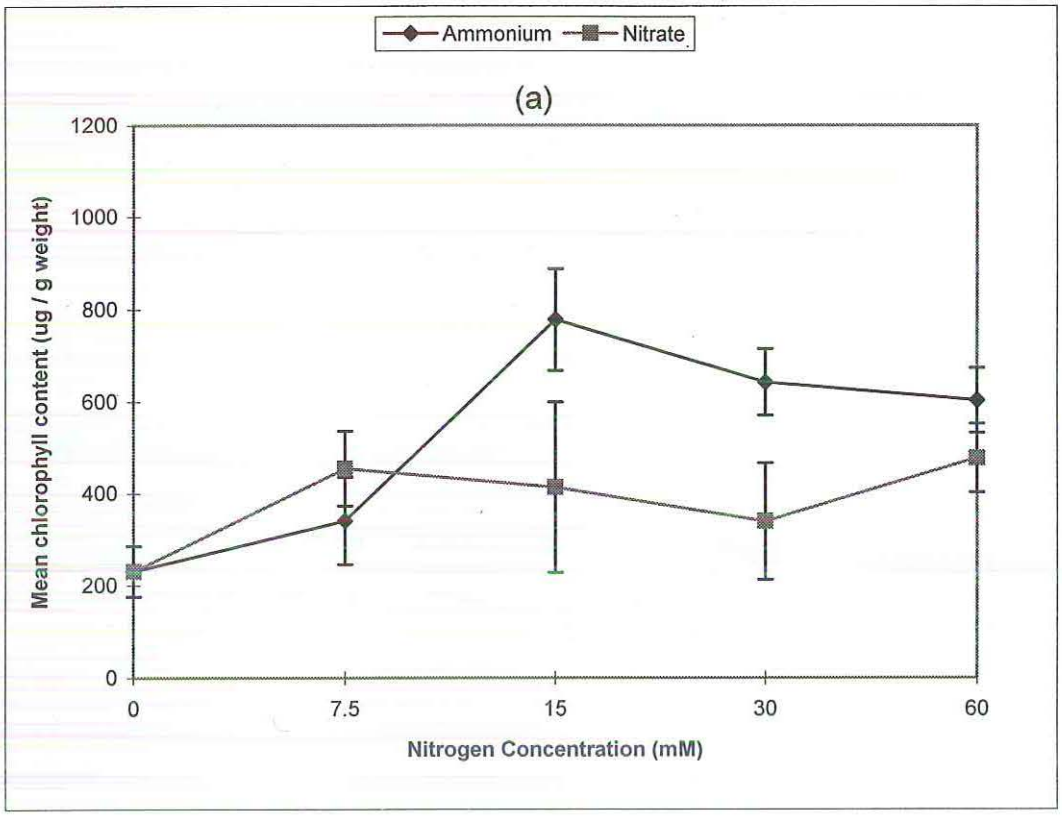


Figure 4.4: The effect of nitrogen source on chlorophyll content for two clones: (a) 1 JN 98 (b) 5 JN 336. Means are for 10 replicates. Vertical bars are standard errors.



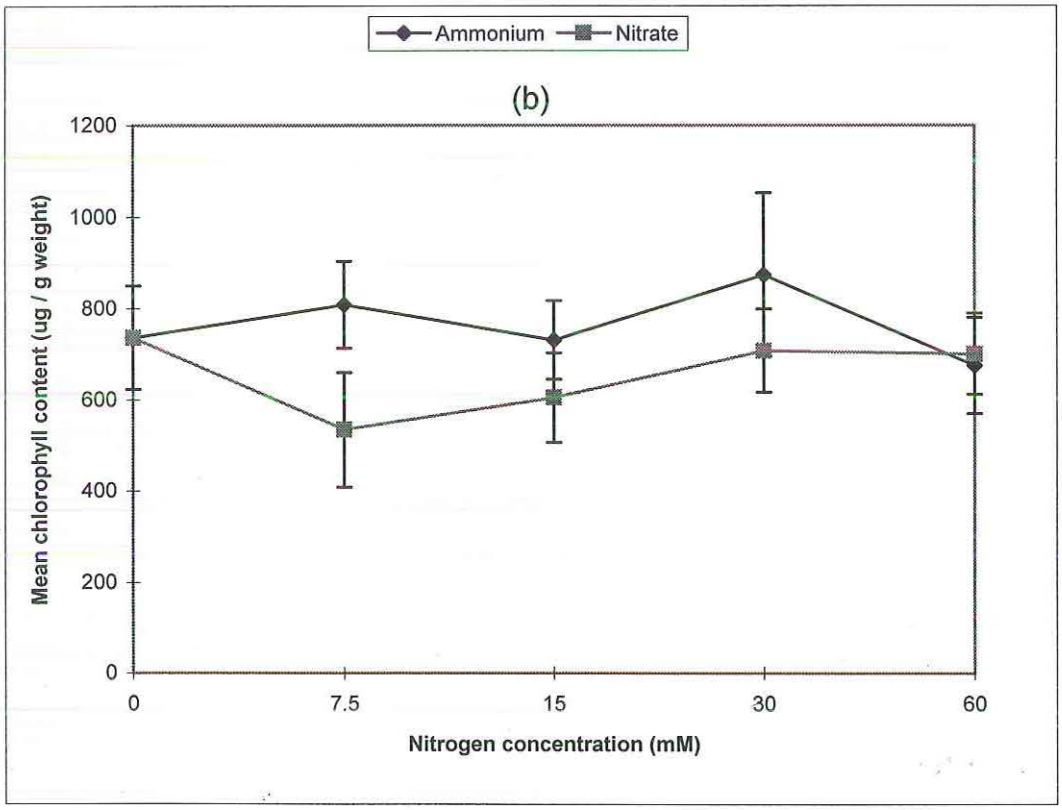
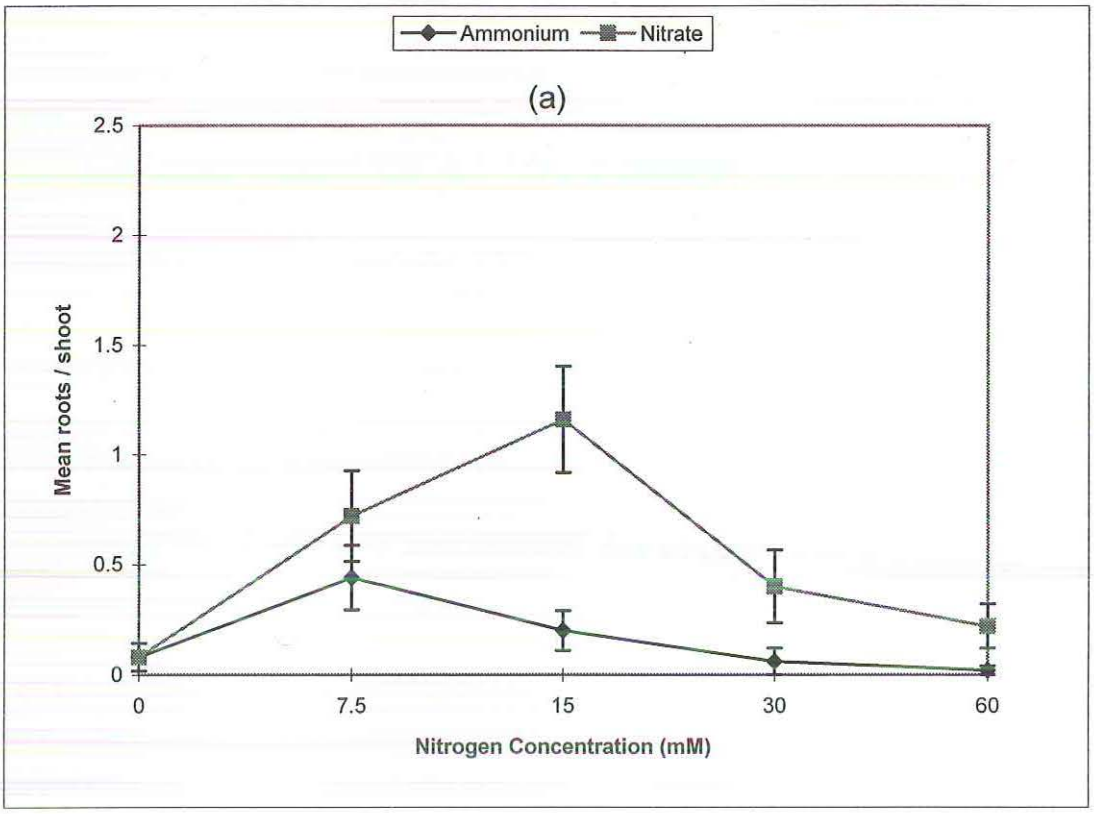
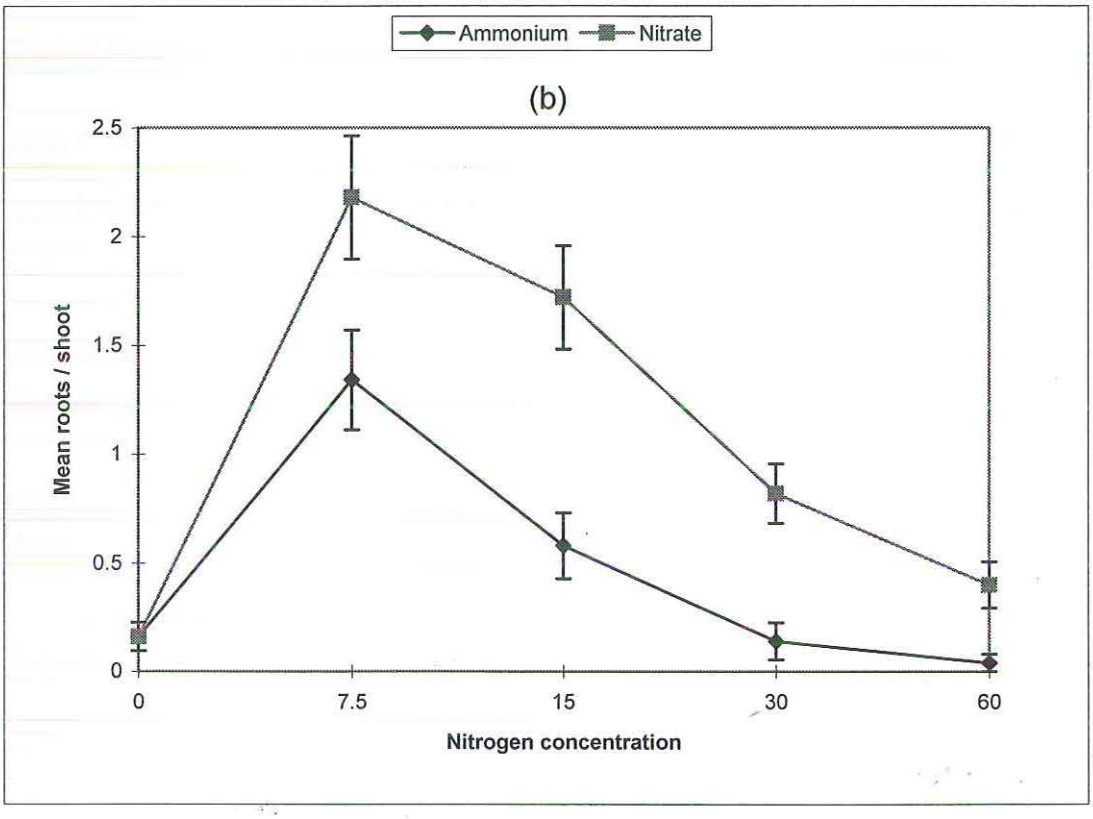


Figure 4.5: The effect of nitrogen source on root induction in two clones (a) 1 JN 98 and (b) 5 JN 336. Values are means for 10 replicates. Vertical bars are standard errors.





grown on 7.5 and 15 mM nitrate, with highest rooting for 1 JN 98 at 15 mM nitrate.

The pH of the media was different between clones and between treatments for both clones (Figures 4.6a and 4.6b). It remained fairly constant for nitrate treatments and decreased in ammonium media.

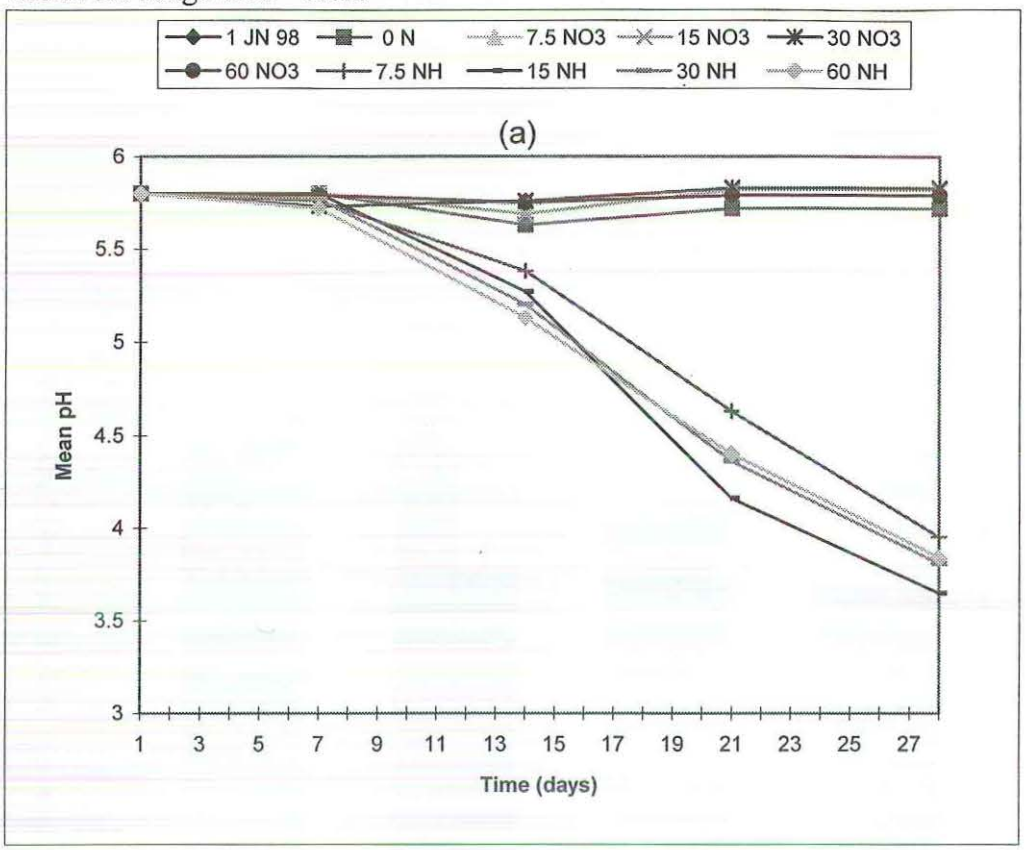
4.3.3 Experiment 3

There was no difference in chlorophyll content between clones or between treatments (Figure 4.7). This indicated that all plants were generally healthy, and that no treatment was having adverse affects on the shoots.

There was a significant difference between clones for the effect of the different nitrogen source and concentration on rooting (Figure 4.8). A significant difference in number of roots per shoot between each treatment was found in clone 5 JN 336 but not in 1 JN 98. The 7.5 mM NO3 treatment gave the highest rooting for both clones, but it was only significantly higher than the other treatments in 5 JN 336. The lowest rooting for both clones was for shoots grown on unbuffered media containing Murashige and Skoog nutrients (ammonium and nitrate).

The pH in the media was not significantly different between clones (Figure 4.9), but was between treatments within both clones. Highest pH was in the

Figure 4.6: The effect of nitrogen source on pH for two clones: (a) 1 JN 98 and (b) 5 JN 336. Values are means for 10 replicates. Standard errors are in the range 0.01 - 0.05.



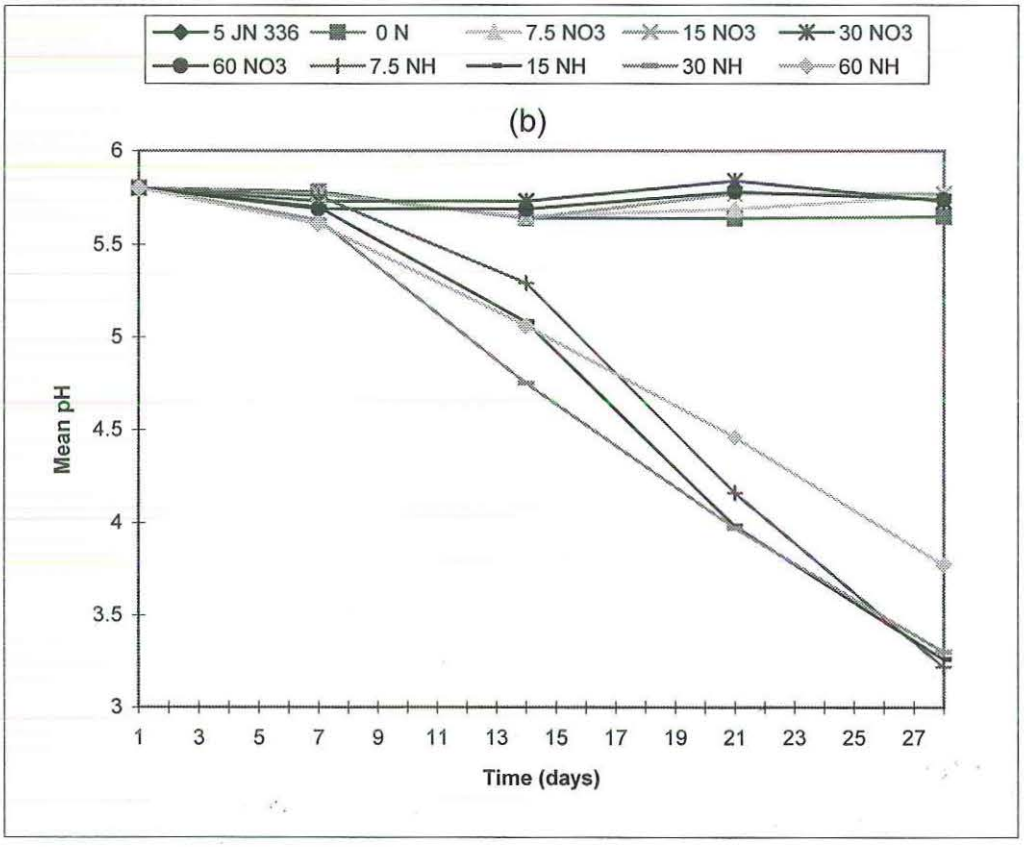


Figure 4.7: The effect of different nitrogen source on chlorophyll content for two clones 1 JN 98 and 5 JN 336. Values are means for 10 replicates. Vertical bars are standard errors.

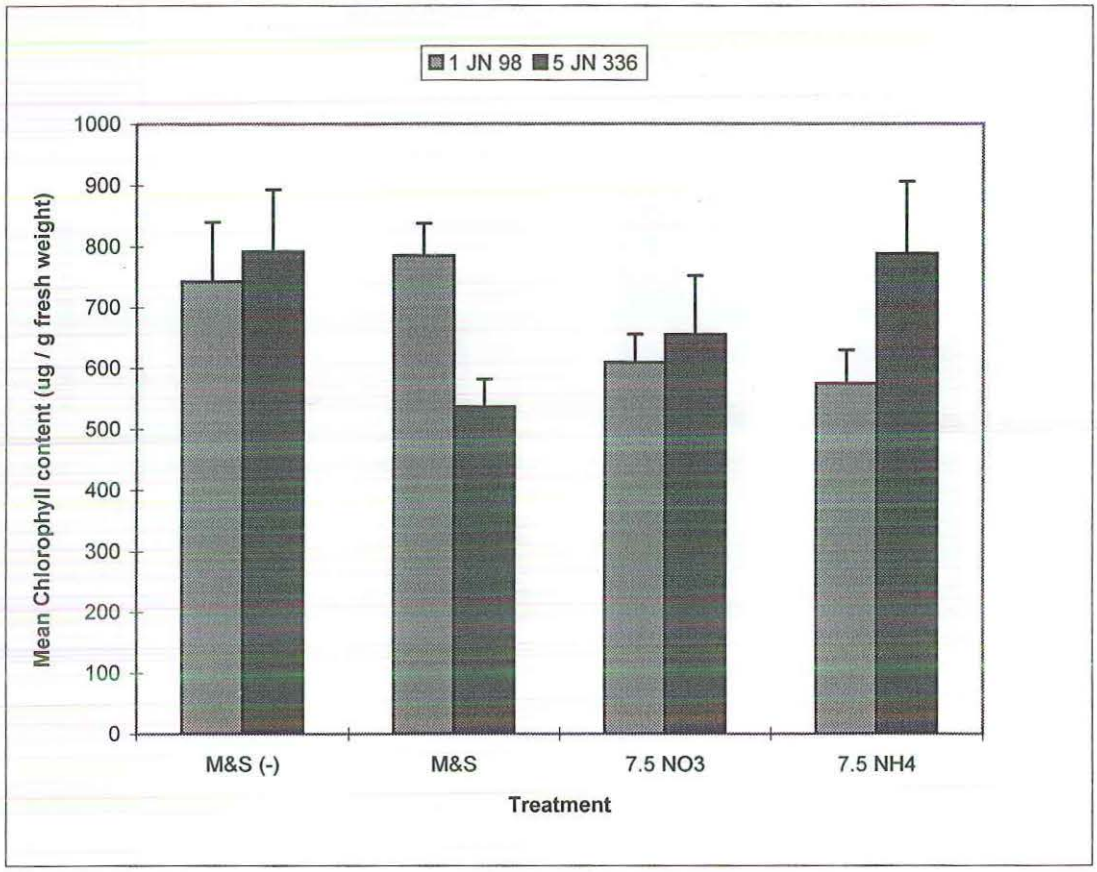


Figure 4.8: The effect of different nitrogen source on rooting for two clones 1 JN 98 and 5 JN 336. Values are means for 50 replicates. Vertical bars are standard errors.

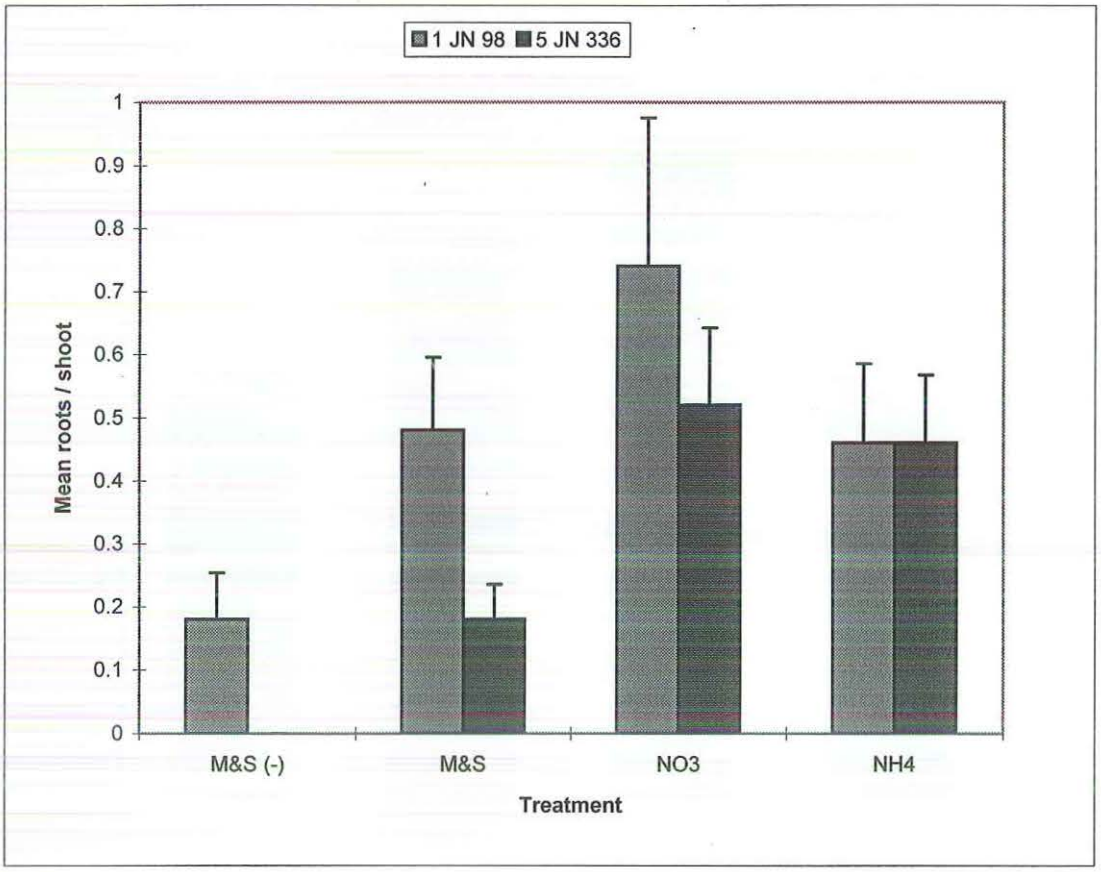
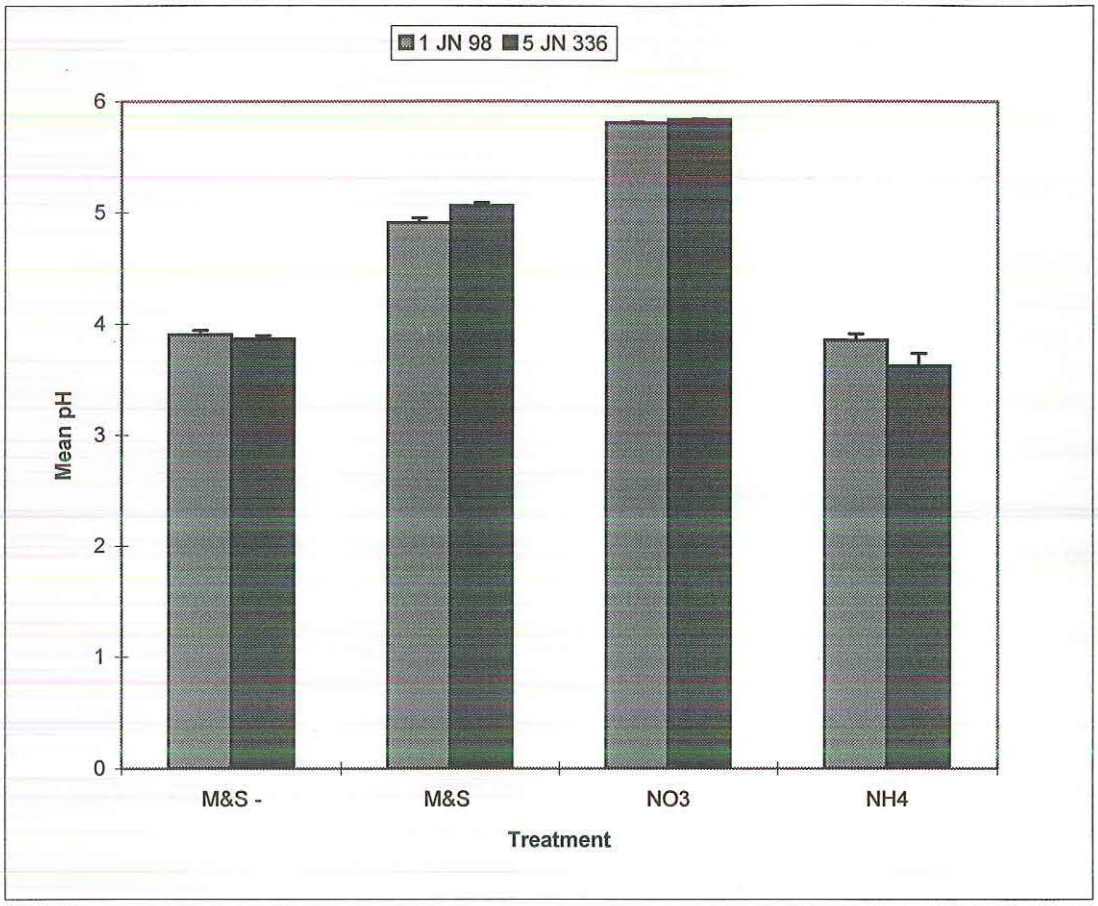


Figure 4.9: The effect of different nitrogen source on pH for two clones, 1 JN 98 and 5 JN 336 after 4 weeks. Means are for 10 replicates. Vertical lines are standard errors.



media containing nitrate, with the lowest being in both the ammonium treatment and also the unbuffered Murashige and Skoog treatment.

4.4 DISCUSSION

4.4.1 Experiment 1

The buffer was effective in stabilising pH, but didn't result in any change in rooting. The difference in rooting between clones indicates a genetic difference in physiology and nutrient requirements. Clone 5 JN 336 had a greater percentage of rooting than clone 1 JN 98.

An unusually high chlorophyll content was found in shoots of clone 1 JN 98 grown on unbuffered media. It is unclear what has caused this value as it is vastly different from the other treatments and from the same treatment in the other clone. The value may be correct, or a measurement error in the chlorophyll determination process may be responsible.

4.4.2 Experiment 2

The differences in rooting between clones at different nitrogen concentration and source again highlights differences in nutrient requirements of different clones. The results suggest that clones not only have differing needs in terms of shoot growth, but also for root induction. The highest rooting in both clones was with nitrate as the sole source, at a lower level (7.5 mM) than the current level (¼ strength M&S, 15 mM), suggesting that this level may be optimum for root induction in *E. marginata*.

Overall, there was more rooting on nitrate medium than on ammonium. While it is possible to achieve root induction in some species with ammonium as the sole nitrogen source (Satharayana & Blake, 1994), *E. marginata* appears to have reduced rooting when grown solely on ammonium. It is unclear whether the reduced rooting was due to ammonium or due to the effect of the pH. Although the media was buffered, the pH in the ammonium treatment still fell by a considerable amount. It seems unlikely that the effects of pH and ammonium can be separated with the use of MES as a buffer, as shoot investigations into the effect of MES on shoot growth suggest that shoot growth is inhibited as buffer concentration is increased. The buffer would have to be supplied in such high amounts that plant growth would be inhibited.

4.4.3 Experiment 3

The lack of difference in chlorophyll content between treatments would seem to indicate that all of the treatments contain sufficient nitrogen, in terms of both source and quantity, to maintain plant health.

The difference in rooting between clones highlights the genetic difference within a species in terms of nutrient requirement and physiology. Nitrogen source appears to have a significant effect on rooting, with nitrate yielding highest rooting. As the level of rooting was higher on nitrate than on Murashige and Skoog (both ammonium and nitrate) medium, it seems possible

that *E. marginata* prefers this as a nitrogen source, rather than both ammonium and nitrate, for rooting.

5. GENERAL DISCUSSION

When the total nitrogen concentration in the medium was lowered, increased shoot growth resulted. Levels of total nitrogen supplied at two-thirds or one-third that of M&S produced significantly higher fresh weight and chlorophyll content than that of higher concentrations of nitrogen. In terms of root induction, there was also a trend found for higher rooting when nitrogen was supplied at half of the total concentration present in M&S (15 mM for root induction). These findings suggest that *E. marginata* prefers lower levels of nitrogen for *in vitro* growth. This seems likely as it has long been recognised that many Australian native plants, and eucalypts in particular, have adapted to living in low nutrient soils (Bowden, 1981; Dell et al., 1987).

This evidence supports further investigation into total nitrogen content in jarrah cultures. One way to test this would be an experiment that uses many levels of nitrogen, ranging from 5 mM through to 60 mM, and analyse shoots for total nitrogen content after a suitable growth period. Time constraints, and unwieldy replication size prevented this from being done for this project.

Previous findings (Chaillou et al., 1991; Sathyanarayana & Blake, 1994; Skirvin et al., 1986) that ammonium causes acidity in the medium and that nitrates cause alkalinity were supported. Ammonium caused a significant fall in pH, even when media was buffered. When nitrates were supplied as the sole source of nitrogen. the pH of nitrate media rose slightly, but the change was

not as great as for that observed in the ammonium media. This was observed for both shoot multiplication and root induction media. It is unclear why such a large fall occurs with ammonium, and why only a slight pH rise occurs with nitrates. It can't be due to lack of nitrate uptake, as shoots grown on this media had greater fresh weight and chlorophyll content. A possible explanation is that MES is more suited to preventing a rise in pH, than a fall.

Another possibility in maintaining a more constant pH is to examine the ratio of ammonium to nitrate. It is the level of ammonium and not that of the nitrate that is responsible for differences in morphology (Chaillou, et al., 1991; Selby & Harvey, 1990). That is, nitrate is not toxic to the plant, while the level of ammonium may cause plant growth irregularities. The nitrate is not metabolised by the plant until it is converted into ammonium, and nitrogen toxicity symptoms are related to ammonium rather than nitrates. Several reports suggest an increase in rooting occurs when the amount of NH_4NO_3 is reduced (Chattopadhyay, Datta & Ray, 1992; Grimes & Hodges, 1990; Sriskandarajah et al., 1990; Evans et al., 1976). In all these reports it was noted that poor buffering in the media was responsible for significant pH changes.

This could be examined by varying the nitrogen ratio in relation to the level of ammonium supplied, with the nitrate being used to make up the total nitrogen required in the media. To achieve this, the 'ratio' of ammonium to nitrate would be varied as well as the total nitrogen, firstly for shoot multiplication and then rooting.

The buffer MES was effective in stabilising the pH of jarrah culture medium, but its effects on shoot growth are unclear. The effect of a slight decrease in fresh weight and chlorophyll content were noted between the shoots grown on buffered and unbuffered medium, where nitrogen source and concentration were unchanged.

It is well recognised that pH affects the availability of nutrients in the medium, with most nutrients becoming limited when pH falls below 5 (Williams, 1993). However, pH was kept at a higher level with buffering, so the problem of lower chlorophyll content is less likely to be caused by pH affecting nutrient availability than it is by the buffer itself. A similar finding with other species has been reported for MES (Parfitt et al, 1988). It is also possible that the higher pH prohibited uptake of nitrates, a possible cause of reduced growth if *E. marginata* prefers this nitrogen source.

MES was effective in maintaining pH in the medium, its effects on plant growth weren't significant and could be overcome by providing a more suitable nitrogen source and concentration.

Nitrogen source was varied in an attempt to increase shoot multiplication and root induction. Previous findings suggested that some eucalypts preferred ammonium to nitrates as a nitrogen source *in vivo* (Shedley et al, 1993). This

was not found to be the case for *E. marginata*, with higher shoot growth and root induction when nitrate was the sole source of nitrogen.

The utilisation of ammonium by plants grown on shoot multiplication media is unclear. Some clones had lower pH on buffered media with low levels of ammonium, than unbuffered medium with higher ammonium. To determine if this is due to greater use of ammonium at lower levels or toxicity at higher levels, a future experiment would need to analyse nitrogen content in shoot material to determine utilisation of nitrates and ammonium. Shoots could be grown on the two different nitrogen sources, and the shoots analysed to determine the total nitrogen present in the plant tissue. This wasn't performed because the number of repetitions required were too large for the scale of this project. The number of repetitions used was already large to enable sufficient testing of pH and chlorophyll content.

Root induction was significantly higher on medium that contained 10 mM MES. There was no difference in shoot condition (measured by chlorophyll content) of these plants when grown on buffered on unbuffered medium. This suggests the incorporation of MES into rooting medium for jarrah cultures may be appropriate.

Despite attempts to separate the effects of pH and ammonium on root and shoot growth by using a buffer, a significant pH fall still occurred in ammonium media. It is not possible to state that the lack of rooting or poor

shoot multiplication in shoots grown on ammonium was due to ammonium toxicity or due to the low pH. Increasing the level of buffer to higher concentrations causes problems of its own, with evidence suggesting that it becomes toxic in different species at a range of 1 - 50 mM (George, 1993), ruling this out as an option.

The genetic difference that exist between clones within a species were highlighted by this investigation. There were significant differences in utilisation of nitrogen source, total nitrogen concentration and effects of pH, both for shoot multiplication and root induction. These differences make it difficult to develop a common protocol for growth of jarrah in culture. Where a clone has characteristics that are of particular importance, it would be feasible to develop a protocol for optimum growth of that clone. To develop a protocol for every clone would be too expensive in terms of both time and financial factors.

The studies have indicated that the current protocol used for growing *Eucalyptus marginata* contains a nitrogen content that is not the optimum for growth *in vitro*. For the species in general, it has been found that increased production in terms of both shoot multiplication and root induction is achieved by reducing the total nitrogen level, supplying more nitrate than ammonium, and with the addition of buffers. While further optimisation needs to be done on the effects of nitrogen, this study has contributed towards improving the protocol for the micropropagation of *Eucalyptus marginata*.

6. REFERENCES

Behrend, J & Mateles, R.I. (1976). Nitrogen metabolism in plant cell suspension cultures. II. Role of organic acids during growth on ammonia. *Plant Physiol* **58**: 510-512

Bell, D. T., McComb, J. A., van der Moezel, P. G., Bennett, I. J., & Kabay, E. D. (1994). Comparisons of selected and cloned plantlets against seedlings for rehabilitation of saline and waterlogged discharge zones in Australian agricultural catchments. *Aust. For.* **57**: 69-75.

Bennett, I. J., & McComb, J. A. (1982). Propagation of jarrah (*Eucalyptus marginata*) by organ and Tissue culture. *Aust. For. Res.* **12**: 121-127.

Bennett, I.J., McComb, J.A. & Bradley, J.S. (1992). Testing the expression of resistance to insect attack: Resistance of jarrah (*Eucalyptus marginata*) to jarrah leafminer (*Perthida glyphopa*). *For. Ecol. Manage.*, **48**: 99-105

Bennett, I.J., McComb, J.A., Tonkin, C.M. (1993). Inoculation of *Eucalyptus marginata* Donn ex Sm. (jarrah) clones with *Phytophthora cinnamomi* Rands in vitro and under glasshouse conditions. *For. Ecol. Manage.*, **57**: 115-124

Bennett, I.J., McComb, J.A., Tonkin, C.M. & McDavid, D.A.J. (1994). Alternating cytokinins in multiplication media stimulated *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Anal. Bot.* **74**: 53-58

Bowden, G.D. (1981). Coping with Low Nutrients. *In The Biology of Australian Plants.* Pate, J.S. & McComb A.J. (Eds). UWA Press. Perth

Cahill, D.M., Bennett, I.J. & McComb, J.A. (1992). Resistance of micropropagated *Eucalyptus marginata* to *Phytophora cinnamomi*. *Plant Disease*, **76**: 630-631.

Chaillou, S., Vessey, J.K., Morot-Gaudry, J.F., Raper C.D. jr, Henry, L.T. & Boutin, J.P. (1991) Expression of characteristics of ammonium nutrition as affected by pH of the root medium. *J. Exp. Bot.* **42**: 189-196

Chattopadhyay, S., Datta, S.K. & Malabika, R. (1992) In vitro effect of NH_4NO_3 on growth and alkaloid content of *Tylophora indica* Merr. *Phytomorphology* **42**: 139-144

De Block, M. (1990). Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid Aspen and Poplar clones. *Plant Physiol.* **93**: 1110-1116

De Little, D.W., Tibbits, W.N., Rassmussen, G.F. & Ravenswood, I.C. (1992). Genetic improvement strategy for APPM eucalypt tree farms in Tasmania. In: *Mass production technology for genetically improved fast growing forest tree species*, Bordeaux, AFOCEL.

Dell, B., Jones,S. & Wilson, S.A. (1987). Phosphorus nutrition of jarrah (*Eucalyptus marginata*) seedlings. *Plant Soil* **97**:369-379

Eldridge, K., Davidson, J., Harwood, C. & van Wyk, G. (1993). Eucalypt Domestication and Breeding. Clarendon Press, Oxford.

Evans, D.A., Sharp, W.R. & Paddock, E.F. (1976) Variation in callus proliferation and root morphogenesis in leaf tissue cultures of *Glycine max* strain T 219[1]. *Phytomorphology* :379-383

Fukunaga, Y., King, J. & Child, J.J. (1978) The differential effects of TCA-cycle acids on the growth of plant cells cultures in liquid media containing various nitrogen sources. *Planta* **139**: 199-202

George, E.F. (1993) Plant propagation by tissue culture. Part 1. The technology. Exegetics Limited, Edington

George, E.F. & Sherrington, P.D. (1984). *Plant propagation by tissue culture.* Hants, England: Exegetics Ltd

Gertsson, U.E. (1988) Influence of macronutrient composition, TIBA and dark treatment on shoot formation and nitrogen content in petiole explants of *Senecio x hybridus*. *J. Hort. Sci.* **63**: 497-502

Grimes, H.D. & Hodges, T.K. (1990) The inorganic NO_3^-:NH_4^+ ratio influences plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.) *J. Plant Physiol.*. **136**: 362-367

Le Roux, J. J. and van Staden, J. (1991). Micropropagation and tissue culture of Eucalyptus - a review. *Tree Physiol* 9: 435-477.

McComb, J. A., Stukely, M., & Bennett, I. J. (1994). Future ecosystems - use of genetic resistance. *J. Royal Soc. West. Aust.* 77:

Moran, R & Porath, D. (1980). Chlorophyll determination in intact tissue using N,N-dimethylformamide. *Plant Physiol.* **65**: 478-479

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plantarum* **15**: 473-497.

Orlikowska, T. (1992). Effects of mineral composition and acidity of media, saccharose level, brand and quantity of agar on rooting of fruit rootstocks *in vitro*. *Biologia Plantarum* **34**: 1-2, 45-52.

Parfitt, D.E., Almehdi, A.A. & Bloksberg, L.N. (1988) Use of organic buffers in plant tissue culture systems. *Sci. Hort.* **36**: 157-163

Salisbury, F.B. & Ross, C.W. (1992). *Plant Physiology*. 4th ed. Wadsworth. California.

Sathyanarayana, B.N. & Blake, J. (1994) The effect of nitrogen sources and initial pH of the meida with or without buffering on in vitro rooting of jackfruit (artocarpus heterophyllus Lam.). In - *Physiology, growth and development of plants in culture. Lumsden, Nicholas & Davies* (eds.). Kluwer Academic Press. Netherlands.

Selby, C. & Harvey, B.M.R. (1990). The influence of composition of the basal medium on the growth and morhpogenesis of cultured Sitka spruce (*Picea sitchensis*) tissues. *Ann. Bot.* **65**: 395-407

Shedley, E., Dell, B. & Grove, T.S. (1993) Effects of inorganic nitrogen forms on growth of *Eucalyptus globulus* seedlings. In: Plant Nutrition - from genetic engineering to field practice. Barrow, N.J. (Ed.) Kluwer Academic Publishers.

Skirvin, R.M., Chu, M.C., Mann, M.L., Young, H., Sullivan, J. and Fermanian, T. (1986). Stability of tissue culture medium pH as a function of autoclaving, time and cultured plant material. *Plant Cell Report* **5**:292-294

Sriskandarajah, S., Skirvin, R.M. & Abu-Qaoud, H. (1990) The effect of some macronutrients on adventitious root development on scion apple cultivars *in vitro*. *Plant Cell Tissue Organ Culture* **21**: 185-189

Stukely, M. J. C., & Crane, C. E. (1994). Genetically based resistance of *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Phytopathology* **84**: 650-656.

Tiburcio, A.F., Gendy, C.A. & Tram Thanh Van, K. (1989) Morphogenesis I tobacco subepidermal cells: Putrescine as marker of root differentiation. *Plant Cell Tissue Organ Culture* **19**: 43-54

Van Der Moezel, P. G., Pearce-Pinto, G. V. N., & Bell, D. T. (1991). Screening for salt and water logging tolerance in *Eucalyptus* and *Melaleuca* species. *For. Ecol. Manage.* **40**: 27-37.

Veliky, I.A. & Rose, D. (1973) Nitrate and ammonium as nitrogen nutrients for plant cell cultures. *Can. J. Bot.* **51**: 1837-1844

Vyskot, B. & Bezdek, M (1984) Stabilisation of synthetic media for plant tissue culture and cell cultures. *Biol. of plants* **26**: 132-143

Williams, R.R. (1993). Mineral Nutrition *in vitro* - A mechanistic approach. *Aust. J. Bot.* **41**:237-51

Williams, R.R., Taji, A.M. & Bolton, J.A. (1985) Specificity and interaction among auxins, light, and pH in rooting of Australian woody species *in vitro*. *Hort. Sci.* **20**(6): 1052-1053

Williams, R.R., Taji, A.M. & Winney, K.A. (1990). The effect of Ptilotus plant tissue on pH of in vitro media. *Plant Cell Tissue Organ Culture* **22**:153-158